

Absence of the Glucagon-Like Peptide-1 Receptor Does Not Affect the Metabolic Phenotype of Mice with Liver-Specific $G_s\alpha$ Deficiency

Min Chen, Eralda Mema, James Kelleher, Nicholas Nemechek, Alta Berger, Jie Wang, Tao Xie, Oksana Gavrilova, Daniel J. Drucker, and Lee S. Weinstein

Metabolic Diseases Branch (M.C., E.M., J.K., N.N., A.B., J.W., L.S.W., T.X.) and Mouse Metabolism Core Laboratory (O.G.), National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892; and Department of Medicine (D.J.D.), Samuel Lunenfeld Research Institute, Mount Sinai Hospital, University of Toronto, Toronto, Ontario M5G 1X5, Canada

The stimulatory G protein α -subunit ($G_s\alpha$) couples hormone and other receptors to the generation of intracellular cAMP. We previously showed that mice with liver-specific $G_s\alpha$ deficiency [liver-specific $G_s\alpha$ knockout (LGsKO) mice] had reduced adiposity and improved glucose tolerance associated with increased glucose-stimulated insulin secretion, pancreatic islet hyperplasia, and very high serum glucagon and glucagon-like peptide 1 (GLP-1) levels. Because GLP-1 is known to stimulate insulin secretion and to have effects on energy balance, we mated LGsKO mice with germline GLP-1 receptor (GLP-1R) knockout mice ($Glp1r^{-/-}$) and compared LGsKO to double-knockout (LGs/ $Glp1r^{-/-}$) mice to determine the contribution of excess GLP-1R signaling to the LGsKO phenotype. Loss of the GLP-1R failed to reverse most of the metabolic features of LGsKO mice, including reduced fat mass, increased glucose tolerance, and second-phase glucose-stimulated insulin secretion, islet cell hyperplasia, and very high glucagon and GLP-1 levels. However, loss of GLP-1R impaired first-phase insulin secretion in mice with or without liver-specific $G_s\alpha$ deficiency. Thus, excess GLP-1 action (or at least through GLP-1R) does not contribute to the LGsKO metabolic phenotype, and other unknown factors involved in the cross talk between the liver $G_s\alpha$ /cAMP pathway and pancreatic islet function need to be further elucidated. (*Endocrinology* 152: 0000–0000, 2011)

Glucose metabolism is regulated by complex neuronal and hormonal systems to maintain blood glucose in a narrow range. In the fed state, insulin stimulates glycolysis, glycogen synthesis, and glucose uptake by peripheral tissues and inhibits hepatic gluconeogenesis to lower blood glucose, whereas in the fasted state, insulin levels are low and glucose levels are maintained by various counterregulatory hormones (e.g. glucagon, epinephrine, and glucocorticoids) that stimulate hepatic glucose production (HGP) via glycogenolysis and gluconeogenesis. Activation of the glucagon receptor in liver stimulates HGP by coupling to the stimulatory G protein α -subunit ($G_s\alpha$) to generate intracellular cAMP, which stimulates cAMP-dependent protein kinase. In addition to directly phosphorylating meta-

bolic enzymes to enhance HGP, cAMP-dependent protein kinase also phosphorylates the transcription factor cAMP-response element-binding protein (CREB). Phospho-CREB stimulates the expression of the transcription factor peroxisome proliferator-activated receptor coactivator 1 α (1, 2), which in concert with coactivators induces expression of gluconeogenic genes (2–4). Insulin counteracts the actions of cAMP/CREB/peroxisome proliferator-activated receptor coactivator 1 α by a variety of mechanisms (5–8). Subjects with type 2 diabetes may have elevated glucagon levels and excessive HGP even when blood glucose levels are high (9).

To study the effects of disrupting glucagon signaling in the liver on glucose metabolism, we previously generated

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Copyright © 2011 by The Endocrine Society

doi: 10.1210/en.2011-0012 Received January 5, 2011. Accepted June 22, 2011.

Abbreviations: AUC, Area under the curve; CREB, cAMP-response element-binding protein; FFA, free fatty acid; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide 1; GLP-1R, GLP-1 receptor; $G_s\alpha$, stimulatory G protein α -subunit; HGP, hepatic glucose production; LGsKO, liver-specific $G_s\alpha$ knockout mice.

mice with liver-specific G_sα deficiency [liver-specific G_sα knockout (LGsKO) mice] (10). LGsKO mice develop a number of abnormalities in glucose and energy metabolism, including reduced adiposity, increased liver weight, improved glucose tolerance associated with increased insulin sensitivity, increased glucose-stimulated insulin secretion, and enlarged pancreatic islets associated with very high serum glucagon and glucagon-like peptide 1 (GLP-1) levels. Although under normal circumstances GLP-1 is primarily secreted by intestinal L cells, results from other mouse models of hepatic glucagon resistance suggest that the high GLP-1 levels in LGsKO mice may result from alternative processing of proglucagon in pancreatic α-cells rather than by increased secretion from the intestine (11, 12).

GLP-1 exerts multiple insulinotropic actions on pancreatic β-cells by activating a receptor that is coupled to the G_sα/cAMP pathway (13). GLP-1 enhances glucose-stimulated insulin secretion by synergizing with glucose to inhibit ATP-sensitive potassium channels (K_{ATP}) (14–16) and stimulates insulin gene expression and insulin biosynthesis (15, 17). In addition, GLP-1 promotes β-cell differentiation, proliferation, and neogenesis (18–20) and reduces β-cell apoptosis through a cAMP signaling pathway (21). However, GLP-1's actions are not limited to the β-cell, and the GLP-1 receptor (GLP-1R) has been shown to be expressed in other tissues including lung, stomach, and brain (22). In the central nervous system, GLP-1 has many actions, including inhibition of food and water intake and promotion of satiety and weight loss, nausea, memory enhancement, and neuronal survival (13). As would be predicted by the important role of GLP-1 in pancreatic β-cell function, mice with germline inactivation of the GLP-1R (*Glp1r*^{-/-}) exhibit glucose intolerance with diminished insulin levels, although they have normal body weight and feeding behaviors (23).

