A GGT Inhibitor Suppresses IL-6 and IL-8 Expressions Enhanced by LPS in Gingival Fibroblasts

GGT阻害剤によるLPS刺激歯肉線維芽細胞におけるIL-6およびIL-8発現抑制

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A GGT Inhibitor Suppresses IL-6 and IL-8 Expressions Enhanced by LPS in Gingival Fibroblasts

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Key words: GGT inhibitor, IL-6, IL-8, LPS, gingival fibroblasts

Abstract

Lipopolysaccharides (LPS) induce reactive oxygen species (ROS) accumulation and oxidative stress in gingival fibroblasts. Glutathione (GSH) plays critical roles in protecting cells from oxidative stress and toxic xenobiotics. Gamma-glutamyl transpeptidase (GGT) is the only cell membrane-bound enzyme of GSH homeostasis that breaks down extracellular GSH. The GGT inhibitor GGsTop® suppresses ROS and induces the production of collagen and elastin in skin fibroblasts. Effects of GGsTop® were studied to human gingival fibroblasts (normal gingival fibroblasts; NGFs) stimulated by Porphyromonas gingivalis LPS. Interleukin (IL)-6 and IL-8 productions were measured using Enzyme-linked Immunosorbent Assay (ELISA). The gene expressions of IL-6 and IL-8 were analyzed by real-time PCR. In addition, the activity of nuclear factor-kappa B (NF-κB) was measured by ELISA. The GGsTop® reduced their gene and protein expressions of IL-6 and IL-8, although NF-κB activity was not affected. It is considered that GGsTop® may be useful for anti-inflammatory in periodontitis.

Introduction

The oral cavity is the primary external opening leading into the gastrointestinal or digestive tract and plays an important role in respiration. It is well known that reduced oral function induces severe illnesses such as aspiration pneumonitis, which can result in death (1). Furthermore, the oral cavity is the first organ that gets invaded by foreign matter such as bacteria and viruses and plays many important roles, including immune function via instant detection and removal of those pathogens from the body (2, 3). The oral commensal bacteria are proposed to be essential for maintaining healthy tissues, having multiple roles including priming immune responses to ensure rapid and efficient defenses against pathogens. However, dysbiosis of the oral microbiome triggers the inflammatory disease periodontitis which is involved in life threatening systemic diseases such as arteriosclerosis, low birth weight infants, diabetes, and respiratory tract infections (4,5). Additionally, tissue weakness, decreased defense due to illnesses, and advanced age result in loss of balance between inflammation and the healing process, leading to tissue damage, thus creating a vicious cycle (6, 7).

Glutathione (GSH) has both antioxidant and detoxifying effects. GSH regulates thiol, antioxidant component, redox transitions in cell signaling, contributing to various illnesses (8). Periodontitis is predominantly due to exaggerated host response to pathogenic microorganisms and their products which causes an imbalance between the reactive oxygen species and antioxidant. Previous studies

showed the low levels of GSH in the blood, serum/plasma, and saliva in cases of severe periodontal disease, and GSH levels are higher when the disease state improved (9, 10). GSH homeostasis is the most essential element of maintaining cell homeostasis. GSH possesses a unique γ-glutamyl bond in its glutamic acid side chain, which is degraded only by gamma-glutamyl transpeptidase (GGT). GGT plays a crucial role in oxidative stress as the enzyme responsible for GSH metabolism and detoxification of foreign matter.

Recently, a GGT inhibitor (GGsTop®; 2-amino-4-{[3-(carboxymethyl) phenoxy] (methoyl) phosphoryl}butanoic acid) is reported to promote the production of collagen, elastin, and hyaluronic acid in the extracellular matrix of skin fibroblasts and to be deeply involved in skin epithelial tissue morphology and the maintenance of its function (11). The epithelial and connective tissue in the skin and oral mucosa are similar, therefore, GGsTop® may contribute to the morphology and maintenance of the function of gingival fibroblasts to protect against tissue damage due to periodontal disease, like its function in the skin. The aim of this study is an investigation of the effect of GGsTop® against the inflammatory response in gingival fibroblasts.

