Effects of H₂O₂ Treatment of Mouse B82 Cells on mtDNA Amounts and Respiratory Function

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[Received May 20, 2005 ; Accepted December 15, 2005]

A mouse fibroblast cell line, B82, was treated with H₂O₂ for examination of the damage to mtDNA and to respiratory function. The results showed that the copy numbers of intact mtDNA molecules reduced rapidly during 15 minutes H₂O₂ treatment, while reduction of respiratory function was observed after 24 hours. The presence of such a time lag, as observed between the damage to mtDNA and respiratory function, suggested that respiration defects were not due to direct damage to polypeptides consisting of respiratory enzyme complexes, but resulted from damage to mtDNA. Moreover, both the reduced amounts of intact mtDNA and the reduced activity of respiratory enzymes were restored in 2-5 days, suggesting that the mtDNA damage was reversible, and thus were not fixed as pathogenic mutations.

Keywords: H₂O₂-induced damage, mouse mtDNA, respiratory function

[International Journal of Sport and Health Science Vol.4, 628-632, 2006]

1. Introduction

It has been presumed that mtDNA accumulates mutations much faster than nuclear DNA (Shay and Werbin, 1987) for the following reasons. First, mitochondria are highly oxygenic organelles producing reactive oxygen species (ROS) owing to their energy producing function. Second, mtDNA in mitochondria lacks histones to protect it from damage by ROS. Finally, its repair systems are limited. Subsequently, the mitochondria theory of aging proposed that a vicious cycle of respiration defects is accelerated by age-associated accumulation of abnormal mtDNAs carrying various somatic mutations, which induce respiration defects and result in the production of greater amounts of ROS, leading to accelerated production of somatic mutations in mtDNA (Linnane, et al., 1989; Miquel, 1991; Wallace, 1992).

However, no reports provided direct evidence that ROS created somatic mutations in mtDNA and induced respiration defects. Moreover, we recently proposed a new idea of mitochondrial genetic complementation, which could avoid direct expression of somatic mutations in mtDNAs

(Inoue, et al., 2000; Nakada, et al., 2001). In fact, extensive and continuous genetic complementation between mitochondria in somatic tissues protected the expression of respiration defects caused by accumulated pathogenic mutations in mtDNA (Hayashi, et al., 1994; Ito, et al., 1999; Nakada, et al., 2001; Ono, et al., 2001). Therefore, it remained possible that mtDNA damage induced by ROS did not fix as somatic mutations in mtDNA, or, even if somatic mutations were fixed in mtDNA, their amounts would not be sufficient for induction of respiration defects, due to the genetic complementation between mitochondria.

This study addressed the issue of whether ROS induces sufficient damage to mtDNA to induce respiration defects, and whether these abnormalities are fixed as somatic mutations in mtDNA. For this, we treated mouse B82 cells with H₂O₂ and subsequently examined its effects on mtDNA amounts, mtDNA sequence, and mitochondrial respiratory enzyme activity.

2. Materials and Methods

2.1. Cells and Cell Culture

Mouse B82 cells were grown in normal medium: RPMI1640 (Nissui Seiyaku, Tokyo) containing 10% fetal calf serum (FCS), 50 mg/ml uridine and 0.1 mg/ml pyruvate.

2.2. H₂O₂ treatment

Mouse B82 cells (2 x 10⁶ cells) in the dish were treated with H₂O₂ (0.1 mM and 1 mM) for 15 minutes in RPMI1640 medium (Nissui Seiyaku, Tokyo) without FCS, because of its antioxidant activity.

2.3. Southern blot analysis of mtDNA

Total cellular DNA (1-2 µg) extracted from 2 x 10⁵ cells was digested with the restriction enzyme *Eco*RI (Toyobo, Japan), and restriction fragments were separated in 0.7% agarose gel, transferred to a NYTRAN membrane, and hybridized with [α -³²P] dATP-labeled mouse mtDNA. [α -³²P] dATP-labeled mouse 18S rDNA encoded by nuclear DNA was used as internal control for normalization of the amounts of mtDNA. Radioactivities of fragments were measured with a bioimaging analyzer, Fujix BAS 5000 (Fuji Film, Japan).

2.4. Sequencing of mtDNAs

Total DNAs extracted from B82 and H₂O₂-treated B82 cells, respectively, were used for amplification of mtDNA fragments. The PCR reactions amplified 2.5-3.0 kbp mtDNA fragments using eight primer pairs designed to generate overlapping fragments of mtDNA (Akimoto, et al., 2004). All PCR amplifications were performed in a 50 µl of solution consisting of 1 x PCR buffer, 0.2 mM dNTPs, 0.6 µM primers, 1U Ampli Taq Gold DNA polymerase (Perkin-Elmer Applied Biosystems), and 1 µg of cellular DNA as template. Reaction conditions were 95°C for 10 minutes with cycle times of 60 seconds for denaturation at 95°C, 60 seconds for annealing at 50-56°C, and 150 seconds for extension at 72°C for 35 cycles. The final extension was for 10 minutes. Amplified mtDNA fragments were separated in 1.0% agarose gel and extracted, and then directly sequenced.

