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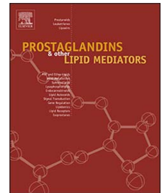
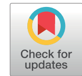


Original Research Article

Control of myogenic tone and agonist induced contraction of intramural coronary resistance arterioles by cannabinoid type 1 receptors and endocannabinoids



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ABSTRACT

It was tested whether intrinsic CB_1R activation modifies myogenic and agonist induced contraction of intramural coronary resistance arteries of the rat. CB_1R protein was detected by immuno-histochemistry and by Western blot, its mRNA by qRT-PCR in their wall. Microsurgically prepared cylindrical coronary segments (~100–150 µm) developed myogenic contraction (~20% of relaxed luminal diameter), from which a substantial relaxation (~15%) in response to WIN55212 (a specific agonist of the CB_1Rs) has been found. CB_1R-mediated relaxation was blocked by O2050 and AM251 (neutral antagonist and inverse agonist of the CB_1R, respectively) and was partially blocked by the NO synthase blocker Nω-nitro-L-arginine. CB_1R blockade enhanced myogenic tone and augmented AngII-induced vasoconstriction (from 17.8 ± 1.2 to 29.1 ± 2.9%, $p < 0.05$). Inhibition of diacylglycerol lipase by tetrahydrolipstatin, (inhibitor of endogenous 2-AG production) also augmented coronary vasoconstriction. These observations prove that vascular endocannabinoids are significant negative modulators of the myogenic and agonist-induced tone of intramural coronary arterioles acting through CB_1Rs.

1. Introduction

Hemodynamic resistance of intramural coronary arterioles with diameters below 200 µm determines local ventricular flow. Such vessels have a substantial spontaneous/myogenic tone which is kept reduced by metabolic factors from surrounding ventricular tissue, beta adrenergic effects, endothelial nitric oxide (NO) and also by vasodilatory prostanoids produced in the wall. Compared to resting coronary vascular flow, during heavy physical exercise and also in hypoxia a 4–5 times increase in cardiac blood flow ("coronary vasomotion") can be achieved [1–7].

It has been revealed, that exogenous tetrahydro-cannabinol (THC) [8], anandamide, 2-arachidonoylglycerol (2-AG) [9,10], different natural and synthetic agonists of the CB_1R (e.g. WIN55212) [11–14] cause substantial vasodilation in several vascular beds, such as in coronary arteries [9–11], cerebral arteries [11], the mesenteric vascular bed [8] and in the aorta [14]. Several hemodynamically and clinically important resistance arteries also responded to cannabinoids [8,15]. Most vasodilatory and hypotensive actions of cannabinoids seem to involve the CB_1R receptors [11,15,16].

Endocannabinoids are produced in the wall of different vessels and by their vasodilatory actions they contribute to local vascular control [15–22]. However, the extent of this contribution can be much different in different vascular areas [18–24]. Exogenous cannabinoids increased coronary flow in isolated rodent hearts [25], dilated larger coronary arteries [9,10,17,26] and even cardioprotective and antiischemic effects [27–29] have been attributed to them. In an earlier work from our laboratory [16], we have demonstrated that continuous production of endocannabinoids (mostly 2-AG) in the wall of skeletal muscle resistance arterioles maintained a reduced spontaneous and agonist induced tone via vascular CB_1 receptors. Pharmacological control of coronary resistance arteries might be much different from that of

Abbreviations: Ach, acetylcholine; 2-AG, 2-arachidonoylglycerol; Ang II, angiotensin II; AT_1R, type 1 angiotensin receptor; BK, bradykinin; CB_1R, type 1 cannabinoid receptor; DAG, diacylglycerol; DAGL, diacylglycerol lipase; EC, endocannabinoid; ERK, extracellular signal-regulated kinase; HPLC, high performance liquid chromatography; KO, knockout; LAD, left anterior descending; LNA, Nω-nitro-L-arginine; MAG, monoacylglycerol; NO, nitric oxide; qRT-PCR, quantitative real-time PCR; PLC, phospholipase C; THC, Δ9-tetrahydro-cannabinol; THL, tetrahydrolipstatin; SNP, sodium-nitroprusside; VSMC, vascular smooth muscle cell

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resistance arteries in other vascular areas [3] and may even differ from larger coronary vessels [5]. Myogenic contraction and tone of resistance-sized arteries involves different agonist-induced and cellular mechanisms [30].

Taking into consideration the unique clinical significance of the coronary vascular system and the specific features of coronary resistance artery control it is of utmost importance to study whether endocannabinoid-mediated vasodilatory mechanisms do exist in coronary resistance arteries and to what extent they are able to modulate the myogenic and agonist induced coronary vascular tone.

