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Endocannabinoid, anandamide in gingival tissue regulates the periodontal inflammation through NF-κB pathway inhibition

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Abstract **Anandamide (AEA) exhibits anti-inflammatory effects. However, its role in the periodontal field remains unknown. Here, we found that gingival crevicular fluid contained a detectable level of AEA. The cannabinoid receptors CB1 and CB2 were expressed by human gingival fibroblasts (HGFs), and markedly upregulated under pathological conditions. AEA significantly reduced the production of pro-inflammatory mediators (IL-6, IL-8 and MCP-1) induced by *Porphyromonas gingivalis* LPS in HGFs, and this effect was attenuated by AM251 and SR144528, selective antagonists of CB1 and CB2, respectively. Moreover, AEA completely blocked LPS-triggered NF-κB activation, implying that AEA may regulate hyperinflammatory reactions in periodontitis.**

Keywords: Anandamide; Cannabinoid receptor; Periodontitis; Cytokine; NF-κB

1. Introduction

Periodontitis is a major chronic inflammatory disease, that destroys the periodontal tissue and eventually causes loss of teeth. One of the main bacterial pathogen of human chronic periodontitis is *Porphyromonas gingivalis* (Pg), which is a gram-negative anaerobic bacterium. The bacterium expresses a number of potential virulence factors including lipopolysaccharide (LPS). LPS has been shown to stimulate host immune cells to produce pro-inflammatory molecules via CD14 protein and TLR4 expressed on the target membranes [1]. These pro-inflammatory molecules are believed to induce several cellular reactions, which eventually lead to an inflammatory status of the periodontal tissue. At the same time, however, protective mechanisms aimed at preventing a pathological outcome of the pro-inflammatory insults are also induced. Hence, the overall balance between the pro- and anti-inflammatory mechanisms is likely to determine the progression and severity of periodontitis. However, the anti-inflammatory mechanism of periodontal diseases remains to be explored.

Endocannabinoids are an emerging class of lipid mediators derived from arachidonic acids and found in several tissues. Anandamide, chemically defined as *N*-arachidonoyl ethanolamine (AEA), and 2-arachidonoylglicerol (2-AG) are the main endocannabinoid described to date [2], and can be generated by activated macrophages via CD14 following exposure to LPS [3]. Recently, endogenous and synthetic cannabinoids have been reported to exhibit immunosuppressive and anti-inflammatory effects [4].

Therefore, we hypothesized that the LPS of periodontopathic bacteria would stimulate host cells to release the endogenous cannabinoid AEA in periodontal tissues, which in turn may reduce the excessive production of pro-inflammatory molecules through the activation of its CB1 and/or CB2 receptors.

The aims of the present study were to examine the levels of AEA in gingival crevicular fluid (GCF) and the expressions of its CB1 and CB2 receptors in gingival fibroblasts and to evaluate the possible role of AEA in periodontitis.

2. Materials and methods

2.1. Reagents

LPS was extracted from lyophilized cells of Pg with phenol–water at 67 °C for 20 min. The pooled extract in the water phase was dialyzed against distilled water and ultracentrifuged at 140 000 × *g* for 3 h to separate LPS. The LPS sediment was resuspended in water and ultracentrifuged twice more by the previous method [5]. AEA and AM251, a CB1-specific antagonist, were purchased from Calbiochem Novabiochem Co. (La Jolla, CA). Affi-Prep polymyxin matrix was purchased from Bio-Rad Laboratories (Hercules, CA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Dojindo (Kumamoto, Japan). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Wako Pure Chemicals Inc. Ltd. (Osaka, Japan). Protease inhibitors (complete cocktail) were purchased from Boehringer Mannheim (Germany). Goat polyclonal antibodies against the CB1(sc-10066) and CB2(sc-10071) receptors were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). SR144528, a CB2-specific antagonist, was a kind gift from Sanofi Recherche (Montpellier, France).

