Multiple mechanisms of GW-9508, a selective G protein-coupled receptor 40 agonist, in the regulation of glucose homeostasis and insulin sensitivity

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Ou HY, Wu HT, Hung HC, Yang YC, Wu JS, Chang CJ. Multiple mechanisms of GW-9508, a selective G protein-coupled receptor 40 agonist, in the regulation of glucose homeostasis and insulin sensitivity. Am J Physiol Endocrinol Metab 67: E668–E676, 2013. First published January 22, 2013; doi:10.1152/ajpendo.00419.2012.—Activation of G protein-coupled receptor 40 (GPR40) by agonists increases insulin release in isolated islets, whereas it is inconclusive whether GPR40 antagonists decrease blood glucose and increase insulin sensitivity. Although some clinical trials indicated that administration of a GPR40 agonist shows benefits in the regulation of blood glucose homeostasis, the pharmacological mechanisms of this receptor in the improvement of glycemic control remain unclear. Therefore, we used a selective GPR40 agonist, GW-9508, to clarify the role of GPR40 in the regulation of blood glucose. Bolus intraperitoneal injection of GW-9508 in mice showed a slight decrease in blood glucose, with an increase in plasma insulin levels under glucose stimuli. However, long-term treatment with low doses of GW-9508 in high-fat diet-induced (HFD) diabetic mice decreased blood glucose with decreased plasma insulin significantly and improved glucose intolerance and insulin resistance. Using small interfering ribonucleic acid to delete GPR40 in HepG2 cells, we demonstrated that GW-9508 reversed palmitate-induced insulin signaling impairment through a GPR40-dependent pathway. We also found that GW-9508 activates the Akt/GSK-3β pathway to increase glycogen levels in HepG2 cells. Furthermore, administration of GW-9508 decreased the hepatic expression of fetuin-A in HFD mice significantly and regulated high-glucose- or palmitate-induced fetuin-A expression to increase insulin sensitivity through a GPR40/PLC/PKC pathway in HepG2 cells. Taken together, GW-9508 exerts a partial agonist effect to regulate blood glucose through multiple mechanisms. Investigation of chemicals that act on GPR40 might be a new strategy for the treatment of diabetes.

diabetes; fetuin-A; G protein-coupled receptor 40; insulin resistance; partial agonist

THE FREE FATTY ACID RECEPTOR (FFAR) family plays critical roles in a variety of physiological and pathophysiological processes, especially in metabolic disorders (12). One member of the FFAR, G protein-coupled receptor 40 (GPR40), also known as FFAR1, functions as a receptor for medium- and long-chain fatty acids (5). GPR40 is expressed abundantly in insulin-producing pancreatic islet cells (13) and is also expressed in the intestine and endocrine cells of the gastrointestinal tract (9) as well as hepatocytes (27, 30). GPR40 couples with a G protein α-sub-unit of the Gq family (12), and its activation in pancreatic islets induces the activity of phospholipase C (PLC) and the hydrolysis of inositol lipids, as well as the intracellular calcium levels (23), and therefore, it increases the secretion of insulin (13, 23).

Although the functions of GPR40 in the regulation of insulin secretion are well known, it is still a matter of debate whether activation of GPR40 would be beneficial for the treatment of type 2 diabetes. In humans, two recent phase 2 clinical trials showed that administration of TAK-875, a GPR40 agonist, in patients with type 2 diabetes improves glucose tolerance (2) and glycemic control significantly (6) without hypoglycemia risk. However, one study showed that GPR40 antagonist improves insulin tolerance and increases insulin signaling in diabetic Zucker rats (32). The findings of these works raise questions about the role of GPR40 in the treatment of diabetes.