2. Materials and methods

2.1. Animals

Male Wistar rats were used (250–350 g, Charles River Laboratories-Semmelweis University, Budapest). All animals were anaesthetized with pentobarbital sodium (Euthasol, ASTfarma 50 mg/kg intraperitoneally) and an extra dose (appr. 10 mg/kg) was additionally given, if necessary. Anesthetized animals were sacrificed by fast bleeding. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* (NIH, 8th edition, 2011) as well as National legal and institutional guidelines for animal care. They were approved by the Animal Care Committee of the Semmelweis University, Budapest and by Hungarian authorities (No. 263/003/2008 and No. 001/2139-4/2012).

2.2. Chemicals

Angiotensin II (Ang II), bradykinin (BK), WIN55212 (a CB_1R agonist), sodium-nitroprusside (SNP), Nω-nitro-L-arginine (LNA, an inhibitor of nitric oxide synthase) and tetrahydrolipstatin (THL, a diacylglycerol lipase inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO, US). The neutral CB_1R antagonist O2050, the inverse CB_1R agonist AM251 were purchased from Tocris Bioscience (Elliswille, MI, US). All other salts and chemicals were purchased from Sigma.

2.3. Immunohistochemistry

Hearts from rats and mice were fixed in 4% paraformaldehyde and placed in 10 and 20% sucrose solutions (15–18 h each) for cryoprotection. The tissues were quickly frozen on dry ice, embedding, sectioning and staining were executed as described [22,31]. Immunostaining was performed with CB_1R primary antibody (Cayman Chemicals, Ann Arbor, MI, overnight). The development of slides was made using the biotin extravidin ABC method (Vector Labs, Burlington, CA). Diaminobenzidine (DAB) was used for visualization [22,31]. Antibody specificity was confirmed preparing aortic slides of wild and CB_1R −/− knockout mice.

2.4. Western blots

Microdissected samples were washed twice in ice cold phosphate buffer solution (PBS, composition in mM, NaCl 137, KCl 2.7, Na_2HPO_4 10.1, KH_2PO_4 1.8, pH 7.4). They were homogenized in glass tubes and lysed in SDS lysis buffer containing 10% merkapto-ethanol and protease inhibitor cocktails (Sigma-Aldrich). In the lysis buffer samples were sonicated, boiled and centrifuged. Proteins from tissue samples were separated with SDS-polyacrylamide gel electrophoresis and were blotted onto the PVDF membrane. Membranes were treated with antibodies against CB_1R (Cayman Chemicals) and beta-actin (Sigma-Aldrich) followed by the treatment with HRP-conjugated secondary antibodies. Visualization was made with SuperSignal West Pico reagent (Promega, Madison, WI) and results were quantitatively evaluated with densitometry.

2.5. RNA extraction and real-time PCR

Vascular tissues were removed by fast and careful microscopic dissection for RNA extraction [16]. In anesthesia, the chest was opened, the heart removed. Intramural coronary arterioles branching from the left anterior descendent coronary artery were isolated. Vessels were placed in cold sterile phosphate buffer solution (PBS as above). Total RNA was extracted (RNeasy mini kit, Qiagen) and reverse transcription was carried out according to the manufacturer's instructions (Fermentas, Ontario, Canada). Real-time PCR assays were performed on LightCycler 480 (Roche Applied Science, Indianapolis, IN) with the SYBR Green method. Primers were designed and synthesized by Sigma-Aldrich. Cycling conditions were: 10 min preincubation at 95 °C, 45–50 cycles of 95 °C 10 s, 62 °C 5 s and 72 °C 15 s. Fluorescence data including melting curves were obtained. For normalization, the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) housekeeping gene was used (ENSRNOG00000004253). Efficiency for each primer pair was determined by using serial dilutions of the PCR product. Fold ratios of gene expression were calculated as follows:

$$\text{Ratio} = E^{\Delta \text{Cttargetgene}}/E^{\Delta \text{CtGAPDH}}$$

Ct was calculated by the second derivative method using LightCycler 480 Software. ΔCt is the difference in Ct values obtained between the reference and tested samples. Relative messenger RNA levels of cannabinoid receptor type 1 (*Cnr1)* were calculated (ENSRNOG00000008223). Primers were for *Cnr1*: forward primer GGACTCAGACTGCCTGCACA, reverse primer ACAAAAGCAGCAG GCTCACA and for *gapdh*: forward primer CCTGCACCACCAAC TGCTTAG, reverse primer CAGTCTTCTGAGTGGCAGTGATG. Tissue gene expression levels were plotted against *gapdh* expression level.

