Repeated treatment with cannabidiol but not Δ^9-tetrahydrocannabinol has a neuroprotective effect without the development of tolerance

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Abstract

Both Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol are known to have a neuroprotective effect against cerebral ischemia. We examined whether repeated treatment with both drugs led to tolerance of their neuroprotective effects in mice subjected to 4 h-middle cerebral artery (MCA) occlusion. The neuroprotective effect of Δ^9-THC but not cannabidiol was inhibited by SR141716, cannabinoid CB_1 receptor antagonist. Fourteen-day repeated treatment with Δ^9-THC, but not cannabidiol, led to tolerance of the neuroprotective and hypothermic effects. In addition, repeated treatment with Δ^9-THC reversed the increase in cerebral blood flow (CBF), while cannabidiol did not reverse that effect. Repeated treatment with Δ^9-THC caused CB_1 receptor desensitization and down-regulation in MCA occluded mice. On the contrary, cannabidiol did not influence these effects. Moreover, the neuroprotective effect and an increase in CBF induced by repeated treatment with cannabidiol were in part inhibited by WAY100135, serotonin $5\text{-}HT_{1A}$ receptor antagonist. Cannabidiol exhibited stronger antioxidative power than Δ^9-THC in an in vitro study using the 1,1-diphenyl-2-picryhydrazyl (DPPH) radical. Thus, cannabidiol is a potent antioxidant agent without developing tolerance to its neuroprotective effect, acting through a CB_1 receptor-independent mechanism. It is to be hoped that cannabidiol will have a palliative action and open new therapeutic possibilities for treating cerebrovascular disorders.
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1. Introduction

Cannabis contains about 60 different cannabinoids, including the psychoactive component, Δ^9-tetrahydrocannabinol (Δ^9-THC) and other major non-psychoactive components, such as cannabidiol, cannabinol and cannabigerol. Δ^9-THC has been demonstrated to produce hypothermia, neuroprotection and tolerance (Wiley and Martin, 2002; Mishima et al., 2005; Hayakawa et al., 2004; Leker et al., 2003; Braida et al., 2003; Rubino et al., 2000). These effects are, at least in part, related to binding to the CB_1 receptor. On the other hand, cannabidiol has a very low affinity (in the micromolar range) for CB_1 and CB_2 receptors and has been found to act as an anticonvulsant in animal models of epilepsy and in humans with epilepsy. Moreover, cannabidiol has been shown to have anti-spasmodic, anxiolytic, anti-nausea and anti-rheumatoid properties (Mechoulam et al., 2002), and to be protective against *N*-methyl-D-aspartate and beta-amyloid peptide toxicity (Iuvone

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et al., 2004), and global and focal ischemic injury (Braida et al., 2003; Hampson et al., 2000). Recently, it has also been reported that cannabidiol had the ability to enhance adenosine signaling through inhibition of uptake (Carrier et al., 2006). These actions are thought to be dependent on a new cannabinoid receptor, such as an abnormal cannabidiol receptor, a non-CB_1 and non-CB_2 receptor, and a GPR55, G-protein-coupled receptor (David et al., 2006; Begg et al., 2005) within the brain. We have previously reported the partial prevention of ischemic damage induced by middle cerebral artery occlusion via 5-HT_{1A} receptors. In addition, cannabidiol increased cerebral blood flow (CBF) to the cortex, and the CBF was partly inhibited by WAY100135 (Mishima et al., 2005). Cannabidiol has also been shown to displace the 5-HT_{1A} receptor agonist, [^{3}H] 8-OH-DPAT, from the cloned human 5-HT_{1A} receptor in a concentration-dependent manner (Russo et al., 2005). In this way, cannabidiol has exerted a wide spectrum of effects, but the neuroprotective mechanism has not been fully explored.

Repeated treatment with Δ^9-THC results in the development of tolerance to its most acute behavioral and pharmacological effects (Abood and Martin, 1992; Sim et al., 1996). Δ^9-THC has been shown to lead to tolerance of hypoactivity, hypothermia, antinociception, catalepsy and pentobarbital-induced sleep prolongation (Sim-Selly and Martin, 2002; Watanabe et al., 1982, 1983). Several studies have attempted to identify the cellular adaptations underlying the development of tolerance to Δ^9-THC. However, the development of tolerance to the neuroprotective effect of Δ^9-THC on cerebral ischemic injury induced by MCA occlusion has not been fully explored. In addition, it has not been shown whether tolerance develops to the neuroprotective effect of non-psychoactive cannabinoids, such as cannabidiol. To explore whether cannabidiol produced the development of tolerance to neuroprotective and other pharmacological effects on locomotor activity and on body temperature, it could contribute to demonstrate these mechanisms of cannabidiol.

2. Materials and methods

2.1. Animals

Male ddY mice (25–35 g, Kiwa Experimental Animal Laboratory, Wakayama, Japan) were kept under a 12 h light/dark cycle (lights on from 07:00 to 19:00 h) in an air-conditioned room (23 ± 2 °C) with food (CE-2; Clea Japan, Tokyo, 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.

2.2. Experimental schedule

Mice were divided into two groups, one is a group of 4-h MCA occluded mice treated with each drugs in twice, immediately before and 3 h after cerebral ischemia. The other is a group of 4-h MCA occluded mice treated with each drugs in twice, immediately before and 3 h after cerebral ischemia at 24 h after 14-day repeated treatment with each drugs once a day.