Because LGsKO mice have very high circulating GLP-1 levels, we wished to determine whether the actions of GLP-1 could explain any of the metabolic phenotype of LGsKO mice, particularly the reduced adiposity, improved glucose tolerance, increased glucose-stimulated insulin secretion, and islet expansion. Accordingly, we generated LGsKO mice that also had germline loss of the *Glp1r* and examined the metabolic phenotype of LGs/*Glp1r*^{-/-} mice. Our findings show that enhanced signaling through GLP-1R probably plays little role in the development of the LGsKO phenotype.

Materials and Methods

Generation of LGsKO and germline LGs/*Glp1r*^{-/-} double-knockout mice

Mice with loxP recombination sites surrounding G_sα exon 1 (E1^{fl/fl}; E1^{fl/+}) were mated with mice carrying an albumin pro-

motor-cre recombinase transgene (Alb-cre) to generate mice with liver-specific G_sα deficiency (LGsKO) as previously described (10). The heterozygous (E1^{fl/+}; Alb-cre⁺) or homozygous (E1^{fl/fl}; Alb-cre⁺) LGsKO mice were then bred with *Glp1r*^{-/-} mice (23) to generate mice that were homozygous for both the liver-specific G_sα deletion and the germline *Glp1r* inactivating mutation (LGs/*Glp1r*^{-/-}). Because the E1^{fl} allele has no effect on G_sα expression or phenotype, all E1^{+/+} or Alb-cre⁻ littermates were used as controls. Mice were genotyped by PCR of mouse tail DNA as previously described (10). The presence of the Alb-cre transgene was determined using an albumin-specific forward primer (5'-AGGAACCAATGAAATGCGAGG-3') and a cre-specific reverse primer (5'-CCCCAGAAATGCCAGATTACG-3'). Disruption of the *Glp1r* gene was determined using the following primers: *Glp1r* forward, 5'-ACATCCACCTGAAACCTGTTTGC-3'; *Glp1r* reverse, 5'-TGATAGGAGAGGAGCCCATCCCCAC-3'; Neo forward, 5'-AGAGGCTATTCCGGCTATGACTG-3'; and Neo reverse, 5'-TTCGTCCAGATCATCCTGATC-3'. Animals were maintained on a 12-h light, 12-h dark cycle (0600–1800 h) and a standard pellet diet (NIH-07, 5% fat by weight). Animal experiments were approved by the National Institute of Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee. Except where noted, all metabolic studies were performed in 2- to 4-month-old male mice unless otherwise indicated. Histological studies were performed on both male and female mice.

Measurement of food intake, body composition, and metabolic rate

Body composition was measured in nonanesthetized mice using the Echo3-in-1 NMR analyzer (Echo Medical Systems, Houston, TX). Oxygen consumption rate was measured by indirect calorimetry using an eight-chamber Oxymax system with one mouse per chamber (Columbus Instruments, Columbus, OH; 2.5-liter chambers with wire mesh floors, using 0.6 liters/min flow rate, 90-sec purge, and 60-sec measure). Total and ambulating motor activities were determined by infrared beam interruption (Opto-Varimex mini; Columbus Instruments). Food intake was measured as previously described (10).

Biochemical assays

Serum insulin, glucagon, leptin, and adiponectin levels were measured using RIA kits, and glucose-dependent insulinotropic polypeptide (GIP) levels were measured using an ELISA kit (Millipore, Billerica, MA). Serum glucagon levels were measured by an RIA with less than 0.1% cross-reactivity to oxyntomodulin and no detectable reactivity to other pancreatic peptides (Millipore; GL-32K). GLP-1 levels were measured using an ELISA kit (Millipore; EGLP-35K) that measures only active forms of GLP-1 (7–36 amide; 7–37) and that does not recognize other inactive forms of GLP-1. Free fatty acids (FFA) and triglycerides were measured using reagents purchased from Roche Diagnostics (Branford, CT) and Thermo Scientific (Middletown, VA), respectively. Serum glucose levels were measured using a Glucometer Elite (Bayer, Tarrytown, NY).

Glucose and insulin tolerance tests

For glucose and insulin tolerance tests, overnight-fasted mice were administered glucose (2 mg/g body weight ip) or insulin (Humulin; 0.75 mIU/g ip), respectively. In each study, tail blood was collected before (time 0) and at indicated times after injection for measurement of glucose using the glucometer. Areas

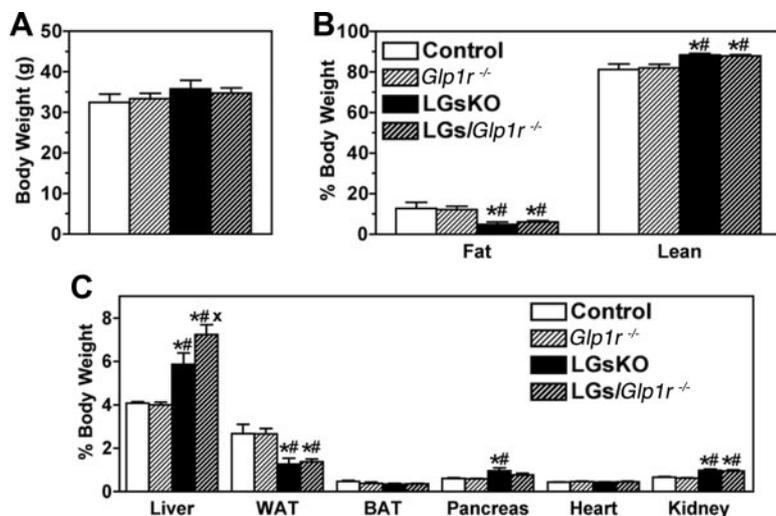


FIG. 1. Body weight, body composition, and organ weights of 6- to 7-month-old male mice. A, Body weight; B, body composition; C, organ weights expressed as percentage of total body weights ($n = 5$ – 10 per group). BAT, Brown adipose tissue; WAT, epididymal white adipose tissue. Results are expressed as mean \pm SEM. *, $P < 0.05$ vs. controls; #, $P < 0.05$ vs. *Glp1r*^{-/-} mice; x, $P < 0.05$ vs. LGsKO mice.

under the curves (AUC) for the glucose tolerance tests were calculated after subtracting the baseline glucose from each time points. AUC for insulin tolerance tests indicate negative excursion of percent glucose from the baseline and were calculated by subtracting each time point from the baseline. To examine short-term glucose-stimulated insulin secretion, glucose (3 mg/g ip) was administered and blood was taken through the tail vein at indicated time points to measure glucose and insulin.

