The Fatty-Acid Receptor GPR40 Plays a Role in Insulin Secretion In Vivo After High-Fat Feeding

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Running title: GPR40 and insulin secretion

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OBJECTIVES - The G protein-coupled receptor GPR40 is expressed in pancreatic β-cells and is activated by long-chain fatty acids (FAs). Gene deletion studies have shown that GPR40 mediates, at least in part, FA-amplification of glucose-induced insulin secretion (GSIS) but is not implicated in GSIS itself. However, the role of GPR40 in the long-term effects of FAs on insulin secretion remains controversial. This study was aimed to test the hypothesis that GPR40 plays a role in insulin secretion after high-fat feeding.

RESEARCH DESIGN AND METHODS- GPR40 knock-out (KO) mice on a C57BL/6 background and their wild-type (WT) littermates were fed a high-fat diet (HFD) for 11 weeks. Glucose tolerance, insulin tolerance, and insulin secretion in response to glucose and Intralipid were assessed during the course of the diet period.

RESULTS- GPR40 KO mice had fasting hyperglycemia. They became as obese, as glucose intolerant and as insulin resistant as their WT littermates under HFD, and developed a similar degree of liver steatosis. Their fasting blood glucose increased earlier than those of control mice during the course of the HFD. The remarkable increase in insulin secretory responses to intravenous glucose and Intralipid seen in WT mice after HFD was of much lower magnitude in GPR40 KO mice.

CONCLUSIONS- GPR40 plays a role not only in fatty-acid modulation of insulin secretion, but also in GSIS after high-fat feeding. These observations raise doubts on the validity of a therapeutic approach based on GPR40 antagonism for the treatment of type 2 diabetes.
As do not initiate insulin release in the absence of glucose but potentiate GSIS upon acute exposure. Their mechanisms of action however are incompletely understood. The discovery of GPR40 as a G-protein coupled receptor highly expressed in pancreatic β-cells and activated by long-chain FAs (1-4) has identified a novel mechanism of action of FAs on insulin secretion. Loss of function of GPR40 via small interfering RNA (2; 5-7), antisense oligonucleotides (8), pharmacological inhibitors (9) or gene deletion in the mouse (10; 11) partially suppresses FA potentiation of GSIS in vitro. We (10) and others (11) have shown that whole-body GPR40 KO mice have normal glucose tolerance and unaltered insulin secretion in response to glucose in vivo and in vitro, but that isolated islets from these mice secrete less insulin in response to FAs. Furthermore, insulin secretion induced by Intralipid in vivo is reduced in GPR40 KO mice, demonstrating a physiological role for GPR40 in FA-potentiation of GSIS (10).

GPR40 has received considerable attention as a potential therapeutic target in type 2 diabetes (12-15). Surprisingly, whether an agonist or antagonist should be developed as a therapeutic agent remains debated (13; 15). This uncertainty stems, in part, from conflicting reports regarding its role on β-cell function (10; 11). Steneberg et al. (11) found that islets isolated from GPR40 KO mice were protected from the inhibitory effects of prolonged FA exposure on GSIS, in contrast to our findings in a different line of GPR40 KO mice (10) and a recent study using GPR40 agonists (16).

Steneberg et al. (11) further showed that GPR40 KO mice were protected from high-fat diet-induced insulin resistance, glucose intolerance and hepatic steatosis. Given these discrepancies and the importance of determining whether an agonist or antagonist approach should be pursued for drug development, the present study was designed to test the hypothesis that GPR40 contributes to the enhancement of insulin secretion after HFD. Specifically, we sought to examine 1) whether GPR40 KO mice are more susceptible to HFD-induced hyperglycemia; if so, 2) whether this is due to changes in insulin secretion in vivo; and 3) whether it is associated with changes in the expression of genes controlling FA metabolism in islets.

**RESEARCH DESIGN AND METHODS**

**Reagents-** Fifty percent dextrose was from McKesson Canada Corp (Montreal, QC), 0.9% saline was from Baxter (Mississauga, ON), and Intralipid was from Fresenius Kabi (Uppsala, Sweden). All other reagents were from Sigma (St. Louis, MO), unless otherwise noted.

**Animals and diets-** GPR40 KO mice were generated as described (10) and backcrossed to the C57BL/6 strain for more than 7 generations at Amgen, Inc. (San Francisco, CA). Animals were housed under controlled temperature (21°C) and a 12-hour light/dark cycle with free access to food and water. At 7 weeks of age male GPR40 KO and WT littermates were fed either high-fat (60% fat, 16% proteins and 24% carbohydrates on caloric basis (#F3282; Bioserv Diets, Frenchtown, New Jersey)) or regular (23% fat, 17% protein and 60% carbohydrates on caloric basis (#2018;
Harlan Teklad, Madison, WI) diet. Mice were housed individually and body weight and food intake determined weekly. All procedures using animals were approved by the Institutional Committee for the protection of Animals at the Centre Hospitalier de l'Université de Montréal.

