Akt1-Mediated Muscle Growth Promotes Blood Flow Recovery After Hindlimb Ischemia by Enhancing Heme Oxygenase-1 in Neighboring Cells

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**Background:** Resistance exercise has beneficial effects for patients with peripheral arterial diseases. The hypothesis that muscle growth promotes angiogenesis by interacting with neighboring cells in ischemic lesions was assessed.

**Methods and Results:** Skeletal muscle-specific inducible Akt1 transgenic (Akt1-TG) mice that induce growth of functional skeletal muscles as a model of resistance training were used. Proteomics analysis identified significant upregulation of heme oxygenase-1 (HO-1) in muscle tissue in Akt1-TG mice compared with control mice. Blood flow recovery after hindlimb ischemia was significantly increased in Akt1-TG mice compared with control mice. Enhanced blood flow and capillary density in Akt1-TG mice were completely abolished by the HO-1 inhibitor, Tin-mesoporphyrin. Immunohistochemistry showed that HO-1 expression was not increased in muscle cells, but it was increased in macrophages and endothelial cells. Consistent with these findings, blood flow recovery after hindlimb ischemia was similar between control mice and skeletal muscle-specific HO-1-knockout mice. Adenoviral-mediated overexpression of Akt1 did not increase HO-1 protein expression in C2C12 myotubes; however, the conditioned medium from Akt1-overexpressing C2C12 myotubes increased HO-1 expression in endothelial cells. Cytokine array demonstrated that a panel of cytokine secretion was upregulated in Akt1-overexpressing C2C12 cells, suggesting paracrine interaction between muscle cells and endothelial cells and macrophages.

**Conclusions:** Akt1-mediated muscle growth improves blood flow recovery after hindlimb ischemia by enhancing HO-1 expression in neighboring cells.

**Key Words:** Angiogenesis; Heme oxygenase-1; Muscle growth
Although the beneficial effect of resistance training on PAD patients has been reported, the molecular mechanisms by which increasing or maintaining muscle mass affect peripheral blood flow are unclear.

Akt is a serine-threonine protein kinase that is phosphorylated and activated by several extracellular stimuli through the phosphatidylinositol 3-kinase pathway, and it modulates diverse cellular responses such as metabolism, growth and proliferation. In muscle tissue, Akt is favorably activated by resistance exercise training, and promotes fast/glycolytic, type II fiber hypertrophy. Using the Tet-on system of gene activation, we have previously generated inducible, muscle-specific Akt1 transgenic (Akt1-TG) mice that can induce functional muscle hypertrophy in the absence of exercise training. We have used these mice as a model of resistance training, to demonstrate that muscle growth improves metabolic parameters in obese mice, attenuates cardiac remodeling after myocardial infarction, and inhibits renal damage in a kidney disease model. We have also shown that Follistatin-like 1, a secreted muscle protein upon Akt activation, promotes endothelial cell function and revascularization in ischemic tissue through a NOS-dependent mechanism. These results suggest that muscle growth governs the disease process in remote or paracrine fashion.

Heme oxygenase-1 (HO-1) is an enzyme that catalyzes the conversion of heme into biliverdin, ferrous iron and carbon monoxide. HO-1 is highly expressed in the liver, spleen and macrophages, and exerts tissue protective properties in response to various extracellular stimuli such as heat shock, heme protein, oxidized lipoprotein, cytokines and endotoxins. It has been reported that in humans, HO-1 transcript expression was significantly increased in response to exhaustive one-legged knee extensor exercise, peaking after 2h of recovery. Regarding angiogenic properties, exogenous HO-1 overexpression has been reported to promote blood flow recovery in hindlimb ischemia model in rats and mice. In contrast, homozygous HO-1-deficient mice show impaired blood flow recovery in ischemic hindlimb compared with their wild-type (WT) littersmates. These results suggest that increased HO-1 expression in muscle tissue in response to resistance exercise facilitates the angiogenic response; however, the mechanisms by which growing muscle activates HO-1 are unclear.

