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Effects of dapagliflozin and/or insulin glargine on beta cell mass and hepatic steatosis in db/db mice

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ABSTRACT

Objective: To explore the beneficial effects of dapagliflozin and/or insulin glargine on the pancreatic beta cell mass and hepatic steatosis in db/db mice.
Methods: Six-week-old db/db mice were assigned to one of four groups: untreated (Placebo), treated with dapagliflozin (Dapa), treated with insulin glargine (Gla), or treated with dapagliflozin and insulin glargine (Dapa+Gla). After 8 weeks of treatment, we determined glucose tolerance, beta cell mass, hepatic lipid content and gene expression.
Results: Glucose tolerance was significantly ameliorated in the three treated groups to the same degree compared with the Placebo group. Immunohistochemical analysis revealed that the pancreatic beta cell mass was significantly maintained in the Dapa and Dapa+Gla groups, but not in the Gla group, compared with the Placebo group (Placebo 2.25 ± 1.44 mg, Dapa 5.01 ± 1.63 mg, Gla 3.79 ± 0.96 mg, Dapa+Gla 5.19 ± 1.78 mg). However, the triglyceride content of the liver was markedly elevated in the Gla group compared with that in the other three groups (Placebo 24.1 ± 11.5 mg, Dapa 30.6 ± 12.9 mg, Gla 128 ± 49.7 mg, Dapa+Gla 54.4 ± 14.1 mg per gram liver). The expression levels of genes related to fatty acid synthesis and lipid storage were significantly upregulated in the Gla group.
Conclusions: Our results showed that beta cell mass was sustained and hepatic steatosis was prevented, after 8 weeks of treatment with either dapagliflozin or dapagliflozin plus insulin glargine, but not with insulin glargine alone, in db/db mice.

1. Introduction

Insulin resistance in tissues including the liver, skeletal muscle and adipose tissue, and insulin secretion abnormalities in pancreatic beta cells are the main stays of pathophysiology of type 2 diabetes [1]. Especially, impaired insulin secretion is a central component in this pathophysiology considering the natural history of type 2 diabetes: beta cells are unable to compensate adequately for emerging peripheral insulin resistance. In this stage, insulin release is insufficient for the degree of insulin resistance, thus mild hyperglycemia develops. Over time, the progressive nature of the beta cell defect results in ongoing loss of secretory function and a further decline in beta cell mass such that severe hyperglycemia develops [2–7]. Moreover, chronic hyperglycemia per se has deleterious effects in many tissues associated with glucose metabolism. In pancreatic beta cells, it causes a reduction in insulin secretion and in beta cell mass, a phenomenon termed glucotoxicity [8,9]. Therefore, elimination of glucotoxicity by ameliorating hyperglycemia via treatment with hypoglycemic agents is crucial for preventing both beta cell dysfunction and loss. Recent studies have revealed that earlier intervention of decreasing blood glucose is important for the prevention of beta cell loss [9–13].

Insulin treatment is usually used to eliminate glucotoxicity in clinical practice. Indeed, it has favorable outcomes on maintenance of beta cell function in patients with type 2 diabetes [14]. Furthermore, islet mass

Abbreviations: SGLT2, Sodium-glucose cotransporter 2; TG, Triglycerides; Fas, Fatty acid synthase; Elovl6, Elongation of very long chain fatty acids protein 6; Scd1, Stearoyl-CoA desaturase; Mogat1, Monoacylglycerol O-Acyltransferase 1; Cidea, Cell death-inducing DFFA-like effector a; Cidec, Cell death-inducing DFFA-like effector c; Pparg, Peroxisome proliferator-activated receptor γ; Irs2, Insulin receptor substrate 2; Srebp1c, Sterol regulatory element-binding protein.

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and insulin content in isolated islets were preserved in db/db mice treated with insulin [15]. Additionally, sodium-glucose cotransporter 2 (SGLT2) inhibitors improve glucose tolerance by suppressing renal glucose reabsorption without direct pharmacological action on pancreatic beta cells. Thus, SGLT2 inhibitors can eliminate glucotoxicity, resulting in the prevention of progressive beta cell dysfunction and loss. In db/db mice, long-term treatment with SGLT2 inhibitors has been reported to protect beta cell function and mass [10,13,16–18]. Some clinical studies have also shown that treatment with SGLT2 inhibitors improved beta cell function as evaluated by model-based measures in patients with type 2 diabetes [19,20]. However, no direct comparison study between an SGLT2 inhibitor and insulin treatment has examined beta cell function or mass in a mouse model of type 2 diabetes.

In addition, we [21] and others [22–24] have shown that SGLT2 inhibitors ameliorated hepatic steatosis in preclinical and clinical studies. However, mechanisms of the anti-steatotic property of SGLT2 inhibitors have not fully elucidated.

In the present study, we examined the effect of one of the SGLT2 inhibitors, dapagliflozin and/or insulin glargine, on pancreatic beta cell function and mass in db/db mice. Furthermore, the effect of these two drugs on hepatic steatosis was examined.

2. Methods

2.1. Animals

We used 6-week-old male BKS. Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J (db/db) mice purchased from Oriental Yeast Co. (Tokyo, Japan). Two or 3 mice were housed per cage for all experiments under controlled ambient conditions with a 12-h light/dark cycle (lights on at 7 am). Mice were divided into four groups: untreated (Placebo), treated with dapagliflozin (Dapa; 1.0 mg/kg/day), treated with insulin glargine (Gla) (Sanofi-Aventis, Paris, France) and treated with insulin glargine and dapagliflozin (0.5 mg/kg/day) (Dapa+Gla) for 8 weeks. We administrated dapagliflozin mixed in the drinking water. Mice in the Gla and Dapa+Gla groups were injected the adjusted dose of insulin glargine subcutaneously to give equivalent glycemic control as the Dapa group. Mice in the Placebo group and Dapa group were injected the same volume of physiological saline as the Gla group. Animals were given free access to drinking water and food, and were maintained at 25 °C. This study was approved by the Animal Use Committee of Hokkaido University Graduate School of Medicine and was conducted in compliance with the Animal Use Guidelines of the Hokkaido University.

2.2. Diet protocol

Standard chow (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) were used, as described previously [25].Dapagliflozin was prepared by AstraZeneca (Cambridge, UK).

2.3. Measurement of biochemical parameters

Blood glucose levels were measured using a Glutestmint portable glucose meter (Sanwa Chemical Co., Nagoya, Japan). Insulin and C-peptide levels were measured using an insulin and C-peptide ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan, and Mercodia, Uppsala, Sweden). Plasma free fatty acids, total cholesterol and triglycerides (TG) were assayed by enzymatic methods (Wako Pure Chemical Industries Ltd., Osaka, Japan).

