Passive Exercise Using Whole-Body Periodic Acceleration Enhances Blood Supply to Ischemic Hindlimb


Objective—Whole-body periodic acceleration (WBPA) has been developed as a passive exercise technique to improve endothelial function by increasing shear stress through repetitive movements in spinal axis direction. We investigated the effects of WBPA on blood flow recovery in a mouse model of hindlimb ischemia and in patients with peripheral arterial disease.

Methods and Results—After unilateral femoral artery excision, mice were assigned to either the WBPA (n = 15) or the control (n = 13) group. WBPA was applied at 150 cpm for 45 minutes under anesthesia once a day. WBPA significantly increased blood flow recovery after ischemic surgery, as determined by laser Doppler perfusion imaging. Sections of ischemic adductor muscle stained with anti-CD31 antibody showed a significant increase in capillary density in WBPA mice compared with control mice. WBPA increased the phosphorylation of endothelial nitric oxide synthase (eNOS) in skeletal muscle. The proangiogenic effect of WBPA on ischemic limb was blunted in eNOS-deficient mice, suggesting that the stimulatory effects of WBPA on revascularization are eNOS dependent. Quantitative real-time polymerase chain reaction analysis showed significant increases in angiogenic growth factor expression in ischemic hindlimb by WBPA. Facilitated blood flow recovery was observed in a mouse model of diabetes despite there being no changes in glucose tolerance and insulin sensitivity. Furthermore, both a single session and 7-day repeated sessions of WBPA significantly improved blood flow in the lower extremity of patients with peripheral arterial disease.

Conclusion—WBPA increased blood supply to ischemic lower extremities through activation of eNOS signaling and upregulation of proangiogenic growth factor in ischemic skeletal muscle. WBPA is a potentially suitable noninvasive intervention to facilitate therapeutic angiogenesis. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: angiogenesis ▪ endothelial function ▪ exercise ▪ peripheral arterial disease ▪ vascular biology

Patients with peripheral arterial disease (PAD) are at risk of limb loss and impairment of quality of life. Exercise therapy is an effective primary nonpharmacologic treatment for patients with PAD. The proposed mechanisms for the beneficial effects of exercise training on PAD include the formation of collateral vessels, improvement in endothelium-dependent vasodilatation and skeletal muscle metabolism, and increased secretion of proangiogenic growth factors. Based on this evidence, the current guidelines recommend supervised exercise for patients with PAD. However, it is sometimes difficult to provide appropriate exercise therapy for PAD patients who are incapable of performing active exercise, such as those with neurodegenerative and rheumatologic diseases or age-related skeletal muscle wasting referred to as sarcopenia.
increases the levels of various vasoactive substrates, which in turn could enhance blood flow and promote blood vessel recruitment. However, the impact of WBPA on blood flow to ischemic legs has not been investigated in detail.

The present study was designed to test the hypothesis that passive exercise using WBPA promotes angiogenesis and increases blood supply in a mouse model of hindlimb ischemia and in patients with PAD. The results indicated that WBPA stimulates ischemia-induced revascularization through the activation of endothelial nitric oxide synthase (eNOS) signaling and upregulation of angiogenic growth factor expression in ischemic hindlimbs.

**Materials and Methods**

**Animal Studies**

Experiments were conducted in male C57BL/6J (wild-type [WT]) mice, eNOS-deficient mice (Jackson Laboratory) and diet-induced obese (DIO) C57BL/6J mice. DIO mice were fed a very-high-fat diet (Diet No. D12492, Research Diets, Inc) from 4 weeks of age. The composition of very-high-fat diet is 34.9% fat, 26.3% carbohydrate, and 26.2% protein. At the age of 8 to 10 weeks, mice were subjected to unilateral hindlimb surgery under anesthesia with sodium pentobarbital (50 mg/kg IP). In this model, the entire left femoral artery and vein were excised surgically. 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).

