The Glucagon-Like Peptide-1 Receptor Regulates Endogenous Glucose Production and Muscle Glucose Uptake Independent of Its Incretin Action

Julio E. Ayala, Deanna P. Bracy, Freyja D. James, Brianna M. Julien, David H. Wasserman, and Daniel J. Drucker

Department of Molecular Physiology and Biophysics (J.E.A., D.P.B., F.D.J., B.M.J., D.H.W.), Vanderbilt University School of Medicine, Nashville, Tennessee 37232; and Department of Medicine (D.J.D.), The Banting and Best Diabetes Centre, Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto, Ontario, Canada M5G 1X5

Glucagon-like peptide-1 (GLP-1) diminishes postmeal glucose excursions by enhancing insulin secretion via activation of the β-cell GLP-1 receptor (Glp1r). GLP-1 may also control glucose levels through mechanisms that are independent of this incretin effect. The hyperinsulinemic-euglycemic clamp (insulin clamp) and exercise were used to examine the incretin-independent glucoregulatory properties of the Glp1r because both perturbations stimulate glucose flux independent of insulin secretion. Chow-fed mice with a functional disruption of the Glp1r ($Glp1r^{-/-}$) were compared with wild-type littermates ($Glp1r^{+/+}$). Studies were performed on 5-h-fasted mice implanted with arterial and venous catheters for sampling and infusions, respectively. During insulin clamps, [3-^{3}H]glucose and 2[^{14}C]deoxyglucose were used to determine whole-body glucose turnover and glucose metabolic index (R_g), an indicator of glucose uptake. R_g in sedentary and treadmill exercised mice was determined using 2[^{3}H]deoxyglucose. $Glp1r^{-/-}$ mice exhibited increased glucose disappearance, muscle R_g, and muscle glycogen levels during insulin clamps. This was not associated with enhanced muscle insulin signaling. $Glp1r^{-/-}$ mice exhibited impaired suppression of endogenous glucose production and hepatic glycogen accumulation during insulin clamps. This was associated with impaired liver insulin signaling. $Glp1r^{-/-}$ mice became significantly hyperglycemic during exercise. Muscle R_g was normal in exercised $Glp1r^{-/-}$ mice, suggesting that hyperglycemia resulted from an added drive to stimulate glucose production. Muscle AMP-activated protein kinase phosphorylation was higher in exercised $Glp1r^{-/-}$ mice. This was associated with increased relative exercise intensity and decreased exercise endurance. In conclusion, these results show that the endogenous Glp1r regulates hepatic and muscle glucose flux independent of its ability to enhance insulin secretion. **(*Endocrinology* 150: 1155–1164, 2009)**

The ingestion of food stimulates the secretion of glucagon-like peptide (GLP)-1 and glucose-dependent insulinotropic polypeptide (GIP) from intestinal enteroendocrine cells. These hormones mediate the increased secretion of insulin in response to oral *vs.* iv glucose delivery, a phenomenon known as the incretin effect (1–3). This occurs via activation of specific G protein-coupled receptors for GLP-1 (Glp1r) and GIP (Gipr) expressed not only in pancreatic islets but also lungs, kidneys, heart, adipose tissue, and the central and peripheral nervous system (4–7). GLP-1, but not GIP, also inhibits glucagon secretion and delays gastric emptying (8–11), thereby diminishing glucose excursions after a meal.

Since its discovery, it has been suggested that GLP-1 exerts glucoregulatory properties that are independent of its ability to modulate pancreatic secretions and gastric emptying. In type 1 diabetic patients, simultaneous peripheral GLP-1 infusion and enteral glucose delivery resulted in increased glucose disappear-

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Abbreviations: AMPK, AMP-activated protein kinase; DG, deoxyglucose; DGP, DG-6-phosphate; DIRKO, double-incretin receptor knockout; endoR_a, endogenous glucose production; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GIP, glucose-dependent insulinotropic polypeptide; Gipr, GIP receptor; GIR, glucose infusion rate; GLP, glucagon-like peptide; Glp1r, GLP-1 receptor; GSK, glycogen synthase kinase; K_g, glucose clearance; MGU, muscle glucose uptake; NEFA, nonesterified fatty acid; R_d, glucose disappearance; RER, respiratory exchange ratio; R_g, glucose metabolic index; SVL, superficial vastus lateralis; VO_2, O_2 consumption; $VO_{2,max}$, maximal VO_2.

ance, even as arterial insulin and glucose levels were clamped (12). Subsequent studies further suggested that GLP-1 enhances glucose utilization and suppresses hepatic glucose production when insulin and glucagon levels are experimentally controlled (13–19). However, other studies suggest that any glucoregulatory effects of GLP-1 are solely due to its ability to regulate the secretion of pancreatic hormones (20–26). Thus, the notion that GLP-1 can directly regulate glucose production and utilization independent of its effects on the pancreas remains disputed.

The aim of the present studies was to determine whether the endogenous GLP-1 receptor regulates hepatic glucose production and muscle glucose uptake (MGU) independent of its ability to enhance insulin secretion. To this end, the hyperinsulinemic-euglycemic clamp (insulin clamp) and exercise were used to stimulate glucose flux in mice lacking Glp1r expression. Glp1r knockout ($Glp1r^{-/-}$) mice exhibit oral glucose intolerance and impaired insulin secretion, but basal glucose metabolism is otherwise normal (27, 28). During an insulin clamp, glucose flux is stimulated by experimentally controlled hyperinsulinemia. Conversely, with exercise, glucose flux is stimulated via insulin-independent mechanisms (29). Thus, these two metabolic perturbations were used to reveal glucoregulatory properties of the Glp1r that are both insulin dependent and insulin independent.

Materials and Methods

Mouse maintenance and genotyping

All procedures performed were approved by the Vanderbilt University Animal Care and Use Committee. At 3 wk of age, wild-type ($Glp1r^{+/+}$) and Glp1r knockout ($Glp1r^{-/-}$) littermates on the C57BL/6 background were separated by sex and were placed on a standard chow diet (Purina 5001; Purina Mills, St. Louis, MO). Genotyping was performed by PCR on genomic DNA obtained from tail biopsies. All experiments were performed on mice at about 4 months of age. Body composition was determined on 5-h-fasted mice using an mq10 nuclear magnetic resonance analyzer (Bruker Optics, The Woodlands, TX). Mice were maintained on a standard light-dark cycle (0600–1800 h light).

Surgical procedures

Catheters were implanted in the left common carotid artery and right jugular vein for sampling and infusions, respectively, as previously described (30, 31), except surgeries were performed under inhaled anesthesia (VetEquip, Pleasanton, CA). Animals were individually housed after surgery and allowed to recover for 5–7 d, during which time body weight was recorded daily. Mice whose weight did not return to within 10% of presurgery weight were excluded.

