Mesenteric Vasodilation Mediated by Endothelial Anandamide Receptors

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Abstract—Cannabinoids, including the endogenous ligand anandamide (arachidonyl ethanolamide), elicit pronounced hypotension in rats via activation of peripherally located CB1 cannabinoid receptors, which have been also implicated in endotoxin (lipopolysaccharide [LPS])-induced hypotension. The present study was designed to test the role of vascular CB1 receptors in cannabinoid- and endotoxin-induced mesenteric vasodilation. In the isolated, buffer-perfused rat mesenteric arterial bed precontracted with phenylephrine, anandamide induced long-lasting (up to 60 minutes) dose-dependent vasodilation (ED_{50}: 79±3 nmol; maximal relaxation: 77±2%), inhibited by 0.5 to 5.0 μmol/L of the selective CB1 receptor antagonist SR141716A. Low doses of the calcium ionophore ionomycin also caused mesenteric vasodilation inhibited by SR141716A. The metabolically stable analogue R-methanandamide elicited mesenteric vasodilation (ED_{50}: 286±29 nmol), whereas the potent synthetic CB1 receptor agonists WIN 55212-2 and HU-210 caused no change in vascular tone or only a minor dilator effect not affected by SR141716A, respectively. The endogenous ligand 2-arachidonyl glycerol caused no change in vascular tone, whereas Δ^9-tetrahydrocannabinol and arachidonic acid caused mesenteric vasoconstriction. After endothelial denudation, the dilator response to anandamide was slightly reduced and was no longer inhibited by SR141716A. In preparations from LPS-pretreated rats, SR141716A alone caused a significant and prolonged increase in perfusion pressure, whereas it had no such effect in control preparations perfused in vitro with or without LPS or after endothelial denudation in preparations from rats pretreated with LPS. We conclude that anandamide-induced mesenteric vasodilation is mediated by an endothelially located SR141716A-sensitive "anandamide receptor" distinct from CB1 cannabinoid receptors and that activation of such receptors by an endocannabinoid, possibly anandamide, contributes to LPS-induced mesenteric vasodilation in vivo. **(*Hypertension*. 1999;33[part II]:429-434.)**

Key Words: vasodilation ■ cannabinoids ■ anandamide ■ endotoxin

Endogenous cannabinoids (or endocannabinoids) represent a novel class of lipid ligands that share receptor binding sites with plant-derived cannabinoids, such as Δ^9-tetrahydrocannabinol (THC), and that mimic the neurobehavioral effects of the latter.[1] Two receptors have been identified by molecular cloning that can recognize cannabinoids with high affinity: the CB1 receptor is present primarily in the brain[2] but also in some peripheral tissues[3,4] and the CB2 receptor is expressed by cells of the immune system.[5] The mRNA of a splice variant of the CB1 receptor, CB1A, has also been identified.[3] Two endocannabinoids have been characterized in some detail: arachidonyl ethanolamide, or anandamide,[6] and 2-arachidonyl glyceride, or 2-AG.[7,8] Neurons in the brain can synthesize anandamide[9] and 2-AG,[10] and both substances have been shown to influence central neural functions, such as long-term potentiation.[10,11] Peripheral tissues also contain anandamide[12–14] and 2-AG.[7,15,16]

Plant-derived cannabinoids can produce cardiovascular effects, including hypotension,[17] that also can be elicited by anandamide[18–20] and 2-AG.[16] Although at first the hypotensive effect of THC was thought to result from centrally mediated sympathoinhibition,[17] more recent evidence implicates peripheral sites of action, such as receptors located on sympathetic nerve terminals,[4,19,21] receptors located in vascular tissue, or both.[16,20,22] In a recent study, the hypotensive potency of cannabinoid agonists, including anandamide, showed a strong positive correlation with the binding affinity of the same ligands to the brain cannabinoid receptor.[22] Furthermore, the CB1 receptor antagonist SR141716A inhibited the neurobehavioral[23] and hypotensive effects of cannabinoids[22] with similar potency. This suggests that cannabinoid-induced hypotension in anesthetized rats is mediated by CB1 receptors.[22]

