Accelerated decline of physical strength in peroxiredoxin-3 knockout mice

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Abstract

As a member of peroxiredoxin family, peroxiredoxin-3 plays a major role in the control of mitochondrial level of reactive oxygen species. During the breeding of experimental mice, we noticed that the peroxiredoxin-3 knockout mice were listless with aging. In the present study, we compared the swimming exercise performance and oxidative status between peroxiredoxin-3 knockout mice ($n = 15$) and wild-type littermates ($n = 15$). At the age of 10 months, the physical strength of peroxiredoxin-3 knockout mice was much lower than the wild-type littermates. Increased oxidative damage and decreased mitochondrial DNA copy number of the animal skeletal muscles were observed in peroxiredoxin-3 knockout mice as compared to that in the wild-type littermates. In addition, we found increased apoptotic cells in the brains of peroxiredoxin-3 knockout mice. Our results suggest that the deficiency of peroxiredoxin-3 induces accelerated oxidative stress and mitochondrial impairment, resulting in the decrease of energy supply and cellular activities. Peroxiredoxin-3 might be involved in the inhibition of aging process.

Keywords: Peroxiredoxin, reactive oxygen species, oxidative stress, mitochondria, aging

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Introduction

Aging is a natural, complex, and multi-factorial biological process that involves gradual function deterioration in various tissues and organs of an individual. As the powerhouse of cells, mitochondria are considered to be the most important cellular organelle to contribute to the aging process. The production of reactive oxygen species (ROS) in elderly subjects is increased and leads to oxidative damage to mitochondrial DNA (mtDNA), lipids, and proteins.^{1,2} The damaged mitochondria in turn produce increased amounts of ROS, leading to progressive augmentation in mitochondrial damage.³ Accumulating evidence has suggested that oxidative stress, mtDNA mutations, and disorganization of mitochondrial structure are associated with aging.^{4–6}

Removal of ROS by antioxidant enzymes plays an important part in limiting this damage. As a member of peroxiredoxin (PRX) family, PRX3 is predominantly located in mitochondria.⁷ PRX3 scavenges H₂O₂ through the conserved reactive cysteines and plays a major role in the control of mitochondrial ROS level.^{7,8} According to the report by Hattori *et al.*,⁹ PRX3 protected against neuronal cell death induced by ROS bursts. In an experiment using

male PRX3-transgenic mice, over-expression of PRX3 could attenuate the progression of left ventricular remodeling and failure after myocardial infarction, a process with ROS-mediated mtDNA damage and dysfunction.^{10,11} Another research conducted by Chen *et al.*¹² indicated that over-expression of PRX3 resulted in significant reduction of mitochondrial H₂O₂ production and improvement of glucose tolerance in mice. These studies suggested the significance of PRX3 in mitochondrial antioxidant defense.^{13–15} During the breeding of experimental mice, we noticed that the PRX3 knockout (PRX3^{-/-}) mice were less active than their wild-type (PRX3^{+/+}) littermates after the age of about 10 months, while the PRX3^{+/-} mice seemed normal in daily activities. The behavior observed in PRX3^{-/-} mice was not sex difference but was increasingly evident with aging. The present study was performed to understand the underlying mechanism for this phenomenon.

Materials and methods

Experimental animals

After approval by the Ethic Committee of Yantai Affiliated Hospital of Binzhou Medical University, we generated

PRX3^{-/-} mice as described before.¹⁶ We mated C57BL/6 mice with PRX3-null mice to generate PRX3-heterozygote offspring, which were then intercrossed to produce PRX3^{-/-}, PRX3^{+/-}, and PRX3^{+/+} littermates for experiments. The genotypes of the mice were determined by PCR on tail DNA of the offspring from heterozygous intercross.¹⁶ The experimental animals included two groups: 2-month old ($n=15$) and 10-month old ($n=15$) PRX3^{-/-} mice. The same number of age-matched PRX3^{+/+} littermates were used as controls.

