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Preconditioning improves muscle regeneration after ischemia-reperfusion injury

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Abstract

Ischemia-reperfusion injury (IRI) is a critical condition associated with serious clinical manifestations. Extensive research has focused on the strategies increasing organ tolerance to IRI. Preconditioning (PC) has been shown to provide protection to various organs toward IRI. However, the underlying mechanisms remain unknown. This study aimed to evaluate the role of PC on muscle regeneration after IRI and the potential underlying mechanisms. Three-month-old male UCP-1 reporter mice underwent unilateral hindlimb IRI with or without PC, the tissue viability and injury index were measured at 24 h after IRI. Hindlimb gait, muscle contractility, muscle histology were analyzed at 2 weeks after IRI. In another group of animals, β3 adrenergic receptor (β3AR) agonist amibegron and β3AR antagonist SR-59230A were administrated before PC/IRI, the hindlimb function and muscle regeneration were evaluated at 2 weeks after IRI. Our results showed that PC has little effect on improving the tissue viability at the acute phase of IRI, but it showed a long-term beneficial role of improving hindlimb function and muscle regeneration as evidenced by increased central nuclei regenerating myofibers. The effects of PC are related to inducing muscle fibro-adipogenic progenitor (FAP) brown/beige-like adipocyte (BAT) differentiation. Amibegron treatment displayed a similar role of PC while SR-59230A abolished the effect of PC. This study suggests PC has a beneficial role in promoting muscle regeneration after IRI through β3AR signaling pathway-stimulated FAP-BAT differentiation.

Keywords

ischemia-reperfusion injury; muscle regeneration; preconditioning; β3 adrenergic receptor

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AUTHOR CONTRIBUTIONS

He Zhang, Hubert T. Kim, Brian T. Feeley, and Xuhui Liu designed the experiments; He Zhang, Mengyao Liu, and Xuhui Liu conducted the experiments, and collected and analyzed the data; He Zhang, Mengyao Liu, Hubert T. Kim, Brian T. Feeley, and Xuhui Liu wrote the manuscript.

1 | INTRODUCTION

Ischemia-reperfusion injury (IRI), which is characterized by deficient oxygen supply and subsequent blood flow restoration, is commonly seen in limb crush injuries, compartment syndrome, vascular injuries, and other orthopedic injuries in skeletal muscle.[1] Long-term consequences of IRI include muscle weakness, atrophy, and temporary or permanent limb function impairment, which negatively impacts patients' quality of life.[2,3] Several methods have been tested to prevent or lessen the harmful effects of IRI.[4] Preconditioning (PC) is the procedure of brief ischemia and reperfusion applied directly or remotely to target organs before subsequent prolonged IRI. PC has been reported to have a protective role in vascular reperfusion, muscle preservation, and decreasing inflammatory infiltrates to various organs, including skeletal muscle.[5,6] However, current studies of PC have conflicting results.[7,8] Much of the current research on PC focuses on its short-term effects on preserving muscle viability,[9,10] but few studies have focused on its long-term role in muscle regeneration and function recovery after IRI.

Fibro-adipogenic progenitors (FAPs) are a muscle residential interstitial progenitor cell population that facilitates muscle regeneration upon injury.[11,12] We recently discovered that FAP can adopt the brown/beige-like adipocyte (BAT) differentiation[13] and promote muscle regeneration after rotator cuff injury.[14–16] Beyond its metabolic role, BAT also has been found to secrete several growth factors that promote muscle growth.[17] BAT is characterized by the expression of its hallmark protein-uncoupling protein-1 (UCP-1). The protective role of UCP-1 has been demonstrated in acute IRI in the kidney through suppressing oxidative stress.[18] Hoeter et al.[19] found that the UCP-1 activity was induced during ischemia-reperfusion and the presence of UCP-1 mitigated reperfusion-induced damage by lowering mitochondrial hyperpolarization at reperfusion. The beta-adrenergic receptor (β-AR) signaling is a major pathway involved in BAT differentiation and thermogenesis. Beta-3 adrenergic receptor (β3AR) is primarily responsible for beige adipocyte induction in pre-existing white adipocytes under adrenergic agonists stimulation, while the cold-induced beiging occurs via β1AR.[20] The role of β3AR agonists in beige fat induction and activation has also been demonstrated in human adipose tissues.[21] In our previous work, we found that β3AR agonist amibegron induces FAP-BAT differentiation, improves muscle quality, and shoulder function after rotator cuff tears in mice.[14,22]

The goal of this study is to test the effect of PC in inducing FAP-BAT differentiation and muscle regeneration after IRI. We also sought to test the role of the β3AR pathway in regulating FAP-BAT differentiation and muscle regeneration after IRI. We hypothesize that PC has a beneficial role in muscle regeneration and function recovery after IRI by stimulating FAP-BAT differentiation through the β3AR pathway.

