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Gpihbp1 deficiency accelerates atherosclerosis and plaque instability in diabetic *Ldlr*$^{-/-}$ mice

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HIGHLIGHTS

- *Gpihbp1*$^{-/-}$ promotes atherosclerosis (AS) and plaques instability in *Ldlr*$^{-/-}$ mice.
- Diabetes accelerates AS and elicits coronary AS and aortic remodeling.
- *Gpihbp1* deficiency aggravates AS by increasing oxidative stress and inflammation.

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ABSTRACT

Background and aims: Glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1) plays a crucial role in triglyceride hydrolysis, and GPIHBP1 deficiency leads to severe hypertriglyceridemia (HTG). *Gpihbp1* knockout (GKO) mice develop mild lesions in the aortic root at the age of 11 months. Herein, we investigated the effect of *Gpihbp1* deficiency on atherosclerosis (AS) under diabetic conditions.

Methods: For experiment 1, diabetes was induced in GKO and wild-type (WT) mice by injection of streptozotocin at 3 months of age and lasted for 4 months. For experiment 2, diabetes was induced in *Gpihbp1*/low-density lipoprotein receptor (*Ldlr*) double-knockout (GLDKO) mice, *Ldlr* knockout (LKO) mice were used as controls. The experiment was continued for 3 or 5 months. Plasma glucose and lipid levels were measured, and atherosclerotic lesions were analyzed at 3 and 5 months during the experiment.

Results: No atherosclerotic lesions were detected in the aorta in GKO mice after 4 months of diabetes. Compared with LKO mice, GLDKO mice manifested enhanced aortic atherosclerotic lesions, decreased plaque stability, and increased oxidative stress and inflammation in plaques at 3 and 5 months after diabetes. Atherosclerotic lesions in the coronary artery and dilated remodeling in the aortic root were also found in GLDKO diabetic mice.

Conclusions: *Gpihbp1* deficiency accelerates the development of AS in the aorta, and the instability of plaques in LKO mice and diabetes promotes these pathologic processes with coronary AS. These findings were probably associated with HTG caused by *Gpihbp1* deficiency and with increased oxidative stress and inflammation in the atherosclerotic lesions.

1. Introduction

Cardiovascular diseases (CVD) have long been recognized as major causes of morbidity and mortality. Atherosclerosis (AS) is the crucial underlying cause of CVD [1]. Furthermore, dyslipidemia is one of the most important risk factors for AS [2–4].

Hypertriglyceridemia (HTG) has been shown to be an independent risk factor for CVD in many studies, and it is causally associated with an increased risk of AS [4–6]. HTG is characterized by high plasma levels of chylomicron (CM), very low-density lipoprotein (VLDL) and their remnants. They play an important role in the development of AS because the small size of the remnants can easily infiltrate the

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subendothelial space [4]. However, several other studies have shown that no independent relationship exists between HTG and the risk of CVD events. Nonetheless, HTG could certainly serve as a synergistic factor in preexisting atherosclerotic cardiovascular disease (ASCVD) events [4,6].

Diabetes increases the risk of developing AS by 2- to 10-fold and accounts for up to 80% of the mortality due to diabetes-related causes [7,8]. Several studies have shown the effects of diabetes on the initiation and progression of AS [9–12]. In addition, diabetic plaques with higher numbers of macrophages exhibited increased oxidative stress and inflammatory gene expression in diabetic low-density lipoprotein receptor deficiency ($Ldlr^{-/-}$, LKO) mice [10]. Inflammatory processes and high oxidative stress levels might play crucial roles in diabetic AS [13]. Interestingly, diabetes increases plaque disruption in advanced lesions through a mechanism that requires triglyceride-rich lipoproteins (TRLs) [14]. HTG plays a significant role in diabetic AS in humans; however, the exact mechanisms are not fully understood [6,15].

Glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1) has shown a considerable effect on the hydrolysis of triglyceride (TG) located exclusively on capillary endothelial cells (ECs). It chaperones lipoprotein lipase (LPL) across the capillary lumen to the apical side [16,17] and acts as a "platform" for hydrolysis of TG by LPL [18]. Furthermore, *Gpihbp1* mutations in patients are associated with severe HTG with impaired intravascular hydrolysis of TG [18–22]. In addition, a woman with *Gpihbp1* mutations was reported to have stenosis and occlusion in the coronary artery [23]. Recently, clinical studies on screening *Gpihbp1* mutations in patients with HTG have shown that the *Gpihbp1* mutation is associated with various diseases, especially CVD [23,24]. However, the association between *Gpihbp1* mutations in humans and diabetic CVD has not been studied.

Gpihbp1 knockout (GKO) mice have severe HTG with mild atherosclerotic lesions in the aortic root at 11–12 months of age and moderate lesions at 16–22 months of age [25]. However, the effects of *Gpihbp1* deficiency on diabetic AS and the stability of atherosclerotic lesions were not mentioned in the study.

In our study, we used *Gpihbp1*-deficient mice with a background of LKO, which is susceptible to AS, to study the effect of *Gpihbp1* on atherosclerotic lesions and the plaque stability of advanced lesions under diabetic conditions. Diabetes was induced by injection of streptozotocin (STZ). The results showed that *Gpihbp1* deficiency accelerated the development of AS in the aorta and instability of plaques in LKO mice with increased oxidative stress and inflammation and that diabetes not only promoted these pathologic processes but also elicited coronary atherogenesis.

2. Materials and methods

2.1. Animals

GKO mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), and littermate wild-type (WT) mice were obtained from Vital River Laboratory (Charles River, Beijing, China). All animals used were on a C57BL/6 background. $Gpihbp1^{-/-}Ldlr^{-/-}$ (GLDKO) mice were generated by crossing GKO mice with LKO mice. Genotyping of these mice was performed by PCR analysis of genomic DNA extracted from their tails [26]. All mice were maintained on a 12-hour light/12-hour dark cycle with free access to water. The study was approved by the Peking University Animal Ethics Committee, Peking University Health Science Center. The Principles of Laboratory Animal Care (NIH Publication, 8th Edition, 2011) were followed.

2.2. Induction of diabetes and experimental design

Type 1 diabetes was induced in 3-month-old male mice by 5–7 successive daily intraperitoneal (i.p.) injections of STZ (Sigma-Aldrich, St Louis, MO, USA) at a dose of 60 mg/kg in citrate buffer as described previously [27]. After the induction of diabetes, only animals with plasma glucose (GLU) levels higher than 300 mg/dL were included in the study. For experiment 1, mice were divided into 4 groups (n = 10): WT and GKO nondiabetic control (Con) groups, and WT and GKO diabetic (Dia) groups. Mice were euthanized at 4 months after the induction of diabetes. For experiment 2, we studied the effect of *Gpihbp1* deficiency on diabetic AS in mice with a LKO background that is susceptible to AS with hypercholesterolemia (HTC). Animals were divided into 4 groups (n = 20): LKO and GLDKO control groups, and LKO and GLDKO diabetic groups. In experiment 2, two weeks after the establishment of diabetes, all mice were fed a modified high-fat diet (HFD). We created the modified HFD by adding 0.05% cholesterol and 5% lard to a chow diet (5.4% fat) in this study, because high mortality was found in GLDKO mice fed a diet of 0.1% cholesterol and 10% or 20% lard added to a chow diet (Supplemental Fig. 2). Mice were euthanized at 3 months (n = 10) or 5 months after induction of diabetes (n = 10). One GLDKO-Dia mouse died in the 3-month group, and two died in the 5-month group. The LKO and GLDKO mice with or without diabetes fed a chow diet were included in a supplemental study for comparison with the modified HFD groups (n = 6).

