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ORIGINAL ARTICLE

Pyruvate dehydrogenase activation precedes the down-regulation of fatty acid oxidation in monocrotaline-induced myocardial toxicity in mice

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Abstract

Fatty acid (FA) oxidation is impaired and glycolysis is promoted in the damaged heart. However, the factor(s) in the early stages of myocardial metabolic impairment remain(s) unclear. C57B6 mice were subcutaneously administered monocrotaline (MCT) in doses of 0.3 mg/g body weight twice a week for 3 or 6 weeks. Right and left ventricles at 3 and 6 weeks after administration were subjected to capillary electrophoresis–mass spectrometry metabolomic analysis. We also examined mRNA and protein levels of key metabolic molecules. Although no evidence of PH and right ventricular failure was found in the MCT-administered mice by echocardiographic and histological analyzes, the expression levels of stress markers such as TNFα and IL-6 were increased in right and left ventricles even at 3 weeks, suggesting that there was myocardial damage. Metabolites in the tricarboxylic acid (TCA) cycle were decreased and those in glycolysis were increased at 6 weeks. The expression levels of FA oxidation-related factors were decreased at 6 weeks. The phosphorylation level of pyruvate dehydrogenase (PDH) was significantly decreased at 3 weeks. FA oxidation and the TCA cycle were down-regulated, whereas glycolysis was partially up-regulated by MCT-induced myocardial damage. PDH activation preceded these alterations, suggesting that PDH activation is one of the earliest events to compensate for a subtle metabolic impairment from myocardial damage.

Keywords Cardiac metabolism · Metabolomics · Pyruvate dehydrogenase · Glycolysis · Monocrotaline

Introduction

A metabolic change is one of the earliest signs in the heart that responds to an internal and/or external stressor, because the heart requires high energy to maintain its pumping function. Therefore, it is important to detect the early changes in metabolism in the heart undergoing some form of stress or damage by over-pressure or cardiotoxic drugs. The healthy heart relies mainly on fatty acid (FA) oxidation to produce cellular energy, accounting for approximately 60–90% of total ATP production. Oxidation of glucose and lactate accounts for the remaining 10–40% of total ATP production [1–3]. To date, a number of studies have demonstrated that cardiac damage impairs energy pathways in mitochondria in the heart, including FA oxidation, the tricarboxylic acid (TCA) cycle, and respiratory chain [4]. Conversely, glycolysis is promoted to compensate for the impairment of energy production [2, 5, 6]. Although studies of metabolic alteration in the cardiac damage have primarily focused on advanced

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end-stage, the key factors of the metabolic alterations in the early phases remain largely unclear.

Recent innovations in mass spectrometry-based methods for comprehensive metabolic profiling have enabled scientists to analyze the alterations in metabolic fuel selection in a variety of cellular and animal models. We have demonstrated chamber-dependent metabolite patterns in the mouse heart by capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) [7]. In the present study, we used this technology to identify the early changes in metabolites of the mouse heart that was exposed to monocrotaline (MCT), a pyrrolizidine alkaloid extracted from the seeds and leaves of *Crotalaria spectabilis*, has been widely used to induce right ventricular failure (RVF) due to pulmonary hypertension (PH) in rats [8–11]. Previous studies have demonstrated that oxygen consumption is decreased and glycolysis is enhanced in the rat right ventricle (RV) under MCT-induced PH [8]. However, metabolic changes in the left ventricle (LV) that is not insulted by MCT-induced PH have not been investigated. In addition, the effect of MCT on metabolism of the mouse heart has not been examined. Mice have been known to be insusceptible to MCT-induced PH, because it is difficult to metabolize MCT to its active metabolite (dehydromonocrotaline) in the mouse liver [12–14]. Therefore, frequent administration of MCT is required for mice to induce PH [11, 14]. Despite this disadvantage, using mice as an animal model has a great advantage, because a considerable body of knowledge on mice is available by genetic manipulations such as genetic knockouts of a specific metabolic enzyme for mice. Therefore, we examined the effect of MCT on myocardial metabolism of both ventricles at an early stage in mice using CE-TOFMS in the present study.

Materials and methods

Animals

All animal care and study protocols were approved by the Animal Ethics Committees of Waseda University, and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No. 85–23, revised 1996). We purchased male C57BL6 mice at the age of 6 weeks from Sankyo Lab Company (Tokyo, Japan). Animals were studied to ensure uniformity among experimental subjects and to minimize variations due to age, gender, and other factors such as dietary habits that might affect cardiac function. The mice were fed normal chow and water ad libitum. Animals were individually housed in Plexiglas cages and were kept in a room with a controlled temperature (22 ± 3 °C) and lighting (lights on from 8:00 AM to 8:00 PM). The bedding of the cages consisted of wood shavings.

Monocrotaline administration

Monocrotaline (Sigma, St Louis, MO, USA) powder was dissolved in 0.1 N HCl, and the pH was adjusted to 7.4 with 0.1 N NaOH and filtered through a 0.22 mm disk filter. Mice at the age of 6 weeks were divided randomly into two groups, control and MCT groups. The MCT group was then divided into two subgroups: one subgroup was subcutaneously administered MCT in doses of 0.3 mg/g body weight twice a week for 3 weeks and another subgroup was administered MCT for 6 weeks. Instead of MCT, physiological saline was administered to the control mice.

Heart isolation

At the 4th or 7th weeks from the first administration of MCT, all mice were killed right afternoon because this time was regarded as a resting time, during which less chow and water were consumed. After cervical dislocation under anesthesia by isoflurane, heart tissues were immediately excised and flushed with PBS to remove blood. The ventricles were separated into the right ventricle (RV) and the left ventricle (LV). They were weighed in the semi-dry condition, immediately frozen with liquid nitrogen, and stored at – 80 °C for extraction of RNA, proteins, and metabolites. In addition, some of the heart tissues were cut along the short axis, and inferior parts of these were stored in 4% paraformaldehyde for histological analysis. The lung tissues were also excised and stored in 4% paraformaldehyde for histological analysis after manual infusion of 4% paraformaldehyde from the trachea into the alveolus. The weight was normalized by tibia length. It should be noted that the interventricular septum was carefully excluded from the ventricular samples to avoid any fusion effect expected from the adjacent lesion.