2.3. Focal cerebral ischemia

Focal cerebral ischemia was induced according to the method described in our previous study (Egashira et al., 2004). The mice were anesthetized with 2% halothane and maintained thereafter with 1% halothane (Flosen, Takeda Chemical Industries, Osaka, Japan). After a midline neck incision, the left common and external carotid arteries were isolated and ligated. A nylon monofilament (8-0; Ethilon, Johnson & Johnson, Tokyo, Japan) coated with silicon resin (Xantopren, Heleus Dental Material, Osaka, Japan) 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 middle cerebral artery (MCA). After then, we stopped 1% halothane anesthesia. We confirmed whether middle cerebral artery was occluded in mice, using indicator of forelimb flexion after awaking from halothane anesthesia. Four hours after occlusion, the mice were re-anesthetized with halothane, and reperfusion was established by withdrawal of the filament.

2.4. 2,3,5-Triphenyltetrazolium chloride (TTC) staining

Twenty-four hours after MCA occlusion, the animals were sacrificed by decapitation. The brains were removed and sectioned coronally into four 2 mm slices using a mouse brain matrix. Slices were immediately stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA). The border between the infarcted and noninfarcted tissue was outlined with an image analysis system (NIH Image, version 1.63), and the area of infarction was measured and the infarction volume was calculated.

2.5. 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. The CBF was measured during 4 h MCA occlusion.

2.6. Western blot analysis

The expression of CB_1 receptor protein was evaluated by Western blotting following sample extraction and SDS-PAGE. Twenty-four hours after 4 h MCA occlusion (a group of 14-day repeated treatment or not), each tissue sample (cortex, striatum and hypothalamus) was homogenized at 4 °C for 1 min in lysis buffer [20 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100] with protease inhibitor cocktail. Tissue extract was centrifuged at 15,000 rpm at 4 °C for 30 min. The supernatant was treated in the same way as the tissue extract.

SDS sample buffer [125 mM Tris (pH 6.8), 2% SDS, 20% glycerol, 0.0001% bromo phenol blue and 10% β-mercaptoethanol] was added to aliquots of tissue extracts containing 15 μg total protein. Samples were heated at 95 °C for 5 min. Protein (15 μg) was separated by SDS-PAGE (12% gel). Blotting was performed at 2 mA/cm² by semi-dry type blotting (BIORAD). The blots were blocked with 5% non-fat dry milk in Tris buffer saline in 0.1% Tween 20 at 4 °C, and incubated with anti-CB1 polyclonal antibodies (1:200) in TBS-T, followed by goat anti-rabbit IgG (H + L) AP conjugate (1:1000) in TBS-T. The blots were visualized by AP color reagents.

Anti-CB1 polyclonal antibodies and anti-GAPDH were purchased from Calbiochem and Santa Cruz, respectively. Goat anti-rabbit IgG (H + L) AP conjugate, AP color reagents A and B were purchased from BIORAD.

The signal intensity of the blots was measured by an image analysis system (NIH Image, version 1.63).

2.7. Measurement of rectal temperature

Rectal temperature was measured immediately before and 1 h after MCA occlusion, using a digital laboratory thermometer (BAT-12, Physitemp Instruments, Clifton, NJ, USA) with a needle-type thermometer at a room temperature of 23 ± 1 °C.

2.8. Blood analysis

Physiological variables (pH, pCo_2, pO_2, hematocrit, potassium and sodium) were measured using a blood analysis system (International Technidyne Co.) at 4 h after MCA occlusion.

2.9. Assessment of antioxidant activity

Modifications were made to the original 1,1-diphenyl-2-picryhydrazyl (DPPH) radical method described by Brand-Williams et al. (1995). EC_{50} is a parameter widely used to express antioxidative power (Yoshida et al., 1989; Kanner et al., 1994). The lower the EC_{50} value, the higher the potential antioxidant activity. DPPH radical 100 μM were formed and the test compounds were prepared in 99% methanol, according to Son and Lewis (2002). The decrease in absorbance of DPPH radical at 520 nm was measured at different time intervals by Sunrise Rainbow Thermo (Wako, Tokyo, Japan) until the reaction reached a plateau. Methanol 99% was used as a blank solution, and DPPH radical solution without test samples. 1,1-Diphenyl-2-picryhydrazyl (DPPH) radical was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Methanol 99% was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

2.10. Drug preparation and administration

Δ^9-THC (isolated by Professor Y. Shoyama, Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyushu University, Japan), cannabidiol [(−)-cannabidiol, 2-[1R,6R]-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol, Sigma–Aldrich], SR141716A (N-(piperidine-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride, a generous gift from Sanofi Recherche, Montpellier, France), AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl) (4-methoxyphenyl)methanone (TOCRIS bioscience, Japan) and WAY100135 (N-tert-butyl-3(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide) (TOCRIS bioscience, Japan) were dissolved in 1% Tween. All these drugs were administered intraperitoneally (i.p.) immediately before and 3 h after MCA occlusion. The repeated treatment with Δ^9-THC and cannabidiol was administered i.p. once a day for 14 days.

2.11. Statistical analysis

These results are expressed as the mean ± SEM. Multiple comparisons were evaluated by Tukey's test after a one-way ANOVA. $P < 0.05$ was considered to be significant.