2.6. Isolation of rat coronary resistance arteries for video microarteriography

During anaesthesia of rats, the chest was quickly opened, the heart removed and placed into cold normal Krebs-Ringer (nKR) solution which contained in mM, NaCl 110, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.0, KH_2PO_4 1.0, $NaHCO_3$ 24 and glucose, 10 (pH 7.4). Intramural coronary arterioles branching from the left anterior descending (LAD) coronary artery were isolated by careful microdissection as described previously [5,32,33]. Distal segments close to the apex with approximately 100–150 μm of inner diameter were prepared. One segment was prepared and measured from each animal. The segment was placed in a tissue bath filled with Krebs solution. It was cannulated at both ends with microcannulas, extended to its in situ length in the glass bottomed tissue bath of a pressure microarteriography chamber (Experimetria, Budapest). Clotted blood was washed out at low perfusion pressure, and then one of the cannulas was closed. Utilizing a pressure-servo syringe reservoir system (Living Systems, Burlington, VT, US) arterioles were pressurized in a no-flow condition. Vessels were checked for leaks by the stability of the inlet pressure when the servo function was turned off. Vessels with observable leaks were discarded. The temperature of the chamber was maintained at 37 °C, bubbled with 21% O_2 and 5% CO_2, balanced with N_2, the pH was kept at 7.4. A continuous superfusion of the bath was applied at a rate of 2.5 mL/min. The cannulated vessel was visualized by video-microscopy and the inner diameter was measured on frozen images (Leica inverted microscope, Leica DFC 320 digital camera, LeicaQWin software) [5,6,16,32]. Calibration was made with a micrometer etalon (Wilde, Heerbrugg, Switzerland). Intraluminal pressure was calibrated with a mercury manometer.

2.7. Experimental protocols of isolated vessel studies

Following a 60-min equilibration period, at 50 mmHg intraluminal pressure in nKR solution, pharmacological responses of the arterial

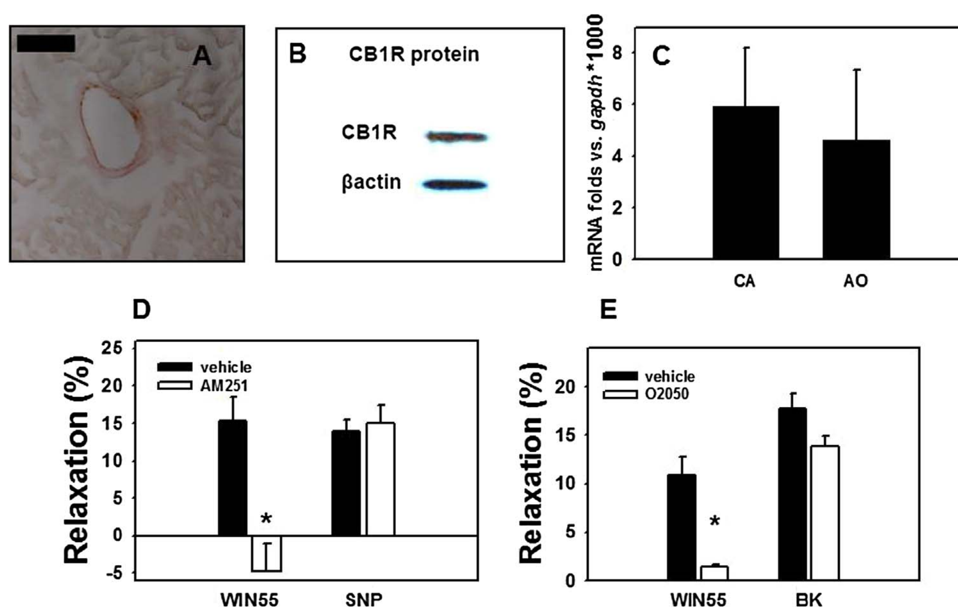


Fig. 1. Molecular and functional evidence of the presence of the CB_1R in the intramural coronary resistance arterioles of the rat. (A) Immuno-histochemical localization of CB_1R receptors. DAB staining for CB_1R protein. Cross section of an intramural coronary arteriole with strong reaction is shown. Scale bar shows 100 μm. (B) Western blot of CB_1R protein from homogenates of microsurgically isolated resistance artery specimens. (C) Expression of mRNA for CB_1R in coronary resistance artery specimens (CA) and in the aorta (AO) of rats as determined by qRT-PCR (n = 5). Expression fold vs. *gapdh* houskeeping gene is plotted. (D, E) Relaxation in response to CB_1R agonist (WIN55212, 1 μM), inhibition of relaxation by the specific CB_1R antagonists O2050 and AM251 (1 μM) of Ang II-precontracted coronary artery segments. SNP-induced (1 μM, an endothelium independent vasodilator, n = 11) and bradykinin-induced (BK, 0.1 μM, an endothelium dependent coronary vasodilator) vasodilations were not affected by CB_1R antagonists (segments precontracted with 1 μM Ang II, n = 8). Mean ± SEM values are shown. *, $P < 0.05$ between control and inhibitor-treated values.