Fetuin-A, also known as α2-Heremans-Schmid glycoprotein, is a glycoprotein synthesized mainly in the liver. Fetuin-A is not only a prominent serum protein but also a major component of mineralized bone. Previous studies have demonstrated that fetuin-A regulates osteogenesis (3) and promotes the deposition of serum minerals within bone collagen fibrils (28). In addition, fetuin-A is an endogenous inhibitor of the insulin receptor tyrosine kinase (21, 25), and fetuin-A knockout mice exhibit increased insulin sensitivity (17). Previous studies found that palmitate increased the expression of fetuin-A in HepG2 cells via activation of nuclear factor κB (NF-κB) (8) or endoplasmic reticulum stress induction (18). In humans, serum fetuin-A concentrations have been shown to be associated with insulin resistance (26), impaired glucose tolerance, type 2 diabetes (20), and hepatic steatosis (19). Given the important role of fetuin-A in insulin sensitivity and glucose metabolism, no pharmacological agent has been developed to decrease fetuin-A levels. Moreover, the role of GPR40 in the regulation of fetuin-A levels remains unknown.

Although the function of GPR40 in the regulation of insulin secretion is well known, whether its activation in hepatocytes regulates insulin action or fetuin-A expression remains unclear. Because both impaired insulin secretion and insulin resistance are important in the pathogenesis of type 2 diabetes, it is necessary to investigate the role of GPR40 agonists in insulin sensitivity before considering them in the treatment of diabetes. In this study, we used high-fat diet (HFD)-induced diabetic mice to evaluate the effects of a GPR40 agonist, GW-9508, on glucose homeostasis, insulin sensitivity, and hepatic fetuin-A expression. In addition, human hepatoma cell line HepG2 cells were also used to identify the possible mechanisms for GPR40 in the regulation of insulin sensitivity and fetuin-A expression.
MATERIALS AND METHODS

Materials. Chelethyrine was purchased from Sigma-Aldrich (St. Louis, MO). GW-9508, BAPTA-AM, U-73122, and U-73343 were purchased from Tocris Bioscience (Ellisville, MO). Primary antibodies, including insulin receptor (Neomarkers, Fremont, CA), phospho-insulin receptor (Upstate Biotechnology, Temecula, CA), Akt, phospho-Akt, GSK-3β and phospho-GSK-3β (Cell Signaling Technology, Beverly, MA), GPR40 (Abcam, Cambridge, UK), and actin (Sigma-Aldrich), were purchased from commercial companies. Insulin ELISA kits were purchased from Mercodia (Uppsala, Sweden).

Animals. C57BL/6J male mice were purchased from the Animal Center of National Cheng Kung University Medical College. Mice were housed in a temperature- (25 ± 1°C) and humidity-controlled (60 ± 5%) room and kept on a 12:12-h light-dark cycle (lights on at 0600). C57BL/6J mice were fed a high-fat diet containing 34.9% fat (wt/wt) for 12 wk (58Y1; TestDiet, Richmond, IN) starting from 8 wk of age. For the short-term treatment experiment, 8-wk-old mice were injected intraperitoneally (ip) with 50 mg/kg GW-9508. For the long-term treatment experiment, the mice were fed a HFD containing GW-9508 at indicated doses for 7 days at the end of 3 mo of HFD. The animal use protocol adopted in this study was reviewed and approved by the Institutional Animal Care and Use Committee of National Cheng Kung University (approval no. 101177). The animal experiments were also conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health as well as the guidelines of the Animal Welfare Act. All of the animal experiments were performed under pentobarbital sodium anesthesia, and all efforts to minimize suffering were made.