Hyperinsulinemic-euglycemic clamps

After 5 d of recovery, insulin clamps were performed on 5-h-fasted mice (30–32). A 5-μCi bolus of [3-^{3}H]glucose was given at t = −90 min before insulin infusion, followed by a 0.05-$\mu Ci \cdot min^{-1}$ infusion for 90 min. Blood samples were obtained via the arterial catheter (30–32). Basal glucose-specific activity was determined from blood samples at t = −15 and −5 min. Fasting insulin and nonesterified fatty acid (NEFA) levels were determined from blood samples taken at t = −5 min. The clamp was begun at t = 0 min with a continuous infusion of human insulin (4 $mU \cdot kg^{-1} \cdot min^{-1}$; Humulin R; Eli Lilly, Indianapolis, IN). The [3-^{3}H]glucose infusion was increased to 0.15 $\mu Ci \cdot min^{-1}$ for the remainder of the experiment. Euglycemia (~150–160 $mg \cdot dl^{-1}$) was maintained by measuring blood glucose every 10 min starting at t = 0 min and infusing 50% dextrose as necessary. Mice received saline-washed erythrocytes from donors throughout the clamp (5–6 $\mu l \cdot min^{-1}$) to prevent a fall of greater than 5% hematocrit. A 12-μCi bolus of 2[^{14}C]deoxyglucose (DG) was given at t = 120 min. Blood samples (80–240 μl) were taken every 10 min from t = 80 to 135 min and processed to determine plasma [3-^{3}H]glucose and 2[^{14}C]DG. Clamp insulin and NEFA was determined at t = 120 min. At t = 135 min, mice were anesthetized with sodium pentobarbital. The soleus, gastrocnemius, superficial vastus lateralis (SVL), liver, diaphragm, heart, and brain were excised, immediately frozen, and stored at −80 C until analyzed. In a separate set of experiments, liver and gastrocnemius from 5-h-fasted mice were excised for determination of basal glycogen content.

Exercise experiments

After 5 d of recovery, mice were acclimated to treadmill running with a single 10-min exercise bout (15.5 $m \cdot min^{-1}$, 0% grade). Exercise experiments were performed 2 d after this acclimation trial. All exercise experiments were performed on 5-h-fasted mice. One hour before the exercise bout, mice were placed in the treadmill for acclimation. At t = 0 min, an arterial sample (100 μl) was taken for the measurement of baseline blood glucose, hematocrit, and plasma insulin and NEFAs. Mice either remained sedentary or ran on the treadmill for 30 min at 16 $m \cdot min^{-1}$, 0% grade. This work intensity is about 75% of maximal oxygen consumption in mice (33). Mice were encouraged to run with the use of an electric grid placed at the back end of the treadmill (1.5 mA, 200 msec pulses, 4 Hz). At t = 5 min, a 12-μCi bolus of 2[^{3}H]DG was administered via the jugular vein catheter. At t = 7, 10, 15, and 20 min, arterial samples (~50 μl) were taken to determine blood glucose and plasma 2[^{3}H]DG. At t = 30 min, an arterial sample (150 μl) was taken for the measurement of blood glucose; hematocrit; and plasma insulin, NEFAs, and 2[^{3}H]DG. Mice were then anesthetized and tissues were excised and stored as in clamp experiments.

Exercise stress and exercise endurance testing

Whole-body O_2 consumption (VO_2) was measured in mice placed in an enclosed treadmill using an Oxymax Deluxe system (Columbus Instruments, Columbus, OH) with an airflow rate of 1 $liter \cdot min^{-1}$. For exercise stress testing, mice were placed in the enclosed treadmill and allowed to acclimate for at least 30 min. Resting VO_2 was determined as the average of measurements over the 10 min before the beginning of the stress test. Mice then began running at 10 $m \cdot min^{-1}$, 0% grade, and the

TABLE 1. Basal (5 h fasted) and insulin clamp characteristics

	$Glp1r^{+/+}$	$Glp1r^{-/-}$
n (male/female)	12 (6/6)	11 (5/6)
Weight (g)	24 ± 1	23 ± 1
Lean mass (g)	19 ± 1	17 ± 1
Fat mass (g)	2.7 ± 0.3	2.5 ± 0.1
Arterial glucose ($mg \cdot dl^{-1}$)		
Basal	143 ± 5	145 ± 8
Insulin clamp	156 ± 3	158 ± 3
Insulin ($ng \cdot ml^{-1}$)		
Basal	0.8 ± 0.1	0.6 ± 0.2
Insulin clamp	3.4 ± 0.4	2.9 ± 0.3
NEFA (mM)		
Basal	1.1 ± 0.1	1.2 ± 0.1
Insulin clamp	0.6 ± 0.1	0.6 ± 0.2
GIR ($mg \cdot kg^{-1} \cdot min^{-1}$)	41 ± 3	53 ± 5[a]
Insulin sensitivity index $(mg \cdot ml) \cdot (ng \cdot kg \cdot min)^{-1}$	14 ± 2	21 ± 4[a]

Basal values represent averages from samples taken at t = −15 and −5 min. Insulin clamp values and GIR represent averages from samples taken at t = 80–120 min. Insulin sensitivity index is calculated as the ratio of GIR to clamp insulin levels.

[a] $P < 0.05$ *vs.* $Glp1r^{+/+}$.