segments were tested according to the specific protocols. Agonists were administered in a dose-dependent manner into the chamber and steady-state diameter was recorded for each dose or in a single (submaximal) dose. 10-min washout periods were applied between drugs. Inhibitors were applied for at least 10 min prior to and during agonist administration. Each inhibitor was applied in separate experimental series, either the pressurized segments were in spontaneous myogenic contraction or their contraction was enhanced with Ang II. In coronary vessels, Ang II is suitable to produce precontraction, since it produces a stable contraction without observable desensitization response in contrast to other type of vessels like gracilis arterioles and aorta [16,22]. After Ang II precontraction (1 μM), SNP (1 μM) was applied to test endothelium-independent vasodilation. Endothelial relaxation was tested by bradykinin (100 nM), which is an appropriate endothelium-dependent vasodilator in coronary resistance arteries of rats [5]. The experiments were terminated by obtaining passive (relaxed) vascular diameter in calcium-free Krebs solution.

In the first group of experiments (n = 14), rat coronary arterioles were subjected to elevated doses of agonists, Ang II (0.1 nM–10 μM), WIN55212 (0.1 nM–1 μM), SNP (0.1 nM–10 μM) and BK (0.1 μM) with the CB_1R neutral antagonist O2050 (1 μM) or only its vehicle being present in the bath. Since WIN55212 is lipid-soluble, it was applied prior to and after CB_1R antagonist in separate vessel segments. In the second group (n = 5), concentration-response to Ang II was obtained before and during the administration of the DAG lipase inhibitor tetrahidrolypstatin (1 μM) or its vehicle. In a third experimental set (n = 6), the effect of CB_1 receptor antagonist (inverse agonist) AM251 (1 μM) (or its vehicle) on the tone of coronary arterioles and on WIN55212- and SNP-induced vasodilations were tested. In a fifth set of experiments (n = 5), the effect of Nω-nitro-L-arginine (an inhibitor of nitric oxide synthase, 50 μM) was applied 20 min prior and during the administration of CB_1R agonist WIN55212 (1 μM) or the endothelium-dependent agonist BK (0.1 μM) in order to test the role of NO in the CB_1R-mediated coronary functions.

2.8. Statistical analysis

Data are presented as means ± SEM. Vascular tone was calculated as percent change from passive diameter. Changes in vascular diameter were also calculated as percent change from control value. For paired data we used the Student's *t*-test, for multiple comparisons one-way and two-way ANOVAs were applied (SigmaStat). Concentration-response curves without and with inhibitors present in the solution were compared using the four parameter logistic function (SigmaPlot). Relative gene expression levels were plotted against reference control (*gapdh*). $P < 0.05$ was considered significant for each comparisons.

3. Results

3.1. Molecular and functional evidence of the expression and presence of CB_1Rs in intramural coronary resistance arteries

CB_1R protein is present in the wall of these rodent microvessels as it has been demonstrated by immunohistochemistry of ventricular tissue. Immunostaining appeared in the wall of intramural coronary arterioles (Fig. 1A). Western blots also proved the expression of the CB_1R protein in the wall of rat small coronary arteries isolated by microdissection (Fig. 1B). In addition, messenger RNA for CB_1 receptors was also detected by qRT-PCR in amounts similar to those found in rat aortic tissue (Fig. 1C). Segments in myogenic contraction relaxed in response to the CB_1R agonist WIN 55212 (1 μM). Further, this relaxation could be inhibited by the specific blockers O2050 (1 μM), and AM251 (1 μM). These CB_1R antagonists, however, did not affect either endothelium independent (SNP, 1 μM) or endothelium dependent (BK, 0.1 μM) relaxations (tested on Ang II precontracted segments, Fig. 1D,E).

3.2. Evidence of existence of an intrinsic CB_1R activation during spontaneous myogenic contraction

Coronary arteries when pressurized, exhibit a substantial spontaneous, myogenic tone in oxygenized nKR solution. This, with all probability mimics the in vivo situation [5,32,33]. We tested whether a basal endocannabinoid release affects the spontaneous tone in these vessels. Vascular diameter of our segments during spontaneous contraction (following an equilibration period) was 138.7 ± 6.6 μm (inner diameter) corresponding to 20.1 ± 2.9% (n = 15) myogenic tone (compared with relaxed controls).

