Rapid Publication

Transgenic Mice Deficient in the LAR Protein-Tyrosine Phosphatase Exhibit Profound Defects in Glucose Homeostasis

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Protein-tyrosine phosphatases (PTPases) play an integral role in the regulation of cellular insulin action. LAR, a transmembrane PTPase expressed in insulin-sensitive tissues, acts as a negative regulator of insulin signaling in intact cell models. The physiological role of LAR was studied in mice in which LAR expression was eradicated by insertional mutagenesis. In the fasting state, adult male homozygous LAR (–/–) mice had significantly lower plasma levels of insulin and glucose, as well as a reduced rate of hepatic glucose production compared with wild-type controls, suggesting a heightened level of insulin sensitivity. In euglycemic clamp studies, the LAR (–/–) mice exhibited a significant resistance to insulin-stimulated glucose disposal and suppression of hepatic glucose output. Examination of hepatic insulin action demonstrated that the major alteration involved a 47% reduction in insulin-stimulated phosphatidylinositol 3′-kinase (PI 3-kinase) activity in the knockout mice, indicating a post-receptor signaling defect. Taken together with previous work on the cellular effects of LAR, the present results are consistent with a physiological role for LAR in the negative regulation of insulin action, with secondary abnormalities that contribute to the resistance to insulin-stimulated signaling in the knockout mice. Overall, these data provide further evidence for an important role for LAR in the regulation of insulin action and glucose homeostasis in intact animals. ***Diabetes*** **47:493–497, 1998**

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ANOVA, analysis of variance; IRS, insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3′-kinase; PTPases, protein-tyrosine phosphatases.

A central role for reversible tyrosine phosphorylation in mediating insulin signaling has been well established; however, detailed knowledge of the regulatory mechanisms that balance the phosphorylation state of the insulin receptor and its post-receptor substrates is lacking, and it is not understood how these pathways are altered in insulin-resistant disease states (1,2). Recently, specific protein-tyrosine phosphatase enzymes (PTPases) have been postulated to regulate insulin signaling in normal physiology and to be involved in the pathogenesis of insulin-resistant disease states (3).

In particular, the receptor-type, transmembrane PTPase LAR has emerged as a candidate insulin receptor PTPase because it is expressed in insulin-sensitive tissues, it is localized to the cell membrane fraction of the cell where insulin receptor dephosphorylation occurs rapidly in situ, and LAR exhibits a relative catalytic specificity for the insulin receptor kinase regulatory domain in vitro (4–6). Studies in intact cells have also shown that LAR can be chemically cross-linked to the insulin receptor at the cell surface and that insulin receptor tyrosine phosphorylation and kinase activity can be significantly enhanced by reduction of LAR mass using antisense techniques (7,8). To further evaluate the potential role of LAR in insulin action in an intact animal model, we used a transgenic mouse line lacking expression of LAR to evaluate the physiological effects of loss of LAR on insulin signaling and glucose homeostasis.

RESEARCH DESIGN AND METHODS

Materials. Anti-phosphotyrosine antibodies were generated and immunopurified as described (9). Anti–insulin receptor substrate (IRS)-1 polyclonal antibody raised to a recombinant rat IRS-1 protein was from Dr. Morris White (Joslin Diabetes Center, Boston, MA). ^{125}I-labeled protein A (>30 μCi/μg) was purchased from ICN (Costa Mesa, CA). Phosphatidylinositol was purchased from Avanti Polar Lipids (Alabaster, AL); phosphatidylinositol 4′-phosphate and protease inhibitors were purchased from Sigma (St. Louis, MO).

Transgenic LAR knockout mice. LAR expression was eradicated by insertional mutagenesis as previously reported (10). Animals were housed and fed ad libitum as described, and Swiss Webster mice (Jackson Laboratory, Bar Harbor, ME) were used as controls (11). The LAR (–/–) mice had no detectable LAR mRNA or protein expression by immunoblot analysis (P.M.L., B.J.G., unpublished observations).