3. Results

3.1. Neuroprotective effects of Δ^9-THC but not cannabidiol were inhibited by the CB_1 antagonist

Both Δ^9-THC 3, 10 mg/kg and cannabidiol 1, 3 mg/kg significantly reduced the infarct volume induced by MCA occlusion in mice [$F(3, 28) = 10.004$, $P < 0.001$, Δ^9-THC 3, 10 mg/kg, $P < 0.01$; $F(3, 22) = 9.839$, $P < 0.001$, cannabidiol 1, 3 mg/kg, $P < 0.01$, Table 1]. Both SR141716 1 mg/kg and AM630 1 mg/kg alone did not change the infarct volume [$F(8, 54) = 9.180$, $P < 0.0001$, Δ^9-THC 10 mg/kg, $P < 0.01$; cannabidiol 3 mg/kg, $P < 0.01$, Fig. 1]. The neuroprotective effect of Δ^9-THC 10 mg/kg but not cannabidiol 3 mg/kg was inhibited by SR141716 1 mg/kg, unlike AM630 1 mg/kg (Fig. 1). The neuroprotective effect of Δ^9-THC 10 mg/kg and cannabidiol 3 mg/kg was not inhibited by AM630 10 mg/kg (data not shown).

3.2. Repeated treatment with Δ^9-THC but not cannabidiol developed tolerance to the neuroprotective and hypothermic effect

After 14-day repeated treatment with Δ^9-THC 10 mg/kg, it significantly increased the infarction. On the other hand,

Table 1
Effect of Δ^9-THC and cannabidiol on cerebral infarction caused by middle cerebral artery occlusion in mice
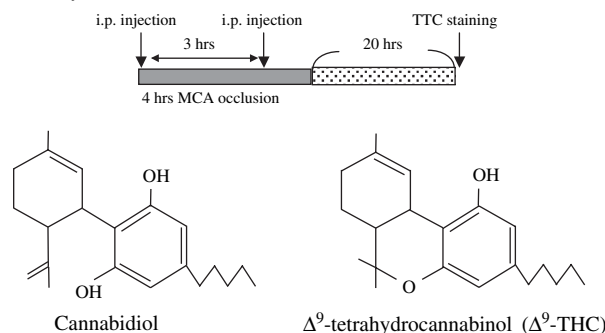
Drug	Dose	Infarct volume (mm^3)
Vehicle	0.1 ml/10 g	91.3 ± 3.5
Δ^9-THC	1 mg/kg	93.9 ± 9.3
	3 mg/kg	53.5 ± 10.4**
	10 mg/kg	46.7 ± 5.9**
Cannabidiol	0.1 mg/kg	94.3 ± 12.1
	1 mg/kg	49.2 ± 10.3**
	3 mg/kg	51.4 ± 7.3**

All drugs were injected i.p. immediately before and 3 h after MCA occlusion. Both Δ^9-THC 3, 10 mg/kg and cannabidiol 1, 3 mg/kg significantly reduced the infarct volume induced by MCA occlusion in mice. Values are expressed as the mean ± SEM ($n = 4$–9). **$P < 0.01$ compared with vehicle (one-way ANOVA test followed by Tukey's test).

14-day repeated treatment with cannabidiol 3 mg/kg did not change the infarction [$F(5.31) = 13.037$, $P < 0.001$, Δ^9-THC 10 mg/kg × 14 days compared with Δ^9-THC 10 mg/kg, $P < 0.05$, Fig. 2A]. In addition, after 14-day repeated treatment with Δ^9-THC 10 mg/kg, it significantly increased the rectal temperature (Δ^9-THC 10 mg/kg, $P < 0.01$ compared with vehicle, Fig. 2B). On the other hand, 14-day repeated treatment with cannabidiol 3 mg/kg did not change the rectal temperature (Δ^9-THC 10 mg/kg × 14 days compared with Δ^9-THC 10 mg/kg, $P < 0.01$, Fig. 2B).

3.3. Repeated treatment with Δ^9-THC and cannabidiol did not change in physiological variable data

In vehicle-treated groups, Δ^9-THC and cannabidiol, there was no significant difference about physiological variables (pH, pCo_2, pO_2, hematocrit, K, and Na). Furthermore, in all 14-day treated groups, there was no difference (Table 2).

3.4. Repeated treatment with Δ^9-THC but not with cannabidiol reversed the increase in cerebral blood flow

CBF was decreased after MCA occlusion by >90%. Both Δ^9-THC and cannabidiol significantly increased CBF during the 4-h MCA occlusion compared with the vehicle-treated group. Repeated treatment with Δ^9-THC but not with cannabidiol significantly reversed the increase in CBF
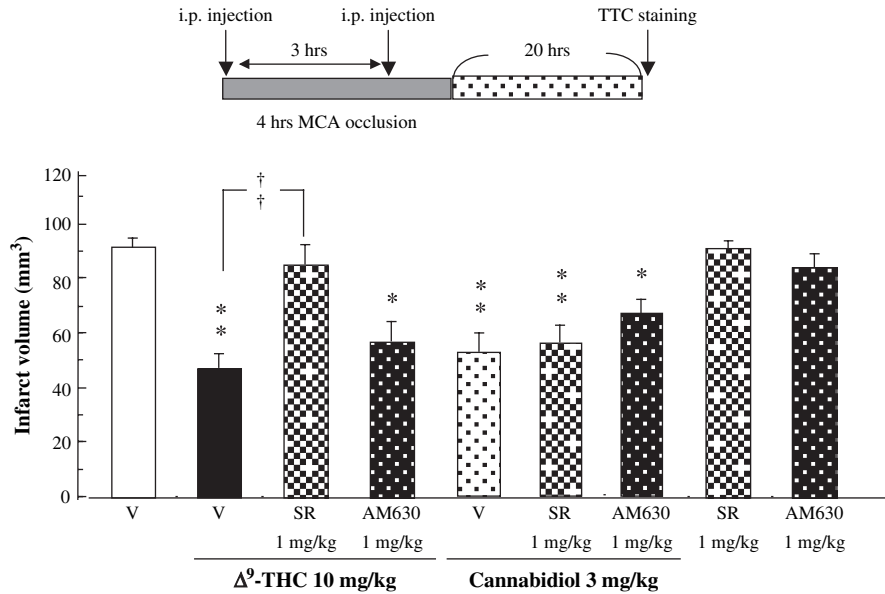
Fig. 1. Effect of SR141716 and AM630 on preventing cerebral infarction by treatment with Δ^9-THC and cannabidiol. All drugs were injected i.p. immediately before and 3 h after MCA occlusion. The neuroprotective effect of Δ^9-THC was inhibited by SR141716 but not by AM630. Values are expressed as the mean $\pm$ SEM ($n = 4–9$). *$P < 0.05$, **$P < 0.01$ compared with vehicle; $^{\dagger\dagger}P < 0.01$ compared with Δ^9-THC 10 mg/kg (one-way ANOVA test followed by Tukey's test).

