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Exercise-induced expression of VEGF and salvation of myocardium in the early stage of myocardial infarction

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Wu G, Rana JS, Wykrzykowska J, Du Z, Ke Q, Kang P, Li J, Laham RJ. Exercise-induced expression of VEGF and salvation of myocardium in the early stage of myocardial infarction. *Am J Physiol Heart Circ Physiol* 296: H389–H395, 2009. First published December 5, 2008; doi:10.1152/ajpheart.01393.2007.—The mechanism of exercise-induced benefit and angiogenesis in ischemic heart disease remains poorly defined. This study was designed to investigate the effects of exercise training on the expression of angiogenic factors and angiogenesis in the infarcted myocardium [myocarial infaction (MI)]. Sixty-three male FVB mice were used for study and were divided into subgroups to test the response to exercise: the time-dependent expression of angiogenic factors to exercise training in normal (*group 1*; $n = 12$) and infarcted myocardium (*group 2*; $n = 15$) and the exercise-induced angiogenic response in normal and infarcted myocardium (*group 3*; $n = 20$) as well as the impact of exercise preconditioning on infarcted myocardium (*group 4*; $n = 26$). Exercise training consisted of daily treadmill exercise for 1 h for 3 days. Expression of VEGF and its receptors Flt-1 and Flk-1 was upregulated by exercise training in mice with MI. Exercise-induced VEGF expression in the MI group was higher than that in the sham (control) group. Cell proliferation assessment showed a significantly higher ($P < 0.05$) number of bromodeoxyuridine-positive cells in post-MI mice in the exercise group as opposed to post-MI mice in the sedentary group. 2,3,5-Triphenyltetrazolium chloride staining revealed a profound difference in the size of MI ($18.25 \pm 2.93\%$) in the exercise group versus the sedentary group ($29.26 \pm 7.64\%$, $P = 0.02$). Moreover, exercise preconditioning before MI promoted VEGF expression at both mRNA and protein levels. In conclusion, activation of VEGF and its receptors occurs in the infarcted mice heart in response to exercise, which results in decreased infarct size and improved angiogenesis.

exercise; angiogenesis; ischemic heart disease; vascular endothelial growth factor

EXERCISE AND PHYSICAL ACTIVITY are known to prevent the development of coronary artery disease and reduce symptoms in patients with established cardiovascular disease (26). In fact, a meta-analysis of 48 randomized trials of cardiac rehabilitation showed that, compared with the usual care, cardiac rehabilitation reduced total mortality by 20% and cardiac mortality by 26% (25). The precise mechanisms by which exercise therapy improves mortality in patients with coronary heart disease has not been fully elucidated (16, 26). Exercise training has been shown to have beneficial effects on the coronary vasculature, including myocardial oxygen demand, endothelial function, autonomic tone, inflammatory markers, and the development of coronary collateral vessels (5, 28). However, the direct effect of exercise on myocardial angiogenesis in ischemic heart disease remains poorly defined.

Angiogenesis is the process of formation of new blood vessels, which is mediated by angiogenic factors such as VEGF. Extensive studies have demonstrated that angiogenesis occurs in skeletal muscle as an adaptive response to exercise that may satisfy the increased tissue requirements for oxygen delivery and metabolic processes (8). VEGF is crucial for myocardial angiogenesis and myocardial salvage after myocardial infarction (MI) (13, 15, 17, 23, 29). Exercise training, in a mouse model, increased endothelial progenitor cells (EPCs) and enhanced angiogenesis (14), and a single episode of exercise could acutely increase the numbers of EPCs and cultured/circulating angiogenic cells in human subjects (21). In addition, exercise training results in increased circulating plasma VEGF levels in both endurance-trained athletes and sedentary men (12). Enhanced angiogenic responses to exercise in skeletal muscle(4) and the healthy myocardium (3) have previously been explored. The present study was designed to investigate the effects of exercise training on angiogenic factors such as VEGF and angiogenesis in the normal and infarcted myocardium (MI) as well as its effect on myocardial salvage post-MI.

MATERIALS AND METHODS

Study design. This study was approved by the Institutional Animal Care and Use Committee. Sixty-three male FVB mice (age: 13 ± 2 wk and weight: 26.8 ± 2.4 g) were used throughout the study. Based on the exercise training protocols (Fig. 1), they were divided into four groups as follows: the time-dependent response of VEGF to exercise training in the intact myocardium (*protocol 1*; $n = 12$) and infarcted heart (*protocol 2A* and *protocol 2B*; $n = 15$), the exercise-induced angiogenic response in the normal myocardium (sham surgery) and infarcted myocardium (*protocol 2A* and *protocol 2B*; $n = 20$), and the impact of preconditioning with exercise on the infarcted myocardium (*protocol 3A* and *protocol 3B*; $n = 26$).

