Regulation of the Endocannabinoid System in Endotoxicosis of Conscious Guinea Pigs

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ABSTRACT Intestinal movements in conscious, unrestrained guinea pigs were observed by telemetry through a force transducer sutured to the taenia caecum. Following administration of lipopolysaccharide (LPS) and cannabinoids, the muscle tension of the taenia caecum decreased dose-dependently. Body temperature decreased in parallel with decreases in tension. Decreases in both these variables were suppressed by pre-treatment with AM281, a cannabinoid CB1 receptor antagonist, but not with AM630, a CB2 receptor antagonist. Following LPS administration, endocannabinoid levels in guinea pig plasma were measured using LC/MS/MS analysis. One hour following LPS administration, 2-arachidonoylglycerol (2-AG) levels had increased significantly, while arachidonoylethanolamide (AEA) levels were below detection limits. For mice, post-LPS survival rates in the presence of AM281 increased. The fact that LPS-induced decrease in muscle tension in taenia caecum is suppressed and post-LPS survival rates improved following administration of a CB1 receptor antagonist, suggests that a signaling pathway associated with the CB1 receptor plays a role in the pharmacological effects of LPS. Increases in blood 2-AG levels one hour after LPS administration suggest regulation of physiological responses by the endocannabinoid system during endotoxicosis.

(Keywords: endocannabinoid, lipopolysaccharide, intestinal muscle, paralytic ileus, CB1 receptor
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Introduction

It is known that in vivo lipopolysaccharide (LPS) induces cytokine production, fever and hypotension, and plays a major role in the pathophysiology of sepsis caused by gram-negative bacteria. Regarding the underlying mechanism, it is thought that LPS activates macrophages and platelets to produce and release bioactive substances which mediate endotoxemia. Cytokines and nitric oxide (NO) have been regarded as the major mediators, but in recent years, endocannabinoids released from cell membranes of macrophages and platelets have been attracting attention as new mediators.

Physiological actions of endocannabinoids are regulated by specific receptors. Cannabinoid CB1 receptors are expressed on central and peripheral nerves and play roles in the regulation of neurotransmission. CB2 receptors are expressed on immunocytes such as lymphocytes and macrophages and play roles in the regulation of inflammatory reactions and immune responses. Endogenous ligands of these receptors that have been reported include arachidonoylethanolamide (anandamide, AEA), 2-arachidonoylglycerol (2-AG), 2-arachidonoylglyceryl ether (noladin ether) and O-arachidonoyl ethanolamine (virodhamine).

LPS-induced increases in circulating endocannabinoid levels were first reported for a rat endotoxemia model. The study demonstrated that in LPS-induced endotoxic shock in rats, circulating levels of anandamide and 2-AG originating from macrophages and platelets increase, activating CB-receptors of blood vessels and lowering blood pressure. The mechanism underlying elevation of anandamide levels was further hypothesized to involve...
LPS suppression of fatty acid amide hydrolase (FAAH), an enzyme that causes the decomposition of anandamide. In a study on the mouse macrophage cell line RAW264.7, anandamide synthesis was dose-dependently facilitated by LPS 1 hour after LPS administration (1-100 μg/mL). LPS stimulation of human dendritic cells increased 2-AG levels but not anandamide levels.

In the present study, the role of endocannabinoids as mediators of endotoxicosis was investigated using LPS-induced intestinal paralysis in conscious guinea-pigs, a model we described in a previous study. Because non-anesthetized animals are used, it is possible to maintain basal tone of the intestinal tract and body temperature at high levels, and as a relatively non-invasive procedure is employed, the model is thought to reasonably reflect endotoxin-induced morbidity. In the present experiments we used guinea pigs, which are highly sensitive to endotoxin, as humans are.

In experiments in which we studied the effects of cannabinoid CB1 receptor antagonists on survival rate after administration of endotoxin, mice were used.

Anandamide and 2-AG were used as the principal cannabinoid receptor ligands and AM281 and AM630 were used as cannabinoid receptor antagonists.

