Original Paper

Protective Effects of Sonic Hedgehog Against Ischemia/Reperfusion Injury in Mouse Skeletal Muscle via AKT/mTOR/p70S6K Signaling

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Key Words

Sonic hedgehog • Ischemia/reperfusion • Skeletal muscle • Apoptosis

Abstract

Background/Aims: Skeletal muscle ischemia/reperfusion (I/R) injury is a common and severe disease. Sonic hedgehog (Shh) plays a critical role in post-natal skeletal muscle regeneration. In the present study, the role of Shh in skeletal muscle I/R injury and the mechanisms involved were investigated. **Methods:** The expression of Shh, AKT/mTOR/p70S6K and apoptosis pathway components were evaluated following tourniquet-induced skeletal muscle I/R injury. Then, mice were subjected to systemic administration of cyclopamine or one-shot treatment of a plasmid encoding the human Shh gene (phShh) to examine the effects of Shh on I/R injury. Moreover, mice were subjected to systemic administration of NVP-BEZ235 to investigate the role of the AKT/mTOR/p70S6K pathway in Shh-triggered skeletal muscle protection. **Results:** We found that the levels of Shh, AKT/mTOR/p70S6K pathway components and Cleaved Caspase 3 and the Bax/Bcl2 ratio initially increased and then decreased at different time points post-I/R injury. Moreover, Shh protected skeletal muscle against I/R injury by alleviating muscle destruction, reducing interstitial fibrosis and inhibiting apoptosis, and these protective effects were abrogated when the AKT/mTOR/p70S6K pathway was inhibited. **Conclusion:** Collectively, these data suggest that Shh signaling exerts a protective role through the AKT/mTOR/p70S6K signaling pathway during skeletal muscle I/R injury. Thus, Shh signaling may be a therapeutic target for protecting skeletal muscle from I/R injury.

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Introduction

Limb ischemia-reperfusion (I/R) injury is a frequent clinical problem, usually secondary to trauma, critical limb ischemia, or revascularization after thromboembolic events or the

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use of a surgical tourniquet [1-3]. Skeletal muscle has high metabolic activity and therefore is sensitive to reperfusion injury after ischemia [4]. Despite rapid restoration of blood flow after severe ischemia, I/R injury causes continuous damage and necrosis of skeletal muscle, which may lead to amputation and multi-system organ dysfunction syndrome [5]. Thus, it is important to develop treatments to minimize skeletal muscle destruction after I/R injury.

Sonic hedgehog (Shh) is a crucial morphogen that regulates epithelial-mesenchymal interactions during embryogenesis [6, 7]. In adults, the Shh pathway has been shown to be up-regulated following skeletal muscle and myocardium ischemia, suggesting that the embryonic Shh pathway can be recruited [8, 9]. Recently, injection of plasmid encoding the human Shh gene (phShh) to facilitate Shh signaling has been proposed to be a potential treatment for certain ischemic cardiovascular diseases. In acute and chronic myocardial ischemia in adults, intra-myocardial phShh injection not only promoted neovascularization and preserved left ventricular function but also reduced fibrosis and cardiac apoptosis [9]. Moreover, the Shh signaling pathway has been shown to play an important role in cerebral, myocardial and renal I/R injury [10-13]. However, the role of the Shh pathway and the therapeutic efficacy of Shh in skeletal muscle I/R injury have not been investigated. In the present study, we found that Shh could be activated postnatally in skeletal muscle subsequent to I/R injury and play a positive role in I/R injury.

Materials and Methods

Animals

Young (10-14 weeks) male C57BL/6 mice were used for this study. All the mice were SPF grade and were obtained from the Department of Laboratory Animal Center of the Chongqing Medical University. Mice were housed under pathogen-free conditions with a 12 h dark/light cycle and provided with food and water *ad libitum*. All animal experimental protocols were implemented according to the instructions of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the ethics committee of the First Affiliated Hospital of Chongqing Medical University. The mice were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) before model construction and skeletal muscle tissue sample collection.

