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Impaired border zone formation and adverse remodeling after reperfused myocardial infarction in cannabinoid CB2 receptor deficient mice

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ABSTRACT

Aims: Reperfusion of myocardial infarction is associated with inflammatory reaction and subsequent myocardial remodeling with a rapid scar formation in mice. The cannabinoid receptor CB2 has been associated with cardioprotection and regulation of macrophage function. We investigated its role in remodeling of reperfused infarction.

Main methods: One hour LAD-occlusion was followed by reperfusion over 6 h and 1, 3 and 7 days in wild-type C57/BL6J (WT) and CB2 receptor-deficient ($Cnr2^{-/-}$) mice (n = 8/group). Hearts were processed for functional, morphological and mRNA/protein analysis, and tissue concentration of endocannabinoids was determined using liquid chromatography-multiple reaction monitoring.

Key findings: In contrast to a rapid formation of granulation tissue and a compacted non-transmural scar in WT mice after 7 days of reperfusion, $Cnr2^{-/-}$ mice showed a non-compacted transmural scar. Millar® left ventricular catheter measurements revealed a significantly worse function in $Cnr2^{-/-}$ mice. We found no compensatory elevation of endocannabinoid concentration in $Cnr2^{-/-}$ hearts. Macrophage infiltration was significantly stronger in $Cnr2^{-/-}$ hearts and affected also the remote septum, when compared to WT hearts. We found a cytokine-driven inflammatory response in $Cnr2^{-/-}$ hearts with no significant induction of chemokines. Immunohistochemistry for thrombospondin-1 revealed a dysfunctional infarction border zone formation in $Cnr2^{-/-}$ hearts. $Cnr2^{-/-}$ hearts showed no significant induction of tenascin C, collagen-Iα or lysil oxidase, thereby indicating adverse myocardial remodeling.

Significance: Endocannabinoids act *via* CB2 receptor in the modulation of inflammatory response and myocardial remodeling after infarction. CB2 receptor plays an important role in the formation of infarction border zone, collagen deposition and organization of stable scar during remodeling.

1. Introduction

Ischemic heart disease may cause myocardial infarction with substantial morphological and functional changes in the heart [1]. Natural course of myocardial infarction is associated with a rapid deterioration of left ventricular function and development of terminal heart failure. The timely onset of the appropriate clinical therapy is therefore crucial for the long-term prognosis. Clinical and experimental studies described myocardial infarct healing as a dynamic biological process initiated by acute inflammatory response and followed by the formation of granulation tissue resulting in a stable scar [2]. Induction of cytokines and chemokines is a prominent feature of inflammatory response after reperfusion and plays an important role in inflammatory leukocyte recruitment [3]. Using a murine model of reperfused infarction we showed a crucial role for the chemokine CCL2 in granulation tissue formation and development of a non-transmural scar [4]. Also, we demonstrated that thrombospondin-1 expression in the infarct border zone might serve as a barrier limiting expansion of granulation tissue and protecting the non-infarcted myocardium from fibrotic

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remodeling [5]. Therefore, we aim at identifying factors modulating inflammatory response and scar formation for potential therapeutic interventions.

The role of the endocannabinoid system in homeostasis and pathology has been established in many organs and systems [6]. An experimental study postulated an anti-fibrotic role for the CB2 receptor in a model of liver fibrosis [7]. A pharmacological study using low-flow ischemia on isolated rat hearts in Langendorff apparatus supports cardioprotective effects of endocannabinoids acting on CB2 receptor [8]. Another study reported a significantly larger infarct size in Cnr2$^{-/-}$ mice after 24 h reperfusion of a myocardial infarction, which went along with a minor ventricular dysfunction after 4 weeks, while they reported recovery to normal left ventricular function in wild type mice [9]. They reported increased apoptosis of cardiomyocytes and higher macrophage density after 3 days of reperfusion in Cnr2$^{-/-}$ mice, as well as higher potential for the differentiation of Cnr2$^{-/-}$ myofibroblasts *in vitro*. Our recent work elaborated the role of CB2 receptor in a model of non-infarcted ischemic cardiomyopathy and revealed the loss of cardiomyocytes with the formation of microinfarctions in Cnr2$^{-/-}$ hearts [10]. We demonstrated a CB2 receptor-mediated mechanism in the regulation of antioxidative enzymes, expression of contractile elements in cardiomyocytes and differentiation of macrophage subtype M2a, leading to the resolution of inflammatory response. These and other experimental studies provided evidence for a cardioprotective role of CB2 receptor and suggested its involvement in myocardial remodeling after infarction. We aimed at further deciphering the CB2 receptor-related mechanisms involved in the modulation of inflammatory response, granulation tissue formation and myocardial scar development.

Here, we provide novel evidence for the role of endocannabinoids acting *via* CB2 receptor in the formation of infarction border zone and collagen deposition, which substantially influences myocardial remodeling after infarction in mice.