Immunohistochemistry and islet morphology

Dissected mouse pancreata were rapidly frozen on dry ice and sectioned at a thickness of 10 μ m. For immunohistochemistry, the cryosections were fixed in 4% formaldehyde for 10 min, washed with PBS, and placed in 3% H₂O₂ for 10 min. After blocking in 10% normal goat serum, the sections were incubated with mouse antiinsulin (Invitrogen, Carlsbad, CA), or rabbit antiglucagon (Lab Vision, Fremont, CA) at 1:500 dilution, 4 C overnight, followed by incubation with secondary antibodies that were labeled with rhodamine or Cy2 (Jackson ImmunoResearch, West Grove, PA). The sections were counterstained with methyl green. For islet morphology, sections were stained with hematoxylin and eosin, and islet size was calculated by using AlphaEaseFC software (Alpha Innotech, Santa Clara, CA).

Statistical analysis

Statistical significance among groups was determined using one- or two-way ANOVA with Tukey's posttest, with differences considered significant at $P < 0.05$.

Results

Effect of *Glp1r* inactivation on body composition, energy balance, serum glucose, and insulin levels in LGsKO mice

Heterozygous or homozygous LGsKO mice were bred with germline GLP-1R receptor knockout mice (*Glp1r*^{-/-}) to

generate liver-specific G_s α and whole-body *Glp1r*^{-/-} double-knockout mice (LGs/*Glp1r*^{-/-}). There were no differences in body weight, body composition, or tissue weights between control and *Glp1r*^{-/-} mice (Fig. 1). Compared with control and *Glp1r*^{-/-} mice, LGsKO and LGs/*Glp1r*^{-/-} mice exhibited no difference in survival (number of expected pups at weaning) or body weight (Fig. 1A) but had reduced fat mass and increased lean mass (Fig. 1B) and reduced epididymal white adipose tissue weights (Fig. 1C). In addition both LGsKO and LGs/*Glp1r*^{-/-} mice had significantly increased liver and kidney weights compared with control and *Glp1r*^{-/-} mice, with liver weights being even higher in LGs/*Glp1r*^{-/-} than in LGsKO mice (Fig. 1C). LGsKO mice had greater pancreas weights than control or *Glp1r*^{-/-} mice (Fig. 1C). Pancreas weights also tended to be higher in LGs/*Glp1r*^{-/-} mice, although the difference did not reach statistical significance. There were no differences in heart or interscapular brown adipose tissue weights between the four groups (Fig. 1C). All the findings con-

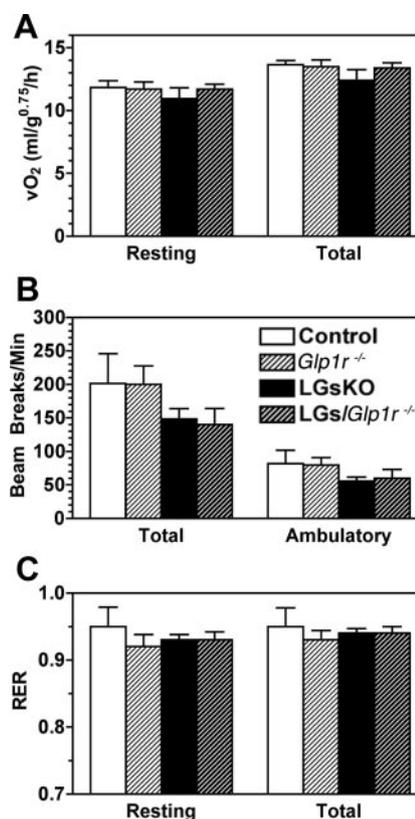


FIG. 2. Energy expenditure and activity studies in 5- to 6-month-old male mice. A, Resting and total O₂ consumption (vO_2); B, total and ambulatory (Amb) activity levels; C, resting and total respiratory exchange ratio (RER, vCO_2/vO_2) measured over 24 h at 22 C ($n = 6$ – 10 per group). Results are expressed as mean \pm SEM.

TABLE 1. Serum chemistries in randomly fed 2- to 3-month-old male mice

	Control	<i>Glp1r</i> ^{-/-}	LGsKO	LGs/ <i>Glp1r</i> ^{-/-}
Glucose (mg/dl)	214 ± 8	250 ± 14	144 ± 16 ^b	185 ± 30
Insulin (ng/ml)	1.58 ± 0.47	2.06 ± 0.90	1.04 ± 0.36	1.89 ± 0.68
FFA (mM)	0.28 ± 0.03	0.28 ± 0.03	0.22 ± 0.04	0.28 ± 0.05
Triglycerides (mg/dl)	212 ± 39	207 ± 21	82 ± 45	235 ± 39
GIP (pg/ml)	77 ± 19	124 ± 25	89 ± 28	87 ± 21
Leptin (mg/dl)	8.90 ± 2.68	9.97 ± 2.60	3.14 ± 1.08	6.66 ± 2.96
Adiponectin (μg/ml)	8.10 ± 0.58	7.69 ± 0.22	4.86 ± 0.56 ^{a,b}	3.88 ± 0.44 ^{a,b}

Data are expressed as mean ± SEM (n = 5–10 per group).

^a P < 0.05 vs. control.

^b P < 0.05 vs. *Glp1r*^{-/-} by one-way ANOVA.

cerning body weight, body composition, and organ weights in LGsKO mice were consistent with our previously published results (10).