Detection of physical strength in the mice

The physical strength of the experimental mice was detected by a test of swimming exercise performance with reference to the protocol described by Huang *et al.*¹⁷ The animals with an overload of 5% body weight attached to animals' tail swan at a cylindrical tank of 45 cm in length and 60 cm in depth. The temperature of the water was approximately 32°C. The exhaustive swimming time was recorded from the beginning to exhaustion as determined by loss of coordinated movements and failure to return to the surface within 7 s.

At the end of experimental period, blood samples were collected and centrifuged at 1500 × *g* and 4°C for 10 min for serum separation. Fatigue-associated biochemical indices including serum lactate and ammonia were determined by an autoanalyzer (Hitachi 747, Hitachi, Tokyo, Japan).

Detection of oxidative state in the skeletal muscles of mice

The expression of nuclear factor (erythroid-derived 2)-like 2 (NRF2) in mouse skeletal muscles was detected by reverse transcriptase PCR (RT-PCR) and Western blotting as previously described.¹⁸ The detected samples included skeletal muscles from PRX3^{-/-} mice aged 2 and 10 months, respectively. The age-matched PRX3^{+/+} mice were used as controls. The primer pairs for RT-PCR were: forward, 5'-CG AGATATACGCAGGAGAGG-3'; reverse, 5'-GCTCGACAA TGTTCTCCAGC-3'. For Western blotting analysis, the primary antibody against NRF2 was purchased from Abcam Company (Cambridge, UK). Visualization was achieved with ECL Western blotting detection system (Amersham, UK). Anti-GAPDH antibody (Santa Cruz Biotechnology, Inc., TX, USA) was used as internal control. The densitometry was performed on pictures using software ImageJ.

In addition, we performed enzyme-linked immunosorbent assay (ELISA) to detect markers for oxidative damage on DNA and lipids. These markers included 8-hydroxy-2'-deoxyguanosine (8-OHdG) and hexanoyl-lysine adduct (HEL). Highly sensitive 8-OHdG check ELISA and HEL ELISA kits were purchased from Japan Institute for the Control of Aging (Fukuroi, Japan). We assayed duplicates of each sample by following the manufacturer's instructions. The absorbance at 450 nm was measured on a spectrophotometer.

Detection of genetic variations in the mtDNA

Using a TaqMan PCR reagent kit and a PRISM 7900HT sequence detection system (Applied Biosystems, CA, USA), we performed real-time PCR to detect the existence of common deletions or mutations of mtDNA in the skeletal muscles of mice. The primer set was located at nucleotide positions 7697–7725 and 12528–12508. The reporter dye 6-carboxyfluorescein (FAM)-labeled TaqMan MGB probe specific for the common deletions was nucleotide positions 7750–7758 and 12455–12464. The primers for wild-type mtDNA were located at nucleotide positions 11933–11952 and 12076–12047. The reporter dye FAM and the quencher dye 6-carboxy-tetramethyl rhodamine (TAMRA)-labeled probe was nucleotide positions 12012–12037. Independent reactions were repeated three times. Sequencing of the displacement loop (D-loop) of mtDNA was also conducted to detect the existence of point mutations in this area.

Detection of mtDNA copy number in the skeletal muscles of mice

DNA was extracted from mouse skeletal muscles, and Southern blot analysis was performed to measure the mtDNA copy number. Primers for the mtDNA probe corresponded to nucleotides 2424–3601 of the mouse mitochondrial genome, and those for the 18S rRNA probe corresponded to nucleotides 438–1949 of the human 18S rRNA genome. The fragments separated by agarose gel electrophoresis were then transferred to nitrocellulose membranes and hybridized with ³²P-dCTP-labeled probes. The radioactivity was measured with a bioimage analyzer Fujix BAS 2000 (Fuji Film, Tokyo, Japan). The mtDNA copy number was normalized to the abundance of the 18S rRNA gene.

