原 著

The Effect of Synthetic Agonists and Antagonists of Cannabinoid Receptors on Migration of Neutrophils Elicited by Leukotriene B_4 in the Microvasculature of Hamster Cheek Pouch

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Abstract

This study was designed to investigate the effects of cannabinoid agonists and antagonists on neutrophil behaviors in response to inflammatory stimulation. The neutrophil behavior was observed in the microvasculature of hamster cheek pouch using a trans-illumination microscope. Superfusion of leukotriene B_4 caused an increase in the number of neutrophils migrating through the endothelium outside the venules. The migration induced by leukotriene B_4 was significantly attenuated in hamsters receiving WIN55212-2, a synthetic non-selective cannabinoid agonist, prior to the leukotriene B_4 superfusion. The inhibitory effect of WIN55212-2 was abolished by AM251, a selective CB_1 antagonist, and also by AM630, a selective CB_2 antagonist. These results suggest that augmentation of the CB_1 and CB_2 cannabinoid system could produce the inhibition of neutrophil migration and contribute to suppression of inflammatory derangement.

Key words; cannabinoid, neutrophil, leukotriene B_4

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Introduction

Cannabinoids are hydrophobic compounds, which are derived from the plant *Cannabis sativa*. To date, two types of the cannabinoid receptors have been identified. These are the CB_1 receptor, first cloned in 1990[1], and the CB_2 receptor, cloned in 1993[2]. The cannabinoid CB_1 receptor is expressed abundantly in the nervous system, especially the brain[1], and has been assumed to play an important role in the attenuation of synaptic transmission[3]. The cannabinoid CB_2 receptor is expressed mainly in various lymphoid tissues[2] and has been postulated to take part in the regulation of inflammatory reactions and immune responses[3]. It has also become clear that CB_1 receptors are also expressed by some non-neuronal cells, including immune cells, and CB_2 receptors by some neurons both within and outside the brain[4,5].

The roles of CB receptors in shock and inflammation are complex[6~8]. It has been reported that a CB_1 receptor antagonist, AM251 improves survival rate of septic shock, suggesting that endogenous CB_1 is involved in the deterioration of the shock[6]. By contrast, it has also been reported that CB_1 and CB_2 receptors have important protective roles in pathophysiological conditions such as shock, ischemia-reperfusion injury and inflammation[7,8]. In this study,

we aimed to investigate the role of cannabinoids in neutrophil migration in response to leukotriene-induced inflammation.

Materials and Methods

Ethical committee for animal experimentation of our institution approved this study. Thirty-five male Golden hamsters, weighing 110～160g at 10～16 weeks old (Nihon SLC, Shizuoka, Japan) were anesthetized with urethane 0.6g/kg and chloral hydrate 0.6g/kg intraperitoneally (ip). A tracheal tube was inserted to facilitate spontaneous respiration with a mixture of oxygen and room air. Arterial pressure and heart rate were measured via a cannula placed in the carotid artery. Another cannula was placed in the femoral vein for infusion.

A. Preparation of hamster cheek pouch

The hamster cheek pouch preparations were set up as previously described[9,10]. The cheek pouches were pulled out, cut longitudinally, and extended. The connective tissue was elaborately dissected away to expose the microvasculature of the mucous layer. The thin mucous membrane tissue was spread out in a plastic chamber.

B. Microscopic observation and recording of leukocyte behavior

The microvasculature of the hamster cheek pouch (unit: 50×100μm) was observed under a transillumination microscope (ECLIPSE, Nikon, Tokyo, Japan), using a W40 water immersion lens (×40, Nikon, Tokyo, Japan) and ×10 eyepieces (Nikon, Tokyo, Japan). Images of the microcirculation were projected onto a color television monitor screen (PVM-20M4J, SONY Co, Tokyo, Japan) via a color TV camera (DXC-5800, SONY Co, Tokyo, Japan) mounted at the top of the microscope. The behavior of the neutrophils in each experiment was recorded with a videotape recorder (SVO-5800, SONY Co, Tokyo, Japan).