Acute hindlimb I/R injury model

The skeletal muscle I/R injury model was constructed according to previous studies [14, 15]. In general, unilateral hindlimb ischemia was induced by placing an orthodontic rubber band at the right hip joint using a McGivney hemorrhoidal ligator for 3 hours followed by removal of the orthodontic rubber band tourniquet for 1, 3, 5, 7 or 14 days of reperfusion. During the pre-ischemic, ischemic, and initial 3 hours of reperfusion intervals, mice were placed on a heating pad to maintain body temperature at 37°C [14]. Tourniquet-induced I/R injury was identified by measuring blood flow to the gastrocnemius muscle as described previously [15, 16]. Blood flow dropped to approximately 2 % of baseline after placement of the tourniquet and remained at a steady level during the 3 hours of ischemia. Upon tourniquet release, a rapid and transient increase in blood flow to approximately 50 % of baseline was observed, which was followed by a decline to a steady state of approximately 30 % of baseline.

phShh treatment

The native full length Shh gene product undergoes an auto-processing reaction during its biogenesis resulting in amino- and carboxy-terminal domain products [17]. The biological activity is contained in the amino-terminal cleavage product. However, during auto-processing, the amino-terminal domain products are cholesterol modified, and this modification causes the amino-terminal protein to be tightly cell associated [18], leaving the protein tethered to the cell that made it. This is disadvantageous for a local gene therapy approach. Therefore, the amino-terminal domain of the human Shh coding sequence (600 bp) was selected to construct phShh with a pCMV-ScriptPCR mammalian expression vector as previously reported [9]. Empty expression vector was used as a control. Both phShh and the empty expression vector were obtained from Genechem (Shanghai, China). Meanwhile, the treatment procedure was performed as previously described

Table 1. Drug treatment protocol. DMSO, dimethyl sulfoxide; PEG 300, polyethylene glycol 300; NMP, N-Methyl pyrrolidone; tiw, three times weekly

	Start time	Dose/frequency (duration)	Vehicle (mixture)	Administration route
Cyclopamine	One day before I/R injury	10 mg/kg/day (8 days)	10% DMSO	Intraperitoneal injection
			30% PEG 300 5% Tween 80 55% ddH ₂ O	
NVP-BEZ235	One week before I/R injury	10 mg/kg tiw (2 Weeks)	10% PEG300 90% NMP	Intraperitoneal injection

[19, 20]. In general, ten minutes after placement of the tourniquet, 0.2 ml of sterile saline containing 200 µg of either phShh or the empty expression vector was injected into five separate sites of the hindlimb (40 µg in each site).

Drug treatment protocol

Cyclopamine, a Smoothed inhibitor (Selleck, Shanghai, China), was used to inhibit the Shh pathway. Experimental group mice received an intraperitoneal injection of cyclopamine (dissolved in 10% DMSO+30% PEG 300+5% Tween 80+55% ddH₂O mixture, at a concentration of 1 mg/ml and a dose of 10 mg/kg/day, starting 1 day before injury) for 8 days until sacrifice, as previously reported [21]. Control group mice were treated intraperitoneally with an equal volume of vehicle (Table 1). NVP-BEZ235, a dual PI3K-mTOR inhibitor (Selleck, Shanghai, China), was used to inhibit the AKT/mTOR/p70S6K pathway. Experimental group mice received an intraperitoneal injection of NVP-BEZ235 (dissolved in 10% PEG 300 + 90% NMP mixture, 100 µl, at a dose of 10 mg/kg, three times a week) for 2 weeks until sacrifice, as previously described [22]. Control group mice were treated intraperitoneally with an equal volume of vehicle (Table 1).

Histology

Hindlimb skeletal muscle tissue samples were collected, fixed in 4% paraformaldehyde, embedded in paraffin wax, cut into 5-µm sections, and stained with hematoxylin and eosin (H&E) and Masson's trichrome staining. The histological degree of skeletal muscle injury in each mouse was evaluated in five random fields from the H&E staining, and the destruction score was calculated according to a previous study [23]. The values for caliber variation, blurring of cell borders, cytoplasmic fragmentation, cell distances, erythrocyte extravasation, infiltration, loss of nuclei and centralization of nuclei were blinded and scored independently by four researchers. Each criterion was graded between 0 points for normal findings to 3 points for very distinctive findings. Masson's trichrome staining (Polyscience, Warrington, PA, USA) was used for observation of muscle fibrosis and was performed as previously described [24]. The collagen area was used for evaluation of the fibrotic tissue and determined by quantification of the area of blue staining. Fiji ImageJ software was used to quantify the collagen area percentage.

Immunofluorescence

Frozen, OCT-embedded mouse skeletal muscle samples were sectioned on a cryostat (Leica CM1900; Leica Microsystems Inc.; Buffalo Grove, IL). Primary antibody from Novus (CO, USA) was diluted 1:200, and the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG was diluted 1:200 (Thermo Scientific, USA). Nuclei were stained with DAPI (Beyotime Biotechnology, China).

