Therapeutic Time Window of Cannabidiol Treatment on Delayed Ischemic Damage via High-Mobility Group Box 1-Inhibiting Mechanism

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Cannabidiol decreases cerebral infarction and high-mobility group box 1 (HMGB1) in plasma in ischemic early phase. However, plasma HMGB1 levels in ischemic delayed phase reach higher concentration with the progressing brain injury. In this study, we investigated the therapeutic time window of cannabidiol on functional deficits, glial HMGB1 and plasma HMGB1 levels in a 4 h mouse middle cerebral artery (MCA) occlusion model. Cannabidiol-treated mice were divided into 3 groups as follows: group (a) treated from day 1, group (b) treated from day 3, group (c) treated from day 5 after MCA occlusion. Moreover, minocycline, microglia inhibitor, and fluorocitrate, an inhibitor of astroglial metabolism, were used to compare with cannabidiol-treated group. Repeated treatment with cannabidiol from 1 and 3 d at the latest after cerebral ischemia improved functional deficits and survival rates. However, cannabidiol from 5 d could not improve the ischemic damage as well as fluorocitrate-treated group. Moreover, both group (a), group (b) and minocycline but not group (c) and fluorocitrate-treated group had a decrease in the number of Iba1 expressing HMGB1 positive cells and HMGB1 levels in plasma. Cannabidiol may provide therapeutic possibilities for the progressing brain injury via HMGB1-inhibiting mechanism.

Key words: cannabidiol; cerebral ischemia; therapeutic time window; high-mobility group box 1

Cannabis contains about 60 different cannabinoids, including the psychoactive component, Δ⁹-tetrahydrocannabinol as well as non-psychoactive components, which include cannabidiol, cannabinol and cannabigerol. Among these components, cannabidiol, a non-psychoactive constituent of cannabis, is known to exert potent anti-inflammatory, immunomodulatory and analgesic effects. In addition, cannabidiol has been shown to be protective against global and focal ischemic injury. Although cannabidiol is generally known to have a very low affinity (in the micromolar range) for the cannabinoid CB₁ and CB₂ receptors, it has many pharmacological actions, including anxiolytic actions and anti-inflammatory and a neuroprotective effect against ischemic injury. These actions are thought to be dependent on a new abnormal cannabinoid receptor-independent mechanism. In addition, cannabidiol has been shown to significantly prevent infarction and myeloperoxidase (MPO) activity after reperfusion via a cannabinoid receptor-independent mechanism. Recently, we have reported that cannabidiol decreased the cerebral infarction via MPO expression high-mobility group box 1 (HMGB1)-inhibiting mechanism. In addition, cannabidiol decreased the level of HMGB1 in plasma in ischemic early phase.

HMGB1, a non-histone DNA-binding protein, is widely expressed in various tissues, including the brain. HMGB1 has been implicated in diverse intracellular functions, including the stabilization of nucleosomal structure and the facilitation of gene transcription. HMGB1 is known to be massively released into the extracellular space by monocytes and macrophages or necrotic cells immediately after an ischemic insult, and induces expression of several genes related to progressive inflammation, leading to apoptosis in the post-ischemic brain. In addition, we have reported that repeated treatment with minocycline, microglia inhibitor, for 14 d improved functional deficits, and decreased plasma levels of HMGB1 and the expression of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) positive cells at 14 d after cerebral ischemia, suggesting that the extracellular HMGB1 level is of considerable importance for the treatment of post-ischemic injury.

Cannabidiol decreased cerebral infarction and HMGB1 in plasma in ischemic early phase. However, plasma HMGB1 levels in ischemic delayed phase reached higher concentration with the progressing brain injury. Therefore, we examined whether cannabidiol can inhibit the progressive inflammation reaction related with HMGB1 and estimated the therapeutic time window of cannabidiol in ischemic delayed phase.

MATERIALS AND METHODS

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 guidelines of the institutional committee for the care and use of animals in biomedical research.
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. Mice were anesthetized with 2% halothane and maintained thereafter with 1% halothane (Flossen, 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). Following occlusion, we stopped the 1% halothane anesthesia. We confirmed occlusion of the MCA by examining forelimb flexion after awakening from halothane anesthesia. Four hours after occlusion, the mice were re-anesthetized with halothane, and reperfusion was established by withdrawal of the filament.