We next examined energy balance in the four groups of mice. There were no differences in food intake measured over 14 d among the four groups of mice (data not shown),

consistent with what was previously reported for LGsKO mice (10) and *Glp1r*^{-/-} mice (23). There were also no significant differences between the four groups in resting or total energy expenditure rates (O₂ consumption), total and ambulatory activity levels, or respiratory exchange ratios (ratio of CO₂ produced to O₂ consumed) measured over 24 h at ambient temperature (22 C), although the activity levels tended to be somewhat lower in both LGsKO and LGs/*Glp1r*^{-/-} mice (Fig. 2). Overall, these results indicate that loss of GLP-1 signaling through its receptor did not affect the phenotype in LGsKO mice with regard to body composition or energy balance.

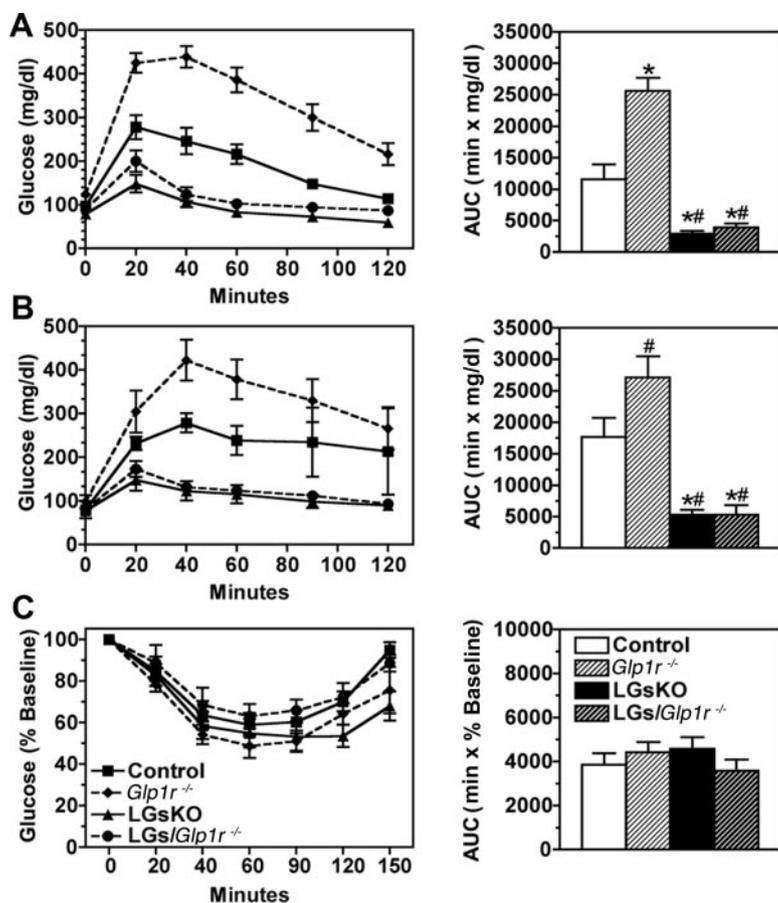


FIG. 3. Glucose and insulin tolerance tests on overnight-fasted 2- to 3-month-old mice. A, Intraperitoneal glucose tolerance test after administration of glucose (2 mg/g body weight ip); B, oral glucose tolerance test after oral glucose administration by gavage (2 mg/g body weight); C, insulin tolerance test after insulin administration (0.75 mU/g body weight ip). Panels on the left show glucose levels at each time point of the studies, and panels on the right show AUC of glucose using time 0 glucose as baseline. For insulin tolerance tests, each time point is the percent decrease from 100% as baseline. Results are expressed as mean ± SEM (n = 4–8 per group). *, P < 0.05 vs. controls; #, P < 0.05 vs. *Glp1r*^{-/-} mice.

As shown in Table 1, we saw no statistical differences between the four groups in serum insulin, FFA, triglyceride, GIP, or leptin levels, although insulin, FFA, triglyceride, and leptin levels tended to be lower in LGsKO mice than in the other groups. Based upon a one-way ANOVA, glucose levels were significantly lower in LGsKO mice compared with *Glp1r*^{-/-} mice but not compared with controls or LGs/*Glp1r*^{-/-} mice. Although the difference between glucose levels of control and LGsKO mice were not significantly different based on one-way ANOVA (Table 1), the data do show a significant effect of the LGsKO genotype by two-way ANOVA (P = 0.004) and a significant difference between these groups when analyzed by t test (P = 0.02), similar to what we previously reported when only these two groups were being studied (10). Glucose, insulin, FFA, triglyceride, and leptin levels tended to be higher in LGs/*Glp1r*^{-/-} mice compared with LGsKO mice. Serum adiponectin levels were similarly reduced in LGsKO and LGs/*Glp1r*^{-/-} mice compared with both controls and *Glp1r*^{-/-} mice.

