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Deletion of GPR40 Impairs Glucose-induced Insulin Secretion in Vivo in Mice Without Affecting Intracellular Fuel Metabolism in Islets

Running title: GPR40 and insulin secretion

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Objective: The G protein-coupled receptor GPR40 mediates fatty-acid potentiation of glucose-stimulated insulin secretion, but its contribution to insulin secretion *in vivo* and mechanisms of action remain uncertain. This study was aimed to ascertain whether GPR40 controls insulin secretion *in vivo* and modulates intracellular fuel metabolism in islets.

Research design and methods: Insulin secretion and sensitivity were assessed in GPR40 knockout (KO) mice and their wild-type (WT) littermates by hyperglycemic clamps and hyperinsulinemic euglycemic clamps, respectively. Transcriptomic analysis, metabolic studies and lipid profiling were used to ascertain whether GPR40 modulates intracellular fuel metabolism in islets.

Results: Both glucose- and arginine-stimulated insulin secretion in vivo were decreased by approximately 60% in GPR40 KO fasted and fed mice, without changes in insulin sensitivity. Neither gene expression profiles nor intracellular metabolism of glucose and palmitate in isolated islets were affected by GPR40 deletion. Lipid profiling of isolated islets revealed that the increase in triglyceride and decrease in lyso-phosphatidylethanolamine species in response to palmitate in vitro was similar in WT and KO islets. In contrast, the increase in intracellular inositol phosphate levels observed in WT islets in response to fatty acids in vitro was absent in KO islets.

Conclusions: These results indicate that deletion of GPR40 impairs insulin secretion *in vivo* not only in response to fatty acids but also to glucose and arginine, without altering intracellular fuel metabolism in islets, via a mechanism that may involve the generation of inositol phosphates downstream of GPR40 activation.

Since its deorphanization in 2003 (1; 2), the G-protein coupled fatty acid (FA) receptor GPR40, highly expressed in pancreatic beta cells, has drawn considerable attention as a potential therapeutic target for type 2 diabetes (T2D). Fatty acids (FA) amplify insulin secretion from the beta cell only in the presence of glucose. This incretin-like effect could be exploited to develop novel therapeutic agents to enhance glucose-stimulated insulin secretion (GSIS) in T2D (3). Previous studies in our laboratory have shown that GPR40 contributes to ~50% of FA-potentiation of insulin secretion *in vitro* and *in vivo* (4). In addition, recent reports (4-7) provide evidence that activation of GPR40 may represent a beneficial approach to enhance insulin secretion in T2D. However, several key questions remain to be answered regarding the role of GPR40 in insulin secretion. Whereas it is clear that GPR40 mediates, at least in part, FA-potentiation of insulin secretion, its contribution to the secretory action of other fuel and non-fuel stimuli *in vivo* is not known. This issue is important because 1- FA are always present in the circulation, and therefore may influence the response to various secretagogues and thereby the overall regulation of insulin secretion *in vivo*; 2- lipid signalling is an integral part of the regulation of insulin secretion in response to glucose and other secretagogues (8); and 3- our previous results suggest impaired insulin secretion in response to glucose in high-fat fed GPR40 knock-out (KO) mice (5), and a recent study reported increased GSIS *in vitro* in islets from GPR40 transgenic mice (9). In addition, the mechanisms of action of GPR40 are incompletely understood. It was shown to couple to the G-protein subunit $G_{\alpha q/11}$ (2) and in turn activate phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-biphosphate into diacylglycerol (DAG) and inositol phosphates (InsPs), which activate protein kinase C and mobilize Ca^{2+} from the endoplasmic reticulum, respectively (reviewed in (10). A rise in cytoplasmic Ca^{2+} has been linked to the activation of dehydrogenases involved in pyruvate mitochondrial metabolism such as pyruvate-, α-ketoglutarate- and isocitrate- dehydrogenase (11), raising the possibility that GPR40 activation modulates intracellular fuel metabolism. The present study was therefore aimed to determine whether deletion of GPR40 1- alters insulin secretion in response to fuel and non-fuel secretagogues and insulin sensitivity *in vivo*; and, 2- affects intracellular fuel metabolism in islets.

RESEARCH DESIGN AND METHODS

Reagents-20% dextrose solution was from Baxter, (Mississauga, ON, CAN), RPMI 1640 and fetal bovine serum (FBS) were from Invitrogen (Burlington, ON, CAN). FA-free bovine serum albumin (BSA) was from Equitech-Bio Inc (Kerrville, TX). Radioactive tracers were from GE Healthcare (Baie d'Urfé, QC, CAN) and all other reagents were from Sigma (St. Louis, MO), unless otherwise noted. FA enzymatic kit was from Wako Chemicals (Neuss, Germany).

Animals- GPR40 KO mice were generated as described (4) and backcrossed to the C57BL/6 strain for more than 7 generations at Amgen, Inc. (San Francisco, CA). All procedures were approved by the Institutional Committee for the Protection of Animals at the Centre Hospitalier de l'Université de Montréal.

Beta cell mass- Freshly excised whole pancreata (from fed 12-14 weeks old mice) were trimmed of fat, weighed, and fixed in 4% buffered paraformadehyde and embedded in paraffin with 5-µm sections mounted on glass slides for immunohistochemical and β-cell mass analyses as previously described (4).

Whole body fat content-The percentage of whole body fat content in fed GPR40 WT and KO (12-14 weeks old) mice was assessed using an EchoMRI-700™ (Houston, TX).

Assessment of insulin secretion and sensitivity by hyperglycemic and euglycemic hyperinsulinemic clamp- One-step hyperglycemic clamps were performed on conscious animals. A 20% dextrose solution was infused through the jugular vein to clamp plasma glucose at ~350 mg/dl for 60 min and was adjusted based on glucose measurements (Roche Accu-Check^{R}, Roche, Indianapolis, IN). At 60 min, an arginine bolus injection was performed (1mmol/kg; Sandoz Canada Inc.) to assess the maximal insulin response. Plasma samples were collected from the tail at several time points during the clamp for insulin measurements using a mouse insulin ELISA kit (Alpco Diagnostics, Salem, NH). Plasma samples for C-peptide measurements were collected at 45 min and analyzed using a mouse C-peptide ELISA kit (Alpco Diagnostics). Two-hour hyperinsulinemic euglycemic clamps were performed in 5 h food-restricted GPR40 WT and KO mice. Following a one minute bolus insulin infusion (85mU/kg; Humulin© R), insulin was infused at 5mU/kg/min. Twenty % dextrose was infused starting 5 min after the insulin infusion to clamp glycemia at ~140 mg/dl. The insulin sensitivity index (M/I) was calculated as the glucose infusion rate (M) divided by the average insulinemia during the last 30 min of the clamp (I).