TUNEL assay

The terminal transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) technique was used to detect apoptosis according to the manufacturer's protocol (Roche Molecular Biology, Mannheim, Germany) [25]. Sections were stained lightly with hematoxylin to allow identification of structural features and to localize TUNEL staining to particular cell types, such as endothelial cells and

muscle fibers. An apoptosis index was used to determine the extent of skeletal muscle apoptosis. Six fields at 400× magnification were randomly selected from two sections in each group, and the apoptosis index was calculated using Image-Pro Plus 6.0 and defined as the number of apoptotic cells/total number of cells counted.

Western blotting analysis

Radio-immunoprecipitation assay buffer (Beyotime Biotechnology, China) was used to extract total protein from the samples in each group according to the manufacturer's instructions. Protein concentrations were measured using a bicinchoninic acid (BCA) kit. Equivalent amounts of protein (60 µg) were loaded into each well, separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electro-transferred onto polyvinylidene fluoride (PVDF) membranes, blocked with 5% dry milk/bovine serum albumin (BSA) and immunoblotted with the indicated primary antibodies overnight at 4°C, followed by incubation with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. Using an enhanced chemiluminescence method (Millipore Corporation, Billerica, MA, USA), protein bands were detected with a Bio-Rad Gel Imaging System (Hercules, CA, USA) and analyzed with Bio-Rad Image Lab software (Hercules, CA, USA). The protein levels were determined by measuring the corresponding band intensities. The relative values of total protein were normalized to β-Tubulin. Antibodies against the following proteins were used in this study: Shh (Novus, CO, USA), Gli2 (LifeSpan, USA), Gli1 and p70S6K (both obtained from Bioworld Technology, Louis Park, MN, USA), phospho-mTOR and mTOR (both obtained from Abcam, Cambridge, MA, USA), phospho-AKT, AKT, phospho-p70S6K, Bcl2, Bax, and Cleaved Caspase 3 (all obtained from Cell Signaling Technology; Danvers, MA, USA).

Statistical analysis

Data are presented as the mean ± SEM. All data were analyzed using SPSS version 19.0 software (SPSS, Inc.). Comparisons between multiple groups were analyzed using one-way analysis of variance (ANOVA). Comparisons between two groups were analyzed using Student's *t*-test. A probability value of < 0.05 was considered statistically significant.

Results

Post-natal recruitment of the Shh pathway in hindlimb skeletal muscle after I/R injury

To investigate whether the Shh pathway was activated during skeletal muscle I/R injury, we established a young mouse model of 3 h hindlimb ischemia with subsequent reperfusion as previously reported [14, 15]. The mice were sacrificed after 1, 3, 5, 7 or 14 days of reperfusion, the skeletal muscle samples were harvested, and the Shh expression level was detected via immunofluorescence and western blotting. Immunofluorescence showed that Shh expression was barely detectable in control and 1 day of reperfusion skeletal muscle. Nevertheless, after 3 days and 5 days of reperfusion, apparent Shh expression was observed in the basement membrane of muscle cells and was co-localized with or adjacent to the myonuclei. After 7 days and 14 days of reperfusion, Shh expression decreased (Fig. 1A). Similar results were found with western blotting, Shh was up-regulated due to I/R injury, peaked at 5 days of reperfusion, and subsequently decreased. In addition, expression of both Gli1 and Gli2 accompanied activation of Shh signaling (Fig. 1B and C). The data suggested that Shh signaling pathway could be recruited following skeletal muscle I/R injury.

Inhibition of the Shh signaling pathway aggravated skeletal muscle damage

To elucidate whether the Shh pathway participated in regulation of skeletal muscle I/R injury, the Shh signaling pathway was inhibited through intraperitoneal injection of cyclopamine. Subsequently, the skeletal muscle 7 days after I/R injury was harvested and evaluated. Shh pathway proteins expression was inhibited following cyclopamine treatment (Fig. 2A and B). Compared to control, infarct areas were observed in the cyclopamine-treated skeletal muscle (Fig. 2C). The degree of skeletal muscle injury was evaluated using destruction scores based on H&E staining [23]. As Fig. 2D shows, significantly higher scores

Fig. 1. The Shh signaling pathway was activated following skeletal muscle I/R injury. (A) Immunofluorescence staining was used to detect Shh expression (green) in uninjured skeletal muscle and in skeletal muscle 1, 3, 5, 7 and 14 days post-I/R injury; nuclei were labeled with DAPI (blue). Scale bars, 50 μ m. n=5/group. (B and C) Western blotting was performed to estimate the protein expression of Shh, Gli1 and Gli2 in skeletal muscle 1, 3, 5, 7

and 14 days post-I/R injury; muscle from the uninjured contralateral limb served as the internal control. n=3/group. All data are presented as the mean $\pm$ SEM; * indicates a statistically significant difference between each I/R group and the control group ($p < 0.05$).

