Effects of sulfonylureas on periodontopathic bacteria-induced inflammation

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Abstract

Interleukin-1β (IL-1β) is an inflammatory cytokine produced by monocytes/macrophages and is closely associated with periodontal diseases. The NLRP3 inflammasome is involved in IL-1β activation through pro-IL-1β processing and pyroptotic cell death in bacterial infection. Recently, glyburide, a hypoglycemic sulfonylurea, has been reported to reduce IL-1β activation by suppressing activation of the NLRP3 inflammasome. Therefore, we evaluated the possibility of targeting the NLRP3 inflammasome pathway by glyburide to suppress periodontal pathogen-induced inflammation. A human monocytes, THP-1 cells, were differentiated to macrophage-like cells by treatment with phorbol 12-myristate 13-acetate and stimulated by periodontopathic bacteria, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, or *Fusobacterium nucleatum,* in the presence of glyburide. IL-1β and caspase-1 expression in the cells and culture supernatants were analyzed by western blotting and ELISA, and cell death was analyzed by the lactate dehydrogenase assay. Stimulation of THP-1 macrophage-like cells with every periodontopathic bacteria induced IL-1β secretion without cell death, which was suppressed by the NLRP3 inhibitor, MCC950, and caspase-1 inhibitor, z-YVAD-FMK. Glyburide treatment suppressed IL-1β expression in culture supernatants and enhanced intracellular IL-1β expression, suggesting that glyburide may have inhibited IL-1β secretion. Subsequently, a periodontitis rat model was generated by injecting periodontal bacteria into the gingiva, which was analyzed histologically. Oral

administration of glyburide significantly suppressed the infiltration of inflammatory cells and the number of osteoclasts in the alveolar bone compared to the control. In addition to glyburide, glimepiride was shown to suppress the release of IL-1β from THP-1 macrophage-like cells, while other sulfonylureas (tolbutamide and gliclazide) or other hypoglycemic drugs belonging to the biguanide family, such as metformin, failed to suppress IL-1β release. Our results suggest that pharmacological targeting of the NLRP3 pathway may be a strategy for suppressing periodontal diseases.

Introduction

Periodontitis is a chronic inflammatory disease characterized by loss of gingival attachment surrounding the teeth and alveolar bone resorption, resulting in tooth loss. Infection of Gram-negative bacteria such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans,* and *Fusobacterium nucleatum* and the host immune response against these bacteria in the periodontal tissue are involved in the pathology of periodontitis (Lamont et al. 2018). Interleukin (IL)-1β, one of the pro-inflammatory cytokines produced by monocytes/macrophages, is associated with periodontal diseases, since IL-1β expression has been shown to be up-regulated in the gingival crevicular fluid, saliva, and gingival tissue of patients with periodontitis as compared to healthy subjects (Tobón-Arroyave et al. 2008; Huang et al. 2015; García-Hernández et al. 2018). Further, it has been demonstrated that IL-1β expression is induced by bacterial stimulation

and its products such as lipopolysaccharide (LPS) (Wang et al. 2019). IL-1β is critical in host defense against bacterial pathogens, regulation of inflammatory responses, and osteoclastogenesis for bone resorption (SCHWARTZ et al. 1997).

IL-1β exerts its biological activity by binding to IL-1 receptor (IL-1R) type 1 on the surface of macrophages. IL-1β/IL-1R interaction induces the phosphorylation of several kinases to activate the transcription factor NF-κB, leading to expression of IL-1β and other inflammatory cytokines such as IL-6, IL-8, and TNFα (Gabay et al. 2010). IL-1 plays a central role in systemic inflammatory diseases, including rheumatoid arthritis and autoinflammatory diseases (Dinarello 2011). Targeting of the IL-1β pathway by biological agents such as IL-1R antagonists (IL-1RA) and anti-IL-1β antibodies has been shown to be effective in improving the pathologies of various inflammatory diseases (Hoffman et al. 2001; Ruperto et al. 2012; Dinarello and Meer 2013; Han et al. 2018). The NLRP3 inflammasome is a cytosolic protein complex consisting of NLRP3, ASC, and pro-caspase-1 and has been reported to be involved in IL-1β activation by bacterial infection, including *P. gingivalis, A. actinomycetemcomitans,* and *F. nucleatum* (Shibata 2018). Recognition of bacteria by pattern recognition receptors such as Toll-like receptors induces pro-IL-1β protein expression in the cytosol via NF-κB activation. Biologically active IL-1β is produced through the processing of pro-IL-1β by caspase-1, which is also processed from pro-caspase-1 by the activation of the NLRP3 inflammasome (He et al. 2016). Caspase-1 cleaves Gasdermin D, and the cleaved N-

terminal fragment forms small holes by forming multimers on the cell membrane, causing inflammatory cell death called pyroptosis (X. Liu et al. 2016). Pyroptosis has been hypothesized to be the cause of IL-1β release from macrophages (Brough and Rothwell 2007).

