EXPERIMENTAL STUDY

Pressure Overload Impairs Cardiac Function in Long-Chain Fatty Acid Transporter CD36-Knockout Mice

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Summary

CD36 is one of the important transporters of long-chain fatty acids (LCFAs) in the myocardium. We previously reported that CD36-deficient patients demonstrate a marked reduction of myocardial uptake of LCFAs, while myocardial glucose uptake shows a compensatory increase, and are often accompanied by cardiomyopathy. However, the molecular mechanisms and functional role of CD36 in the myocardium remain unknown.

The current study aimed to explore the pathophysiological role of CD36 in the heart. Methods: Using wild type (WT) and knockout (KO) mice, we generated pressure overload by transverse aortic constriction (TAC) and analyzed cardiac functions by echocardiography. To assess cardiac hypertrophy and fibrosis, histological and molecular analyses and measurement of ATP concentration in mouse hearts were performed.

By applying TAC, the survival rate was significantly lower in KO than that in WT mice. After TAC, KO mice showed significantly higher heart weight-to-tibial length ratio and larger cross-sectional area of cardiomyocytes than those of WT. Although left ventricular (LV) wall thickness in the KO mice was similar to that in the WT mice, the KO mice showed a significant enlargement of LV cavity and reduced LV fractional shortening compared to the WT mice with TAC. A tendency for decreased myocardial ATP concentration was observed in the KO mice compared to the WT mice after TAC operation.

These data suggest that the LCFA transporter CD36 is required for the maintenance of energy provision, systolic function, and myocardial structure.

Key words: Transverse aortic constriction, ATP, Glucose, Cardiomyopathy, S6 kinase

Cardiomyocytes are known to mainly utilize fatty acids and glucose. Decreased availability of fatty acids results in increased utilization of glucose. CD36 is an 88-kDa class B scavenger glycoprotein receptor that is expressed in several organs and tissues, such as the heart, skeletal muscles, microvascular endothelial cells, adipose tissues, enterocytes, macrophages, and platelets. As we previously reported, CD36-deficient patients present with higher serum triglyceride levels and lower HDL cholesterol levels, as well as impaired glucose metabolism due to insulin resistance. Although patients with CD36 deficiency also demonstrate a marked reduction in the myocardial uptake of [123I]15-(p-iodophenyl)-(R, S)-methylpentadecanoic acid (BMIPP), which is an analog of long-chain fatty acids (LCFAs), the myocardial uptake of [18F]fluorodeoxy-glucose (FDG) is increased. Tanaka, et al. reported a high prevalence of CD36 deficiency in patients with hypertrophic cardiomyopathy (HCM), and we also found an association between CD36 deficiency and either hypertrophic or dilated cardiomyopathy (DCM) among patients. These data suggest that impaired fatty acid utilization due to CD36 deficiency may be related to one of the etiologies of genetic cardiomyopathy. However, the molecular mechanisms underlying cardiomyopathy in association with CD36 deficiency remain unknown.

To investigate the association between depressed myocardial fatty acid uptake and cardiac hypertrophy or dysfunction, Kasuka, et al. investigated the effects of...
sulfo-N-succinimidyl palmitate (SSP) on the rat heart and showed that SSP irreversibly inhibited fatty acid transport in rat heart muscle. SSP treatment caused a 12% increase in heart weight. These findings suggest that decreased cardiac fatty acid uptake may result in cardiac hypertrophy.\textsuperscript{11} Cardiac manifestations in several animal models with impaired fatty acid utilization have been reported. Loss of lipoprotein lipase-derived fatty acids leads to severe cardiac fibrosis in old-aged mice and higher mortality under pressure overload compared to wild-type mice.\textsuperscript{12} Investigation of heart-type fatty acid binding protein (H-FABP)-knockout mice has revealed that decreased fatty acid utilization results in a low tolerance to exercise.\textsuperscript{13} However, these transgenic mice depend on glucose utilization and do not show any cardiac dysfunction without stress. Although we did not observe a decline in cardiac function or blood pressure in CD36-KO mice under non-stress conditions, cardiac dysfunction may occur under some stresses such as pressure overload. In the current study, we investigated the pathophysiologic effects of CD36 deficiency on cardiac function under pressure overload. The findings will provide insight into the molecular mechanisms involved in the development of cardiomyopathy associated with a variety of metabolic disorders.

