In 3T3-L1 adipocytes, hyperosmotic stress was found to inhibit insulin signaling, leading to an insulin-resistant state. We show here that, despite normal activation of insulin receptor, hyperosmotic stress inhibits both tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and IRS-1-associated phosphoinositide 3 (PI 3)-kinase activity in response to physiological insulin concentrations. Insulin-induced membrane ruffling, which is dependent on PI 3-kinase activation, was also markedly reduced. These inhibitory effects were associated with an increase in IRS-1 Ser307 phosphorylation. Furthermore, the mammalian target of rapamycin (mTOR) inhibitor rapamycin prevented the osmotic shock-induced phosphorylation of IRS-1 on Ser307. The inhibition of mTOR completely reversed the inhibitory effect of hyperosmotic stress on insulin-induced IRS-1 tyrosine phosphorylation and PI 3-kinase activation. In addition, prolonged osmotic stress enhanced the degradation of IRS proteins through a rapamycin-insensitive pathway and a proteasome-independent process. These data support evidence of novel mechanisms involved in osmotic stress-induced cellular insulin resistance. Short-term osmotic stress induces the phosphorylation of IRS-1 on Ser307 by an mTOR-dependent pathway. This, in turn, leads to a decrease in early proximal signaling events induced by physiological insulin concentrations. On the other hand, prolonged osmotic stress alters IRS-1 function by inducing its degradation, which could contribute to the down-regulation of insulin action.

Insulin regulates blood glucose levels through multiple regulatory mechanisms such as suppression of endogenous glucose production in liver and stimulation of glucose uptake into muscle and adipocytes (1). Glucose transport in muscle and adipose tissues is caused by the translocation of the glucose transporter Glut 4 from an intracellular pool to the plasma membrane. These biological responses require tyrosine phosphorylation of IRS-1, which, in turn, binds and activates PI 3-kinase. Downstream effectors of PI 3-kinase such as protein kinase B (PKB) or atypical PKC could be involved in Glut 4 translocation (2, 3). Further, it has recently been shown that insulin-induced Glut 4 translocation also requires the activation of the adaptor protein containing PH and SH2 domain (APS/Chl-associated protein (CAP)/Cbl-Crk-II/TC10 pathway independent of PI 3-kinase activation (4–6). Other stimuli, such as osmotic shock, can promote Glut 4 translocation. However, osmotic shock only partly mimics the insulin effect on Glut 4 translocation and glucose uptake. The effect induced by osmotic stress requires the tyrosine phosphorylation of the adaptor protein Grb2-Associated binder-1 and is independent of PI 3-kinase/PKB activation (7–10). We have recently shown that both osmotic shock and insulin share the Crk-II/TC10 pathway to stimulate Glut 4 translocation (10). However, like several other insulinomimetic agents, hyperosmolality not only partly activates several insulin-specific biological responses but also induces a state of insulin resistance. Indeed, in rat epididymal fat cells, hyperosmotic stress markedly reduces insulin-induced glucose transport (11). In perfused rat liver, hyperosmolality impairs insulin-mediated cell swelling and reverses the proteolysis inhibition induced by insulin (12). In 3T3-L1 adipocytes, pre-treatment with sorbitol strongly decreases the ability of insulin to stimulate glucose uptake, lipogenesis, and glycogen synthesis (13). The molecular mechanism by which hyperosmotic stress antagonizes insulin-mediated responses has not yet been fully elucidated. A recent report has shown that hyperosmolality prevents insulin-induced activation of PKB. This could be mediated by the stimulation of calyculin A- or okadaic acid-sensitive protein phosphatases acting at the level of PKB in response to hyperosmotic stress (13). Thus, hyperosmolality-induced insulin resistance could partially result from the stimulation of a PKB phosphatase that maintains PKB in an inactive state. However, because the role of PKB in insulin-stimulated glucose transport is still controversial (14–17), further possibilities include the involvement of PKB in the hyperosmolality-induced insulin resistance. Indeed, it has recently been shown that insulin resistance caused by short- and long-term stress could be caused by an increase in the serine/threonine phosphorylation of IRS-1, with a subsequent inhibition of IRS-1 tyrosine phosphorylation. For instance, the inflammatory cytokine tumor necrosis factor-α

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induces the phosphorylation of IRS-1 on its Ser<sup>307</sup> (18). This serine residue is located near the phosphotyrosine binding (PTB) domain of IRS-1, and an interaction between this domain and the activated insulin receptor is required for the tyrosine phosphorylation of IRS-1 (2). IRS-1 phosphorylation on Ser<sup>307</sup> prevents this interaction and thus inhibits tyrosine phosphorylation of IRS-1 providing a potential mechanism to explain, at least in part, the insulin resistance induced by cellular stress (19). Whereas short-term osmotic stress could be a short-term mechanism involved in the negative regulation of IRS-1 function, regulated degradation of IRS-1 might also promote long-term insulin resistance (2).

