Pressure Overload–Induced Cardiomyopathy in Heterozygous Carrier Mice of Carnitine Transporter Gene Mutation

Ryotaro Takahashi, Toru Asai, Hisashi Murakami, Ryuichiro Murakami, Michitaka Tsuzuki, Yasushi Numaguchi, Hideo Matsui, Toyoaki Murohara, Kenji Okumura

Abstract—Primary systemic carnitine deficiency is an autosomal recessive disorder caused by a decreased renal reabsorption of carnitine because of mutations of the carnitine transporter OCTN2 gene, and hypertrophic cardiomyopathy is a common clinical feature of homozygotes. Although heterozygotes for OCTN2 mutations are generally healthy with normal cardiac performance, heterozygotes may be at risk for cardiomyopathy in the presence of additional risk factors, such as hypertension. To test this hypothesis, we investigated the effects of surgically induced pressure overload on the hearts of heterozygous mutants of a murine model of OCTN2 mutation, juvenile visceral steatosis mouse (jvs/+). Eleven-week-old jvs/+ mice and age-matched wild-type mice were used. At baseline, there were no differences in physical characteristics between wild-type and jvs/+ mice. However, plasma and myocardial total carnitine levels in jvs/+ mice were lower than in wild-type mice. Both wild-type and jvs/+ mice were subjected to ascending aortic constriction with or without 1% L-carnitine supplementation for 4 weeks. At 4 weeks after ascending aortic constriction, jvs/+ mice showed an exaggeration of cardiac hypertrophy and pulmonary congestion, further increased gene expression of atrial natriuretic peptide in the left ventricles, further deterioration of left ventricular fractional shortening, reduced myocardial phosphocreatine:adenosine triphosphate ratio, and increased mortality compared with wild-type mice; L-carnitine supplementation prevented these changes in jvs/+ mice subjected to ascending aortic constriction. In conclusion, cardiomyopathy and heart failure with energy depletion may be induced by pressure overload in heterozygotes for OCTN2 mutations and could be prevented by L-carnitine supplementation. (Hypertension. 2007;50:497-502.)

Key Words: cardiomyopathy ■ genes ■ heart failure ■ hypertension ■ hypertrophy

Mitochondrial β-oxidation of fatty acids is the main source of energy for the heart.1 Inborn errors in myocardial fatty acid oxidation have been reported to be important causes of inherited cardiomyopathies, which are typically hypertrophic with diminished systolic function.2 Carnitine is essential for the transport of long-chain fatty acids into the mitochondrial matrix for β-oxidation and plays an important role in cellular energy metabolism.3 Carnitine deficiency leads to energy an metabolism disorder because of impaired fatty acid oxidation. Primary systemic carnitine deficiency (SCD; Online Mendelian Inheritance in Man 212140) is an autosomal recessive disorder caused by decreased renal reabsorption of carnitine because of mutations of the carnitine transporter OCTN2 gene.4 Progressive cardiomyopathy is a common clinical manifestation of SCD and could be prevented by high-dose L-carnitine supplementation.5,6

The juvenile visceral steatosis (JVS) mouse was established as an excellent murine model of SCD.7 SCD in JVS mice is caused by decreased renal reabsorption of carnitine because of a spontaneous mutation in the OCTN2 gene, as has been reported in human SCD as well.4 In homozygous mutants of JVS mice, carnitine levels in the myocardium are markedly reduced compared with the levels in wild-type (WT) mice.8 Homozygous JVS mice develop cardiomyopathy characterized by marked cardiac hypertrophy without fibrosis, myocardial energy metabolism disorder,8 and progressive cardiac dysfunction.9

It was reported that myocardial carnitine levels were also lower in heterozygous mutants of JVS mice compared with WT mice.10 Although there were no differences in the heart weight:body weight ratio and the mortality rate between WT mice and heterozygous JVS mice, heterozygous mice showed an age-associated increase in left ventricular myocyte diameters compared with WT mice.11 In humans, a genetic epidemiological study in Japan demonstrated that heterozygotes for OCTN2 mutations have consistently low serum carnitine concentrations, suggesting that heterozygosity for this gene may predispose to cardiomyopathy and heart failure with energy depletion.12

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carnitine phenotypes, and the estimate of the overall prevalence of heterozygotes was 1.01%. In this study, an echocardiographic analysis of the families of patients with SCD revealed that heterozygotes for OCTN2 mutations were predisposed to late-onset cardiac hypertrophy with normal cardiac performance compared with the WT mice.

