International Journal of Cardiology 177 (2014) 894–901

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Impact of remote physiological ischemic training on vascular endothelial growth factor, endothelial progenitor cells and coronary angiogenesis after myocardial ischemia

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ARTICLE INFO

Article history:
Received 26 February 2014
Received in revised form 8 September 2014
Accepted 18 October 2014
Available online 22 October 2014

Keywords:
Physiological ischemic training
Myocardial ischemia
Coronary collateral circulation
Capillary density
Endothelial progenitor cells
Vascular endothelial growth factor

ABSTRACT

Objective: This experimental study investigates the potential role of physiological ischemic training (PIT) of remote limbs on vascular endothelial growth factor (VEGF), endothelial progenitor cells (EPCs) and myocardial angiogenesis after myocardial ischemia.
Methods: Forty-two rabbits were assigned into six groups at random: sham-operated (SO), training only (TO), myocardial ischemia (MI), PIT, EPC promoter (PIT+), and EPC inhibitor (PIT−). MI was experimentally induced by implanting a constrictor around the left ventricular branch. The PIT procedure included three 3-min cycles of cuff inflations on the hind limbs followed by a 5 min reperfusion. VEGF mRNA, protein and EPC numbers were measured in plasma and myocardium. Capillary density (CD), coronary blood flow (CBF) and coronary collateral blood flow (CCBF) were also determined.
Results: Groups were compared using non-parametric statistics and associations between agents were explored with fractional polynomial regression. VEGF-mRNA and -protein levels were highest in PIT+ and PIT. PIT differed significantly from SO, TO, MI, and PIT− regarding VEGF-mRNA and -protein in plasma and VEGF-protein in myocardium. EPCs were highest in PIT+ followed by PIT. PIT differed significantly from SO, TO, MI, and PIT− regarding plasma EPCs. CD, CCBF and CCBF/CBF were significantly increased in PIT+ and PIT as compared to controls. PIT− did not differ significantly from SO and TO. VEGF explained up to 43% of variance in EPCs. EPCs explained up to 87% of variance in CD. CD explained up to 97% of variance in CCBF and CCBF/CBF.
Conclusion: PIT stimulates VEGF-mediated mobilization of EPCs as well as angiogenesis and might be proven as a new treatment strategy for patients with coronary heart disease.

1. Introduction

Coronary heart disease (CHD) has been the leading cause of death around the world for the past decades [1]. Revascularization plays a pivotal role in patients with CHD. Coronary angioplasty and surgical coronary artery bypass are the two recommended invasive revascularization options for patients with CHD on the price of some short and long-term clinical risks. Other methods aiming at angiogenesis such as the application of isolated or purified stem and progenitor cells are still under investigation [2,3].

A previous research demonstrated that transient ischemic stimulation of remote organs, such as the kidney, mesentery and skeletal muscles can induce cardiac protection during myocardial ischemia [4–6]. Physiological ischemic training (PIT) is a non-pharmacological and non-myocardial ischemia mediated intervention exploiting this phenomenon. PIT features an isometric contraction training of normal skeletal muscles which is supposed to trigger a physiological, i.e. functional non-pathological process improving angiogenesis in remote ischemic areas [7]. Endothelial progenitor cells (EPCs) may play an important role in this process. "EPCs are primitive bone marrow cells that have the capacity to proliferate, migrate, and differentiate into cells that line the lumen of blood vessels" [8]. EPCs can be recruited into the blood circulation by specific stimuli such as drugs, ischemia, or exercise. Once circulating, EPCs home in on target organs and participate in the maintenance of the endothelial cell layer [9]. Lambiase et al. demonstrated that patients with inadequate collateral circulation as compared to those with adequate collateral support tended to have less EPCs in vitro [10].

Vascular endothelial growth factor (VEGF) has been identified as one of the most important contributors to regulating the proliferation,

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http://dx.doi.org/10.1016/j.ijcard.2014.10.034
0167-5273/

mobilization, and differentiation of EPCs [11]. Adams and colleagues found that a short episode of myocardial ischemia in CHD patients was sufficient to induce a considerable increase in the number of circulating EPCs and that this increase seemed to be related to the expression of plasma VEGF [12]. A previous research demonstrated that intermittent exercise at ischemia threshold intension could safely promote coronary collateral formation and increased VEGF expression in a porcine model [13]. However, the potential role of PIT on VEGF, EPC and myocardial angiogenesis has not yet been investigated.

