Introduction

Cannabinoid-1 (CB1) receptors, which bind with a major, biologically active component of marijuana, Δ9-tetrahydrocannabinol (THC), were first identified in 1990 by Matsuda et al. (1). Cannabinoid receptors have since been shown to have two subtypes, CB1 and CB2 receptors (2). CB1 receptors are expressed at high levels in the brain (1); and they are present in peripheral tissues, including heart tissue (3), vascular endothelium (4, 5), and smooth muscle cells (6). The expression of CB2 receptors has been identified in immune and hematopoietic cells (2), acting to modulate cytokine release, with biological implications for pathological conditions such as inflammation and chronic pain (7 – 9). Yoshihara et al. (10) reported that CB2 receptors, which physiologically inhibit the activation of capsaicin-sensitive afferent sensory nerves, might be involved in the clinical effects of

Full Paper

Anandamide Induces Endothelium-Dependent Vasoconstriction and CGRPergic Nerve–Mediated Vasodilatation in the Rat Mesenteric Vascular Bed

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Abstract. An endogenous cannabinoid anandamide (N-arachidonoylethanolamide) has been shown to cause vasodilatation in vitro and a brief vasoconstriction followed by prolonged depressor response in vivo. This study investigated the vascular effects of anandamide and underlying mechanisms in rat mesenteric vascular beds. In preparations with an intact endothelium and active tone, anandamide at low concentrations (0.1 – 1 nM) caused a concentration-dependent decrease in perfusion pressure due to vasodilatation, but at high concentrations (10 nM – 1 μM) elicited an initial and sharp increase in perfusion pressure due to vasoconstriction followed by long-lasting vasodilatation in a concentration-dependent manner. Treatment with SR141716A [cannabinoid-1 (CB1)-receptor antagonist] blunted both the vasoconstrictor and vasodilator responses. Also, removal of the endothelium and indomethacin (cyclooxygenase inhibitor), but not adrenergic denervation with 6-hydroxydopamine (adrenergic neurotoxin), markedly inhibited the vasoconstrictor response to anandamide, while these treatments did not affect vasodilatation. The vasodilatation, but not vasoconstriction, in response to anandamide was markedly attenuated by capsazepine [selective antagonist for transient receptor potential vanilloid-1 (TRPV1)], pretreatment with capsaicin [calcitonin gene–related peptide (CGRP)ergic-nerve depletor], or cold-storage denervation. These results suggest that in rat mesenteric vascular beds, anandamide causes CB1-receptor– and prostanoid-mediated endothelium-dependent vasoconstriction and perivascular capsaicin-sensitive CGRPergic nerve–mediated vasodilatation.

Keywords: anandamide, cannabinoid-1 receptor, transient receptor potential vanilloid-1, CGRPergic nerve, prostanoid
anti-asthmatic compounds. The fact that CB₁ receptors exist in the body resulted in the isolation of an endogenous ligand, the first endocannabinoid anandamide (N-arachidonoyl ethanolamide), which is the ethanolamide of arachidonic acid, from the porcine brain (11).

Subsequent studies have obtained evidence that anandamide is generated in the vascular endothelium (4), brain neurons, and other peripheral tissues. Moreover, anandamide has been shown to cause vasodilatation and modulate regional blood flow and arterial blood pressure and to reduce heart rate (12–15). Interestingly, a recent study has shown that an increase in the local concentration and life-span of anandamide in circulation is beneficial in cases of hypertension (15).

Randall et al. (12) reported that vasodilatation in response to anandamide was antagonized by the CB₁-receptor antagonist SR141716A in the isolated rat mesenteric artery, thus implying the involvement of CB₁ receptors in this response. Additionally, the hypotensive action of anandamide has been shown to be absent in mice lacking CB₁ receptors (16). However, Pratt et al. (17) and Wagner et al. (18) have shown that the vasodilator response to anandamide is not mediated by CB₁ receptors in the bovine coronary artery or rat mesenteric artery. Furthermore, Zygmunt et al. (19) and Ho and Hiley (20) reported that anandamide induces vasodilatation by activating transient receptor potential vanilloid type 1 receptor (TRPV1) on perivascular sensory nerves, causing the release of calcitonin gene–related peptide (CGRP). Additionally, anandamide-induced vasodilatation has been reported to be mediated by the release of nitric oxide (NO) from perfused renal arterial segments (4). Furthermore, Randall et al. (21) and Randall and Kendall (22) hypothesized that anandamide is produced by endothelial cells and acts as an endothelium-derived hyperpolarizing factor (EDHF). Thus, multiple explanations for anandamide-induced vascular responses have been proposed; however, the actual mechanism remains unknown.

