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Downregulation of adipose triglyceride lipase in the heart aggravates diabetic cardiomyopathy in *db/db* mice

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ABSTRACT

Adipose triglyceride lipase (ATGL) was recently identified as a rate-limiting triglyceride (TG) lipase and its activity is stimulated by comparative gene identification-58 (CGI-58). Mutations in the ATGL or CGI-58 genes are associated with neutral lipid storage diseases characterized by the accumulation of TG in multiple tissues. The cardiac phenotype, known as triglyceride deposit cardiomyovasculopathy, is characterized by TG accumulation in coronary atherosclerotic lesions and in the myocardium. Recent reports showed that myocardial TG accumulation is significantly higher in patients with diabetes and is associated with impaired left ventricular diastolic function. Therefore, we investigated the roles of ATGL and CGI-58 in the development of myocardial steatosis in the diabetic state. Histological examination with oil red O staining showed marked lipid deposition in the hearts of diabetic fatty *db/db* mice. Cardiac triglyceride and diglyceride contents were greater in *db/db* mice than in *db/+* control mice. Next, we determined the expression of genes and proteins that affect lipid metabolism, and found that ATGL and CGI-58 expression levels were decreased in the hearts of *db/db* mice. We also found increased expression of genes regulating triglyceride synthesis (sterol regulatory element-binding protein 1c, monoacylglycerol acyltransferases, and diacylglycerol acyltransferases) in *db/db* mice. Regarding key modulators of apoptosis, PKC activity, and oxidative stress, we found that Bcl-2 levels were lower and that phosphorylated PKC and 8-hydroxy-2′-deoxyguanosine levels were higher in *db/db* hearts. These results suggest that reduced ATGL and CGI-58 expression and increased TG synthesis may exacerbate myocardial steatosis and oxidative stress, thereby promoting cardiac apoptosis in diabetic mice.

1. Introduction

People with diabetes are at significantly increased risk of developing cardiomyopathy and heart failure compared with nondiabetic individuals. Furthermore, numerous epidemiologic studies have shown that a large proportion of patients with heart failure have diabetes [1]. Although patients with diabetes are at increased risk of structural heart disease as a result of vascular complications, they are also at increased risk of congestive heart failure independent of the presence of underlying macroscopic coronary disease [2]. Although diabetic cardiomyopathy is characterized by the presence of myocardial dysfunction in the absence of coronary artery disease [3], the pathogenesis of diabetic cardiomyopathy is still poorly understood. Therefore, better understanding of this disease is urgently needed, as are the possible treatments. Myocardial triglyceride (TG) content is significantly higher in patients with prediabetes or diabetes than in healthy individuals [4,5] and is associated with impaired left ventricular diastolic function [4]. Neutral lipid storage diseases (NLSDs) are characterized by the presence of intracellular TG accumulation in most tissues, and are caused by mutations in adipose triglyceride lipase (ATGL) [6] or comparative gene identification-58 (CGI-58) [7]. ATGL catalyzes the first step in the hydrolysis of TG stored within lipid droplets [8], while CGI-58 stimulates ATGL activity by up to 20-fold [9]. Triglyceride deposit cardiomyovasculopathy (TGCV), a cardiac phenotype of NLSD, is characterized by massive accumulation of TG in the coronary arteries and myocardium, and ultimately leads to chronic heart failure [10]. Because a previous report showed that insulin suppresses ATGL expression in adipocytes, possibly

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through FoxO1 [11], ATGL may play some roles in the development of cardiomyopathy in insulin-resistance states, including diabetes. In this report, we determined TG and diglyceride (DG) contents, and the expression of genes and proteins involved in TG metabolism, oxidative stress, and apoptosis in the heart of *db/db* mice, a rodent model of type 2 diabetes.