Heterozygotes for OCTN2 mutations may be at risk for cardiomyopathy in the presence of risk factors such as hypertension in addition to aging. To test this hypothesis, we investigated the effects of surgically induced pressure overload on the hearts of heterozygous mutants of JVS mice.

Methods

An expanded Materials and Methods section can be found in the online supplemental data available at http://hyper.ahajournals.org.

Experimental Animals and Study Protocol

Heterozygous mutants of 11-week-old male JVS mice of the C3H strain were used. Heterozygous JVS mice (jvs/+ ) were created by mating homozygous mutants and heterozygous mutants of JVS mice. Heterozygous mutants were distinguished from homozygous mutants, which have swollen fatty livers recognized through the abdominal wall at 5 days after birth. Age-matched male WT mice of the C3H strain were used as controls. Both WT and jvs/+ mice were divided into the following 3 groups: (1) sham operation; (2) ascending aorta constriction (AAC); and (3) 1% L-carnitine supplementation (10 g of L-carnitine per kilogram of chow) for 4 weeks after AAC. L-Carnitine was not included in the ingredients of the standard chow. AAC was created by ligation of the ascending aorta in a 27-gauge needle using 7–0 silk suture in anesthetized (50 mg/kg of pentobarbital IP) and ventilated mice. Sham-operated mice underwent the same surgical procedure without the ligation of the aorta. All of the animals were studied 4 weeks after the operation. Animals were anesthetized with diethyl ether before sacrifice. For survival analysis, cages were inspected daily for deceased animals during the study period.

This study conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All of the protocols described were approved by the Nagoya University Animal Ethics Committee.

Echocardiographic and Hemodynamic Measurements

On the day of sacrifice, the systolic blood pressure and heart rate were determined in each animal. The tail-cuff method was used, employing a photoelectric tail cuff detection system, Softtron BP-98A (Softtron). Left ventricular function was evaluated by transthoracic echocardiography using SONOS 7500 (Philips Medical Systems) with a 10-MHz imaging transducer. To evaluate the degree of stenosis, the pressure gradient across the constriction was assessed by Doppler echocardiography at 3 days after operation.

Plasma and Myocardial Total Carnitine Levels

Plasma and myocardial total carnitine levels were determined by an enzymatic method described elsewhere.

Histological Analysis

Heart tissue was examined by means of light microscopy. The tissue was fixed in 10% formaldehyde in phosphate buffer and was embedded in paraffin. Transverse sections (4-µm thickness) at the level of papillary muscle were stained with hematoxylin/eosin or with Masson’s trichrome. The myocyte cross-sectional area and the extent of fibrosis were evaluated with the use of the image analysis software WinROOF (Mitani Corp).

Real-Time Quantitative RT-PCR

Total RNA was extracted from the left ventricle, and reverse transcription was performed by a method described previously. To examine the relative mRNA expression of atrial natriuretic peptide, quantitative RT-PCR analysis was performed using the Stratagene Mx3000P QPCR System (Stratagene) and the Power SYBR Green PCR Master Mix (Applied Biosystems).

Myocardial High-Energy Phosphate Content

Animals were anesthetized with diethyl ether, and the hearts were rapidly excised and snap-frozen with liquid nitrogen. The frozen tissue was homogenized in 0.5 mol/L of ice-cold perchloric acid for deproteinization and then neutralized with 2 mol/L of K2CO3. After centrifugation, the supernatant was filtered, and an aliquot of 20 µL was used for measurement. Phosphocreatinine (PCr) and adenosine triphosphate (ATP) contents were measured by high-performance liquid chromatography, as described previously.

Statistics

All of the values are expressed as mean±SEM. The survival analysis was performed by the Kaplan-Meier method, and between-group differences in survival were tested by the log-rank test. Between-group comparisons were assessed by 1-way ANOVA followed by the Bonferroni posthoc test. A value of P<0.05 was considered to be statistically significant.

