Glucose is preferentially utilized for biomass synthesis in pressure-overloaded hearts: evidence from fatty acid-binding protein-4 and -5 knockout mice

Yogi Umbarawan1,2†, Mas Rizky A.A. Syamsunarno1,3†, Norimichi Koitabashi1, Aiko Yamaguchi4, Hirofumi Hanaoka4, Takako Hishiki5,6, Yoshiko Nagahata-Naito6, Hideru Obinata7, Motoaki Sano8, Hiroaki Sunaga1, Hiroki Matsui9, Yoshito Tsushima10,11, Makoto Suematsu5, Masahiko Kurabayashi1, and Tatsuya Iso1,12*

1Department of Cardiovascular Medicine, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan; 2Faculty of Medicine; 3Department of Biochemistry and Molecular Biology, Universitas Padjadjaran, Jatinangor, West Java 45363, Indonesia; 4Department of Bioimaging Information Analysis, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan; 5Department of Biochemistry; 6Clinical and Translational Research Center, Keio University School of Medicine, Shinjuku-ku, Tokyo 160-8582, Japan; 7Gunma University Initiative for Advanced Research (GIAR), Maebashi, Gunma 371-8511, Japan; 8Department of Cardiology, Keio University School of Medicine, Shinjuku-ku, Tokyo 160-8582, Japan; 9Department of Laboratory Sciences, Gunma University Graduate School of Health Sciences, Maebashi, Gunma 371-8511, Japan; 10Division of Integrated Oncology Research, Research Program for Diagnostic and Molecular Imaging, Maebashi, Gunma 371-8511, Japan; and 11Education and Research Support Center, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan

Received 23 June 2017; revised 13 January 2018; editorial decision 2 March 2018; accepted 12 March 2018; online publish-ahead-of-print 15 March 2018

Time for primary review: 50 days

Aims

The metabolism of the failing heart is characterized by an increase in glucose uptake with reduced fatty acid (FA) oxidation. We previously found that the genetic deletion of FA-binding protein-4 and -5 [double knockout (DKO)] induces an increased myocardial reliance on glucose with decreased FA uptake in mice. However, whether this fuel switch confers functional benefit during the hypertrophic response remains open to debate. To address this question, we investigated the contractile function and metabolic profile of DKO hearts subjected to pressure overload.

Methods and results

Transverse aortic constriction (TAC) significantly reduced cardiac contraction in DKO mice (DKO-TAC), although an increase in cardiac mass and interstitial fibrosis was comparable with wild-type TAC (WT-TAC). DKO-TAC hearts exhibited enhanced glucose uptake by 8-fold compared with WT-TAC. Metabolic profiling and isotopomer analysis revealed that the pool size in the TCA cycle and the level of phosphocreatine were significantly reduced in DKO-TAC hearts, despite a marked increase in glycolytic flux. The ingestion of a diet enriched in medium-chain FAs restored cardiac contractile dysfunction in DKO-TAC hearts. The de novo synthesis of amino acids as well as FA from glycolytic flux was unlikely to be suppressed, despite a reduction in each precursor. The pentose phosphate pathway was also facilitated, which led to the increased production of a coenzyme for lipogenesis and a precursor for nucleotide synthesis. These findings suggest that reduced FA utilization is not sufficiently compensated by a robust increase in glucose uptake when the energy demand is elevated. Glucose utilization for sustained biomass synthesis further enhances diminishment of the pool size in the TCA cycle.

Conclusions

Our data suggest that glucose is preferentially utilized for biomass synthesis rather than ATP production during pressure-overload-induced cardiac hypertrophy and that the efficient supplementation of energy substrates may restore cardiac dysfunction caused by energy insufficiency.

Keywords

Energy metabolism • Heart failure • Cardiac hypertrophy • Glucose • Fatty acid

† The first two authors contributed equally to the study.
* Corresponding author. Tel: +81 27 220 8145; fax: +81 27 220 8158, E-mail: isot@gunma-u.ac.jp
The Author(s) 2018. Published by Oxford University Press on behalf of the European Society of Cardiology.
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
1. Introduction

The heart preferentially uses fatty acids (FAs) as energy-providing substrates; >70% of cardiac adenosine triphosphate (ATP) is derived from the oxidation of FAs, with the remaining sources being glucose, lactate, ketone bodies, and amino acids.1–3 Because the storage of ATP within the cardiac myocyte is minimal, impairments in the process of ATP production may rapidly induce contractile dysfunction. Patients with heart failure have derangements of energy metabolisms that are characterized by a profound switch from FA oxidation to increased reliance on glucose as substrate.1–4 These metabolic changes, referred to as ‘metabolic remoulding’, have a foetal-like metabolic pattern and improved myocardial oxygen efficiency. To date, however, the effects of the substrate shift from FA to glucose on cardiac function and the underlying mechanisms leading to energy depletion during transition to heart failure remain poorly understood.

FA-binding protein 4 (FABP4, also known as aP2) and FABP5 are abundantly expressed in adipocytes and macrophages, and studies in mice with the genetic deletion of both FABP4 and FABP5 [double knockout (DKO)] provided evidence that FABP4 and FABP5 function as critical mediators of the inflammatory process, both locally and systemically.5,6 Recent studies from our laboratory and others indicate that both FABP4 and FABP5 are also expressed in capillary endothelial cells in the heart and skeletal muscle.7–9 We further found that capillary endothelial FABP4/5 play a significant role in FA transport from circulation into these tissues (referred to as trans-endothelial FA transport), especially in the heart.7 In DKO hearts, FA uptake is reduced by 30%, while glucose uptake is 20-fold higher compared with wild-type (WT) hearts. Because cardiac myocytes per se do not express FABP4/5, the difference in the metabolic profile between DKO and WT hearts should be ascribed to the difference in FA uptake via endothelial cells, and the possibility that cardiac myocytes from DKO mice have intrinsically defective FA uptake is excluded.7 Thus, DKO hearts display the reprogramming of myocardial fuel utilization without apparent cardiac dysfunction, which prompted us to employ DKO mice as a useful model to study the effects of the substrate shift from FA to glucose in the heart.