Glyburide (also known as Glibenclamide) is an oral hypoglycemic drug classified under sulfonylureas and widely used globally for the treatment of type II diabetes mellitus without serious side effects (Sola et al. 2015; Roglic and Norris 2018). Glyburide reduces blood glucose by inhibiting ATP-sensitive potassium channels (Sur1-Kir6.2) in pancreatic β cells, resulting in an increase in intracellular calcium levels and subsequently stimulating insulin release (Ashcroft 2005). In addition to the hypoglycemic effect, the anti-inflammatory role of glyburide has been shown in patients and in experimental animal models of inflammatory diseases such as melioidosis, cystitis, pancreatitis, and inflammatory bowel disease (L. Liu et al. 2016). For example, melioidosis, which is caused by the Gram-negative bacterium *Burkholderia pseudomallei*, results in bacteremia, abscesses in multiple organs, and causes pneumonia. Patients with melioidosis and diabetes treated with glyburide have shown decreased serum levels of inflammatory cytokines and lower mortality compared to patients without diabetes (Koh et al. 2011). Furthermore, it has been reported that glyburide reduces IL-1β production in mouse macrophages after LPS and ATP stimulation by inhibiting the NLRP3 inflammasome activation pathway (Lamkanfi et al. 2009). Considering the role of the NLRP3

inflammasome in IL-1β activation, inhibition of the NLRP3 pathway may effectively suppress inflammation in periodontal diseases. In this study, we evaluated the potential of glyburide and other sulfonylureas to control periodontal inflammation by inhibiting the IL-1β activation.

Materials and Methods

Bacteria

In this study, *P. gingivalis* strain ATCC 33277, *A. actinomycetemcomitans* Y4, and *F. nucleatum* ATCC10953 were used as periodontopathic bacteria, and non-oral bacterial *Escherichia coli* MC4100 and *Aerococcus viridans* ATCC 10400 were used as references for Gram-negative and Gram-positive bacteria, respectively. Culturing conditions of these bacteria were described previously (Hara et al. 1996). After the washing procedure, these bacteria were freeze-dried and kept at -80℃. These bacteria were resuspended in PBS, boiled for 10 min, and sonicated before use.

Cell preparation and stimulation

THP-1, a human monocyte, was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone), 100 U/mL penicillin, and 100 μg/mL streptomycin. THP-1 cells were plated in a 96-well plate at 1×10^5 cells/100 μL and differentiated into macrophage-like cells by exposure to 10 nM phorbol 12-myristate 13-acetate (PMA) for 48 h.

Differentiated THP-1 macrophage-like cells were stimulated with bacterial samples for 24 h or, for NLRP3 activation, with 500 ng/mL of ultrapure *E. coli* LPS (Invivogen) for 24 h, followed by 10 nM of nigericin (Sigma-Aldrich) for 45 min. Wherever indicated, the cells were treated with MCC950 (NLRP3 inhibitor, Cayman Chemical), z-YVAD-FMK (caspase-1 inhibitor, BioVision) or glyburide (sigma-Aldrich). To investigate whether other sulfonylureas and other types of hypoglycemic drugs on NLPR3 activation, the cells were also pretreated with other sulfonylureas (tolbutamide, gliclazide, and glimepiride) (Sigma-Aldrich), or metformin (Sigma-Aldrich). None of the inhibitors and hypoglycemic drugs showed any negative impact on the growth of THP-1 macrophage-like cells, as assessed by an MTT assay (Appendix Fig. 1).

Enzyme-linked immunosorbent assay (ELISA)

The amount of IL-1β proteins in the culture supernatants was measured using human-specific IL-1β ELISA kits (R&D Systems) according to the manufacturer's instructions.

Assessment of cell death

Lactate dehydrogenase (LDH) activity in the culture supernatants was measured using CytoTox 96® Non-Radioactive Cytotoxicity assay kit (Promega) according to the manufacturer's instruction. Cells were lysed using lysis buffer to maximize LDH release. Percentage of cell death was calculated by the following equation: (sample LDH -

unstimulated LDH)/(maximum LDH - unstimulated LDH) × 100.

Western Blotting

THP-1 macrophage-like cells in 10-cm culture dishes (3×10^6 cell/10 mL) were stimulated with bacterial samples or nigericin in the presence or absence of glyburide. Cell lysates were prepared by direct lysis with lysis buffer consisting of 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, and protease inhibitors (Complete EDTA-free, Roche). The protein content in the supernatant was measured by acetone precipitation and resuspension in lysis buffer. Protein concentration of the samples was determined using protein assay reagent (DC™ Protein Assay, Bio-Rad Laboratories). The samples were electrophoresed under reducing conditions on a 12.5% sodium dodecyl sulfate poly-acrylamide gel. After transfer to a PVDF membrane (0.2 μm), membranes were blocked with 5% BSA in TBST (0.01 M Tris-HCl pH 7.5 containing 0.15 M NaCl, and 0.1% Tween 20) for 1 h at 22°C and subsequently incubated with rabbit anti-IL-β (Cell Signaling Technology), anti-caspase-1 (Abcam), or anti-β-actin (Cell Signaling Technology) primary antibodies diluted in 5% BSA in TBST for 1 h at RT. After washing, membranes were incubated with secondary anti-rabbit IgG-HRP (Cell Signaling Technology) in 5% BSA in TBST for 1 h and developed using ECL western blotting substrate (GE Healthcare).