Results

Baseline Characteristics of Heterozygous JVS Mice

Table S1 demonstrates the characteristics of jvs/+ mice at basal condition. At baseline, there were no differences in the gravimetric, hemodynamic, and echocardiographic data between WT and jvs/+ mice. However, the plasma and myocardial total carnitine levels in jvs/+ mice were lower than in WT mice (52.8±0.9 µmol/L versus 70.2±1.1 µmol/L, P<0.01; 349.2±25.9 nmol/g versus 642.3±30.1 nmol/g, P<0.01, respectively).

Assessment of Cardiac Hypertrophy, Pulmonary Congestion, and Hemodynamics

Gravimetric and hemodynamic data at 4 weeks after AAC are shown in the Table. jvs/+ -AAC mice showed a further increase in the heart weight:body weight ratio, the lung weight:body weight ratio, and the wet lung weight:dry lung weight ratio compared with WT-AAC mice. Systolic blood pressure was further reduced in jvs/+ -AAC mice compared with WT-AAC mice. L-Carnitine supplementation prevented these changes in jvs/+ -AAC mice. There was no difference in heart rate among all of the groups.

Echocardiographic Examination

Echocardiographic data are demonstrated in Figure 1. There was no difference in aortic pressure gradient among all of the groups at 3 days after AAC (Figure 1E). Four weeks after operation, jvs/+ -AAC mice showed a further increase in left ventricular end-systolic dimension and a further deterioration of left ventricular fractional shortening compared with WT-AAC mice (Figure 1C and 1D). L-Carnitine supplementation prevented these changes in jvs/+ -AAC mice. However, there was no difference in left ventricular end-diastolic dimension between WT-AAC and jvs/+ -AAC mice (Figure 1B).
Histopathologic Analysis and Relative Atrial Natriuretic Peptide mRNA Expression in the Heart

Transverse sections of the hearts are shown in Figure 2A. jvs/+ -AAC mice showed an exaggeration of cardiomegaly compared with WT-AAC mice. l-Carnitine supplementation prevented the cardiomegaly in jvs/+ -AAC mice. jvs/+ -AAC mice showed an exaggeration of cardiomyocyte hypertrophy compared with WT-AAC mice (Figure 2B and 2C). l-Carnitine supplementation also prevented the cardiomyocyte hypertrophy in jvs/+ -AAC mice. The relative mRNA expression of atrial natriuretic peptide, a molecular marker of cardiac hypertrophy, in the left ventricles of jvs/+ -AAC mice increased more than in WT-AAC mice; l-carnitine supplementation prevented this change in jvs/+ -AAC mice (Figure 2D). The extent of fibrosis was similar between WT-AAC and jvs/+ -AAC mice (Figure S1A and S1B).

Survival Study

The acute mortality rate within 72 hours after AAC was not different among all of the mice that were subjected to AAC (16% of WT-AAC mice, 16% of WT-AAC mice treated with l-carnitine, 17% of jvs/+ -AAC mice, and 18% of jvs/+ -AAC mice treated with l-carnitine; P value was not significant for all). None of the mice died after the sham operation. In mice that survived the acute periopeative period after AAC, 27% of jvs/+ -AAC mice (4 of 15) died in the following 4 weeks, whereas none of the mice in the other groups died during the same period (Figure 3).
Myocardial High-Energy Phosphate Content

At 4 weeks after AAC, jvs/+ -AAC mice showed a significant decrease in myocardial PCr/ATP, an index of the myocardial energy reserve, whereas PCr/ATP in WT-AAC mice was preserved (Table). L-Carnitine supplementation prevented the reduction of PCr/ATP in jvs/+ -AAC mice.

Figure 2. A, Hematoxylin/eosin staining of heart tissues at 4 weeks after AAC. Bars indicate 1 mm. B, Masson’s trichrome staining of heart tissues at 4 weeks after AAC. Bars indicate 20 μm. C, Quantitative data of cardiomyocyte cross-sectional area. Values are mean±SEM. n=4 for each group. *P<0.01 vs WT-sham; †P<0.01 vs jvs/+ -sham; ‡P<0.01 vs WT-AAC; §P<0.01 vs jvs/+ -AAC. D, Relative expression of atrial natriuretic peptide (ANP) mRNA in the hearts. Data are normalized to r18S mRNA content and expressed as fold increase over WT-sham mice. Values are mean±SEM. n=4 for each group. Values are mean±SEM. *P<0.01, †P<0.05 vs WT-sham; ‡P<0.01, §P<0.05 vs jvs/+ -sham; #P<0.01 vs WT-AAC; **P<0.01 vs jvs/+ -AAC.

