

Angiotensin II Induces Vascular Endocannabinoid Release, Which Attenuates Its Vasoconstrictor Effect via CB₁ Cannabinoid Receptors*

Received for publication, January 25, 2012, and in revised form, July 10, 2012. Published, JBC Papers in Press, July 11, 2012, DOI 10.1074/jbc.M112.346296

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Background: In expression systems diacylglycerol (DAG) produced during AT₁ angiotensin receptor signaling can be converted to 2-arachidonoylglycerol.

Results: Inhibition of CB₁ receptors and DAG lipase augmented angiotensin II-induced vasoconstriction in resistance arteries.

Conclusion: Angiotensin II-induced vasoconstriction is attenuated via 2-arachidonoylglycerol release and consequent CB₁ receptor activation.

Significance: This is the first demonstration that angiotensin II-induced endocannabinoid release can modulate vasoconstriction.

In the vascular system angiotensin II (Ang II) causes vasoconstriction via the activation of type 1 angiotensin receptors. Earlier reports have shown that in cellular expression systems diacylglycerol produced during type 1 angiotensin receptor signaling can be converted to 2-arachidonoylglycerol, an important endocannabinoid. Because activation of CB₁ cannabinoid receptors (CB₁R) induces vasodilation and reduces blood pressure, we have tested the hypothesis that Ang II-induced 2-arachidonoylglycerol release can modulate its vasoconstrictor action in vascular tissue. Rat and mouse skeletal muscle arterioles and mouse saphenous arteries were isolated, pressurized, and subjected to microangiometry. Vascular expression of CB₁R was demonstrated using Western blot and RT-PCR. In accordance with the functional relevance of these receptors WIN55212, a CB₁R agonist, caused vasodilation, which was absent in CB₁R knock-out mice. Inhibition of CB₁Rs using O2050, a neutral antagonist, enhanced the vasoconstrictor effect of Ang II in wild type but not in CB₁R knock-out mice. Inverse agonists of CB₁R (SR141716 and AM251) and inhibition of diacylglycerol lipase using tetrahydrolipstatin also augmented the Ang II-induced vasoconstriction, suggesting that endocannabinoid release modulates this process via CB₁R activation. This effect was independent of nitric-oxide synthase activity and endothelial function. These data demonstrate that Ang II stimulates vascular endocannabinoid formation, which attenuates its vasoconstrictor effect, suggesting that endocannabinoid release

from the vascular wall and CB₁R activation reduces the vasoconstrictor and hypertensive effects of Ang II.

Angiotensin II (Ang II)² is the key effector molecule of the renin-angiotensin system. It plays a crucial role in physiologic and pathologic control processes such as aldosterone secretion, vasoconstriction, cell proliferation, inflammation, atherosclerosis, and vascular remodeling (1–4). Ang II exerts its biological actions by activating type 1 (AT₁R) and type 2 (AT₂R) angiotensin receptors. The most important short and long term physiological and pathophysiological actions of the hormone are mediated by AT₁R (1–4). AT₁R is a G protein-coupled receptor, and its signal transduction is characterized by diverse, G protein-dependent and independent signaling mechanisms (2–5). The main signal transduction mechanism of AT₁Rs is activation of G_{q/11} proteins, which elevates intracellular calcium levels, stimulates diacylglycerol (DAG) formation, and activates downstream signaling molecules such as tyrosine kinases and mitogen-activated protein kinases (1–6). Calcium signal generation has been linked to endocannabinoid formation in neurons and other cells (7, 8).

Endocannabinoids serve as endogenous ligands for cannabinoid receptors and participate in tissue-specific paracrine regulatory mechanisms such as retrograde control of neurotransmitter release (7). To date several endocannabinoid compounds have been identified including arachidonoyl ethanolamide (anandamide), 2-arachidonoylglycerol (2-AG), and 2-arachidonoylglycerol ether (7, 9–11). Cannabinoid receptors were originally named after their affinity for Δ⁹-tetrahydrocan-

* This work was supported by grants from the Hungarian National Science Foundation (OTKA NK-100883, NK-72661, OTKA TO32019), Hungarian Ministry of National Resources (ETT 495/09), and from the National Development Agency, Hungary (TÁMOP 4.2.1.B-09/1/KMR-2010-0001).

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² The abbreviations used are: Ang II, angiotensin II; Ach, acetylcholine; 2-AG, 2-arachidonoylglycerol; AT₁R, type 1 angiotensin receptor; CB₁R, type 1 cannabinoid receptor; DAG, diacylglycerol; LNA, N^ω-nitro-L-arginine; THL, tetrahydrolipstatin.

nabinol, the main active ingredient of the extracts of *Cannabis sativa*. Endocannabinoid receptors include CB₁ cannabinoid receptors (CB₁Rs), which are characteristically present in neural tissues, and CB₂ receptors (CB₂Rs), which occur mostly in immune cells (7). However, both receptors were identified in a number of other tissues, and the existence of other cannabinoid receptors has also been proposed (10, 12). CB₁Rs in the central nervous system typically occur in presynaptic locations, and they modulate synaptic transmission. During stimulation by neurotransmitters such as glutamate and acetylcholine, endocannabinoid-mediated CB₁R activation mediates important physiological functions such as depolarization-induced retrograde synaptic inhibition (7, 9, 13).

In addition to the key functions of endocannabinoids in the central nervous system, their roles in peripheral tissues are also increasingly in the focus of interest (12, 14). It has been shown that the cannabinoid system plays a role in cardiovascular, inflammatory, gastrointestinal, and peripheral metabolic regulatory mechanisms (10, 12, 14). In the cardiovascular system, negative inotropic, vasodilator, and hypotensive actions of cannabinoids have been reported (14). The growing number of studies with compounds that modulate the endocannabinoid system may lead to novel therapeutic approaches in a number of metabolic and cardiovascular disorders (10, 12, 14). Although CB₁ receptors have key roles in the central neural control of appetite and evidence for the tonic activity of the endocannabinoid/CB₁ receptor system in obesity provide a rationale for the use of CB₁ receptor antagonists as anti-obesity agents, these molecules were withdrawn from the market due to their central side effects (12). However, there is still hope that selective targeting of peripheral CB₁ receptors has potential therapeutic value in metabolic and other diseases (12).

We have reported recently in transient expression systems that Ang II-induced activation of AT₁R can lead to CB₁R activation in cells coexpressing both AT₁ and CB₁ receptors (8, 15). Moreover, we have shown that stimulation of AT₁Rs and other G_{q/11} protein-coupled G protein-coupled receptors can cause 2-AG-mediated paracrine transactivation of CB₁Rs (8). The Ang II-induced CB₁R activation was inhibited by DAG lipase inhibitors, suggesting that DAG generated from phosphoinositides can be converted to 2-AG by DAG lipase during the signaling of Ca²⁺-mobilizing hormones and neurotransmitters (8, 15). Although the physiological relevance of this mechanism has not been fully established, our preliminary data suggest that Ang II-induced endocannabinoid release may mediate the central hypertensive effect of Ang II in the paraventricular nucleus (16).

Based on our earlier findings on the mechanism of Ang II-induced paracrine transactivation of CB₁ receptors, we hypothesized that the vasoconstrictor effect of Ang II may be attenuated by vascular endocannabinoid formation and consequent CB₁R activation, and this mechanism can have a significant modulatory effect during the regulation of the vascular tone. This hypothesis was tested in this study.