**WBPA**

The mice were placed longitudinally on a linear shaker that produces repetitive head-to-foot direction movements of the body (model KS-6320, Marysol, Tokyo, Japan). In the WBPA group, WBPA was applied at 150 cpm for 45 minutes under anesthesia with sodium pentobarbital (25 mg/kg IP) once a day until the last day of the experiment. Mice of the control group remained in a static position under the same dose of anesthesia for 45 minutes.

**Laser Doppler Perfusion Imaging**

Excess hair was removed from the hindlimbs using a depilatory cream. Mice were first placed on a heating plate set at 37°C. This was followed by measurement of hindlimb blood flow with a laser Doppler Imager (Moor Instruments). To minimize the effects of other variables including ambient light and temperature, the calculated perfusion was expressed as the ratio of ischemic to nonischemic hindlimb. Under pentobarbital anesthesia, laser Doppler perfusion imaging was performed at baseline; immediately after surgery; and at 1, 3, 5, 7, and 14 days after surgery, as described previously.

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**Figure 1.** Whole-body periodic acceleration (WBPA) promotes blood flow recovery in response to hindlimb ischemia. **A**, Representative laser Doppler perfusion images of the control and WBPA groups. **B**, Time course of computer-assisted analysis of laser Doppler perfusion flow ratio. **C**, Representative photographs of adductor muscles stained for CD31 and 4′,6-diamidino-2-phenylindole (DAPI). Control group, n=4; WBPA group, n=6. **D**, Capillary density was expressed as the proportion of CD31-positive cells relative to the control. Data are mean±SEM. *P<0.05 vs control.
Measurements of Metabolic Parameters
Blood glucose was assayed with an Accu-Chek glucose monitor (Roche Diagnostics, Manheim, Germany). Glucose tolerance test was performed in 12-hour-fasted mice as described previously. Glucose tolerance test was performed in 18-hour-fasted mice as described previously. Insulin sensitivity test was performed in 18-hour-fasted mice as described previously. Mice were intraperitoneally injected with D-glucose (1.5 g/kg of body weight), and blood glucose levels were determined immediately before and 30, 60, and 120 minutes after injection. Insulin sensitivity test was performed in 18-hour-fasted mice as described previously. Mice were intraperitoneally injected with insulin (0.5 U/kg of body weight), and blood glucose levels were determined immediately before and 20, 40, and 60 minutes after injection. Total serum cholesterol and triglycerides levels were analyzed by an online dual-enzymatic method for simultaneous quantification of cholesterol and triglycerides by high-performance liquid chromatography at Skylight Biotech Inc (Akita, Japan).

Clinical Study
Ten patients with PAD (age, 69 ± 7 years; 9 men, 1 woman) underwent a single-session WBPA at 140 cpm for 45 minutes, as described previously. Another group of 5 patients (age, 64 ± 3 years, 4 men, 1 woman) underwent 45-minute WBPA daily for 7 days. WBPA was performed after >3 hours of fasting. All subjects were asked to abstain from smoking and alcohol congestion for 24 hours before the study. The skin blood flow of the lower extremity was estimated before and immediately after WBPA with the AT-101 (Non-Invasive Monitoring Systems, Inc, Miami, FL). The regions of interest were placed on the toe of the ischemic and nonischemic lower extremities, and the ischemic/nonischemic ratio was then calculated in a fashion analogous to the animal study. The study was approved by Institutional Review Board of the Osaka Ekisaikai Hospital, and each subject provided informed consent before participation.

Statistical Analysis
All data are presented as mean ± SEM. Differences between data of 2 experimental groups were tested for statistical significance by the Student t test. Data of multiple groups were compared by 1-way analysis of variance (ANOVA). A probability value less than 0.05 was accepted as statistically significant.
Results