Additionally, several studies demonstrated that anandamide produces endothelium-dependent vasodilatation as a result of its catabolism from arachidonic acid to vasodilator prostanoids (17, 23, 24). Some studies showed that THC, an exogenous ligand for CB₁ receptors, induced a vasoconstrictor response in rat mesenteric vascular beds (18) or in rabbit ear arteries (25). In addition, in anesthetized rats, both THC and anandamide have been shown to cause a brief pressor and prolonged depressor response (26), indicating that anandamide has cardiovascular effects similar to those of THC.

Therefore, the aim of this study is to investigate the mechanisms underlying anandamide-induced vascular responses in mesenteric resistance arteries of the rat.

Materials and Methods

Animals

Male Wistar rats (purchased from Shimizu Laboratory Supplies Co., Shizuoka), weighing 280–350 g, were used in this study. Animals were given food and water ad libitum. They were housed in the Animal Research Center of Okayama University at a controlled ambient temperature of 22°C ± 2°C with 50% ± 10% relative humidity and with a 12-h light / 12-h dark cycle (lights on at 8:00 a.m.). This study was carried out in accordance with the Guidelines for Animal Experiments at Okayama University Advanced Science Research Center, the Japanese Government Animal Protection and Management Law No. 105, and the Japanese Government Notification on Feeding and Safekeeping of Animals No. 6. Every effort was made to minimize the number of animals used and their suffering.

Perfusion of mesenteric vascular beds

The animal was anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), and the mesenteric vascular bed was isolated and prepared for perfusion as described previously (27, 28). Then the rats were killed by rupturing the diaphragm and exsanguinations. The isolated mesenteric vascular bed was placed on a water-jacketed organ bath maintained at 37°C and perfused with Krebs solution at a constant flow rate of 5 mL/min with a peristaltic pump and superfused with the same solution at a rate of 0.5 mL/min to prevent drying. The Krebs solution was bubbled with a mixture of 95% O₂ plus 5% CO₂ before passage through a warming coil maintained at 37°C. The modified Krebs solution had the following composition: 119.0 mM NaCl, 4.7 mM KCl, 2.4 mM CaCl₂, 1.2 mM MgSO₄, 25.0 mM NaHCO₃, 1.2 mM KH₂PO₄, 0.03 mM EDTA-2Na, and 11.1 mM glucose (pH 7.4). Changes in the perfusion pressure were measured with a pressure transducer (model TP-400T; Nihon Kohden, Tokyo) and recorded using a pen recorder (model U-228; Nippon Denshi Kagaku, Tokyo).
**Periarterial nerve stimulation and bolus injection of agonists**

Periarterial nerve stimulation (PNS) was applied for 30 s using bipolar platinum ring electrodes placed around the superior mesenteric artery. Rectangular pulses of 1 ms and a supramaximal voltage (50 V) were applied at 1 Hz using an electronic stimulator (model SEN 3301, Nihon Kohden).

Some agonists such as acetylcholine and CGRP were injected directly into the perfusate proximal to the arterial cannula with an infusion pump (model 975; Harvard Apparatus, Holliston, MA, USA). A volume of 100 μL was injected over a period of 12 s.

**Perfusion of anandamide**

After the basal perfusion pressure had been allowed to stabilize, preparations were perfused with Krebs solution containing methoxamine (α1-adrenoceptor agonist) at a concentration of 2 – 15 μM to induce submaximal vasoconstriction. After stabilization of the elevated perfusion pressure, the preparations were subjected to perfusion of anandamide. The Krebs solution containing methoxamine and anandamide at a final concentration of 0.1, 1, 10, 100, or 1000 nM was perfused for 1 min.

