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High-cholesterol feeding aggravates cerebral infarction via decreasing the CB_1 receptor

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Abstract

We examined how feeding conditions affect the CB_1 receptor and cerebral infarction caused by cerebral ischemia. Mice were divided into the following three groups: normal diet (ND), caloric restriction (CR) and high-cholesterol-enriched diet (HCD), and were kept for 6 weeks. After 6 weeks, we measured both serum and brain cholesterol and the expression level of cannabinoid CB_1 receptor within the brain in intact mice. In addition, middle cerebral artery (MCA) was occluded for 2 h following reperfusion. Serum cholesterol significantly increased in the HCD group in comparison with both the ND and CR groups. However, brain cholesterol decreased in the HCD group. Then, the expression level of CB_1 receptor significantly decreased in the HCD group, while that of the CR group clearly increased in comparison with the ND group in intact mice. In MCA-occluded mice, The HCD group produced the most severe cerebral infarction, while cerebral infarction was significantly decreased in the CR group. These results suggest that CR prevents infarction by increasing CB_1 receptor expression, while high-cholesterol feeding aggravates cerebral infarction both by hypercholesterolemia in serum and by decreasing CB1 receptor expression modulated by hypocholesterolemia within the brain.

Keywords: Cerebral ischemia; High-cholesterol; Caloric restriction; CB_1 receptor

Hypercholesterolemia is a modifiable risk factor for coronary artery and peripheral vascular diseases because its effects on large arterial vessels (atherosclerosis) increase the likelihood that tissues will experience an ischemic episode [25,6,28]. In addition, it has been reported that the inflammatory response induced by cerebral ischemia in the cerebral microvasculature is exaggerated in mice with hypercholesterolemia [18,4], and that a high-cholesterol diet (HCD) causes progression of experimental cerebral ischemia [7]. On the other hand, calorie restriction (CR) has been known to extend life span. CR delays a wide spectrum of diseases in different experimental animals, for example, kidney disease, several neoplasias, autoimmune disease and diabetes [11,12,20]. In addition, CR reduces neuronal loss in most mouse models of neurodegenerative disorders such as Parkinson's disease or Alzheimer's disease [9,29]. Moreover, CR has also been shown to reduce the oxidative damage [22] and to prolong the life span through an increase in the SIR2 gene [21]. It has been shown that insulin receptor knock out mice are not viable, and that the receptor might be a central regulator of mammalian lifespan [17]. However, it has not yet been fully reported how an HCD and CR affect cerebral infarction induced by middle cerebral artery (MCA) occlusion in mice.

We focused on the cannabinoid CB_1 receptors, which have been reported as key receptors in the systems that regulate feeding and prevention of ischemic injury caused by cerebral ischemia [19,24,8,13]. The endocannabinoids mediate hyperphargic actions induced by CB_1 receptor agonists, and have been reported to reliably induce overeating [13]. These actions

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are inhibited by the CB_1 receptor antagonist SR141716, but not SR144258, an antagonist of peripherally expressed CB_2 receptors [27]. In addition, it has also been reported to increase the endocannabinoid 2-arachidonoyl glycerol (2-AG) in the hypothalamus of CR mice [23], and to enhance CB_1 receptor expression in the nodose ganglion of food-restricted rats [15]. The expression of the CB_1 receptors is inhibited by cholecystokinin, which modulates the effect of satiety signals on food intake, by satiation [5]. In addition, high-cholesterol exposure reduces the binding efficiency and signaling of CB_1 receptors in nerve cells [3]. In this way, cholesterol modulates the variable function of CB_1 receptor at plasma membrane.

The expression level of CB_1 receptor has been reported to depend on a neuroprotective role and a potential therapeutic role in stroke for drugs, such as endocannabinoid, anandamide and 2-AG, that activate CB_1 receptor [19]. We have also shown a neuroprotective effect of Δ^9-tetrahydrocannabinol on cerebral infarction induced by MCA occlusion in mice, and this effect is inhibited by CB_1 receptor antagonist SR141716 [16]. Therefore, we hypothesize that an HCD attenuates the expression level of CB_1 receptors and results in aggravating the ischemic injury induced by focal cerebral ischemia. On the other hand, CR might enhance CB_1 receptor expression within the brain and prevent ischemic injury. It has not yet been reported how an HCD and CR affect CB_1 receptors and ischemic injury in vivo.