C. Animal groups

The animals were assigned into seven groups by the type of administered agents; ①Vehicle; normal saline, ip (n=5), ②Non-selective CB agonist; WIN55212-2 (3.5mg/kg, ip) (n=5), ③WIN55212-2 (5mg/kg, ip) (n=5), ④CB_1 antagonist; AM251 (3mg/kg, ip) (n=5), ⑤CB_2 antagonist; AM630 (1mg/kg, ip) (n=5), ⑥WIN55212-2 (5mg/kg, ip) +AM251 (3mg/kg, ip) (n=5), ⑦WIN55212-2 (5mg/kg, ip) + AM630 (1mg/kg, ip) (n=5).

D. Chemotactic agent

Leukotriene B_4 (Paesel, GMBH, Frankfurt, Germany) was used as a chemo-attractant[11]. A stock solution of leukotriene B_4 (30μM in absolute ethanol) was kept at −80℃ and diluted to 300nM with Tyrode's solution immediately before use. Ten minutes after initiating the administration of cannabinoid compounds or saline, leukotriene B_4 (300nM) was applied to the microvasculature at the observation site with a 50μl micropipette.

E. Count of migrated neutrophils

Neutrophils could be individually visualized as bright white cells against the dark background of the blood stream, since they rolled slowly on the endothelial wall. Migration of neutrophils was determined when neutrophils were moving from the venular wall into the interstitial space (unit: 50μ×100μm).

F. Data analysis

All values are expressed in mean±SD. Data were statistically analyzed with ANOVA and Student's t-test was used for comparisons between the two groups, and paired t-test for comparisons before and after interventions in the same group. $P<0.05$ was considered as a statistically significant difference.

Results

A. Systolic blood pressure and heart rate

Table 1 and **2** show systolic blood pressure and heart rate, respectively, before the administration of agents (baseline), at the time of leukotriene B_4 application, and 30, 60, and 90 min after leukotriene B_4 application. Both systolic blood pressure and heart rate gradually decreased as the time passed approximately by 10～20% at the end in all groups including the vehicle group. The values of systolic blood pressure and heart rate were not significantly different among groups.

Table 1 Systolic blood pressure (mmHg) at baseline and after leukotriene B4 application

	baseline	0 min	30 min	60 min	90 min
Vehicle	106±4	107±6	107±4	93±14	83±23*
WIN (3.5mg)	100±6	97±8	90±8	87±8	97±27
WIN (5.0mg)	97±4	88±8	86±11	84±11	78±7*
AM251	107±16	105±18	108±14	88±14	84±12*
AM630	102±6	97±9	94±7	84±4	77±6*
WIN+AM251	106±6	98±18	94±13	85±11	83±8*
WIN+AM630	103±12	95±15	104±11	93±6	92±4*

*$p < 0.05$ vs baseline

Table 2 Heart rate (beats per min) at baseline and after leukotriene B4 application

	baseline	0 min	30 min	60 min	90 min
Vehicle	430±33	424±29	435±17	422±42	372±49
WIN (3.5mg)	437±15	415±20	407±33	401±43	398±53
WIN (5.0mg)	423±14	402±20	406±19	397±17	377±26*
AM251	440±16	442±18	449±14	422±14	395±12*
AM630	432±19	440±20	441±23	433±37	392±29*
WIN+AM251	396±28	400±45	408±44	389±45	371±41
WIN+AM630	427±31	407±33	430±28	414±13	395±15

*$p < 0.05$ vs baseline

B. Migration of neutrophils

Before the application of leukotriene B4, several neutrophils were observed to move slowly along the vascular endothelium. The application of leukotriene B4 caused a transient increase and a subsequent decrease in rolling of neutrophils. There were no significant differences in the alterations in rolling and adhesion of neutrophils among the seven groups.

Fig. 1～3 show time course of numbers of migrated neutrophils in seven groups. Migration of neutrophils started at 30 min after leukotriene B4 application in all groups. In the vehicle group, 1.4±0.2 counts/unit of neutrophils were migrated at 90 min after leukotriene B4 (**Fig. 1**). WIN55212-2 caused significant inhibitions of migrated neutrophils as compared with the vehicle group in a dose-dependent manner (**Fig. 1**). The numbers of migrated neutrophils in the AM251 and AM630 groups were not different from the vehicle group (**Fig. 2**). The decreases in migrated neutrophils by WIN55212-2 (5mg/kg) were abolished in WIN55212-2+AM251 and WIN55212-2+AM630 (**Fig. 3**).

