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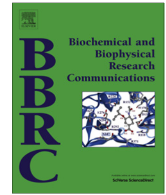

Downregulation of adipose triglyceride lipase in the heart aggravates diabetic cardiomyopathy in *db/db* mice

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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.

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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′-GGGACACTGTGATGGT-ATTC-3′; CGI-58, 5′-TGACAGTGATGCGGAAGAAG-3′ and 5′-AGAT-CTGGTCGCTCAGGAAA-3′; hormone sensitive lipase (HSL), 5′ ACTCAGACCAGAAGGCACTA-3′ and 5′-TAGTTCCAGGAAGGAGTT GA-3′; sterol regulatory element-binding protein 1c (SREBP1c),5′-CGCGGAAGCTGTCGGGGTAG-3′ and 5′-AAATGTGCAATCCATGGC TCCGTGGTC-3′; monoacylglycerol acyltransferase (MGAT)1, 5′-CTGGTTCTGTTTCCCGTTGT-3′ and 5′-TGGGTCAAGGCCATCTTAAC-3′; MGAT2, 5′-GTGTGGGATTAGGGGGACTT-3′ and 5′-TCCCTG TTTGTCCTTTGGTC-3′; diacylglycerol acyltransferase (DGAT)1, 5′-TTCCGCCTCTGGGCATT-3′ and 5′-AGAATCGGCCCACAATCCA-3′; DGAT2, 5′-AGTGGCAATGCTATCATCATCGT-3′ and 5′-TCTTCT GGACCCATCGGCCCCAGGA-3′; microsomal triglyceride transfer protein (MTP), 5′-TGAGCGGCTATACAAGCTCAC-3′ and 5′-CTGGAA-GATGCTCTTCTCGC-3′; Bax, 5′-TGCAGAGGATGATTGCTGAC-3′ and 5′-GATCAGCTCGGGCACTTTAG-3′; Bcl-2, 5′-ACCGTCGTGACTTCGC AGAG-3′ and 5′-GGTGTGCAGATGCCGGTTCA-3′; and β-actin, 5′-TGACAGGATGCAGAAGGAGA-3′ and 5′-GCTGGAAGGTGGACAGT-GAG-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%

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