We recently reported that SR141716A antagonized the hypotension of hemorrhagic[14] and endotoxic[16] shock in rats, conditions that also induced circulating macrophages to generate anandamide[14,16] and platelets to generate 2-AG.[16] When isolated from animals in shock, these blood cells elicited SR141716A-sensitive hypotension in normal recipient rats, which suggested that macrophage- and platelet-

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derived endocannabinoids are likely contributors to shock-related hypotension.[14,16] Although activated macrophages and platelets are known to adhere to the vascular wall, the mechanism by which mediators generated by these cells elicit relaxation of vascular smooth muscle and the potential additional role of the vascular endothelium have remained unclear. This is an important question because there is evidence that endocannabinoids may also be generated in the vascular endothelium.[13,15] Furthermore, findings in the isolated perfused rat mesenteric arterial bed have led to the proposal that the elusive endothelium-derived hyperpolarizing factor may be an endocannabinoid,[24,25] although this proposal has been challenged.[26–28] The purpose of the present study was to test the role of CB1 receptors and of the vascular endothelium in cannabinoid- and endotoxin-induced mesenteric vasodilation.

Methods

Preparation of Rat Isolated Buffer-Perfused Mesenteric Arterial Bed

Male Sprague-Dawley rats (300 to 350 g) anesthetized with ether were laparotomized, and a second-order branch of the superior mesenteric artery was cannulated using a PE60 polyethylene cannula. The mesenterium was isolated, separated from the intestines, and placed in a water-jacketed perfusion chamber maintained at 37°C.[29] The preparation was perfused at 2 mL/min with Krebs-Henseleit solution pregassed with 95%O_2/5%CO_2, with a peristaltic pump (Rainin). Perfusion pressure was monitored via a T tube inserted between the pump and the inflow cannula and connected to a pressure transducer (Abbott) and physiograph (Astromed). After a 30-minute equilibration period, the perfusion pressure was raised from 30 to 40 mm Hg to approximately 80 to 100 mm Hg by the inclusion of phenylephrine (15 μmol/L) in the medium. Once the perfusion pressure had stabilized, endothelium-dependent and -independent vasodilator responses were tested by bolus intra-arterial injections of acetylcholine and sodium nitroprusside, respectively. The magnitude of vasodilation (seen as a decrease in perfusion pressure) was expressed as percent relaxation of established tone. Agonists were injected as a 100-μL bolus into the artery over a period of 5 seconds, with their dose expressed as nmol of drug in 100 μL vehicle. Each preparation was tested with no more than 3 doses of an agonist. Antagonists were dissolved in the perfusion medium at the indicated concentrations (in μmol/L). In some experiments, rats received 15 mg/kg *Escherichia coli* (*E. coli*) lipopolysaccharide (LPS) IP 2 hours before removal of the mesenteric bed.

Endothelial Denudation

To achieve endothelial denudation, the preparation was perfused with distilled water in the absence of phenylephrine for 3 to 6 minutes. On resuming perfusion with Krebs-Henseleit buffer containing phenylephrine, perfusion pressure returned to the same level as before distilled water perfusion. Functional denudation was considered to be achieved when the maximal dilator response to acetylcholine was reduced to <20% of control or converted to a pressor response, whereas the maximal dilator response to sodium nitroprusside remained unchanged. Only those preparations that met these criteria were used for further testing.

