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Exendin-4 and exercise improve hepatic glucose homeostasis by promoting insulin signaling in diabetic rats

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Abstract

Recently, it has been reported that a long-acting glucagon-like peptide–1 (exendin-4) and physical exercise improve hepatic insulin action in diabetic rats. However, this phenomenon remains poorly understood. We investigated the long-term effect that exendin-4 and exercise had on hepatic insulin resistance through the modulation of hepatic and/or hypothalamic insulin signaling in 90% pancreatectomized diabetic rats fed 40% energy fat diets. The rats were divided into 4 groups: exendin-4 only, exendin-4 plus exercise training, saline (control), or exercise training only. Rats in the exendin-4 groups were administered with 150 pmol/kg exendin-4 twice a day for 8 weeks, whereas those in the exercise groups ran on an uphill treadmill with a 15° incline at 20 m/min for 30 minutes 5 days a week. Exendin-4 reduced serum glucagon levels in overnight-fasted rats. Exendin-4 treatment by itself decreased hepatic glucose output at hyperinsulinemic states, and exercise without exendin-4 treatment also had the same effect. Exendin-4 promoted hepatic insulin signaling by potentiating tyrosine phosphorylation of the insulin receptor substrate–2 without changing hypothalamic insulin signaling. Exendin-4 also enhanced hypothalamic glucose sensing. However, exercise improved both hepatic and hypothalamic insulin signaling by activating the phosphorylation of cyclic adenosine monophosphate–responding element binding proteins to induce insulin receptor substrate–2 expression. Exendin-4 and exercise decreased the expression of phosphoenolpyruvate carboxykinase, which in turn reduced hepatic glucose output. Exendin-4 in combination with exercise had no additive effects. In conclusion, exendin-4 and exercise improve hepatic glucose homeostasis by promoting hepatic insulin signaling in diabetic rats.

1. Introduction

Type 2 diabetes mellitus is a heterogeneous metabolic disorder characterized by insulin deficiency and peripheral insulin resistance [1,2]. When insulin resistance occurs in the liver, skeletal muscles, and adipose tissues as a result of conditions such as obesity, stress, inflammation, and aging, a sufficient amount of insulin secretion from pancreatic β-cells compensates for this resistance and maintains normoglycemia [1,3]. The imbalance between glucose-stimulated insulin secretion and peripheral insulin resistance causes the development of type 2 diabetes mellitus [1,2]. Among peripheral tissues, the liver, because of its ability to store glucose as glycogen and produce glucose from glycogen breakdown or gluconeogenic precursors, plays an important role in regulating glucose homeostasis by chiefly maintaining the normal concentration of blood glucose. [4]. *Hepatic insulin resistance* is defined as the failure of insulin to adequately suppress hepatic glucose production by attenuating insulin signaling. As a result, hepatic insulin resistance induces basal and postprandial hyperglycemia and increases hepatic fat accumulation, independently of body mass index or intraabdominal obesity [5]. Thus, the reduction of hepatic insulin resistance contributes to relieving diabetic symptoms.

Glucagon-like peptide–1 (GLP-1) belongs to the large glucagon superfamily of peptide hormones, and it is secreted into the circulation by L-cells located in the distal ileum and colon during a meal [6]. It impedes gastric functioning, suppresses appetite, and causes a reduction in body weight by acting on the hypothalamus [7,8]. In addition, GLP-1 has been known to promote glucose-stimulated insulin gene transcription, biosynthesis, and secretion from the pancreatic β-cells and to suppress

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glucagon secretion from the α-cells in rodents and humans [9,10]. Glucagon-like peptide–1 receptor knockout mice showed fairly consistent results. They exhibit mild fasting hyperglycemia without significant changes in fasting insulin messenger RNA (mRNA) and contents; and when feeding, they have impaired glucose tolerance with a modest reduction in insulin contents [11]. Thus, fasting hyperglycemia in GLP-1 receptor knockout mice may be associated with hepatic glucose intolerance.

However, GLP-1 cannot be used as a therapeutic drug because GLP-1 is quickly inactivated by dipeptidyl peptidase IV upon its secretion. Several GLP-1 receptor agonists have been discovered for use in relieving diabetic symptoms: the activity of exendin-4 continues for 12 hours and has similar biological effects to that of GLP-1 [12]. The injection of exendin-4, a peptide isolated from the saliva of Gila monsters (*Heloderma suspectum*), increases first-phase insulin secretion and *β*-cell mass in rodents or people with type 2 diabetes mellitus; and this compensates for peripheral insulin resistance associated with type 2 diabetes mellitus [12]. Furthermore, recent studies have revealed that exendin-4 enhances peripheral insulin sensitivity, especially in the liver [13,14]. Serum glucose and hepatic steatosis were significantly reduced in exendin-4–treated *ob*/*ob* mice. The GLP-1-treated hepatocytes resulted in a significant increase in cyclic adenosine monophosphate (cAMP) production as well as a reduction in mRNA expression of stearoyl–coenzyme A desaturase–1 and genes associated with fatty acid synthesis; the converse was true for genes associated with fatty acid oxidation [14]. Thus, exendin-4 appears to effectively reverse hepatic insulin resistance in *ob*/*ob* mice by improving insulin sensitivity; but the mechanism by which this is achieved remains poorly understood.

Physical exercise was found to be associated with a reduced risk of developing type 2 diabetes mellitus and is a well-recognized remedy in improving insulin sensitivity at the skeletal muscle level [15]. In addition, regular aerobic exercise reduces visceral fat mass and body weight without decreasing lean body mass and also ameliorates insulin sensitivity. However, the effect of physical training on hepatic insulin action in patients with type 2 diabetes mellitus has not received much attention. Some studies have revealed that regular exercise improves hepatic and hypothalamic insulin signaling in diabetic rats, but this process remains unclear [16-19]. Therefore, we investigated the effect of exendin-4 and exercise on hepatic insulin resistance in Sprague-Dawley 90% pancreatectomized (Px) male diabetic rats. In addition, we inquired into how their molecular mechanism, by modifying hepatic and hypothalamic insulin signaling, modulated hepatic insulin resistance in Px diabetic rats. However, the involvement of hypothalamic insulin signaling in hepatic glucose homeostasis remains controversial. As previous studies have shown that the Px diabetic rat is a good example of mild and nonobese type 2 diabetes mellitus and displays both insulin resistance and insulin deficiency, it was deemed to be an appropriate type 2 diabetes mellitus animal model [20,21].