2.5. Biochemical analysis of COX and citrate synthase activities

COX activity was measured by examining the rate of cyanide-sensitive oxidation of reduced cytochrome *c* (Seligman, et al., 1968) with modifications. Biochemical analysis was based on the procedure described before (Miyabayashi, et al., 1984).

Biochemical assay for citrate synthase follows the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 minus 360nm, coupled to the reduction of CoA by the citrate synthase reaction in the presence of oxaloacetate. The reduction of DTNB is followed at 412 minus 360 nm in a 1-ml plastic cuvette at 30°C. The reaction mixture of 0.1 M Tris-HCl, 10 mM acetyl CoA, 1 mM DTNB, and 10 µg cell mitochondrial protein is incubated at 30°C for 10 minutes. The reaction is initiated by the addition of 10 mM oxaloacetate, and the absorbance change is monitored for 1 minute.

3. Results and Discussion

A mouse fibroblast cell line, B82, was treated with H₂O₂ (0.1 mM or 1 mM) for 15 minutes. Then, we removed H₂O₂ from the cultivation medium, and examined its effects on the amounts of mtDNA and respiratory function using Southern blot analysis of mtDNA and biochemical assay for cytochrome *c* oxidase (COX) activity, respectively (**Figure 1**).

Southern blot analysis showed that the amounts of intact mtDNA reduced progressively to 10-30% of normal amounts after 15 minutes treatment of H₂O₂, while those of nuclear DNA remained constant (**Figure 1A and B**). On the contrary, biochemical analysis of COX activity showed that respiratory function did not reduce during H₂O₂ treatment, but reduced to 40-60% of normal activity 24 hours after treatment (**Figure 1C**). However, the reduction of the amounts of mtDNA and COX activity was restored in 2-5 days to normal levels (**Figure 1**).

Considering that COX consisted of both mtDNA- and nuclear DNA-encoded subunits, the presence of the time lag between the damage to mtDNA and to COX activity after H₂O₂ treatment suggested that the reduction of COX activity was not caused by H₂O₂-induced damage to COX subunits, but resulted from the damage to mtDNA. This idea was supported by the observations that activity of mitochondrial citrate synthase, which was

Figure 1 Effects of H₂O₂ treatment on mtDNA and respiratory function
A, Southern blot analysis of mtDNA and nuclear DNA after H₂O₂ treatment; B, Quantitative estimation of the Southern blot signals of mtDNA and nuclear DNA. Amounts of intact mtDNA were normalized by the amounts of 18S rDNA (n=3); C, COX activity after H₂O₂ treatment (n=3). Hatched bars, untreated B82 cells; open bars, B82 cells treated with 0.1 mM H₂O₂; solid bars, B82 cells treated with 1 mM H₂O₂.

exclusively encoded by nuclear DNA, did not change substantially after H₂O₂ treatment. Probably, damage to mtDNA induced by H₂O₂ treatment suppressed mitochondrial transcription followed by suppression of the translation of mtDNA-encoded subunits required for expression of normal respiratory function, such as COI, COII, and COIII of the COX subunits. To provide direct evidence that H₂O₂-induced damage to mtDNA are responsible for H₂O₂-induced respiration defects (**Figure 1**), further experiments have to be carried out in order to show a coordinated decrease in mitochondrial transcription and translation after H₂O₂ treatment.

Rapid restoration of the mtDNA amounts and COX activity after H₂O₂ treatment (**Figure 1**) suggests that the H₂O₂-induced mtDNA damage is reversible as long as 1mM H₂O₂ treatment was carried out, and not fixed as genetic changes, such as creation of pathogenic mutations, in mtDNA. Recovery from the damage could be explained by the repair of H₂O₂-induced strand breaks in mtDNA and/or by accelerated replication of the remaining intact mtDNA.

Our preliminary experiment for direct sequencing of mtDNA prepared from H₂O₂ treated and untreated B82 cells showed that mtDNA sequence did not change after H₂O₂ treatment. Of course, it remains possible that H₂O₂ treatment induced somatic mutations in a very small proportion of mtDNA that was not detectable by direct sequencing and was not sufficient to induce observed respiration defects. Thus, we are investigating whether H₂O₂ treatment induces somatic mutations in mtDNA using cloning and sequencing of PCR products of mtDNAs prepared from H₂O₂-treated and untreated mouse B82 cells.

Acknowledgment

This work was supported in part by Grants-in-Aid for Creative Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to J.-I.H.

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