Metabolomic analysis

We analyzed metabolites of the ventricular samples as described previously [7, 15]. Briefly, frozen ventricular samples were immediately plunged into methanol containing internal standards (300 μM each of methionine sulfate for cations and MES for anions) and homogenized to inactivate enzymes. Then, deionized water was added, chloroform was added, and the solution was thoroughly mixed. The solution was centrifuged and the upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter to remove proteins. The filtrate was lyophilized and dissolved in 50 μl of Milli-Q water containing reference compounds (200 μM each of 3-aminopyrrolidine and trimmed) prior to CE-TOFMS. Metabolites were simultaneously measured

using 4–5 each ventricular sample from the control or MCT-administered mice. However, the measurements for 3-week or 6-week group were performed on different dates. All CE-TOFMS was performed using an Agilent CE capillary electrophoresis system (Agilent Technologies, Santa Clara, CA). Analyses of cationic metabolites, anionic metabolites, and nucleotides were performed as previously described [15, 16].

Quantitative real time-PCR analysis

Total RNA was extracted from the ventricles using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), as recommended by the manufacturer. cDNAs were synthesized with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA, USA) and real time-PCR was performed using the StepOne™ Real Time PCR System with Fast SYBR® Green Master Mix (Applied Biosystems Inc., Foster City, CA, USA) as recommended by the manufacturer. The sequences of PCR primers were designed based on the mouse nucleotide sequences in PrimerBank. We used the following primers: 18s ribosomal RNA (18s rRNA), carnitine palmitoyltransferase (CPT)-1b, CPT-2, long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), pyruvate dehydrogenase kinase-4 (PDK-4), pyruvate dehydrogenase phosphatase-1 (PDP-1), tumor necrosis factor-alpha (TNFα), interleukin-6 (IL-6), caspase-3, atrial natriuretic peptide (ANP), beta-myosin heavy chain (β-MHC), CD68, and CD163.

Western blot analysis

Total proteins extracted from the ventricles were used for Western blot analyzes using antibodies against pyruvate dehydrogenase (PDH) E1-α (Abcam, Cambridge, MA, USA), and phosphorylated PDH (Ser293) (Abcam), as described previously [7].

Echocardiography

PH and cardiac function were assessed by echocardiography, as described previously [17]. Mice were anesthetized with an intraperitoneal injection of Avertin (250 μg/g). Since we have observed that the heart rates of mice decrease after intraperitoneal injection of Avertin, reaching stable minimal levels around 15–20 min after injection, we obtained the echocardiographic data around 15–20 min after injection of Avertin.

Masson trichrome staining

The effects of MCT on pulmonary vessels and RV were assessed by Masson trichrome staining. The lungs and the heart were fixed in 4% paraformaldehyde and embedded in paraffin blocks, and sections of 5 μm were cut along the short-axis. The stained sections were examined using a Keyence microscope (Keyence Corporation, Osaka, Japan) and photographed.

Statistical analysis

Group data are indicated as mean ± SEM. Statistical analysis was performed using the unpaired two-tailed Student's *t* test. A *P* value of less than 0.05 was considered significant.

Supplementary materials: The following are available online at www.mdpi.com/link, Figure S1: Body weight of the MCT-administered mice.

Results

MCT-administered mice exhibited myocardial damage with no evidence of PH at the earlier stages

At 3-week and 6-week intermittent administration of MCT, we did not observe pulmonary arterial medial hypertrophy and stenosis of pulmonary vessels in the lung tissues in mice (Fig. 1a). Accordingly, the echocardiographic study revealed that the ratio of acceleration time per ejection time (AT/ET) in the RV, an indicator of PH, was not changed in MCT-administered mice (Fig. 1b). In addition, the weight of the RV or LV was not increased (Fig. 1c), and the RV and LV were not histologically hypertrophic in the MCT-administered mice (Fig. 1d). Thus, these results indicated that twice-a-week administration of MCT for 3 or 6 weeks was not enough to induce apparent PH, which is consistent with previous findings showing that mice are resistant to MCT-induced PH [13]. Interestingly, abundant CD68-positive cells were found in not only the RV but also the LV in MCT-administered mice, although no apparent fibrotic region was observed around these CD68-positive cells (Fig. 1e). Quantitative RT-PCR analyzes revealed that the expression levels of CD68 mRNA were significantly increased in the RV of MCT-administered mice at 3 weeks and in the LV of MCT-administered mice at 6 weeks (Fig. 2a). On the other hand, the expression levels of CD163 mRNA, which is a marker of M2 macrophages, were significantly decreased in the RV of MCT-administered mice at 3 weeks and in the LV of MCT-administered mice at 6 weeks (data not shown just for reviewers). These results indicated that M1 macrophages were increased and inflammatory response occurred in the heart of MCT-administered mice. However,