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Abbreviations: AEA, *N*-arachidonoyl ethanolamine; HGFs, human gingival fibroblasts; Pg, *Porphyromonas gingivalis*; GCF, gingival crevicular fluid; PMB, polymyxin B; HPLC, high-performance liquid chromatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay

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2.2. Clinical diagnoses and tissue sampling

Periodontitis, gingivitis and healthy periodontium were diagnosed as previously described [6]. Briefly, periodontitis exhibited a probing pocket depth of 4–10 mm, alveolar bone loss, and bleeding on probing. Gingivitis exhibited a probing pocket depth of less than 4 mm, no alveolar bone loss, and bleeding on probing. Healthy periodontium exhibited a probing pocket depth less than 4 mm, with no alveolar bone loss or bleeding on probing. Informed consent was obtained from the subjects before resection of the gingival tissues.

2.3. GCF sampling

GCF was collected using periopaper (Proflow Inc., Amityville, NY) as previously described [7]. Briefly, periopaper was inserted into the periodontal pocket for 30 s. After removing the paper from the site, the GCF volume was recorded with a Periotron 8000 (Proflow Inc.) and the paper was transferred to 1 ml of saline. Following an interval of 60 s, the procedure was repeated twice.

2.4. AEA measurement by HPLC analysis

Taking advantage of the ability of polymyxin B (PMB) to bind to AEA [8], we measured the levels of AEA in biological fluids after selective absorption onto PMB-immobilized beads. The eluate from the beads was directly fractionated using reverse-phase high-performance liquid chromatography (HPLC). The fractions corresponding to authentic AEA were collected, derivatized with a fluorogenic reagent and quantified by HPLC with fluorometric detection as described previously [9].

2.5. Cell culture for HGFs

Human gingival fibroblasts (HGFs) were obtained from healthy and inflamed gingival tissues according to a previously described method [10]. Tissue samples were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 125 μg/ml of streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells growing out from the tissues were subcultured and used for later experiments.

2.6. Immunohistochemistry

The expressions of CB1 and CB2 in inflamed human gingival tissues were examined by immunohistochemistry using the labeled streptavidin biotin (LSAB) technique according to the manufacturer's instruction. Briefly, the inflamed gingival tissues were sectioned and stained with antibodies against the CB1(1:80 dilution) and CB2(1:80 dilution) receptors overnight at 4 °C. Normal goat IgG was used as a negative control. Next, the sections were incubated with a biotinylated anti-goat antibody followed by incubation with peroxidase-conjugated streptavidin (LSAB Kit Peroxidase; DakoCytomation). The antibody localization was determined using 3,3′-diaminobenzidine (DAB) as the substrate and counterstained with hematoxylin.

2.7. RT-PCR

HGFs obtained from gingival tissues with different disease statuses (1×10^6 cells/ml) were seeded onto 100 mm cell culture dishes in DMEM supplemented with 10% FBS with or without 100 ng/ml LPS for the indicated times. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The purified total RNA (2 μg) was converted to cDNA by reverse transcriptase (RT; Gibco BRL, Gaithersburg, MD) and amplified by RT-PCR. The amplification profile consisted of an initial denaturation of 1 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 2 min at 62 °C and 2 min at 72 °C, and a final extension of 5 min at 72 °C. The following primers (purchased from Kurabo Industries Ltd., Osaka, Japan) were used: CB1 sense: 5′-CGTGGGCAGCC-TGTTCCTCA-3′; CB1 antisense: 5′-CATGCGGGCTTGGTCTGG-3′; CB2 sense: 5′-CGCCGGAAGCCCTCATACC-3′; and CB2 antisense: 5′- CCTCATTCGGGCCATTCCTG-3′. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene.

