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Original article

# Atheroprotection via cannabinoid receptor-2 is mediated by circulating and vascular cells in vivo

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ABSTRACT

Low-dose oral tetrahydrocannabinol (THC) reduces progression of atherosclerosis in mice. THC activates central cannabinoid-1 receptors (CB1) with subsequent psychoactive effects as well as peripheral cannabinoid-2 receptors (CB2). In order to dissect the underlying mechanisms, we performed experiments under selective CB2 stimulation as well as after genetic disruption of the CB2 receptor. Atherosclerosis prone apolipoprotein E-deficient mice were crossed with cannabinoid receptor-2 deficient mice to obtain ApoE −/− CB2 −/− double knockout mice. After 8 weeks of a high-cholesterol diet, immunohistochemical stainings of the aortic root revealed that vascular leukocyte infiltration in atherosclerotic plaques was accelerated in ApoE −/− CB2 −/− mice compared with ApoE −/− mice. This was accompanied by increased release of reactive oxygen species as measured using L012-enhanced chemiluminescence, and by decreased endothelial function as assessed in isolated aortic rings in organ chamber experiments. ApoE −/− mice treated with the selective CB2 agonist JWH 133 during a high-cholesterol diet showed decreased atherosclerotic lesion formation, improved endothelial function and reduced levels of reactive oxygen species. To assess whether CB2 expression in circulating cells influences atherosclerosis, irradiated ApoE −/− mice were repopulated with bone marrow-derived cells from ApoE −/− and ApoE −/− CB2 −/− mice and were fed a high-cholesterol diet for 8 weeks. CB2 deficiency in bone marrow-derived cells increased leukocyte infiltration into the vessel wall, but had no impact on plaque formation. Cell culture experiments revealed that CB2 activation diminishes ROS generation in vascular cells. Selective CB2 receptor stimulation modulates atherogenesis via impact on both circulating proinflammatory and vascular cells.



## 1. Introduction

Atherosclerosis is a chronic inflammatory disease and represents the primary cause of heart failure and death in the western world [1]. In addition to the established therapy, new efficient therapeutic substances are urgently required, but the development and discovery are challenging and an arduous task. Recently, it has been suggested that low dose oral cannabinoid therapy with tetrahydrocannabinol (THC) reduces the progression of atherosclerosis in mice [2]. It has been posited that this effect may be based on anti inflammatory properties due to the stimulation of peripheral CB2 receptors, but direct evidence for this mechanism is lacking [2]. To date, it remains unclear to what extent vascular cells, such as endothelial cells and smooth muscle cells, contribute to CB2-mediated vasculoprotection. Effects of cannabinoids on inflammation and proinflammatory circulating cells have been described [3,4]. THC simultaneously activates central CB1 receptors with subsequent psychoactive effects that limit its therapeutic potential. The CB1 receptor is primarily expressed in neurons in the central nervous system whereas the CB2 receptor has primarily been found on peripheral immune cells [5,6]. Both receptors are heterotrimeric GTP-binding protein-coupled receptors, which are not only activated by the cannabinoid THC but also by endogenous ligands such as 2-arachidonoylglycerol (2-AG) and arachidonylethanolamide (AEA or anandamide) [7]. Moreover, several synthetical substances have been characterized that either activate both CB receptors or activate selectively either subtype 1 or 2. In particular, substances selectively targeting the peripheral CB2 receptor such as JWH 133 and HU 308 are of high interest as they are devoid of neurobehavioral adverse effects but exert potentially beneficial anti inflammatory effects [8]. In this context it has been shown that JWH 133 attenuates autoimmune uveoretinitis in a rodent model by provoking the suppression of antigen presentation through TLR4 signaling down-regulation and inhibition of leukocyte trafficking [9]. Activation of the CB2 receptor suppressed autoimmunity and inflammation in various animal

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**Table 1**
Cholesterin, blood pressure, and heart rate.

| | ApoE −/− | ApoE −/− CB2 −/− | ApoE −/− + JWH 133 | ApoE −/− + vehicle |
|---|---|---|---|---|
| Total cholesterol, mg/dl | 1155 ± 248 | 1005 ± 327 | 1137 ± 180 | 1184 ± 406 |
| SBP, mm Hg | 112 ± 22 | 110 ± 19 | 111 ± 17 | 116 ± 24 |
| Heart rate, bpm | 688 ± 175 | 709 ± 144 | 655 ± 139 | 709 ± 188 |

Total cholesterol, systolic blood pressure and heart rate were determined in the animals groups after a high-fat diet containing 1.25% cholesterol. Values are mean ± SEM.

models including collagen-induced arthritis, experimental autoimmune encephalomyelitis, and multiple sclerosis [10–12]. Until now, the approach of selective CB2 stimulation has not been considered in the context of atherosclerosis. Thus, we tested whether genetic deletion or selective stimulation of CB2 influences progression of atherosclerosis in mice.

## 2. Methods

A detailed description of the applied methods is available in the data supplement.