Animal Studies

**WBPA Promotes Blood Flow Recovery Following Hindlimb Ischemia**

To test whether WBPA promotes blood flow recovery, we created a mouse model of hindlimb ischemia by femoral artery excision. Immediately after surgery, blood flow to the ischemic hindlimb decreased to approximately 90% of the nonischemic hindlimb in both the WBPA group (n=15) and the control group (n=13). WBPA mice showed significant blood flow recovery in the ischemic limb compared with the control mice (day 7: 0.54±0.06 versus 0.33±0.04; day 14: 0.61±0.05 versus 0.51±0.03, respectively, \(P<0.05\); Figure 1A and 1B). To investigate the extent of revascularization at the capillary artery level, capillary density was measured in histological sections harvested from muscles of the ischemic limb. Figure 1C shows representative images of the adductor muscles of the WBPA and control mice stained with CD31, an endothelial marker. Quantitative analysis indicated a significant increase in capillary density at day 7 in WBPA mice compared with control mice (Figure 1D), suggesting that WBPA promotes angiogenesis at a microcirculatory level.

**Stimulatory Action of WBPA on Revascularization Is Dependent on eNOS Signaling**

Because WBPA is reported to increase eNOS phosphorylation, as well as nitric oxide production,\(^{17}\) Western blot analysis using ischemic adductor muscle lysates obtained at 3 to 12 hours after a single session of WBPA was conducted to investigate the effect of WBPA on eNOS activation. Compared with the still-control mice, the level of phosphorylation of eNOS in the ischemic muscle of the WBPA mice was significantly increased at 3, 6, and 12 hours after a single-session WBPA (Figure 2A and 2B). We also performed Western blot analysis using ischemic muscle lysates obtained after 7 or 14 days WBPA and found that eNOS activation was sustained in the chronic phase of WBPA (Figure 2C and 2D). The level of phosphorylation of Akt in the ischemic muscle of the WBPA mice was not changed at 3, 6, and 12 hours after WBPA (Supplemental Figure I). In the next set of experiments in eNOS-deficient mice, we examined the causal role of eNOS activation in WBPA-induced blood flow improvement. Hindlimb ischemia was surgically induced in eNOS-deficient mice. Postoperatively, the mice were divided into the control (n=7) and WBPA (n=7) groups. Consistent with previous results,\(^{18}\) eNOS-deficient mice exhibited impairment of ischemia-induced neovascularization, compared with the WT mice. WBPA did not increase the ischemic/nonischemic side blood perfusion ratio in eNOS-deficient mice at day 14 (Figure 2E and 2F). As shown in Figure 2G and 2H, the density of capillaries in ischemic hindlimb was not different between the control and WBPA groups at 14 days after hindlimb ischemia surgery. These results suggest that the stimulatory action of WBPA on revascularization in vivo is dependent on eNOS signaling pathway.

**WBPA Upregulates the Expression of Angiogenic Growth Factors in Ischemic Skeletal Muscle**

We examined the expression of molecules involved in angiogenesis in ischemic adductor muscle of WT mouse. Quantitative real-time polymerase chain reaction analysis showed significant increases in expression of vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), stromal cell–derived factor-1 (SDF-1) and platelet-derived growth factor-B (PDGF-B) in WBPA mice compared with control mice after a single session of WBPA (Figure 3A). There was a trend toward increases in the expression levels of VEGF, FGF2, and PDGF-B after daily sessions of WBPA for 7 days, although the differences were not statistically significant (Supplemental Figure IIA).
Next, the effect of eNOS signaling pathway on the expression levels of angiogenic growth factors was analyzed in the ischemic muscle of eNOS-deficient mouse. WBPA increased FGF2, PDGF-B, and stromal cell–derived factor-1α (SDF-1α), but not VEGF, in eNOS-deficient mice after a single session of WBPA (Figure 3B). WBPA did not affect the expression levels of these angiogenic growth factors after 7 or 14 days of daily sessions of WBPA in eNOS-deficient mice (Supplemental Figure IIB).