2.4. Oral glucose tolerance test (OGTT)

For the OGTT, mice were fasted for 16 h before being orally loaded with glucose (1.0 mg/g body weight). Blood samples were collected from the tail vein at 0, 15, 30, 60 and 120 min after glucose administration to determine the blood glucose levels. The total area under the blood glucose concentration curve (AUC) was determined from time 0 to 120 min after glucose loading.

2.5. Intraperitoneal insulin tolerance test

Mice were injected intraperitoneally with insulin (2 units/kg body weight) and blood was collected from the tail vein at 0, 30, 60, 90 and 120 min to measure glucose levels. The total AUC was determined from time 0 to 120 min after insulin administration.

2.6. Beta cell morphology and immunohistochemistry

Isolated pancreatic tissues were immersion-fixed in 4% paraformaldehyde at 4 °C overnight. Tissues were then roughly paraffin-embedded and 5-μm sections were mounted on glass slides using standard procedures. Sections were immersed for 15 min in methanol containing 0.3% (vol/vol) H_2O_2 to deactivate endogenous peroxidase activity. After rinsing with PBS, sections were immunostained with rabbit anti-human insulin antibody (diluted 1:250) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Beta cell mass was calculated as: beta cell mass (mg) = beta cell area / pancreatic area × pancreas weight (mg) [10,25,26]. We also evaluated beta cell mass by point-counting [27].

For immunofluorescence, tissue sections were incubated overnight at 4 °C with the primary antibodies listed in Supplementary Table 1. After rinsing with PBS, tissues were incubated with secondary antibodies for 60 min (diluted 1:200) using previously described procedures [10] (Supplementary Table 1). Immunofluorescence images were acquired using a BZ-II analyzer (Keyence, Osaka, Japan) according to the manufacturer's instructions.

Beta cell proliferation was identified by staining sections with Ki67 antibody, as described previously [10]. Immunohistochemical detection of Ki67 was performed by double immunostaining with an anti-insulin antibody (diluted 1:1). Beta cell apoptotic cell death was identified using a fluorometric terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) system (Deadend; Promega, Madison, WI, USA). Ki67-positive and TUNEL-positive beta cells were quantitatively assessed as a percentage of the total number of beta cells.

2.7. Islet isolation

Islets were isolated using collagenase from *Clostridium histolyticum* (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. To measure insulin content, isolated islets were incubated in acid ethanol and the insulin concentration in the assay buffer was measured using an insulin ELISA kit (Morinaga Institute of Biological Science).

2.8. Oil Red O staining and TG content of the liver

Lipid accumulation was assessed by Oil Red O staining of 10-μm frozen liver sections fixed in phosphate-buffered 4% paraformaldehyde. Liver specimens were washed once for 1 min with H_2O. After additional washing for 1 min with 60% isopropanol, the liver sections were stained for 10 min at 37 °C in freshly diluted Oil Red O solution. We quantified hepatic tissues by hematoxylin and eosin staining on the basis of NASH/NAFLD Clinical Research Network scoring system definition and scores [28], and also evaluated steatosis in these hepatic tissues using point counting system [29]. To evaluate the triglyceride content of the liver, tissue homogenate was extracted with 2:1 (vol/vol) chloroform/methanol. Chloroform/methanol was added to the homogenate and the mixture was shaken for 15 min. After centrifugation at 14,000 rpm for 10 min, the organic layer was collected. This process was repeated three times, and the collected samples were dried, resuspended in 1% Triton X-100/ethanol and the measurement was conducted using a Cholestest TG kit (Sekisui Medical Co. Ltd., Tokyo, Japan). Lipid