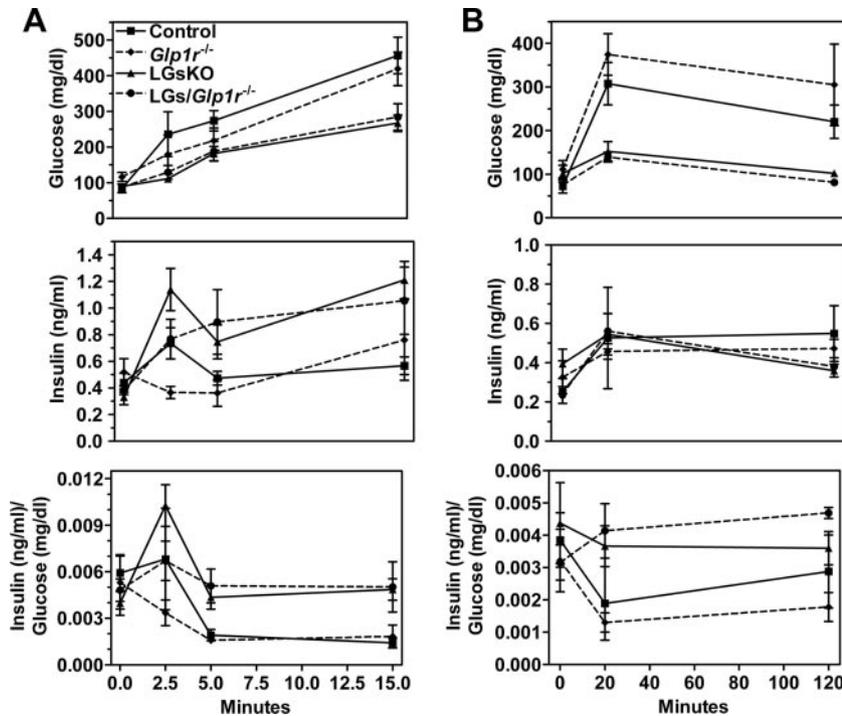


FIG. 4. Glucose-stimulated insulin responses. A, Short-term insulin response to high-dose glucose (3 mg/g body weight ip); B, longer-term insulin response to glucose (2 mg/g body weight ip) showing glucose (upper panels), insulin levels (middle panels), and insulin to glucose ratios (lower panels) for each study. Results are expressed as mean \pm SEM (n = 4–11 per group). Two-way ANOVA showed that both the LGsKO and *Glp1r*^{-/-} independently affected first-phase insulin secretion (insulin levels and insulin to glucose ratios at 2.5 min), whereas at later time points representing second-phase insulin secretion, only the LGsKO mutation had a significant effect.

Effect of *Glp1r* inactivation on glucose tolerance and glucose-stimulated insulin secretion in LGsKO mice

We performed ip and oral glucose tolerance tests on male mice. Consistent with previously reported results (23), *Glp1r*^{-/-} mice had significantly impaired glucose tolerance compared with controls after either ip or oral glucose administration (Fig. 3, A and B). In contrast, LGsKO mice had markedly improved glucose tolerance in both the ip and oral glucose tolerance tests, consistent with our previously published results (10). LGs/*Glp1r*^{-/-} mice exhibited glycemic excursion profiles comparable to LGsKO mice with very similar AUC on both the ip and oral glucose tolerance tests, indicating that improved glucose tolerance in LGsKO mice is not a reflection of enhanced signaling through the GLP-1R.

Intraperitoneal insulin tolerance tests showed similar overall insulin sensitivity among the four groups with no differences in the AUC between groups (AUC being calculated using the percent change from baseline at each time point) (Fig. 3C). We also saw no difference in the AUC between groups when calculated using absolute glucose values as opposed to percentage of baseline glucose (data not shown). At the later time points (120 and 150 min), glucose

remained lower in the LGsKO mice and, to a lesser extent in the LGs/*Glp1r*^{-/-} mice, consistent with an impaired counterregulatory response.

To measure the rapid insulin response to glucose, insulin levels and insulin/glucose ratios were measured at 0, 2.5, 5, and 15 min after administration of a higher dose of glucose (3 mg/g body weight ip) (Fig. 4A). Control mice had a rapid first-phase insulin response with a peak at 2.5 min, and this peak was enhanced in LGsKO mice, as previously reported (10). The presence of the *Glp1r*^{-/-} mutation reduced the first-phase response to a similar extent in control and LGsKO mice, and two-way ANOVA confirmed that both LGsKO and *Glp1r*^{-/-} mutations had independent effects on first-phase insulin secretion with no interaction between them. As previously noted (10), LGsKO mice had enhanced second-phase insulin secretion (insulin levels and insulin/glucose ratios at 5 and 15 min). However, the presence of the *Glp1r*^{-/-} mutation had no independent effect on second-phase insulin secretion in mice either with or without the LGsKO mutation. Therefore, the enhanced second-

phase insulin response in LGsKO mice does not appear to be dependent on GLP-1R signaling, although GLP-1R signaling has an independent effect on first-phase response in both LGsKO mice and mice without disruption of liver *G α* expression.

We also examined the increase in insulin in response to a somewhat lower glucose dose over longer time periods (2 mg/g ip, Fig. 4B). In this experiment, both LGsKO and LGs/*Glp1r*^{-/-} mice showed comparable glucose and insulin responses, with insulin levels similar to those of control and *Glp1r*^{-/-} mice despite the fact that lower glucose levels were achieved, consistent with what we previously observed in LGsKO mice (10). Insulin to glucose ratios after 20 min were higher in LGsKO and LGs/*Glp1r*^{-/-} mice than in controls and *Glp1r*^{-/-} mice. This confirms that LGsKO mice have greater insulin responses than might be expected based upon the rise in glucose but this difference was not the result of excess GLP-1 action at its receptor.

Glp1r deletion does not reverse the high serum glucagon and GLP-1 levels and pancreatic islet hyperplasia in LGsKO mice

We had previously shown that LGsKO mice have markedly elevated serum glucagon levels, presumably a