2. Materials and methods

2.1. Experimental animals

All rats were housed individually in stainless steel cages in a controlled environment (23°C and a 12-hour light and dark cycle). All surgical and experimental procedures were performed in accordance with the recommendations found in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (USA) and approved by the Institutional Animal Care and Use Committee of Hoseo University, Korea. Male Sprague-Dawley rats had 90% of their pancreas removed using the technique of Hosokawa et al [20] or received a sham pancreatectomy (sham). After a 90% pancreatectomy, the remaining pancreas was only within 2 mm of the common bile duct and extended from the duct to the first part of the duodenum. Because the pancreas regenerated itself by up to 50% within 2 weeks, Px rats with random fed serum glucose levels of less than 9.4 mmol/L were excluded 2 weeks after surgery to select only those rats that showed mild type 2 diabetes mellitus symptoms. The Px rats included in the experiment exhibited nearly normal fasting serum glucose levels, but postprandial levels were greater than 10 mmol/L because of insulin deficiency and peripheral insulin resistance. Our previous studies revealed that whole-body glucose infusion rates of Px diabetic rats were approximately 50% to 60% lower than those of sham (normal) rats during euglycemic-hyperinsulinemic clamp with 20 pmol kg^{-1} min^{-1} insulin infusion but that the hepatic glucose output of Px rats in a hyperinsulinemic clamped state was about 2 times higher than that of sham rats [18,21,22]. This indicated that the peripheral insulin resistance of Px rats was almost 2 times higher than that of sham rats. Therefore, Px rats have common characteristics of mild type 2 diabetes mellitus.

2.2. Experimental design

All experimental rats had free access to a 40% energy fat diet (Sam Yang, Kangwon-Do, Korea) and water throughout the experimental periods. Pancreatectomized rats weighing 186 ± 25 g were divided into 4 groups: (1) exendin-4, (2) exendin-4 plus exercise, (3) saline (control), and (4) exercise. During the 8-week experimental period, rats in the exercise groups ran on an uphill treadmill with a 15° incline at 20 m/min for 30 minutes 5 times a week. Rats in the exendin-4 groups were subcutaneously administered with 150 pmol/kg exendin-4 (Sigma, St Louis, MO) twice a day, whereas those in the control group were given saline in the same manner as those administered with exendin-4. The dosage of exendin-4 administered to the rats in this experiment was 6 to 10 times higher than that present in humans. In this way, we were able to consider the difference in metabolic rates between humans

and rodents. Sham rats were included as a normal control group. Overnight-fasted serum glucose levels, food intake, and body weight were measured every Tuesday at 10:00 AM.

2.3. Insulin resistance

After 7 weeks of treatment, catheters were surgically implanted into the right carotid artery and left jugular vein of rats anesthetized with intraperitoneal injections of ketamine and xylazine (100 mg and 10 mg/kg body weight, respectively). After 5 to 6 days of implantation, a euglycemic-hyperinsulinemic clamp was performed on fasted conscious rats to determine insulin resistance as previously described [3]. [3-^{3}H] glucose (NEN Life Science, Boston, MA) was continuously infused during a 4-hour period at the rate of 0.05 μCi/min. Basal hepatic glucose output was measured in blood collected at 100 and 120 minutes after initiation of the [3-^{3}H] glucose infusion. Afterward, a primed continuous infusion of human regular insulin (Humulin; Eli Lilly, Indianapolis, IN) was initiated at a rate of 20 pmol kg^{-1} min^{-1} to raise plasma insulin concentration to approximately 1100 pmol/L. Glucose (25%) was infused at variable rates as needed to clamp glucose levels at about 6 mmol/L. While blood glucose levels were steady between 200 and 240 minutes, total glucose appearance and whole-body glucose uptake were determined as the ratio of the [^{3}H] glucose infusion rate (disintegrations per minute [dpm] per minute) to the specific activity of plasma glucose (dpm per micromole). Clamped hepatic glucose output was calculated by subtracting glucose infusion rates from the rates of glucose appearance. Whole-body glucose infusion was expressed in terms of the milligram of glucose per kilogram of body weight per minute that was required to maintain euglycemia during hyperinsulinemia [3]. At the end of the clamp, the rats were anesthetized with the mixture of ketamine and xylazine and were killed by decapitation. Tissues were rapidly dissected, weighed, frozen in liquid nitrogen, and stored at −70°C until further analysis could be performed. Serum glucose levels were analyzed with a Glucose Analyzer II (Beckman, Palo Alto, CA). Serum insulin and leptin levels were measured by radioimmunoassay (RIA; Linco Research, St Charles, MO).

To determine the glycogen content in the liver, its lysates were centrifuged at 3000 rpm for 10 minutes; and the supernatants were deproteinized with 1.5 N perchloric acid. The glycogen content was calculated from glucose concentrations derived from glycogen hydrolyzed by α-amyloglucosidase in an acid buffer [18]. Triglyceride was extracted with chloroform-methanol (2:1, vol/vol) from the liver and resuspended in pure chloroform [23]. Triacylglycerol concentration was determined using a Trinder kit (Young Dong Pharmaceutical, Seoul, Korea).

2.4. Immunoblot analysis

Five rats from each group that had not been subjected to a euglycemic-hyperinsulinemic clamp were used for immunoblotting assay. Overnight-fasted nondiabetic rats and diabetic rats treated with exendin-4 and/or exercised for 8 weeks were anesthetized with ketamine and xylazine mixture by intraperitoneal injection. Human insulin (5 U/kg body weight) was injected through the inferior vena cava to stimulate insulin action. The liver, soleus muscles, and hypothalamus of rats were dissected after 10 minutes of stimulation and immediately frozen with liquid nitrogen. Frozen tissues were lysed with a 20-mmol/L Tris buffer (pH 7.4) containing 2 mmol/L EDTA, 137 mmol/L NaCl, 1% NP40, 10% glycerol, and 12 mmol/L α-glycerol phosphate and protease inhibitors. After 30 minutes on ice, the lysates were centrifuged for 10 minutes at 12 000 rpm at 4°C. Lysates containing equal amounts of protein (30-50 μg) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis; and immunoblotting was performed with specific antibodies against insulin receptor substrate (IRS) 1, IRS2 (UBI, Waltham, MA), protein kinase B (or Akt), phosphorylated protein kinase B^{Ser473}, p85 (subunit of phosphoinositide 3-kinases), cAMP responding element binding protein (CREB), phosphorylated CREB^{Ser133} (Cell Signaling Technology, Beverly, MA), AMP kinase (AMPK), phosphorylated AMPK^{Thr172}, glucose transporter–2 (GLUT2; Santa Cruz, Santa Cruz, CA), and phosphoenolpyruvate carboxykinase (PEPCK) as previously described [24]. The intensity of protein expression was determined using Imagequant TL (Amersham Biosciences, Piscataway, NJ). These experiments were repeated 3 times for each group.

