Tetrahydropteridine deficiency impairs mitochondrial function in *Dictyostelium discoideum* Ax2

Hye-Lim Kim\(^{a,1}\), Yong Kee Choi\(^{a,1}\), Do Hyung Kim\(^{a}\), Sun Ok Park\(^{a}\), Jin Han\(^{b}\), Young Shik Park\(^{a,}\ast\)

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**Abstract** A putative cellular function of tetrahydropteridines (L-erythro-tetrahydrobiopterin and D-threo-tetrahydrobiopterin) was investigated in *Dictyostelium discoideum* Ax2 using a mutant disrupted in the gene encoding sepiapterin reductase (SR). The SR mutant, which produces about 3% of tetrahydropteridines if compared to wild-type, was elucidated to have several functional defects related to mitochondrial and oxidative stress: retarded growth, poor spore viability, impaired mitochondrial function, and increased susceptibility to oxidative stress induced by hydroxyamphetamine or cumene-hydroperoxide. However, the physiological defects were almost completely rescued by extrachromosomal expression of *Dictyostelium* SR. The results strongly suggested that tetrahydropteridines in *Dictyostelium* are associated with mitochondrial function, probably via direct protection against oxidative stress.

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**Keywords:** Tetrahydrobiopterin; Tetrahydrodictyopterin; Antioxidant; Oxidative stress; Mitochondria; Spore viability

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1. Introduction

A multicellular slime mold *Dictyostelium discoideum* Ax2 is notorious for the synthesis of high amounts of L-erythro-tetrahydrobiopterin (BH4) and its isomer D-threo-BH4 (DH4), although their function(s) remained unknown [1,2]. In higher animals, BH4 is a well-known cofactor for aromatic amino acid hydroxylation and nitric oxide synthesis, which are crucial for neuropsychiatric and endothelial functions [3]. BH4 also suggested that tetrahydropteridines in *Dictyostelium* are associated with mitochondrial function, probably via direct protection against oxidative stress.

Considerable attention has been focused on the role of BH4 in the pathologic states of endothelial and neuronal cells developing under oxidative stress [4]. As a tight regulator of NOS, BH4 deficiency causes NOS uncoupling, where NOS transfers electron to molecular oxygen to form oxidant species such as superoxide and peroxynitrite instead of nitric oxide (NO) production. The reactive oxygen species (ROS) further increase oxidative stress, which induces mitochondrial impairment, finally leading to endothelial dysfunction. NOS uncoupling also increases neuronal vulnerability to hypoxia [5]. On the other hand, BH4 was also reported to exert direct protective effect against the cell injury induced by H\(_2\)O\(_2\) in endothelial cells [6] and against mitochondrial superoxide in dopaminergic neurons [7]. Its physiological significance, however, remains elusive because of low in situ concentration of BH4.

In *Dictyostelium* DH4 is dominant over BH4, constituting more than 95% of the total and the intracellular concentration is at least two orders of magnitude higher than that of BH4 in animal cells [2], possibly indicating that the amount is more than enough solely for catalytic function. In addition, so far animal NOS activity has not been found in *Dictyostelium* and the finished genome sequence [8] did not reveal any NOS homolog. Therefore, it was postulated that tetrahydropteridines in *Dictyostelium* might have NOS-independent antioxidant function.

We previously created a *Dictyostelium* knockout mutant disrupted in the gene encoding sepiapterin reductase (SR), the enzyme catalyzing the last step of BH4 synthesis [2]. The mutant was found viable in spite of remarkable decrease in tetrahydropteridine synthesis to about 3% of wild-type (18.1% of BH4 and 0.6% of DH4) [2,9,10]. The mutant was rescued with *Dictyostelium* SR to recover normal pteridine synthesis (72% of BH4 and 99.4% of DH4) [11]. In a purpose of elucidating the putative cellular function of tetrahydropteridines, we investigated the putative functional defects of SR mutant and report here the results demonstrating mitochondrial dysfunction and increased susceptibility to ROS.