2. Materials and methods

2.1. Animals

Male C57BL/KsJ *db/db* mice and their age-matched lean littermates, *db/+* mice, were purchased from Clea Japan Inc. (Tokyo, Japan). All mice were bred under pathogen-free conditions at Kyushu University Animal Center (Fukuoka, Japan). The animals had free access to tap water and standard chow (Clea) containing 50.1% carbohydrate, 25.1% protein, 7.1% mineral, 4.5% fat, and 4.3% cellulose. At 10 weeks of age, the mice were fasted for 16 h and blood samples were obtained from the retro-orbital venous plexus. Blood samples were used to measure plasma insulin concentrations using an enzyme-linked immunosorbent assay (Morinaga Institute of Biological Science, Yokohama, Japan). Cardiac gene expression and immunostaining were studied in 10-week-old mice, and cardiac TG content was determined in 10- and 20-week-old mice. All mice were anesthetized by pentobarbital (0.1 mg/g intraperitoneal injection) and killed. The heart was rapidly dissected, frozen in liquid nitrogen, and kept at −80 °C until use. All protocols were reviewed and approved by the Committee on the Ethics of Animal Experiments, Graduate School of Medical Sciences, Kyushu University.

2.2. Tissue preparation and histological analysis

Serial 10 μm-thick sections of each heart were prepared using a sliding Coldtome (Sakura Fine Technical Co. Ltd., Tokyo, Japan). For histological analysis, the sections were collected on glass slides, stained with oil red O, and counterstained with hematoxylin to identify intramyocardial lipid deposits. The stained sections were observed under a fluorescent light microscope (BZ-9000, Keyence, Osaka, Japan).

2.3. Measurement of cardiac TG and DG contents

Heart TG content was assayed using a Triglyceride Quantification Kit (BioVision, Mountain View, CA, USA), in accordance with the manufacturer's instructions. Briefly, the heart tissue was perfused with phosphate-buffered saline and homogenized in 5% Triton-X100 in water. Samples were slowly heated to 80 °C for 5 min. Insoluble materials were removed by centrifugation. The TG concentration in the supernatant was determined using the enzyme-based colorimetric assay.

For biochemical analysis of DGs, lipids were extracted from heart tissue using the Folch partition method [12]. Briefly, the heart tissue was homogenized in 2 ml of 100% methanol for 30 s. After adding 2 ml of chloroform and 1 ml of H_2O to the homogenates, the samples were allowed to stand for 30 min at room temperature. After centrifugation at 500×*g* for 10 min, the lower phase was collected. The upper phase was mixed with 4 ml chloroform and DGs were re-extracted as outlined above. The lower phases (containing lipid) from both centrifugation steps were combined and dried under nitrogen gas. The total DG content and the amounts of specific DGs were measured by high-performance liquid chromatography–tandem mass spectrometry as previously described [13,14].

2.4. RNA extraction and quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen heart tissue using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Extracted RNA (1 μg) was converted to single-stranded cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). The mRNA levels were quantified by quantitative RT-PCR using an iTaq SYBR Green mix (Bio-Rad, Hercules, CA, USA) with the Bio-Rad Chromo 4/Opticon system. The following (sense and antisense) primer pairs were used: ATGL, 5′-ATTTATCCCGGTGTACTGTG-3′ and 5′-GGGACACTGTGATGGTATTC-3′; CGI-58, 5′-TGACAGTGATGCGGAAGAAG-3′ and 5′-AGATCTGGTCGCTCAGGAAA-3′; hormone sensitive lipase (HSL), 5′ ACTCAGACCAGAAGGCACTA-3′ and 5′-TAGTTCCAGGAAGGAGTTGA-3′; sterol regulatory element-binding protein 1c (SREBP1c),5′-CGCGGAAGCTGTCGGGGTAG-3′ and 5′-AAATGTGCAATCCATGGCTCCGTGGTC-3′; monoacylglycerol acyltransferase (MGAT)1, 5′-CTGGTTCTGTTTCCCGTTGT-3′ and 5′-TGGGTCAAGGCCATCTTAAC-3′; MGAT2, 5′-GTGTGGGATTAGGGGGACTT-3′ and 5′-TCCCTGTTTGTCCTTTGGTC-3′; diacylglycerol acyltransferase (DGAT)1, 5′-TTCCGCCTCTGGGCATT-3′ and 5′-AGAATCGGCCCACAATCCA-3′; DGAT2, 5′-AGTGGCAATGCTATCATCATCGT-3′ and 5′-TCTTCTGGACCCATCGGCCCCAGGA-3′; microsomal triglyceride transfer protein (MTP), 5′-TGAGCGGCTATACAAGCTCAC-3′ and 5′-CTGGAAGATGCTCTTCTCGC-3′; Bax, 5′-TGCAGAGGATGATTGCTGAC-3′ and 5′-GATCAGCTCGGGCACTTTAG-3′; Bcl-2, 5′-ACCGTCGTGACTTCGCAGAG-3′ and 5′-GGTGTGCAGATGCCGGTTCA-3′; and β-actin, 5′-TGACAGGATGCAGAAGGAGA-3′ and 5′-GCTGGAAGGTGGACAGTGAG-3′. The linearity of the amplifications as a function of cycle number was tested in preliminary experiments. The mRNA expression levels of each gene were normalized to the expression levels of the housekeeping gene β-actin.

