β-Carotene Suppresses *Porphyromonas gingivalis* Lipopolysaccharide-mediated Cytokine Production in THP-1 Monocytes Cultured with High Glucose Condition

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Short title: β-Carotene Suppresses Cytokine Production

Abbreviation list: lipopolysaccharide, LPS; *Porphyromonas gingivalis*, Pg; high glucose, HG; normal glucose, NG; Toll-like receptors, TLR; protein kinase C, PKC.

Abstract

Periodontitis is associated with development of diabetes mellitus. Although lipopolysaccharide (LPS) of *Porphyromonas gingivalis* (Pg), a major pathogen of periodontitis, may lead the progression of diabetes complications, the precise mechanisms are unclear. We, therefore, investigated the effects of β-carotene on production of Pg LPS-induced inflammatory cytokines in human monocytes cultured high glucose (HG) condition. THP-1 cells were cultured under 5.5 mM or 25 mM glucose conditions, and cells were stimulated with Pg LPS. To investigate the productivity of TNF-α, IL-6 and MCP-1, cell supernatants were collected for ELISA. To examine the effects of NF-kB signals on cytokine production, Bay11-7082 was used. HG enhanced Pg LPS-induced production of TNF-α, IL-6 and MCP-1 *via* NF-kB signals in THP-1. β-carotene suppressed the enhancement of the Pg LPS-induced cytokine production in THP-1 *via* NF-κB inactivation. Our results suggest that β-carotene might be a potential anti-inflammatory nutrient for circulating Pg LPS-mediated cytokine production in diabetic patients with periodontitis.

Key Words: β-carotene; *Porphyromonas gingivalis* LPS; diabetic complications; glucose; monocyte

1. Introduction

Periodontitis is an infectious disease caused by periodontopathic bacteria such as Gram-negative bacteria *Porphyromonas gingivalis* (Pg) (Darveau et al., 1997). In diabetic patients, it is well-known that dentists experience severe periodontitis frequently. Recently, it has been reported that periodontitis, persistent low-grade infection of Gram-negative bacteria, mainly Pg, is associated with development of systemic disease such as diabetes and atherosclerosis because of increasing of invasion of the pathogens to the circulation (Zelkha et al., 2010). Indeed, many investigators have reported previously that periodontitis patients have elevated systemic inflammatory markers caused by bacteremia, such as C-reactive protein and interleukin 6 (IL-6), resulting in development of diabetes and atherosclerosis (Teeuw et al., 2014; Moutsopoulos et al., 2006). However, it is still unclear whether the pathophysiology of these systemic diseases is regulated by invaded Pg-induced cytokine production.

Bacterial endotoxin lipopolysaccharide (LPS) is a major component of the cell walls of Gram-negative bacteria (Shoji et al., 2016). High doses of LPS are responsible most prominently for septic shock, whereas low doses of circulating LPS are common in chronic disease settings, and may contribute to the development of persistent, low-grade, non-resolving inflammation (Morris et al., 2014). Ao et al. reported that Pg LPS exacerbates free fatty acid-induced endothelial injury in aortal walls of obese individuals (Ao et al., 2014). Circulating Pg may colonize the deep intima and promote the development of atherosclerosis through additive local production of pro-inflammatory cytokines such as TNF-α, IL-6 and MCP-1.

Carotenoids belong among phytochemicals that are responsible for different colors

of the foods (Rao et al., 2007). These natural diets are widely present in many fruits and vegetables. β-carotene, one of the main carotenoids, is a vitamin A precursor that has been shown to exert antioxidant and anticancer effects. Higuchi et al. reported that higher serum β-carotene levels, associated with higher intake of green and yellow vegetables probably, confer beneficial effects against insulin resistance (Higuchi et al., 2015). Amengual et al. also reported that a diet supplemented with β-carotene reduces body adiposity, size of adipocytes and circulating leptin levels in mice (Amengual et al., 2011). Although β-carotene is recognized as playing an important role in the prevention of human diseases and maintaining good health, the clinical usefulness is only now beginning to be investigated.