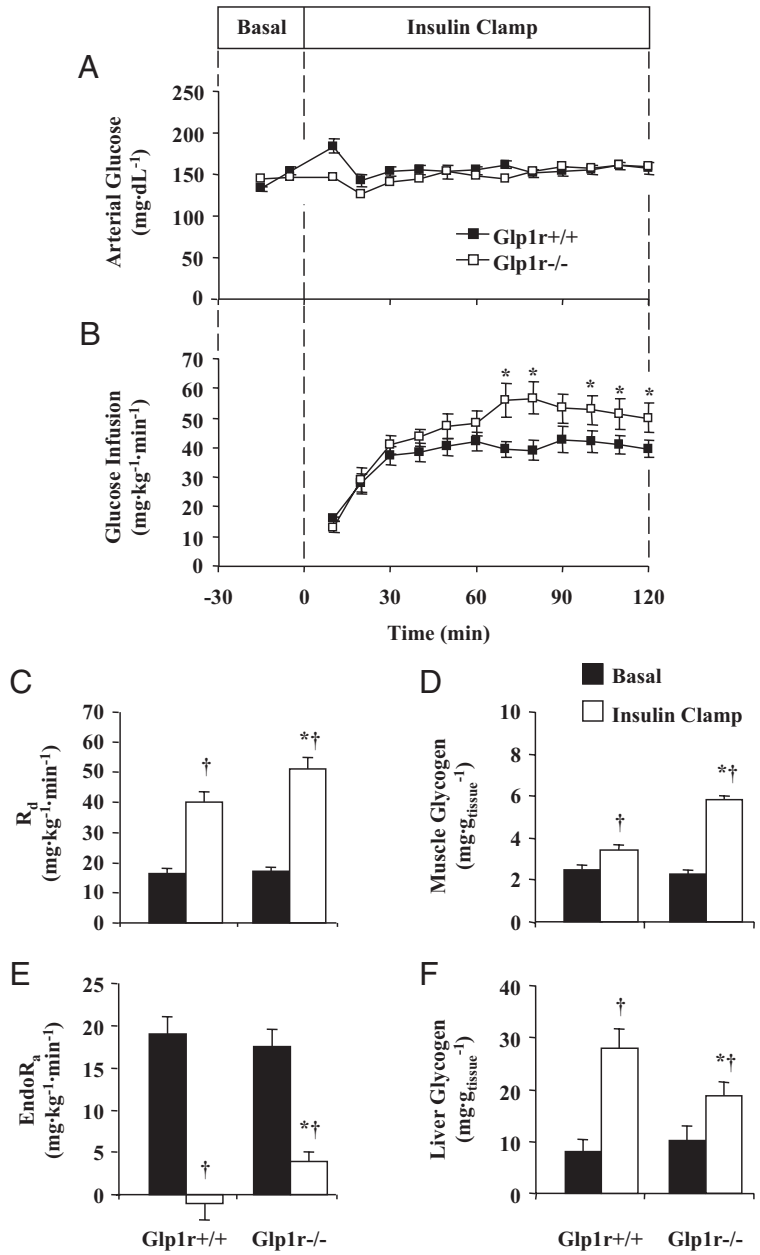

FIG. 1. Insulin clamp experiments in 5-h-fasted mice. Arterial glucose (A) and GIR (B) during insulin clamps are presented for Glp1r$^{+/+}$ (*black squares*) and Glp1r$^{-/-}$ (*white squares*) mice. R_d (C), muscle (gastrocnemius) glycogen (D), endoR_a (E), and liver glycogen (F) from 5-h-fasted (basal, *black bars*) mice and mice from clamp experiments (insulin clamp, *white bars*) are presented. Data are mean ± SEM for 11–12 mice/genotype. *, $P < 0.05$ *vs.* Glp1r$^{+/+}$; †, $P < 0.05$ *vs.* basal within the same genotype.

speed was increased by 4 m · min^{-1} every 3 min until exhaustion. Mice were encouraged to run with the use of an electric grid placed at the back end of the treadmill (1.5 mA, 200 msec pulses, 4 Hz). Mice were defined as exhausted when they spent more than 5 continuous seconds on the electric grid. Maximal VO_2 ($VO_{2,max}$) was achieved when VO_2 no longer increased despite an increase in work rate.

Endurance capacity was determined at least 5 d after maximal testing. Mice were placed in the enclosed treadmill to acclimate and resting VO_2 was determined as before. Mice were run at 20 m · min^{-1} until exhausted, with exhaustion defined as before. Exercise VO_2 was determined as the average of measurements over the 10 min before exhaustion. Postexercise VO_2 was determined as the average of measurements over the 10 min immediately after exhaustion. Respiratory exchange ratio (RER) was calculated as CO_2 production/VO_2.

Processing of plasma and tissue samples

Insulin levels were determined by ELISA (Linco, St. Charles, MO). NEFAs were measured spectrophotometrically by an enzymatic colorimetric assay (Wako NEFA HR(2) kit; Wako Chemicals, Richmond, VA). Plasma [3-^{3}H]glucose and 2[^{14}C]DG (insulin clamps) or plasma 2[^{3}H]DG (exercise) and tissue 2[^{14}C]DG-6-phosphate 2[^{14}C]DGP or tissue 2[^{3}H]DG-6-phosphate (2[^{3}H]DGP) radioactivity were determined as previously described (31, 32). The accumulation of 2[^{14}C]DGP or 2[^{3}H]DGP was normalized to tissue weight. Liver and gastrocnemius glycogen was determined by the method of Chan and Exton (34).

Protein immunoblots

Liver and muscle (gastrocnemius) tissue (20–40 mg) was homogenized in 10 μl · mg^{-1} tissue extraction buffer [50 mM Tris, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100 (pH 7.5)] supplemented with protease (Pierce, Rockford, IL) and phosphatase (Sigma, St. Louis, MO) inhibitor cocktails. Homogenates were centrifuged (20 min, 4500 × *g*, 4 C), pellets were discarded, and supernatants were retained for protein determination. Protein content was determined using a BCA protein assay kit (Bio-Rad, Hercules, CA). Whole-cell (20–100 μg) extracts were separated on 10% Bis-Tris SDS-PAGE gels (Invitrogen, Carlsbad, CA), followed by electrophoretic transfer to polyvinylidene fluoride membranes. Primary antibodies were incubated with the membranes overnight at 4 C. Secondary antibodies were incubated at room temperature for 1 h. Imaging and densitometry were performed using the Odyssey imaging system (LI-COR, Lincoln, NE). Antibodies for Akt, phosphorylated Akt (Ser^{473}), glycogen synthase kinase (GSK)-3β, phosphorylated GSK-3β (Ser^9), total (α1/α2) AMP-activated protein kinase (AMPK), and phosphorylated AMPK (Thr^{172}) were from Cell Signaling (Beverly, MA). Antibody for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Abcam (Berkeley, CA).

Calculations

Whole-body glucose appearance (R_a) and disappearance (R_d) were determined using Steele nonsteady-state equations (35, 36). Endogenous glucose production (endoR_a) was determined by subtracting the glucose infusion rate (GIR) from total glucose appearance. Glucose metabolic index (R_g) and glucose clearance (K_g) were calculated as previously described (37, 38) and were normalized to values obtained in the brain.

Statistical analysis

Data are presented as means ± SEM. Differences between groups were determined by one-way ANOVA followed by Tukey's *post hoc* tests or by two-tailed *t* test as appropriate. The significance level was $P < 0.05$.

Results

Glp1r$^{-/-}$ mice exhibit enhanced insulin-stimulated MGU but impaired suppression of endoR_a

Baseline characteristics in 5-h-fasted mice undergoing insulin clamps are shown in Table 1. Total, fat, and muscle mass was not

different between genotypes. There were no differences in fasting glucose, insulin, and NEFA levels among genotypes.