Chemicals

SR141716A (N-[piperidin-1-yl]-5-[4-chlorophenyl]-1-[1,2-dichlorophenyl]-4-methyl-1H-pyrazole-3-carboxamide HCl) was from Sanofi Co; WIN 55212-2 ([R]-[+]-[2,3-dihydro-5-methyl-3-{[4-morpholinyl]methyl}pyrrolol[1,2,3-*de*]-1,4-benzoxazin-6-yl]–1 [naphtalenyl] methanone mesylate), R(+)-methanandamide, and N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) were from RBI; THC (Δ^9-tetrahydrocannabinol) and anandamide (arachidonyl ethanolamide) were provided by Dr Billy R. Martin; HU-210 ([-]-11-OH-Δ^9-THC) was from Dr Raphael Mechoulam; and 2-AG (2-arachidonyl-glyceride) was from Deva Biotech. Acetylcholine, sodium nitroprusside, phenylephrine, arachidonic acid, indomethacin, ionomycin, *E. coli* LPS (0127:B8), and phenylmethylsulfonyl fluoride (PMSF) were from Sigma Chemical Co. SR141716A, THC, anandamide, 2-AG, and HU-210 were dissolved in 1:1:18 emulphor:ethanol:saline. WIN 55212-2 was dissolved in 1:1:18 emulphor:DMSO:saline. Emulphor is a polyoxyethylated vegetable oil.

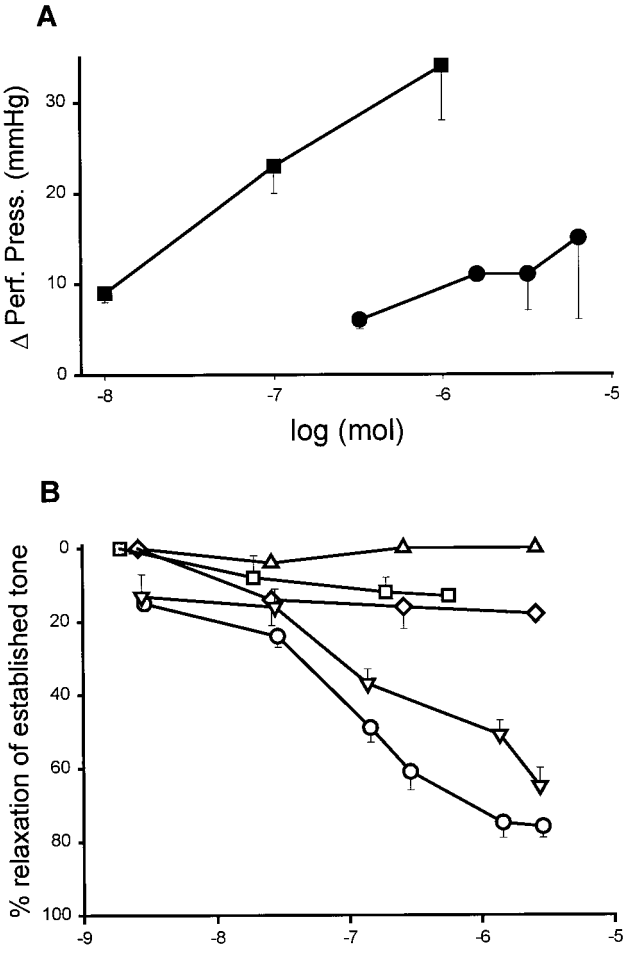

Figure 1. Dose-dependent mesenteric vasoconstrictor (A) or vasodilator (B) action of cannabinoid agonists and of arachidonic acid. Drugs were injected intra-arterially in a 100-μL bolus. Isolated, precontracted mesenteric arterial bed preparations were perfused as described in "Methods." Points and vertical lines represent mean±SE from 4 to 6 separate experiments. The drugs tested were THC (●), arachidonic acid (■), anandamide (○), R-methanandamide (▽), HU-210 (◇), WIN 55212-2 (□), and 2-AG (△).

Statistical Analysis

The paired *t* test was used to compare agonist effects tested in the same preparations in the absence and in the presence of an antagonist. The statistical package of Tallarida and Murray[30] was used to determine agonist ED_{50} values from graded dose-response curves.