MI model. Mice were anesthetized by an intraperitoneal injection of Avertin (0.2 ml/10 g body wt), and mechanical ventilation was carried out with tidal volume of $0.4–0.5$ ml. The heart was exposed, and the left anterior descending coronary artery (LAD) was ligated $1.5–2.0$ mm away from the left auricle. Each animal was given 24 h of recovery before exercise training. In the sham group, the chest was opened, but the LAD was left patent.

Fig. 1. Exercise protocols. All animals were given pre-exercise training to familiarize animals with the treadmill [low speed of 5 m/min (mpm) for 5 min twice daily]. The next day, animals entered the formal exercise protocols. There was a 24-h recovery period after myocardial infaction (MI). Before MI, exercise speed was 17 m/min. However, running speed was changed to 12 m/min after MI. Mice in *protocol 1* underwent exercising training without MI; mice with left anterior coronary artery ligation (MI) were randomly assigned to one of four protocol groups. *Protocol 2A* (P2A) consisted of mice kept sedentary for 3 days both before and after MI as a control group; *protocol 2B* (P2B) consisted of mice that were kept sedentary before MI but exercised for 3 days post-MI (12 m/min, 10° incline, 1 h/day) 1 day after MI. *Protocol 3A* (P3A) consisted of mice that exercised before MI followed by 4 sedentary days; *protocol 3B* (P3B) consisted of mice that exercised (17 m/min, 10° incline, 1 h/day) before MI and also after MI (12 m/min, 10° incline, 1 h/day) 1 day after MI.
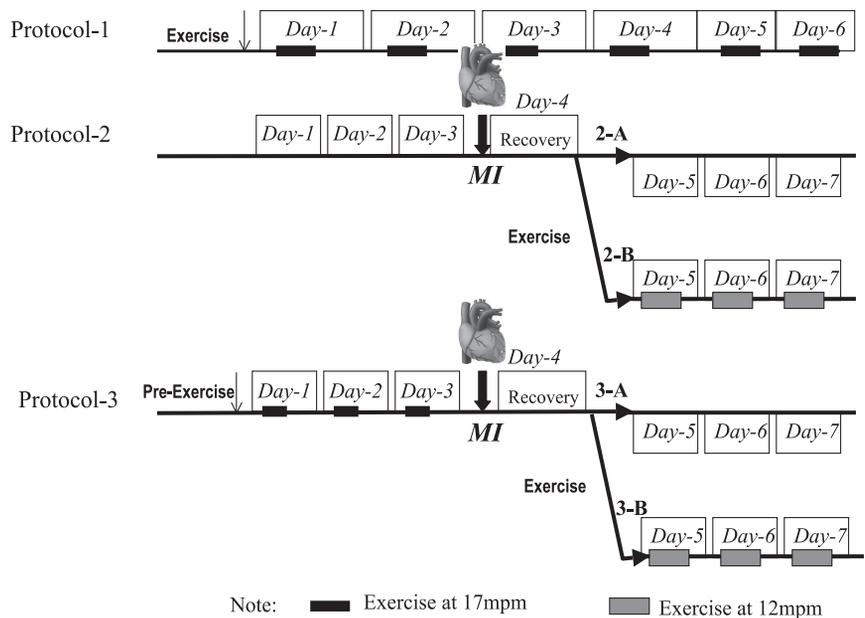
Exercise training protocol. Before the study began, all mice were familiarized with the rodent treadmill by walking them at 5 m/min, at 10% grade, for 5 min. First, the time-dependent expression of VEGF after exercise was determined (*protocol 1*). In *protocols 2* and *3*, MI was induced on *day 3*. Before MI, mice exercised for 1 h daily at 17 m/min; after MI, the speed was reduced to 12 m/min for 1 h daily. Semiquantitative PCR, Northern blot analysis, and Western blot analysis were used to determine the expression of angiogenic factors.

Tissue preparation. Under deep Avertin anesthesia (0.2 ml/10 g body wt), animals were killed by excessive chloroform in a sealed tank 1 h after exercise in the exercise group. The whole heart was excised and weighed. The left ventricle was then cut into three sections transversally with the middle section at the ligation level. The middle section was fixed in 10% formalin and used for morphological and immunohistochemical analyses. The other two sections were snap frozen, immediately placed into liquid nitrogen, and later transferred to −80°C for further analysis.