Insulin action was assessed in conscious, unrestrained mice via the insulin clamp. This approach was used to determine the role of the Glp1r in the regulation of hepatic glucose production (endo R_a) and MGU under controlled hyperinsulinemic conditions. Arterial glucose was clamped at similar levels (~150 mg · dl^{-1}) in Glp1r$^{+/+}$ and Glp1r$^{-/-}$ mice (Fig. 1A). The GIR necessary to maintain euglycemia was significantly higher in Glp1r$^{-/-}$ mice (Fig. 1B and Table 1). Clamp insulin and NEFA levels were not different between genotypes (Table 1). Normalizing the GIR to clamp insulin levels, an index of whole-body insulin sensitivity, shows that disruption of Glp1r expression results in an approximately 1.5-fold increase in insulin sensitivity (Table 1). This was due to an effect on insulin-stimulated R_d, which was enhanced in Glp1r$^{-/-}$ mice (Fig. 1C). Paralleling this, muscle glycogen accumulation was significantly greater in Glp1r$^{-/-}$ mice (Fig. 1D). In contrast to the effects on R_d, suppression of endoR_a was significantly impaired in Glp1r$^{-/-}$ mice (Fig. 1E). This was associated with decreased glycogen accumulation in the liver (Fig. 1F).

R_g was measured to assess the effect of disrupting Glp1r expression on glucose uptake in specific tissues. As shown in Fig. 2, A–C, R_g in hindlimb skeletal muscles was significantly elevated in Glp1r$^{-/-}$ mice. R_g in the diaphragm and heart was not significantly different between genotypes (Fig. 2, D and E). Taken together, these results show that disruption of Glp1r expression results in enhanced insulin-stimulated MGU and muscle glycogen accumulation but impaired suppression of endogenous glucose production and hepatic glycogen accumulation.

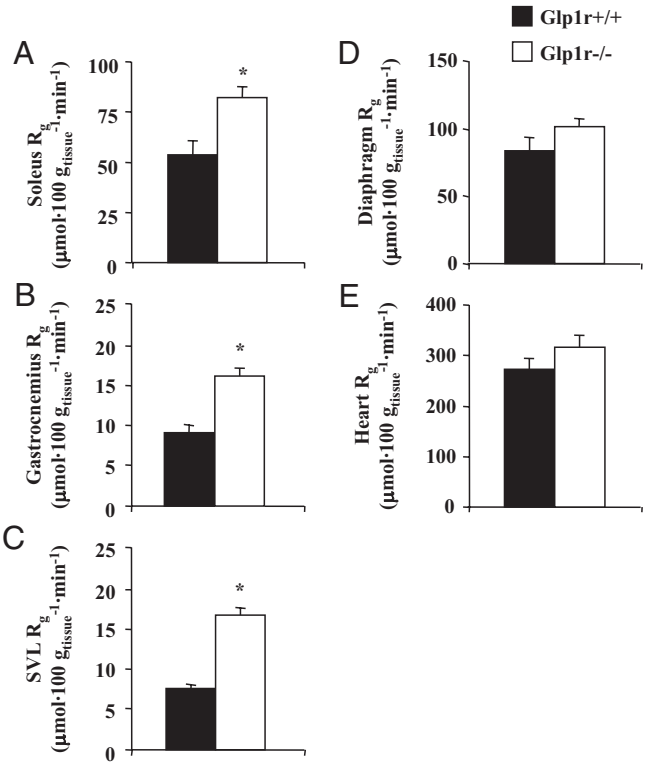

FIG. 2. R_g after insulin clamp experiments in 5-h-fasted mice. R_g in soleus (A), gastrocnemius (B), SVL (C), diaphragm (D), and heart (E) are presented for Glp1r$^{+/+}$ (*black bars*) and Glp1r$^{-/-}$ (*white bars*) mice. Data are mean ± SEM for 11–12 mice/genotype. *, $P < 0.05$ *vs.* Glp1r$^{+/+}$.

Disruption of Glp1r expression does not enhance muscle insulin signaling but does impair hepatic insulin signaling

To determine whether increased muscle R_g in Glp1r$^{-/-}$ mice was due to enhanced insulin action, activation of insulin signaling proteins was assessed (Fig. 3). Disruption of Glp1r expression did not increase levels of phosphorylated or total Akt in muscle (Fig. 3B). Thus, activation of muscle Akt, defined as the ratio of phosphorylated to total Akt, was not different between genotypes (Fig. 3C). Phosphorylation of muscle GSK-3β, which inactivates this enzyme and promotes glycogen synthesis, was also not affected by the lack of Glp1r expression (Fig. 3, B and C). These results show that increased muscle R_g and muscle glycogen accumulation in Glp1r$^{-/-}$ mice is not associated with enhanced insulin signaling.

In contrast with muscle phenotypes, the inability of insulin to completely suppress endoR_a in Glp1r$^{-/-}$ mice does correlate with impaired hepatic insulin signaling. Although Akt activation was not different between genotypes, levels of phosphorylated and total Akt were decreased in Glp1r$^{-/-}$ mice (Fig. 3, E and F). Furthermore, decreased hepatic glycogen accumulation in Glp1r$^{-/-}$ mice was associated with decreased phosphorylation of GSK-3β (Fig. 3, E and F).

Glp1r$^{-/-}$ mice exhibit hyperglycemia during exercise even as MGU is normal

Exercise stimulates glucose flux in the absence of an increase in insulin levels. This physiological intervention was used to further characterize the glucoregulatory properties of the Glp1r that are independent of its ability to stimulate insulin secretion. During moderate exercise, increased glucose use by the working muscle is typically matched by an increase in hepatic glucose production (29). The consequence of this is that arterial glucose levels do not vary appreciably during exercise, as was observed in Glp1r$^{+/+}$ mice (Fig. 4A). Such was not the case in Glp1r$^{-/-}$ mice, which exhibited exercise-induced hyperglycemia (Fig. 4A). This could not be attributed to an effect on the dynamics of insulin because both genotypes showed a similar decrease in the levels of this hormone (Fig. 4B). Plasma NEFA levels were not different between the genotypes in sedentary or exercised mice (Fig. 4C). Similarly, muscle and liver glycogen levels were not different between genotypes in either sedentary or exercised mice (Fig. 4, D and E).

To assess whether exercise-induced hyperglycemia in Glp1r$^{-/-}$ mice was due to a defect in MGU, R_g was measured in various muscle types (Fig. 5). R_g in hindlimb skeletal muscles was not different between genotypes in sedentary mice and was equally increased in response to exercise (Fig. 5, A–C). Whereas R_g in the diaphragm did not increase with exercise in Glp1r$^{+/+}$ mice, it was significantly increased in Glp1r$^{-/-}$ mice (Fig. 5D). Conversely, R_g in the heart was stimulated by exercise in Glp1r$^{+/+}$ mice but was unaffected in Glp1r$^{-/-}$ mice (Fig. 5E). This likely is due to the fact that sedentary R_g in the heart was elevated in Glp1r$^{-/-}$ mice, such that exercise did not increase it further. In

