

Cannabidiol Prevents Cerebral Infarction Via a Serotonergic 5-Hydroxytryptamine_{1A} Receptor-Dependent Mechanism

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Background and Purpose—Cannabidiol has been reported to be a neuroprotectant, but the neuroprotective mechanism of cannabidiol remains unclear. We studied the neuroprotective mechanism of cannabidiol in 4-hour middle cerebral artery (MCA) occlusion mice.

Methods—Male MCA occluded mice were treated with cannabidiol, abnormal cannabidiol, anandamide, methanandamide, cannabidiol plus capsazepine, and cannabidiol plus WAY100135 before and 3 hours after MCA occlusion. The infarct size was determined after 24 hours (2,3,5-triphenyltetrazolium chloride staining). Cerebral blood flow (CBF) was measured at, before and 1, 2, 3, and 4 hours after MCA occlusion.

Results—Cannabidiol significantly reduced the infarct volume induced by MCA occlusion in a bell-shaped curve. Similarly, abnormal cannabidiol but not anandamide or methanandamide reduced the infarct volume. Moreover, the neuroprotective effect of cannabidiol was inhibited by WAY100135, a serotonin 5-hydroxytryptamine_{1A} (5-HT_{1A}) receptor antagonist but not capsazepine a vanilloid receptor antagonist. Cannabidiol increased CBF to the cortex, and the CBF was partly inhibited by WAY100135 in mice subjected to MCA occlusion.

Conclusions—Cannabidiol and abnormal cannabidiol reduced the infarct volume. Furthermore, the neuroprotective effect of cannabidiol was inhibited by WAY100135 but not capsazepine, and the CBF increased by cannabidiol was partially reversed by WAY100135. These results suggested that the neuroprotective effect of cannabidiol may be related to the increase in CBF through the serotonergic 5-HT_{1A} receptor. (*Stroke*. 2005;36:1071-1076.)

Key Words: cannabidiol ■ cerebral infarction ■ middle cerebral artery ■ serotonin receptor ■ 5HT_{1A}

Cannabis contains ≈80 different cannabinoids, including the psychoactive component Δ⁹-tetrahydrocannabinol, and nonpsychoactive components, which include cannabidiol, cannabinol, and cannabigerol. In those components, cannabidiol, a nonpsychoactive constituent of cannabis, was found to be an anticonvulsant in animal models of epilepsy and in humans with epilepsy. Moreover, cannabidiol has been shown to have antispasmodic, anxiolytic, antiemetic, and antirheumatoid arthritic properties.¹ In addition, cannabidiol has been shown to be protective against global and focal ischemic injury.^{2,3}

Cannabidiol is generally known to have a very low affinity (in the micromolar range) for the cannabinoid CB1 and CB2 receptors but also to have many pharmacological actions,⁴ an anticonvulsant, anxiolytic action, and a neuroprotective effect against ischemic injury. These actions are thought to be dependent on a new cannabinoid receptor within the brain. In fact, as for new cannabinoid receptors, an abnormal canna-

bidiol receptor, a non-CB1 receptor, and a non-CB2 receptor are assumed to exist, but no cloning of them has been performed and the physiological roles are mostly unknown.

In our previous study, we reported that Δ⁹-tetrahydrocannabinol, a CB1 agonist, and cannabidiol prevented cerebral infarction,¹⁴ suggesting that Δ⁹-tetrahydrocannabinol exerted its neuroprotective action by inducing hypothermia through the CB1 receptor, whereas cannabidiol prevented cerebral infarction by mild hypothermia through a CB1 receptor-independent mechanism. However, the neuroprotective mechanisms of cannabidiol remain unclear. In other reports, cannabidiol has been reported to inhibit anandamide amidase⁵ and the reuptake of anandamide.^{6,12} Moreover, cannabidiol was found recently to be a full, although weak, agonist of human vanilloid receptor type 1 (VR1),¹² which has been found in the brain,^{1,13} particularly in the hippocampus, and cannabidiol was desensitized to the VR1 by capsaicin.¹² Therefore, we examined whether anan-