The tricarboxylic acid (TCA) cycle is a metabolic hub that links a number of metabolic pathways.2,3,10 A central role of the TCA cycle is to oxidize acetyl-CoA derived from FAs, carbohydrates, ketone bodies, and amino acids to produce NADH and FADH2 for ATP synthesis in the respiratory chain. Under normal conditions, nearly all ATP is generated from the mitochondrial oxidation of FA and glucose through the TCA cycle and given unrestricted access to water and standard chow (CE-2, Clea Japan, Inc.). An 8- to 10-week-old mice were used. The Institutional Animal Care and Use Committee (Gunma University Graduate School of Medicine) approved all studies. Animal experiments were performed conform the NIH guidelines (Guide for the Care and Use of Laboratory Animals).

2. Methods

2.1 Mice

Mice doubly deficient for Fabp4 and Fabp5 (DKO mice) with the C57BL6j background were generated from the intercross between Fabp4(-/-) and Fabp5(-/-) mice as described previously in.12 Mice were housed in a temperature-controlled room with a 12-h light/12-h dark cycle and given unrestricted access to water and standard chow (CE-2, Clea Japan, Inc.). An 8- to 10-week-old mice were used. The Institutional Animal Care and Use Committee (Gunma University Graduate School of Medicine) approved all studies. Animal experiments were performed conform the NIH guidelines (Guide for the Care and Use of Laboratory Animals).

2.2 TAC and echocardiography

Pressure overload was produced by constricting the transverse aorta as previously described in.13 In vivo cardiac morphology and function were assessed by transthoracic echocardiography (EUB-7500, Hitachi Medical Systems, Tokyo) in conscious mice.

2.3 Histology

Fibrosis and capillary density were estimated as described previously in.7

2.4 RNA isolation and real-time PCR

Total RNA isolation and quantitative real-time PCR were performed as described previously in.7

2.5 Biodistribution of [15-(p-iodophenyl)-3-(R, S)-methyl pentadecanoic acid] and 2-fluorodeoxyglucose

The biodistributions of 15-(p-iodophenyl)-3-(R, S)-methyl pentadecanoic acid (125I-BMIPP) and 2-fluorodeoxyglucose (18F-FDG) were determined as described previously in.14,15

2.6 Western blot analysis

Western blot analyses were carried out as described elsewhere.7

2.7 Metabolome analysis by capillary electrophoresis-mass spectrometry

The heart samples were freeze-clamped using aluminium blocks precooled in liquid nitrogen and maintained at -80°C until use. Metabolome analyses were carried out as described in the Supplementary material online.7,16

2.8 Tracing study with 13C6-glucose

Twelve hours after fasting, 13C6-glucose (1 mg/g)16 was intraperitoneally injected into mice. Ten minutes later, the ventricles were isolated, and metabolome analyses were conducted as described in the Supplementary material online.16

2.9 Statistical analysis

The statistical analyses were performed using an unpaired two-tailed Student’s t-test with Welch correction for two groups. An unpaired Student’s t-test was performed for each pair of four groups and subsequent multiple comparisons were made with use of the
Bonferroni method. A P value < 0.05 was considered significant (\(^{*}P < 0.05, {*}{*}P < 0.01, \text{and} {*}{*}{*}P < 0.001\)).

3. Results

3.1 Severe cardiac dysfunction in DKO-TAC mice

To investigate whether myocardial reprogramming that limits FA uptake and enhances glucose uptake renders the heart susceptible to heart failure under pressure overload, DKO and WT mice are subjected to TAC, which are referred to as DKO-TAC and WT-TAC, respectively. Three DKO-TAC mice but no WT-TAC mice died within 8 weeks after TAC procedure, but this difference was not statistically significant (Figure 1A). However, cardiac function measured by echocardiography in DKO-TAC mice was more compromised compared with that in WT-TAC mice. At all measured time points after 1 week, DKO-TAC mice showed significantly decreased fractional shortening and significantly increased left ventricular diameter (Figure 1B and see Supplementary material online, Figure S1A). The thickness of the interventricular septum in the diastole was more increased in WT mice 8 weeks after TAC (Figure 1B).

Essentially, the same cardiac dysfunction was observed in female DKO-TAC mice (see Supplementary material online, Figure S1B, Tables S1 and S2). When compared with DKO-TAC mice, the ablation of either the FABP4 or FABP5 gene in mice did not deteriorate cardiac dysfunction by TAC (see Supplementary material online, Figure S1C and D, Tables S2 and S3), indicating that the loss of circulating FABP4 or FABP5 was not associated with cardiac contractile dysfunction. Furthermore, bilateral nephrectomy, in which the serum levels of FABP4 were elevated by 10-fold, did not influence cardiac function in WT mice, both at baseline and 1 week after TAC (see Supplementary material online, Figure S1E and Table S4). These results suggest that cardiac dysfunction by pressure overload is caused by the combined effects of the simultaneous disruption of FABP4/5, but circulating FABP4 or FABP5 may not play a role in such processes.

3.2 An increase in cardiac mass and fibrosis are comparable between WT-TAC and DKO-TAC hearts

We analysed mice at 1 week after TAC, and a significant difference in cardiac function between WT-TAC and DKO-TAC mice was observed. The HW and HW/BW ratios were comparable (Figure 1C and see Supplementary material online, Table S5). Although several reports suggested a possible role of FABP4 in angiogenesis,17–19 capillary density was not different between WT-TAC and DKO-TAC (Figure 1D). Fibrosis evaluated by Masson’s trichrome stain (Figure 1E and see Supplementary material online, Figure S2A) and the gene expression levels of fibrosis markers (see Supplementary material online, Figure S2B) were similarly observed in WT-TAC and DKO-TAC mice. These data suggest that cardiac dysfunction by pressure overload is deteriorated in DKO mice, despite similar levels of structural remodelling.

