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

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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 tetrahydrocannabinol (THC) 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 AT1R is activation of Gq/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-

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2The 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-nitro-l-arginine; THL, tetrahydrodipstatin.
nabniln, the main active ingredient of the extracts of Cannabis sativa. Endocannabinoid receptors include CB1 cannabinoid receptors (CB_1Rs), which are characteristically present in neural tissues, and CB2 receptors (CB_2Rs), 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). CB_1Rs 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 CB2R activation in cells coexpressing both AT1 and CB2 receptors (8, 15). Moreover, we have shown that stimulation of AT1R,Rs and other Ga11 protein-coupled G protein-coupled receptors can cause 2-AG-mediated paracrine transactivation of CB2Rs (8). The Ang II-induced CB2R 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 CB2 receptors, we hypothesized that the vasoconstrictor effect of Ang II may be attenuated by vascular endocannabinoid formation and consequent CB2R 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, Hungary). 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, Cnr1tm1zim) 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 Nα-nitro-l-arginine (LNA) (a nitric oxide (NO)-synthase inhibitor) were purchased from Sigma. CB1R antagonists O2050 (6a,R10aR)-3-(1-methanesulfonylaminio-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

**TABLE 1**

**Geometric values of isolated arteries**

| Prepared segments | CD (μm) | PD (μm) | Tone (m%) |
|-------------------|---------|---------|-----------|
| Rat gracilis arteriole | 123.7 ± 5.4 | 152.9 ± 6.6 | 18.5 ± 1.7 38 |
| Mouse gracilis arteriole | 119.4 ± 7.0 | 135.8 ± 5.4 | 12.3 ± 1.8 5 |
| Mouse a. saphena (control mice) | 185.4 ± 15.0 | 216.7 ± 17.0 | 13.7 ± 3.3 10 |
| Mouse a. saphena (CB1 ko mice) | 179.4 ± 17.2 | 210.1 ± 21.4 | 13.9 ± 2.2 5 |
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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, and vasodilator capacity of rat gracilis arterioles (0.1–100 nM). The CB1R inhibitor O2050 was applied for at least 10 min before agonist and vasodilator capacity of rat gracilis arterioles (0.1–100 nM). The CB1R inhibitor 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. In these experiments CB1R agonist WIN55212 (1 μM) 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 μM). In similar protocols, responses to Ang II were also obtained with DAG lipase inhibition by THL (1 μM, 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 μM, 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 μM) 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/6). 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 segments 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 arteries 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 (ENSRNOG0000008223). Primers for Cnr1 were forward (GGAAGCTCAGCTGCTGACA) and reverse (ACAAAAAGACAGGGCTCACA) and for Gapdh were forward (CCTCGACCAACACATGCTTAG) and reverse (CAGTCTCTTGAGTGCGATG). 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 activity was developed by using the ABC method (Vector Laboratories, Burlingame, 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 arteries 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 ± S.E. values. Vascular responses are expressed as percent changes in vessel diameter from baseline values. Comparisons were made with one- or two-way analysis of variance tests followed by Tukey’s or Holm-Sidak post hoc tests. Vasoconstrictor responses were also analyzed with nonlinear curve-fitting (four-parameter logistic curves). Effective maximal responses (Emax) and half-maximal responses (half-effective concentration, EC50) 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 CB1R Exerts Vasodilatory Effects in Rat Gracilis Arterioles—In rat gracilis arterioles, WIN55212, a CB1R agonist, relaxed arterioles by 18.2 ± 2.5% (Fig. 1A), showing that CB1Rs are functionally present on these vessels and that their activation induces relaxation. Disruption of the endothelium did not interfere with the vasoconstrictor 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 CB1R mRNA and Protein—The above-described functional data suggest that CB1Rs are present in rat gracilis arterioles. CB1R mRNA expression was detected in rat gracilis arterioles and was compared with expression in the rat aorta. The relative expression levels of CB1R, mRNA and Gapdh are shown in Fig. 1B. The presence of CB1R protein and its localization have also been confirmed in the vascular tissue using a Western blot (Fig. 1C). Immunohistochemistry shows the localization of CB1R in rat and mouse gracilis muscle tissue. CB1R staining was not observed in the vessels prepared from CB1R knock-out mice, which confirms the specificity of the antibody used in our studies (Fig. 1D).