Plasma assays and euglycemic clamp studies. Plasma glucose was analyzed by the glucose oxidase method (Glucose Analyzer II, Beckman Instruments, Fullerton, CA) and plasma glucagon and insulin was analyzed by double antibody

TABLE 1
Plasma metabolite and hormone levels in fasted male LAR (–/–) and control mice at 8 weeks of age

	Wild-type	LAR (–/–)
Body wt (g)	29 ± 2 (24)	17 ± 1 (5)*
Glucose (mg/dl)	122 ± 4 (40)	57 ± 5 (5)*
Insulin (ng/ml)	0.30 ± 0.01 (54)	0.00 (5)*
Glucagon (pg/ml)	45 ± 4 (18)	116 ± 37 (5)*
Lactate (mg/dl)	39 ± 10 (10)	27 ± 3 (5)
Free fatty acids (mmol/l)	1.3 ± 0.2 (7)	1.6 ± 0.2 (5)
Triglycerides (mg/dl)	163 ± 43 (24)	108 ± 13 (5)
Glutamine (mmol/l)	0.33 ± 0.03 (5)	0.36 ± 0.13 (5)
Alanine (mmol/l)	0.05 ± 0.00 (5)	0.32 ± 0.02 (5)*

Data are means ± SE (n). *$P < 0.05$ vs. wild-type by Student's t test.

radioimmunoassays using human and rat standards, respectively (11). Plasma chemistry determinations were performed using an automated analyzer (Roche Diagnostics, Nutley, NJ) and diagnostic reagent kits (Sigma Chemical, St. Louis, MO). Euglycemic clamp studies employed a two-step technique initially developed in rats and adapted for mice exactly as previously reported (11,12).

Analysis of insulin-stimulated hepatic protein tyrosine phosphorylation. Insulin action was evaluated in the transgenic mice by the intraperitoneal injection of 5 g recombinant human insulin (Sigma) per 30 µg body weight or saline in the control injections. At 15 min post-injection, the mice were killed, and liver tissue was removed and homogenized with three passes of 20 s each using a Polytron (Brinkman Instruments, Westbury, NY) at a setting of 4. The homogenization buffer was ice-cold 50 mmol/l HEPES, pH 7.5, containing 150 mmol/l NaCl, 1 mmol/l Na_3VO_4, 10 mmol/l NaF, 10 mmol/l phenylmethylsulfonyl fluoride (PMSF), and 10 µg/ml each of antipain, pepstatin A, aprotinin, and leupeptin. Triton X-100 was added to a final concentration of 1% (vol/vol), and the homogenate was solubilized on ice for 20 min. Insoluble material was removed by centrifugation at 12,000*g* for 20 min, and the supernatant was stored in aliquots at –80°C before use.

For Western immunoblotting, a portion of each solubilized tissue sample (30 µg of protein) was denatured by boiling for 4 min in Laemmli gel sample buffer containing 100 mmol/l dithiothreitol and fractionated on 7.5% (wt/vol) polyacrylamide gels containing SDS (13). After blotting onto nitrocellulose filters (90 V for 90 min) in 154 mmol/l glycine, 20% (vol/vol) methanol, and 0.02% (wt/vol) SDS in 20 mmol/l Tris-HCl, pH 8.3 (14), blots were probed for 1 h each with polyclonal antibodies and 2 µCi ^{125}I–protein A in a buffer containing 5% (wt/vol) BSA, 0.9% (wt/vol) NaCl, and 0.01% (wt/vol) NaN_3 in 10 mmol/l Tris-HCl, pH 7.2, and quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

PI 3-kinase assay. The solubilized tissue homogenate was immunoadsorbed with polyclonal anti-phosphotyrosine antibodies, and PI 3-kinase activity was assayed as described (15).

Statistical methods. The data are presented as means ± SE for the indicated number in each group. Student's t test was used for comparisons between two groups and multiple comparisons were performed with analysis of variance (ANOVA) using Bonferroni's correction, with a significance level set at $P < 0.05$.

RESULTS

Phenotype and basal hormone and substrate concentrations. The LAR (–/–) mice appeared grossly normal but their body weight was reduced to 59% of the controls (Table 1). Fasting glucose levels were reduced to 47% of control in the LAR (–/–) mice. This was associated with a profound decrease in fasting insulin levels from 0.30 ng/ml in the control mice to below the limits of detection by the assay in the knockout mice.

The LAR (–/–) mice also exhibited an enhanced counterregulatory hormone response, with a 2.6-fold increase in fasting glucagon concentrations over control levels, and increased gluconeogenic substrates as evidenced by fasting alanine levels that were 6.4-fold higher than control values. Lactate levels were mildly reduced in the LAR (–/–) mice, but no significant difference was noted in glutamine, free fatty acid, or triglyceride levels.