Figure 3. Kaplan–Meier survival analysis of WT and jvs/+ mice subjected to AAC with or without L-carnitine supplementation. n=14 to 16 for each group.

Discussion

In the present study, we clearly demonstrated the increased susceptibility to pathological cardiac hypertrophy and heart failure in heterozygous mice of OCTN2 mutation with relatively low carnitine levels when subjected to pressure overload. jvs/+ -AAC mice showed an exaggeration of cardiac hypertrophy and heart failure and a decrease in myocardial PCr/ATP compared with WT-AAC mice, and these changes were prevented by L-carnitine supplementation, suggesting the involvement of the reduced carnitine levels and the perturbation of cardiac energy metabolism in the pathogenesis of cardiomyopathy in jvs/+ -AAC mice.

Animal studies have demonstrated a reduction in fatty acid oxidation rates and increased glucose uptake and use in the hypertrophied heart because of pressure overload.17-19 This alteration of myocardial energy substrate metabolism may be an adaptive mechanism for maintaining the cardiac function. Liao et al20 reported that increasing glucose use in hypertrophied hearts protects against contractile dysfunction and LV dilatation after chronic pressure overload in mice. The manipulation of the myocardial substrate metabolism by chronic inhibition of fatty acid oxidation has been shown to have
therapeutic potential as a treatment for heart failure in animal models and in humans.\textsuperscript{21,22} However, in the present study, heterozygous JVS mice with reduced carnitine levels showed an exaggeration of cardiac hypertrophy and heart failure and impaired myocardial energy metabolism when subjected to pressure overload. Previously, we have demonstrated that homozygous JVS mice are hypoglycemic,\textsuperscript{9,15,23} which indicates the energy substrate metabolism shift toward glucose metabolism because of fatty acid oxidation disorder. Although plasma glucose levels or myocardial energy substrate metabolism were not assessed in the present study, the myocardial energy substrate metabolism may shift from fatty acid oxidation toward glucose metabolism also in heterozygous JVS mice because of the relatively low carnitine levels. In heterozygous JVS mice, the glucose metabolism may be further enhanced in the heart when subjected to pressure overload. The exaggeration of heart failure and the reduction of PCT/ATP observed in jvs/+ -AAC mice suggest that this increase in glucose metabolism may be insufficient to support the myocardial energy metabolism. The results of our study indicate that the presence of substantial levels of carnitine to support the fatty acid oxidation capacity may be required to maintain the cardiac function in mice subjected to pressure overload for 4 weeks. However, myocardial energy substrate metabolism was not assessed in the present study. Further investigation is required to elucidate the mechanism underlying the energy depletion in heterozygous JVS mice subjected to pressure overload.

Although several early studies had initially suggested that myocardial high-energy phosphate contents were unaltered in heart failure,\textsuperscript{24,25} more recent studies have provided evidence that there is an energy deficit in the failing heart and that the failing heart is “energy starved.”\textsuperscript{26,27} In terms of animal models of pressure-overloaded hearts, myothematic measurements demonstrated that heat production was lower in pressure-overloaded hearts than in normal hearts in rabbits.\textsuperscript{28} In mice subjected to chronic pressure overload (8 weeks after AAC), myocardial PCT/ATP was significantly reduced compared with sham-operated mice.\textsuperscript{20} In our study, WT mice subjected to AAC for 4 weeks did not show any decrease in myocardial PCT/ATP. However, heterozygous JVS mice showed a decrease in myocardial PCT/ATP and an exacerbation of cardiac dysfunction and heart failure when subjected to AAC for 4 weeks; these changes were prevented by L-carnitine supplementation. Whether heart failure or cardiac remodeling is caused by a deficit in energy production or a deficit in energy use has been a continuing debate. During the compensatory stage in cardiac remodeling, modifications in the cellular apparatus that are responsible for energy production could account for further impairment of myocardial function.\textsuperscript{29} The results of our study support this hypothesis and suggest that the reduction in carnitine leading to myocardial energy depletion plays a causal role for the exacerbation of cardiac dysfunction and heart failure in pressure-overloaded hearts.