\textbf{Methods}

\textbf{CD36-KO mice:} CD36-KO mice generated on a C57BL/6J background were kindly provided by Mason W. Free-\textsuperscript{man, MD, PhD, Professor at Harvard Medical School.\textsuperscript{14} CD36-KO (KO) mice and C57BL/6J (WT) mice were housed in a temperature- and humidity-controlled facility with a 12-hour light/dark cycle and fed a normal chow diet (MF, Oriental Bio Laboratories, Chiba, Japan). The mice used for the isolation of hearts were anesthetized by intraperitoneal injection of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). Sufficient anesthetic depth was ensured by monitoring the respiratory rate and the absence of response to a paw pinch. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine and was conducted in accordance with the Public Health Service (PHS) Policy on the Humane Care and Use of Laboratory Animals, which was incorporated into the Institute for Laboratory Animal Research (ILAR) Guide for the Care and Use of Laboratory Animals.

\textbf{Transverse aortic constriction (TAC):} Seven- to eleven-week-old mice were subjected to pressure overload by TAC under isoflurane anesthesia as previously described.\textsuperscript{15} Briefly, the transverse aortic arch was visualized through median sternotomy, and a 7-0 silk ligature was tied around the aorta (27-gauge constriction for severe TAC or 25-gauge constriction for mild TAC) between the right brachiocephalic and left common carotid arteries.\textsuperscript{16} Sham-operated mice underwent an identical procedure, including isolation of the transverse aortic arch, but without banding.

\textbf{Transthoracic echocardiography:} Transthoracic echocardiography was performed on conscious mice with a Vevo 770 high-resolution imaging system equipped with a 25-MHz transducer (Visual Sonics, Toronto, Canada). M-mode images were obtained for measurements of left ventricular (LV) wall thickness, LV diameter at diastole (LVDd), and LVD at systole (LVDs). Fractional shortening (FS), which is a measure of systolic function, was calculated according to the following equation: F (%) = (LVDd - LVDs/LVDd) × 100.

\textbf{Quantitative real-time PCR:} Gene expression in cardiac tissues was measured with quantitative real-time polymerase chain reaction (qRT-PCR). Briefly, total mRNA was extracted from snap-frozen, homogenized heart tissue using Trizol reagent (Life Technologies Co., Carlsbad, CA, USA). RNA was treated with DNase using a TURBO DNA-free Kit (Life Technologies Co.) and reverse-transcribed using SuperScript VILO cDNA Synthesis Kit (Life Technologies Co.). Diluted cDNA was used as the template to quantify the relative concentration of mRNA. qRT-PCR was performed using a Universal Probe Library (Roche, Basel, Switzerland) and a Light Cycler 480 system (Roche). The relative gene expression values were normalized to those of ribosomal protein S28 gene expression using the comparative Ct (threshold cycle) method according to the manufacturer’s instructions.\textsuperscript{17}

\textbf{Protein analysis:} Cardiac tissues were homogenized in cold RIPA lysis reagent containing 25 mM Tris HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 10 mM okaic acid, protease inhibitors (Complete Mini EDTA-free, Roche, Basel, Switzerland), and phosphatase inhibitors (PhosSTOP, Roche), and then, the lysate was centrifuged at 20,000 g for 15 minutes. The supernatant was used for SDS-PAGE and immunoblotting. Proteins were applied to an SDS PAGE gel (Tefco, Tokyo, Japan), separated using SDS running buffer, and then transferred onto polyvinylidene difluoride membranes. The membranes were incubated for 30 minutes at room temperature in Blocking One blocking buffer (Nacalai Tesque, Kyoto, Japan). They were then probed with a polyclonal anti-phospho-Akt (Thr308) antibody (Cell Signaling Technology Inc., Danvers, MA, USA), an anti-Akt antibody (Cell Signaling Technology Inc.), an anti-phospho-AMPK-α (Thr172) antibody (Cell Signaling Technology Inc.), an anti-AMPK-α antibody (Cell Signaling Technology Inc.), an anti-phospho-S6 (Ser240/244) antibody (Cell Signaling Technology Inc.), an anti-Akt antibody (Cell Signaling Technology Inc.), and an anti-S6 antibody (Cell Signaling Technology Inc.) or an anti-S6 antibody (Cell Signaling Technology Inc.) in TBS with 0.1% Tween-20 (TBS-T) overnight at 4°C. A corresponding anti-rabbit secondary antibody was obtained from Jackson Immuno Research Laboratories. The reactions were developed with ECL Plus Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA). Images were analyzed using the ImageQuant LAS 4000 mini (GE Healthcare, Uppsala, Sweden).