At the end of each experiment, the preparations were perfused with 100 μM papaverine to induce complete relaxation. Vasodilator activity is expressed as a percentage of the perfusion pressure at maximum relaxation induced by papaverine. Vasoconstrictor responses were expressed as a percentage of the perfusion pressure before anandamide perfusion.

**Chemical removal of vascular endothelium**

To remove the vascular endothelium, preparations with resting tone were perfused with a 1.8 mg/mL solution of sodium deoxycholate in saline for 30 s as described by Takenaga et al. (29). This caused a transient increase (20 – 30 mmHg) in perfusion pressure. The preparations were then rinsed with sodium deoxycholate-free Krebs solution for 40 min. After the preparations were contracted by perfusion with Krebs solution containing methoxamine, chemical removal of the endothelium was assessed by the lack of a relaxant effect after the injection of 1 nmol acetylcholine.

**Cold-storage denervation, chemical adrenergic denervation with 6-hydroxydopamine, and CGRPergic nerve depletion with capsaicin**

Cold-storage denervation was achieved by storing isolated mesenteric vascular beds in cold Krebs solution at 4°C for 72 h according to the method described by Kawasaki et al. (28). To determine the intact responsiveness of the smooth muscle, a bolus of acetylcholine or CGRP was injected to cause vasodilatation. Successful denervation of periarterial nerves was confirmed by a lack of PNS-induced vasoconstriction (8 and 12 Hz) and vasodilatation (1 Hz).

In vitro adrenergic denervation was carried out by incubation with 6-hydroxydopamine as described by Zhang et al. (30). The isolated mesenteric vascular bed was incubated in Krebs solution containing 6-hydroxydopamine (2 mM) for 20 min twice with a 30-min interval in 6-hydroxydopamine-free Krebs solution. Successful denervation of adrenergic nerves was confirmed by a lack of PNS-induced vasoconstriction (8 and 12 Hz) in the resting tension.

In vitro depletion of CGRPergic nerves was performed according to the method described by Kawasaki et al. (27, 31). The isolated mesenteric vascular bed was perfused with Krebs solution containing capsaicin (1 μM) for 20 min and then rinsed with capsaicin-free Krebs solution. After being rinsed for 30 min, the preparation was contracted by perfusion with Krebs solution containing methoxamine (2 – 5 μM). Successful loss of CGRPergic nerve function was confirmed by the lack of a relaxant effect on PNS (1 Hz).

**Experimental protocols**

An active tone was produced by methoxamine (7 μM), and after the elevated perfusion pressure stabilized, Krebs solution containing methoxamine and the final concentration of anandamide was perfused for 1 min as a control. To assess the underlying mechanisms of the vascular effect of anandamide, the effects of various antagonists and inhibitors were examined. In preparations with an intact endothelium, the Krebs solution containing methoxamine and 3 or 5 μM SR141716A (CB1-receptor antagonist), 1 or 5 μM capsazepine (TRPV1 antagonist), or 1 or 10 μM indomethacin (cyclooxygenase inhibitor) was perfused to produce active tone and then Krebs solution containing methoxamine, the final concentration of anandamide and SR141716A, capsazepine, or indomethacin was perfused for 1 min.

To investigate the influence of the endothelium, the anandamide-induced vascular responses were examined in preparations without an endothelium. In these preparations, active tone was produced by perfusion with Krebs solution containing methoxamine (2 μM).

In another series of experiments, the vascular effect of anandamide was examined in preparations with an intact endothelium and treated with cold-storage, 6-hydroxydopamine, or capsaicin.

**Statistical analysis**

All experimental results were presented as the mean ± S.E.M. The statistical analysis was performed...
with the Student’s unpaired t-test. A value of $P < 0.05$ was considered statistically significant.