Discussion

The results of this study demonstrated that WIN55212-2, a synthetic non-selective CB cannabinoid agonist, inhibited leukotriene B4-induced migration of neutrophils. Leukotriene B4 is a pro-inflammatory mediator synthesized in myeloid cells from arachidonic acid[12]. It induces recruitment and activation of neutrophils, monocytes and eosinophils by stimulating the production of a number of proinflammatory cytokines and mediators[11,12]. In our study, superfusion of leukotriene B4 could also augment migration of neutrophils in the microvasculature of hamster cheek pouch. Pharmacological inhibition studies support a role for leukotriene B4 in the pathogenesis of neutrophil mediated tissue damage, and treatments that can reduce the production or block the effects of leukotriene B4 may prove beneficial in neutrophil mediated inflammatory diseases[12～14].

Since neutrophil migration elicited by superfusion of leukotriene B4 was inhibited by pretreatment of WIN55212-2 in this study, it is suggested that exogenous administration of cannabinoids can be beneficial in prevention of neutrophil-mediated tissue damage. It is also suggested that endogenous cannabinoids are not involved in the leukotriene B4-induced neutrophil migration because neither pretreatment of AM251, a selective CB_1 antagonist, nor AM630, a selective CB_2

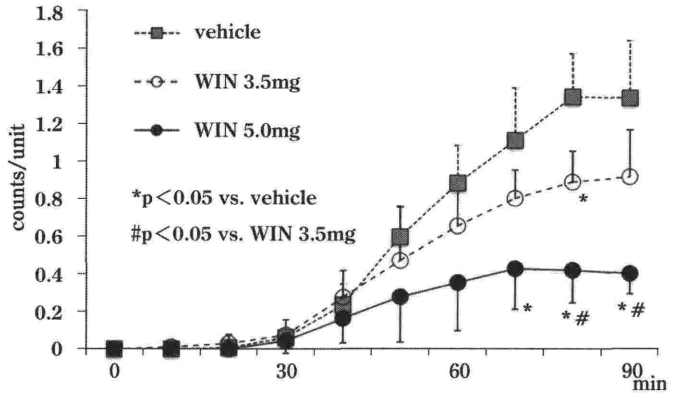

Figure 1 Changes in the number of migrating neutrophils after superfusion of leukotriene B4.
The abscissa indicates the time lapse after the application of leukotriene B4. The ordinate indicates the number of migrating neutrophils in the detection window (counts/unit). WIN55212-2 significantly inhibited the migration of neutrophils elicited by leukotriene B4 superfusion ($p<0.05$) as compared with the vehicle group in a dose-dependent manner. Values are shown as mean±SD. Error bars are shown only one direction for simplicity.

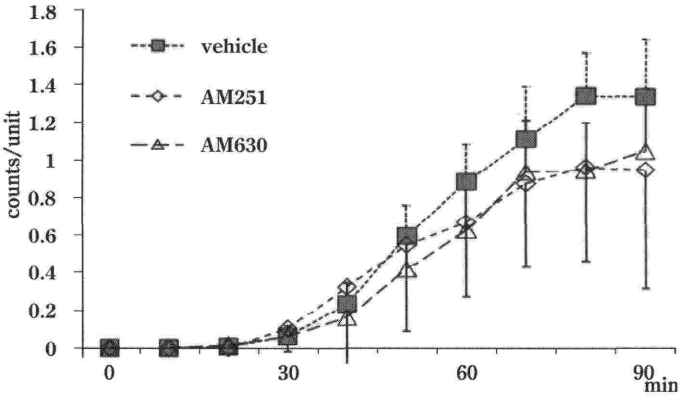

Figure 2 Changes in the numbers of migrating neutrophils after superfusion of leukotriene B4.
AM251 and AM630 did not significantly affect the neutrophil migration. Values are shown as mean±SD. Error bars are shown only one direction for simplicity.

