Changes in distinct species of 1,2-diacylglycerol in cardiac hypertrophy due to energy metabolic disorder

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Abstract

Objective: The juvenile visceral steatosis (JVS) mouse, a genetic model of systemic carnitine deficiency resulting from carnitine transport mutation, develops cardiac hypertrophy. We determined two putative lipid messengers, 1,2-diacylglycerol (DAG) and ceramide, in JVS and carnitine palmitoyltransferase-I (CPT-I) inhibitor etomoxir-treated mice because these lipids function as co-messengers in the myocardium via modification of protein kinase C activity. Methods: JVS mice were evaluated at 4 and 8 weeks of age. The effect of long-term etomoxir treatment (45 mg/day) (ET) on mice was investigated in control mice from 4 to 8 weeks of age. As a model of inhibited cardiac hypertrophy, carnitine-treated JVS (CT) mice were produced. Myocardial DAG and ceramide levels and their fatty acid composition were measured. Results: The heart/body weight ratio increased by 100\% in JVS mice compared with that in controls, while that of CT mice was normalized in comparison with controls at 8 weeks of age. DAG markedly increased in both JVS and ET mice compared with that in controls (1677±684, 1258±49, and 585±58 ng/dry wt, respectively; $P<0.01$ for controls versus JVS or ET mice), whereas it was decreased significantly in CT mice compared with that in JVS mice (1066±54 ng/dry wt, $P<0.01$). Furthermore, the fatty acid composition of DAG was similar in JVS and ET mice; in particular, 18:1 and 18:2 were significantly elevated in the myocardium ($P<0.01$ versus controls). On the other hand, that of DAG in CT mice was similar to that of the control group. In contrast, no difference was observed in myocardial ceramide levels among the groups. Conclusions: Pharmacological intervention with etomoxir mimics changes in the lipid second messenger characteristic of genetic JVS mice. The results suggest that the increases in distinct DAG species might be involved in the pathogenesis of cardiac hypertrophy as a result of disorder of fatty acid transport.

Keywords: Hypertrophy; Lipid metabolism; Second messengers; Signal transduction

1. Introduction

Disorders of cardiac energy metabolism, which include defects in fatty acid oxidation, induce cardiac hypertrophy [1]. However, the mechanisms of cardiomyopathy in these disorders are not yet entirely understood. Recently, the juvenile visceral steatosis (JVS) mouse, a genetic model of systemic carnitine deficiency, was established [2]. The JVS mouse results from mutations of the renal carnitine transporter gene [3], and renal conservation of carnitine is impaired. Carnitine plays an important role in the transport of long-chain fatty acids into the mitochondrial matrix for $\beta$-oxidation [4]. Thus, disorder of mitochondrial $\beta$-oxidation is present in the JVS mouse and leads to the development of marked cardiac hypertrophy [5]. In addition, carnitine treatment has also been shown to inhibit cardiac hypertrophy in JVS mice.

Etomoxir, a hypoglycemic agent with no acute side-effects, is an oxirane carboxylic acid derivative that specifically inactivates carnitine palmitoyltransferase I (CPT-I), the key enzyme for the transport of long-chain acyl-CoA compounds into mitochondria. Etomoxir pro-
motes cardiac growth and increases myosin isoenzyme V₁ [6]. Cardiac growth induced by etomoxir in non-overloaded ventricles is associated with concentric hypertrophy [7]. The mechanisms underlying this etomoxir-induced hypertrophic growth with a unique myocardial phenotype are unknown, but they are assumed to be related to CPT-1 inhibition. Since both the JVS mouse and etomoxir-treated mice show a disorder of fatty acid β-oxidation and lipid accumulation in myocardial tissue, the evaluation of lipid second messengers seems to be of importance to elucidate the pathogenesis of cardiac hypertrophy in these models.