EXPERIMENTAL PROCEDURES

Animals—Male Wistar rats were used (300–350 g, Charles River Laboratories, Semmelweis University, Budapest, Hun-

TABLE 1

Geometric values of isolated arteries

Control (CD) and passive (PD, relaxed) diameter (μm) and spontaneous tone values of rat and mouse gracilis arterioles and mouse saphenous arteries (mouse a. saphena) are shown. Mean \pm S.E. values and number of segments (n) are indicated for each vessel type. Vascular tone was calculated as percent contraction related to passive diameter. Values for rat saphenous arteries were separated as of wild type (control) and CB₁R knockout mice.

Prepared segments	CD	PD	Tone	n
	μm	μm	%	
Rat gracilis arteriole	123.7 \pm 5.4	152.9 \pm 6.6	18.5 \pm 1.7	38
Mouse gracilis arteriole	119.4 \pm 7.0	135.8 \pm 5.4	12.3 \pm 1.8	5
Mouse a. saphena (control mice)	185.4 \pm 15.0	216.7 \pm 17.0	13.7 \pm 3.3	10
Mouse a. saphena (CB ₁ ko mice)	179.4 \pm 17.2	210.1 \pm 21.4	13.9 \pm 2.2	5

gary). C57BL/6J (21–25 g) mice were obtained from the animal facility of the Department of Physiology. CB₁R knock-out (–/–, CB₁R-KO) and wild type (+/+, C57BL/6J, Cnr1tm1zlm) mice (21–25 g) were kindly provided by Professor Andreas Zimmer, University of Bonn (9). Animals were anesthetized with pentobarbital sodium (Euthasol, ASTfarma, 50 mg/kg intraperitoneally) and immediately sacrificed by fast bleeding. All procedures conform with the Guide for the Care and Use of Laboratory Animals (NIH, 1996) legal and institutional guidelines for animal care and were approved by the Animal Care Committee of Semmelweis University, Budapest and by Hungarian authorities (no. 263/003/2008).

Chemicals—Angiotensin II, norepinephrine, sodium nitroprusside, acetylcholine (Ach), WIN55212 (R-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone mesylate (a CB₁R agonist), tetrahydrolipstatin (a diacylglycerol lipase inhibitor) and N_ω-nitro-L-arginine (LNA) (a nitric oxide (NO)-synthase inhibitor) were purchased from Sigma. CB₁R antagonists O2050 (6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H dibenzo [*b,d*]pyran), a silent antagonist, and inverse agonist AM251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) were purchased from Tocris Bioscience (Ellisville, MI), and SR141716 (rimonabant, 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide) was purchased from Cayman Chemicals (Tallinn, Estonia). AT₁R antagonist candesartan was purchased from Toronto Research Chemicals (Toronto, Canada).

Isolation of Vessels for Pressure Video Microangiography—Rat and mouse skeletal muscle (gracilis) arterioles (~120 μm in diameter) and mouse saphenous arteries (~180 μm in diameter, Table 1) were prepared under microscopic guidance. For the preparation of gracilis arterioles the adductor muscles were removed, placed into cold Krebs solution that contained 110 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgSO₄, 1 mM KH₂PO₄, 24 mM NaHCO₃, and 10 mM glucose, pH 7.4, and prepared as described before (17, 18). For the preparation of saphenous arteries, the surface of the leg was cleared from the skin, and the arterial segment was further isolated *in situ* using a microscope (19). Segments were cannulated in a vessel chamber (Experimetria) and subjected to pressure microangiography (Living Systems, Burlington, VT) as also described previously (20–22). The cannulated vessel was visualized by digital videomicroscopy, and the inner diameter was measured using

Leica DFC 320 digital camera and LeicaQWin software (Leica, Wetzlar, Germany) (20). Arterial segments developed substantial (10–18%) vascular tone (Table 1).

Experimental Protocols for Pressure Microangiography—Pressurized segments were allowed to equilibrate for 30 min at 50 mm Hg intraluminal pressure, and pharmacological responses of the arterial segments were tested according to the specific protocols. Pharmacological agonists were administered in a concentration-dependent manner into the chamber, and steady-state diameter was recorded for each concentration or in a single (submaximal) concentration. 10-Min washout periods were applied between drugs. Precontraction of segments before vasodilator treatment was made with norepinephrine (50–100 nM). Endothelial integrity was tested by Ach (10 μ M, NO-dependent vasodilator) in all experiments. The experiments were terminated by obtaining passive (relaxed) vascular diameter in calcium-free Krebs solution.

According to specific protocols on rat gracilis arterioles, concentration-responses to Ang II were obtained in vehicle or with CB₁R inhibitors O2050 (neutral antagonist, 1 μ M), SR141716, and AM251 (inverse agonists 1–1 μ M) in three different experimental sets for each inhibitor (nine, five, and five animals, respectively). Ang II-induced concentration-responses were repeated on separate segments to prevent desensitization (data not shown), which can be attributed to the rapid agonist-induced AT₁R-internalization (3). Because AM251, an inhibitor of CB₁Rs was reported to inhibit also constitutive activity of CB₁Rs previously (8), we also applied the neutral antagonist of CB₁R, O2050. Inhibitor was applied for at least 10 min before and during agonist administration. In these experiments CB₁R agonist WIN55212 (1 μ M) was also applied. Endothelial integrity was tested by Ach, and vasodilator capacity of rat gracilis vessels was also tested by sodium nitroprusside (up to 10 μ M). In similar protocols, responses to Ang II were also obtained with DAG lipase inhibition by THL (1 μ M, n = 6). DAG lipase inhibition was applied to clarify the role of endogenously produced endocannabinoids in the vascular CB₁R activity. In separate experiments, simultaneous administration of THL and O2050 was also applied (additional three rats), and effects of Ang II were also obtained. In additional experiments, the effects of WIN55212 and Ang II were also obtained with LNA (10 μ M, n = 5) and with endothelial disruption (performed with intraluminal administration of a bubble for 10 minutes, n = 6) to test the NO/endothelial dependence of the cannabinoid effects. In additional experiments (n = 4), AT₁R blocker candesartan (10 μ M) was also applied to repeat Ang II concentration-responses to test the AT₁R dependence of the Ang II response on rat gracilis arterioles (0.1–100 nM). The CB₁R inhibitor O2050 was also tested on mouse gracilis arterioles (n = 4). To clarify the role of CB₁Rs in the control of vascular tone, a similar protocol was also performed on saphenous arteries from CB₁R knock-out (n = 4) and wild type (n = 4) mice with inhibition of CB₁Rs (O2050). In an additional set of experiments (n = 6) the effect of THL on the vasoactive responses was also tested on mouse saphenous arteries of control mice of the CB₁R KO tribe (C57BL/6J). Control and passive diameter and spontaneous tone of the prepared segments are summarized (Table 1). The number of animals used in each set is given (n), and 1–2 seg-

ments were prepared from each animal. In all experimental settings vehicles were also tested, and time control was also applied.

RNA Extraction and Real-time PCR—Segments of thoracic aorta were removed and gracilis arterioles were removed under microscopic guidance as described above from 4 rats. Samples were washed and placed in cold sterile phosphate buffer solution (PBS, with 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). RNA was extracted and further processed for real-time PCR (Roche Applied Science) as described (23). RT-PCR assays were performed using the SYBR Green method. Primers were designed and synthesized by Sigma. Cycling conditions were: 10 min of preincubation at 95 °C, 45–50 cycles of 95 °C for 10 s, 62 °C for 5 s, and 72 °C for 15 s. Fluorescence data including melting curves were obtained. For normalization, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), a housekeeping gene, was used (ENSRNOG00000004253). Relative messenger RNA levels of cannabinoid receptor type 1 gene (*Cnr1*) were calculated (ENSRNOG00000008223). Primers for *Cnr1* were forward (GGACTCAGACTGCCTGCACA) and reverse (ACAAAAGCAGCAGGCTCACA) and for *Gapdh* were forward (CCTGCACCACCAACTGCTTAG) and reverse (CAGTCTTCTGAGTGGCAGTGTG). Tissue gene expression levels were plotted against *Gapdh* expression levels. Amplicons obtained from gel electrophoresis and relative CB₁R mRNA values are shown in Fig. 1B.

