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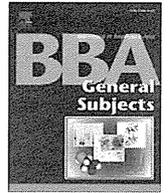
Preface

Biochemistry of mitochondria, life and intervention 2010

Mitochondrial research and medicine have been continuously expanded for the last 40 years. Since mitochondria play a central role in the metabolism of carbohydrates, lipids, and amino acids, alterations of mitochondrial functions have been implicated in various human disorders, such as mitochondrial myopathy, diabetes mellitus, aging-process, Alzheimer's disease, Parkinsonism, cancer, atherosclerosis, obesity, and metabolic syndrome.

Recent developments of clinical research and medicine indicate that the many human disorders have a link to mitochondrial function and possible to indicate the therapeutic application for cure of the disorders. ROS production from the respiratory chain plays pivotal roles not only in the control of proliferation and differentiation of cells but also in the regulation of mitochondrial mass in the cell. ROS is also related with aging and cartinogenesis. Molecular phathophysiology of maintenance of mitohondria is also discovered as fission and fusion mechanism, which are related to the quality control of mitochondria (mitophagy) seen in Parkinsonism. Many animal models are created by KO mice and are investigated the pathophysiology of disorders. Therapeutic clinical approaches are also investigated such as L-arginine on MELAS, sodium pyruvate for lactic acidosis, and hydrogen water for mitochondrial disorders. Assisted reproductive technology for mitochondrial disease patients is well developed in the fields to apply the clinical application. Such fundamental studies of mitochondrial bioenergetics could apply the new therapeutic indication for mitochondrial disorders.

In this special issue of BBA-general on " Biochemistry of Mitochondria, Life and Intervention 2010" which contains selected papers from 7th annual meeting of Asian Society for Mitochondrial Research and Medicine and 10th J-mit (Japanese Society of Mitochondrial Research and Medicine), we discuss the new aspect of mitochondrial functions relating to human disorders, and possible and on-going therapeutic approach of human disorders. This issue is organized in five chapters as follows: (i) Update mitochondrial research field, (ii) Mitophagy (fission and fusion), (iii) Animal model of mitochondrial disorders, (iv) Therapeutic approach of mitochondrial disorders, and (v) Mitochondrial pathophysiology in atherosclerosis, cancer, and aging.

Dr. Yasutoshi Koga is a professor of Pediatrics and Child Health, Kurume University Graduate School of Medicine, Japan. After he completed the MD and PhD, he joined the Mitochondrial Research Group in 1990 as a post doctoral research fellow granted by Muscular Dystrophy Association at the Department of Neurology, College of Physicians and Surgeons of Columbia University (Profs. DiMauro and Schon EA), where he directed his research to mitochondrial genetics especially pathogenic mechanism of MELAS. This led to the development of rho-zero cybrid system in mitochondrial research in 1992. He is the vice-president of Asian Society of Mitochondrial Research and Medicine and is organizing the Joint Symposium of 7th Asian Society of Mitochondrial Research and Medicine, and 10th Japanese Society for Mitochondrial Research and Medicine in 2010 at Fukuoka, Japan. He pioneered the development of a novel therapeutic procedure for MELAS and has completed the investigator-mediated clinical trial of L-arginine on MELAS. He received the Kelsey Wright Award from United Mitochondrial Disease Association (USA) in 2008. He now become a core committee member of International Mitochondrial Research and Medicine especially therapeutic division.

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Mitochondriology
Molecular Biology on Oxidative stress
Hydrogen medicine
On November 18, 2011

Dr. Yau-Huei Wei graduated in June 1974 from the Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan. He joined the laboratory of the late Professor Tsoo E. King in 1976 and earned his PhD degree in 1980 from the Department of Chemistry, State University of New York at Albany, New York, USA. He returned to Taiwan after one year of postdoctoral training at the Departments of Chemistry and Physics, SUNY-Albany. He was appointed as an associate professor during 1981–1985 at the Department of Biochemistry, National Yang-Ming Medical College, Taipei, Taiwan. He was promoted to full professor in 1985 and served as the chairman of the department until 1991. He also served as the Director, Common Instrumentation Center (1986–1989) and the Dean of Student Affairs (1989–1991) of the College. Dr. Wei was appointed as the Director General, Department of Life Sciences, National Science Council of Taiwan, 2001–2005. He served as the Dean of Academic Affairs (2006–2008) and was a Distinguished Professor (2007–2009), National Yang-Ming University. In August 2009, Dr. Wei was appointed the founding President of Mackay Medical College. He has actively participated in the promotion of international collaboration in biomedical research and mitochondrial medicine. Dr. Wei was one of the founding members of Asian Society for Mitochondrial Research and Medicine, and was the Vice-President (2002–2005) and President (2005–2008) of the Society. He has been the President of Taiwan Society for Mitochondrial Research and Medicine (2006–2012). Since 2006, Dr. Wei has served on the editorial board of Biochimica et Biophysica Acta-General Subjects. Dr. Wei's major research has focused on "Molecular and cellular biology studies of mitochondrial diseases, cancer and age-related diseases" and "The cross-talk between mitochondria and the nucleus and metabolic shift in the differentiation of stem cells". He was among the few investigators to show that mitochondrial function decline and mitochondrial DNA mutations are important contributory factors of human aging. His research team was one of the earliest groups to demonstrate that oxidative stress and oxidative damages elicited by mitochondrial DNA mutations contributes to the pathophysiology of many mitochondrial disorders. In the past few years, Dr. Wei and his students have established that mitochondrial biogenesis and respiratory function as well as antioxidant enzymes are upregulated in a coordinate manner in the process of differentiation of stem cells. Dr. Wei and his students have published in SCI journals ~300 research papers and ~30 review articles and book chapters in the fields of bioenergetics, mitochondrial medicine, free radical biology and medicine, molecular and cellular biology, male infertility, and aging research.

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Original Paper

Evaluation of Systemic Redox States in Patients Carrying the MELAS A3243G Mutation in Mitochondrial DNA

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Abstract
Background/Aims: To clarify the change of systemic redox states in patients carrying the A3243G mutation in mitochondrial DNA (A3243G), we evaluated oxidative stress and antioxidant activity in the serum of patients. *Methods:* Oxidative stress and antioxidant activity in the serum samples obtained from 14 patients carrying A3243G and from 34 healthy controls were analyzed using the diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests, respectively. *Results:* The mean d-ROMs level of all patients was significantly greater than that of the controls (p < 0.005), and the mean BAP/d-ROMs ratio of all patients was significantly lower than that of the controls (p < 0.02). In the patients with a history of stroke-like episodes (n = 10), both mean d-ROMs and BAP levels were increased compared with those of the controls (both p < 0.01). The mean BAP level of the patients without a history of stroke-like episodes (n = 4) was significantly decreased compared with that of the controls (p < 0.001), but the mean d-ROMs levels were not significantly different. *Conclusion:* d-ROMs and BAP tests indicated that patients carrying A3243G are always exposed to underlying oxidative stress, even at a remission state of stroke-like episodes.

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Introduction

Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome is the most common type of mitochondrial disease, and is mainly caused by an A-to-G transition mutation at nucleotide position 3243 (A3243G) in mitochondrial DNA (mtDNA) [1]. MELAS is characterized by stroke-like episodes that occur repeatedly and provoke neurological symptoms (e.g. headache, epilepsy, hemiparesis, and dementia) due to 'stroke-like' brain lesions [2]. In other words, stroke-like episodes are diagnostic symptoms of MELAS, and are crucial factors determining the prognosis of patients with this syndrome [2].

In addition, A3243G is responsible for not only stroke-like episodes but also mitochondrial cardiomyopathy or diabetes mellitus (DM) [1, 3–6]. Conversely, some patients carrying A3243G present with typical MELAS syn-

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Table 1. Demographic characteristics of patients and controls

Subject	Patients			Normal controls
	all	'stroke type'	'non-stroke type'	
Number	14	10	4	34
Gender (males/females)	7/7	5/5	2/2	20/14
Mean age at examination, years	32.1 ± 14.7	27.8 ± 12.5	42.8 ± 15.8	34.6 ± 7.4
Clinical features				
Stroke-like episodes, n	10	10	0	0
Cardiomyopathy, n	6	2	4	0
Diabetes, n	3	0	3	0
Under antioxidant therapy, n	11	10	1	0

Values are mean ± SD.

dromes with stroke-like episodes, and others present with only cardiomyopathy or DM without stroke-like episodes. However, the pathophysiological difference of phenotypes between the presence and absence of stroke-like episodes in patients carrying A3243G remains obscure.

Recent studies using cells cultured in vitro demonstrated increased oxidative stress in cells with impaired mitochondria due to A3243G [7–10]. Oxidative stress is provoked by reactive oxygen species (ROS) generation exceeding antioxidant defenses, such as manganese superoxide dismutase and glutathione peroxidase, and damages nucleic acids, proteins and lipids, which leads to cellular dysfunction. Indeed, previous pathological or imaging studies demonstrated enhanced regional oxidative stress in lesions of both stroke-like episodes and cardiomyopathy in patients carrying A3243G [11–13]. Therefore, there is a high possibility that oxidative stress participates in the pathogenesis caused by A3243G, and influences the phenotypic diversity. In other words, redox (reduction-oxidation) states should be evaluated in patients carrying A3243G both with and without a history of stroke-like episodes to clarify the role of oxidative stress in the emergence of stroke-like episodes.

To perform such an investigation, a rapid and reliable method of evaluating redox states in patients carrying A3243G is needed. Direct measurement of oxidative stress and antioxidant activity in living humans has been difficult; redox states have thus not been clearly evaluated in patients carrying A3243G to date. Recently, the diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests have been used to evaluate redox states in serum. The d-ROMs level reflects the intensity of oxidative stress, and the BAP level indicates the activity of endogenous antioxidants [14, 15]. Their effectiveness as clinical markers has been reported in various diseases [16–22]. We evaluated redox states in fresh serum of both patients carrying A3243G and healthy volunteers using d-ROMs and BAP tests, and clarified the change of redox states due to A3243G and the pathophysiological difference in phenotypes with or without stroke-like episodes.