2.3. Measurement of plasma glucose and lipid levels

Mice were fasted for 4 h, blood samples were taken from the retro-orbital plexus, and plasma was separated by centrifugation for 10 min at 4000 rpm. The concentrations of plasma GLU, total cholesterol (TC) and TG were measured by enzymatic colorimetric methods using commercial kits (Sigma).

2.4. Atherosclerotic lesion analysis

Mice were anesthetized by i.p. injection of 1% sodium phenobarbital (45 mg/kg) and flushed with 20 mL 0.01 M phosphate-buffered solution (PBS) through the left ventricle. After perfusion with 4% paraformaldehyde, the total aorta and heart were harvested and fixed. The *en face* aortic root preparation and quantification of the lesion areas in the whole aorta were performed as described previously [28–30]. For *en face* analysis, the whole aorta was cut open and then stained with oil red O (ORO) (Sigma). For lesion assessment in the individual regions of the whole aorta, the 3 segments defined as the aortic arch (aortic root to below the left subclavian), thoracic aorta (the region between the end of the arch and the last intercostal branch) and abdominal aorta (the region between the end of the thoracic aorta segment and the iliac bifurcation) [31]. For aortic root analysis, the heart was embedded in O.C.T. compound, snap-frozen in liquid nitrogen and cross-sectioned serially at the aortic root level at a 7 μm thickness [28]. The cryosections were then stained with ORO and counterstained with hematoxylin. Images were obtained with a Leica graphic analysis system, and quantification was determined with ImageJ [30].

For coronary atherosclerosis analysis, the heart was cross-sectioned at a 7 μm thickness from the level of the aortic sinus to that of the papillary muscle at an interval of 300 μm. For each heart, six levels were obtained and analyzed individually after ORO staining as described previously [32–34]. The severity of coronary atherosclerosis was divided into four categories as follows: none, < 50% occluded, > 50 (50–95%) occluded, and 100% occluded. Data are presented as the percentage of the number of coronary arteries (CAs) with the same degree of occlusion to the total number of CAs for the level of sinus or papillary muscle.

Analysis of atherosclerotic plaque cell composition and evidence of damage from inflammation and lipid peroxidation were conducted by immunohistochemistry (IHC) in the aortic root. Macrophages and smooth muscle cells (SMCs) were stained with CD68 antibody (ab53444, diluted at 1:200; Abcam, Cambridge, UK) and α-SMA antibody (A5228, diluted at 1:200; Sigma), respectively. Inflammation and

lipid peroxidation were analyzed by staining for vascular cell adhesion molecule 1 (VCAM1) and 4-hydroxynonenal (4HNE) using VCAM1 antibody (ab134047, diluted at 1:200; Abcam) and 4HNE antibody (ab48506, diluted at 1:200; Abcam, Cambridge, UK), respectively. The cryosections were fixed (10 min) in 4% paraformaldehyde solution and rinsed (10 min) with PBS (0.1 M, pH 7.4) supplemented with 3% hydrogen peroxide. After washing and incubation (30 min) in blocking solution (PBS containing 10% goat serum), the sections were incubated overnight at 4 °C with the primary antibody, diluted in blocking solution, and washed three times with PBS. The cryosections were then incubated with the appropriate biotinylated secondary antibodies (1:200, ABC Vectastain; Vector Laboratories, Burlingame, CA, USA) in 2% normal blocking serum and visualized using 3, 3′-diaminobenzidine (DAB, Vectastain; Vector Laboratories) [35]. The results of the plaque cell composition and IHC were represented by the percentage staining of the total plaque area. Dihydroethidium (DHE) staining was used to evaluate oxidative stress levels in the aortic sinus (n = 6). Freshly frozen aortic sections were incubated with DHE (Bestbio, Shanghai, China; 10 μM, 45 min, 37 °C) in the dark and visualized by an immunofluorescence microscope (Leica DMI 3000B, Wetzlar, Germany) using the same exposure for every section [26]. DHE fluorescence was represented by the percentage of fluorescence intensity to the aortic root area using ImageJ software [36].

For plaque stability analysis, the areas containing collagen (red on Sirius red stain), lipid-rich cores (red on ORO stain), SMC (α-SMA-positive area) and macrophage accumulation (CD68-positive area) were estimated by the percentage staining of the total plaque area. Plaque stability was evaluated by the formula plaque stabilization score = (SMC area + collagen area)/(macrophage area + lipid area) [37]. The surface area of the aortic root was also determined to evaluate arterial remodeling.

2.5. Statistical analysis

All data are presented as the means ± SEM. Statistical comparison between the groups was performed using two-way ANOVA followed by Tukey's test or the Mann-Whitney *U* test for nonparametric data. GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA) was used for the statistical analyses. A value of $p < 0.05$ was considered indicative of statistical significance.

3. Results

3.1. Changes in plasma glucose and lipid levels and atherogenesis in GKO mice after diabetes

To study the contribution of *Gpihbp1* deficiency to atherogenesis under diabetic conditions, diabetes was induced in GKO mice. Plasma GLU levels were increased to ~400 mg/dL after diabetes induction, confirming the successful establishment of diabetes. *Gpihbp1* deficiency did not influence plasma GLU levels in either the control or diabetic group (Fig. 1A). Extremely high plasma TG levels (3000–4000 mg/dL) and increased plasma TC levels were detected in GKO mice compared to those in WT mice ($p < 0.001$, Fig. 1B and C). These results were consistent with a previous study [16]. Moreover, we found that plasma TG levels were further increased in GKO mice 4 months after induction of diabetes compared with those in GKO control mice (Fig. 1B). However, we did not observe atherosclerotic lesions in the aorta or aortic root in GKO mice at 7 months of age, 4 months after induction of diabetes (Supplemental Fig. 1).