Materials and methods

Microorganisms

Porphyromonas gingivalis strain ATCC33277 and *Candida albicans* strain ATCC90029 were used

to investigate whether GGsTop® has antibacterial or antifungal effects. *P. gingivalis* was cultured in trypticase soy medium containing phytone peptone (5 mg/ml), NaCl (5 mg/ml), cysteine (0.1%), hemin (5 mg/ml) and menadione (0.01%). *C. albicans* was cultured in brain heart infusion broth (BD, USA). The bacterial and fungal counts in cell suspensions were measured using a spectrophotometer (BioSpectrometer; Eppendorf, Germany) at a wavelength of 600 nm by measuring their opacity and adjusted (OD_{600}=1.0) for our experiments. *P. gingivalis* was seeded on blood agar plates (Modified-CDC ANAEROBE 5% Sheep Blood agar BD, USA) with 300 μg/ml gentamicin and 200 μg/ml metronidazole or GGsTop®. *C. albicans* was seeded on Sabouraud's agar plates (BD, USA) containing 1 μg/ml amphotericin B or GGsTop®. GGsTop® was purchased from FujiFilm Wako Pure Chemical Corporation (Japan) and used according to manufacturer's recommendation.

Cell culture

Clinical isolated gingival fibroblasts without inflammation (Normal gingival fibroblasts; NGFs) were used in our experiments. The NGFs were established by the methods of Somerman et al (12). Cells were passaged in Dulbecco's modified Eagle's medium (Sigma, USA; D-MEM) containing 10% Fetal bovine serum (FBS) and antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin; GIBCO, USA) at 37°C in 95% air and 5% CO_2. Informed consent from donors were obtained before starting the experiments (ethical approval number: EC15-010).

The detection of endotoxin unit in LPS from P. gingivalis

The lipopolysaccharide (LPS) was extracted by hot phenol-water method (13). Endotoxin unit of LPS from *P. gingivalis* was detected using an EndoZyme Endotoxin Test Kit (Funakoshi Co., Ltd.). *Escherichia coli* strain K12 (Sigma, USA) was used as a positive control. In addition, the effect of GGsTop® on the endotoxin unit in LPS from *P. gingivalis* was studied.

Addition of LPS and GGsTop®

NGFs were seeded in 6-well culture plates at 2×10^5 cells/well. After culturing them for 18 hours, the culture medium was replaced with fresh D-MEM without FBS and antibiotics. GGsTop® (0.1-500 μg/ml) was added in fresh medium. The cultured cells were collected after 24 hours, and the number of the viable cells were counted by using Invitrogen™ Countess™ (Invitrogen, U.S.A.).

LPS (1.0 μg/ml) and GGsTop® (0.1 or 100 μg/ml) were added simultaneously to NGFs for 8 hours. Interleukin (IL) -8 production in the culture supernatant was measured using a Human IL-8 ELISA Ready-SET-Go!™ Kit (Affymetrix, USA) to decide the GGsTop® concentration.

Measuring IL-6 and IL-8 protein productions

NGFs were cultured with LPS and GGsTop® for 24 hours, and the culture supernatants were

collected. Both IL-6 and IL-8 protein productions were measured using an Human IL-6 or IL-8 ELISA Ready-SET-Go!™ Kit (Affymetrix, USA).

IL-6 and IL-8 mRNA expression analysis

At 8 hours after adding LPS and GGsTop® to NGFs, total RNAs were extracted using the RNeasy Mini kit (Qiagen, Germany), and cDNAs were synthesized using a QuantiTect Reverse Transcription Kit (Qiagen, Germany). The mRNA expressions were analyzed by real-time PCR (Eco^{TM}Real Time PCR System; illumina, USA) using a KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, USA) with primers (Table 1) and cDNAs.

Measuring the activity of nuclear factor-kappa B (NF-κB)

Cells were seeded in 96-well culture plates at 2×10^4 cells/well and cultured for 18 hours in D-MEM without FBS and antibiotics. The cells were washed with phosphate buffered saline, and D-MEM with 1.0 μg/ml LPS and 100 μg/ml GGsTop® were added to the cells. After culturing for 30 minutes, NF-κB activity was measured using a Phospho-RelA/NFkB p65 (S536) Cell-Based ELISA (R&D Systems, USA).