Subjects and Methods

Subjects

Fourteen Japanese patients (7 men and 7 women; mean age 32.1 ± 14.7 years) carrying A3243G were recruited at the University of Fukui Hospital, Fukui, and at the Kurume University Hospital, Fukuoka, Japan (table 1). Patients were classified by the presence or absence of stroke-like episodes into 'stroke type' and 'non-stroke type'. Ten patients with a history of stroke-like episodes were categorized as 'stroke type', and the other 4 patients who presented with mainly cardiomyopathy without a history of stroke-like episodes were categorized as 'non-stroke type'. Eleven patients were treated by antioxidant therapy such as coenzyme Q_{10} (CoQ_{10}; daily dose 30–90 mg) and/or vitamin E (daily dose 100 mg) administration; 10 of these patients were 'stroke type', and the other patient was 'non-stroke type'. Eight patients categorized as 'stroke type' were also treated with an oral administration of L-arginine (daily dose 14–21 g). All patients were in remission, free from exacerbation of symptoms or acute stroke-like episodes, when they were examined. Functional status was evaluated using the performance status rating (mean rating 1.3 ± 1.0). In 'stroke type' patients, the mean age of the first stroke-like episode was 21.1 ± 15.2 years, and the mean duration between the examination and the last stroke-like episode was 14.2 ± 9.2 months. 'Stroke type' patients had headaches and/or vomiting on average twice a month, but almost none had convulsions. Thirty-four Jap-
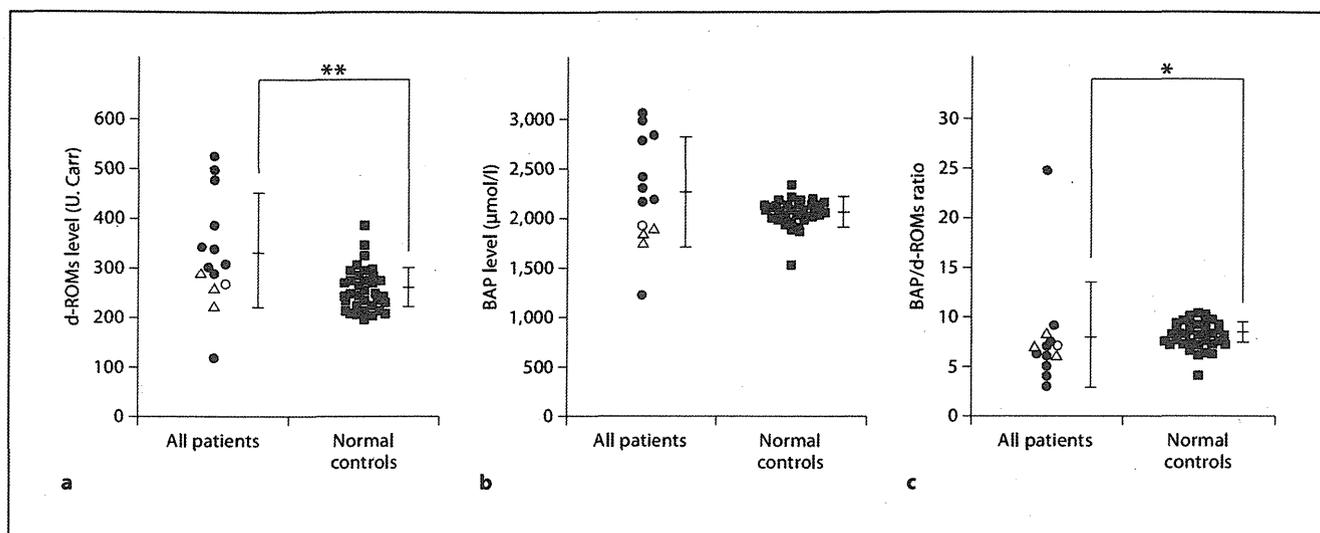
Fig. 1. Scatter plots portraying the levels of d-ROMs (**a**) and BAP (**b**) and BAP/d-ROMs ratios (**c**) in all the patients and controls. Circles and triangles correspond to the patients with and without antioxidant administration, respectively. In addition, closed and open diagrams correspond to the 'stroke type' patients and 'non-stroke type' patients, respectively. * p < 0.02, ** p < 0.005, according to the two-tailed Mann-Whitney U test. Bars indicate mean ± SD.

anese healthy volunteers (20 men and 14 women; mean age 34.6 ± 7.4 years) were also recruited as normal controls from the local community (table 1). This study was approved by the Ethics Committee of the University of Fukui. All subjects provided written informed consent to participate in the study.

Measurement of Oxidative Stress Levels

The oxidative stress levels were evaluated by measuring the quantity of hydroperoxides (R-OOH) in fresh serum samples using the d-ROMs test with the Free Radical Analytical System 4 (FRAS4[R]; H&D srl, Parma, Italy) automatically [14]. Blood sampling was performed at fasting and at rest. Hydroperoxides consist of dehydrogenized and peroxidized proteins, lipids and fatty acids produced by ROS. In the d-ROMs test, hydroperoxides are turned to radicals by the Fenton reaction in an acid medium, and these generated radicals oxidize N,N-diethyl-para-phenylenediamine (DEPPD). Oxidized DEPPD quantity is determined by an absorbency measurement (white light 505 nm). The sequence of these methods is automated, and oxidative stress levels can be evaluated easily and quickly. The values are expressed as U. Carr, where 1 U. Carr corresponds to 0.8 mg/l H_2O_2.

Measurement of Antioxidant Activity Levels

The antioxidant activity levels were evaluated by measuring the quantity of molecules with antioxidative potency in fresh serum samples using the BAP test in the FRAS4[R] automatically [15, 17]. Blood sampling was performed at fasting and at rest. In the BAP test, serum molecules with antioxidative potency reduce and decompose compounds of ferric chloride ($FeCl_3$) and thiocyanate derivative (AT) to $FeCl_2$ and free AT. Free AT is achromatized and dissociates from compounds, and is quantified by an absorbency measurement (white light 505 nm). The sequence of these methods is automated, and antioxidant activity levels can be evaluated easily and quickly. The results are expressed as μmol/l.

Statistical Analysis

The BAP-to-d-ROMs ratio (BAP/d-ROMs ratio) was calculated from the ratio of the BAP levels and d-ROMs levels for each subject. Data are presented as means ± standard deviations (SD). The resultant differences between normal controls and all patients were analyzed by means of a two-tailed Mann-Whitney U test. Since the subject number of each group was small, a nonparametric Kruskal-Wallis test was used for multiple data comparison and a post hoc Dunn test was performed to evaluate differences among normal controls, 'stroke type' patients and 'non-stroke type' patients. All statistical analyses were performed in SPSS Statistics Version 17.0 (SPSS Japan Inc., Tokyo, Japan), and p < 0.05 was considered significant.

Results

The levels of serum d-ROMs and BAP, and BAP/d-ROMs ratios of all the patients and controls are shown in figure 1, and those of the 'stroke type' patients, 'non-stroke type' patients and controls are shown in figure 2. The mean age of each group demonstrated no significant differences.

The mean d-ROMs level of all patients (332.6 ± 110.7 U. Carr) was significantly higher than that of the controls
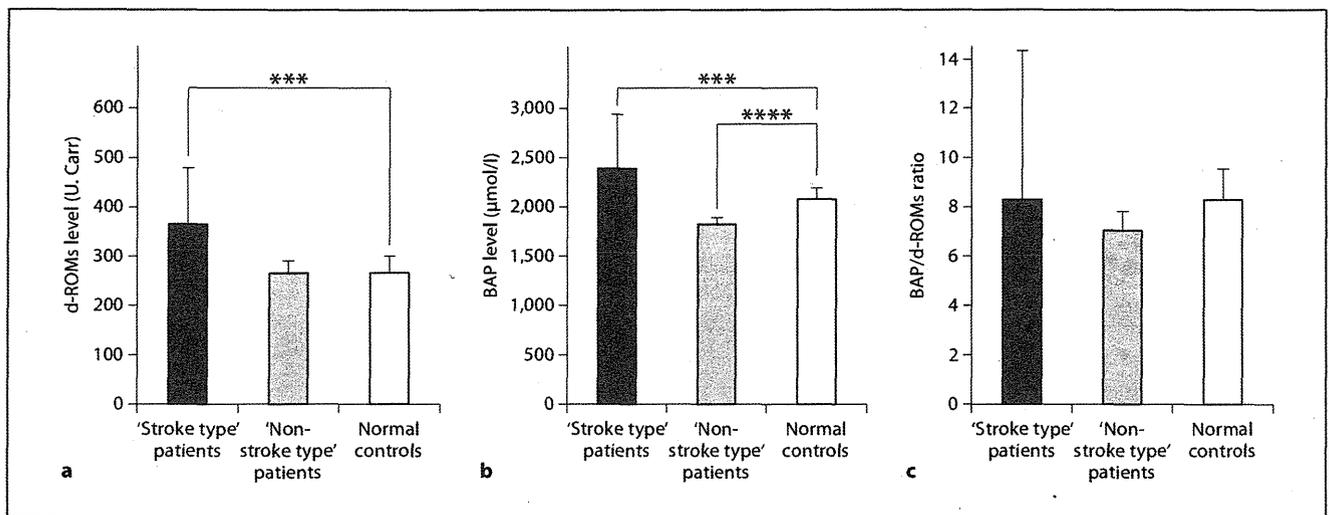
Ikawa/Arakawa/Hamano/Nagata/
Nakamoto/Kuriyama/Koga/Yoneda

Fig. 2. The mean d-ROMs (**a**) and BAP (**b**) levels and mean BAP/d-ROMs ratio (**c**) in the 'stroke type' patients (black), 'non-stroke type' patients (grey) and controls (white). *** $p < 0.01$, **** $p < 0.001$, according to the Dunn test. Bars indicate mean ± SD.

(259.1 ± 42.0 U. Carr; $p < 0.005$) (fig. 1a). In particular, the mean d-ROMs level of the 'stroke type' patients (361.0 ± 119.6 U. Carr) was significantly greater than that of the controls ($p < 0.01$) (fig. 2a). Meanwhile, the mean d-ROMs level of 'non-stroke type' patients (261.5 ± 28.0 U.Carr) demonstrated no significant differences compared with those of the controls and 'stroke type' patients (fig. 2a).

The mean BAP level of all patients (2,258.9 ± 517.7 μmol/l) was not significantly different compared with that of the controls (2,057.6 ± 149.5 μmol/l) (fig. 1b). However, compared with the controls, 'stroke type' patients (2,428.9 ± 523.1 μmol/l) demonstrated significantly high BAP levels ($p < 0.01$), and 'non-stroke type' patients (1,834.0 ± 59.2 μmol/l) demonstrated significantly low BAP levels ($p < 0.001$) (fig. 2b). There was no significant difference between 'stroke type' patients and 'non-stroke type' patients in terms of the mean BAP levels.

The mean BAP/d-ROMs ratio of all patients (7.87 ± 5.05) was significantly lower than that of the controls (8.13 ± 1.30; $p < 0.02$) (fig. 1c). However, there were no significant differences among the controls and patient groups (fig. 2c).