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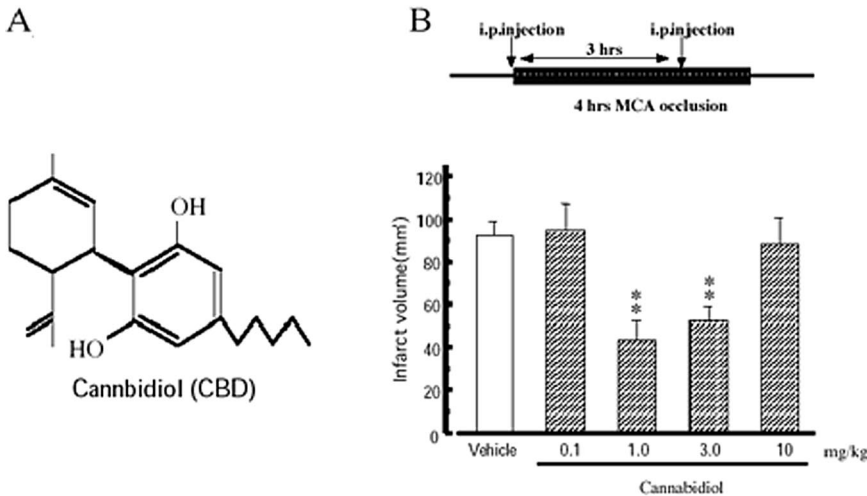


Figure 1. Chemical structure of cannabidiol and effect of cannabidiol (CBD) on cerebral infarction. A, Chemical structure of cannabidiol. B, Cannabidiol was injected intraperitoneally (i.p.) immediately before and 3 hours after the occlusion. Cerebral infarction was significantly reduced in a dose-dependent bell-shaped curve. Values are expressed as the mean \pm SEM ($n=6$ to 8). ** $P<0.01$ (Dunnett test).

damide and methanandamide, a stable anandamide analog, can prevent cerebral infarction induced by middle cerebral artery (MCA) occlusion and whether the neuroprotective effect of cannabidiol can be inhibited by capsazepine, a VR1 antagonist.

Furthermore, we focused on the anxiolytic action of cannabidiol. Cannabidiol has been shown to have anxiolytic properties,¹ and we formed a hypothesis that the 5-hydroxytryptamine_{1A} (5-HT_{1A}) receptor may be related to the neuroprotective effect of cannabidiol. It has been reported that the 5-HT_{1A} receptor played a critical role in the pathophysiology of anxiety and depression as well as in the mode of action of anxiolytic and antidepressant drugs.⁷ Moreover, the 5-HT_{1A} receptor agonist has been reported to have a neuroprotective effect on the cerebral ischemia model.^{8,28} Therefore, we examined whether the 5-HT_{1A} receptor is related to the neuroprotective effect of cannabidiol by treatment with WAY100135, a 5-HT_{1A} receptor antagonist.

Materials and Methods

Animals

Male MCA occluded mice (25 to 35 g; Kiwa Experimental Animal Laboratory; Wakayama, Japan) were kept under a 12-hour light/dark cycle (lights on from 7:00 AM to 7:00 PM) in an air-conditioned room ($23\pm 2^\circ\text{C}$) with food (CE-2; CLEA Japan) and water available ad libitum. All procedures regarding animal care and use were performed in compliance with the regulations established by the experimental animal care and use committee of Fukuoka University.

Focal Cerebral Ischemia

Focal cerebral ischemia was induced according to the method described in our previous study.⁹ Mice were anesthetized with 2% halothane and maintained thereafter at 1% halothane (Flossen; Takeda Chemical Industries). After a midline neck incision, the left common and external carotid arteries were isolated and ligated. A nylon monofilament (8-0; Ethilon; Johnson & Johnson) coated with silicon resin (Xantopren; Heleus Dental Material) was introduced through a small incision into the common carotid artery and advanced to a position 9 mm distal from the carotid bifurcation for occlusion of the MCA. During the surgery, the body temperature was maintained in the physiological range with a warming pad. Four hours after the occlusion, mice were reanesthetized with halothane, and reperfusion was established by withdrawal of the filament. Twenty-four hours after MCA occlusion, the animals were euthanized by decapitation. Brains were removed and sectioned coronally into 4 2-mm slices

using a mouse brain matrix. Slices were immediately stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma). The border between the infarct volume was calculated.