**WBPA Alters Expression Profile of Angiogenesis-Related Proteins**

WBPA is reported to increase serum levels of various vasoactive substrates. Therefore, mouse angiogenesis antibody array analysis was performed to determine the effects of WBPA on circulating angiogenesis-related proteins (Supplemental Figure IIIA). Supplemental Figure IIIB shows representative images of angiogenesis antibody array analysis of mouse sera of control and 3, 6, and 12 hours after a single session of WBPA. Significant differences in the expression levels of 9 proteins were noted in mice of the WBPA group at 1 or more time points (Supplemental Figure IIIC). Several proangiogenic proteins were differentially upregulated, including amphiregulin, angiogenin, epidermal growth factor, osteopontin, and PDGF-AB/BB. On the other hand, WBPA significantly upregulated angiostatic proteins, such as ADAMTS1 and Delta-like protein 4, compared with the control. To determine whether these factors are derived from the ischemic hindlimb, we examined the mRNA expression levels of these proteins in ischemic skeletal muscles. Consistent with the protein array data, PDGF-B and ADAMTS1 expression levels were increased by WBPA. However, the expression profile of other factors did not correlate with their protein array data (Supplemental Figure IIIID). These results suggest that WBPA upregulates the expression of circulating angiogenic-related growth factors not only in

**Figure 4.** Whole-body periodic acceleration (WBPA) promotes blood flow recovery in response to hindlimb ischemia in diet-induced obese (DIO) mice. A, Effect of WBPA on glucose tolerance at 10 days after daily sessions of WBPA. Wild-type (WT) mice, n=5; DIO mice, n=7; WBPA-DIO mice, n=8. B, Effect of WBPA on insulin sensitivity at 14 days after daily sessions of WBPA. WT mice, n=5; DIO mice, n=6; WBPA-DIO mice, n=8. C, Effect of WBPA on lipid profile at 14 days after daily sessions of WBPA. WT mice, n=5; DIO mice, n=6; WBPA-DIO mice, n=8. D, Representative laser Doppler perfusion images of DIO and WT mice. E, Time course of computer-assisted analysis of laser Doppler perfusion flow ratio. WBPA DIO mice, n=8; control DIO mice, n=7; WBPA-WT mice, n=5; control WT mice, n=5. Data are mean±SEM. *P<0.05 WT vs WT + WBPA. **P<0.05 DIO vs DIO + WBPA. †P<0.05 WT vs DIO. ‡P<0.05 WT + WBPA vs DIO + WBPA. n.s. indicates not significant.
the ischemic hindlimb but also in tissues remote from the ischemic site.

**WBPA Promotes Blood Flow Recovery in Response to Hindlimb Ischemia in DIO Mice**

Diabetes mellitus is an important and major cause of PAD, and associated with impaired angiogenesis. Therefore, we assessed the effects of WBPA on glucose metabolism and ischemia-induced revascularization in DIO mice. There was no difference in body weight between the WBPA and control groups at 10 days after surgery (control: 23.1 ± 0.12 g; WBPA: 23.1 ± 0.3 g). Blood flow recovery was impaired in DIO mice compared with WT mice at 7, 14, and 21 days after hindlimb surgery (0.18 ± 0.02 versus 0.33 ± 0.04, 0.27 ± 0.01 versus 0.41 ± 0.02, and 0.56 ± 0.03 versus 0.66 ± 0.03, respectively, P<0.05; Figure 4A). In DIO mice, daily sessions of WBPA for 10 days did not affect glucose tolerance (Figure 4A), and WBPA for 14 days did not affect insulin sensitivity (Figure 4B). Total cholesterol but not triglyceride level was significantly lower after 14 days of WBPA (Figure 4C). WBPA significantly enhanced blood flow recovery at 7 and 14 days after hindlimb surgery in DIO mice compared with non-WBPA mice (0.39 ± 0.05 versus 0.27 ± 0.01, 0.65 ± 0.02 versus 0.57 ± 0.03, respectively, P<0.05; Figure 4D and 4E). These results suggest that WBPA-induced accelerated flow recovery is independent of glucose tolerance and insulin sensitivity.