Results

Pharmacological Characterization of Cannabinoid-Induced Mesenteric Vasodilation

Bolus doses of anandamide produced vasodilation that lasted 40 to 60 minutes (Figure 1). The ED_{50} of anandamide was

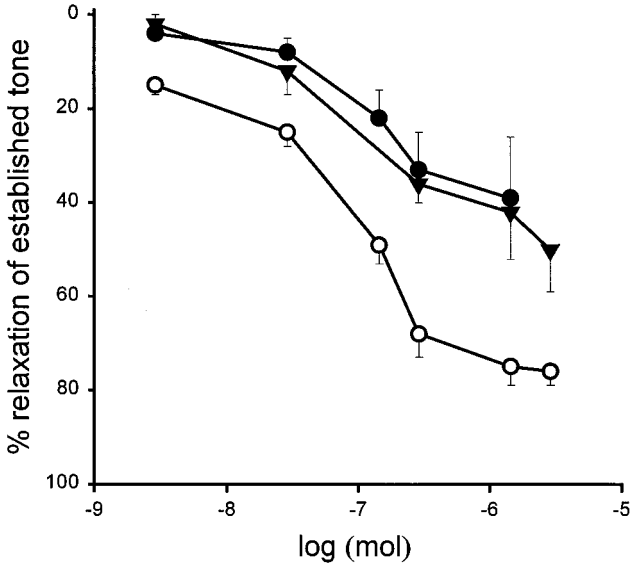

Figure 2. Inhibition of the mesenteric vasodilator action of anandamide by SR141716A. Anandamide was administered as a bolus intra-arterial injection of the indicated dose in the absence (○) or presence (▼) of 0.5 μmol/L or 5 μmol/L (●) of SR141716A present in the perfusion medium. The antagonist was added to the medium 15 minutes before the injection of anandamide. Points and vertical bars represent mean±SE from 4 to 5 separate experiments.

79±3 nmol, the peak effect was 77±2%. Once perfusion pressure had returned to its preinjection level, a second injection of the same dose of anandamide produced a similar response. The metabolically stable analogue of anandamide, R-methanandamide,[31] also caused dose-dependent relaxation, although it was somewhat less efficacious than anandamide (ED_{50}: 286±29 nmol, Figure 1). Because 2-AG was found to elicit a hypotensive response similar to that of anandamide,[16] it was also tested in the mesenteric preparation. In bolus doses of 2.6 to 2600 nmol, 2-AG failed to alter perfusion pressure (Figure 1). In doses of 320 to 6400 nmol, THC caused only increases in perfusion pressure, without any evidence of vasodilation (Figure 1). HU-210, a synthetic cannabinoid 3 orders of magnitude more potent than anandamide in causing profound hypotension in rats,[22] caused only a very minor dilator effect at the ultrahigh bolus dose of 2.6 μmol, which was the same in the absence (18±2%, n=5) or presence of 1 μmol/L SR141716A, a selective CB1 receptor inhibitor[23] (16±6%, n=4). Another potent hypotensive cannabinoid WIN 55212-2[22] was also ineffective in eliciting mesenteric vasodilation in the dose range of 2 to 570 nmol (Figure 1).

Because these agonist effects did not fit the pharmacological profile of CB1 receptors, we tested whether the CB1 receptor antagonist SR141716A inhibited the vasodilator effect of anandamide. In the presence of 0.5 μmol/L SR141716A, the anandamide dose-response curve was right-shifted by a factor of 8.3 (ED_{50}: 663±4 nmol, $P<0.001$, Figure 2), which is comparable to SR141716A antagonism of CB1 receptor–mediated effects.[23] However, when the concentration of SR141716A was increased 10-fold (to 5 μmol/L), the degree of inhibition increased only 2-fold (ED_{50} of anandamide: 1415±6 nmol, $P<0.001$, Figure 2). This suggests that additional SR141716A-insensitive mechanisms also contribute to the vasodilator effect of anandamide. Because anandamide may act on the endothelium, on the vascular smooth muscle, or both, we tested the effects of anandamide in endothelium-denuded preparations.

Effects of Endothelial Denudation on Anandamide-Induced Mesenteric Vasodilation

Figure 3 illustrates the effects of denudation on the responses to acetylcholine and sodium nitroprusside. Anandamide retained its vasodilator action after denudation, although the effect was somewhat reduced ($P<0.05$, Figure 4). However, this reduced effect was no longer influenced by SR141716A, as illustrated in Figure 4. This suggests that, although anandamide is capable of causing vasodilation by a direct effect on vascular smooth muscle, this effect is not mediated by an SR141716A-sensitive mechanism.