The generation of second messengers from precursor molecules is one of the earliest changes in cells stimulated by hormones and growth factors. 1,2-DAG is one such second messenger and is an endogenous activator of protein kinase C (PKC) [8]. PKC regulates many biological processes, such as differentiation and mitosis. Ceramide is a product of sphingomyelin breakdown by sphingomyelinase and also acts as a lipid second messenger to mediate the effects of extracellular agents on differentiation, growth inhibition, and apoptosis [9]. Given that sphingolipid metabolites, including ceramide, have been demonstrated to inhibit PKC activity, ceramide may counter 1,2-DAG-mediated action [10].

In the present study, we investigated the difference between two disorder models of β-oxidation, the heredity and pharmacological interventional models, and carnitine-treated JVS mice, the model of inhibited cardiac hypertrophy. It is important to investigate the changes in 1,2-DAG and ceramide mass in experimental models in which PKC may be involved. In this study, we measured myocardial lipid levels, including 1,2-DAG and ceramide, by means of Iatroscan thin-layer chromatography with flame ionization detection (TLC/FID) [11], and investigated the involvement of these lipid messengers in cardiac hypertrophy in these models.

2. Methods

2.1. Animal preparation

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). The JVS mice were of the C3H strain [3], and were kindly donated by Dr. Nikaido, Institute for Experimental Animals, Kanazawa University School of Medicine, Kanazawa, Japan. All animals were maintained under specific pathogen-free conditions.

Homozygous mutants (jvs/jvs) had swollen fatty livers that were recognizable through the abdominal wall 5 days after birth. We treated the homozygous mutants with 5 μmol of 1-carnitine from 11 to 20 days after birth in order to prolong their lives according to a previously reported method [12]. In addition, we produced carnitine-treated JVS mice with inhibited cardiac hypertrophy; 1% carnitine (10 mg/kg) was administered from 4 to 8 weeks of age. (+)-Etomoxir sodium salt (45 mg/kg/d) was administered to normal mice in their drinking water for 4 weeks, from 4 to 8 weeks of age. The dose was maintained by monitoring the animals’ daily water consumption and body weight. Wild-type animals of the C3H strain were used as the control.

2.2. Blood pressure and echocardiographic measurements

On the day of sacrifice, the systolic blood pressure and heart rate were determined for each animal. The tail-cuff method was used, employing a photoelectric tail-cuff detection system (Softron BP-98A; Softron, Tokyo, Japan). Left ventricular (LV) contractile function was evaluated by transthoracic echocardiography using a EUB 8000 CV (Hitachi, Tokyo, Japan) with a 10-MHz imaging transducer. In brief, each animal was slightly sedated with sodium pentobarbital (20 mg kg⁻¹ i.p.). M-mode images of the LV at the level of the chordae tendineae were recorded, and LV dimensions at end-diastole (EDD) and end-systole (ESD) were measured by means of the leading edge method. For each measurement, data from three cardiac cycles were averaged, and fractional shortening was calculated as

\[ \%FS = \frac{(EDD - ESD)}{EDD} \times 100 \]

2.3. Tissue sampling

Each animal was anesthetized with diethyl ether. The hearts were rapidly excised and washed thoroughly with cold saline, then immediately frozen in liquid nitrogen and lyophilized.

2.4. Lipid analysis

The lyophilized heart samples were homogenized in 5 ml of a chilled chloroform/methanol mixture (2:1, by vol) containing 0.01% butylated hydroxytoluene as an antioxidant and cholesteryl acetate as an internal standard. The phospholipid content was quantified using the TLC/FID method as previously described [11]. Simultaneous quantitation of 1,2-DAG and ceramide levels was also performed by the TLC/FID method as previously described [13]. In brief, 1 μl of a lipid extract solution containing ceramides, neutral lipids, and free fatty acids was dissolved in chloroform and applied carefully to silica gel using 75 μm precoated thin-layer rods (Chromarod-SIII, Iatron Lab., Tokyo, Japan). The first development was carried out in a solvent system of chloroform/methanol/H₂O (57:12:0.6, by vol) until the solvent front had migrated approximately 2.5 cm. The second development was carried out in 1,2-dichloroethane/chloroform/acetic acid (46:6:0.05, by vol)
until the solvent had migrated approximately 9 cm. The third development consisted of a repetition of the second development. The fourth development was carried out in n-hexane/diethyl ether/acetic acid (98:1:1, by vol) until the solvent front had migrated approximately 11.5 cm. The Chromarods were then scanned in an Iatroscan MK-5 analyzer (Iatron Lab.). Each sample was analyzed with three Chromarods, and the results were averaged.