were observed with respect to blurring of cell borders, cytoplasmic fragmentation and centralization of nuclei in the cyclopamine treatment group, indicating that skeletal muscle damage caused by I/R injury deteriorated after inhibition of the Shh pathway. In addition, Masson's trichrome staining was performed to quantify intramuscular collagen deposition to estimate the degree of skeletal muscle fibrosis. There was a significant increase in the percentage of area occupied by collagen in the cyclopamine treatment group compared to the control group (42% vs. 25%, $p < 0.05$, Fig. 2E). These results indicated that inhibition of the Shh pathway aggravated skeletal muscle I/R injury.

Shh gene therapy exerted a protective effect on skeletal muscle after I/R injury

To further test whether the Shh pathway exerted a protective function following I/R injury, Shh was over-expressed through intramuscular injection of phShh. The immunofluorescence results showed that Shh expression was enhanced after phShh treatment (Fig. 3A). Moreover, the western blotting results showed that Shh, Gli1 and Gli2 expression was up-regulated due to phShh treatment (Fig. 3B). The destruction scores and collagen area percentage were used to measure the degree of skeletal muscle injury and fibrosis. As shown in Fig. 3 C, D and E, significantly lower scores of caliber variation, cell distance and centralization of nuclei

Fig. 2. Inhibition of Shh aggravated skeletal muscle destruction and fibrosis in response to I/R injury. (A and B) In muscle, 7 days after I/R injury, the protein expression of Shh, Gli1 and Gli2 in the cyclophosphamide treatment group, vehicle treatment group and untreated group was estimated by western blotting; muscle from the uninjured contralateral limb served as the control group. n=3/group. (C) Representative image and H&E staining of a muscle 7 days post-I/R injury treated with cyclophosphamide in comparison with the vehicle treatment group. (C left, infarct areas were marked with yellow arrows; C middle, magnification = $\times 40$, infarct areas are marked with black dashed line, Scale bars, 500 μm ; C right, magnification = $\times 400$, Scale bars, 50 μm). n=5/group. (D) Destruction scores from H&E staining were used to estimate the level of skeletal muscle damage. (E) Representative images of Masson's trichrome staining of skeletal muscle in cyclophosphamide-treated and vehicle-treated groups. The collagen area, determined by quantification of the blue staining area, was used to evaluate the fibrotic tissue. Fiji Image J software was used to quantify the percentage of the collagen area. n=3/group. All data are presented as the mean $\pm$ SEM; * indicates a statistically significant difference between the cyclophosphamide treatment group and the vehicle treatment group ($p < 0.05$).

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as well as less collagen deposition (28% vs. 16%, $p < 0.05$) were observed in phShh-treated skeletal muscle, which indicated a decrease in skeletal muscle destruction and fibrosis. These data suggested that Shh plays a protective role in skeletal muscle I/R injury.

The AKT/mTOR/p70S6K signaling pathway was activated after skeletal muscle I/R injury

To investigate the time-course of anabolic activity in the skeletal muscle I/R injury model, we detected local expression of AKT, mTOR and p70S6K in skeletal muscle after I/R

Fig. 3. Effects of phShh treatment on skeletal muscle destruction and fibrosis in response to I/R injury. (A) Skeletal muscle was treated with phShh after ischemic injury, and immunofluorescence staining for Shh (green) was performed on cross sections of phShh-treated and control vector-treated skeletal muscle 7 days after I/R injury; nuclei were labeled with DAPI (blue). Scale bars, 50 μ m. $n=5$ /group. (B) Western blotting was performed to estimate the protein expression of Shh, Gli1 and Gli2 in phShh-treated, control vector-treated and untreated skeletal muscle 7 days after I/R injury. Muscle from the uninjured contralateral limb served as the control group. $n=3$ /group. (C) H&E staining and Masson's trichrome staining were performed on muscle sections from the phShh treatment group and the control vector treatment group 7 days after I/R injury (C left, H&E staining, magnification = $\times 400$; C right, Masson's trichrome staining, magnification = $\times 400$). Scale bars, 50 μ m. $n=3$ /group. (D) Destruction scores determined by H&E staining of skeletal muscle were used to estimate the degree of muscle damage. (F) The collagen area, based on Masson's trichrome staining, was used to evaluate the fibrotic tissue and was determined by quantification of the blue staining area. Fiji ImageJ software was used to quantify the percentage of collagen area. All data are presented as the mean $\pm$ SEM; * indicates a statistically significant difference between the phShh treatment group and the control vector treatment group ($p < 0.05$).

