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Increased ROS generation and SOD activity in heteroplasmic tissues of transmitochondrial mice with A3243G mitochondrial DNA mutation

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ABSTRACT. The mitochondrial A3243G tRNALeu(UUR) mutation associated with a variety of mitochondrial disorders results in a severe respiratory deficiency, an increase in reactive oxygen species (ROS) production and activities of anti-oxidative enzyme *in vitro*. However, the phenotypic implications of this mutation have not been described *in vivo*. Here, mitochondria carrying A3243G transition from the peripheral blood of diabetes mellitus patients were microinjected into zygotes. Influence of this mutation on mitochondrial respiratory enzyme activities, ROS generation, and anti-oxidative enzyme activities in the heteroplasmic tissues of transmitochondrial mice was evaluated. The chimeric mice exhibited a subtle impaired oxidative phosphorylation, reduced activity of complex I/IV, increased activity of superoxide dismutase, and in turn, enhanced ROS generation. Our results suggest that mitochondrial

A3243G mutation may be responsible for the high ROS production *in vivo*. Increased generation of ROS caused by mtDNA mutation may also play a role in the pathogenesis of the A3243G mutation-associated diseases.

Key words: Mitochondria; Mitochondrial DNA; A3243G mutation; Mitochondrial disorders; Transmitochondrial mice; Transmitochondrial

INTRODUCTION

Mitochondrial dysfunction caused by mitochondrial DNA (mtDNA) mutations is associated with a wide spectrum of human diseases. Although over 100 (Meulemans et al., 2006) types of different mtDNA mutations have been documented, point mutations in mitochondrial tRNA are particularly frequent (Kasraie et al., 2008). Among them, the mitochondrial A3243G tRNALeu(UUR) mutation is the most frequent (Urata et al., 1998) and has proven to be associated with various of single- or multi-organ syndromes, such as mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes, etc. (Janssen et al., 2006; Mkaouar-Rebai et al., 2007). The occurrence of more than one mtDNA sequence variants in a single cell or individual is called as 'heteroplasmy'. The ratio of normal/mutant mtDNA determines the onset of mitochondrial diseases or disorders. When mutated mtDNA exceeds a minimum critical level or so-called "threshold", deleterious effects become possible (McKenzie et al., 2004; Gardner et al., 2007). Although heteroplasmy or threshold can partly explain the pathological phenotypes observed in patients, emerging evidence suggests no definite correlation between the clinical severity and the proportion of mtDNA mutation (Wang et al., 2002).

Apart from ATP generation, mitochondria are also the major source of reactive oxygen species (ROS) (Petrozzi et al., 2007). Emerging evidence from patients and *in vitro* studies suggests that mitochondrial dysfunction, either a primary (e.g., respiratory chain abnormalities) or a secondary (elevated ROS and control of apoptosis) event, plays an important role in the pathogenesis of mtDNA-based diseases (Ishikawa et al., 2005; Vives-Bauza et al., 2006; McKenzie et al., 2004, 2007). Patients carrying A3243G mutation exhibited an impaired oxidative phosphorylation with a deficient activity of respiratory chain complexes, increased ROS production, and significant induction of heme oxygenase-1 (Ishikawa et al., 2005). *In vitro* studies revealed the A3243G mutation impaired RNA processing, aminoacylation, post-transcriptional tRNA modification, and translation (Mkaouar-Rebai et al., 2007). Transmitochondrial cybrids carrying A3243G showed decreased growth rate, lower oxygen consumption, defect complex I/IV, reduced electronic transfer chain activities and ATP synthesis, increased ROS production, decreased superoxide dismutase (SOD), and other antioxidant defense systems (Vives-Bauza et al., 2006; McKenzie et al., 2007). While A3243G mutation has been linked with mitochondrial dysfunction *in vitro*, the pathogenesis of A3243G mutation in animal models has not been investigated due to the limitation of transmitochondrial techniques.

In this study, the deleterious effects of A3243G mutation on mitochondrial respiratory, ROS generation, and anti-oxidative enzymatic activities in the heteroplasmic tissues (hearts and brains) of transmitochondrial mice model were elucidated. Our results

showed that the heteroplasmic tissues carrying A3243G mutation exhibited a subtle mitochondrial respiratory deficiency and a mild reduction of the complex I/IV enzymatic activities, indicating that the A3243G mutation is responsible for the respiratory deficiency and the subsequent high ROS generation and increased SOD activity.

MATERIAL AND METHODS

Animals

C57BL/6J female mice were used to generate zygotes via superovulation. ICR female mice were used to produce pseudo-pregnant mice. All animals were housed in groups under an artificial 12:12 lighting conditions with food and water available *ad libitum* and were maintained in a specific pathogen-free barrier facility. All procedures adhered to the Chinese Veterinary Medical Association Guide.