2.8. Immunoblot analysis

HGFs obtained from gingival tissues with different disease statuses (1×10^6 cells/ml) were seeded onto 100 mm cell culture dishes in DMEM supplemented with 10% FBS with or without 100 ng/ml LPS, and analyzed for their expressions of the CB1 and CB2 receptors as described previously [11]. Briefly, at the end of the culture, whole-cell lysates were prepared by washing the cells with ice-cold PBS and lysing them in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 1 mM PMSF, 0.5 mM Na_2VO_3 and protease inhibitors). Proteins were separated by 12% SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk and 1% bovine serum albumin for 1 h at room temperature and then sequentially probed with an appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody. The membranes were washed with Tris-buffered saline containing 0.1% Tween-20 and developed with a Western blotting ECL kit (Amersham Biosciences, Buckinghamshire, UK).

2.9. Evaluation of cell viability

Healthy HGFs were seeded at a density of $2–3 \times 10^4$ cells/well in 96-well dishes and treated with AEA (20 μM) for 24 h. Next, the cell viability was examined using an MTT assay as previously described [12].

2.10. ELISA

Healthy HGFs (1×10^4 cells) were cultured in DMEM containing 1% FBS in the absence or presence of the indicated concentrations of AEA for 2 h and then exposed to LPS (100 ng/ml) for 24 h. In some experiments, HGFs were pre-incubated with AM251 (1 μM) or SR144528 (1 μM) for 1 h and then incubated with or without AEA for 2 h before further stimulation with LPS (100 ng/ml) for 24 h. At the end of the treatment, the levels of IL-6, IL-8 and MCP-1 in the culture media were examined using appropriate ELISA Kits (Biosource, Camarillo, CA) in triplicate. The minimum detectable doses of IL-6, IL-8 and MCP-1 were less than 2, 5 and 20 pg/ml, respectively.

2.11. Electrophoretic mobility shift assay

Healthy HGFs were treated with 10 μM AEA in DMEM containing 1% FBS for 2 h prior to incubation with or without 100 ng/ml of LPS for 2 h. Next, nuclear extracts were prepared and subjected to electrophoretic mobility shift assay (EMSA) as described previously [13]. Briefly, double-stranded deoxyoligonucleotides containing the NF-κB consensus recognition site (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were labeled with [γ^{-32}]ATP using T4 polynucleotide kinase (Promega, Madison, WI). Next, a 10 μg sample of each nuclear extract was incubated with 0.2 mg poly(dI-dC)/ml in a binding buffer (5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl, pH 7.5, 20% glycerol) for 20 min at room temperature after the addition of a labeled probe. The mixtures were then electrophoresed, and the gel was dried and exposed to X-ray film at −80 °C.

2.12. Statistical analysis

The data were presented as means ± S.D. and analyzed statistically using Student's t tests. Differences were considered significant at $P < 0.01$.

3. Results and discussion

3.1. Detection of AEA in human GCF

The roles of endogenous cannabinoids in the pathogenesis of various diseases in different organs have been studied over recent years. Some of the studies have indicated that these endocannabinoids have anti-inflammatory properties [4].

In the current study, we investigated whether or not human GCF contains AEA for the first time. As shown in Table 1, there was a detectable level of AEA in human GCF and its concentration and amount were 4.63 ± 2.82 μg/ml and 16.4 ± 9.30 ng, from pathologic condition, respectively. Since there was little collectable GCF from healthy condition, we could not conclude these AEA concentration was pathologic or not. There are two possible sources of the AEA in GCF. Specifically, AEA may exude from the blood and/or arise through local release from periodontal tissues as a result of

Table 1
AEA detection in human gingival crevicular fluid

Poket depth (mm)	Clinical atouch level (mm)	Amount of GCF (μl)	Concentration of AEA (μg/ml)	Amount of AEA (ng)
7.33 ± 1.56	4.86 ± 3.29	4.00 ± 2.24	4.63 ± 2.82	16.4 ± 9.30

$n = 12$. Data are the means ± S.D.

infection by gram-negative microorganisms. In vitro studies have shown that stimulation with bacterial LPS increases the production of AEA and 2-AG by immune cells, including macrophages [3].