The neutral CB_1 receptor antagonist (O2050, 1 μM) moderately (2.6 ± 1.7%, n.s.), the inverse CB_1R agonist AM251 (1 μM) significantly increased coronary myogenic tone (6.0 ± 1.0%, $p < 0.05$). The DAG lipase enzyme inhibitor THL (1 μM) effectively increased basal coronary tone (11.2 ± 3.9% contraction, $p < 0.05$). This effect was additive with the similar effect of AM251: a substantial diameter reduction of 24.6 ± 5.3% ($p < 0.05$) was reached when both

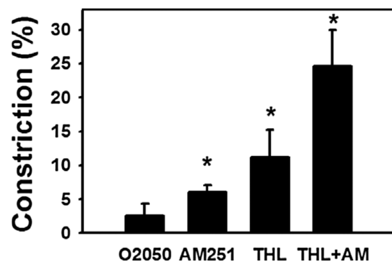


Fig. 2. Intrinsic CB_1R activity keeps myogenic tone reduced. The type 1 cannabinoid receptor (CB_1R)-antagonists O2050 (a neutral antagonist, 1 μM) and AM251 (an inverse agonist, 1 μM) and also the diacylglycerol (DAG) lipase-inhibitor tetrahydrolypstatin (THL, 1 μM, inhibits the production of 2-AG) substantially increase the spontaneous tone of pressurized rat coronary arteries (n = 5 or 6 for each group). Mean ± SEM values are shown. *, $P < 0.05$ between control and inhibitor-treated values.

antagonists were applied together (Fig. 2.). Products of the enzyme DAG lipase must be the main endocannabinoids in this tissue. These experiments indirectly prove that spontaneous myogenic tone of these vessels is kept under continuous control by endogenously produced 2-AG. This control can be considered fairly effective as the myogenic contraction of these vessels practically doubles if 2-AG synthesis and endocannabinoid action on CB_1Rs are both inhibited at the same time.

3.3. Effect of exogenous cannabinoid on coronary arterial tone

Adding angiotensin II to the bath further contracts coronary arteries, this contraction is added to the myogenic tone. The specific CB_1R agonist WIN55212 very effectively relaxed coronary arteriole segments precontracted with close-to-maximal concentrations of Ang II (1 μM). The concentration-dependent vasodilation by the CB_1R agonist WIN 55212 is shown on Fig. 3A and C, which is effectively inhibited by the CB_1R antagonists O2050 and AM251. This record proves that agonist induced contraction of coronary resistance arterioles can be inhibited by exogenous stimulation of the cannabinoid receptors. However, the

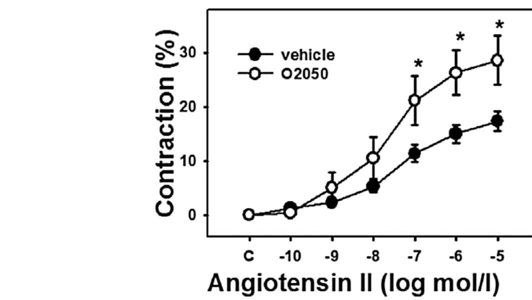
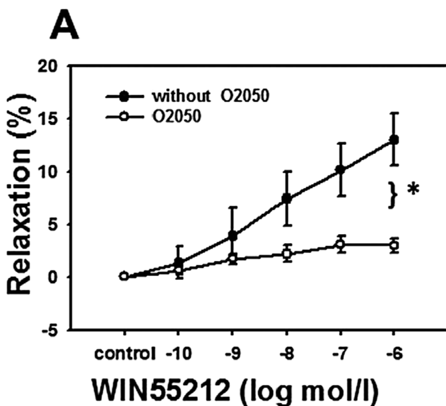


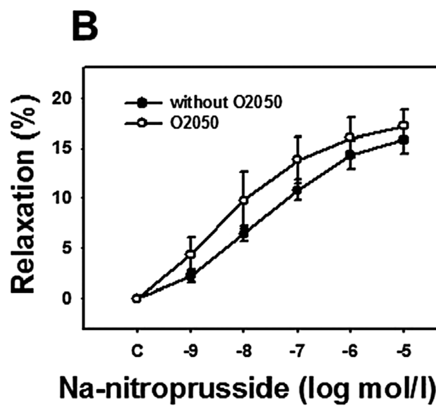
Fig. 4. CB_1R-mediated modulation of agonist-induced contraction. Enhancement of concentration dependent Ang II contraction in the presence of the cannabinoid receptor (CB_1R) antagonist O2050 (1 μM) in the tissue bath (n = 10 and 8). *, $P < 0.05$ between control and inhibitor-treated values.

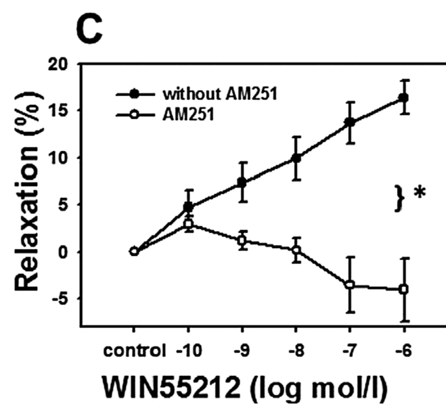
concentration-dependent vasodilation by the NO donor sodium nitroprusside (SNP) was not affected by these CB_1R antagonists (Fig. 3B and D). Fig. 3. and Fig. 1D demonstrate an interesting fact: WIN55212, a CB_1R agonist is an almost as effective a coronary vasodilator as the well-known NO donor SNP is.