Total RNA and Northern blot analysis. About 100 mg of frozen tissue were homogenized in TRI reagent (Sigma, St. Louis, MO) buffer, and total RNA was extracted following the manufacturer's instructions. Primers for VEGF and GAPDH were designed using Biology Workbench online. Probes of targets were prepared by PCR. The product of PCR was then purified using a QIAquick PCR purification kit (QIAGEN, Valencia, CA) as the probe of targets. Total RNA (10 μg) was then fractionated on a 1.3% formaldehyde-agarose gel and transferred to a GeneScreen Plus membrane (New England Nuclear, Boston, MA). [α-^{32}P]dCTP-labeled VEGF and GAPDH probes were hybridized in QuikHyb solution (Stratagene, La Jolla, CA). Autoradiographical signals were quantified by densitometry using ImageQuant software and adjusted to GAPDH density and the 28S band (shown).
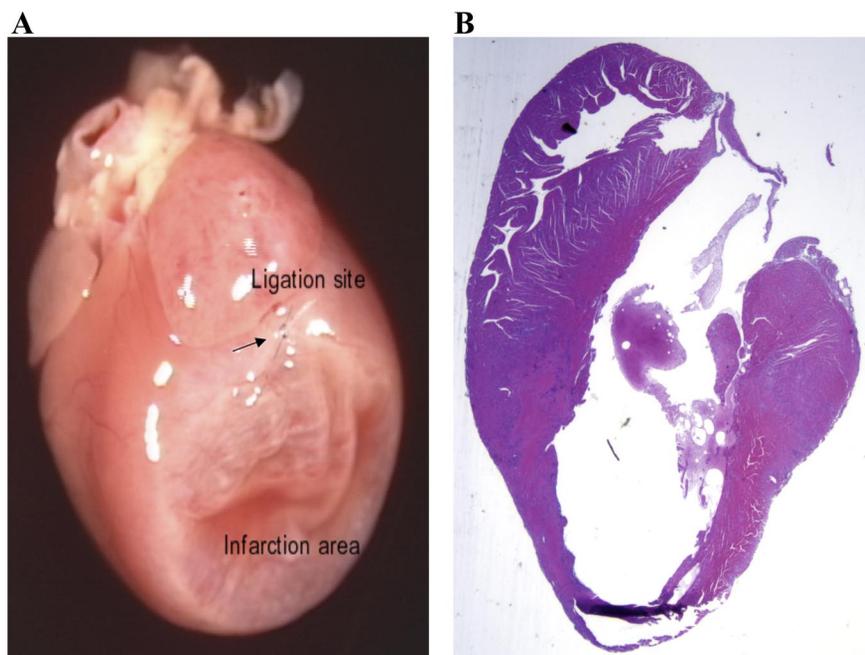

Fig. 2. Mice model of MI performed by left anterior descending coronary artery ligation 1.5–2 mm distal to the left atrium (*A*; the arrow points to the site of ligation). This resulted in an anterior myocardial infarct shown 4 days later on gross examination (*A*) and histology [hematoxylin and eosin staining (H&E); *B*].

Semiquantitative PCR. Expression of VEGF receptors Flk-1 and Flt-1 was assessed by RT-PCR. Single-strand cDNA as a PCR template was synthesized using an oligo(dT)$_{12-18}$ primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA fragments of Flk-1 and Flt-1 as well as the 18S primer pair (Ambion, Austin, TX) as an internal standard were amplified using *Taq* DNA polymerase. Primers were designed based on the murine species using Biology Workbench online and were as follows: VEGF, forward 5′-CAGGCTGCTGTAACGATGAA-3′ and reverse 5′-AGGAATCCCAGAAACAACCC-3′; Flt-1, forward 5′-CAGCT-TCCAAGTGGCTAAGG-3′ and reverse 5′-CATAATGGAATTT-GGGGTCG-3′; Flk-1, forward 5′-TGAGGAGCTTTCACCGAACT-3′ and reverse 5′-GCCTCCTGTTTGCTCTGTTC-3′; and GAPDH, forward 5′-AACTTTGGCATTGTGGAAGG-3′ and reverse 5′-TGT-GAGGGAGATGCTCAGTG-3′. PCR (30 cycles) was performed on 30 ng of cDNA from each sample using GeneAmp PCR Systems 9700 (Applied Biosystems). Reaction conditions were as follows : 1× (94°C, 5 min), 30× (94°C, 30 s; 58°C, 1 min; and 72°C, 1 min), and 1× (72°C, 7 min). Products were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide. The expression of 18S was referenced to an endogenous internal standard. Densitometry was used to quantify gene expression normalized to the 18S product.

Protein extraction and Western blot analysis. Heart tissue samples were homogenized, lysed by RIPA buffer (Boston Bioproducts, Ashland, MA), and fractionated by 10% SDS-polyacrylamide gels. Protein extracts were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The VEGF signal was detected with a polyclonal VEGF antibody (Oncogene, Cambridge, MA). In addition, β-actin and GAPDH antibodies were also used to normalize VEGF protein expression. Other parameters studied included FGF receptor 1 (FGF-R1). Immunoblots were visualized by ECL Western blot detection reagents (Amersham Life Science, Arlington Heights, IL).