In this report, we provide evidences of new mechanisms, apart from PKB inactivation, that contribute to osmotic stress-induced insulin resistance. We show that short-term osmotic stress inhibits IRS-1 function by an mTOR-dependent phosphorylation of IRS-1 on Ser<sup>307</sup>, whereas prolonged osmotic stress promotes the degradation of IRS proteins by an mTOR- and proteasome-independent mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**— Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum, and calf serum were obtained from HyClone. Hyclone, Logan, UT. Penicillin, streptomycin, and amphotericin B (100 U/ml) were purchased from Sigma. [γ-<sup>32</sup>P]ATP was purchased from Amersham Biosciences. Polyvinylidene difluoride membranes were purchased from Millipore. BCA reagent was obtained from Pierce. Protease inhibitors mixture was purchased from Roche Diagnostics. All other chemical reagents were purchased from Sigma. Polyclonal anti-phospho-IRS-1 (Ser<sup>307</sup>) antibody (α-pS<sup>307</sup>) was raised in rabbit against a synthetic peptide (ESITATp<sup>307</sup>PSAVGKK) flanking Ser<sup>307</sup> that is conserved among mouse, rat, and human (Eurogentec, Seraing, Belgium) (18). Antibodies against phosphotyrosine (clone p-Tyr-100) and phospho-p70 S6-kinase (Thr<sup>389</sup>) were purchased from Cell Signaling. Antibodies against IRS-2, IRS-3, and the p85 subunit of PI 3-kinase were purchased from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Enhanced chemiluminescence reagent was purchased from PerkinElmer Life Sciences.

**Cell Culture**—3T3-L1 fibroblasts were grown in 35- or 100-mm dishes in DMEM, 25 mM glucose, and 10% calf serum and induced to differentiate in adipocytes as described previously (10, 20). Differentiation medium was removed after 2 days and replaced with DMEM, 25 mM glucose, and 10% fetal calf serum supplemented with insulin for 2 more days. The cells were fed every 2 days with DMEM, 25 mM glucose, and 10% fetal calf serum. 3T3-L1 adipocytes were used 8–15 days after the beginning of the differentiation protocol.

**Immunoprecipitation Assays**—3T3-L1 adipocytes were serum-starved overnight in DMEM/0.5% bovine serum albumin. 3T3-L1 adipocytes were incubated in serum-free medium supplemented or not with 600 mM sorbitol and subsequently treated with or without insulin, as indicated in figure legends. To study the effect of pharmacological inhibitors, the cells were pretreated for 30 min with various inhibitors in serum-free medium followed by incubation in the serum-free medium with or without 600 mM sorbitol and pharmacological inhibitors. Cells were then washed twice with ice-cold PBS, 0.5% BSA and 0.1% Triton X-100 before insulin stimulation (0.2 nM). IRS-1 was immunoprecipitated with an anti-IRS-1 antibody. Samples were analyzed by SDS-PAGE using a 7.5% resolving gel and transferred to polyvinylidene difluoride membranes. Membrane was blocked with saline buffer (10 mM Tris, pH 7.4, and 140 mM NaCl) containing 5% (w/v) bovine serum albumin for 2 h at room temperature and blotted overnight at 4°C with the indicated antibodies at the dilution indicated by the manufacturer’s instructions and at 0.5 μg/ml for the anti-pS<sup>307</sup>. IRS1 antibody. After incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were detected by enhanced chemiluminescence. In some cases, the membrane was stripped for 30 min at 50°C in 62 mM Tris, pH 7.6, 100 mM 2-mercaptoethanol, and 2% SDS, and reprobed with the indicated antibodies.