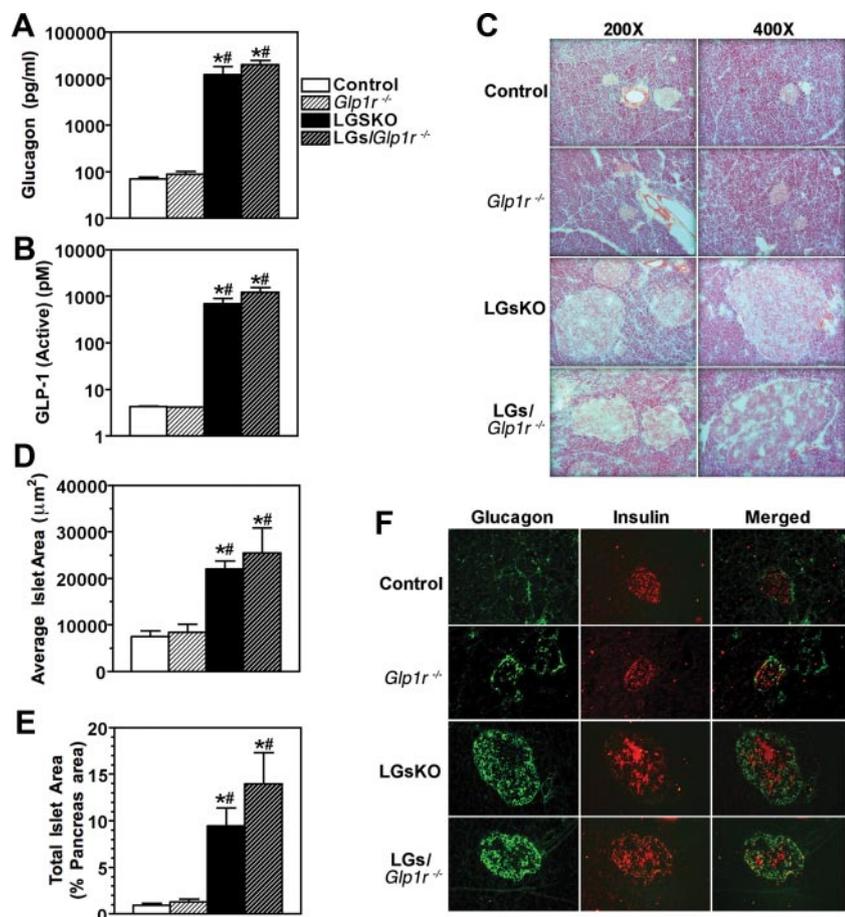


FIG. 5. Serum glucagon and active GLP-1 levels and islet histology in 3- to 4-month-old male mice. A and B, Serum glucagon (A) and active GLP-1 (B) levels (in log scale) in fed mice ($n = 5-10$ per group); C, representative hematoxylin- and eosin-stained sections showing islets at $\times 200$ and $\times 400$ original magnification; D and E, average islet area (D) and total islet area (E) as percentage of total pancreatic area ($n = 4-6$ per group); F, representative immunohistochemistry of islets stained for glucagon (green, left column), insulin (red, middle column), and merged images (right column). Results are expressed as mean \pm SEM. *, $P < 0.05$ vs. controls; #, $P < 0.05$ vs. *Glp1r*^{-/-} mice.

response to hepatic glucagon resistance, as well as extremely elevated active GLP-1 levels that, based upon findings in similar models, may be due to aberrant processing of excess proglucagon to GLP-1 in pancreatic α -cells (10). Our present results confirmed the marked elevation in glucagon and active GLP-1 levels in LGsKO mice and showed that both glucagon and active GLP-1 levels were similarly increased in LGs/*Glp1r*^{-/-} mice (Fig. 5, A and B). Therefore, elevation of the levels of these circulating hormones in LGsKO mice does not appear to result from increased GLP-1 action. Moreover, loss of the GLP-1R did not lead to a further increase in serum active GLP-1 levels. There were no significant differences in glucagon or GLP-1 levels between *Glp1r*^{-/-} and control mice. Similar results were obtained when active GLP-1 was measured in plasma rather than serum (data not shown).

Our previous study showed that LGsKO mice developed markedly enlarged islets with an increased proportion of α -cells (10). Histological analysis in the present

study revealed markedly enlarged islets in both LGsKO and LGs/*Glp1r*^{-/-} mice compared with control and *Glp1r*^{-/-} mice (Fig. 5C). Quantitative analysis demonstrated that average and total islet areas were similarly increased in both LGsKO and LGs/*Glp1r*^{-/-} mice, whereas there were no differences in average or total islet areas between control and *Glp1r*^{-/-} mice (Fig. 5, D and E). Immunohistochemistry of islets using glucagon- and insulin-specific primary antibodies showed the typical distribution pattern of cells within the islet in control and *Glp1r*^{-/-} mice, with the majority of centrally located cells being insulin-secreting β -cells and peripherally located cells being glucagon-secreting α -cells. In contrast, islets from both LGsKO and LGs/*Glp1r*^{-/-} mice exhibited greater glucagon immunopositivity (85 and 82% of total islet area vs. 5 and 9% in control and *Glp1r*^{-/-} mice, respectively), with glucagon-staining cells now expanding from the periphery into the central portion of the islet (Fig. 5F). There also were a greater number of insulin-staining cells per islet. Hence, excess GLP-1 action is not required for islet and α -cell expansion in LGsKO mice.

Discussion

The purpose of this study was to determine the contribution of significantly elevated GLP-1 levels and the GLP-1R in LGsKO mice to the metabolic phenotype resulting from loss of $G_{s\alpha}$ signaling in the liver. To do this, we introduced a whole-body inactivation of the *Glp1r* gene into LGsKO mice. It appears that the classical GLP-1R does not play a substantial role in the LGsKO phenotype, because in most respects the features of LGsKO mice, including reduced adiposity, improved glucose tolerance, increased second-phase insulin secretion, excess glucagon and GLP-1 secretion, and pancreatic islet hyperplasia, were not reversed in LGs/*Glp1r*^{-/-} mice. Our previous work showed LGsKO mice to have reduced adiposity, although the mechanism was unclear because we were unable to observe differences in food intake or energy expenditure (10). Although GLP-1 is known to work via the central nervous system to promote increased satiety and weight loss (13), GLP-1

action through the known GLP-1R appears not to play a role in the reduced adiposity of LGsKO mice, because this effect was not reversed in LGs/*Glp1r*^{-/-} mice. One caveat of our study is that we cannot rule out the possibility that excess GLP-1 is affecting the LGsKO phenotype through mechanisms independent of GLP-1R.