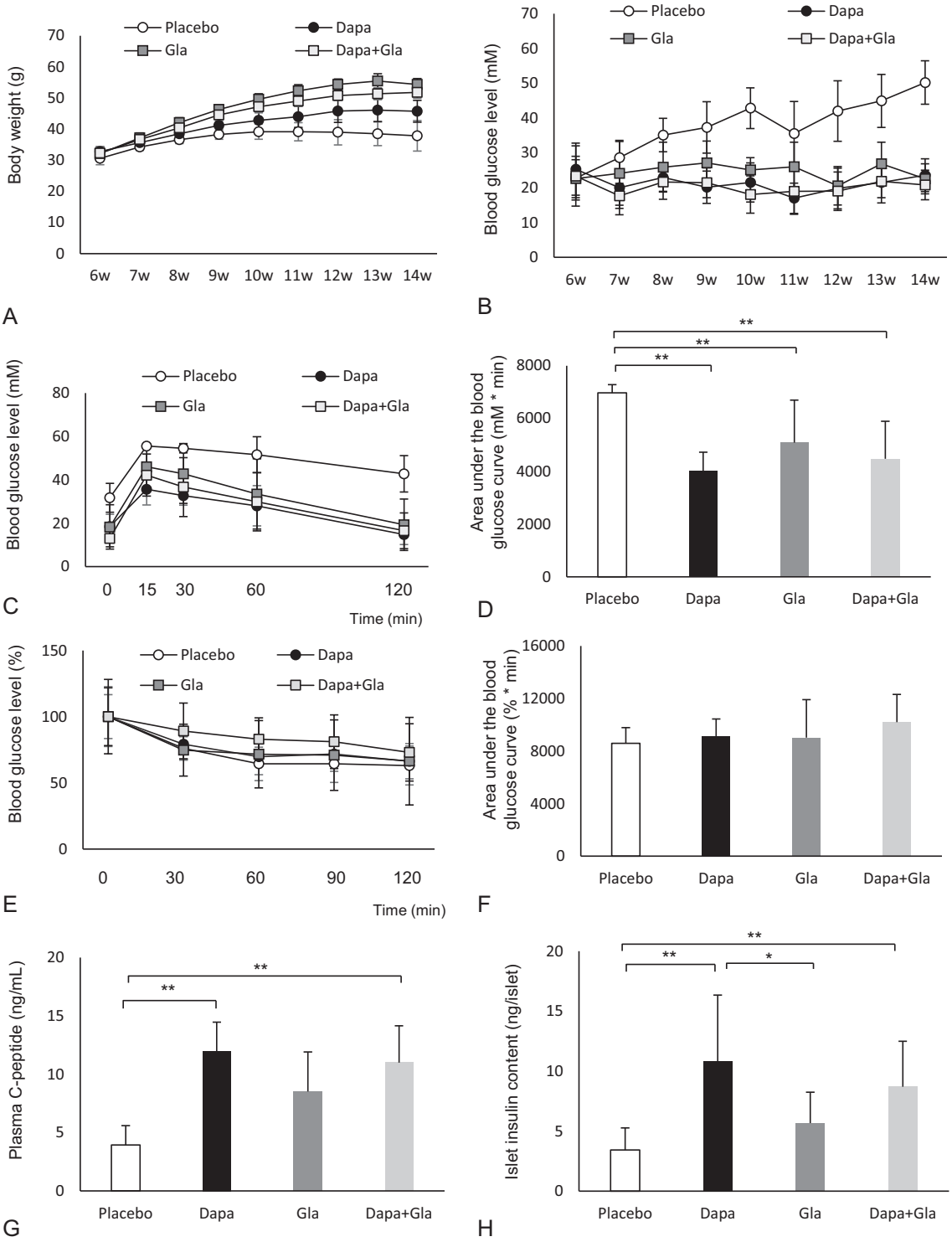


Fig. 1. Effects of dapagliflozin and/or insulin glargine on metabolic changes and glucose tolerance in db/db mice. A, B: Changes in (A) body weight and (B) blood glucose levels in db/db mice untreated (Placebo group; white circles), treated with dapagliflozin (1.0 mg/kg/day; Dapa group; black circles), treated with insulin glargine (Gla group; gray squires) or treated with dapagliflozin and insulin glargine (0.5 mg/kg/day; Dapa+Gla group; light gray squires) for 8 weeks ($n = 9$–10). C: Blood glucose levels during the oral glucose tolerance test (OGTT) in the Placebo group (white circles), the Dapa group (black circles), the Gla group (gray squires) and the Dapa+Gla group (light gray squires) after a 16-h fast at 13 weeks of age ($n = 9$–10). D: Area under the curve of glucose excursion during the OGTT in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) after a 16-h fast at 13 weeks of age ($n = 9$–10). E: Blood glucose levels during the intraperitoneal insulin tolerance test corrected with the baseline being 100% in the Placebo group (white circles), the Dapa group (black circles), the Gla group (gray squires) and the Dapa+Gla group (light gray squires) at 13 weeks of age ($n = 9$–10). F: Area under the curve of blood glucose levels during the intraperitoneal insulin tolerance test in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) at 13 weeks of age ($n = 9$–10). G: Plasma C-peptide in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) at 13 weeks of age ($n = 4$). H: Islet insulin content in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) at 14 weeks of age ($n = 4$). Values are mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$.

extraction from samples was executed by bead mill homogenization with TissueLyser (Qiagen, Inc., Hilden, Germany). The obtained supernatant was saponified in KOH. The saponified samples were dissolved in 100 ng/mL 18O2-containing methanol. The LC system, LC-20ADXR (Shimadzu Co., Ltd., Kyoto, Japan), was coupled with LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA). Fourier transform mass spectrometry detection was carried out in full scan mode at a resolution of 30,000 and a range of m/z 140–600. Fatty acids were detected by obtaining the extracted ion chromatograms of deprotonated ions ([M-H]-) at a mass tolerance of 10 ppm. Desaturation index corresponded to the ratio of monounsaturated fatty acids: oleate C18:1/saturated fatty acid: palmitate C16 plus stearate C18 (MUFA/SFA) ratio [30].

2.9. Microarray analysis

Total RNA from the liver was isolated with an RNeasy Mini Kit (Qiagen). mRNA expression profiles were determined using a Clariom S Mouse Gene 2.0 ST array (Thermo Fisher Scientific Inc., Waltham, MA, USA). Differentially expressed genes were defined as genes that showed at least a 1.5-fold change.

2.10. Real-time quantitative PCR

Total RNA was isolated from liver using an RNeasy mini kit (Qiagen) and was used as the starting material for cDNA preparation. Real-time PCR was performed in duplicate using a 7500 Fast Real Time PCR system with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primer sequences used are listed in Supplementary Table 2.

2.11. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Individual comparisons between more than two groups were assessed using one-way analysis of variance (ANOVA) followed by *post-hoc* Bonferroni's test. When the normality was rejected using the Shapiro-Wilk test, analysis was performed using Kruskal-Wallis test, followed by *post-hoc* Dunn's test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of dapagliflozin and/or insulin glargine on metabolic changes and glucose tolerance in db/db mice

Changes in body weight and fed blood glucose levels are shown in Fig. 1A and B. Although there were no differences in food intake between the four groups, the body weight was significantly higher in the treatment groups (Dapa, Gla and Dapa+Gla) compared with the Placebo group after 8 weeks of treatment (Fig. 1A and Supplementary Fig. 1A). Fed blood glucose levels increased over time in the Placebo group. However, in the treatment groups they similarly decreased (Fig. 1B). In the Placebo group, urine volume and urinary glucose excretion increased markedly due to prominent hyperglycemia (Supplementary Fig. 1B and C). However, they were clearly inhibited in the Gla group. In the Dapa and Dapa+Gla groups, urinary glucose excretion after 1 week of treatment was similar to that in the Placebo group. In contrast, after 4 and 8 weeks of treatment, it decreased in parallel with the blood glucose level (Supplementary Fig. 1C). Next, we evaluated glucose tolerance by OGTT after 7 weeks of treatment. Glucose tolerance evaluated by the $AUC_{0-120min}$ for blood glucose was significantly lower in the treatment groups (Dapa, Gla and Dapa+Gla) compared with the Placebo group (Fig. 1C and D). To examine the effects of dapagliflozin and insulin glargine on insulin sensitivity in db/db mice, we performed an intraperitoneal insulin tolerance test and found that there were no differences in insulin sensitivity in the four groups (Fig. 1E and F). Furthermore, to assess the effects of dapagliflozin and insulin glargine on insulin secretion, we measured plasma C-peptide and insulin content in isolated pancreatic islets. Both the plasma C-peptide and insulin content in the Dapa and Dapa+Gla groups were significantly higher than those in the Placebo group (Fig. 1G and H). These results indicated that the treatments with dapagliflozin for 8 weeks not only ameliorated glucose tolerance, but also improved the endogenous insulin secretion capacity in db/db mice.

3.2. Effects of dapagliflozin and/or insulin glargine on islet morphology in db/db mice

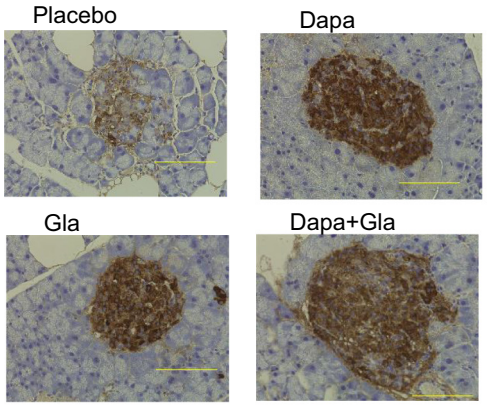
The improvement in endogenous insulin secretion capacity prompted us to investigate the effects of dapagliflozin and insulin glargine on beta cell mass after 8 weeks of treatment. Beta cell mass in the Dapa and Dapa+Gla groups was significantly higher, compared with the Placebo group (Fig. 2A and B). Similar results were obtained by point-counting. Next, we evaluated beta cell proliferation using immunohistochemical detection of Ki67. The ratio of Ki67-positive beta cells in the Dapa and Dapa+Gla groups was significantly higher, compared with the Placebo group (Fig. 2C). Additionally, the proportion of apoptotic beta cells in the three treatment groups, analyzed by the TUNEL assay, was about half that of the Placebo group, although the difference was not significant (Fig. 2D).