2.5. Western blotting analysis

To prepare total protein extracts for western blotting analysis of ATGL, CGI-58 and phospho-HSL, heart tissues were homogenized in lysis buffer (0.25 M sucrose, 1 mM EDTA) supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and phosphatase inhibitors (Sigma), and centrifuged for 5 min at 16,000 rpm. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Then 30 μg protein/lane was separated on discontinuous 4%–15% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinyl difluoride membranes (Bio-Rad). After blocking nonspecific binding, the membranes were incubated overnight at 4 °C with anti-ATGL (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-CGI-58 (1:1000; Abnova, Taipei, Taiwan), anti-phosphorylated HSL (1:1000; Abcam, Cambridge, UK), anti-phosphorylated pan protein kinase C (PKC) (1:1000; Cell Signaling Technology), anti-pan PKC (1:1000; Cell Signaling Technology), or anti-β-actin mouse polyclonal (1:10,000; Santa Cruz, Santa Cruz, CA, USA) antibodies, followed by horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (1:10,000; Amersham Pharmacia Biosciences, Buckinghamshire, UK) or donkey anti-rabbit IgG antibody (1:10,000; Amersham) as secondary antibodies. We used the ECL Plus system (Amersham) to detect the protein bands.

2.6. Immunohistochemistry

Heart tissues were fixed in 10% formaldehyde and embedded in paraffin. Sections (5 μm thick) were deparaffinized and dehydrated with xylene and ethanol. Antigen retrieval was carried out in 10 mM citrate buffer with 0.1% Nonidet P-40 (Sigma) in a microwave oven. Endogenous peroxidase was inactivated with 3%

Table 1
Body weight, blood glucose levels, and plasma insulin levels in *db/+* and *db/db* mice.

	Age (weeks)	*db/+*	*db/db*
Body weight (g)	10	27.67 ± 0.21	44.90 ± 0.56*
	20	30.83 ± 0.31	54.70 ± 1.16*
Blood glucose (mg/dl)	10	128.7 ± 13.6	544.9 ± 14.7*
	20	122.5 ± 14.4	473.9 ± 18.3*
Plasma insulin (ng/ml)	10	0.23 ± 0.051	2.47 ± 0.147*

Data are means ± SEM.
* $p < 0.0001$ *vs. db/+* mice.

H_2O_2 in methanol. After blocking with 10% normal rabbit serum, the sections were immunostained with anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) mouse monoclonal antibody (4 μg/ml) (Japan Institute for the Control of Aging, Fukuroi, Japan) overnight at 4 °C. The sections were then incubated with biotinylated antimouse immunoglobulin serum for 30 min, followed by incubation with peroxidase-labeled streptavidin using a Histofine SAB-PO kit (Nichirei, Tokyo, Japan) for 15 min at room temperature. The peroxidase was then visualized with diaminobenzidine.