3.3 Extreme reliance on glucose in DKO-TAC hearts

We next examined the effects of TAC on the uptake of \(^{125}\text{I}-\text{BMIPP}, \text{an FA tracer, and}^{18}\text{F-FDG, a glucose tracer. In WT mice, the uptake of}^{18}\text{F-FDG was increased, and the uptake of}^{125}\text{I}-\text{BMIPP tended to be decreased by TAC, indicating the shift of substrate preference in pressure-overloaded heart (Figure 2A). More interestingly, we observed a significant further increase in}^{18}\text{F-FDG uptake, while}^{125}\text{I}-\text{BMIPP uptake remained decreased in DKO hearts (Figure 2A). These findings suggest that substrate use in DKO-TAC hearts shifts more towards glucose. We next measured the serum levels of the biochemical parameters (Figure 2B). The serum levels of glucose were lower in DKO mice compared with WT, although the insulin levels were comparable. Interestingly, elevated levels of \(\beta\)-hydroxybutyrate (BOH) in DKO mice at baseline were reduced by TAC to the same levels of WT-TAC mice, which suggest that BOH utilization is enhanced in hearts after TAC. These findings imply that the supply of circulating energy substrates is not disturbed in DKO-TAC hearts compared with WT-TAC hearts.

3.4 Reduction in gene expression for FA use in WT-TAC and DKO-TAC hearts

We next examined the levels of mRNAs for a variety of genes relevant to FA metabolism. The mRNA expression of peroxisome proliferator-activated receptor \(\alpha\) (Ppara) and its co-activator, PPARY-co-activator 1 (Pgc1), both of which are central regulators of proteins involved in FA uptake and oxidation,20 was significantly decreased in WT-TAC and DKO-TAC hearts to the same degree (Figure 3). Consistently, the expression of the battery of genes regulated by the PPAR\(\alpha\)/PGC-1 complex was decreased to similar levels in WT-TAC and DKO-TAC mice. These results suggest that the difference in FA uptake through endothelial FABP4/5, but not FA \(\beta\)-oxidation activity, may account for severe cardiac dysfunction in DKO-TAC hearts.

3.5 Regulation of glucose uptake and glycolytic flux in WT-TAC and DKO-TAC hearts

To address the questions of how enhanced glucose uptake in DKO-TAC hearts is regulated, we studied the expression of the myocardial glucose transporters Glut1 and Glut4 (Figure 4A). An increase in Glut4 expression in DKO hearts was accompanied by its protein expression (Figure 4B), which could account for the marked increase in glucose uptake at least partly. The increase in GLUT4 expression in DKO hearts was not enhanced by TAC, suggesting that certain mechanisms other than the increased expression of GLUT4 are involved to enhance glucose uptake by TAC.

To assess whether an increase in glucose uptake in DKO hearts leads to an increase in glycolysis, we next assessed metabolic profiling for the glycolysis pathway by metabolome analysis (see Supplementary material online, Figure S7). The total metabolites involved in glycolysis (G1P to pyruvate) were comparable, despite a marked increase in glucose uptake in DKO hearts (Figure 4C). They were marginally suppressed by TAC, but there was no significant difference before and after TAC in both mice. The enzymatic activity of phosphor-fructokinase 1, a rate-limiting enzyme for glycolysis, was comparable between WT and DKO mice with or without TAC (see Supplementary material online, Figure S3A). The pyruvate levels were significantly lower in DKO hearts compared with WT hearts, both at baseline and under TAC. These findings suggest that glycolytic flux rate and the conversion of pyruvate to lactate or acetyl-CoA in the TCA cycle may be accelerated in DKO hearts. Consistent with this idea, tracer studies with \(^{13}\text{C}_{2}\)-glucose revealed that the levels of \(^{13}\text{C}_{2}\)-lactate (Figure 4D) and \(^{13}\text{C}_{2}\)-malate (Figure 5B) were significantly increased in DKO hearts irrespective of TAC. The increase in \(^{13}\text{C}_{2}\)-malate in DKO hearts was 9-fold higher than that in WT at baseline, whereas the increase in \(^{13}\text{C}_{2}\)-lactate was only 3.2-fold higher.
strongly suggesting that glycolytic products are preferentially utilized in the TCA cycle.

We next studied the phosphorylation levels of pyruvate dehydrogenase (PDH), which lacks the enzymatic activity to covert pyruvate into acetyl-CoA. Western blot analyses showed that the phosphorylated form of PDH was significantly reduced in DKO-TAC hearts (see Supplementary material online, Figure S3B). Consistent with this finding, the mRNA expression of PDH kinase 4 (Pdk4), a target gene of PPARα/Capillary/Myocyte

Figure 1  Cardiac contraction was reduced by TAC in DKO mice without difference of cardiac hypertrophy and fibrosis. (A) The survival curves of WT and DKO mice after TAC. The survival rate was not significantly different (n = 20) (P = 0.075). (B) Cardiac function was estimated by echocardiography before and 1, 4, and 8 weeks after TAC. Cardiac contraction was reduced in DKO mice with enlarged LV diameter after TAC. FS, fractional shortening; IVSd, thickness of interventricular septum in diastole; LVDd, diastolic diameter of left ventricle; LVDs, systolic diameter of left ventricle (n = 10–12). (C) Mice were sacrificed 1 week after TAC to isolate hearts after a 12 h fast. Heart weight was measured after removing atria. The heart weight/body weight ratio (HW/BW) was comparable between WT and DKO mice in the presence or absence of TAC (n = 6). (D) The capillary density estimated by isoelectin B4 and wheat germ agglutinin staining was comparable (n = 6). (E) Fibrosis area estimated by Masson’s trichrome stain was comparable (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001. For Figure 1B, the data were analysed with an unpaired two-tailed Student’s t-test with Welch correction. For Figure 1C–E, an unpaired Student’s t-test was performed for each pair of four groups and subsequent multiple comparisons were made with use of the Bonferroni method.
PGC1 complex and an inhibitor of PDH activity via phosphorylation, was significantly reduced in DKO mice at baseline and under TAC (see Supplementary material online, Figure S3C). These results suggest that glycolytic flux into the TCA cycle is not suppressed at the PDH level in DKO hearts.