2 | MATERIALS AND METHODS

2.1 | Animal and procedures

Experiments were performed on 3-month-old male UCP-1 reporter mice (Stock No: 026690; Jackson Laboratory Corp.), in which a luciferase-tdTomato cassette was inserted into the first exon of UCP-1. The overall experimental design is depicted in Figure 1. The mice were

randomly divided into an IRI group and a PC + IRI group. After general anesthesia was achieved by l%–5% isoflurane in oxygen, mice underwent unilateral hindlimb PC with a rubber band on the base of the right thigh for 10 min followed by a 10-min break for three cycles for mice in PC + IRI group. The ischemic injury was performed on the same hindlimb at 24 h after PC by applying the rubber band on the base of the thigh for 3 h.[23] There were four mice in each group that were harvested at 24 h and eight mice in each group that were harvested at 2 weeks post IRI (four mice for muscle contractility test and four mice for histology analysis). In the β3AR antagonist treatment group, mice were then divided into two groups: dimethyl sulfoxide (DMSO) + PC + IRI ($n = 4$) and SR-59230A+ PC + IRI (SR+PC + IRI, $n = 4$). In the β3AR agonist treatment group, mice were randomly divided into DMSO + IRI ($n = 4$) and amibegron + IRI (Ami + IRI, $n = 4$) group. Amibegron (10 mg/kg; β3AR agonist), 10 mg/kg SR-59230A (β3AR antagonist) were administered to mice 24 h before PC/IRI through intraperitoneal injection; DMSO was used as vehicle control. All the mice were housed in a neutral temperature environment on a 12/12 h light/dark cycle and were provided with standard laboratory food and water. All experiments were approved by the Institutional Animal Care and Use Committee of our institution.

2.2 | Gait analysis

Gait analysis was conducted using a DigiGait system (Mouse Specifics) to assess hindlimb function at 2 weeks after the ischemia injury. All the mice walked at 10 cm/s for 10 s on the DigiGait system as described previously.[14,24] Swing, propel, stance, stride, and stride length were recorded to assess hindlimb function,[23,25] all the parameters were normalized by the baseline to evaluate the difference pre- and post-injury.

2.3 | Muscle contractility test

The muscle contractility test was conducted using 3-in-1 Whole Animal System (Aurora Scientific) at 2 weeks after the ischemia injury. All instrumentations were turned on at least 30 min before testing for proper calibration and to minimize thermal drift of the force transducer. Mice were under general anesthesia with 2% isoflurane in oxygen inhalation and placed on a heated platform (37°C) during the whole testing process. An incision was made on the skin anterior to the ankle and the tendon of the tibialis anterior (TA) muscle was carefully isolated. A 4.0 Ethicon silk nonabsorbable suture was tied to the tendon. The other side of the suture was attached to a load cell as described previously.[26,27] After the optimal length of the muscle is achieved, the maximally fused tetanic contraction was obtained at 150 Hz at this length. The maximal specific force (mN)/muscle weight (mg) was used to evaluate muscle contractility.

2.4 | Muscle harvesting and histology

Mice were killed at either 24 h or 2 weeks after the initial injury. Muscle specimens were flash-frozen by immersion in liquid nitrogen cool isopentane and sectioned at the thickness of 7 μm with a cryostat for histology analysis. Slides were fixed in cold acetone for 10 min and rinsed in distilled water followed by hematoxylin and eosin (H&E) staining. After dehydrated with xylene, slides were mounted with resinous mounting media (Permount). The slides were reviewed by two blinded reviewers and the injury index (the percentage of damaged fibers/total fibers) was calculated. For immunofluorescence staining, slides were

fixed with 4% paraformaldehyde and rinsed in phosphate-buffered saline (PBS), incubated in blocking solution (0.1% Triton X-100, 2% bovine serum albumin in PBS) for 30 min at room temperature and then primary antibody (rabbit anti-Laminin, 1:200, Sigma-Aldrich L9393) at 4°C overnight. Slides were then washed with PBS, and treated with secondary antibody (donkey anti-rabbit IgG Alexa Fluor® 488, ab150073, 1:200; Abcam). Tissue sections were stained with DAPI (4′,6-diamidino-2-phenylindole) and then mounted with Fluoromount-G. Cross-sectional area (CSA) was measured from at least four different locations in the muscle belly for each sample. Regenerating muscle was measured with center nucleation fiber rate (number of fibers with central nuclei/total fiber numbers × 100%). To evaluate the percentage of UCP-1-positive cells, the UCP-1(+) cell numbers were divided by total cell number × 100%. The minimal Feret's diameter (MFD) which is defined as the closest possible distance between the two parallel tangents of muscle fiber was measured by CellProfiler (3.1.9) as previous described.[28]

2.5 | Muscle viability assay

Muscle viability was measured using methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (Sigma-Aldrich M5655). TA muscles were cut into pieces and washed with ice-cold PBS. Muscle pieces were then incubated in 300-μl MMT solution (1 mg/ml) at 37°C for 3 h with shaking. After incubation, muscles were washed with deionized water then incubated with 2-propanol overnight at 37°C with shaking. 2-Propanol (200 μl) solution was then taken out for absorbance testing at OD = 590 nm with a microplate reader.

2.6 | Statistical analysis

Independent t test was applied on tissue viability, gait parameters, muscle contractility, muscle CSA, center nucleation, MFD, and UCP-1-positive cells percentage between PC and non-PC IRI groups. The number of animals required for this study was determined based upon power analysis using the effect side of 0.2 (20% difference) and standard deviation of 0.1, type I error rate $\alpha = .05$, type II error rate $\beta = .20$. Based on those assumptions, we determined that four mice per group would be sufficient to demonstrate significant differences between the PC and non-PC groups. All data were shown as mean ± *SD*. Statistical difference was determined when $p < .05$.