**Neurological Score** The score was divided into 5 groups: 0; normal motor function, 1; flexion of torso and of contralateral forelimb upon lifting of the animal by the tail, 2; circling to the ipsilateral side but normal posture at rest, 3; circling to the ipsilateral side, 4; rolling to the ipsilateral side, 5; leaning to the ipsilateral side at rest (no spontaneous motor activity). Neurological score was measured at 1, 7, and 14 d after cerebral ischemia.

**Rota-Rod Test in MCA-Occluded Mice** Motor coordination was measured using the rota-rod test as described previously. Mice were placed on a rotating rod (diameter: 3 cm; Neuroscience Inc., Tokyo, Japan) with a non-skid surface, and the latency to fall was measured for up to 2 min. The rotation speed was 10 rpm.

**Fluorescent Immunostaining** Mice (n=5 in each group) were sacrificed by decapitation after perfusion using saline and 4% paraformaldehyde at 1, 3, 7, and 14 d after MCA occlusion. The brains were removed of fat and water by an auto-degussing unit (RH-12, Sakura Seiko Co., Tokyo, Japan) and then embedded in paraffin. Subsequently, 5-μm sections were mounted on slides and dried at 37 °C for 1 d. After deparaffinization and rehydration, these sections were re-anesthetized with halothane, and reperfusion was established by withdrawal of the filament.

**TUNEL Staining** After deparaffinization and rehydration, these sections were assayed for TUNEL using direct binding of fluorescein-conjugated dUTP (green fluorochrome) with anti-mouse NeuN (Chemicon International, Temecula, CA, U.S.A.) providing the red counterstain, and using the FITC-Apoptosis detection system (Promega, Madison, Wisconsin, U.S.A.). The coverslips were mounted then analyzed by fluorescence microscopy.

**HMGB1 Measurements** Plasma samples were fractionated by SDS-PAGE, and HMGB1 levels were determined by immunoblotting using a standard curve for recombinant HMGB1 as a reference (Sigma-Aldrich, St. Louis, MO, U.S.A.).

**Drug Preparation** Cannabidiol (TOCRIS bioscience, Bristol, U.K.) was dissolved at 0.3 mg/ml in 1% Tween. Cannabidiol (0.3 mg/ml) was administered intraperitoneally (i.p. 0.1 ml/10 g) at 3 mg/kg on the following schedule depending on the group: group (a) treated daily for 14 d from day 1, group (b) treated daily for 12 d from day 3, group (c) treated daily for 10 d from day 5 after MCA occlusion. Minocycline (1 mg/ml, Sigma-Aldrich, St. Louis, MO, U.S.A.), microglia inhibitor, was administered intraperitoneally (i.p. 0.1 ml/10 g) from 5 d after cerebral ischemia. In addition, fluorocitrate (1 mmol/site, Sigma-Aldrich, St. Louis, MO, U.S.A.), an inhibitor of astroglial metabolism, was injected intracortically from 5 d after cerebral ischemia.

The fluorocitrate solution for intracortical injection was prepared as follows: 8 mg of D,L-fluorocitric acid, Ba, salt (Sigma-Aldrich, St. Louis, MO, U.S.A.) was dissolved in 1 ml of 0.1 M HCL. Two to three drops of 0.1 M Na2SO4 was added to precipitate Ba2+. Two milliliters of 0.1 M NaHPO4 was added, and the suspension was centrifuged at 1000 g for 5 min. The supernatant was diluted with 0.9% NaCl to the final concentration, and pH was adjusted to 7.4.

The fluorocitrate solution was microinjected stereotaxically into the sensory-motor cortex region (anterior: −0.22 mm; lateral: 2.5 mm from bregma; depth: 2 mm from the skull surface). The coordinates were based on the mouse brain atlas of Franklin and Paxinos. One microliter was injected continuously at a rate of 0.25 μl/min through a stainless steel cannula (28 gauge) connected to a 25-μl syringe driven by a slow-injection pump.