### 3.6 Reduced metabolites in the TCA cycle in WT-TAC and DKO-TAC hearts

We next assessed the amounts of metabolic intermediates in the TCA cycle by metabolome analysis (Figure 5A and see Supplementary material online, Figure S7). Interestingly, the pool size in the TCA cycle (acetyl-CoA to malate) was significantly reduced in DKO mice compared with WT mice at baseline (Figure 5A) and was reduced further by TAC in both WT and DKO mice. A similar reduction was observed in the former and latter parts of the TCA cycle. Consistent with these, each intermediate in the TCA cycle, such as citrate, α-KG and malate, was lower in DKO mice at baseline and was reduced further by TAC. However, isotopomer analysis with $^{13}$C$_2$-glucose revealed that the levels of $^{13}$C$_2$-α-KG and $^{13}$C$_2$-malate in DKO mice were markedly elevated at baseline and were not significantly altered by TAC (Figure 5B). The elevated levels of $^{13}$C$_2$-citrate in DKO hearts at baseline were not significantly reduced in DKO-TAC hearts by unknown mechanism. The levels of $^{13}$C$_2$-citrate, $^{13}$C$_2$-α-KG, and $^{13}$C$_2$-malate were elevated in WT-TAC hearts compared with those at baseline. These findings demonstrated that an increase in glucose uptake in DKO hearts led to increased glycolytic flux into the TCA cycle, but a marked increase in glycolytic flux into the TCA cycle cannot compensate for the reduced supply of FAs in DKO hearts, resulting in a reduced pool size in the TCA cycle. Our data in WT mice also show that increased glycolytic flux into the TCA cycle by TAC is insufficient to maintain the pool size in the TCA cycle in WT-TAC hearts.

### 3.7 The creatine phosphate energy shuttle in WT-TAC and DKO-TAC hearts

Pathological cardiac hypertrophy is associated with the depletion of energy reserves manifested as maintained ATP levels but also a reduction in reserve energy, phosphocreatine (PCr). As compensated hypertrophy advances to overt heart failure, ATP levels are also reduced, which is well-known as the energy starvation hypothesis. Since the pool size in the TCA cycle was markedly diminished in DKO-TAC hearts, we assumed that the depletion of ATP and/or PCr in the creatine phosphate (CP) energy shuttle was enhanced in DKO-TAC hearts. As expected, the levels of PCr were significantly reduced in DKO-TAC hearts, although the ATP levels were not significantly different (Figure 5C), which suggests that ATP synthesis is reduced in DKO-TAC hearts and that measurable ATP levels are maintained by the buffering effect of PCr. These findings raise a plausible scenario that the reduced supply of total fuels causes a reduction in ATP synthesis, which in turn leads to cardiac contractile dysfunction in DKO-TAC hearts.

### 3.8 The restoration of cardiac function by the ingestion of medium-chain FAs in DKO-TAC hearts

A recent study using cardiomyocyte-specific CD36 KO mice demonstrated that feeding the mice a diet enriched in medium-chain FA (MCFA), which bypasses CD36 for entry into the cardiomyocytes, is able to protect these mice from developing heart failure via pressure overload by TAC. Because the reduction in FA uptake by the heart is more modest in DKO mice than in CD36 KO mice at baseline (see Supplementary material online, Figure S4), we assumed that MCFA might also be able to bypass FABP4/5-mediated trans-endothelial FA uptake. Therefore, we employed the same approach by feeding the mice a MCFA-rich diet to rescue cardiac contractile dysfunction in DKO-TAC hearts. As expected, feeding the mice a MCFA-rich diet significantly improved cardiac contraction in DKO-TAC hearts compared with standard chow (Figure 5D and see Supplementary material online, Table S6). Interestingly, improved cardiac contraction was reduced 12 h after fasting, even in mice fed an MCFA-rich diet. The reduction in cardiac contraction by fasting was improved again by refeeding an MCFA-rich diet but not by standard chow. Thus, metabolic intervention by feeding the mice an MCFA-rich diet successfully restored cardiac contractile dysfunction in DKO-TAC hearts, which strongly suggests that energy insufficiency by reduced uptake of long-chain FAs directly causes cardiac contractile dysfunction in our model.

As described earlier, our data show that the total supply of energy substrates and ATP synthesis seem to be reduced in DKO-TAC hearts,
despite a comparable increase in cardiac mass and fibrosis. It is well-known that glucose is utilized in multiple collateral pathways, not only for the catabolic process to synthesize ATP but also for the anabolic process to produce amino acids, nucleic acids and FAs. These findings suggest that a portion of glucose taken up by DKO-TAC hearts may be preferentially utilized in biomass synthesis pathways rather than mitochondrial ATP production. Accordingly, we next studied the glucose-derived de novo synthesis of cellular building blocks (i.e. the biosynthesis of amino acids, nucleotides and FAs).

3.9 The biosynthesis of amino acids and the malate-aspartate shuttle in WT-TAC and DKO-TAC hearts

Non-essential amino acids are generated from glucose via glycolysis and the TCA cycle (see Supplementary material online, Figure S7). Two amino acids, glutamate and aspartate, are directly synthesized from $\alpha$-KG and OAA in the TCA cycle, respectively. Interestingly, the steady-state levels of both glutamate and aspartate were significantly elevated in DKO-TAC hearts compared with those at baseline (Figure 6A), despite a reduction in the pool size in the TCA cycle, especially a marked reduction in $\alpha$-KG, a glutamate precursor (Figures 5A and 6B). The levels of other non-essential amino acids, such as proline and glycine, were also elevated in DKO-TAC hearts. Although the levels of alanine in DKO hearts at baseline were lower than those in WT hearts (Figure 6A), the levels of alanine relative to pyruvate was elevated in DKO-TAC hearts (Figure 6B). Considering greater utilization of amino acids as building blocks during development of cardiac hypertrophy, the increased levels of amino acids in DKO-TAC hearts compared with those at baseline suggest that de novo amino acid synthesis from glucose is accelerated in DKO-TAC hearts, despite the shortage of fuel supply against increased workload.