In the present study, we examined how an HCD and CR for 6 weeks can affect serum cholesterol, brain cholesterol in striatum and in hypothalamus, and CB_1 receptor expression. Moreover, we measured the size of the cerebral infarcts 24 h after 2 h MCA occlusion.

Male ICR mice at 6 weeks of age (Experimental Animal Laboratory, Kyudo, Japan) were kept under a 12-h light/12-h dark cycle (lights on from 07:00 to 19:00 h) in an air-conditioned room ($23 \pm 2\,^{\circ}C$) with 9 g food per day (CE-2, 346.8 kcal/100 g, protein 24.9%, fat 4.6%, carbohydrate 6.7%; Clea Japan, Tokyo, Japan) and water available ad libitum for 6 weeks (Normal diet, ND). CR was initiated at the same age at 3 g/day for 6 weeks. HCD was initiated at the same age with high-cholesterol food (F2HFD1, 414 kcal/100 g, protein 22%, fat 36%, carbohydrate 42%; Kyudo, Japan) and water available ad libitum for 6 weeks. 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, Japan.

Focal cerebral ischemia was induced according to the method described in our previous study [10]. 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 MCA. Two hours after occlusion, the mice were re-anesthetized with halothane, and reperfusion was established by withdrawal of the filament. 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 (Sigma, St. Louis, MO, USA). The border between the infarcted and non-infarcted tissue was outlined with an image analysis system (NIH Image, version 1.63), and the infarction area and volume were calculated.

The expression of CB_1 receptor protein was evaluated by Western blotting following sample extraction and SDS-PAGE. Six weeks after feeding, tissue samples (cortex, striatum and hypothalamus) from each group were homogenized at $4\,^{\circ}C$ for 1 min in lysis buffer [20 mM Tris (pH 7.4), 100 mM EDTA, 100 mM EGTA, 0.1% Triton X-100] with 1% protease inhibitor cocktail. Tissue extracts were centrifuged at 15,000 rpm at $4\,^{\circ}C$ for 30 min. The supernatant were treated in the same way as the tissue extracts.

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 10 μg total protein. Samples were heated at $95\,^{\circ}C$ for 5 min. Protein (10 μg) was separated by SDS-PAGE (12% gel). Blotting was performed at $2\,mA/cm^2$ by semi-dry type blotting (BIORAD, Japan). The blots were blocked with 5% non-fat dry milk in Tris buffer saline in 1% Tween 20 (TBS-T) at $4\,^{\circ}C$, 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 (US and Canada) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Goat anti-rabbit IgG (H + L) AP conjugate, AP color reagent 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). Total cholesterol in mouse blood was measured using a cholesterol analysis system (SPOTCHEM EZ, sp-4430 ARKRAY, Japan) and the cholesterol content within the brain was measured by means of cholesterol oxidase, as previously described [3].

The results are expressed as the mean ± S.E.M. Multiple comparisons were evaluated by Tukey's test after one-way ANOVA. $P < 0.05$ was considered to be statistically significant.

All groups had identical body weight at the beginning of each experiment, namely 28.3 ± 0.14 g. At the end of the study, body weight of mice in the ND, CR and HCD groups was 40.9 ± 0.99, 30.1 ± 0.53 and 43.2 ± 0.77 g, respectively. HCD but neither ND nor CR significantly increased total blood cholesterol [$F(2, 17) = 129.627$, $P < 0.001$, one-way ANOVA, ND, 81.6 ± 5.3 mg/dL; CR, 86.7 ± 6.5 mg/dL; HCD, 221.4 ± 8.8 mg/dL, $P < 0.01$ compared with ND, Tukey's test]. However, HCD but neither ND nor CR significantly decreased cholesterol in striatum and tended to decrease in hypothalamus within the brain [striatum; $F(2, 10) = 6.672$, $P < 0.05$, one-way ANOVA, ND, 12.2 ± 1.4 μg/mg; CR, 8.2 ± 1.6 μg/mg; HCD, 4.3 ± 0.5 μg/mg, $P < 0.05$ compared with ND, Tukey's test: hypothalamus; ND, 7.2 ± 0.7 μg/mg; CR, 8.5 ± 1.2 μg/mg; HCD, 4.4 ± 0.4 μg/mg]. The expression level of CB_1 receptors