**Statistical Analysis** Results are expressed as the mean ± S.E.M. Multiple comparisons were evaluated by Tukey–Kramer’s test after one-way ANOVA. p<0.05 was considered to be statistically significant.

**RESULTS**

**The Expression Changes of HMGB1 on Neutrophils, Microglia, and Astrocytes Following Cerebral Ischemia** HMGB1 expressing MPO-positive cells were observed at 1 and 3 d after cerebral ischemia. Similarly, Iba1 expressing HMGB1 positive cells were observed at 1, 3, 7 and 14 d in the ischemic core including the striatum after cerebral ischemia. On the other hand, GFAP expressing HMGB1 positive cells were observed at 7 and 14 d in the ischemic penumbra. TUNEL positive cells increased in the ischemic core at a peak 7 d after cerebral ischemia, but they were rarely observed in the ischemic penumbra except for 7 d after cerebral ischemia (Fig. 1).

**Effect of Cannabidiol on Neurological Impairment after Cerebral Ischemia** The mice subjected to MCA occlusion had a significantly impaired neurological func-
Cannabidiol-treated group (a) and group (b) had an improved neurological score 14 d after cerebral ischemia compared with the vehicle treated group. On the other hand, group (c) did not show an improved survival rate compared with the vehicle treated group. On the other hand, group (c) did not have an improved neurological score [F(4,20)=11.767, p<0.01, one-way ANOVA; vehicle treated group, group (a), group (b), group (c), **p<0.01 compared with sham, 1 d after cerebral ischemia. F(4,20)=8.463, p<0.01, one-way ANOVA; vehicle treated group, group (a), group (b), group (c), **p<0.01 compared with vehicle treated group, 7 d after cerebral ischemia. F(4,20)=14.200, p<0.01, one-way ANOVA; vehicle treated group, group (a), group (c), **p<0.01 compared with sham. Group (a), group (b), **p<0.01 compared with vehicle treated group, 14 d after cerebral ischemia, *p<0.05, **p<0.01 compared with vehicle treated group. Tukey–Kramer’s test (Fig. 2B).

Effect of Cannabidiol on Motor Coordination after Cerebral Ischemia The mice subjected to MCA occlusion had a significantly impaired motor coordination. Cannabidiol-treated group (a) and group (b) showed improved motor coordination on a rota-rod test compared with the vehicle treated group. On the other hand, group (c) did not show improved motor coordination [F(4,20)=86.283, p<0.01, one-way ANOVA; vehicle treated group, group (a), group (b), group (c), ***p<0.01 compared with sham, 1 d after cerebral ischemia. F(4,20)=19.092, p<0.01, one-way ANOVA; vehicle treated group, group (a), group (b), group (c), ***p<0.01 compared with sham, 7 d after cerebral ischemia. F(4,20)=12.775, p<0.01, one-way ANOVA; vehicle treated group, group (c), ***p<0.01 compared with sham, group (a), *p<0.05 compared with vehicle treated group, 14 d after cerebral ischemia (Fig. 2C).

Effect of Cannabidiol on the Survival Rate after MCA Occlusion Cannabidiol-treated group (a) and group (b) had an improved survival rate compared with the vehicle treated group. On the other hand, group (c) did not show an improved survival rate (Fig. 2D).

Effect of Microglia Inhibitor, Minocycline, and Fluorocitrate, on Neurological Impairment, Motor Coordination, and Survival Rate in MCA Occluded Mice It is shown the schedule of minocycline 10 mg/kg and fluorocitrate 1 nmol/site after MCA occlusion (Fig. 3A). Minocycline-treated group but not fluorocitrate-treated group showed improved neurological score, motor coordination and survival rates. [Neurological score: F(3,22)=14.083, p<0.01, one-way ANOVA; vehicle treated group, fluorocitrate-treated group, **p<0.01, *p<0.05 compared with sham, 14 d after cerebral ischemia (Fig. 3B). Motor coordination: F(3,22)=240.010, p<0.01, one-way ANOVA; vehicle treated group, minocycline-treated group, fluorocitrate-treated group, **p<0.01 compared with sham. Minocycline-treated group, *p<0.01 compared with vehicle-treated group, 14 d after cerebral ischemia (Fig. 3C)].

