Electrical stimulation induces mitochondrial autophagy via activating oxidative stress and Sirt3 signaling pathway

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To the Editor: Mitochondrial function is one of the most cutting-edge fields in the current research and further study is needed to investigate the specific signaling mechanisms of mitochondrial function. A previous study has suggested that exercise increases energy metabolism via regulating mitochondrial dynamics by multiple mechanisms.[1] A recent study has demonstrated that electrical stimulation (ES) regulated mitochondrial function by the increase of reactive oxygen species (ROS) production.[2] However, whether the effects of ES on mitochondrial function are associated with mitochondrial autophagy and the potential signaling pathways remain unknown.

In the current study, C2C12 (C3H muscle myoblast of the mouse) cells were used, since the differentiated C2C12 myotubes have the same contraction as *in vivo* skeletal muscle while avoiding the interference of other organs, tissues, cells, and hormones *in vivo*. Therefore, the mitochondrial function in C2C12 myotubes can be studied with different treatments in real-time. In order to ensure the stability of the myotubes during the experiment, we took photos of differentiated myotubes from day 0 to day 7 [Figure 1A]. The results indicated that differentiation for 7 days had the same contractility as *in vivo* muscles. Thus, C2C12 myotubes at day 7 were employed for further experiments.

Any change in mitochondria will directly or indirectly affect the function of skeletal muscle, which is the most important part of energy metabolism. The decrease of mitochondrial membrane potential (MMP) is the precursor of cellular oxidative damage. It has been demonstrated that aerobic exercise is beneficial to maintain the MMP of myocardial cells in a 12-week aerobic exercise study.[3] To investigate the effects of ES on mitochondrial function, on the 7th day of differentiation, C2C12 myotubes were given ES exposure (15 V, 3 Hz, 30 ms, Grass S-48 stimulator) according to the established methods in our laboratory (unpublished). We first detected the MMP, ROS, and malondialdehyde (MDA; Beyotime Biotechnology, Shanghai, China) levels in the research. Compared with the control group, MDA and ROS were increased significantly at 120 and 180 min after ES ($P < 0.05$) [Figure 1B and 1C]. Notably, ROS was also increased at 60 min after ES ($P < 0.05$) [Figure 1C]. We also observed that the MMP was decreased slightly at 60 min ($P < 0.05$) and significantly at 120 and 180 min ($P < 0.01$) after ES [Figure 1D]. Together, these findings implicate that the oxidative stress of mitochondria can be induced by ES. A prior study showed that ROS was increased after ES *in vitro*,[2] and our data advance these results to show that C2C12 myotubes exhibited a significant increase in ROS production during prolonged exposure to comparable intensities of ES. Our findings suggest that ES at high voltage intensity on mitochondrial function may have similar effects to the high intensity of exercise. Exercise-induced ROS production in skeletal muscle was shown to be a critical factor for muscle adaptation during exercise training, which indicates that skeletal muscle responses to oxidative stress are involved in muscle adaptation during intense exercise.

Autophagy in skeletal muscle is peculiar compared to other important metabolic tissues. Most tissues show a transient activation of autophagy that only lasts for few hours during fasting. In contrast, skeletal muscle shows a persistent generation of autophagosomes that continues for days. These results suggest that the formation of autophagosomes may be affected by different signaling pathways during the short-term or long-term induction of autophagy. In the present study, we examined the mitochondrial microstructure by transmission electron microscopy and autophagy-related proteins by Western