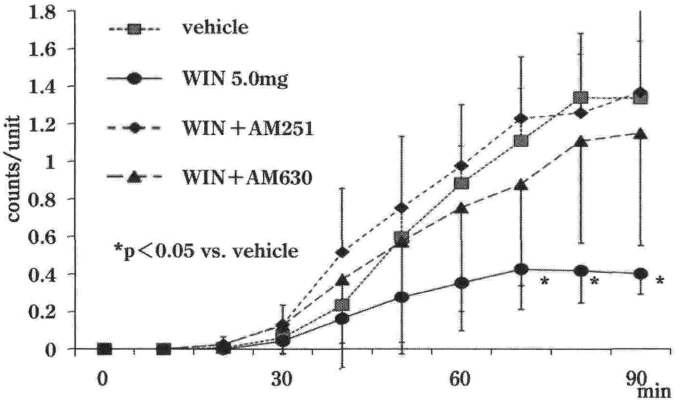

Figure 3 Changes in the numbers of migrating neutrophils after superfusion of leukotriene B4.
The inhibition of migrating neutrophils elicited by WIN55212-2 was reversed both in the WIN55212-2+AM251 group and the WIN55212-2+AM630 group. Values are shown as mean±SD. Error bars are shown only one direction for simplicity.

antagonist, changed the migration.

Our result of the anti-inflammatory activity of WIN55212-2 is consistent with a previous study[15] in which WIN55212-2 has been shown to suppress the infiltration of leukocytes induced by 12-*O*-tetradecanoylphorbol 13-acetate. However, the effects of cannabinoids on inflammatory reactions are controvertial. Some reports have shown the stimulatory effects, whereas the others inhibitory effects. It has been reported that 2-arachidonoylglycerol (2-AG), an endogenous ligand for the cannabinoid receptors augmented production of chemokines in HL-60 cells[16] and induced the migration of mouse splenocytes[17] via cannabinoid CB_2 receptor-dependent mechanisms. It has been also reported that cannabinoid CB_2 receptor antagonists/inverse agonists, suppress carrageenan-induced mouse paw edema[18]. These observations strongly suggest that the cannabinoid CB_2 receptors are involved in the stimulation of several types of inflammatory reactions and immune responses.

On the other hand, several investigators reported that 2-AG and the cannabinoid CB_2 receptor are involved in the attenuation of inflammatory reactions and immune responses. Ouyang et al.[19] demonstrated that 2-AG suppresses interleukin 2 (IL-2) secretion and inhibits IL-2 promoter activity. Chang et al.[20] demonstrated that 2-AG suppresses the production of IL-6 in J774 macrophage-like cells, and Gallily et al.[21] reported that 2-AG attenuates tumor necrosis factor α (TNF-α) production in lipopolysaccharide-stimulated mouse macrophages. However, it is not clear whether the effects of 2-AG are actually mediated via the cannabinoid CB_2 receptor. Several investigators also demonstrated that cannabinoid receptor agonists, such as WIN55212-2, HU-308, AM1241, CT-3, nabilone and anandamide, suppress inflammatory reactions *in vivo*[22,23].

Although in most cases, the cannabinoid CB_2 receptor was suggested to be involved in inflammation, our study shows that the inhibitory effect of WIN55212-2 on neutrophil migration was blocked by AM251, a selective CB_1 antagonist, as well as by AM630, a selective CB_2 antagonist. It is therefore suggested that both CB_1 and CB_2 receptors are involved in the inhibitory effect of WIN55212-2 on neutrophil migration. The contribution of CB_1 receptors to cytokine modulation and inflammation has also been reported[24]. The inhibitory effects of WIN55212-2 on the production of inflammatory cytokines elicited by endotoxin[24] was blocked by SR141716A, a highly selective cannabinoid CB_1 receptor antagonist, but not by SR144528, a selective cannabinoid CB_2 receptor antagonist. This observation indicated that cytokine modulation by the cannabinoid receptor agonists occurred through activation of cannabinoid CB_1 receptors. While cytokine responses appeared to be modulated through central cannabinoid receptors, other reports have demonstrated a role for peripheral cannabinoid CB_1 receptors in the anti-inflammatory effects of cannabinoid receptor agonists[25,26]. Inflammation is a complex biological process involving a variety of pathophysiological events such as edema, redness, heat, and pain. These responses are known to be regulated by diverse biological systems including the immune system, vascular system, peripheral nervous system, and the central nervous system in complex and sophisticated manners. Further detailed studies are thus necessary for a thorough elucidation of the mechanism underlying cannabinoid receptor agonist-mediated suppression of inflammation.

In conclusion, this study demonstrated that a synthetic non-selective cannabinoid reduced neutrophil migration elicited by leukotriene B_4 in the microvasculature of hamster cheek pouch, representing a new therapeutic strategy for neutrophil-mediated tissue damage.

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