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Global metabolomic analysis of heart tissue in a hamster model for dilated cardiomyopathy

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ABSTRACT

Dilated cardiomyopathy (DCM), a common cause of heart failure, is characterized by cardiac dilation and reduced left ventricular ejection fraction, but the underlying mechanisms remain unclear. To investigate the mechanistic basis, we performed global metabolomic analysis of myocardial tissues from the left ventricles of J2N-k cardiomyopathic hamsters. This model exhibits symptoms similar to those of human DCM, owing to the deletion of the δ-sarcoglycan gene. Charged and lipid metabolites were measured by capillary electrophoresis mass spectrometry (MS) and liquid chromatography MS(/MS), respectively, and J2N-k hamsters were compared with J2N-n healthy controls at 4 (presymptomatic phase) and 16 weeks (symptomatic) of age. Disturbances in membrane phospholipid homeostasis were initiated during the presymptomatic phase. Significantly different levels of charged metabolites, occurring mainly in the symptomatic phase, were mapped to primary metabolic pathways. Reduced levels of metabolites in glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle, together with large decreases in major triacylglycerol levels, suggested that decreased energy production leads to cardiac contractile dysfunction in the symptomatic phase. A mild reduction in glutathione and a compensatory increase in ophthalmate levels suggest increased oxidative stress in diseased tissues, which was confirmed by histochemical staining. Increased levels of 4 eicosanoids, including prostaglandin (PG) E2 and 6-keto-PGF1α, in the symptomatic phase suggested activation of the protective response pathways. These results provide mechanistic insights into DCM pathogenesis and may help identify new targets for therapeutic intervention and diagnosis.

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1. Introduction

Dilated cardiomyopathy (DCM), a common cause of heart failure and a prevalent cardiomyopathy [1], is characterized by left ventricular dilation, impaired cardiac pump function, and a thin cardiac wall, which result in severe contractile dysfunction. β-Blockers constitute a common treatment [2], but severely affected patients may undergo heart transplantation or implantation of left ventricular assist devices. While the underlying etiological factors remain largely unknown, and both familial and non-familial factors are associated with DCM, some proposed disease mechanisms include coronary artery disease, genetic mutation, and viral infection [3]. Mutations in sarcomeric and cytoskeletal genes cause hypertrophic and dilated cardiomyopathies, respectively [4]. Some familial DCM cases are caused by mutations in genes encoding components of the dystrophin–glycoprotein complex (DGC), which spans the sarcolemma linking the extracellular matrix and cytoskeleton and provides mechanical strength for contraction [5]. Mutations in dystrophin, a major cytoskeletal component of the DGC, lead to a high incidence of X-linked DCM in patients with

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Duchenne or Becker muscular dystrophy. Mutations in other DGC genes, including *δ-sarcoglycan*, are also associated with human DCM [1].

Oxidative stress is also reported to be involved in DCM pathogenesis. Patients with DCM exhibit increased plasma glutathione levels and lipid peroxidation products such as malondialdehyde [6], and total plasma peroxide levels are inversely correlated with the cardiac ejection fraction [7]. However, a contrasting study found that human left ventricular DCM tissue showed normal glutathione peroxidase and superoxide dismutase activities and malondialdehyde levels similar to those found in healthy control tissue [8]. Thus, the role of oxidative stress in DCM pathogenesis remains to be elucidated.

Animal models with a pathophysiology similar to human DCM are useful for investigating pathogenic mechanisms. A J2N-k DCM hamster and J2N-n control line were established by repeated sib mating of J2N(N8), produced by cross-breeding BIO14.6 cardiomyopathic and normal golden hamsters [9]. J2N-k hamsters are deficient in δ-sarcoglycan and are an animal model of human limb-girdle muscular dystrophy–associated cardiomyopathy. They begin showing heart tissue fibrosis and exhibit moderate cardiac dysfunction at 8–9 weeks of age. At 20 weeks, J2N-k hamsters exhibit considerable fibrosis, a reduced number of cardiomyocytes, and hypertrophic changes in the remaining cardiomyocytes; no such changes occur in J2N-n heart tissues [9]. Accordingly, the life span of J2N-k hamsters (ca. 298 days) is much shorter than that of J2N-n hamsters (ca. 788 days). Besides the *δ-sarcoglycan* gene, J2N-k and J2N-n hamsters have very similar genetic backgrounds. Since mutations in *δ-sarcoglycan* are also detected in DCM patients, J2N-k hamsters are an ideal DCM disease model.

To gain an insight into the DCM in metabolic pathway basis, we performed global metabolomic analysis of myocardial tissues from the left ventricles of J2N-k and J2N-n hamsters. Capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS) [10] and liquid chromatography (LC)-TOFMS or triple quadrupole MS/MS were used to measure levels of charged (e.g., amino acids) and lipid (e.g., phospholipids) metabolites, respectively. We identified significant changes in several metabolite levels in age-matched J2N-k and J2N-n hamsters.