Furthermore, we performed double immunostaining using anti-insulin and anti-glucagon antibodies as described before [10] (Fig. 2E). The ratio of the number of beta cells to alpha plus beta cells in pancreatic islets, in the Dapa group was significantly higher compared with that in the Placebo group (Fig. 2F). However, there were no differences in the fasting plasma glucagon levels among the four groups (Supplementary Fig. 1D). These results suggest that the dapagliflozin and dapagliflozin plus insulin glargine treatments, but not the insulin glargine treatment, preserved beta cell mass in db/db mice.

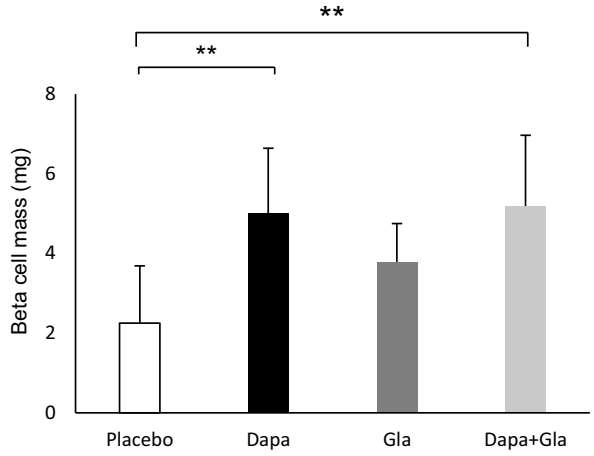
3.3. Effects of dapagliflozin and/or insulin glargine on liver steatosis in db/db mice

We measured the weight of visceral and subcutaneous fat, and the liver relative to the body weight. Although the total visceral fat weight was slightly higher only in the Dapa+Gla group compared with that in the Placebo group, the subcutaneous fat weight in the three treatment groups was significantly higher than that in the Placebo group after 8 weeks of treatment (Supplementary Fig. 2A). In contrast, the liver weight in the Dapa and Dapa+Gla groups, but not in the Gla group, was significantly lower than that in the Placebo group after 8 weeks (Fig. 3A). While the plasma TG levels were significantly lower in the three treatment groups, the plasma non-esterified fatty acid levels were significantly lower only in the Gla group compared with the Placebo group (Fig. 3B and C). Hematoxylin and eosin staining and Oil Red O staining of the liver revealed high fat and lipid droplets accumulation in the Gla group in the higher extent compared with that in the other three groups (Fig. 3D and E). When we quantified hepatic

Fig. 2. Effect of dapagliflozin and/or insulin glargine on beta cell morphology in db/db mice. A: Immunohistological analysis of pancreatic islets from the four groups (Placebo, Dapa, Gla and Dapa+Gla) of 14-week-old db/db mice. Beta cells are stained brown. Yellow scale bars, 100 μm. B: Quantitation of beta cell mass in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) ($n = 9–10$). C: Proliferation rate of beta cells assessed by Ki67 staining in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) ($n = 9–10$). D:Apoptosis rate of beta cells assessed using the fluorometric terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) ($n = 6–10$). E: Representative insulin (green) and glucagon (red) immunofluorescent staining of pancreas sections from the four groups (Placebo, Dapa, Gla and Dapa+Gla) of 14-week-old db/db mice. Yellow scale bars, 100 μm. F: The ratio of the number of beta cells to the sum of alpha plus beta cells in pancreatic islets from the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) ($n = 5$). Values are mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$.


Placebo
Dapa
Gla
Dapa+Gla

A


**
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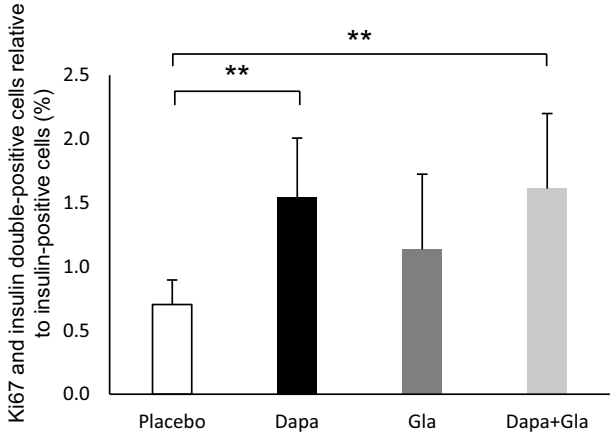
Beta cell mass (mg)
8
6
4
2
0
Placebo
Dapa
Gla
Dapa+Gla

B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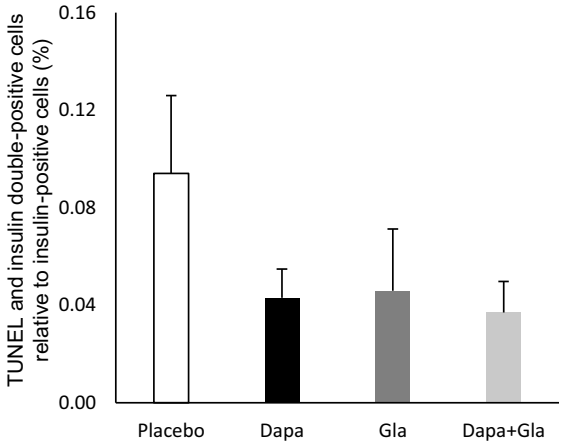
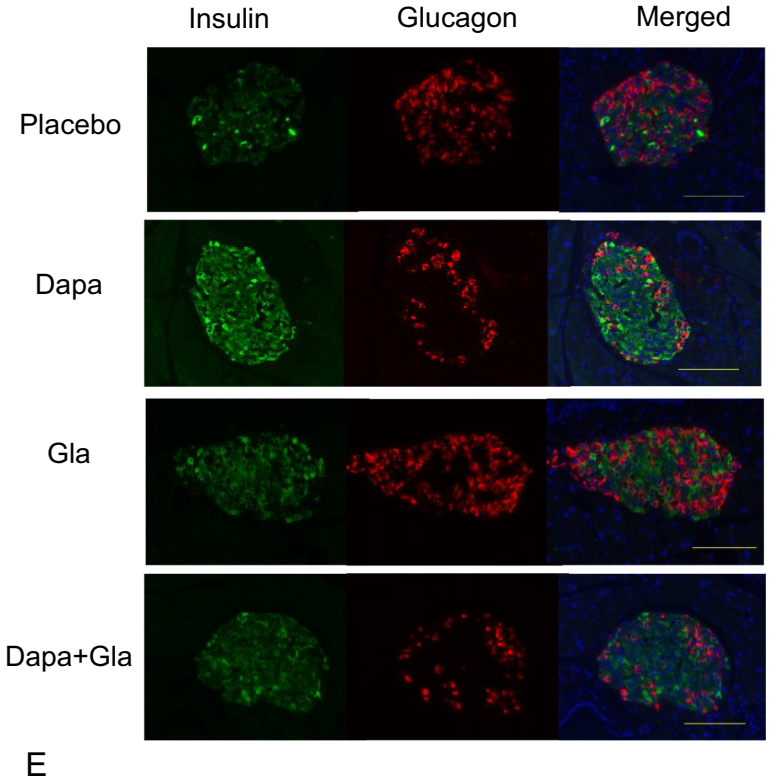
Ki67 and insulin double-positive cells relative to insulin-positive cells (%)
2.5
2.0
1.5
1.0
0.5
0.0
Placebo
Dapa
Gla
Dapa+Gla