Transcriptomic study- Islets were isolated from GPR40 WT and KO fed mice (13-15 weeks old, n = 5 for each genotype) as previously described (4). Total RNA were extracted from pancreatic islets using RNeasy Micro kit (Qiagen) in accordance with the manufacturer's protocol as previously described (5). Total RNA were stored at -80°C for microarray hybridization (See Supplemental Methods in the online appendix available at http://diabetes.diabetesjournal.org).

Metabolic studies in islets- Islets were isolated from 24 h-fasted or ad libitum-fed mice (12-14 weeks old) as previously described (4). Freshly isolated islets were recovered at 37°C for 30 min in KRBH/0.25% BSA supplemented by 2.8 mM glucose. Palmitate and control solutions were prepared as previously described (12). For measurements of glucose oxidation and utilization, batches of 20 islets were incubated in KRBH/0.25% BSA containing 1μCi D-[U-^{14}C]-glucose (250 mCi/mmol) and 0.5 μCi D-[5-^{3}H]-glucose (16 Ci/mmol) at 2.8 or 16.7 mM glucose for 2 h. Glucose oxidation was measured by the generation of KOH-trapped $^{14}CO_2$ and glucose utilization was determined by measuring the amount of $^{3}H_2O$ generated as described previously (13). To measure FA oxidation and incorporation in total lipids, batches of 50 islets were incubated in KRBH/0.25% BSA, 0.1 mM palmitate, 1mM carnitine, 2μCi/ml [9,10(n)-^{3}H] palmitate (51 Ci/mmol) and 2.8 or 16.7 mM glucose for 2 h. Supernatants were transferred to eppendorf tubes for separation of $^{3}H_2O$ from labelled FA as previously described (14). The remaining islets were washed and sonicated in water. Total lipids were extracted using the Folch method. Radioactivity incorporation was counted on the lipid soluble fractions.

FA esterification- Islets isolated from fed mice were cultured overnight in RPMI supplemented with 10% FCS (complete RPMI) at 11 mM glucose. FA esterification was determined in batches of 70 islets incubated for 4 h in the same conditions as described above. After the incubation, total lipids were extracted and subjected to separation by thin-layer chromatography as described (15).

Lipid profiling- Islets isolated from fed mice were cultured overnight in complete RPMI medium at 11 mM glucose. Batches of 250 islets each were incubated for 1 h at 2.8

mM glucose in RPMI, and then exposed to 16.7 mM glucose with or without 0.5 mM palmitate for 1 h. Total lipids were extracted using the Folch method, dried, and stored at -80°C for LC-MS analysis (See Supplemental Methods).

Arachidonic acid release- Islets isolated from fed mice were cultured overnight in complete RPMI medium at 5.6 mM glucose and 4 μCi/ml [5, 6, 8, 9, 11, 12, 14, 15(n)-^{3}H] arachidonic acid (^{3}H-AA , 250 Ci/mmol). After prelabeling, ^{3}H-AA release was measured in islets incubated at 16.7 mM glucose, with or without 0.5mM palmitate or 100μM carbachol in KRBH/0.6% BSA for 30 min as described (16). After incubation, islets were lysed to measure incorporation of the total radioactivity.

Intracellular inositol phosphate accumulation- Islets isolated from fed mice were cultured overnight in complete RPMI medium at 11.1 mM glucose. Batches of 40 islets were prelabeled with 7.5 μCi of [^{3}H]myo-inositol (95 Ci/mmol) in KRBH/0.01% BSA for 3 h. After prelabeling, InsPs accumulation was measured in islets incubated at 16.7 mM glucose, with or without 30μM oleate or 1 mM carbachol in KRBH/0.01% BSA/10 mM LiCl for 20 min. After incubation, islets were lysed in 20 mM formic acid (150μl) and ^{3}H-InsPs content was measured using Yttrium Silicate scintillation proximity assay (YSi SPA) beads (GE Healthcare) (17). Briefly, YSi SPA beads were diluted 8-fold with water, 100 μl of diluted beads were added to each well of a Wallac Isoplate-96 white plate (Perkin Elmer, Woodbridge, ON, CAN) followed by 50μl of islets extracts, and radioactivity was measured using a MicroBeta TriLux scintillation counter (Perkin Elmer, Woodbridge, ON, CAN) after a 2-h incubation.

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, as appropriate. $P < 0.05$ was considered significant.

RESULTS

Insulin secretion is impaired in GPR40 KO mice during hyperglycemic clamps. Basal metabolic parameters were first compared between 24-h fasted and ad libitum-fed male GPR40 KO mice (back-crossed onto the C57Bl/6J for 7 generations) and their wild-type (WT) littermates (Table 1). As expected, plasma FA levels were significantly increased, while both glucose and insulin levels were significantly decreased, in fasted vs. fed mice. Blood glucose, FA, insulin, and C-peptide levels were similar in GPR40 KO and WT mice in both the fed and fasted state, consistent with our previous report (4). Insulin secretion in response to glucose and arginine was assessed *in vivo* using 1-h hyperglycemic clamps (Fig. 1A&D). As expected, the glucose infusion rate was markedly lower in fasted than in fed animals, although it was not affected by the genotype when taking into account the prevailing insulin levels (Table 2). Insulin secretion during the clamp was markedly decreased in both fasted and fed GPR40 KO mice (Fig.1B&E). The areas under the curve for insulin over the first 60 min of the clamp were reduced in fasted and fed GPR40 KO mice by ~2 fold and ~3 fold, respectively (Table 2). Accordingly, C-peptide concentrations 45 min into the clamp were significantly lower in GPR40 KO fed animals vs. WT (1.45 ± 0.13 ng/ml (n=7) vs. 2.04 ± 0.17 ng/ml (n=9); $p<0.05$). Arginine-potentiation of GSIS was also decreased by ~3 fold in fasted and fed GPR40 KO mice (Table 1, Fig.1C and F). Similar observations were made in female GPR40 KO mice (not shown). Beta-cell mass (Table 1) and islet size distribution (not shown) were not different between GPR40 WT and KO mice. Insulin sensitivity was then examined in hyperinsulinemic-euglycemic clamps. Both the glucose infusion rate (Fig.

2B) required to maintain glycemia at ~140 mg/dl (Fig. 2A) during the clamp and the insulin sensitivity index (Fig. 2C) were similar in GPR40 WT and KO mice. Thus, GPR40 deletion does not affect whole-body insulin sensitivity, consistent with previous reports using insulin tolerance tests (4; 18). Altogether, our data demonstrate that GPR40 deletion impairs insulin secretion *in vivo* in response to glucose and arginine without altering insulin sensitivity.