**RESULTS**

**Hyperosmotic Stress Impairs IRS-1 Tyrosine Phosphorylation, Subsequent PI 3-Kinase Activation, and Membrane Ruffling Induced by Insulin**—Insulin effect on glucose uptake requires the tyrosine phosphorylation of IRS-1 and the activation of PI 3-kinase (3). Because hyperosmotic stress antagonizes insulin-mediated Glut 4 translocation and glucose uptake (13), we investigated the effect of hyperosmotic stress on insulin-mediated tyrosine phosphorylation of IRS-1. IRS-1 was immunoprecipitated from 3T3-L1 adipocytes pre-treated or not with 600 mM sorbitol for 40 min before insulin stimulation using a physiological or a supraphysiological insulin concentration (0.2 and 100 nM, respectively). IRS-1 tyrosine phosphorylation was analyzed by immunoblotting with phosphotyrosine antibodies. As shown in Fig. 1, sorbitol alone did not induce IRS-1 tyrosine phosphorylation. Sorbitol treatment inhibited insulin-induced tyrosine phosphorylation of IRS-1 by 50% at physiological insulin concentration (0.2 nM), but did not modify IRS-1 tyrosine phosphorylation induced by 100 nM insulin. We then investigated whether this decrease in IRS-1 tyrosine phosphorylation also impairs its ability to recruit the p85 subunit of the PI 3-kinase and to activate the enzyme. IRS-1 was immunoprecipitated from 3T3-L1 adipocytes pretreated with or without 600 mM sorbitol for 40 min before 0.2 nM insulin stimulation. The level of p85 subunit of the PI 3-kinase co-immunoprecipitated with IRS-1 was detected with an anti-p85 antibody. As shown in Fig. 2A, treatment of cells with sorbitol induced a 57% inhibition in the amount of p85 associated with IRS-1 in response to a physiological insulin concentration. This was correlated with a reduction in PI 3-kinase activity (Fig. 2B). Indeed, in absence of sorbitol, PI 3-kinase activity associated to IRS-1 was increased 14-fold by insulin, and sorbitol...
induced a 50% inhibition of insulin effect. This indicates that osmotic stress inhibits both the tyrosine phosphorylation of IRS-1 and subsequent PI 3-kinase activation.

Because the activation of PI 3-kinase (22–24) and not PKB (25) plays an important role in insulin-induced membrane ruffling, we investigated the effect of hyperosmotic stress on this insulin effect. 3T3-L1 adipocytes were incubated with or without sorbitol before insulin stimulation. Cells were then fixed and permeabilized, and F-actin structures were visualized using Texas red coupled-phalloidin by confocal fluorescence microscopy as described under “Experimental Procedures.” Representative fields obtained in each condition are shown in Fig. 3A. Quantification of several experiments (Fig. 3B) indicates that sorbitol induced an increase of nearly 2-fold in the number of cells with membrane ruffles (12% in unstimulated cells versus 22% in sorbitol-treated cells). Insulin stimulation promoted a 4-fold increase in the number of cells harboring membrane ruffles (12% in unstimulated cells versus 47% in insulin-stimulated cells), an effect that was totally abolished by sorbitol treatment. This indicates that sorbitol pretreatment markedly inhibits the insulin-induced membrane ruffling, suggesting that inhibition of IRS-1-associated PI3-kinase activity by hyperosmotic stress appears sufficient to alter this insulin effect.

Hyperosmotic Stress Does Not Alter the Activation of Insulin Receptor—The marked reduction in insulin-induced IRS-1 phosphorylation after pretreatment with sorbitol could result from an alteration in insulin receptor kinase activity. To test this hypothesis, we determined the tyrosine phosphorylation level of insulin receptor from cells preincubated with or without sorbitol before insulin stimulation (0.2 nM). As shown in Fig. 4, hyperosmotic stress did not impair the ability of insulin to stimulate the autophosphorylation of its receptor. Thus, in sorbitol-treated cells, the decrease in insulin-stimulated IRS-1 function did not result from an alteration in insulin-mediated receptor activation.