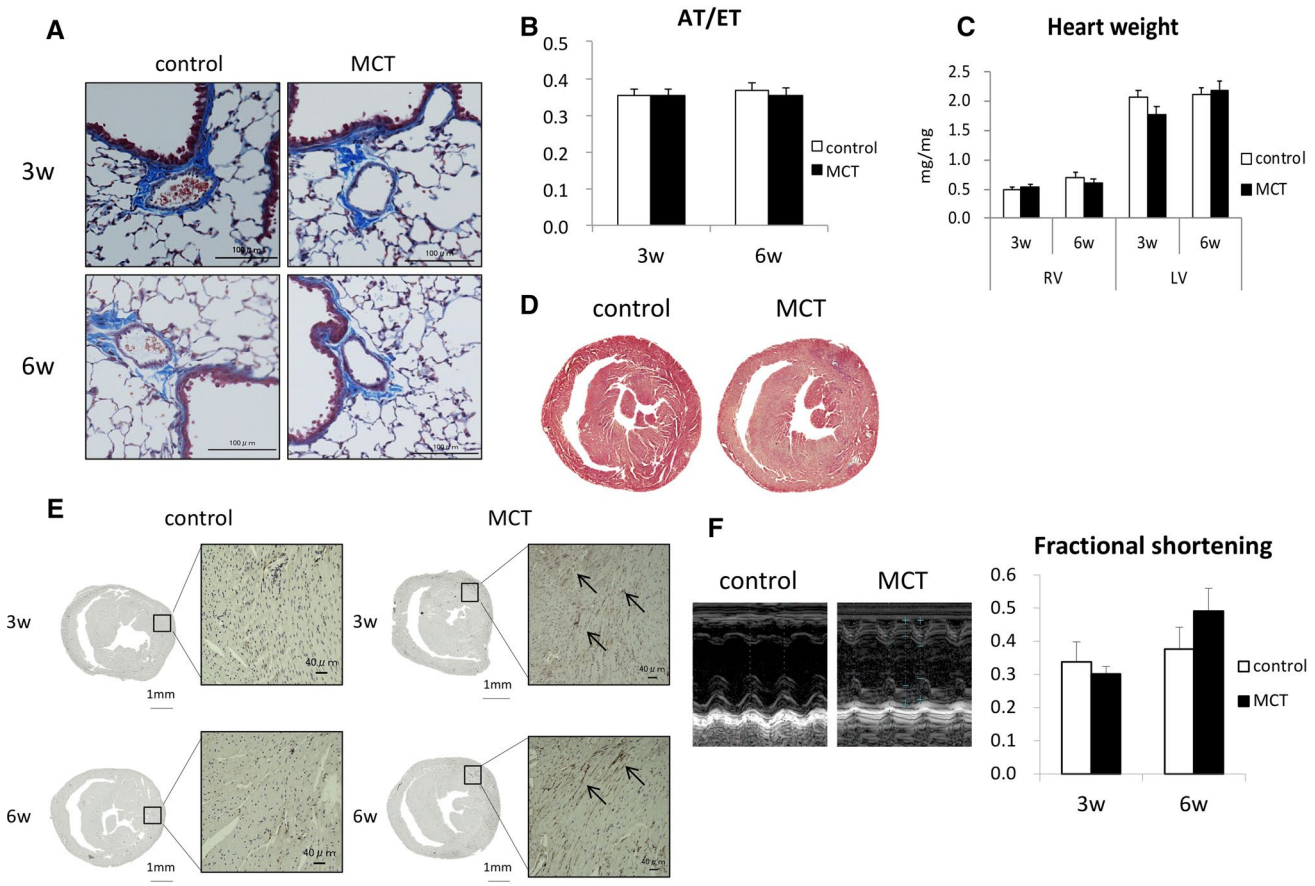

Fig. 1 Histological and echocardiographic analysis. **a** Masson trichrome staining did not show signs of vascular injury or PH in the lung tissue of MCT-administered mice. **b** AT/ET was not altered in either 3-week or 6-week MCT-administered mice. **c** RV hypertrophy did not develop in 6-week MCT-administered mice. **d** The picture represents the heart structure in a 6-week MCT administered mouse. **e** Panels show the representative cross sections stained with anti-CD68 antibody. Arrows indicate the CD68 staining areas. Strong CD68 staining was detected in 3-week and 6-week MCT-administered mice. **f** Left, representative M-mode images of control and MCT-administered mice at 6 weeks. Right, fractional shortening at 3 weeks and 6 weeks. Fractional shortening was not altered in either the 3-week or the 6-week MCT-administered mice. Values are expressed as mean ± SEM. $n = 4–5$ in each group. *3w* 3-week administration, *6w* 6-week administration

LV function assessed by fractional shortening using echocardiography was not altered in the MCT-administered mice at 3 and 6 weeks (Fig. 1f). All MCT-administered mice showed decreased body weight when they received MCT twice a week for more than 3 weeks (data not shown just for reviewers), as a loss of body weight is usually seen in MCT-administered rats [9].

MCT administration induced latent myocardial damage

Although there was no apparent PH in MCT-administered mice at 3 and 6 weeks, the abundant CD68-positive cells in the heart (Fig. 1e) and up-regulation of CD68 transcripts (Fig. 2a) suggest that the direct cardiotoxic effect of MCT on the myocardium in which a latent inflammatory response was induced. Therefore, we examined several inflammatory markers of the heart. We found that the expression levels of TNFα and IL-6 mRNAs were significantly increased in the LV of 3-week MCT administered mice (Fig. 2b, c). In the 6-week MCT-administration group, the expression levels of TNFα and IL-6 mRNAs were increased in both ventricles, although the increase was statistically significant only for the RV. In addition, the expression levels of caspase-3 mRNAs were significantly increased in both ventricles of 3-week and 6-week MCT-administered mice (Fig. 2d). As cardiac stress markers, the expression levels of atrial natriuretic factor (ANF) mRNA were increased in LV, but not RV of 3-week and 6-week MCT-administered mice (Fig. 2e), whereas β myosin heavy chain (β-MHC) mRNA was up-regulated in both RV and LV of 3-week and 6-week MCT-administered mice (Fig. 2f).