2.5. Measurement of the fatty acid composition of 1,2-DAG

Following the quantification of 1,2-DAG and ceramide, the remaining lipids were separated using silica gel plates (20×20 cm, Kieselgel 60 F254, Merck, Darmstadt, Germany). The area corresponding to 1,2-DAG was identified by exposure of 1,2-diolein to iodine vapor, and scraped into a 2 ml chloroform/methanol mixture (9:1, v/v). The extract was evaporated to dryness under a stream of N₂ gas, and the fatty acid moieties in this fraction were transesterified with boron fluoride–methanol [14]. Methyl fatty acids were analyzed on a gas chromatograph (Model GC 14-A, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and an HR-SS-10 fused-silica capillary column (30 m×0.25 mm internal diameter, Shinwakakoh, Kyoto, Japan). Peaks were identified by comparison to standards (Nu-Chek-Prep, Elysian, MN, USA), and the peak areas were calculated.

2.6. Morphological study

Cardiac tissue was examined by means of light microscopy. Tissue was fixed in 10% formaldehyde in phosphate buffer, and paraffin sections at a thickness of 4 μm were examined after staining with hematoxylin–eosin.

2.7. Statistics

Results are expressed as mean±S.E. Between-group comparisons were assessed by one-way ANOVA and Scheffe’s test. For the fatty acid composition of 1,2-DAG, the Mann–Whitney U-test was used. \( P<0.05 \) was considered statistically significant.

3. Results

The heart weight in the JVS group was elevated from 4 weeks of age and comparable to that of the control group. At 8 weeks of age, the heart weight in both the JVS and the etomoxir-treated control group was significantly increased compared with that in the control group. The heart weight in the carnitine-treated JVS group was significantly decreased compared with that in the JVS group. However, no difference was observed in body weight among the groups. The heart weight/body weight ratios in both the etomoxir-treated and JVS groups were much higher than that in the control group at 8 weeks of age, but that in the carnitine-treated JVS group was significantly decreased compared with that in JVS group (Table 1).

3.1. Myocardial performance

In the JVS and carnitine-treated JVS groups, systolic blood pressure and fractional shortening of the left ventricle as measured by echocardiography were significantly decreased compared with those in the control group. However, in the carnitine-treated JVS group, systolic blood pressure was elevated and fractional shortening of the left ventricle was slightly, but not significantly, improved compared with these factors in the JVS group. Furthermore, these parameters showed a tendency to decrease to the values in the etomoxir-treated group (Table 2).

3.2. Histological analysis

In transverse sections, the walls of the left and right

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Heart weight (mg)</th>
<th>Body weight (g)</th>
<th>Heart weight/body weight (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4W</td>
<td>8W</td>
<td>4W</td>
</tr>
<tr>
<td>Control</td>
<td>62.1±1.9</td>
<td>79.5±4.4</td>
<td>14.5±0.8</td>
</tr>
<tr>
<td>JVS</td>
<td>80.3±3.2*</td>
<td>168.4±5.1³</td>
<td>14.2±0.5</td>
</tr>
<tr>
<td>Etomoxir</td>
<td>108.5±1.8³</td>
<td>20.0±0.2</td>
<td>17.4±0.5</td>
</tr>
<tr>
<td>Carnitine</td>
<td>81.2±2.2³</td>
<td>17.4±0.5</td>
<td>17.4±0.5</td>
</tr>
</tbody>
</table>