In the present study, we used skeletal muscle-specific inducible Akt1-TG mice as a model of resistance exercise training. We performed comprehensive proteomic analysis to find the factors that promote angiogenesis, and investigated the potential mechanism by which muscle growth enhances angiogenesis by interacting with neighboring cells in ischemic lesions.

**Methods**

**Animals**

Muscle creatine kinase-reverse tetracycline trans-activator transgenic mice (1256 [3Emut] MCK-rTA TG) were crossed with tetracycline responsive element-myristoylated Akt1 transgenic mice (TRé-myrAkt1-TG) to generate double transgenic mice (Akt1-TG mice). The MCK promoter construct used in the driver line is mutated and the transgene is expressed in a muscle subset, but no expression occurs in the heart. For Akt1 transgene expression, Akt1-TG mice were treated with doxycycline in the drinking water (0.5 mg/mL).

Skeletal muscle-specific HO-1 knockout mice were generated by crossing HO-1 Floxed mice (B6J129P2-Hmox1<tm1<1Mym>) with ACTA1-Cre mice [B6.Cg-Tgt(ACTA1-cre)79Jme/J]. HO-1 Floxed mice were obtained from RIKEN BioResource Center (Tsukuba, Japan), and ACTA1-Cre mice were obtained from Jackson Laboratory.

Male, 8- to 12-week-old mice were used in this study. All procedures were performed in accordance with the Kumamoto University animal care guidelines, which conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996).

**Proteomic Analysis of Muscle Tissue**

Two weeks after doxycycline treatment, gastrocnemius muscle samples from Akt1-TG and control mice were subjected to comprehensive analysis using iTRAQ methods.

**Hindlimb Ischemia Model**

Hindlimb ischemia was generated as described previously. In this model, the entire left femoral artery and vein were excised surgically. Under pentobarbital anesthesia, Laser Doppler perfusion imaging was performed at baseline, immediately after surgery and 3, 7 and 14 days after surgery, as described previously. In some experiments, the HO-1 inhibitor, Tin-mesoporphyrin (SnMP; 20 mg/kg), was intraperitoneally injected immediately after hindlimb surgery, and then every other day.

**Histological Analysis**

The soleus muscle was assessed in 10 μm-thick frozen sections after staining with antibodies specific for HO-1, CD31 and CD206. Section images were obtained using a confocal microscope Leica TCS SP8 (Leica microsystems). We analyzed 5-7 sections per animal. Analysis was performed digitally using Luminaw Vision software (version 2.2; Mitani Corp, Tokyo, Japan).

**Cell Culture and Adenoviral Infection**

C2C12 mouse myoblasts (American Type Culture Collection, Manassas, VA, USA) were maintained in growth medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum), which was replaced with differentiation medium (DMEM supplemented with 2% heat-inactivated horse serum) for 5 days to induce differentiation. C2C12 myocytes were infected with adenoviruses expressing myristoylated-Akt1 (Ad-Akt1), HO-1 (Ad-Hmox1) or β-galactosidase (Ad-LacZ) at a multiplicity of infection (m.o.i.) of 100 for 16 h followed by incubation with serum-free DMEM for 24 h. Cell or tissue lysates, or culture media were resolved by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In some experiments, culture media were transferred to human umbilical vein endothelial cells (HUVECs) or THP-1 macrophages to evaluate HO-1 expression.

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was prepared using a Qiagen RNeasy Fibrous Mini Kit, according to the manufacturer's protocol, and cDNA was produced using the PrimeScript RT-PCR System (Takara, Otsu, Japan). Quantitative real-time PCR was performed as described previously. Transcript expression levels were determined as the number of transcripts relative to that of 18S or β-actin.
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Comparison tests, the P value was adjusted by the Bonferroni method. Quantitative blood flow imaging data were compared by 2-way repeated-measures ANOVA. The significance level of a statistical hypothesis test was set at 0.05.