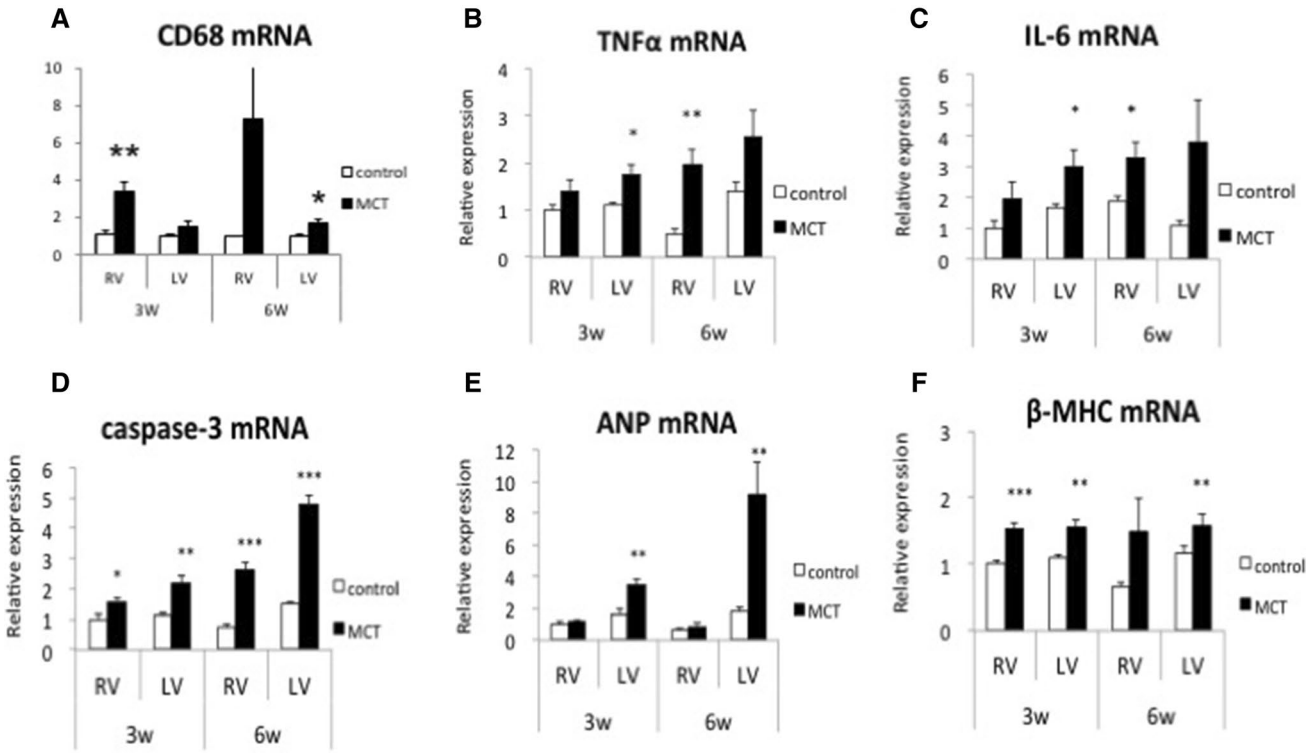

Fig. 2 The expression levels of inflammatory and stress markers. **a** The expression levels of CD68 were significantly increased in the RV of 3-week and the LV of 6-week MCT-administered mice. $n=3$–6 in each group except RV at 6-week control mice. Only one sample was available for the RV at 6-week control mice. **b**–**d** The expression levels of TNFα, IL-6, and caspase-3 were increased in the both ventricles of 3-week and 6-week MCT-administered mice. $n=4$–8 in each group. **d** The expression levels of ANP were increased in both LV of 3-week and 6-week MCT-administered mice. $n=4$–8 in each group. **e** The expression levels of β-MHC were increased in both ventricles of 3-week and 6-week MCT-administered mice. $n=4$–8 in each group. 18s rRNA was used as an internal control. Values are expressed as mean ± SEM. *RV* right ventricle, *LV* left ventricle, *3w* 3-week administration, *6w* 6-week administration. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to control group

The metabolites in the TCA cycle were decreased and those in glycolysis were increased in 6-week MCT-administered mouse ventricles

We then measured the metabolites of both ventricles in 3-week and 6-week mice. Because the samples from 3-week and 6-week mice were separately measured, the data were compared among the samples at the same age as the relative changes in metabolites from the control mice LVs (Fig. 3). MCT administration induced no significant alteration in metabolites in both ventricles at 3 weeks (Fig. 3). After 6 weeks of MCT administration, a significant decrease in the relative amounts of metabolites of the TCA cycle was observed in both ventricles (Fig. 3). Among the substrates in the TCA cycle, succinate and malate were significantly decreased in both ventricles, and citrate, isocitrate, and fumarate were decreased only in the RV of 6-week MCT-administered mice. On the other hand, the total content of metabolites in glycolysis was significantly increased in the LV of the 6-week MCT-administered mice. Fructose 1,6-bisphosphate was significantly increased in both ventricles of the 6-week MCT-administered mice. The content of pyruvate and lactate was not altered between the control and MCT-administered ventricles. The present CE-TOFMS analysis could not detect lipid metabolites and other TCA cycle-related metabolites that are not displayed in Fig. 3. Furthermore, because of a technical problem, acetyl-CoA was not measured in the ventricles of 6-week mice.

CE-TOFMS analysis also demonstrated that the total content of ATP, ADP, and AMP was not altered in both 3-week and 6-week MCT-administered mice (Fig. 4a). The ratio of CoA/acetyl CoA was not altered in the 3-week administered group and not measured in the 6-week administration group due to a technical problem (Fig. 4b). In addition, there was no difference in the ratios of NAD^+/NADH (Fig. 4c) and $NADP^+$/NADPH (Fig. 4d).

Among 20 amino acids that serve as the building blocks of proteins, we found that several amino acids were significantly increased or decreased compared to controls at 3 weeks and 6 weeks (Fig. 5). For example, alanine, glutamate, isoleucine, lysine, and valine were significantly decreased in both ventricles at 6-week MCT-administered