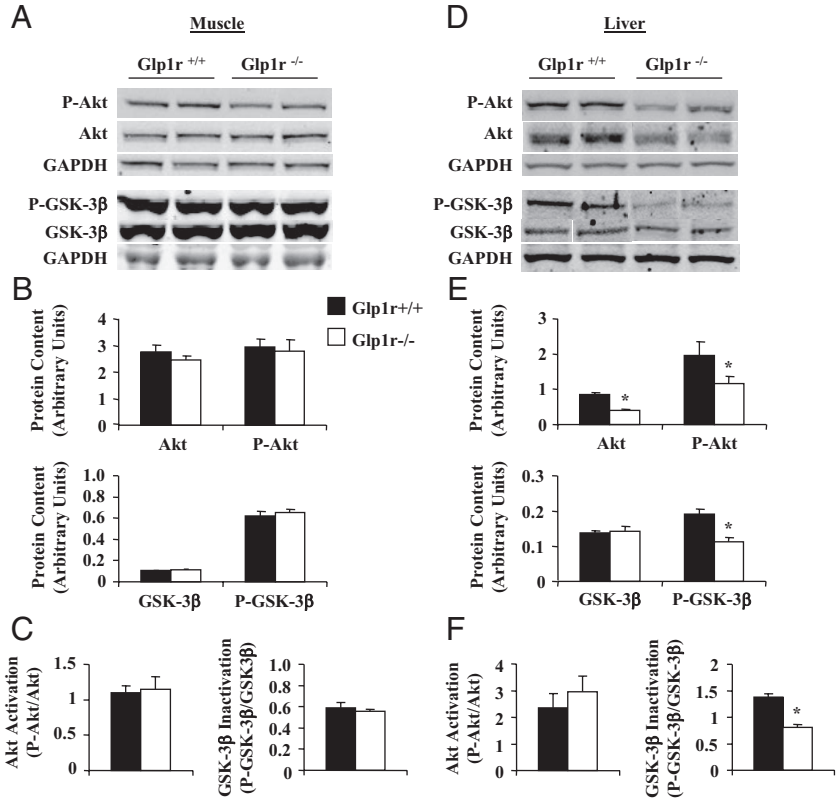

FIG. 3. Immunoblots for insulin signaling proteins after insulin clamp experiments in 5-h-fasted mice. Representative immunoblots from gastrocnemius muscle (A) and liver (D) extracts are shown. Protein content and protein activation is presented for Glp1r$^{+/+}$ (*black bars*) and Glp1r$^{-/-}$ (*white bars*) mice. Protein content of Ser^{473}-phosphorylated Akt (P-Akt), total Akt, Ser^{9}-phosphorylated GSK-3β (P-GSK-3β), and total GSK-3β (GSK-3β) is shown from muscle (B) and liver (E) extracts. Protein content is normalized to the GAPDH loading control. Akt activation (P-Akt/Akt) and GSK-3β inactivation (P-GSK-3β/GSK-3β) are also shown from muscle (C) and liver (F) extracts. Inactivation of GSK-3β promotes glycogen synthesis. Data are mean ± SEM for 11–12 mice/genotype. *, $P < 0.05$ *vs.* Glp1r$^{+/+}$.

summary, hyperglycemia during exercise in Glp1r$^{-/-}$ mice was not due to an impairment in MGU because exercise-induced R_g was not different or even elevated compared with Glp1r$^{+/+}$ mice.

Measurement of R_g is dependent on glucose concentration (37). Because glucose levels were higher in Glp1r$^{-/-}$ mice during exercise, this could inflate values of R_g. K_g is an index of glucose uptake that is independent of glucose concentration (37). As shown in Fig. 5, F–J, sedentary and exercise-stimulated values of K_g recapitulated the results obtained via measurement of R_g. Thus, K_g in hindlimb muscles was not different between genotypes in sedentary and exercised mice (Fig. 5, F–H). Furthermore, exercise-stimulated K_g in the diaphragm and sedentary K_g in the heart were elevated in Glp1r$^{-/-}$ mice (Fig. 5, I and J). Taken together, the results of exercise studies show that exercise-induced hyperglycemia in Glp1r$^{-/-}$ mice is not due to an impairment in MGU. This suggests an inappropriate increase in hepatic glucose production beyond the requirements of the working muscle.

Disruption of Glp1r expression enhances phosphorylation of AMP-activated protein kinase in the muscle during exercise

Activation of the AMPK is proposed to be involved in the stimulation glucose uptake during exercise (39–41). Interestingly, although MGU during exercise did not differ between genotypes, muscle AMPK activation was significantly enhanced in Glp1r$^{-/-}$ mice (Fig. 6, A–C). This was only evident in the muscle, as activation of AMPK in the liver was not different between the genotypes (Fig. 6, D–F).

Glp1r$^{-/-}$ mice exercise at a relatively higher intensity and have impaired exercise endurance

High intensity exercise is characterized by increased arterial glucose levels and enhanced skeletal muscle AMPK activation (29, 42, 43). To assess whether Glp1r$^{-/-}$ mice exercise at different relative intensities for a given treadmill speed, exercise stress and exercise endurance tests were performed. Capacity for maximum intensity exercise was not different between the two genotypes, as noted by the running rates during an exercise stress test. However, $VO_{2,max}$ was significantly higher in Glp1r$^{-/-}$ mice (Table 2). During an exercise endurance test, mice from both genotypes ran at the same absolute speed (20 m · min^{-1}). As shown in Table 2, this work intensity corresponded to about 78% of $VO_{2,max}$ in Glp1r$^{+/+}$ mice but about 88% of $VO_{2,max}$ in Glp1r$^{-/-}$ mice. This shows that at a given absolute work intensity, Glp1r$^{-/-}$ mice exercise at a higher relative intensity. This was associated with a decrease in endurance capacity, measured as time to exhaustion during treadmill exercise at 20 m · min^{-1} (Table 2).

Discussion

The role of the Glp1r to enhance insulin secretion in response to nutrient intake has been clearly established. It was thus assumed that this incretin action mediated through the β-cell was solely responsible for any effects of Glp1r activation on hepatic glucose production and MGU. Whereas it has been proposed that the Glp1r can regulate glucose production and utilization independent of its effect on pancreatic hormone secretion (13–19), this idea remains controversial (20–26). In the present study, insulin clamps and exercise were used to stimulate glucose flux under conditions in which circulating insulin levels were experimentally controlled (insulin clamps) or decreased (exercise). This approach was taken to assess the potential role of the Glp1r to regulate MGU independent of its ability to stimulate endogenous insulin secretion. Given its role in mediating the incretin effect, it was surprising to observe that Glp1r$^{-/-}$ mice exhibited enhanced skeletal muscle glucose uptake during an insulin clamp. Conversely, suppression of hepatic glucose production was impaired in these mice. These effects were paralleled by increased muscle glycogen accumulation and decreased hepatic glycogen accumulation. In response to exercise, Glp1r$^{-/-}$ mice became