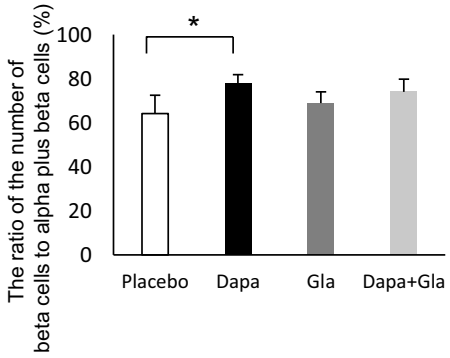
C


TUNEL and insulin double-positive cells relative to insulin-positive cells (%)
0.16
0.12
0.08
0.04
0.00
Placebo
Dapa
Gla
Dapa+Gla

D


Insulin
Glucagon
Merged
Placebo
Dapa
Gla
Dapa+Gla

E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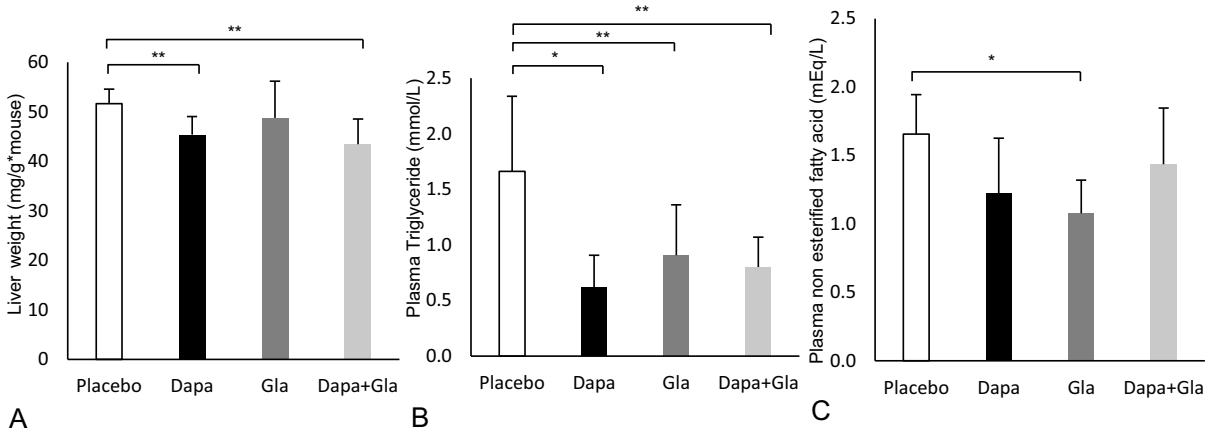
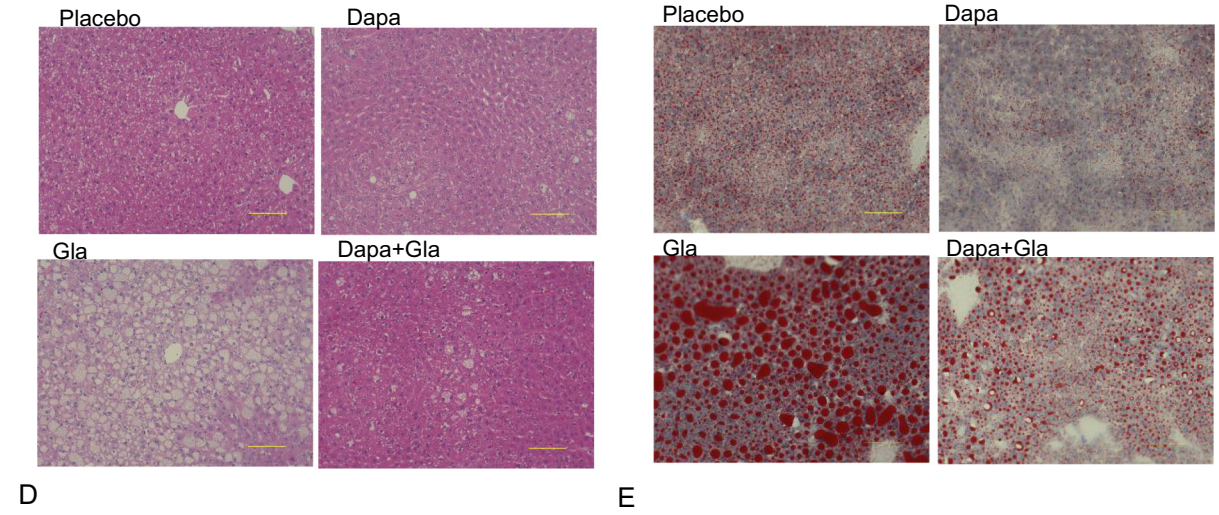
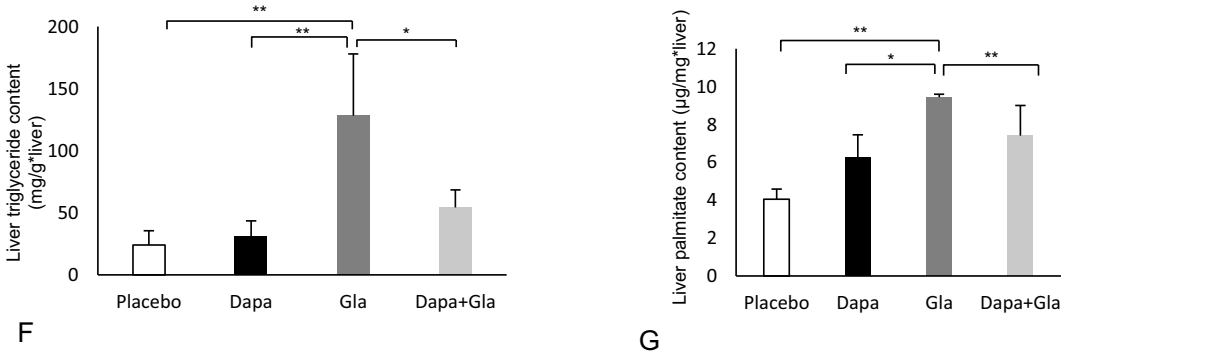
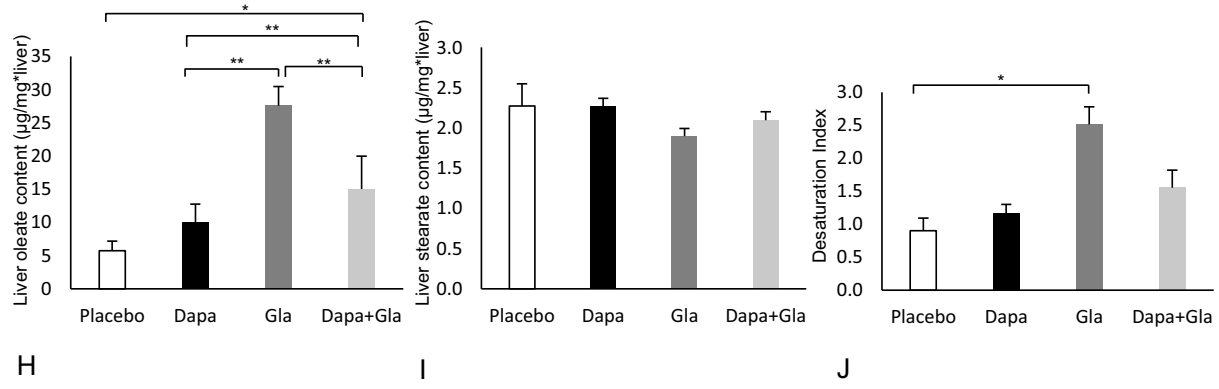
The ratio of the number of beta cells to alpha plus beta cells (%)
100
80
60
40
20
0
Placebo
Dapa
Gla
Dapa+Gla