Intracellular metabolism of glucose and FA in islets is not affected by GPR40 deletion. Transcriptomic analysis, metabolic studies and lipid profiling were employed to address whether GPR40 modulates intracellular fuel metabolism in islets. We first assessed the impact of GPR40 deletion on the islet transcriptome using expression microarrays. Approximately 1770 genes were significantly modulated (1109 downregulated and 661 upregulated) in KO vs.WT ($p < 0.05$). However, expression of none of these genes was changed more than 2 fold (Suppl. Fig. 1 in the online appendix). Thus, GPR40 deletion does not have a major impact on the islet transcriptome. To explore the functional impact of GPR40 deletion on fuel partitioning, glucose and palmitate metabolism were assessed in islets isolated from 24-h fasted and fed mice. Raising glucose from 2.8 to 16.7 mM significantly increased glucose utilization and oxidation, decreased palmitate oxidation by ~ 3 fold, and increased palmitate esterification by ~ 1.5 fold, in both fasted and fed WT islets (Fig. 3A-D). No differences were observed between WT and KO islets for any of these measurements (Fig 3A-D). Consistent with previous studies (19; 20), fasting decreased glucose oxidation and palmitate esterification and increased palmitate oxidation (Fig. 3A-D).

Palmitate esterification into phospholipids (PL), triacylglycerol (TAG), DAG, and FA was assessed in islets isolated from fed WT and KO mice. Raising glucose from 2.8 to 16.7 mM increased palmitate esterification into DAG and TAG and increased FA content similarly in WT and KO islets (Fig. 4A-B). The effect of elevated glucose on palmitate esterification in PL, DAG, TAG, and on intracellular FA levels was not different between WT and KO islets. These data indicate that GPR40 deletion does not affect glucose or palmitate metabolism in islets, nor does it contribute to changes in intracellular fuel metabolism in response to fasting.

The islet lipid profile is modulated acutely by palmitate independently of GPR40. Data presented in Fig. 3 and 4 indicate that GPR40 deletion does not alter fluxes through major glucose and palmitate metabolic pathways. This does not rule out the possibility that GPR40 might modulate the abundance of individual lipid species in islets in response to palmitate. This possibility was tested using liquid chromatography-mass spectrometry (LC-MS)-based lipid profiling. Islets isolated from WT and KO mice were incubated for 1 h at 16.7 mM glucose ± 0.5 mM palmitate. Lipid-soluble fractions were separated by LC followed by tandem MS analysis as described in Supplemental Methods. The data are presented as relative abundance (i.e. comparative mass measurements across samples) after log2 transformation (Fig. 5). Palmitate treatment significantly modulated the relative abundance of 59 lipid species in WT islets and 62 in KO islets ($p < 0.05$) (see Suppl. Fig. 2 in the online appendix), among which 13 were identified based on their match with the accurate mass and time tag database (see Supplemental Methods). Several TAG species were markedly increased by palmitate treatment both in WT and KO islets (Fig. 5A). For example, the abundance of TAG 48:1 was increased 5 fold (2.3 log2 units) in WT islets in response to palmitate. Although the effect of palmitate was more pronounced for some TAG in WT vs. KO islets, when calculated as

fold increase over control, no significant differences were observed between WT and KO islets. These results indicate that acute palmitate treatment induces a robust increase in several TAG species in islets independently of GPR40. Palmitate treatment decreased the abundance of all identified lyso-phosphatidylethanolamine (LPE) species (Fig. 5B), but did not have a major effect on lyso-phosphatidylcholines (LPC; Fig. 5B), phosphatidylethanolamines (PE, Suppl. Fig. 3A I the online appendix), phosphatidylcholines (PC, Suppl. Fig. 3A in the online appendix), or sphingomyelins (Suppl. Fig. 3B in the online appendix). Although the effect of palmitate was more pronounced for some LPE in KO vs. WT islets (Fig. 5B), when calculated as fold decrease over control, no difference was observed between the 2 genotypes. These data indicate that palmitate modulates LPE abundance independently of GPR40. Several lines of evidence suggest that hydrolysis of membrane PL by phospholipase A2 (PLA2) into lyso-PL and arachidonic acid (AA) participates in the control of insulin secretion (21-24). We therefore tested whether palmitate lowering of LPE content results from PLA2-mediated PL hydrolysis. PLA2 activity was indirectly assessed in islets by measuring the release of AA as previously described (16). The muscarinic agonist carbachol stimulated AA release to a similar extent in WT and KO islets, while palmitate had no effect (Fig. 6A). We conclude that 1- Palmitate potentiation of GSIS is independent of AA release; and 2- Activation of the PLA2 pathway is not affected by GPR40 deletion.

Deletion of GPR40 abolishes intracellular accumulation of inositol phosphates in response to oleate. Previous studies suggest that GPR40 couples to the G-protein subunit $G_{\alpha q/11}$ (2) and in turn activates PLC-mediated hydrolysis of phosphatidylinositol into DAG and InsPs (25). Furthermore, pharmacological inhibition of $G_{\alpha q}$ (4; 26) or PLC (26; 27) in beta cells prevents FA-potentiation of GSIS. However, whether GPR40 mediates FA-induced InsPs generation in islets is unknown. To address this question, we compared InsPs generation from tritiated phosphoinositides in response to oleate in islets from WT and GPR40 KO mice (Fig. 6B). Remarkably, the increase in InsPs accumulation observed in WT islets in response to oleate was absent in KO islets. In contrast, InsPs accumulation in response to the muscarinic agonist carbachol was similar in both groups (1.9 ± 0.3 fold increase in KO islets vs. 2.0 ± 0.7 fold increase in WT islets; n=3; NS). These data indicate that FA-induced InsPs accumulation is dependent upon GPR40 in mouse islets.

DISCUSSION

The objectives of this study were to examine whether deletion of GPR40 impairs insulin secretion *in vivo* and modulates intracellular fuel metabolism in islets. We found that in GPR40 KO mice insulin secretion in response to both glucose and arginine is impaired in fed and fasted animals during hyperglycemic clamps, without changes in insulin sensitivity, as measured by hyperinsulinemic-euglycemic clamps. This was associated with a complete absence of FA-induced intracellular InsPs accumulation in islets, without detectable changes in intracellular glucose or FA metabolism. We conclude that GPR40 signalling is implicated in the regulation of insulin secretion *in vivo*, independently from changes in fuel metabolism.