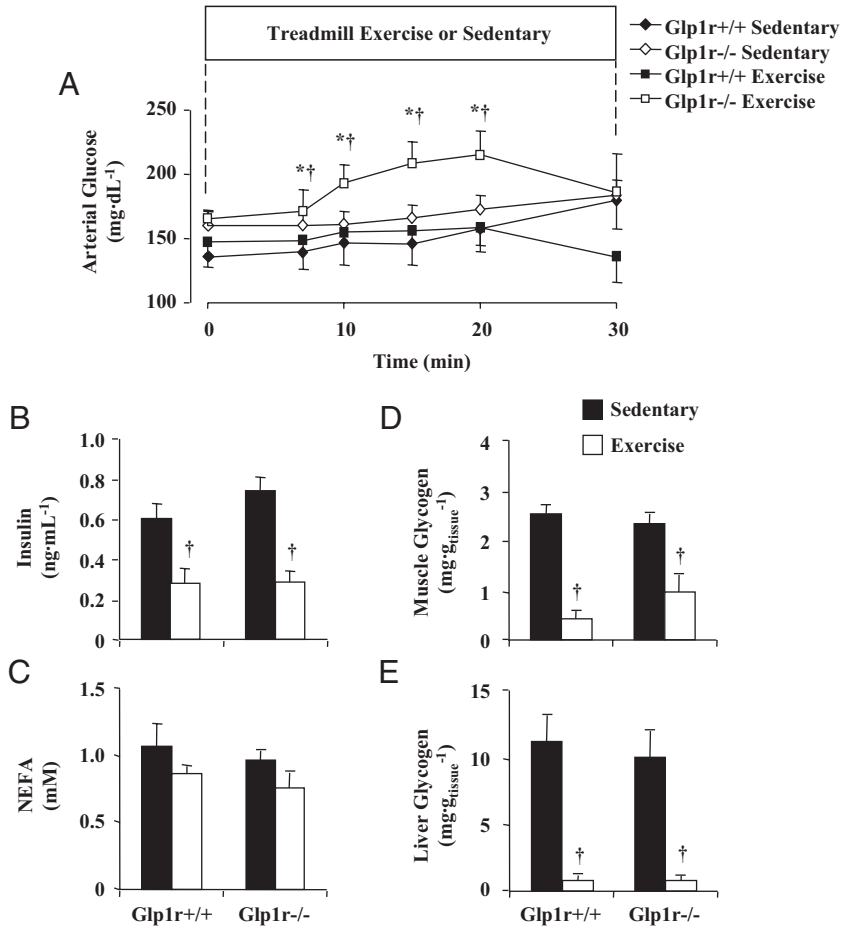

FIG. 4. Exercise experiments in 5-h-fasted mice. Arterial glucose (A) levels are presented for Glp1r$^{+/+}$ (*black diamonds* and *squares*) and Glp1r$^{-/-}$ (*white diamonds* and *squares*) mice that remained sedentary (*diamonds*) or underwent treadmill exercise (*squares*) for 30 min. Insulin (B), NEFA (C), muscle (gastrocnemius) glycogen (D), and liver glycogen (E) levels in sedentary (*black bars*) and exercised (*white bars*) Glp1r$^{+/+}$ and Glp1r$^{-/-}$ mice are shown. Data are mean ± SEM for 14–16 mice/genotype. *, $P < 0.05$ *vs.* Glp1r$^{+/+}$ exercise; †, $P < 0.05$ *vs.* Glp1r$^{-/-}$ sedentary.

hyperglycemic. This was not due to a defect in glucose uptake as MGU was normal or increased in Glp1r$^{-/-}$ mice. Taken together, these results demonstrate that, under conditions of increased glucose flux, the endogenous Glp1r is a regulator of glucose production and MGU independent of its ability to stimulate endogenous insulin secretion.

We have previously shown that disruption of both the Glp1r and the Gipr in double-incretin receptor knockout (DIRKO) mice preserves insulin action in response to chronic high-fat feeding, a dietary intervention that precipitates insulin resistance in C57BL/6 mice (32). DIRKO mice are also protected from high-fat diet-induced obesity due to increased energy expenditure (32, 44). Disruption of the Gipr increases fat oxidation and decreases adiposity in genetic and dietary rodent models of obesity (45–47). This results in improved glucose tolerance and insulin sensitivity (45–48). Thus, the enhanced insulin action in DIRKO mice is likely due, at least in part, to the improved metabolic profile resulting from the loss of Gipr expression. In the present studies, we show that fat mass and muscle mass is normal in Glp1r$^{-/-}$ mice. Therefore, the phenotypes observed in Glp1r$^{-/-}$ mice are not secondary to an effect on body composition.

Our findings from insulin clamp and exercise studies reveal a novel role for the Glp1r in the regulation of hepatic glucose production and MGU during conditions of increased glucose flux. A direct effect of GLP-1 on the liver and muscle is not likely because neither tissue expresses the Glp1r. Recent evidence indicates that Glp1r action in the brain, particularly in the hypothalamus, can acutely regulate glucose production and use. Knauf *et al.* (49) showed that glucose requirements during a hyperinsulinemic-hyperglycemic clamp were higher in Glp1r$^{-/-}$ mice and mice receiving an intracerebroventricular infusion of the Glp1r antagonist exendin (9–39). Furthermore, hepatic glycogen levels were lower, whereas muscle glycogen levels were higher, in Glp1r$^{-/-}$ mice and mice receiving intracerebroventricular infusions of exendin (9–39). A similar phenotype was observed in the present studies using Glp1r$^{-/-}$ mice and was associated with impaired suppression of endoR_a but enhanced MGU. The effect of central Glp1rs to regulate hepatic and muscle insulin action appears to be limited to receptors in the arcuate nucleus of the hypothalamus. Infusion of GLP-1 into the arcuate nucleus, but not the paraventricular nucleus, has recently been shown to enhance suppression of endoR_a but impair stimulation of R_d during insulin clamps in rats (50). This is in agreement with the present studies, which show that disruption of Glp1r expression impairs suppression of endoR_a but enhances stimulation of R_d.

Enhanced insulin-stimulated MGU and muscle glycogen accumulation in Glp1r$^{-/-}$ mice was not associated with increased activation of insulin signaling proteins. This is also consistent with the findings of Knauf *et al.* (49), who showed that increased muscle glycogen accumulation via inhibition of central Glp1rs does not require expression of the insulin receptor in muscle. This effect of central Glp1r action did require innervation of the muscle because severing of the sciatic nerve blunted muscle glycogen accumulation (49). Contrasting the effects observed in the muscle, the hepatic phenotypes observed in Glp1r$^{-/-}$ mice were associated with an effect on insulin signaling. Thus, the impaired ability of insulin to suppress endoR_a in Glp1r$^{-/-}$ mice correlated with decreased levels of total and phosphorylated Akt. Furthermore, decreased hepatic glycogen storage in these mice was associated with decreased phosphorylation of GSK-3β.