Statistical analysis

Values were calculated as the mean±standard deviation (S.D.). Student's t-test was used for all statistical analyses. Significantly differences between groups were considered at P values of <0.05.

Results

The antibiotic effect of GGsTop®

P. gingivalis did not grow on a blood agar plate with gentamicin and metronidazole, however, *P. gingivalis* grew on the plate containing 100 μg/ml GGsTop® (Fig.1 A).

C. albicans did not grow on a Sabouraud's agar plate with Amphotericin B, however, *C. albicans* was grown on the plate in the presence of 100 μg/ml GGsTop® (Fig. 1B).

The effect of GGsTop® on endotoxin unit in LPS from P. gingivalis

The endotoxin unit in LPS from *P. gingivalis* was not significantly different from that of *E. coli* K12, which was used as a positive control (Fig. 2). We measured the endotoxin unit after treatment with GGsTop®. In the control group, purified water was added instead of GGsTop®. There were no significant differences in the endotoxin unit of LPS between GGsTop® and control groups (Fig. 3).

The effect of GGsTop® on the cell viability of NGFs

There were no significant differences between GGsTop® and control groups (Fig. 4).

The effect of GGsTop® concentration on NGFs

IL-8 production in the culture supernatant of NGFs with GGsTop®-only was not significantly different from control group, but significantly lower in the LPS and 0.1 μg/ml or 100 μg/ml GGsTop® group compared to the LPS-only group (Fig. 5). There was not significant difference between 0.1 μg/ml GGsTop® group and 100 μg/ml GGsTop® group. The GGsTop® at 100 μg/ml was added to NGFs in subsequent experiments.

The effect of LPS and GGsTop® on NGFs

The productions of IL-6 and IL-8 in the culture supernatant of NGFs with GGsTop® were not significantly different from the control group, however, productions increased significantly in the LPS-only and LPS and GGsTop® groups. Moreover, both IL-6 and IL-8 productions decreased in the LPS and GGsTop® group compared to that in the LPS-only group (Fig. 6 A, B).

As for the IL-6 and IL-8 gene expressions in NGFs after adding LPS and GGsTop®, similar to protein production results, there was no difference in the GGsTop® group compared to the control group. However, there was a significant increase in the LPS-only group, and a substantial decrease from those levels in the LPS and GGsTop® group (Fig. 7 A, B).

The effect of LPS and GGsTop® to NGFs on NF-κB activity

The activity of NF-κB at 30 minutes after adding GGsTop® was not significantly different from control, however, increased significantly in the LPS-only group. There was no considerable difference between the LPS-only group and the LPS and GGsTop® group (Fig. 8).

Discussion

Periodontal bacteria can cause damage the periodontal tissue directly and indirectly (14-17). It is important for wound healing to converge the inflammatory phase. We focused on GSH which play important role to maintain cellular homeostasis. GGT is indispensable for the synthesis of intracellular GSH. However, GGT in the periodontal pocket promotes the differentiation of cells into osteoclasts (18), so regulation of excessive GGT activity may be the key to promote healing in damaged tissue. Here, we used GGsTop® as a GGT inhibitor for experiments in this study, as it is highly stable even in water, and non-toxic.

First, we studied the anti-bacterial and anti-fungal effects of GGsTop® against *P. gingivalis* and *C. albicans.* The results showed that it does not have the anti-microbial or anti-fungal ability. Second, the effect of GGsTop® on endotoxin unit in LPS from *P. gingivalis* was studied. GGsTop® does not affect it. These results indicated that GGsTop® was useful property for clinical application, since considering long-term oral administration in the future, it is unlikely to affect the normal bacterial composition in

the oral cavity or creation of resistant bacteria.

It is essential to accelerate differentiation into epithelial cells for repair (19), and epithelial growth factors produced by the connective tissues assist in this process (20). This feature explains the reason for our choice of fibroblasts, not epithelial cells as a target in this study. The number of viable cells was not changed in NGFs added with all various concentration of GGsTop®. This result indicated that The GGsTop® did not have toxicity to NGFs. In addition, we added low (0.1 μg/ml) or high (100 μg/ml) concentration of GGsTop® with/without LPS (1.0 μg/ml) to determine the suitable GGsTop® concentration in subsequent experiments. The IL-8 production was significantly decreased with LPS and GGsTop® compared to that with LPS. There was not significant difference between low and high concentrations of GGsTop®. These results indicated that GGsTop® did not work concentration-dependently.