Cerebral Blood Flow

Cerebral blood flow (CBF) was monitored by laser-Doppler flowmetry (LDF) using a probe (diameter 0.5 mm) of a laser-Doppler flowmeter (ALF2100; Advance Co.) inserted into the left cortex (anterior -0.22 mm; lateral 2.5 mm from bregma; depth 2 mm from the skull surface) through a guide cannula.¹⁰

Drugs

Cannabidiol and anandamide (Sigma Aldrich), abnormal cannabidiol and methanandamide (Tocris Cookson), and the capsazepine VR1 antagonist (Sigma Aldrich) were dissolved in 1% Tween. WAY100135, a 5-HT_{1A} antagonist (Tocris Cookson), was dissolved in 0.9% saline. All other drugs were administered intraperitoneally immediately before and 3 hours after MCA occlusion.

Blood Analysis

Physiological variables (pH, PCO₂, PO₂, and hematocrit) were measured using a blood analysis system (International Technidyne Co.).

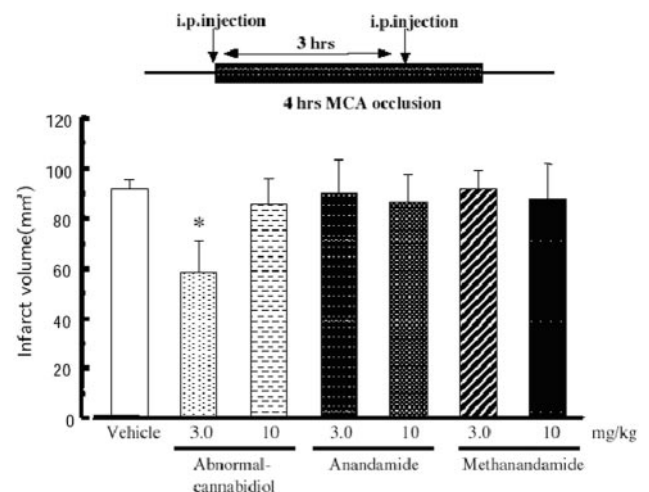


Figure 2. Effect of abnormal cannabidiol and anandamide/methanandamide on cerebral infarction. Abnormal cannabidiol reduced the infarct volume as effectively as did cannabidiol by treatment immediately before and 3 hours after MCA occlusion. Anandamide and methanandamide reduced the infarction. Values are expressed as the mean \pm SEM ($n=4$ to 7). * $P<0.05$ (Dunnett test).

TABLE 1. Physiological Variable Data

	Sham	4-Hour MCAO			
		Vehicle	Cannabidiol	CBD+WAY	WAY100135
Physiological variables (n=4)					
pH	7.3±0.03	7.2±0.02	7.2±0.03	7.2±0.06	7.3±0.01
Pco ₂	49.7±4.04	50.5±2.68	44.4±1.52	46.8±0.67	39.7±1.25
PO ₂	49.0±8.85	57.3±6.16	58.8±6.93	60.7±1.79	54.8±6.90
Hct (%)	38.0±1.12	38.1±1.56	35.9±1.71	34.8±0.34	32.3±0.31
Blood pressure (n=6)					
Maximum	126.0±6.83	133.8±24.4	152.4±5.75	146.4±9.9	151.3±12.12
Minimum	66.0±7.34	54.9±7.26	99.9±4.54	87.0±6.12	71.8±10.2
Mean	80.3±7.31	81.4±12.89	70.5±10.8	63.8±6.48	98.3±10.17
Heart rate	520.9±17.98	496.6±43.02	511.8±12.61	523.4±13.69	563.2±53.88

Physiological variable data were obtained immediately before reperfusion, and drugs were administered immediately before and 3 hours after cerebral ischemia (n=4). Blood pressure data were obtained 2 hours after cerebral ischemia, and drugs were administered only immediately before MCA occlusion (n=6).