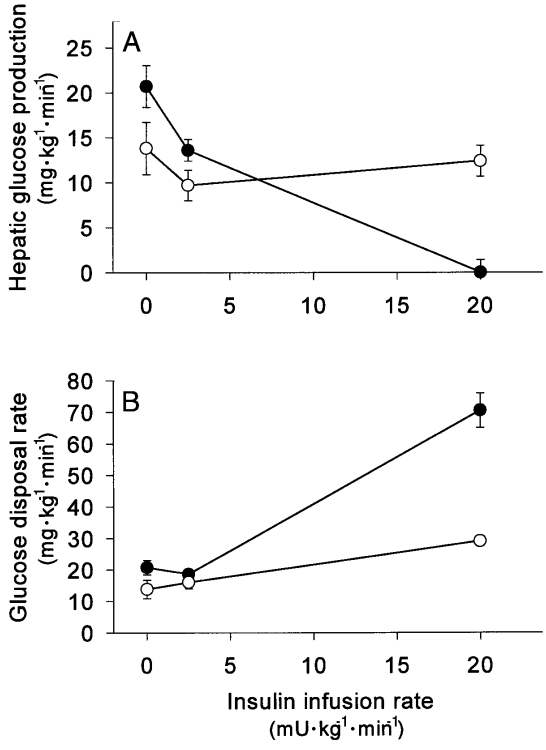

FIG. 1. Euglycemic clamp studies in fasting, awake male LAR (–/–) mice and controls. Insulin infusions were performed as described in METHODS. *A*: Insulin suppression of hepatic glucose production; *B*: insulin stimulation of whole-body glucose disposal. ●, data from wild-type mice; ○, data from LAR (–/–) mice.

Euglycemic glucose clamp studies. At steady state, before insulin infusion, the fasted LAR (–/–) mice had 33% lower rates of whole-body glucose disposal and hepatic glucose production, compared with control mice ($P < 0.05$; Fig. 1). In the control mice, insulin infusion enhanced glucose disposal dramatically to a maximal level of 3.4 times the basal rate at the high insulin infusion (20 mU · kg^{-1} · min^{-1}). In contrast, the LAR (–/–) mice exhibited a significantly diminished glucose disposal response to the insulin infusion, with only a 2.1-fold increase above the basal level, which was only 41% of the maximal level observed in the control animals at the high insulin infusion ($P < 0.05$).

In the control mice, insulin infusion lowered hepatic glucose production as expected, with virtually complete suppression of glucose output at the high insulin infusion rate. In contrast, in the LAR (–/–) mice, insulin infusion had little or no effect on hepatic glucose output, which remained close to the level observed before insulin stimulation ($P < 0.05$ vs. control mice).

Studies on cellular insulin signaling. To gain insight into the biochemical mechanisms underlying the alterations in whole-body glucose metabolism in the knockout mice revealed by the clamp studies, several aspects of insulin action were examined. At baseline and 15 min after injection of insulin into the portal vein of fasted mice, liver tissue was homogenized and processed to measure insulin-stimulated