We next addressed whether the Glp1r regulates MGU in the absence of an increase in insulin levels. Exercise is a useful tool to address this question because it stimulates MGU via insulin-independent mechanisms. Whereas arterial glucose levels did not vary appreciably during exercise in Glp1r$^{+/+}$ mice, Glp1r$^{-/-}$ mice exhibited exercise-induced hyperglycemia. This was not due to an impairment in MGU, which was normal in hindlimb

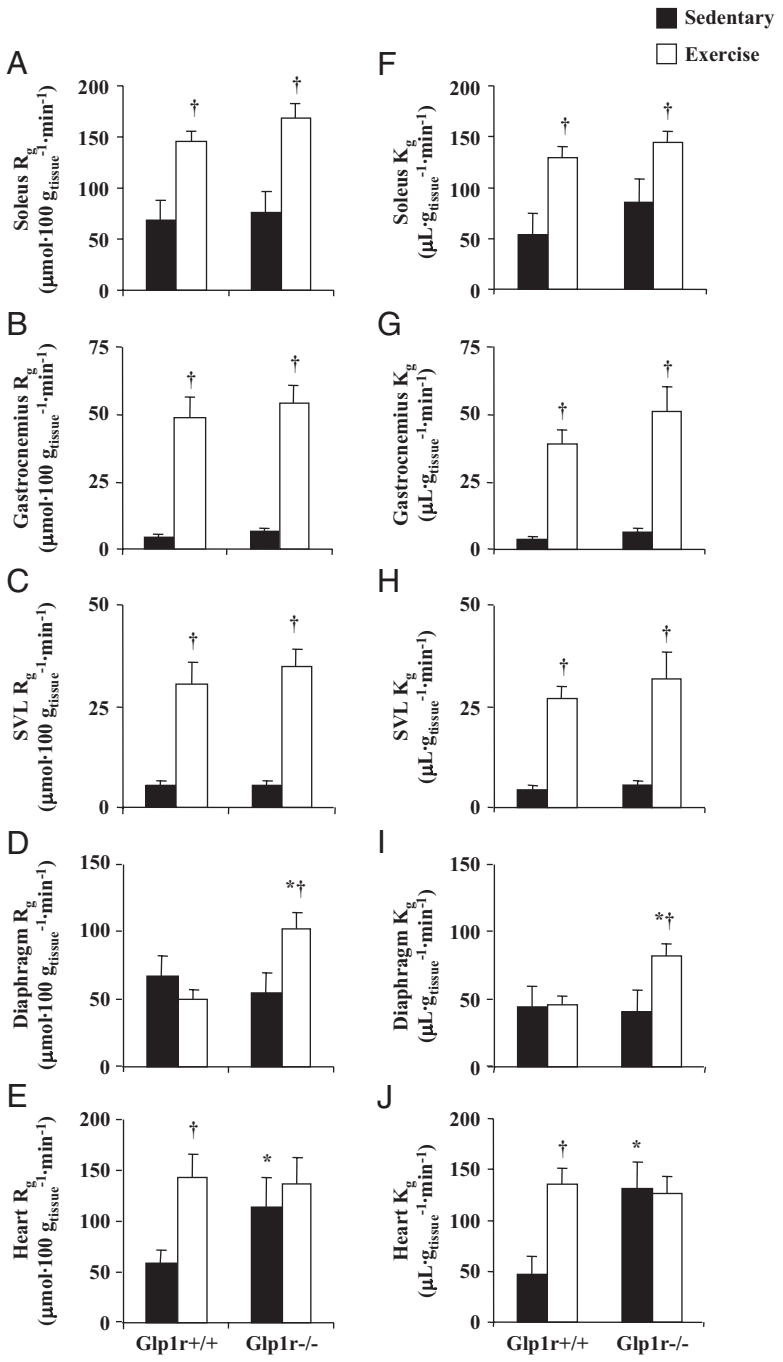

FIG. 5. R_g and K_g in 5-h-fasted sedentary (*black bars*) and exercised (*white bars*) Glp1r$^{+/+}$ and Glp1r$^{-/-}$ mice. R_g is shown for soleus, gastrocnemius, SVL, diaphragm, and heart (A–E) is shown. K_g is shown for the same tissues (F–J). Data are mean ± SEM for 14–16 mice/genotype. *, $P < 0.05$ *vs.* Glp1r$^{+/+}$ within the same experimental condition; †, $P < 0.05$ *vs.* sedentary within the same genotype.

prevailing hyperinsulinemia. These results suggest that the endogenous Glp1r normally inhibits hepatic glucose production in response to increased glucose flux. Because activation of the Glp1r normally occurs concurrent with increased insulin (*e.g.* after a meal), then a role for the Glp1r in the regulation of hepatic glucose production during insulin clamps makes physiologic sense. Such a role for the Glp1r during exercise is less clear because exercise is characterized by an increase in hepatic glucose production, and activation of Glp1rs would counter this hepatic response. Venous levels of GLP-1 increase with exercise (51), raising the possibility that activation of the Glp1r is involved in the normal response to exercise.

Glucagon is a major driving force behind the increase in endoRa during exercise (52). Because activation of the Glp1r suppresses glucagon secretion (8, 9, 11), it is possible that increased hepatic glucose production in Glp1r$^{-/-}$ mice during insulin clamps and exercise results from increased glucagon secretion. Previous studies have shown normal fasting glucagon levels, as well as normal suppression of glucagon by oral glucose loading, in Glp1r$^{-/-}$ mice (27, 53, 54). However, these measurements of plasma glucagon were made from cardiac blood samples. Because glucose clearance occurs in the liver, portal vein glucagon levels would more accurately represent changes in the secretion of this hormone. Thus, it is still possible that increased glucagon secretion plays a role in the hepatic phenotypes observed in the present studies.