Tissue Immunohistochemistry—Tissue perfusion was performed under anesthesia. Rat and mouse gracilis muscles were removed, washed in ice-cold PBS, fixed with 4% paraformaldehyde subjected to cryoprotection in sucrose solutions, then quickly frozen on dry ice as described previously (23, 24). Cryostat sections of gracilis muscles were mounted on Super Colorfrost slides (Fisher). Sections were blocked with 1% bovine serum albumin, and endogenous peroxidase activity was blocked with 3% H₂O₂ solution. Staining was performed with primary CB₁R antibody (Cayman Chemicals, Tallinn, Estonia), and biotin-extravidin-peroxidase was developed by using the ABC method (Vector Laboratories, Burlington, CA) (24). Brain samples of CB₁R knock-out and wild type mice were used to check immunoreactivity in a CB₁R-specific tissue (data not shown).

Western Blotting of CB₁R Protein—Five rats were anesthetized and sacrificed. Thoracic aortas and gracilis arterioles were removed as described above. Samples were washed twice in ice-cold PBS solution-modified Krebs-Ringer buffer (see above). Aortas were cut into pieces, and tissue samples weighing 20–30 mg were homogenized in glass tubes and lysed in SDS lysis buffer containing 10% mercaptoethanol and protease inhibitor cocktails (Sigma). In the lysis buffer samples were sonicated, boiled, and centrifuged. Proteins from both tissue, and cell samples were separated with SDS-polyacrylamide gel electrophoresis and were blotted onto PVDF membranes. Membranes were treated with antibodies against CB₁R (Cayman Chemicals) and β -actin (Sigma) 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.

Data Analysis—Data are presented as the mean \pm S.E. values. Vascular responses are expressed as percent changes in vessel diameter from base-line values. Comparisons were made with one- or two-way analysis of variance tests followed by Tukey's and Holm-Sidak post hoc tests. Vasoconstrictor responses were also analyzed with nonlinear curve-fitting (four-parameter logistic curves). Effective maximal responses (E_{\max}) and half-maximal responses (half-effective concentration, EC_{50}) were calculated. Graphic presentations and statistical calculations were made using the Sigmaplot and Sigmastat program packages (Systat Software, Inc. San Jose, CA). For paired data, Student's *t* test was applied. Probability levels $p < 0.05$ were taken as statistically significant.

RESULTS

Activation of CB_1R Exerts Vasodilatory Effects in Rat Gracilis Arterioles—In rat gracilis arterioles, WIN55212, a CB_1R agonist, relaxed arterioles by $18.2 \pm 2.5\%$ (Fig. 1A), showing that CB_1R s are functionally present on these vessels and that their activation induces relaxation. Disruption of the endothelium did not interfere with the vasodilator effect of WIN55212, whereas it completely inhibited Ach-induced vasodilation (Fig. 1A), proving the presence of pharmacologically active endothelium in our preparations.

Vascular Expression of CB_1R mRNA and Protein—The above-described functional data suggest that CB_1R s are present in rat gracilis arterioles. CB_1R mRNA expression was detected in rat gracilis arterioles and was compared with expression in the rat aorta. The relative expression levels of CB_1R s and *Gapdh* are shown in Fig. 1B. The presence of CB_1R protein and its localization have also been confirmed in the vascular tissue using a Western blot (Fig. 1C). Immunohistochemistry shows the localization of CB_1R in rat and mouse gracilis muscle tissue. CB_1R staining was not observed in the vessels prepared from CB_1R knock-out mice, which confirms the specificity of the antibody used in our studies (Fig. 1D).

Effects of the CB_1R Inhibitor O2050 (Neutral Antagonist) on Ang II-induced Vasoconstriction and the Effects of Vasodilators in Gracilis Arterioles—Both in rat and mouse gracilis arterioles, WIN55212, a CB_1R agonist, relaxed arterioles by approximately 15% (Fig. 2, A and B). The neutral antagonist of CB_1R O2050 had no significant effect on the basal tone of gracilis arterioles (Table 2) but completely inhibited the WIN55212-induced vasodilations (Fig. 2, A, B, and E). O2050 did not modulate endothelium-dependent and -independent vasodilatory responses; it had no significant effect on Ach-induced (Fig. 2, A and B) or sodium nitroprusside-induced (Fig. 2F) vasodilation of gracilis arterioles. Ang II caused concentration-dependent vasoconstriction (Fig. 2, C and D). CB_1R inhibition by O2050 significantly enhanced Ang II-induced vasoconstrictions both in isolated rat and mouse gracilis arterioles (Fig. 2, C and D). At 1 nM Ang II, O2050 augmented the constriction from 11.4 ± 2.1 to $46.7 \pm 6.5\%$ in rat gracilis arterioles. In rat gracilis arterioles, nonlinear curve-fitting showed that in the presence of the CB_1R inhibitor, O2050, the E_{\max} value of Ang II-induced contraction significantly increased from 30.3 ± 4.4 to $47.5 \pm 4.5\%$ ($p < 0.05$, $n = 5-6$), whereas changes in log EC_{50} did not reach the level of statistical significance (Fig. 2C). In mouse gracilis arterioles, in