This animal experiment investigates the above described mechanism from the genetic, molecular and cellular up to the tissue level, particularly focusing on the potential role of VEGF and EPCs in remote PIT-induced revascularization of the myocardium.

2. Methods

2.1. Model of controlled myocardial ischemia

Experimental models of myocardial ischemia were successfully established in 45 male New Zealand White rabbits (2.1–2.5 kg; aged 3 months). We simulated cardiac disease by experimentally inducing myocardial ischemia through the following surgical procedure. All rabbits were anesthetized with 3% (by volume) sodium pentobarbital (1 ml/kg of body weight) injected into the marginal ear vein. Supplemental anesthesia was provided when necessary. Postoperatively, all rabbits were closely monitored and given 4×10^5 units of penicillin (intramuscular) for three days. Under sterile surgical conditions, the heart was exposed through an incision in the pericardium and a water balloon constrictor was placed around the left ventricular branch of the circumflex artery, 0.5–1 cm below the left atrial appendage and secured with a 5-0 silk suture. To produce complete occlusion of the vessel, 0.1–0.3 ml of water was injected into the balloon. The dose was dependent on a limb lead electrocardiogram (ECG) response. An ST segment depression or elevation by 0.1 mV was considered sufficient. Initially, we injected 2 ml methylene blue into the rabbits' femoral artery at the same time to confirm that the ischemic region of the myocardium was colored red and the non-ischemic region blue indicating complete occlusion of the circumflex coronary artery. The size of the ischemic region was about 0.5 cm by 0.5 cm on average.

Ischemia was maintained for 2 min, after which the water was drained from the balloon, followed by 10 min reperfusion. This procedure was started one week after the implantation of the balloon and for the next four weeks it was repeated twice per day, five days per week. Further ECG monitoring was performed twice per week to confirm that the balloon was in the correct position [14–16]. This procedure has been used elsewhere and was shown to produce constant ischemia [16]. The rabbits were sacrificed at the study endpoint to obtain tissue samples from the myocardium.

2.2. Remote physiological ischemic training protocol

A blood pressure cuff was placed around both hind limbs of the rabbits. The cuff was inflated to 200 mm Hg until the femoral artery pulse disappeared for 3 min. In the experiments randomized to myocardial ischemia and PIT, after two cycles of 2 min myocardial ischemia and 10 min of myocardial reperfusion, PIT was applied for three times with 5 min hind limb reperfusion intervals. The procedure was performed five days per week, over a period of 4 weeks [16].

2.3. Randomization and experimental groups

The rabbits were randomized into six groups. 1) The sham-operated group (SO, n = 6) was implanted a balloon constrictor but no water was injected. This group also received no intervention. 2) The training only group (TO, n = 6) received physiological ischemic training on the hind limbs only and had not been exposed to myocardial ischemia. 3) The myocardial ischemia group (MI, n = 9) had myocardial ischemia only. 4) The physiological ischemic training group (PIT, n = 9) had been exposed to myocardial ischemia and received physiological ischemic training on the hind limbs. 5) The atorvastatin group (PIT+, n = 7) had myocardial ischemia and received PIT as well as atorvastatin (10 mg/kg/day), a promoter of EPCs, by oral feeding [17]. 6) The rapamycin group (PIT−, n = 8) had myocardial ischemia, and received PIT as well as rapamycin (0.5 mg/kg/day), an EPC inhibitor by oral feeding [18]. Three animals died during the training period: one in the MI group due to ventricular fibrillation, one in the PIT due to pericarditis, and one in the PIT− group due to diarrhea.

All animal experimental protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health, United States of America) and were approved by the Ethics Committees of the Nanjing Medical University and the Jiangsu Province Hospital. The experimental protocol is given in Fig. 1.

2.4. Plasma and tissue measurements of VEGF-mRNA

Total RNA was isolated from the plasma and left ventricular ischemic myocardial tissue samples. The expression of plasma and tissue VEGF-mRNA was analyzed by real-time reverse-transcription (RT) polymerase chain reaction (PCR) with the specific primers listed in Table 1.

2.5. Plasma and tissue measurements of VEGF-protein

Plasma expression of VEGF-protein was measured with a highly sensitive rabbit VEGF enzyme-linked immune sorbent assay (ELISA; obtained from BPB Biomedical, USA). Results were compared with standard curves and each measurement was duplicated.