$[F(5,12) = 11.166$, $P < 0.001$, Δ^9-THC, $P < 0.05$ compared with vehicle; cannabidiol, $P < 0.01$ compared with vehicle; 14-day repeated treatment with Δ^9-THC, $P < 0.05$ compared with Δ^9-THC; 14-day repeated treatment with cannabidiol, $P < 0.05$ compared with 14-day repeated treatment with vehicle, Table 2].

3.5. Repeated treatment with Δ^9-THC but not cannabidiol decreased the expression of CB_1 receptor protein in MCA occluded mice

At 24 h after MCA occlusion, the expression of CB_1 receptor decreased in striatum and cortex but not hypothalamus in
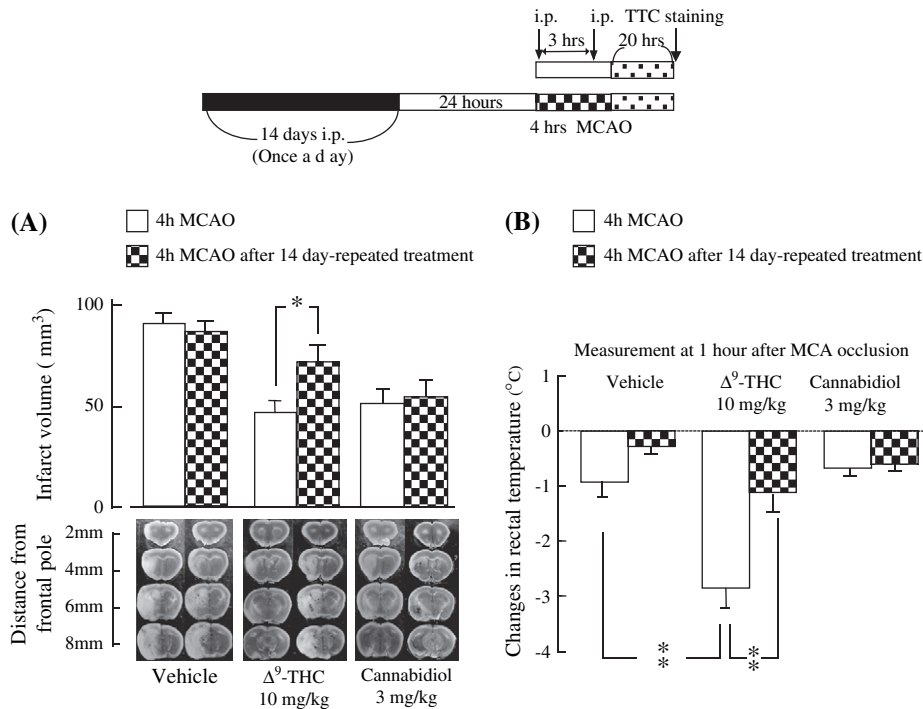

Fig. 2. Effect of repeated treatment with Δ^9-THC and with cannabidiol on cerebral infarction and rectal temperature in MCA occluded mice. Twenty-four hours after 14-day repeated treatment, Δ^9-THC and cannabidiol were injected i.p. immediately before and 3 h after MCA occlusion. Both neuroprotective effect (A) and hypothermic effect (B) of Δ^9-THC, but not cannabidiol, were inhibited by the 14-day repeated treatment. Values are expressed as the mean $\pm$ SEM ($n = 5–9$). *$P < 0.05$, **$P < 0.01$ compared with vehicle (one-way ANOVA test followed by Tukey's test).

Table 2
Physiological variable data
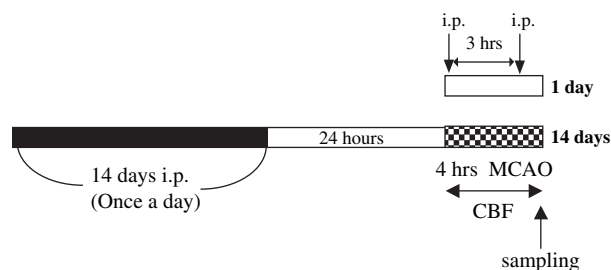
	Vehicle		Δ^9-THC 10 mg/kg		Cannabidiol 3 mg/kg	
	1 day	14 days	1 day	14 days	1 day	14 days
pH	7.19 ± 0.02	7.20 ± 0.01	7.29 ± 0.04	7.21 ± 0.04	7.20 ± 0.03	7.20 ± 0.03
pCo_2 (mmHg)	50.5 ± 2.7	52.3 ± 1.9	51.2 ± 3.2	48.8 ± 1.0	44.4 ± 1.5	50.7 ± 1.4
pO_2 (mmHg)	57.3 ± 6.2	56.9 ± 6.4	61.6 ± 4.0	61.5 ± 6.3	58.9 ± 6.9	60.0 ± 2.8
Hct (%)	38.1 ± 1.6	36.3 ± 1.0	35.1 ± 1.2	36.5 ± 1.6	35.9 ± 1.7	36.6 ± 0.3
Na (mM)	150.3 ± 2.1	154.6 ± 1.6	153.2 ± 6.0	150.8 ± 2.4	147.9 ± 2.0	154.9 ± 5.7
K (mM)	6.3 ± 1.5	5.4 ± 0.3	4.0 ± 0.7	4.9 ± 0.2	4.9 ± 1.1	4.9 ± 0.3
CBF (%) (average during 4 h MCAo)	14.0 ± 1.2	11.7 ± 1.3	$45.5 \pm 4.4^*$	$19.1 \pm 3.8^\dagger$	$52.0 \pm 11.2^{**}$	$40.5 \pm 0.7^\#$