2.7. Statistical analysis

All data are expressed as means ± SEM. Between-group comparisons were madeusing Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Myocardial lipid content

As shown in Table 1, the body weights and blood glucose levels of 10- and 20-week-old *db/db* mice were significantly greater than those of *db/+* mice. Plasma insulin levels were also significantly higher in 10-week-old *db/db* mice than in age-matched *db/+* mice. We first examined intramyocardial lipid deposition using oil red O staining. This experiment showed that lipid deposition was more pronounced in 10- and 20-week-old *db/db* mice than in age-matched *db/+* mice (Fig. 1A). Consistent with these histological findings, heart TG content was 1.5 to 2.0-fold higher in *db/db* mice than in *db/+* mice (Fig. 1B). Additionally, total DG content and the amounts of specific DGs, as measured by high-performance liquid chromatography–tandem mass spectrometry, were significantly greater in the hearts of *db/db* mice than in *db/+* mice (Fig. 1C).

3.2. Expression of genes involved in lipid metabolism in the hearts of db/db mice

ATGL hydrolyzes the first ester bond of TG [8], and is the rate-limiting lipase in hormone-stimulated TG hydrolysis [15]. The resulting DG is hydrolyzed by HSL. The mRNA and protein levels of ATGL were decreased in the hearts of *db/db* mice as compared with *db/+* mice (Fig. 2A). Post-translational regulation of ATGL activity is mediated by CGI-58 [9]. Although the mRNA level of CGI-58 did not change, CGI-58 protein expression was significantly decreased in the hearts of *db/db* mice (Fig. 2B). By comparison, there were no changes in HSL mRNA or phosphorylated HSL protein expression levels (Fig. 2C). Next, we examined the expression of genes that affect TG and DG synthesis. MGATs and DGATs catalyze two consecutive steps in the synthesis of DG and TG [16]. The mRNA levels of MGAT1 and DGAT1 were increased in the hearts of *db/db* mice (Fig. 3). We also found that the mRNA expression of SREBP-1c, which upregulates the genes required for *de novo* lipogenesis, was also increased (Fig. 3). The myocardial mRNA expression of MTP, which catalyzes triglyceride transport and very low density lipoprotein assembly/secretion, was almost identical in *db/db* and *db/+* mice (Fig. 3).

3.3. Effects of diabetes on PKC activity, oxidative stress, and apoptosis in the heart

Lipid overload activates the DG–PKC pathway, which produces reactive oxygen species [17]. Therefore, we performed immunoblotting of phosphorylated PKC, and found that it was increased in the hearts from *db/db* mice (Fig. 4A). We also performed immunostaining for 8-OHdG in left ventricular sections to evaluate oxidative stress status in the heart. The staining intensity of 8-OHdG was stronger in *db/db* mice than in *db/+*mice (Fig. 4B). PKC activation and oxidative stress stimulate cellular apoptosis [18], and apoptosis has major pathogenic roles in a variety of cardiovascular diseases, including diabetic cardiomyopathy [19]. Therefore, we determined the expression levels of two key modulators of apoptosis, Bax and Bcl-2, in the hearts of *db/+* and *db/db* mice. Although there was no difference in Bax expression between the two genotypes, the expression of the anti-apoptotic protein Bcl-2 was significantly decreased in the hearts from *db/db* mice (Fig. 4C).

4. Discussion

ATGL is the causative gene of TGCV, which is characterized by TG accumulation in the coronary arteries and the myocardium, leading to concentric and diffuse coronary atherosclerotic lesions and chronic heart failure, which are common in patients with diabetes. Elevated levels of TG substrates, especially glucose and fatty acids, promotes TG accumulation in most tissues in the diabetic state. Lingvay et al. [20] reported that increased myocardial TG content is also associated with ventricular diastolic dysfunction. Because a previous report showed that insulin suppresses the expression of ATGL in adipocytes, possibly through FoxO1 [11], the higher plasma insulin levels in the insulin-resistant state may reduce cardiac ATGL expression. Therefore, we examined the possibility that reduced ATGL expression aggravates diabetic cardiomyopathy. The present study revealed marked cardiac TG accumulation in *db/db* mice, together with decreased ATGL and CGI-58 expression levels in the heart of these mice. It was reported that cardiac lipid accumulation caused by ATGL gene deletion severely affects systolic and diastolic function in mice [21], while ATGL overexpression improves cardiac function [22,23]. Thus, decreased expression of ATGL and CGI-58 in the *db/db* heart may facilitate lipid accumulation, leading to cardiac dysfunction. We also found enhanced expression of several genes that regulate TG synthesis in the *db/db* heart. These results suggest that increased TG synthesis and decreased TG hydrolysis may lead to the accumulation of TG in the *db/db* heart. Notably, cardiac mRNA expression of MTP, which catalyzes the excretion of excess TG from the heart as lipoproteins [24], was almost identical in *db/+* and *db/db* mice (Fig. 3). This impaired compensatory increase in very low density lipoprotein assembly/secretion may also aggravate TG accumulation in the hearts of *db/db* mice.