3.2. Changes in plasma glucose and lipid levels in GLDKO mice after diabetes

To study the effects of *Gpihbp1* deficiency on the development of AS, GLDKO mice were used in this study. *Gpihbp1* deficiency did not influence plasma GLU levels in either the control or diabetic group (Fig. 1D). GLDKO mice had higher plasma TG and TC levels than LKO mice throughout the experiment in both the control and diabetic groups. Compared with basal levels, plasma TG levels began to increase one month after initiation of the modified HFD (0.05% cholesterol and 5% lard to the chow diet) in the nondiabetic control group, plasma TC levels were increased only at 5 months after initiation of the modified HFD in GLDKO mice ($p < 0.05$, Fig. 1E and F). For the diabetic group, plasma TG and TC levels were increased one month after induction of diabetes and modified HFD compared to basal status and remained similar until the end of the study. Diabetes did not further increase plasma TG and TC levels in GLDKO mice (Fig. 1E and F).

3.3. Gpihbp1 deficiency promoted atherogenesis in the aorta and CA in GLDKO mice after diabetes

The whole aortas and aortic roots were stained with ORO for quantification of lesions after 3 and 5 months of diabetes. In the *en face* analysis at 3 months, we detected that the total plaque areas were greater in GLDKO mice than in LKO mice in both the control and diabetic groups. Diabetes increased the total plaque areas in the LKO group ($p < 0.001$) but not in the GLDKO group. Further analysis of plaque areas in different aortic sections showed that the plaque areas of both the aortic arch and abdominal aorta were greater in GLDKO-Con mice than in LKO-Con mice ($p < 0.01$, $p < 0.01$). Notably, diabetes increased the plaque areas only in the abdominal aorta in the GLDKO group at 3 months ($p < 0.05$, Fig. 2A–E). In the 5-month study, the total plaque areas were greater in the GLDKO-Con group than in the LKO-Con group ($p < 0.01$), and diabetes increased the total plaque areas in the GLDKO group ($p < 0.05$). In different sections of the aorta, we observed that the plaque areas of the thoracic and abdominal aorta in the GLDKO-Dia group were increased compared with those in the GLDKO-Con ($p < 0.01$, $p < 0.05$) and LKO-Dia groups ($p < 0.01$, $p < 0.01$, Fig. 2F–J).

For the quantification analysis of plaque areas in the aortic root at 3 months, the plaque areas were greatly increased in the GLDKO-Dia group compared with those in the GLDKO-Con and LKO-Dia groups ($p < 0.001$, Fig. 2K and M). At 5 months, GLDKO mice had considerably larger plaque areas than LKO mice in both the control and diabetic groups ($p < 0.001$). However, diabetes did not aggravate atherosclerotic lesions in aortic roots in GLDKO mice, probably due to a saturation state of plaque lesions in the root at 5 months (Fig. 2L and N). Overall, diabetes increased the plaque areas in the aortic root rather than in the total aorta in GLDKO mice at 3 months after induction of diabetes. As AS progressed, contradictory results were detected at 5 months after induction of diabetes. A diabetes-induced increase in atherosclerotic lesions in GLDKO mice mainly occurred in the abdominal segment at 3 months, while it was dominant in the thoracic segment at 5 months.

Notably, atherosclerotic lesions were detected in more than 80% of the CAs at the aortic sinus level in the GLDKO-Dia group at 3 months, 22.2% of CAs were > 50% occluded, and 66.7% were < 50% occluded (Fig. 3A and B). In the GLDKO-Dia group at 5 months, 37.5% of CAs were totally occluded, 25% were > 50% occluded and 25% were < 50% occluded (Fig. 3A and B). There were fewer CA lesions at the papillary muscle level compared with that at the aortic sinus level: only 11.1% or 37.5% of CA were < 50% occluded in the GLDKO-Dia group at 3 or 5 months, respectively (Fig. 3C and D). CA lesions at the other four levels between the aortic sinus and the papillary muscle were quite similar to those of the papillary muscle level. No CA lesions were found in the other 3 groups at the aortic sinus (Fig. 3A and B) or papillary muscle level (Fig. 3C and D).

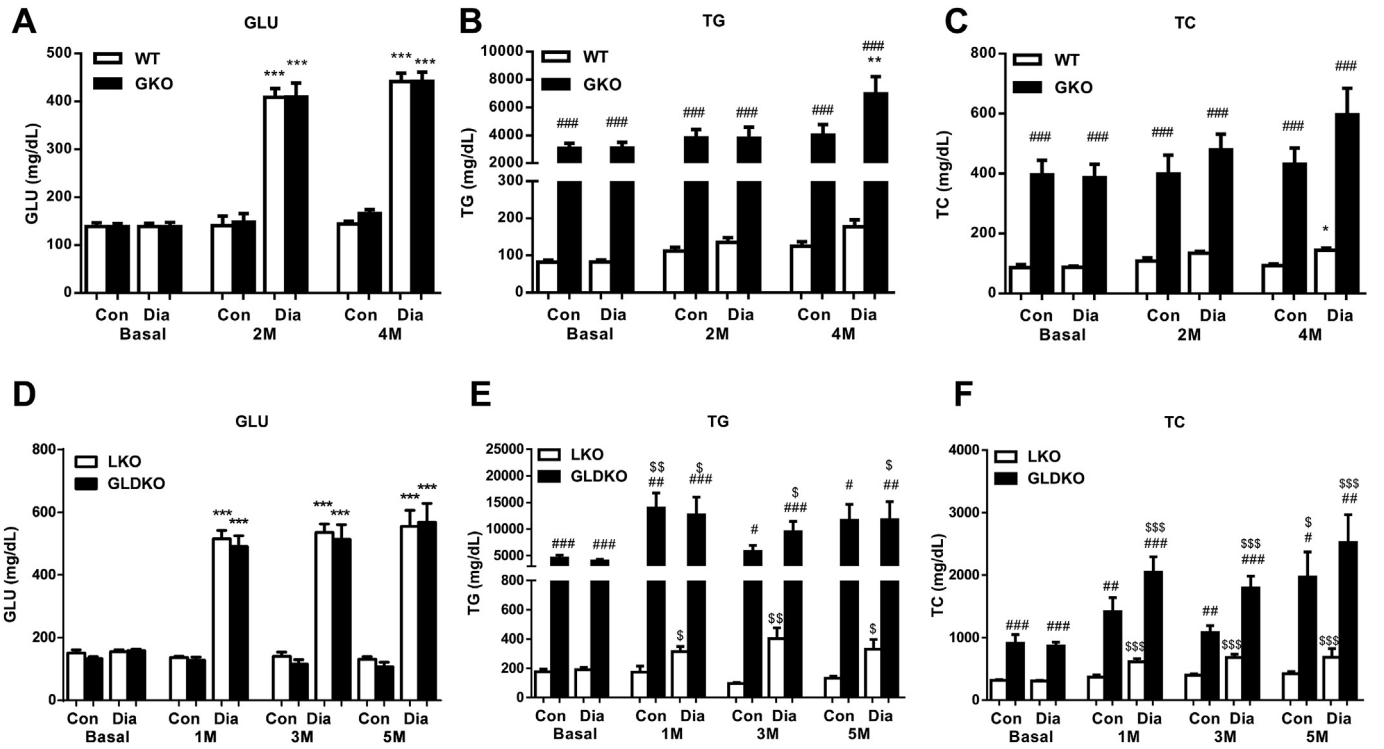

Fig. 1. Analysis of plasma glucose and lipids in GKO and GLDKO mice under diabetic conditions.
(A) Plasma GLU, (B) TG and (C) TC levels before, 2 and 4 months of diabetes in GKO mice. (D) Plasma GLU, (E) TG and (F) TC levels before, 1, 3 and 5 months of diabetes with the modified HFD in GLDKO mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, effect of genotype; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, effect of diabetes; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, effect of time-course, compared with the basal levels; HFD, high fat diet; Con, control; Dia, diabetes; WT, wild type; GKO, *Gpihbp1* knockout; LKO, *Ldlr* knockout; GLDKO, *Gpihbp1* and *Ldlr* double knockout.