3.4. Agonist induced contraction goes on with parallel CB_1R activation

Ang II elevated coronary arteriole tone in a dose-dependent manner, at maximal concentrations inducing about a 20% further reduction of diameter of segments in spontaneous contraction. This effect reached a maximum level around 10 μM of Ang II concentration. Inhibition of CB_1 receptors by O2050, significantly enhanced Ang II-induced contractions of coronary arterioles (significant over 100 nM Ang II, $p < 0.05$, Fig. 4). The four parameter logistic analysis has shown that there was not significant change in log EC50% values for Ang II contraction (−7.36 ± 0.19 vs. −7.54 ± 0.21 without and with the antagonist, respectively, n.s.), while maximum contraction values significantly elevated (17.8 ± 1.2 vs. 29.1 ± 2.9%, $p < 0.05$). These







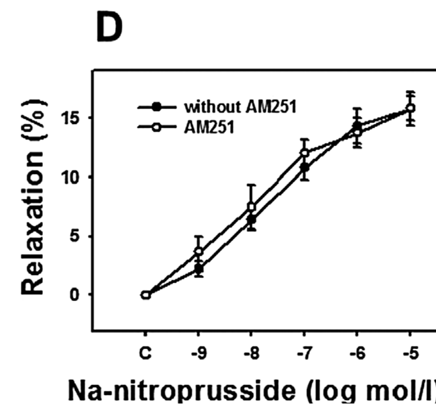


Fig. 3. CB_1R-mediated modulation of agonist-induced contraction. (A) Concentration dependent dilation of Ang II (1 μM) precontracted coronary arteriole segments in response to different concentrations of the CB_1R agonist WIN55212. The relaxation is almost completely inhibited in the presence of the specific CB_1R blocker O2050 (1 μM, n = 5–6). (B) For comparison, concentration-dependent vasodilation effect of NO donor sodium nitroprusside on coronary artery segments, which was not affected by the CB_1R antagonist treatment (n = 6–8). (C and D) Similar responses were detected with the CB_1R inverse agonist AM251 (1 μM, n = 6–8). *, $P < 0.05$ between control and inhibitor-treated values.

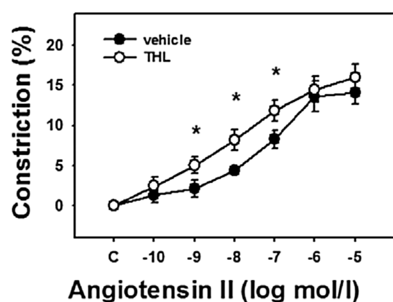


Fig. 5. Identification of the vasodilatory effect of a diacylglycerol lipase product. Enhancement of concentration-dependent Ang II-induced contraction in the presence of the DAG lipase blocker tetrahydrolipstatin (THL, 1 μM) in the tissue bath (n = 6 and 5). *, $P < 0.05$ between control and inhibitor-treated values.

observations prove that during agonist induced contraction, a continuous and stimulated endocannabinoid production is present in the wall which modulates contraction due to its effect on CB_1Rs.

3.5. Effect of inhibition of DAG lipase enzyme on the agonist-induced response

The DAG lipase blocker THL also augmented Ang II-induced contractile response in the coronaries (Fig. 5, $p < 0.05$ at 1 nM–100 nM of Ang II). The difference in Ang II-induced contraction with and without THL reached 5% of diameter at 10 nM Ang II concentration. Based on this observation, it is highly probable that 2-AG is the endocannabinoid whose production is elevated by Ang II in this vascular preparation and the co-stimulation of CB_1Rs produced limits then Ang II contraction [22].

3.6. Role of endothelial nitric oxide in CB_1R induced coronary vasodilation

The coronary dilatory effect of the CB_1R agonist Win 55212 on Ang II precontracted segments was partially suspended by the NO synthase blocker Nω-nitro-L-arginine (50 μM), suggesting a partially NO mediated effect (Fig. 6, $p < 0.05$). By comparison, the dilatory effect of bradykinin, an endothelial coronary vasodilator, was fully diminished by the same concentration of LNA ($p < 0.001$).