In general, although it is well known that cellular responses to elevated extracellular glucose in periodontitis are thought to contribute to the development of diabetes complications (Moutsopoulos et al., 2006), the molecular mechanisms remain unknown. Increased persistent micro-inflammation induced by invasion of periodontal pathogens into blood stream might be a key etiological factor in the development of many diabetes complications. In the present study, therefore, we examined the regulation of cytokines production in THP-1 monocytes stimulated with Pg LPS under high glucose (HG) condition, and evaluated the inhibitory effects of β-carotene on Pg LPS-mediated cytokines production in THP-1 monocytes.

2. Materials and methods

2.1. Reagents

Ultrapure Pg LPS was obtained from Invivogen (San Diego, USA). Antibodies against phospho-p65, p65, phospho-IκB, IκB were obtained from Cell Signaling Technology (Beverly, MA). Antibody against β-actin, MTT (3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium Bromide) and β-carotene were obtained from Sigma (St. Louis, MO). Selective NF-κB inhibitor Bay 11-7082 was obtained from Selleck (Houston, Tx).

2.2. Cell culture

The human monocytic cell line THP-1 (American Type Culture Collection, Manassas, VA, USA) was maintained in RPMI 1640 medium containing 5.5 mM glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (Invitrogen), and were divided into normal glucose (NG) group incubated in RPMI containing 5.5 mM glucose or HG group incubated in RPMI containing 25 mM glucose as described in previous report (Dasu et al., 2008; Omori et al., 2008). THP-1 monocytes were cultured in NG or HG condition, and were stimulated with Pg LPS (1 μg/mL) at indicated periods. To examine the effect of NF-κB pathway on cytokine production, Bay11-7082 (50 μM) was added 60 min prior and during stimulations. To examine the effect of β-carotene on cytokines production, β-carotene (10 μM) was added 60 min prior and during stimulations.

2.3. Cell viability

Cell proliferation activity was examined using MTT assay as described previously

(Yamaguchi et al., 2008). Briefly, THP-1 monocytes (5 x 10^5 cells/well) were seeded in each well of a 96-well plate in a final volume of 100 μL of RPMI medium containing 5.5 mM or 25 mM glucose supplemented with 0.5 % FBS. After 24 hours, the cells were treated with 1 μg/mL of Pg-LPS for 24 h. At the end of the treatments, MTT (final concentration: 0.5 μg/mL) was added to each well and incubated for 4 h in a humidified atmosphere prior to the addition of 100 μL of the extraction buffer (10% SDS in PBS) into each well. The reaction mixture on each well of the 96-well culture plate was measured fluorometrically using a micro plate reader (iMARK^{TM}: Bio-Rad, Hercules, CA; excitation at 595 nm), with the extraction buffer as blank. Next, to examine the effects of carotenoids on cell cytotoxicity, THP-1 monocytes were cultured under NG condition supplemented with β-carotene (10 μM) for 24 hours, and then trypan blue (0.2% w/v) was added to cells in a 1:1 volume, and the percentage viable (excluding dye) was estimated with a hemocytometer.

2.4. Intracellular signaling

To examine the intracellular signaling, cells cultured under NG or HG condition were treated with Pg LPS (1 μg/mL) for 30 min. Total cell lysate was extracted with a lysis buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 % NP-40 and protease inhibitor cocktail (Complete^{TM}; Roche Diagnosis, Berkeley, CA)). The total proteins (10 μg each) were separated in a denaturing 10 % polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) as described elsewhere. The membranes were then blocked with 5 % skim milk in TTBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05 % Tween 20) and subsequently incubated with antibodies against phospho-p65, total-p65, phospho-IκBα,

total-IκBα. Immunoreactive proteins were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL reagents (Amersham, Arlington Heights, IL). β-carotene (10 μM) was added 60 min prior and during stimulations. β-actin was used as an internal control of loading proteins.

2.5. Cytokines production

To investigate the cytokines production, cells cultured under NG or HG condition were treated with Pg LPS (1 μg/mL) for 24 hours. Supernatants were collected and stocked at -80 °C until use. β-carotene (10 μM) was added 60 min prior and during stimulations. The amount of target proteins (TNF-α, IL-6 and MCP-1) was measured using sandwich ELISA kits (R&D Systems).