Histological examination and apoptotic detection of mouse tissues

Brains, hearts, kidneys, and skeletal muscles were removed from the mice at the age of 10 months. Two pathologists without knowledge of PRX3 genotypes performed histological examination on the paraffin-embedded sections after staining with hematoxylin and eosin. To investigate the possible occurrence of apoptosis in mouse tissues, we used the *In situ* Apoptosis Detection Kit (Takara, Tokyo, Japan. MK500) to perform the TUNEL (terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling) assay according to the manufacturer's protocols. Briefly, deparaffinized sections were microwaved in 0.01 mol/L citrate buffer (pH 6.0) for 5 min. Free 3' hydroxyl ends of fragmented DNA were then labeled with FITC-conjugated dUTP by incubating the sections with terminal deoxynucleotidyl transferase for 70 min at 37°C. FITC-labeled cells were detected by incubation with peroxidase-conjugated anti-FITC antibody and development with 3,3'-diaminobenzidine. Counterstaining was performed with either methyl green or 4',6-diamidino-2-phenylindole (DAPI; 30 nM solution in PBT). All sections were stained simultaneously. Photographs were taken with a digital camera under a light microscopy or a fluorescence microscopy (Olympus, Tokyo, Japan).

Statistic analysis

The physical strength, oxidative damage, and the copy number of mtDNA were compared between PRX3^{-/-} mice and PRX3^{+/+} littermates by analysis of variance. Comparison of apoptotic cells was performed by chi-square test. $P < 0.05$ was considered to be statistically significant.

Results

Physical strength

As indicated in Table 1, there was no significant difference in the endurance time between two-month-old PRX3^{-/-} and PRX3^{+/+} mice undergoing an exhaustive swimming test. In the animals at the age of 10 months, the endurance time was shorter in PRX3^{-/-} than in PRX3^{+/+} mice (Table 1, PRX3^{-/-} vs. PRX3^{+/+}; $P < 0.05$). Although the serum levels of lactate and ammonia were approximately equal between the two genotype animals at the age of two months, the difference between PRX3^{-/-} and PRX3^{+/+} mice reached statistically significant at the age of 10 months (Table 1, PRX3^{-/-} vs. PRX3^{+/+}; $P < 0.05$).

Oxidative markers in the skeletal muscles of mice

Since NRF2 has been referred to as the master regulator of antioxidant defenses,¹⁹ we detected the expression of NRF2 in mouse skeletal muscles. As shown in Figure 1, the levels of NRF2 in the skeletal muscles of 10-month-old PRX3^{-/-} mice were significantly higher than in the control samples. We then performed ELISA to detect oxidative damage in the skeletal muscles of the experimental mice. As indicated in Table 2, we observed significant increase of 8-OHdG and HEL in the skeletal muscles of PRX3^{-/-} mice (PRX3^{-/-} vs. PRX3^{+/+}; $P < 0.05$).

To know whether the oxidative stress was a local or systemic existence, we detected the serum levels of 4-hydroxy-2-nonenal (HNE) using an OxiSelect™ HNE Adduct Competitive ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA). As indicated in Table 2, the level of circulating HNE was significantly higher in PRX3^{-/-} mice than that in PRX3^{+/+} littermates (PRX3^{-/-} vs. PRX3^{+/+}; $P < 0.01$).

The mutation and copy number of mtDNA in mouse skeletal muscles

To clarify whether the oxidative stress resulted in mtDNA mutations, we examined the common deletions and point mutations of mtDNA in mouse skeletal muscles.

Although we found occasional 4977-bp deletions or D-loop base substitutions of mtDNA in the skeletal muscles of either PRX3^{+/+} or PRX3^{-/-} mice, there was no significant difference in the proportions of mtDNA mutations

Figure 1 Analysis for NRF2 expression in mouse skeletal muscles. (a) A representative result of RT-PCR analysis showed increased expression of NRF2 in 10-month-old PRX3^{-/-} mice as compared to age-matched wild-type animals. We did not notice significant difference in the NRF2 expression between two-month-old PRX3^{-/-} and PRX3^{+/+} mice. (b) A representative result of Western blotting analysis showed that the signal for NRF2 protein was significantly enhanced in PRX3^{-/-} mice aged 10 months. (c) Quantitative comparison for Western blotting results obtained from 15 mice of each group indicated a significant increase of NRF2 expression in 10-month-old PRX3^{-/-} mice (PRX3^{-/-} vs. PRX3^{+/+}; * $P < 0.05$).