3 | RESULTS

3.1 | PC does not improve tissue viability in acute phase after IRI

MTT test showed that after 24 h of IRI, PC did not improve the muscle viability compared with no PC IRI group (1.3 ± 0.2 vs. 1.2 ± 0.2; $p = .583$). However, histology grading based on H&E staining showed that TA muscle from mice treated with PC exhibited a significantly decreased injury index after IRI compared with no PC IRI group (7.2 ± 14.8 vs. 15.3 ± 11.3; $p = .008$; Figure 2).

3.2 | PC improves muscle regeneration in the chronic phase of IRI

Two weeks after IRI, mice treated with PC showed improved hindlimb function recovery as evidenced by less swing time (0.008 ± 0.009 vs. 0.021 ± 0.012; $p = .03$) and higher relative maximum specific muscle force (maximum specific force of injured TA/maximum specific

force of non-injured contralateral TA × 100%) compared with no PC IRI group (79.3 ± 17.3% vs. 58.1 ± 16.3%; $p = .036$; Figure 3A,B). As a marker of muscle regeneration, the central nucleation index was significantly upregulated with PC treatment compared with no PC IRI group (78.1 ± 8.8 vs. 63.4 ± 16.3; $p = .042$). However, no statistical difference was found in muscle CSA (1181.9 ± 408.0 vs. 1097.7 ± 512.9; $p = .722$) and MFD (31.2 ± 4.0 vs. 29.1 ± 7.6; $p = .494$; Figure 3D).

3.3 | PC-mediated muscle regeneration is related to FAP-BAT differentiation

Although no difference was found in the percentage of UCP-1-positive cells in total cells at the 24-h time point when comparing PC + IRI group to IRI group (3.3 ± 2.2 vs. 2.5 ± 2.0; $p = .212$), PC significantly induced the UCP-1(+) FAP percentage at 2 weeks after IRI in TA muscle (10.1 ± 2.6 vs. 6.5 ± 0.8; $p = .002$; Figure 4).

3.4 | β3AR antagonist SR-59230A blocks the effect of PC

SR-59230A, a specific β3AR antagonist, abolished the effect of PC as evidenced by significantly less propel time (−0.089 ± 0.054 vs. −0.012 ± 0.025; $p = .042$) and reduced percentage of maximum muscle force relative to their contralateral side (63.9 ± 20.0% vs. 101.2 ± 12.1%; $p = .019$) in SR-59230A-treated group compared with the vehicle-treated control group (Figure 5A,B). In addition, the percentage of UCP-1-positive cells was also significantly down-regulated with SR-59230A injection at 2 weeks after PC and IRI (1.5 ± 0.6 vs. 10.6 ± 2.0; $p = .0001$). No significant difference in the regenerative muscle central nucleation was found between the SR-59230A treatment group and the vehicle-treated control group (2.6 ± 1.4 vs. 32.6 ± 28.6; $p = .127$; Figure 5C).

3.5 | β3AR agonist amibegron simulates the effect of PC

Mice treated with amibegron, a specific β3AR agonist significantly improved hindlimb function recovery compared with the vehicle-treated control group as evidenced with significantly more propel (0.022 ± 0.030 vs. −0.066 ± 0.041; $p = .014$)/stance (0.029 ± 0.013 vs. −0.047 ± 0.036; $p = .018$)/stride time (0.035 ± 0.030 vs. −0.054 ± 0.032; $p = .006$) and longer stride length (0.375 ± 0.299 vs. −0.525 ± 0.275; $p = .004$), as well as higher muscle contractility (104.7 ± 11.2 vs. 75.1 ± 12.0; $p = .011$) at 2 weeks after IRI (Figure 6A,B). Amibegron treatment also increased the percentage of UCP-1-positive cells (11.4 ± 1.1 vs. 8.2 ± 2.1; $p = .032$) and central nucleation (93.3 ± 4.5 vs. 81.1 ± 3.5; $p = .005$) compared with the vehicle group at 2 weeks after IRI (Figure 6C).

4 | DISCUSSION

Despite acute damage to muscular and nonmuscular tissues, skeletal muscle IRI can also cause damaging long-term consequences including muscle weakness, fibrosis, and disability to affected patients. Short-term treatment for IRI focuses on the preservation of muscle viability and minimization of local and remote organ damage. However, when muscle damage is irreversible, promoting muscle regeneration will become the focus to alleviate the consequence of IRI. Although most PC studies focused on its role in short-term muscle preservation, we focused on the long-term of PC on muscle regeneration after IRI in this study. In the acute phase of IRI, we found that PC has a minimal role in the preservation

of muscle viability even though it reduced the injury index histologically. However, in the chronic phase of IRI, we found that PC significantly improved muscle regeneration and improved muscle function. Results from the current study provided preclinical evidence of long-term beneficial role of PC on muscle regeneration after IRI.

Regeneration of muscle to improve body or limb function is the eventual goal of regenerative muscle studies. In this study, we found that PC significantly increased TA muscle contractility and improved hindlimb gait after IRI. Gait abnormalities are associated with decreased muscle function. The study has reported that the loss of muscle strength and mass induced by muscular dystrophy could lead to gait disturbances.[29] Our previous work has also demonstrated forelimb gait is directly related to rotator cuff muscle atrophy and fatty degeneration.[14,22,24] Thus, results from these functional tests suggest that PC stimulates functional muscle regeneration after IRI. It is interesting that muscle contractility improvement was not accompanied with an increase in muscle fiber size. However, there are several mechanisms by which muscle contractility can improve without increased muscle size. One possible explanation for this is that the effects of PC may relate to improved nerve-muscle junction regeneration and function, rather than increased muscle size. A previous study reported that PC activates endogenously released factors including neurotransmitters.[30] Cruz et al.[31] and Hyngstrom et al.[32] reported increased neuromuscular activation as one of the main effectors of PC. Dong et al.[33] showed that dissecting the femoral nerve abolished the myocardial infarct-limiting effect of remote limb PC. This effect of PC target organ innervation was also confirmed by a study by Ding et al.,[34] in which they found that the cardioprotection provided by remote PC in a rabbit model was abolished when the renal nerve was sectioned before renal IRI. Future work is warranted to determine the relationship of PC on muscle innervation.