Loss of GLP-1R signaling had no effect on the increased glucose tolerance observed in LGsKO mice, even though the *Glp1r*^{-/-} phenotype was associated with significantly impaired glucose tolerance. This finding is partially explained by the fact that LGsKO mice have increased insulin sensitivity (10), a factor that did not appear to be reversed in LGs/*Glp1r*^{-/-} mice. In addition, the increase in second-phase insulin secretion and β -cell mass observed in LGsKO mice was not affected by loss of the GLP-1R, despite the fact that GLP-1 is an insulinotropic agent that promotes insulin secretion and synthesis and β -cell mass expansion (24). One potential explanation for the lack of a significant change in phenotype attributable to loss of the GLP-1R in LGsKO mice would be compensatory up-regulation of the other incretin GIP, which has been previously observed in *Glp1r*^{-/-} mice (25). However, we saw no increase in circulating GIP levels in LGs/*Glp1r*^{-/-} mice. An alternative explanation is that GLP-1, or GLP-1-derived peptides, continue to enhance insulin sensitivity and promote weight loss through GLP-1R-independent mechanisms (26, 27). On the other hand, the enhanced first-phase insulin response observed in LGsKO mice was reduced in LGs/*Glp1r*^{-/-} mice, and the first-phase response observed in controls was absent in *Glp1r*^{-/-} mice, indicating that GLP-1R signaling is critical for first-phase insulin secretion regardless of the physiological context.

The excess glucagon and GLP-1 secretion and α -cell hyperplasia observed in LGsKO mice was also not reversed in LGs/*Glp1r*^{-/-} mice, indicating that this phenotype is not the direct result of excess GLP-1 action mediated through the GLP-1R. The α -cell hyperplasia in LGsKO mice is not likely to be due to excess glucagon action on α -cells because glucagon receptor knockout mice with impaired glucagon action at the liver and hyperglucagonemia also develop α -cell hyperplasia despite a loss of glucagon receptors in all tissues including islets (11). Moreover, it has been shown that there is little or no expression of glucagon receptors in most pancreatic α -cells (28). Finally, prohormone convertase 2-deficient (PC2^{-/-}) mice, which have low glucagon levels due to disrupted processing of proglucagon, also develop α -cell hyperplasia (29), which is reversed by glucagon replacement (30). It therefore appears that α -cell hyperplasia in LGsKO mice and similar models is the result of chronic hypoglycemia and/or other feedback signals from the liver to the islet in response to reduced glucagon signaling.

It is known that changes in hepatic signaling pathways can affect glucose homeostasis via altering pancreatic islet function. In addition to the effects of impaired hepatic glucagon signaling on α -cell growth outlined above, it has also been shown that disruption of hepatic insulin signaling in liver-specific insulin receptor knockout mice causes increased islet and β -cell mass and hyperinsulinemia (31). Mechanisms underlying the potential inter-organ communication between the liver and pancreatic islets have not been well elucidated. A recent study has shown that activation (phosphorylation) of ERK in liver induces pancreatic β -cell proliferation through neuronal signals involving afferent fibers from the liver in the splanchnic nerve and vagal efferents to the pancreas (32). It seems likely that the disruption of hepatic G_s/cAMP signaling in the liver can trigger multiple hormonal, metabolic, or neuronal signals that feed back onto the pancreatic islets.

Acknowledgments

We thank William Jou for assistance in collection of serum samples for insulin assay and Tatyana Chanturiya for assistance in measurements of serum chemistries.

Address all correspondence or request for reprints to: Min Chen, Metabolic Diseases Branch, Building 10 Room 8C101, National Institute for Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-1752. E-mail: minc@intra.niddk.nih.gov.

This work was supported by the Intramural Research Program of the National Institute for Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, U.S. Department of Health and Human Services and by Canadian Institutes of Health Research Grants 93749 and 82700 (to D.J.D.).

Present address for E.M.: Stony Brook University Medical Center, Stony Brook, New York 11794.

Present address for J.K.: New Jersey Medical School, Newark, New Jersey 07101.

Present address for T.X.: Food and Drug Administration, Bethesda, Maryland 20892.

Disclosure Summary: D.J.D. has served as an advisor or consultant within the past 12 months to Amylin Pharmaceuticals, Arena Pharmaceuticals Inc., Arisaph Pharmaceuticals Inc., Eli Lilly Inc., Glaxo Smith Kline, Hoffmann-La Roche Inc., Merck Research Laboratories, Metabolix Inc., Novartis Pharmaceuticals, Novo Nordisk Inc., and Transition Pharmaceuticals Inc. Neither he nor his family members hold stock directly or indirectly in any of these companies. All other authors have nothing to disclose.