Table 1 Physical strength and fatigue-associated indices in experimental mice ($n = 15$ in each group) as tested by exhaustive swimming performance

Genotype	Endurance time (min)		Fatigue-associated indices			
			Lactate (mmol/L)		Ammonia (μ mol/L)	
	2-Month	10-Month	2-month	10-month	2-month	10-month
PRX3 ^{+/+}	11.5 $\pm$ 1.12	10.7 $\pm$ 1.32	5.38 $\pm$ 0.62	6.23 $\pm$ 0.81	121 $\pm$ 18.3	141 $\pm$ 21.5
PRX3 ^{-/-}	11.0 $\pm$ 1.51	6.2 $\pm$ 0.91*	5.51 $\pm$ 0.77	10.12 $\pm$ 1.1*	118 $\pm$ 22.4	234 $\pm$ 27.2*

* $P < 0.05$.

Table 2 Oxidative damage in skeletal muscles and peripheral blood of experimental mice ($n = 15$ in each group)

Genotype	Oxidative damage in skeletal muscles					
	8-OHdG (ng/mL)		HEL (nmol/mL)		Serum HNE (nmol/mL)	
	2-month	10-month	2-month	10-month	2-month	10-month
PRX3 ^{+/+}	0.25 ± 0.02	0.28 ± 0.02	1.8 ± 0.11	2.21 ± 0.18	1.56 ± 0.05	1.82 ± 0.06
PRX3 ^{-/-}	0.24 ± 0.02	0.48 ± 0.05*	2.03 ± 0.16	6.5 ± 0.32 [†]	1.61 ± 0.05	4.28 ± 0.09 [†]

* $P < 0.05$; [†] $P < 0.01$.

Figure 2 Southern blot analysis for mtDNA copy number in mouse skeletal muscles. (a), A representative result showed that there was no difference in the signals between two-month-old PRX3^{-/-} and PRX3^{+/+} mice. However, the signal for 10-month-old PRX3^{-/-} mice was much weaker than that for age-matched PRX3^{+/+} mice. (b), Quantification for the Southern blot results from 15 mice of each group showed an evident decline of mtDNA copy number in the skeletal muscles of 10-month-old PRX3^{-/-} mice (PRX3^{-/-} vs. PRX3^{+/+}; * $P < 0.05$)

between the two genotype mice. We then detected the copy number of mtDNA in mouse skeletal muscles. As shown in Figure 2, the mitochondrial copy number in the skeletal muscles of PRX3^{-/-} mice was significantly decreased as compared to that in PRX3^{+/+} mice (PRX3^{-/-} vs. PRX3^{+/+}; $P < 0.05$).

Histological changes and apoptosis in mouse tissues

We previously reported that there were no significant anatomical abnormalities in the major tissues of eight-week-old PRX3^{-/-} mice.¹⁶ In the present study, we performed pathological examinations on the tissues of experimental mice at the age of 10 months. We did not find significant difference between PRX3^{-/-} and PRX3^{+/+} mice in the examined tissues except in the case of brains, in which degenerative features such as vacuolation were noticed in the hippocampus cells of PRX3^{-/-} mice (Figure 3). Our TUNEL assay showed that the number of apoptotic cells in the hippocampus of PRX3^{-/-} mice was $9.3 \pm 0.3\%$, which was significantly higher than that ($1.1 \pm 0.2\%$) in PRX3^{+/+} mice (Figure 3, PRX3^{-/-} vs. PRX3^{+/+}; $P < 0.01$). Although we found sporadic apoptotic cells in other tissues including hearts, kidneys, and skeletal muscles of the experimental mice, the quantitative difference between the two genotype mice did not reach statistical significance (data not shown).