Next, we investigated the effect of the addition of LPS and GGsTop®. IL-6 and IL-8 productions in the culture supernatant for 24 hours, were increased significantly with LPS compared to the control and decreased with the addition of GGsTop®. Furthermore, the gene expressions of IL-6 and IL-8, at 8 hours after the addition of GGsTop® increased significantly with LPS compared to the control and decreased with the addition of GGsTop®, showing that GGsTop® might suppress the cellular inflammatory response caused by LPS. We expected that the increases in gene and protein expressions of IL-6 and IL-8 in LPS stimulated NGFs were due to an increase the activity of NF-κB which plays a central role in inflammation via the LPS receptors, Toll like receptors (TLRs) same as previous

studies (21, 22). However, there were no significant changes in NF-κB phosphorylation with/without GGsTop®. According to previous studies, GGT promotes the activation of NF-κB, and the GGT inhibitor Acivicin was reported to suppress that response (23). Acivicin is highly toxic with low target selectivity and its targets are glutamine amidotransferases. Whereas, GGsTop® is a cell membrane-bound enzyme which hydrolyzes γ-glutamyl bonds between Glu and Cys-Gly, so despite both being GGT inhibitors, it was possible that they might have different effects. Moreover, studies of Acivicin used the human histiocytic lymphoma-derived monocytic cell line U937; the gingival fibroblasts used in this study differ from carcinoma. Besides, it is conceivable that GGsTop® decreased the expressions of IL-6 and IL-8 via another pathway, p38 mitogen-activated protein kinase (MAPK), not via NF-κB. It is possible, GGsTop® suppressed reactive oxygen species (ROS) produced by LPS (24) and signal transduction via p38 MAPK pathway with apoptosis signal-regulating kinase 1 (ASK1) (25-28) was impaired, causing decreased IL-6 and IL-8 expression. GGsTop® might indirectly suppress ROS production via maintaining intracellular GSH which has antioxidant effect. To prove this, in future studies we need to determine the effect of GGsTop® on the p38 MAPK pathway via ASK1 and ROS production due to an LPS stimulation. It thought to be necessary for the improvement of periodontal disease status to maintain GSH, which plays a central role in intracellular redox homeostasis. Due to this, the regulation of GGT activation, which contributes to ROS production and GSH biosynthesis, is essential for controlling the inflammatory response and protection against systemic diseases.

Taken together, our results suggested that GGsTop® may have potential future clinical application to reduce inflammation in periodontal disease.

Conclusions

The GGT inhibitor, GGsTop®, has no either antibacterial or antifungal effects against *P. gingivalis* or *C. albicans*. Additionally, GGsTop® has no cytotoxic activity in NGFs and exhibits a suppressive effect on increased IL-6 and IL-8 expression in NGFs treated with a *P. gingivalis*-derived LPS stimulation. These results suggested that GGsTop® may have potential future clinical application to reduce inflammation in periodontal disease.

Conflict of interest

The authors of this study received funding for this research provided by NAHLS corporation, the company which developed GGsTop®. Although there is a conflict of interest at the time of writing (December 2018), it in no way hinders its publication

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Table 1.

Primers in this study

Gene name	Forward (5'-3')	Reverse (5'-3')
IL-6	AAGCCAGAGCTGTGCAGATGAGTA	TGTCCTGCAGCCACTGGTTC
IL-8	ATTGCATCTGGCAACCCTAG	CTGCGCCAACACAGAAATTA
GAPDH	GTAGAGGCAGGGATGATGT	TCCAAAATCAAGTGGGGCGA