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**Abbreviations:** BH4, L-erythro-tetrahydrobiopterin; DH4, D-threo-tetrahydrobiopterin; SR, sepiapterin reductase; NO, nitric oxide; ROS, reactive oxygen species

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2. Materials and methods

2.1. Cells and growth conditions

Axenic strain Ax2 cells were grown at 22 °C in HL5 medium (15.4 g glucose, 7.15 g yeast extract, 14.3 g protease peptone, 0.485 g KH\(_2\)PO\(_4\), 1.28 g Na\(_2\)HPO\(_4\), 12H\(_2\)O, pH 6.5, per liter) with 100 μg/ml streptomycin sulfate and 100 U/ml benzylpenicillin potassium [12]. The SR
knockout mutant (spr−) was grown in HL5 containing 10 μg/ml Blasticidin S. The SR mutant rescued with Dictyostelium SR (spr+) was grown in HL5 containing 10 μg/ml G418. The mutants were created in the previous research [2,11].

2.2. Developmental condition

Cells growing exponentially in axenic HL5 (2·3·10⁶ cells/ml) were harvested by centrifugation at 350 x g for 3 min, resuspended in 12 mM cold phosphate buffer, pH 6.5 (PB), and washed three times. Finally, the cell suspension was adjusted to a density of 1·10⁶ cells/ml and deposited for 24 h onto a Whatman #50 filter paper, which was placed on a non-nutrient agar plate containing PB.

2.3. Spore viability assay

Two days after fruiting bodies appeared, spores were harvested by banging the inverted plates on the bench and collected with cold PB. The spore suspension was adjusted to 1·10⁶ cells/ml. An aliquot of a hundred spores was incubated with 1 ml Escherichia coli at 37 °C for 30 min to germinate and then spread on a nutrient agar plate [13]. After incubation for 3 days at 22 °C, plaques were counted as viable spores. Ovoid phase-dark spores were also counted with a hemacytometer under phase contrast microscope. Protein concentration was determined using Bradford reagent.

2.4. Oxidative stress assay

Exponentially growing cells (1.5·10⁶ cells/ml) were mixed with hydroxylamine (0·2 mM) or cumene-hydroperoxide (0·2 mM) for 1 h, washed twice with HL5, and incubated at 22 °C for 12 h with shaking at 150 rpm. Viable cells were counted by Trypan blue exclusion method. 0·5 ml of cell suspension was mixed with 0·1 ml of 0·4% trypan blue dye in phosphate buffered saline. After 5 min the cells were examined for dye penetration with a light microscope.

2.5. Analyses of mitochondrial transmembrane potential and H₂O₂ production

JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanide iodide) was used to evaluate the membrane potential. To remove indigenous fluorescent molecules in cells, cells (2–3·10⁶ cells/ml) were incubated in same medium for 30 min. Cells were stained in the same medium containing 5 μM JC-1. The JC-1 stained cells were analysed by flow cytometry under phase contrast microscope. Protein concentration was determined using Bradford reagent.

2.6. Measurement of cytochrome c oxidase activity

Cytochrome c oxidase (COX) of the electron transport chain complex IV was assayed using a kit (CYTOCOX-11) from Sigma following the product manual. Isolated mitochondria were disintegrated in 1 ml n-dodecyl β-maltoside. The reaction mixture containing 10 mM Tris–HCl, pH 7·0, 120 mM KCl, 10 mM cytochrome c, and mitochondrial homogenate equivalent to 5 μg protein was incubated at 25 °C. The oxidation of cytochrome c was measured by absorbance decrease at 550 nm for 2 min. One unit was defined as 1·0 μmol of oxidized ferricytochrome c per min at 25 °C. Mitochondria were prepared from the exponentially growing cells using a Sigma kit (MITO-ISO1).

