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Original Article
Mitochondrial biogenesis in ventral spinal cord and nerve following sciatic nerve axotomy

Wei Huang1*, Tianbing Wang1*, Baoguo Jiang1, Ming Zheng2
1Department of Trauma and Orthopaedics, Peking University People’s Hospital, Beijing, China; 2Department of Physiology and Pathophysiology, Health Science Center, Peking University, Beijing, China. *Equal contributors.
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Abstract: In the nerve system, mitochondria play pivotal roles in regulating various cellular processes and provide over 90% ATP supply to nerve activities. After nerve injury, cell bioenergetic status and energy metabolism related genes were significantly changed, but it is not clear whether mitochondrial biogenesis is involved in post-injury nerve degradation and regeneration processes. Here we show that mitochondrial biogenesis was down-regulated in spinal cord and up-regulated in proximal nerve after sciatic nerve injury, as indicated by the decreased or increased expressions of biogenesis biomarkers PGC-1α, NRF-1, and TFAM. Mitochondrial to nuclear DNA ratio decreased in spinal cord and increased in proximal nerve. Moreover, mitochondrial morphology and ATP production were changed accordingly after nerve injury, with less, irregular mitochondria and reduced ATP content in spinal cord, and more, condensed mitochondria and elevated ATP content in proximal nerve. Our study revealed important but distinct changes of mitochondrial biogenesis in ventral spinal cord and nerve after peripheral nerve injury, and may provide treatment strategies for peripheral nerve injury through adjusting mitochondrial biogenesis and metabolic status.

Keywords: Mitochondrial biogenesis, nerve, motoneuron, energy metabolism, nerve regeneration

Introduction
Peripheral nerves degenerate after injury by Wallerian degeneration, a process defined as the degeneration of the axon distal to the injury site [1, 2]. The neurons then re-initiate proximal axonal growth, known as regeneration, to repair the injured connection of the nerves with their targets. Enormous effort was made to promote nerve regeneration by improving the surgical technique, increasing neuronal survival and the rate of axon regeneration or retarding Schwann cell and basal lamina atrophy. However, emerging evidence has shown that peripheral nerve injury also triggers a number of cellular and molecular events in neurons which might be essential for nerve regeneration [3, 4]. The study by Hu et al [4] revealed that some genes expression related to cell apoptosis increased and genes associated with neuronal survival or function decrease. Indeed, axonal regeneration is a coherent process of neurons, both proximal and distal axon, and even the Schwann cells, and the functional and metabolic status of these parts determine the degree of neurological recovery [5].

Mitochondria are important organelles, playing central roles in regulating various cellular processes including production of ATP, modulation of calcium signaling, generation of reactive oxygen species, and even determination of cell fates. In neuron, mitochondria provide more than 90% of total amount of ATP through oxidative phosphorylation [6], to supply the establishment of membrane excitability, the synthesis of neurotransmitter and excitability conduction, and the regeneration of nerves [7]. Under different physiological and pathological conditions, energy demands are different, the morphology, function and amount of mitochondria in neurons are regulated accordingly. After root avulsion, most of the gene expression associated with energy metabolism decreased in motor neurons [4]. However, it is not clear whether genes related to mitochondrial biogenesis of the ventral neuron and proximal nerve stump area changed after peripheral nerve injury.

In this study, we analyzed the molecular, morphological, and bioenergetic changes of mitochondria in proximal sciatic nerve and spinal
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We found that mitochondrial biogenesis biomarkers PGC-1α, TFAM, NRF-1 and mitochondrial to nuclear DNA ratio were oppositely regulated in post-injury spinal cord and proximal nerve, with decreased expression in spinal cord and increased expression in proximal nerve. Consistently, sciatic nerve injury reduced mitochondrial amount and ATP content in spinal cord while elevated mitochondrial amount and ATP content in proximal sciatic nerve.

Materials and methods

Animals and surgical procedures

All experimental programs were approved by Ethics Committee of Peking University People’s Hospital. Sprague Dawley adult male rats of 220-250 g were used in the present study. Firstly, rats were anesthetized using sodium pentobarbital, 30 mg/kg and then the right sciatic nerves were exposed from the muscle gap and cut off 5 mm distal to the lower edge of piriformis. The ventral horn of the lesion side of cord and proximal sciatic nerve (5 mm proximal side to the lesions site of sciatic nerves) at the lesion side were taken. B. Ulcer of the limb at sciatic nerve injured side.