Lipid overload results in the accumulation of lipid intermediates such as DG, which activates PKC [25], and the production of reactive oxygen species [17], which can promote apoptosis [26]. It was reported that oxidative stress and apoptosis are important pathogenic factors in the development of diabetic cardiomyopathy [19,27,28]. In the present study, we found that DG content, phosphorylated PKC, and oxidative stress were increased, while the expression of the anti-apoptotic protein Bcl-2 was decreased in the hearts of *db/db* mice. We previously reported that reduced expression of ATGL activates PKC through increased fatty acid up-

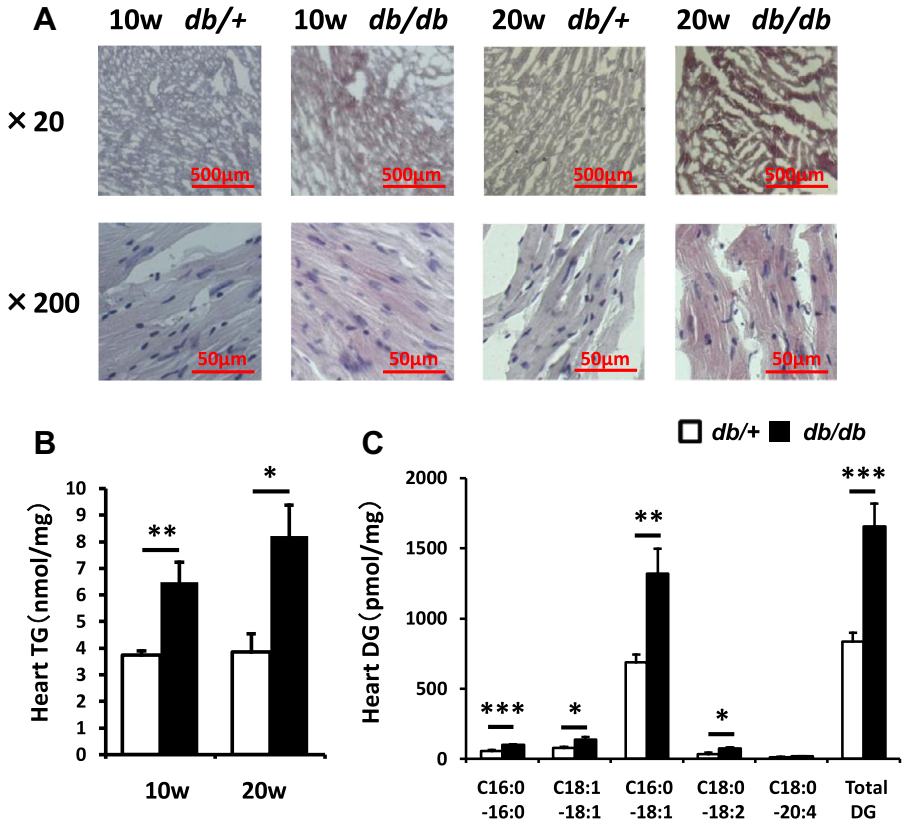

Fig. 1. (A) Morphological changes in the hearts of *db/db* and *db/+* mice. Tissue sections were stained with oil red O and counterstained with hematoxylin to identify lipids in red. (B,C) TG (B) and DG (C) contents in the hearts of *db/db* and *db/+* mice. Heart TG and DG contents were normalized for heart tissue wet weight. Bars represent means + SEM ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$ *vs. db/+* mice.