**Clinical Study**

**WBPA Improves Blood Flow in the Lower Extremity of Patients With PAD**

We also assessed the effect of WBPA on blood supply in the lower extremity of patients with PAD. Table 2 summarizes the clinical characteristics of 15 patients with PAD. No hemodynamic or mechanical complications were observed during the procedure or follow-up (Figure 5A). Single-session WBPA significantly improved the ischemic/nonischemic ratio of blood flow in the lower extremity from 0.74 ± 0.21 to 0.87 ± 0.21 (P<0.05, Figure 5B and 5C). There were no significant differences in hemodynamics and laboratory data, including NO and VEGF, between before and after the procedure (Table 3).

In the 5 patients who received daily WBPA for 7 days, the ischemic/nonischemic ratio of blood flow in the lower extremity increased from 0.85 ± 0.04 to 0.99 ± 0.08 (P=0.007, Figure 5D). Interestingly, the extent of blood flow improvement after 1-week WBPA was similar to that after a single-session WBPA (0.14 ± 0.06 versus 0.13 ± 0.12, P=0.9), but the variability of the response decreased by 50%. Specifically, the ischemic/nonischemic ratio of blood flow in the lower extremity was slightly decreased or unchanged after a single-session WBPA in 2 of 10 (20%) patients, whereas blood flow improved in all patients (100%) after 1-week WBPA.

**Discussion**

In the present study, we demonstrated that passive exercise using WBPA stimulates revascularization in a murine model of hindlimb ischemia through an eNOS-dependent mechanism. Histological analysis confirmed that WBPA promotes angiogenesis at a microcirculatory level, suggesting that this mode of exercise facilitates both arteriogenesis and angiogenesis. The stimulatory effect of WBPA on blood flow was also documented in a mouse model of diabetes despite there being...
no alteration in glucose tolerance and insulin sensitivity. Furthermore, both a single-session and 7-day repeated sessions of WBPA improved blood flow in the lower extremities of patients with PAD. Our findings suggest that WBPA could be a potentially useful noninvasive intervention to facilitate therapeutic angiogenesis.

Exercise is known to improve vascular function of the peripheral and coronary endothelia, leading to a reduction in cardiovascular events. In the clinical setting, however, it is sometimes difficult to apply exercise therapy in patients with chronic cardiovascular diseases. Passive exercise refers to exercise performed without volitional control that can be viewed as an alternative and complementary modality for active exercise. Passive exercise is known to increase blood flow by mechanical pump effects or reflex activation. For example, passive movement using a motor-driven knee extensor device enhanced the secretion of proangiogenic growth factor and increased capillary density in skeletal muscle. Passive leg cycle exercise in the sitting position increased vascular blood flow velocity in the paralyzed legs of patients with paraplegia. On the other hand, passive leg movements and cycling did not affect on the arterial peripheral circulation assessed by echo Doppler measurements in subjects with spinal cord injury. In the present experimental and clinical studies, we demonstrated a clear enhancement of blood flow by WBPA. Our results are in agreement with the findings of Adams et al, who demonstrated that WBPA increased regional blood flow in anesthetized pig model.

In our clinical study, both single-session and daily administration of WBPA for 7 days significantly improved blood flow in the lower extremities of patients with PAD. Interestingly, the extent of blood flow improvement after 7-day WBPA was similar to that after a single session of WBPA, although the variability of the response diminished by 50%. Our recent clinical study showed that a single session of WBPA significantly improved coronary microcirculation in patients with coronary artery disease. Recently, Miyamoto et al reported that 4-week WBPA in patients with angina enhanced exercise capacity, ameliorated myocardial ischemia, and improved left ventricle function. In agreement with these reports, our findings provide definitive proof that WBPA improves vascular function both in the acute and chronic phases.