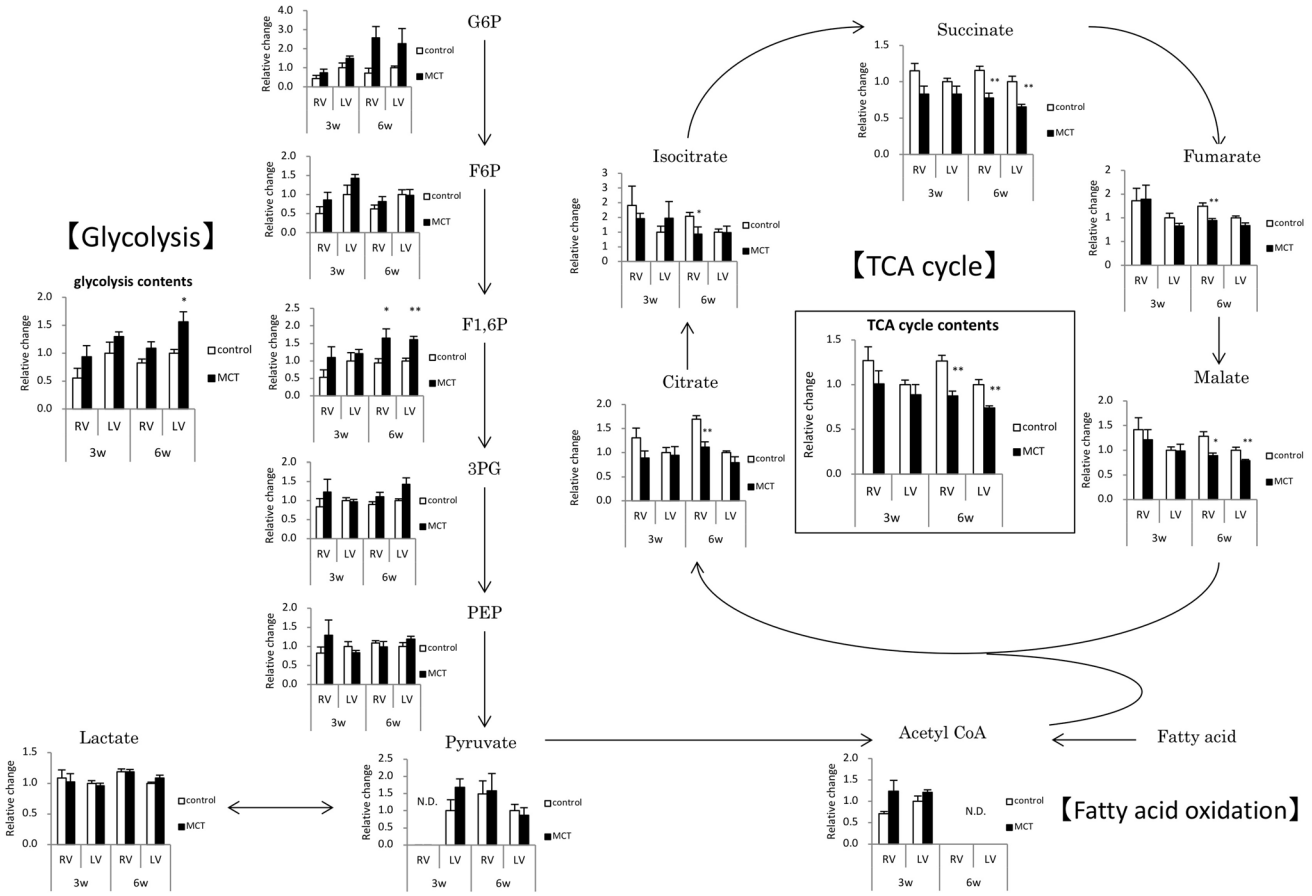

Fig. 3 The profile of glycolysis and TCA cycle. In 3-week administered mice, there was no significant metabolomic alteration in glycolysis and the TCA cycle. In both ventricles of the 6-week MCT-administered mice, the total content of metabolites in the TCA cycle were significantly lower. On the other hand, the total content of metabolites in glycolysis was significantly increased in the LV. The values were compared among the samples at the same age as the relative changes in metabolites from the control LV. Values are expressed as mean ± SEM. $n = 4$–5. *RV* right ventricle, *LV* left ventricle, *G6P* glucose 6-phosphate, *F6P* fructose 6-phosphate, *F1,6P* fructose 1,6-bisphosphate, *3PG* 3-phosphoglycerate, *PEP* phosphoenolpyruvate. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group

mice. On the other hand, glutamine, proline, and tyrosine were significantly increased in both ventricles at 6-week MCT-administered mice.

Phosphorylation level of PDH was decreased in 3-week and 6-week MCT-administered mice

PDH is an enzyme that converts pyruvate into acetyl-CoA and links the glycolysis and TCA cycle. Dephosphorylated PDH by PDH phosphatases (PDPs) is active, whereas phosphorylated PDH by PDH kinases (PDKs) is inactive. Although PDH has 3 phosphorylation sites (sites 1–3) in the E1 subunit, phosphorylation of only one site is enough for PDH inactivation. Because phosphorylation of site 1 is the most rapid, which indicates the importance of the site 1 for acute regulation, we examined the phosphorylation level of PDH at site 1. We found that the phosphorylation level at site 1 was significantly decreased in the RV of 3-week MCT-administered mice (Fig. 6a), suggesting that PDH became more active in the MCT-administered RV. In addition, PDH was significantly dephosphorylated in both ventricles of 6-week MCT-administered mice. In the heart, PDPs have 2 isoforms, PDP-1 and PDP-2, and PDKs have 4 isoforms, PDK-1–PDK-4. Because previous studies have demonstrated that PDP-1 and PDK-4 are the predominant isoforms in the heart [5, 7], we examined their expression by qRT-PCR analysis. The expression levels of PDP-1 mRNA were not altered between control and MCT-administered mice (Fig. 6b). On the other hand, the expression levels of PDK-4 mRNA were significantly decreased in the LV of MCT-administered mice, which is consistent with the phosphorylation level of PDH (Fig. 6c). These expression levels were also decreased in the RV of MCT-administered mice, although they did not reach a statistical significance.