**Drugs**
The following drugs were used: acetylcholine chloride (Daiichi Sankyo, Tokyo; anandamide (Alexis Biochemicals, San Diego, CA, USA); capsaicin, capsazepine, 6-hydroxydopamine hydrobromide, sodium deoxycholate (Sigma-Aldrich Japan, Tokyo); indomethacin (Nacalai Tesque, Tokyo), methoxamine hydrochloride (Nihon Shinyaku, Kyoto); papaverine hydrochloride (Dainippon Sumitomo, Osaka); rat CGRP (Peptide Institute, Osaka); and SR141716A \( \text{N}(\text{piperidine-1-yl})-5-(4\text{chlorophenyl})-1-(2,4\text{dichlorophenyl})-4\text{methyl-1H-pyrazole-3-carboxamide hydrochloride} \), gift from Sanofi-Aventis Recherche & Développement, Montpellier, France. All drugs, except for capsaicin and sodium deoxycholate, were dissolved in 99.5% ethanol and diluted with Krebs solution. Capsaicin was dissolved in 50% ethanol and diluted with Krebs solution (final alcohol concentration, 0.4 mg/mL). Sodium deoxycholate was dissolved in 0.9% saline. Acetylcholine and rat CGRP were diluted with Krebs solution containing 2 – 15 μM methoxamine.

**Results**

**Vascular responses to anandamide**

In the preparation with an intact endothelium and active tone produced by perfusion with Krebs solution containing 7 μM methoxamine, PNS at 1 Hz caused a transient increase in perfusion pressure due to vasoconstriction followed by a long-lasting decrease in perfusion pressure due to vasodilatation, as shown in Fig. 1. The transient vasoconstrictor response has been shown to be mediated by adrenergic nerves (32), and the long-lasting vasodilatation is mediated by CGRPergic nerves (27, 29). The injection of acetylcholine caused a sharp drop in perfusion pressure, due to vasodilatation (Fig. 1A).

In this preparation, perfusion with anandamide at lower concentrations (0.1 – 1 nM) for 1 min induced vasodilatation (Fig. 1: A and C), while higher concentrations (10 – 1000 nM) caused a biphasic vascular response; the first phase (Phase 1) being a transient vasoconstriction and the second phase (Phase 2), a long-lasting vasodilatation (Fig. 1), which lasted for 5 – 10 min before a return to the pre-perfusion level within 20 min. The anandamide-induced phase-1 and 2 responses were concentration-dependent (Fig. 1: B and C).

**Effect of removing the endothelium on vascular responses to anandamide**

The concentration of methoxamine required to raise the tone was reduced to 2 μM after denudation, since removing the endothelium augmented methoxamine-induced vasoconstriction (33). In this preparation, the acetylcholine-induced vasodilatation was abolished, indicating that the endothelium was successfully removed.
(Fig. 2A). However, PNS (1 Hz) induced an initial small vasoconstriction followed by vasodilatation (Fig. 2A).

As shown in Fig. 2A, in preparations without the endothelium, anandamide at 10 – 100 nM did not induce vasoconstriction (Fig. 2B), while anandamide at 1 μM caused a small vasoconstriction. However, the vasodilator response to anandamide was not affected by endothelium removal, as shown in Fig. 2, A and C.

**Effects of SR141716A and capsazepine on vascular responses to anandamide**

As shown in Fig. 3A, in preparations with intact endothelium, vasoconstrictor responses to anandamide at concentrations of 100 and 1000 nM were markedly attenuated by SR141716A (3 and 5 μM). In the presence of SR141716A (3 and 5 μM), anandamide at 1000 nM caused a small vasoconstriction. Also, SR141716A (3 and 5 μM) significantly attenuated vasodilator responses to anandamide at higher concentrations (100 – 1000 nM), but not lower concentrations (0.1 – 1 nM) (Fig. 3B).

As shown in Fig. 3D, capsazepine (1 and 5 μM) concentration-dependently inhibited vasodilatation in response to anandamide (10 – 1000 nM), but it did not affect the vasoconstrictor response to anandamide at any of concentrations (Fig. 3C).

