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Differential Regulation of Insulin-Stimulated Tyrosine Phosphorylation of IRS-1 and SHC by Wortmannin in Intact Cells

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Wortmannin is an inhibitor of phosphatidylinositol (PI) 3′-kinase, a cellular kinase activated by docking to phosphotyrosyl residues of insulin receptor substrate-1 (IRS-1) that can also phosphorylate serine residues on IRS-1 *in vitro.* After treatment of hepatoma cells with 100 nM wortmannin, the tyrosine phosphorylation of IRS-1 in response to insulin was increased by 38.3 ± 3.3% while its phosphoserine/threonine content was reduced by 19%. Treatment with 1 μM wortmannin further increased IRS-1 tyrosine phosphorylation to 180% of control, while under these conditions, tyrosine phosphorylation of the IR substrate p52 Shc was reduced to less than 50% of control. Thus, alteration of the serine phosphorylation of IR substrates by a wortmannin-sensitive kinase may regulate post-insulin receptor signaling pathways by differential modulation of their insulin-stimulated tyrosine phosphorylation.

Insulin receptor substrate (IRS)-1 is the major cellular protein in insulin-sensitive tissues that is rapidly phosphorylated on tyrosine residues by the activated insulin receptor kinase (1). Although tyrosine phosphorylation of IRS-1 has been intensively studied, IRS-1 is also phosphorylated on serine and threonine residues by mechanisms that are poorly characterized (2–4). Phosphatidylinositol 3′-kinase (PI 3′-kinase) has been implicated in the signaling mechanism of insulin and of other mitogenic and non-mitogenic stimuli that act through tyrosine kinases (5). PI 3′-kinase is a heterodimeric enzyme composed of an 85-kDa subunit that binds tyrosyl-phosphorylated proteins via its SH2 domains and a 110-kDa catalytic subunit that possesses both lipid and serine kinase activities (6–11). In the insulin signalling pathway, PI 3′-kinase is activated by interaction of the SH2 domains of its p85 regulatory subunit with tyrosine-phosphorylated IRS-1 (12–14). Several laboratories reported previously that the kinase activity of PI 3′-kinase is responsible for *in vitro* serine phosphorylation of IRS-1 (15–17). However, the physiological significance of IRS-1 serine phosphorylation by PI 3′-kinase is still unknown.

Wortmannin is a fungal metabolite that irreversibly binds to the p110 catalytic subunit of PI 3′-kinase and inhibits its serine kinase and lipid kinase activity in isolated rat adipocytes (15) and 3T3 L1 cells (16,18). The effects of wortmannin on cell metabolism have been shown to be dose-dependent. At concentrations of 100 nM or greater, wortmannin completely inhibits the ability of insulin to stimulate glucose uptake and lipolysis in isolated rat adipocytes (19); however, in skeletal muscle, up to 1 μM wortmannin is required to achieve maximal inhibition of PI 3′-kinase activity and glucose uptake (20).

In the present work, we treated rat hepatoma cells with wortmannin and found that inhibition of PI 3′-kinase activity reduced the serine phosphorylation of IRS-1 and was associated with a reciprocal increase in the insulin-stimulated tyrosine phosphorylation of IRS-1. Interestingly, under conditions of PI 3′-kinase inhibition, insulin-stimulated tyrosine phosphorylation of another insulin receptor substrate, Shc, was reduced, suggesting that PI 3′-kinase may serve to differentially regulate the phosphorylation state of the two major insulin receptor substrates in liver cells.

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MATERIALS AND METHODS

Materials. The KRC-7 rat hepatoma cell line was kindly provided by Dr. John Koontz (University of Tennessee) (21). Rabbit polyclonal anti-phosphotyrosine antibody was generated and affinity-purified as described (22). Rabbit polyclonal anti-IRS-1 antibody was a generous gift from Dr. Morris White (Joslin Diabetes Center, Boston, MA). Rabbit polyclonal anti-Shc antibody was from Transduction Laboratories (Lexington, KY).