3.4. *Gpihbp1* deficiency aggravated atherosclerotic plaque instability in GLDKO mice after diabetes

To further investigate whether the lack of *Gpihbp1* had an adverse effect on atherosclerotic plaque cell composition and stability, consecutive sections at the aortic root level were estimated with various staining techniques. Percentage of lipid-rich core areas in plaque (ORO %) were significantly higher in the GLDKO-Con group compared with the LKO-Con group at 5 months (Fig. 2L and O, $p < 0.01$). Diabetes promoted an increase in lipid-rich core areas in GLDKO mice at 3 months (Fig. 2K and O, $p < 0.01$). In the diabetic group, the lipid-rich core areas were significantly larger in GLDKO mice than in LKO mice at 3 and 5 months ($p < 0.001$). In all groups, these areas were significantly enlarged at 5 months compared with those at 3 months ($p < 0.05$, Fig. 2K, L and O).

Percentage of collagen positive staining in plaque (collagen %) were decreased by diabetes in GLDKO mice ($p < 0.05$) at 3 months. In the diabetic group, the GLDKO mice had significantly fewer collagen % than the LKO mice at 3 and 5 months ($p < 0.05$). In addition, the GLDKO-Dia group had fewer collagen % areas at 5 months than at 3 months ($p < 0.05$, Fig. 4A and D).

Percentage of SMC positive staining in plaque (SMC %) were smaller in the GLDKO-Dia group than in the GLDKO-Con group ($p < 0.01$) and the LKO-Dia group at 3 months ($p < 0.05$). The GLDKO-Con and LKO-Dia groups had fewer SMC % at 5 months than at 3 months ($p < 0.05$, $p < 0.05$, Fig. 4B and E).

Percentage of infiltrated macrophage in plaque (macrophage %) were larger in the GLDKO-Dia group than in the LKO-Dia group at 5 months ($p < 0.05$). The GLDKO-Dia group had increased macrophage % at 5 months than at 3 months ($p < 0.05$, Fig. 4C and F).

The histological plaque stability score revealed that the GLDKO-Con group had a lower plaque stability score than the LKO-Con group at 5 months ($p < 0.05$). Diabetes could promote the instability of plaques in LKO mice and GLDKO mice at 3 months ($p < 0.05$). Moreover, in the diabetic groups, AS was less stable in the GLDKO group than in the LKO group at 3 and 5 months ($p < 0.05$). The stability of AS in the GLDKO-Con, LKO-Dia and GLDKO-Dia groups was remarkably decreased at 5 months compared to that at 3 months ($p < 0.05$, Fig. 4G).

Collectively, we found that *Gpihbp1* deficiency on the LKO background promoted atherosclerotic plaque instability at 3 and 5 months under diabetic conditions. In nondiabetic mice, the effect of *Gpihbp1* deficiency on plaque instability was detected only at 5 months.

3.5. *Gpihbp1* deficiency increased oxidative stress and inflammation in atherosclerotic plaques and induced aortic remodeling in GLDKO mice after diabetes

To investigate whether *Gpihbp1* deficiency leads to an increase in inflammation in the plaques, IHC staining against VCAM1 was performed in the aortic root. VCAM1 staining was increased in the GLDKO-Con group compared with that in the LKO-Con group at 5 months ($p < 0.001$). Diabetes increased VCAM1 staining in both LKO mice and GLDKO mice (3 months: GLDKO, $p < 0.01$; 5 months: LKO, $p < 0.01$, GLDKO, $p < 0.05$). In the diabetic groups, VCAM1 staining was increased in the GLDKO group compared with that in the LKO group at 3 and 5 months ($p < 0.01$). VCAM1 staining in all groups was remarkably increased at 5 months compared with that at 3 months (LKO-Con, $p < 0.001$; GLDKO-Con, $p < 0.001$; LKO-Dia, $p < 0.01$; GLDKO-Dia, $p < 0.01$, Fig. 5A and D).

Evidence of oxidative stress was measured by DHE (Fig. 5B and E). Oxidative stress was increased significantly in the GLDKO group compared with the LKO group at 3 and 5 months with or without diabetes ($p < 0.001$). Diabetes increased oxidative stress in GLDKO mice at 3 ($p < 0.05$) and 5 months ($p < 0.001$). Oxidative stress was increased at 5 months compared to that at 3 months in GLDKO-Con ($p < 0.01$), GLDKO-Dia and LKO-Dia groups ($p < 0.001$). Furthermore, the lipid