Hyperosmotic Stress Promotes the Phosphorylation of IRS-1 on Serine 307—A potential mechanism involved in the decrease in insulin-induced IRS-1 tyrosine phosphorylation is an increase in its serine/threonine phosphorylation (2, 26, 27). Several recent reports have pointed out the role of the Ser307 phosphorylation in IRS-1 as an inhibitory mechanism to trigger the decrease in insulin-induced IRS-1 tyrosine phosphorylation (18, 19, 28). To determine whether Ser307 is phosphorylated after sorbitol stimulation, 3T3-L1 adipocytes were treated with or without 600 mM sorbitol and IRS-1 was immunopurified and immunoblotted with a phosphospecific antibody against Ser307.
were pretreated with a MEK1 inhibitor (40 nM PD-98059) or stimulated (gray bars) with 600 mM sorbitol for 40 min before 20 min of insulin stimulation (0.5 nM). The cells were then fixed and subjected to confocal fluorescent microscopy using Texas red-phalloidin. A, representative fields of cells are shown. B, results are expressed as the percentage of positive cells. Bars represent the means ± S.E. of three independent experiments in which at least 200 cells were scored in each condition (see “Experimental Procedures”). *, p < 0.05 compared with basal; †, p < 0.01 compared with insulin-stimulated control.

Phosphorylation of Ser307 in Response to Hyperosmotic Stress Is Prevented by Inhibition of mTOR—IRS-1 could be phosphorylated by several serine/threonine kinases (2). We wanted to determine the signaling pathway involved in the phosphorylation of IRS-1 in response to sorbitol, which could result from an increase in its serine/threonine phosphorylation. Immunoblotting with α-pSer307 antibody indicates that the phosphorylation of this serine residue was very low in unstimulated cells but markedly increased after sorbitol treatment. Taken together, these results indicate that hyperosmotic stress increases the serine phosphorylation of IRS-1 and that one of the phosphorylated sites is the Ser307.

Fig. 4. Hyperosmotic stress does not alter insulin receptor autophosphorylation. 3T3-L1 adipocytes were treated with (gray bars) or without (empty bars) sorbitol (600 mM) for 40 min before a 5-min insulin stimulation (0.2 nM). Cell lysates were then immunoprecipitated (IP) using anti-insulin receptor (α-IR) antibodies, and immunoprecipitated proteins were resolved by SDS-PAGE and blotted (IB) using anti-phosphotyrosine (α-pY) antibodies. The membrane was then stripped and probed using anti-insulin receptor (IR) antibodies. Top, representative autoradiographs are shown. Bottom, Tyr-phosphorylation of insulin receptor was quantified by densitometry scanning analysis and normalized for the total insulin receptor amounts. Data are expressed as percentage of insulin effect and presented as the means ± S.E. of three independent experiments.

As shown in Fig. 5A, when IRS-1 was immunoblotted with an anti-IRS-1 antibody, we observed a reduction in the electrophoretic mobility of IRS-1 in response to sorbitol, which could result from an increase in its serine/threonine phosphorylation. Immunoblotting with α-pSer307 antibody indicates that the phosphorylation of this serine residue was very low in unstimulated cells but markedly increased after sorbitol treatment. Taken together, these results indicate that hyperosmotic stress increases the serine phosphorylation of IRS-1 and that one of the phosphorylated sites is the Ser307.

Inactivation of mTOR by Rapamycin Prevents the Inhibitory Effect of Hyperosmotic Stress on Insulin-mediated IRS-1 Tyrosine Phosphorylation and PI 3-Kinase Activation—Because mTOR seems to be involved in the serine phosphorylation of IRS-1 in response to sorbitol, we determined whether inhibition of mTOR could prevent the inhibitory effect of sorbitol on insulin signaling. 3T3-L1 adipocytes were incubated with or without rapamycin before sorbitol and insulin stimulation. Both the IRS-1 tyrosine phosphorylation level (Fig. 6A) and PI 3-kinase activity associated to IRS-1 (Fig. 6B) were measured as described under “Experimental Procedures.” As previously shown, sorbitol treatment reduced the insulin-induced IRS-1 tyrosine phosphorylation and its associated PI 3-kinase activ-

Fig. 3. Hyperosmotic stress strongly inhibits insulin-induced membrane ruffling. After serum starvation, 3T3-L1 adipocytes were either left untreated (empty bars) or stimulated (gray bars) with 600 mM sorbitol for 40 min before 20 min of insulin stimulation (0.5 nM). The cells were then fixed and subjected to confocal fluorescent microscopy using Texas red-phalloidin. A, representative fields of cells are shown. B, results are expressed as the percentage of positive cells. Bars represent the means ± S.E. of three independent experiments in which at least 200 cells were scored in each condition (see “Experimental Procedures”). *, p < 0.05 compared with basal; †, p < 0.01 compared with insulin-stimulated control.