Our results demonstrate that deletion of GPR40 impairs insulin secretion in response to glucose and arginine *in vivo* under fasted and fed conditions. Since neither beta-cell mass nor insulin content (not shown) were affected by GPR40 deletion, we infer that the decrease in insulin levels in GPR40 KO mice results from a *bona fide* secretory defect. This conclusion is further supported

by the observed decrease in circulating C-peptide levels during the hyperglycemic clamp. Since FA are always present in the circulation *in vivo*, we suggest that GPR40 mediates their potentiating action on glucose- and arginine-stimulated insulin secretion. Consistent with this view, GSIS *ex-vivo* was similar in WT and KO islets isolated from fasted mice (not shown). In addition, we have previously shown that the islet response to glucose alone in the absence of exogenous FA is not affected by GPR40 deletion, whereas the response to FA is markedly decreased (4). The observed impairment of insulin secretion in this study using hyperglycemic clamps is in apparent contradiction with our previous findings that insulin secretion was similar in WT and KO mice when assessed by intravenous glucose tolerance tests (IVGTT) (4; 5). One possible explanation for this discrepancy is that the single bolus of glucose in the IVGTT mostly stimulates first-phase insulin release, which relies less on the amplification pathway of insulin secretion than the full, first- and second-phase secretory profile induced under clamp conditions. Also, sustained glucose stimulation during the clamp evokes a greater integrated insulin response than the IVGTT bolus, and is therefore more likely to detect secretory defects.

Using transcriptomic, metabolic, and lipid profiling approaches, we tested the hypothesis that impaired insulin secretion in GPR40 KO mice involves modulation of intracellular fuel metabolism in islets. First, microarray analyses indicated that GPR40 deletion does not alter the expression of islet genes by more than two fold, suggesting that the defect in insulin secretion is not related to major changes in islet phenotype. Second, neither glucose nor palmitate metabolism (as assessed by tracer studies) was modified in fasted or fed GPR40 KO islets, nor were there any significant differences in lipid profiles using LC-MS between WT and KO islets in response to palmitate. Third, AA generation was unaltered by GPR40 deletion. In contrast, InsPs accumulation in response to oleate was completely absent in GPR40 KO islets. Together with previous studies form our (4) and other (26; 27) groups using pharmacological inhibitors of the $G_{\alpha q/11}$ signalling pathway, these data therefore reinforce the notion that the mode of regulation of insulin secretion by GPR40 involves receptor-mediated signalling rather than fuel-derived metabolic signals. This conclusion has important implications for the understanding of the mechanisms of action of FA on the beta cell. On the one hand, molecular and pharmacological approaches have shown that FA activation to fatty acyl-CoA and intracellular metabolism are required for their potentiating effects on GSIS (8). On the other hand, several studies have reported that blockade of GPR40 signalling prevents – at least in part – FA-potentiation of GSIS (2; 4; 26; 28; 29). This suggested that perhaps these 2 mechanisms of action (intracellular metabolism and GPR40 activation) were interrelated. The results of the present study show that, to the contrary, FA signalling through GPR40 is independent from their intracellular metabolism. They further suggest that in this context, the GPR40-dependent rise in intracellular Ca^{2+} levels does not significantly affect the activity of the TCA cycle, as measured by fuel oxidation. Based on our previous observation that GPR40 deletion results in a 50% decrease in insulin secretion in response to FA *in vivo* (4), we propose that GPR40 signalling contributes approximately half of the potentiating effects of FA on GSIS.

LC-MS-based lipid profiling showed that acute palmitate treatment mardekly increased several TAG species and decreased LPE species independently from GPR40. To our knowledge, this is the first direct demonstration that acute exposure of islets to palmitate rapidly result in a dramatic (5 fold

for some species) increase in different TAG species. It suggests that intermediates generated along the TAG synthesis pathway might be implicated in GPR40-independent amplification of GSIS by FA. Whether or not this is also associated with increased rates of lipolysis and an increase in TAG-FA cycling (30) remains to be determined. A potential mechanism for the palmitate-induced decrease in LPE involves LPE re-esterification by lyso-PL-acyltransferase (Lands cycle) (31). Such a mechanism is consistent with the observed increase in PE 36:4 and PE 18:0p/20:4 (Suppl Fig. 3A in the online appendix). Whether or not this mechanism plays a role in FA-potentiation of GSIS remains to be investigated. LPC was recently shown to potently enhance GSIS via activation of the GPR119 receptor (32; 33). Both LPE and LPC are generated by plasma membrane-associated PLA2 activities known to stimulate insulin secretion (34)), and a recent report suggested that FA modulate the PLA2 pathway and the generation of AA via GPR40 in primary hepatocytes (35). However, although we have not directly measured the activity of the enzyme, our finding that AA release is not increased by palmitate does not support the hypothesis that the effects of palmitate on GSIS are mediated by PLA2.

GPR40 expression has been detected in the ileum (1; 2), monocytes (1), pancreatic alpha cells (36), some areas of the brain (1; 37), entero-endocrine cells (38), and osteoclasts (39), albeit at much lower levels than in beta cells. Consequently, we cannot exclude that deletion of GPR40 in these tissues in our global KO model could have contributed to the insulin secretory defect. However, there was not apparent change in body weight, food intake, or insulin sensitivity in these mice, and the main phenotype appears restricted to the beta cell. Therefore, we believe that the contribution of non beta-cells to the observed phenotype, if any, is minimal, although a tissue-specific KO model would be required to formally exclude this possibility.

In conclusion, this study identifies GPR40 as necessary for insulin secretion *in vivo*. The impaired insulin secretion observed in GPR40 KO mice is independent of nutrient metabolism in islets and more likely involves the canonical GPR40 signalling pathway.

Supplemental data: Supplemental Data include three figures, Supplemental Experimental Procedures and Supplemental References.

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FIGURE LEGENDS

Fig. 1. Hyperglycemic clamps in 24 h-fasted and ad libitum fed GPR40 WT and KO mice. Glucose (A and D) and insulin levels (B and E) during the course of the hyperglycemic clamp in fasted and fed GPR40 WT and KO mice. Insulin levels (C and F) in response to an arginine bolus (1mmol/kg). Values are expressed as mean ± SE of 7-9 mice/group.

Fig. 2. Hyperinsulinemic euglycemic clamps in GPR40 WT and KO mice. Glucose levels (A) and glucose infusion rate (GIR) (B) during the course of the hyperinsulinemic clamp in 5 h food restricted GPR40 WT and KO mice. The insulin sentivity index (M/I) (C) was calculated as the glucose infusion rate (M) divided by the average insulinemia during the last 30 min of the clamp (I). Values are expressed as mean ± SE of 5-6 mice/group.