**Effects of cold-storage denervation and chemical adrenergic denervation on vascular responses to anandamide perfusion**

In preparations with an intact endothelium and resting tone, PNS at 8 and 12 Hz caused a sharp increase in perfusion pressure due to vasoconstriction (data not shown). The PNS-induced vasoconstrictor response has been shown to be due to stimulation of vascular adrenergic nerves (34, 35). In preparations subjected to cold-storage, PNS induced neither a vasoconstrictor response at the resting tone (8 and 12 Hz) nor a vasodilator response at the active tone (1 Hz) (data not shown), indicating that the perivascular nerves were effectively denervated. In this preparation, anandamide produced a concentration-dependent vasoconstriction similar to control responses (Fig. 4A). However, anandamide-induced vasodilatation was markedly attenuated by cold-storage denervation (Fig. 4B).

Treatment with 6-hydroxydopamine abolished vasoconstrictor responses to PNS (8 and 12 Hz) at resting tone, but did not affect the vasodilator response to PNS (1 Hz) at active tone (data not shown). It has been shown that the release of noradrenaline and vasoconstriction evoked by PNS (12 Hz) were markedly reduced after 6-hydroxydopamine treatment (32), indicating selective...
Anandamide-Induced Vascular Responses

adrenergic denervation. As shown in Fig. 4, C and D, neither the vasoconstriction nor vasodilatation were affected by adrenergic denervation with 6-hydroxydopamine.

Effects of capsaicin on vasculature responses to anandamide

In perfused mesenteric vascular beds with an intact endothelium, capsaicin treatment abolished the vasodilator response to PNS (1 Hz) without affecting the vasoconstrictor response to PNS (1 Hz), indicating the successful depletion of periarterial CGRPergic nerves (Fig. 5A). In this preparation, capsaicin treatment did not affect the vasoconstrictor response to anandamide at any of concentrations (Fig. 5B). However, vasodilator response to anandamide (10, 100, and 1000 nM) was markedly attenuated by capsaicin treatment, as shown in Fig. 5C.

Effect of indomethacin on vascular responses to anandamide

In preparations with an intact endothelium, indomethacin at concentrations of 1 and 10 μM significantly inhibited the anandamide (100 and 1000 nM)-induced vasoconstriction in a concentration-dependent manner (Fig. 6: A and B). However, indomethacin did not affect the vasodilator response to anandamide (Fig. 6: A and C).

Discussion

The present study demonstrated that anandamide caused a concentration-dependent vascular response consisting of two phases, a transient and sharp vasoconstriction followed by a long-lasting vasodilatation, in the rat mesenteric vascular bed. Previous studies in vitro have found that anandamide caused vasodilatation in isolated blood vessels including cat cerebral artery (6), bovine coronary artery (17), rat hepatic artery (17), rat mesenteric artery (20 – 22, 37), and rat mesenteric artery (20 – 22, 37), but no vasoconstrictor response. A previous study in vivo using anesthetized rats showed that anandamide caused a brief pressor and prolonged depressor response (26). This is in accordance with the present findings that anandamide induced vaso-
Fig. 4. Effect of cold-storage denervation (4°C for 72 h) (A and B) and 6-hydroxydopamine pre-treatment (C and D) on vascular responses to anandamide in rat mesenteric vascular beds with an intact endothelium (E+) and active tone. Each point indicates the mean ± S.E.M. *P < 0.05, **P < 0.01, compared with the control.

Fig. 5. A typical recording (A) and line graphs (B and C) showing effects of capsaicin on vascular responses to anandamide in rat mesenteric vascular beds with an intact endothelium (E+) and active tone. In panel A, solid circle and inverted triangle indicate the injection of acetylcholine (ACh) and periarterial nerve stimulation (PNS), respectively. PPV, perfusion with papaverine. In panels B and C, phase 1 and phase 2 show vasoconstrictor and vasodilator responses to anandamide, respectively. Each point indicates the mean ± S.E.M. **P < 0.01, compared with the control.
constrictor and vasodilator responses in the rat mesenteric vascular bed.