ides other than Ser307. In contrast, cell treatment with rapamycin before sorbitol stimulation not only prevented the decrease in the electrophoretic mobility of IRS-1 but also abrogated its phosphorylation on Ser307 (Fig. 5A), indicating that a mTOR signaling pathway was the major pathway involved in sorbitol-induced phosphorylation of IRS-1. IRS-1 has been shown to be a potential substrate of both mTOR and its effectors, the p70 S6-kinases (29). Using a phosphospecific antibody, we did not detect the phosphorylation of threonine 389 in p70 S6-kinases in sorbitol-treated cells (Fig. 5B). Because phosphorylation of this site in p70 S6-kinases is correlated with their activation (30), it was unlikely that these kinases were involved in the phosphorylation of IRS-1 during sorbitol treatment. This suggests that mTOR could be the kinase responsible for Ser307 phosphorylation of IRS-1 after osmotic stress.

Experimental Procedures.
ity. More importantly, inactivation of mTOR by rapamycin prevented these inhibitory effects of sorbitol (Fig. 6). As shown in Fig. 7, mTOR inhibition by rapamycin did not reverse the inhibitory effect of sorbitol on insulin-induced membrane ruffling. Taken together, these results show that mTOR plays a crucial role in the serine phosphorylation of IRS-1 and, thus, in the negative regulation of IRS-1 function. However, other mechanisms are also involved in the inhibition of insulin-induced membrane ruffling by hyperosmotic stress.

Long-term Hyperosmotic Stress Enhances the Degradation of Both IRS-1 and IRS-2—It has been reported recently that long-term insulin stimulation could trigger IRS-1 degradation by proteasome in 3T3-L1 adipocytes. Moreover, it has been suggested that a rapamycin-sensitive IRS-1 phosphorylation on serine allows for this degradation (31–34). Because hyperosmolarity also promotes the serine phosphorylation of IRS-1 by a rapamycin-dependent pathway, we investigated the effect of long-term hyperosmolarity on IRS protein expression.

Fig. 5. Hyperosmotic stress triggers the phosphorylation of IRS-1 on serine 307 via a rapamycin-sensitive pathway. A, 3T3-L1 adipocytes were incubated with vehicle, PD-98059 (10 μM, PD), rapamycin (40 nM, Rapa), or wortmannin (100 nM, W) for 30 min. The cells were either left untreated (empty bars) or stimulated (gray bars) with 600 mM sorbitol for 20 min at 37°C. Cell lysates were then immunoprecipitated (IP) using anti-IRS-1 (α-IRS1) antibodies. Immunoprecipitated proteins were resolved by SDS-PAGE and blotted (IB) using anti-pSer307IRS-1 (α-pS307) antibodies. The membrane was then stripped and probed with anti-IRS-1 antibodies. Top, representative autoradiographs are shown. Bottom, phosphorylation of Ser307 was quantified by densitometry scanning analysis and normalized for the total IRS-1 amounts. Data are expressed as percentage of insulin effect and presented as the means ± S.E. of three independent experiments.

Fig. 6. Inactivation of mTOR prevents the inhibitory effect of hyperosmotic stress on insulin-mediated IRS-1 tyrosine phosphorylation and subsequent PI 3-kinase activity. After serum starvation, 3T3-L1 adipocytes were incubated with vehicle or rapamycin (40 nM) for 30 min. The cells were left untreated (empty bars) or were stimulated (gray bars) with 600 mM sorbitol for 40 min before a 5-min insulin stimulation (0.2 nM). Cell lysates were immunoprecipitated using anti-IRS-1 (α-IRS1) antibodies. A, immunoprecipitated (IP) proteins were resolved by SDS-PAGE and blotted using indicated antibodies. α-pS6K, anti-phospho-S6 kinase; p70 S6Kp-T389, phospho-p70 S6 kinase (Thr389).
growth arrest caused by hyperosmotic stress (35). We examined the effect of mTOR and proteasome inhibitors on IRSs expression levels. 3T3-L1 adipocytes were preincubated with a proteasome inhibitor (20 μM lactacystin) or mTOR inhibitor (40 nM rapamycin) before stimulation with either sorbitol (600 mM) or insulin (100 nM) for 4 h. As shown in Fig. 8A, cell treatment with rapamycin or lactacystin largely prevented the insulin-induced IRS-1 degradation as described previously (31–34). In contrast, both inhibitors were without effect on hyperosmotic stress-induced IRS-1 or IRS-2 degradation. This indicates that although both long-term insulin treatment and hyperosmotic stress induce the degradation of IRS-1, both agents mediate this response through different processes. Long-term insulin treatment promotes the serine/threonine phosphorylation of IRS-1, which is correlated with IRS-1 degradation via a proteasome-dependent process (31–34). In contrast, after a long-lasting hyperosmotic stress, the degradation of both IRS-1 and IRS-2 occurs through an mTOR- and proteasome-independent process.