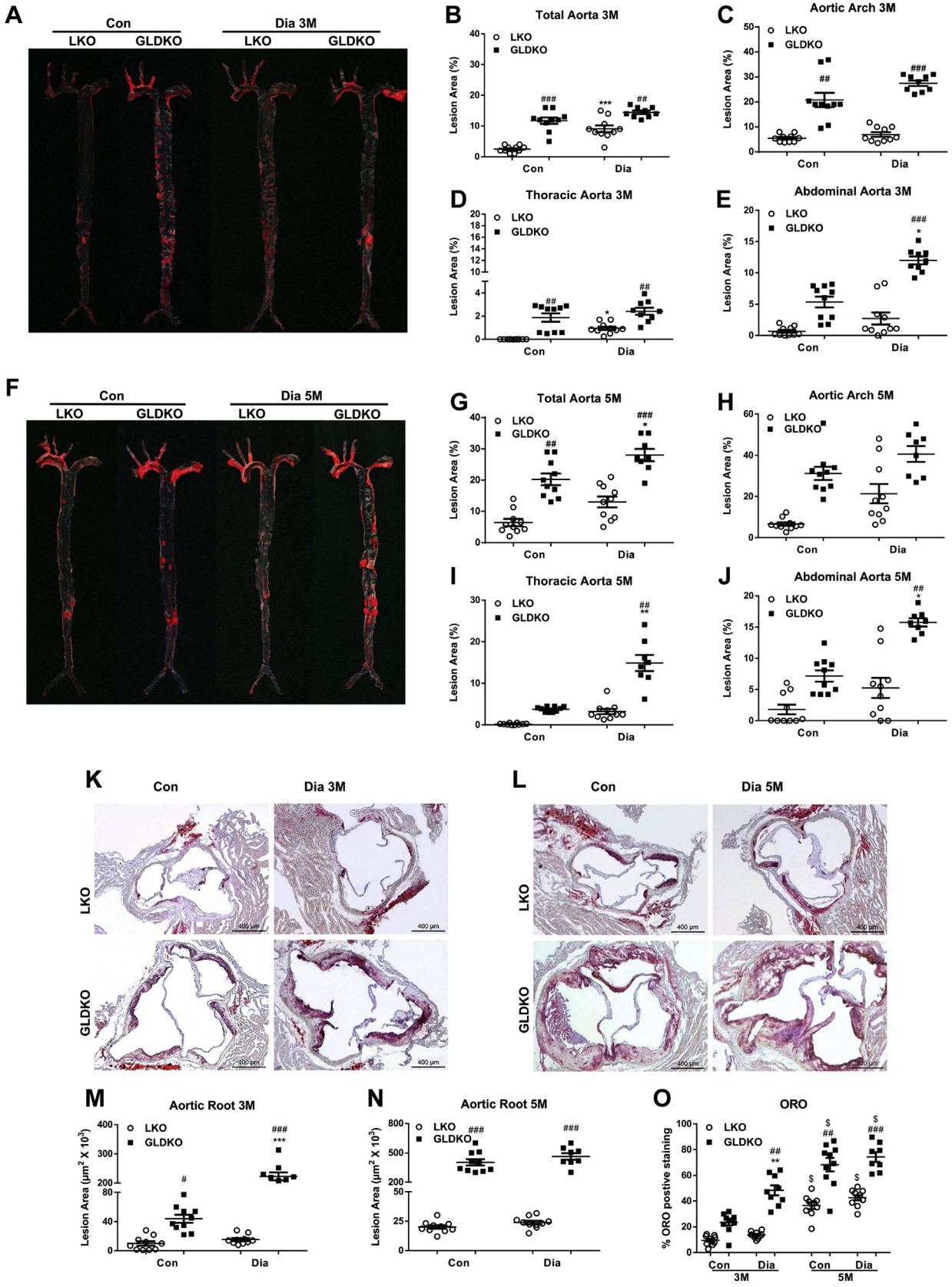

Fig. 2. Analysis of aortic atherogenesis in GLDKO mice under diabetic conditions.
(A, F) Representative images of Oil red O (ORO) staining of the total aorta in GLDKO mice at 3 and 5 months of diabetes. Quantification of plaque areas in the (B, G) total aorta, (C, H) aortic sections of aortic arch, (D, I) thoracic and (E, J) abdominal in GLDKO mice at 3- and 5-month of diabetes. (K, L) Representative images of ORO staining of the aortic root in GLDKO mice at 3 and 5 months of diabetes. (M, N) Quantification of plaque areas in aortic roots in GLDKO mice at 3 and 5 months of diabetes. (O) Quantification of % positive staining areas/plaque areas in aortic root using staining of ORO. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, effect of genotype; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, effect of diabetes; $^{\$}p < 0.05$, effect of time-course; Con, control; Dia, diabetes; LKO, *Ldlr* knockout; GLDKO, *Gpihbp1* and *Ldlr* double knockout.

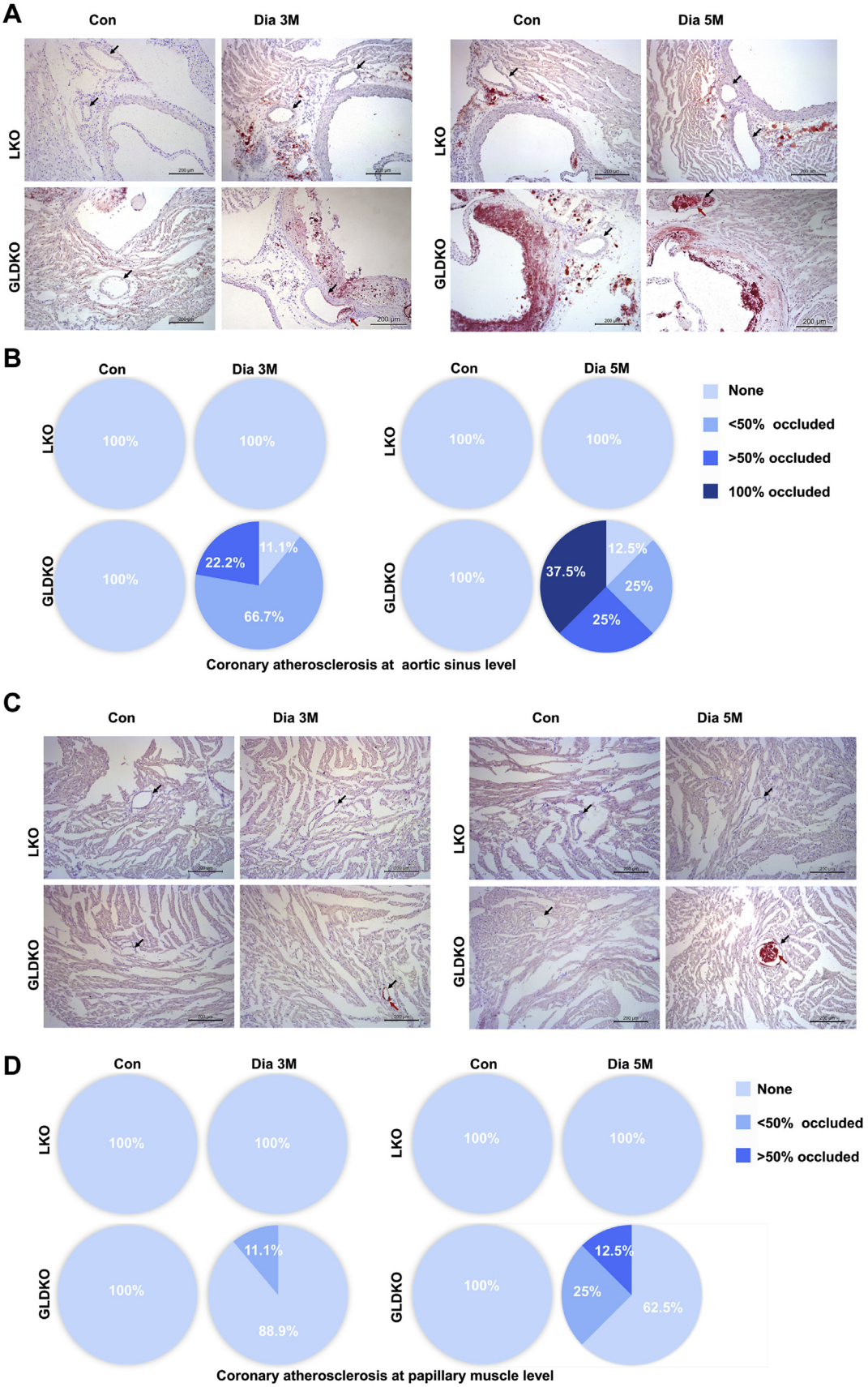

Fig. 3. Analysis of coronary atherogenesis in GLDKO mice under diabetic conditions.
(A, B) Representative images and quantitative analysis of the coronary atherosclerotic lesions by ORO staining at aortic sinus level in GLDKO mice at 3 and 5 months of diabetes. (C, D) Representative images and quantitative analysis of the coronary atherosclerotic lesions by ORO staining at papillary muscle level in GLDKO mice at 3 and 5 months of diabetes. "black arrows" indicate coronary artery, "red arrows" indicate plaque of coronary artery; Con, control; Dia, diabetes; LKO, *Ldlr* knockout; GLDKO, *Gpihbp1* and *Ldlr* double knockout. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