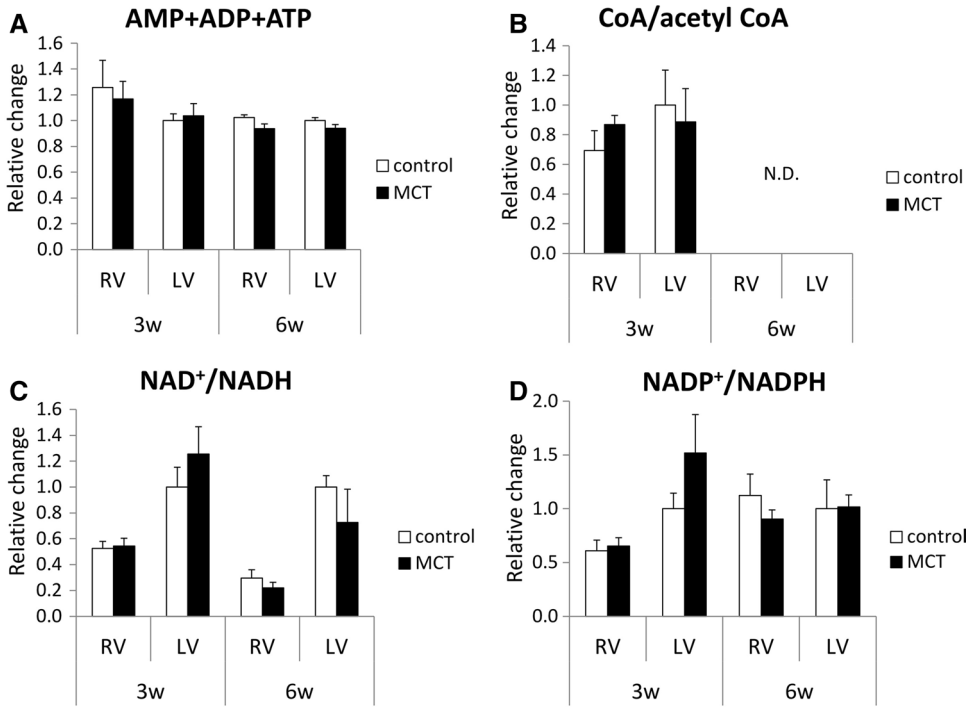

Fig. 4 The high-energy phosphate pool and the ratios of NAD^+/NADH, $NADP^+$/NADPH, and CoA/acetyl CoA. **a** In 3-week and 6-week MCT-administered mouse groups, the total content of ATP, ADP, and AMP was not altered by CE-TOFMS analysis. **b** In 3-week administered mice, the ratio of CoA/acetyl CoA was not altered. It could not be detected in the 6-week administered mice due to a technical problem. **c**, **d** In 3-week and 6-week administered mice, there was no alteration in NAD^+/NADH and $NADP^+$/NADPH. The values were compared among the samples at the same age as the relative changes in metabolites from the control LV. Values are expressed as mean ± SEM. $n = 3–5$. *RV* right ventricle, *LV* left ventricle, *3w* 3-week administration, *6w* 6-week administration

Down-regulation of FA oxidation-related genes in 6-week MCT-administered mice

The present CE-TOFMS analysis could not detect the metabolites of FA oxidation. Therefore, to examine the FA oxidation, the expression levels of FA oxidation-related genes were analyzed. CPTs transport FA from the cytosol into the mitochondria, and are rate-limiting enzymes for FA oxidation. CPT-1 is located at the mitochondrial outer membrane, whereas CPT-2 is located at the inner membrane. Although there are two CPT-1 isoforms (CPT-1a and -1b), CPT-1b is known to be a predominant isoform in the heart [7]. LCAD and MCAD mediate the first step of β-oxidation. Whereas the expression level of MCAD mRNA was not altered, the expression levels of a CPT-1b, CPT-2, and LCAD mRNAs were significantly decreased in both ventricles of 6-week, but not 3-week, MCT-administered mice (Fig. 7a–d). Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α plays a role in the regulation of both carbohydrate and lipid metabolism. The expression levels of PGC-1α mRNA were significantly decreased in the RV of 6-week MCT-administered mice (Fig. 7e).

Discussion

In the present study, there was no evidence showing that PH and RVF were developed in 3-week and 6-week MCT-administered mice by histological and echocardiographic findings. The reason why MCT showed little effect on the mouse pulmonary vessels in the present study is explained to be due to the inability of mice to metabolize MCT to its active metabolite (dehydromonocrotaline) in the mouse liver [12–14]. However, cardiac stress markers were up-regulated not only in the RV but also in the LV in MCT-administered mice. In addition, we found abundant CD68-positive cells and up-regulation of CD68, TNFα, and IL-6 mRNAs in both RV and LV in MCT-administered mice. Therefore, we thought that MCT directly exerted a cardiotoxic effect on the mouse heart. In this regard, MCT is known to be not only pneumotoxic but also toxic to other organs such as the liver and kidney [18–20]. MCT also has been reported to have a direct cardiotoxic effect in adult rats [13, 21]. Thus, it is possible that MCT injures cardiomyocytes in both ventricles through coronary blood flow and induces inflammation. Therefore, we thought that the metabolic alterations observed in the present study are