3.2. Periodontal tissues express CB1 and CB2 receptors

Next, we investigated the expressions of cannabinoid receptors in periodontal tissues by immunohistochemistry. As shown in Fig. 1, both CB1 and CB2 were expressed in a number of fibroblasts, endothelial cells and macrophage like cells in the gingival connective tissue whereas no expression of CB1 and CB2 was observed in the negative control in which normal goat IgG was used instead of primary antibodies against CB1 and CB2 (Fig. 1C), indicating the specificity of antibodies for CB1 and CB2. Although endothelial cells and macrophages have previously been reported to express CB1 and CB2 receptors [14,15], to the best of our knowledge there have no reports to date on cannabinoid receptor expression in gingival

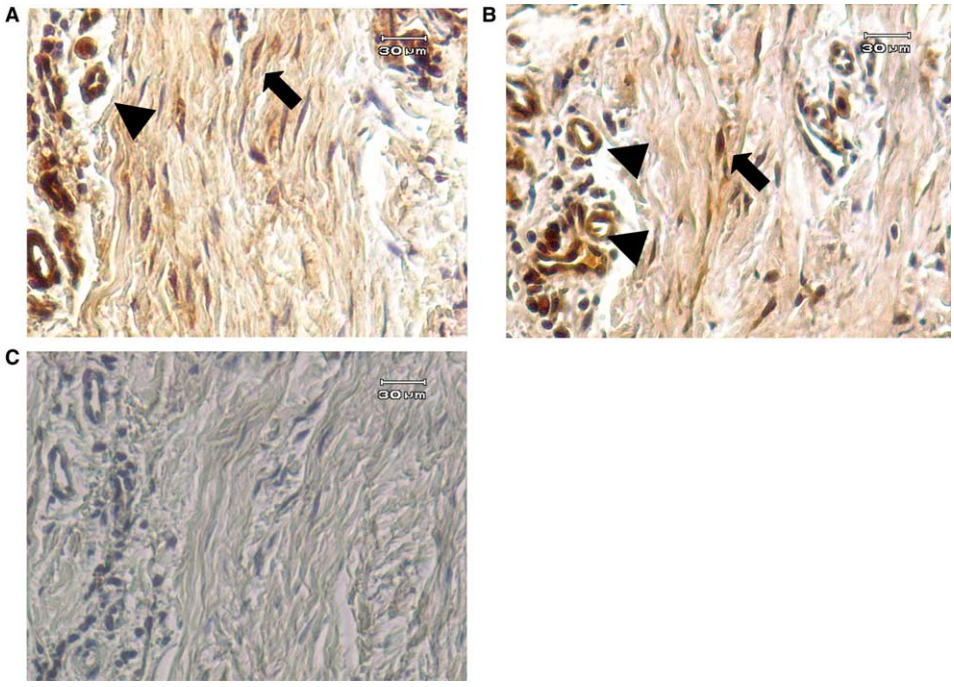

Fig. 1. Immunohistochemical detection of CB1 and CB2 expressions in inflamed periodontal connective tissues. Sections of periodontal connective tissues were incubated with biotinylated goat antibodies against CB1 and CB2, and then with peroxidase-conjugated streptavidin. DAB was used as a substrate for color development. (A) CB1; (B) CB2; (C) normal goat IgG. Arrows: HGFs; arrowheads: endothelial cells.