Blood pressure was measured using a blood pressure manometer (TK-370C; Neuroscience).

Statistical Analysis

Results are expressed as means±SEM. Dunnett’s test and Tukey’s test after one-way ANOVA were used for the infarct volume and average of CBF. A P value of <0.05 was considered significant.

Results

Effect of Cannabidiol on Cerebral Infarction

Treatment of cannabidiol immediately before and 3 hours after MCA occlusion significantly reduced the infarct volume induced by MCA occlusion in mice with a dose-dependent bell-shaped curve (vehicle 91.3±3.5 mm³; 0.1 mg/kg canna-

bidol 94.3±12.1 mm³; 1.0 mg/kg 49.2±10.32 mm³, P<0.01; 3.0 mg/kg 51.4±7.3 mm³, P<0.01; 10 mg/kg, 87.0±12.5 mm³; F_(4,27)=6.553, P<0.001, one-way ANOVA; Figure 1).

Effect of a Cannabidiol Analog and Anandamide Analogs on Cerebral Infarction

Abnormal cannabidiol, a cannabidiol analog, reduced the infarct volume as effectively as cannabidiol by treatment immediately before and 3 hours after the MCA occlusion (abnormal cannabidiol 3.0 mg/kg, 58.2±12.2 mm³, P<0.05; 10 mg/kg 84.8±10.6 mm³). Anandamide and methanandamide did not reduce the infarction (anandamide 3.0 mg/kg 89.6±13.2 mm³; 10 mg/kg 85.7±11.4 mm³; methanandamide 3.0 mg/kg 90.9±7.7 mm³; 10 mg/kg 87.3±14.2 mm³; F_(6,32)=1.191, P<0.5, one-way ANOVA; Figure 2).

Neuroprotection of Cannabidiol Was Inhibited by the 5-HT_{1A} Antagonist

The neuroprotective effect of cannabidiol was not inhibited by SR141716, a CB1 receptor antagonist (cannabidiol 3.0 mg/kg and SR141716 1.0 mg/kg 54.9±6.7 mm³, P<0.01), and capsazepine, a VR1 antagonist (cannabidiol 3.0 mg/kg and capsazepine 10 mg/kg 57.2±13.2 mm³, P<0.01). Meanwhile, the neuroprotective effect of cannabidiol was inhibited by WAY100135, the 5-HT_{1A} antagonist (cannabidiol 3.0 mg/kg and WAY100135 10 mg/kg, 81.1±9.3 mm³; F_(4,36)=6.310, P<0.001; one-way ANOVA). Capsazepine and WAY100135 alone did not change the infarct volume (data not shown; Figure 3).

Cannabidiol Increased the CBF in Mice Subjected to the MCA

CBF was decreased after MCA occlusion by ≥90% compared with the vehicle-treated group using the LDF method. Cannabidiol at 3.0 mg/kg significantly increased CBF during the 4-hour MCA occlusion compared with the vehicle-treated group. On the other hand, WAY100135 at 10 mg/kg significantly and partially reduced an increase in the CBF induced by cannabidiol at 3.0 mg/kg, but it was smaller than canna-