Results

HO-1 Expression Is Increased in Muscle Tissue in Akt1-TG Mice

To identify the factors that promote the angiogenic response for keeping up with muscle growth, we performed comprehensive proteomic analysis of gastrocnemius muscle samples from Akt1-TG and control mice, 2 weeks after doxycycline treatment by iTRAQ methods (Figure 1A). The ratio of p-Akt to t-Akt was 7.1-fold (P=0.028, n=4; Figure 1B) increased in Akt1-TG mice 14 days after doxycycline treatment, and activation of Akt1 signaling in myofibers led to an increase in muscle mass, assessed by gastrocnemius muscle weight (16.3±1.8 vs. 9.8±0.3 mg/g, 95% confidence interval).
Serine-threonine protein kinase (Akt1)-mediated muscle growth promotes blood flow recovery by a heme oxygenase-1 (HO-1)-dependent mechanism. (A) Schematic illustration of the experimental protocol and doxycycline-treatment time course. Akt1-TG and control mice were treated with vehicle or the HO-1 inhibitor, Tin-mesoporphyrin (SnMP). (B) Time course of computer-assisted analysis of laser Doppler perfusion flow ratio in Akt1-TG and control mice with or without treatment by the HO-1 inhibitor. The perfusion ratio was determined by using the contralateral non-ischemic limb of the same animal. (C) Capillary density was expressed as the ratio of CD31-positive cells relative to muscle fiber. (D) Representative immunoblots of HO-1, p-Akt, t-Akt and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein expression in ischemic and non-ischemic gastrocnemius muscle, 2 weeks after surgery and doxycycline treatment. (E) Representative images of HO-1-stained ischemic and non-ischemic gastrocnemius muscle, 2 weeks after surgery and doxycycline treatment. (F) Representative immunoblots of HO-1 and p-Akt protein expression in ischemic gastrocnemius muscle, 2 weeks after surgery and doxycycline treatment with or without SnMP. Results are presented as mean±SEM.

Myogenic Akt1 Activation Promotes Blood Flow Recovery by a HO-1-Dependent Mechanism

To test whether Akt1-mediated muscle growth enhances blood flow, a hindlimb ischemia model was generated by unilateral femoral artery resection in Akt1-TG and control mice (Figure 2A). Laser Doppler imaging showed significant improvement in blood flow recovery following hindlimb ischemia in Akt1-TG mice compared with control mice (Figure 2B). To investigate the extent of revascularization at the microcirculatory level, the capillary density was measured in histological sections harvested from the adductor muscle of the ischemic limb. Quantitative analysis demonstrated significantly higher CD31-positive cells at day 14 post-ischemia in Akt1-TG mice compared with WT mice (1.58±0.07 vs. 0.90±0.10/fiber, P<0.01, n=7; Figure 2C). In contrast, capillary density was not different between P=0.003, n=3 and 5 respectively; Figure 1C). At this time point, 60 proteins were over 2.0-fold upregulated in the muscle tissue in Akt1-TG mice compared with control mice (Figure S1). We validated this protein expression profile by western blot analysis and identified that HO-1 was the most upregulated protein in Akt1-TG mice compared with control mice (Figure 1D).
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To investigate the causal relationship between the upregulated HO-1 expression and the augmented angiogenic response, Akt1-TG and control mice were treated with the HO-1 inhibitor, Tin-mesoporphyrin (SnMP). Treatment with SnMP increased HO-1 protein expression, suggesting the presence of a compensatory mechanism (Figure 2F).

Improved blood flow recovery observed in Akt1-TG mice was attenuated by SnMP treatment, as revealed by Laser Doppler imaging (Figure 2B). At the microcirculatory level, the capillary density in Akt1-TG mice was significantly reduced by SnMP treatment (1.32±0.09 vs. 1.58±0.07, /field, P<0.05, n=7; Figure 2C). These data indicate that Akt1-mediated muscle growth enhanced blood flow recovery in response to hindlimb ischemia, at least in part, a HO-1-dependent manner.