Immunoprecipitation and Western blotting analysis. KRC-7 cells were serum-starved overnight in DMEM medium containing 0.1% (w/v) BSA and were subconfluent at the time of analysis. After treatment with wortmannin or carrier DMSO for 30 min, cells were stimulated with 100 nM insulin for 2 or 15 min. Cells were lysed into homogenization buffer (150 mM NaCl, 2 mM EDTA, 10 mM NaF, 2 mM Na_3 VO_4, 1% Triton X-100, 10 mM PMSF and 10 μg/ml of antipain and leupeptin in 50 mM HEPES, pH 7.5), clarified at 12,000 × *g* for 20 min and protein levels were measured by the method of Bradford (23). For immunoprecipitation, cell lysates were absorbed for 1 hr each with polyclonal anti-IRS-1 antibody and Trisacryl protein A beads, and pellets were washed three times with lysis buffer. Samples were then denatured by boiling in gel buffer and applied to 7.5% (w/v) polyacrylamide gels containing sodium dodecyl sulfate (24). Separated proteins were transferred onto nitrocellulose filters, probed for 1 hr each with polyclonal anti-phosphotyrosine antibody and 2 μCi [^{125}I]protein A (25) prior to visualization and data quantitation by Phosphorimager analysis (Molecular Dynamics).

Phosphoamino acid analysis of phosphorylated IRS-1. After overnight incubation in phosphate-free DMEM medium, the cells were labeled with 0.2 mCi/ml of [^{32}P]orthophosphate for 2 hr. Labeled proteins were precipitated with IRS-1 antibody and transferred to PVDF membranes following gel electrophoresis. The labeled IRS-1 bands were hydrolyzed with 5.7 N HCl at 110°C for 1 hr. Digested samples were lyophilized and then subjected to one-dimensional thin layer electrophoresis as described (26). The migration of phosphoamino acid standards was calibrated by staining with ninhydrin.

RESULTS

Effect of wortmannin on the insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1. In insulin-stimulated KRC-7 hepatoma cells treated with 100 nM wortmannin for 30 min, the tyrosine phosphorylation of high molecular mass insulin receptor substrates ("pp185") was significantly increased while the level of insulin receptor autophosphorylation was essentially unchanged in response to 100 nM insulin stimulation for either 2 or 15 min (Fig. 1A). Western blotting of immunoprecipitated IRS-1 with anti-phosphotyrosine antibody revealed that the increased insulin-stimulated tyrosine phosphorylation of the high M_r receptor substrates was mainly contributed by IRS-1 (Fig. 1B). Phosphorimager analysis revealed that treatment of the cells with 100 nM wortmannin for 30 min and 100 nM insulin for 2 min increased IRS-1 tyrosine phosphorylation by 38.3 ± 3.3% while insulin receptor autophosphorylation was increased by only 4.0 ± 1.0% (Fig. 2). Raising the concentration of wortmannin to 1 μM further increased tyrosine phosphorylation of IRS-1 and insulin receptor to 186% and 118% of controls, respectively (Fig. 2), indicating that the effect was dose-dependent. Wortmannin had no effect on the abundance of IRS-1 or insulin receptor protein in the treated hepatoma cells, as assessed by immunoblotting (data not shown).

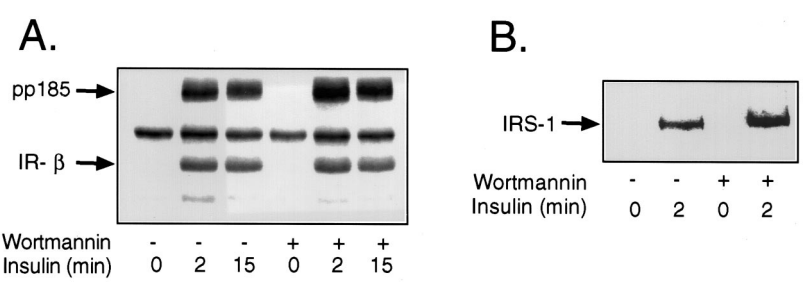

FIG. 1. Effect of wortmannin on insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1. *Panel A*, KRC-7 cells were treated with DMSO or 100 nM wortmannin for 30 min prior to stimulation with 100 nM insulin as shown for 2 or 15 min. Cell lysates were then analyzed by electrophoresis and immunoblotting with antiphosphotyrosine antibody. *Panel B*, After the indicated treatment, cell lysates were immunoprecipitated with IRS-1 antibody and immunoblotting analysis with antiphosphotyrosine antibody was performed. The migration positions of the insulin receptor β-subunit (IR-β), pp185 and IRS-1 are indicated.