Discussion

During the aging process, chronic increase of ROS production in the mitochondria is responsible for the age-associated mitochondrial dysfunction.^{20,21} In the present study, we found that the NRF2 levels in the skeletal muscles of 10-month-old PRX3^{-/-} mice were significantly higher than in the control samples, implying the existence of oxidative stress in the animals deficient in PRX3. 8-OHdG is a product of oxidatively damaged DNA formed by ROS, while HEL and HNE are useful biomarkers for lipid peroxidation. We demonstrated significant increases of 8-OHdG and HEL in the skeletal muscles of PRX3^{-/-} mice. Together with the elevated levels of serum HNE, our results indicated serious oxidative damage of DNA and lipids in the animals, suggesting the important role of PRX3 as an antioxidant enzyme in mitochondria.^{22,23}

Oxidative stress may cause macromolecule impairment and trigger cell death.^{24–27} Brains are mitochondrion-abundant and metabolism-exuberant, producing large amount of ROS. The brain tissue is also characterized by low contents of antioxidant systems.²⁸ In the present study, we showed more apoptotic cells in the hippocampus than in other tissues of PRX3^{-/-} mice, indicating that brain tissues were particularly susceptible to oxidative damage. Since the lack of introns and protective histones, and the limitation of DNA repair capacity, mitochondria are more prone to oxidative damage. The mtDNA 4977-bp deletion located between nucleotides 8469 and 13447 is a frequent

Figure 3 Histological changes and apoptosis in the brains of mice at the age of 10 months. A, The hippocampus of PRX3^{-/-} mice showed degenerative features such as vacuolation in the cells. TUNEL assay showed a fair amount of apoptotic cells in the hippocampus of PRX3^{-/-} mice (the insert). B, Sporadic apoptotic cells were observed in the hippocampus of PRX3^{+/+} mice (the inserts). Original magnification: $\times 400$

occurrence in tissues such as brain, skeletal, and cardiac muscles with aging.²⁹ However, we did not detect remarkable mtDNA mutations (including mtDNA 4977-bp deletion and D-loop point mutations) in the skeletal muscles of 10-month-old PRX3^{-/-} mice. Interestingly, the mitochondrial copy number in the skeletal muscles of PRX3^{-/-} mice was significantly decreased as compared to the PRX3^{+/+} littermates. According to the report by Shokolenko *et al.*, mtDNA exposed to H₂O₂ tended to be strand breaks instead of base lesions. The oxidatively damaged mtDNA was fragmented and ultimately eliminated, which was a unique response of mitochondria to oxidative stress.³⁰ The mtDNA fragments contained high level of 8-OHdG, a marker commonly used in the analysis of base oxidation.³¹ Our result showing increased 8-OHdG in the skeletal muscles of PRX3^{-/-} mice is consistent with the previous findings. It is possible that the gradual increase of ROS production with age (chronic oxidative stress) induces mitochondrial degradation. In addition, Huh *et al.*³² reported that PRX3 knockout mice showed defect in mitochondrial biogenesis in adipocytes, leading to metabolic alterations such as increased fat mass and impaired glucose tolerance. We suppose that both reduced mtDNA replication and increased oxidative damage to mitochondria contributed to low copy number of mtDNA.

Mengel-From *et al.*³³ previously demonstrated that elderly human beings with low mtDNA copy number had poorer outcomes in terms of cognitive performance, physical strength, self-rated health, and higher all-cause mortality than the subjects with high mtDNA copy number. In the present study, the PRX3^{-/-} mice exhibited decreased physical strength, increased fatigue-related biochemical variables, and accelerated decline of mtDNA copy number. These findings were in accordance with the notion that the aging process was associated with accumulation of damaged organelles and alterations in the integrity of mitochondria.³⁴ Therefore, our results have provided new evidence for the significance of PRX3 in aging, a process in which ROS is increasingly produced with age. The inactive

behavior of PRX3^{-/-} mice might be a consequence of low mtDNA copy number and insufficient energy supply, which implied a state of premature senility due to oxidative microenvironment in the tissues.

Conclusion

In conclusion, our findings suggest that PRX3 plays a crucial role in the mitochondrial homeostasis. The decline of mtDNA copy number implied that the mitochondria were impaired in an accelerated rate in the absence of PRX3. The “laziness” of PRX3^{-/-} mice represents a kind of adaptive response to the low energy supply in the animals.

Author contributions: YGZ detected physical strength of the mice and the oxidative state in the skeletal muscles of the mice. LW performed the genotyping and histological examination of the experimental mice. TK performed the apoptotic detection. JL and XL detected the mutations and copy number of mtDNA in the skeletal muscles of mice. LL contributed to the experimental design, analysis of the results, and preparation of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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