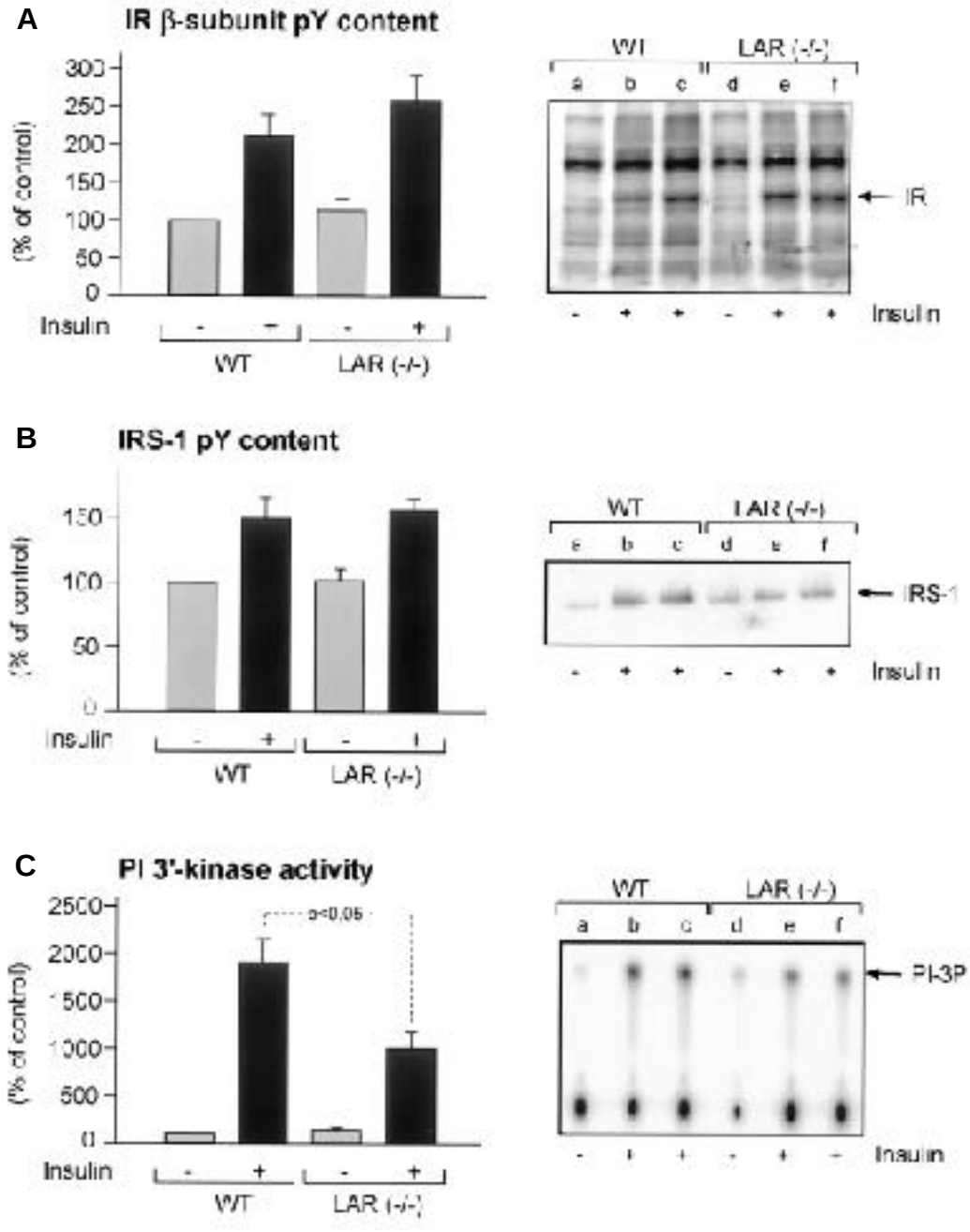

FIG. 2. In vivo insulin action in control and LAR (–/–) mice. After intraperitoneal insulin injection, liver homogenates were analyzed for basal and insulin-stimulated insulin receptor β-subunit (IR-β) phosphotyrosine (pY) content (*A*), IRS-1 pY content (*B*), and anti-pY immunoprecipitable PI 3-kinase activity (*C*), using methods described in the text. Representative primary experimental data are shown (right), with the corresponding data tabulated from three to five experiments summarized in the bar graphs (left). Statistical analysis was performed with ANOVA using Bonferroni's correction for multiple comparisons.

receptor autophosphorylation, IRS-1 phosphorylation, and activation of PI 3-kinase (Fig. 2). By immunoblot analysis, levels of protein abundance for the insulin receptor, IRS-1, Shc, and the p85 subunit of PI 3-kinase were unchanged in the knockout mice as compared with control mice (not shown). Basal tyrosine phosphorylation of the insulin receptor and IRS-1 was not significantly different in the control and LAR knockout mice. After insulin infusion, however, the tyrosine phosphorylation of the insulin receptor β-subunit was increased to 210 and 259% of basal, respectively, representing on average a 23% higher response in the LAR (–/–) mice compared with control mice. However, because of experimental variation, the difference in receptor autophosphorylation between the control and knockout mice was not statistically significant. The tyrosine phosphorylation of IRS-1 was also increased similarly by insulin stimulation to 149 and 155% of control in the wild-type and LAR knock-out mice.

Before insulin infusion, the mean anti-phosphotyrosine immunoprecipitable PI 3-kinase activity was increased by

45%, suggesting enhanced basal activation of this signaling pathway in the knockout mice; however, this result was not statistically significant (Fig. 2). In contrast, after insulin stimulation, the PI 3-kinase activity was increased 19-fold over the basal PI 3-kinase activity in the control mice, but only 10-fold over the basal level of activation in the knockout mice, representing a significant decrease of 47%. These results clearly indicated a major post-receptor defect in insulin signaling in the LAR knockout mice.