Figure 3. Serine-threonine protein kinase (Akt1)-mediated muscle growth enhances heme oxygenase-1 (HO-1) expression in neighboring macrophages and endothelial cells. (A) Representative images of HO-1 and CD206 double-stained ischemic adductor muscle sections, 2 weeks after surgery and doxycycline treatment. (B) Representative images of HO-1 and CD31 double-stained ischemic adductor muscle sections, 2 weeks after surgery and doxycycline treatment. (C) Time course of computer-assisted analysis of laser Doppler perfusion flow ratio in skeletal muscle-specific HO-1-deficient mice and control littermates. (D) Representative immunoblots of HO-1 and p-Akt protein expression in C2C12 cells infected with adenoviruses expressing myristoylated Akt1 (Ad-Akt1), HO-1 (Ad-Hmox1) or β-galactosidase (Ad-LacZ) at a multiplicity of infection of 100 for 16 h. (E) Left: Representative immunoblots of HO-1, endothelial nitric oxide synthase (eNOS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein expression in human umbilical vein endothelial cells (HUVECs) treated with conditioned medium from Ad-LacZ- or Ad-Akt1-overexpressing C2C12 cells. Right: Quantitative analysis of immunoblots (n=4 per experimental group). Results are presented as mean±SEM.

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WT and Akt1 TG mice 7 days after surgery (Figure S2), suggesting that initial blood flow recovery was due to increased arteriogenesis but not angiogenesis.

Western blot analysis demonstrated that HO-1 protein expression was increased in Akt1-TG mice both in non-ischemic and ischemic muscle tissue (Figure 2D). As shown in Figure 2E, histological analysis also showed that HO-1-positive cells in non-ischemic hindlimb was significantly higher in Akt1-TG mice than in control mice (48.7±13.0 vs. 24.7±10.2, /field, P<0.01; n=3). HO-1 expression was further increased in response to hindlimb ischemic surgery in Akt1-TG mice (206.3±80.1 vs. 71.0±10.6, /field, P<0.01, n=3). The difference in western blot results and immunohistochemistry might be due to the heterogenous Akt1 transgene expression in our mice model. We previously reported that the Akt1 transgene was not expressed in all subsets of skeletal muscle because of the specificity of the 1256 [3Emut] MCK promoter fragment.
Akt1-overexpressing myocytes enhance HO-1 expression

These results indicate that the secreted factor derived from Akt1-overexpressing C2C12 cells increased HO-1 protein expression in endothelial cells and macrophages.

**Figure 4.** Schematic diagram of the proposed mechanisms of Akt1-mediated muscle growth that promotes angiogenesis. Akt1-mediated muscle growth promotes blood flow recovery in ischemic lesions by enhancing HO-1 expression in neighboring endothelial cells and macrophages.

**HO-1 Expression Is Not Increased in Muscle Cells, But in Macrophages in Ischemic Hindlimb**

To investigate the cellular source of the upregulated HO-1, we performed immunohistochemistry using cell type-specific marker antibodies. Unexpectedly, in ischemic hindlimb, HO-1 was not upregulated in myocytes, but in macrophages and endothelial cells (Figure 3A,B). Consistent with these results, the blood flow recovery and capillary density after hindlimb ischemia were similar between skeletal muscle-specific HO-1-deficient mice and control littermates (Figure 3C). These results suggest that Akt1-mediated muscle growth enhances HO-1 expression in surrounding macrophages, but not in myocytes.

**Myocyte-Derived Secreted Factors Enhance HO-1 Expression in Endothelial Cells**

To investigate the mechanism by which myogenic Akt1 activation enhances HO-1 expression in neighboring cells, we performed intravital experiments using C2C12 cells as a model of muscle cells. Consistent with the in vivo experiments, overexpression of Akt1 in C2C12 cells did not enhance HO-1 expression (Figure 3D). However, conditioned medium from Akt1-overexpressing C2C12 cells increased HO-1 protein expression in endothelial cells (1.70±0.35 vs. 1.0±0.41, P<0.05, n=4), which was accompanied by upregulation of phosphorylated eNOS (Figure 3E). These results indicate that the secreted factor derived from Akt1-overexpressing myocytes enhances HO-1 expression in endothelial cells.