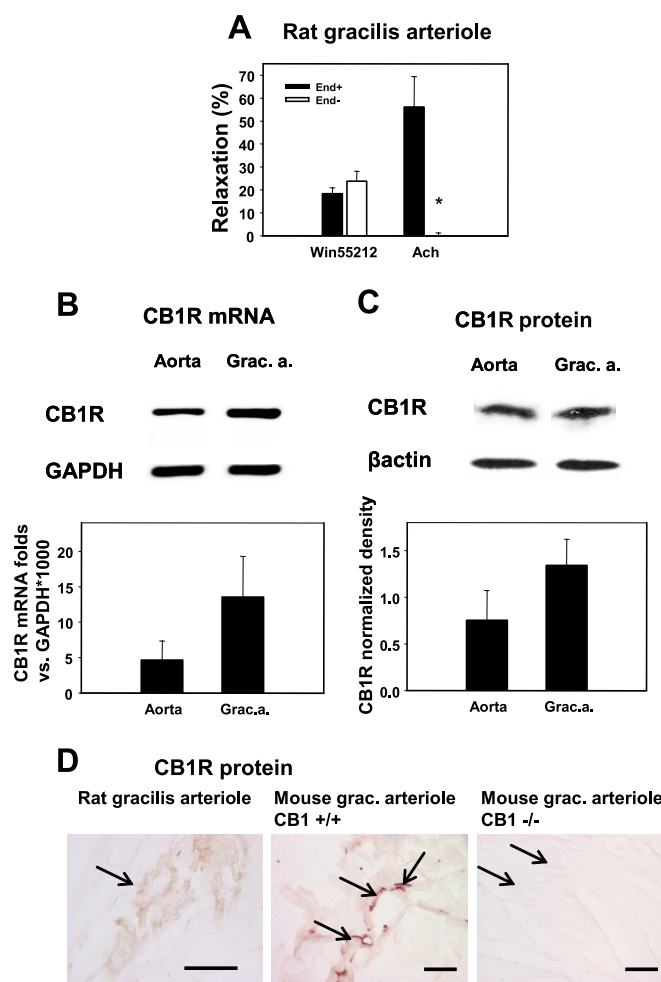


FIGURE 1. CB_1R receptor expression and function in rat skeletal muscle arterioles. A, relaxations induced in rat gracilis arteriole segments by the CB_1R agonist WIN 55212 (1 μ M) and by the endothelial relaxant Ach (10 μ M) as well as the effects of de-endothelization (5–5 segments) are shown. Values were calculated as percent change of diameter compared with control. Mean \pm S.E. values are shown. The asterisk indicates significant change of agonist-induced tone in response to inhibitor treatment ($p < 0.05$). B, shown is gel electrophoresis of the mRNA amplicons and relative quantification of mRNA expression. Messenger RNA expression of CB_1R (*Cnr1* gene) was normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, $n = 4$). Grac. a., gracilis arterioles. C, shown is Western blot detection of CB_1R protein from tissue homogenates. Expression of CB_1R protein in rat aorta and gracilis arterioles was quantitatively detected by densitometry ($n = 4$) of HRP-induced fluorescence. D, immunohistochemical localization of the CB_1R protein (arrows) in rat gracilis arterioles and in mouse gracilis vessels is shown. No staining was detected in CB_1R knock-out (–/–) mice. Left bar, 50 μ m. Bars in middle and right, 100 μ m.

the presence of O2050, the E_{\max} value also significantly increased from 24.4 ± 1.5 to $42.1 \pm 6.1\%$ ($p < 0.05$, $n = 4$, Fig. 2D), and changes in log EC_{50} were not significant. These data show that endocannabinoid release reduces the maximum of the Ang II contraction effect.

Effects of O2050 on Ang II-induced Vasoconstrictor Responses in CB_1R Knock-out and Wild Type Mice—To provide independent genetic evidence for the role of CB_1R s in the modulation of Ang II-induced vasoconstriction, the above angiometric tests were also performed in saphenous arteries of CB_1R -KO and corresponding wild type mice. Mouse saphenous arteries, similar in size to downstream rat gracilis arterioles, were pre-

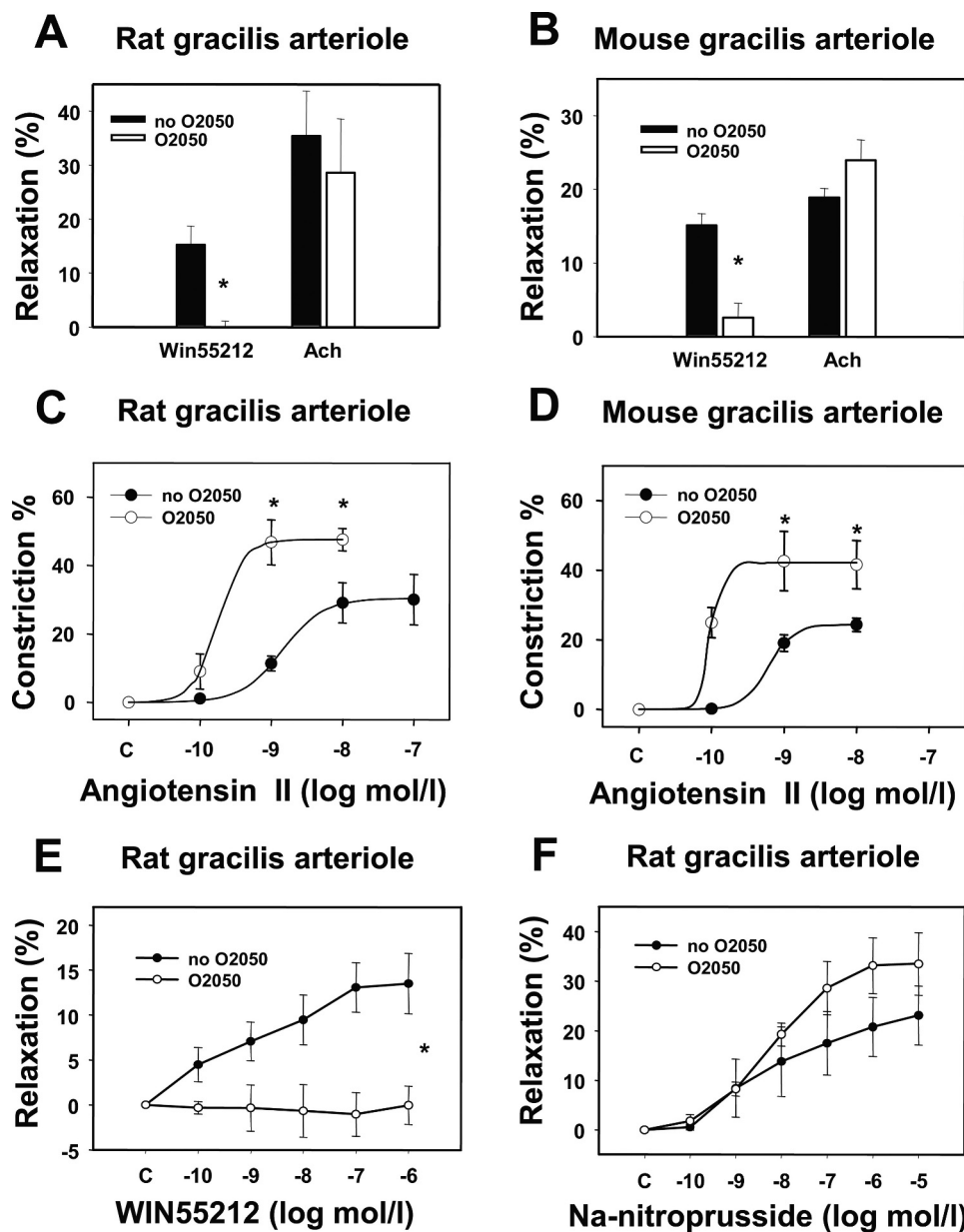


FIGURE 2. Effects of CB₁R inhibition on agonist-induced responses of gracilis arterioles. A and B, shown are the effects of the CB₁R antagonist O2050 (1 μ M) on the relaxations induced by the CB₁R agonist WIN 55212 (1 μ M) and by the endothelial relaxant Ach (10 μ M) in rat gracilis arteriole segments (A, $n = 4$) and in mouse gracilis arteriole segments (B, $n = 4$). Values were calculated as percent change of diameter from control. Mean \pm S.E. values are shown. The asterisk indicates significant change of agonist-induced tone in response to inhibitor treatment ($p < 0.05$). C and D, cumulative log-concentration-effect curves for Ang II were taken by pressure arteriography in rat (C, $n = 5-6$) and mouse (D, $n = 4$) gracilis arterioles. Effects of the CB₁R antagonist O2050 (1 μ M) are shown. E and F, shown are cumulative log-concentration-effect curves induced by WIN 55212 (0.1 nM–1 μ M) and by sodium nitroprusside (0.1 nM–10 μ M) in rat gracilis arterioles ($n = 4-7$). Effects of the CB₁R antagonist O2050 (1 μ M) are shown. Values were calculated as percent change of diameter from the incubated control. Mean \pm S.E. values are shown. The asterisk indicates significant change of vasoconstrictor-induced contraction in response to inhibitor treatment ($p < 0.05$). Data obtained with curve fitting method showed that in rat gracilis arterioles E_{\max} values were 30.3 ± 4.4 and $47.5 \pm 4.5\%$ ($p < 0.05$, $n = 5-6$) in the absence and in the presence of O2050, respectively. Log EC₅₀ values were -8.8 ± 0.2 and -9.7 ± 0.29 in the absence and presence of O2050, respectively. In mouse arterioles E_{\max} values were 24.3 ± 1.5 and $42.1 \pm 6.1\%$ in the absence and presence of O2050, respectively ($p < 0.05$, $n = 4$). Log EC₅₀ values were -9.2 ± 0.2 and -10.0 ± 3.4 in the absence and presence of O2050. Changes in EC₅₀ values were not significant.