Fig. 1. Bolus injection of GW-9508 increases insulin secretion to regulate blood glucose levels in wild-type mice. Eight-week-old wild-type mice were fasted for 8 h. Each group of mice was then administered 50 mg/kg GW-9508 (●; A) or saline intraperitoneally (ip) (○; B). Another 2 groups of mice were injected ip with 50 mg/kg GW-9508 or saline prior to the injection of 1 g/kg glucose for 30 min (C and D). The blood samples were then collected from the retroorbital sinus and centrifuged at 12,000 rpm for 5 min for the determination of plasma insulin (C) and glucose (D) levels. Data are expressed as means ± SE from 8–10 animals of each group. *P < 0.05 compared with saline-injected control group. AUC, area under the curve.
Insulin tolerance test. The mice were fasted for 8 h, and then their insulin tolerance was tested and initial blood glucose determined. The mice were then given insulin (1.0 mU/kg ip; Novo Nordisk, Bagsvaerd, Denmark), and the blood samples were collected from the retroorbital sinus of each mouse at 0, 30, 60, 90, and 120 min postinjection for blood glucose measurements. The glucose disappearance constant ($K_g$), a measure of insulin sensitivity, was calculated as the slope of the natural log of glucose from 0 to 60 min, expressed as the percentage of change per minute.

Intraperitoneal glucose tolerance test. The mice were fasted for 8 h and then given glucose (1.0 g/kg ip). Blood samples were collected from the retroorbital sinus at regular intervals, and then the glucose concentration was measured. Integration of the glucose and insulin tolerance test curves (i.e., areas under the curve) were calculated by the standard trapezoid method (15).

Cell culture. HepG2 cell line was purchased from the Bioresource Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan). The cells were cultured at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (100 IU/ml penicillin, 100 mg/ml streptomycin). The cells were grown in six-well culture dishes at a density of 2 × 10⁵ cells/well.

Small-interfering RNA transfection. HepG2 cells were transfected with duplexed RNA oligonucleotides (Stealth RNAi; Invitrogen) of human GPR40 or scrambled siRNA (as a negative control), using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The cells were treated with GW-9508 at 48-h posttransfection.

Western blot analysis. After starvation for 24 h, the mice were given 5 units of insulin (Novo Nordisk) in the inferior vena cava. After 5 min, the liver samples were removed and then homogenized with ice-cold phosphate-buffered saline containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein lysates (30 μg) of liver tissues or cells were separated using 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). After they had been blocked with 10% milk in TBS-T [10 mM Tris (pH 7.6), 150 mM NaCl, and 0.05% Tween-20], the membranes were incubated with appropriate primary antibodies. The relative signal intensity was quantified using ImageJ software from W. Rasband.

RESULTS

Single bolus injection of GW-9508 potentiates glucose-dependent insulin secretion significantly in wild-type mice. To investigate the effects of GW-9508 in vivo, C57BL/6J mice received a single bolus intraperitoneal injection of 50 mg/kg GW-9508 to detect the blood glucose and plasma insulin levels (Fig. 1). Injection of GW-9508 alone showed no effects on the blood glucose (Fig. 1A) or plasma insulin levels (Fig. 1B) of the mice. However, administration of GW-9508 enhanced glucose-induced plasma insulin levels significantly (Fig. 1C) and thus decreased glucose injection-induced hyperglycemia (Fig. 1D).

Improvement of insulin resistance in a dose-related manner in HFD-induced insulin-resistant mice after being fed a HFD containing GW-9508 for 7 days. We further investigated the effect of GW-9508 in HFD-induced insulin-resistant mice (HFD mice) after a long-term administration. Administration of GW-9508 for 7 days did not significantly affect daily food intake (control group, 2.32 ± 0.38 g; GW-9508 group, 2.18 ± 0.29 g; P = not significant) or body weight of the HFD mice (Fig. 2A). Consistent with the results of single bolus injection of GW-9508 in wild-type mice, mice fed a high-fat diet that contained GW-9508 for 7 days had significantly decreased blood glucose levels (Fig. 2B). However, in contrast to the
increase in plasma insulin levels after bolus injection of GW-9508, administration of GW-9508 for 7 days significantly decreased the plasma insulin levels in a dose-related manner (Fig. 2C). In addition, the values of HOMA-IR were dose-dependently decreased by GW-9508, implying that the increase in insulin sensitivity was caused by the long-term administration of GW-9508 in HFD mice (Fig. 2D). Therefore, we further confirmed that GW-9508 improved insulin resistance (Fig. 3). The fasting blood glucose levels were decreased significantly after administration of GW-9508 for 7 days (Fig. 3A). In addition, both the glucose utility (Fig. 3A) and insulin sensitivity (Fig. 3B) were improved. Moreover, HFD-induced impairment of insulin signaling in the liver (Fig. 3C) and muscle (Fig. 3D) was also improved by GW-9508.