**Comprehensive Analysis of Muscle Cell-Derived Cytokines and Chemokines**

To investigate the factors that enhance HO-1 expression in neighboring cells, we performed cytokine array analysis using culture medium from Akt1-overexpressing and control C2C12 myocytes. This analysis demonstrated that the expression of a panel of cytokines was upregulated in Akt1-overexpressing C2C12 cells (Figure S3). These upregulated cytokines included interferon γ (IFN-γ), interleukin (IL)-1β, IL-13, IL-17, IL-23, C-X-C motif chemokine ligand 9 (CXCL9) and Chemokine ligand 3 (CCL3). Collectively, these data suggest that the effect of Akt1-mediated muscle growth on HO-1 expression in endothelial cells and macrophages is mediated by multiple factors including these candidate cytokines.

**Discussion**

In addition to aerobic exercise training, resistance training aimed at maintaining or increasing muscle mass has been reported to have a beneficial effect on PAD patients. However, the molecular mechanism of the beneficial effect was unclear, partially because of the lack of an appropriate animal model, which can induce muscle growth in an inducible manner. In the present study, we used conditional, skeletal muscle-specific Akt1 transgenic mice and demonstrated that: (1) Akt1-mediated muscle growth promoted blood flow recovery following hindlimb ischemia, (2) myogenic Akt1 activation enhanced HO-1 expression in neighboring endothelial cells and macrophages; and (3) augmented angiogenic response in Akt1-TG mice was completely abolished by treatment with an HO-1 inhibitor. These data indicate that Akt1-mediated muscle growth, independent of exercise, can facilitate angiogenesis in response to hindlimb ischemia through a HO-1-mediated mechanism (Figure 4).

Supervised exercise therapy is the most fundamental intervention for PAD patients, and recommended as Class I in clinical guidelines.

Aerobic training is generally recommended; however, severe PAD patients cannot perform sufficient exercise. For these patients, alternative exercise intervention is required. As one possibility, we have previously shown that whole-body periodic acceleration, a non-invasive, passive exercise modality, increased blood supply to ischemic lower extremities by upregulation of pro-angiogenic growth factors and activation of eNOS signaling in ischemic skeletal muscle.

These findings suggest that skeletal muscles secrete various factors to activate eNOS in remote cells.

Another possibility is resistance-type exercise training, which aims at maintaining or increasing muscle mass.

Muscle hypertrophy plays an important role in the adaptive response to physical exercise. This process is associated with blood vessel recruitment, such that the capillary density is either maintained or increased in the growing muscle.

Relative organic ischemia should be provoked when muscle growth initially occurs. These hypoxic conditions induce angiogenesis, a mainly HIF-1-mediated mechanism.

It has been shown that angiogenesis and compensatory muscle hypertrophy are temporally coupled, suggesting that these 2 processes are controlled by common regulatory mechanisms.

It has also been shown that myogenic Akt signaling regulates blood vessel recruitment during myofiber growth.
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in vitro and in vivo. Hypertrophy of cultured C2C12 myotubes resulted in a marked increase in the secretion of vascular endothelial growth factor (VEGF). These data suggest that myogenic Akt signaling controls both fiber hypertrophy and VEGF synthesis, illustrating a mechanism through which blood vessel recruitment is coupled to normal tissue growth. However, additional growth factors are likely to participate in the recruitment of blood vessels during muscle growth.