pared to study this effect on segments from CB₁R-deficient and wild type mice. In saphenous arteries of wild type mice, O2050 alone did not change the basal tone (Table 2) but inhibited WIN55212-induced vasodilation (Fig. 3A, $p < 0.05$). This result is in accordance with data obtained in rat gracilis arterioles (shown above). However, WIN55212-induced vasodilation was entirely absent in CB₁R-deficient mice (Fig. 3B), suggesting that these vessels of wild type mice contain functionally relevant

CB₁Rs. Furthermore, in saphenous arteries of both CB₁R-deficient and wild type mice Ach-induced vasodilation was unaffected by CB₁R inhibition (Fig. 3, A and B), which is also in agreement with the above observations. Similar to the results obtained in gracilis arterioles (see Fig. 2, A and B), O2050 significantly increased Ang II-induced contractile responses in wild type mice by increasing Ang II-induced contraction (E_{\max} value was increased from 20.4 ± 3.7 to

TABLE 2
Effects of specific inhibitors on the basal vascular tone

Summary of the effects of specific inhibitors, CB₁ receptor antagonists O2050 ($n = 9$, 1 μM), AM251 ($n = 7$, 1 μM), and SR141716 (SR, $n = 5$, 1 μM), diacylglycerol lipase inhibitor THL ($n = 7$, 1 μM), AT₁R inhibitor candesartan (Cand, $n = 4$, 10 μM), and nitric-oxide synthase inhibitor LNA ($n = 4$, 10 μM) on the basal vascular tone of rat gracilis arterioles and effects of O2050 ($n = 4$, 1 μM) and THL, $n = 7$, 1 μM) on mouse saphenous arteries. Note that CB₁ receptor antagonist (inverse agonist) AM251 and NO synthase inhibitor LNA cause a significant elevation of the basal vascular tone, whereas the other inhibitors do not change the tone of these vessels in the applied doses. Data are the mean \pm S.E. values. Differences in diameter values with inhibitors from control were considered as significant if $p < 0.05$. NS, not significant.

Prepared segments	O2050	AM251	SR	THL	Cand	LNA
Gracilis arterioles						
Contraction %	0.06 \pm 2.3	4.82 \pm 1.2	2.17 \pm 2.1	7.36 \pm 5.2	2.11 \pm 1.4	11.0 \pm 5.1
n	9	7	5	7	4	4
Statistics	NS	$p < 0.05$	NS	NS	NS	$p < 0.05$
Saphenous arteries						
Contraction %	-3.3 \pm 1.3			4.71 \pm 3.9		
n	4			7		
Statistics	NS			NS		

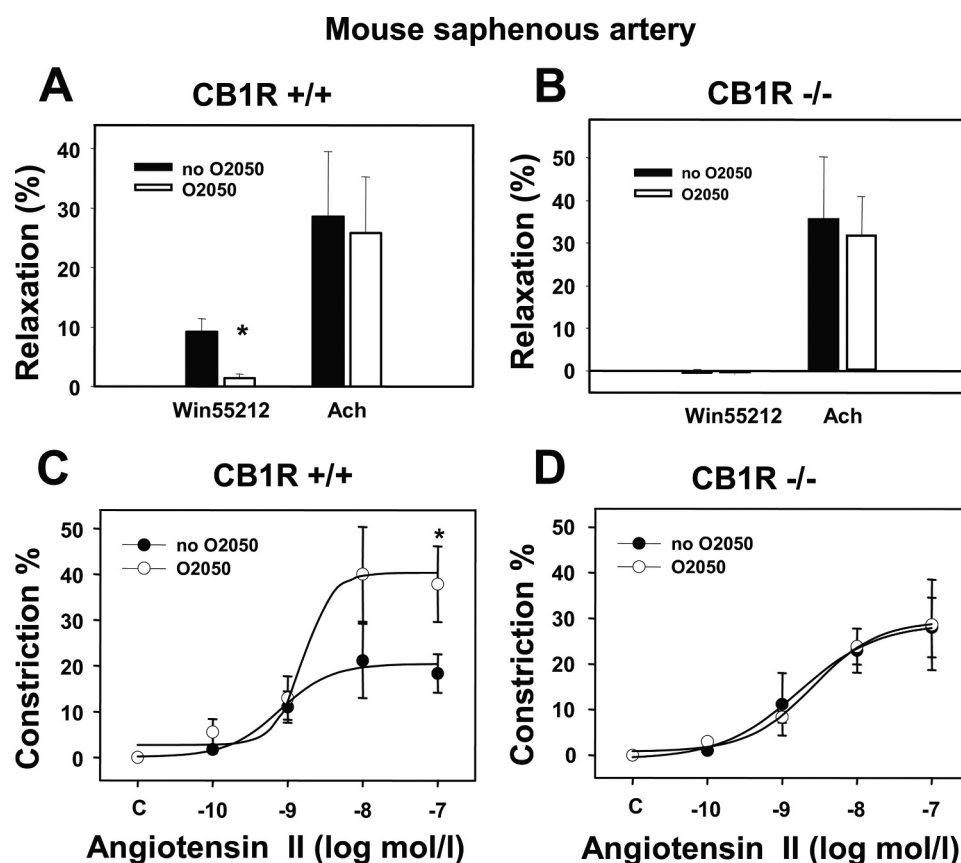


FIGURE 3. Comparison of agonist-induced responses in CB₁R knock-out and wild type mice. Vasodilations induced by the CB₁R agonist WIN55212 (1 μM) and by the endothelial vasorelaxant Ach (10 μM) as well as the effects of CB₁R inhibition by the O2050 (1 μM) are shown in saphenous arteries of wild type (A, +/+) and of CB₁R-deficient (B, -/-) mice ($n = 4-6$). C and D, shown is modulation of Ang II concentration-response curves by O2050 (1 μM) in the two mice strains ($n = 4-5$). Mean \pm S.E. values are shown. The asterisk indicates significant effect of agonist or antagonist, $p < 0.05$. Data obtained with curve-fitting method showed that in wild type mice E_{max} values were 20.4 ± 3.7 and $40.3 \pm 6.9\%$ ($p < 0.05$, $n = 4-5$), and log EC_{50} values were -9.1 ± 0.3 and -8.8 ± 0.4 M in the absence and in presence of O2050, respectively. In CB₁R-KO mice E_{max} values were 28.7 ± 7.1 and $29.4 \pm 6.4\%$ in the absence and presence of O2050, respectively. Changes in EC_{50} values were not significant.

$40.3 \pm 6.9\%$, $p < 0.05$, $n = 4-5$). However, O2050 had no significant effect on the log EC_{50} values of the Ang II response (-9.1 ± 0.3 and -8.8 ± 0.4 M, respectively; not significant, Fig. 3C). However, in contrast to wild type mice, the Ang II-induced vasoconstriction was not affected by the CB₁R inhibitor in CB₁R-KO mice (E_{max} values 28.7 ± 7.1 and $29.4 \pm 6.4\%$ in the absence and presence of O2050, respectively; Fig. 3D). These data provide genetic evidence for the role of CB₁R in the attenuation of Ang II-induced vasoconstriction in mouse saphenous arteries.