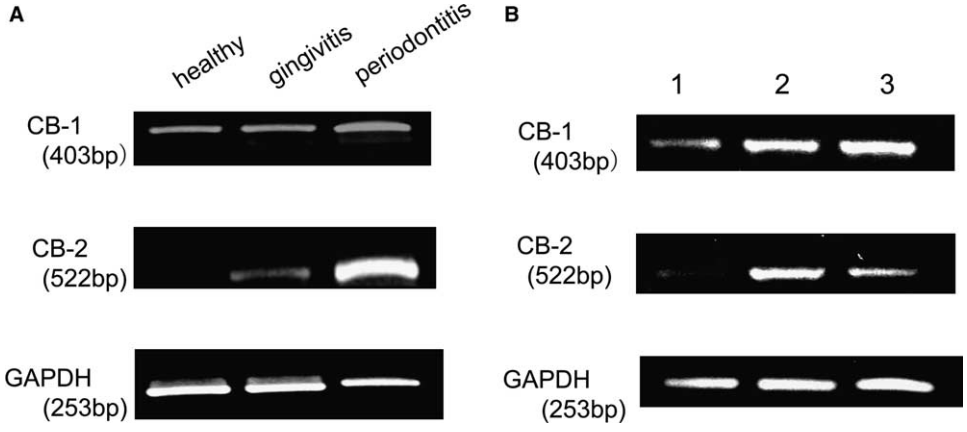

Fig. 2. RT-PCR analysis of CB1 and CB2 mRNA expressions in HGFs. Total RNA was isolated from HGFs obtained from healthy, gingivitis and periodontitis sites (A) or healthy HGFs exposed to Pg LPS (100 ng/ml) for 24 and 48 h (B). The mRNAs were reverse-transcribed and amplified by PCR using primers specific for CB1 and CB2. A photograph of the ethidium bromide-stained amplicons is shown. Lane 1: control, 48 h; lane 2: LPS, 24 h; lane 3: LPS, 48 h. The results are representative of 3 independent experiments. GAPDH was used as a control gene.

fibroblasts. Thus, the present results provide the first evidence that gingival fibroblasts express CB1 and CB2 receptors. Moreover, we were interested to note that some, but not all, of the fibroblasts were positive for CB1 and CB2 receptors. Therefore, we next investigated whether or not the expressions of CB1 and CB2 were correlated with the inflammatory status of clinically diagnosed gingival tissues. For this purpose, the expressions of CB1 and CB2 in HGFs obtained from various stages of periodontal disease as well as from healthy volunteers were evaluated by RT-PCR (Fig. 2A) and immunoblotting analysis (Fig. 3A).

CB1 mRNA expression was detectable in all types of HGFs, but significantly higher in HGFs from gingivitis and periodontitis patients (Fig. 2A). On the other hand, CB2 mRNA was poorly detected in healthy HGFs, but showed striking upregulation in HGFs from gingivitis and periodontitis patients (Fig. 2A). Consistent with these RT-PCR results, Western blot analysis also revealed significant upregulation of CB1 and CB2 receptors in HGFs from gingivitis and periodontitis patients (Fig. 3A). We further examined whether LPS treatment affected the expression patterns of CB1 and CB2 receptors. As shown in Figs. 2B and 3B, healthy HGFs exposed to Pg LPS showed marked upregulation of CB1 and CB2 mRNAs at 24 h as determined by RT-PCR analysis and significant upregulation of the corresponding proteins at 48 h as revealed by immunoblot analysis. Several lines of evidence have indicated that the activation of immune cells in response to LPS or other pro-inflammatory stimuli also upregulates the expression of

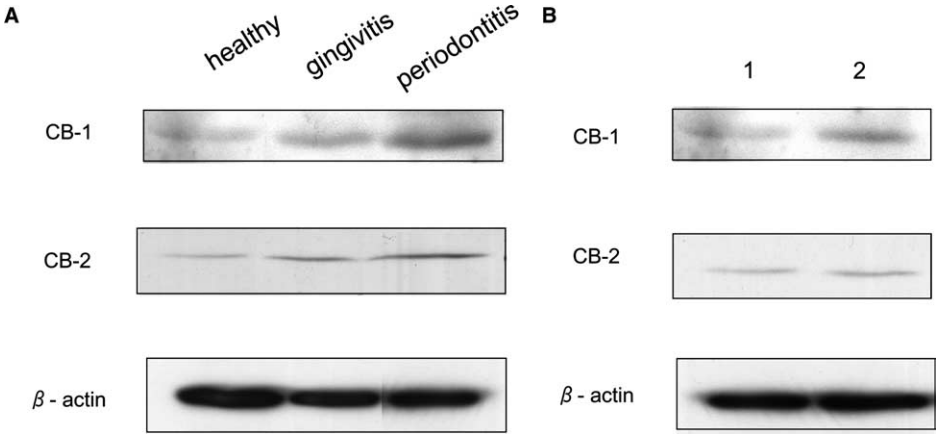

Fig. 3. Immunoblot analysis of CB1 and CB2 protein expressions in HGFs. HGFs obtained from healthy, gingivitis and periodontitis sites (A) and healthy HGFs exposed to Pg LPS (100 ng/ml) for 48 h (B) were analyzed. Cell lysates were prepared and analyzed by immunoblotting using antibodies against CB1 and CB2. Lane 1: control, 48 h; lane 2: LPS, 48 h. The blots are representative of 3 independent experiments.