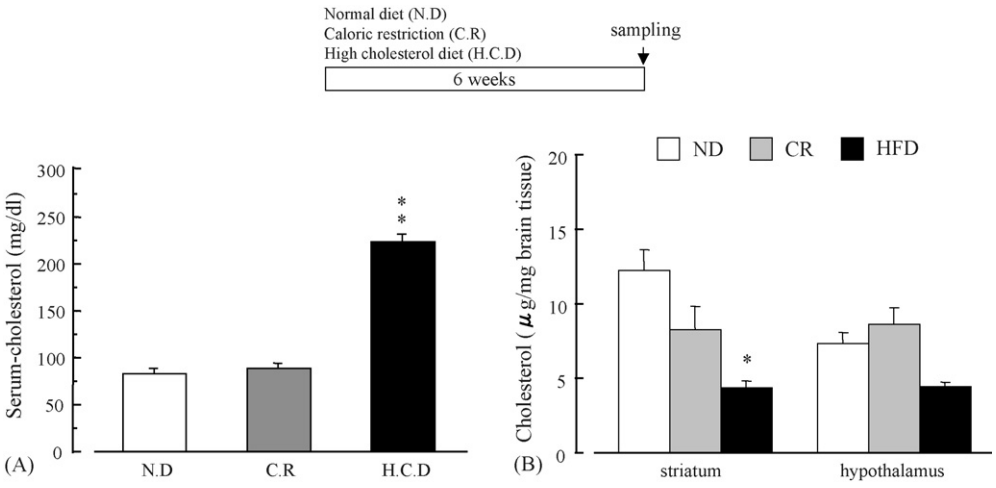

Fig. 1. Total serum cholesterol was significantly increased in the HCD group for 6 weeks but not in either the ND or CR groups (A). While, cholesterol in striatum significantly decreased and cholesterol in hypothalamus tended to decrease in the HCD group for 6 weeks (B). There was no difference in total cholesterol between the CR and ND groups. Values are expressed as the mean ± S.E.M. ($n = 7$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. ND group (one-way ANOVA followed by Tukey's test).

at the striatum and the hypothalamus was significantly decreased in the HCD group, while that in the CR group was clearly increased in comparison with that in the ND group, in intact mice before MCA occlusion [striatum, $F(2, 9) = 22.248$, $P < 0.001$, one-way ANOVA, ND, $16.3 \pm 1.5\%$; CR, $23.8 \pm 1.9\%$, $P < 0.05$ compared with ND; HCD, $9.5 \pm 1.2\%$, $P < 0.05$ compared with ND, hypothalamus, $F(2, 9) = 15.5449$, $P < 0.01$, one-way ANOVA, ND, $12.7 \pm 1.6\%$; CR, $20.6 \pm 2.0\%$, $P < 0.05$ compared with ND; HCD, $8.8 \pm 0.8\%$, Tukey's test]. The HCD group produced the most severe cerebral infarction, while the CR group showed the mildest infarction [$F(2, 17) = 12.362$, $P < 0.001$, one-way ANOVA, ND, $53.1 \pm 4.5\,mm^3$; CR, $24.9 \pm 4.8\,mm^3$, $P < 0.05$ compared with ND; HCD, $81.5 \pm 11.7\,mm^3$, $P < 0.05$ compared with ND, Tukey's test] (Figs. 1–3).

The present study shows that high-cholesterol feeding increased serum cholesterol and decreased both brain cholesterol content and CB_1 receptor expression. On the other hand, CR enhanced CB_1 receptor expression without changing both serum and brain cholesterol, in comparison with ND. Infarction was greater in the HCD group compared with that in the ND group, while infarction in the CR group was milder than that in the ND group. These results suggest that high-cholesterol feeding may aggravate cerebral infarction by down-regulating CB_1 receptor expression, while CR may prevent cerebral infarction by up-regulating CB_1 receptor expression, and provide evidence of a new action of cholesterol on cannabinoid CB_1 receptors and on ischemic injury in vivo.