F


Liver weight (mg/g*mouse)
Plasma Triglyceride (mmol/L)
Plasma non esterified fatty acid (mEq/L)
Placebo
Dapa
Gla
Dapa+Gla
A
B
C


Placebo
Dapa
Gla
Dapa+Gla
D
E


Liver triglyceride content (mg/g*liver)
Liver palmitate content (μg/mg*liver)
Placebo
Dapa
Gla
Dapa+Gla
F
G


Liver oleate content (μg/mg*liver)
Liver stearate content (μg/mg*liver)
Desaturation Index
Placebo
Dapa
Gla
Dapa+Gla
H
I
J

tissues on the basis of NASH/NAFLD Clinical Research Network scoring system definition and scores [28], the total score significantly elevated in the Gla group compared with that in the other three groups (Supplementary Fig. 2B). We also evaluated steatosis in these hepatic tissues using point counting system [29], indicating a significant increase of steatosis in the Gla group compared with that in the other three groups (Supplementary Fig. 2C). Strikingly, the liver TG content was markedly elevated in the Gla group compared with that in the other three groups (Fig. 3F). Although the plasma total cholesterol levels was higher in the Gla group compared with the Placebo and Dapa+Gla groups, there were no differences in liver total cholesterol content among the four groups (Supplementary Fig. 2D and E). We next investigated the composition of the liver fatty acid contents and found that the palmitate and oleate contents were significantly increased in the Gla group compared with the other three groups (Fig. 3G, H), although there was no difference in the liver stearate content among the four groups (Fig. 3I). The desaturation index, which was calculated by the level of these three fatty acids, was higher in the Gla group compared with the Placebo group (Fig. 3J). An increase in this index is typically observed when lipogenesis is induced. Additionally, we evaluated the composition of the plasma fatty acid contents and found that there were no differences in plasma palmitate, oleate or stearate contents among the four groups (Supplementary Fig. 2F—H). However, there was a significant increase in the desaturation index in the Gla group compared with the Dapa and Dapa+Gla groups (Supplementary Fig. 2I). These results indicated that administration of insulin glargine, but not dapagliflozin, induced liver steatosis in db/db mice.

3.4. Effects of dapagliflozin and/or insulin glargine on gene expression in the liver of db/db mice

We investigated the expression levels of genes associated with liver steatosis in db/db mice treated with dapagliflozin and/or insulin glargine. First, we performed a microarray analysis of genes expressed in the liver of mice from the four groups. Eighty-nine genes in the Dapa group, 158 in the Gla group and 219 in the Dapa+Gla group were significantly upregulated (fold change ≥1.5 and $p < 0.05$), compared with the Placebo group. As shown in Table 1, *Cidea*, *Cidec* and *Mogat1*, which are mainly related to fatty acid storage and synthesis, were upregulated in the Gla group compared with the Placebo group. These genes were not upregulated in the Dapa or Dapa+Gla group. Real-time quantitative PCR revealed that the expression of genes associated with fatty acid synthesis, such as *Fas*, *Elovl6*, *Scd1* and *Mogat1*, was significantly upregulated in the Gla group compared with the Placebo group (Fig. 4A). As for the expression levels of genes related to fatty acid uptake and storage, *Cidea* and *Cidec* were significantly elevated in the Gla group compared with the Placebo group. *Pparg2*, which is a target gene associated with *Fas*, *Elovl6*, *Scd1*, *Mogat1* and *Cidec*, was significantly upregulated in the Gla group compared with the Placebo group (Fig. 4B). However, there were no significant differences in the expression levels of genes involved in fatty acid oxidation nor inflammation among the four groups (Fig. 4C and D). These results suggest that insulin glargine promoted fatty acid synthesis, uptake and storage in the liver of db/db mice, which led to fatty acid accumulation and lipid droplets formation.

Table 1
Microarray analysis of liver gene expression: the top ten upregulated genes in the Dapa, Gla and Dapa+Gla groups compared with the Placebo group.