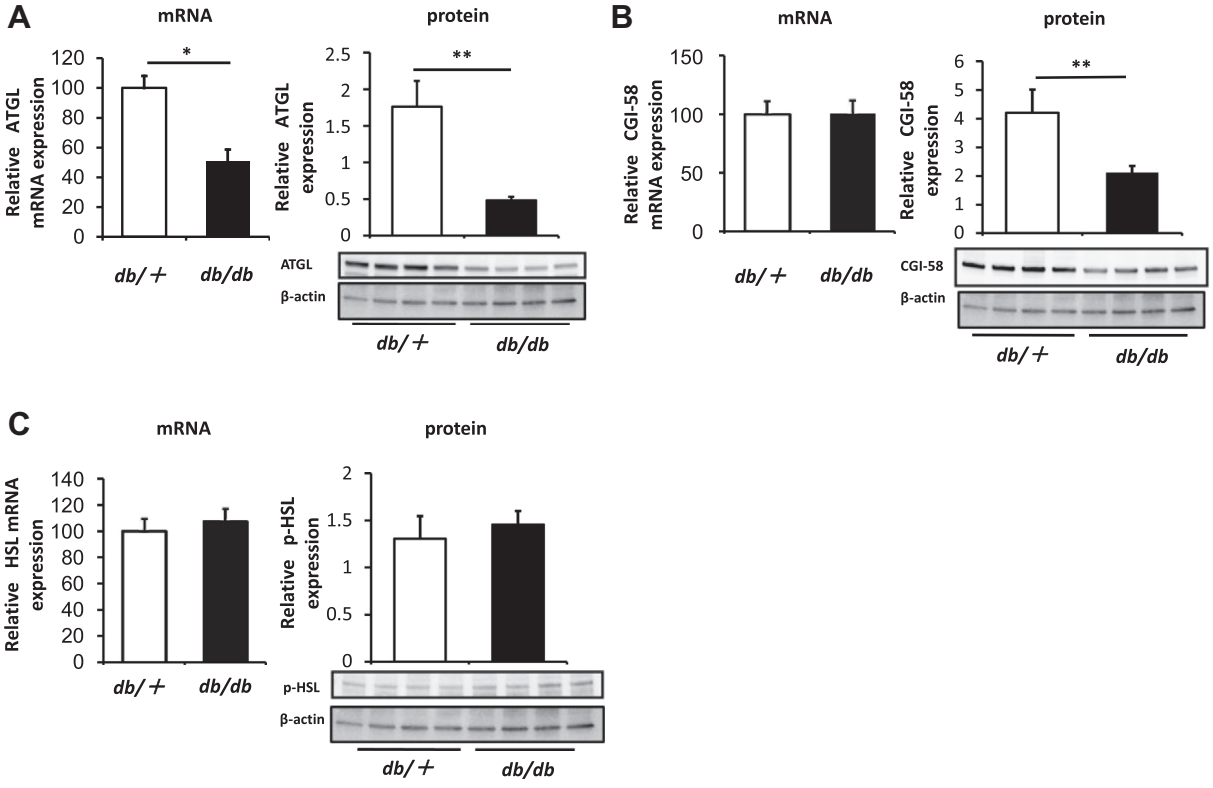

Fig. 2. Expression of genes and proteins that regulate TG hydrolysis in the hearts of *db/db* mice. The mRNA expression levels of ATGL, CGI-58, HSL, and β-actin were determined by real-time RT-PCR. Bars represent means + SEM ($n = 5$). Western blotting was performed using anti-ATGL, anti-CGI-58, anti-phosphorylated (p)-HSL, and anti-β-actin antibodies. Bars represent means + SEM ($n = 4$). $^{*}p < 0.005$ and $^{**}p < 0.0005$ *vs. db/+* mice.