Physiological variable data were obtained immediately before reperfusion, and drugs were administered immediately before and 3 h after cerebral ischemia. Δ^9-THC and cannabidiol, there was no significant difference about physiological variables (pH, pCo_2, pO_2, hematocrit, K, and Na). Furthermore, in all 14-day treated groups, there was no difference. Both Δ^9-THC and cannabidiol significantly increased CBF during the 4-h MCA occlusion compared with the vehicle-treated group. Repeated treatment with Δ^9-THC but not with cannabidiol significantly reversed the increase in CBF. Values are expressed as the mean $\pm$ SEM ($n = 3$–4). $*P < 0.05$, $**P < 0.01$ compared with vehicle, $^\dagger P < 0.05$ compared with 1 day Δ^9-THC 10 mg/kg, $^\# P < 0.05$ compared with the repeated treatment with vehicle (one-way ANOVA test followed by Tukey's test).

vehicle-treated group [striatum, $F(3,9) = 11.257$, $P < 0.01$, vehicle $P < 0.01$ compared with control; cortex, $F(3,9) = 4.039$ $P < 0.05$, vehicle $P < 0.05$ compared with control, Fig. 3A]. Δ^9-THC and cannabidiol did not change in CB_1 receptor

expression (Fig. 3A). But repeated treatment with Δ^9-THC significantly decreased the expression level of CB_1 receptor at striatum, cortex and hypothalamus. On the other hand, cannabidiol had no effect on CB_1 receptor expression [striatum,
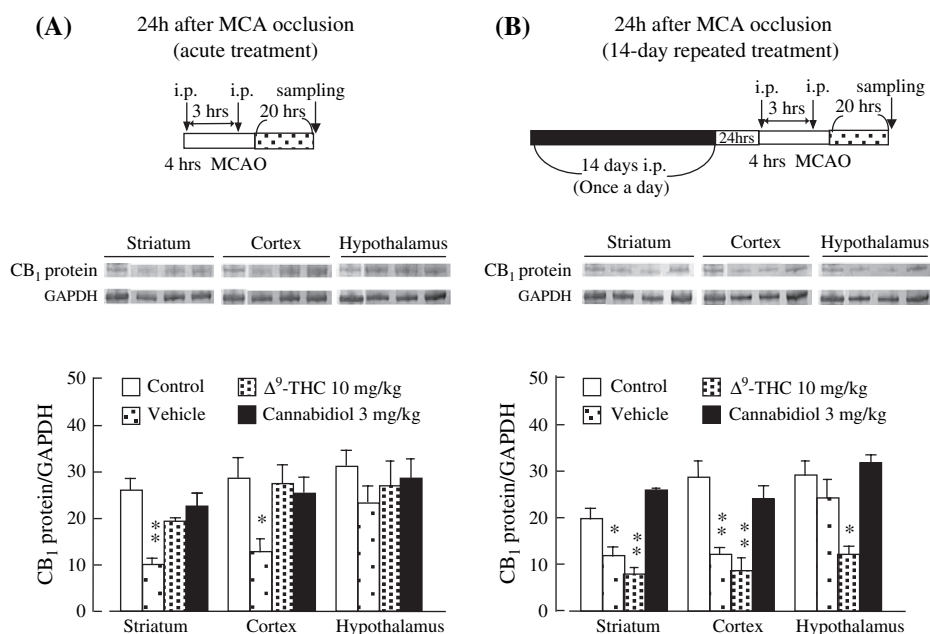

Fig. 3. Effect of repeated treatment with Δ^9-THC and with cannabidiol on CB_1 receptor expression at 24 h after MCA occlusion. At 24 h after MCA occlusion, the expression of CB_1 receptor decreased in striatum and cortex but not in hypothalamus in vehicle-treated group. Δ^9-THC and cannabidiol had no change in CB_1 receptor expression (A). But repeated treatment with Δ^9-THC significantly decreased the expression level of CB_1 receptor at striatum, cortex and hypothalamus. On the other hand, cannabidiol had no effect on CB_1 receptor expression (B). Values are expressed as the mean $\pm$ SEM ($n = 3$). $*P < 0.05$ compared with vehicle (one-way ANOVA test followed by Tukey's test).

$F(3,8) = 29.069$, $P < 0.001$, vehicle $P < 0.05$, Δ^9-THC $P < 0.01$ compared with control; cortex, $F(3,8) = 12.753$, $P < 0.01$, vehicle $P < 0.01$, Δ^9-THC $P < 0.01$ compared with control; hypothalamus, $F(3,8) = 9.855$, $P < 0.01$, Δ^9-THC $P < 0.05$ compared with control, Fig. 3B].