### 2.1. Animals and procedures

Wildtype mice (C57BL/6J; Charles River, Wilmington, USA), ApoE −/− mice (C57BL/6J genetic background; Charles River, Wilmington, USA), and age-matched ApoE −/− CB2 −/− mice (C57BL/6J genetic background; generated by Molecular Psychiatry, University of Bonn, Germany) were used for this study. All mice were fed a high-fat, cholesterol-rich diet for 8 weeks that contained 21% fat, 19.5% casein, and 1.25% cholesterol (Ssniff), starting at the age of 12 weeks. Several groups of mice were established. First, ApoE −/− mice and ApoE −/− CB2 −/− mice were fed the high-cholesterol diet to dissect the role of genetic disruption of the CB2 receptor on the course of atherosclerosis. Second, ApoE −/− mice treated with JWH 133 10 mg/kg body weight every second day by intraperitoneal injection (i.p.) were fed the high-cholesterol diet and compared with ApoE −/− mice treated with vehicle (Tocrisolve, Tocris Bioscience, Bristol, UK). Third, ApoE −/− mice and ApoE −/− CB2 −/− mice were treated with JWH 133 (Tocris Bioscience, Bristol, UK) 10 mg/kg body weight every second day by intraperitoneal injection to confirm that the observed effects were mediated through the CB2 receptor. Finally, ApoE −/− mice were lethally irradiated and transplanted with ApoE −/− CB2 −/− or ApoE −/− bone marrow. After the treatment period, mice were sacrificed and tissue samples and blood were collected immediately.

## 3. Results

### 3.1. Cholesterol and blood pressure

Mice were fed a high-cholesterol diet for 8 weeks. Serum cholesterol levels, blood pressure, and heart rate are depicted in Table 1. There were no significant differences between the groups.

### 3.2. Reactive oxygen species

Reactive oxygen species (ROS) were measured by L012 chemiluminescence assays in intact aortic segments of wildtype, ApoE −/−, ApoE −/− CB2 −/−, and in ApoE −/− mice treated either with JWH 133 or vehicle. Fig. 1A shows that superoxide release was increased 2-fold in ApoE −/− CB2 −/− mice compared with ApoE −/− mice (481 ± 71 vs. 224 ± 29, P<0.05). Treatment of ApoE −/− mice with the selective CB2 agonist JWH 133 reduced ROS release to control levels of wildtype animals (210 ± 47 vs. 84 ± 28, P<0.05) (Fig. 1B). Fig. 1C shows that treatment of ApoE −/− CB2 −/− mice with JWH 133 did not influence the superoxide generation whereas ApoE −/− mice treated with JWH 133 displayed reduced levels of superoxide release (361 ± 94 vs. 133 ± 22, P<0.05) demonstrating CB2 receptor specificity of the observed effect.

### 3.3. Endothelial function

Vascular function was measured in isolated intact aortic rings. First, ApoE −/− mice and ApoE −/− CB2 −/− mice were compared showing a significantly impaired endothelial-dependent vasorelaxation compared to wildtype mice (P<0.05). Endothelial-dependent vasorelaxation was already almost completely abolished in ApoE −/− mice, ApoE −/− CB2 −/− mice displayed therefore no further decline (Fig. 2A). Endothelial-independent vasorelaxation assessed after stimulation with nitroglycerine was comparable between all groups (Fig. 2B). In a second experimental set-up, ApoE −/− mice were treated with either the selective CB2 agonist JWH 133 or vehicle. Endothelial-dependent vasorelaxation was significantly improved in ApoE −/− mice treated with JWH 133 (P<0.05) compared with ApoE −/− mice that received vehicle (Fig. 2C). Endothelial-independent vasorelaxation was not different between both groups (Fig. 2D). In order to confirm specificity of the used models, endothelial function was compared in ApoE −/− mice and ApoE −/− CB2 −/− mice both treated with the CB2 agonist (Fig. 2E). Whereas JWH 133 improved endothelial dependent vasorelaxation in ApoE −/− mice, the compound had no effect in ApoE −/− CB2 −/− mice. Endothelial-independent vasorelaxation did not reveal significant differences (Fig. 2F).

### 3.4. Atherosclerosis development

Atherosclerotic lesions were detected after 8 weeks of a high-fat, cholesterol-rich diet by means of oil red O staining and subsequent quantification. ApoE −/− mice developed large plaques in the aortic sinus. In age matched ApoE −/− CB2 −/− mice, atherosclerotic lesion formation tended to be enhanced compared with ApoE −/− mice, but the effect did not reach statistical significance (ratio: 0.55 ± 0.04 vs. 0.50 ± 0.02, P>0.05) (Fig. 3A) (lesion size: 0.254 ± 0.042 $mm^2$ vs. 0.201 ± 0.019 $mm^2$, P>0.05) (Supplementary Fig. 7A). This is probably related to the already extensive disease at the time of investigation. In a second approach, the influence of selective CB2 stimulation on vascular plaque size in ApoE −/− mice was investigated (Fig. 3B). Treatment with JWH 133 significantly reduced atherosclerotic lesion formation in ApoE −/− mice (ratio: 0.40 ± 0.03 vs. 0.51 ± 0.02, p<0.05) (lesion size: 0.137 ± 0.014 $mm^2$ vs. 0.203 ± 0.023 $mm^2$, P<0.05) (Supplementary Fig. 7B). Interestingly, treatment with JWH 133 did not impact atherogenesis in ApoE −/− CB2 −/− mice (ratio: 0.52 ± 0.03 vs. 0.48 ± 0.03, P>0.05) indicating a CB2 receptor specific effect (Fig. 3C and Supplementary Fig. 7C).

Leukocyte recruitment into the vessel wall contributes to vascular inflammation in atherosclerosis. Using MOMA-2 staining, we assessed vascular accumulation of monocytes and macrophages. Wildtype mice showed no macrophage infiltration into the vessel wall. As expected, ApoE −/− mice displayed severe leukocyte infiltration (Fig. 4A). In ApoE −/− CB2 −/− mice macrophage recruitment was even more intense (0.40 ± 0.04 vs. 0.53 ± 0.03, P<0.05). After treatment of ApoE −/− mice with JWH 133 recruitment of MOMA-2 positive cells was reduced if compared with ApoE −/−