Materials and Methods

In vivo recording of longitudinal muscle movement in the caecum

Male Hartley guinea pigs (weight, 350 to 400 g) were used after approval of the Animal Experimental Ethical Review Committee of Nippon Medical School (No. 15-17). Guinea pigs underwent laparotomy under anesthesia with pentobarbital sodium (30 mg/kg, i.p.). Longitudinal muscle movement was recorded using a method previously described by Ninomiya et al. Briefly, a force transducer (3 x 5 mm, F-041S, Star Medical, Tokyo, Japan) was sutured to the taenia caecum. A cylindrical electric transmitter (15 x 35 mm, IMT-10T; Star Medical) connected to the transducer via a cable was embedded subcutaneously in the dorsal region of the animal and sutured in place. Signals from the transmitter were detected by a receiver (IMT-10RA; Star Medical) directly under the animal cages. Received output was stored in a personal computer, providing a continuous record of changes in tension of the longitudinal muscle. After the operation, animals were allowed to eat and drink freely. Cannabinoids or LPS were injected intraperitoneally 4 to 5 days after the operation, at the doses indicated, as longitudinal muscle movement of the taenia was recorded continuously. AM281 or AM630 was administered intraperitoneally 10 min before cannabinoids or LPS injection.

Measurement of body temperature

After depilation between the right and left scapulae on the dorsum of the animal, a plate-type thermosensor (PTP-50; Unique Medical, Tokyo, Japan) was fixed tightly to the skin. Skin temperature was recorded continuously by a temperature monitor (PTC-301; Unique Medical).

Measurement of plasma anandamide and 2-AG

Guinea pigs were anesthetized with sodium pentobarbital (40 mg/kg, body weight, i.p.) at 1, 2, 3 and 4 h after treatment with LPS or vehicle, and blood samples were collected from the abdominal aorta in plastic tubes containing heparin as anticoagulant. Blood samples were immediately centrifuged (10 min, 1630 x g, 4°C) and the separated plasma samples were stored at -80°C until analysis. Each plasma sample (0.2 mL) was mixed with acetonitrile (50 μL) / saline (0.8 mL) / diethylether (5 mL) containing 1 ng/μL of octa-deuterated (d8)- anandamide and 2 ng/μL of octa-deuterated (d8)- 2-AG as internal standard. The lipid-containing organic phase was dried at 40°C under a nitrogen stream and resolved with methanol/H2O (80:20 volume/volume), and an aliquot of 10 μL was analyzed by liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (LC-APCI-MS-MS) using a high-performance liquid chromatography (HPLC) apparatus (HP1100, Agilent Technologies, Inc., USA) coupled to a mass spectrometer (API3000, AB MDS Sciex, USA). The HPLC column was an L-column ODS (2.1 mm i.d. x 150 mm). Anandamide and 2-AG as quasi-molecular ions were quantified by isotope dilution with the above-mentioned deuterated standard and its amount in ng/mL of lipid extract.

Mortality of mice injected with LPS with or without AM281

Male ddy mice (30-40 g body weight) were used after approval by the Animal Experimental Ethical Review Committee of Nippon Medical School (No.15-66). Mice were used for experiments to obtain survival rates, because the animals have been used frequently in acute toxicity studies to determine LD90 and they are not costly. Mice were injected intraperitoneally with LPS (50 mg/kg) alone or LPS and AM281 (1, 3, or 10 mg/kg) in a total volume of 30-40 μL. AM281 was administered intraperitoneally 10 min before LPS injection. Mortality was assessed at 6, 12, 18, 24 and 30 h.
Chemicals

The following chemicals were used. 2-AG (2-arachidonoyl glycerol, Cayman Chemical, Ann Arbor, MI), AM281 [(1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morphphonyl-1H-pyrazole-3-carboximide), Tocris Cookson, Bristol, UK], AM630 {6-indo-2methyl-1-[2-(4-morpholinyl)ethyl]-1H-indo-3-yl} (4-methoxyphenyl) methanone, Tocris Cooksson, Bristol, UK}, anandamide (Cayman Chemical), lipopolysaccharide (LPS, Escherichia coli serotype O111:B4, Sigma Chemical Co. St. Louis, MO), pentobarbital sodium (Schering Plough, Kenilworth, NJ), octa-deuterated (d8)-2-AG (Cayman Chemical), octa-deuterated (d8)-anandamide (Cayman Chemical).