JVS, juvenile visceral steatosis mice; etomoxir, etomoxir-treated control mice; carnitine, carnitine-treated JVS mice. Values are mean±S.E. of eight animals. *\( P<0.01 \) compared with control group at 4 weeks of age; ³\( P<0.01 \) compared with control group at 8 weeks of age; ³\( P<0.01 \) compared with JVS group.
ventricles in both the JVS and etomoxir-treated groups were increased in thickness compared to those in the control group. No apparent difference in pathologic histology was seen between the control and carnitine-treated JVS groups (Fig. 1). Myocardial fibrosis in the JVS and etomoxir-treated groups was not obviously present in comparison with the control group based on a comparison of myocardial tissues after examination by means of Masson’s trichrome staining (data not shown).

3.3. Myocardial lipid content

Myocardial triglyceride (TG) contents were significantly increased in both the JVS and etomoxir-treated groups compared with those in the control group. Those in the carnitine-treated JVS group were decreased compared with those in the JVS group. However, myocardial sphingomyelin contents were significantly decreased in the JVS group compared with those in the control group. No differences in other lipid contents were observed among the four groups (Table 3).

3.4. Myocardial 1,2-DAG and ceramide contents

Fig. 2 shows myocardial 1,2-DAG contents of the four groups. There were no statistically significant differences in the amounts of 1,2-DAG between the JVS and control groups at 4 weeks of age (730 ± 75 ng/mg dry weight in control group versus 975 ± 140 ng/mg dry weight in JVS...
group, NS). However, at 8 weeks of age, the amounts of 1,2-DAG were significantly elevated in both the JVS and etomoxir-treated groups (585 ± 58 ng/mg dry weight in the control versus 1677 ± 54 ng/mg dry weight in the JVS group and 1258 ± 109 ng/mg dry weight in the etomoxir-treated group; *P < 0.01) (Fig. 2a). In the carnitine-treated JVS group, the mass of 1,2-DAG was decreased compared with that of the JVS group. No difference in the amounts of ceramide was observed among the four groups (Fig. 2b).

3.5. Fatty acid composition of 1,2-DAG

We compared the fatty acid composition of 1,2-DAG between the control and JVS groups at 4 weeks of age (Fig. 3a); furthermore, that of 1,2-DAG among the control, JVS, carnitine-treated JVS and etomoxir-treated groups were compared at 8 weeks of age (Fig. 3b). There was a significant difference in the fatty acid profile of 1,2-DAG between the control and JVS groups, but the fatty acid

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**Fig. 2.** (a) Myocardial 1,2-DAG contents in JVS and control groups at 4 and 8 weeks of age, and etomoxir-treated control mice and carnitine-treated JVS mice at 8 weeks of age. *P < 0.01 compared with the control group. §P < 0.01 compared with the JVS group. (b) Myocardial ceramide contents in JVS and control groups at 4 and 8 weeks of age, and etomoxir-treated control mice and carnitine-treated JVS mice at 8 weeks of age. Data are the mean ± S.E.M. of seven animals per group.

**Fig. 3.** (a) Fatty acid composition of 1,2-DAG in hearts in the JVS and control groups at 4 weeks of age. (b) Fatty acid composition of 1,2-DAG in hearts in JVS, control, etomoxir-treated control and carnitine-treated JVS mice at 8 weeks of age. Fatty acids are expressed as the area percentage of the chromatograms. Data are means ± S.E.M. *P < 0.01 compared with the control group at age 4 weeks. §P < 0.01 compared with the control group at age 8 weeks. ¶P < 0.01 compared with JVS group at age 8 weeks.
composition of 1,2-DAG in carnitine-treated JVS mice was similar to that in the control, except for 18:1(n-9) and 18:2(n-6) fatty acids. Moreover, the profile of the fatty acid composition of 1,2-DAG in the etomoxir-treated control group was similar to that in the JVS group. In the JVS and control groups, a consistent pattern was observed between 4 and 8 weeks of age. The percentages of 14:0, 16:0, 22:1 and 22:6(n-3) fatty acids were significantly decreased in both the JVS and etomoxir-treated groups; those of 18:1(n-9) and 18:2(n-6) fatty acids were much higher in both the JVS and etomoxir-treated groups than in other groups. Other fatty acid species of 1,2-DAG remained unchanged.