2.6. Statistical analysis

All analyses were performed with SPSS Statistics version 20 (IBM, Chicago, IL). Statistical significances were determined by Student's *t*-test or ANOVA Tukey-HSD analysis. P value less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of HG condition on Pg LPS-mediated cell proliferation

Cell morphology did not change throughout the experiments. To investigate the effects of HG on cell proliferation, MTT assay was performed (Fig. 1). No significant differences were found in cell proliferative activity of the THP-1 monocytes treated with or without Pg LPS in both NG and HG conditions. On the other hand, HG condition suppressed significantly in the cell proliferative activity ($P<0.05$, Student's t-test).

3.2. Effects of HG condition on NF-κB activation in THP-1 monocytes treated with Pg LPS

As shown in Fig. 2, induction of phosphorylation of p65 and IκBα was found in THP-1 monocytes treated with Pg LPS. Furthermore, the phosphorylation of p65 and IκBα by Pg LPS in HG condition was dramatically enhanced compared with that in NG condition.

3.3. Increase of Pg LPS-induced cytokines production under HG condition and inhibitory effects of NF-κB inhibitor

To determine the effects of HG condition on cytokines production in THP-1 monocytes, the levels of cytokines secreted into the culture medium were measured using specific ELISA methods. As shown in Fig. 3A, B and C, Pg LPS significantly increased TNF-α, IL-6 and MCP-1 production in THP-1 monocytes, even in NG condition ($P<0.01$ vs. untreated cells, ANOVA Tukey-HSD). Furthermore, Pg LPS dramatically increased TNF-α, IL-6 and MCP-1 production in HG condition ($P<0.01$ vs. NG, ANOVA Tukey-

HSD), corresponding to the results of NF-κB activation. To confirm the effects of NF-κB activity on Pg LPS-induced cytokines production, it was examined whether specific IκB inhibitor, Bay 11-7082 inhibits TNF-α, IL-6 and MCP-1 production. As shown in Fig. 3D, E and F, Bay 11-7082 significantly inhibited TNF-α, IL-6 and MCP-1 production in THP-1 monocytes treated with Pg LPS ($P<0.01$ vs. Pg LPS stimuli without Bay, ANOVA Tukey-HSD).

3.4. Effects of β-carotene on cytokines production and NF-κB activation in THP-1 monocytes treated with Pg LPS in both NG and HG conditions

At first, cell cytotoxicity by treatment of β-carotene (10 μM, 24 h) was investigated. As shown in Fig. 4A, no significant differences were found in cell cytotoxicity of the THP-1 monocytes treated with or without β-carotene in both NG and HG conditions. Next, we demonstrated that the phosphorylation of p65 induced by Pg LPS in THP-1 monocytes was inhibited markedly by treatment of β-carotene, corresponding to the results of inhibition of cytokine production (Fig. 4B). The inhibitory effects by β-carotene were not changed between NG and HG conditions. Finally, we found that β-carotene (10 μM) inhibited partially TNF-α, IL-6 and MCP-1 production in THP-1 monocytes treated with Pg LPS ($P<0.01$ vs. Pg LPS stimuli without β-carotene, ANOVA Tukey-HSD) (Fig. 5A, B and C).

4. Discussion

Cellular responses to elevated extracellular glucose contribute to the development of diabetes complications (Yan et al., 2008), although the precise mechanism remains elusive. Shi et al. reported that MCP-1 is involved in renal inflammation and fibrosis in diabetic models (Shi et al., 2008). Funatsu et al. also reported that aqueous levels of IL-6 were significantly correlated with the severity of diabetic retinopathy (Funatsu et al., 2001). Although Pg LPS, a major pathogen of periodontitis, may lead the progression of diabetes complications, the precise action is unclear. Recent studies suggested that oral infections such as periodontitis increase the risk of various systemic diseases, after inflammatory cytokines entered in the blood stream (Teeuw et al., 2014; Moutsopoulos et al., 2006). Thus, IL-6 and MCP-1 produced by monocytes exposed by Pg LPS may lead to the progression of diabetes complications. Suitable control of the periodontal inflammation might exert beneficial effects on Pg LPS-mediated diabetes complications.