Effects of Inverse Agonists of CB₁R (SR141716 and AM251) on Ang II-induced Vasoconstriction and the Vasodilatory Responses in Gracilis Arterioles—SR141716 (rimonabant) and AM251 effectively inhibited WIN55212-induced vasodilation of rat gracilis arterioles (Fig. 4A). These inhibitors of CB₁R are “inverse agonists,” because they can inhibit the constitutive activity of CB₁Rs (15). In gracilis vessels, AM251 slightly elevated the basal tone (Table 2). This can indicate a slight vascular constitutive activity of the CB₁Rs in this preparation (on the other hand, the neutral antagonist, O2050, had no effect on the

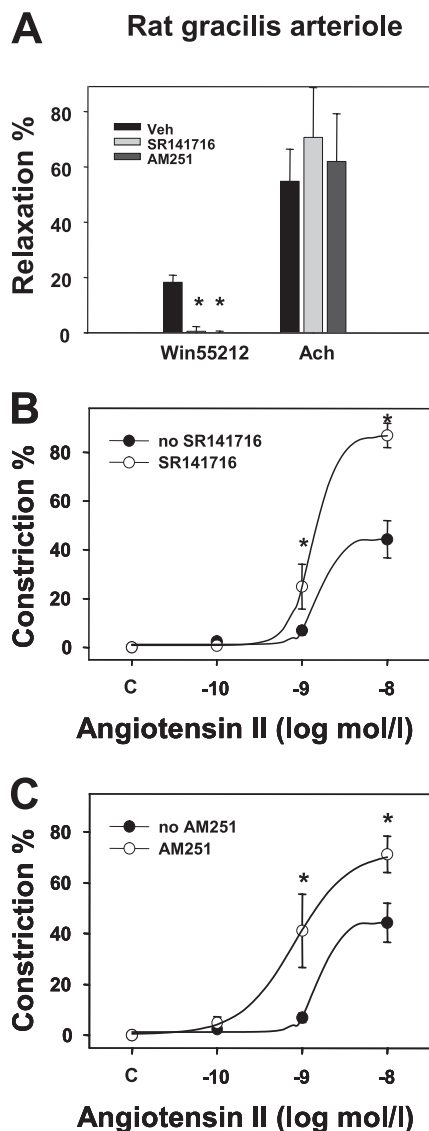


FIGURE 4. Effects of CB₁R inhibition on agonist-induced responses of gracilis arterioles. A, shown are the effects of the CB₁R antagonists AM251 (1 μ M) and SR141716 (1 μ M) on the relaxations induced by the CB₁R agonist WIN 55212 (1 μ M) and by the endothelial relaxant Ach (10 μ M) in rat gracilis arteriole segments (A, $n = 4-6$). Values were calculated as percent change of diameter from control. Mean \pm S.E. values are shown. Asterisks indicate significant change of agonist-induced tone in response to inhibitor treatment ($p < 0.05$). SR141716, rimonabant. B and C, shown are cumulative log-concentration-effect curves for Ang II taken by pressure arteriography in rat gracilis arterioles and the effects of the CB₁R antagonists SR141716 (B, $n = 5-6$) and AM251 (C, $n = 4-6$) on them. Values were calculated as percent change of diameter from incubated control. Mean \pm S.E. values are shown. Asterisks indicate significant changes in vasoconstrictor-induced contraction after inhibitor treatment ($p < 0.05$). Data obtained with the curve-fitting method showed that E_{\max} values were 44.3 ± 4.9 and $87.1 \pm 5.1\%$ ($p < 0.05$, $n = 5-6$), and $\log EC_{50}$ values were -8.8 ± 0.9 and -8.9 ± 0.3 M in the absence and presence of SR141716, respectively. In the presence of AM251, the E_{\max} value of the Ang II response was $72.3 \pm 6.4\%$ ($p < 0.05$ from control, $n = 4-6$). Changes in EC_{50} values were not significant.

basal tone, Table 2). However, similar to the effect of O2050 (Fig. 2, C and D), CB₁R inhibition with SR141716 and AM251 significantly enhanced Ang II-induced vasoconstrictions in isolated rat gracilis arterioles (Fig. 4, B and C). Nonlinear curve-fitting shows that SR141716 significantly increased the E_{\max}

value of the Ang II response from 44.3 ± 4.9 to $87.1 \pm 5.1\%$ ($p < 0.05$, $n = 5-6$), whereas $\log EC_{50}$ varied non-significantly from -8.8 ± 0.9 to -8.9 ± 0.3 M (Fig. 4B). Similar to the effect of SR141716, AM251 also significantly increased the E_{\max} value of the Ang II response to $72.3 \pm 6.4\%$ ($p < 0.05$, $n = 4-6$) without affecting its $\log EC_{50}$ value (Fig. 4C). These data show that CB₁R antagonists consequently augment the vasoconstrictor response of Ang II by increasing the efficacy of the agonist response without significantly affecting its potency. The differences in the kinetics of Ang II response in the presence of the different inhibitors may reflect their differential effects on constitutive CB₁R activity.

Effects of DAG Lipase Inhibition on Ang II-induced Vasoconstrictor Responses—DAG lipase inhibition using THL induced a slight (4–7%), but not significant, increase in the basal vascular tone (Table 2) and caused a significant enhancement of Ang II-induced vasoconstriction in rat gracilis arterioles (Fig. 5A, $p < 0.05$ at 100 nM Ang II) without significantly modulating the Ach-induced vasodilation (data not shown). These data suggest the role of DAG lipase in Ang II-induced endocannabinoid formation in these vessels. Nonlinear curve-fitting showed that in the presence of the THL, E_{\max} value significantly increased from 29.7 ± 4.9 to $47.0 \pm 7.6\%$ ($p < 0.05$, $n = 5-6$), and $\log EC_{50}$ varied from -8.9 ± 0.2 to -8.9 ± 0.2 M (statistically not significant, Fig. 5A). A similar effect of THL was observed in mouse saphenous arteries, where the E_{\max} value significantly increased from 37.1 ± 6.8 to $66.1 \pm 5.1\%$ ($p < 0.05$, $n = 4-5$) and $\log EC_{50}$ varied from -8.8 ± 0.5 to -8.8 ± 0.2 M (not significant, Fig. 5B). The effect of THL and O2050 on Ang II-induced vasoconstriction was not additive in rat gracilis arterioles (Fig. 5C), which is consistent with the role of DAG lipase in Ang II-induced endocannabinoid formation.

Effects of NO Synthase Inhibition and Endothelial Disruption on Endocannabinoid Modulation of Ang II Vasoconstriction—Inhibition of NO synthesis by N_{ω} -nitro-L-arginine significantly increased the basal tone of gracilis arterioles (by $\sim 10\%$, Table 2, $p < 0.05$). LNA also effectively inhibited Ach-induced vasodilation ($p < 0.05$), but it did not significantly modified Ang II-induced vasoconstriction and did not affect WIN55212-induced vasodilation (Fig. 6, A and B). Disruption of the endothelium did not interfere with the augmentation of the Ang II-induced vasoconstriction by O2050 (Fig. 6C). These data suggest that the CB₁R-mediated modulation of the Ang II-induced vasoconstriction is independent of NO production and endothelial function.

Effect of AT₁R Inhibitor Candesartan on Ang II-induced Vasoconstriction—Presence of the specific AT₁R blocker candesartan (10 μ M) in the bath inhibited the Ang II-induced (0.1–100 nM) vasoconstriction of rat gracilis arterioles. Maximum responses ($30.7 \pm 2.4\%$) were reduced to $3.8 \pm 1.9\%$ ($p < 0.05$), indicating that the Ang II-induced vasoconstriction was mediated by AT₁Rs (Fig. 7).