A major finding of this study is that the anandamide-induced vasoconstriction was almost abolished when the endothelium was removed, suggesting that the initial response is dependent on an intact endothelium. Furthermore, SR141716A, a CB1-receptor antagonist, markedly inhibited the vasoconstriction. Therefore, it is likely that the vasoconstrictor response to anandamide is mediated by the activation of CB1 receptors in the endothelium. Additionally, the present study showed that the cyclooxygenase inhibitor, indomethacin, concentration-dependently inhibited the vasoconstriction. Thus, it appears that the vasoconstrictor response to anandamide is mediated by the activation of CB1 receptors in the endothelium. Moreover, SR141716A, a CB1-receptor antagonist, markedly inhibited the vasoconstriction. Therefore, it seems likely that anandamide has a direct action on the vascular smooth muscle to induce vasoconstriction.

It has been reported that THC induced vasoconstriction in a perfused artery in the ear of rabbits through the release of noradrenaline from adrenergic nerve terminals (25). However, in the present study, neither cold-storage denervation nor selective destruction of perivascular adrenergic neurons by 6-hydroxydopamine affected the vasoconstriction, suggesting that perivascular sympathetic adrenergic nerves are not responsible for the vasoconstrictor response to anandamide.

The present study also demonstrated that anandamide causes long-lasting vasodilatation in rat mesenteric resistance artery, which was significantly inhibited by SR141716A and capsazepine, a TRPV1 antagonist.
Therefore, these findings suggest that anandamide causes vasodilatation via the activation of both CB1 receptors and TRPV1. This notion is supported by previous findings that anandamide caused vasodilatation through the stimulation of TRPV1 and CB1 receptors (37). CB1 receptors (4, 5) and TRPV1 (40) have been shown to be located on the vascular endothelium. However, the present study showed that anandamide-induced vasodilatation is not affected by the endothelium removal. Therefore, it is unlikely that CB1 receptors and TRPV1 located on the vascular endothelium are involved in the vasodilator response to anandamide in the rat mesenteric artery.

There is strong evidence that vascular tone is regulated not only by sympathetic adrenergic vasoconstrictor nerves but also by non-adrenergic non-cholinergic vasodilator nerves in various species (27, 41 – 44). We previously reported that CGRPergic nerves, releasing a potent vasodilator neuropeptide, CGRP, were the main non-adrenergic non-cholinergic nerves innervating the rat mesenteric artery (27). Capsaicin, a TRPV1 agonist, has been shown to release CGRP from primary sensory neurons (45), which leads to the depletion of capsaicin-sensitive primary nerves when employed at a concentration above 1 µM. In the present study, capsaicin treatment, cold-storage denervation, and capsaizpine resulted in more marked inhibition of anandamide-induced vasodilatation than SR141716A treatment, suggesting that the vasodilator response to anandamide is sensitive to capsaicin and that capsaicin-sensitive nerves are mainly responsible for the response via TRPV1. Furthermore, Eguchi et al. (46) reported that TRPV1 was distributed in CGRPergic nerves of the rat mesenteric artery. In addition, anandamide has been shown to release CGRP via activation of TRPV1 in rat dorsal root ganglia (47). Therefore, it is likely that anandamide induces vasodilatation by activating TRPV1 on CGRPergic nerves innervating rat mesenteric arteries. In contrast, Ishac et al. (48) reported that anandamide inhibits the exocytotic release of noradrenaline by presynaptic CB1 receptors in peripheral adrenergic nerves. However, this was not the case in the present study, since chemical adrenergic denervation with 6-hydroxydopamine did not alter the vasoconstrictor and vasodilator responses to anandamide. It seems likely that the activation of CB1 receptors in CGRPergic nerves is also involved in the vasodilator responses to anandamide. However, the distribution and function of CB1 receptors in CGRPergic nerves need to be clarified.

In conclusion, these results suggest that anandamide causes endothelium-dependent vasoconstriction, which is mediated by CB1 receptors on the vascular endothelium and vasoconstrictor prostanoids. Also, it is suggested that perivascular capsaicin-sensitive CGRPergic nerves act-

ing via TRPV1 in rat mesenteric vascular beds mainly mediate anandamide-induced vasodilatation.

References
16. Ledent C, Valverde O, Cossu G, Petitet F, Aubert JF, Beslot F,