There was no relationship between the functional status evaluated by performance status rating and the d-ROMs level or BAP level or BAP/d-ROMs ratio.

Discussion

In the present study, the d-ROMs and BAP tests were applied to evaluate the redox states in serum of patients carrying A3243G. These tests demonstrated that oxidative stress represented by the d-ROMs levels was increased and redox balance represented by the BAP/d-ROMs ratios was decreased (tendency for oxidation) in the patients compared with those of the controls (fig. 1). These findings suggested that an imbalance of redox states due to mitochondrial dysfunction affects the pathogenesis in patients carrying A3243G.

In the 'stroke type' patients in particular, both d-ROMs levels (oxidative stress) and BAP levels (antioxidant activity) were increased compared with those of the controls (fig. 2a, b). In vitro studies previously demonstrated that A3243G enhances ROS generation leading to oxidative stress [7–10], and enhanced oxidative stress is proportional to mitochondrial dysfunction [7, 23]. In the present study, all of the 'stroke type' patients have been treated with antioxidants, and 8 out of 10 patients were also treated with an oral administration of L-arginine. Although serum antioxidant activity may be increased by antioxidants and L-arginine therapy, serum oxidative stress was still increased in 'stroke type' patients. Increased oxidative stress even with increased antioxidant activity suggested a severe deterioration of mitochondrial function in patients with a history of stroke-like epi-

sodes, and that oxidative stress plays a crucial role not only in the brain lesions of stroke-like episodes [11, 12] but also systemically in these patients. In other words, a history of stroke-like episodes indicates that patients who have these episodes are exposed to underlying oxidative stress.

In the 'non-stroke type' patients, the mean d-ROMs level (oxidative stress) was not significantly different compared with that of the controls (fig. 2a). Meanwhile, the BAP levels (antioxidant activity) were significantly decreased (fig. 2b). Only 1 of 4 patients was treated with antioxidants, and antioxidant therapy may not affect antioxidant activity in 'non-stroke type' patients. These findings may reflect that antioxidants are consumed in order to prevent increase of oxidative stress in these patients. In addition, the difference of profiles in redox states between 'stroke type' and 'non-stroke type' suggested phenotypic diversity in patients carrying A3243G.

In the present study, we presented redox states in the serum of patients carrying A3243G using the d-ROMs and BAP tests. Rapid evaluation of redox states in serum has been difficult to date. To assay oxidative stress in serum, the spin trap method using electron spin resonance (ESR) has been the most reliable method [24]. However, performing ESR is cumbersome, thus it is difficult to apply this method in clinical practice. The d-ROMs test can evaluate oxidative stress in serum by measuring oxides due to hydroperoxides, and this test has been validated by ESR [25]. Likewise, each endogenous antioxidant can be measured, but there has been no method estimating the whole activity of endogenous antioxidants in serum to date. The BAP test provides a reliable indicator of the antioxidant activity in serum by measuring the ability to reduce ferric to ferrous ions [15]. Moreover, the d-ROMs and BAP tests only need a small amount of blood, and require only 15 min for measurement. Therefore, these methods are prompt and reliable, and suitable for evaluating redox states in patients.

Previous studies using postmortem organs or positron emission tomography imaging have demonstrated regional enhancement of oxidative stress in the brain lesions of stroke-like episodes and the heart lesions of cardiomyopathy in patients carrying A3243G [11–13]. Although enhanced oxidative stress due to A3243G has been proven in these lesions, systemic oxidative stress in patients carrying A3243G has not been evaluated to date. The present study demonstrated a systemic and underlying imbalance of redox states in these patients.

The present study has some limitations. (1) The 'non-stroke type' group included only 4 patients. (2) The mean age of 'non-stroke type' patients was likely older than that of 'stroke type' patients. (3) The 'stroke type' group included only 2 of 10 patients with cardiomyopathy or diabetes, which might affect the systemic redox states. (4) All of the 10 'stroke type' patients received antioxidant therapy, but only 1 of the 4 'non-stroke type' patients received antioxidant therapy. (5) This study did not show any significant difference in either value of oxidative stress or antioxidant activity between the 'stroke type' and 'non-stroke type' groups. (6) The possibility that the 'non-stroke type' patients in this study will also subsequently develop stroke-like episodes cannot be ruled out. Further studies are necessary to confirm our preliminary results.

Taken together, the d-ROMs and BAP tests clearly demonstrated an abnormality of redox states in patients carrying A3243G. In particular, enhanced oxidative stress in patients with a history of stroke-like episodes may reflect severe mitochondrial dysfunction, which would contribute to the emergence of stroke-like episodes. In addition, in patients without stroke-like episodes, consumption of antioxidant activity may indicate latent oxidative stress. These findings suggested that patients carrying A3243G are always exposed to underlying oxidative stress, and further antioxidant therapy would be beneficial to prevent an intensification of the symptoms.

Disclosure Statement

The authors report no conflicts of interest.

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JOURNAL OF MEDICAL
CASE REPORTS

CASE REPORT   Open Access

A two-day-old hyperthyroid neonate with thyroid hormone resistance born to a mother with well-controlled Graves' disease: a case report

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Junko Nishioka[1] and Yasutoshi Koga[1]

Abstract

Introduction: Resistance to thyroid hormone is a syndrome caused by thyroid hormone receptor β mutations, which are usually inherited in an autosomal-dominant pattern.

Case presentation: Our patient, a Japanese neonate boy, showed hyperthyroid symptoms at age two days. Although our patient was diagnosed as having resistance to thyroid hormone, his hyperthyroid symptoms continued for two weeks. Therefore, our patient was treated with methimazole and iodine for two weeks from birth, showing no side effects and no symptoms upon treatment. At age 70 days, an R243W mutation in thyroid hormone receptor β was detected in our patient; while absent in his mother, the mutation was present in his father, who never showed any symptoms.

Conclusions: To the best of our knowledge this is the first case report of a resistance to thyroid hormone in a neonate presenting with hyperthyroid symptoms born to a mother with Graves' disease and treated with methimazole and iodine. These results suggest that methimazole and iodine may be a good short-term option for treatment.

Keywords: Hyperthyroid symptoms, Maternal Graves' disease, Symptomatic neonate with resistance to thyroid hormone, Treatment for resistance to thyroid hormone

Introduction

Resistance to thyroid hormone (RTH) is an autosomal dominant (AD) syndrome in which an individual's response to thyroid hormone (TH) is decreased due to mutations in the TH receptor β gene (*TRβ*) [1,2]. Patients with RTH have increased serum TH levels and increased or normal thyroid-stimulating hormone (TSH) levels. The clinical characteristics of RTH vary strikingly, as even the characteristics of various tissues within the same individual or family members who carry identical mutations differ notably [3]. While most patients are asymptomatic, some are symptomatic and show main clinical features such as goiter, hyperactivity, and tachycardia [1]. However, although RTH has been well

investigated recently, the response of patients with RTH to treatment remains unclear.

A neonate born to a mother with Graves' disease (GD) has an increased risk of developing neonatal GD, a rare condition that affects 1% to 5% of babies born to mothers who have hyperthyroidism during pregnancy. Most babies are asymptomatic because the mother normally receives treatment for her GD. In contrast, patients with RTH do not usually receive treatment because they show no symptoms. Indeed, even when they do show symptoms, the results of treatment have been discouraging. Kim *et al.* treated one symptomatic patient with RTH with methimazole (MMI) and T_4 treatment but had to cease treatment because a large goiter developed [4], improving upon treatment withdrawal.

Here, we describe a symptomatic neonate with an R243W *TRβ* mutation inherited from his non-symptomatic father. Our patient, who was born to a

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mother with well-controlled GD, continued to show hyperthyroid symptoms for two weeks, at which point we administered MMI and iodine for another two weeks and monitored our patient's symptoms and thyroid function tests.

Case presentation

Our patient's parents were non-consanguineous and of Japanese origin, with an unremarkable family history except for the mother, who had thyroid symptoms. The mother had an onset of GD at 23 years of age and was subsequently treated for hyperthyroidism with 30mg/day MMI and 50mg/day iodine potassium. After two weeks of treatment, our patient's mother experienced side effects from the MMI, and the regimen was therefore changed to 300mg/day of propylthiouracil (PTU). The PTU dose was then reduced gradually as thyroid hormone levels improved. The mother became pregnant seven months after the GD diagnosis and was treated with 50mg PTU every two days. Thyroid hormones and antibodies related to GD in the mother were within normal ranges throughout the pregnancy (Table 1).

Our patient was born at 38 weeks into the pregnancy following a non-problematic gestation period. His birth weight was 2910g. Our patient exhibited visible hyperthyroid symptoms two days after birth, including tachycardia, frequent bowel movements, and hyper-irritability. A complete blood cell count and blood chemistry examination revealed normal levels with the exception of increased thyroid hormone levels (Table 1). Antibodies associated with thyroid disease were within normal ranges (Table 1). Electrocardiography primarily showed a regular sinus rhythm, and our patient's sleeping heart rate was slightly elevated at 150 to 160 beats per minute compared with the normal range of 120 to 140 beats per minute. Ultrasonography revealed the thyroid to be normal in size with no nodules. A TSH-secreting adenoma