\textbf{Histological analyses:} Hearts were isolated and fixed with formalin. Paraffin sections (5-μm thick) were stained with hematoxylin and eosin (WAKO, Tokyo, Japan) and Masson trichrome (Sigma-Aldrich, St Louis, USA) to evaluate cell surfaces and fibrosis areas.

\textbf{Measurement of ATP concentration in the heart:} The concentration of ATP in the heart was measured using high-performance liquid chromatography (HPLC) as described in a previous report.\textsuperscript{18} Briefly, hearts were isolated
and immediately frozen in liquid nitrogen. Frozen tissues were dried in a vacuum dryer. Freeze-dried samples were weighed and homogenized with 0.5 mL of 0.6 N perchloric acid and centrifuged at 5,000 rpm for 10 minutes at 4°C. The supernatant was neutralized with KOH to pH 5.0-7.0 and centrifuged at 8,000 g for 5 minutes at 4°C. The supernatant was used for HPLC (Shiseido Co., Ltd., Tokyo, Japan) with a STM-ODS-M column (4.6 mm × 250 mm; Shimadzu, Kyoto, Japan).

In vivo measurement of myocardial glucose uptake: Myocardial glucose uptake 1 week after a sham or a severe TAC operation was measured. In vivo cardiac glucose uptake was measured as described previously. Briefly, after a 6-hour fast, 3μCi of 2-deoxy-D (1-13C) glucose (Daiichi Clarity, Chiba, Japan) was injected intravenously via tail vein. At 60 minutes after injection, hearts were perfused with PBS, isolated, and homogenized with Solution350 (PerkinElmer Life and Analytical Science, Waltham, MA). The radioactivity of these solutions was counted, using liquid scintillation counter, Tri-Carb3100 TR (PerkinElmer).

Statistical analyses: The data were expressed as the means ± SEM. The Kaplan-Meier method with a log-rank test was used for the survival analysis. Two-group comparisons were performed with Student t-test, the Welch t-test, or the Mann-Whitney U test as appropriate. A P value of < 0.05 was considered statistically significant. Statistical analysis was performed using JMP Pro 13 software.

Results

KO mice decompensate under pressure overload: We performed severe-TAC operation by banding the transverse aorta with a 27-gauge needle. After severe-TAC for 4 weeks, the survival rate was significantly lower in the KO mice than in the WT mice (Figure 1A). One week after the severe-TAC operation, the KO mice showed significantly greater gross heart weights on imaging and lower LV FS (Figure 1B and C). With severe-TAC, the heart weight-to-tibial length ratio was significantly higher in the KO mice than in the WT mice, and a tendency for greater lung weight was observed in the KO mice compared to the WT mice (Figure 1D).

Impaired LV systolic function and increased heart and lung weights in KO mice with TAC: Because the survival rate was rapidly decreased by applying severe-TAC in KO mice, we next applied mild TAC using a 25-gauge needle to band the aorta for analysis using transthoracic echocardiography for 12 weeks after the mild-TAC procedure. At baseline, no significant differences in LV wall thickness and LV FS were observed between the KO and WT mice. After pressure overload was induced via mild-TAC, although the LV wall thickness in the KO mice was similar to that in the WT mice, the KO mice showed a significant enlargement of the LV cavity and reduced LV FS compared to the corresponding values in the WT mice with mild-TAC (Figure 2A and B). Twelve weeks after the mild-TAC procedure, the difference of the heart weight-to-tibial length ratios between the WT and KO mice was enhanced. A tendency for greater lung weight was observed in the KO mice compared to the WT mice with mild TAC (Figure 2C). These data suggest that CD36-deficient hearts develop heart failure due to systolic dysfunction with eccentric hypertrophy under pressure overload.