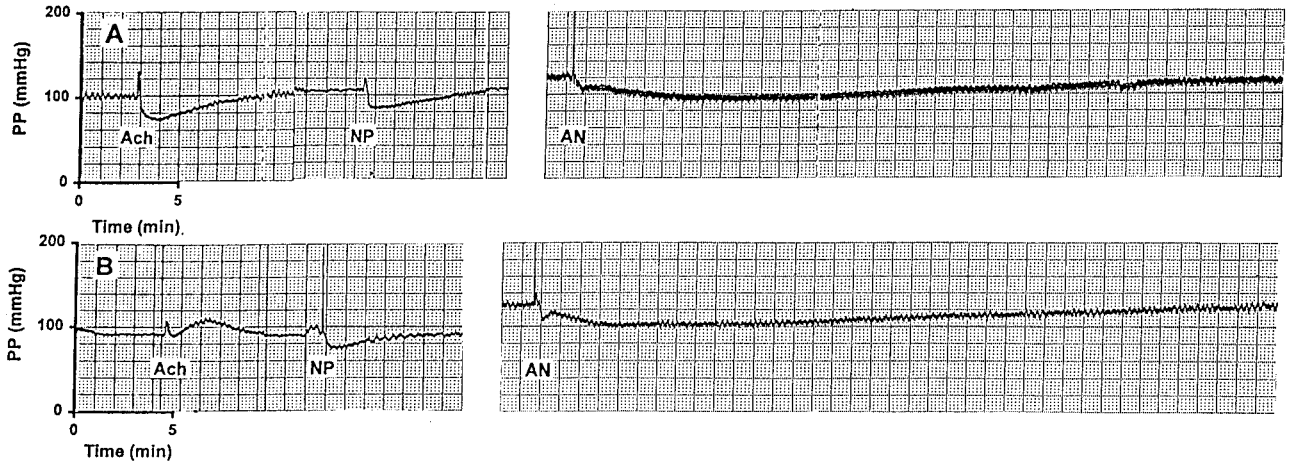

Figure 3. The effects of acetylcholine (ACh, 110 nmol), sodium nitroprusside (NP, 15 nmol), and anandamide (AN, 145 nmol) on vascular tone in an isolated, buffer-perfused mesenteric arterial bed preparation before (A) and after endothelial denudation (B). Endothelial denudation was achieved by a 4-minute perfusion with distilled water, as described in "Methods." Note that the effect of acetylcholine is converted from dilation to constriction, whereas the response to sodium nitroprusside remains unaffected and that of anandamide is only slightly reduced after denudation. PP indicates perfusion pressure.

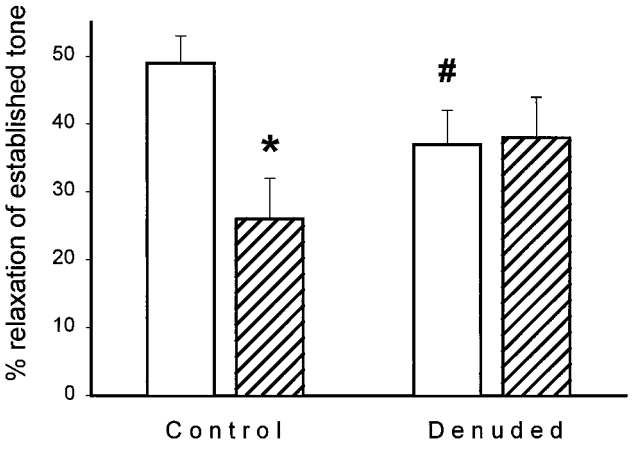

Figure 4. The effect of endothelial denudation on the mesenteric vasodilator response to anandamide. The effect of 145 nmol anandamide was tested in control (left) and endothelium denuded (right) preparations, in the absence (open columns) or presence (shaded columns) of 5 μmol/L SR141716A. * indicates a significant difference ($P<0.05$) from corresponding values in the absence of antagonist. # indicates a significant difference from corresponding values in control preparations. Columns and vertical bars represent mean±SE from 5 to 7 experiments.