DISCUSSION

The present work demonstrates that adult mice carrying homozygous mutations in the LAR gene and lacking detectable LAR expression develop profound abnormalities in glucose homeostasis and insulin action. The adult LAR (–/–) mice exhibit lower fasting levels of both insulin and glucose compared with controls, suggesting a heightened level of insulin sensitivity. These initial findings might be expected from the loss of a PTPase that has been shown in cellular studies to exert a negative influence on the balance of insulin receptor activation and signaling, and provide strong evidence for a major role of the LAR PTPase in the regulation of insulin action in an intact animal model.

Careful analysis of tissue responsiveness to insulin infusion by glucose clamp studies, however, revealed significantly decreased insulin effects on hepatic glucose output and whole-body glucose disposal. Analysis of insulin signaling in tissues from the transgenic mice suggested an enhancement of insulin-stimulated receptor autophosphorylation and basal PI 3-kinase activity, consistent with the loss of a negative effect of LAR on early steps in insulin action in the knockout mice. However, the major alteration involved a significant impairment of insulin-stimulated PI 3-kinase activation in the LAR (–/–) mice. Since post-receptor effects of decreased insulin signaling were not evident in short-term studies in transfected hepatoma cells with reduced LAR abundance (8), this finding in the LAR (–/–) mice suggests that secondary compensatory changes in insulin action have occurred at a post-receptor site in the adult mice, which may account for the observed resistance to insulin-stimulated glucose disposal and suppression of hepatic glucose output in the clamp studies.

Without further assessment of the ontogeny of the biochemical abnormalities during the adult development of the mice, it may not be possible to further delineate the molecular basis for the abnormal glucose metabolism, especially with the likely occurrence of secondary signaling alterations in the adult mice. On the basis of available data, we hypothesize that loss of the negative influence of LAR on insulin signaling in the knockout mice causes an early enhancement of insulin action, which eventually leads to a downregulation of post-receptor signaling. This phenomenon is in some ways similar to the effects on post-receptor signaling observed in cells undergoing chronic insulin stimulation (16). The LAR knockout mouse is clearly unique, however, as a mouse model exhibiting a discordance between basal glucose homeostasis with apparent insulin sensitivity, possibly associated with enhanced basal PI 3-kinase activity, and evidence of resistance to infused insulin that is due, at least in part, to deficient activation of PI 3-kinase. The low circulating insulin levels in the knockout mice might also have contributed to the observed post-receptor defect. A related defect has been reported in patients with insulin-deficiency in poorly controlled type 1 diabetes, where the site of abnormal insulin action in target tissues has not been identified at a molecular level (17).

While these results implicate LAR in the regulation of insulin signaling, other work has also suggested that additional PTPases might act additively or synergistically with LAR to regulate insulin action. Modulation of the cellular activity of PTP1B has shown that this enzyme can serve as a negative regulator of insulin signaling acting, at least in part, directly at the level of the insulin receptor itself (18,19), and PTP1B has been shown to physically associate with the insulin receptor in intact cells (20). A few studies in transfected cell systems have also suggested that RPTP-α, and the closely related transmembrane enzyme RPTP-ε, can act as negative regulators of the insulin receptor tyrosine kinase (21,22). Further work will better define how these PTPases may interact, possibly in a synergistic fashion, to balance the steady-state activation of the insulin receptor kinase. Also, the cellular PTPases that regulate the reversible phosphorylation of IRS-1, IRS-2, Shc, and other post-receptor substrates in the insulin-signaling pathway have not been identified.

Alterations in the amount and distribution of specific PTPases, acting as negative regulators, may also be involved in the pathogenesis of insulin-resistant disease states, including obesity and type 2 diabetes. Recent work from our laboratory has implicated LAR in clinical insulin resistance, since tissue PTPase activity is increased in adipose tissue and skeletal muscle of obese human subjects; this is associated with increased LAR abundance that is normalized after immunodepletion of this PTPase from the tissue homogenates (23,24). Furthermore, after weight loss in obese human subjects, increased sensitivity to insulin was associated with reduced adipose tissue PTPase activity and a decrease in the abundance of LAR and PTP1B (25). The reversibility of the increased PTPase abundance and activity is consistent with the finding that the clinical defects in insulin signaling may be acquired, since they have been observed to revert to near-normal in adipose tissue and muscle of subjects with obesity after weight reduction (26,27). Further work in this area may help to pave the way for the development of agents that can modulate the activity of individual PTPases involved in insulin action in order to augment insulin signaling in states characterized by resistance to the tissue action of insulin.

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