(TSHoma) was ruled out through magnetic resonance imaging (MRI) scans of the pituitary gland, and our patient's human chorionic gonadotropin β (hCG-β) levels were found to be normal at admission. Our patient was suspected of having RTH rather than neonatal GD due to unsuppressed TSH and high free T_4 (FT_4) and T_3 (FT_3) levels.
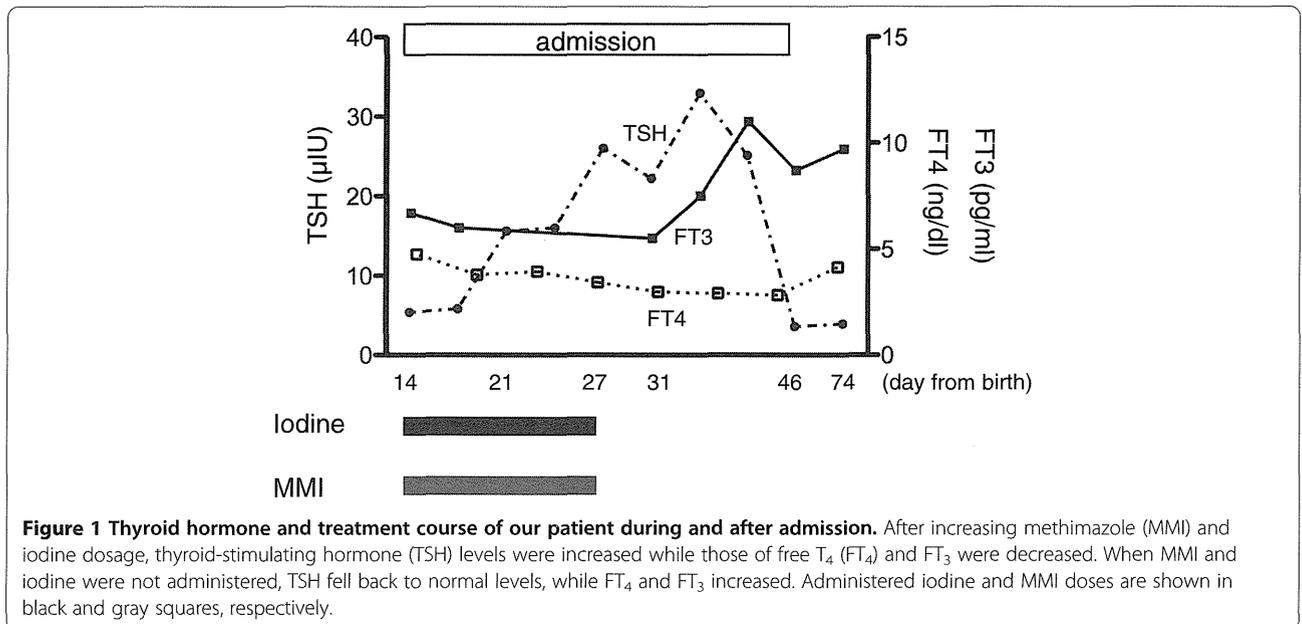
At 14 days old, our patient still continued to show hyperthyroid symptoms and was therefore treated with 0.65mg/kg/day MMI and 12.6mg/day iodine. Our patient responded to the therapy clinically. His irritability diminished, and his sleeping heart rate reduced to 130 to 150 beats per minute. Expectedly, his TSH level increased, and his FT_4 and FT_3 levels decreased (Figure 1). During this course, our patient presented no elevation of antibodies related to GD, and an abnormal thyroid hormone profile continued without goiter. MMI and iodine were discontinued at age 28 days, as our patient's symptoms, particularly hyper-irritability and frequent bowel movements, were improved. In the two-week treatment period, our patient showed neither severe nor worsening symptoms. Our patient was hospitalized for a total of 33 days before being discharged, at which point our patient was confirmed to be euthyroid; follow-up was conducted every three months using thyroid function tests. Today, our patient is three years old and remains clinically euthyroid without the use of therapeutic drugs after discharge. Our patient has also reached developmental milestones appropriate for his age.

Our patient's bone development was also normal, and his electrocardiography, Holter electrocardiography, and echocardiogram findings were all within normal limits, with no severe cardiac complications observed. Our patient's father had never experienced symptoms of hypothyroid or hyperthyroidism, although inappropriate thyroid hormone levels were seen in laboratory tests (Table 1).

Table 1 Thyroid hormone profile of our patient and his mother and father

Profile and normal range	Onset	P0	P5	P8	Delivery	Patient's father	Patient
TSH (μIU/mL) (0.4 to 4.0)	0	0	0.61	0.27	0.04	0.59	5.38 (1.0 to 38.9)
FT₄ (ng/dL) (0.8 to 1.9)	5.64	1.53	0.83	0.9	1.16	2.87	4.76 (2.0 to 4.9)
FT₃ (pg/mL) (2.2 to 4.1)	22.38	2.65	1.93	2.27	NA	4.88	6.7 (2.0 to 6.1)
TgAb (IU/mL) (<28)	0.3	0.3	0.3	0.3	NA	0.3	<0.1 (<28)
TPOAb (IU/mL) (<16)	9.1	0.7	0.3	0.3	NA	<0.3	<0.1 (<16)
TRAb (IU/L) (<1)	15	4	1.2	1	NA	<0.1	<0.1 (<1)
TSAb (%) (<180)	188	239	135	132	NA	130	173 (<180)
Tg (ng/mL) (<32.7)	540	130	68	NA	NA	NA	NA

Thyroid hormone profile of the mother (onset of GD to child delivery), the father, and our patient at two days after birth. P0 is zero months' pregnant, P5, five months' pregnant, P8, eight months' pregnant. Values at onset and P0 indicate typical Graves' disease (GD). GD was well controlled during pregnancy by propylthiouracil. Reference values for adults are used for the mother and the father; reference values for neonates are used for our patient. In the father and our patient, all values for thyroid antibodies were negative, indicating resistance to thyroid hormone. Thyroid-stimulating antibody (TSAb) was measured using radioimmunoassay (RIA), while all other values were measured using electrochemiluminescence immunoassay (ECLIA).
FT₃/T₄, free T₃/T₄; Tg, thyroglobulin; TgAb, thyroglobulin antibody; TPOAb, thyroid peroxidase antibody; TRAb, TSH receptor antibody; NA, not available.

Figure 1 Thyroid hormone and treatment course of our patient during and after admission. After increasing methimazole (MMI) and iodine dosage, thyroid-stimulating hormone (TSH) levels were increased while those of free T_4 (FT_4) and FT_3 were decreased. When MMI and iodine were not administered, TSH fell back to normal levels, while FT_4 and FT_3 increased. Administered iodine and MMI doses are shown in black and gray squares, respectively.

Blood samples were obtained from our patient and both his parents, and genomic deoxyribonucleic acid (DNA) was isolated from leukocytes using standard protocols. Sequencing the patient's *TRβ* gene revealed a missense mutation that causes an R243W substitution within the receptor's T_3-binding domain. While the mother's *TRβ* gene did not contain this mutation, the father's did.

Discussion

This case report describes a baby boy with a *TRβ* R243W mutation born to a mother with no RTH mutation and with no family history of RTH. Our patient's asymptomatic father carried the same mutation as our patient.

The R243W point mutation, first detected by Pohlenz *et al.* [5], has a mechanism of action differing from that of other *TRβ* mutations, as the R243W receptor has normal T_3-binding affinity but transactivates poorly upon binding T_3, thereby conferring a dominant-negative effect [6,7]. The resulting phenotype is usually euthyroid with occasional hypothyroidism being observed. Regardless, clinical features of both hypothyroidism and hyperthyroidism are expected due to variable resistance in the different tissues of an individual. In the present case, a small amount of PTU from the mother transmitted to the fetus may have induced hyperthyroidism just after birth. Since such quantities of PTU may slightly suppress thyroid hormones in the fetus, a sudden release of thyroid hormones after birth may have occurred, causing our patient to be initially diagnosed as having hyperthyroidism.

The clinical phenotype for R243W also differs among families and individuals. In fact, the same mutation can cause either generalized RTH or pituitary RTH in different individuals within the same family. For example, a boy who had slight attention-deficit hyperactivity disorder and the R243W mutation was born to a mother who remained clinically euthyroid with the same mutation [8]. Additionally, only a weak correlation has been observed between a given mutation and the development of RTH [1,3,9]. In rare cases, RTH coexists with GD [10,11]. Considering the mother's condition in the present case, it would be normal for our patient to be suspected of having neonatal GD. However, since our patient showed no suppressed TSH levels and had high FT_4 and FT_3 levels with no antibodies related to GD, our patient was diagnosed as having RTH.

Patients with RTH are not usually treated because many patients do not have significant symptoms. Kim et al. administered MMI plus T_4 treatment for a symptomatic 11-month-old patient who had hyperthyroidism secondary to RTH [4]. However, a large goiter developed without clinical improvements, and after withdrawing treatment, the goiter then improved. In the present case, we treated our patient with MMI and iodine for two weeks, after which our patient showed improved hyperthyroid symptoms and no goiter. In the report by Kim *et al.*, the patient had severe symptoms, including a failure to thrive, verbal delays and tachycardia. Our patient had symptoms of tachycardia, diarrhea, and hyperirritability but showed no failure to thrive or developmental delay, an aspect we attribute to our treatment program.

The differential diagnoses in the present case were neonatal GD and TSHoma. TSHoma can be discovered relatively easily using magnetic resonance imaging

(MRI); however, when a pregnant mother has GD, a neonate with hyperthyroid symptoms is typically expected to have neonatal GD, which can often lead to a misdiagnosis of RTH [10]. Consulting previous papers, which showed how to diagnose RTH with thyroid diseases [12,13], would have avoided such a misdiagnosis.

Conclusions

We describe the case of a neonate presenting with hyperthyroid symptoms. In hyperthyroid neonates born to mothers with GD, it is important to examine the thyroid hormone levels of both parents. Had serum TSH receptor antibody (TRAb) or thyroid-stimulating antibody (TSAb) been elevated in both our patient and his mother, diagnosis of RTH would have been further delayed. To the best of our knowledge this is the first report of a symptomatic neonate with RTH born to a mother with GD and treated by MMI and iodine in the neonatal period without side effects. MMI and iodine, therefore, may make for optimal short-term treatment in hyperthyroid RTH neonates.

Consent

Written informed consent was obtained from the patient's next-of-kin for publication of this case report and any accompanying images. A copy of the written consent is available for review from the Editor-in-Chief of this journal.

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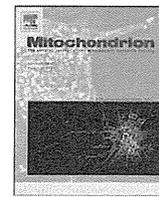# Metabolomic profiling rationalized pyruvate efficacy in cybrid cells harboring MELAS mitochondrial DNA mutations

Kenjiro Kami [a,b,1], Yasunori Fujita [c,1], Saori Igarashi [a], Sayaka Koike [a], Shoko Sugawara [a], Satsuki Ikeda [a], Naomi Sato [a], Masafumi Ito [c], Masashi Tanaka [d,*], Masaru Tomita [a,b,e], Tomoyoshi Soga [a,b,e]

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ABSTRACT

Pyruvate treatment was found to alleviate clinical symptoms of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome and is highly promising therapeutic. Using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS), we measured time-changes of 161 intracellular and 85 medium metabolites to elucidate metabolic effects of pyruvate treatment on cybrid human 143B osteosarcoma cells harboring normal (2SA) and MELAS mutant (2SD) mitochondria. The results demonstrated dramatic and sustainable effects of pyruvate administration on the energy metabolism of 2SD cells, corroborating pyruvate as a metabolically rational treatment regimen for improving symptoms associated with MELAS and possibly other mitochondrial diseases.