Animals

Sixty-four, 7-week-old male Lewis rats (Oriental Yeast) were used in this study (n = 8/group). The rats were maintained under specific pathogen-free conditions in the Animal Center of Fukuoka Dental College. Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Fukuoka Dental College (Permission No. 16016).

In vivo experimental protocol

Appendix Fig. 2A shows the experimental protocol for the *in vivo* experiments. *P. gingivalis, A. actinomycetemcomitans,* or *E. coli* (0.6 μg/3 μL of PBS) was injected into the palatal gingiva of the maxillary first molar under isoflurane anesthesia every 24 h. Glyburide in 20% ethanol (20 mg/kg weight of rats) or vehicle (Mock) was orally administered to each group every 24 h using a tube. Rats were euthanized 24 h after the third injection of bacterial solution.

Preparation of tissues

The maxilla of each rat was removed immediately after death and fixed in 4% paraformaldehyde in PBS at 4°C for 10 h, decalcified with 10% EDTA for 3 weeks, and embedded in paraffin by the AMeX method (acetone, methyl benzoate, and xylene) (Sato et al. 1986). Bucco-paratal serial sections (4 μm thickness) at the central root levels from

the upper first molar were obtained.

Histopathological and histometric studies

Five groups of serial sections, each containing 10 subsections, were obtained from each specimen. The first subsection from each group was stained with hematoxylin and eosin (H&E) for histopathological observation. The number of infiltrated inflammatory cells in the 500 μm × 300 μm area of connective tissue above the bone crest was counted using image analysis software (Image J). The distance between the cement-enamel junction (CEJ) and the alveolar bone crest was measured to asses bone resorption (Appendix Fig. 2B).

To identify osteoclasts, the second section from each group was stained with tartrate-resistant acid phosphatase (TRAP) (Katayama et al. 1972). The number of TRAP-positive cells in a 1,000-μm-wide area on the alveolar bone surface on the periodontal ligament side was counted under the light microscope (Appendix Fig. 2B).

To detect IL-1β production, the third subsections from each group was immunohistologically stained using the VECTASTAIN Elite ABC-PO kit (Vector Laboratories) according to the manufacturer's instructions. In brief, sections were deparaffined, incubated in PBS with 0.3% Triton X-100 (PBST) for 20 min at room temperature, and treated with 3% hydrogen peroxide in distilled water to inhibit endogenous peroxidase activity. Sections were then pre-treated with 2% normal goat

serum in PBST for 30 min, and incubated with the primary antibodies against anti-IL-1β (rabbit polyclonal, 1:200, Abcam) in PBS for 1 h at room temperature. The sections were then incubated with biotinylated anti-rabbit antibody and ABC solution for 30 min, followed by treatment with 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (ImmPACTTM DAB Peroxidase Substrate kit, Vector Laboratories). All sections were counterstained with hematoxylin.

Statistical analyses

Data are expressed as mean ± standard deviation. One-way ANOVA, followed by Tukey's, and unpaired t-tests were used to determine statistical significance between groups. P values < 0.05 were considered to be statistically significant.

Results

IL-1β levels in the culture supernatants of THP-1 macrophage-like cells stimulated with each bacterium were measured by ELISA. All Gram-negative bacteria (including periodontal pathogens), but not Gram-positive *A. viridans,* stimulated THP-1 macrophage-like cells to release IL-1β in a dose-dependent manner (Fig. 1A). Although *A. actinomycetemcomitans* and *F. nucleatum* showed comparable activity as *E. coli*, *P. gingivalis* showed the weakest activity among the Gram-negative bacteria. The NLRP3 stimulator (LPS + nigericin) stimulated robust release of IL-1β (Fig. 1B). Expression

levels of pro- and mature forms of IL-1β and pro-caspase-1 were up-regulated in the cell lysate stimulated with each bacterium or LPS + nigericin, as shown by western blotting (Fig. 1C). The mature form of IL-1β (p17) was detected in the supernatant from cell cultures stimulated with every bacterium, except *A. viridans,* and in the supernatant of the LPS + nigericin stimulation group. Cleaved fragment of caspase-1 (p20) was only detected in the supernatant from cell cultures stimulated with LPS + nigericin.

Since caspase-1 activation has been reported to induce pyroptotic cell death, the LDH assay was conducted to determine the cell death of THP-1 macrophage-like cells stimulated with individual bacteria or LPS + nigericin. Although LPS + nigericin stimulation showed about 60% cell death, bacterial stimulation did not significantly induce cell death, even at the maximum bacterial concentration of 10 μg/mL (Fig. 1D).