Development of chimeric mice

Mitochondria with A3243G mutation were isolated from the peripheral blood of diabetes mellitus patients following a method described by Pinkert et al. (1997). Briefly, tissue samples were suspended in 1X MS buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 7.5), homogenized for 20 times, and centrifuged at 1500 *g* for 10 min. The supernatant was then spun at 9800 *g* for 10 min to pellet mitochondria and the pellet was washed with 150 mM KCl solution, recollected by centrifuging at 9800 *g* for 10 min, and resuspended in 40 µL 150 mM KCl. Microinjections were performed using inverted microscope (Pinkert et al., 1997).

All founders were genotyped by polymerase chain reaction (PCR) followed with *Apa*I digestion. The primers 5' CCT CCC TGT ACG AAA GGA CA 3' at position 3116-3135 and 5' GCC TAG GTT GAG GTT GAC CA 3' at position 3667-3647 were used for PCR (Zhang et al., 2005). The thermal profile consisted of denaturation of DNA at 95°C for 45 s, annealing of DNA with primer at 56°C for 45 s, and primer extension at 72°C for 56°C. Amplification was usually done for 30 cycles and PCR-amplified DNA fragment was digested with the restriction enzyme *Apa*I (Promega). The *Apa*I-digested PCR products were then subjected to electrophoresis on a 1.5% agarose gel.

Measurement of respiratory chain complex I-IV activities, ROS production, and antioxidant enzyme activities

The respiratory chain complexes were assessed as described previously (Lionetti et al., 2004; Panov et al., 2005; Bonora et al., 2006; Meany et al., 2006; Long et al., 2007). For the assessment of ROS production, tissue samples were washed with PBS, digested with 0.5% trypsin and 0.1% collagenase for 40 min, centrifuged at 600 rpm for 5 min and resuspended in D-Hank's buffer. Approximately 1×10^6 cells were washed with PBS and resuspended in D-Hank's buffer. Forty microliters dichlorofluorescein diacetate (DCFH-DA; 2.5 mmol/L) was added to each sample and incubated for 30 min at 37°C in the dark. Fluorimetric analysis was performed at 488 nm excitation and 535 nm emission.

The antioxidant enzyme activities were measured using an SOD, glutathione peroxidase (GSH-Px), and catalase activity detection kit (Nanjing Jiancheng Bioengineer Institute, China) following the manufacturer instructions.

Statistical analysis

The results are reported as the mean ± SD. Statistical analysis was performed using the Student *t*-test.

RESULTS

A3243G mutation in tissues of transmitochondrial mice

To generate transmitochondrial mice carrying A3243G mutation, the mutated mitochondria isolated from diabetes mellitus patients were microinjected into C57BL/6J mice. Previous studies (Zhang et al., 2005) reported that the A3243G mutation, but not the wild-type mtDNA, can be recognized by *Apa*I. Therefore, restrict enzyme digestion by *Apa*I was performed to identify the positive mice. As shown in Figure 1, the digestion of mitochondria isolated from the transmitochondrial mice generated 423- and 130-bp fragments, respectively (Figure 1, lanes 2, 3 and 4). The mutant mtDNA was observed in the hearts and brains of two heteroplasmic mice (data not shown). However, due to the relatively poor transferring rate, only two male animals carrying the mutant mtDNA were obtained.