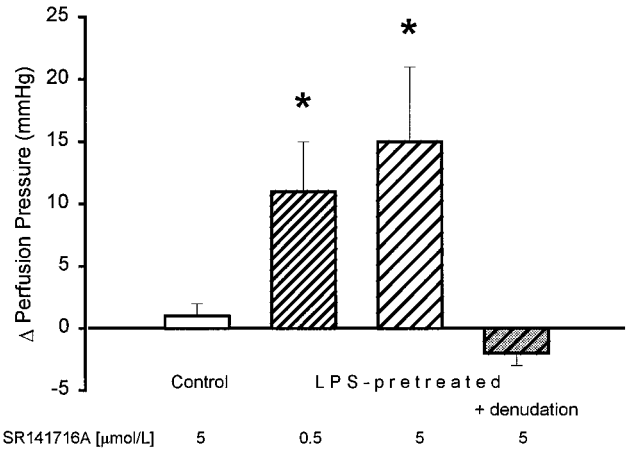

Figure 5. The effect of SR141716A on perfusion pressure in mesenteric arterial preparations from control (open column) and LPS-pretreated (shaded columns) rats. Columns and vertical bars represent mean±SE from 4 to 6 separate preparations in each group. * indicates significant difference ($P<0.05$) from value in control preparations.

Is Anandamide-Induced Vasodilation Due to a Degradation Product of Anandamide?

Anandamide is rapidly degraded by an amidohydrolase,[9] and the arachidonic acid thus released may account for noncannabinoid receptor–mediated effects.[28] However, this possibility could be ruled out in the mesenteric preparation. First, the serine protease inhibitor PMSF, which inhibits anandamide hydrolysis,[32] did not prevent the dilator response to anandamide. In intact preparations, the dilator response to 29 nmol anandamide (14±1%, n=4) was actually enhanced in the presence of 200 μmol/L PMSF (28±5%, n=4, $P<0.05$), whereas in endothelium-denuded preparations a borderline potentiation was observed (16±7% versus 24±5%, n=4, $P=0.07$). Second, the metabolically stable analogue R-methanandamide (1.38 μmol) caused a 54±8% dilation after denudation (n=4), which was similar to its effect in intact preparations (51±4%, n=5, see Figure 1). Third, in bolus doses of 10 to 1000 nmol, arachidonic acid caused no dilation and only dose-dependent increases in perfusion pressure (Figure 1).

Role of the Endothelium in Endotoxin-Induced Mesenteric Vasodilation

In view of the postulated role of endocannabinoids and SR141716A-sensitive cannabinoid receptors in endotoxin-induced hypotension,[16] we examined the possible involvement of the vascular endothelium in this effect. Perfusion of the phenylephrine precontracted mesenteric vasculature with 5.0 μmol/L SR141716A did not influence vascular tone (1±1 mm Hg, n=7). However, when the mesenteric arterial bed was isolated from rats pretreated with 15 mg/kg LPS, IP, 2 hours before they were killed, the inclusion of 0.5 or 5.0 μmol/L SR141716A in the perfusion medium caused a sustained pressor response (Figure 5). After such preparations were first denuded, SR141716A when subsequently administered failed to affect perfusion pressure (Figure 5). In vitro perfusion of control preparations with LPS, 100 μg/mL for 60 minutes (n=5), failed to affect perfusion pressure. These findings suggest that LPS induces an SR141716A-sensitive endothelium-dependent dilatory response in the in situ blood-perfused mesenteric vasculature.