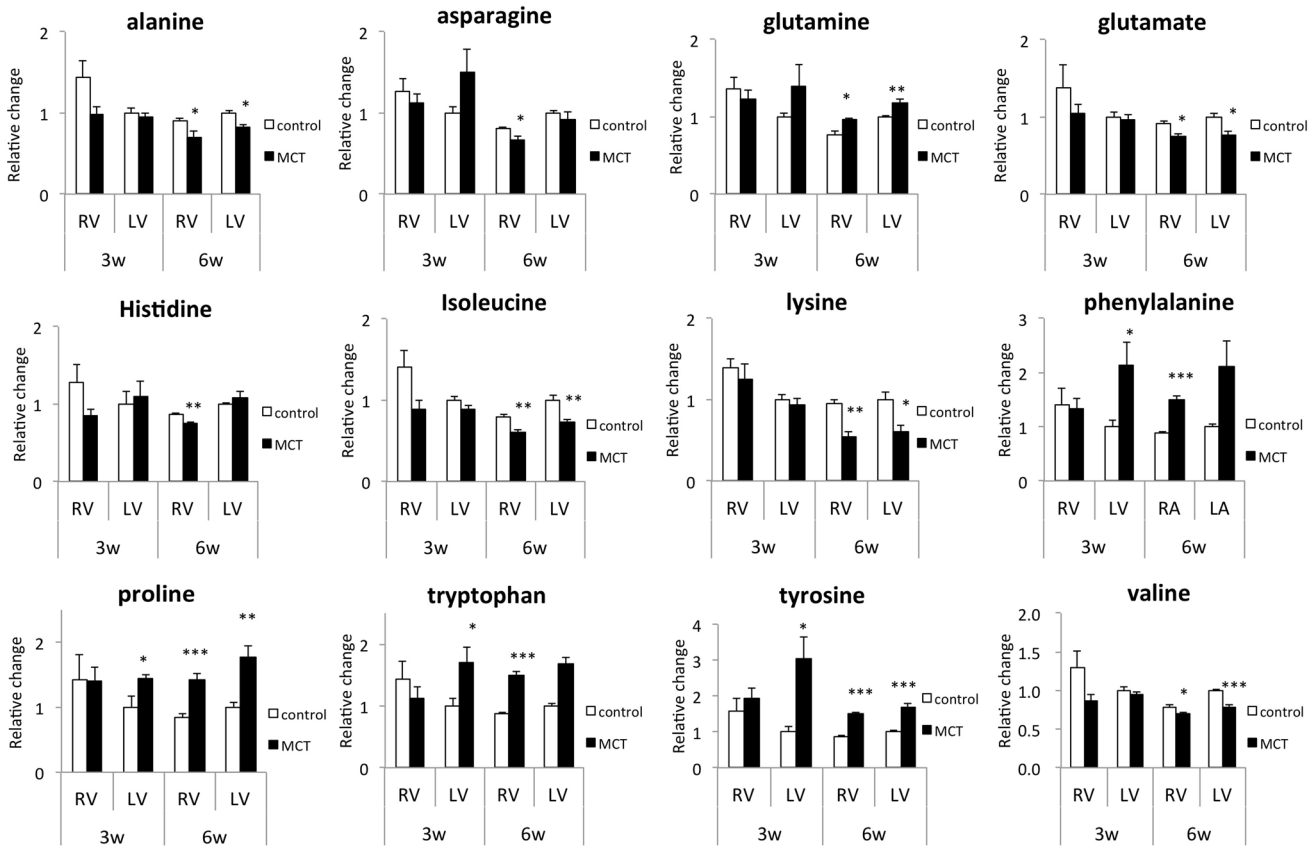

Fig. 5 The content of amino acids. Amino acids that were significantly increased or decreased in MCT-administered mice are shown. The values were compared among the samples at the same age as the relative changes in metabolites from the control LVs. Values are expressed as mean ± SEM. $n = 4$–5. *RV* right ventricle, *LV* left ventricle. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control group

mainly due to cardiotoxicity-induced myocardial damage by MCT.

The main finding of the present study is that, when myocardial damage is induced by MCT administration, PDH activation precedes the apparent changes in the metabolites in glycolysis and the TCA cycle at an earlier stage detected by CE-TOFMS analysis, which is an important difference between our study and previous studies. In previous studies, it was observed that PDH is phosphorylated and inactivated in advanced pathological conditions such as HF [8, 22–24]. In our study, however, the PDH phosphorylation level was decreased, and PDH was activated at an even earlier stage of myocardial damage when cardiac function and energy content were preserved. We think that this difference in PDH activity may be due to a difference in the timing of the analysis. We analyzed cardiac metabolism at an early phase, when only the gene expression levels of stress markers were altered without cardiac dysfunction. It is possible that PDH might be inactivated at a point later than 6 weeks, if the cardiac dysfunction might also become apparent. We also think that the PDH activation observed in the present study might be related to the compensatory mechanism of cardioprotection in response to MCT-induced cardiac toxicity.

It is known that PDH activity is regulated by the levels of its products and substrates such as pyruvate, acetyl-CoA, CoA, NAD^+, and NADH [5, 25, 26]. Higher pyruvate level and higher values of the ratio of both NAD^+/NADH and CoA/acetyl CoA inhibit PDK, which results in higher PDH activity. As shown in Fig. 3, pyruvate content tended to be higher in the LV of 3-week MCT-administered mice, although it did not reach a statistical significance. In addition, the ratios of NAD^+/NADH and CoA/acetyl CoA were not altered in both 3-week MCT-administered mice (Fig. 4b, c). These data suggest that PDH activation was not regulated by its products and substrates in the present study. Instead, considering the decreased PDK-4 expression levels at 3 weeks, we think that the PDK-4 down-regulation is also an important factor in promoting PDH activity in the damaged myocardium at an early stage. Although PDH activity was increased in the MCT-administered heart at 3 weeks, there was no change in pyruvate or lactate. We speculated that lactate could be supplied from blood to compensate the energy demand,

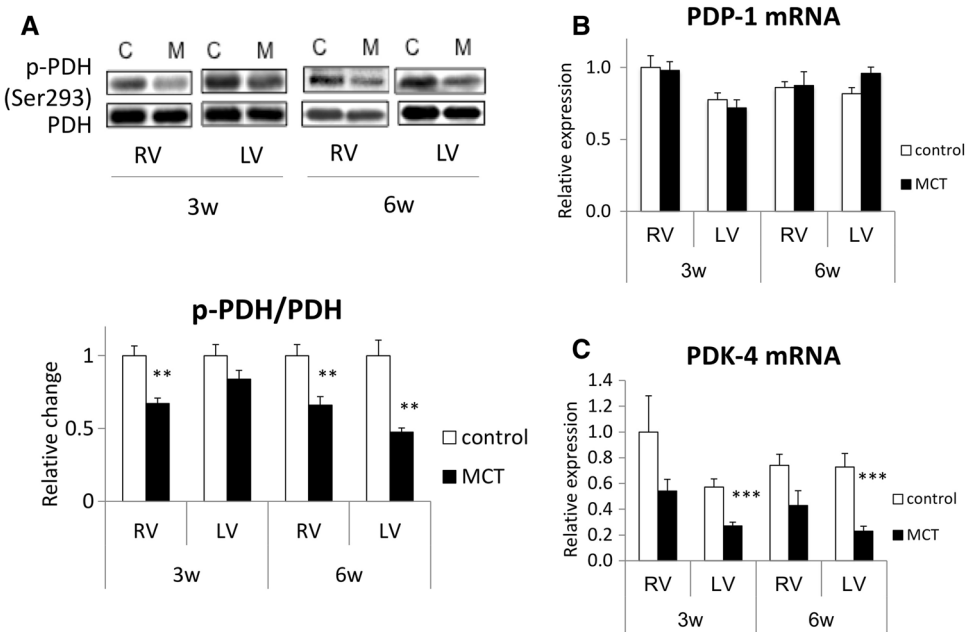

Fig. 6 The phosphorylation level of PDH and the gene expression levels of PDK-4 and PDP-1. **a** The phosphorylation level of PDH tended to be decreased in both ventricles of 3-week MCT-administered mice. It was decreased significantly in both ventricles of 6-week MCT-administered mice. Relative fold change in each control group was set as 1. **b** The gene expression level of PDK-4 tended to be decreased in both ventricles of 3-week and 6-week MCT-administered mice. **c** No alteration was observed in the gene expression level of PDP-1. 18s rRNA was used as an internal control. Values are expressed as mean ± SEM. **a***n* = 6 in each group. **b**, **c***n* = 5–8. *C* control group, *M* MCT group, *RV* right ventricle, *LV* left ventricle, *3w* 3-week administration, *6w* 6-week administration. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control group