2.5. RNA isolation and reverse transcription polymerase chain reaction

Total RNA was isolated from the liver of the rats from each group using a monophasic solution of phenol and guanidine isothiocyanate (Trizol reagent; Gibco-BRL, Rockville, MD), followed by extraction and precipitation with isopropyl alcohol [16]. The complementary DNA was synthesized from equal amounts of total RNA with SuperScript III (Invitrogen, Carlsbad, CA) reverse transcriptase, and polymerase chain reaction was performed with high-fidelity Taq DNA polymerase. The primers used to detect rat PEPCK and 18S genes were the following: PEPCK—forward 5′ CAG GAA GTG AGG AAG TTT GTG G 3′, reverse 5′ ATG ACA CCC TCC TCC TGC AT 3′; 18S—forward 5′-agttgctgcagttaaaaagc-3′, reverse 5′-actcagctaagagcatcgag-3′. The primers were designed to sandwich at least 1 intron to distinguish between the products derived from mRNA and genomic DNA.

2.6. Statistical analysis

Statistical analysis was performed using the SAS (Cary, NC) statistical analysis program, and all results are expressed as mean ± standard deviation. Because there was no significant interaction between exendin-4 and exercise, their 2 main effects were determined by 2-way analysis of variance. Significant differences in the main effects among groups were identified by Tukey tests at a $P < .05$.

Table 1
Metabolic parameters at the end of experimental periods

	Ex4 (n = 15)	Ex4 + Exe (n = 15)	Control (n = 15)	Exe (n = 15)	Sham control (n = 10)
Body weight (g)	356 ± 23[a,c]	360 ± 19[c]	393 ± 28[b]	382 ± 24[b,*]	437 ± 28**
Epididymal fat pads (g)	2.8 ± 0.5[c]	2.7 ± 0.5[c]	5.1 ± 0.7[b]	3.3 ± 0.8[c,*]	5.3 ± 0.7
Caloric intakes (kcal/d)	88.5 ± 11.4[c]	95.7 ± 10.9[c]	118.2 ± 15.4[b]	113.5 ± 15.9[b,*]	89.7 ± 12.4**
Overnight-fasted leptin levels (ng/mL)	2.9 ± 0.5[c]	3.0 ± 0.5[c]	3.9 ± 0.5[b]	3.1 ± 0.4[c,*]	6.3 ± 0.8***
Overnight-fasted serum glucose (mmol/L)	5.7 ± 0.6[c]	6.0 ± 0.7[c]	7.1 ± 0.7[b]	6.2 ± 0.8[c,*]	4.7 ± 0.7**
Overnight-fasted serum insulin (ng/mL)	0.85 ± 0.14	0.88 ± 0.19	0.75 ± 0.17	0.83 ± 0.21	1.39 ± 0.25***
Overnight-fasted serum glucagon (pg/mL)	108.3 ± 15.4[c]	104.5 ± 16.2[c]	140.4 ± 19.3[b]	125.1 ± 21.4[bc,*]	101.6 ± 15.6**

[a] Values are mean ± SD. Ex4 indicates exendin-4 treatment; Exe, exercise. Means with different alphabets ([b,c]) were significantly different at $P < .05$ according to the Tukey test.

* Significant difference among the groups at $P < .05$ according to 1-way ANOVA.