To evaluate glycolytic flux to amino acid synthesis directly, isotope analysis with $^{13}$C$_6$-glucose was carried out. Unexpectedly,
$^{13}$C$_2$-glutamate and $^{13}$C$_2$-aspartate in DKO hearts were 17.2- and 7.4-fold higher compared with those in WT hearts at baseline, respectively (Figure 6C). The remarkable enrichment of $^{13}$C$_2$-glutamate and $^{13}$C$_2$-aspartate could be accounted for by the well-known metabolic response of the malate-aspartate shuttle,$^{24,25}$ which is activated to supply cytosolic NAD$^+$ when glycolysis is accelerated (see Section 4 and see Supplementary material online, Figure S5 in detail). The moderate enrichment of other amino acids, such as glutamine, asparagine and alanine, was also observed in DKO hearts at baseline, suggesting that a large amount of glucose is utilized through de novo amino acid synthesis.

Pressure overload by TAC further enhanced the enrichment of aspartate and asparagine in DKO hearts. Enrichment of these amino acids was also observed in WT-TAC hearts compared with those at baseline. Thus, these findings suggest that in addition to ATP synthesis, a large amount of the components from glycolytic flux are utilized through amino acid synthesis in DKO hearts and under increased workload.

3.10 The biosynthesis of nucleic acid precursors and the production of nicotinamide adenine dinucleotide through PPP in WT-TAC and DKO-TAC hearts

The pentose phosphate pathway (PPP) is a major collateral pathway for glucose metabolism (see Supplementary material online, Figure S7). The PPP produces precursors for the de novo synthesis of nucleic acids, such as 5-phosphoribosyl pyrophosphate (PRPP), inosine monophosphate, and nicotinamide adenine dinucleotide (NADPH), a critical coenzyme for cellular oxidative stress and lipid synthesis.$^{1–3}$ We then investigated...
Figure 5 The pool size in the TCA cycle and PCr was significantly reduced in DKO-TAC hearts despite a marked increase in glycolytic flux. Mice were sacrificed 1 week after TAC to isolate hearts with a prior 12 h fast. (A) The metabolic profiling in the TCA cycle. Note that oxaloacetate (OAA) cannot be detected by this method. TCA cycle, tricarboxylic acid cycle; a-KG, α-ketoglutarate (n = 5–7). (B) A tracer study with 13C6-glucose. After a 12 h fast, the enhanced glucose use for biomass synthesis in the heart.
whether the levels of products in PPP were altered in DKO-TAC hearts. The levels of PRPP were significantly elevated in DKO-TAC hearts compared with those at baseline (Figure 7A). Isotopomer analysis with $^{13}$C$_6$-glucose revealed the enrichment of $^{13}$C$_5$-ribose 5-phosphate, a precursor for nucleic acid synthesis, in DKO hearts, which was further elevated by TAC (Figure 7B). In addition, the myocardial generation of NADPH was significantly increased in DKO-TAC hearts (Figure 7C). These findings suggest that glucose consumption through PPP was also accelerated in DKO-TAC hearts to supply nucleic acids precursors and NADPH, presumably for the hypertrophic response.

3.11 The biosynthesis of FAs in WT-TAC and DKO-TAC hearts
Malonyl-CoA, a precursor of FA synthesis, is produced from citrate in the TCA cycle (see Supplementary material online, Figure S7).$^{11}$ Cis-aconitate is the metabolite next to citrate, and isocitrate is the metabolite next to cis-aconitate in the TCA cycle.$^{11}$ The precursor for the three intermediates is citrate itself. Metabolome analysis showed that the malonyl-CoA levels were comparable, whereas the levels of cis-aconitate and isocitrate were significantly reduced in DKO-TAC hearts (Figure 7D). Interestingly, the level of malonyl-CoA relative to citrate was significantly increased (1.5-fold vs. WT-TAC), while the levels of cis-aconitate relative to citrate and iso-citrate relative to citrate were not altered in DKO-TAC hearts (Figure 7D), suggesting the facilitation of the lipogenic pathway rather than the TCA cycle. Further, western blot analysis showed that the levels of two lipogenic enzymes, ACLy, which is required for the synthesis of acetyl-CoA from citrate, and FASN, which is a multifunctional enzyme involved in the synthesis of long-chain FA from malonyl-CoA,$^{11,26,27}$ were significantly induced in both WT-TAC and DKO-TAC hearts (Figure 7E). In addition, the levels of NADPH, a key coenzyme for FASN, were increased as described above (Figure 7C). Given that phospholipids are also required for hypertrophic response as building blocks of lipid bilayers for plasma membranes and organelles, our data suggest that FAs can be produced from glucose, even in the heart, which combusts a large number of FAs.

4. Discussion
Despite the well-described shift to greater glucose utilization in the hypertrophied and failing hearts than normal hearts, a question of whether this alteration of nutrient utilization is favourable for cardiac function remains to be addressed. This study demonstrates that the compensatory increase in glucose use with reduced uptake of FA renders hearts more susceptible to cardiac dysfunction upon pressure overload. Our results suggest that the total fuel supply for ATP synthesis is reduced in DKO-TAC hearts as estimated by pool size in the TCA cycle and the amount of PCr. The restoration of cardiac function by the ingestion of an MCFA-rich diet strongly supports our hypothesis that energy insufficiency causes cardiac contractile dysfunction. Other important findings are that biomass synthesis (i.e. synthesis of amino acids, nucleic acids and FAs) from glycolytic flux for hypertrophic growth is comparable or even facilitated in DKO-TAC hearts, despite a reduction in ATP synthesis (see Supplementary material online, Figures S6 and S7). These findings imply that glucose is preferentially utilized for anabolic reactions rather than ATP synthesis during the development of cardiac hypertrophy, even under conditions where the total fuel supply is limited. We also suggest that FAs are the central energy substrate for ATP synthesis to maintain cardiac contractile function, even under increased workload, and accordingly, limited FA use can cause cardiac dysfunction under such conditions.