VEGF-protein expression in homogenates of the left ventricular ischemic myocardium was quantified by Western blotting. Antibodies against VEGF (Santa Cruz Biotechnology, USA) were used afterwards. Beta-actin (Santa Cruz Biotechnology, USA) was used as the loading control. Relative VEGF expression levels were quantified with the Gel-Doc2000 system (Bio-Rad Laboratories, USA) after densitometric scanning of the exposed films.

2.6. Plasma and myocardium measurements of EPCs

At the study endpoint, 5 ml blood from the marginal veins of the ear and 100 mg left ventricular ischemic myocardium tissue samples were obtained and frozen at −70 °C. The number of EPCs was analyzed by fluorescence-activated cell sorting (FACS) with the following mouse antibodies as rabbit antibodies were not available at the time of the study: PerCP-conjugated anti-mouse CD45 (BD Biosciences, USA), PE-conjugated anti-mouse CD34 (BD Biosciences, USA) and APC-conjugated anti-mouse CD309 (BD Biosciences, USA). The variability of FACS measurements was less than 0.5% for single-positive events and less than 1% for double-positive events.

2.7. Measurement of capillary density

Tissue samples from the myocardium supplied by the left ventricular branch were collected and stored in 4% neutral formaldehyde for immunohistochemistry, which was performed using CD31 mouse-antibodies (Santa Cruz Biotechnology, USA). A commercial kit (ABC Vector, USA) was used to identify endothelial cells. A capillary was defined as a brown round structure with a central lumen and a diameter less than 10 μm under light microscopy (magnification factor of 200). The CD count was the mean of observations in five random fields of each slice.

2.8. Measurement of coronary collateral blood flow

Dye-Trak microspheres (Triton Technology Company, USA) were injected into the left atrium before and after myocardial ischemia at baseline and the study endpoint. Caution was taken to harvest only the portion of the left ventricular tissue served by the left ventricular branch. Each sample was weighed, cut into small pieces, decayed and digested in 1 mol/l KOH at 60 °C overnight with periodic shaking [13]. Microspheres were measured with a spectrophotometer (Ultrospec2000, Pharmacia Biotech Co., USA). The number of microspheres was calculated from their absorbance using a matrix inversion technique supplied by Triton Technology [19]. The number of microspheres before and after occlusion represented coronary blood flow (CBF) and coronary collateral blood flow (CCBF), respectively. To account for baseline differences in CBF, the relation of CCBF to CBF was calculated using the following equation: (CCBF / CBF) × 100 = blood flow after occlusion / blood flow before occlusion in percent with 100 indicating a complete restoration [20,21].

2.9. Statistical analysis

Data on VEGF, EPCs and change in CCBF were not normally distributed. We thus used non-parametric methods to test for group differences. First, we calculated a non-parametric test for trend across the ordered groups after Cuzick [22], according to our hypothesis that outcomes were lowest in the PIT− group followed by SO, TO, MI, PIT, and finally PIT+ group. Second, we performed Wilcoxon-rank sum tests to determine differences between individual groups. We used Benjamini and Hochberg's method to correct for alpha error accumulation due to multiple testing. This method is less conservative than Bonferroni's method and appropriate since we have multiple groups and non-parametric testing already comes at a loss of statistical power. Benjamini and Hochberg's method defines an upper confidence bound for the false discovery rate, i.e. the minimal confidence level we are willing to accept in order to assume that a rejected null hypothesis is really false. Based on this confidence bound, a corrected critical p-value (smaller than 0.05) is calculated. A null-hypothesis is rejected if and only if the corresponding p-value is smaller than the corrected critical p-value [23]. To visualize the data we used Box–Whisker plots. Whiskers were defined as the highest or lowest value that is less than 1.5 times the upper quartile or less than −1.5 times the lower quartile, respectively. Outliers were defined as any values outside the whiskers.

To model the supposed mechanism we performed regressions of EPC numbers on VEGF, CD on EPC numbers, and changes in CCBF and CCBF/CBF. We used third degree fractional polynomials to account for potential non-linear relationships [24]. All possible functions using a predefined set of powers (−2, −1, −0.5, 0, 0.5, 1, 2, 3; with 0 representing the logarithmic transformation) are compared regarding their fit in predicting the outcome. Repeated powers are possible. Simple models are preferred. More complicated functions are accepted only if they fit far better. We report p-values of beta coefficients and percentage of variance explained through these regression models, i.e. r-squared.

All analyses were performed with Stata13 (Stata corporation, USA). Data were analyzed by JR who was blinded to the experiments.