Fig. 3. Glucose and palmitate metabolism in GPR40 WT and KO islets. Glucose utilization (A), glucose oxidation (B), palmitate oxidation (C) and palmitate incorporation into total lipids (D) in islets isolated from fasted or fed mice incubated at 2.8 or 16.7 mM glucose for 2 h. Data are expressed as mean ± SE of 3 to 5 independent experiments. *, ** and ***, $p < 0.05$, $p < 0.01$, or $p < 0.001$ respectively, versus 2.8 mM glucose. $, $$ and $$$, $p < 0.05$, $p < 0.01$, or $p < 0.001$ respectively, versus fasted.

Fig. 4. Palmitate esterification in GPR40 WT and KO islets. Palmitate esterification into triacylglycerol (A), diacylglycerol (B), non-esterified fatty acid (C) and phospholipids (D) in islets incubated at 2.8 or 16.7 mM glucose for 4 h. Data are expressed as mean ± SE of 5 independent experiments. * and **, $p < 0.05$ or $p < 0.01$, respectively, versus 2.8 mM glucose.

Fig. 5. Lipid profiles in GPR40 WT and KO islets. Log 2 relative abundances of triacylglycerols (A) and lyso-phospholipids (B) in islets incubated at 16.7 mM glucose with or without 0.5 mM palmitate for 1 h. Lipid extracts were subjected to LC-MS analysis (see Suppl. Methods). Data are expressed as mean ± SE of 5 independent experiments as relative abundance (i.e. comparative mass measurements across samples) after log2 transformation. *, ** and ***, $p < 0.05$, $p < 0.01$, or $p < 0.001$ respectively, versus WT Control. #, ## and ###, $p < 0.05$, $p < 0.01$, or $p < 0.001$ respectively, versus KO Control.

Fig. 6. Arachidonic acid release and InsPs accumulation in GPR40 WT and KO islets. (A) Arachidonic acid (AA) release in islets incubated during 30 min at 16.7 mM glucose with or without 0.5 mM palmitate and 100µM carbachol. AA efflux is expressed as the percentage release of total incorporated radioactivity. Data are expressed as mean ± SE of 3 to 4 independent experiments. **, $p < 0.01$ versus Control. (B) InsPs accumulation in islets incubated during 20 min at 16.7 mM glucose with or without 30 µM oleate. InsPs accumulation is expressed as fold changes over control conditions (16.7 mM glucose). Data are expressed as mean ± SE of 3 independent experiments. *, $p < 0.05$ versus Control.

Table 1: Metabolic parameters of GPR40 WT and KO mice and beta-cell mass.

	WT	KO
Basal 24 h fasted mice		
Body weight (g)	24.1 ± 0.2	25.1 ± 0.5
Basal glycemia (mg/dl)	120 ± 5	119 ± 9
Free fatty acids (mM)	0.50 ± 0.03	0.55 ± 0.03
Basal insulinemia (ng/ml)	0.06 ± 0.01	0.08 ± 0.01
C-peptide (ng/ml)	0.51 ± 0.04	0.49 ± 0.01
Basal fed mice		
Body weight (g)	28.2 ± 0.9 *	27.8 ± 0.5 *
Basal glycemia (mg/dl)	151 ± 3 **	154 ± 5 **
Free fatty acids (mM)	0.09 ± 0.01 **	0.11 ± 0.02 **
Basal insulinemia (ng/ml)	0.48 ± 0.01 **	0.51 ± 0.03 **
C-peptide (ng/ml)	0.64 ± 0.03 *	0.71± 0.02 *
Beta-Cell mass (mg)	1.55 ± 0.09	2 ± 0.22
Fat (% of body weight)	12 ± 1	10 ± 1

Values are mean ± SE. n = 7-9 mice/group. * and **, $p < 0.05$ or $p < 0.01$, respectively, versus fasted.

Table 2: Insulin secretion in GPR40 WT and KO during hyperglycemic clamps.

	WT	KO
Hyperglycemic Clamp in 24 h fasted mice		
AUC Insulin T0-60 min	51.6 ± 9.6	24.9 ± 6.9 *
AIR Max (ng/ml) T61 min	5.7 ± 1.3	2.1 ± 0.6 *
GIR (mg/kg/min)	16 ± 5	10.5 ± 1
Hyperglycemic Clamp in fed mice		
AUC Insulin T0-60 min	65.4 ± 17.2	19.3 ± 6.9 *
AIR Max (ng/ml) T61 min	11.8 ± 1.9	3.9 ± 0.5 **
GIR (mg/kg/min)	71 ± 9 ##	39 ± 4 ## **

Values are mean ± SE. n = 7-9 mice/group. AUC, area under the curve; AIR max, maximal arginine-induced insulin response; GIR, Glucose infusion rate. *, ** and ***, $p < 0.05$, $p < 0.01$, or $p < 0.001$ respectively, versus WT. ##, $p < 0.01$ versus fasted.