GW-9508 increases hepatic glycogen content in HFD mice and improves palmitate-induced insulin signaling impairment through GPR40. We further investigated the effect of GW-9508 on hepatic glycogen content. The results show that

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Fig. 3. Long-term administration of GW-9508 increases glucose utility and insulin sensitivity in HFD-induced insulin-resistant mice. Each group of mice was fasted for 8 h and then injected ip with 1 g/kg glucose for glucose tolerance test (A) or 1 U/kg insulin for insulin sensitivity test (B). The blood samples were then collected to determine plasma glucose levels. Glucose AUC and glucose disappearance constant were calculated accordingly. After fasting for 24 h, the mice were given 5 units of insulin in the inferior vena cava. After 5 min, the liver (C) and muscle (D) were removed for the detection of phospho-insulin receptor (p-IR) or phospho-Akt levels. Data are expressed as means ± SE from 8 animals in each group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the normal chow or indicated groups.
GW-9508 treatment for 7 days can increase the hepatic glycogen content significantly (Fig. 4A). Similarly, the glycogen content in HepG2 cells was dose-dependently increased (Fig. 4C), and this effect was further diminished by deletion of GPR40 (Fig. 4D). We also found that GW-9508 itself slightly induced the phosphorylation of insulin receptors at 5 min and then declined at 60 min. The phosphorylation of the insulin receptors reached another peak at 360 min. Following the phosphorylation of the insulin receptors, the phosphorylation of Akt was observed at 15 min and GSK-3β at 30 min after the treatment of GW-9508 in HepG2 cells, implying an increase in insulin sensitivity (Fig. 4B). We then investigated the role of GPR40 in palmitate-induced insulin signaling impairment. Treatment of HepG2 cells with palmitate impaired insulin signaling significantly, as determined by the decrease in insulin-induced phosphorylation of insulin receptors and Akt. Pretreatment with GW-9508 reversed palmitate-induced insulin signaling impairment significantly (Fig. 5A). The effect of GW-9508 on palmitate-induced insulin signaling impairment was reversed partially by deletion of GPR40 (Fig. 5B). These results suggest that GW-9508 improved palmitate-induced insulin signaling impairment partially through GPR40 in HepG2 cells.

GW-9508 decreases high-glucose-induced fetuin-A expression through the GPR40 pathway. GW-9508 treatment for 7 days dose-dependently decreased hepatic fetuin-A expression (Fig. 6A) in HFD mice. High glucose-induced fetuin-A expression was dose- (Fig. 6B) and time-dependently decreased (Fig. 6C) after treatment with GW-9508. Deletion of GPR40 by siRNA reduced the effect of GW-9508 on the expression of fetuin-A (Fig. 6D). We further investigated the role of the downstream signaling of GPR40 in the expression of fetuin-A. Pretreatment with the inhibitors of PLC (U-73122), PKC (chelerythrine), and calcium (BAPTA-AM) reversed the effect of GW-9508 on fetuin-A expression (Fig. 6E), whereas pretreatment with the inactive analog of U-73122, U-73343, had

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**Fig. 4. GW-9508 activates Akt/GSK-3β pathway to increase glycogen content in HepG2 cells.** Eight-week-old mice were fed a HFD for 3 mo and supplied with indicated doses of GW-9508 in their diet for 7 days. A: the hepatic glycogen content of mice was determined at the end of the experiment. HepG2 cells were starved overnight and then treated with 10 μM GW-9508. B: cell lysates were collected at the indicated times after GW-9508 treatment for the detection of p-IR, p-Akt, or p-GSK-3β levels. C: HepG2 cells were treated with the indicated doses for 24 h and harvested for the determination of cellular glycogen contents. D: cells were transfected with duplexed RNA oligonucleotides of G protein-coupled receptor 40 (GPR40) or scrambled small-interfering RNA and treated with 10 μM GW-9508 for 24 h and then starved for the detection of the glycogen content. Data are expressed as means ± SE and obtained from 3 individual experiments. *P < 0.05 and **P < 0.01 compared with the control group.
no effect on the decrease in GW-9508-induced fetuin-A expression.