2-AG was originally received in acetonitrile, which was evaporated under a gentle stream of nitrogen. 2-AG, anandamide, AM281 and AM630 were dissolved in ethanol, tween80, and saline (1:1:18) immediately before i.p. administration. This solvent was without effect on muscle contractility. LPS was diluted in sterile saline.

Statistical analysis

All experimental results are shown as mean ± SEM. Statistical differences were determined by repeated measures analysis of variance (ANOVA). A paired t-test was used to evaluate differences between two groups. P-values less than 0.05 were considered statistically significant. To compare survival data, a Kaplan-Meyer test was performed using log rank statistics.

Results

Effects of anandamide, 2-AG and LPS on tension of colonic longitudinal muscles and body temperature

Following intraperitoneal administration into guinea pigs of anandamide 3mg/kg or 2-AG 1mg/kg, but not vehicle, the tension of the colonic longitudinal muscle immediately began to decrease. (Fig. 1). The decrease peaked approximately 30 min after administration of either anandamide or 2-AG, reaching 67 ± 11.9% and 64.9 ± 5.7% of basal levels respectively (Fig. 2A). The tension returned to about pre-administration levels 2 to 3 h after administration.

Following administration of LPS 0.3 mg/kg, intestinal tension began to decrease after approximately 2 hours of latency. Thereafter, changes in the tension progressed in a similar manner as after anandamide and 2-AG. Muscle tone 3 hours after administration was 66.5 ± 5.2% of the basal level (Fig. 2A).

Following administration of anandamide and 2-AG, body temperature decreased in near synchronization with decreases in intestinal tension (Fig. 2B).

Effects of AM281 on decreases in intestinal muscle tone and body temperature

In guinea pigs receiving AM281 intraperitoneally 10 min before administration of LPS, decreases in intestinal tension by LPS were suppressed (Fig. 3B, 4A). AM630, a cannabinoid CB2 receptor antagonist, had no effect on the decrease (Fig. 3C). Neither AM281 nor AM630 had any effect on the muscle tension by itself (data not shown).

Similarly, decreases in body temperature induced by LPS were suppressed by AM281 (Fig. 3B, 4B). AM630 had no effect on the decrease (Fig. 3C). Neither AM281 nor AM630 had any effect on body temperature by itself (data not shown).

In experiments in which AM281 was administered 1 hour after LPS administration, decreases in intestinal tension and in body temperature induced by LPS were suppressed (Fig. 3D).

Successive changes in blood concentrations of 2-AG and AEA after LPS administration

Guinea pig plasma concentrations of 2-AG and anandamide were measured using liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS). Guinea pig plasma concentrations of 2-AG increased significantly 1 hour after LPS administration (Fig. 5), while levels of AEA were below detection limits.

Effects of AM281 on survival rates following LPS administration

LPS-associated mortality of mice receiving AM281 (1 mg/kg or 3 mg/kg) 10 min before LPS administration decreased dose-dependently (Fig. 6)

Discussion

In conscious, unrestrained guinea pigs, intestinal tension and body temperature decreased immediately after intraperitoneal administration of the cannabinoids, AEA and 2-AG. That this observation closely resembled what was observed 2 to 3 hours after LPS administration suggested that an endocannabinoid-mediated pathway is involved in the mechanism of development of endotoxicosis. Following intraperitoneal administration of 2-AG, blood level of 2-AG is thought to peak immediately. The de-
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Fig. 1. Typical recordings of muscle tension induced by injection of cannabinoids. Individual guinea pigs were injected intraperitoneally with 3 mg/kg of AEA (A), 1 mg/kg of 2-AG (B) or the solvent for AEA and 2-AG (C) at the time points indicated by the arrows. The horizontal axis shows time (hr) after administration of cannabinoids.