** Significantly different from Px control at $P < .05$.

*** Significantly different from Px control at $P < .01$.

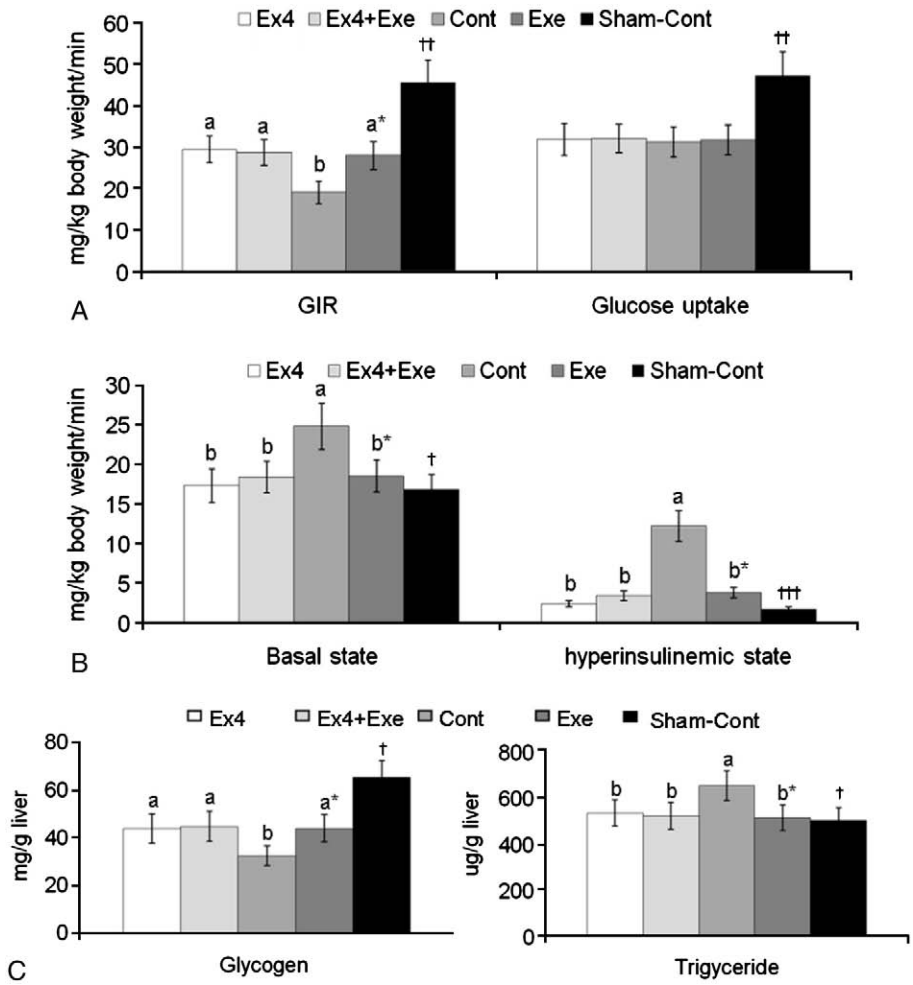

Fig. 1. Metabolic parameters under a euglycemic-hyperinsulinemic clamp. After an 8-week treatment period with exendin-4 and/or exercise , a euglycemic-hyperinsulinemic clamp was performed in conscious, free-moving, and overnight-fasted rats to determine whole-body insulin resistance. After an infusion of [3-^{3}H] glucose for 2 hours to measure basal hepatic glucose output, a primed continuous infusion of human regular insulin was initiated at a rate of 20 pmol kg^{-1} min^{-1} to raise plasma insulin concentration to approximately 1100 pmol/L. As clamp glucose levels at about 6 mmol/L were required, a 25% glucose solution was infused at variable rates. To measure [3-^{3}H] glucose and ^3H_2O, glucose uptake and clamped hepatic glucose output were determined. The number of animals in each group was 10, and the results were expressed as mean ± SD. A, Whole-body glucose infusion rates and glucose uptake. B, Hepatic glucose output at the basal and clamped states. C, Hepatic glycogen and triglyceride storage. *Significant difference among the groups of Px rats at $P < .05$ according to 1-way ANOVA. Means with different alphabets ([a,b]) were significantly different at $P < .05$ according to the Tukey test. †Significantly different from Px control at $P < .05$, ††at $P < .01$, and †††at $P < .001$. Ex4 indicates exendin-4; Exe, exercise; GIR, glucose infusion rate.

3. Results

3.1. Exendin-4 and exercise alter body weight; caloric intake; and serum glucose, insulin, and glucagon levels

Although sham rats consumed fewer calories on a daily basis, their body weight was greater than Px rats; but there was no significant difference in epididymal fat pads between sham and Px rats (Table 1). Serum leptin levels were significantly higher in sham rats than in Px rats. These results may be partly due to factors such as urinary glucose excretion and insulin deficiency. Insulin deficiency reduced leptin secretion, which increased food intake. These results suggested that the synergistic interaction of insulin and leptin is necessary to regulate body weight, fat accumulation, and food intake. Subcutaneous injection of exendin-4 decreased