WBPA is known to cause internal shifts of blood; the motion platform accelerates and decelerates blood flow by

| Table 3. Hemodynamics and Laboratory Data Before and After a Single Session of WBPA |
|---------------------------------|------------------|------------------|------------------|------------------|
|                                | Before WBPA      | After WBPA       | P Value          |
| **Hemodynamics**               | (n=10)           | (n=10)           |                  |
| Heart rate, beats/min      | 63±5.1           | 66±5.4           | 0.5              |
| Systolic blood pressure, mm Hg | 139±5.7          | 139±8.2          | 0.9              |
| **Laboratory data**           |                  |                  |                  |
| WBC, per mm³                | 6001±556         | 5985±639         | 0.9              |
| High-sensitive CRP, mg/dL   | 0.7±0.35         | 1.19±0.75        | 0.4              |
| Total cholesterol, mg/dL    | 171.3±11.4       | 168.0±12.4       | 0.5              |
| LDL cholesterol, mg/dL      | 97.1±9.4         | 94.3±9.8         | 0.4              |
| HDL cholesterol, mg/dL      | 42.8±2.5         | 43.0±2.6         | 0.7              |
| Triglyceride, mg/dL         | 153.5±14.1       | 137.5±17.4       | 0.2              |
| Glucose, mg/dL              | 136.2±15.2       | 153.0±24.1       | 0.4              |
| Nitric oxide, μmol/L        | 58.3±9.4         | 57.2±10.2        | 0.6              |
| VEGF, pg/mL                 | 143.6±25.4       | 146.9±32.4       | 0.8              |

Data are mean±SEM. WBPA indicates whole-body periodic acceleration; WBC, white blood count; CRP, C-reactive protein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; VEGF, vascular endothelial growth factor.
adding pulses to the circulation, thereby increasing shear stress to the endothelium.8 Braith et al30 recently demonstrated that enhanced external counterpulsation, representing a noninvasive outpatient treatment option for patients with ischemic heart diseases, improves peripheral artery flow-mediated dilatation, increases endothelial-derived vasoactive agents, and decreases the production of proinflammatory cytokines. The enhanced external counterpulsation increases shear stress and thus stimulates endothelial NO release via the activation of eNOS. Evidence suggests that eNOS signaling participates in the regulation of endothelial cell function and blood vessel growth under conditions of ischemic stress.31 In the present study, we demonstrated that the level of phosphorylation of eNOS in skeletal muscle was significantly increased by WBPA in both the acute and chronic phases, and the proangiogenic effect of WBPA on ischemic limb was blunted in eNOS-deficient mice, indicating that the stimulatory action of WBPA on revascularization is dependent, at least in part, on eNOS.

The eNOS signaling pathway plays a pivotal role in growth factor–stimulated angiogenesis.18 Numerous studies reported that eNOS is an important downstream mediator of several angiogenic growth factors, such as FGF2, PDGF-B, stromal cell–derived factor-1α, and VEGF.32 On the other hand, it was reported that NO, derived from eNOS, acts as upstream promoter of VEGF expression in certain cell types.33,34 In the present study, WBPA did not increase the mRNA expression levels of VEGF in eNOS-deficient mice in the acute phase of hindlimb ischemia, and WBPA had virtually no proangiogenic effect in eNOS-deficient mice. Along with the previous report showing that WBPA causes the release of significant amounts of NO into the peripheral circulation,31 our findings suggest that WBPA promotes angiogenesis through eNOS-dependent mechanism, and NO-dependent VEGF upregulation facilitates angiogenesis in skeletal muscles in the acute phase of hindlimb ischemia.

Diabetes is an important risk factor for the progression of PAD.19 Diabetes also impairs skeletal muscle angiogenesis through several proteins of the angiogenesis signaling pathway.20–23 Especially, eNOS signaling plays an important role in endothelial dysfunction and vascular inflammation in the presence of insulin resistance.35,36 It was reported that eNOS-dependent NO production is essential for the activation of insulin signaling.37 Therefore, it is reasonable to speculate that WBPA could improve glucose tolerance and insulin sensitivity. In this study, proangiogenic effects of WBPA on ischemic limb were observed in a mouse model of diabetes; however, glucose tolerance and insulin sensitivity did not change after 2 weeks of WBPA. One reason for the lack of improvement of glucose and insulin tolerance is perhaps the relatively short duration of the study. Additional long-term experiments are warranted to investigate the effects of WBPA on glucose and lipid metabolism.