4.1 FAs as central fuel for the heart
The salient finding in this study is that cardiac dysfunction was associated with a reduction in pool size in the TCA cycle and levels of PCr in DKO-TAC hearts in vivo. Without stress, the total fuel supply with compensatory glucose uptake seems to be sufficient to maintain cardiac function in DKO hearts. When the energy demand against increased workload is enhanced; however, DKO-TAC hearts become energetically compromised because enhanced glucose uptake does not meet the increased energy demand. Glucose utilization for biomass synthesis (i.e. amino acids, nucleic acids, and FAs) could also cause the further diminishment of pool size in the TCA cycle. Consistent with our findings, Sung et al. recently reported that reduced FA uptake in CD36-deficient hearts is detrimental for the compensated hypertrophic heart, which is improved by an MCFA-rich diet.$^{23}$ Our study also exhibited the successful restoration of cardiac dysfunction by the ingestion of an MCFA-rich diet. Moreover, cardiac energetics and function can be preserved, even under pressure overload, by sustaining FA oxidation in mice lacking acetyl-CoA carboxylase 2 (ACC2).$^{28}$ These findings support the notion that FAs are useful fuel, even for overloaded hearts and are not toxic under such conditions. Recent studies have also demonstrated that in addition to FAs as central fuels, other fuels, including carbohydrates, ketone bodies and amino acids, are also crucial for cardiac function. All mice lacking the capability of the utilization of any fuels show depressed cardiac function under stressed conditions,$^{29}$ which implicates that the loss of any fuel selection can impair the stress response. All these findings suggest that the ATP synthesis capacity seems to be more important than the substrate selection for sustaining cardiac energetics and function.

4.2 Enhancement of glycolytic flux in DKO hearts
The precise mechanism underlying a compensatory increase in glucose uptake in DKO hearts has yet to be determined, but it may be that the multifactorial and glucose-FA cycle, the so-called Randle cycle, plays a significant role. In this concept, the heart prefers FAs as a primary fuel and intermediates of FA oxidation restrict glucose metabolism.$^{30}$ In DKO hearts, however, limited FA use causes a reduction in intermediates in the TCA cycle and ATP synthesis, which could accelerate glycolysis by allos-teric regulation. A robust increase in the GLUT4 levels can also contribute to the enhancement of glycolytic flux at baseline. Further studies are necessary to clarify the mechanisms underlying enhanced glycolysis.
Figure 6  The biosynthesis of non-essential amino acids seems to be enhanced in DKO-TAC hearts. Mice were sacrificed 1 week after TAC to isolate hearts with a prior 12 h fast. (A) The levels of non-essential amino acids in hearts. Glutamate (Glu), aspartate (Asp), glutamine (Gln), asparagine (Asn), and proline (Pro) can be generated from metabolites in the TCA cycle. Serine (Ser), glycine (Gly), and alanine (Ala) can be produced from glycolytic intermediates (n = 5–7). (B) Glu/α-KG, glutamate/α-ketoglutarate ratio; Ala/pyruvate, alanine/pyruvate ratio (n = 5–7). (C) A tracer study with 13C6-glucose. After a 12 h fast, hearts were isolated 10 min after an intraperitoneal injection of 13C6-glucose (n = 5–7). Data are normalized to WT control. *P < 0.05, **P < 0.01, and ***P < 0.001. An unpaired Student’s t-test was performed for each pair of four groups and subsequent multiple comparisons were made with use of the Bonferroni method.
4.3 Significance of malate-aspartate shuttle in DKO hearts

The most unexpected findings in this study were a robust increase in the flux of $^{13}$C$_2$-glutamate and $^{13}$C$_2$-aspartate in DKO hearts compared with WT. To our knowledge, a similar massive flux of glutamate and aspartate from glucose has never been reported in hearts in any animal models. We assume that these glutamate and aspartate pools should be exploited by the malate-aspartate shuttle (see Supplementary material online, Figure S5). Higher rates of glycolysis in DKO hearts are confirmed by higher levels of glycolytic products, such as $^{13}$C$_3$-lactate (Figure 7).
4D), 13C2-citrate (Figure 5B), and 13C3-alanine (Figure 6C). The high-rate glycolysis produces a large amount of NADH via the enzymatic reaction of GAPDH. Because the lactate dehydrogenase reaction, which also requires NAD+/NADH, is bidirectional under aerobic conditions, the malate-aspartate shuttle has a predominant role in the provision of cytosolic NAD+/NADH to maintain glycolytic flux (see Supplementary material online, Figure S5). The enrichment of 13C2-glutamate (17.2-fold) and 13C2-aspartate (7.4-fold) was higher than that of their interconvertible molecules, 13C2-glutamine (3.3-fold) and 13C2-asparagine (2.9-fold), respectively, which also suggests the functional significance of the enrichment of glutamate and aspartate from glucose. It is reported that the malate-aspartate shuttle activity is enhanced in embryonic heart and pressure-overloaded heart with increasing glycolysis.31,32 Our data strongly suggest for the first time that the malate-aspartate shuttle is facilitated in animal models where compensatory glucose use with reduced FA uptake is accelerated in the heart. Thus, it is very likely that the enhanced glycolytic flux and malate-aspartate shuttle are tightly coupled for balancing the cytosolic NAD+/NADH redox pair in general.