A human study using echocardiography showed that variations in the peroxisome proliferator-activated receptor-\(\alpha\), a key transcriptional regulator of the expression of fatty acid oxidation pathways genes, influence the hypertrophic growth in response to both exercise and hypertension.\textsuperscript{30} It was also reported that the myocardial fatty acid metabolism is an independent predictor of left ventricular mass in hypertension and in left ventricular dysfunction in humans.\textsuperscript{31} These observations provide evidence that the perturbation of myocardial fatty acid oxidation may play a causal role in the pathogenesis and/or modulation of the hypertrophic phenotype because of hypertension. Consistently, the present study suggest that the reduction in carnitine, possibly leading to impaired myocardial fatty acid oxidation, may have been involved in the exaggeration of cardiac hypertrophy and heart failure in heterozygous JVS mice subjected to pressure overload.

We have reported previously that homozygous JVS mice develop pathological cardiac hypertrophy with vast lipid accumulation and that the increase in myocardial 1,2-diacylglycerol, a lipid second messenger, and the alteration of fatty acid composition of 1,2-diacylglycerol were implicated in the pathogenesis of cardiac hypertrophy in homozygous JVS mice.\textsuperscript{9,15,23,32} In the present study, we have also carried out myocardial lipid analysis in heterozygous JVS mice subjected to pressure overload (Table S2). In contrast to homozygous JVS mice, jvs/+ -sham mice showed only a modest but significant increase in myocardial triacylglycerol and 1,2-diacylglycerol levels compared with WT-sham mice. However, in mice subjected to AAC, the myocardial triacylglycerol level was significantly reduced to identical levels in both WT and jvs/+ mice, and the 1,2-diacylglycerol level in jvs/+ -mice was significantly reduced to WT mice levels. In contrast to the dramatic alteration of the fatty acid composition of myocardial 1,2-diacylglycerol in homozygous JVS mice as demonstrated in our previous reports,\textsuperscript{9,15,23,32} jvs/+ -sham mice did not show significant alteration in the fatty acid composition of 1,2-diacylglycerol. Although mice subjected to AAC showed changes in fatty acid composition of myocardial 1,2-diacylglycerol compared with sham-operated mice, there was no difference between WT-AAC and jvs/+ -AAC mice. L-Carnitine supplementation did not alter the myocardial triacylglycerol or 1,2-diacylglycerol levels and also did not change the fatty acid composition of 1,2-diacylglycerol in both WT-AAC and jvs/+ -AAC mice. These results suggest that myocardial lipid moieties may not be involved in the pathogenesis of cardiomyopathy and heart failure in heterozygous JVS mice subjected to pressure overload.

Perspectives
A genetic epidemiological study in the Akita prefecture of Japan demonstrated that the estimate of the overall prevalence of heterozygotes for \textit{OCTN2} mutations was 1.01\%, and an echocardiographic analysis revealed that heterozygotes were predisposed to late-onset cardiac hypertrophy with normal cardiac performance.\textsuperscript{12} Although heterozygotes of SCD are generally healthy without cardiac complications, it has been proposed that heterozygotes may be at risk for cardiomyopathy in the presence of additional risk factors, such as hypertension.\textsuperscript{13} The results of our study support this hypothesis and suggest that L-carnitine supplementation may be a potential therapy for preventing cardiomyopathy and heart failure in heterozygotes for \textit{OCTN2} mutations with
hypertension. The prevalence of heterozygotes for OCTN2 mutations seems to be higher than expected, suggesting that heterozygotes are often overlooked clinically. Further examination and epidemiological studies are necessary to stratify the risk for cardiomyopathy in hypertensive patients with OCTN2 mutations. The identification of OCTN2 mutations in hypertensive patients and a prospective study to investigate the efficacy of L-carnitine supplementation may contribute to the development of novel strategies for the prevention and treatment of cardiomyopathy and heart failure in these subjects.

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Disclosures
None.