Skeletal muscles are known to secrete factors that influence the behavior of neighboring or remote cells.38 For example, VEGF is secreted from skeletal muscles to maintain tissue perfusion, illustrating a mechanism through which blood vessel recruitment can be coupled to normal tissue growth.39 Using myocyte-specific VEGF-deficient mice, Olf-}

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**Disclosures**

None.

**References**


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Materials and Methods

Western blot analysis

Western blotting was performed with a SDS-PAGE Electrophoresis System as described previously\(^1\). Briefly, 20-μg protein samples were resuspended in a reduced sample buffer, and then electrophoresed on a 7.5 to 10% Tris gel with Tris running buffer; blotted to PVDF membrane; and sequentially probed with primary antibodies against phosphorylated eNOS (Ser 1177, p-eNOS), eNOS (Cell Signaling Technology, Beverly, MA), phosphorylated-Akt (ser-473) and Akt. A horseradish peroxidase-conjugated goat anti-rabbit antibody was then added, and secondary antibodies were detected through autoradiography using enhanced chemiluminescence (ECL Plus, General Electric Healthcare, Milwaukee, WI). \(\alpha\)-Tubulin was used as a protein loading control. Values for phosphorylated eNOS and phosphorylated Akt protein were normalized to the total eNOS and Akt protein, respectively.

Quantitative real-time PCR

Total RNA was prepared by Qiagen using the protocol provided by the manufacturer. In this method, cDNA was produced using ThermoScript RT-PCR Systems (Invitrogen, Carlsbad, CA). Real-time PCR was performed as described previously\(^2\). Transcript levels of vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), stromal cell–derived factor 1\(\alpha\) (SDF-1\(\alpha\)), platelet-derived growth factor-B (PDGF-B) epidermal growth factor (EGF), Amphiregulin, Delta-like protein 4
(DLL4), NOV, ADAMTS1, Angiogenin and Osteopontin were determined as the number of transcripts relative to those of 18S, and normalized to the mean value of the control. Table 1 and Supplemental Table I lists the sequences of primers used in this study.

**Immunohistochemistry**

Capillary density in the adductor muscle was assessed on 6 μm-thick, frozen sections after staining with a monoclonal rat antibody specific for murine CD31 (PECAM-1, BD Biosciences). The number of arterioles was assessed after staining with DAPI against cell nuclei. The number of CD31-immunopositive cells was counted manually on 7 random microscope fields per section (200 x magnification).

**Angiogenesis antibody array analysis**

The expression profile of 53 angiogenesis-related proteins was analyzed with mouse angiogenesis antibody arrays (R&D Systems Inc., Minneapolis, MN). Blocking, hybridization of the array filters, washing conditions, and chemiluminescent detection steps were performed according to the instructions supplied by the manufacturer.
Legends for Supplemental Figures

Supplemental Figure I. Effects of WBPA on Akt activation in skeletal muscle.
(A) Representative immunoblots of p-Akt and Akt expression using ischemic adductor muscle in control and WBPA mice after a single session of WBPA. (B) Quantitative analysis of western blotting. Data are mean±SEM of 3 to 5 mice.

Supplemental Figure II. Effects of WBPA on angiogenic growth factors expression in ischemic skeletal muscle at chronic phase. (A) VEGF, FGF2, PDGF-B and SDF-1 transcript expression in adductor muscle of wild-type mouse after 7 or 14 days daily WBPA assessed by quantitative real-time PCR. (B) VEGF, FGF2, PDGF-B and SDF-1 transcript expression levels in adductor muscle of eNOS-deficient mouse after 7 or 14 days daily WBPA assessed by quantitative real-time PCR. Data are mean±SEM of 4 mice.