2.7. Quantitative real-time PCR

The ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) was determined by quantitative real-time PCR, essentially as described by others. Total DNA was isolated from exponentially growing cells by using LaboPass Genomic DNA isolation Kit (Cosmo Co. Ltd., Korea). The nuclear gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the mitochondrial gene cytochrome c oxidase subunit I (COX-I) were quantified separately using Rotor-Gene 3000 (Corbett Life Sci., Australia). The PCR reactions were carried out using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). A typical 10 μl reaction mixture contained 5 μl SYBR Green PCR Master Mix, 500 ng DNA, 0·5 μmol of specific primers: GAPDH upper, 5′-aactgactcccaataaag; GAPDH lower, 5′-cctgaaggctgtttaaa; COX-I upper, 5′-aacaataacggtggaacg; COX-I lower, 5′-ttaaattgaeecccaacag. An initial denaturation step (10 min 95 °C) was followed by 40 cycles (30 s 95 °C, 30 s 55 °C, 20 s 72 °C).

2.8. Statistical analysis

All the experiments described above were performed at least in triplicates and the results were expressed as means ± S.D.

3. Results

3.1. Growth and spore viability

We observed growth and development of SR mutant in comparison with wild-type Ax2 and the rescued SR mutant. SR mutant reproducibly exhibited retarded growth during exponential phase, while the rescued mutant completely recovered to normal (Fig. 1). The development of SR mutant looked normal, completing in 24 h with normal appearances (data not shown). However, the number of phase-dark spores of unhealthy character increased in SR mutant but returned to wild-type level in the rescued mutant (Fig. 2A). The appearance of phase-dark regions in non-viable spores was known to originate from the damaged mitochondria [14], strongly suggesting that SR mutant cells may have mitochondrial dysfunction. The putative problem in SR mutant spores was confirmed by spore viability assay (Fig. 2B): SR mutant spores survived much less, whereas the rescued mutant recovered almost. As it was reported that DH4 synthesis occurs during spore germination in Dictyostelium [15], we further examined spore viability of SR mutant after supplementing with either BH4 or DH4. They did not improve the spore viability at all (Fig. 2C), implying that the poor viability of SR mutant spores was not attributable simply to tetrahydropteridine deficiency itself. Taken together, these functional observations strongly

Fig. 1. Analysis of vegetative growth. The cells equivalent to 2·10⁶ were grown in HL5 medium and the growth was measured by counting cells using hemacytometer.

suggested that SR mutant has an irreversible problem in mitochondria.

3.2. Mitochondrial function

In order to examine mitochondrial dysfunction in SR mutant we carried out a series of experiments. Vegetative cells harvested from exponential growth were stained with JC-1 and counted using FACS (Fig. 3A). The cells exhibiting higher transmembrane potential was a little less in SR mutant than wild-type and the rescued mutant. In consistent with the result, COX activity was significantly lower in SR mutant (Fig. 3B), clearly supporting that mitochondria are impaired in SR mutant. Since mitochondrial dysfunction was well-known to accompany oxidative stress, resulting in reduced mitochondrial DNA abundance, we subsequently determined cellular ROS using DCFH-DA staining and mtDNA abundance by quantitative real-time PCR. Compared with wild-type and the rescued, a larger amount of SR mutant cells displayed higher level of fluorescence intensity (Fig. 3C), attesting increased cellular ROS. The copy number of COX-I mtDNA relative to GAPDH nDNA was also less in SR mutant (Fig. 3D). Thus, the results verified mitochondrial dysfunction in SR mutant.

3.3. Oxidative stress

The above results implicated a protective function of tetrahydropteridines against the oxidative damage to mitochondria. In order to see the antioxidant role, we compared the sensitivities of SR mutant and wild-type cells to hydroxylamine or cumene-hydroperoxide (Fig. 4): hydroxylamine acts as an inhibitor of catalase, thereby increasing in situ hydrogen peroxide [16], while cumene-hydroperoxide causes direct ROS increase and lipid peroxidation [17]. In a preliminary experiment with different concentrations of the chemicals, SR mutant was about two times more vulnerable than wild-type and cumene-

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Fig. 2. Spore viability. (A) Phase-dark spores counted under phase contrast microscope. (B) Viability measured after germination. An aliquot of a hundred spores was incubated with E. coli to germinate and then spread on a nutrient agar plate to count plaques on the bacterial lawn. (C) Viability of SR mutant spores after supplementation with BH4 or DH4.