In this study, we observed a significant increase in the percentage of UCP-1-positive FAPs in the muscle after PC, indicating that PC can induce FAP-BAT differentiation after IRI. BAT is highly metabolically active and utilizes chemical energy for heat production,[35] and UCP-1 plays a central role in BAT activation. Recent studies have shown that the transplantation of brown fat in muscle can significantly increase muscle mass and contractile force production upon cardiotoxin injury,[36] suggesting its role in muscle regeneration. We have previously shown that transplantation of FAP-BAT or activation of FAPs with amibegron, improves muscle function in a chronic rotator cuff repair model.[13] BAT is also considered to be an endocrine organ in the body that secretes adipokines such as adiponectin and FGF21.[37,38] Gunawardana et al.[39] has reported elevation of the promyogenic factor IGF1 level in embryonic BAT transplanted mice with type 1 diabetes. Follistatin, which has been demonstrated to be the mediator of functional interaction between FAPs and muscle satellite cells during muscle regeneration,[12] is also highly expressed in BAT and skeletal muscle.[40] Future work is needed to define if BAT-differentiated FAPs secrete promyogenic cytokines that are the mediators of PC in protecting muscle from IRI.

β3AR is a subtype of β-adrenergic receptors which are members of the G protein-coupled receptor family.[41] This receptor family has garnered considerable attention on regulating metabolic processes since it was first discovered. β3AR has emerged as a leading molecular target for activating brown or beige adipocytes.[42] A previous study has reported that β3AR-

KO mice showed impaired cold-induced thermogenesis with a reduction in white adipocyte beiging.[43] In addition, FAPs can be induced and differentiated into BAT by a β3AR agonist.[44] Our results showed that PC could induce the expression of UCP-1 after IRI. We further found that the effect of PC was abolished with β3AR antagonist SR59230A injection as evidenced by poor gait and significantly reduced muscle contractility in this treatment group. The percentage of UCP-1-positive cells also significantly decreased. However, we did not see a significant difference in central nucleation. Conversely, amibegron treatment showed improved hindlimb gait and muscle contractility with upregulation in UCP-1-positive cells percentage and central nucleation, indicating that amibegron treatment has an equivalent role of PC in the chronic phase of IRI. Amibegron is a selective β3AR agonist with high affinity and potency for β3AR but interact with β1- and β2-ARs only weakly.[45] SR59230A is one of the most widely used β3AR antagonists. It is first listed as a putative β3AR antagonist in rat gut while also displaying interesting properties in rat brown adipose tissue.[46] These data suggest that the β3AR signaling may be the underlying mechanism of PC in regulating muscle regeneration and function recovery in the chronic phase of IRI.

There are some limitations of this study. First, due to the fact that UCP-1 driven luciferase-tdTomato reporter gene was inserted into the Y chromosome, only male mice were used in this study. Though no gender difference of muscle IRI has been reported so far, future work is warranted to include female mice to investigate the effect of gender on muscle regeneration after IRI. Second, to reduce animal numbers, only one time point (2 weeks after IRI) was used in this study. Though 2 weeks post-injury has been proven as a time point with robust muscle regeneration after IRI in our previous and current studies, more time points will be adopted in future works to address the time course of muscle regeneration. Third, we only tested TA muscle, a typical glycolytic muscle in this study. The skeletal muscle metabolic phenotype importantly modulates the deleterious effects of lower limb IR. The glycolytic muscle has been reported to be less protected compared with oxidative skeletal muscles against ischemia-reperfusion due to its lower antioxidant capacity.[47] Thus, we only collected TA muscles for histology analysis or tissue viability assay in this study. Last but not the least, though amibegron and SR-590230A are relatively highly selective to β3AR, off-target effects may exist and potentially influence the results. β3AR knockout mice may be employed in future studies to avoid this problem.

In conclusion, this study revealed a beneficial role of PC in promoting muscle regeneration, a role beyond its typical muscle protection after IRI. We further defined that the role of PC in muscle regeneration is related to FAP-BAT differentiation through the β3AR signaling pathway.