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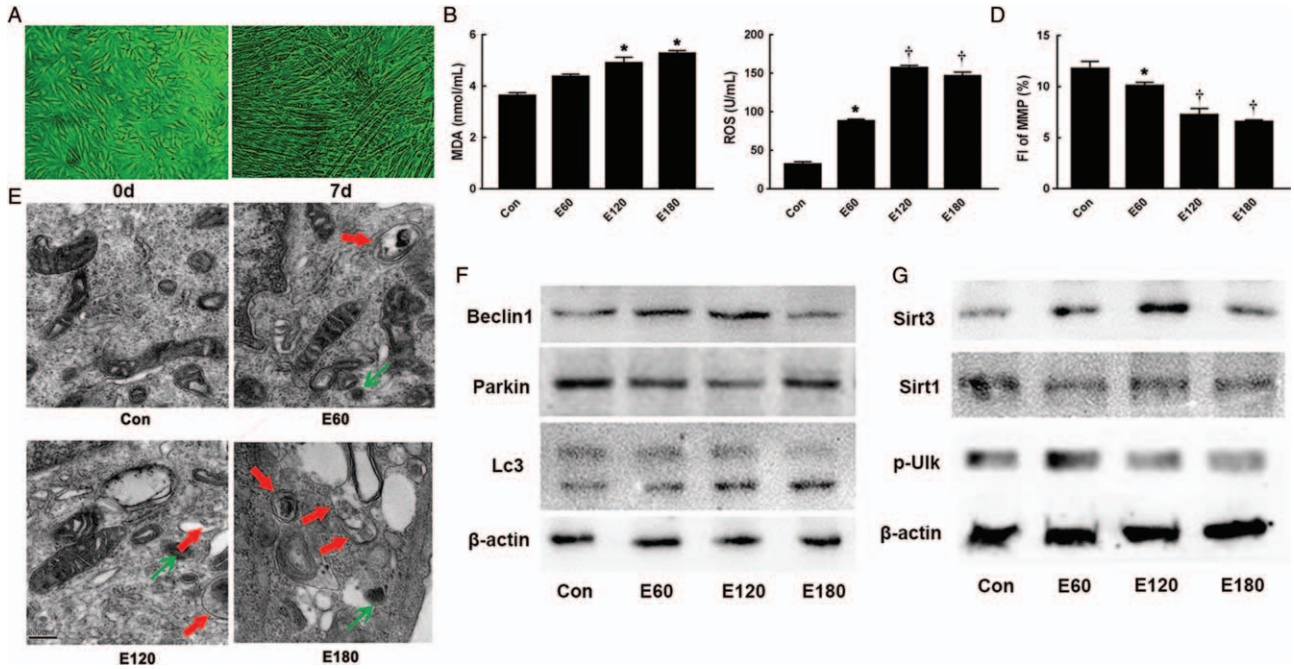

Figure 1: Electrical stimulation induces mitochondrial autophagy via activating oxidative stress and the Sirt3 signaling pathway. (A) C2C12 cells differentiated for 7 days were employed in the study. (Original magnification, ×100). (B) MDA was increased at 120 (E120) and 180 min (E180) after electrical stimulation compared to the control group (Con). (C) ROS was increased significantly at 60 (E60), 120, and 180 min after electrical stimulation compared to the control group. (D) MMP was decreased slightly at 60 min and significantly at 120 and 180 min after electrical stimulation. (E) TEM analysis showed enlarged lysosomes and abnormal autophagosome-like structures were increased and normal mitochondria were reduced as electrical stimulation time-points increases. Red arrows: autophagosome-like structures; green arrows: lysosomes. (Original magnification, ×50,000). (F) The level of Beclin1 was increased after 60 and 120 min of electrical stimulation; Parkin expression levels were decreased after 60 and 120 min of electrical stimulation; Lc3 expression levels were enhanced after 120 and 180 min of electrical stimulation. (G) The expression level of Sirt3 increased significantly after 60 and 120 min of electrical stimulation; Sirt1 level was decreased after 60 min of electrical stimulation; the level of p-Ulk was increased significantly after 60 min of electrical stimulation. The data are shown as mean ± standard deviation. $^{*}P < 0.05$ *vs.* Con; $^{\dagger}P < 0.01$ *vs.* Con. Con: Control group; E60: Electrical stimulation for 60 min; E120: Electrical stimulation for 120 min; E180: Electrical stimulation for 180 min; FI: Fluorescence intensity; MDA: Malondialdehyde; MMP: Mitochondrial membrane potential; ROS: Reactive oxygen species; TEM: Transmission electron microscopy.

blotting (WB) since autophagy of mitochondria is essential for maintaining healthy skeletal muscles. As showed in Figure 1E, ES for 60 min induced the formation of abnormal autophagosome-like structures in C2C12 myotubes compared to the control group. Furthermore, there were more abnormal autophagosome-like structures, swelling mitochondria, and enlarged lysosomes in the ES for 120 min group. After 180 min of ES, fewer mitochondria, more enlarged lysosomes, and abnormal autophagosome-like structures were observed.