In the present study, we demonstrated for the first time that HG exposure increased significantly the production of Pg LPS-induced inflammatory cytokines, TNF-α, IL-6 and MCP-1 in THP-1 monocytes (Fig. 3), although HG suppressed the cell proliferative activity (Fig. 1). It has been generally accepted that the intracellular signaling induced by Pg LPS include the Toll-like receptors (TLR)/NF-κB pathway (Yan et al., 2015). Since NF-κB is involved in the pathogenesis of several chronic inflammatory diseases (Patel et al., 2009), suppression of NF-κB-dependent inflammatory cascades may be considered as an effective therapeutic strategy for preventing the progression of diabetes complications. Interestingly, we found that phosphorylation of p65 and IκBα was significantly enhanced by Pg LPS in THP-1 monocytes cultured under HG condition (Fig.

2). Dasu et al. reported that HG induces TLR2/4 expression *via* protein kinase C (PKC)-α and PKC-δ, respectively, in THP-1 monocytes (Dasu et al., 2008). The over-expression of TLRs is probably not related to the osmotic effects of HG, since the same concentration of mannitol had no effects on TLRs expression. Although HG might enhance the TLRs-mediated NF-κB pathway dramatically in Pg LPS-treated THP-1 monocytes, it has been reported previously that HG induced oxidative burst in monocytes (Hayashi et al., 2007). It remains unclear whether the released oxidants lead to the enhancement of Pg LPS-induced NF-κB activation and thus are involved in TLR2/4 induction by HG exposure. To clarify the molecular mechanisms more in depth, further experiments will be needed.

Chronic HG exposure induces the inflammatory responses directly in several tissues/organs such as kidney and retina through blood stream and therefore plays an important role in the development and progression of diabetes complications. Concretely, the inflammatory responses such as macrophage/lymphocytes infiltration or overexpression of inflammatory cytokines (i.e. TNF, IL-6), contribute to the pathogenesis of diabetic nephropathy or retinopathy. Since LPS is a significant inflammation-related molecule (Su et al., 2015), synergistic effects of HG exposure and circulating Pg LPS on blood cells such as monocytes might lead to severe stage of diabetes complications. Therefore, investigating treatment strategies using anti-inflammatory agents may offer new approaches for preventing the unwanted diabetes complications.

β-carotene, a naturally occurring provitamin A, is the most prominent member of the carotenoids that is present in the human diet (von Lintig et al., 2004). A prospective study reported that high circulating levels of β-carotene have been strongly associated with a decreased risk of diabetes and cardiovascular diseases (Hak et al., 2004). Furthermore, Zhu et al. reported that β-carotene (50 μM) increased significantly the number of

apoptotic cells in human squamous cell carcinoma cells *via* NF-kB pathway (Zhu et al., 2016). Since the concentration of β-carotene in our *in vitro* experiments was quite low (10 μM), no apoptotic cells were observed throughout our experiments (see Fig. 4A). Importantly, we have shown for the first time that β-carotene suppressed the enhancement of Pg LPS-induced TNF-α, IL-6 and MCP-1 production in THP-1 monocytes under HG conditions without cell damage (Fig. 5). Corresponding to the results of cytokine production, β-carotene also suppressed enhancement of Pg LPS-induced phosphorylation of p65 in THP-1 monocytes under HG conditions (Fig. 4B). One of natural compounds, β-carotene which is included in fruits, vegetables and plants, might have received special attention due to the potential to interfere with diabetes complications by targeting NF-κB safely. To confirm the general usefulness of β-carotene, appropriate clinical trial will be required in the future.