Measurement of MI size. For the purpose of MI size identification, 2,3,5-triphenyltetrazolium chloride (TTC) was applied to identify viable tissue. Briefly, the heart was sectioned transversely into five sections with one section being made at the site of the ligature. All sections were immediately rinsed in 2% TTC, incubated for 30 min at 37°C, and thereafter preserved in 10% formaldehyde for further image analysis. The viable myocardium stained brick red, and the infarct appeared as pale white. Sections were photographed, and the infarct size was marked and calculated in Image J1.29X. Infarct size was expressed as a percentage of the infarct area (unstained) versus the total left ventricle area. For each animal, three serial sections including one containing the ligature were analyzed, and the mean value of all sections in one heart was treated as one value for statistical analysis.

Morphometric analysis. Formalin-fixed myocardial tissues were embedded in paraffin and cut in 4-μm transverse sections for the detection of the exercise-induced angiogenic response in the heart. For the purpose of fully visualizing and understanding myocardial angiogenesis, a rat anti-mouse PECAM-1 monoclonal antibody (BD Biosciences, San Jose, CA) was used at a dilution of 1:50. Biotinylated rabbit anti-rat IgG, mouse absorbed (the second antibody), was combined with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) to detect myocardial microvessels. In the negative control sections for each tissue sample, primary antiserum was replaced with 1 mg/ml BSA (Sigma, Milwaukee, WI). Quantitative measurements of myocardial capillary density were determined from five random microscopic fields (×200) with the Image J1.29X tool using computer-assisted morphometry. The number of microvessels identified from 5 fields/section were averaged and expressed as the number of microvessels per 0.4 mm^2.

Cell proliferation analysis by bromodeoxyuridine incorporation. Mice were injected intraperitoneally with bromodeoxyuridine (BrdU; 100 mg/kg body wt) once a day for 3 consecutive days before death. The heart was fixed with 4% formalin, paraffin embedded, and sectioned, including locations of both the infarcted area and borderline

territory. The incorporation of BrdU was detected with a mouse monoclonal anti-BrdU antibody (Calbiochem, Cambridge, MA). The number of BrdU-positive nuclei per 200 viewing fields was counted and averaged with five random fields from the section.

Statistical analysis. Results are presented as means ± SD. Paired and unpaired Student *t*-tests and ANOVA for multiple comparisons with the Bonfferoni correction were used where applicable. *P* values of <0.05 were considered statistically significant.

RESULTS

MI model and animal care during exercise training. All animals survived MI 24 h after the ligation of the LAD (Fig. 2). However, two mice in the sedentary group and one mouse in the exercise group died after 2 days of MI (on the treadmill). Autopsy showed aneurysm formation in the hearts of the mice from the sedentary group and apical rupture in the mouse from the exercise group. Vigorous exercise could have precipitated rupture, and exercise post-MI was reduced to 12 from 17 m/min at 10° slope. All animals before the exercise protocol
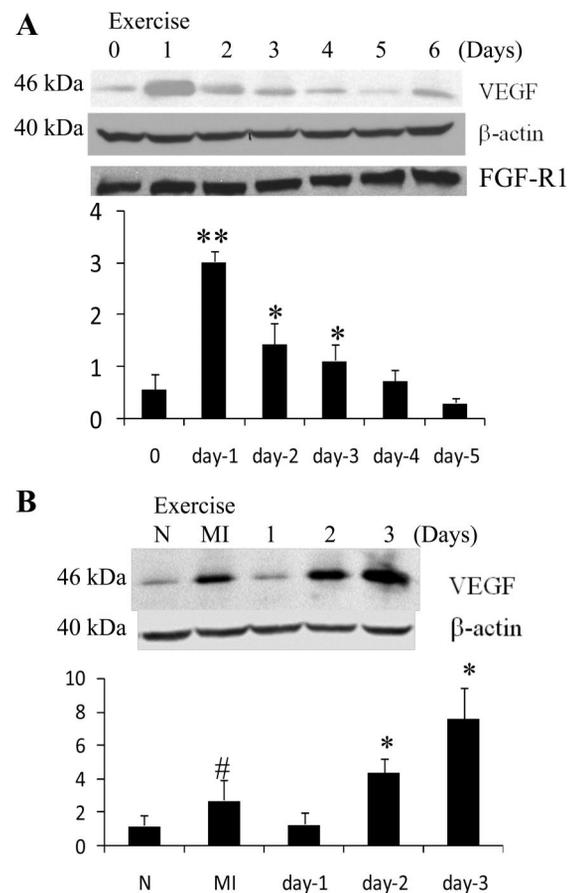