Figure 1. Surgical procedures of generation of sciatic nerve injury model. A. The right sciatic nerve was exposed and sectioned 5 mm distal to the lower edge of piriformis. The ventral horn of the lesion side of cord and proximal sciatic nerve (5 mm proximal side to the lesions site of sciatic nerves) at the lesion side were taken. B. Ulcer of the limb at sciatic nerve injured side.

experiments, at time points of 3, 7, 14, 21, and 28 days after operation (Figure 1A).

ATP measurement

ATP contents in ventral horn of the right side of the cord or proximal sciatic nerve were determined by an ATP Assay System (Vigorous, China) according to the principle of luciferin-luciferase following the manufacturer’s instructions. Briefly, the spinal cord and sciatic nerves were harvested, homogenized in tissue lysis buffer, and then centrifuged at 10000 g for 15 min at 4°C. Supernatant was collected and added to the ATP measuring reagent. The light emission was determined with Luminometer (Bio-Rad). ATP concentration of the samples was calculated according to ATP standard curve. All data was normalized with the protein abundance by using the Bradford method.

Transmission electron microscopic examination

The processing of ventral part of the spinal cord of lesioned side or sciatic nerves for electron microscopic analysis was performed as previously reported [8]. Briefly, ventral cord specimens and the proximal stump of the sciatic nerves were obtained and fixed in 2.5% glutaraldehyde with 0.1 M phosphate buffer (pH, 7.2) for four hours. After fixation in osmium tetroxide and dehydration by gradient acetone, tissues were embedded in Epon-812-resin, sectioned by ultramicrotome for semithin sections and stained with toluidine blue. The sections were examined with a light microscope to locate motor neurons at ventral horn and nerve fibers. Then ultrathin sections were harvested and examined with a transmission electron microscope (JEM-1230, Tokyo, Japan) equipped with a digital photography.

RNA isolation and reverse transcription (RT), real-time PCR analysis

Total RNA extraction from ventral part of the spinal cord or proximal sciatic nerve was com-
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Results

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Figure 2. mRNA and protein changes of mitochondrial biogenesis biomarkers in spinal cord. mRNA levels of PGC-1α (A), TFAM (B), and NRF-1 (C) were suppressed at indicated days after sciatic nerve injury. D. Representative western blot showing the decreased protein level of PGC-1α, TFAM, and NRF-1 at 7 days after injury, and (E) the average data of (D). n=6 sides for each group. *P<0.05, **P<0.01 vs. control.

Materials and Methods

Real-Time PCR analysis

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with 3 ug total RNA at 25°C for 5 min, 42°C for 60 min, 70°C for 15 min using Oligo (dT) and GoScript II reverse transcriptase (Promega).

Western blot analysis

Tissue samples were homogenized in lysis buffer (containing 0.15 mol/l NaCl, 0.1 mol/l Tris-Cl, 1 mmol/l EDTA, 1 mmol/l PMSF, 10 μg/ml Aprotinin, 10 μg/ml Pepstatin A, 1% TritonX-100); lysates were then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was collected and total protein was measured using Bradford Assay Kit. An equal volume of 2× sample buffer (100 mM Tris-HCl pH 6.8, 2.5% SDS, 20% glycerol, 0.006% bromophenol blue and 10% β-mercaptoethanol) was added. Samples were boiled and then separated by 8-12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat milk for 2 hour at room temperature, then probed with primary antibodies (PGC-1α, NRF-1, TFAM or GAPDH) at 4°C overnight, and then incubated with horseradish-peroxidase-conjugated secondary antibodies. GAPDH was used as a loading control.

DNA isolation and real-time PCR analysis

Genome DNA from ventral part of the spinal cord or proximal sciatic nerve was isolated by using the Universal Genome DNA Kit (Beijing Zoman Biotechnology, China). The mtDNA copy number was determined by PCR through the relative number of Cyt B to genome β-actin [9, 10].