Rank	Ratio	Gene symbol	Gene description	*p* value
Dapa vs. Placebo				
1	3.78	*Gck*	Glucokinase	0.01
2	2.64	*Pcsk9*	Proprotein convertase subtilisin/kexin type 9	0.04
3	2.57	*Ifit3*	Interferon-induced protein with tetratricopeptide repeats3	<0.01
4	2.56	*Ifit3b*	Interferon-induced protein with tetratricopeptide repeats3B	<0.01
5	2.40	*Gm10972*	Predicted gene 10972	0.01
6	2.39	*Fdps*	Farnesyl diphosphate synthase	0.04
7	2.34	*Cyp17a1*	Cytochrome P450, family 17, subfamily a, polypeptide 1	<0.01
8	2.33	*Gm10198*	Predicted gene 10198	0.03
9	2.31	*Nudt7*	Nudix-type motif 7	0.04
10	2.20	*Zfp125*	Zinc finger protein 125	<0.01
Gla vs. Placebo				
1	7.14	*Cidec*	Cell death-inducing DFFA-like effector c	0.02
2	6.73	*Cidea*	Cell death-inducing DFFA -like effector A	0.046
3	5.40	*Gck*	Glucokinase	<0.01
4	3.81	*Pltp*	Phospholipid transfer protein	0.04
5	3.48	*Aqp8*	Aquaporin 8	0.04
6	3.29	*Abcd2*	ATP-binding cassette, sub-family D (ALD), member 2	0.02
7	3.24	*Osbpl3*	Oxysterol binding protein-like 3	0.02
8	3.02	*Slc10a2*	Solute carrier family 10, member 2	<0.01
9	2.91	*Mogat1*	Monoacylglycerol O-acyltransferase 1	0.048
10	2.89	*Klk1b4*	Kallikrein 1-related peptidase b4	<0.01
Dapa+Gla vs. Placebo				
1	4.43	*Mvd*	Mevalonate (diphospho) decarboxylase	<0.01
2	4.20	*Gck*	Glucokinase	<0.01
3	4.12	*Pltp*	Phospholipid transfer protein	0.02
4	4.07	*Acly*	ATP citrate lyase	<0.01
5	3.97	*Fasn*	Fatty acid synthase	0.02
6	3.77	*Cyp51*	Cytochrome P450, family 51	0.02
7	3.66	*Me1*	Malic enzyme 1, NADP(+)-dependent, cytosolic	<0.01
8	3.63	*Aqp8*	Aquaporin 8	0.01
9	3.50	*H60b*	Histocompatibility 60b	0.01
10	3.37	*Hmgcr*	3-Hydroxy-3-methylglutaryl-Coenzyme A reductase	0.047

4. Discussion

The db/db mice represent well the characteristics of the natural history of type 2 diabetes as these mice show progressively decreased beta cell function and mass. According to our previous study [10], pancreatic beta cell function and mass in db/db mice gradually decreased from 6 weeks of age. Thus, in this study we administered dapagliflozin and insulin glargine from 6 weeks. We found that compared with the Placebo group, body weight augmented by administration of these agents (Fig. 1A). Although it is generally considered that treatment with SGLT2 inhibitors does not increase body weight [31], we found that compared with the Placebo group, body weight augmented by administration of these agents (Fig. 1A), even in the Dapa group. Similar results of the effect of SGLT2 inhibitors on body weight in db/db mice were observed in the previous reports [17,32].

Because of the marked hyperglycemia followed by increased catabolism, mice in the Placebo group tended to lose body weight as time

Fig. 3. Effects of dapagliflozin and/or insulin glargine on liver steatosis in db/db mice. A: Liver weight as a proportion of body weight in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) at 14 weeks of age ($n = 9$–10). B: Fasting plasma triglyceride level in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) at 13 weeks of age ($n = 4$). C: Fasting plasma non-esterified fatty acid level in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) at 13 weeks of age ($n = 4$). D, E: (D) Hematoxylin and eosin staining, and (E) Oil Red O staining of liver sections from the four groups (Placebo, Dapa, Gla and Dapa+Gla) at 14 weeks of age. Yellow scale bars, 100 μm. F: Liver triglyceride content in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) at 14 weeks of age ($n = 4$). G–I: Liver (G) palmitate, (H) oleate and (I) stearate content in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) at 14 weeks of age ($n = 4$). J: Liver desaturation index in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) at 14 weeks of age ($n = 4$). Values are mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$.