Hypertrophic and fibrotic changes in KO mice with TAC: The mRNA expression levels of nppa and nppb were significantly higher in KO sham mice than in WT sham mice. These differences were enhanced under the pressure overload condition. Although the gene expression levels of collagen 1α1 and collagen 3α1 in the KO mice were comparable to those in the WT mice, these gene expression levels were higher in the KO mice than in the WT mice following mild-TAC application (Figure 3A). We observed myocardial signals that induce cell growth-promoting transcriptional activity. The protein expression level of phosphorylated S6 was increased in the KO mice compared to the WT mice, even when the sham operation was performed. These differences in mRNA expression and signal activity suggest that KO mice have a potential for cardiac hypertrophy. Although phosphorylated Akt and AMPK affect S6 kinase activity, those in the KO mice were comparable to the WT mice (Figure 3B). The KO mice with mild-TAC showed a larger cross-sectional area of cardiomyocytes (Figure 4A, B, and E) and larger fibrosis areas than the WT mice with mild-TAC (Figure 4C, D, and F). These data revealed pro-hypertrophic and pro-fibrotic effects of CD36 deficiency during pressure overload.

Decreased ATP concentrations in KO mouse hearts after TAC: The concentration of ATP in mouse hearts was evaluated to explore the mechanisms of impaired heart function with pressure overload. ATP concentrations in the KO mouse hearts after the severe-TAC procedure were decreased compared to those after the sham operation, whereas ATP concentrations in the WT mouse hearts after the severe-TAC procedure were comparable to those after the sham operation. A tendency for decreased myocardial ATP concentration was observed in the KO mice compared to those in the WT mice after the severe-TAC procedure (Figure 5A). Next, we examined myocardial glucose uptake. The KO mice showed an 11.6-fold increase in glucose uptake in the heart compared to the WT mice without pressure overload. The increase in glucose uptake in the WT mice was greater than that in the KO mice under pressure overload (7.7- and 1.4-fold changes, respectively; Figure 5B). Because the capacity for glucose utilization was lower in KO mice than that in WT mice under pressure overload conditions, ATP concentrations in the heart might be decreased in the KO mice.

Discussion

CD36 is an LCFA transporter, and we reported that CD36-deficient patients show a decreased uptake of LCFA and an increased uptake of glucose in the heart, which are often associated with cardiomyopathy. As we reported previously, CD36-deficient mouse hearts depend on glucose utilization; however, KO mice do not present with cardiomyopathy and heart failure under no-stress conditions.

Irie, et al. observed myocardial recovery from ische-
Figure 1. CD36 protein is essential for the protective response against pressure overload on the heart. A: Kaplan-Meier survival analysis of WT and KO mice 4 weeks after the sham or severe-TAC operation (WT sham, 7; KO sham, 8; WT TAC, 14; KO TAC, 10). *P = 0.0018 KO TAC compared to WT TAC. B: Representative gross images of hearts 1 week after the sham or severe-TAC operation in WT and KO mice. C: Representative M-mode images of WT and KO hearts after 1 week of the sham or severe-TAC operation. D: Heart weight (HW) and lung weight (LW)-to-tibial length ratio in WT and KO mice 2 weeks after the sham or severe-TAC operation (WT sham, 3; KO sham, 3; WT TAC, 7; KO TAC, 5). Data are shown as means ± SEM. *P < 0.01 KO TAC compared to WT TAC.

mia in CD36-deficient mice. They revealed decreased LCFA oxidation, compensatory increase in glucose utilization, and impaired myocardial recovery from ischemia in KO mice. In contrast to their report, Kuang, et al. exhibited that KO mouse hearts were not functionally and energetically sensitive to ischemia. The discrepancy between these results may be explained by the difference of perfusate used in working heart examination. Because these findings were observed in ex vivo working mouse hearts, it is not certain whether these data reflect in vivo pathological change or not.