Fig. 3. *A*: Western blot analysis of exercise-induced VEGF protein expression in intact mice (17 m/min, 10° slope, 1 h daily). VEGF protein levels were normalized by β-actin at each time point and are expressed as means ± SD. FGF-R1, FGF receptor 1. *P* < 0.05 and **P* < 0.01 compared with control. *B*: VEGF protein response to exercise in the infarcted myocardium. Protein signals were normalized by β-actin and are expressed as means ± SD based on the sample of each time point. After MI (6 h after surgery), VEGF protein expression was significantly increased compared with controls (sham surgery). Thereafter, it was downregulated by exercise after 1 day. With the continuation of exercise, VEGF levels increased. #*P* < 0.05 compared with the normal (N) heart; *P* < 0.05 and **P* < 0.01 compared with the MI group.

change were excluded from data analysis to minimize inappropriate comparisons.

Time-dependent response of VEGF in intact and infarcted hearts. VEGF protein expression was significantly upregulated after 1 day of exercise training in intact mice and gradually returned to baseline after 4 days (Fig. 3*A*). In MI mice, upregulation of VEGF protein started at *day 2* of exercise training and continued to increase significantly at *day 3* (Fig. 3*B*).

Response of VEGF and its receptors Flk-1 and Flt-1 to exercise training after MI. VEGF mRNA expression was enhanced significantly by 3 days of exercise training in mice with MI. There was a slight increase of VEGF mRNA after exercise in the sham group, but it was not statistically significant (Fig. 4*A*). Short-term exercise training promoted upregulation of Flk-1 and Flt-1 in both the MI and sham groups. However, the extent of expression of these two receptors was significantly higher in the MI group (Fig. 4, *B* and *C*).

Exercise training and salvation of infarcted myocardium. To investigate the role of short-term exercise training on limiting infarct size in post-MI mice, TTC staining was used (Fig. 5, *A*–*C*). There was a profound difference in infarct size with exercise (*protocol 2B*, 18.2 ± 2.9%) compared with the sedentary group (*protocol 2A*, 29.3 ± 7.6%, $P = 0.02$; Fig. 5*C*).

Angiogenic response to exercise training in mice with MI. After the upregulation of VEGF and its receptors as a result of exercise training in post-MI mice had been assessed, myocardial capillary density was investigated by PECAM-1 immunohistochemical staining. There was increased capillary density in the post-MI exercise group (*protocol 2B*, 16.5 ± 3.4/0.4
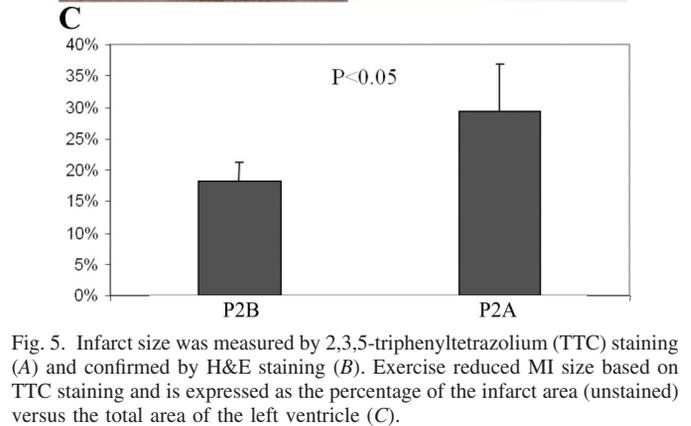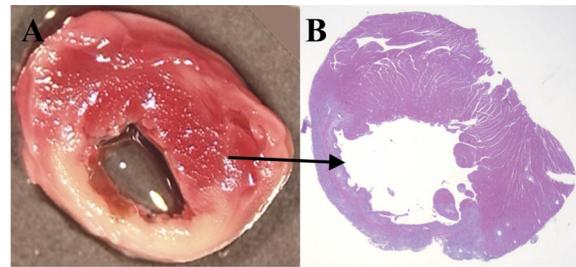

Fig. 5. Infarct size was measured by 2,3,5-triphenyltetrazolium (TTC) staining (*A*) and confirmed by H&E staining (*B*). Exercise reduced MI size based on TTC staining and is expressed as the percentage of the infarct area (unstained) versus the total area of the left ventricle (*C*).

mm²) versus the post-MI sedentary group (*protocol 2A*, 10 ± 2.1/0.4 mm², $P = 0.029$; Fig. 6, *A*–*C*).