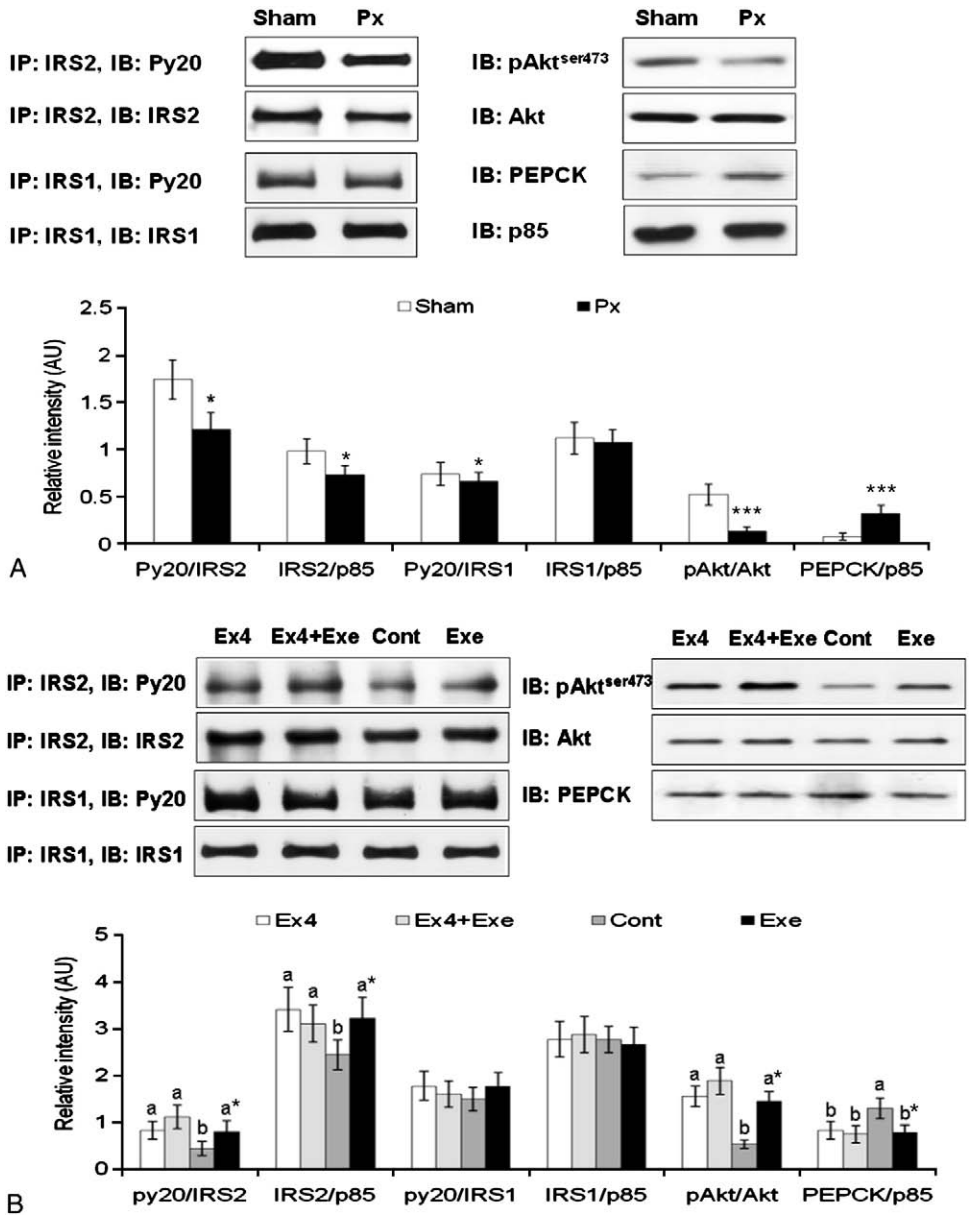

Fig. 2. Insulin signaling cascade in the liver. At the end of the experimental period, the rats in each group were injected with human insulin (5 U/kg body weight) through the inferior vena cava to stimulate insulin action. After 10 minutes of stimulation, the liver was obtained and immediately frozen with liquid nitrogen. Frozen tissues were lysed with a 20-mmol/L Tris lysis buffer. An equal amount of protein was used for immunoblotting analysis. Immunoprecipitation was performed before immunoblotting analysis, when tyrosine phosphorylation and protein expression of IRS1 and IRS2 were revealed. The phosphorylation and expression of other proteins were detected with specific antibodies by immunoblotting analysis only. The number of animals in each group was 4, and the results were expressed as mean ± SD. A, Phosphorylation and expression of IRS1, IRS2, Akt, and PEPCK involved in the insulin signaling cascade in nondiabetic and diabetic rats. B, Phosphorylation and expression of IRS1, IRS2, Akt, and PEPCK involved in the insulin signaling cascade in diabetic rats. C, Expression of glucokinase, GLUT2, and AMPK in diabetic rats. *Significant difference among the groups of Px rats at $P < .05$ according to 1-way ANOVA. Means with different alphabets (^{a,b}) were significantly different at $P < .05$ according to the Tukey test.

body weight in Px rats because of a lowered caloric intake at the end of the 8-week experimental period, whereas exercise training had no effect on either body weight or caloric intake (Table 1). However, simultaneous treatment of exendin-4 injection and exercise decreased epididymal fat pads, representing visceral fat storage, in comparison with the control group (Table 1).

Overnight-fasted serum glucose levels of Px rats were higher than sham rats. This increase was accompanied by a concomitant decrease in serum insulin levels (Table 1). Serum glucose and insulin levels indicated that Px rats exhibited mild diabetic symptoms due to decreased insulin secretion. Overnight-fasted serum glucose levels of Px rats administered exendin-4 and/or exercised were lower than those of Px rats administered saline. Exendin-4 plus exercise did not have any cumulative effects in serum glucose concentrations. Conversely, overnight-fasted serum insulin levels were not different among groups in Px diabetic rats. By contrast, serum glucagon levels were reduced in exendin-4–treated rats; and exercise also tended to decrease levels but not by a significant margin.

3.2. Exendin-4 and exercise improve insulin sensitivity

During euglycemic-hyperinsulinemic clamp, exogenous insulin was infused into the rats of each group to maintain serum insulin levels at the clamped states at approximately 1100 pmol/L; and the serum insulin levels were not significantly different among the groups. In addition, serum glucagon levels were reduced by approximately 60% at the hyperinsulinemic clamped state; and they were not significantly different among the groups (data not shown). Serum glucose levels at the clamped state reached approximately 5.5 mmol/L among the rats in each group by infusing exogenous glucose. As depicted in Fig. 1, whole-body glucose infusion rates, representing peripheral insulin sensitivity, decreased in Px rats as compared with sham rats. Before the clamp, overnight-fasted serum glucose levels were higher in the control group of Px rats than the other groups (Table 1). At the hyperinsulinemic clamp state, glucose infusion rates were elevated in exendin-4, exercise, and exendin-4 plus exercise groups by 56%, 41%, and 44%, respectively, compared with the those in the control group (Fig. 1A). Exendin-4 plus exercise did not have any additive incremental effect on glucose infusion rates in diabetic rats (Fig. 1A).

During euglycemic-hyperinsulinemic clamp, hepatic glucose output in basal and hyperinsulinemic clamped states was higher in Px rats than sham rats, indicating that the output was not suppressed in diabetic rats as much as nondiabetic rats regardless of serum insulin levels (Fig. 1B). Exendin-4, exercise, and exendin-4 plus exercise suppressed hepatic glucose output in the basal (fasting) state compared with the control group, consistent with the reduction of serum glucose levels that occurred in a fasting state (Fig. 1B). Increased hepatic glucose output reflects the impairment of insulin action as exhibited during a hyperinsulinemic state [1,5]. In addition, during a clamped state, at about 1100-pmol/L serum insulin levels, exendin-4, exercise, and exendin-4 plus exercise similarly lowered hepatic glucose output compared with the control group (Fig. 1B). This was related to a simultaneous increase of glycogen storage and decrease of triglyceride accumulation in the liver (Fig. 1C). Thus, either exendin-4 or exercise was partially able to overcome insulin resistance induced by the partial removal of the pancreas.

3.3. Exendin-4 and exercise improve hepatic insulin signaling

The euglycemic-hyperinsulinemic clamp experiment revealed that exendin-4 and exercise decreased hepatic

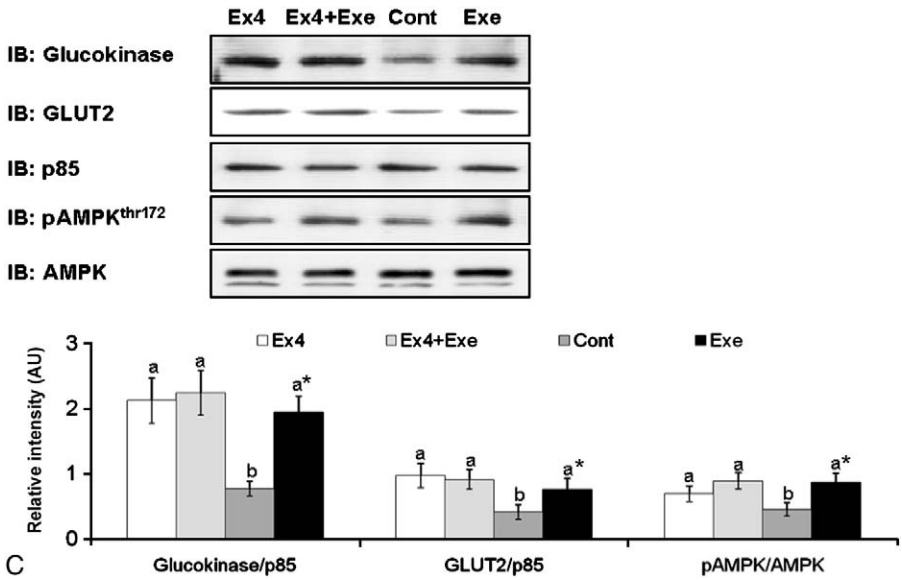

Fig. 2. continued

glucose output and increased glycogen storage in a hyperinsulinemic state. This suggests that the long-term administration of exendin-4 and exercise inhibits glucose output by potentiating hepatic insulin action at hyperinsulinemic states, in which process IRS1, IRS2, Akt, PEPCK, GLUT2, and glucokinase might be involved. After 10 minutes of stimulation with 5 U insulin per kilogram of body weight, IRS2 protein in the liver was reduced in Px rats; and its tyrosine phosphorylation was concomitantly attenuated compared with sham rats (Fig. 2A). However, tyrosine phosphorylation of IRS1 was slightly and insignificantly decreased without any modulation of protein levels in Px rats