FIGURE 1

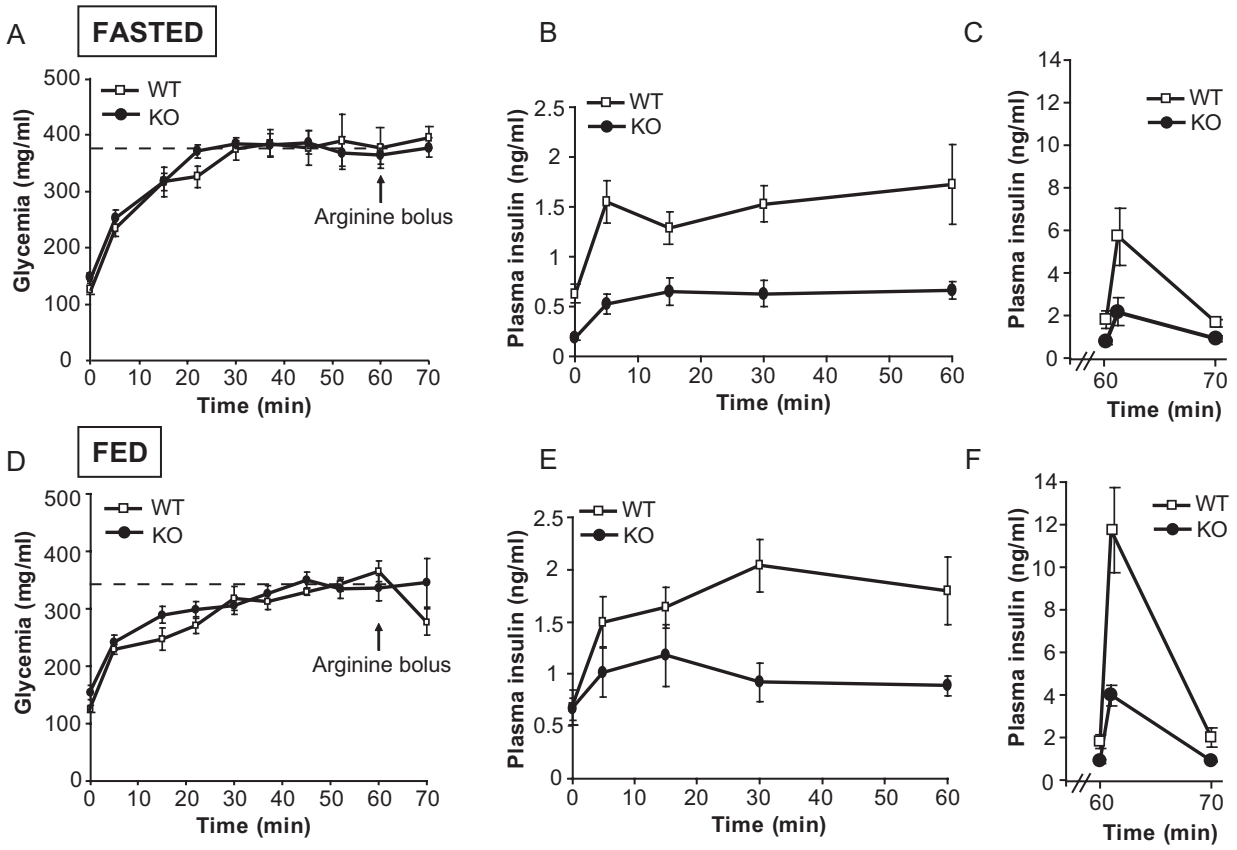


FIGURE 2

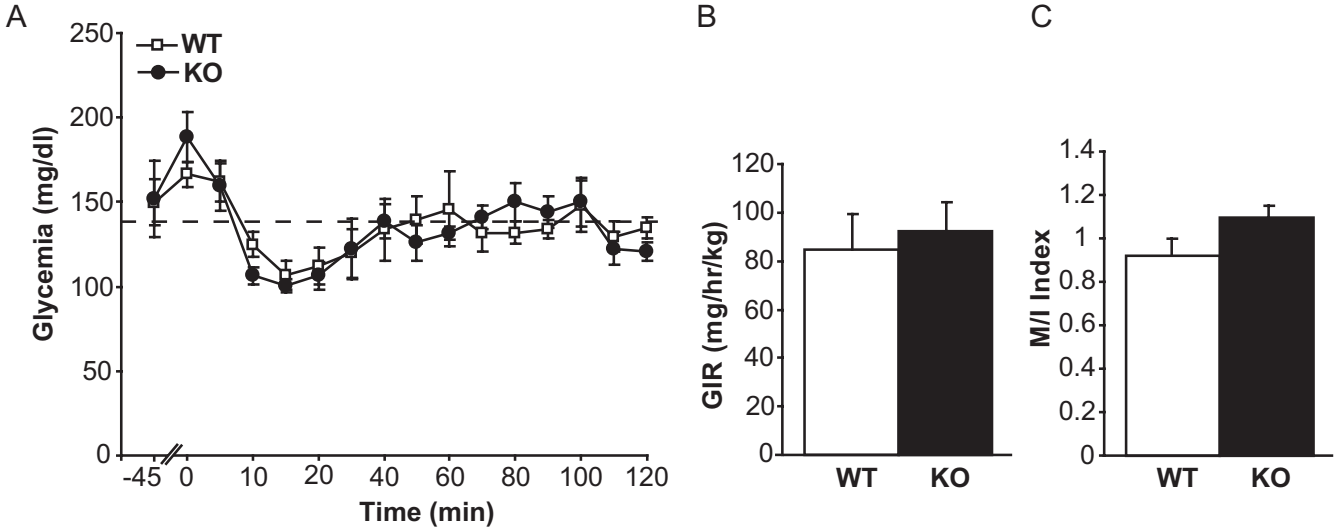


FIGURE 3

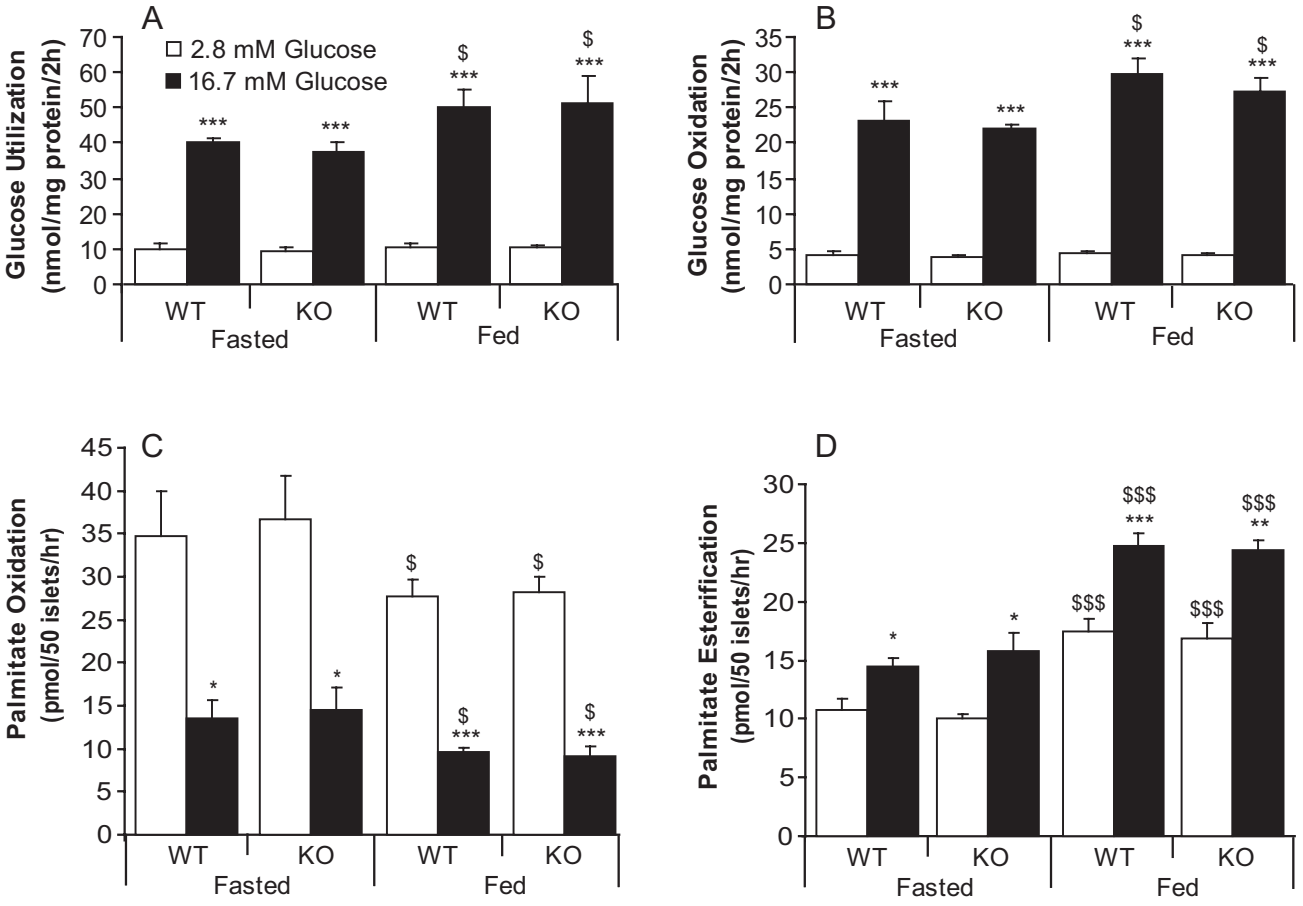

A
□ 2.8 mM Glucose
■ 16.7 mM Glucose
Glucose Utilization (nmol/mg protein/2h)
WT
KO
WT
KO
Fasted
Fed
B
Glucose Oxidation (nmol/mg protein/2h)
C
Palmitate Oxidation (pmol/50 islets/hr)
D
Palmitate Esterification (pmol/50 islets/hr)