4.4 Significance of FA synthesis in the heart

All tissues obtain lipids from free FAs associated with albumin, lipoproteins, and de novo synthesis.33 De novo FA synthesis is accelerated mainly in adipose tissue and the liver when an excess amount of fuel is provided. Although de novo synthesis is thought to play a minor role in heart lipid metabolism, an increase in the level of malonyl-CoA relative to citrate and the enhanced expression of two lipogenic enzymes in DKO-TAC hearts led us to assume that de novo lipogenesis might be enhanced under increased workload where the demand of membrane phospholipids synthesis is increased, especially when FA availability is limited. Indeed, a study of the deletion of FASN in the heart showed that de novo lipid synthesis is important to maintain cardiac function during aortic constriction and ageing.34 Furthermore, a study of the cardiac-specific deletion of ACC2, which produces malonyl-CoA, demonstrated an attenuation of cardiac hypertrophy by TAC, which also suggests the involvement of de novo lipogenesis in membrane phospholipid synthesis during hypertrophic response.28 Given that amino acid synthesis is also accelerated in DKO-TAC hearts, despite a reduction in fuel provision, we assume that certain mechanisms are involved in promoting the de novo synthesis of amino acids and FAs during the hypertrophic response, irrespective of energy status. Mechanical stretch-associated signalling could be a possible candidate to promote them in the heart. It is also important to address the question of whether maintained malonyl-CoA is directly linked to phospholipid synthesis in overloaded hearts. Further studies are warranted to address these intriguing possibilities.

4.5 Does FABP4 have a positive or negative inotropic effect?

Lamounier-Zepter et al.35 identify FABP4 as adipocyte-derived protein that has a negative inotropic effect on cardiomyocytes in vitro. However, the effect of FABP4 on cardiac function in vivo remained to be determined. We confirmed that there is no significant difference in cardiac function between WT and FABP4 single KO mice after TAC. In addition, the significant elevation of circulating FABP4 by the nephrectomy did not affect cardiac function. Because we found in the previous study that isolated cardiomyocytes are not energetically compromised,7 functional characteristics of isolated cardiomyocytes were not assessed. Taken together, these findings strongly suggest that circulating FABP4 has little, if any, effect on cardiac contractile function in vivo.

In conclusion, a shift in energy substrate from FA to glucose use results in maladaptive metabolic remodelling during pressure-overload-induced cardiac hypertrophy. The heart favours anabolic reactions rather than ATP production during hypertrophic response, which results in contractile dysfunction because of insufficient ATP synthesis. Future studies will be required to develop the novel therapeutic strategy to regulate metabolic pathways, so that cardiac myocytes can adapt to pathophysiological situations by increasing ATP synthesis capacity.

### Supplementary material

#### Supplementary material

Supplementary material is available at Cardiovascular Research online.

#### Acknowledgements

We thank Miki Matsui, Yukio Tosaka, Keiko Matsuura, and Takako Kobayashi for their excellent technical help.

#### Conflict of interest

None declared.

#### Funding

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (to M.K. and T.J.), a grant from the Japan Cardiovascular Foundation, a grant from Gunma University Initiative for Advanced Research (to M.K.), and a grant from Takeda Science Foundation (to T.J.). The CE/MS-based metabolome analysis was supported by Biobank Japan Project by AMED. M.S. was the leader of the ERATO Project until March 2015.

#### References

1. Taegtmeyer H, Young ME, Lopaschuk GD, Abel ED, Brunengraber H, Darley-Usmar V, Des Rosiers C, Gerszten R, Glatz JF, Griffin JL, Gropler RJ, Holzhuetter HG, Kizer JR, Lewandowski ED, Malloy CR, Neubauer S, Peterson LR, Portman MA, Recchia FA, Van Eyk JE, Wang Tj. Assessing cardiac metabolism: a scientific statement from the american heart association. Circ Res 2016;118:1659–1701.
2. Kolwicz SC. Jr., Purohot S, Tan R. Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes. Circ Res 2013;113:603–616.
3. Dosen T, Nguyen TD. Abel ED. Cardiac metabolism in heart failure: implications beyond atp production. Circ Res 2013;113:709–724.
4. Neubauer S. The failing heart—an engine out of fuel. N Engl J Med 2007;356:1140–1150.
5. Hotamisligil GS, Bernlohr DA. Metabolic functions of fabps–mechanisms and therapeutic implications. Nat Rev Endocrinol 2015;11:592–605.
6. Furuhashi M, Hotamisligil GS. Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. Nat Rev Drug Discov 2008;7:489–503.
7. Iso T, Maeda K, Hanaoka H, Suga T, Goto K, Syamsunoro MR, Hishiti T, Nagahata Y, Matsu H, Arai M, Yamaguchi A, Abumrad NA, Sano M, Suematsu M, Endo K, Hotamisligil GS, Kurabayashi M. Capillary endothelial fatty acid binding proteins: 4 and 5 play a critical role in fatty acid uptake in heart and skeletal muscle. Arterioscler Thromb Vasc Biol 2013;33:2549–2557.
8. Elmasri H, Karaaslan C, Teper Y, Gheifi E, Weng M, Ince TA, Kozakewich H, Bischoff J, Catapetto S. Fatty acid binding protein 4 is a target of vegf and a regulator of cell proliferation in endothelial cells. fsebio 2009;37:3865–3873.
9. Massouye I, Hagens G, Van Kuppevelt TH, Madsen P, Saurat JH, Veeranki JH, Pepper MS, Siegenthaler G. Endothelial cells of the human microvasculature express epidem- mal fatty acid-binding protein. Circ Res 1997;81:297–303.
10. Des Rosiers C, Labarthe F, Lloyd SG, Chatham JC. Cardiac anaplerosis in health and disease: food for thought. Cardiovasc Res 2011;90:210–219.
11. Salwy JG. Metabolism at a glance. 3rd ed. Blackwell Publishing; 2004.
12. Maeda K, Cao H, Kono K, Gorgun CZ, Furuhashi M, Uysal KT, Cao Q, Assumi G, Malone H, Krishnan B, Minokoshi Y, Kahn BB, Parker RA, Hotamisligil GS. Adipocytokine/macrophage fatty acid binding proteins control integrated metabolic responses in obesity and diabetes. Cell Metab 2005;1:107–119.
13. Kojitabashi N, Danner T, Zaiman AL, Pinto YM, Rowell J, Mankowski J, Zhang D, Nakamura T, Takimoto E, Kass DA. Pivotal role of cardiomyocyte tgf-beta signaling in the murine pathological response to sustained pressure overload. J Clin Invest 2011;121:2301–2312.
14. Coburn CT, Knapp FF, Jr., Febrero M, Beets AL, Silverstein RL, Abumrad NA. Defective uptake and utilization of long chain fatty acids in muscle and adipose tissues of cd36 knockout mice. J Biol Chem 2000;275:32523–32529.