Activation of muscle AMPK has been implicated as necessary for the increase in MGU during exercise (39–41). Interestingly, phosphorylation of muscle AMPK, a marker of activation, was significantly greater in Glp1r$^{-/-}$ mice during exercise, even as MGU was not different from that in Glp1r$^{+/+}$ mice. This suggests that activation of AMPK only partially mediates the increase in MGU during exercise. Indeed, studies have shown normal contraction-induced stimulation of MGU in the absence of functional increases in AMPK activity (55–58). Activation of muscle AMPK increases with exercise intensity (42, 43). Results from maximal and endurance exercise tests performed in the present studies suggest that for the same absolute speed, Glp1r$^{-/-}$ mice exercise at a greater intensity than Glp1r$^{+/+}$ mice. Thus, at a speed of 20 m · min^{-1}, the speed used in endurance tests in the present studies, Glp1r$^{+/+}$ mice were exercising at about 78% of their $VO_{2,max}$, whereas Glp1r$^{-/-}$ mice were exercising at about 88% of their $VO_{2,max}$. This was

muscles and even elevated in the diaphragm. These findings suggest that exercise-induced hyperglycemia in Glp1r$^{-/-}$ mice results from an inappropriate increase in hepatic glucose production. During moderate exercise, hepatic glucose production typically increases to match glucose use by the contracting muscle (29). The phenotype observed in Glp1r$^{-/-}$ mice suggests an added drive to further increase glucose production beyond what the working muscle demands. This is similar to what was observed in the insulin clamp studies, in which the disruption of the Glp1r resulted in an inappropriate increase in endoR_a for the

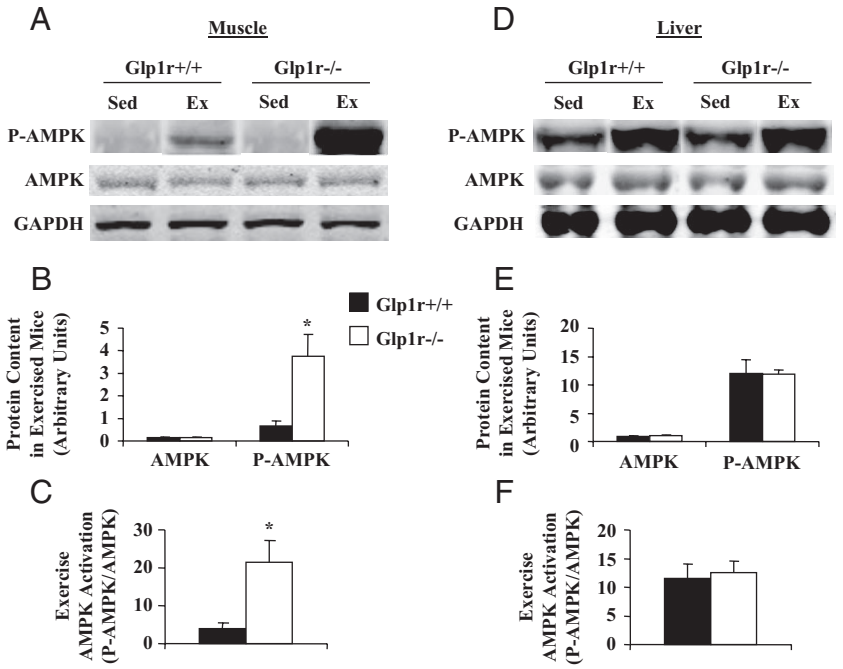

FIG. 6. Immunoblots for AMPK in 5-h-fasted sedentary and exercised Glp1r. Representative immunoblots are shown from gastrocnemius muscle (A) and liver (D) extracts. Protein content and protein activation is presented for Glp1r$^{+/+}$ (*black bars*) and Glp1r$^{-/-}$ (*white bars*) mice. Protein content of Thr^{172}-phosphorylated AMPK (P-AMPK), and α1/α2 AMPK (AMPK) is shown from muscle (B) and liver (E) extracts from exercised mice. Protein content is normalized to the GAPDH loading control. AMPK activation (P-AMPK/AMPK) is also shown from muscle (C) and liver (F) extracts from exercised mice. Data are mean ± SEM for eight to 10 mice/genotype. *, $P < 0.05$ *vs.* Glp1r$^{+/+}$.

associated with lower endurance time in Glp1r$^{-/-}$ mice. Furthermore, Glp1r$^{-/-}$ mice exhibited increased glucose uptake into the diaphragm, suggesting increased breathing and effort. Taken together, these results suggest that the metabolic response to exercise in mice lacking Glp1r expression resembles that of high intensity exercise. Interestingly, one of the metabolic features of high intensity exercise is an increase in glucose production that exceeds the increase in glucose use, leading to a rise in circulating glucose levels (29).

The Glp1r also exerts regulatory control on cardiovascular function (59). Activation of central Glp1rs increases heart rate and blood pressure (60, 61). Recent studies also demonstrate a protective role for the Glp1r against cardiac injury and heart failure (62–68). Disruption of Glp1r expression results in defects in cardiac morphology and function, including increased left ventricular thickness and impaired left ventricular contractility (69). These cardiac phenotypes are often associated with a preference for glucose over fatty acid oxidation in the heart (70–72). In support of this, sedentary cardiac glucose uptake was increased in Glp1r$^{-/-}$ mice in the present studies. It is thus possible that these phenotypes associated with impaired cardiac function explain the decreased exercise capacity in Glp1r$^{-/-}$ mice.

In conclusion, the present studies show that the Glp1r regulates glucose production and use independent of its ability to stimulate insulin secretion. The findings from the insulin clamp studies suggest an important role for the Glp1r in the proper disposal of meal-derived glucose. By enhancing hepatic insulin action at the expense of MGU, activation of Glp1rs ensures that hepatic glucose clearance is increased and hepatic glycogen stores are replenished. This is crucial for the maintenance of glucose homeostasis during the postabsorptive period. Results from exercise studies suggest that activation of Glp1rs also occurs during exercise. Although endoR_a was not directly measured, the fact that glucose levels rose during exercise in Glp1r$^{-/-}$ mice in the absence of a defect in MGU suggests an increase in glucose production exceeding the rate of use. This demonstrates a role for the Glp1r in the regulation of glucose production independent of insulin. Taken together, these results extend the importance of GLP-1 action in regulating glucose homeostasis beyond the pancreas and show it to be a key determinant of glucose fate during times of increased flux.

TABLE 2. Results from exercise stress and exercise endurance tests

	Glp1r$^{+/+}$	Glp1r$^{-/-}$
n (male/female)	14 (9/5)	17 (10/7)
Maximum running rate ($m \cdot min^{-1}$)	36.3 ± 1.3	34.0 ± 1.0
$VO_{2,max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	132 ± 4	138 ± 2
Endurance time (min)	120 ± 13	89 ± 9[a]
VO_2 ($ml \cdot kg^{-1} \cdot min^{-1}$)		
Rest	90 ± 5	90 ± 4
Exercise	103 ± 3	121 ± 4[a]
After exercise	61 ± 5	66 ± 3
RER		
Rest	0.75 ± 0.04	0.78 ± 0.01
Exercise	0.77 ± 0.03	0.83 ± 0.02
After exercise	0.70 ± 0.02	0.73 ± 0.01

Maximum running rate and $VO_{2,max}$ were determined from exercise stress tests. Endurance time VO_2 and RER were determined from exercise endurance tests.

[a] $P < 0.05$ *vs.* Glp1r$^{+/+}$.

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Address all correspondence and requests for reprints to: Julio E. Ayala, Ph.D., 2200 Pierce Avenue, 702 Light Hall, Nashville, Tennessee 37232. E-mail: julio.ayala@vanderbilt.edu.

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