Fig. 4. Shh activation combined with AKT/mTOR/p70S6K signaling up-regulation following skeletal muscle I/R injury. (A) Western blotting was used to estimate the protein expression of Shh, the phosphorylation and total protein expression of AKT, mTOR, and p70S6K after 3 hours of unilateral hindlimb ischemia and 1, 3, 5, 7, or 14 days of reperfusion; muscle from the uninjured contralateral limb served as the control. (B) Densitometry was used to quantify the immunoblot images, and the results are shown as the ratio of p-AKT to total AKT, p-mTOR to total mTOR and p-p70S6K to total p70S6K. $n=3/\text{group}$. All data are presented as the mean $\pm$ SEM; * indicates a statistically significant difference between the I/R group and the control group ($p < 0.05$).

Fig. 5. phShh treatment increased AKT/mTOR/p70S6K signaling following skeletal muscle I/R injury. (A) Skeletal muscle was treated with phShh after ischemic injury, and then, western blotting was used to estimate the protein expression of Shh, the phosphorylation and total protein expression of AKT, mTOR, and p70S6K in phShh-treated, control vector-treated, and untreated skeletal muscle 7 days after I/R injury. Muscle from the uninjured contralateral limb served as an internal control. (B) Densitometry was used to quantify the immunoblot images, and the results are shown as the expression of Shh and the ratio of p-AKT to total AKT, p-mTOR to total mTOR and p-p70S6K to total p70S6K. $n=3/\text{group}$. All data are presented as the mean $\pm$ SEM; * indicates a statistically significant difference between the phShh treatment group and the control vector treatment group, # indicates a statistically significant difference between the I/R group and the control vector group ($p < 0.05$).

injury. As shown in Fig. 4A and B, accompanied by the rapid induction of Shh expression, the phosphorylation levels of AKT, mTOR and p70S6K were all significantly increased in the

Fig. 6. The protective effects of phShh treatment were abrogated by inhibition of the AKT/mTOR/p70S6K pathway. (A, B and C) phShh treatment was used to over-express the Shh pathway, and NVP-BEZ235 was used to inhibit the AKT/mTOR/p70S6K pathway. Subsequently, in muscle 7 days after I/R injury, western blotting was used to estimate the expression of Shh, Gli1 and Gli2 and the phosphorylation and total protein expression of AKT, mTOR, and p70S6K in the control vector treatment, phShh treatment, vehicle treatment, NVP-BEZ235 treatment, phShh+NVP-BEZ235 treatment and untreated groups. Muscle from the uninjured contralateral limb served as the control group. Densitometry was used to quantify the immunoblot images. $n=3/\text{group}$. (D) H&E

staining and Masson's trichrome staining were performed on muscle sections from the phShh treatment group and phShh+NVP-BEZ235 treatment group 7 days after I/R injury (D top, H&E staining, magnification = $\times 400$; D Bottom, Masson's trichrome staining, magnification = $\times 400$. Scale bars, 50 μm). $n=3/\text{group}$. (E) The collagen area, based on Masson's trichrome staining, was used for evaluation of the fibrotic tissue and was determined by quantification of the blue staining area. Fiji ImageJ software was used to quantify the percentage of collagen area. (F) The destruction scores of skeletal muscle, determined by H&E staining, were used to estimate the degree of muscle damage. All data are presented as the mean $\pm$ SEM; * indicates a statistically significant difference; # indicates a statistically significant difference between each I/R group and the control group ($p < 0.05$).

early phase of recovery. Indeed, the ratio of p-AKT/AKT peaked at 4.6-fold and the ratio of p-p70S6K/p70S6K peaked at 7.9-fold after 3 days of reperfusion and decreased thereafter. The ratio of p-mTOR/mTOR peaked at 3.4-fold after 5 days of reperfusion and subsequently