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Fig. 3. Mitochondrial function of vegetative cells. (A) Mitochondrial transmembrane potential measured by JC-1 staining. The active red fluorescent cells were analyzed at 575 nm using FACS. (B) Cytochrome c oxidase assay. The oxidation of cytochrome c was measured by absorbance decrease at 550 nm. (C) ROS measured by DCFH-DA staining. The fluorescent cells were analyzed at 525 nm using FACS. (D) Quantitative real-time PCR. The nuclear GAPDH gene and the mitochondrial COX-I gene were quantified separately using Rotor-Gene 3000 (Corbett Life Sci., Australia). Quantitative results were normalized for the level of GAPDH from wild-type.
ROS (Fig. 4). The functional role, as a direct ROS scavenger, idines by presenting increased vulnerability of SR mutant to [18,19].

Incorporation of tetrahydropteridines in Dictyostelium might have deteriorated energy metabolism to exhibit defects of mtDNA-encoded gene expression and respiratory chain complex enzymes. Mitochondrial dysfunction in SR mutant might have deteriorated energy metabolism to exhibit the delayed growth rate (Fig. 1) and the increased spore sterility (Fig. 2). In support of this, Dictyostelium cells treated with ethidium bromide, a potent inhibitor of mtDNA replication and transcription, exhibited delayed growth and incomplete development depending on the concentration [18,19].

We demonstrated an antioxidant function of tetrahydropteridines by presenting increased vulnerability of SR mutant to ROS (Fig. 4). The functional role, as a direct ROS scavenger, was already assumed from the results of mitochondrial dysfunction (Fig. 3). Insufficient tetrahydropteridines in SR mutant would have generated an oxidative cellular environment, which was detrimental to mitochondria. Together with the results, the high intracellular concentration of tetrahydropteridines may also signify such a quantitative role as an antioxidant against endogenous or exogenous ROS. Although NO generation was shown in Dictyostelium [20], no NOS activity or protein homolog has been found so far. In the absence of NOS, a major endogenous ROS source in Dictyostelium may be mitochondria. Interestingly, Dictyostelium usually resists to ultraviolet (UV)-light, γ-radiation, and DNA-damaging chemicals [21]. ROS are not only produced endogenously as by-products of normal aerobic metabolism but also can result from exposure to exogenous agents such as UV, γ-radiation, and a wide variety of environmental oxidants [22]. Dictyostelium might have evolved to utilize tetrahydropteridines as an antioxidant.

Taken together, we elucidated a cellular function of tetrahydropteridines in Dictyostelium by demonstrating mitochondrial dysfunction and increased susceptibility to ROS in SR mutant, which produces about 3% of tetrahydropteridines in wild-type. The results strongly suggest that tetrahydropteridines in Dictyostelium have a direct antioxidant function to protect mitochondria against oxidative stress. This is the first experimental evidence demonstrating the functional role of tetrahydropteridines in association with mitochondria and oxidative stress in Dictyostelium.

4. Discussion

Through comparative investigation of SR mutant with wild-type, we found that tetrahydropteridine deficiency in Dictyostelium resulted in decreased vegetative growth (Fig. 1), reduced spore viability (Fig. 2), impaired mitochondrial function (Fig. 4), and increased vulnerability to oxidative stress (Fig. 3). These physiological defects disappeared near completely in the rescued SR mutant, validating the functional consequences of tetrahydropteridine deficiency.

The mitochondrial dysfunction in SR mutant was verified by several lines of evidence. Both mitochondrial transmembrane potential (Fig. 3A) and COX activity decreased (Fig. 3B). ROS generation was higher (Fig. 3C) but mtDNA/nDNA ratio was lower (Fig. 3D). There is accumulation of evidence that mitochondria are a major cellular source of ROS and could also be a major target for ROS-mediated damage, inducing mtDNA damage and consequently leading to defects of mtDNA-encoded gene expression and respiratory chain complex enzymes. Mitochondrial dysfunction in SR mutant might have deteriorated energy metabolism to exhibit the delayed growth rate (Fig. 1) and the increased spore sterility (Fig. 2). In support of this, Dictyostelium cells treated with ethidium bromide, a potent inhibitor of mtDNA replication and transcription, exhibited delayed growth and incomplete development depending on the concentration [18,19].

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