3.6. Both the neuroprotective effect of cannabidiol and the CBF increased by cannabidiol was inhibited by WAY100135, 5-HT$_{1A}$ receptor antagonist

The neuroprotective effect of cannabidiol and CBF increased by cannabidiol was significantly inhibited by WAY100135, 5-HT$_{1A}$ receptor antagonist [infarct volume; $F(3,27) = 5.283$, $P < 0.01$; cannabidiol, $P < 0.01$, compared with vehicle, CBF; $F(3,8) = 29.788$, $P < 0.001$; cannabidiol, $P < 0.01$, compared with vehicle; cannabidiol, $P < 0.01$, compared with cannabidiol + WAY100135 10 mg/kg, Fig. 4A]. After 14-day repeated treatment with cannabidiol, the effects of cannabidiol were inhibited by WAY100135, in MCA occluded mice. [infarct volume; $F(3,13) = 4.192$, $P < 0.05$; cannabidiol, $P < 0.05$, compared with vehicle, CBF; $F(3,8) = 32.007$, $P < 0.001$; cannabidiol, $P < 0.01$, compared with vehicle; cannabidiol, $P < 0.01$, compared with cannabidiol + WAY100135 10 mg/kg, Fig. 4B].

3.7. Antioxidant activity

Cannabidiol exhibited stronger antioxidative power ($EC_{50} = 89.2$ μM) than Δ^9-THC ($EC_{50} = 464.2$ μM) (Fig. 5).

4. Discussion

Both Δ^9-THC and cannabidiol significantly reduced the infarct volume in a mouse MCA occlusion model and the neuroprotective effect of Δ^9-THC was inhibited by CB$_1$ receptor antagonist SR141716, but not by CB$_2$ receptor antagonist AM630. Cannabidiol was not inhibited by either antagonist. Twenty-four hours after the 14-day repeated treatment with Δ^9-THC, but not with cannabidiol, tolerance developed to the neuroprotective effects and hypothermic effect in MCA occluded mice. In addition, Δ^9-THC and cannabidiol significantly increased the CBF in MCA occluded mice and repeated treatment with Δ^9-THC but not with cannabidiol reversed the increase in the CBF. In MCA occluded mice, repeated treatment with Δ^9-THC caused CB$_1$ receptor desensitization and down-regulation. After 14-day repeated treatment with cannabidiol, the neuroprotective effect of cannabidiol and the CBF increased by cannabidiol was inhibited by WAY100135, 5-HT$_{1A}$ receptor antagonist. In addition, cannabidiol suppressed
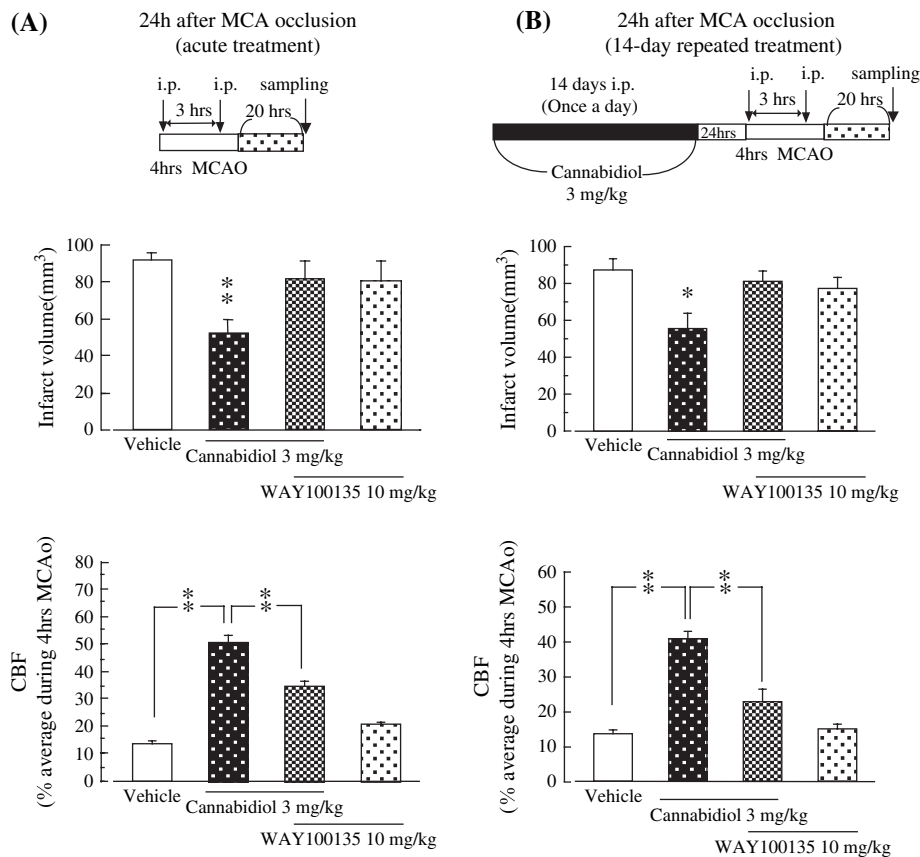