3.6. Fatty acid composition of phosphatidylcholine, phosphatidylinositol and triglyceride

With respect to the fatty acid composition of 1,2-DAG, the percentages of 18:1(n-9) and 18:2(n-6) fatty acids were significantly increased and those of 16:0 and 22:6(n-3) fatty acids were significantly decreased in the JVS as compared with the control group. These differences be-

Fig. 4. Fatty acid composition of 1,2-DAG, phosphatidylcholine, phosphatidylinositol and triglyceride in hearts in the JVS and control groups. Fatty acids are expressed as percentages of the chromatograms. Data are means±S.E.M. *P<0.05, **P<0.01 compared with the control group.
between the JVS and control groups were more similar to those in phosphatidylcholine (PC) than those in phosphatidylinositol (PI). In contrast to PC, only a slight difference in the fatty acid composition of PI was seen between the control and JVS groups, except for slight increases in 18:1(n−9) and 18:2(n−6) in the JVS group. With respect to the fatty acid composition of TG, the percentage of 18:1(n−9) was significantly increased, but that of 18:2(n−6) was no different between the control and JVS groups (Fig. 4).

4. Discussion

Mitochondrial β-oxidation of fatty acids is the main source of energy for the heart. It is well known that a disorder of β-oxidation causes cardiac hypertrophy [1]. However, the precise mechanisms of cardiac hypertrophy in such disorders are poorly understood. In this study, we used two models of cardiac hypertrophy resulting from the disorder of β-oxidation: the JVS mouse, which exhibits hereditary systemic carnitine deficiency, and the etomoxir-treated control mouse, in which fatty acid utilization is impaired by inhibiting CPT-1. Moreover, a model of inhibition of cardiac hypertrophy, carnitine-treated JVS mice, was used for comparison with these β-oxidation disorder models. In both of these β-oxidation disorder models, the concentrations of total lipids in the heart were significantly increased compared with those in the control and carnitine-treated JVS models. Therefore, we consider that the evaluation of lipid second messengers might be important for elucidating the pathogenesis of cardiac hypertrophy. There have been few reports regarding the level of 1,2-DAG directly measured in the hypertrophied heart [15,16]. In this study, we evaluated myocardial 1,2-DAG and ceramide levels by means of the TLC/FID technique.

To our knowledge, this is the first report to demonstrate that the amounts of 1,2-DAG are significantly increased in the hypertrophied heart as a result of energy metabolic disorder. Moreover, distinct fatty acids such as 18:1(n−9) and 18:2(n−6) were significantly increased in both the JVS and etomoxir-treated mice. The fatty acid profile of 1,2-DAG was similar between the JVS and etomoxir-treated groups, whereas in carnitine-treated JVS mice, the amount of 1,2-DAG was significantly decreased and the fatty acid composition of 1,2-DAG was different compared with that in JVS mice. Previous reports have suggested that 1,2-DAG, which is considered to be only one of the physiological activators of PKC, might exert cardiac hypertrophy by both its increased total level and by the altered composition of particular molecular species [8]. These results also demonstrate that the amounts of 1,2-DAG have been elevated and the fatty acid compositions of 1,2-DAG have been altered. This suggests that the specific molecular species composition of 1,2-DAG, 18:1(n−9) and 18:2(n−6), are important as a signal related to cardiac hypertrophy. It has been reported that the amounts of 1,2-DAG are elevated in response to a stimulating event in vivo and in vitro [15,16]. We previously reported that the amounts of 1,2-DAG are elevated in the hypertrophic heart in the Bio 14.6 hamster [17]. In addition, Milano et al. demonstrated the elevation of 1,2-DAG in cardiac hypertrophy of overexpressing constitutively active mutant α1b-adrenergic receptor transgenic mice [18]. In vitro, many experiments have demonstrated that the 1,2-DAG level is raised by the stimulation of cultured cells. Also, Eskildsen-Helmond et al. showed that the distinct molecular species composition of 1,2-DAG is related to the activation of PKC [19]. The above findings, taken together, lead us to believe that the increase in, and altered composition of, the total level of 1,2-DAG may play a role in activating the PKC pathway, resulting in the development of cardiac hypertrophy.