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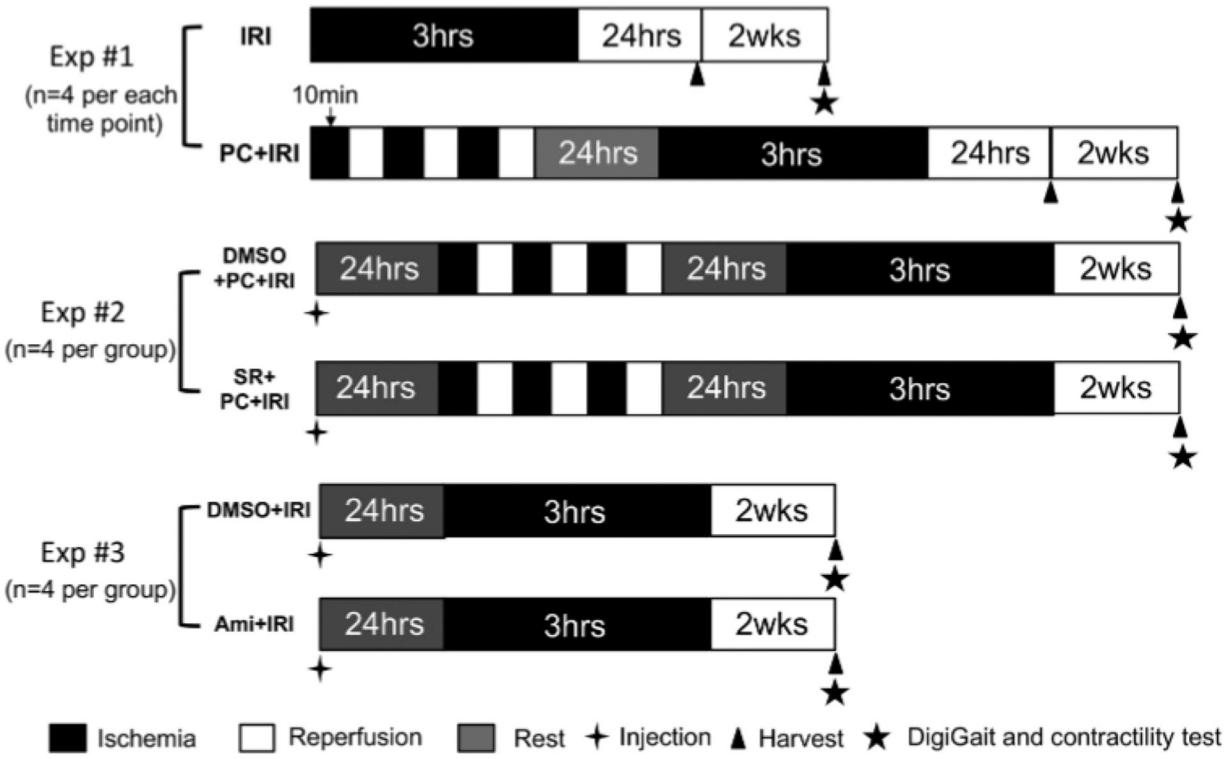

FIGURE 1.

Flow diagram of experimental design. Four mice in each group received ischemia-reperfusion injury (IRI) with or without preconditioning (PC) treatment and harvested at 24 h later, four mice in each group received the same treatment and were harvested at 2 weeks later. Four mice in each group received SR-59230A, amibegron, and dimethyl sulfoxide (DMSO) injection along with PC and IRI, then harvested at 2 weeks