References
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Expanded Materials and Methods

Experimental Animals and Study Protocol

Heterozygous mutants of 11-week-old male JVS mice of the C3H strain were used. Heterozygous JVS mice (jvs/+) were created by mating homozygous mutants and heterozygous mutants of JVS mice. Heterozygous mutants were distinguished from homozygous mutants, which have swollen fatty livers recognized through the abdominal wall at 5 days after birth.\(^1\) Age-matched male WT mice of the C3H strain were used as controls. Both WT and jvs/+ mice were divided into the following 3 groups: 1) sham operation; 2) ascending aorta constriction (AAC); 3) 1% L-carnitine supplementation (10 g L-carnitine per kg chow) for 4 weeks after AAC. L-Carnitine was not included in the ingredient of the standard chow. AAC was created by ligation of the ascending aorta with a 27-gauge needle using 7-0 silk suture in anesthetized (pentobarbital 50 mg/kg IP) and ventilated mice. Sham-operated mice underwent the same surgical procedure without the ligation of the aorta. All animals were studied 4 weeks after the operation. Animals were anesthetized with diethyl ether before sacrifice. For survival analysis, cages were inspected daily for deceased animals during the study period.

This study conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). All protocols described were approved by the Animal Ethics Committee of Nagoya University, Nagoya, Japan.

Echocardiographic and Hemodynamic Measurements

On the day of sacrifice, the systolic blood pressure and heart rate were determined in each animal. The tail-cuff method was used, employing a photoelectric tail cuff detection system, Softron BP-98A (Softron). Left ventricular function was evaluated by transthoracic
echocardiography using SONOS 7500 (Philips Medical Systems) with a 10-MHz imaging transducer. Briefly, each animal was slightly sedated with pentobarbital (20 mg/kg IP). M-mode images of the left ventricle (LV) were recorded, and left ventricular dimensions at end-diastole (LVEDD) and end-systole (LVESD) were measured by the leading edge method. For each measurement, data from three cardiac cycles were averaged, and left ventricular fractional shortening (LVFS) was calculated as LVFS (%) = (LVEDD-LVESD)/LVEDD x 100.

To evaluate the degree of stenosis, the pressure gradient across the constriction was assessed by Doppler echocardiography at 3 days after operation. The transducer was placed at the apex and oriented toward the proximal ascending aorta. The peak velocity (m/s) was measured, and the maximum instantaneous gradient (mmHg) was calculated by use of the Bernoulli equation: pressure gradient = 4 x (velocity)^2.

**Plasma and Myocardial Total Carnitine Levels**

Plasma and myocardial total carnitine levels were determined by an enzymatic method described elsewhere.²

**Histological Analysis**

Heart tissue was examined by means of light microscopy. The tissue was fixed in 10% formaldehyde in phosphate buffer and was embedded in paraffin. Transverse sections (4 μm thickness) at the level of papillary muscle were stained with hematoxylin-eosin (H-E) or with Masson’s trichrome. The myocyte cross-sectional area of LV was measured from myocytes that were cut transversely and exhibited both a nucleus and an intact cell membrane. At least 200 myocytes per LV were assessed and the average value was used for analysis. The extent of fibrosis was evaluated by calculating the ratio of Masson’s trichrome-stained fibrotic area to the total area of the myocardium with the use of the image analysis software WinROOF (Mitani Corp.).
Real-Time Quantitative RT-PCR

Total RNA was extracted from the left ventricle and reverse transcription was performed by a previously described method. To examine the relative mRNA expression of atrial natriuretic peptide (ANP), quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis was performed using the Stratagene Mx3000P™ QPCR System (Stratagene) and Power SYBR® Green PCR Master Mix (Applied Biosystems). Sequences of the sense and antisense primers used for amplification were: ANP, 5’-CGTCTTGCCCTTTGCT-3’ and 5’-CCAGGTGGTAGCAGTTTCT-3’, product size: 104 bp; r18S, 5’-GTAACCGTTGAACCCCATT-3’ and 5’-CCATCCAATCGGTAGTAGCG-3’, product size: 151 bp. Standard curves were generated by full sequence plasmids of known concentrations. Gene expression of ANP mRNA was normalized to r18S and was expressed as fold increase over sham-operated WT mice.