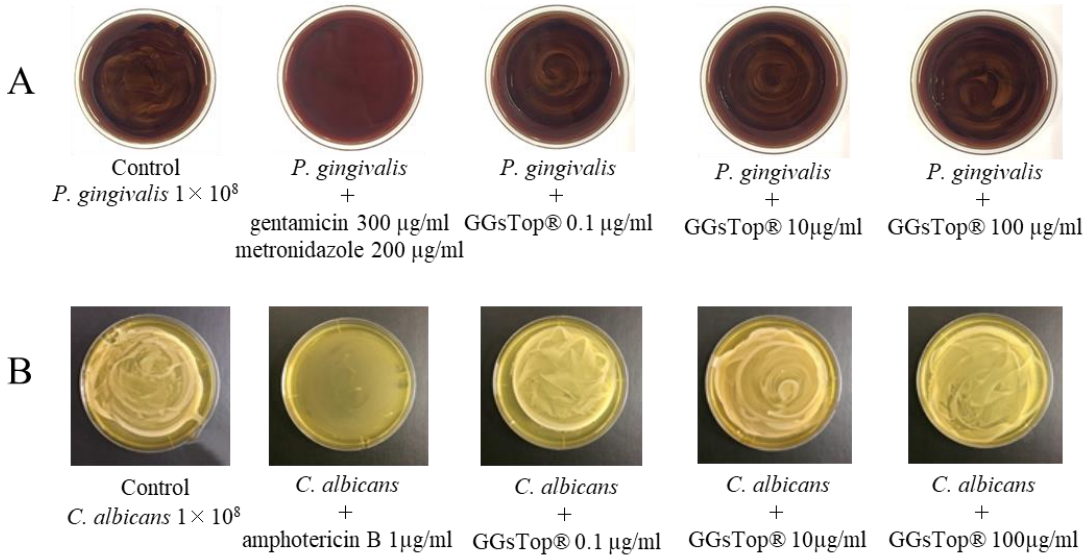

Fig.1. The antibiotic effect of GGsTop®

P. gingivalis (A) and *C. albicans* (B) were seeded on each selective agar plate. They grew on each control plate without any reagents. (A) *P. gingivalis* did not grow on blood agar plate with gentamicin and metronidazole. *P. gingivalis* grew on plates with various concentrations of GGsTop®. (B) *C. albicans* grew on the plate not with amphotericin B but with various concentrations of GGsTop®.

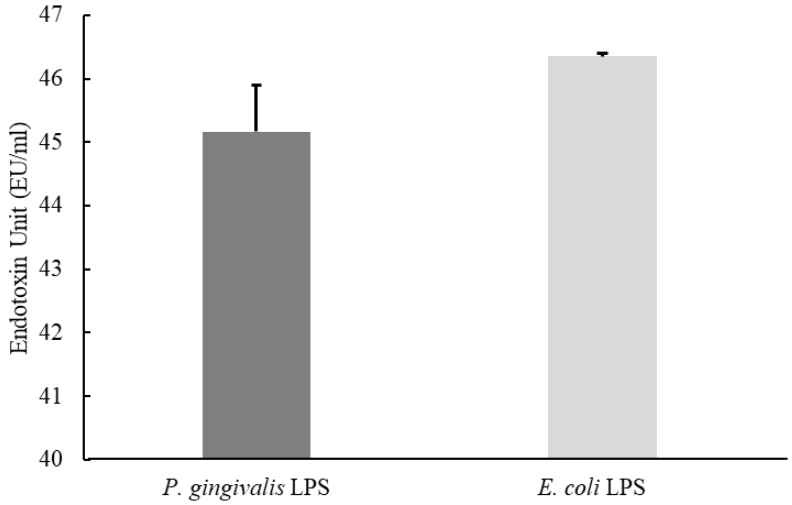

Fig. 2. The endotoxin unit of LPS from *P. gingivalis*

The endotoxin unit of LPS from *P. gingivalis* was observed using EndoZyme Endotoxin Test Kit. There was no significant difference between *P. gingivalis* and *E. coli* on the endotoxin unit.