Periodontal lesion provides a rich source of inflammation-related molecules such as Pg LPS in the blood. In the present study, we demonstrated that HG indirectly increases production of inflammatory cytokines such as TNF-α, IL-6 and MCP-1 *via* NF-κB signals in THP-1 monocytes treated with Pg LPS (Fig. 6). This pathway might be an attractive target for clarifying the systemic effects of circulating Pg LPS on the development of diabetes complications. Furthermore, we demonstrated that β-carotene inhibits significantly the production of inflammatory cytokines. Exactly, with the appropriate treatment and recommended lifestyle changes, many people with diabetes would be able to prevent or delay the onset of the complications. However, it is often difficult clinically to improve the poor control of blood glucose levels in diabetic patients. Taken together, β-carotene might be an effective and useful nutrient for a novel treatment

strategy to prevent the development of diabetes complications in diabetic patients with periodontitis.

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Author's contributions

Y.K. conducted experiments, analyzed the data and wrote initial draft of the manuscript, Y. N. and J. H. L. helped with the experiments, J. K. and T.N. participated in interpretation of the data, and K.N. designed the experiments, wrote the final manuscript and supervised the study.

Conflict of interest

The authors have no conflicts of interest.

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Figure Legends

Figure 1. Effects of HG on cell proliferation

After THP-1 monocytes were cultured under NG (5.5 mM) and HG (25 mM), the cells were treated with Pg LPS (1 μg/ml) for 24 h. The cell proliferative activity was determined by MTT assay. Data represents as the mean ± SD from 3 independent experiments. NG, normal glucose; HG, high glucose. **, $P<0.01$, ANOVA Tukey-HSD.

Figure 2. HG enhances Pg LPS-induced phosphorylation of p65 and IκBα.

THP-1 monocytes were cultured under NG (5.5 mM) or HG condition and stimulated by Pg LPS (1 μg/ml) for 30 mins. Signaling molecules were detected by Western blotting. Cell lysates were resolved by SDS-PAGE and analyzed using Western blotting probed with antibodies against phospho-p65, phospho-IκBα. Equal loading of total lysates (10 μg) was confirmed by re-probing with antibodies against each molecule p65, IκBα and β-actin. This figure shows a result of representative 3 independent experiments. Quantitation of the phosphorylated protein levels was performed by densitometric scanning of each band using Image J software (NIH, Washington DC, USA), and fold changes of each band were expressed as a ratio of control. NG, normal glucose; HG, high glucose.

Figure 3. HG enhances Pg LPS-induced production of inflammatory cytokines via NF-*k*B pathway.

THP-1 monocytes were cultured under NG or HG condition and treated by Pg LPS (1 μg/ml) for 24 hours. The levels of target molecules: (A) TNF-α, (B) IL-6, (C) MCP-1

were measured using ELISA Kits. Next, to confirm the Pg LPS-mediated signaling pathway, NF-κB inhibitor Bay11-7082 (50 μM) was added 60 min prior and during stimulations. The levels of target molecules: (D) TNF-α, (E) IL-6, (F) MCP-1. Data represents as the mean ± SD from 3 independent experiments. NG, normal glucose; HG, high glucose; Bay, Bay11-7082. **, P<0.01, ANOVA Tukey-HSD.

Figure 4. Effects of β-carotene on Pg LPS-induced phosphorylation of p65 and IκBα. (A) Effects of β-carotene on cell viability. Cells were treated with β-carotene (10 μM) for 24 hours, cell viability response to β-carotene was assessed with trypan blue dye exclusion. The viability was expressed as a percentage. NS, not significant differences, ANOVA Tukey-HSD. (B) THP-1 monocytes were cultured under NG (5.5 mM) or HG condition and stimulated by Pg LPS (1 μg/ml) for 30 mins. To confirm the effects of β-carotene on Pg LPS-induced phosphorylation of p65, β-carotene (10 μM) was added 60 min prior and during stimulations. Signaling molecules were detected by Western blotting. Cell lysates were resolved by SDS-PAGE and analyzed using Western blotting probed with antibodies against phospho-p65. Equal loading of total lysates (10 μg) was confirmed by re-probing with antibodies against each molecule p65 and β-actin. This figure shows a result of representative 2 independent experiments. Quantitation of the phosphorylated protein levels was performed by densitometric scanning of each band using Image J software (NIH, Washington DC, USA), and fold changes of each band were expressed as a ratio of control. NG, normal glucose; HG, high glucose. β-Caro, β-carotene.