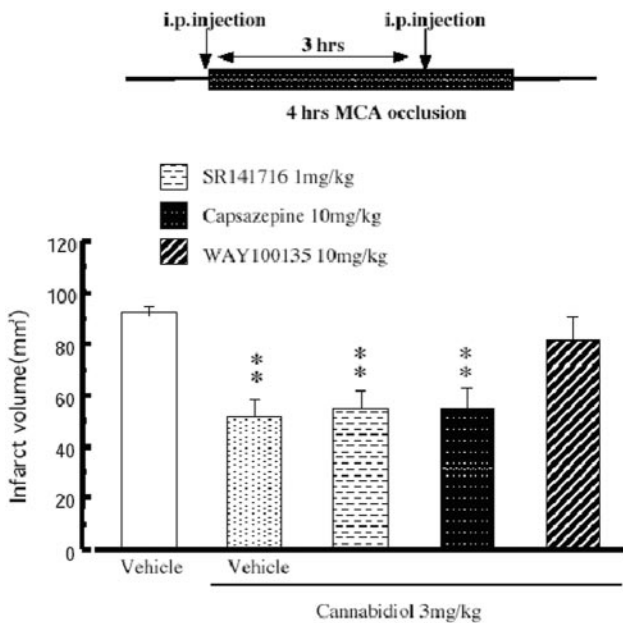


Figure 3. The neuroprotective effect of cannabidiol was inhibited by the 5-HT_{1A} antagonist. The neuroprotective effect of cannabidiol was inhibited by WAY100135, the 5-HT_{1A} antagonist, but not by SR141716, the CB1 receptor antagonist, and capsazepine, the VR1 antagonist. Values are expressed as the mean±SEM (n=6 to 10). **P<0.01 (Dunnett test).

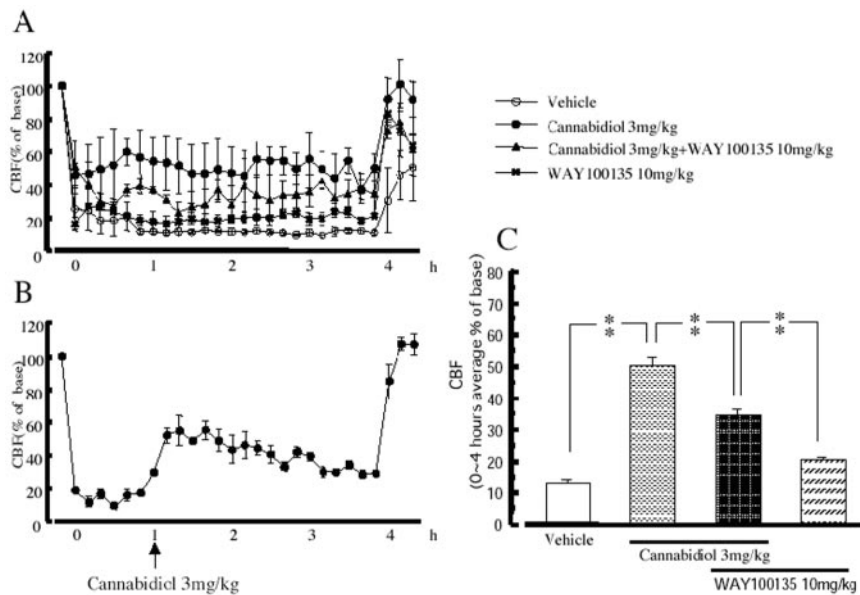


Figure 4. Cannabidiol increased the CBF in mice subjected to MCA occlusion. Cannabidiol increased the CBF during the 4-hour MCA occlusion, and the increased CBF was reduced by WAY100135, 5-HT_{1A} antagonist (A). Cannabidiol was treated 1 hour after cerebral ischemia, and then the CBF increased until reperfusion (B). WAY100135 significantly and partially reduced the average of the increased CBF of cannabidiol during the MCA occlusion (C). Values are expressed as the mean \pm SEM (n=3). ** P <0.01 (Tukey test).

bidiol at 3 mg/kg alone (average CBF during the 4-hour MCA occlusion: vehicle $13.3 \pm 1.0\%$; cannabidiol 3 mg/kg $50.3 \pm 2.5\%$, $P < 0.01$ versus vehicle; cannabidiol 3.0 mg/kg plus WAY100135 10 mg/kg $34.5 \pm 1.8\%$ $P < 0.01$ versus cannabidiol 3.0 mg/kg and vehicle; WAY100135 10 mg/kg; $20.51 \pm 0.58\%$, $P < 0.01$ versus cannabidiol 3.0 mg/kg plus WAY100135 10 mg/kg; $F_{(3,96)} = 70.70$; $P < 0.0001$; one-way ANOVA. Immediately after the reperfusion, the LDF reading of the vehicle-treated group returned to $30.7 \pm 19.5\%$, whereas that of cannabidiol at 3.0 mg/kg returned to $92.3 \pm 12.3\%$. Moreover, the cannabidiol at 3 mg/kg plus WAY100135 at 10 mg/kg treatment group returned to $73.3 \pm 5.1\%$ (Figure 4).