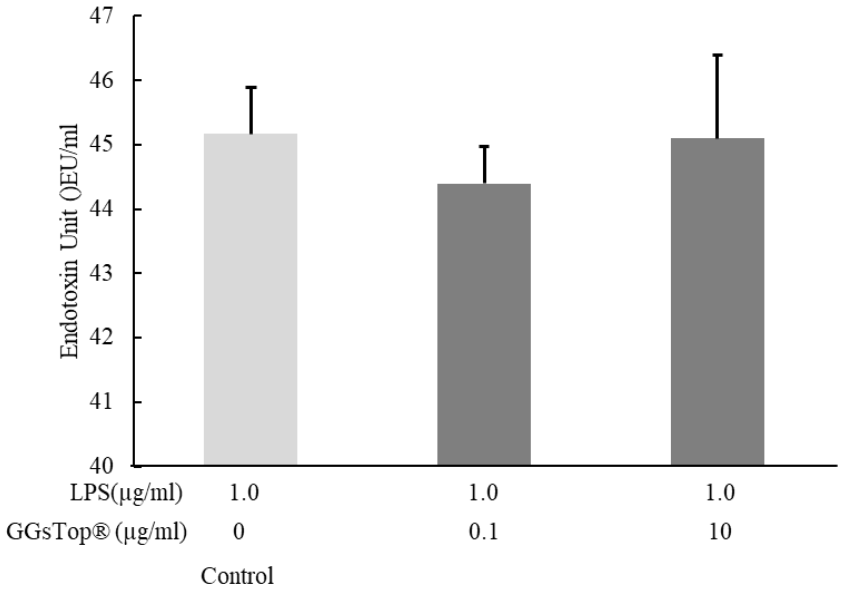

Fig. 3. The effect of GGsTop® to the endotoxin unit of LPS

The endotoxin unit of LPS derived from *P. gingivalis* with/without GGsTop® was observed. GGsTop® was added to LPS at concentration of 0.1 or 10.0 μg/ml. There was no significant difference between control and LPS with GGsTop® on the endotoxin unit.

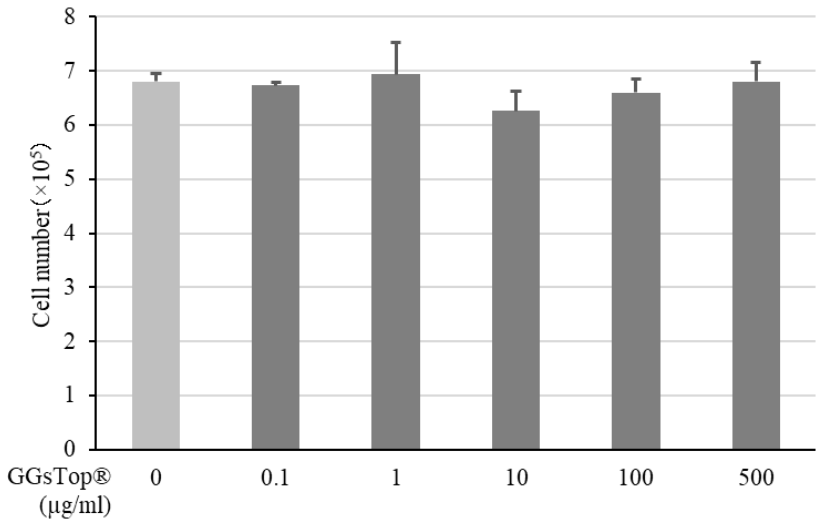

Fig. 4. The effect of GGsTop® concentration on NGFs viability.

The number of NGFs was counted after treatment of various GGsTop® concentration. The cell number at all concentration was same as control. There was not significant difference between control and each GGsTop®.