Fig. 2. Effects of cannabinoids and LPS on muscle tension of taenia caecum and body temperature in guinea pigs. The graph shows data indicating muscle tension (A) or body temperature (B) after treatment with anandamide (AEA, 3 mg/kg, n=3), 2-AG (1 mg/kg, n=5) or LPS (0.3 mg/kg, n=5).

Mean ± SEM are shown. * p<0.05, ** p<0.01 compared with basal levels of muscle tension.
Fig. 3. Typical recordings of muscle tension and body temperature induced by injection of LPS and CB-receptor antagonists. Individual guinea pigs were injected intraperitoneally with 0.3 mg/kg of LPS without antagonist (A), LPS with AM281 pretreatment (B), LPS with AM630 pretreatment (C), LPS with AM281 post-treatment (D), at the time points indicated by the arrows. The upper trace shows muscle tension (g) of the taenia caecum. The lower trace shows body temperature (°C). The horizontal axis shows time (hr) after administration of LPS.

Fig. 4. Effects of AM281 on muscle relaxation (A) and hypothermia (B) induced by LPS. Individual guinea pigs were injected with LPS at 0.3 mg/kg intraperitoneally at time point 0. AM281 3 mg/kg was injected intraperitoneally 10 min before injection of LPS. * p<0.05 compared with control values (LPS-injected group) at the corresponding time point. Points and bars respectively show the mean ± SEM (n = 3-5 per group).

○ - ○ LPS (0.3 mg/kg), ● - ● AM281 (3 mg/kg) + LPS (0.3 mg/kg)
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LPS 0.3mg/kg

Fig. 5. Concentration of 2-AG in guinea pig plasma after the injection of LPS. Plasma levels of 2-AG increased significantly 1 hour after LPS administration. **p<0.01 compared with the basal level of 2-AG. Mean ± SEM are shown (n=4 per group).

Fig. 6. Survival rates of mice injected with LPS with or without AM281. Survival rates of 7 to 9 mice per group were calculated every 6 hours. The post-LPS survival rates improved in mice receiving AM281. Statistical significance was analyzed using the Kaplan-Meyer test.

* p<0.05 compared with control group (LPS-injected group).
- ● - Saline + LPS 50 mg/kg
- △ - AM281 1 mg/kg + LPS 50 mg/kg
- ○ - AM281 3 mg/kg + LPS 50 mg/kg

crease in muscle tension reached peak after a 30 to 40 min time lag (Fig. 1B). The above finding suggested that following intraperitoneal administration of LPS, plasma level of 2-AG reached peak within 2 hr of the administration (Fig. 5), and there was time lag before the decrease in muscle tension reached peak approximately 160 min after administration of LPS (Fig. 4B). Platelets have been reported to be 2-AG producing cells. It remains unclear, however, which signal transmission mechanism is involved in release of 2-AG following LPS administration. Our results suggest that synthesis and release of 2-AG takes place during a time lag of approximately 2 hours between LPS administration and the beginning of intestinal relaxation and decreases in body temperature. In fact, blood concentrations of 2-AG increased significantly 1 hour after LPS administration. As 2-AG, anandamide and LPS are all highly lipophilic and can pass easily through the blood-brain barrier, effects of these agents may include reactions in the central nervous system.

Lien and Bertler argued that LPS acts through toll-like receptors (TLR), that is, LPS in vivo forms a complex with LPS binding protein (LBP) in blood. In this view, the complex binds to membrane binding CD14 (mCD14) on the surface of macrophage cells. Then, as LPS binds with TLR-4, LPS signals are transmitted to the inside of cells through the product of the MyD88 gene, an intracellular adapter molecule. In the cells, IRAK (IL-1 receptor associated kinase) is activated through the MyD88 gene product. Next, transcription factor NFKcB is activated through TRAF6, tumor necrosis factor (TNF) receptor associated factor 6, IKK (IκB kinase) and IκB. Ultimately, inflammatory cytokines are produced. However, effects of these cytokines alone cannot fully explain the actions of LPS. In addition, the signal transduction system involved in cannabinoid production after LPS binding has not been elucidated.