The biosynthesis of anandamide is calcium dependent, and ionomycin has been used to induce anandamide synthesis in various cell types.[9,12,14] In micromolar doses, ionomycin was found to elicit irreversible mesenteric vasoconstriction, probably due to a direct effect on vascular smooth muscle (results not shown). However, when used at extremely low doses of 10 to 100 pmol per 100 μL bolus, ionomycin elicited reversible and reproducible dilator responses, which were significantly inhibited in the presence of 5 μmol/L SR141716A (Figure 6), similar to the observations of Randall et al.[24] In all experiments with ionomycin, the perfusion buffer contained 100 μmol/L L-NAME and 10 μmol/L indomethacin to block nitric oxide synthase and cyclooxygenase, respectively. Endothelial denudation reduced the dilator response to ionomycin, and the residual response was no longer affected by SR141716A (Figure 6).

Discussion

The present findings indicate that THC and the highly potent synthetic cannabinoids HU-210 and WIN 55212-2, which produce profound and long-lasting hypotension in anesthetized rats via activation of CB1 cannabinoid receptors,[22] are devoid of mesenteric vasodilator activity or, in the case of HU-210, caused a minor dilator response not mediated by CB1 receptors. On the other hand, the endogenous ligand anandamide, which causes relatively modest and brief (<10 minutes) hypotension in anesthetized rats in vivo,[18,19,22] elicits near-maximal and long-lasting (>30 minutes) mesenteric vasodilation. The metabolically stable analogue R-methanandamide has a similar effect. Another endogenous ligand, 2-AG, which can also elicit hypotension in anesthetized rats,[16] has no mesenteric vasodilator activity. These findings indicate that, first, the mesenteric vasculature does not significantly contribute to the profound hypotension

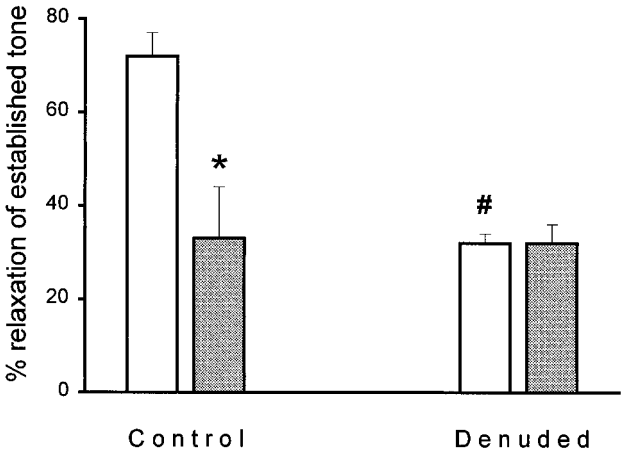

Figure 6. The endothelium-dependent vasodilator effect of ionomycin is antagonized by SR141716A. The effect of a bolus injection of 100 pmol ionomycin was tested in control (left) and endothelium-denuded (right) preparations, in the absence (open columns) or presence (gray columns) of 5 μmol/L SR141716A. In both cases, the perfusion buffer contained 100 μmol/L L-NAME and 10 μmol/L indomethacin. * indicates a significant difference ($P<0.05$) from corresponding value in the absence of SR141716A; # indicates a significant difference ($P<0.01$) from corresponding values in control preparations. Columns and vertical bars represent mean±SE from 4 to 5 experiments.

caused in vivo by plant-derived or synthetic cannabinoids and that, second, the receptors mediating the mesenteric vasodilator response to anandamide and R-methanandamide are distinct from CB1 receptors. These receptors should preferably be called anandamide receptors rather than cannabinoid receptors, to reflect their insensitivity to compounds derived from or related to cannabis.

The role of CB1 receptors in cannabinoid-induced hypotension was indicated by earlier findings with agonists and antagonists.[22] The hypotensive potency of a series of agonists, including those tested in the present study, displayed a strong positive correlation with their analgesic potency or their binding K_d to the brain cannabinoid receptor.[22] Furthermore, the inhibitory potency of the highly selective CB1 receptor antagonist SR141716A[23] was similar for cannabinoid-induced hypotension[33] and neurobehavioral effects.[23] Although SR141716A did inhibit the mesenteric vasodilator response to anandamide, the inhibition was unusual in that a 10-fold increase in the concentration of the antagonist resulted in a much smaller than expected increase in inhibition. Such "ceiling" effects, which result in anomalous Schild plots, can arise if more than one mechanism is involved in the action of the agonist, only one of which is sensitive to the antagonist. Indeed, SR141716A failed to inhibit anandamide-induced vasodilation in endothelium-denuded preparations. This suggests that mesenteric vasodilation by anandamide has 2 components: one mediated by a SR141716A-sensitive non–CB1 receptor site located on the endothelium and the other by an SR141716A-resistant direct action on vascular smooth muscle. The relative contribution of these 2 components to the net response to anandamide may depend on the species and tissue used and on the condition of the endothelium, which may explain conflicting reports in the literature about the ability[24,25] or inability[26–28] of SR141716A to block the vasorelaxant effect of anandamide.