Statistical analysis

SPSS 17.0 software (SPSS, Chicago, IL, USA) was used for data analysis. Data were presented as mean ± SEM. Differences in the means between control group and experimental groups were analyzed using Student’s t test. P<0.05 was considered statistically significant.
Results

Generation of sciatic nerve injury model

Comparing with sham limbs, the operated limbs after sciatic nerve transection displayed denervation, characterized by dropping ankles and claudication. Among rats under sciatic nerve injury, some rats bitted their toes and the limbs appeared ulcer, even all of the toes disappeared 1 week after operation (Figure 1B). The amount of rats with limb ulcers increased with time and most ulcers gradually aggravated. The operated limbs couldn’t walk 4 weeks after surgical operation, indicating the effective modeling. In control group, the rats could walk normally.

Suppressed mitochondrial biogenesis in spinal cord after sciatic nerve injury

To determine if sciatic nerve axotomy disturbs mitochondrial biogenesis in spinal cord, we examined the gene expression levels of several mitochondrial biogenesis biomarkers, PGC-1α, TFAM, and NRF-1, in tissue samples taken from ventral cord (L4-L6) of axon injury side or sham control side, by real time PCR (RT-PCR). Interestingly, we found that after sciatic nerve surgery, the mRNA levels of PGC-1α, TFAM, and NRF-1 in spinal cord from injury side showed similar downward trend as comparing with before operation (Figure 2A-C). The mRNA levels of three mitochondrial biogenesis biomarkers slightly decreased at as early as 3 days after nerve injury, and significantly decreased 7 days after injury, to a level of 70.5±5.0% for PGC-1α (Figure 2A), 66.3±7.1% for TFAM (Figure 2B), and 53.6±11.6% for NRF-1 (Figure 2C), respectively, as compared to tissues from sham control rats. And at 14 days after sciatic nerve injury, the mRNA levels of the three mitochondrial biogenesis biomarkers decreased more, to 42.7±5.9% for PGC-1α (Figure 2A), 44.5±8.5% for TFAM (Figure 2B), and 47.2±6.0% for NRF-1 (Figure 2C), comparing with control sides. Moreover, the low mRNA levels kept till at least one month after surgery.

In consistent with mRNA levels, protein levels of PGC-1α, TFAM, and NRF-1 in spinal cord from injury side also decreased at 7 days after sciatic nerve transection comparing with control side, as indicated by western blotting (Figure 2D). And highly comparable to the decreased mRNA levels, protein levels of PGC-1α, TFAM, and NRF-1 in the injury side of spinal cord decreased to 51.8±8.6%, 44.1±4.4%, and 58.7±9.1% to that of the control side, respectively (Figure 2E).

Figure 3. mRNA and protein changes of mitochondrial biogenesis biomarkers in proximal nerve stump. Real time PCR (RT-PCR) analysis for PGC-1α (A), TFAM (B), and NRF-1 (C) in proximal nerve stump at indicated days after sciatic nerve injury. (D) Representative western blot showing the increased protein levels of PGC-1α, TFAM, and NRF-1 at 3 days after injury. (E) The average data of (D). n=6 sides for each group. *P<0.05, **P<0.01 vs. control.
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Elevated mitochondrial biogenesis in proximal sciatic nerve after sciatic nerve injury

Next, we sought to know if mitochondrial biogenesis was disturbed in nerve after sciatic nerve injury. We also examined the mRNA levels of PGC-1α, TFAM, and NRF-1 by RT-PCR (Figure 3A-C). Surprisingly, in contrast to that in spinal cord, we found the mRNA levels of PGC-1α, TFAM, and NRF-1 in proximal nerve significantly increased from as early as 3 days after sciatic nerve injury, to 1.49±0.17, 1.41±0.13, and 1.94±0.22 folds of control nerve. The increased mRNA levels of PGC-1α, TFAM, and NRF-1 persisted at 7, 14, 21, 28 days after sciatic nerve injury, to 2.21±0.14, 1.72±0.12, 2.10±0.13, and 1.91±0.13 folds for PGC-1α, 2.17±0.21, 2.41±0.28, 2.22±0.14, and 2.28±0.17 folds for TFAM, 2.14±0.23, 2.57±0.25, 2.18±0.27, and 2.34±0.20 folds for NRF-1, respectively, as compared to that of control nerve.