DISCUSSION

We previously proposed that Ang II can cause the release of 2-AG, an endocannabinoid, followed by paracrine or autocrine transactivation of cannabinoid receptors. Phospholipase C activation generates diacylglycerol from inositol lipids enriched in

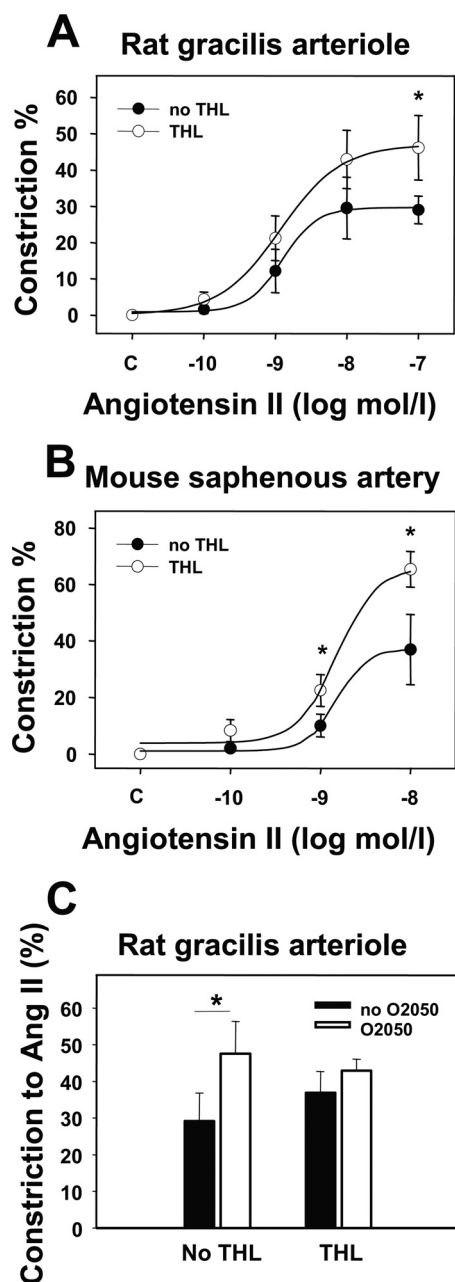


FIGURE 5. Effects of DAG lipase inhibition on Ang II-induced arteriolar tone. A and B, shown is the effect of the DAG lipase inhibitor THL (1 μ M) on the Ang II-induced vasoconstriction in rat gracilis arterioles (A, $n = 5-6$) and on mouse saphenous arteries (B, $n = 4-5$), taken by pressure arteriography. Values were calculated as percent change of diameter compared with control. Mean \pm S.E. values are shown. Asterisks indicate significant changes of vasoconstrictor-induced tone after inhibitor treatment ($p < 0.05$). C, shown is the effect of CB₁R inhibitor (O2050, 1 μ M) on the Ang II-induced (10 nM) vasoconstriction with and without THL pretreatment in rat gracilis arterioles (4-5 segments of each treatment). Mean \pm S.E. values are shown. Asterisks indicate significant changes of the vasoconstrictor effect in the presence of the inhibitor ($p < 0.05$). Data obtained with the curve-fitting method showed that E_{max} values were 29.7 ± 4.9 and $47.0 \pm 7.6\%$ ($p < 0.05$, $n = 5-6$), and log EC_{50} values were -8.9 ± 0.2 and -8.9 ± 0.2 M in the absence and presence of THL, respectively. In mouse saphenous arteries E_{max} values were 37.1 ± 6.8 and $66.1 \pm 5.1\%$ ($p < 0.05$, $n = 4-5$), and log EC_{50} values were -8.8 ± 0.5 and -8.8 ± 0.2 M in the absence and presence of THL, respectively. Changes in EC_{50} values were not significant.

arachidonic acid, which in turn can be converted to 2-AG by the DAG lipase (8, 15). This study provides the first direct demonstration of the physiological relevance of this mechanism in

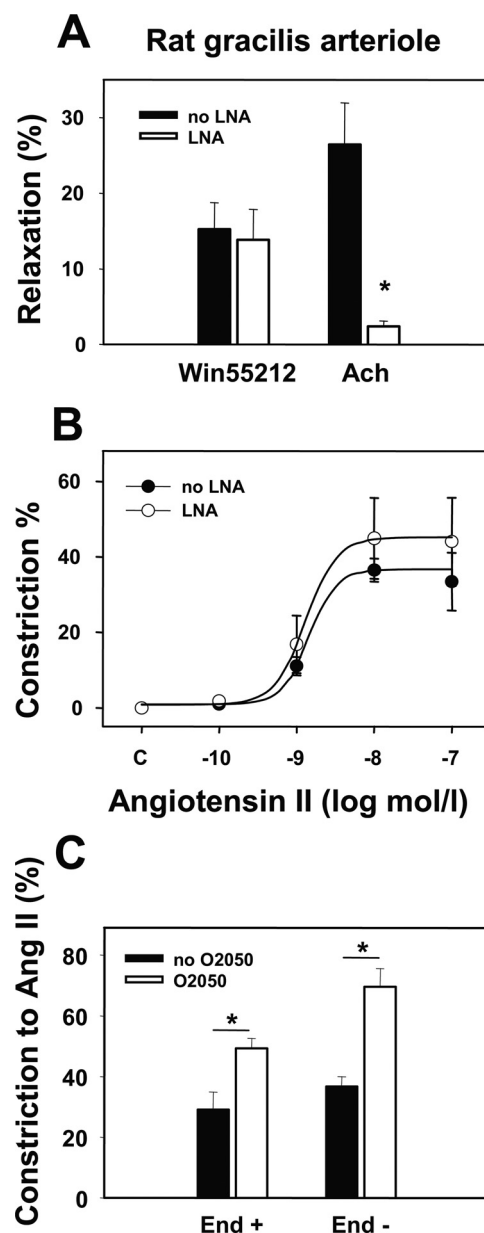


FIGURE 6. Endothelial contribution to agonist-induced responses. Data were obtained by pressure arteriography on rat gracilis arterioles. A, shown is the effect of the NO synthesis inhibitor LNA (10 μ M) on WIN55212 (1 μ M) and Ach (10 μ M)-induced vasodilations ($n = 4-5$). B, shown is the effect of pretreatment with LNA on the angiotensin II (Ang II)-induced vasoconstriction ($n = 4-5$). C, shown is the effect of the CB₁R antagonist O2050 (1 μ M) on the Ang II-induced (10 nM) vasoconstriction in segments with intact endothelium and after endothelial removal ($n = 4-5$). Values were calculated as percent change of diameter from control. Mean \pm S.E. values are shown. Asterisks indicate significant changes of vessel tone in response to inhibitor treatment ($p < 0.05$).

vascular tissues. It shows that endocannabinoid release can modulate the vasoconstrictor action of Ang II in resistance vessels by parallel activation of cannabinoid CB₁Rs. A physiological mechanism, exerting an inherent braking effect on the vasoconstrictor and acute hypertensiogenic activity of this important physiological endocrine and local agonist, has thus been first identified.