GW-9508 decreases palmitate-induced fetuin-A expression through the GPR40 pathway. GW-9508 not only reversed high-glucose-induced fetuin-A expression but also dose- (Fig. 7A) and time-dependently reversed (Fig. 7B) the palmitate-induced fetuin-A expression. Deletion of GPR40 by siRNA diminished the effect of GW-9508 on fetuin-A expression (Fig. 7C). Pretreatment with the inhibitors of PLC, PKC, and calcium reversed the effect of GW-9508 on fetuin-A expression (Fig. 7D), whereas pretreatment with the inactive analog of U-73122, U-73343, had no effect on the decrease in GW-9508-induced fetuin-A expression.

DISCUSSION

To the best of our knowledge, this is the first study to investigate the mechanism of GPR40 in the regulation of glucose homeostasis and insulin sensitivity. We found that activation of GPR40 by GW-9508 can improve insulin resistance and glucose intolerance in HFD mice. In addition, we further demonstrated that GW-9508 increased the phosphorylation of insulin signaling-related proteins in HepG2 cells to increase intracellular glycogen content. GW-9508 also decreased hepatic fetuin-A expression through a PLC/PKC pathway to increase insulin sensitivity. Our results suggest that GPR40 can regulate blood glucose homeostasis through both insulin receptor-independent and dependent pathways in addition to its insulinotropic effect. This insulin-sensitizing effect supports the use of GPR40 agonists in the treatment of type 2 diabetes.

Consistent with a previous study (31), we found that acute administration of GW-9508 significantly enhanced glucose-induced plasma insulin levels in vivo (Fig. 1); however, we found that the plasma insulin levels were decreased after long-term treatment with GW-9508 without the body weight of HFD mice being affected (Fig. 2). The decreased HOMA-IR value induced by GW-9508 suggests an improvement in insulin sensitivity (Fig. 2D). Therefore, we followed Kamei et al. (14) and confirmed the increase in insulin sensitivity by detection of downstream insulin signaling. Administration of GW-9508 in HFD mice increased not only glucose utility and insulin sensitivity but also the phosphorylation of Akt and insulin receptor in the liver and muscle (Fig. 3). Our results are similar to those of Zhang et al. (32), which indicated that a GPR40 antagonist decreased plasma insulin levels and increased insulin sensitivity in diabetic rats. These results imply that GW-9508 may have a partial agonist effect in the regulation of glucose homeostasis and are in agreement with the findings of Briscoe et al. (4), which indicated that GW-9508 is a partial agonist of GPR40.

We then used HepG2 cells to investigate the possible mechanisms of GW-9508 to improve insulin sensitivity (Fig. 4). Conversion of glucose into glycogen is a major pathway for the removal of blood glucose by the liver during the postprandial state (1). Hepatic glycogen synthesis and breakdown play important roles in blood glucose homeostasis (24). An increase in glycogen levels in the liver improves hyperglycemia in type 2 diabetic mice (33). Insulin reportedly increases the activity of glycogen synthase, the rate-limiting enzyme for glycogen synthesis, by decreasing the phosphorylation of glycogen synthase through insulin-mediated phosphorylation of GSK-3β and inhibiting its activity (11). In addition to insulin signaling, activation of the PKC pathway possibly activates the Akt/GSK-3β pathway to increase glycogen content (16). In this
study, we found that administration of a HFD in mice increases the glycogen content significantly because of the excess nutrition (29), and administration of GW-9508 further increases the content of glycogen in liver (Fig. 4A). GW-9508 activated Akt to phosphorylate GSK3β (Fig. 4B) and further increased the glycogen content in HepG2 cells, implying that GW-9508 works to increase insulin sensitivity (Fig. 4C). In addition, although it has been shown in both functional and binding assays to be at least 100-fold more selective for GPR40 than for 220 other G protein-coupled receptors, 60 kinases, 63 proteases, seven integrins, and 20 nuclear receptors, including PPARs (4), deletion of GPR40 in HepG2 cells, as carried out in a previous study (30), diminished GW-9508-induced glycogen levels, indicating that GW-9508 increased glycogen levels through a GPR40-dependent mechanism (Fig. 4D). Notably, GW-9508 significantly increased the phosphorylation of insu-
lin receptors in a late time course at 6 h (Fig. 4B). Therefore, we investigated the mechanism that increases the phosphorylation of the insulin receptors.