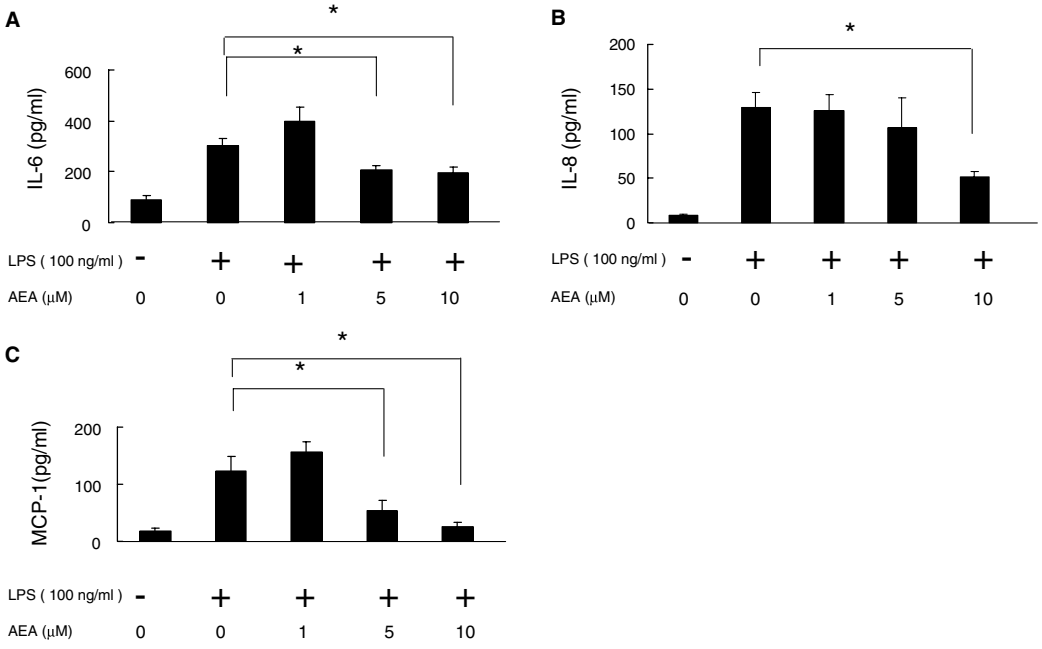

Fig. 4. Effects of AEA on LPS-induced productions of IL-6, IL-8 and MCP-1 in healthy HGFs. Cells were pretreated with the indicated concentrations of AEA for 2 h before the addition of 100 ng/ml Pg LPS for 24 h. The levels of IL-6 (A), IL-8 (B) and MCP-1 (C) in the culture media were determined by ELISA. The data represent the means ± S.D. of 3–6 experiments. $^{*}P < 0.01$.

CB1 and CB2 [16,17]. However, there are also some contradictory results indicating downregulation of these receptors [18,19]. The discrepancies among these results may be due to differences in the cell types or stimuli examined.