Myocardial High-Energy Phosphate Content

Animals were anesthetized with diethyl ether and the hearts were rapidly excised and snap-frozen with liquid nitrogen. The frozen tissue was homogenized in ice-cold 0.5 mol/L perchloric acid for deproteinization, and then neutralized with 2 mol/L K2CO3. After centrifugation, the supernatant was filtered and an aliquot of 20 µL was used for measurement. Phosphocreatinine (PCr) and adenosine triphosphate (ATP) contents were measured by high-performance liquid chromatography (HPLC), as previously described. Briefly, HPLC was performed with the use of the Hewlett Packard Model 1100 HPLC system consisting of an 1100 quaternary pump, variable wavelength UV-VIS detector, Hypersil ODS C18 reversed-phase column (250 mm x 2.1 mm i.d., particle size 5 µm), and ChemStation software. The mobile phase was composed of 215 mmol/L potassium dihydrogen phosphate, 2.3 mmol/L tetrabutyl ammonium hydrogen sulfate, 4% acetonitrile, and 4 mmol/L potassium hydroxide, and the flow rate was 300 µL/min. The absorbance was monitored at 220 nm.
Myocardial Lipid Analysis

The lyophilized tissue samples were homogenized in 5 mL of a chilled chloroform-methanol mixture (2:1, v/v) containing 0.01% butylated hydroxytoluene as an antioxidant and cholesteryl acetate as an internal standard. Simultaneous quantitation of 1,2-diacylglycerol and triacylglycerol was performed by the TLC–FID method, as previously described. In brief, 1 µL of a lipid extract solution containing ceramides, neutral lipids, and free fatty acids was dissolved in chloroform and applied carefully to silica gel using 75-µm precoated thin-layer rods (Chromarod-SIII, Iatron Lab., Inc.). The first development was carried out in a solvent system of chloroform–methanol–H₂O (57:12:0.6, v/v). The second and the third developments were carried out in 1,2-dichloroethane–chloroform–acetic acid (46:6:0.05, v/v). The fourth development was carried out in n-hexane–diethyl ether–acetic acid (98:1:1, v/v). The Chromarods were then scanned in an Iatroscan MK-5 analyzer (Iatron Lab., Inc.). The fatty acid composition of 1,2-diacylglycerol obtained by TLC was determined by gas chromatography (model GC 14-A, Shimadzu) using an HR-SS-10 fused silica capillary column (30 m x 0.25 mm internal diameter, Shinwakakoh), as previously described.

Statistics

All values are expressed as mean±SEM. The survival analysis was performed by the Kaplan-Meier method, and between-group differences in survival were tested by the log-rank test. Between-group comparisons were assessed by one-way ANOVA, followed by the Bonferroni post hoc test. A value of $P<0.05$ was considered to be statistically significant.
Results of Myocardial Lipid Analysis

Table S2 shows the results of myocardial lipid analysis at 4 weeks after operation. In sham-operated mice, the myocardial triacylglycerol level was 1.6-fold higher and the 1,2-diacylglycerol level was 1.7-fold higher in jvs/+ mice than in WT mice. However, in mice subjected to AAC, the myocardial triacylglycerol level was significantly reduced to identical levels in both WT and jvs/+ mice, and the 1,2-diacylglycerol level in jvs/+ mice was significantly reduced to WT mice levels. L-Carnitine supplementation did not alter the myocardial triacylglycerol or 1,2-diacylglycerol levels in both WT and jvs/+ mice subjected to AAC.

In sham-operated mice, the fatty acid composition of myocardial 1,2-diacylglycerol showed that C14:0 and C18:0 were modestly reduced, and C18:2n-6 was modestly increased in jvs/+ mice compared to WT mice. However, these changes were not significant. In mice subjected to AAC, C18:2n-6 and C20:1n-9 were significantly reduced in both WT and jvs/+ mice compared to sham-operated groups. C14:0 and C18:0 were significantly increased in AAC groups compared to sham-operated jvs/+ mice. L-Carnitine supplementation did not alter the fatty acid composition of myocardial 1,2-diacylglycerol in both WT and jvs/+ mice subjected to AAC.
References