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1. Introduction

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome is one of the mitochondrial cytopathies first introduced by Pavlakis et al. (1984). Complex pathologies observed in MELAS patients primarily stem from the adenine-to-guanine transition mutation at position 3243 of the mitochondrial genome (A3243G) located in the mitochondrial tRNA$^{Leu(UUR (R=A or G))}$ gene (Goto et al., 1990), which accounts for about 80% of all cases of MELAS (Koga et al., 2012b) and is estimated to be carried by as high as 0.06% of the general population (Sproule and Kaufmann, 2008). The mutated mitochondrial tRNA$^{Leu(UUR)}$ gene recognizes the UUA but not the UUG codon and causes protein synthesis defects due to a shortened life-span of tRNA$^{Leu(UUR)}$, a lowered ratio of aminoacyl- to uncharged-tRNA$^{Leu(UUR)}$, accumulations of leucine aminoacylation and processing intermediates, and a defect in modifying uridine to 5-taurinomethyluridine at the first position of the anticodon (Koga et al., 2012b). Due to the frequent appearance of the UUG codons

in the coding sequence of NADH dehydrogenase subunit 6, mitochondria in the skeletal muscle of MELAS patients often exhibit defects in activities of complex I (Ichiki et al., 1989) and complex IV and others in severe cases (Iizuka and Sakai, 2005; Yoneda et al., 1989). The defects in these respiratory complexes induce impaired oxidative phosphorylation, increased generation of free radicals, and a decreased level of free nitric oxide (Hussein et al., 2009). Disordered ATP production has also been confirmed as a pathogenesis of MELAS by studies using cytoplasmic hybrids, or "cybrids," which are human cell lines containing the patient's mitochondria with mutated genomes (DiMauro and Schon, 2003). Most treatment regimens for mitochondrial diseases including MELAS are designed to mitigate the cellular consequences of dysfunction of the respiratory chain by supplementation with electron acceptors and reactive oxygen species scavengers such as creatine, coenzyme Q10, α-lipoic acid, and vitamins (riboflavin, thiamine, vitamin C, vitamin E, and biotin); however, the clinical efficacy of these supplements remains limited or doubtful (Sproule and Kaufmann, 2008). Recently, pyruvate treatment was found to alleviate muscle impairment in patients with not only MELAS (Tanaka et al., 2007) but also other mitochondrial diseases such as Leigh's syndrome (Koga et al., 2012a; Komaki et al., 2010) and mitochondrial depletion syndrome (Saito et al., 2012) without causing notable side effects, and thus is considered promising as an alternative therapeutic. Metabolic mechanisms of pyruvate efficacy in MELAS mutant cells, however, are not clearly understood. We thus aimed to

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elucidate the metabolic responses of pyruvate-supplemented MELAS mutant cells by performing time-course metabolome analysis using capillary electrophoresis time-of-flight mass spectrometry or CE-TOFMS (Soga et al., 2003, 2006). Metabolomics technologies make it possible to simultaneously identify and quantify hundreds of metabolites contained in cells (Ishii et al., 2007; Ohashi et al., 2008), tissues (Hirayama et al., 2009; Soga et al., 2006) or body fluids (Soga et al., 2006; Sugimoto et al., 2010). Among other analysis platforms frequently used in metabolomics research, such as liquid chromatography or gas chromatography combined with mass spectrometry (LC-MS and GC-MS, respectively) and nuclear magnetic resonance (NMR) analysis, CE-TOFMS specializes in a comprehensive measurement of charged compounds (Soga et al., 2003) and thus is best suited to quantitatively analyze alterations of energy metabolism in cells. In addition, the use of ^{13}C-labeled compounds and quantification of the resulting ^{13}C-labeled isotopomers enable an investigator to trace small amounts of labeled compounds, thus facilitating our understanding of metabolic fluxes. Therefore, we used this approach to measure and quantify time-course changes in 161 intracellular metabolites in, and 85 metabolites released into the medium (hereafter referred to as medium metabolites) by, cybrid human 143B osteosarcoma cells containing normal mitochondria (2SA cells) or MELAS mutant mitochondria (2SD cells) cultured with 10 mM [3-^{13}C] pyruvate. Since most MELAS patients exhibit a symptom of lactic acidosis in addition to seizures and stroke-like events (e.g., 94 of 101 (94%), 97 of 102 (96%), and 106 of 107 (99%) patients, respectively (Hirano and Pavlakis, 1994)), the medium was supplemented with 10 mM [3-^{13}C] lactate as a comparative control condition. The resulting metabolomic profiles highlighted the basal metabolic differences between 2SA and 2SD cells and their metabolic alterations and flux patterns in response to a high dose of lactate or pyruvate. In particular, constantly low ATP levels and poor energy charge characterized the basal metabolism of lactate-supplied 2SD cells and were likely due to impaired oxidative phosphorylation; however, pyruvate administration improved the lactate-to-pyruvate ratio ([Lac]/[Pyr]) and NADH-to-NAD^{+} ratio ([NADH]/[NAD]) in 2SD cells, which enhanced glycolysis and replenished TCA cycle intermediates for maintaining the ATP at a level as high as that in 2SA cells. These results demonstrated a dramatic and favorable effect of pyruvate administration on the energy metabolism of 2SD cells, supporting the idea that balancing the [NADH]/[NAD] ratio is crucial for facilitating active glycolysis and replenishing TCA intermediates in MELAS mutant cells for a sufficient and stable energy production.

2. Materials and methods

2.1. Cell culture

The 2SA and 2SD cybrid cell lines carrying 100% wild-type and 94% A3243G mutant mtDNA, respectively, were established by fusion of mtDNA-deficient $\rho^0$206 cells generated from human 143B osteosarcoma cell line with enucleated myoblasts derived from a MELAS patient (Chomyn et al., 1992; Tanaka et al., 2002; Yoneda et al., 1994). The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 0.4 mM uridine and maintained under 5% CO_2 at 37 °C. The experiments were initiated by replacing the medium with DMEM supplemented with 10% fetal bovine serum, 0.4 mM uridine, and 10-mM [3-^{13}C] lactate or 10-mM [3-^{13}C] pyruvate.

2.2. Metabolite extraction

Cell and medium samples were obtained 0, 1, 2, and 4 h after 10 mM [3-^{13}C] lactate or 10 mM [3-^{13}C] pyruvate administration. The sample medium was mixed with methanol containing 50 μM internal standards (3-aminopyrolidine, L-methionine sulfone, trimesate, 2-morpholinoethanesulfonic acid, and D-camphor-10-sulfonic acid), Milli-Q water, and CHCl$_3$ in the ratio of 1:4:2:5. The cells were washed

twice with 5% mannitol solution and covered with methanol (1 ml) containing 25 μM internal standards for enzyme inactivation. The methanol and cell mixtures were collected and mixed with Milli-Q water and CHCl$_3$ in the ratio of 2:1:2. Both the medium and cell sample solutions were then centrifuged at 20,000 ×g for 15 min, and the aqueous layers were collected for centrifugal filtration through a 5-kDa-cutoff filter at 9,000 ×g for 2.5 h. The extracted metabolites were concentrated with a centrifugal concentrator (Tomy, Tokyo, Japan) and stored at − 80 °C until analysis could be performed. The appropriate volume of Milli-Q water was added for the dissolution of the concentrated metabolites immediately before the sample injection into the CE-TOFMS.

2.3. Metabolome analysis

Concentrations of all the charged compounds were measured by CE-TOFMS using the methods developed by (Soga et al., 2006, 2003). Briefly, for analyzing cations (Soga et al., 2006), a fused silica capillary (50 μm i.d. × 100 cm total length) was used with 1 M formic acid as both the running and preconditioning buffer. Each sample (approximately 3 nl) was injected by applying a pressure of 50 mbar for 3 s and a continuous voltage of + 30 kV. A solution of 5 mM ammonium acetate and 0.5 μM reserpine in 50% (v/v) methanol in water was used as the sheath liquid at a flow rate of 10 μl/min. For analyzing anions (Soga et al., 2006), a commercially available cationic capillary, SMILE(+) (Nacalai Tesque, Kyoto, Japan), was used with 50 mM ammonium acetate solution (pH 8.5) as the running buffer and 50 mM acetic acid (pH 3.4) as the preconditioning buffer. Each sample (approximately 30 nl) was injected by applying a pressure of 50 mbar for 30 s and a continuous voltage of − 30 kV. For analyzing nucleotides and coenzyme A compounds (Soga et al., 2007), the fused silica capillary and 50 mM ammonium acetate (pH 7.5) were used. A voltage of − 30 kV was applied to the inlet capillary, along with pressure of 50 mbar, to maintain the conductive liquid junction at the capillary outlet.

2.4. Metabolome data processing

The CE-TOFMS data were preprocessed by our proprietary software, MasterHands, which calculates accurate m/z, quantifies peak areas from the electropherogram, and aligns the peaks of multiple datasets. We quantified the concentrations of 161 intracellular and 85 medium metabolites including ^{13}C-labeled isotopomers involved in primary energy metabolism such as glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, urea cycle, and the metabolism of amino acids and nucleotides. The average amount of each metabolite per cell was evaluated based on the number of viable cells, which was counted at each sampling time by using a Countess Automated Cell Counter (Invitrogen, Carlsbad, California, US). For pyruvate, lactate, phosphoenolpyruvate, Gly, Ala, Ser, Asn, Asp, citrate, isocitrate, 2-oxoglutarate, succinate, fumarate, and malate, intracellular amounts and medium concentrations of their isotopomers were evaluated, taking into account the natural isotope abundance of C, H, and O atoms, according to the method of van Winden et al. (2002). Subsequently, z-values were evaluated for each compound on the basis of the average values of the time-course data and presented as a heat map in relation to the values determined at t = 0 followed by Euclidean distance-based hierarchical clustering using MeV (Saeed et al., 2003). The processed datasets were also comprehensively visualized on metabolic pathways constructed by using VANTED software (Junker et al., 2006).

3. Results

3.1. Heat map representation of time-series metabolome data from 2SA and 2SD cells

The concentrations of 161 intracellular and 85 medium metabolites including ^{13}C-labeled isotopomers were comprehensively mapped onto

metabolic pathways to enhance viewability (Supplementary Fig. 1A and B for the cell and medium metabolome data, respectively). Overall trends of the intracellular metabolomic changes in 2SA and 2SD cells were analyzed by Euclidean-distance-based hierarchical clustering analysis, and the results were presented as a heat map (Fig. 1), which highlighted the following intriguing features: First, the metabolomic profiles of lactate- and pyruvate-supplied 2SA cells were analogous to each other except for the changes in the metabolites in cluster 2 including glycolytic and PPP intermediates such as sedoheptulose 7-phosphate, dihydroxyacetone phosphate, ribulose 5-phosphate, and glucose 1-phosphate. Second, the metabolomic profiles of the lactate-supplied 2SA and 2SD cells were significantly different, particularly regarding the changes in metabolites in cluster 1, such as essential amino acids and those in cluster 3, such as ATP and 2-oxoglutarate, which showed nearly opposite trends. Third, the trends of the pyruvate-supplied 2SA and 2SD cells, however, significantly resembled each other except for the changes in only a few TCA cycle intermediates and amino acids. This resemblance was made even clearer from the results of the principal component analysis of the time-course metabolome data (Fig. 2A), which illustrates that the metabolomic profiles of lactate-supplied 2SD cells were located in the 3rd quadrant and thus deviated from the other profiles, whereas those of pyruvate-supplied 2SA and 2SD cells were aggregated mostly in the 4th quadrant. Pyruvate administration thus exhibited a dramatic effect on the energy metabolism of the MELAS mutant 2SD cells, redressing their overall metabolomic profile such that it resembled the profile of 2SA cells as a whole.