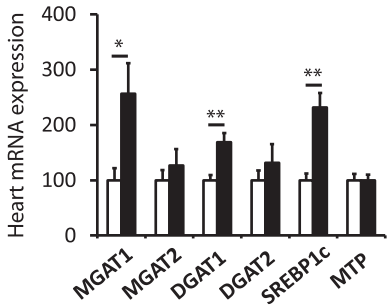

Fig. 3. Expression of genes that regulate triglyceride synthesis in the hearts of *db/db* mice. The mRNA expression levels of MGAT, DGAT, SREBP1c, and MTP were determined by real-time RT-PCR. Bars represent means + SEM ($n = 5$). $^{*}p < 0.05$ and $^{**}p < 0.005$ *vs. db/+* mice.

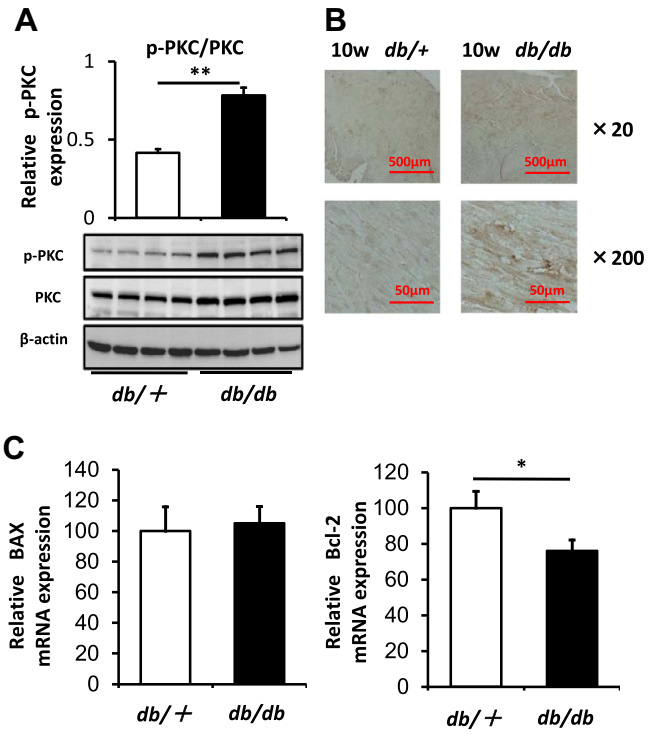

Fig. 4. Phosphorylation of PKC, and expression of oxidative stress-related and apoptosis-related markers in the hearts of *db/db* mice and *db/+* mice. (A) Western blotting was performed using anti-phosphorylated (p)-PKC, anti-PKC, or anti-β-actin antibodies. Bars represent means + SEM ($n = 4$). (B) Heart sections from *db/db* and *db/+* mice were immunostained with anti-8-OHdG antibody. Brown, 3,3′-diaminobenzidine tetrahydrochloride stain. (C) Bax and Bcl-2 mRNA expression was determined by real-time RT-PCR. Bars represent means + SEM ($n = 5$). $^{*}p < 0.05$ and $^{**}p < 0.001$ *vs. db/+* mice.

take and DG synthesis in cultured aortic endothelial cells [14]. Therefore, decreased ATGL expression in the *db/db* heart may also activate the DG–PKC pathway. Furthermore, because ATGL hydrolyzes TG, DG, and monoglyceride, the reduction in ATGL-mediated DG hydrolysis in the absence of a compensatory increase in HSL may facilitate the increases in DG content and PKC activity. The increase in oxidative stress and apoptosis, which is at least partly caused by TG accumulation and activation of the DG–PKC pathway following downregulation of ATGL, could increase the risk of heart disease among patients with type 2 diabetes.

In conclusion, our results show for the first time that the expression levels of ATGL and CGI-58 are reduced in *db/db* mice, and these events may be involved in the development of myocardial steatosis and cardiac apoptosis. These results also imply that cardiac ATGL and CGI-58 are novel clinical targets to control or prevent diabetic cardiomyopathy and that *db/db* mice are a useful model for future studies of diabetes-related TGCV.

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