Fig. 4. Both the neuroprotective effect of cannabidiol and the CBF increased by cannabidiol was inhibited by WAY100135, 5-HT$_{1A}$ receptor antagonist. The neuroprotective effect of cannabidiol was inhibited by WAY100135, 5-HT$_{1A}$ receptor antagonist, in MCA occluded mice. In addition, the CBF increased by cannabidiol was inhibited by WAY100135, 5-HT$_{1A}$ receptor antagonist, in MCA occluded mice (A). The neuroprotective effect and the increase in CBF of repeated treatment with cannabidiol were also inhibited by WAY100135 (B). Values are expressed as the mean ± SEM ($n = 3-5$). *$P < 0.05$, **$P < 0.01$ compared with vehicle group which was treated cannabidiol 3 mg/kg repeatedly (one-way ANOVA test followed by Tukey's test).
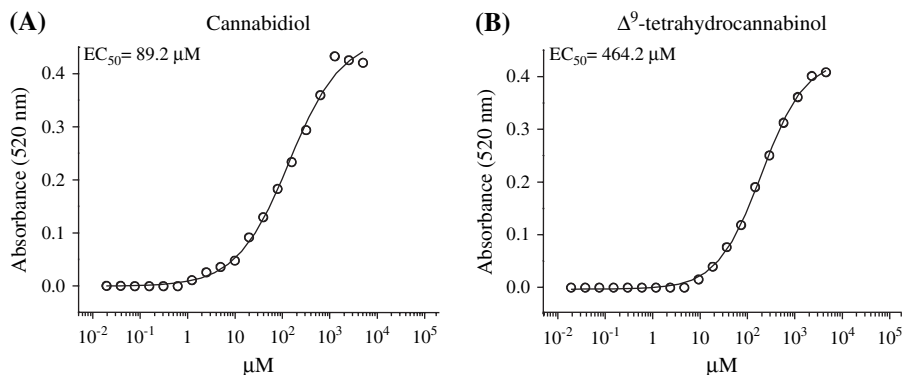
(A)   Cannabidiol

$EC_{50} = 89.2 \ \mu M$

Absorbance (520 nm)

(B) Δ^9-tetrahydrocannabinol

$EC_{50} = 464.2 \ \mu M$

Absorbance (520 nm)

Fig. 5. Antioxidant activity. The lower the EC_{50} value, the higher the potential antioxidant activity. Cannabidiol exhibited stronger antioxidative power ($EC_{50} = 89.2 \ \mu M$) than Δ^9-THC ($EC_{50} = 464.2 \ \mu M$).

the DPPH radical and was more active than Δ^9-THC. The results presented here support the view that cannabidiol exerts cerebroprotective activity without tolerance different from that of Δ^9-THC with tolerance to the neuroprotective effect.

Δ^9-THC is known to produce hypothermia, neuroprotection, increase in regional CBF and tolerance via the cannabinoid CB_1 receptor (Wiley and Martin, 2002; Mishima et al., 2001; Leker et al., 2003; Braida et al., 2003; Rubino et al., 2000; Mathew et al., 1999, 2002). On the other hand, cannabidiol, a non-psychoactive constituent of *Cannabis*, has been shown to be protective against global and focal ischemic injury, in agreement with the present study (Braida et al., 2003). The neuroprotective mechanism of cannabidiol remains unclear, but novel non-CB_1 and non-CB_2 receptors have been proposed, because cannabidiol has a very low affinity (in the micromolar range) for CB_1 and CB_2 receptors and has many pharmacological actions (Wiley and Martin, 2002). In this study, Δ^9-THC was shown to have a neuroprotective effect on cerebral injury induced by MCA occlusion via the CB_1 receptor but not the CB_2 receptor. On the contrary, cannabidiol was not inhibited by either CB_1 or CB_2 receptor antagonists. These results suggest that Δ^9-THC exerts its neuroprotective action through the CB_1 receptor, while cannabidiol prevents cerebral infarction via a CB_1 and CB_2 receptor-independent mechanism.

The neuroprotective effect of Δ^9-THC and other cannabinoids is related to the CB_1 receptor-mediated inhibition of voltage-sensitive Ca^{2+} channels, which reduces Ca^{2+} influx, glutamate release and excitotoxicity (Iuvone et al., 2004) and increases regional CBF (Mathew et al., 1999, 2002). Moreover, in this study, we showed that the neuroprotective effect of Δ^9-THC was inhibited by SR141716 but not AM630. Moreover, Δ^9-THC increased the CBF in MCA occluded mice, while repeated treatment with Δ^9-THC reversed the increase in CBF. In addition, repeated treatment with Δ^9-THC caused CB_1 receptor desensitization and down-regulation in mice subjected to MCA occlusion, which might correlate with tolerance of CB_1 receptor activity induced by repeated treatment with Δ^9-THC. These results suggest that the neuroprotective effect of Δ^9-THC is induced via cannabinoid CB_1 receptor in MCA occluded mice, and long-term stimulation

of CB_1 receptor leads to development of tolerance to these pharmacological effects.

Cannabidiol showed a neuroprotective effect via CB_1 and CB_2 receptor-independent mechanism. It has been reported that post-ischemic treatment with cannabidiol prevented electroencephalographic flattening, hyperlocomotion and neuronal injury in gerbils in agreement with the present study (Braida et al., 2003). Moreover, cannabidiol has been shown to be protective against *N*-methyl-D-aspartate and beta-amyloid peptide toxicity (Iuvone et al., 2004) and global and focal ischemic injury (Hampson et al., 1998, 2000). However, the neuroprotective mechanisms of cannabidiol remain unclear, but the novel non-CB_1 and non-CB_2 receptors have been proposed, because cannabidiol has a very low affinity (in the micromolar range) for CB_1 and CB_2 receptors and has many pharmacological actions (Mechoulam et al., 2002). Our previous report demonstrated that the neuroprotective effect of cannabidiol was not inhibited, both by CB_1 receptor antagonist and by warming (Hayakawa et al., 2004). It has been reported that warming increased pro-inflammatory agents (Forsyth and Levinsky, 1990; Kurabayashi et al., 1997). Cannabidiol inhibited the leukocyte secretion of TNF and IL-1 in vitro study (Watzl et al., 1991). Moreover, cannabidiol inhibited phorbol ester plus calcium ionophore-stimulated interleukin-2 production by mouse splenocytes via cannabinoid receptor-independent mechanisms (Kaplan et al., 2003). These reports suggest that the neuroprotective effect produced by cannabidiol involves a potent anti-inflammatory mechanism, which might be cannabinoid receptor-independent.