Assessment of glucose homeostasis and insulin secretion- Fasting blood glucose was measured weekly. Oral glucose tolerance was assessed in overnight-fastened animals after administration of 1g/kg glucose by gavage (10). Insulin tolerance was measured in 5-hour fasted animals after intraperitoneal administration of 1U/kg of human insulin (10). For measurements of insulin secretion in vivo, a catheter was inserted into the right jugular vein under general anesthesia. Insulin secretion in response to intravenous (IV) glucose (0.5g/Kg) or Intralipid (100µl of a 20% solution preceded by 30 U of heparin) was measured as described (10).

Tissue harvesting and histology- Mice were sacrificed after 12 weeks of diet and islets were isolated as described (10). Livers were snap-frozen in liquid nitrogen, embedded in OCT, pre-frozen in anhydrous ethyl alcohol (100% ethanol), and stained with Oil Red O.

Gene expression studies- Total RNA was extracted from approximately 150 islets and RT-PCR was carried out using QuantiTect SYBR Green PCR kit (Qiagen) as described (17). Results are expressed as the ratio of target mRNA to β-actin mRNA and normalized to the levels in islets from RD-fed WT mice, arbitrarily set as 1.

Analytical measurements- Plasma glucose and FA levels were measured enzymatically (Wako Chemicals, Neuss, Germany). Plasma insulin was measured using a mouse ELISA kit (Alpco Diagnostics, NH).

Expression of data and statistics- Data are expressed as mean ± SE. Intergroup comparisons were performed by ANOVA with post-hoc adjustments for 2-by-2 comparisons or Student's t-test, where appropriate. P < 0.05 was considered significant. In Table 1 and Figure 3, glucose clearance and insulin secretion are expressed as the area under the curve (AUC), calculated above basal values and expressed as Arbitrary Units (AU).

RESULTS AND DISCUSSION

Metabolic characteristics of GPR40 KO mice on a C57BL/6 background- Seven-week old GPR40 KO mice on a C57BL/6 background had similar body weight as their WT littermates (22.5 ± 0.9 vs. 21.6 ± 0.9 g; n=10 each; NS) but showed fasting hyperglycemia (135.8 ± 5.3 vs. 115.4 ± 4.1 mg/dl; n=19-22 per group; p< 0.05; Fig. 1A). Glucose and insulin tolerance were similar in GPR40 KO and WT mice at 7 weeks of age (Supplementary Fig. S1). Thus, on a pure C57BL/6 background, deletion of GPR40 leads to fasting hyperglycemia despite normal glucose and insulin tolerance, suggesting that GPR40 contributes to the maintenance of blood glucose levels during fasting. In the fasting state, circulating levels of FAs increase and the β-cell uses FAs as its main energy source, with a corresponding increase in FA oxidation (18). Our results therefore
suggest that GPR40-mediated FA signaling contributes to the maintenance of basal insulin secretion – and thereby normoglycemia – during fasting.

**Effects of HFD on glucose and insulin tolerance in GPR40 KO mice** - Body weight, fat pad weight (white and brown adipose tissue), and food intake increased to similar levels in GPR40 KO and WT mice under HFD (Table 1). As expected, WT mice developed fasting hyperglycemia after 8 weeks of HFD. Fasting blood glucose increased earlier in GPR40 KO mice during the course of the HFD (Fig. 1B). Thus, after 3 weeks on diet, blood glucose levels of the KO mice on HFD were significantly higher than those on RD (123.8 ± 5.2 vs. 101.9 ± 5.8 mg/dl; n= 8-11 per group; P<0.006), whereas WT mice on HFD and RD had similar blood glucose values (102.5 ± 3.5 vs. 93.3 ± 1.7 mg/dl, n= 10-11 per group; P>0.08). Both GPR40 KO and WT animals were hyperglycemic after 8 weeks of HFD. Oral and IV glucose tolerance were impaired to a similar level in WT and KO mice after HFD (Fig. 2A and Table 1) compared to mice on RD (Supplementary Fig. S2A). Similarly, the glucose-lowering effect of insulin was reduced to the same extent in both genotypes on HFD (Fig. 2B) compared to mice on RD (Supplementary Fig. 2B). Liver steatosis developed in all animals examined in both genotypes under HFD (Fig. 2C). Thus, GPR40 KO mice on a C57BL/6 background have fasting hyperglycemia which is further aggravated during HFD. Furthermore, HFD in both GPR40 KO mice and their WT littermates is associated with insulin resistance and glucose intolerance. Although we have not performed euglycemic-hyperinsulinemic clamps to directly measure insulin sensitivity, the observed decrease in the glucose-lowering effects of insulin, combined with the presence of liver steatosis, indicate that GPR40 KO mice were not protected from the development of insulin resistance during HFD. We suspect that the discrepancies between our results and those of Steneberg et al. (11) might be related to the genetic background of the mice, an important determinant of susceptibility to glucose intolerance (19). The mice used by Steneberg et al. (11) were on a mixed background, as opposed to the C57BL/6 background used in the present study.