References

1. Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman B, Montminy M

- 2001 CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* 413:179–183
2. Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelman G, Stafford J, Kahn CR, Granner DK, Newgard CB, Spiegelman BM 2001 Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* 413:131–138
 3. Puigserver P, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, Kitamura Y, Altomonte J, Dong H, Accili D, Spiegelman BM 2003 Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1 α interaction. *Nature* 423:550–555
 4. Koo SH, Flechner L, Qi L, Zhang X, Sreaton RA, Jeffries S, Hedrick S, Xu W, Boussouar F, Brindle P, Takemori H, Montminy M 2005 The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. *Nature* 437:1109–1111
 5. Dentin R, Liu Y, Koo SH, Hedrick S, Vargas T, Heredia J, Yates 3rd J, Montminy M 2007 Insulin modulates gluconeogenesis by inhibition of the coactivator TORC2. *Nature* 449:366–369
 6. Liu Y, Dentin R, Chen D, Hedrick S, Ravnskjaer K, Schenk S, Milne J, Meyers DJ, Cole P, Yates 3rd J, Olefsky J, Guarente L, Montminy M 2008 A fasting inducible switch modulates gluconeogenesis via activator/coactivator exchange. *Nature* 456:269–273
 7. Zhou XY, Shibusawa N, Naik K, Porras D, Temple K, Ou H, Kaihara K, Roe MW, Brady MJ, Wondisford FE 2004 Insulin regulation of hepatic gluconeogenesis through phosphorylation of CREB-binding protein. *Nat Med* 10:633–637
 8. Rodgers JT, Haas W, Gygi SP, Puigserver P 2010 Cdc2-like kinase 2 is an insulin-regulated suppressor of hepatic gluconeogenesis. *Cell Metab* 11:23–34
 9. Reaven GM, Chen YD, Golay A, Swislocki AL, Jaspan JB 1987 Documentation of hyperglucagonemia throughout the day in nonobese and obese patients with noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 64:106–110
 10. Chen M, Gavrilova O, Zhao WQ, Nguyen A, Lorenzo J, Shen L, Nackers L, Pack S, Jou W, Weinstein LS 2005 Increased glucose tolerance and reduced adiposity in the absence of fasting hypoglycemia in mice with liver-specific G_sα deficiency. *J Clin Invest* 115:3217–3227
 11. Gelling RW, Du XQ, Dichmann DS, Romer J, Huang H, Cui L, Obici S, Tang B, Holst JJ, Fledelius C, Johansen PB, Rossetti L, Jelicks LA, Serup P, Nishimura E, Charron MJ 2003 Lower blood glucose, hyperglucagonemia, and pancreatic α -cell hyperplasia in glucagon receptor knockout mice. *Proc Natl Acad Sci USA* 100:1438–1443
 12. Sloop KW, Cao JX, Siesky AM, Zhang HY, Bodenmiller DM, Cox AL, Jacobs SJ, Moyers JS, Owens RA, Showalter AD, Brenner MB, Raap A, Gromada J, Berridge BR, Monteith DK, Porksen N, McKay RA, Monia BP, Bhanot S, Watts LM, Michael MD 2004 Hepatic and glucagon-like peptide-1-mediated reversal of diabetes by glucagon receptor antisense oligonucleotide inhibitors. *J Clin Invest* 113:1571–1581
 13. Drucker DJ 2007 The role of gut hormones in glucose homeostasis. *J Clin Invest* 117:24–32
 14. Holz 4th GG, Kühtreiber WM, Habener JF 1993 Pancreatic β -cells are rendered glucose-competent by the insulinotropic hormone glucagon-like peptide-1(7–37). *Nature* 361:362–365
 15. Drucker DJ, Philippe J, Mojsov S, Chick WL, Habener JF 1987 Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proc Natl Acad Sci USA* 84:3434–3438
 16. Mojsov S, Weir GC, Habener JF 1987 Insulinotropin: glucagon-like peptide I (7–37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas. *J Clin Invest* 79:616–619
 17. Fehmann HC, Habener JF 1992 Insulinotropic hormone glucagon-like peptide-I(7–37) stimulation of proinsulin gene expression and proinsulin biosynthesis in insulinoma β TC-1 cells. *Endocrinology* 130:159–166
 18. Xu G, Stoffers DA, Habener JF, Bonner-Weir S 1999 Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased β -cell mass and improved glucose tolerance in diabetic rats. *Diabetes* 48:2270–2276
 19. Edvell A, Lindström P 1999 Initiation of increased pancreatic islet growth in young normoglycemic mice (Umeå +/?). *Endocrinology* 140:778–783
 20. Perfetti R, Zhou J, Doyle ME, Egan JM 2000 Glucagon-like peptide-1 induces cell proliferation and pancreatic-duodenum homeobox-1 expression and increases endocrine cell mass in the pancreas of old, glucose-intolerant rats. *Endocrinology* 141:4600–4605
 21. Jhala US, Canettieri G, Sreaton RA, Kulkarni RN, Krajewski S, Reed J, Walker J, Lin X, White M, Montminy M 2003 cAMP promotes pancreatic β -cell survival via CREB-mediated induction of IRS2. *Genes Dev* 17:1575–1580
 22. Thorens B 1992 Expression cloning of the pancreatic β cell receptor for the gluco-incretin hormone glucagon-like peptide 1. *Proc Natl Acad Sci USA* 89:8641–8645
 23. Scrocchi LA, Brown TJ, McClusky N, Brubaker PL, Auerbach AB, Joyner AL, Drucker DJ 1996 Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. *Nat Med* 2:1254–1258
 24. Drucker DJ 2006 The biology of incretin hormones. *Cell Metab* 3:153–165
 25. Pederson RA, Satkunarajah M, McIntosh CH, Scrocchi LA, Flamez D, Schuit F, Drucker DJ, Wheeler MB 1998 Enhanced glucose-dependent insulinotropic polypeptide secretion and insulinotropic action in glucagon-like peptide 1 receptor $-/-$ mice. *Diabetes* 47:1046–1052
 26. Tomas E, Wood JA, Stanojevic V, Habener JF 2011 GLP-1-derived nonapeptide GLP-1(28–36)amide inhibits weight gain and attenuates diabetes and hepatic steatosis in diet-induced obese mice. *Regul Pept* 169:43–48
 27. Tomas E, Habener JF 2010 Insulin-like actions of glucagon-like peptide-1: a dual receptor hypothesis. *Trends Endocrinol Metab* 21:59–67
 28. Kieffer TJ, Heller RS, Unson CG, Weir GC, Habener JF 1996 Distribution of glucagon receptors on hormone-specific endocrine cells of rat pancreatic islets. *Endocrinology* 137:5119–5125
 29. Wang J, Xu J, Finnerty J, Furuta M, Steiner DF, Verchere CB 2001 The prohormone convertase enzyme 2 (PC2) is essential for processing pro-islet amyloid polypeptide at the NH₂-terminal cleavage site. *Diabetes* 50:534–539
 30. Webb GC, Akbar MS, Zhao C, Swift HH, Steiner DF 2002 Glucagon replacement via micro-osmotic pump corrects hypoglycemia and α -cell hyperplasia in prohormone convertase 2 knockout mice. *Diabetes* 51:398–405
 31. Michael MD, Kulkarni RN, Postic C, Previs SF, Shulman GI, Magnuson MA, Kahn CR 2000 Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol Cell* 6:87–97
 32. Imai J, Katagiri H, Yamada T, Ishigaki Y, Suzuki T, Kudo H, Uno K, Hasegawa Y, Gao J, Kaneko K, Ishihara H, Nijijima A, Nakazato M, Asano T, Minokoshi Y, Oka Y 2008 Regulation of pancreatic β -cell mass by neuronal signals from the liver. *Science* 322:1250–1254