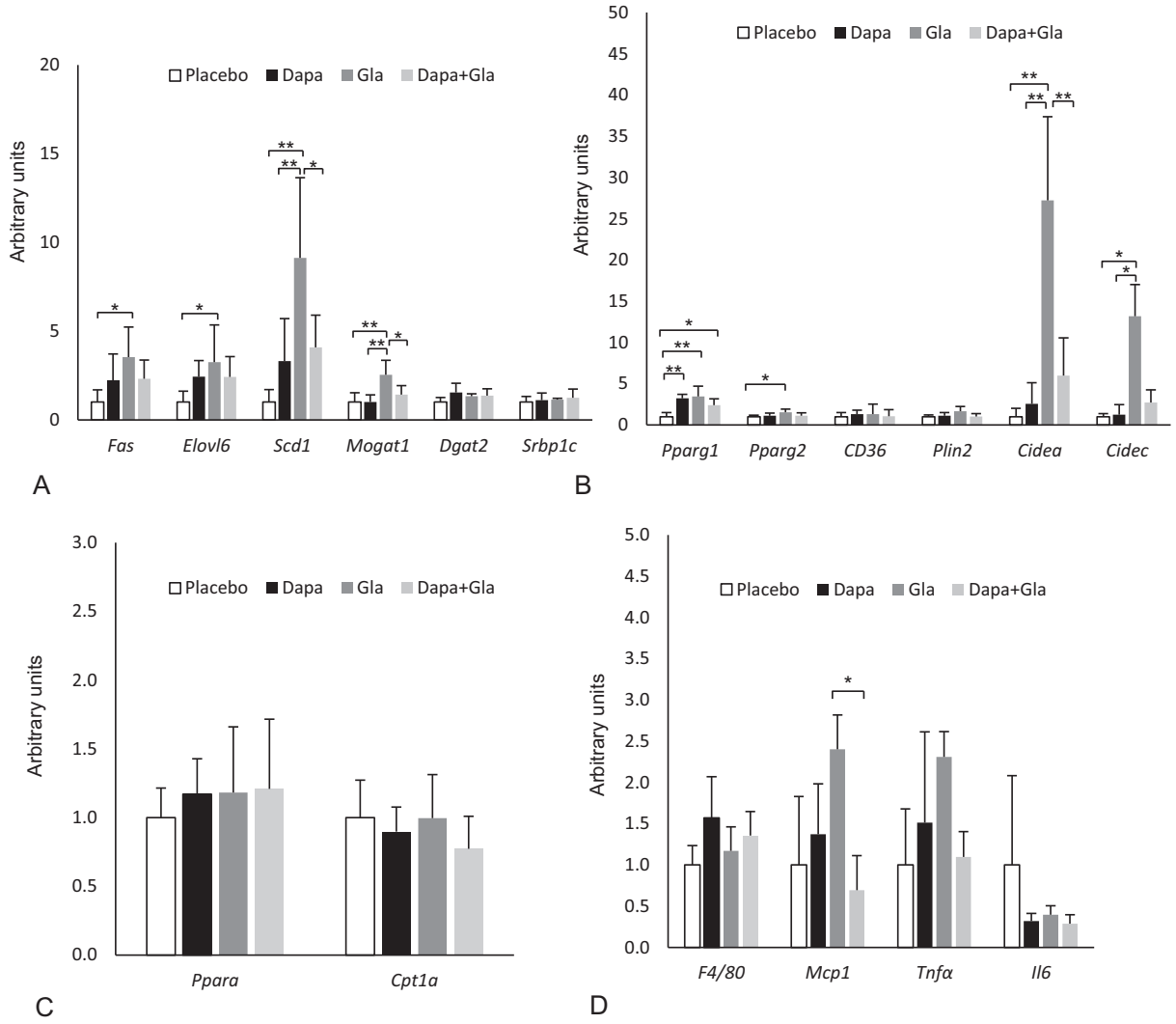


Fig. 4. Effects of dapagliflozin and/or insulin glargine on gene expression in the liver of db/db mice. A–D: Changes in hepatic expression levels of genes involved in (A) fatty acid synthesis, (B) fatty acid uptake and storage, (C) fatty acid oxidation and (D) inflammation in the Placebo group (white bar), the Dapa group (black bar), the Gla group (gray bar) and the Dapa+Gla group (light gray bar) at 14 weeks of age. These genes were measured by real-time quantitative PCR. Data have been normalized to *beta actin* expression ($n = 6$). Values are mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$.

proceeds (Fig. 1A). In contrast, mice in the other three treatment groups (Dapa, Gla and Dapa+Gla) gained body weight because catabolism was prevented by ameliorating hyperglycemia. Moreover, the body weight of the Gla group was the highest because treatment with insulin delivers glucose into the body rather than promote urinary glucose excretion (Supplementary Fig. 1C).

The primary aim of this study was to investigate the effect of dapagliflozin and/or insulin glargine on the beta cell mass. The beta cell mass in the Dapa group was significantly retained, compared with the placebo group (Fig. 2A and B), which was consistent with previous reports [10,17]. The reduction of glucotoxicity is considered to be an important mechanism for the preserving effect on beta cell mass [32].

Why was the beta cell mass not retained in the Gla group whose blood glucose levels were equivalent to those of the Dapa and Dapa +Gla groups? One plausible explanation is that the blood glucose levels in the Gla group were largely varied. We administered insulin by subcutaneous injection, which may not be able to achieve a stable hypoglycemic effect. The action of insulin glargine peaks, which may result in increased blood glucose fluctuation. However, the daily profile of blood glucose (Supplementary Fig. 3) showed that there were no significant differences in the mean blood glucose levels or blood glucose fluctuations among the three treatment groups. Another possible explanation is differences in lipid profiles, which may be related to lipotoxicity. However, there were no differences in the plasma TG nor free fatty acid levels among the three treatment groups (Fig. 3B and C). Furthermore, our microarray data of pancreatic islets revealed that the expression level of insulin receptor substrate 2 (Irs2) in the Gla group was significantly decreased compared with the Placebo group (0.35 fold, $p = 0.04$), while those in the Dapa and Dapa+Gla groups were not significantly changed (data not shown). Given that Irs2 plays an important role in regulating pancreatic beta cell mass [33–35], which might affect beta cell mass in the Gla group in spite of maintaining the blood glucose as the same level as the Dapa and Dapa+Gla groups.

Of particular interest, severe liver TG accumulation was observed in the Gla group (Fig. 3E and F). Although SGLT2 inhibitors have been reported to prevent liver steatosis [21–24], there was no difference in liver TG content between the Dapa and the Placebo groups. As mentioned earlier, owing to marked hyperglycemia, catabolism progressed in the Placebo group, resulting in reduced TG content in the liver. To elucidate the mechanism of liver steatosis in the Gla group, we performed microarray analysis and real-time quantitative PCR. The results revealed that groups of genes involved in fatty acid synthesis and lipid accumulation were significantly upregulated in the Gla

group, while no significant change was observed in terms of genes involved in fatty acid oxidation (Fig. 4). It is generally recognized that *Srebp1c* is the key molecule in fatty acid synthesis [36]. Despite the upregulated expression of molecules such as *Fas*, *Elovl6* and *Scd1*, which are downstream of *Srebp1c*, the expression of *Srebp1c* was not upregulated in the Gla group (Fig. 4A). Therefore, the mechanism of liver steatosis induced by insulin glargine appears to be independent of *Srebp1c* [37]. Our results also indicated that the expression level of *Pparg2* was significantly elevated in the Gla group compared with the Placebo group (Fig. 4B). Pparg2 plays an important role in liver steatosis [36–38]. *Mogat1* and *Cidec*, which are regulated by *Pparg* [36], were also upregulated in the Gla group compared with the Placebo group. Furthermore, it has been reported that insulin administration is responsible for *Pparg* upregulation [39], thus treatment with insulin glargine appears to induce liver steatosis through a Pparg-related pathway.

Type 2 diabetes is caused by progressive beta cell dysfunction, mainly due to hyperglycemia. To prevent beta cell impairment, chronic hyperglycemia should be alleviated. There are two strategies for improving glycemic control: one is to stimulate insulin secretion from pancreatic beta cells and the other is to reduce the demand for insulin. The latter is called "beta cell rest" and is more favorable for protecting beta cell sustainably [40]. Insulin and SGLT2 inhibitors contribute to this "beta cell rest", which prompted us to compare these two agents. Herein, we found that treatment with dapagliflozin or dapagliflozin plus insulin glargine, but not insulin glargine alone, prevented beta cell dysfunction and ultimately liver steatosis in db/db mice. Considering nonalcoholic fatty liver disease induces nonalcoholic steatohepatitis or hepatocellular carcinoma, SGLT2 inhibitors could be an effective alternative for improving hyperglycemia. However, it should be noted that SGLT2 inhibitors may cause ketoacidosis under conditions such as marked impairment of insulin secretion. In such cases, the combination therapy of an SGLT2 inhibitor and insulin would be recommended as our results indicated that this combination therapy was as effective as SGLT2 inhibitor monotherapy. Further study is required to examine the efficacy and safety of these treatments including the combination therapy in randomized clinical trials.

In conclusion, our results showed that beta cell mass was preserved and hepatic steatosis was prevented in db/db mice, after 8 weeks of treatment with dapagliflozin or dapagliflozin plus insulin glargine, but not with insulin glargine. This study would provide new insight into the treatment of type 2 diabetes.

Declaration of Competing Interest

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Author contribution

All authors conceived and designed the study, and participated in the analysis and interpretation of the data. K.O. and A.N. drafted the manuscript and all other authors revised it critically for intellectual content. K.C.H. evaluated hepatic tissues pathologically. R.T. gave a useful statistical advice in analyzing data. All authors approved the final version of the paper.

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.06.006.

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