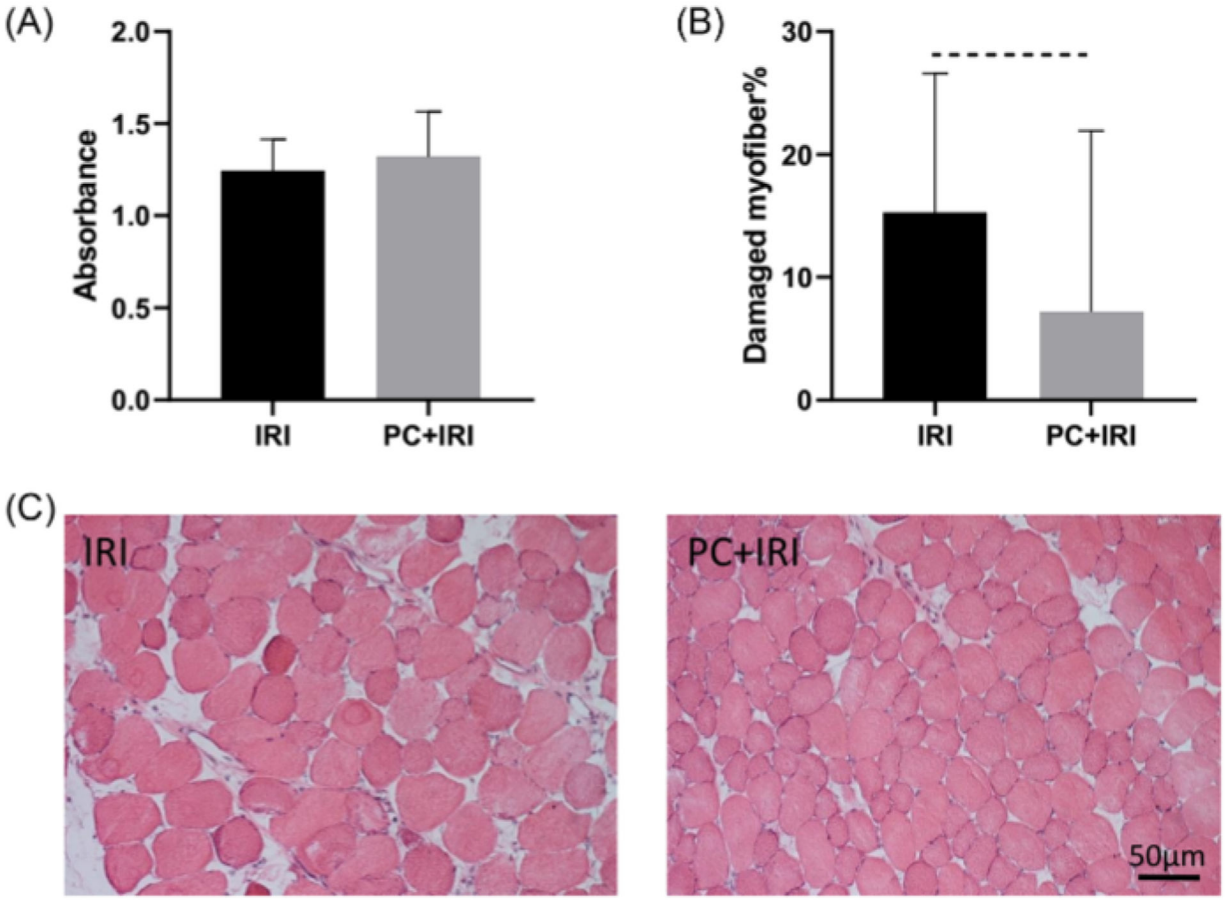

FIGURE 2.

Preconditioning (PC) showed no effect on tissue viability (A) at 24 h after ischemia-reperfusion injury (IRI). However, PC reduced tibialis anterior (TA) muscle injury index (damaged myofiber %) histologically (B). (C) Typical images of hematoxylin and eosin staining of TA muscle from mice with and without PC at 24 h after IRI. Dashed lines indicate $p < .01$ ($N = 4$ in each group)

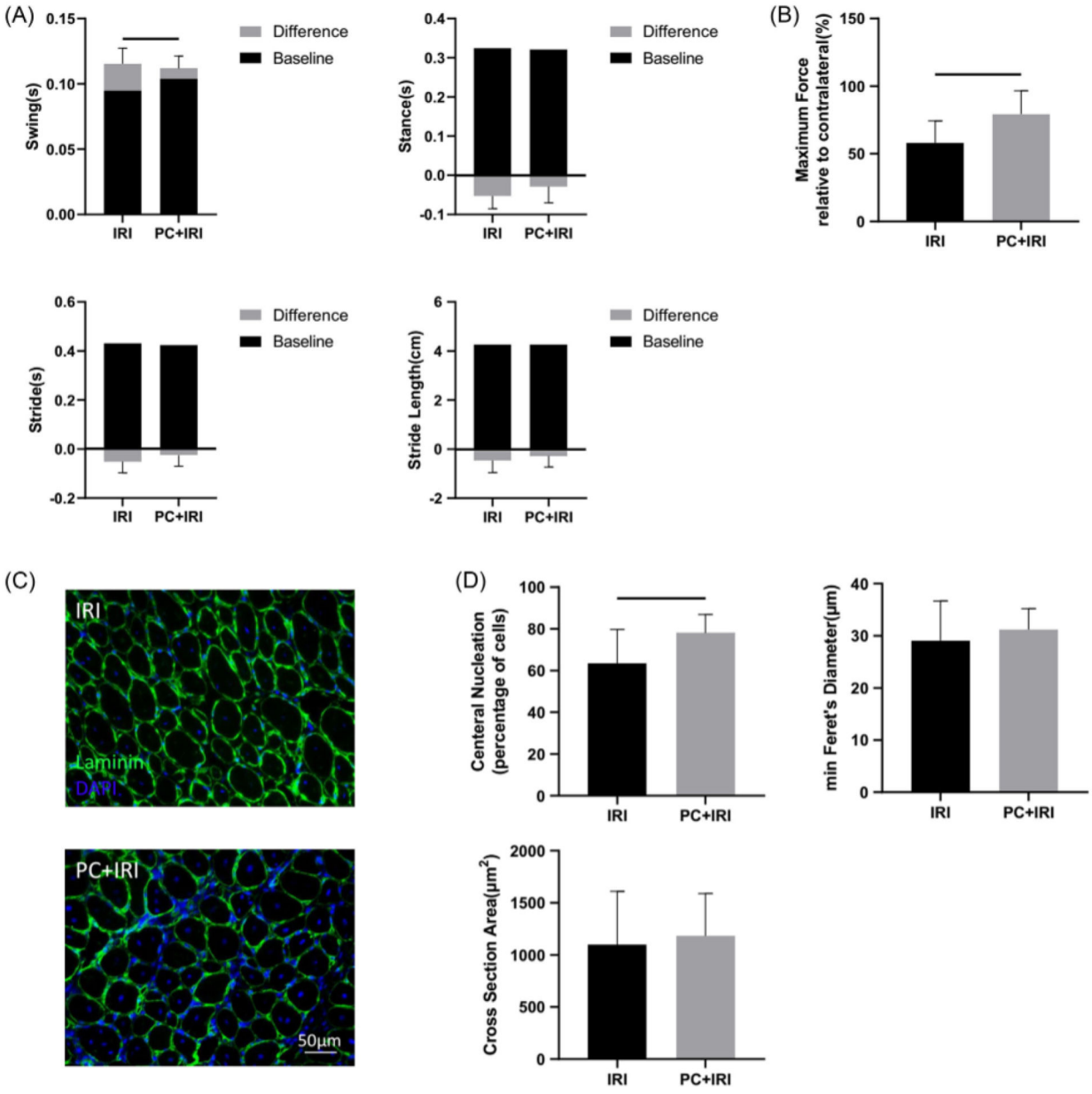

FIGURE 3.