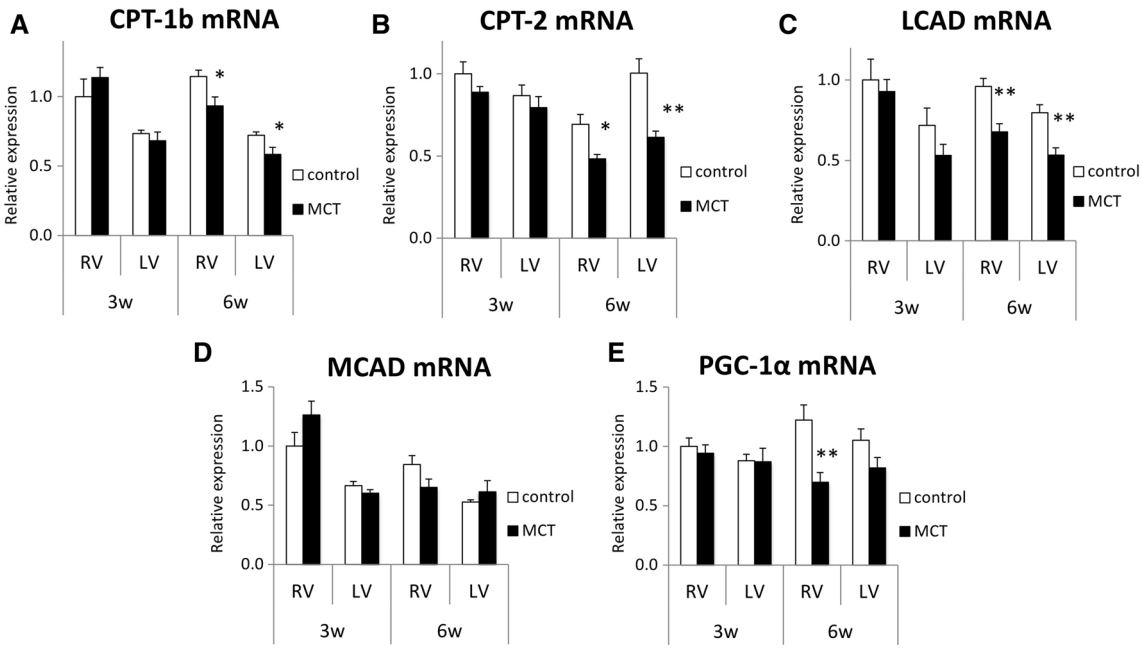

Fig. 7 The expression levels of FAO-related factors. The expression levels of CPT-1β, CPT-2, LCAD, and PGC-1α were decreased in both ventricles of 6-week MCT-administered mice. That of MCAD was not altered. 18s rRNA was used as an internal control. Values are expressed as mean ± SEM. *n* = 4–8 in each group. *RV* right ventricle, *LV* left ventricle, *3w* 3-week administration, *6w* 6-week administration. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group

which would mask the decrease in pyruvate and lactate by the activation of PDH in glycolysis.

When the myocardial damage was advanced, we found that the metabolites in the TCA cycle were significantly decreased, probably due to impaired FA oxidation and partially activated glycolysis, because the intermediate metabolites such as F-1,6-BP were increased, but 3PC and PEP were not. The down-regulation of FA oxidation-related genes might be induced as an adaptive and cardioprotective response. This idea is supported by previous studies, which reported that FA oxidation inhibition has a beneficial effect on delaying the progression of HF and improving cardiac function in HF [25, 27–29]. For example, CPT-1 has been recognized as a metabolic target for the treatment of HF. According to recent pharmacological studies, metabolic manipulation away from FA oxidation toward greater glucose oxidation can improve cardiac function and has therapeutic potential for the treatment of HF [5]. Moreover, the down-regulation of FA oxidation increases carbohydrate uptake and oxidation [30]. The down-regulation of FA oxidation-related genes might be one factor contributing to increased glucose oxidation at 6 weeks. Interestingly, there was a time lag between changes in the level of FA oxidation-related genes and PDH activation. The adaptive response via PDH activation may be more acutely induced than the down-regulation of FA oxidation. However, PDH-mediated glucose oxidation may not be effectively used because glycolysis was partially activated. Under continuous stress, cardiomyocytes may further promote carbohydrate oxidation by down-regulating FA oxidation and fully activation of glycolysis, and thus, they gain energy more efficiently.

We also found the changes in several amino acids in the MCT-administered heart. Interestingly, branched chain amino acids such as isoleucine and valine were decreased. We speculate that the decrease would be due to the increase in acetyl-CoA in the TCA cycle via catabolism of these amino acids for compensation of energy reduction. On the other hand, phenylalanine, proline, tryptophan, and tyrosine that are required oxygen for their catabolism were increased in the MCT-administered heart. This suggests that catabolism of these amino acids was avoided to reduce oxygen consumption. These possibilities should be investigated in a future study.

Although CE-TOFMS enabled us to comprehensively analyze the amount of metabolites, it should be noted that there was a limitation in the present study in measuring lipid metabolites with CE-TOFMS. As for FA oxidation activity, we examined the gene expression levels of FA oxidation-related factors by real-time PCR. Further study is required to clarify whether the activity of FA oxidation is impaired at an earlier stage of myocardial damage.

In conclusion, PDH activation precedes the metabolic alterations in FA oxidation, TCA cycle, and glycolysis in the MCT-induced damaged heart. These data suggest that PDH activation might be one of the earliest compensative metabolic responses of the heart to cardiotoxic stress.

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Author contributions SM and NG conceived and designed the experiments; GN, DS, and KU performed the experiments; GN, DS, and TS analyzed the data; IK and QJ contributed reagents/materials/analysis tools; GN, DS, and SM wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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