4. Discussion

4.1. Substantial modulator effect found both in myogenic and in agonist induced contraction

Our observations demonstrated the existence and a substantial functional role of CB_1Rs in rodent intramural coronary arterioles, vessels that basically determine local ventricular flow. The presence of CB_1R protein (with immune-histochemistry and Western blot, Fig. 1A and B) and of its mRNA (Fig. 1C) have been first proven in this vessel-

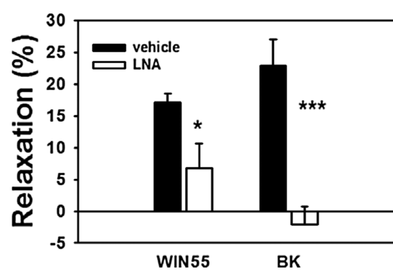


Fig. 6. Endothelial component of CB_1-R vasodilation. The CB_1R agonist WIN55212 (1 μM)-induced vasodilation was partially blocked by the presence of Nω-nitro-L-arginine in the bath (50 μM, n = 5). For comparison, the full inhibition of BK induced relaxation by LNA is demonstrated to the right. Mean ± SEM values are shown. *, $P < 0.05$ and *** $P < 0.01$ between control and inhibitor-treated values.

type. We have also proved that endocannabinoids acting on such receptors play an important role in forming myogenic and agonist induced tone of these vessels. The specific CB_1R agonist WIN55212 induced a substantial concentration-dependent vasodilation (Fig. 3A and C) which was prevented by the CB_1R neutral antagonist O2050 (Fig. 3A) and by the inverse agonist AM251 (Fig. 3C). AM251 and the DAG lipase enzyme inhibitor THL substantially increased coronary arteriole myogenic tone (Fig. 2). Ang II induced tone was augmented both by O2050 and THL (Figs. 4 and 5). We have a sound foundation to declare that a DAG lipase product endocannabinoid, with all probability 2-AG is continuously synthesized in the wall of these vessels, and the amount produced is enhanced by Ang II. Both the spontaneous myogenic and Ang II-induced contractions are attenuated by its effect. As neither SNP nor BK relaxations were effected by CB_1R antagonists (Fig. 1E, Fig. 3B and D), we can conclude that simply altering the contractile state of a coronary arteriole segment will not automatically alter its endocannabinoid production.

4.2. Coronary cannabinoid-mediated relaxation

Our findings are in accord with an earlier publication by White et al. [9] who found that isolated rings prepared from the left anterior descendent coronary artery of the rat (not a resistance sized artery) and precontracted with 5-HT, dilated in response to exogenous anandamide. This dilation could be inhibited by the CB_1R blocker SR141716A. It is interesting to note that they have found the more specific CB_1R blocker AM251 less effective. There seems to be some contradiction also with the observations by Mair et al. [26]. On main coronary artery branches of the rat, precontracted with U46619 (a thromboxane agonist), they have found that relaxation occurred through CB_2Rs due to activation of the ERK1/2 pathway and vasodilatory prostanoids. Our opinion is that tissue composition and function of larger coronary arteries viable for wire myographic studies can be substantially different from the real resistance vessels studied by us using the pressure arteriography technique.

4.3. Coronary endocannabinoid production and relaxation

Endocannabinoid production has been detected in different vascular beds and also in heart [15–22]. Several types of cells of different vascular tissues can be sources of endocannabinoids: endothelial cells, smooth muscle cells, perivascular neurons, platelets, leukocytes, monocytes, macrophages etc. [18,34–36]. Endogenous release of 2-AG has been observed from human vascular endothelial cells. The 2-AG released was supposed to be an intrinsic vasomodulator [37]. In bovine coronary arteries, metacholine stimulates endothelial 2-AG release through PLC and DAGL activation and serves as an intermediate for vasodilatory eicosanoid release [34]. Not only endothelial cells but also vascular smooth muscle cells can release 2-AG [22]. We have found previously that Ang II-stimulated 2-AG-release from vascular smooth muscle cells has been attenuated by the inhibition of DAG lipase and augmented by monoacylglycerol (MAG) lipase inhibition [22]. In conformity with the vascular production of 2-AG, we have also found that inhibition of DAG lipase augmented, while inhibition of MAG lipase attenuated vasoconstriction of the aorta. This indicates the role of locally released 2-AG in the control of vascular tone in this vessel [22]. Similarly, a previous observation found that 2-AG production in rat aorta was enhanced by carbachol [38].