(A, B) Preconditioning (PC) improved hindlimb gait (A) and muscle contractility (B) at 2 weeks after ischemia-reperfusion injury (IRI). (C) Typical images of tibialis anterior (TA) muscle at 2 weeks after IRI. (D) PC significantly increased regenerating central nuclear myofibers, but did not increase fiber diameter or cross-sectional area in TA muscle at 2 weeks after IRI. Solid lines indicate $p < .05$ ($N = 4$ in each group)

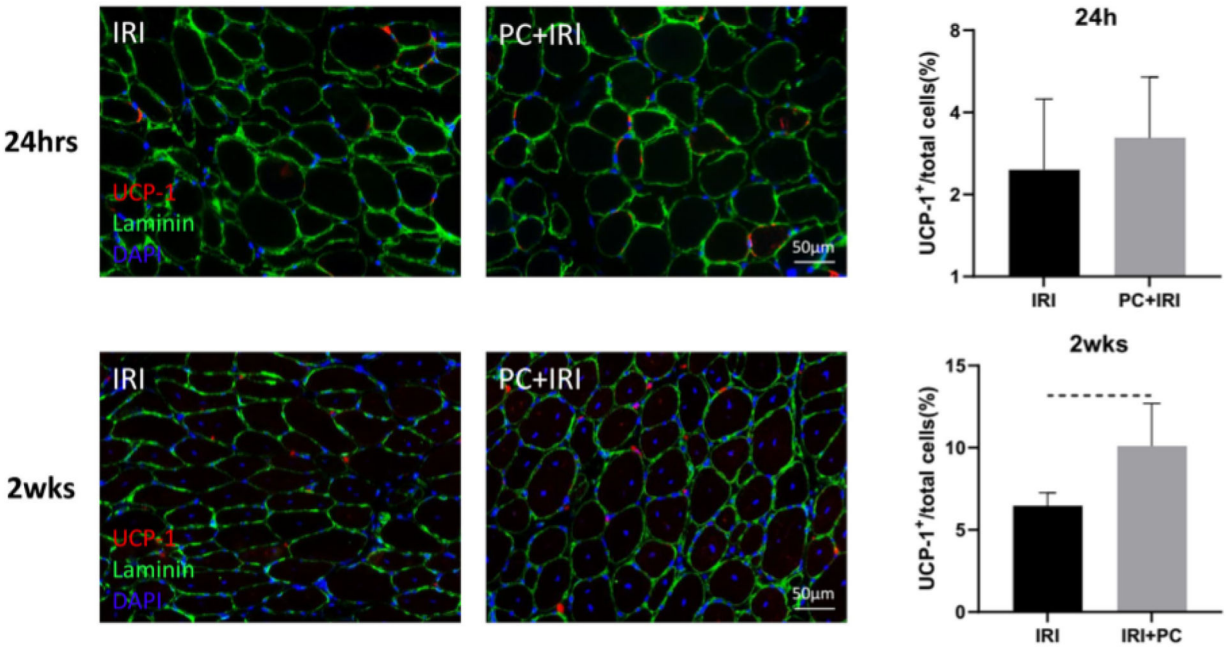

FIGURE 4.

Preconditioning (PC) significantly increased the percentage of UCP-1-positive cells in tibialis anterior muscle at 2 weeks after ischemia-reperfusion injury (IRI). Dashed lines indicate $p < .01$ ($N = 4$ in each group)