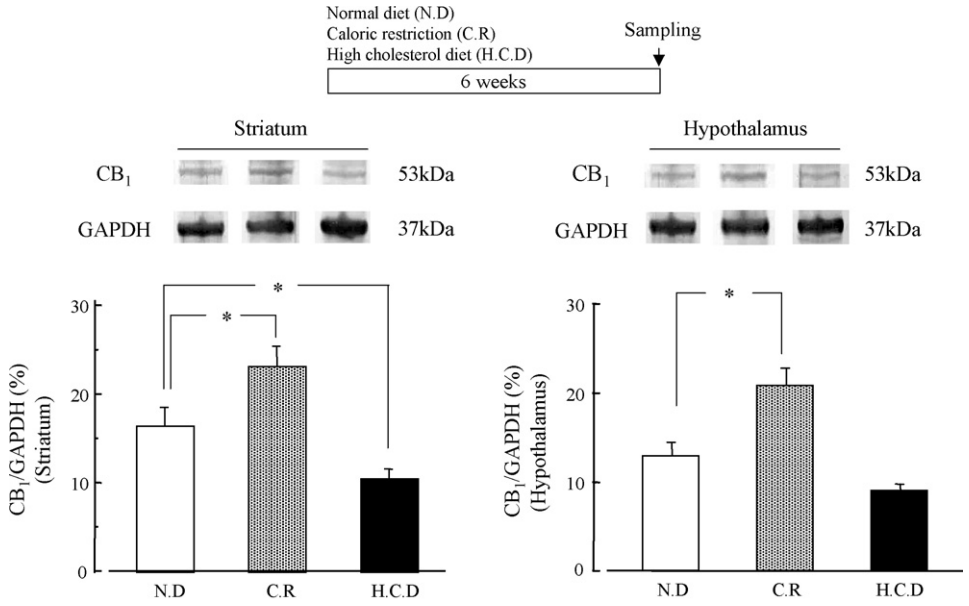

Fig. 2. CB_1 receptor expression at the striatum and the hypothalamus was significantly decreased in the HCD group, while that in the CR group was clearly increased in comparison with that in the ND, in intact mice before MCA occlusion. Values are expressed as the mean ± S.E.M. ($n = 4$). $^{*}P < 0.05$ vs. ND group (one-way ANOVA followed by Tukey's test).

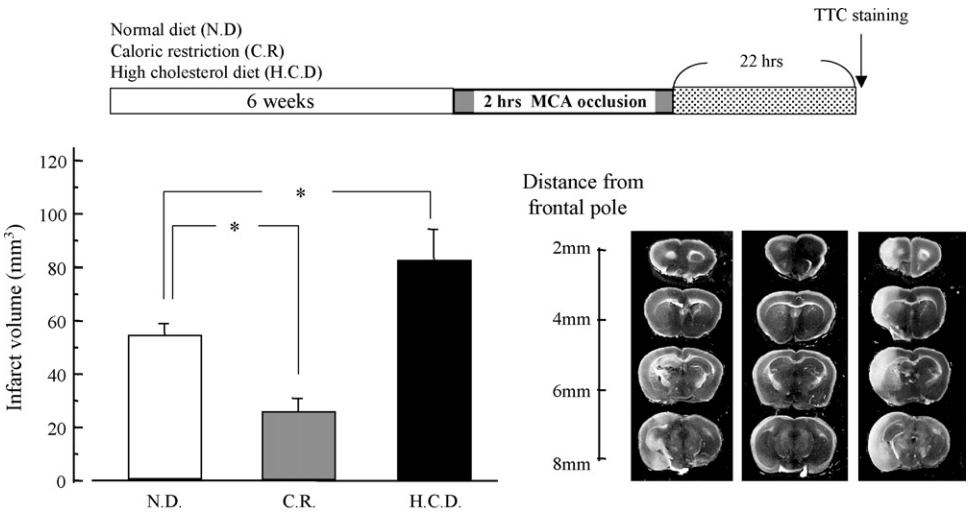

Fig. 3. Effect of both HCD and CR on the cerebral infarction caused by MCA occlusion in mice. The HCD group produced greater infarction than the ND group, while the CR group showed weaker infarction. Values are expressed as the mean ± S.E.M. ($n = 5$–6). $^{*}P < 0.05$ vs. ND group (one-way ANOVA followed by Tukey's test).