2. Material and methods

2.1. Study animals

All mouse experiments were performed with 18–25 g and 8–12 weeks old mice in accordance to the animal protocol approved by the local governmental authorities and according to the EU Directive 2010/63/EU for animal research. We used both male and female mice in an equal distribution for all experiments. Wild-type (WT-) C57BL/6J mice (Charles River, Sulzfeld, Germany), homozygote CB2 receptor-deficient Cnr2$^{-/-}$ mice were used, as published previously [11]. All mice were sacrificed by cervical dislocation.

2.2. Mouse model of reperfused myocardial infarction

Anesthesia was induced with 3% isoflurane (Forene®, Abbott) and maintained with 0.8% isoflurane and 100% O_2. For initial surgery, left parasternal thoracotomy was performed and an 8-0 Prolene suture (Ethicon, Norderstedt, Germany) was placed around the left descending coronary artery (LAD). Suture ends were threaded through a sterile PE-10 tube (Becton Dickinson, Franklin Lakes, NJ, USA), exteriorized through the thoracic wall and then stored subcutaneously in order to avoid systemic inflammatory effects of surgical trauma during ischemia protocol. After chest closure, metamizol (100 mg/kg; Novalgin) was given for analgesia in a mixture with cefuroxim for antibiotic prophylaxis i.p. (100 mg/kg, Zinacef; Bristol-Myers Squibb, Munich, Germany). Mice were allowed to recover for 7–10 days from initial surgery intervention.

Myocardial infarction was induced under isoflurane anesthesia as described above. Therefore ligature ends were connected to metal picks and LAD occlusion for 60 min was achieved by pulling the picks apart as described before [3]. Myocardial ischemia was confirmed by continuous visualization of ST segment elevation in EKG lead II of Einthoven during the LAD occlusion procedure. Coronary artery occlusion was followed by reperfusion for 6 h, 1, 3, or 7 days.

After reperfusion the hearts were excised, dissected from the atria and great vessels and subsequently washed in ice-cold cardioplegic solution in order to rinse out blood and to allow the relaxation of the myocardium. Afterwards the hearts were fixated for 24 h in zinc-paraformaldehyde (Z-fix, 4%; Anatech, Battle Creek, MI, USA) for histology, quick-frozen using liquid nitrogen and stored at −80 °C for endocannabinoid measurements or stored in RNA-later (Qiagen, Hilden, Germany) for gene expression analysis.

2.3. Hemodynamic parameters measured with Millar® pressure–volume catheter

Hemodynamic parameters were measured with a Millar® pressure/volume catheter (Millar® Instruments, Houston, TX, USA) [12,13] after 1 h of LAD occlusion and 7 days of reperfusion. Anesthesia was induced with isoflurane as described above. A 1.4-Fr PV-catheter was inserted through the right carotid artery into the left ventricle across the aortic valve. After the stabilization of hemodynamic parameters, pressure volume loops were recorded while anesthesia was maintained with 0.7 to 0.8 vol.% isoflurane. All pressure–volume loop data were analyzed using a cardiac pressure–volume analysis program (PVAN3.5, Millar Instruments) and a minimum of 15 representative loops.

2.4. Histology and immunohistochemistry

Fixated hearts were embedded in paraffin and at 250 μm intervals, a block of 10 × 5 μm sections was mounted on glass slides [14]. Only sections below the papillary muscles were further used for staining in order to guarantee comparative analysis of infarcted areas within different mice. Basic histological evaluation using hematoxylin and eosin as well as quantitative planimetric analysis of collagen stained area was performed as previously published [15].

Antigen-specific immunohistochemical staining using primary antibodies was performed using Vectastain Elite ABC kits and diaminobenzidine (AXXORA, Lörrach, Germany). M.O.M. immunodetection kit (AXXORA) was used for mouse-derived antibodies. Cell density was calculated using cell count of specific antibody staining. M.O.M. immunodetection kit (AXXORA) was used for mouse-derived antibodies. The following primary antibodies were used: alpha smooth muscle actin (α-smac) mouse anti-mouse monoclonal antibody (clone 1A4; Sigma-Aldrich, St. Louis, MO, USA), galectin-3 (MAC-2) rat anti-mouse monoclonal antibody (clone 3/38, AXXORA), MCA 771G rat anti-mouse monoclonal antibody (clone 7/4, Serotec, Kidlington, UK), and thrombospondin (TSP-1) mouse anti-mouse monoclonal antibody (clone A6.1, Thermo-Fisher Scientific, Schwerte, Germany).

All images from immunological staining were taken with the Olympus BX 41 microscope in combination with the DP70 camera and analyzed planimetrically using analySIS® software (all from Olympus, Hamburg, Germany) [16].

2.5. Endocannabinoid quantification by liquid chromatography-multiple reaction monitoring

2.5.1. Chemicals and standard solutions

Anandamide (AEA), 2-arachidonoyl glycerol (2-AG), arachidonic acid (AA), and their deuterated analogues AEA-d4, 2-AG-d5, and AA-d8 were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Water, acetonitrile (ACN), formic acid (FA), ethylacetate, and hexane (all from Fluka LC-MS grade) were obtained from Sigma-Aldrich.

2.5.2. Endocannabinoid extraction

For eCB extraction heart tissues were first transferred to extraction tubes containing cold steel beads. Spiking solution of deuterated eCBs