Figure S1. A, Masson’s trichrome staining of the hearts at 4 weeks after ascending aorta constriction (AAC). Bars indicate 100 µm. B, Quantitative data of the area of fibrosis in the hearts. Values are mean±SEM. n=4 each group. *P<0.01 vs WT-sham; †P<0.01 vs (jvs/+) sham.
| TABLE S1. Gravimetric, Hemodynamic, and Echocardiographic Measurements, and Total Carnitine Levels at Baseline |
|---------------------------------------------------|----------------|----------------|
| Mice/Parameter | WT | jvs/+ |
| BW, g | 23.1±0.1 | 23.1±0.4 |
| HW, mg | 94.8±2.2 | 94.5±2.0 |
| HW/BW, mg/g | 4.10±0.10 | 4.09±0.13 |
| Wet LW/BW, mg/g | 5.18±0.14 | 5.46±0.04 |
| Wet LW/dry LW | 4.63±0.03 | 4.70±0.05 |
| Systolic BP, mmHg | 117±1 | 116±4 |
| HR, bpm | 561±4 | 561±7 |
| LVEDD, mm | 2.9±0.1 | 2.8±0.1 |
| LVESD, mm | 1.2±0.1 | 1.2±0.1 |
| LVFS, % | 56.5±0.4 | 55.7±0.9 |
| Total carnitine levels | | |
| Plasma, µmol/L | 70.2±1.1 | 52.8±0.9* |
| Myocardium, nmol/g | 642.3±30.1 | 349.2±25.9* |

Data are mean±SEM. n=6 to 8 for each group. BW indicates body weight; HW, heart weight; LW, lung weight; BP, blood pressure; HR, heart rate; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LVFS, left ventricular fractional shortening.

*P<0.01 vs WT.


**TABLE S2. Myocardial Lipid Analysis at 4 Weeks After AAC**

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<thead>
<tr>
<th>Operation and mice/Parameter</th>
<th>Sham</th>
<th>AAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT jvs/+</td>
<td>WT WT+CAR</td>
</tr>
<tr>
<td>Triacylglycerol, µg/mg dry weight</td>
<td>10.0±1.2</td>
<td>2.6±0.2*†</td>
</tr>
<tr>
<td></td>
<td>15.9±2.1*</td>
<td>2.5±0.3*†</td>
</tr>
<tr>
<td>1,2-Diacylglycerol, ng/mg dry weight</td>
<td>218±27</td>
<td>195±64†</td>
</tr>
<tr>
<td></td>
<td>383±83*</td>
<td>195±64†</td>
</tr>
<tr>
<td>Fatty acid composition of 1,2-diacylglycerol, % of total fatty acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>10.9±1.3</td>
<td>13.0±1.1†</td>
</tr>
<tr>
<td>C16:0</td>
<td>23.2±1.8</td>
<td>23.1±1.2</td>
</tr>
<tr>
<td>C18:0</td>
<td>10.9±1.5</td>
<td>13.0±2.0†</td>
</tr>
<tr>
<td>C18:1n-7,9</td>
<td>16.2±1.3</td>
<td>13.9±0.7</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>19.1±2.9</td>
<td>11.5±2.2*†</td>
</tr>
<tr>
<td>C20:1n-9</td>
<td>1.7±0.2</td>
<td>1.1±0.1*†</td>
</tr>
<tr>
<td>C20:4n-6</td>
<td>2.3±0.1</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>C24:1n-9</td>
<td>1.6±0.3</td>
<td>1.8±0.2</td>
</tr>
</tbody>
</table>

Data are mean±SEM. n=4 to 6 for each group. AAC indicates ascending aorta constriction; CAR, L-carnitine.

*P<0.05 vs WT-sham; †P<0.05 vs (jvs/+)-sham.
Figure S1

A

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>AAC</th>
<th>AAC + L-Carnitine</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td><img src="image1" alt="WT Sham" /></td>
<td><img src="image2" alt="WT AAC" /></td>
<td><img src="image3" alt="WT AAC + L-Carnitine" /></td>
</tr>
<tr>
<td>jvs/+</td>
<td><img src="image4" alt="jvs/+ Sham" /></td>
<td><img src="image5" alt="jvs/+ AAC" /></td>
<td><img src="image6" alt="jvs/+ AAC + L-Carnitine" /></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>jvs/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Carnitine</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>Sham</td>
<td><img src="image7" alt="Sham" /></td>
<td><img src="image8" alt="Sham" /></td>
</tr>
<tr>
<td>AAC</td>
<td><img src="image9" alt="AAC" /></td>
<td><img src="image10" alt="AAC" /></td>
</tr>
</tbody>
</table>

Area of fibrosis (%)

* * † † † †