Figure 5. Effects of β-carotene on Pg LPS-induced production of inflammatory cytokines

THP-1 monocytes were cultured under NG or HG condition and treated by Pg LPS (1 μg/ml) for 24 hours. To examine the effects of β-carotene on Pg LPS-induced production of inflammatory cytokines, β-carotene (10 μM) was added 60 min prior and during stimulations. The levels of target molecules: (A) TNF-α, (B) IL-6, (C) MCP-1, were measured using ELISA Kits. Data represents as the mean ± SD from 3 independent experiments. NG, normal glucose; HG, high glucose; β-Caro, β-carotene. *, $P<0.05$; **, $P<0.01$, ANOVA Tukey-HSD.

Figure 6. Schematic representation of circulating Pg LPS-induced production of inflammatory cytokines from monocyte: inhibitory effects of β-carotene on progression of diabetic complications

HG enhances circulating Pg LPS-induced cytokine production, resulting in progression of diabetic complications. In addition to periodontitis treatment clinically, persistent β-carotene intake may prevent the progression of these unwanted cascades.

Kajiura et al.

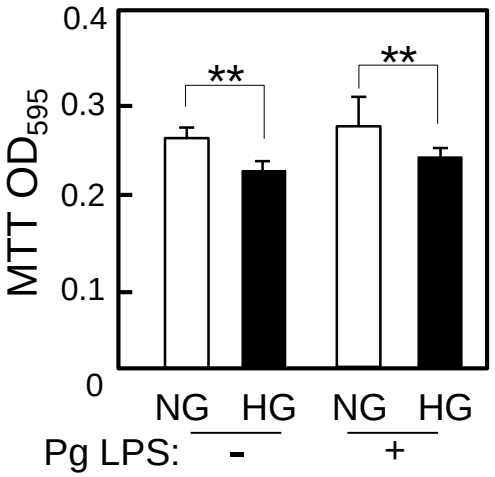

Figure 1

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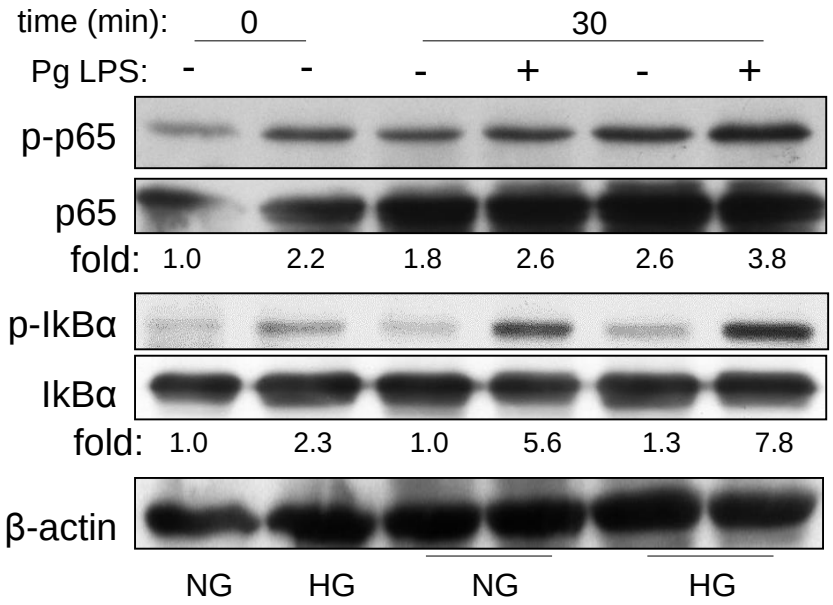