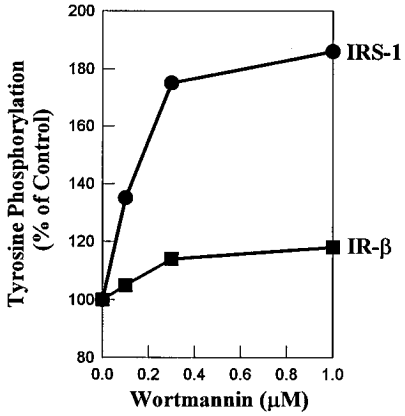

FIG. 2. Dose-dependence of the effect of wortmannin on tyrosine phosphorylation of IRS-1 and the insulin receptor. KRC-7 cells were treated with the indicated concentration of wortmannin for 30 min prior to stimulation with insulin for 2 min, and insulin-stimulated tyrosine phosphorylation of IRS-1 and insulin receptor were measured by immunoblotting.

Effect of wortmannin on serine phosphorylation of IRS-1 response to insulin. In order to determine whether the wortmannin effect on tyrosine phosphorylation of IRS-1 was related to a reduction of the serine phosphorylation of IRS-1 catalyzed by PI 3′-kinase, KRC-7 hepatoma cells were labeled with [^{32}P]orthophosphate, followed by treatment of the cells with wortmannin and insulin as described above. Under control conditions without wortmannin, phosphoamino acid analysis of the IRS-1 band indicated that most of the [^{32}P]orthophosphate was incorporated into serine residues (Fig. 3). Wortmannin treatment increased insulin-dependent tyrosine phosphorylation of IRS-1 by 25%, but at the same time, this resulted in a reduction of IRS-1 serine phosphorylation by 19%.

Effect of wortmannin on the insulin-stimulated tyrosine phosphorylation of Shc. KRC-7 cells express all three protein isoforms of Shc (27) but insulin receptor kinase elicits the tyrosine phosphorylation of only p52^{Shc}. After treatment with 1 μM wortmannin for 30 min, the tyrosine phosphorylation of Shc stimulated by 100 nM insulin was reduced by at least 50% (Fig. 4). There was no change in the abundance of Shc protein isoforms in both control and wortmannin treated cells (Fig. 4). Therefore, reduced tyrosine phosphorylation was a net result of either inability of p52 Shc phosphorylated by insulin receptor or increased dephosphorylation of p52 Shc by protein-tyrosine phosphatases.

DISCUSSION

Wortmannin, an inhibitor of cellular PI 3′-kinase activity, at concentrations as low as 100 nM, induced a significant increase in the insulin-stimulated tyrosine phosphorylation of IRS-1. Previous

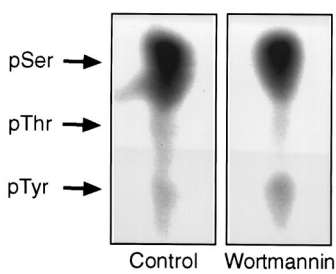

FIG. 3. Effect of wortmannin on phosphoamino acid content of IRS-1. KRC-7 cells were prelabeled with [^{32}P]orthophosphate and treated with 100 nM wortmannin for 30 min followed by 100 nM insulin for 2 min. Phosphoamino acid analysis of immunoprecipitated IRS-1 from cell lysates was performed as described in the Methods section.