3.2. Energy status and metabolic parameters

The energy charge in the lactate-supplied 2SD cells decreased to 0.92 in 4 h from the initiation of the experiment but was otherwise maintained at ~0.96 under the other conditions (Fig. 2B). In these lactate-supplied 2SD cells, the levels of ATP and resulting total adenylates also dropped but ADP and AMP levels slightly increased (Fig. 2B), which characterizes a defective energy production in 2SD cells when cultured with a high dose of lactate. A low basal ATP level in 2SD cells was also identified in a previous study of ours (Fujita et al., 2007). Since no significant difference was observed in the reduced- to oxidized-glutathione ratio ([GSH]/[GSSG]) or in the NADPH-to-NADP$^+$ ratio ([NADPH]/[NADP]), the redox status of the 2SD cells appeared to be relatively well-maintained even under lactate-supplied condition (Fig. 2B). In contrast, [Lac]/[Pyr] and [NADH]/[NAD] ratios significantly increased only in the lactate-supplied 2SD cells (Fig. 2B), which implies an enhancement of lactic acidosis, NAD$^+$ shortage, and resulting stagnations of NAD$^+$-dependent reactions. Indeed, levels of fructose 1,6-bisphosphate and dihydroxyacetone phosphate, metabolites upstream of the glyceraldehyde 3-phosphate dehydrogenase reaction, which requires NAD$^+$ as a cofactor, significantly increased in level only in the lactate-supplied 2SD cells (Supplementary Fig. 1A). Glycolytic ATP production thus might have been stagnated due to the high [NADH]/[NAD] ratio. With pyruvate treatment, however, the 2SD cells retained [Lac]/[Pyr] and [NADH]/[NAD] ratios as low as those in 2SA cells (Fig. 2B). Taken together, these results indicate that the energy and redox statuses of 2SA cells were relatively robust in response to a high dose of either lactate or pyruvate whereas those of 2SD cells were vulnerable and susceptible to a high dose of lactate but were well-maintained and greatly approximated to those of 2SA cells as a result of pyruvate treatment, which facilitated efficient ATP production and improved the energy status by decreasing the [Lac]/[Pyr] ratio and maintaining the [NADH]/[NAD] ratio.

3.3. Isotopomer distribution and amino acid metabolism

The changes in lactate or pyruvate metabolism of 2SA and 2SD cells were interpreted by examining the distribution of isotopomers in the cells (Fig. 3) and medium (Supplementary Fig. 2), which were derived from the [3-^{13}C] lactate or [3-^{13}C] pyruvate used to supplement the medium. The initial intracellular lactate level in 2SD cells (at t = 0) was more than twice as high as that in 2SA cells (47.9 and 21.2 fmol/cell, respectively; Fig. 3). The lactate level in 2SD cells abruptly dropped 1 h after the medium replacement but gradually increased afterwards under both lactate- and pyruvate-supplied conditions, whereas that in 2SA cells was almost constant and remained low. The percentage of ^{13}C-labeled lactate to the total lactate in pyruvate-supplied 2SD cells was ~35% and unexpectedly higher than under the other conditions (~25%), which indicates that the proportion of lactate production from the medium-derived [3-^{13}C] pyruvate increased in the pyruvate-supplied 2SD cells. Intracellular pyruvate, in contrast, significantly increased only under the pyruvate-supplied condition (8.7- and 13.2-fold in 4 h in 2SA and 2SD cells, respectively) but was remained low and almost constant in lactate-supplied conditions. Accordingly, the [Lac]/[Pyr] ratio in pyruvate-supplied 2SD cells was lowered not by the decrease in lactate but primarily by the significant increase in pyruvate, which thus boosted the flux from pyruvate to lactate and thereby balanced the [NADH]/[NAD] ratio. In addition, the significant increase in unlabeled pyruvate in pyruvate-supplied 2SD cells implies that pyruvate treatment enhanced not only the influx of pyruvate from the medium into the cells but also the pyruvate production from other routes such as glycolysis. Similar to the trend of the pyruvate level, 2-oxoglutarate, fumarate, and malate levels in 2SD cells were also significantly higher under pyruvate-supplied condition than under the lactate-supplied condition, as were the proportions of ^{13}C-labeled isotopomers. Pyruvate administration thus enhanced the replenishment of these TCA cycle intermediates, which were produced partly from [3-^{13}C] pyruvate (~37% and ~3% in 2SA and 2SD cells, respectively, for 2-oxoglutarate and ~40% and ~18% in 2SA and 2SD cells, respectively, for both fumarate and malate) and largely from other metabolites. Nevertheless, this finding does not necessarily imply that the TCA cycle activity of 2SD cells was enhanced by pyruvate treatment, since ^{13}C$_2$-isotopomers of most TCA cycle-intermediates, which were supposedly produced from [3-^{13}C] pyruvate after the 2nd or more cycles of the TCA cycle, were quantifiable in 2SA cells but were largely below the detection limits in 2SD cells, probably due to the impaired oxidation of NADH by the respiratory chain in the 2SD cells. Intracellular and medium levels of Ala under the pyruvate-supplied condition were also higher than those under the lactate-supplied one, with a high proportion of ^{13}C$_1$-labeled isotopomers in both cell lines (~51% and ~50% in 2SA and 2SD cells, respectively). The increase in the concentrations of both labeled and unlabeled medium Ala in the pyruvate-supplied 2SD cells (Supplementary Fig. 2) is of particular significance among other changes of medium amino acids (Supplementary Fig. 3). These data imply that pyruvate treatment enhanced conversion of [3-^{13}C] pyruvate to ^{13}C$_1$-alanine coupled with the glutamate to 2-oxoglutarate conversion by alanine aminotransferase, which might have contributed to the increase in 2-oxoglutarate (Fig. 3) while excreting Ala into the medium as a byproduct, especially in the pyruvate-supplied 2SD cells. Pyruvate treatment appears also to have enhanced Asp production in both cell lines, but the basal level of Asp was higher in 2SA cells than in 2SD ones. Although the levels of both ^{13}C-labeled and unlabeled Asp isotopomers increased in both cell lines, the ^{13}C$_2$-labeled Asp isotopomer, which would have been produced from ^{13}C$_2$-labeled

Fig. 1. Metabolomic profiles of 2SA and 2SD cells supplemented with 10-mM lactate or 10-mM pyruvate for 161 intracellular metabolites and 85 metabolites in the medium. The metabolite names preceded by "(m)" indicate the medium levels of the corresponding metabolites and the others indicate the intracellular levels. The total concentration of ^{13}C-labeled and -unlabeled isotopomers was used for pyruvate, lactate, phosphoenolpyruvate, Gly, Ala, Ser, Asn, Asp, citrate, isocitrate, 2-oxoglutarate, succinate, fumarate, and malate. Features of the clusters 1–3 are explained in the text.
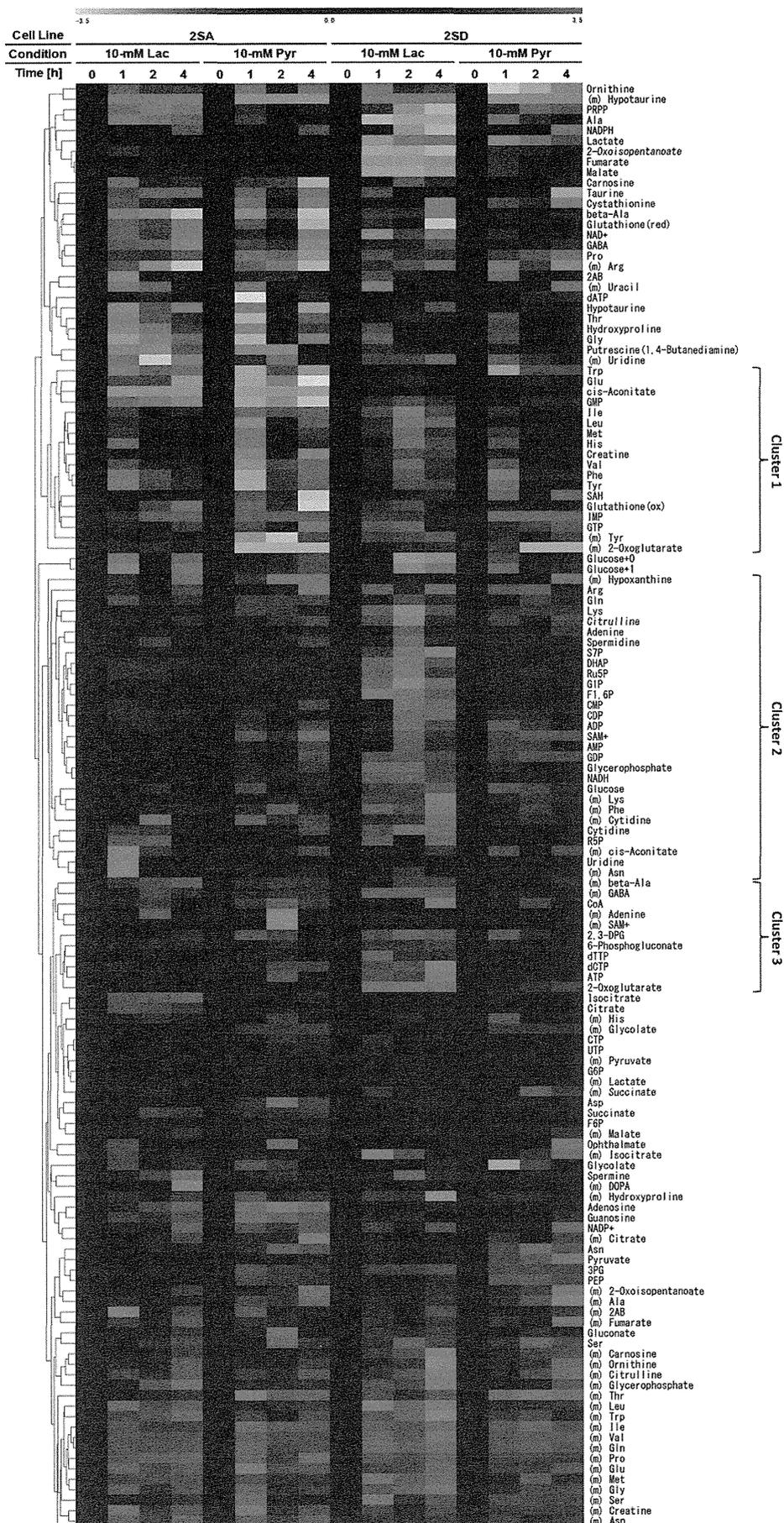
oxaloacetate, for example, was barely observed in 2SD cells, representing again their stagnated TCA cycle activity. Among other amino acids (Fig. 4 for cells and Supplementary Fig. 3 for medium), the Pro level was overall higher in 2SA cells than in 2SD cells. Despite our previous finding of the up-regulation of the asparagine synthetase (ASNS) gene in MELAS mutant cells through the elevation of ATF4 expression and its binding to NSRE-1 (Fujita et al., 2007), the change in the Asn level was not significantly different between cell lines but rather was dependent on lactate or pyruvate treatment in this study. Interestingly, the levels of all the essential amino acids except Thr were slightly but consistently higher in lactate-supplied 2SD cells than in those under the other conditions (Fig. 4), and this trend nevertheless disappeared by pyruvate treatment.