Cell proliferation. Cell proliferation was assessed by BrdU incorporation. The number of BrdU-positive cells was significantly higher ($P < 0.05$) in the post-MI exercise group

Fig. 4. Impact of short-term exercise on the mRNA expression of VEGF and its receptors, Flk-1 and Flt-1, in mice with or without MI. Exercise was at 12 m/min, 10° incline, 1 h/day, for 3 days after 24 h of recovery from MI; control animals were sham operated with or without exercise. $n = 5$ for all groups. *A*: Northern blot analysis demonstrated that exercise significantly increased VEGF mRNA expression in mice with MI. VEGF mRNA expression increased slightly with exercise in sham-operated animals and significantly increased in with exercise in MI animals. Data are expressed as means ± SD. *B* and *C*: exercise training promoted Flk-1 and Flt-1 mRNA expression, as demonstrated by semiquantitative PCR analysis, in both groups (MI and sham operation). All data were normalized by 18S rRNA and are expressed as means ± SD. P2A-C: P2A after sham surgery (control, sedentary); P2B-C, P2B after sham surgery (control, exercise). *Top right*: representative Northern blot of VEGF expression; *bottom right*: representative PCR of Flt-1 expression.
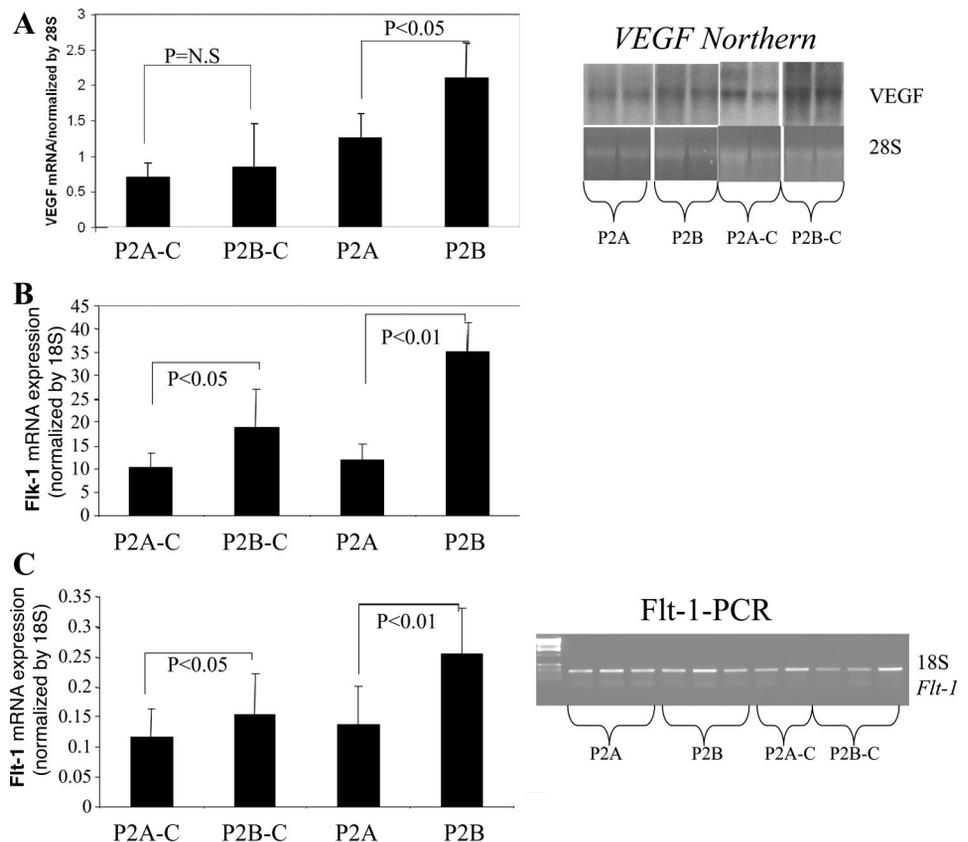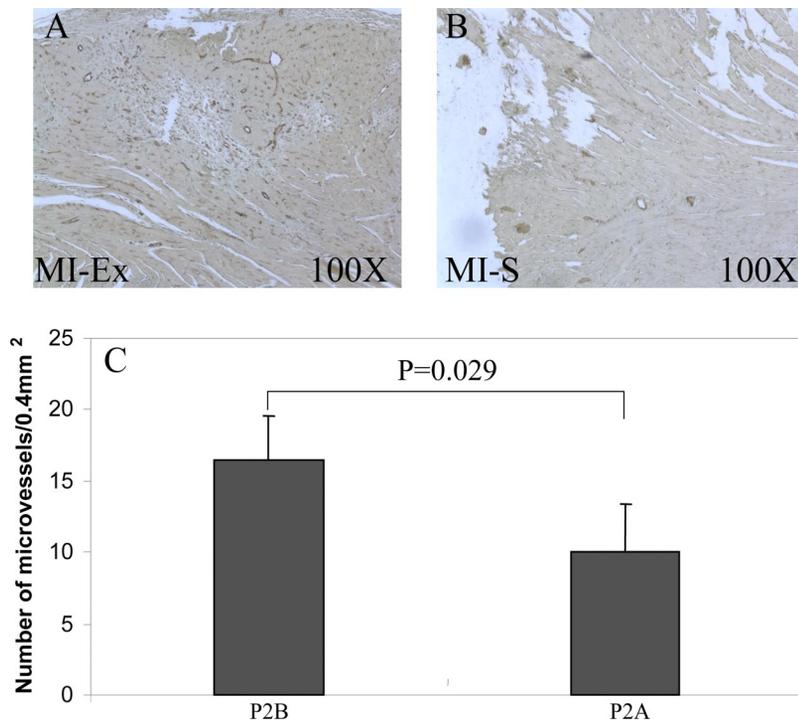
Fig. 6. PECAM-1 (CD31) immunostaining demonstrated significantly more microvessels in the MI group with exercise (MI-Ex; P2B; *A*) compared with the MI sedentary group (MI-S, P2A; *B*; $P < 0.05$) as shown in *C*. Magnification: ×100.