**Effects of HFD on insulin secretion in GPR40 KO mice** - To examine whether GPR40 plays a role in insulin secretion after HFD, we measured insulin release in response to glucose and Intralipid after 10 and 11 weeks, respectively, of HFD or RD. Insulin secretion in response to IV glucose was similar in RD-fed GPR40 KO and WT mice (Fig. 3A, C, D). After administration of HFD, the second phase insulin secretion to IV glucose was greatly enhanced in WT mice (Fig 3B&D) and this increase was significantly blunted in GPR40 KO mice (Fig 3B&D). These results suggest GPR40 becomes rate-limiting for GSIS after HFD. These findings are consistent with the observation that enhancement of intracellular lipid signaling plays a role in β-cell compensation for insulin resistance (20). Interestingly, the marked differences in GSIS between GPR40 KO and WT mice after HFD were not accompanied by changes in glucose clearance, in keeping with the notion that glucose clearance after an IV load in mice is mostly
determined by insulin-independent mechanisms (21; 22).

As shown in Fig. 3E&G, GPR40 KO mice on a C57BL/6 background have reduced insulin secretion in response to Intralipid, as previously shown in mice on a mixed background (23). Thus, first-phase insulin secretion was lower in GPR40 KO mice in response to Intralipid compared to WT littermates (6.72 ± 1.59 vs. 11.10 ± 1.47 AU; n=6-7; P<0.05). After 11 weeks on HFD, WT mice had a markedly increased response to IV Intralipid compared to RD-fed mice (Fig 3F-H). In contrast, the increase in first-phase insulin release in response to Intralipid after HFD was markedly reduced in GPR40 KO mice (Fig. 3F-H). These findings show that GPR40 not only plays a role in insulin secretion in response to FAs under basal conditions, but is also essential for the β-cell to mount a compensatory increase in insulin secretion in response to both glucose and FAs in the face of HFD-induced insulin resistance.

**Effects of HFD on gene expression in GPR40 KO mice**- To gain insight into the molecular basis for the role of GPR40 in insulin secretion, mRNA levels of genes involved in FA transport and metabolism were measured in islets from GPR40 KO and WT mice after HFD or RD (Table 1). GPR120 is another receptor for long-chain FAs expressed in enteroendocrine cells (23). We found GPR120 mRNA to be expressed in isolated mouse islets, although its expression did not increase in compensation for the absence of GPR40 in GPR40 KO islets (Table 1). CD36 is a FA transport protein which plays an important role in FA uptake and FA-potentiation of GSIS (24). Carnitine palmitoyl-transferase-1 (CPT-1) catalyzes the rate-limiting step in FA oxidation, i.e. transport of long-chain acyl-CoAs across the mitochondrial membrane (25). GPR40 KO mice on RD displayed an approximately 5- and 7-fold increase in islet CD36 and CPT-1 mRNA expression, respectively, as compared to WT islets (Table 1), suggesting an attempt to compensate for the absence of GPR40 by enhancing FA transport and intracellular metabolism. Although expression of neither CD36 nor CPT-1 was significantly affected by HFD in WT animals, in KO islets the increase in CD36 and CPT1 gene expression was markedly reduced by HFD. This suggests that enhanced expression of CD36 and CPT1 perhaps contributes to the maintenance of normal glucose tolerance despite the absence of GPR40 under basal conditions, but that the absence of such an increase under HFD results in an inability of the mice to sustain normal GSIS.