Blood Analysis

In group vehicle and 3 mg/kg cannabidiol, there was no difference significantly about physiological variables (pH, PCO₂, PO₂, and hematocrit). Furthermore, in 3 mg/kg cannabidiol plus 10 mg/kg WAY100135-treated group, there was no difference significantly compared with group vehicle treated and cannabidiol treated. Moreover, there was no difference in blood pressure (Table 1).

Discussion

In our study, cannabidiol significantly reduced the infarct volume in a mouse MCA occlusion model with a dose-dependent bell-shaped curve. Abnormal cannabidiol, cannabidiol analog, exerted a neuroprotective effect with a dose-dependent bell-shaped curve as potently as did cannabidiol. Anandamide and methanandamide did not reduce the infarction. The neuroprotective effect of cannabidiol was inhibited by the 5-HT_{1A} receptor antagonist but not by the CB1 receptor antagonist or the VR1 antagonist. Moreover, cannabidiol at 3 mg/kg increased the CBF of the cortex more than that of vehicle-treated group. The 5-HT_{1A} receptor antagonist significantly reduced an increase in the CBF induced by cannabidiol. These results suggest that cannabidiol may partially

have a neuroprotective action via the 5-HT_{1A} receptor but not the CB1 receptor and VR1.

The neuroprotective effect of cannabidiol showed a dose-dependent bell-shaped curve. Abnormal cannabidiol, a cannabidiol analog, produced the same dose-dependent bell-shaped curve as cannabidiol, and a higher dose of abnormal cannabidiol did not reduce the infarct volume. The dose-dependent bell-shaped curve of cannabinoids is not surprising because a similar pattern has been seen for the anti-inflammatory effect of cannabidiol in a murine collagen-induced arthritis model.¹⁶ Moreover, it has been shown for the reinforcing properties of CP55,940 using an intracerebroventricular self-administration paradigm in rats.¹⁷ Moreover, it has been reported that cannabidiol produced a dose-dependent bell-shaped curve for the electroencephalographic flattening of total power in gerbils subjected to cerebral ischemia.²¹ Although cannabidiol has been reported to be a full, although weak, agonist of human VR1,¹² in the present study, the neuroprotective effects of cannabidiol were not inhibited by capsazepine, a VR1 antagonist. This does not support the result that the bell-shaped curve obtained by cerebral infarction with increasing doses is related to VR₁. As for the other reason, it has been reported that cannabidiol produces carbon monoxide¹⁸ because 2 phenol hydroxyl groups (Resorcin structure) in cannabidiol participated in the production of carbon monoxide. Moreover, carbon monoxide has been significantly associated with ischemic stroke mortality.¹⁹ As for the results of these reports, they suggest that at higher doses, cannabidiol may produce a large infarction by MCA occlusion by promoting the production of carbon monoxide, whereas at lower doses, cannabidiol may not be able to prevent the infarct volume induced by the severe condition (ie, a 4-hour MCA occlusion). In fact, in the present study, higher or lower doses of cannabidiol enlarged the infarct volume induced by the 4-hour MCA occlusion. The dose-response curve of cannabidiol should be examined in detail.