The SR141716A-resistant effect of anandamide cannot be attributed to arachidonic acid or its metabolites because in endothelium-denuded preparations PMSF, which prevents the degradation of anandamide, did not antagonize anandamide-induced vasodilation, and the metabolically stable R-methanandamide retained its vasorelaxant property. It is unclear whether the SR141716A-resistant effect of anandamide is unrelated to cannabinoid receptors, such as a direct opening of potassium channels, or whether it is mediated by an as-yet-unidentified cannabinoid receptor in vascular smooth muscle cells. However, CB2 receptors are unlikely to be involved because (1) anandamide binds to but does not activate CB2 receptors,[34] (2) the potent CB2 agonist WIN 55212-2[35] failed to cause mesenteric vasorelaxation, and (3) SR141716A, which has a K_d of 700 nmol/L at CB2 receptors,[23] should have inhibited the response at the concentration used.

In mesenteric preparations from LPS-pretreated rats, the endothelium-dependent pressor response to SR141716A indicates a role for the endothelium in endotoxin-induced mesenteric vasodilation. This finding, together with the observation that low doses of a calcium ionophore elicit endothelium-dependent SR141716A-sensitive mesenteric vasodilation, suggests that an endocannabinoid may be released from the endothelium of mesenteric arterioles. Indeed, anandamide has been identified in endothelial cells in the renal vasculature,[13] and 2-AG was found to be present in endothelial cells from a human umbilical vein.[15] However, in the rat mesenteric arteriolar bed, 2-AG does not cause dilation, and the SR141716A-sensitive component of the dilatory response to anandamide is endothelium dependent. Therefore, if anandamide is the cannabinoid released from mesenteric endothelial cells by ionomycin or LPS, its primary site of action must also be the endothelium, which implies its luminal release. Because of the presence of cholinacetyltransferase in vascular endothelial cells, a similar mechanism has been proposed for the endothelium-dependent vasodilatory effect of acetylcholine.[36] However, in contrast to the effect of acetylcholine, the putative endothelial mediator of SR141716A-sensitive vasodilation is unlikely to be nitric oxide, prostacyclin, or both, because the effect of ionomycin was observed in the presence of L-NAME and indomethacin.

An alternative or additional source of anandamide in the LPS-pretreated preparations may be circulating macrophages. LPS treatment has been shown to induce the production of anandamide by macrophages,[16] and activated macrophages may actually become incorporated into the endothelium in which they can form tight junctions with endothelial cells.[37] Anandamide released from such resident macrophages may act in a juxtacrine manner to activate endothelial cannabinoid receptors. That macrophages may be required for the pressor effect of SR141716A in the LPS-treated preparations is suggested by the inability of LPS to induce mesenteric vasodilation in vitro, in a buffer-perfused preparation. Although LPS can directly activate the vascular endothelium, this mechanism is far less potent than the indirect pathway of endothelial activation dependent on circulating macrophages.[38]

In summary, the present findings indicate that anandamide induces mesenteric vasodilation in the rat via a unique receptor located on endothelial cells, which is not activated by other cannabinoids but can be inhibited by the CB1-selective antagonist SR141716A. This endothelial receptor is thus distinct from CB1 receptors that mediate the profound hypotensive effect of synthetic cannabinoids in vivo. Activation of endothelial anandamide receptors may contribute to mesenteric vasodilation in endotoxic shock.

Acknowledgments

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