3.3. AEA reduces the production of pro-inflammatory mediators induced by LPS in HGFs

AEA has been shown to inhibit the production of inflammatory cytokines in mouse macrophage cultures [20] and cortical astrocytes [21]. Recently, many functional roles of cannabinoid receptors have been demonstrated, such as the regulation of immune responses [4]. We investigated whether or not AEA affects LPS-induced cytokine/chemokine production in HGFs. As shown in Fig. 4, AEA dose-dependently reduced LPS-induced production of IL-6, IL-8 and MCP-1 in HGFs. Moreover, the effect of AEA was attenuated by pretreatment with potent and selective antagonists of the cannabinoid CB1 and CB2 receptors, namely AM251 (1 μM) and SR144528 (1 μM), respectively (Fig. 5), indicating the involvement of cannabinoid receptors in mediating the AEA-induced anti-inflammatory effects in HGFs. These results are consistent with recent reports demonstrating that cannabinoids inhibit atherosclerosis, which is a chronic inflammatory disease, by acting through CB2 receptors [22]. It has also been reported that activation of CB1 and the endogenous cannabinoid system is an early and important physiological step during self-protection of the colon against inflammation [17]. Since AEA has been reported to affect cell viability in several cell types [12], we next examined whether the suppressed release of pro-inflammatory mediators by AEA was accompanied by loss of HGF cell viability. Cell viability, as evaluated by MTT assays, was unaffected by treatment with AEA up to 20 μM for 24 h (data not shown). Therefore, AEA-induced cytotoxicity may depend on the degree of receptor expression, cell type and fatty acid amide hydrolase (FAAH) enzyme (an AEA-hydrolyzing enzyme) status, etc.

3.4. AEA blocks LPS-induced NF-κB activation

It is now well documented that the transcription factor NF-κB plays a central role in most inflammatory processes by augmenting the expression of inflammatory genes, including those for cytokines and chemokines. The results presented above showed that 10 μM AEA significantly reduced LPS-induced production of IL-6, IL-8 and MCP-1. Therefore, we next investigated the effect of AEA on LPS-induced NF-κB activation in HGFs. We observed that AEA (10 μM) almost completely blocked LPS-induced NF-κB activation (Fig. 6), indicating that AEA suppressed pro-inflammatory mediators through blocking NF-κB activation. These results are supported by a previous report that AEA inhibits NF-κB activation [23]. Furthermore,