1,2-DAG is generated through several pathways: de novo synthesis [20,21] and hydrolysis of PC [22–24], PI [25–27] or other lipids such as phosphatidylethanolamine [28]. In general, the production of 1,2-DAG shows a biphasic pattern after stimulation [29]. The initial phase of increase in 1,2-DAG is predominantly derived from PI, whereas the late phase of 1,2-DAG increase is produced from PC [30]. DAGs derived from PC, which usually show a more sustained peak than do those from PI, have been suggested to lead to a sustained PKC activation [19]. The fatty acid patterns of PC and PI do not change after agonist stimulation, and the DAGs derived from PC can be distinguished from those derived from PI based on their fatty acid composition profiles [31]. In this study, 1,2-DAG in the hypertrophied heart may be derived mainly from PC, judging from the fatty acid composition of 1,2-DAG. Since cardiac hypertrophy developed gradually in both hereditary and drug-induced models, sustained stimuli may be necessary to cause it. Moreover, the fatty acid pattern of TG is not basically coincident with that of 1,2-DAG. Therefore, the elevated 1,2-DAG does not appear to be derived from TG. Only a few studies have been reported about the function of TG as a signal.

Furthermore, the 18:1(n−9) and 18:2(n−6) fatty acid compositions of PI were increased in the JVS group of cardiac hypertrophy, suggesting that the phosphatidylinositol 4,5-bisphosphate phospholipase C (PIP₂−PLC) pathway may also participate, to some extent, in regulating 1,2-DAG production. It is known that 1,2-DAG derived from PIP₂−PLC activates PKCs ubiquitously. Therefore, the possibility cannot be excluded that this increased 1,2-DAG may be derived from PI and PC together. At present, although the accumulated 1,2-DAG under different conditions was characterized differently, previous studies have not sufficiently revealed whether different 1,2-DAG species are active, or where these lipids are located and how they contribute to the specificity.

Three potential pathways of PC utilization result in
monounsaturated phosphatidic acids (PAs) exhibit activity as signaling molecules [42] and Wakelam et al. demonstrated that DAG derived from the PLD/PAP pathway may not be involved in PKC activation in vivo [43]. The increased fatty acids of 1,2-DAG in this study were 18:1(\(n-9\)) (monounsaturated) and 18:2(\(n-6\)) (polyunsaturated). The 1,2-DAGs mainly showing increases in 18:2(\(n-6\)) fatty acid are likely to activate the PKC pathway. On the other hand, in our in vivo study, we could not determine whether the 1,2-DAG showing an increase in the 18:1(\(n-9\)) fatty acid belonged to the metabolites of PAs via the PC–PLD pathway or production through other pathways. Because PAs, as signal molecules, are rapidly converted to DAG both in vivo and in vitro, monounsaturated PA may be changed into monounsaturated DAG. In any event, we believe that not only the increase in the total level of 1,2-DAG, but the altered composition of distinct fatty acids, may activate the PKC pathway.

In conclusion, changes in the lipid second messenger 1,2-DAG and ceramide in the hypertrophied heart resulting from a \(\beta\)-oxidation disorder are similar between hereditary and pharmacological interventional models. 1,2-DAG may be involved in the pathogenesis of cardiac hypertrophy resulting from an energy metabolic disorder.

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