FIGURE 4

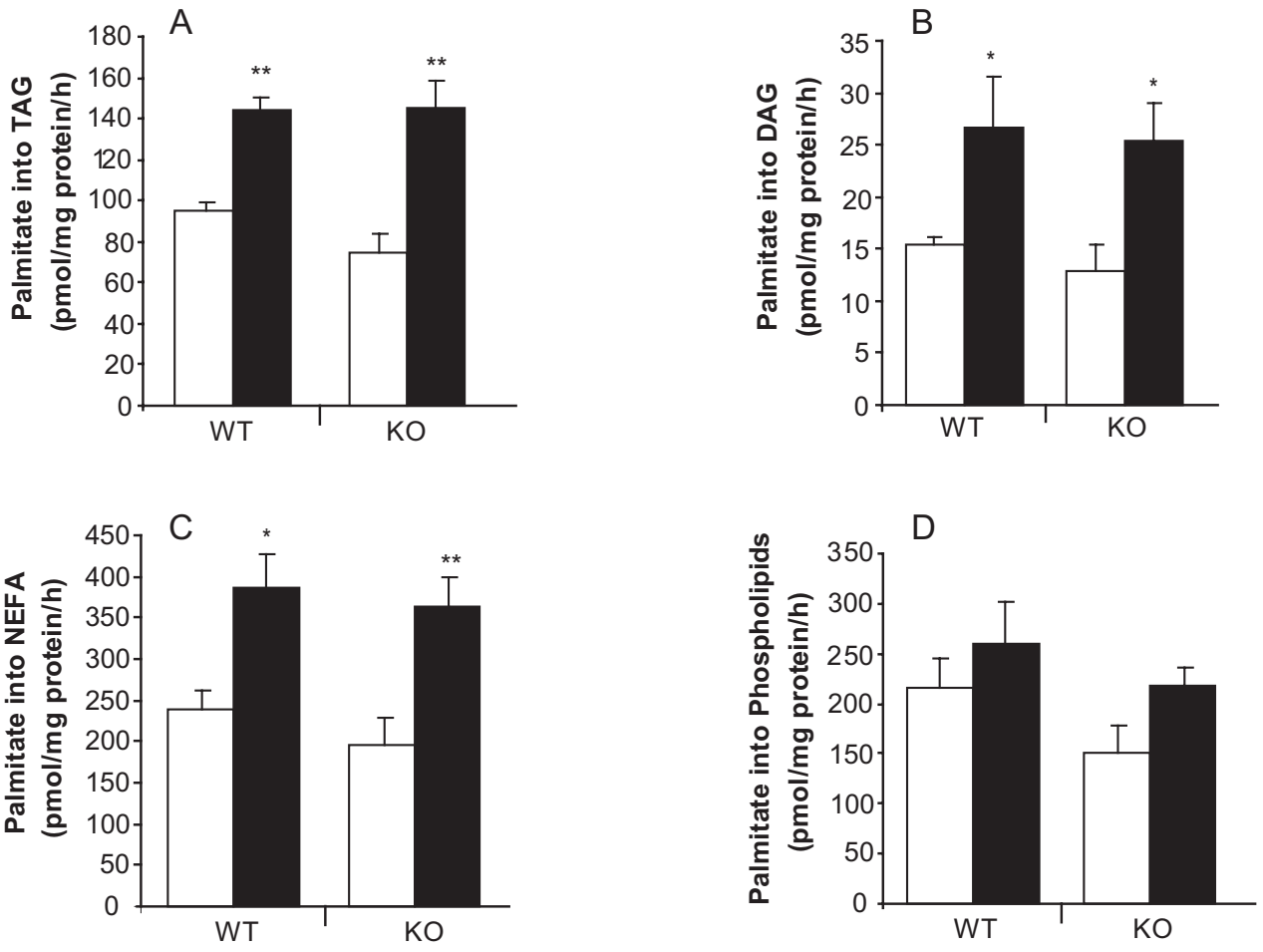

A
Palmitate into TAG (pmol/mg protein/h)
180
160
140
120
100
80
60
40
20
0
**
**
WT
KO
B
Palmitate into DAG (pmol/mg protein/h)
35
30
25
20
15
10
5
0
*
*
WT
KO
C
Palmitate into NEFA (pmol/mg protein/h)
450
400
350
300
250
200
150
100
50
0
*
**
WT
KO
D
Palmitate into Phospholipids (pmol/mg protein/h)
350
300
250
200
150
100
50
0
WT
KO

