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Original article

Ischemic preconditioning-mediated cardioprotection is disrupted in heterozygous Flt-1 (VEGFR-1) knockout mice

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Abstract

This study attempts to address an important clinical issue by identifying potential candidates of VEGF signaling through Flt-1 receptor that trigger angiogenic signal under ischemic stress. To determine the significance of VEGF-Flt-1 (VEGFR1) signaling in ischemic preconditioned (PC) myocardium, we used heterozygous Flt-1 knockout (KO) mice to dissect the pathway and identify candidate genes involved in VEGF signaling. DNA microarrays were employed to detect, characterize and distinguish altered myocardial gene expression by comparing between wild type (WT) CD-1 and heterozygous Flt-1 KO mice when exposed to ischemia (30 min) and reperfusion (2 h). Moreover, KO mice demonstrated reduced beneficial effects of PC when compared to the WT with PC. In the KO and WT mice, the % recovery of the left ventricular developed pressure and the maximum first derivative of the developed pressure after ischemia/reperfusion without PC were similar. However, when animals were subjected to PC, the left ventricular functional recovery throughout the reperfusion period was significantly lower in KO mice than in WT mice. These results indicate for the first time that in the heterozygous Flt-1 KO mice, PC is not as effective as that found in WT. This observation may be due to downregulation of several important genes such as growth-regulated oncogene 1 (Gro1), heat shock proteins (HSP), I kappa B kinase β (IKKβ), colony-stimulating factor-1 (CSF-1) and annexin A7, suggesting the importance of VEGF-Flt-1 receptor signaling during PC.

Keywords: Ischemia; Reperfusion; VEGFR-1/Flt-1; Cardioprotection; Microarray; Gene expression; Gene knockout

1. Introduction

The process of angiogenesis is regulated by signals from the transmembrane receptor tyrosine kinases (RTKs) and non-RTKs on endothelial cells. Flk-1/KDR and Flt-1 are two such RTKs, which together with their ligand VEGF, control blood vessel development during embryogenesis [1,2]. This receptor–ligand system augments neovascularization [3–6]. Studies in a rat myocardial infarction (MI) model have indicated rapid induction of VEGF (275%), Flk-1 (375%), and Flt-1 (400%) mRNA expression throughout the entire heart after infarction [6]. Although attention has focused on studies of VEGF expression and its function in myocardial ischemia/hypoxia [7–12], relatively little is known regarding the VEGF receptors, Flk-1 and Flt-1.

Functional differences between Flk-1 and Flt-1 have been reported in endothelial cells [6]. Genetically manipulated homozygous Flk-1 knockout (KO) are subjected to early embryonic death due to inhibition of vasculogenesis, whereas, homozygous Flt-1 KO mice fail to form vascular channels [1,2]. In addition, staining intensity for both receptors increased significantly in the hypoxia/reoxygenation group

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compared to the corresponding normoxic control group [11]. In a systemic whole body hypoxic rat model followed by 24 h of reoxygenation, we found that the expression of Flk-1 after 1 h hypoxic treatment was significant and remained elevated even after 2 h of hypoxia, but was reduced after 4 h of hypoxia. These data suggest a role for Flk-1 in the initial rather than later stages of the early angiogenic process. In contrast, Flt-1 expression increased after 1 h and remained elevated after 4 h of treatment, indicating a more continuous role in the early angiogenic process [13]. The mammalian heart can adapt to ischemia after repeated exposure to short-term reversible ischemia followed by short durations of reperfusion (this phenomenon is called ischemic preconditioning, PC). We have concluded that ischemic preconditioning-mediated VEGF expression is cardioprotective because of signaling via its Flt-1 and Flk-1 receptors [14].