**DISCUSSION**

The preincubation of 3T3-L1 adipocytes with sorbitol decreases the ability of insulin to stimulate glucose uptake, lipogenesis, and glycogen synthesis (13). The molecular mechanisms by which hyperosmolarity induces cellular insulin resistance have not yet been fully elucidated. These alterations could result, in part, from the stimulation of phosphatases that maintain PKB in an inactive state (13).

In the present study, we show that hyperosmotic stress antagonizes the effect of physiological insulin concentrations on IRS-1 tyrosine phosphorylation, the subsequent PI 3-kinase activation and membrane ruffling. This is associated with an increase in the phosphorylation of IRS-1 on Ser307 in sorbitol-treated cells. The Ser307 residue is located close to the PTB domain. It has been proposed that the phosphorylation of the Ser307 prevents the association between IRS-1 and the insulin receptor, leading to a decrease in IRS-1 tyrosine phosphorylation (19). Several stimuli, such as fatty acids (36), tumor necrosis factor-α (18, 28), or long-term administration of insulin or IGF-1 (18), induce the phosphorylation of Ser307, leading to the reduction in both IRS-1 tyrosine phosphorylation and IRS-1-associated PI 3-kinase activity. The importance of the Ser307 in the negative regulation of IRS-1 seems to be a general mechanism. However, although hyperosmotic stress promotes the phosphorylation of Ser307, it inhibits the tyrosine phosphorylation of IRS-1 and the subsequent PI 3-kinase activation only when physiological insulin concentrations were used. Osmotic stress did not inhibit insulin-induced IRS-1 tyrosine phosphorylation when a supraphysiological concentration of insulin (100 versus 0.2 nM) was used (Fig. 1 and Ref. 13). Because high insulin concentrations activate IGF-1 receptors, the lack of sorbitol inhibitory effect could indicate that phosphorylation of Ser307 in IRS-1 induced by hyperosmotic stress does not prevent its tyrosine phosphorylation by IGF-1 receptors. However, this explanation seems unlikely because 3T3-L1 adipocytes contain only low amounts of IGF-1 receptors compared with insulin receptors (10,000 versus 200,000) (37). Moreover, at 13 nM of IGF-1, the tyrosine phosphorylation of IRS-1 is similar to the phosphorylation observed at the 0.2 nM insulin concentration (data not shown). When MCF-7 cells were treated with an activator of the c-Jun NH2-terminal kinase pathway, which strongly phosphorylates IRS-1 on Ser307, IGF-1-induced PI 3-kinase-dependent PKB activation and cell

**FIG. 7.** Inactivation of mTOR does not prevent the inhibitory effect of hyperosmotic stress on insulin-induced membrane ruffling. After serum starvation, 3T3-L1 adipocytes were incubated with vehicle or rapamycin (40 nM) for 30 min. The cells were then fixed and subjected to confocal fluorescent microscopy using Texas red-phalloidin and analyzed as described in the legend to Fig. 3. Results are expressed as percentage of insulin effect. Bars represent the means ± S.E. of three independent experiments in which at least 200 cells were scored in each condition (see “Experimental Procedures”). *p < 0.01 compared with insulin alone and is not significantly modified by rapamycin.