Similarly, protein levels of PGC-1α, TFAM, and NRF-1 increased comparably to mRNA levels (Figure 3D), to 1.44±0.12, 1.54±0.15, and 1.41±0.09 folds of that in control nerve fibers, at 7 days after sciatic nerve transection (Figure 3E).

Mitochondrial to nuclear DNA ratios changes after sciatic nerve injury

In order to evaluate mitochondria biogenesis from another aspect, we tested mitochondria DNA. At spinal cord, the mitochondrial to nuclear DNA ratio was 665.27±41.43 in control group, and significantly decreased 7 days after injury, to a level of 390.56±42.29 (Figure 4A). The decreased mitochondrial to nuclear DNA ratio persisted at 14, 21, 28 days after sciatic nerve injury, to 300.46±54.21, 374.35±39.98 and 346.62±60.27 (Figure 4A). At proximal nerve, the mitochondrial to nuclear DNA ratio was 24.79±5.64 in control group, and significantly increased 3 days after injury, to a level of 68.10±7.13 (Figure 4B). The increased mitochondrial to nuclear DNA ratio persisted at 7, 14, 21, 28 days after sciatic nerve injury, to 62.86±10.3, 72.10±8.30, 61.27±8.11 and 62.10±8.39 (Figure 4B).

Ultrastructural alterations of mitochondria after sciatic nerve injury

We then performed transmission electron microscopic (TEM) examination on ventral horn neurons and proximal sciatic nerve to visualize the ultra-structural changes of mitochondria. While numerous mitochondria with roughly similar length around 1 μm was observed in ventral horn neurons from control side (Figure 5A), less but enlarged mitochondria with length ranging from 1 μm to 3 μm was found in ventral horn neurons from injury side at 7 days after sciatic nerve transection (Figure 5B), supporting the finding of declined mitochondrial biogenesis, and further suggesting the dysfunction of mitochondrial in neuronal bodies, even though the injury occurred in peripheral nerve fiber. In contrast to the decreased number of mitochondria in neuronal bodies, the number of mitochondria in Schwann cells around proximal...
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Mal sciatic nerve after injury increased significantly 3 days after sciatic nerve transection (Figure 5D), as compared with those in Schwann cells from control side (Figure 5C), indicating an essential role of mitochondria in proximal Schwann cells during axon regeneration. Together, our TEM data here are in general agreement with above mentioned down-regulated mitochondrial biogenesis in ventral spinal cord and up-regulated mitochondrial biogenesis in proximal nerve after sciatic nerve injury.

Bioenergetic changes after sciatic nerve injury

Mitochondria are the main source of intracellular ATP production, so we measured ATP contents in both spinal cord and in proximal sciatic nerve. In parallel with the decreased mRNA and protein levels of mitochondrial biogenesis biomarkers, ATP contents in ventral horn of injured side reduced from two weeks after injury, to 57.2±11.0% of that in control side, and the declined ATP content persisted till at least one month (Figure 5E). Moreover, ATP content in the injured proximal sciatic nerve increased to 1.33±0.11 fold of control nerve at 3 days after injury, up to 1.77±0.17 fold at 7 days after injury, and keep at the high level till at least one month (Figure 5F).

Discussion

In this study, we found that mitochondrial function was oppositely regulated in post-injury ventral spinal cord and nerve proximal to the site of injury at least one month after nerve transection, with a lower bioenergetic level in spinal cord and higher level in proximal nerve area. In post-injury spinal cord, mitochondrial biogenesis biomarkers PGC-1α, NRF-1, TFAM and mitochondrial to nuclear DNA ratio were down-regulated at both mRNA and protein levels, and consistently, less mitochondria and decreased ATP content were observed. In contrast with the changes in spinal cord, mitochondrial biogenesis markers and ATP production were up-regulated in post-injury sciatic nerve area, and more mitochondria were found. In addition, the changes of mitochondria at proximal nerve area were earlier than at neuronal cell bodies,
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3 days compared with 7 days after axotomy. Collectively, our data indicated the important but distinct roles of mitochondria in regulating degradation and regeneration in neuronal cell body and nerve after peripheral nerve injury.