(*protocol 2B*) compared with the post-MI sedentary group (*protocol 2A*; Fig. 7).

Preconditioning with exercise enhances VEGF expression. To explore the impact of preconditioning with exercise (3 days, 1 h/day at 17 m/min) on the infarcted myocardium in mice, 26 mice were randomly allocated to 4 subgroups: exercise and nonexercise training after MI with or without exercise preconditioning (Fig. 1). The results demonstrated that exercise preconditioning before MI promoted VEGF expression at both mRNA and protein levels after MI (Fig. 8). Particularly,

exercise preconditioning (*protocol 3A*) significantly increased VEGF protein and mRNA expression compared with *protocol 2A*, underscoring the effect of exercise before MI (12.2 ± 4.2 vs. 8.7 ± 3.2 for mRNA and 0.62 ± 0.31 vs. 0.31 ± 0.23 for protein expression, $P < 0.05$ for both comparisons).

DISCUSSION

Our study demonstrated that VEGF expression is upregulated in a time-dependent fashion by exercise training in mice
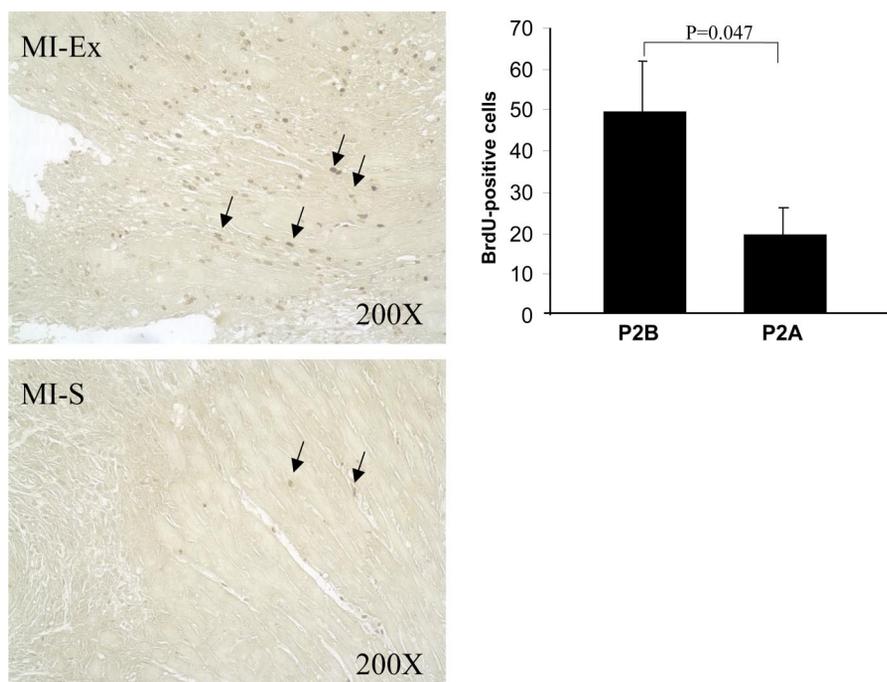