In the current study, we performed pressure overload in the hearts of KO mice and observed survival rates, cardiac function, alterations in gene expression, histological changes in cardiomyocytes, and ATP concentrations in hearts. The KO mouse hearts showed an impaired tolerance to pressure overload, decreased ATP concentrations, eccentric hypertrophy without prior concentric hypertro-
Cardiomyocytes utilize glucose and fatty acids as substrates for ATP generation. Several reports have revealed impaired fatty acid utilization and compensatory increase in glucose utilization in CD36-deficient mouse heart.22,23 In the current study, we observed the amount of ATP in the hearts and myocardial glucose uptake. Gener-
Figure 3. Increased mRNA expressions of hypertrophic and fibrotic markers in KO mouse hearts investigated 12 weeks after mild-TAC. Enhanced phospho-S6 signal in KO mouse heart. A: The mRNA values of nppa, nppb, collagen1α1, and collagen3α1 normalized to 28S and reported as the fold change from WT mice and KO mouse hearts after the sham or mild-TAC operation (WT sham, 4; KO sham, 4; WT TAC, 6; KO TAC, 6). Data are shown as means ± SEM. *P < 0.05 KO sham compared to WT sham. #P < 0.05 KO TAC compared to WT TAC. B: Immunoblots of p-Akt, p-AMPK, and p-S6 signaling in sham and severe-TAC operated mice 2 weeks after each operation.

Pressure overload condition and our data showed KO heart had poor intolerance to pressure overload. These unfavorable effects may have resulted in impaired systolic function. Because previous studies have shown some agents have favorable effects on myocardial energy metabolism,24,25 there might be some interventions that improve the energy efficiency of CD36-deficient heart under pressure overload.

A major regulator of cardiomyocyte size is mTOR kinase, which activates S6 kinase to induce transcriptional activity that promotes cell growth.26 Activated mTOR was observed in several models showing cardiac hypertro-
In the present study, phosphorylated S6 were enhanced in KO mice compared to WT mice, even when the sham operation was performed. These data suggest that KO mice have latency for cardiac hypertrophy. Although Akt activity induces S6 kinase phosphorylation through mTOR kinase, activated AMPK diminishes S6 kinase activity. Akt was not increased and phosphorylated AMPK was not decreased in KO mice. Although the reason for activated S6 kinase in the KO mice remains uncertain in the current study, Sharma, et al. showed that S6 phosphorylation occurred after cardiomyocytes were exposed to 2-deoxyglucose, which can be phosphorylated. Ellis, et al. demonstrated that mouse cardiac acyl coenzyme A synthetase 1 deficiency impairs fatty acid oxidation and com-
pensatory glucose catabolism, leading to S6 kinase activation and cardiac hypertrophy. Our data suggest that impaired fatty acid metabolism and increased glucose utilization may consequently increase the glycolytic flux in KO mice, possibly contributing to enhanced mTOR activity and cardiac hypertrophy. The direct mechanisms for the pro-hypertrophic and pro-fibrotic change are not known in the present study; however, the loss of lipoprotein lipase-derived fatty acids leads to impaired myocardial fatty acid metabolism, severe cardiac fibrosis in old-aged mice, and higher mortality under pressure overload compared to WT mice. Impaired fatty acid metabolism in the heart might play a critical role in cardiac remodeling and tolerance to pressure overload.

The limitations of the current study were as follows: (1) The differences in the analysis shown in Figure 5 were not statistically significant, and these might be significant if the number of samples increased. However, the number of mice could not be increased due to the limited resource for the study. (2) The KO mice used for this study were conventional knockout mice, and the CD36 protein may have effects on tissues other than heart tissues. (3) The amount of ATP was not directly measured by the real-time quantification in live animals; ATP may be decreased after isolation of the heart. P-MRS that can measure ATP levels in the heart in vivo and in live animals might be adequate. (4) Eccentric hypertrophic changes were observed in KO mouse hearts under a pressure overload condition. Increased cardiomyocyte length may lead to extreme chamber enlargement; however, cardiomyocytes were not isolated to analyze contraction and myocyte length. (5) In the current study, we used CD36-deficient mice. Whether patients with CD36 deficiency have a low tolerance for afterload is uncertain.

Further investigations are warranted to clarify the mechanisms of decreased systolic function and histological changes under stress conditions in the heart in the context of impaired LCFA utilization and to analyze a tolerance to pressure overload in CD36-deficient patients.

Conclusion

We investigated whether alterations occur in CD36-KO mouse hearts under a stress condition compared to WT mouse hearts. We found that CD36 deficiency was associated with a low tolerance to pressure overload, eccentric hypertrophy, fibrotic changes, and decreased ATP levels in the heart, resulting in heart failure. These data suggest that the LCFA transporter CD36 is required for the maintenance of energy production, systolic function, and myocardial structure.

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Disclosures

Conflicts of interest: None.

References