To evaluate the involvement of NLRP3 or caspase-1 in the periodontal bacterial-stimulated IL-1β release, THP-1 macrophage-like cells were pretreated with an NLRP3 inhibitor, MCC950, or caspase-1 inhibitor, z-YVAD-FMK, before bacterial stimulation. Pretreatment of THP-1 macrophage-like cells with MCC950 or z-YVAD-FMK decreased IL-1β release stimulated by each periodontal bacterium or *E. coli* in a dose-dependent manner (Fig. 2A, B). Further, we investigated the effect of glyburide on the release and activation of IL-1β. Pretreatment of THP-1 macrophage-like cells with glyburide effectively inhibited the release of IL-1β stimulated by each bacterium or LPS + nigericin (Fig. 2C, D). Western blotting analysis of the cell lysate and supernatant of THP-1

macrophage-like cells stimulated with each bacterium or with LPS + nigericin in the absence or presence of glyburide has been shown in Fig 2E. Consistent with the ELISA results, protein level of the mature form of IL-1β (p17) in the supernatant was inhibited by glyburide. Further, expression of the cleaved form of caspase-1 (p20) was inhibited in the supernatant from cell cultures stimulated with LPS + nigericin. Interestingly, expression of the mature form of IL-1β (p17) in the cell lysate was increased by glyburide treatment.

Next, we evaluated the effects of glyburide administration on inflammation of periodontal tissue and osteoclastogenesis using an experimental rat periodontitis model, in which bacterial suspensions were injected into the palatal gingiva of the upper first molar. *P. gingivalis* and *A. actinomycetemcomitan*s were used in this *in vivo* experiment because these bacteria have been shown to be closely associated with chronic periodontitis and aggressive periodontitis, respectively. Blood glucose levels significantly decreased after oral administration of glyburide on day 5, suggesting the hypoglycemic effect of glyburide (Appendix Fig. 3). Figure 3A shows histological findings of the specimen at the site of bacterial injection, and Fig. 3B shows the number of inflammatory cells at the evaluation site, as depicted in Appendix Fig. 2B. Although injection of PBS alone induced slight accumulation of inflammatory cells, injection of *A. actinomycetemcomitans* or *E. coli* significantly increased the number of inflammatory cells. Injection of *P. gingivalis* also increased the number of inflammatory cells, but

without statistical significance. Oral administration of glyburide significantly decreased the number of inflammatory cells in rats injected with *A. actinomycetemcomitans* or *E. coli*. Figures 3C and 3D show TRAP staining and histological analysis of the number of TRAP-positive cells on the bone surface at the periodontal ligament between the alveolar bone crest and 1,000 μm below the alveolar bone crest (Appendix Fig. 2B). Significantly increased number of TRAP-positive cells was observed in rats injected with *A. actinomycetemcomitans* or *E. coli* compared to Mock + PBS. Injection of *P. gingivalis* also increased the number of TRAP-positive cells, without statistical significance. Oral administration of glyburide significantly decreased the number of TRAP-positive cells in rats injected with *A. actinomycetemcomitans* or *E. coli.* Further, the distance between the CEJ and the alveolar bone crest was statistically increased in rats injected with *A. actinomycetemcomitans* or with *E. coli*, suggestive of the alveolar bone resorption. Oral administration of glyburide significantly decreased the distance between the CEJ and the alveolar bone crest in rats injected with each bacterium (Fig. 3E). Figure 3F shows histological immunostaining of IL-1β. Although injection of PBS alone did not induce significant IL-1β expression, its expression was increased in the gingiva with injection of *P. gingivalis*, *A. actinomycetemcomitans,* or *E. coli*. Oral administration of glyburide decreased the IL-1β expression in the gingiva of rats injected with each bacterium.

Furthermore, to address whether other sulfonylureas and hypoglycemic drugs belonging to the viganide family, such as metformin, inhibited IL-1β release similarly to

glyburide, we stimulated THP-1 macrophage-like cells with *E. coli* in the presence of other sulfonylureas (tolbutamide , gliclazide, and glimepiride) or metformin. Similar to glyburide, glimepiride suppressed IL-1β release in THP-1 macrophage-like cells stimulated with *E. coli* (Fig. 4A). In contrast, tolbutamide, gliclazide, and metformin failed to suppress IL-1β release, even at the highest concentration. Glimepiride inhibited IL-1β release from THP-1 macrophage-like cells stimulated with each of the periodontopathic bacteria (Fig. 4B). Conversely, tolbutamide significantly increased IL-1β release from THP-1 macrophage-like cells stimulated with *F. nucleatum,* and gliclazide increased IL-1β release from THP-1 macrophage-like cells stimulated with *A. actinomycetemcomitans* or *F. nucleatum*.

Discussion

Although pro-IL-1β and pro-caspase-1 were constitutively and weakly expressed in THP-1 macrophage-like cells, their expression was upregulated by stimulation with each of the periodontal bacteria used in this study. The mature forms of IL-1β (p17) and caspase-1 (p20) were observed in the cell lysates and the supernatants only when THP-1 macrophage-like cells were stimulated with the periodontopathic bacteria. This IL-1β release from THP-1 macrophage-like cells was inhibited by pretreatment with MCC950 or z-YVAD-FMK, suggesting involvement of the NLRP3 inflammasome. These results are consistent with previous studies showing that periodontopathic bacterial infection

causes activation of the NLRP3 inflammasome and IL-1β secretion (Park et al. 2014; Shenker et al. 2015; Bui et al. 2016; Tan et al. 2018).