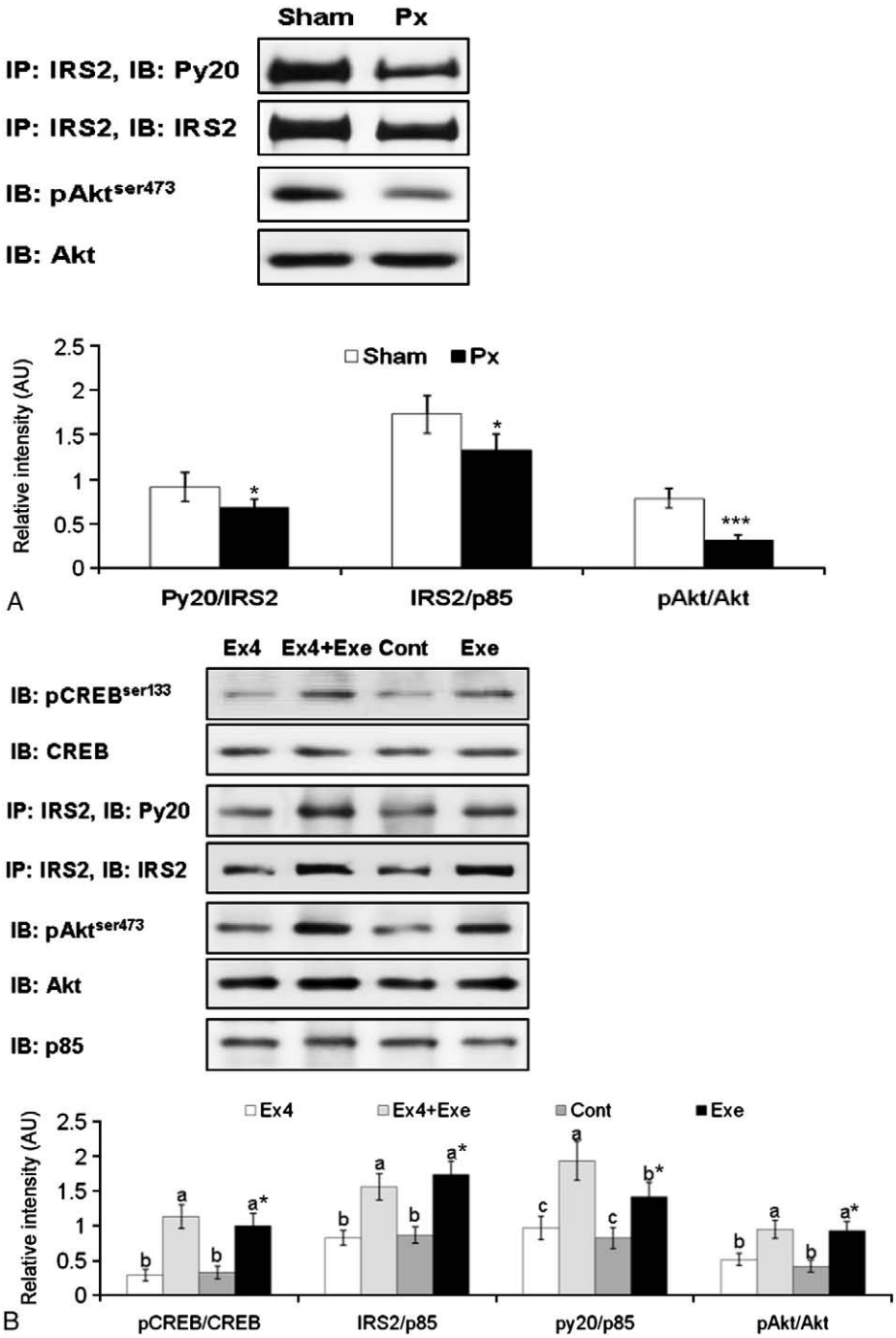

Fig. 3. Insulin signaling cascade in the hypothalamus. The experimental procedure was the same as in Fig. 2, and the hypothalamus instead of the liver was used for the samples. A, Phosphorylation and expression of IRS2 and Akt involved in the insulin signaling cascade in nondiabetic and diabetic rats. B, Phosphorylation and expression of CREB, IRS2, and Akt involved in the insulin signaling cascade in diabetic rats. C, Expression of glucokinase and GLUT2 in diabetic rats. *Significant difference among the groups of Px rats at $P < .05$ according to 1-way ANOVA. Means with different alphabets (^{a,b}) were significantly different at $P < .05$ according to the Tukey test.

compared with sham rats (Fig. 2A). The phosphorylation of Akt, a downstream mediator of IRS2, was also more attenuated in Px rats compared with sham rats (Fig. 2A). This attenuation contributed to an increase in the expression of PEPCK, one of the major enzymes that regulate gluconeogenesis, which led to elevated hepatic glucose output in Px rats (Fig. 2A). Both exendin-4 (150 pmol/kg body weight twice a day) and regular exercise training potentiated tyrosine phosphorylation of IRS2, with a slight increase of IRS2 protein levels in the liver. The enhancement of tyrosine phosphorylation of IRS2 was delivered into serine^{473} phosphorylation of Akt, the downstream regulator of IRS2 in insulin signaling (Fig. 2B). Thus, IRS2 induction was brought on by the separate administration of exendin-4 or exercise and sufficiently enhanced insulin signaling in the liver. However, the concomitant treatment of exendin-4 and exercise together did not improve signaling more than that which resulted from the separate treatment of exendin-4 or exercise.