Effect of Cannabidiol on Microglia- and Astrocyte-Expressing HMGB1 and TUNEL Positive Cells Cannabidiol-treated group (a) and group (b) had a decrease in the number of Iba1 expressing HMGB1 positive cells and TUNEL positive cells in the ischemic core. On the other hand, group (c) did not show a decrease in these cells. Group (b) showed no changes in the GFAP expressing HMGB1 positive cells and TUNEL positive cells in the ischemic penumbra. Cannabidiol-treated group (a) displayed the morphology of fibrous astrocytes. In addition, TUNEL positive cells were rarely observed. On the other hand, treatment of group (c) and fluorocitrate resulted in a disruption in the morphology of the astrocytes and the number of TUNEL positive cells did not decrease in the ischemic penumbra 14 d after cerebral ischemia (Fig. 4A). The amount of HMGB1 released into the plasma was significantly increased at 14 d after cerebral ischemia. Group (a), group (b) and minocycline-treated group did not
Fig. 2. Effect of Cannabidiol on Neurological Impairment, Motor Coordination, and Survival Rate in MCA Occluded Mice

It is shown the schedule of repeated treatment with cannabidiol (3 mg/kg, i.p.) after MCA occlusion (A). MCA occluded mice exhibited a significantly impaired neurological function and motor coordination, and decreased survival rate. Cannabidiol-treated group (a) and group (b) showed improved neurological score (B), motor coordination (C) and survival rates (D). On the other hand, group (c) did not show improved functional deficits and survival rate. Values are expressed as the mean ± S.E.M. (B) **p<0.01 compared with sham group, ##p<0.01 compared with 14d vehicle treated group. (C) **p<0.01 compared with sham group, *p<0.05 compared with 14d vehicle treated group (n=6—8). (Tukey-Kramer’s test).

Fig. 3. Effect of Microglia Inhibitor, Minocycline, and Gliotoxin, Fluorocitrate, on Neurological Impairment, Motor Coordination, and Survival Rate in MCA Occluded Mice

It is shown the schedule of minocycline 10 mg/kg and fluorocitrate 1 nmol/site after MCA occlusion (A). Minocycline-treated group but not fluorocitrate-treated group showed improved neurological score (B), motor coordination (C) and survival rates (D). Values are expressed as the mean ± S.E.M. (B) **p<0.01 compared with sham group. (C) ***p<0.01 compared with sham group, *p<0.01 compared with 14d vehicle treated group (n=6—8). (Tukey-Kramer’s test).
increase the HMGB1 level in plasma [F(6,21) = 6.380, p < 0.01, one-way ANOVA; vehicle treated group and group (c), fluorocitrate-treated group, **p < 0.01 compared with the sham-operated group, Fig. 4B].

DISCUSSION

Repeated treatment with cannabidiol (3 mg/kg, i.p.) from 1 d or 3 d after cerebral ischemia improved the functional deficits, survival rates and decreased the HMGB1 level in plasma and the number of Iba1 expressing HMGB1 positive cells. Additionally, both groups decreased TUNEL positive cells in ischemic core after cerebral ischemia. On the other hand, cannabidiol from 5 d but not minocycline from 5 d after cerebral ischemia did not improve the functional deficits as well as fluorocitrate-treated group. Our results suggest that cannabidiol will open the therapeutic possibilities for treatment of post-ischemic injury via modulating glial HMGB1.

In this study, Iba1 expressing HMGB1 and TUNEL positive cells increased during 1—14 d after cerebral ischemia in the ischemic core with the impairment of neurological functions. On the other hand, astrocyte expressing HMGB1 positive cells were observed during 7—14 d after cerebral ischemia in the penumbra region, and TUNEL positive cells were rarely observed in this region compared with ischemic core region. These results suggest that the role of microglia
expressing HMGB1 would be different from that of astrocyte expressing HMGB1 because the number of TUNEL positive cells was different in each of the ischemic regions.