There is little information available regarding physiologic control of angiogenesis by VEGF receptors during myocardial protection processes. Our goal is to document the candidate genes involved in VEGF signaling through Flk-1 and Flt-1 receptors in PC-mediated angiogenesis in the MI model. In this report, using a mouse with targeted KO of the Flt-1 gene, we examine whether the cardioprotective effect of PC is due to the VEGF-specific tyrosine kinase receptor, Flt-1. Inactivation of the functional Flt-1 gene results in both impaired postischemic left ventricular functional recovery and increased infarct size after PC in heterozygous Flt-1 KO mice compared with wild type (WT) mice, suggesting that heterozygous Flt-1 KO mice are unable to precondition the myocardium to the same degree as do WT mice. When subjected to ischemia/reperfusion, heterozygous Flt-1 KO mice also have significant alteration in expression of a number of genes as determined by DNA microarray analysis.

2. Materials and methods

2.1. Experimental animals

All animals received care in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH). The heterozygous Flt-1 KO mice were generated as previously described [1] and maintained on a CD-1 background. Briefly, the first exon and splice donor site were replaced by a cassette containing neomycin phosphotransferase and the *Escherichia coli* Lac Z gene, resulting in disruption of Flt-1 coding and expression of beta-galactosidase [13]. Flt-$1^{+/-}$ mice were identified by beta-galactosidase staining and age- and sex-matched CD-1 littermates were used as WT control. We have used heterozygous Flt-1 KO mice since the life span of Flt-1 homozygous KO mice is only 8 embryonic days.

2.2. Langendorff preparation

Isolated heart perfusion was performed as previously described [15]. The CD-1 (WT) mice and $Flt^{+/-}$ (KO) mice, weighing 30–35 g, were anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg). The heart was excised rapidly, and the aorta was cannulated and perfused at a constant pressure of 65 mmHg with gassed (5% CO_2, 95% O_2) Krebs–Hensleit buffer. A fluid-filled polyethylene balloon was inserted into the left ventricle and inflated to an end-diastolic pressure (LVEDP) of 8–10 mmHg. Left ventricular developed pressure (LVDP = LVSP – LVEDP), the maximal value of LVdp/dtmax, heart rate (HR) and coronary flow (CF) rate were recorded.

2.3. Experimental protocol

CD-1 (WT) mice and Flt-$1^{+/-}$ (KO) mice were both randomized into two groups. For Group I (WT), after a 20-min stabilization, hearts were perfused for 60 min, followed by exposure to zero-flow normothermic global ischemia for 30 min followed by 120 min of reperfusion (I/R) and, for Group II with ischemic preconditioning (WT + PC), after stabilization, hearts were subjected to four episodes of 5-min global ischemia followed by 10-min reperfusion before I/R. For Group III (KO), hearts were perfused for 60 min before I/R and for Group IV (KO + PC), hearts were subjected to the same protocol as WT + PC. Six animals per group were used for functional analysis and estimation of infarct size, and three animals per group for microarray analysis and real-time reverse-transcription polymerase chain reaction (RT-PCR).

2.4. Infarct size

The infarct size was measured as previously described [15]. After reperfusion; hearts were immediately perfused with 1% triphenyltetrazolium chloride. The area of infarcted (unstained) and viable (stained) tissue were measured by computed planimetry.

2.5. RT-PCR analysis for Flt-1, Flk-1 and VEGF

cDNAs were amplified in PCR buffer containing 1 unit Taq DNA polymerase, 200 μM dNTPs and 50 pmol Flt-1 sense (GGC CCA GCT TCT GCT TCT CCA GCT GT) and antisense (GGT CAC GAA AAG CAG CTG GCT CCT GT), 50 pmol Flk-1 sense (GGG ACC TGG CAG CAC GAA ACA) and antisense (ACC GAA AGA CCA CAC ATC GCT C), or 10 pmol of VEGF-sense (TTC TGC TCT CTT GGG TGC ACT GGA C) and antisense (CGC CTT GGC TTG TCA CAT CTG CA), and for internal control β-actin sense (CAA TAG TGA TGA CCT GGC CGT) and antisense (AGA GGG AAA TCG TGC GTG AC) primers. Amplification for Flt-1 and Flk-1 was for 35 cycles and for VEGF, 30 cycles.

2.6. Quantitative real-time RT-PCR

RT was performed on 10 μg total RNA isolated from left ventricular tissue of WT and Flt-$1^{+/-}$ heterozygous KO mice subjected to I/R with or without PC. Real-time RT-PCR analy-