The mechanism underlying the suppressed activation and release of IL-1β by glyburide has not yet been elucidated. Since potassium efflux or even low intracellular potassium concentration has been reported to trigger NLRP3 activation in macrophages/monocytes (Pétrilli et al. 2007; Muñoz-Planillo et al. 2013), inhibition of ATP-sensitive potassium channels that prevent depletion of cytosolic potassium might be a plausible mechanism of glyburide-mediated suppression of the NLRP3 inflammasome (Hughes et al. 2013). However, Lamkanfi et al. demonstrated that ATP-sensitive potassium channels were dispensable for the inhibition of NLRP3 inflammasome activation by LPS + ATP using mice deficient for ATP-sensitive potassium channels components Kir6.1, Kir6.2, or SUR2. (Lamkanfi et al. 2009). In addition, another sulfonylurea drug, glipizide, inhibited ATP-sensitive potassium channels but failed to inhibit NLRP3 inflammasome activation. Therefore, the authors speculated that glyburide could inhibit NLRP3 inflammasome activation specifically. In this study, although glyburide inhibited IL-1β (p17) release from THP-1 macrophage-like cells stimulated with periodontal bacteria, its protein expression was conversely augmented in the cell lysate, suggesting that NLRP3 activation occurs in the cytosol. In addition, cell death is not associated with IL-1β release by periodontal pathogen stimulation. These results suggest that glyburide may inhibit the secretory pathway of IL-

1β. The reason for this discrepancy is not clear; however, differences in stimulators and animals used in these studies may underlie the differences. When pyroptosis does not occur, glyburide can inhibit IL-1β release. Since IL-1β does not contain a signal sequence, it does not follow the conventional endoplasmic reticulum (ER)/Golgi secretion route. Therefore, passive secretion by pyroptotic cell death is thought to be a major secretory system of IL-1β. However, several unconventional pathways have been shown for IL-1β release (Monteleone et al. 2015), active caspase-1 plays an important role in the regulation of unconventional protein secretion for multiple proteins (Keller et al. 2008). Therefore, caspase-1 could be inhibited by glyburide via an unconventional secretory pathway of IL-1β. In this study, the expression of the mature form of IL-1β (p17) in the cell lysate was increased by glyburide treatment, which may be the result of inhibition of the unconventional IL-1β secretory pathway.

Glyburide, which has been shown to inhibit the NLRP3 pathway, supressed inflammation, osteoclast formation, the alveolar bone resorption, and the IL-1β protein expression in the periodontal tissue of rats injected with the periodontal pathogens. Consistent with these results, Yamaguchi et al. reported that the gingival expression of proinflammatory cytokines (IL-1β and IL-18) and the alveolar bone resorption were significantly decreased in the NLRP3-deficient mice infected with *P. gingivalis* compared to that in the wild-type mice (Yamaguchi et al. 2016). Furthermore, expression of the NLRP3 inflammasome components such as NLRP3, ASC, and caspase-1 was up-

regulated in periodontitis patients (Huang et al. 2015; García-Hernández et al. 2018; Higuchi et al. 2019), suggesting that the NLRP3 inflammasome may play an important role in regulating periodontal inflammation and bone resorption.

We found that glimepiride also had similar activity to glyburide, while gliclazide, tolbutamide, and metformin, the biguanide therapeutic drugs, had no such activity. Consistent with this result, glimepiride was reported to reduce LPS-induced secretion of cytokines, including IL-1β, IL-6, and TNF-α (Ingham et al. 2014). Based on the chemical structure of sulfonylureas used in this study, glyburide and glimepiride possessed similar functional groups, benzamide and pyrrolamide, respectively. Moreover, the sulfonylurea backbone is an essential structure for binding to ATP-sensitive potassium channels, resulting in insulin release (Appendix Table 1). The presence of both benzamide and sulfonyl groups in glyburide was shown to be the minimum structure required to inhibit the NLRP3 inflammasome (Lamkanfi et al. 2009). These results suggest differences in the structural requirements for hypoglycemic action and the NLRP3 inflammasome inhibition. Future efforts should focus on the development of synthetic compounds specifically to inhibit the NLRP3 inflammasome, but not ATP-sensitive potassium channels.

In summary, glyburide was shown to inhibit the release of mature IL-1β from THP-1 macrophage-like cells stimulated with periodontopathic bacteria *in vitro*. Furthermore, glyburide inhibited inflammatory cell infiltration, osteoclast formation, and bone

resorption in experimental periodontitis rats induced by injection of periodontopathic bacteria. Collectively, these results suggest that the NLRP3 may be a therapeutic target to inhibit periodontal diseases.

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Figure legends

Fig. 1

(A, B) THP-1 macrophage-like cells stimulated with different kinds of bacteria or 500 ng/mL of LPS for 24 h, followed by 10 μM of nigericin for 30 min. IL-1β concentrations in the supernatant were measured by ELISA. The closed bar and error bar represent the average and standard deviation, respectively. The experiments were conducted in triplicate for three independent experiments, and representative data have been shown in the figures. (C) THP-1 macrophage-like cells stimulated with different bacteria (10 μg/mL, 24 h) or LPS (1 μg/mL, 24 h) + nigericin (10 μM, 45 min). Protein expression of IL-1β and caspase-1 in the supernatant and lysate was analyzed by Western blotting. The experiment was repeated three times. (D) THP-1 macrophage-like cells stimulated with different bacteria for 24 h and cell death were measured by LDH activity. The experiments were conducted in triplicate for three independent experiments, and representative data have been shown in the figures. *P. g.*, *P. gingivalis*; *A. a.*, *A. actinomycetemcomitans*; *F. n.*, *F. nucleatum*; *E. c.*, *E. coli*; *A. v.*, *A. viridans*, Nig, nigericin. Statistical analyses were performed using one-factor ANOVA, followed by the Tukey's test. *$P < 0.05$ vs (-), **$P < 0.01$, ***$P < 0.001$.