Consistent with the attenuation of hepatic insulin signaling, Px rats had higher PEPCK protein levels in the liver than sham rats (Fig. 2A). The PEPCK expression decreased in the exendin-4, exercise, and exendin-4 plus exercise groups of diabetic rats (Fig. 2B). In addition, mRNA levels of PEPCK (the ratio of PEPCK and 18S) were higher in Px rats than sham rats (2.8 ± 0.5 vs 1.3 ± 0.4, $P < .01$); and exendin-4, exercise, and exendin-4 plus exercise reduced mRNA levels of PEPCK (the ratio of PEPCK and 18S) in Px rats in comparison with the control (1.8 ± 0.4, 1.7 ± 0.4, 1.6 ± 0.4 vs 2.9 ± 0.5). Because many studies have shown that PEPCK expression can represent PEPCK activities without a measurement of those activities being taken, PEPCK activities were not measured in the present study [24,25]. This suggests that exendin-4 and exercise reduced hepatic glucose output at hyperinsulinemic states by reducing PEPCK expression by activating insulin signaling. In addition, the potentiated insulin signaling cascade stimulated glucose sensing in the liver. The expression of glucokinase and GLUT2 was similarly raised in the exendin-4, exercise, and exendin-4 plus exercise groups; and this elevation stimulated glucose sensing in the liver (Fig. 2C). In addition, serine phosphorylation of AMPK, an energy sensor that is known to stimulate fatty acid oxidation by regulating acetyl coenzyme A carboxylase activity [26,27], was stimulated in the exendin-4 and exercise groups of diabetic rats (Fig. 2C). No further increment of AMPK phosphorylation was shown to result from the concomitant treatment of exendin-4 and exercise.

3.4. Only exercise improves hypothalamic insulin signaling, but both exercise and exendin-4 enhance hypothalamic glucose sensing

It has recently been reported that a decrease in hypothalamic insulin signaling could also account for hepatic insulin resistance, resulting from the impairment of hepatic insulin signaling [28,29]. Pancreatectomized rats had reduced tyrosine phosphorylation of IRS2 as a result of a reduction in IRS2 protein contents in the hypothalamus (Fig. 3A), which indicated that hypothalamic insulin signaling was attenuated in Px rats. In diabetic rats, only exercise, not exendin-4, increased CREB phosphorylation in the hypothalamus of diabetic rats, which in turn raised IRS2 protein levels (Fig. 3B). The tyrosine phosphorylation of IRS2 increased the most in the exendin-4 plus exercise group among the groups of Px rats, whereas exercise by

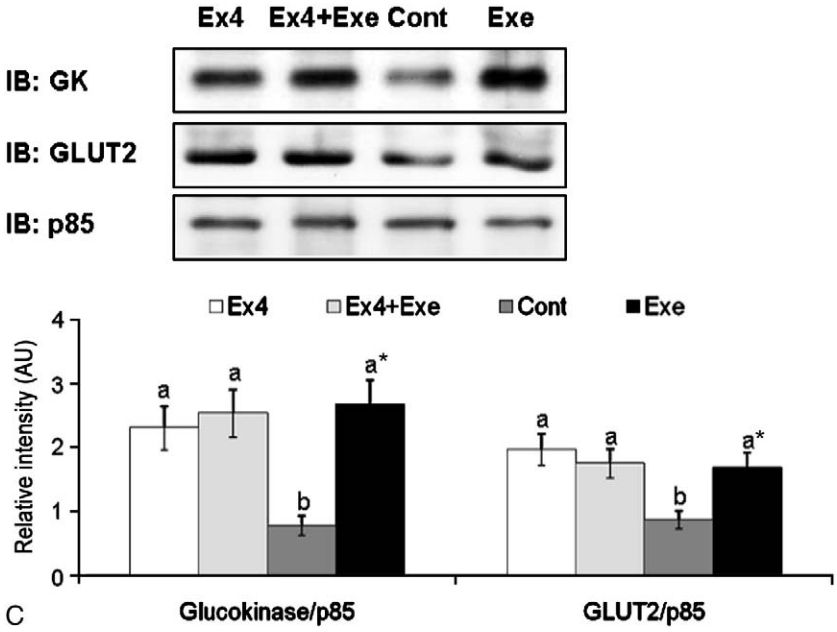

Fig. 3. continued

itself elevated phosphorylation more than the control. However, exendin-4 by itself did not lead to IRS2 induction and did not potentiate tyrosine phosphorylation. Thus, tyrosine phosphorylation of IRS2 in the liver and the hypothalamus depended on IRS2 protein levels during the treatment program of exendin-4 treatment combined with exercise. The enhancement of IRS2 tyrosine phosphorylation activated serine phosphorylation of Akt in the exercise groups, indicating that exercise, but not exendin-4, potentiated hypothalamic insulin signaling (Fig. 3B). However, although exendin-4 did not enhance hypothalamic insulin signaling, exercise and exendin-4 increased the expression of glucokinase and GLUT2, which are involved in glucose sensing. This indicated that exercise improved glucose sensing possibly by potentiating insulin signaling in the hypothalamus of diabetic rats but that exendin-4 enhanced the sensing by some other pathway (Fig. 3C).

4. Discussion

Type 2 diabetes mellitus is characterized by insulin resistance in peripheral tissues and functional failure of pancreatic β-cells [1]. Hepatic insulin resistance induces hyperglycemia in fasted and fed states by impairing insulin signaling [1,30]. These defects in turn result in reduced glycogen synthase activity with insulin stimulation, resulting in decreased insulin-stimulated hepatic glucose uptake and increased hepatic glucose production with and without insulin stimulation [31]. Consistent with the general characteristics of type 2 diabetes mellitus, in this present study, Px rats increased their hepatic glucose output, whereas their hepatic insulin signaling was attenuated. These results are consistent with other studies conducted on this topic. [20-22]. Therefore, the Px rat is a good animal model for investigating the effect that exendin-4 and exercise have on relieving hepatic insulin resistance and the mechanism by which it occurs.