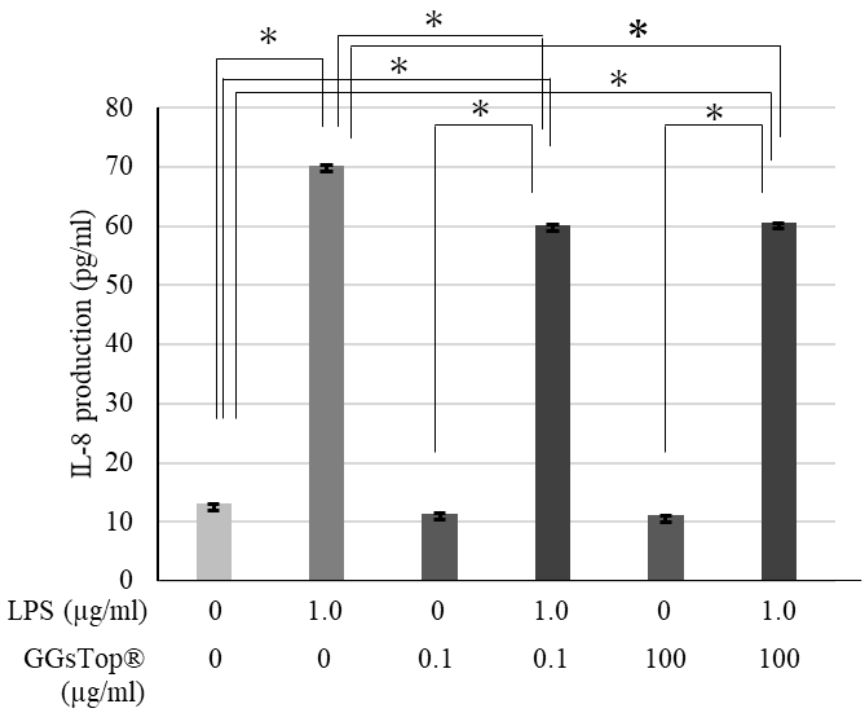

Fig. 5. The effect of GGsTop® concentration on IL-8 production of NGFs

IL-8 production in the supernatant of NGFs cultured with 1.0 μg/ml LPS and/ or 0.1, 100 μg/ml GGsTop® for 8 hrs were measured by ELISA. IL-8 production of NGFs with GGsTop® was same as control. The IL-8 production of NGFs with LPS was significantly higher compared with control and that was significantly decreased in NGFs with LPS and GGsTop®. ∗:$p<0.05$

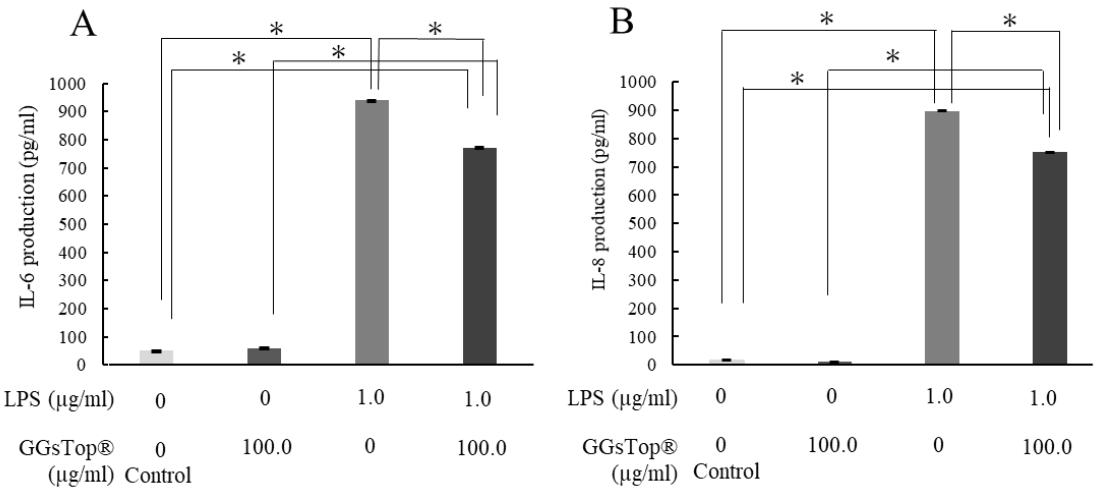

Fig. 6. The effect of GGsTop® on IL protein productions of on NGFs

IL-6 (A) and IL-8 (B) productions in the culture supernatant of NGFs with 1.0 μg/ml LPS and/or 100μg/ml GGsTop® for 24 hours were measured by ELISA. IL-6 and IL-8 protein productions with only GGsTop® were not increased and were significantly increased at treatment of LPS. The increased IL-6 and IL-8 protein productions by LPS were significantly decreased at addition of GGsTop®. * : $p<0.05$