Repeated treatment with Δ^9-THC results in the development of tolerance to most acute behavioral and pharmacological effects produced by Δ^9-THC (Abood and Martin, 1992; Sim et al., 1996). Δ^9-THC has been shown to induce tolerance to hypoactivity, hypothermia, antinociception, catalepsy and pentobarbital-induced sleep prolongation (Sim et al., 1996; Watanabe et al., 1982, 1983). Twenty-four hours after the 14-day repeated treatment with Δ^9-THC, but not with cannabidiol, developed tolerance to the neuroprotective effect, the increase in cerebral blood flow and hypothermic effect in mice subjected to MCA occlusion without changing the physiological variable data. In a previous study, the neuroprotective and hypothermic

effects of Δ^9-THC were also completely inhibited by SR141716 and by warming of the animals (Hayakawa et al., 2004). Moreover, other artificial cannabinoids, HU210 and WIN55,212-2, caused neuroprotection and hypothermia via a CB_1 receptor (Leker et al., 2003; Bonfils et al., 2006). In addition, the neuroprotective effect of HU210 was inhibited by warming (Leker et al., 2003). Thus, we hypothesized that the neuroprotective effect of CB_1 receptor agonists might be mediated, at least in part, by hypothermia through the hypothalamic CB_1 receptor, primarily in regions associated with thermoregulation. At 24 h after MCA occlusion, the expression level of CB_1 receptor protein in the hypothalamus was attenuated by repeated treatment with Δ^9-THC but not vehicle and cannabidiol. In addition, the repeated treatment with Δ^9-THC reversed the hypothermic effect. This result supports that the neuroprotective effect of Δ^9-THC might be mediated by hypothermia through the hypothalamic CB_1 receptor. As well as the hypothalamic CB_1 receptor, the 14-day repeated treatment with Δ^9-THC decreased the expression of CB_1 receptor protein in the cortex and striatum at 24 h after the MCA occlusion. It has been reported that the expression level of CB_1 receptor depends on a neuroprotective and potential therapeutic role in stroke for drugs that activate the CB_1 receptor (Jin et al., 2000). Therefore, decreasing expression of CB_1 receptors may attenuate the neuroprotective effect of Δ^9-THC on cerebral ischemic injury.

On the other hand, cannabidiol did not lead to development of tolerance to the neuroprotective effect on infarction and increase in the CBF in MCA occluded mice. We suggest that the neuroprotective effect of cannabidiol might be, at least in part, related to the activation of the $5\text{-}HT_{1A}$ receptor (Mishima et al., 2005). A $5\text{-}HT_{1A}$ receptor agonist has been shown to reduce the cortical infarct volume induced by permanent MCA occlusion, and to have a neuroprotective effect in vitro (Semkova et al., 1998). In addition, an agonist of the $5\text{-}HT_{1A}$ receptor has played an important role as a vasodilator (Hill et al., 2003; Adnot et al., 1995). A $5\text{-}HT_{1A}$ receptor full agonist, 8-OH-DPAT, has shown the development of tolerance to the hypothalamic effect (Renyi et al., 1992), while a $5\text{-}HT_{1A}$ receptor partial agonist such as buspirone has not shown (Young et al., 1993). In fact, after repeated treatment with cannabidiol, the neuroprotective effect of cannabidiol and CBF increased by cannabidiol was in part inhibited by $5\text{-}HT_{1A}$ receptor antagonist, WAY100135. Repeated treatment with cannabidiol did not cause development of tolerance to the neuroprotective effect, and the increase in CBF, therefore, cannabidiol might be a partial, but not a full, agonist for the $5\text{-}HT_{1A}$ receptor.

Both cannabidiol and Δ^9-THC suppressed the oxidation potential measured by cyclic voltammetry, with cannabidiol being more active (Hampson et al., 2000), suggesting that cannabidiol may be a neuroprotective antioxidant. We also examined the effects of cannabidiol and Δ^9-THC on the 1,1-diphenyl-2-picryhydrazyl (DPPH) radical in an in vitro study. The results showed that both drugs suppressed the DPPH radical and cannabidiol was more active than Δ^9-THC. Thus, cannabidiol is a potent antioxidant agent, acting through a CB_1 cannabinoid receptor-independent mechanism, and without developing tolerance to its neuroprotective effect, which

cannabidiol might have a potent anti-inflammatory, antioxidative and partial $5\text{-}HT_{1A}$ receptor agonistic mechanism.

In conclusion, the findings of the present study show that cannabidiol exerts a neuroprotective effect through a CB_1 and CB_2 receptor-independent mechanism. In addition, repeated treatment with Δ^9-THC led to development of tolerance to neuroprotective effect and hypothermic effect through CB_1 receptor-dependent mechanism. On the other hand, cannabidiol did not lead to development of tolerance to the neuroprotective effect and the increase in CBF in MCA occluded mice. In addition, cannabidiol can suppress the DPPH radical and was more active than Δ^9-THC. Cannabidiol shows cerebroprotective activity different from that of Δ^9-THC. It is to be hoped that cannabidiol will have a role in palliative treatment and open new therapeutic options for treating cerebrovascular disorders.

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