15. Hajri T, Han XX, Bonen A, Abumrad NA. Defective fatty acid uptake modulates insulin responsiveness and metabolic responses to diet in cd36-null mice. J Clin Invest 2002;109:1381–1389.

16. Umbarawan Y, Syamsunarno M, Obinata H, Yamaguchi A, Sunaga H, Matsu H, Hishiki T, Matsuda T, Koizumi K, Nakao H, Hase A, Kinugasa F, Tsushima Y, Suyama M, Kurosawa M, Iso T. Robust suppression of cardiac energy catabolism with marked accumulation of energy substrates during lipopolysaccharide-induced cardiac dysfunction in mice. Metab Clin Exp 2017;77:47–57.

17. Elmasri H, Ghelfi E, Yu CW, Traphagen S, Cernadas M, Cao H, Shi GP, Plutzky J, Salim M, Horamisigil G, Cataltepe S. Endothelial cell-fatty acid binding protein 4 promotes angiogenesis: role of stem cell factor/c-kit pathway. Angiogenesis 2012;15:457–468.

18. Ghelfi E, Yu CW, Elmasri H, Terwelp M, Lee CG, Bhandari V, Comhair SA, Erzurum SC, Hotamisligil GS, Elias JA, Cataltepe S. Fatty acid binding protein 4 regulates Vegf-induced airway angiogenesis and inflammation in a transgenic mouse model: implications for asthma. Am J Pathol 2013;182:1425–1433.

19. Yu CW, Liang X, Lipsky S, Karaaslan C, Kozakewich H, Hotamisligil GS, Bischoff J, Cataltepe S. Dual role of fatty acid-binding protein 5 on endothelial cell fate: a potential link between lipid metabolism and angiogenic responses. Angiogenesis 2016;19:95–106.

20. Rowe GC, Jiang A, Arany Z. PGC-1 coactivators in cardiac development and disease. Circ Res 2010;107:825–838.

21. Mandard S, Muller M, Kersten S. Peroxisome proliferator-activated receptor alpha target genes. Cell Mol Life Sci 2004;61:393–416.

22. Opie LH. Heart Physiology. 4th ed. Lippincott Williams & Wilkins; 2004. pp. 306–354.

23. Sung MM, Byrne NJ, Kim TT, Levasseur J, Masson G, Boisvenue JJ, Febrero M, Dyck JR. Cardiomyocyte-specific ablation of cd36 accelerates the progression from compensated cardiac hypertrophy to heart failure. Am J Physiol Heart Circ Physiol 2017;312:H552–H560.

24. Carley AN, Taegtmeyer H, Lewandowski ED. Matrix revisited: mechanisms linking energy substrate metabolism to the function of the heart. Circ Res 2014;114:717–729.

25. Ussher JR, Jaswal JS, Lopaschuk GD. Pyridine nucleotide regulation of cardiac intermediary metabolism. Circ Res 2012;111:628–641.

26. Kroezer G, Pouyssegur J. Tumor cell metabolism: cancer’s achilles’ heel. Cancer Cell 2008;13:472–482.

27. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the warburg effect: the metabolic requirements of cell proliferation. Science 2009;324:1029–1033.

28. Kolwicz SC, Jr., Olson DP, Marney LC, Garcia-Menendez L, Synovec RE, Tian R. Cardiac-specific deletion of acetyl coa carboxylase 2 prevents metabolic remodeling during pressure-overload hypertrophy. Circ Res 2012;111:728–738.

29. Ritterhoff J, Tian R. Metabolism in cardiomyopathy: every substrate matters. Cardiovasc Res 2017;113:411–421.

30. Hue L, Taegtmeyer H. The randle cycle revisited: a new head for an old hat. Am J Physiol Endocrinol Metab 2009;297:E578–E591.

31. Lewandowski ED, O’Donnell JM, Scholz TD, Sorokina N, Buttrick PM. Recruitment of nadh shuttling in pressure-overloaded and hypertrophic rat hearts. Am J Physiol Cell Physiol 2007;292:C1880–C1886.

32. Griffin J, O’Donnell JM, White LT, Hajjar RJ, Lewandowski ED. Postnatal expression and activity of the mitochondrial 2-oxoglutarate-malate carrier in intact hearts. Am J Physiol Cell Physiol 2000;279:C1704–C1709.

33. Goldberg IJ, Treul CM, Schulze PC. Lipid metabolism and toxicity in the heart. Cell Metab 2012;15:805–812.

34. Razani B, Zhang H, Schulze PC, Schilling JD, Vertske J, Lodhi IJ, Tokarczuk VK, Feng C, Coleman T, Kovacs A, Kelly DP, Safi J. E. Dorn GW, 2nd, Nichols CG, Semenovich CF. Fatty acid synthase regulates homoeostatic responses to myocardial stress. J Biol Chem 2011;286:30949–30961.

35. Lamounier-Zepter V, Look C, Alvarez J, Christ T, Ravens U, Schunk WH, Ehrhart-Bornstein M, Bornstein SR, Morano I. Adipocyte fatty acid-binding protein suppresses cardiomyocyte contraction: a new link between obesity and heart disease. Circ Res 2009;105:326–334.