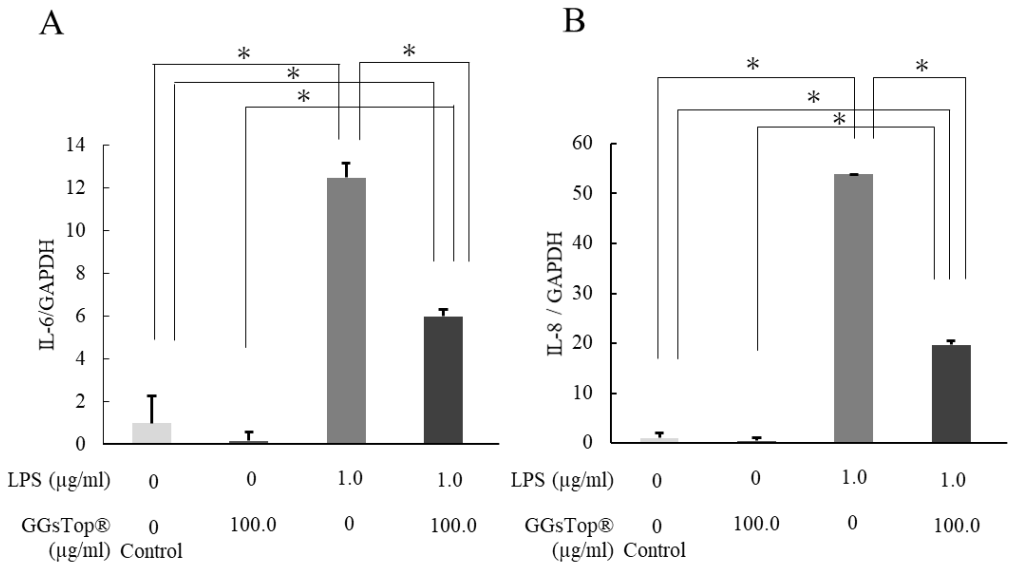

Fig. 7. The effect of GGsTop® on the mRNA expressions in NGFs

IL-6 (A) and IL-8 (B) mRNA expressions in NGFs treated with 1.0 μg/ml LPS and/or 100 μg/ml GGsTop® for 8 hours were analyzed using real-time PCR. The level of mRNA expressions of IL-6 and IL-8 in NGFs with only GGsTop were same as control. The enhancement of IL-6 and IL-8 mRNA expressions by LPS were significantly decreased by GGsTop®.∗: $p<0.05$

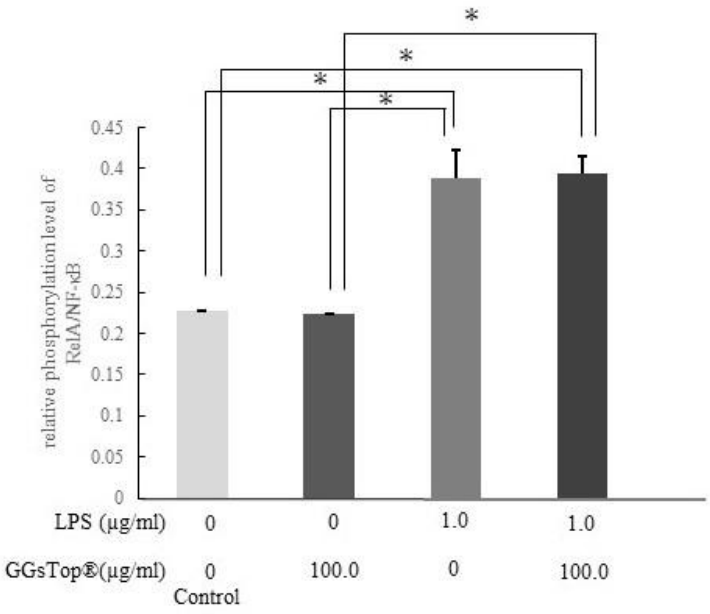

Fig. 8. The effect of GGsTop® to NF-κB activity in NGFs

The NF-κB activity in NGFs treated with LPS and/or GGsTop® for 30 minutes was measured by ELISA. The NF-κB activity in NGFs with only LPS was same as control. The enhancement of NF-κB activity by LPS in NGFs was not changed by GGsTop®. ∗: $p<0.05$