Supplemental Figure III. WBPA alters the expression profiles of angiogenesis-related proteins.
(A) Template for the mouse angiogenesis protein array. (B) Representative protein arrays of four independent experiments. (C) Time course of protein array. The expression of 9 proteins was significantly different between the control and WBPA mice at one or multiple time points. Data are mean±SEM of 4 independent experiments. (D) The time course of ADAMTS1, Amphiregulin, Angiogenin, NOV, osteopontin, DLL4 and EGF transcript expression in adductor muscle after WBPA assessed by quantitative real-time PCR. Data are mean±SEM of 4 to 5 mice, each.
*P<0.05 vs. control.
References for Supplemental Material


**Supplemental Table I.** Sequences of Primers Used for Quantitative Real-Time PCR.

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Supplemental Figure I

A.

P-AKT

T-AKT

Tubulin

WBPA

- + + +

3h 6h 12h

Hours after WBPA

B.

P-AKT/T-AKT

WBPA

- + + +

3h 6h 12h

Hours after WBPA

0 0.5 1.0 1.5

n.s

n.s

P-AKT/T-AKT
Supplemental Figure II

A. VEGF/18S

VEGF/18S

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<th>Days after surgery</th>
<th>7d</th>
<th>14d</th>
</tr>
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<td>-</td>
<td>n.s</td>
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</tr>
<tr>
<td>+</td>
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FGF2/18S

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<th>Days after surgery</th>
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<th>14d</th>
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</thead>
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<tr>
<td>-</td>
<td>n.s</td>
<td></td>
<td></td>
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<tr>
<td>+</td>
<td>n.s</td>
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PDGF-B/18S

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<th>Days after surgery</th>
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<th>14d</th>
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</thead>
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<tr>
<td>-</td>
<td>n.s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>n.s</td>
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SDF-1α/18S

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<th>14d</th>
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<tr>
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<td>n.s</td>
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Days after surgery:
- 7d
- 14d
Supplemental Figure II

B. VEGF/18S

- n.s
- n.s

Days after surgery:
- 7d
- 14d

WBPA:
- -
- +

FGF2/18S

- n.s
- n.s

SDF-1α/18S

- n.s
- n.s

Days after surgery:
- 7d
- 14d

WBPA:
- -
- +

PDGF-B/18S

- n.s
- n.s

WBPA:
- -
- +

Days after surgery:
- 7d
- 14d
Supplemental Figure III

A.

<p>| | | | | | | |</p>
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<td>4</td>
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<td>IL-10</td>
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<td>PD-ECGF</td>
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<td>IGFBP-1</td>
<td>Endostatin/Collagen X</td>
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<td>VEGF</td>
<td>PIGF-2</td>
<td>MMP-3</td>
<td>IGFBP-2</td>
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<td>Prolactin</td>
<td>MMP-8</td>
<td>IGFBP-3</td>
<td>FGF acidic</td>
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<td>Proliferin</td>
<td>MMP-9</td>
<td>IL-1α</td>
<td>FGF basic</td>
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<td>IL-1β</td>
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<td>positive control</td>
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</table>

B.

(control) 3h 6h 12h
Supplemental Figure III

C.

**FGF basic**

**DLL4**

**Osteopontin**

**Angiogenin**

**EGF**

**ADAMTS1**

**PDGF-AB/PDGF-BB**

**Amphiregulin**

**NOV**
Supplemental Figure III

D. EGF/18S

Amphiregulin/18S

n.s

n.s

D. DLL4/18S

NOV/18S

n.s

n.s

D. 3h 6h 12h

WBPA

Hours after WBPA

WBPA

Hours after WBPA

WBPA

Hours after WBPA

WBPA

Hours after WBPA

Hours after WBPA

0 1.0 2.0

0 1.0 2.0

0 1.0 2.0

0 1.0 2.0
Supplemental Figure III

D. ADAMTS1/18S

- WBPA
  - 3h
  - 6h
  - 12h

- WBPA +
  - 3h
  - 6h
  - 12h

P<0.05

n.s

Angiogenin/18S

- WBPA
  - 3h
  - 6h
  - 12h

- WBPA +
  - 3h
  - 6h
  - 12h

n.s

Osteopontin/18S

- WBPA
  - 3h
  - 6h
  - 12h

- WBPA +
  - 3h
  - 6h
  - 12h

n.s