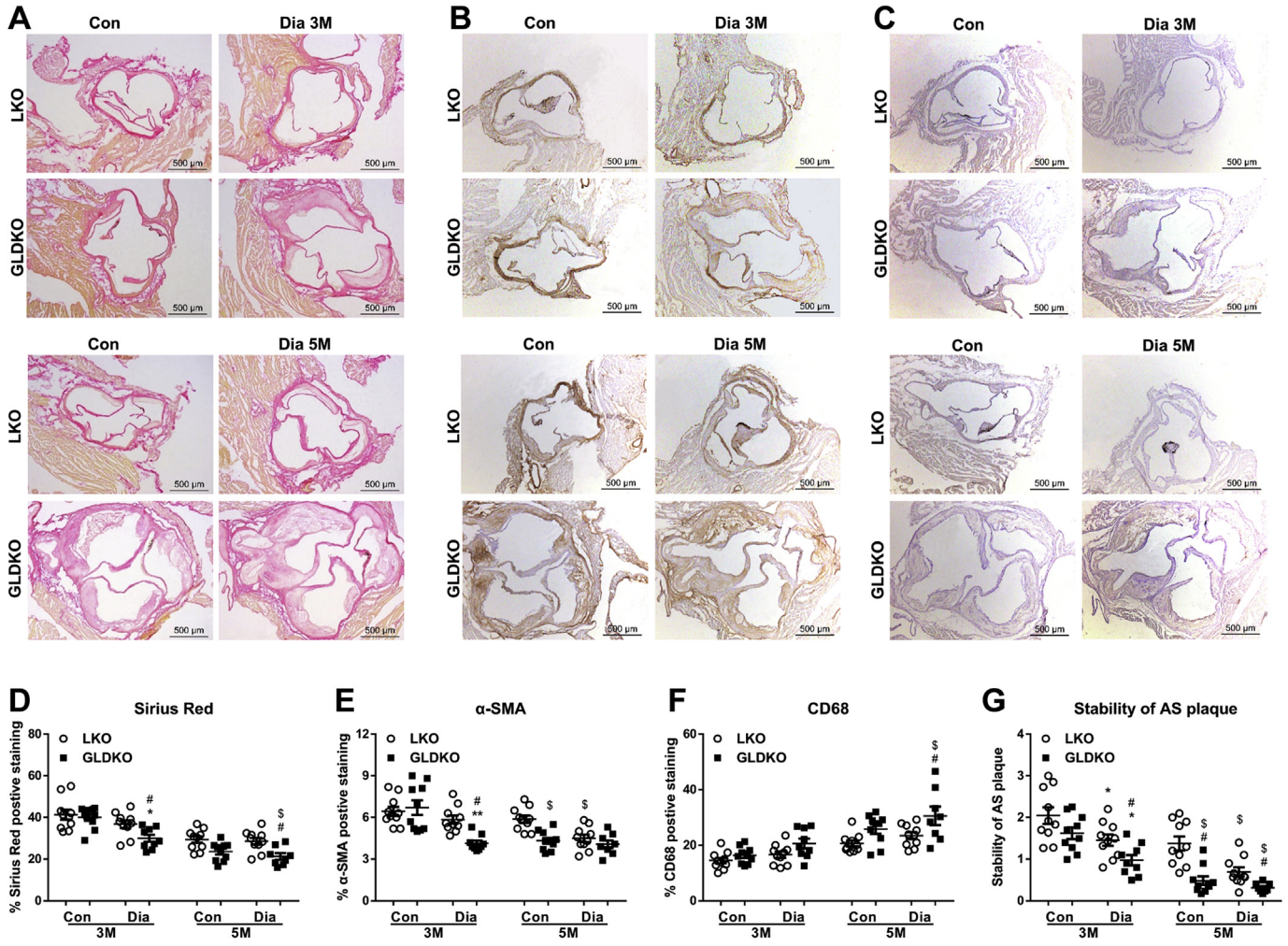

Fig. 4. Estimation of plaque stability in GLDKO mice under diabetic conditions.
(A, B) Representative images and quantitative analysis of the coronary atherosclerotic lesions by ORO staining at aortic sinus level in GLDKO mice at 3 and 5 months of diabetes. (C, D) Representative images and quantitative analysis of the coronary atherosclerotic lesions by ORO staining at papillary muscle level in GLDKO mice at 3 and 5 months of diabetes. "black arrows" indicate coronary artery, "red arrows" indicate plaque of coronary artery; Con, control; Dia, diabetes; LKO, *Ldlr* knockout; GLDKO, *Gpihbp1* and *Ldlr* double knockout. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

peroxidation product was measured with 4HNE staining (Fig. 5C and F). The results were similar to those of the DHE staining, however, diabetes did not further increase lipid peroxidation in GLDKO mice at 3 months, and no difference was found in GLDKO-con group at 5 months compared to that at 3 months.

Interestingly, we found that the aortic root surface areas in GLDKO mice were obviously enlarged without thickening of the vessel wall (Fig. 2K and L, Fig. 4A–C, Fig. 5A–C). Quantitative analysis revealed that the areas were increased in GLDKO mice compared with LKO mice in both the control and diabetic groups at 3 and 5 months and that enlarged areas became more remarkable at 5 months than at 3 months. Diabetes increased the areas in GLDKO mice at 3 months (Fig. 5G).

3.6. Plasma glucose and lipid levels and atherogenesis in GLDKO diabetic mice fed a chow diet

For the supplemental study, a chow diet was fed to LKO and GLDKO mice with or without diabetes for 3 months. Higher plasma TG and TC levels were found in GLDKO mice compared to those in LKO mice throughout the experiment in both the control and diabetic groups ($p < 0.001$, Supplemental Figs. 3B and C). Plasma TG and TC levels were increased at 3 months after induction of diabetes in GLDKO mice compared with the basal levels ($p < 0.05$, Supplemental Figs. 3B and C).