2. Methods

2.1. Animals

Male 3- and 15-week-old J2N-k cardiomyopathic hamsters and age-matched J2N-n controls were purchased from Nihon SLC Inc. (Hamamatsu, Japan). All animals were maintained in a specific pathogen-free facility under controlled conditions (20–24 °C and 40–70% humidity) with a 12-h light cycle and were given free access to standard laboratory rat chow (MF, Oriental Yeast, Tokyo, Japan) and tap water. After 1 week of habituation, 4- and 16-week-old animals were anesthetized by intraperitoneal injection of pentobarbital (Dainippon Sumitomo Pharma, Osaka, Japan) at a dose of 50 mg/kg, and the left ventricle was excised. The isolated tissue was processed for either histological analysis (N = 4) or for metabolomic and western blot analysis (N = 7). For metabolomic analysis, tissue was randomly divided into 2 samples and minced on ice to measure charged and lipid metabolites. The tissue samples were weighed and snap frozen in liquid nitrogen before being stored at −80 °C. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Guidelines for Animal Experimentation and under the control of the Ethics Committee of Animal Care and Experimentation of the National Cerebral and Cardiovascular Center, Japan (Approval number, 12056).

2.2. Echocardiography and histochemical staining

Cardiac function was assessed by echocardiography measurements as shown in the supplementary information. Following this procedure, J2N-k and J2N-n hamsters were sacrificed as described above, and ventricle tissue (from both hamster lines) was processed for Masson's trichrome staining to detect fibrosis, 4-hydroxynonenal (4-HNE) staining to estimate lipid peroxidation [11], and dihydroethidium (DHE) staining to approximate superoxide production [12] as described in the supplementary information.

2.3. Metabolite extraction and quantification

Detailed information regarding the extraction and quantification of charged and lipid metabolites has been provided in the supplementary information. Briefly, charged metabolites were extracted by homogenizing myocardial tissue in methanol and subjected to CE-TOFMS, as previously described [10,13,14]. Lipid metabolite extraction was performed using the Bligh and Dyer method [15] with minor modifications. Lower organic and upper aqueous layers were analyzed by LC-TOFMS and LC-MS/MS for phospholipids/sphingolipids/triacylglycerols and oxidative fatty acids, respectively. Structural analysis of phospholipids (PLs) and sphingomyelins (SMs) was performed as previously described [16].

2.4. Data analysis

Datasets obtained from CE-TOFMS were processed using our proprietary software, MasterHands [17] as shown in the supplementary information. Hydrophilic metabolite concentrations have been provided as the amount of metabolite (μmol) per gram of tissue.

LC-TOFMS data were processed using the 2DICAL software (Mitsui Knowledge Industry, Tokyo, Japan) [18] as described in the supplementary information. Extracted ion peaks were normalized using internal standards (ISs). Metabolites eluting from 0.1 to 37.5 min and from 37.5 to 60 min for LC were normalized to 1, 2-dipalmitoyl-[$^2H_6$]-sn-glycero-3-phosphocholine (16:0-16:0PC-d6; Larodan Fine Chemicals, Malmo, Sweden) and 1,2-caprylin-3-linolein, respectively. Some oxidative fatty acids were quantified using commercially available standards.

2.5. Statistical and multiple classification analyses

Student's *t*-test was used for two-class comparisons between J2N-n and J2N-k at each growth stage (4 and 16 weeks), and $p<0.05$ was deemed as statistically significant. The multiple testing correction was not applied since metabolite levels are not exclusive but rather related with each other, and we focused on revealing overall metabolic changes (such as pathways or metabolite groups) in the cardiomyocytes from J2N-k hamsters compared to J2N-n cardiomyocytes. In addition, data were imported into the SIMCA-P+ software (Version 12.0; Umetrics, Umeå, Sweden), pareto-scaled, and subjected to principal component analysis (PCA; short explanation is provided in the supplementary information). Cluster analysis and heatmap representations were obtained using the Spotfire software (Version 7.1; TIBCO, MA, USA).

3. Results

3.1. Cardiac function and pathophysiology of J2N-n and J2N-k hamsters

In this study, the 4- and 16-weeks of ages were selected as DCM presymptomatic and symptomatic phases for J2N-k (and its control J2N-n) hamsters according to the previous paper [9]. First, we examined cardiac function of both hamsters at these time points. Echocardiograph measurements of J2N-k hamsters at 16 weeks revealed a significant increase in the internal diameter of the left ventricle (LVID) during both diastolic and systolic states; however, this

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