Fig. 2

(A, B) THP-1 macrophage-like cells stimulated with 10 μg/mL of different bacteria for 24 h in the presence of (A) MCC950 and (B) Z-YVAD-FMK and IL-1β release in the

supernatant measured by ELISA. (C, D) THP-1 macrophage-like cells stimulated with 10 μg/mL of different bacteria or 500 ng/mL of LPS for 24 h, followed by 10 μM of nigericin for 45 min in the presence of glyburide. IL-1β release in the supernatant was analyzed by ELISA. All ELISA experiments were conducted in triplicate for three independent experiments, and representative data have been shown in the figures. (E) THP-1 macrophage-like cells stimulated with 10 μg/mL of different bacteria or 1 μg /mL of LPS for 24 h, followed by 10 μM of nigericin for 45 min in the presence or absence of glyburide. Protein expression of IL-1β and caspase-1 in the supernatant and lysate was analyzed by Western blotting. Western blotting was performed in three independent experiments, and representative data have been shown in the figures. *P. g*., *P. gingivalis*; *A. a.*, *A. actinomycetemcomitans*; *F. n.*, *F. nucleatum*; *E. c.*, *E. coli*. Nig, nigericin; Gly, Glyburide. Statistical analyses were performed using one-factor ANOVA, followed by the Tukey's test. *$P < 0.05$ vs DMSO, **$P < 0.01$, ***$P < 0.001$.

Fig. 3

(A, B) Histological findings of H&E staining and the number of inflammatory cells in the connective tissue. (C, D) Histological findings of TRAP staining and the number of TRAP-positive cells on the bone surface. (E) Histological findings of IL-1β immunological staining. (F) The distance between the cement enamel junction and the alveolar bone crest. *P. g.*, *P. gingivalis*; *A. a.*, *A. actinomycetemcomitans*; *F. n.*, *F.*

nucleatum; *E. c.*, *E. coli*. Bar = 200 μm. Statistical analysis was performed using unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001 vs Mock. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 vs PBS/Mock.

Fig. 4

(A) THP-1 macrophage-like cells stimulated with 1 μg/mL of *E. coli* for 24 h in the presence of various sulfonylureas or metformin and IL-1β release in the supernatant measured by ELISA. (B) THP-1 macrophage-like cells stimulated with 1 μg/mL of *P. gingivalis, A. actinomycetemcomitans,* and *F. nucleatum* for 24 h in the presence of tolbutamide, gliclazide, or glimepiride, and IL-1β release in the supernatant measured by ELISA. All experiments were conducted in triplicate for three independent experiments, and representative data have been shown in the figures. *P. g.*, *P. gingivalis*; *A. a.*, *A. actinomycetemcomitans*; *F. n.*, *F. nucleatum*; *E. c.*, *E. coli*.; Tol, Tolbutamide; Glic, Gliclazide; Glim, Glimepiride; Met, Metformin. Statistical analyses were performed using one-factor ANOVA, followed by the Tukey's test. *P < 0.05 vs DMSO, **P < 0.01, ***P < 0.001.