The plaque areas in the total *en face* aorta and aortic sinus were increased in GLDKO mice compared with LKO mice in both the control and diabetic groups ($p < 0.001$, Supplemental Figs. 3D–G). Diabetes increased the plaque areas in the total *en face* aorta ($p < 0.05$) and aortic sinus ($p < 0.01$) in GLDKO mice (Supplemental Figs. 3D–G). No evident atherosclerotic lesions were found in the chow diet-fed LKO mice with or without diabetes. Fewer atherosclerotic lesions were detected in GLDKO mice fed a chow diet compared with those fed the modified HFD at 3 months after induction of diabetes. Neither advanced lesions in the sinus nor CA lesions were found in chow diet-fed GLDKO-Dia mice (data not shown). In our study, GLDKO-Dia mice fed with modified HFD were used to evaluate the role of *Gpihbp1* in plaque stability and CA lesions.

4. Discussion

We demonstrate for the first time that *Gpihbp1* deficiency accelerates the development of AS in the aorta and instability of plaques in LKO mice. Diabetes promotes these pathologic processes with coronary atherogenesis. Moreover, we found that *Gpihbp1* deficiency leads to vascular dilated remodeling in LKO mice.

HTG resulting from *Lpl* or *Gpihbp1* deficiency promotes only mild

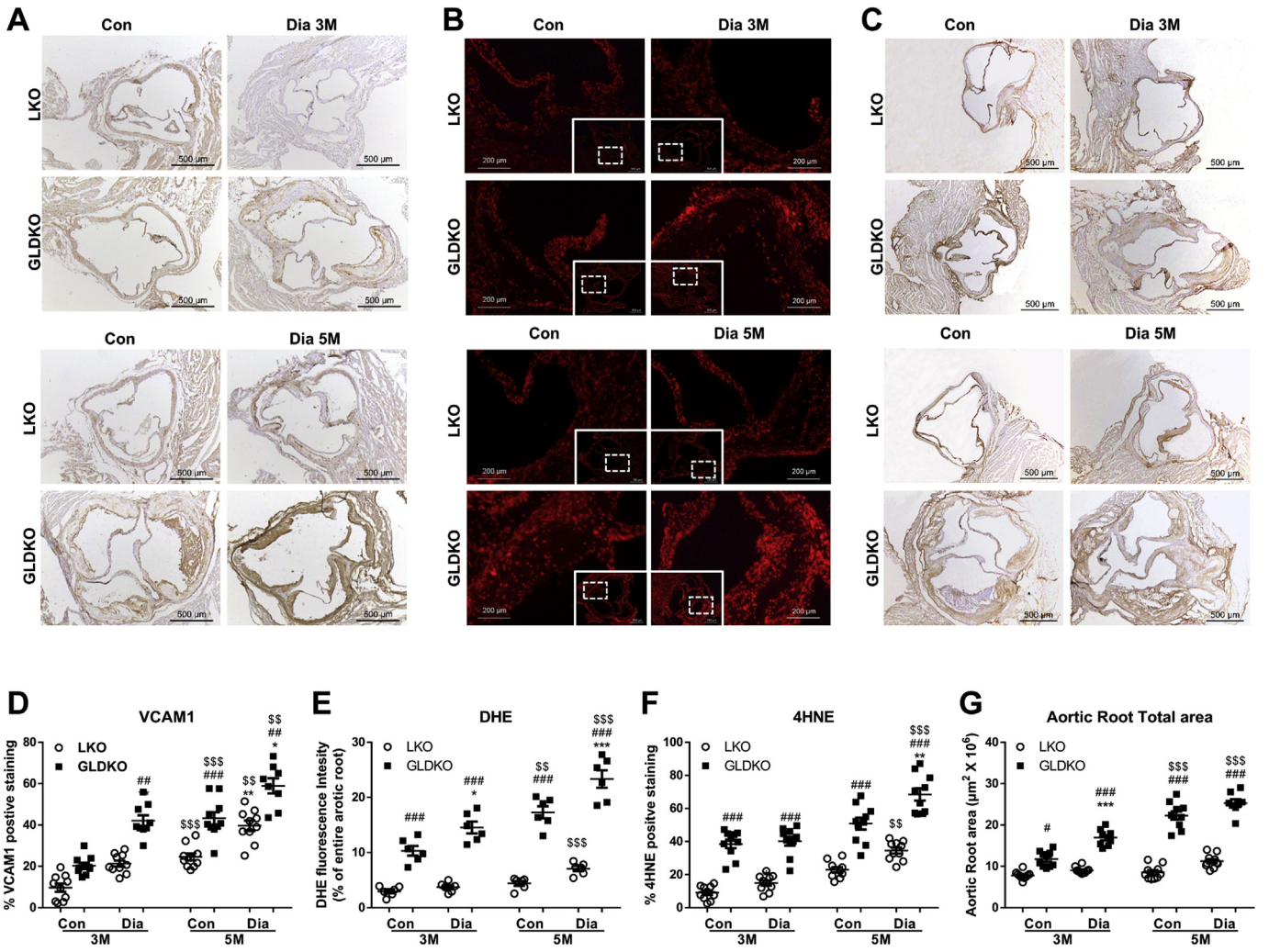

Fig. 5. Plaque inflammation and oxidative stress and aortic surface areas in the aortic root in diabetic GLDKO mice.
Representative images of IHC for (A) VCAM1 and (B) DHE and (C) 4HNE analysis in the aortic root. Quantification of % positive staining areas/plaque areas in aortic root by (D) VCAM1 and (F) 4HNE staining in the aortic root; Quantification of % positive staining areas/entire aortic root by (E) DHE. Scale bar represents 500 μm for VCAM1 and 4HNE staining; For DHE staining, scale bar represents 200 μm and the scale bar in inset represents 500 μm. (G) Quantification of aortic root total area. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, effect of genotype; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, effect of diabetes; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, effect of time-course; Con, control; Dia, diabetes; LKO, *Ldlr* knockout; GLDKO, *Gpihbp1* and *Ldlr* double knockout. IHC, immunohistochemistry.

spontaneous AS in > 15- or 11-month-old mice, respectively [25,30]. GKO mice exhibited extremely high plasma TG levels of 3000–4000 mg/dL, which were mainly characterized by CMs and VLDL as described previously [25]. However, we did not observe atherosclerotic lesions in GKO mice at 7 months of age, even 4 months after induction of diabetes. Diabetes seemed to have no promoting effect on the initiation of AS in our study. We found that *Gpihbp1* deficiency promoted AS in LKO mice. *Lpl*-deficient aged mice exhibit HTG and develop foam cell-rich atherosclerotic lesions mediated by the oxidation of CM and the activation of inflammation, including VCAM1 and monocyte chemoattractant protein 1 (MCP1), in ECs [30]. HTG in Apolipoprotein CIII (ApoCIII) transgenic mice promotes restenosis and AS, and TRLs rich in ApoCIII could promote SMC proliferation and activation of oxidative stress and inflammation with increasing VCAM1 and macrophage infiltration in lesion sites [26]. Moreover, GKO aged mice exhibit macrophage filtration in plaques [25]. In our study, *Gpihbp1* deficiency with HTG promoted AS, which was also related to increased oxidative stress and inflammation in LKO mice (Fig. 5). The oxidation of increased CMs might play an important role in the development of AS in GLDKO mice, as detected in a study of *Lpl* deficiency mice [30].