4. Discussion

MELAS syndrome is one of the most frequently occurring, maternally inherited mitochondrial disorders that devastatingly affect multiple organs including brain, nervous system, and muscles, as well as cognitive abilities. Although the efficacy of most treatment regimens has remained limited or doubtful (Sproule and Kaufmann, 2008), pyruvate was recently identified as an effective, safe, and affordable therapeutic agent that exhibits favorable effects on symptoms associated with not only MELAS (Tanaka et al., 2007) but also other mitochondrial diseases (Komaki et al., 2010; Saito et al., 2012). In order to elucidate the therapeutic mechanisms of pyruvate treatment from a viewpoint of energy metabolism, we used CE-TOFMS to investigate the metabolic profiles of the 2SA cells or MELAS mutant 2SD cells treated with 10 mM lactate or 10 mM pyruvate. The results revealed significant differences between the metabolomic profiles of 2SA and 2SD cells under the lactate-supplied condition and contrasting remarkable resemblances under the pyruvate-supplied condition. We previously reported that 2SD cells show up-regulated expression of genes associated with growth arrest (GADD45A, GADD45B, and CHOP) and exhibit a slower growth rate than their parental strain (Fujita et al., 2007). A recent study using human cybrid cells harboring the A3243G mutation also showed an increasing susceptibility of the cells to apoptosis and a high level of mutated mtDNA (Liu et al., 2004). Although no difference was observed between the cell viability of 2SA and 2SD cells in 4 h of culturing under the lactate- or pyruvate-supplied condition in this study (Supplementary Fig. 4), an unsound energy status of 2SD cells characterized by consistently low ATP levels and energy charges was observed under the lactate-supplied condition (Fig. 1), rationalizing their low cell viability in long-term cultures. Due to their limited capacity for oxidative phosphorylation-dependent ATP production, the lactate-supplied 2SD cells might have relied more on GTP and GTP → ATP conversion, which can be backed by relatively high GTP levels and significantly higher GTP-to-ATP ratios ([GTP]/[ATP]), than the cells in the other 3 groups (Supplementary Fig. 4). The increased IMP only in the lactate-supplied 2SD cells suggests a possibility of enhanced purine degradation via AMP deaminase. The energy metabolism of 2SD cells treated with lactate was thus not only defective in maintaining stable energy production but also unordinary in terms of their purine turnover.

The metabolomic profiles of the lactate-supplied 2SD cells exemplified the basal metabolism of MELAS patients with the symptom of lactic acidosis. With impaired mitochondrial complexes and a limited capacity for oxidative phosphorylation, 2SD cells are considered to rely inevitably on enhanced anaerobic glycolysis for ATP generation, which leads to increased flux from pyruvate to lactate and thus exacerbates lactic acidosis. ATP production via glycolysis is known to be stagnated when [Lac]/[Pyr] exceeds 25.6 (Voet and Voet, 1995). In both cell lines, the [Lac]/[Pyr] ratio was maintained below 25.6 under the pyruvate-supplied condition but not under the lactate-supplied one, under which the [Lac]/[Pyr] ratio in the normal 2SA cells was almost constant and ~43.1 but that in 2SD cells continuously increased and eventually exceeded 100 in 4 h. The 2SA cells can potentially shift their metabolism to oxidative phosphorylation for energy production

when glycolysis is stagnated. In contrast, 2SD cells are left with ineffective glycolysis and intrinsically defective oxidative phosphorylation, and this situation impedes the production of sufficient ATP. Under the lactate-supplied condition, the increasing rate of the total (labeled and unlabeled) lactate concentration in the medium, which can be estimated by assuming a linear change in time and considering only the initial and final concentrations, was 731 and 919 fmol/cell/h in 2SA and 2SD cells, respectively, and that of unlabeled lactate in the medium was 765 and 1133 fmol/cell/h in 2SA and 2SD cells, respectively. Thus, the excretion rate of unlabeled lactate, which is probably the by-product of anaerobic glycolysis, appears to have been much higher in 2SD cells than in 2SA cells. Similarly, the decreasing rate (or incorporation rate into the cells) of $^{13}C_1$-labeled medium lactate was estimated and was again much higher in 2SD cells than in 2SA cells (206 and 32.6 fmol/cell/h, respectively), which thus shows a quick turnover of lactate in the lactate-supplied 2SD cells. Even under the pyruvate-supplied condition, the increasing rate of lactate (the total of labeled and unlabeled) was higher in 2SD cells than in 2SA cells (1310 and 676 fmol/cell/h, respectively). Unexpectedly, this increase in 2SD cells was even higher with pyruvate treatment in comparison with lactate treatment, indicating that pyruvate administration did not lower but rather enhanced lactate production. Although the trend of the total pyruvate concentration in the medium in 2SA and 2SD cells was analogous under the pyruvate-supplied condition, the turnover of medium pyruvate was also significantly higher in 2SD cells than in 2SA cells. The decreasing rate of $^{13}C_1$-labeled medium pyruvate was 353 and 536 fmol/cell/h in pyruvate-supplied 2SA and 2SD cells, respectively, while the increasing rate of unlabeled medium pyruvate was 271 and 424 fmol/cell/h in pyruvate-supplied 2SA and 2SD cells, respectively. Accordingly, pyruvate treatment significantly enhanced the incorporation of [3-^{13}C] pyruvate from and the excretion of lactate into the medium of 2SD cells, resulting in a balanced [NADH]/[NAD] and a sustainable ATP production primarily via anaerobic glycolysis in these cells. The [Lac]/[Pyr] ratio was therefore maintained low primarily by the alteration of pyruvate levels rather than lactate levels.