FIGURE 5

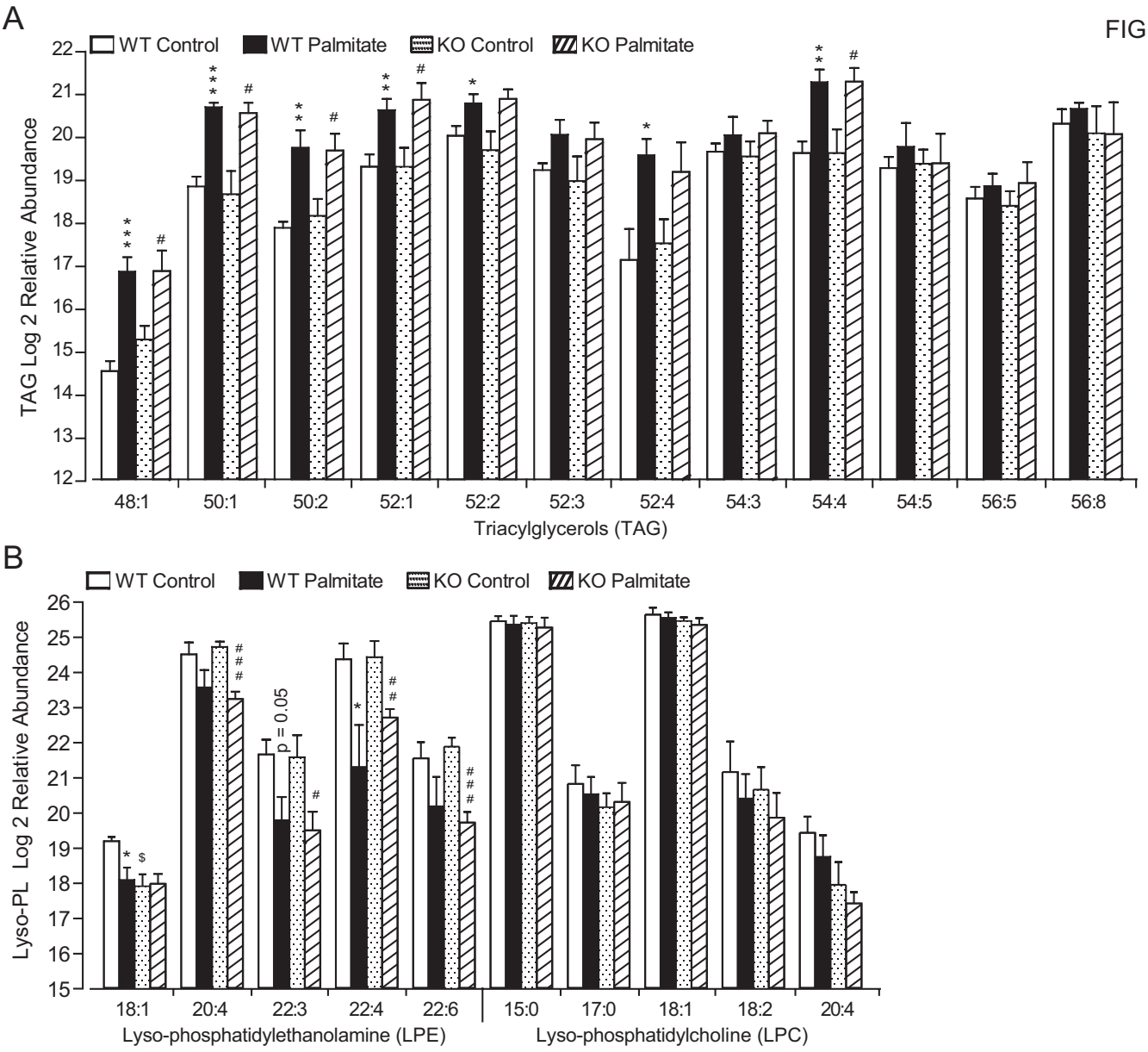
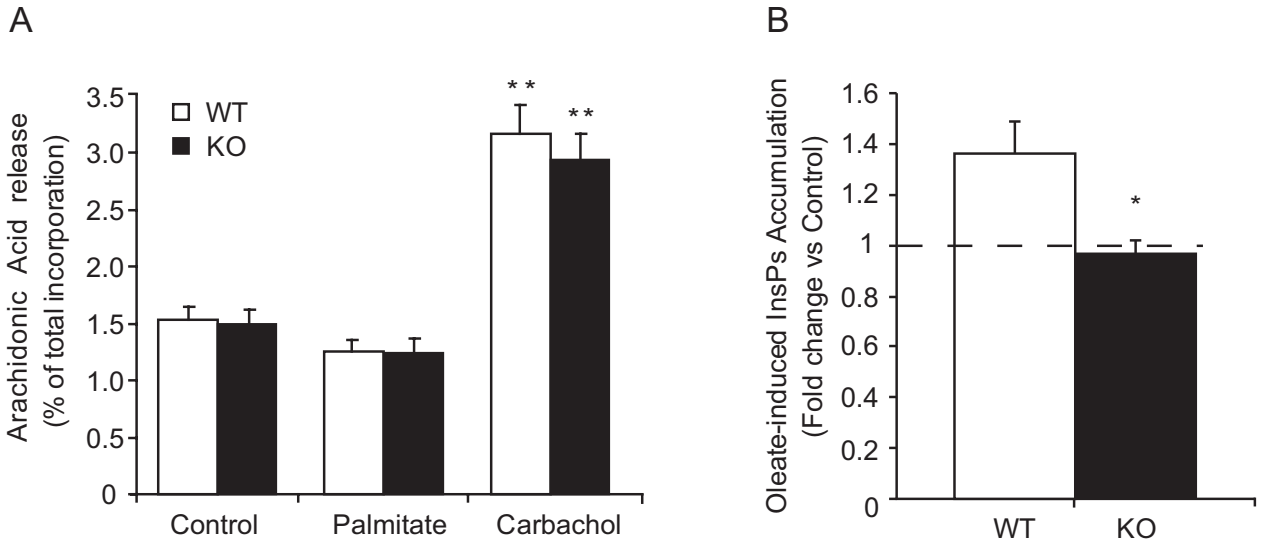

A
WT Control
WT Palmitate
KO Control
KO Palmitate
TAG Log 2 Relative Abundance
22
21
20
19
18
17
16
15
14
13
12
48:1
50:1
50:2
52:1
52:2
52:3
52:4
54:3
54:4
54:5
56:5
56:8
Triacylglycerols (TAG)
B
WT Control
WT Palmitate
KO Control
KO Palmitate
Lyso-PL Log 2 Relative Abundance
26
25
24
23
22
21
20
19
18
17
16
15
p = 0.05
18:1
20:4
22:3
22:4
22:6
15:0
17:0
18:1
18:2
20:4
Lyso-phosphatidylethanolamine (LPE)
Lyso-phosphatidylcholine (LPC)

FIGURE 6


A
Arachidonic Acid release
(% of total incorporation)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
WT
KO
* *
* *
Control
Palmitate
Carbachol
B
Oleate-induced InsPs Accumulation
(Fold change vs Control)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
WT
KO