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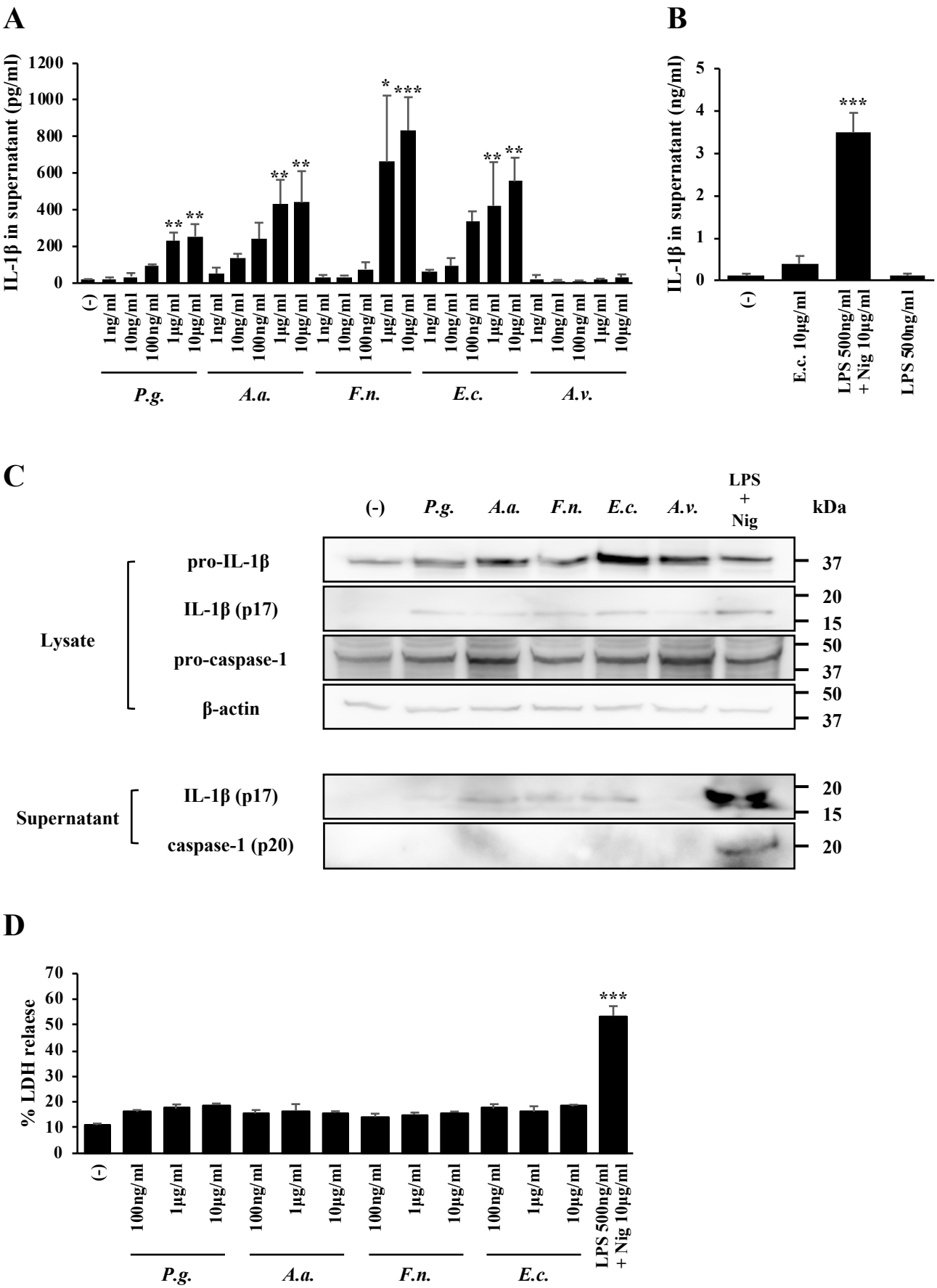


Fig. 1 Kawahara et al.