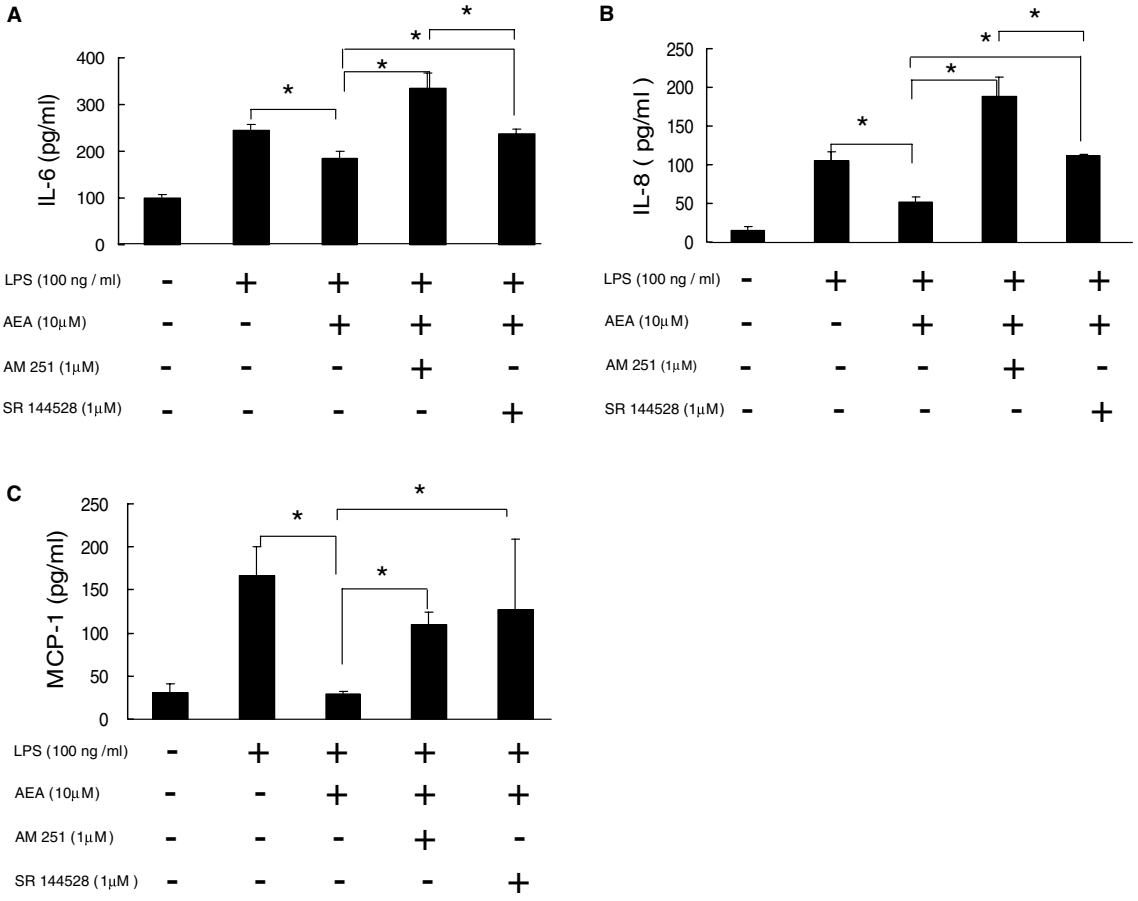

Fig. 5. Effects of CB1 and CB2 antagonists on LPS-induced IL-6, IL-8 and MCP-1 productions in healthy HGFs. Cells were pretreated with AM251 (1 μM) or SR144528 (1 μM) for 1 h and then incubated with or without AEA (10 μM) for 2 h before further stimulation with LPS (100 ng/ml) for 24 h. At the end of the treatment, the levels of IL-6 (A), IL-8 (B) and MCP-1 (C) in the culture media were examined by ELISA. The data represent the means ± S.D. of 3 experiments. $^{*}P < 0.01$.

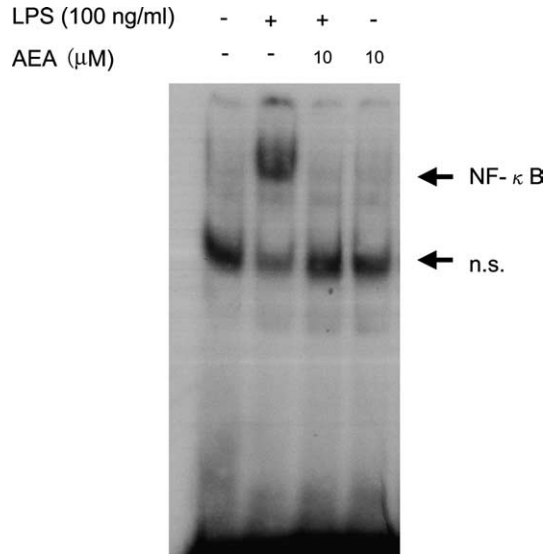

Fig. 6. AEA inhibits the NF-κB activation induced by LPS in HGFs. HGFs were pretreated with AEA (10 μM) for 2 h and then incubated with or without 100 ng/ml Pg LPS for 2 h. After harvesting of the cells, nuclear extracts were prepared and analyzed by EMSAs. Note that AEA completely inhibits the NF-κB activation. The result shown is representative of 3 independent experiments.

cannabinoids were reported to inhibit NF-κB activation and reduce the upregulation of the NF-κB target genes TNFα and IL-6 [24].

In conclusion, the present results demonstrate that AEA and its receptors, CB1 and CB2, are present in periodontal tissues, and indicate that these cannabinoid systems may be physiologically involved in protecting these tissues against excessive inflammation by regulating cellular pathways leading to inflammatory responses. Further studies are needed to examine the roles of endogenous cannabinoid systems in periodontal diseases in more detail. Investigating the analgesic roles of endogenous cannabinoid systems is also an issue for future research. Such studies may lead to the development of novel periodontal therapies and strategies for public oral health.

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