The contribution of the endocannabinoid mechanism to lumen control in these important vascular segments is substantial, we have a good reason to suppose a physiological impact. We have found here that inhibition of DAGL, the enzyme responsible for the production of a substantial part of tissue endocannabinoids, augmented coronary resistance artery tone by around 10% even without adding any stimulants. The double inhibition of the endocannabinoid effect (both CB_1R and DAGL inhibition) induces 24.6 ± 5.3% contraction, that

practically doubles the approximately 20% spontaneous myogenic tone (Fig. 2). We can conclude that similarly to endogenous prostanoids and NO, a continuous production of vasodilatory endocannabinoids in the resistance artery wall counterbalances the myogenic tone in coronary resistance arteries. The concentration-dependent Ang II-induced contraction of these segments has also been accompanied by a stepwise elevation of the moderating effects of endogenous cannabinoids. Both the CB_1R antagonist O2050 and the DAG lipase blocker THL augmented Ang II-induced contraction when compared with contraction without the antagonist (Figs. 4 and 5). This makes it highly probable, that "basal" endocannabinoid release (and its vasorelaxation effect) is increasing with increasing concentrations of the contracting agonist. According to a previous observation from our laboratory, in non-vascular cells co-expressing type 1 angiotensin receptor (AT_1R) and CB_1R, Ang II-induced stimulation of AT_1Rs via the activation of PLC and DAG-production led to the transactivation of CB_1 receptors by endocannabinoid release [39]. The Ang II-induced CB_1 receptor activity was inhibited by DAGL inhibitor suggesting, that 2-AG production from DAG plays an important role in the mediation of this action [40]. Similarly, our previous observation on skeletal muscle arterioles indicated that inhibition of CB_1 receptors augmented Ang II-induced vasoconstriction by increasing both efficacy and the potency of the Ang II-induced responses [16].

Considering the mechanism of this relaxing effect, our present studies on coronary arterioles suggest a mechanism analogous that found in skeletal muscle arterioles. The AT_1R, G_q, PLC, DAG pathway produces a substrate for the DAG lipase, the 2-AG produced co-activates CB_1Rs in the neighborhood, inducing, by a not fully identified yet action, relaxation, limiting the contraction exerted. How does the co-activation of CB_1Rs relax these vessels? K^+ channel-mediated cell hyperpolarization [9,10,17,41], depression of Ca^{2+} influx [41,42], modulation of endogenous prostanoid production [9,10,26,35], activation of $G_{i/o}$ or other G proteins [3,9,40] are the mechanisms suggested in the literature by different authors.

The role of endothelium and endothelium-mediated nitric oxide in the cannabinoid-induced vasodilatory response seems to be different in different vascular territories. Previously, in gracilis arterioles we found that WIN55212-induced vasodilation was not attenuated by the blocker of NO synthase nitro-L-arginine, while it effectively inhibited acetylcholine-induced vasodilation [16]. Also, we found that the removal of endothelium did not change the augmented vasoconstrictor effect induced by the CB_1R blocker O2050 in gracilis vessels. Sources of vascular endocannabinoids can be heterogenous: 2-AG production has been detected both from endothelial cells and from vascular smooth muscle cells [22,35,37,38]. These observations are in good accordance with our present observations on coronary resistance vessels, inhibition of NO synthesis caused a partial (by approx. 50%) attenuation of the CB_1R agonist WIN55212-induced vasodilation.

4.4. Potential physiological role

We can remark here that such intrinsic "braking" feed-backs frequently occur in complex physiological control networks. In case of the coronary resistance artery control mechanisms, the endothelial NO and endogenous prostanoid production, stimulated by agonists, can be mentioned. We and other laboratories have also found earlier that myogenic and agonist-induced tone of coronary resistance arteries are fine-tuned by altered NO and vasoactive prostanoid production occurring in the wall of these vessels [3,6,7]. Now, we can add to this a similar new mechanism: endogenous cannabinoid production. One can theorize on the potential physiological benefits: Full contraction closing the lumen can be prevented by them or, certain contractile effects can be neutralized. Anyway, they yield a more complex and more versatile lumen control mechanism, which is able to respond to diverse physiological needs in a more diverse manner.

5. Conclusion

Our studies have proven the existence of the CB_1R protein, its mRNA and also its functional relevance to induce substantial vasodilation in the wall of intramural coronary resistance arteries, vessels that basically determine ventricular local flow. Further, we provide clear evidence that a vasorelaxant endocannabinoid system is present in these vessels, which substantially contributes to the physiologically important lumen controlling mechanisms here through vascular CB_1 receptors. This mechanism is active both during spontaneous myogenic contraction and also in Ang II-induced contraction. We suggest, that to the known physiological mechanisms that reduce the substantial spontaneous/myogenic tone of coronary resistance vessels (metabolic factors, beta adrenergic stimuli, vasodilatory prostanoids and endothelial NO) the endogenous cannabinoids should be added.

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Disclosures, conflicts of interests

None is declared.

Author contributions

M.Sz. and L.H.: conception and design of research. M.Sz. and G.L.N. performed experiments. M.Sz., G.L.N. and E.S-K. performed analysis. M.Sz. and G.L.N. interpreted results of experiments. M.Sz. and E.S-K. prepared figures. M.Sz. drafted manuscript. L. H. edited and revised manuscript. L. H., M.Sz., G.L.N. and E.S-K. approved final version of manuscript.

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