Repeated treatment with cannabidiol from 1 d or 3 d after cerebral ischemia improved the functional deficits, such as neurological score and motor coordination, and survival rates. In addition, both groups did not increase the HMGB1 level in plasma, and decreased the number of Ibal1 expressing HMGB1 positive cells and TUNEL positive cells. However, treatment with cannabidiol from 5 d after cerebral ischemia did not improve them. From these results it was determined that the therapeutic time window of cannabidiol in ischemic delayed phase is at the latest 3 d after ischemic insult through an activated microglia-expressing HMGB1 inhibiting mechanism.

In previous study, cannabidiol produced a cerebroprotective effect that was mediated by inhibition of MPO activity after cerebral ischemia, via a CB₁ and CB₂ receptor-independent mechanism in ischemic acute phase.⁹ In addition, although the cerebroprotective effect of cannabidiol was partly inhibited by 5-HT1A receptor antagonist, WAY100135,¹⁸ but not the MPO inhibition of cannabidiol. Therefore, the effect of cannabidiol would depend on antioxidant effect or other effects. Recently, it has been reported that cannabidiol inhibits microglial cell migration,²⁰ and prevents astroglial activation via inhibiting the expression of GFAP mRNA and protein.²¹ Moreover, it has also been reported that cannabidiol has the ability to enhance adenosine signaling through inhibition of uptake,²⁵ suggesting that it leads to a decrease of ATP induction. Taken together, cannabidiol might inhibit glial activation both microglia and astrocyte via a decrease of ATP induction, and then cannabidiol might inhibit the expression of HMGB1 in both glial cells. We have reported that repeated treatment with minocycline, a microglia inhibitor, for 14 d from 1 d after cerebral ischemia decreased activated microglia expressing HMGB1 within the brain, and also decreased plasma HMGB1 at 14 d after MCA occlusion. Additionally, minocycline significantly decreased the number of TUNEL positive cells at 14 d after cerebral ischemia.²³ However, minocycline did not affect reactive astrocytes expressing HMGB1 on the same time frame. Next, to inhibit reactive astrocytes induced in ischemic delayed phase, we used fluorocitrate, an inhibitor of astroglial metabolism.

Fluorocitrate (1 nmol/site) temporary inhibits astroglial metabolism but not neuron without destruction of the astroglial cells, and 24 h after its injection, the astroglial cells appear to have largely recovered.²² As the results, repeated treatment with fluorocitrate at once per 2 d from 5 d inhibited reactive astrocytes expressing HMGB1 and caused neurological deterioration at 14 d after cerebral ischemia. In addition, plasma HMGB1 did not be affected by the injection of fluorocitrate. Taken together, these suggest that activated microglia expressing HMGB1 releases the large amount of HMGB1 into extracellular space, and it may be related to progressive inflammatory reaction in the ischemic core in ischemic early phase. In contrast, reactive astrocytes expressing HMGB1 partially release HMGB1 and influence the long-term recovery after brain injury; through neurite outgrowth, synaptic plasticity, or neuron regeneration.²³ Actually, stimulated astrocytes released HMGB1 protein and induced neuroblastoma cell differentiation.²⁶ Recent evidence identifies HMGB1 as a cytokine-like mediator of delayed endotoxin lethality.¹²,¹⁴ In addition, high serum levels of HMGB1 in patients with sepsis or hemorrhagic shock have been reported to be associated with increased mortality and disease severity.¹⁴,²⁴ In this study, plasma HMGB1 levels were correlated with the extent of brain injury, which suggests that plasma HMGB1 levels would be a marker of progressive brain injury after ischemic insult.

In conclusion, we are the first to demonstrate that repeated treatment with cannabidiol from 3 d after cerebral ischemia has cerebroprotective effect and attenuates microglia expressing HMGB1 without affecting reactive astrocytes expressing it. Our results suggest that cannabidiol will provide therapeutic possibilities for progressively expanding inflammatory responses after stroke via modulating microglial HMGB1.

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