In the present study, we demonstrated that HO-1, a pro-angiogenic factor, was upregulated in endothelial cells and macrophages adjacent to hypertrophied muscle cells, and that the angiogenic property of Akt1-mediated muscle growth was HO-1-dependent. It has been reported that VEGF supplementation to endothelial cells enhances HO-1 expression; therefore, it is reasonable to speculate that muscle-derived VEGF contributes to HO-1 upregulation in these cells. Rowe et al have demonstrated that Secreted phosphoprotein 1 was secreted from muscle cells upon genetic activation of PGC-1α or aerobic exercise, and that it activated macrophages to secrete chemokines, which then activated adjacent endothelial cells. In our study, cytokine array data indicated that various chemokines, which potentially activate HO-1 in endothelial cells, were secreted from C2C12 myotubes in response to Akt1 activation (Figure 4). Consistently, overexpression of IL-13 has been reported to increase HO-1 expression in HUVECs. Further studies are required to determine which factors are causally involved in HO-1 activation in endothelial cells and macrophages, and in induction of the angiogenic properties of the Akt1-mediated skeletal muscle growth. eNOS activation is recognized as a central mechanism of HO-1-mediated angiogenesis in endothelial cells. HO-1 catalyzes the conversion of heme to biliverdin, ferrous iron and carbon monoxide. Carbon monoxide and biliverdin activate eNOS. In this study, we found that conditioned medium from Akt1-activated C2C12 cells increased HO-1 expression, which was accompanied by enhanced eNOS phosphorylation (Figure 3D). We have reported that the expression and secretion of VEGF and Follistatin like-1, which exert angiogenic properties by activating eNOS in endothelial cells, were increased by myogenic Akt1 activation in vitro and in vivo. Furthermore, we have reported that Akt1-mediated muscle growth attenuates cardiac remodeling and renal damage in a murine model of myocardial infarction and obstructive kidney disease, and these improvements appear to be mediated by an increase in eNOS-signaling in the heart and kidney. Collectively, these data suggest that Akt1-mediated muscle growth induced eNOS activation in neighboring or remote cells, and exerted its pro-angiogenic properties through, at least in part, HO-1-dependent mechanisms.

It has been reported that exogenous HO-1 gene delivery into skeletal muscle reduced infarct size and improved cardiac function in a murine model of myocardial infarction; therefore, we assessed the possibility that HO-1 itself acts as a muscle-derived secreted factor. However, Akt1 overexpression in C2C12 cells did not increase the HO-1 protein level (Figure 3C). In addition, we did not observe a significant difference in circulating HO-1 levels between PAD and non-PAD patients (2.9±0.83 vs. 3.12±1.35 ng/mL, P=NS, n=8 and 72 respectively). Based on these data, it is less likely that HO-1 itself acts as a muscle-derived secreted factor. The present study has certain limitations. Because HO-1 activity does not always correlate with HO-1 protein and transcript levels, it is desirable to measure HO-1 activity. We could not obtain convincing data in the present study because of technical issues. Recently, a novel and sensitive assay for HO-1 activity has been developed, thus future studies would clarify the relationship between myogenic Akt1 activation and HO-1 activity in the muscle tissue. In conclusion, Akt1-mediated muscle growth promotes blood flow recovery in ischemic lesions by enhancing HO-1 expression in neighboring endothelial cells and macrophages. Because Akt1 signaling is preferentially activated by resistance-type exercise training in skeletal muscle, our data strongly support the notion that maintaining or increasing muscle mass could be an alternative exercise intervention for PAD patients.

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Disclosure

The authors declare no conflicts of interest.

References


Supplementary Files

Supplementary File 1

Figure S1. Proteomic analysis showing over 2-fold upregulated proteins, 2 weeks after serine-threonine protein kinase (Akt1) activation in gastrocnemius muscle.

Figure S2. Capillary density was not different between wild-type (WT) and skeletal muscle-specific inducible Akt1 transgenic (Akt1-TG) mice 7 days after surgery.

Figure S3. Comprehensive analysis of muscle cell-derived cytokines and chemokines. Please find supplementary file(s): http://dx.doi.org/10.1253/circj.CJ-18-0135