Nerve transection causes a variety of modifications in gene expression and cellular metabolism in neuron body, which likely are parts of the process to protect the cell, regenerate the injured axon and restore cellular functions [11-14]. However, whether mitochondria are involved in this process is still unclear. The present study provides evidence showing that mitochondria in both ventral spinal cord and proximal nerve are profoundly involved in cellular modification after nerve transection. In spinal cord, less and enlarged mitochondria was found at one week after sciatic nerve transection, in consistent with previous reports of the swelling and irregularly arranged mitochondria in dorsal root ganglion and spinal cord motoneuron after sciatic nerve axotomy [15, 16]. Moreover, decreased expressions of genes related to mitochondrial metabolism were also reported [3]. The changed mitochondrial morphology and metabolism related genes in neuron body after nerve transection suggest the dysfunction of mitochondria. Indeed, in the present study, we found that ATP content in spinal cord decreased two weeks after nerve injury. The mechanisms underlying the dysfunctional mitochondria and the subsequent decreased energy supply in spinal cord after nerve transection is currently unclear and needs further investigation. However, the suppressed mitochondrial function may prevent the neural regeneration after injury, and causing poor functional recovery following nerve axotomy, particularly when surgical repair is delayed post injury. In contrast to the dysfunctional mitochondria in spinal cord, we found mitochondrial ATP production in nerve proximal to injury site was largely increased, indicating an active mitochondrial metabolism status responding to nerve injury, which may contribute to the proximal axon regeneration. The finding that more condensed mitochondria appeared in Schwann cells around nerve fiber area further supports this view.

Mitochondrial regeneration is a very complex process, which is regulated by mitochondrial and nuclear genes. While nuclear transcription factors, mainly nuclear respiratory factor 1 and 2 (NRF-1 and -2), and coactivator such as PGC-1α regulates the expression of nuclear-gene encoded mitochondrial proteins [17], the expression of mitochondria-gene encoded proteins are mainly controlled by the mitochondrial transcription factor A (TFAM) [17-19]. In our present study, we found that, in spinal cord 7 days after nerve transection, both mRNA and protein levels of PGC-1α, NRF-1, and TFAM largely decreased, suggesting decreased mitochondrial biogenesis after peripheral nerve injury. Less and enlarged or vacuolated mitochondria with loose cristae in spinal cord support the down-regulation of mitochondrial biogenesis. Moreover, energy supply in neuron body concurrently down-regulated, indicating that the regulation and balance of these biogenesis related genes determine mitochondrial oxidative capacity. In agreement with our findings, it has been reported that deprivation of each one of these genes leads to decreased mitochondria amount and lower neuronal activity [20]. Interestingly, opposite changes occurred in nerve. Expression levels of PGC-1α, NRF-1, and TFAM were significantly increased companied with increased ATP content. There are two possible explanations for the increased ATP supply. One is that mitochondrial transportation from neuron body to proximal nerve increased largely after axotomy [21]. Another is the up-regulated mitochondrial biogenesis in Schwann cells, which promote the re-growth of the axon. Anyway, the higher bioenergetic level at proximal nerve fiber area during early stage of sciatic nerve injury may serve as a protective response of sciatic nerve to injury.

In summary, our data have shown that mitochondrial biogenesis were oppositely regulated in post-injury spinal cord and proximal nerve following sciatic nerve injury, with decreased level in spinal cord and increased level in proximal nerve. And consistently, ATP content reduced in spinal cord while elevated in proximal sciatic nerve. These findings not only extend our understanding of changes of the mitochondrial biogenesis and bioenergetic metabolism after peripheral nerve injury, but also may provide treatment strategies for peripheral nerve injury through adjusting mitochondrial biogenesis and metabolic status.

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Disclosure of conflict of interest
None.

Address correspondence to: Dr. Ming Zheng, Department of Physiology and Pathophysiology, Health Science Center, Peking University, Beijing 100191, China. Tel: 86-10-8280-2043; E-mail: zhengm@bjmu.edu.cn; Dr. Baoguo Jiang, Department of Trauma and Orthopaedics, People’s Hospital, Peking University, Beijing 100044, China. Tel: 86-10-8832-6550; E-mail: jiangbaoguo@vip.sina.com

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