Glucagon-like peptide–1 and exendin-4, a GLP-1 receptor agonist, are both well known to have an insulinotropic action, that is, inducing the activation of β-cell function and mass [11,31]. However, the removal or activation of GLP-1 receptors showed extrapancreatic effects in the liver, muscles, and adipose tissues that modulate peripheral insulin action in type 1 and type 2 diabetes mellitus patients and animals [13-15,32]. Consistent with the present study, the long-term administration of exendin-4 has also been shown to stimulate glycogen storage, to reduce triglyceride contents, and to suppress glucose output in the liver possibly by enhancing hepatic insulin signaling [13,14,32]. In addition, physical exercise enhances hepatic insulin signaling and inhibits the activity of PEPCK, which catalyzes a regulatory step in gluconeogenesis in diabetes-prone *Psammomys obesus* [16] and in obese Zucker rats [17]. However, the molecular mechanism by which exendin-4 and exercise operate in the liver has not yet been clearly established. The present study suggests that exendin-4 and exercise decreased hepatic glucose production at basal and hyperinsulinemic states in diabetic rats. The reduction at the basal state was related to the lowering of serum glucagon levels in the overnight-fasted state, whereas the decrease at the hyperinsulinemic state resulted from increased glycogen storage and decreased triglyceride accumulation subsequent to an enhancement in hepatic insulin signaling. In addition to the effects on the liver, Ayala et al [32] also demonstrated that GLP-1 knockout mice exhibited increased glucose loss, increased muscle glucose disposal rates, and increased muscle glycogen levels during euglycemic-hyperinsulinemic clamp, symptoms which are not associated with enhanced muscle insulin signaling [32]. Our preliminary study also showed that insulin signaling (tyrosine phosphorylation of IRS1 → serine phosphorylation of Akt) in gastrocnemius muscle was not affected by exendin-4 but that the signaling was slightly increased by exercise (data not shown). However, we cannot conclude that exendin-4 does not affect muscle glucose utilization because other pathways besides insulin signaling are involved in muscle glucose utilization and various types of muscles displayed different reactions. Further experiments are necessary to find out how exendin-4 affects muscles.

The regulation of hepatic gluconeogenesis is an important process in the adjustment of the blood glucose level, and the impairment of hepatic glucose production is a central characteristic in type 2 diabetes mellitus [30]. The key gluconeogenic enzymes, PEPCK and glucose-6-phosphatase (G-6-Pase), play an important role in the regulation of hepatic glucose production. The activity of the PEPCK and G-6-Pase is regulated by transcriptional and nontranscriptional mechanisms [30]. Glucagon and glucocorticoids are known to increase hepatic gluconeogenesis by inducing the expression of PEPCK and G-6-Pase. Conversely, insulin is the most important hormone that inhibits gluconeogenesis for the most part by suppressing the expression of PEPCK and G-6-Pase via the activation of phosphoinositide 3-kinases [33]. In the present study, exendin-4 reduced serum glucagon levels; and this observation is supported by other studies [33]. This reduction also acted as a factor in decreasing the PEPCK activity in addition to activating hepatic insulin signaling. In the present study, we did not measure G-6-Pase expression; but it may also, like PEPCK, be reduced because exendin-4 reduced serum glucagon levels and hepatic insulin action were increased through tyrosine phosphorylation of IRS2 → pAkt.

Glucagon-like peptide–1 and exendin-4 are known to be involved in multiple signaling pathways such as protein kinase A, extracellular signal–regulated kinase, c-Jun *N*-terminal kinase, and/or Wnt signaling [33-35]. However, there has been no evidence presented to support the contention that exendin-4 affects the insulin receptor binding affinity and/or efficiency. Our previous study showed that exendin-4 activated protein kinase A and extracellular signal–regulated kinase to activate CREB in the islets and

that CREB phosphorylation induced an increase in IRS2 expression [35]. The elevation of IRS2 expression enhanced insulin/IGF-1 signaling in the islets [18,36,37]. Similar to exendin-4 action in islets, the present study revealed that both exendin-4 and regular exercise increased IRS2 induction in the liver, which was related to CREB phosphorylation. The IRS2 induction potentiated tyrosine phosphorylation of IRS2 and serine phosphorylation of Akt and decreased PEPCK expression. Both exendin-4 and exercise decreased hepatic glucose output in hyperinsulinemic clamped states, and this was associated with the elevation of insulin-stimulated glycogen storage and the decrease of PEPCK expression through potentiating hepatic insulin signaling. They also reduced hepatic triglyceride accumulation, which has a beneficial action on hepatic insulin sensitivity. It is known that the potentiation of insulin signaling activates glycogen synthase through the phosphorylation of glycogen synthase kinase-3 and decreases PEPCK expression by increasing the phosphorylation of forkhead box protein O [30]. Recently, it was reported that exenatide administration in an obese mouse model reversed hepatic steatosis by increasing fatty acid oxidation and/or inhibiting de novo lipogenesis [14,38]. The correction of hepatic steatosis relieved hepatic insulin resistance. The results of Li et al [38] supported our findings that long-term treatment with exenatide prevented a high-fat diet–induced deterioration in peripheral and hepatic insulin sensitivity in euglycemic-hyperinsulinemic clamps. In addition, they revealed that exenatide treatment decreased tyrosine phosphorylation of IRS1 in fat and skeletal muscles. Chang et al [17] also found that exercise decreased PEPCK gene expression in obese Zucker rats. This is related to changes in the hepatic insulin-IRS2 pathway. By disrupting hepatic IRS2, insulin cannot suppress gluconeogenesis because of the attenuation of insulin signaling of IRS2 → Akt → Forkhead transcription factor in hepatocytes [36,39]. Furthermore, hepatic IRS2 overexpression attenuates gluconeogenesis and reduces fasting glucose levels [39]. However, although hepatic-specific IRS1 knockout did not alter hepatic glucose production, liver-specific IRS1 and IRS2 knockout mice had worsened hepatic glucose homeostasis in comparison with hepatic IRS2 knockout mice [40]. This indicated that IRS proteins in the liver were redundant and that IRS2 played a more crucial role in regulating hepatic glucose production than IRS1. In the present study, tyrosine phosphorylation and the expression of IRS2, not IRS1, were potentiated in rats undergoing exendin-4 and exercise treatment. Thus, the increase in hepatic IRS2 expression, at least partly, has a direct impact on the modulation of glucose homeostasis in the liver.

In summary, exendin-4 and exercise improved glucose homeostasis; but only exendin-4 reduced overnight-fasted serum glucagon levels. Both exendin-4 and exercise increased whole-body glucose infusion rates and decreased hepatic glucose output at hyperinsulinemic states. The reduction of hepatic glucose production was due to decreased PEPCK expression, which was partly the result of enhanced insulin signaling through IRS2 induction in the liver. Because exendin-4 in combination with exercise has no additive effects in hepatic insulin action, both partly have a common pathway. Only exercise improved hepatic insulin action and potentiated both hepatic and hypothalamic insulin signaling, but exendin-4 enhanced hepatic insulin action without any improvement in hypothalamic insulin signaling. However, exendin-4 did improve hypothalamic glucose sensing. Thus, exendin-4 and exercise improve hepatic insulin action in addition to enhancing hypothalamic glucose sensing.

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