Figure 2

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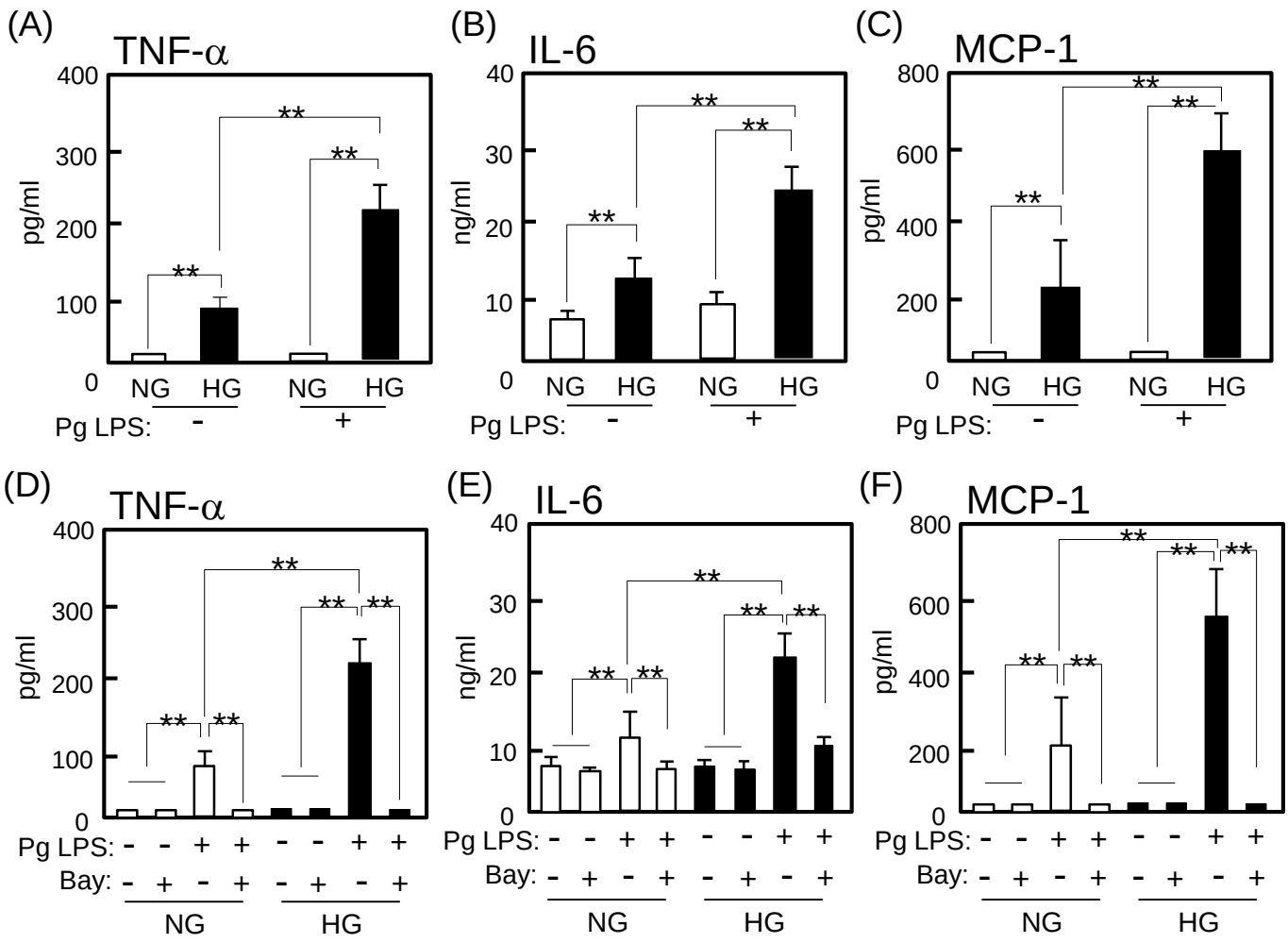