**Conclusions**- GPR40 KO mice on a C57BL/6 background have fasting hyperglycemia and are not protected from HFD-induced insulin resistance, and that GPR40 is implicated not only in insulin secretion in response to FAs, but also in GSIS after HFD. These findings suggest that the mechanisms by which the β-cell mounts a compensatory response to HFD-induced insulin resistance involve, at least in part, signaling through GPR40. As such, our results raise doubts on the validity of a therapeutic approach based on GPR40 antagonism for the treatment of type 2 diabetes.
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|                          | WT-RD     | WT-HF     | KO-RD     | KO-HF     |
|--------------------------|-----------|-----------|-----------|-----------|
| Weight gain (g)          | 6.1 ± 0.7 | 14.5 ± 1.3*| 5.6 ± 0.8 | 11.5 ± 1.9*|
| Food intake (kcal/g/day) | 0.58 ± 0.02| 0.64 ± 0.05| 0.61 ± 0.02| 0.62 ± 0.05|
| Subcutaneous fat (g)     | 0.19 ± 0.02| 1.12 ± 0.13*| 0.11 ± 0.02| 0.84 ± 0.16*|
| Infra-renal fat (g)      | 0.04 ± 0.00| 0.39 ± 0.07*| 0.03 ± 0.00| 0.29 ± 0.06*|
| Brown adipose tissue (g) | 0.07 ± 0.01| 0.15 ± 0.02*| 0.11 ± 0.01| 0.20 ± 0.05*|
| AUC_glucose OGGT week 8  | 11,715 ± 1,104| 18,517 ± 1,612*| 13,183 ± 1,496| 17,847 ± 997*|
| AUC_glucose IVGT week 10 | 12,613 ± 1,491| 19,024 ± 2,445*| 10,949 ± 1,049| 15,348 ± 3,189*|
| GPR40 mRNA               | 1.0 ± 0.2 | 0.4 ± 0.2 | ND        | ND        |
| GPR120 mRNA              | 1.0 ± 0.4 | 0.7 ± 0.3 | 1.3 ± 0.4 | 0.7 ± 0.2 |
| CD36 mRNA                | 1.0 ± 0.3 | 1.4 ± 0.2 | 5.2 ± 0.4**| 2.6 ± 0.5 |
| CPT-1 mRNA               | 1.0 ± 0.2 | 0.9 ± 0.2 | 6.7 ± 1.0**| 1.1 ± 0.2 |

**Table 1**: Metabolic parameters of GPR40 KO and WT mice fed RD or HFD, and gene expression levels in islets. Values are expressed as mean ± SE of n=9-12 animals per group *: P<0.05 vs. RD and **: P<0.05 vs. WT.
FIGURE LEGENDS

Figure 1: Fasting blood glucose levels of GPR40 KO and WT mice (A) before and (B) during feeding a high-fat or regular diet. *: P<0.05

Figure 2: A: Glucose levels during oral glucose tolerance tests after 8 weeks of HFD. n=10-11 mice per group. B: Glucose levels during insulin tolerance tests after 9 weeks of HFD. n=8-10 mice per group. C: Oil Red O staining of liver sections of KO and WT mice fed regular or high-fat diet. Images are representative of 3-4 animals in each group.

Figure 3: Insulin levels in response to IV glucose after 10 weeks of RD (A) or HFD (B). Area under the curve for insulin over the first 10 min (C) and from 10-60 min (D) following glucose injection. Values are expressed as mean ± SE of 8-10 mice per group. Insulin levels in response to IV Intralipid after 11 weeks of RD (E) or HFD (F). Area under the curve for insulin for the first 10 min (G) and from 10-60 min (H) following Intralipid injection. Values are expressed as mean ± SE of 6-8 mice per group. *: P<0.
Figure 1

A

**Fasting Blood glucose (mg/dl)**

WT (n=22)  KO (n=19)

B

**Fasting Blood glucose (mg/dl)**

- WT RD (n=10)
- WT HF (n=11)
- KO RD (n=8)
- KO HF (n=11)

Time (weeks):

- 1
- 3
- 8

* indicates significant difference.
Figure 2

A. OGTT - Week 8

Blood glucose (mg/dl) vs. Time (min) for WT (n=11) and KO (n=10).

B. IPITT - Week 9

Blood glucose (% change from basal) vs. Time (min) for WT (n=10) and KO (n=8).

C. Histological images of KO and WT tissues.
Figure 3

A. IVGTT - Week 10 - RD

B. IVGTT - Week 10 - HFD

C. AUC First phase

D. AUC Second phase
E  IVILTT - Week - 11 - RD

Plasma Insulin (ng/ml)

- WT (n=6)
- KO (n=7)

Time (min)

F  IVILTT - Week - 11 - HFD

Plasma Insulin (mg/dl)

- WT (n=6)
- KO (n=8)

Time (min)

G  AUC First phase

AUC_{\text{insulin 0-10 min (ng/ml)}}

- WT
- KO

RD  HFD

H  AUC Second phase

AUC_{\text{insulin 10-60 min (mg/dl)}}

- WT
- KO

RD  HFD