Fig. 7. phShh treatment exerted an anti-apoptotic effect following skeletal muscle I/R injury. (A) The time-course expression of Cleaved Caspase 3, Bax and Bcl2 after 3 hours of unilateral hindlimb ischemia and 1, 3, 5, 7, or 14 days of reperfusion were estimated with western blotting. $n=3/\text{group}$. * indicates a statistically significant difference between each I/R group and the control group ($p < 0.05$). (B) phShh treatment was used to over-express Shh, and/or cyclopamine treatment was used to inhibit the Shh pathway. Then, the expression of Cleaved Caspase 3 protein, and the ratio of Bax/Bcl2 in skeletal muscle 7 days after I/R injury were estimated by western blotting. $n=3/\text{group}$. # indicates a statistically significant difference between the I/R+phShh group and the I/R+control vector group, * indicates a statistically significant difference between each I/R group and the I/R+phShh+cyclopamine group ($p < 0.05$). (C) TUNEL staining was used to examine the apoptosis of muscle cells in the phShh-treated and control vector-treated skeletal muscle 7 days after I/R injury. In addition, the apoptotic index was used to quantify the level of apoptosis. $n=3/\text{group}$. (D) phShh treatment was used to over-express Shh, and/or NVP-BEZ235 treatment was used to inhibit the AKT/mTOR/p70S6K pathway. Then, the expression of cleaved caspase-3 protein and the ratio of Bax/Bcl2 in skeletal muscle 7 days after I/R injury were estimated by western blotting. $n=3/\text{group}$. * indicates a statistically significant difference between each I/R group and the I/R+phShh+NVP-BEZ235 group ($p < 0.05$). All data are presented as the mean $\pm$ SEM.

decreased (Fig. 4A and B). The results indicated that the AKT/mTOR/p70S6K signaling pathway was activated in skeletal muscle in the early recovery phase of I/R injury.

Shh gene therapy increased local expression of the AKT/mTOR/p70S6K signaling pathway

To further test whether the AKT/mTOR/p70S6K pathway was involved in the process of Shh-triggered skeletal muscle protection following I/R injury, over-expression of the Shh pathway was induced using phShh as previously described, and the expression of AKT/mTOR/p70S6K pathway protein was detected after 7 days of reperfusion. As shown in Fig. 5A and B, the ratios of p-AKT/AKT, p-mTOR/mTOR and p-p70S6K/p70SK were significantly higher in the phShh treatment group than in the control vector group. From these data, it can be concluded that phShh treatment increased the anabolic activity in skeletal muscle following I/R injury, suggesting that Shh-mediated skeletal muscle protection is dependent on the AKT/mTOR/p70S6K pathway.

Inhibition of PI3K-mTOR abrogated Shh-induced skeletal muscle protection

To further investigate the role of the AKT/mTOR/p70S6K pathway in Shh-induced skeletal muscle protection, phShh was used to over-express Shh, and/or NVP-BEZ235 was used to inhibit the AKT/mTOR/p70S6K pathway. Subsequently, the expression of Shh and AKT/mTOR/p70S6K pathway protein, skeletal muscle destruction scores and intramuscular collagen deposition at 7 days of reperfusion were evaluated. As shown in Fig. 6A, B and C, although Shh, Gli1 and Gli2 expression was unaffected, the up-regulation of AKT, mTOR and p70S6K phosphorylation induced by Shh was abolished with NVP-BEZ235 treatment. Meanwhile, H&E staining and Masson's trichrome staining indicated that the protective effect of Shh on skeletal muscle I/R injury was also abrogated (Fig. 6D, E and F). These data confirmed the hypothesis that Shh protection of skeletal muscle after I/R injury is dependent on the AKT/mTOR/p70S6K pathway.

Shh exerted an anti-apoptotic effect on skeletal muscle, which was mediated by the AKT/mTOR/p70S6K pathway

The time-course expression of the pro-apoptotic protein Cleaved Caspase 3 and Bax, and the anti-apoptotic protein Bcl2 in I/R injured skeletal muscle were detected with western blotting. Early after reperfusion injury, the level of Cleaved Caspase 3 and the Bax/Bcl2 ratio increased, and both peaked at 7 days of reperfusion and subsequently decreased (Fig. 7A). To further investigate the anti-apoptotic role of Shh in skeletal muscle following I/R injury, phShh was used to over-express Shh and/or cyclopamine was used to inhibit the Shh pathway. Then, the level of Cleaved Caspase 3 and the Bax/Bcl2 ratio in skeletal muscle 7 days after I/R injury were examined by western blotting. Our data showed that phShh treatment decreased Cleaved Caspase 3 expression and the Bax/Bcl2 ratio, but cyclopamine treatment increased Cleaved Caspase 3 expression and the Bax/Bcl2 ratio, which indicated that Shh plays an anti-apoptotic role. Furthermore, the anti-apoptotic effect of phShh was completely blocked by cyclopamine (Fig. 7B). TUNEL staining was also used to evaluate apoptosis of skeletal muscle cells, and the results showed that the apoptotic index of the skeletal muscle was significantly lower in the phShh treatment group than in the control vector treatment group (30% vs. 54%, $p < 0.05$, Fig. 7C). Taken together, these data suggested that Shh can also decrease the level of apoptosis in skeletal muscle cells after I/R injury.