There are many reports on in vitro actions of cannabinoids on the digestive tract in which isolated intestinal tissues were used. Cannabinoids, through CB1 receptors in the gastrointestinal nerve plexus, suppress the release of contractile neurotransmitter and excitatory transmission. An in vivo study also reported on cannabinoid-induced suppression of intestinal movement. Specifically in experiments on colonic transportation in mice, transportation of contents in the intestine principally by contraction of the circular muscles was suppressed by cannabinoids and involved CB1 receptors. In a mouse paralytic ileus model produced by intraperitoneal administration of acetic acid, the density of CB1 receptors in the myenteric plexus and nerve bundles increased. Anandamide levels in the small intestine also increased, suggesting that endocannabinoids play a role in suppression of intestinal movement. In addition, Harada et al. demonstrated that, following administration of 2-AG, intestinal muscle tension in conscious guinea pigs decreases dose-dependently. The present study demonstrates that cannabinoids and LPS suppress tension of longitudinal muscles of guinea pig colon, and that the suppression is
mediated by CB1 receptors.

The present study also found that cannabinoids and LPS administered to the guinea pig induce hyperthermia, indicating that the reaction is mediated by CB1 receptors. It is known that the anterior nucleus of the hypothalamus plays an important role in temperature regulation24,25). A recent study using rats reported that intraperitoneal administration of WIN55212-2 induced hyperthermia in association with activation of CB1 receptors26). It remains unclear which functional sites are involved in temperature decreases following LPS administration.

In summary, decreases in intestinal tension and body temperature following LPS administration in conscious, unrestrained guinea pigs are inhibited significantly by pretreatment with a CB1 receptor antagonist. This finding suggests that decreases in intestinal tension and body temperature by LPS are mediated by CB1 receptors. Increases in post-LPS survival rates following administration of AM281 in mice, suggests the possibility that CB1 receptor antagonists could be used as therapeutic agents for endotoxosis.

Although various therapeutic agents have been proposed for the treatment of sepsis27), no therapeutic agent has been established as being effective. The present in vivo experiments using guinea pigs, which have a sensitivity to LPS as high as humans do, demonstrate the possibility that endocannabinoids contribute to intestinal paralysis and decreases in body temperature at the early phase of sepsis. Further studies will be needed regarding application of cannabinoid receptor antagonists to the treatment of sepsis in emergency care.

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References


覚醒モルモットのエンドトキシン血症における内因性カンナビノイド系の調節

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要旨 意識下・無拘束モルモットの腸管運動を、結腸網に取り付けたforce transducerを介したテレメトリー法で観察した。lipopolysaccharide (LPS) 投与および cannabinoïds 投与により、濃度依存性の結腸網張力低下が観察された。意識下・無拘束で同時に測定した体温は、LPS および cannabinoïds 投与による結腸網張力低下と同じ時間経過で低下した。LPS および cannabinoïds 投与による結腸網張力低下反応と体温低下反応は、cannabinoid CB1 receptor antagonist である AM281 前処置により抑制されたが、CB2 receptor antagonist AM630 により抑制されなかった。LPS 投与後のモルモット血漿中の内因性カンナビノイド濃度をLC/MS/MS分析により測定した。LPS 投与後の内因性カンナビノイド濃度は、2-arachidonoylglycerol (2-AG) 濃度が LPS 投与後 1 時間で有意に上昇したが、arachidonoylethanolamide (AEA) 濃度は検出限界以下だった。また、AM281 存在下・非存在下において LPS 投与後のマウス生存率を比較した。AM281 投与マウスでは、LPS による生存率が上昇した。以上、CB1 receptor antagonist 投与により LPS 投与後 1 時間で有意に上昇したが、LPS 投与による生存率が上昇したことから、LPS 作用発現の情報伝達系に CB1 を介する系が関与していることが示唆された。また、LPS 投与後 1 時間で血中 2-AG 濃度が上昇したことから、エンドトキシン血症における内因性カンナビノイド系の関与が考えられた。

キーワード：内因性カンナビノイド, lipopolysaccharide, 平滑筋, イレウス, CB1 receptor

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