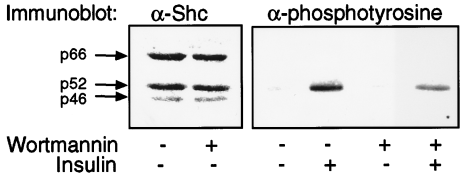

FIG. 4. Differential regulation of p52 Shc tyrosine phosphorylation by wortmannin. KRC-7 cells were treated with 1 μM wortmannin where indicated for 30 min prior to incubation with 100 nM insulin as shown. Cell lysates were analyzed by immunoblotting with Shc antibody to visualize the Shc protein isoforms (*left panel*) or immunoprecipitated with Shc antibody and then immunoblotted with antiphosphotyrosine antibody to quantitate the tyrosine phosphorylation state of p52 Shc (*right panel*).

studies have demonstrated that at this concentration, wortmannin does not inhibit a number of other cellular kinases, including the insulin-receptor tyrosine kinase (19), myosin light-chain kinase, protein kinase A, protein kinase C or cyclic-GMP-dependent protein kinase (28), the components of the MAP kinase cascade and $p70^{S6\ kinase}$ (29,30). Treatment of isolated adipocytes with 100 nM wortmannin did not alter the phosphorylation state of the insulin receptor or IRS-1 although this was an effective concentration to inhibit the serine kinase activity of PI 3′-kinase *in vitro* (15). In skeletal muscle, a complete inhibition of insulin-induced glucose uptake by wortmannin required a concentration of up to 1 μM. Maximum inhibition of mitogen-activated protein kinase in neutrophils was also achieved at 1 μM wortmannin (31). Our dose-response experiment (Fig. 2) showed that the effect of 1 μM wortmannin was maximal. Therefore, it seemed that the activity profile of wortmannin may differ *in vivo* and *in vitro*, as well as in different cell types.

Previous studied have provided evidence that serine phosphorylation of IRS-1 could regulate the extent of its tyrosine phosphorylation by insulin receptors. Treatment of 3T3-L1 adipocytes with okadaic acid, a serine/threonine phosphatase inhibitor, could reduce tyrosine phosphorylation of IRS-1 in response to insulin without modification of insulin receptor kinase activity (32). Hotamisligil *et al.* (33) reported recently that tumor necrosis factor (TNF)-α of adipocytes could induce serine phosphorylation of IRS-1. They also demonstrated that after serine phosphorylation, IRS-1 was resistant to further tyrosine-phosphorylation and actually became an inhibitor of the insulin receptor kinase. Considering the relative specificity of wortmannin for PI 3′-kinase, our results suggested that the increase in tyrosine phosphorylation of IRS-1 caused by this compound was most likely mediated by an inhibition of the serine kinase activity of PI 3′-kinase, the only cellular serine kinase that has been physiologically associated with IRS-1 to date. This hypothesis is substantiated by our phosphoamino acid analysis of IRS-1 protein from the insulin-stimulated cells treated with wortmannin, which showed that the increase in IRS-1 tyrosine phosphorylation was associated with diminished serine phosphorylation (Fig. 3). At high wortmannin concentrations, we also found that autophosphorylation of the insulin receptor was also enhanced (Fig. 2). It is possible that reduced serine phosphorylation of IRS-1 can ameliorate its inhibition of the insulin receptor kinase activity, as discussed above (33).

In contrast, tyrosine phosphorylation of Shc, another substrate of the insulin receptor kinase (34), was inhibited more than 50% by 1 μM wortmannin without a change in the abundance of Shc protein (Fig. 4). Shc can directly associate with the insulin receptor (35,36) and the binding site was identified as phosphorylated Tyr-960 in the receptor juxtamembrane region (37). Removal of Tyr-960 form the insulin receptor impaired Shc (38) and IRS-1 (39,40) tyrosine phosphorylation by the insulin receptor kinase, consistent with the notion that both Shc and IRS-1 compete for this tyrosine residue in their association with phosphorylated insulin receptor (41). Thus, it is possible that in its reduced serine phosphorylation state, IRS-1 has an enhanced affinity for binding to the insulin receptor and is more competitive than Shc, whose tyrosine phosphorylation by the receptor kinase is effectively diminished. Alternatively, accelerated dephosphorylation of Shc by protein-tyrosine phosphatases cannot be excluded.

In summary, our data suggest that reducing the level of serine phosphorylation of IRS-1 by inhibition of a wortmannin-sensitive kinase, most likely PI 3′-kinase, leads to enhanced IRS-1 tyrosine phosphorylation. Under the same conditions, the tyrosine phosphorylation state of Shc in reduced. These data suggest that a feedback mechanism mediated by serine phosphorylation of cellular insulin receptor substrates may, in turn, regulate post-receptor insulin-stimulated signal transduction pathways.

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