Effects of the CB1R 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 CB1R agonist, relaxed arterioles by approximately 15% (Fig. 2, A and B). The neutral antagonist of CB1R O2050 had no significant effect on the basal tone of gracilis arterioles (Table 2) but completely inhibited the WIN55212-modulated 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). CB1R 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 ± 6.5% in rat gracilis arterioles. In rat gracilis arterioles, nonlinear curve-fitting showed that in the presence of the CB1R inhibitor, O2050, the Emax value of Ang II-induced contraction significantly increased from 30.3 ± 4.4 to 47.5 ± 4.5% (p < 0.05, n = 5–6), whereas changes in log EC50 did not reach the level of statistical significance (Fig. 2C). In mouse gracilis arterioles, in the presence of O2050, the Emax value also significantly increased from 24.4 ± 1.5 to 42.1 ± 6.1% (p < 0.05, n = 4, Fig. 2D), and changes in log EC50 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 CB1R Knock-out and Wild Type Mice—To provide independent genetic evidence for the role of CB1Rs in the modulation of Ang II-induced vasoconstriction, the above angiometric tests were also performed in saphenous arteries of CB1R-KO and corresponding wild type mice. Mouse saphenous arteries, similar in size to downstream rat gracilis arterioles, were pre-

FIGURE 1. CB1R receptor expression and function in rat skeletal muscle arterioles. A, relaxations induced in rat gracilis arteriole segments by the CB1R agonist WIN55212 (1 μM) and by the endothelial relaxant Ach (10 μM) as well as the effects of de-endothelialization (5–5 segments) are shown. Values were calculated as percent change of diameter compared with control. Mean ± 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 CB1R (Cnr1 gene) was normalized to glyceraldehyde-3-phosphate dehydrogenase (Gapdh, n = 4). Grac. a., gracilis arterioles. C, shown is Western blot detection of CB1R protein from tissue homogenates. Expression of CB1R protein in rat aorta and gracilis arterioles was quantitatively detected by densitometry (n = 4) of HRP-induced fluorescence. D, immunohistochemical localization of the CB1R protein (arrows) in rat gracilis arterioles and in mouse gracilis vessels is shown. No staining was detected in CB1R knock-out (−/−) mice. Left bar, 50 μm. Bars in middle and right, 100 μm.
pared to study this effect on segments from CB₁-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 CB₁-deficient mice (Fig. 3B), suggesting that these vessels of wild type mice contain functionally relevant CB₁Rs. Furthermore, in saphenous arteries of both CB₁-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 ($E_{\text{max}}$ value was increased from 20.4 ± 3.7 to 30.3 ± 4.4 and 47.5 ± 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 $E_{\text{max}}$ values were 24.3 ± 1.5 and 42.1 ± 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.

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 μM) on the relaxations induced by the CB₁R agonist WIN55212 (1 μM) and by the endothelial relaxant Ach (10 μM) 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 ± 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 μM) are shown. E and F, shown are cumulative log-concentration-effect curves induced by WIN 55212 (0.1 nM–1 μM) and by sodium nitroprusside (0.1 nM–10 μM) in rat gracilis arterioles (n = 4–7). Effects of the CB₁R antagonist O₂050 (1 μM) are shown. Values were calculated as percent change of diameter from the incubated control. Mean ± 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 $E_{\text{max}}$ values were 30.3 ± 4.4 and 47.5 ± 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 $E_{\text{max}}$ values were 24.3 ± 1.5 and 42.1 ± 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.
However, O2050 had no significant effect on the logEC50 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 ± 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
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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 \( E_{\text{max}} \) value of the Ang II response from 44.3 ± 4.9 to 87.1 ± 5.1% (\( p < 0.05, n = 5–6 \)), whereas logEC50 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 \( E_{\text{max}} \) value of the Ang II response to 72.3 ± 6.4% (\( p < 0.05, n = 4–6 \)) without affecting its logEC50 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, \( E_{\text{max}} \) value significantly increased from 29.7 ± 4.9 to 47.0 ± 7.6% (\( p < 0.05, n = 5–6 \)), and log EC50 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 \( E_{\text{max}} \) value significantly increased from 37.1 ± 6.8 to 66.1 ± 5.1% (\( p < 0.05, n = 4–5 \)) and log EC50 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 L-arginine significantly increased the basal tone of gracilis arterioles (by −10%, Table 2, \( p < 0.05 \)). L-N monomethylarginine also effectively inhibited Ach-induced vasodilation (\( p < 0.05 \)), but it did not significantly modify 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 μM) in the bath inhibited the Ang II-induced (0.1–100 nM) vasoconstriction of rat gracilis arterioles. Maximum responses (30.7 ± 2.4%) were reduced to 3.8 ± 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
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 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-
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**FIGURE 7.** AT,R dependence of the effect of Ang II on arterial tone. Effect of the specific AT,R inhibitor candesartan (Cand, 10 μM) on the Ang II-induced (0.1–100 nM) vasoconstriction in rat gracilis arterioles (n = 4–5). Mean ± S.E. values are shown. Asterisks indicate significant effects of the inhibitor on Ang II-induced vasoconstriction (p < 0.05).

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 CB1R 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 AT1R, 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 inhibitors 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-
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significantly 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<sub>i/o</sub> 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<sup>⁺</sup> 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 CB<sub>1</sub>Rs, and concomitant G<sub>i/o</sub> 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 CB<sub>1</sub>Rs. This mechanism raises the possibility that, similar to endothelial NO-dependent vasodilatation, endocannabinoid release from the vascular wall and CB<sub>1</sub>R 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.

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