The role of the AKT/mTOR/p70S6K pathway in the Shh-induced anti-apoptotic effect was estimated as well. phShh was used to over-express Shh, and/or NVP-BEZ235 was used to inhibit the AKT/mTOR/p70S6K pathway. The results showed that NVP-BEZ235 treatment aggravated the level of apoptosis in skeletal muscle and partially blocked the anti-apoptotic effect of phShh treatment (Fig. 7D).

Discussion

The role of the Shh pathway in skeletal muscle I/R injury was previously unknown. Several studies have reported that exogenously driven Shh expression could induce robust angiogenesis and myogenesis in the setting of hindlimb ischemia [8, 21, 26], and the

angiogenesis and myogenesis are also thought to be of great importance in regeneration of skeletal muscle following I/R injury. Thus, we speculated that the Shh pathway was perhaps involved in the regulation of skeletal muscle I/R injury and played a protective role in this pathological condition. We found that the Shh pathway was activated following skeletal muscle I/R injury. The Shh pathway level transiently peaked and then decreased. Subsequently, we demonstrated that Shh exerted protective effects against skeletal muscle I/R injury by alleviating skeletal muscle destruction, reducing skeletal muscle fibrosis and inhibiting apoptosis. Meanwhile, we found that the skeletal muscle protection triggered by Shh was dependent on the AKT/mTOR/p70S6K pathway. Taken together, our findings elucidate one aspect of the role Shh plays in skeletal muscle I/R injury and provide potential opportunities to test novel therapeutic options.

We showed that in normal mouse skeletal muscle, the Shh gene was barely detectable. However, clear but not sustained Shh expression was induced in skeletal muscle after I/R injury. Comparable results were recently observed in other experimental models, including a cardiotoxin (CTX)-induced injury model [27], skeletal muscle mechanical crush model [21] and hindlimb ischemia model [20]. All of the above studies and our data collectively indicate that Shh, an important regulator in embryo development, is recruited postnatally in specific pathological conditions. Moreover, in the present study we also observed that up-regulation of Gli1 and Gli2 accompanied the Shh response to I/R injury. Among the Glis, Gli1 is the principal transcription factor in the Shh pathway, and many studies have shown that it is of great importance in skeletal muscle regeneration [20, 27, 28]. Furthermore, Gli2 activity has been confirmed to be sufficient and required for efficient MyoD treatment during skeletal myogenesis [29], which plays a major role in regulating muscle differentiation during embryogenesis and adult muscle regeneration [30]. Based on these data, we speculate that both Gli1 and Gli2 are crucial for the process by which Shh modulates skeletal muscle I/R injury.

Although previous studies have already outlined the beneficial effects of Shh in skeletal muscle after hindlimb ischemia, they mainly focused on its promotion of angiogenesis in relatively late recovery phase of skeletal muscle injury [8, 20]. However, our study turned the attention to the effects of Shh on muscle destruction and muscle fibrosis after I/R injury. The autologous up-regulation of Shh signaling subsequent to I/R injury prompted us to wonder whether this I/R-induced Shh expression was a protective factor. We used intraperitoneal injection of cyclopamine to inhibit the Shh pathway [12, 21, 31] and intramuscular injection of phShh to over-express the Shh pathway [19, 20]. We found that cyclopamine-treated mice showed a significantly greater proportion of fibers with two or three central nuclei and a higher degree of cell border blurring and cytoplasmic fragmentation, which is a sign of delayed muscle fiber regeneration [23]. In addition, cyclopamine treatment apparently aggravated skeletal muscle fibrosis, which suggested impaired recovery of skeletal muscle function, as increased deposition of connective tissue has a negative impact on contractile muscle function by decreasing myofiber occupancy [32]. Conversely, we also showed that activation of the Shh signaling pathway via exogenous Shh expression decreased muscle destruction and fibrosis. All these novel findings clearly show that Shh serves as a protector of skeletal muscle regeneration against I/R injury.