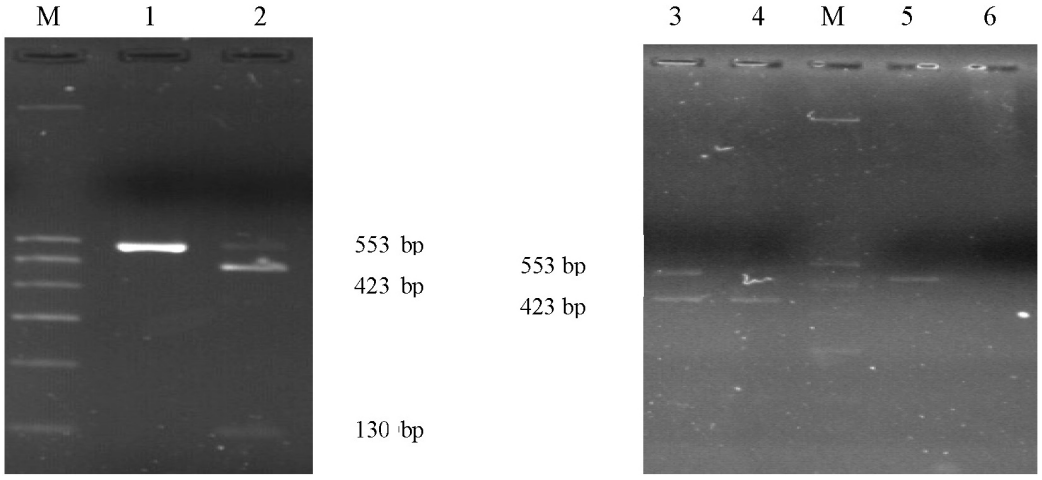

Figure 1. Genotype analysis of chimera mice by polymerase chain reaction (PCR) and *Apa*I digestion. mtDNA fragment amplified by PCR was digested with *Apa*I and size fractionated on agarose gel. *Lanes 1* and *5* = restriction pattern of control individual; *lane 2* = diabetes mellitus patient with A3243G mutation; *lanes 3* and *4* = chimera mice carrying A3243G mutation; *lane 6* = wild-type C57BL/6J mouse. M = marker.

Chimera mice carrying A3243G mutation exhibited low mitochondrial complex I and IV activities

To address if A3243G mutation in the heteroplasmic hearts and brains influences mitochondrial respiratory chain function, the enzymatic activities of complex I (NADH: ubiquinone oxidoreductase), II (succinate:ubiquinone oxidoreductase), III (ubiquinol:cytochrome c oxidoreductase), and IV (cytochrome c oxidase) in chimeric mice (N = 2) and age-matched wild-type C57BL/6J mice (N = 5) were assayed. As shown in Table 1, in terms of respiratory enzymatic activities, no significant difference between chimeric mice and controls was observed (Table 1), probably due to small samples used. However, the heteroplasmic heart and brain tissues exhibited mild decrease of complex I and IV activities, while the activity of complex II and complex III remained unchanged (Table 1). As shown in Table 1, activity of complex I was reduced by 25% in the heteroplasmic hearts (P = 0.072) and by 32% (P = 0.106) in the heteroplasmic brains. Activity of complex IV was reduced by 27% in the heteroplasmic hearts (P = 0.105) and by 40% in the brains (P = 0.092), compared with control tissues. These data suggested that the A3243G mutation in the heteroplasmic tissues caused a subtle defect in respiratory chain function, and the severity of mitochondrial respiratory deficiency in the brains was higher possibly owning to the higher proportion of mutant mtDNA. The finding of impaired respiratory function in the chimeric mice is in agreement with previous reports that the primary consequence of this mutation is a reduction in combined complex I/IV activities (Ishikawa et al., 2005; McKenzie et al., 2004, 2007).

Table 1. Activities of mitochondrial complex I-IV in the heteroplasmic tissues of chimera mice.

Group	Heart				Brain			
	Complex I	Complex II	Complex III	Complex IV	Complex I	Complex II	Complex III	Complex IV
C57/BL6J (N = 5)	33.91±3.99	72.82±14.26	1368±143	3595±624	23.19±4.73	72.17±11.4	1323±115	1911±453
Chimera mice (N = 2)	25.28±6.21	82.69±4.15	1383±108	2628±397	15.82±4.84	78.03±3.30	1321±51	1151±359
P value	0.072	0.402	0.907	0.105	0.106	0.527	0.986	0.092

Values are reported as $nmol \cdot min^{-1} \cdot mg^{-1}$ Pro.

Chimeric mice carrying A3243G mutation exhibited increased ROS production and anti-oxidative enzymatic activities

We next asked if A3243G mutation influences ROS synthesis *in vivo*. To this end, ROS levels, SOD, GSH-Px, and catalase activities in the heteroplasmic heart and brain tissues were detected. As shown in Table 2, ROS level increased 14% (P = 0.139) in the heteroplasmic heart and 18% (P = 0.059) in the heteroplasmic brain tissues, compared with controls. At the same time, total SOD activity in the heteroplasmic heart and brain tissues increased by 18% (P = 0.093) and 19% (P = 0.114), respectively (Table 3). To our surprise, the GSH-Px and catalase activities remained unchanged (Table 3). Taken together, these data indicate that the A3243G mtDNA mutation increases ROS generation and total SOD activity in the heteroplasmic tissues.

Table 2. Reactive oxygen species (ROS) levels in the heteroplasmic tissues of chimera mice.

Group	ROS production (DCF FI)	
	Heart	Brain
C57/BL6J (N = 5)	53.56 ± 4.13	51.95 ± 3.43
Chimera mice (N = 2)	60.91 ± 7.55	61.24 ± 7.52
P value	0.139	0.059

Values are reported as fluorescence intensity $\cdot 1 \times 10^6$ cells^{-1}. DCF FI = dichlorofluorescein fluorescence intensity.

Table 3. Activities of anti-oxidase enzyme in the heteroplasmic tissues of chimera mice.