Exogenously applied cannabinoids have been shown to have complex cardiovascular effects both by modulating the auto-

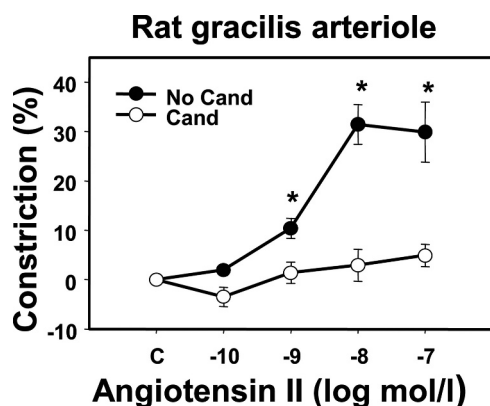


FIGURE 7. **AT₁R dependence of the effect of Ang II on arterial tone.** Effect of the specific AT₁R inhibitor candesartan (Cand, 10 μ M) on the Ang II-induced (0.1–100 nM) vasoconstriction in rat gracilis arterioles ($n = 4–5$). Mean \pm S.E. values are shown. Asterisks indicate significant effects of the inhibitor on Ang II-induced vasoconstriction ($p < 0.05$).

nomic outflow and also through their direct cardiac and vascular effects (14, 25–28). Targets of cannabinoids in the vascular tissue have been identified as endothelial and vascular smooth muscle cells and perivascular neuronal fibers (14, 27, 29–34). *In vivo* studies and studies in isolated vessel segments have shown that natural cannabinoids (e.g. anandamide, 2-AG, Δ^9 -tetrahydrocannabinol) and synthetic cannabinoid agonists (e.g. WIN55212) cause vasodilation in many vascular beds, such as in aorta, coronary, cerebral, and mesenteric arteries (27, 29, 30, 32, 33, 35–40). The vascular effects of endocannabinoids in different vessel types may involve CB₁R and Transient Receptor Potential Vanilloid (TRPV) receptors (26, 30, 37, 38) or other unidentified receptors (35, 36). In this study we observed substantial vasodilation to CB₁R agonist WIN55212 in gracilis arterioles and saphenous arteries (Figs. 2, A and B, and 3A). The action of this agonist was fully inhibited by CB₁R antagonists and was absent in the CB₁R knock-out mice, demonstrating the presence of functionally relevant CB₁Rs in these vessels (Figs. 3, A and B, and 4A). Similar to previous studies, we could confirm the vascular expression of the CB₁R in rodents (26, 34, 41, 42). We also found that the neutral antagonist O2050, which does not influence constitutive activity of CB₁Rs, did not modulate basal vascular tone. In contrast, the inverse agonist AM251, which also inhibits the constitutive activity of CB₁Rs (8), slightly increased the basal vascular tone of gracilis vessels.

Ang II is a potent vasoconstrictor (1, 18, 43–45), and its effect is mediated by AT₁Rs, as it was demonstrated in isolated vessels from several vascular beds (1, 45). In accordance with previous studies in gracilis arterioles by others (45), the vasoconstrictor effect of Ang II observed in this study was mediated by AT₁R, as candesartan, an AT₁R blocker, prevented this effect (Fig. 7). Our data demonstrate that the Ang II-induced vasoconstriction was augmented by the concomitant blockade of CB₁Rs in rat skeletal muscle resistance vessels, suggesting that activation of cannabinoid receptors substantially attenuates the vasoconstrictor effect of Ang II. This effect was shown in this study using three separate CB₁R inhibitors (O2050, SR141716, AM251) but was absent in vessels from CB₁R knock-out mice. The latter finding provided additional genetic evidence for the relevance of this mechanism. Although all three CB₁R inhibi-

tors had a marked effect on the Ang II-induced vasoconstriction, a neutral CB₁R inhibitor (O2050) or inverse agonists (SR141716, AM251) had no or minimal effects on the basal vascular tone, respectively, demonstrating that they interfere with the effects of Ang II-induced endocannabinoid production in the vessels. 2-AG release may attenuate the calcium response via multiple mechanisms (16, 40).

The difference in the kinetics of the Ang II-induced contractile response observed between gracilis and saphenous arteries, as seen in Figs. 2 and 3, showing the concentration-response to Ang II in gracilis arterioles shifted to the left, can be attributed to their different individual properties. Gracilis arterioles are smaller in size and more peripheral in location compared with saphenous arteries. It has been observed that in the same vascular bed smaller resistance vessels may show augmented metabolic responses compared with larger and conduit vessels, which is in accordance with their physiological role in the control of tissue perfusion (e.g. Ref. 17, 21). The stronger effect of CB₁R inhibition on the augmentation of the AT₁R response in gracilis arterioles can be attributed to larger AT₁R signaling-dependent augmentation of the contractile effects.

The inhibition of DAG lipase also enhanced the Ang II-induced vasoconstriction (Fig. 5) and diminished the modulating effect of the CB₁R antagonist (O2050) in these vessels. These data specifically point at 2-AG being the endocannabinoid, which modulates Ang II-induced vasoconstriction in rat skeletal muscle resistance vessels. The effect of Ang II on 2-AG production was previously detected in CHO cells using mass spectrometry (8). In vascular tissue, several types of cells can be sources of endocannabinoids, such as vascular endothelial cells, perivascular neurons, platelets, leukocytes, monocytes, and macrophages, etc. (14, 27, 29, 46). Agonist-induced release of 2-AG endocannabinoid has been detected from vascular endothelial cells (47) from rat aorta (48) and from bovine coronaries (46). In bovine coronary arteries metacholine-stimulated 2-AG release was suspected to be derived from endothelium (46). Earlier studies have also suggested that NO release and endothelium-dependent hyperpolarization contributes to cannabinoid-induced vasodilation (14, 35, 39, 49), and anandamide-induced vasodilation was found to be endothelium-independent (36). Our results suggest that CB₁R-mediated vasodilation in skeletal muscle resistance vessels is not mediated by the release of NO and independent of the endothelium. This conclusion is in accordance with earlier findings showing that endocannabinoid content of cerebral arteries was not influenced by endothelium denudation (40). Similar to the effect of Ang II observed by us, another G protein-coupled receptor agonist U46619 was shown to increase anandamide and 2-AG content of cerebral vessels (40) that was proposed to interfere with the vasoconstrictor effect of U46619, suggesting a possible physiological feedback mechanism exerted by endocannabinoids on the thromboxane receptor action (16, 40).

A growing number of studies suggest that vascular endocannabinoids may have important physiological control functions (16, 40, 46–48). However, our data provide the first evidence that in skeletal muscle resistance vessels, which have important functions in local blood flow and systemic blood pressure control processes, Ang II-induced endocannabinoid release signif-

icantly modulates the vascular tone. Additional studies are needed to elucidate the cellular mechanism of cannabinoid-induced CB₁R-mediated vasodilatory action in vascular tissue. CB₁R-mediated G_{i/o} signaling involves the inhibition of L-type calcium channels, which has been observed in cerebral vessels (11, 42). It has also been proposed that cannabinoid-induced vasodilation in coronary arteries involves the activation of calcium-activated K⁺ channels (36), which were also shown to be activated by atypical cannabinoid ligands in endothelial cells (50).

It is important to note that the mechanism observed by us in peripheral vessels shows an analogy with the retrograde synaptic inhibition exerted by the same substances on neuronal cells in the central nervous system (7, 13). This involves 2-AG-release induced by calcium signal generation and/or DAG formation, activation of CB₁Rs, and concomitant G_{i/o} activation (11).

In conclusion, our study reveals that Ang II-stimulated release of endocannabinoids from the vascular wall exerts a substantial relaxing effect on the vessels through activation of vascular smooth muscle cell CB₁Rs. This mechanism raises the possibility that, similar to endothelial NO-dependent vasodilation, endocannabinoid release from the vascular wall and CB₁R activation counteracts the vasoconstrictor effect of Ang II. Thus, in skeletal muscle resistance arteries, endocannabinoid release can serve as an endothelium-independent, important control mechanism of the vascular tone by the agonist-induced attenuation of the contraction.

Acknowledgments—We are grateful to Drs. Csaba Ádori, Attila Mócsai, and Péter Várnai (Semmelweis University, Budapest), Andreas Zimmer (University of Bonn) and István Katona (Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary) for helpful discussions and to Judit Rácz, Ildikó Oravec, Anikó Schulcz, and Edina Simon for expert assistance.

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