Palmitate is a saturated fatty acid that induces lipid accumulation and inhibits signaling downstream of the insulin receptor (22). Although palmitate, as well as other medium- to long-chain saturated and unsaturated fatty acids, is able to increase intracellular calcium in a concentration-dependent manner in human GPR40-expressing human embryonic kidney-293 cells (5), the substrate specificity of the GPR40 receptor is not congruent with the pattern of fatty acid toxicity. Saturated and unsaturated fatty acid exerted completely different effects on /H9252-cell viability. Long-chain saturated fatty acids show a strong cytotoxic effect, whereas unsaturated long-chain fatty acids are well tolerated. Binding to GPR40 cannot explain these different effects, because both saturated and unsaturated long-chain fatty acids are ligands for GPR40. In addition, some studies with GPR40 KO mice showed that GPR40 does not mediate lipotoxicity (10). Therefore, we used palmitate to evaluate the effect of GW-9508 on palmitate-induced insulin signaling impairment in HepG2 cells (Fig. 5), and the results show that it significantly reversed palmitate-induced insulin signaling impairment. Administration of GW-9508 recovered the phosphorylation of Akt in palmitate-induced insulin signaling impairment, whereas it had no effect on the phosphorylation of insulin receptors (Fig. 5A). Deletion of GPR40 diminished the effect GW-9508 had on Akt phosphorylation, indicating that GW-9508 increased insulin sensitivity through a GPR40-dependent pathway (Fig. 5B).

Fetuin-A levels are positively associated with impaired glucose tolerance and diabetes in humans (20). It has also been reported that fetuin-A is an endogenous inhibitor of the insulin receptor tyrosine kinase (21), and fetuin-A knockout mice exhibit increased insulin sensitivity with decreased plasma insulin levels (17). Using recombinant mouse fetuin-A expressed from a recombinant baculovirus, Cintrón et al. (7) demonstrated that mouse fetuin-A inhibits insulin-stimulated insulin receptor autophosphorylation in vitro. In this study, we observed that GW-9508 increased the phosphorylation of insulin receptor at both 5 min and 6 h. Consistent with this result, GW-9508 significantly decreased the expression of fetuin-A in HFD mice (Fig. 6A) as well as in HepG2 cells in a dose-related manner at 6 h after the treatment (Fig. 6, B and C). We further evaluated the role of GPR40 in the GW-9508-induced decline in fetuin-A. Deletion of GPR40 in HepG2 cells significantly reduced the GW-9508-induced decrease in fetuin-A expression (Fig. 6D). In addition, pretreatment with PLC, PKC, or calcium inhibitors reversed the effect of GW-9508 on fetuin-A expression, implying that GPR40 inhibits fetuin-A expression through a PLC/PKC and calcium-dependent pathway (Fig. 6E).

Taken together, the results presented in this work demonstrate that GW-9508 may have a partial agonist effect to regulate blood glucose homeostasis. GW-9508 activates Akt and decreases fetuin-A levels to enhance insulin signaling. Therefore, the potential effects of GW-9508 with regard to the treatment of diabetes in humans deserve further attention in future research.
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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