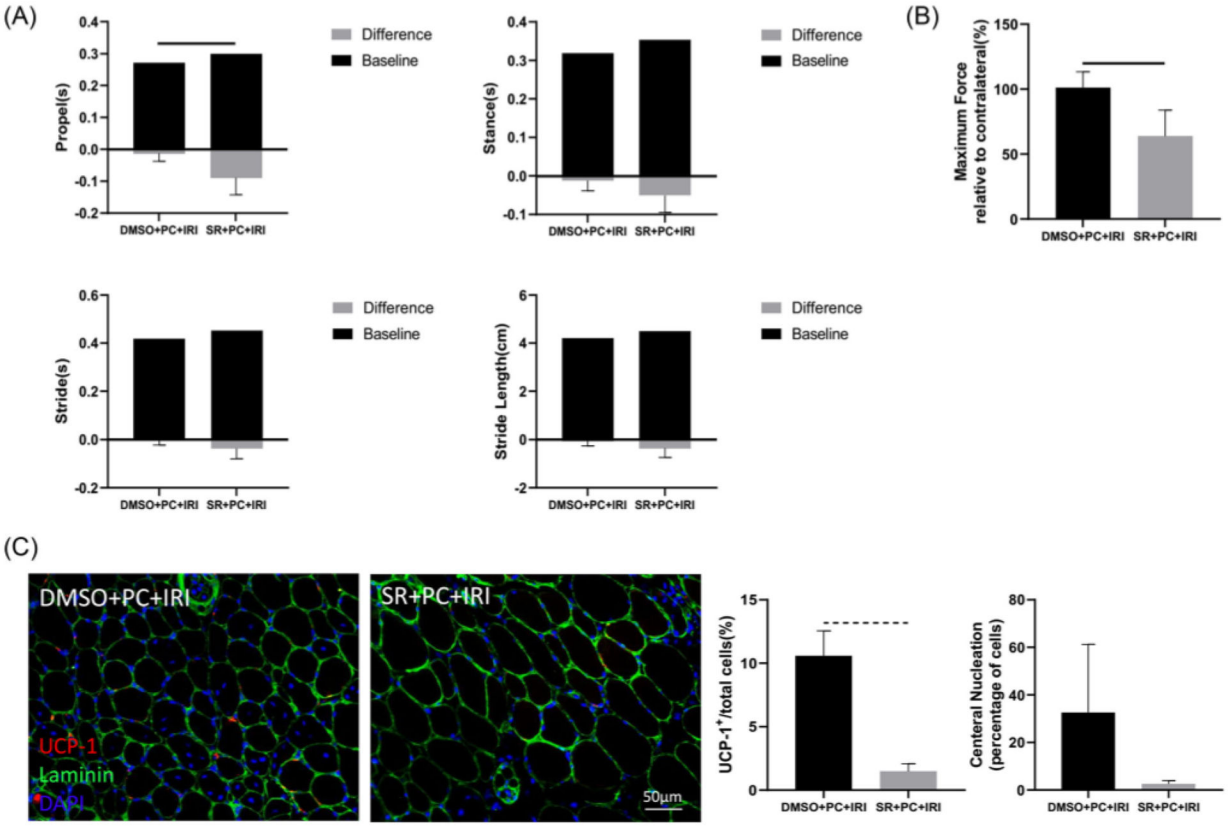

FIGURE 5.

(A, B) β3AR antagonist SR-59230A significantly reduced propel time (A) and muscle contractility (B). (C) SR-59230A significantly decreased the percentage of UCP-1-positive cells, however, no significant difference was found in central nucleation in TA muscle at 2 weeks after IRI. Solid lines indicate $p < .05$ and dashed lines indicate $p < .01$ ($N = 4$ in each group). DMSO, dimethyl sulfoxide; IRI, ischemia-reperfusion injury; PC, preconditioning; TA, tibialis anterior

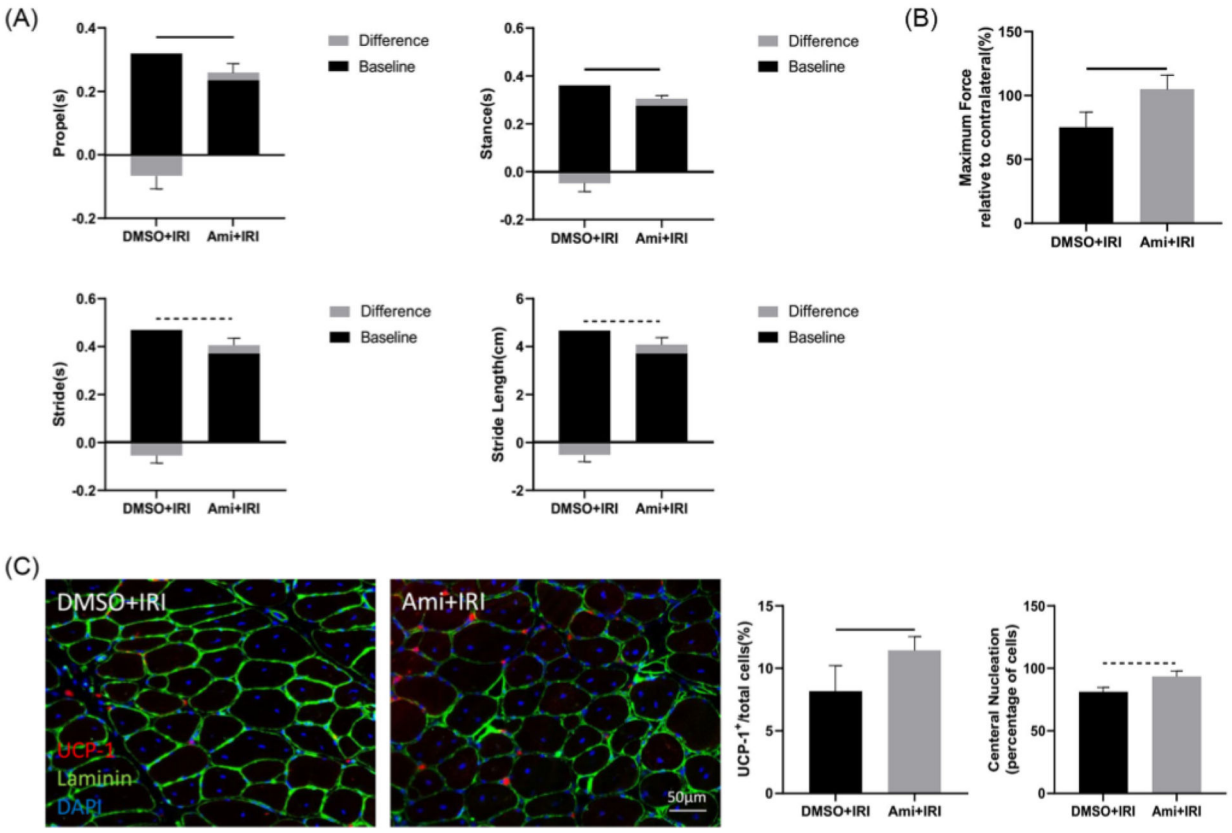

FIGURE 6.

(A, B) β3AR agonist amibegron improved hindlimb gait (A) and muscle contractility (B) at 2 weeks after IRI. (C) Amibegron significantly increased the percentage of UCP-1-positive cells and central nucleation in TA muscle. Solid lines indicate $p < .05$ and dashed lines indicate $p < .01$ ($N = 4$ in each group). Ami, amibegron; DMSO, dimethyl sulfoxide; IRI, ischemia-reperfusion injury; PC, preconditioning; TA, tibialis anterior