Beclin1 and Lc3 are involved in the formation of autophagosomes which is an important step in autophagy. Parkin is an E3 ubiquitin ligase that regulates protein degradation and signaling pathways by mediating the ubiquitination of proteins. Studies have shown that Parkin maintains mitochondrial integrity by regulating different mitochondrial functions, including membrane potential, internal environment stability, and mitochondrial respiratory activity. Results of WB showed that the expression level of Beclin1 (Cell Signaling Technologies, Danvers, MA, USA) was increased after 60 and 120 min of ES ($P < 0.05$) [Figure 1F]; Lc3 expression level was also enhanced after 120 and 180 min of ES ($P < 0.05$) [Figure 1F]. While Parkin expression level was decreased after 60 and 120 min of ES ($P < 0.05$) [Figure 1F], suggesting the mitochondrial integrity was impaired by ES. Altogether, these studies demonstrated that ES acts on mitochondria of C2C12 myotubes to induce autophagy. Our findings suggest that autophagy may be an important factor for mitochondrial adaptation in skeletal muscle. Therefore, it stands to reason that autophagy could rapidly facilitate the elimination of pre-existing structures and proteins in order to structural remodeling.

Autophagy could be regulated by activation of several common signaling cascades, including Sirt1, Sirt3, and UNC-51-like kinase (ULK1/2) signaling. Sirt1 and Sirt3 are members of the sirtuin family and participate in the regulation of diverse biological processes, such as survival, apoptosis, autophagy, and metabolism.[4] Sirt3 is a NAD-dependent deacetylase located mainly in the mitochondria. Current research has found that Sirt3 regulates autophagy levels through multiple pathways. For example, Sirt3 up-regulates many antioxidant enzymes by deacetylating FOXO3a to increase ROS removal, including catalase, Mn-superoxide dismutase, and nicotinamide adenine dinucleotide phosphate, and so on. Besides, Sirt3 regulates the production of ROS by deacetylating the components of the mitochondrial electron transport chain, including nicotinamide adenine dinucleotide (NADH) dehydrogenase, succinate dehydrogenase, and complex III. ULK1/2 has been featured as autophagy-specific regulators via phosphorylating downstream targets, such as the Beclin1 complex. To examine the mechanisms by which ES induced mitochondrial autophagy, we measured several signaling pathways related to autophagy. As shown in Figure 1G, compared with the control group, the

expression level of Sirt3 and p-Ulk were increased significantly, while Sirt1 was decreased after 60 min of ES. After 120 min of ES, the expression level of Sirt3 increased significantly [Figure 1G]. However, there were no changes in the levels of all proteins mentioned above after 180 min of ES [Figure 1G].

In this study, we investigated the probable signaling pathways in enhanced autophagy by measuring the protein levels of Sirt1, Sirt3, and p-Ulk. The results showed that the level of Sirt3 was stably increased after continuous moderate ES. Previous reports have shown the correlation between Sirt1/Sirt3 and mitochondrial ROS production under different pathological and physiological conditions.[4] Both Sirt3 and Sirt1 can promote mitochondrial biosynthesis and fatty acid oxidation via PGC1, but the mechanism was different. Sirt3 activates PGC1 while Sirt1 promotes the deacetylation of PGC1.[5] Sirt3 is differentially expressed *in vivo*, mainly elevated in metabolically active tissues like skeletal muscle, where Sirt3 is dynamically regulated by different environmental conditions, suggesting that exercise training regulates Sirt3 but not Sirt1 expression in muscle. At present, it remains to be considered how these two key sirtuin enzymes work cooperatively in certain tissues in response to environmental signals.

In conclusion, in this study, using *in vitro* muscle contraction model, we demonstrated that muscle contraction is caused by ES-induced oxidative stress and mitochondrial autophagy in C2C12 myotubes. In addition, we explored the effects of ES on mitochondrial autophagy were varied from different time-courses. Here, we identified that 120 min of ES showed the optimum efficiency. We further demonstrated that Sirt3 signaling was the major pathway in moderate ES-induced mitochondrial autophagy. In the following study, siRNA interference will be used to observe the role of Sirt3 and Sirt1 in the mitochondrial function of skeletal muscle and to more precisely study the mechanism of the effects of oxidative stress on mitochondrial function of skeletal muscle.

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Conflicts of interest

None.

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