Group	SOD		GSH-Px		Catalase	
	Hearts	Brains	Hearts	Brains	Hearts	Brains
C57/BL6J (N = 5)	49.04 ± 6.28	117.53 ± 11.57	22.52 ± 6.17	29.11 ± 5.39	3.10 ± 0.59	4.77 ± 0.41
Chimera mice (N = 2)	58.38 ± 3.63	138.91 ± 14.95	21.67 ± 1.82	25.58 ± 2.79	3.34 ± 0.18	5.03 ± 0.11
P value	0.114	0.093	0.863	0.435	0.613	0.43

Values are reported as U·mg Pro^{-1}·min^{-1}. SOD = superoxide dismutase; GSH-Px = glutathione peroxidase.

DISCUSSION

mtDNA mutations cause a wide range of clinical phenotypes characterized by mitochondrial respiratory chain dysfunctions. However, it still remains controversial whether mutated mtDNA would contribute to ROS generation. Even though several previous studies have shown that mtDNA mutation is related to augmented ROS production (Lionetti et al., 2004; Petrozzi et al., 2007; Carelli et al., 2007), controversial results have also been reported. For example, it has been shown that mice with mutant mtDNA do not exhibit increased ROS levels in spite of carrying a high mutational burden (Meissner, 2007). Another report has demonstrated that mutant cybrids carrying A3243G mtDNA from a patient suffering from MELAS have reduced activity of cytochrome c oxidase significantly, lower ATP level and decreased mitochondrial membrane potential, but the endogenous levels of ROS are very similar in all cybrids regardless of whether they carry the mtDNA defects or not (Sandhu et al., 2005). Therefore, up to date the interplay between A3243G mtDNA mutation and ROS has not been fully clarified.

We reported here that A3243G mutation is responsible for the impaired respiration enzymatic activities, increased ROS generation and SOD activity in the heteroplastic tissues of transmitochondrial mouse model. These results strongly suggest that A3243G mtDNA mutation is linked with the disorder of ATP and ROS generation *in vivo*. Therefore, the mitochondrial dysfunction is a primary event in the A3243G mutation-based diseases' process. Dysfunction of the mitochondrial respiratory chain leading to decrease of energy production primarily affects tissues with high energy requirements and renders cells unable to adapt to conditions of reduced mitochondrial energy supply (Janssen et al., 2006). This might explain why the deleterious effects of A3243G mutation were detected both in brains and hearts, but not in other heteroplasmic tissues such as the kidneys and spleens.

Mitochondria are not only a major source of ROS, but they also possess vigorous antioxidant defense including free radical scavengers and antioxidant enzymes such as SOD and GSH-Px (Hervouet et al., 2007). Besides the energy defect, A3243G mutation also causes ROS associated diseases' pathophysiology (Ishikawa et al., 2005; Vergani et al., 2007). For example, the A3243G mutation has been shown to increase total SOD and catalase activities in myoblasts (Rusanen et al., 2000). Increased ROS production was also reported in the endomyocardial tissues of a patient with A3243G mutation (Maechler and de Andrade, 2006). In agreement with the findings cited above, we observed enhanced ROS generation and SOD activity in the chimeric mouse models carrying A3243G mutation, but no change of GSH-Px and catalase activities was observed. SOD is an essential enzyme detoxifying superoxide radicals to hydrogen peroxide. Increase in SOD may result in the enhanced formation of hydrogen peroxide. This combined with unchanged in catalase activity could lead to increased hydrogen peroxide accumulation and thus inducing oxidative stress. The elevated ROS generation detected by DCFH-DA may be interpreted as increased oxidative stress in the heteroplasmic tissues. Therefore, ROS is a secondary event in the pathogenesis of A3243G mutation. The increase in ROS production in the heteroplasmic tissues is compatible with the decrease in activity of complex I, of which the flavin mononucleotide group has been proposed as the main physiologically relevant site of ROS production (Wei et al., 2004). The complex I inhibitor rotenone has been shown to increase H_2O_2 generation in prepared mouse mitochondria from the brain, heart, but not kidney (Beretta et al., 2006; Carelli et al., 2007). Comparative expression in warts and lymphocyte defect induces mitochondrial outgrowth as a consequence of increase ROS production (Bonora et al., 2006). It is therefore tempting to speculate that the clinic phenotypes of patients with A3243G mutation are in part due to stimulation of mitochondrial proliferation by ROS.

Taken together, these data support the "vicious" circle hypothesis. The A3243G mutations affect synthesis of respiratory chain subunits, resulting in respiratory dysfunction and enhanced ROS production. In turn, increased ROS production induces damage to lipids, proteins and DNA and enhances mtDNA mutations. This vicious cycle is proposed to cause an exponential increase of mtDNA mutations over time, leading to diseases (Trifunovic et al., 2005; Petrozzi et al., 2007; Long et al., 2007; Meissner, 2007).

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