Figure 3

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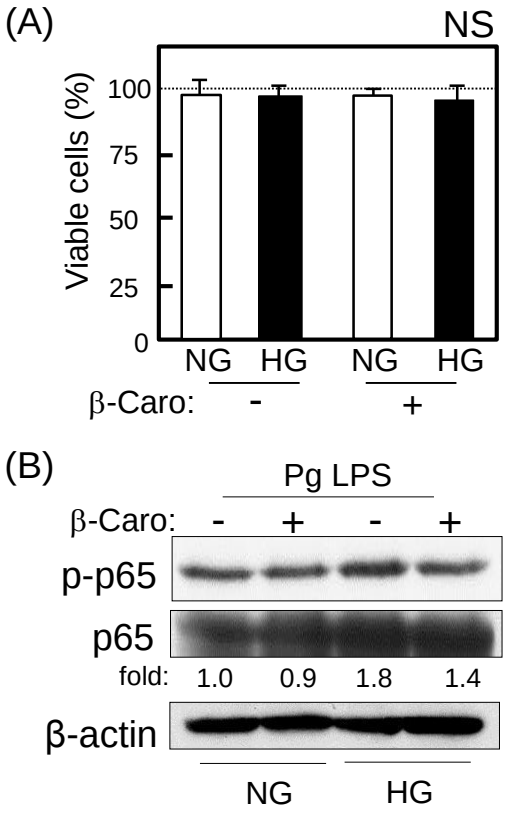

Figure 4

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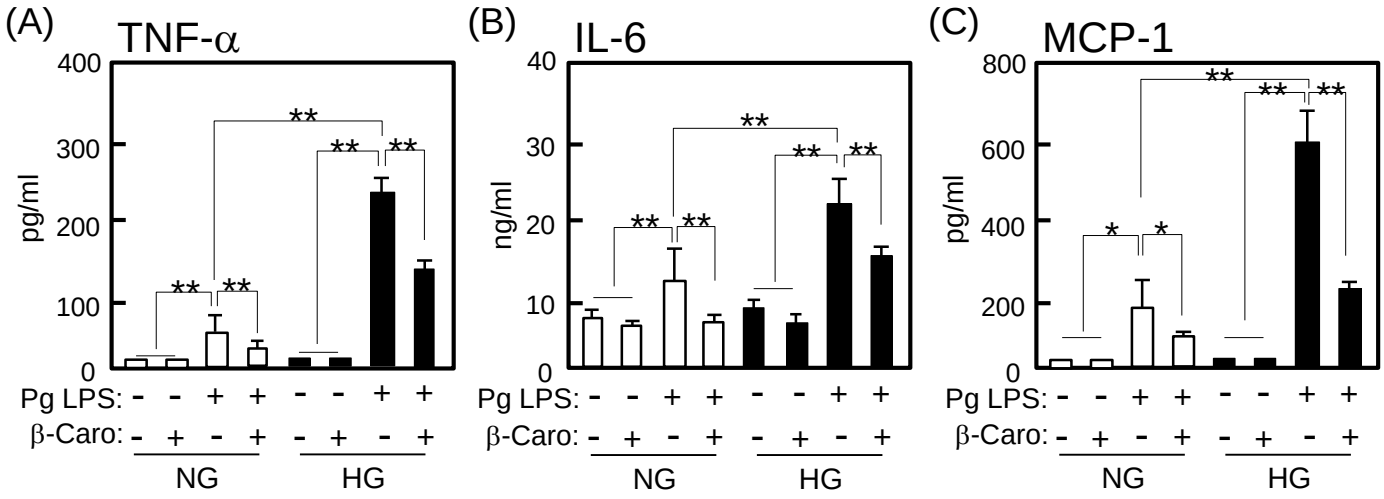

Figure 5

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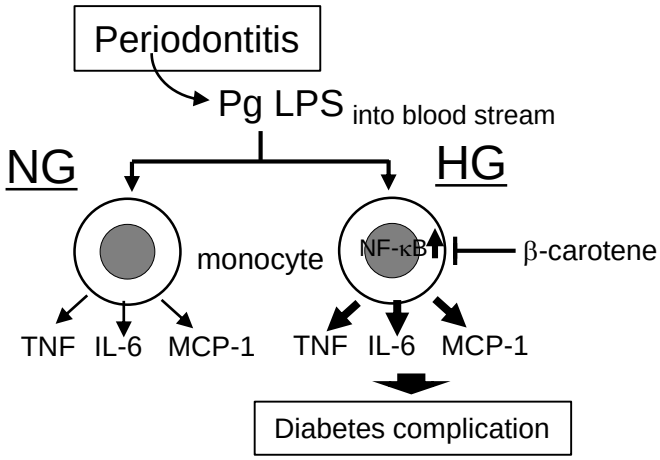

Figure 6