It has been reported that postischemic treatment with cannabidiol prevented electroencephalographic flattening, hyperlocomotion, and neuronal injury in gerbils, in agreement with the present study.²¹ Moreover, cannabidiol has been shown to be protective against *N*-methyl-D-aspartate and β -amyloid peptide toxicity.¹¹ However, the neuroprotective mechanisms of cannabidiol remain unclear, but the novel non-CB1 and non-CB2 receptors have been proposed because cannabidiol has a very low affinity (in the micromolar range) for CB1 and CB2 receptors and has many pharmacological actions.¹ Our previous report demonstrated that the neuroprotective effect of cannabidiol was not inhibited by the CB1 receptor antagonist SR141716, and by warming. These results indicated that cannabidiol was found to have a CB1 receptor-independent mechanism, unlike Δ^9 -tetrahydrocannabinol, in agreement with the present study. It is possible that cannabidiol has another mechanism independent of hypothermia because the neuroprotective effect of cannabidiol was not abolished by warming. Cannabidiol has been reported to inhibit anandamide amidase⁵ and the reuptake of anandamide.^{6,12} Therefore, we examined whether anandamide and methanandamide can prevent cerebral infarction. The results demonstrated that they could not prevent cerebral infarction. Furthermore, it was reported recently that cannabidiol was found to be a full, although weak, agonist of VR1 and has the ability to desensitize VR1 to the action of capsaicin.¹² Therefore, we examined whether capsazepine, a selective VR1 antagonist, can inhibit the neuroprotective effect of cannabidiol on the cerebral infarction. We found that capsazepine could not inhibit the neuroprotection. These results demonstrated that anandamide and VR1 are not likely to be related to the neuroprotective effect of cannabidiol on the cerebral infarction in the MCA occlusion model.

We measured quantification of brain water content of 4-hour MCA occlusion mice. Cannabinoids have been reported to have neuroprotective effects with reducing brain edema caused by brain injury and cerebral ischemia.²⁹ We studied whether the neuroprotective effect of cannabidiol is due to reduce the edema. However, there was no significant change (data not shown). Cannabidiol may directly have neuroprotective effect without reducing brain edema.

Cannabidiol has been shown to have anxiolytic properties.¹ Moreover, cannabidiol exerts a neuroprotective effect through its antioxidant, antispasmodic, and antiemetic activity,¹ and vasorelaxation through G_i/G_o to the phosphatidylinositol 3-kinase/Akt signaling pathway.¹⁵ We noticed that these pharmacological actions of cannabidiol resemble that of the 5-HT_{1A} receptor agonists. The 5-HT_{1A} receptor has been related to the aggressive and anxious behavior of male mice with various aggressive experiences,²² and 5-HT_{1A} receptor expression has played a critical role in the pathophysiology of anxiety and depression, as well as in the mode of action of anxiolytic and antidepressant drugs.⁷ In addition, an agonist of the 5-HT_{1A} receptor has played an important role as a vasodilator.^{20,23–25} Furthermore, a 5-HT_{1A} receptor agonist has reduced the cortical infarct volume induced by permanent MCA occlusion and also shown a neuroprotective effect in vitro.⁸ Moreover, activation of the 5-HT_{1A} receptor has reduced Ca(2+) and glutamatergic receptor-evoked arachi-

donic acid and NO/cGMP release in the adult hippocampus²⁶ and protected against *N*-methyl-D-aspartate-induced apoptotic cell death in the primary mesencephalic neurons that were exposed to the Parkinsonian toxin MPP⁺ (*N*-methyl-4-phenylpyridine).²⁷ Therefore, we examined whether the neuroprotective effect of cannabidiol could be related to 5-HT_{1A} receptor activation. The results demonstrated that the neuroprotective effect of cannabidiol was inhibited by WAY100135, a 5-HT_{1A} receptor antagonist, suggesting that the neuroprotective effect of cannabidiol may be at least in part related to the activation of the 5-HT_{1A} receptor. Moreover, because cannabidiol increased the CBF and the increased CBF was in part inhibited by WAY100135, cannabidiol may have a pharmacological action as well as being a 5-HT_{1A} receptor agonist. The neuroprotective effect of cannabidiol may be in part related to its effect via the 5-HT_{1A} receptor.

Conclusion

We applied a new approach to the investigation of the neuroprotective mechanism of cannabidiol to identify with a CB1 receptor antagonist, a VR1 antagonist, and a 5-HT_{1A} receptor antagonist. The neuroprotective effect of cannabidiol was inhibited by the 5-HT_{1A} receptor antagonist but not by the CB1 receptor antagonist or the VR1 antagonist. Furthermore, the increased CBF caused by cannabidiol was also in part reduced by the 5-HT_{1A} receptor antagonist. These results suggest that cannabidiol may have, at least in part, a neuroprotective effect via the 5-HT_{1A} receptor toward cerebral ischemia.

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