**FIG. 8.** Degradation of both IRS-1 and IRS-2 is enhanced by chronic hyperosmotic stress by a mechanism which is mTOR and proteasome independent. A, 3T3-L1 adipocytes were incubated with either sorbitol (600 mM) for 0.5 or 4 h, as indicated, or with insulin (Ins) for 4 h. Total cell lysates (50 μg of protein) were blotted using indicated antibodies. IB, immunoblots. B, 3T3-L1 adipocytes were incubated with vehicle, lactacystin (Lact) (10 μM), or rapamycin (Rapa) (40 nM) for 30 min. The cells were left untreated or were stimulated with either 600 mM sorbitol or 100 nM insulin for 4 h. Total cell lysates (50 μg of protein) were blotted using indicated antibodies. Typical autoradiographs representative of three to four experiments are shown.
survival responses were largely reduced (38). Furthermore, treatment of 3T3-L1 adipocytes with 13 nM IGF-1 also led to the phosphorylation of IRS-1 on Ser\textsuperscript{307} (18). This indicates that phosphorylation of IRS-1 on Ser\textsuperscript{307} inhibits both insulin and IGF-1 signaling. Thus, activation of IGF-1 receptors by a high insulin concentration cannot explain this observation (Fig. 1 and Ref. 13).

A more conceivable explanation could be that the inhibitory effect of the phosphorylation of the Ser\textsuperscript{307} in IRS-1 could depend on the number of activated insulin receptors. Both PH and PTB domains of IRS-1 participate in the efficient tyrosine phosphorylation of IRS-1 by the insulin receptor. In cells expressing a high level of receptors, either the PH or the PTB domain is sufficient to promote IRS-1 tyrosine phosphorylation. In cells expressing a low level of receptors, both domains are required for efficient tyrosine phosphorylation of IRS-1 (39, 40). It is thus possible that at high insulin concentration, the PH domain of IRS-1 could be sufficient to compensate for the inhibitory effect of the phosphorylation of the Ser\textsuperscript{307} on the PTB domain function (19). In contrast, at low insulin concentration, when fewer insulin receptors are activated, the interaction of IRS-1 with insulin receptor would require both PH and PTB domains. In this case, hyperosmotic stress-induced phosphorylation of Ser\textsuperscript{307} in IRS-1 by inhibiting the function of the PTB domain could reduce the coupling between IRS-1 and the insulin receptor leading to a partial inhibition of the tyrosine phosphorylation of IRS-1. A delicate balance between positive IRS-1 tyrosine phosphorylation versus negative IRS-1 serine phosphorylation could regulate IRS-1 function depending on the number of activated receptors.

Ser\textsuperscript{307} can be phosphorylated by different kinases, such as c-Jun NH\textsubscript{2}-terminal kinase (28), MEK-dependent kinase (18), inhibitor \textalpha B kinase (41), or PI 3-kinase-dependent pathway (18). Inactivation of extracellular signal-regulated kinases (by MEK1 inhibitor), PI 3-kinase (Fig. 5A), or c-Jun NH\textsubscript{2}-terminal kinase (data not shown) by specific inhibitors did not prevent hyperosmotic stress-mediated Ser\textsuperscript{307} phosphorylation. In contrast, inhibition of mTOR by rapamycin completely abrogated the phosphorylation of Ser\textsuperscript{307} in response to sorbitol. Furthermore, mTOR-induced serine phosphorylation of IRS-1 seems to play a major role in the inhibitory effect of osmotic stress on insulin signaling. Indeed, rapamycin prevents the shift in the apparent molecular weight of IRS-1 in sorbitol-treated cells, and this was concomitant with the suppression of the inhibitory effect of hyperosmotic stress on insulin-induced IRS-1 tyrosine phosphorylation and PI 3-kinase activation. Because phosphorylation of Ser\textsuperscript{307} is sensitive to rapamycin, this indicates that mTOR and/or downstream kinases such as p70 S6-kinases could be responsible for IRS-1 phosphorylation. It is unlikely that p70 S6-kinases are involved in Ser\textsuperscript{307} phosphorylation. Indeed, as shown in Fig. 5B, sorbitol fails to activate the p70 S6-kinases and instead may lead to the stimulation of phosphatases, which maintain these kinases in an inactive state (42). Insulin treatment is also able to induce the phosphorylation of IRS-1 on Ser\textsuperscript{307} by an mTOR-dependent pathway (43, 44) but independent of p70 S6-kinase (43). We can hypothesize that Ser\textsuperscript{307} is directly phosphorylated by mTOR. In favor of such an hypothesis, it has been shown that mTOR and IRS-1 were constitutively associated (45). Moreover, mTOR catalyzes the phosphorylation of a set of Ser/Thr-Pro sites that have a proline in the +1 position, as is the case for Ser\textsuperscript{307} in IRS-1 (46). Thus, we demonstrate that the mTOR-signaling pathway is involved in the phosphorylation of Ser\textsuperscript{307}. Moreover, our results strengthen the observation that distinct kinases mediated by different stimuli might converge at Ser\textsuperscript{307} to inhibit insulin response. Although we have identified Ser\textsuperscript{307} in IRS-1 as a site phosphorylated in response to osmotic stress, we cannot exclude the possibility that other serine or threonine residues are also phosphorylated in sorbitol-treated cells. In agreement with this, it has been reported that mTOR is able to phosphorylate IRS-1\textsuperscript{311–372} \textit{in vitro}, leading to the inhibition of JAK-1-dependent IRS-1 tyrosine phosphorylation induced by interferon-\textalpha (29). Furthermore, tumor necrosis factor-\textalpha is also able to induce the phosphorylation of IRS-1 on Ser\textsuperscript{368–369} by a mTOR-dependent pathway (45).