Fig. 7. Cell proliferation analysis by bromodeoxyuridine (BrdU) incorporation. Mice were injected intraperitoneally with BrdU (100 mg/kg body wt) once a day for 3 consecutive days before death. Hearts were fixed with 4% formalin, paraffin embedded, and sectioned including locations of both the infarcted area and borderline territory. The incorporation of BrdU was detected with a mouse monoclonal anti-BrdU antibody. The number of BrdU-positive nuclei (arrow) was counted and averaged by 5 random fields from the section and showed significantly more BrdU uptake with exercise.
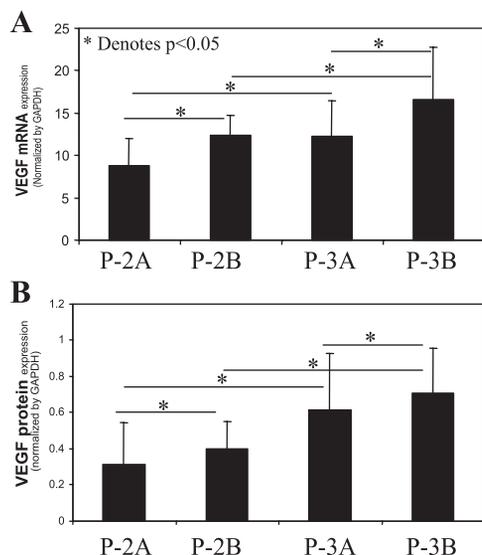
Fig. 8. Exercise-induced VEGF mRNA (*A*) and protein (*B*) expression in mice with MI. Preconditioning with 3 days of exercise (P3B) significantly increased VEGF protein and mRNA expression. Particularly, exercise preconditioning (P3A) significantly increased VEGF protein and mRNA expression compared with P2A, underscoring the effect of exercise before MI (12.2 ± 4.2 vs. 8.7 ± 3.2 for mRNA and 0.62 ± 0.31 vs. 0.31 ± 0.23 for protein expression, $P < 0.05$ for both comparisons).

with MI. VEGF receptors Flt-1 and Flk-1 were induced maximally in MI mice exposed to exercise training. In addition, post-MI exercise resulted in a significant decrease in infarct size and a significant increase in capillary density and BrdU uptake in MI mice. Finally, exercise preconditioning before MI promoted VEGF expression at all stages at both mRNA and protein levels.

Our results confirm previous studies and suggest that exercise programs before and after ischemic events have beneficial effects, as noted clinically. This may be related to the expression of angiogenic cytokines and their receptors, such as VEGF, Flt-1 and Flk-1.

VEGF is a key growth factor in physiological angiogenesis and induces angiogenesis in myocardial ischemia and MI (13, 15, 17, 23, 29). Exercise has been shown to enhance myocardial tolerance to ischemia in a rat ischemia-reperfusion injury model through a PKC-mediated mechanism (30). However, previously, swimming exercise has been shown to have no effect on remodeling of infarcted or noninfarcted myocardium (1). The lack of benefit in this study could have been related to the vigorous exercise with swimming, which resulted in early mortality in out-pilot experiments and made us reduce the exercise level after MI. This, coupled with data on the causal role of VEGF in the growth of coronary collaterals and myocardial salvage after MI, suggest a causal effect (18, 27, 31).

The beneficial role of exercise on the risk of coronary heart disease is well established. In fact, in a 12-mo program, regular physical exercise in selected patients with stable coronary artery disease resulted in superior event-free survival and exercise capacity to percutaneous revascularization, notably owing to reduced rehospitalizations and repeat revascularizations (10). Among the potential mechanisms underlying this beneficial effect of exercise is increased collateral development, as seen experimentally (6, 19, 24). Clinical studies have

shown that in patients with coronary atherosclerosis, exercise training partially improves endothelial function of large coronary conduit and resistance vessels (11) and leads to improved agonist-mediated endothelium-dependent vasodilation (9). Exercise training in ischemic cardiomyopathy leads to improved collateralization (2). Other benefits of exercise may include a decrease in inflammation, which may favorably affect coronary disease (22).

Furthermore, exercise has been shown to reduce brain damage in rats subjected to transient middle cerebral artery occlusion, and the reduced brain injury was associated with angiogenesis as well as the cellular expression of nerve growth factor (7). These and our findings may explain the physiological improvements in myocardial perfusion and function seen in many of the placebo-equivalent angiogenesis studies, where it is hypothesized that patients feel better, exercise more, and develop more ischemia and collaterals, leading to improved perfusion and function: "physiological placebo" (20).

There are several limitation of this study. Although the data suggest a causal relationship between exercise-induced VEGF expression and myocardial salvage post-MI, this is not directly demonstrated and other unstudied cytokines may have contributed to that effect.

Our data show comprehensively that after MI, exercise minimizes infarct size, increases the number of microvessels and capillaries, and upregulates the expression of angiogenic factors like VEGF and its receptors Flt-1 and Flk-1. Furthermore, exercise preconditioning induces VEGF expression in post-MI mice with exercise training significantly more than the groups that either received no preconditioning or no post-MI exercise. These findings provide direct evidence of exercise-induced angiogenesis and myocardial salvage in the ischemic myocardium.

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