In lactate-supplied 2SD cells, the levels of essential amino acids increased (cluster 1 in Fig. 1), whereas 2-oxoglutarate and nucleoside triphosphates such as ATP, dTTP, and dCTP decreased (cluster 3 in Fig. 1); however, these characteristics were alleviated by pyruvate treatment, and the profile closely resembled that of the pyruvate-supplied 2SA cells. The differences in these metabolite profiles were rather trivial between lactate- and pyruvate-supplied 2SA cells. Supposedly, lactate was actively converted to pyruvate and then to TCA cycle intermediates for efficient oxidative phosphorylation in the 2SA cells, whereas lactate was hardly converted to pyruvate and rather accumulated in and out of the 2SD cells due to a high [NADH]/[NAD] ratio. In short, pyruvate treatment of 2SD cells facilitated their pyruvate-to-lactate conversion, decreased their [Lac]/[Pyr] ratio, and normalized their [NADH]/[NAD] ratio, and thus geared up their glycolysis for boosting ATP production and energy charge, without significantly changing the intracellular lactate level. This important finding implies that the decrease in lactate level is not necessarily essential to ameliorate the energy status of the MELAS mutant cells but rather that the lactate-to-pyruvate ratio and resulting [NADH]/[NAD] ratio are considered to be crucial. TCA cycle intermediates such as 2-oxoglutarate, fumarate, and malate significantly increased in level in the pyruvate-supplied 2SD cells. This increase might have been due to a potentially high activity of succinate dehydrogenase in 2SD cells, since a strong expression of succinate dehydrogenase in blood vessels is a well-known characteristic of MELAS patients (Hasegawa et al., 1991). The $^{13}C_1$-labeled isotopomers of these TCA cycle intermediates derived from ^{13}C-pyruvate accounted for ~2% of 2-oxoglutarate and ~17% of both fumarate and malate, and these proportions did not change considerably over a 4-h period; whereas the increase in unlabeled isotopomers contributed more to the increase in the total levels of these metabolites. Citrate and succinate, however, did not increase as much as the other TCA cycle intermediates in response to pyruvate treatment. These results imply that the addition of
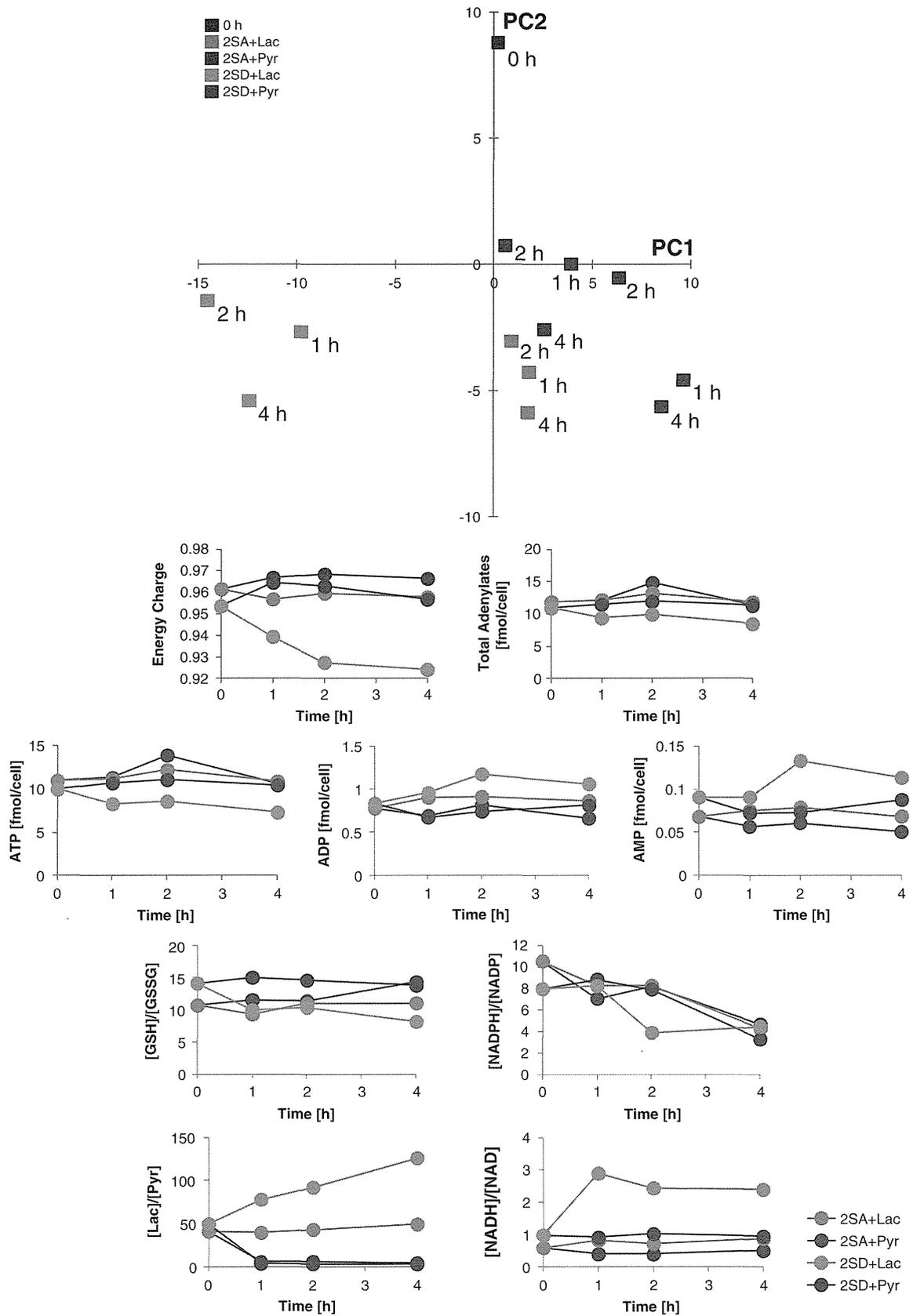
Fig. 2. A. Distributions of the principal component scores 1 (x-axis) and 2 (y-axis) of time-course metabolome data based on the intracellular and medium metabolites in 2SA and 2SD cells cultured with 10 mM lactate or 10 mM pyruvate. B. Time-course changes in metabolic parameters representing the energy status of 2SA and 2SD cells cultured with 10 mM lactate or 10 mM pyruvate. Energy charge was evaluated by $([ATP] + 0.5 \times [ADP])/([ATP] + [ADP] + [AMP])$; and total adenylates indicate the sum of ATP, ADP, and AMP levels.
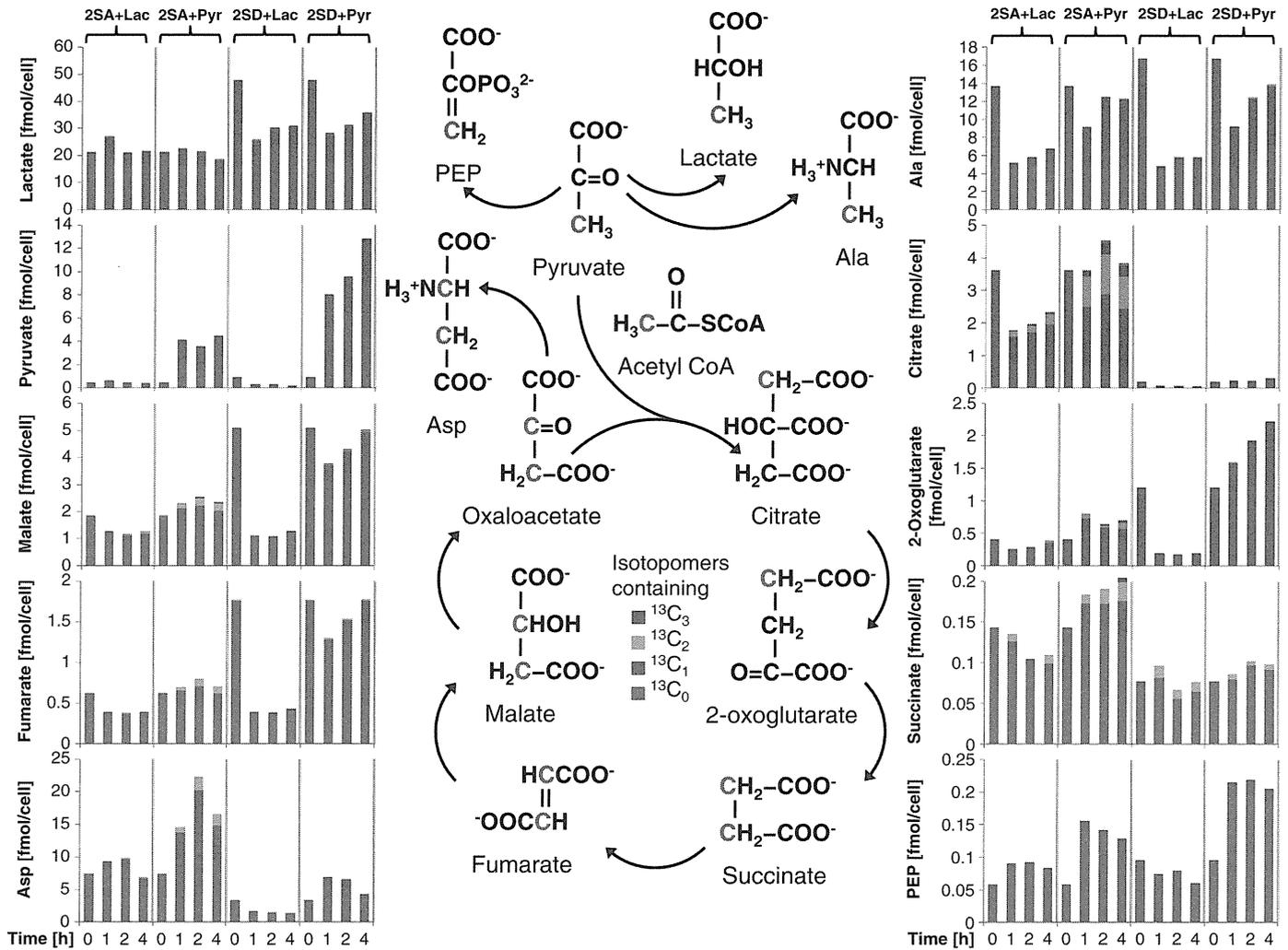
Fig. 3. Time-dependent changes in isotopomer proportions of intracellular pyruvate, lactate, PEP, Ala, Asp, and TCA cycle intermediates in 2SA and 2SD cells cultured with 10 mM lactate or 10 mM pyruvate. The colors of the bars represent the number of ^{13}C replaced with ^{12}C in the metabolites and isotopomers examined. The carbon atoms shown in black and red in each chemical structure represent the expected positions of ^{12}C and ^{13}C, respectively, in the first rotation of the TCA cycle. There are 2 possibilities considered for the ^{13}C position in succinate, fumarate, malate, oxaloacetate, and Asp.

pyruvate increased the metabolic flux not mainly from the pyruvate but from other sources to 2-oxoglutarate, fumarate, and malate. In this perspective, the balanced [NADH]/[NAD] ratio upon pyruvate treatment possibly enhanced the reactions involving NAD^+ as a cofactor. For example, the improved [NADH]/[NAD] ratio would have activated the glyceraldehyde 3-phosphate → 1,3-bisphosphoglycerate reaction by glyceraldehyde 3-phosphate dehydrogenase, which requires NAD^+ as a cofactor, and this would be the key for an optimal glycolytic ATP production in the pyruvate-supplied 2SD cells. In addition, the transaminase reaction involving the oxaloacetate + Glu → Asp + 2-oxoglutarate conversion might have been enhanced and contributed to the increased Asp and 2-oxoglutarate levels in the pyruvate-supplied 2SD cells. Since the malate dehydrogenase reaction also involves NAD^+, the significant increase in Asp, 2-oxoglutarate, fumarate, and malate levels in the pyruvate-supplied 2SD cells might have been due to the enhanced transamination cycle coupled with the urea cycle. Moreover, the glutamate → 2-oxoglutarate reaction by glutamate dehydrogenase and 2-oxoglutarate → succinyl-CoA reaction by 2-oxoglutarate dehydrogenase also require NAD^+ as a cofactor; thus, the activation of these reactions may have facilitated the succeeding succinyl CoA → succinate conversion for GTP production. This replenishment of TCA cycle intermediates and the balanced [NADH]/[NAD] ratio are considered essential for a limited but steady production of ATP via oxidative phosphorylation in 2SD cells, given that the expression of respiratory

complexes I, III, and IV in 2SD cells is known to be decreased but not lost and that the expression of complex V is as high as that in their parental cells (Fujita et al., 2007). Although the pyruvate → acetyl CoA reaction by pyruvate dehydrogenase also involves NAD^+ as a cofactor, the pyruvate treatment did not significantly increase the intracellular citrate level. This result might have been due to a shortage of oxaloacetate, which combines with acetyl CoA for citrate production, or alternatively to a defect in pyruvate dehydrogenase and/or citrate synthase. In fact, pyruvate dehydrogenase deficiency and resulting altered oxidative phosphorylation function have been reported in a MELAS patient (Wilichowski et al., 1998), whereas citrate synthase activity in such a patient is reportedly nearly normal (Yoneda et al., 1989).

In the lactate-supplied 2SD cells, the levels of intracellular essential amino acids such as Ile, Leu, Met, His, Val, and Phe (cluster 1 in Fig. 1) and those of essential amino acids in the medium, such as Lys and Phe (cluster 2 in Fig. 1), accumulated significantly. The alteration of intracellular free amino acid pools in MELAS mutant cells was proposed based on our previous finding that ASNS gene expression is up-regulated in these cells (Fujita et al., 2007). MELAS mutant cybrids are known to exhibit autophagic cell death triggered by a combination of nitrosative and metabolic stress (Sandhu et al., 2005); thus, 2SD cells may have a constitutively high level of autophagic activity, and this might also have contributed to the generation of free amino acids by autophagic degradation of proteins and the resulting accumulation of essential