High serum cholesterol levels have been associated with increased risk of ischemic stroke [18,7,26]. Diet-induced hypercholesterolemia causes the cerebral microvasculature to undergo oxidative stress and to assume a proinflammatory and prothrombogenic phenotype [18]. On the other hand, CR has been associated with decreased risk of ischemic stroke and with life span [22,21,17]. In this study, the HCD group induced the most severe infarction, including a decrease in CB_1 receptor expression, in comparison with the ND group, while the CR group showed the mildest infarction, including an increase in CB_1 receptor expression, in comparison with the ND group. These results are supported by previously published studies. In addition, expression level of CB_1 receptor may be very important for modulating cerebral infarction in the HCD- or CR-feeding conditions.

The cannabinoid CB_1 receptors are G-protein-coupled receptors that mediate several actions of the endocannabinoids, such as anandamide and 2-AG, in the central nervous system. It has been reported that endocannabinoids reduce infarct volume caused by traumatic brain injury [24], and that the expression level of CB_1 receptor would depend on a neuroprotective effect [19]. We have also shown that the neuroprotective effect of Δ^9-tetrahydrocannabinol on cerebral infarction is inhibited by the CB_1 receptor antagonist, SR141716, and by warming [16]. These results suggest that the neuroprotective effect of cannabinoids is induced by hypothermia, via the CB_1 receptor at the hypothalamus. In this study, CB_1 receptor expression at the striatum and the hypothalamus were increased in the CR group. Although it is unclear that the endocannabinoids prevent cerebral infarction induced by MCA occlusion, they might contribute to the prevention of cerebral infarction. This is based on the fact that decreasing expression of CB_1 receptors leads to aggravation of cerebral ischemic injury, while increasing their expression leads to prevention of ischemic injury. It would need to study on potential relationship of endocannabinoid system and CB_1 receptor function, including pre- and post-synaptic CB_1 receptor.

Serum cholesterol significantly increased, but brain cholesterol decreased in striatum and in hypothalamus (no significantly) in the HCD group but not in either the ND or CR groups. However, how serum cholesterol associates with brain cholesterol content is unknown. It might decrease brain cholesterol content in compensation for the high concentration of serum cholesterol. CB_1 receptor expression decreased in the HCD group compared with that in the ND group. On the other hand, CB_1 receptor expression in the CR group was increased compared with that in the ND group, without any difference in cholesterol from that in the ND group. Membrane cholesterol has been shown to regulate critical events in the central nervous system. CB_1 receptors is dependent on lipid rafts composed of cholesterol and sphingolipid [2]. Moreover, the function of CB_1 receptor relates with membrane fluidity [1]. In this study, brain cholesterol content significantly decreased in HCD group. Then, the expression of CB_1 receptor also decreased in striatum and in hypothalamus. These results suggest that the reduced membrane fluidity resulting from degradation of membrane cholesterol may decrease CB_1 receptor expression at the plasma membrane.

The present study shows that CB_1 receptor expression was enhanced by CR at the striatum and the hypothalamus. There was no difference in serum cholesterol and in brain cholesterol between the CR and ND groups. There are indications that endocannabinoids are key components of the systems that regulate feeding. For example, endocannabinoids appear to be crucial for neonatal suckling [14], and they are involved in feeding responses across the phylogenetic scale [8]. Recently, it has been reported that the effect of endogenous cannabinoids on appetite is mediated by vagal afferent neurons, suggesting a role in modulating gut-brain signaling, and CB_1 receptor expression is enhanced in the nodose ganglion of food-restricted rats [15]. Therefore, presumably CB_1 receptor expression is enhanced by several actions at both central and peripheral sites. CB_1 receptor expression might be enhanced within the brain, and an increase

in expression might lead to prevention of the cerebral infarction caused by cerebral ischemia.

In conclusion, the findings of the present study show that CB_1 receptor expression is down-regulated by hypocholesterolemia within the brain, and hypercholesterolemia in serum promotes ischemic injury, in HCD group, and results in aggravating cerebral infarction, while CB_1 receptor expression is increased by fasting, and results in infarction prevention. The data provide a new insight into the action of cholesterol on cannabinoid CB_1 receptor and on ischemic injury.

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