Compared with LKO mice, GLDKO mice showed a 25-fold increase in plasma TG levels, which was further elevated to 37-fold after diabetes and modified HFD (0.05% cholesterol and 5% lard added to chow diet). This finding suggested that the acceleration of atherogenesis in GLDKO mice compared with that in LKO mice was associated with high TG levels. Diabetes could induce oxidative stress and inflammation, which play significant roles in diabetic AS [13]. In this study, diabetes further promoted AS in GLDKO mice. However, the plasma TG levels remained unchanged after diabetes in GLDKO mice. This result suggested that the promoting effect of diabetes on AS in GLDKO mice was not associated with high TG levels but instead was related to the diabetes-induced enhancement of inflammation and oxidative stress in GLDKO mice. Whether *Gpihbp1* deficiency has an independent effect on HTG in terms of the development of AS requires further investigation.

Type 1 diabetes has been reported to promote the disruption of advanced atherosclerotic plaques in LKO mice [14]. Although hyperglycemia might contribute to plaque disruption, it was clearly not sufficient. The authors found that diabetes significantly elevated TG in LKO mice, similar to inadequately treated humans with type 1 diabetes. Aggressive lowering primarily of TRLs by Ad-VLDLR prevented both plaque disruption and an increase in the proinflammatory biomarker

S100A9 in diabetic atherosclerotic lesions. This finding indicates the important link between diabetes, plaque disruption and HTG [14]. In our study, *Gpihbp1* deficiency contributed to atherosclerotic plaque instability by increasing lipid deposition in LKO mice without diabetes. Diabetes further increased plaque instability with increased infiltration of lipids and macrophages and decreased collagen and SMC in GLDKO mice compared to those in LKO mice. The increased TRLs can simply infiltrate the subendothelial space, leading to the formation of lipid-rich plaques, and diabetes might enhance oxidative and glycosylated TRLs and inflammation, result in the generation of foam cells and decrease SMCs as well as collagen content in GLDKO mice. Surprisingly, we found that *Gpihbp1* deficiency induced dilated aortic remodeling in GLDKO mice, and diabetes further increased this remodeling at 3 months. Vulnerable lesions have been shown to be more frequently associated with dilated remodeling, and inflammation is an etiologic factor in arterial enlargement [38]. These results indicate that arterial remodeling might aggravate plaque instability in GLDKO mice. No evidence showed a relationship between aortic sinus dilatation and HTG. A clinical meta-analysis showed that TG levels remained convincingly associated with abdominal aortic aneurysm [39]; however, another MR study obtained a controversial result [40]. In addition to valvular dysfunction, the turbulent flow generated by stenosis appeared to contribute to aortic dilation in a clinical study [41]. The exact mechanism for aortic sinus dilatation requires further investigation.

GKO mice develop mild coronary atherosclerotic lesions at the age of 18 months [25]. We identified obvious coronary atherosclerotic lesions in GLDKO mice at the age of 6 months, which was 3 months after diabetes induction, and occlusion of the coronary artery in GLDKO mice was detected at 5 months after diabetes induction (Fig. 3). The proatherogenic effect of diabetes seems to be dominant in coronary arteries with HTG. Furthermore, the coronary artery is well known to typically be lesion-free in mice unless it undergoes coronary artery ligation. However several mouse models develop AS in the coronary artery [42], scavenger receptor class B type I (*Srb1*)/*Apoe* double KO mice exhibited aggravated AS and even occlusive coronary lesions. Unfortunately, 50% mortality has been reported in these mice at 6 weeks old [43]. Recently, a study established a diabetic mouse model, Western-diet-fed male $Apoe^{-/-}$;$Ins2^{+/Akita}$ mice, with profound coronary AS resulting in a shortened lifespan. Only 20% of these mice survived until 25 weeks of age [44]. In our study, GLDKO diabetic mice presented a high survival rate with the progression of coronary lesions. GLDKO mice might be an ideal animal model for the study of diabetic coronary artery disease.

In a preliminary study, we fed different levels of the HFD to GLDKO diabetic mice for 6 weeks. The GLDKO diabetic mice all died within 5 weeks with high or medium levels of HFD (Supplemental Fig. 2). In these mice, the plasma TC levels were approximately 2000 mg/dL, and TG levels were greater than 20000 mg/dL after 2 weeks of HFD feeding (data not shown). High mortality was found in diabetic LKO mice and $Apoe^{-/-}$;$Ins2^{+/Akita}$ mice as a result of coronary occlusion [9,44]. In our study, we found 37.5% occluded coronary arteries in GLDKO diabetic mice after 5 months of the modified HFD (Fig. 3A and B). Patients with Gpihbp1 deficiency and severe HTG have recurrent severe acute pancreatitis [45]. Therefore, we speculate that the cause of death in GLDKO diabetic mice within 5 weeks after high and medium levels of HFD might be related to coronary atherosclerosis or acute pancreatitis. The exact cause of death needs further investigation.

No AS was detected in GKO mice at 4 months after induction of diabetes; however, we detected higher plasma TC levels with ~400 mg/dL in GKO mice, while $Apoe^{-/-}$ mice develop AS with similar plasma TC levels at 4 months of age in the previous studies. The results of fast protein liquid chromatography (FPLC) showed that the increased TC was mainly CMs and VLDL in GKO mice [25], while $Apoe^{-/-}$ mice had mainly remnant particles of CMs and VLDL, which more easily infiltrate the subendothelial space. In our study, plasma TC levels increased to approximately 900 mg/dL in GLDKO mice. These levels increased in the same proportion during the same period of study in both GKO and GLDKO mice compared to those in the control mice. This evidence supported that the aggravated AS caused by *Gpihbp1* deficiency in LKO mice was related to increased CMs and VLDL, rather than low-density lipoprotein cholesterol (LDL-C), which plays an important role in AS.

In summary, *Gpihbp1* deficiency accelerates the development of AS in the aorta and instability of plaques in LKO mice, and diabetes promotes these pathologic processes with coronary atherogenesis. These findings are probably associated with HTG caused by *Gpihbp1* deficiency and increased oxidative stress and inflammation in atherosclerotic lesions. Moreover, we found that *Gpihbp1* deficiency leads to vascular remodeling in LKO mice. GLDKO mice develop obvious coronary artery lesions and might be an ideal model for the study of diabetic coronary artery disease.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

X. J. Liu designed the experiments and wrote the paper. W. Huang supervised the experimental design, data analysis and manuscript preparation. X. M. Huang, J. Y. Li and Y. H. Wang performed the genotyping. Q. Shen performed Sirius red staining. J. W. Liao and H. Wang analyzed the plasma lipid levels. X. J. Liu performed the rest of the experiments and analyzed the data. J. Y. Li performed the supplemental study. W. Huang, W. Kong and G. Liu revised the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

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