The PI3K/AKT associated signaling pathway is a classical protective pathway against I/R injury [33-35]. A study conducted by Hammers *et al.* reported that tourniquet-induced skeletal muscle I/R injury could activate expression of IGF-1 and further stimulate AKT, mTOR, and FoxO3 expression, which was beneficial to skeletal muscle regeneration [36]. Here, we also observed obvious activation of the AKT/mTOR/p70S6K signaling pathway accompanied by Shh signaling following tourniquet-induced skeletal muscle I/R injury. While the peak expression time points of AKT and mTOR in the present study were earlier than those in Hammers *et al.* study, we think that this may be due to the difference in ischemia duration in these two experimental models. Unexpectedly, we found that mTOR was phosphorylated after AKT and p70S6K. We suggest that the explanation may be related to recent studies demonstrating that mTOR phosphorylation at the Ser2448 site is not a

direct target of AKT [37], and a negative feedback loop exists between p70S6K and mTOR phosphorylation at Ser2448 [38]. However, these observations raised the question of how Shh interferes in muscle regeneration following I/R injury. As we know, the canonical Shh pathway involves Shh binding to its receptor Patched1, releasing the inhibitory effect of Patched1 on the effector Smo, which transduces a signal that activates Gli family members. However, the Shh signaling pathway also demonstrates complex crosstalk with multiple signaling pathways, including the AKT/mTOR/p70S6K signaling pathway [39]. Dafna *et al.* found that recombinant N-terminus active Shh (N-Shh) treatment activated AKT phosphorylation, which was abrogated by cyclopamine, in C2 mouse myogenic cells, and the specific PI3K inhibitor Ly294002 blocked Shh-mediated induction of muscle-specific protein expression, suggesting that the effects of Shh on cell differentiation are directly mediated by the PI3K/AKT pathway downstream of Smo [40]. We therefore explored whether Shh plays a role in the present I/R model via regulation of the AKT/mTOR/p70S6K cascades. Herein, we first showed that phShh treatment increases the phosphorylation of AKT/mTOR/p70S6K pathway proteins. This indicated that an enhanced protein synthetic activity was induced by Shh, which further confirmed the beneficial role of Shh. Subsequently, we demonstrated that the protective effect of phShh was abrogated when the AKT/mTOR/p70S6K pathway was inhibited with the dual PI3K and mTOR inhibitor NVP-BEZ235. These data suggest that AKT/mTOR/p70S6K signaling pathway as a downstream mediator contributes to Shh-triggered skeletal muscle protection.

It is generally recognized that apoptosis is an inevitable phase of I/R-induced cell death [41, 42], especially in skeletal muscle [43]. M. Koleva *et al.* indicated that Shh inhibited muscle satellite cell Caspase 3 activation and apoptosis induced by serum deprivation, which could be reversed by simultaneous administration of cyclopamine [44]. Similarly, we demonstrated that exogenous phShh treatment diminished apoptosis of skeletal muscle cells by down-regulating the levels of the pro-apoptotic proteins Cleaved Caspase-3 and Bax and up-regulating the anti-apoptotic protein Bcl2, while cyclopamine treatment neutralized Shh anti-apoptotic activity. This suggested a functional role for Shh in controlling the fate of skeletal muscle cells. Some studies have indicated that the PI3K/AKT pathway regulates cell survival through Bcl2 family proteins [45, 46]. Recently, the protective effect of Shh has been attributed to activation of the PI3K/AKT/Bcl2 pathway [47]. Meanwhile, our results also showed that exogenous phShh treatment was capable of driving expression of AKT/mTOR/p70S6K pathway proteins. Thus, we speculate that the up-regulation of the AKT/mTOR/p70S6K pathway induced by Shh may be responsible for the anti-apoptotic effect of Shh against I/R injury. We found that the anti-apoptotic effect of Shh was partially reversed, when facilitating Shh signaling with phShh but blocking the AKT/mTOR/p70S6K pathway with the inhibitor NVP-BEZ235. Therefore, we suggest that Shh prevents apoptosis in skeletal muscle, in part, through the AKT/mTOR/p70S6K pathway. Nevertheless, these results cannot rule out the possibility of crosstalk between Shh and other pathways in regulating skeletal muscle apoptosis for limited information, such as the ERK1/2 MAPK signaling pathway [46]. The underlying mechanism by which Shh modulates apoptosis in skeletal muscle needs to be further studied.

Conclusion

We provide evidence that Shh can be postnatally recruited in the setting of skeletal muscle I/R injury. Autologous expression of Shh following hindlimb I/R injury can influence skeletal muscle recovery. In addition, Shh gene therapy exerts protective effects against skeletal muscle I/R injury including alleviating skeletal muscle destruction and reducing skeletal muscle fibrosis through the AKT/mTOR/p70S6K pathway, and exhibits an anti-apoptotic effect on skeletal muscle that partially depends on the AKT/mTOR/p70S6K pathway. These findings highlight the pleiotropic effects of Shh in alleviating skeletal muscle I/R injury and thus provide a novel therapeutic strategy.

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Disclosure Statement

The authors declare that they have no conflicts of interest regarding the contents of this article.

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