Whereas inhibition of mTOR completely reversed the inhibitory effect of hyperosmotic stress on insulin-induced IRS-1 tyrosine phosphorylation and PI 3-kinase activation, cell treatment with rapamycin did not prevent the inhibition of insulin-induced membrane ruffling by sorbitol. This could indicate that other mechanisms, in addition to inhibition of IRS-1 function, are involved in the inhibitory mechanism of insulin-induced response by hyperosmolality. Insulin-induced membrane ruffling may involve the activation of the PI 3-kinase/Rac/Protein kinase N pathway (22–24, 47). Activation of protein kinase N in response to insulin is also dependent of the PI 3-kinase/PDK1 pathway (47). In contrast, sorbitol promotes Rac activation and membrane ruffling through a PI 3-kinase-independent manner (9, 48). Osmotic stress negatively regulates PKB and p70-S6-kinase by activating phosphatases (13, 42). An attractive explanation could be that osmotic stress also inhibits the Protein kinase N activity by the same mechanism.

Although serine phosphorylation is usually considered a short-term inhibitory mechanism, regulated degradation of IRS proteins may also promote long-term insulin-resistance. As described previously, prolonged insulin treatment reduces the level of IRS-1 through a proteasome-dependent process. Moreover, it has been proposed that the mTOR-dependent IRS-1 phosphorylation on serine could allow its degradation by the proteasome (31–34, 44). As described previously (33, 34, 49), we did not detect a significant reduction of IRS-2 amount after long-term exposure of 3T3-L1 adipocytes to insulin, although Rui et al. (50) reported its degradation. We also show that long-term hyperosmotic stress stimulates the degradation not only of IRS-1 but also of IRS-2, as described in Fao cells (50). This effect was completely insensitive to mTOR and proteasome inhibitors, suggesting that hyperosmotic stress induces IRS-1 degradation through a lysosomal process in 3T3-L1 adipocytes. On the other hand, in Fao cells, IRS-2 degradation induced by osmotic stress seems to require proteasome activity (50). Thus, depending on cell types, mechanisms induced by osmotic stress to promote IRS-2 degradation could be different. Although both insulin and osmotic stress induced the serine phosphorylation of IRS-1 through an mTOR-dependent pathway, other events activated specifically by insulin treatment could be required to trigger the IRS-1 degradation by the proteasome. Because the N-terminal region of IRS-1 contains a structural element that is crucial for the specificity of ubiquitination and proteasome degradation in response to insulin (51), another region of this protein could be required for its osmotic stress-induced degradation.

In summary, short-term osmotic stress can inhibit insulin action by distinct mechanisms including the activation of a phosphatase, which dephosphorylates PKB (13) and p70 S6 kinases (13, 42). We provide first evidence for another mechanism demonstrating that the hyperosmotic stress inhibits IRS-1 function by increasing its serine phosphorylation. mTOR could play a role in both mechanisms. Indeed, mTOR is partly associated with mitochondria and senses osmotic stress via mitochondrial dysfunction resulting in an activation of phosphatases (52). On the other hand, mTOR kinase activity could phosphorylate IRS-1 on Ser\textsuperscript{307}, inducing its decoupling with...
insulin receptor. Both pathways could allow the cells to integrate a variety of stress signals and to adapt their metabolism to the environmental modifications. A long-term hyperosmotic stress reduces the IRS-1 expression level by stimulating a degradation process.

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