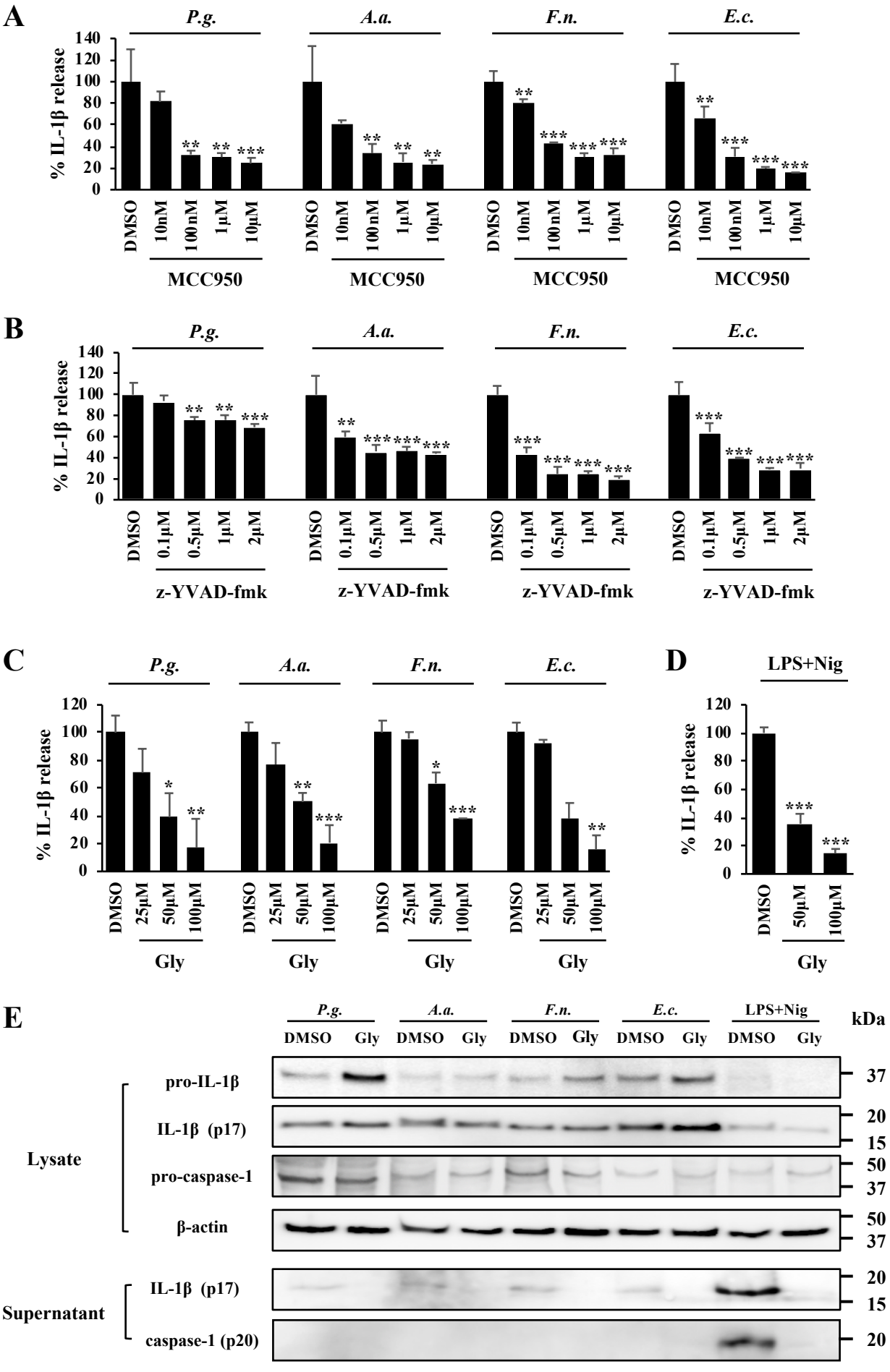
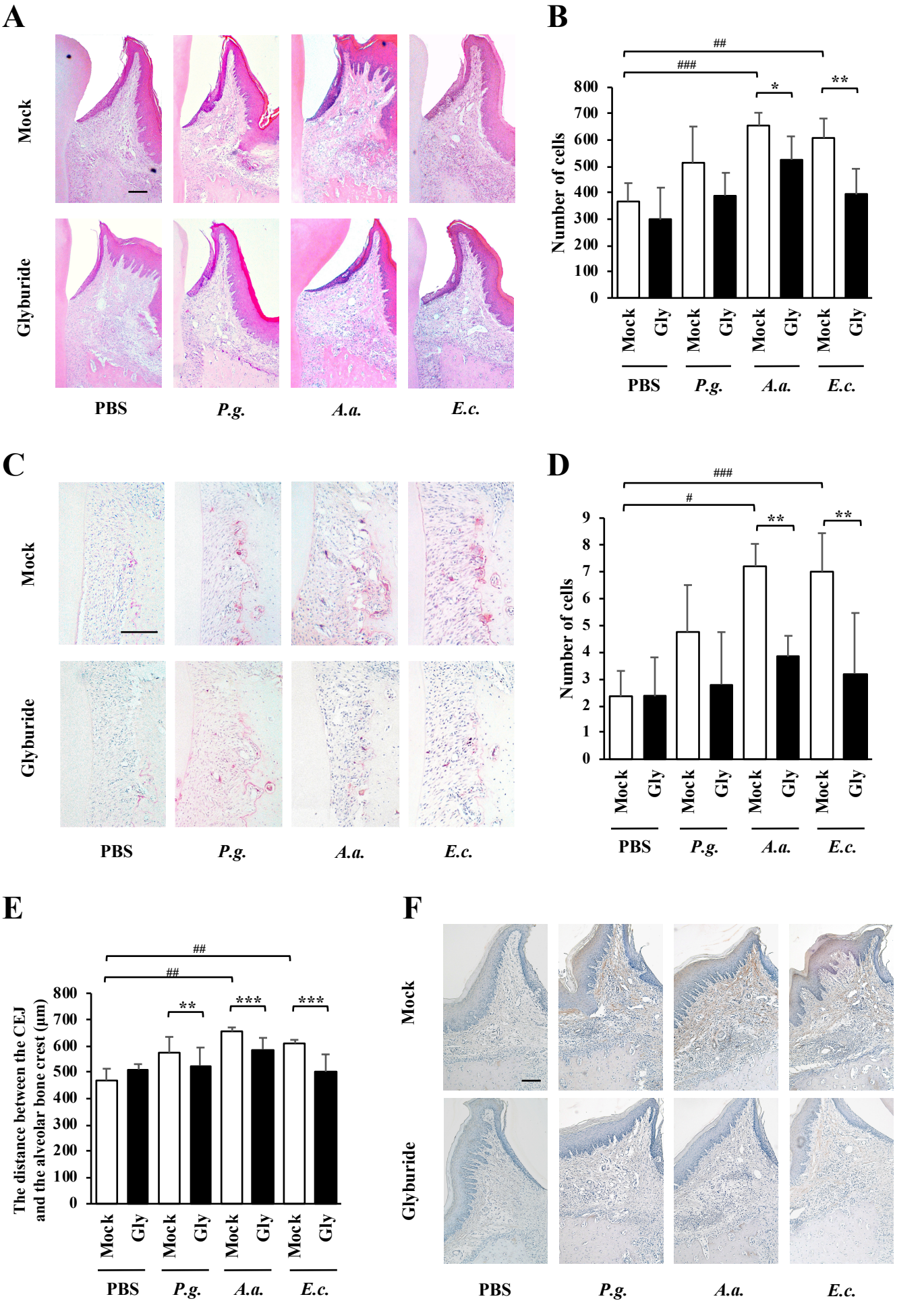


Fig. 2 Kawahara et al.



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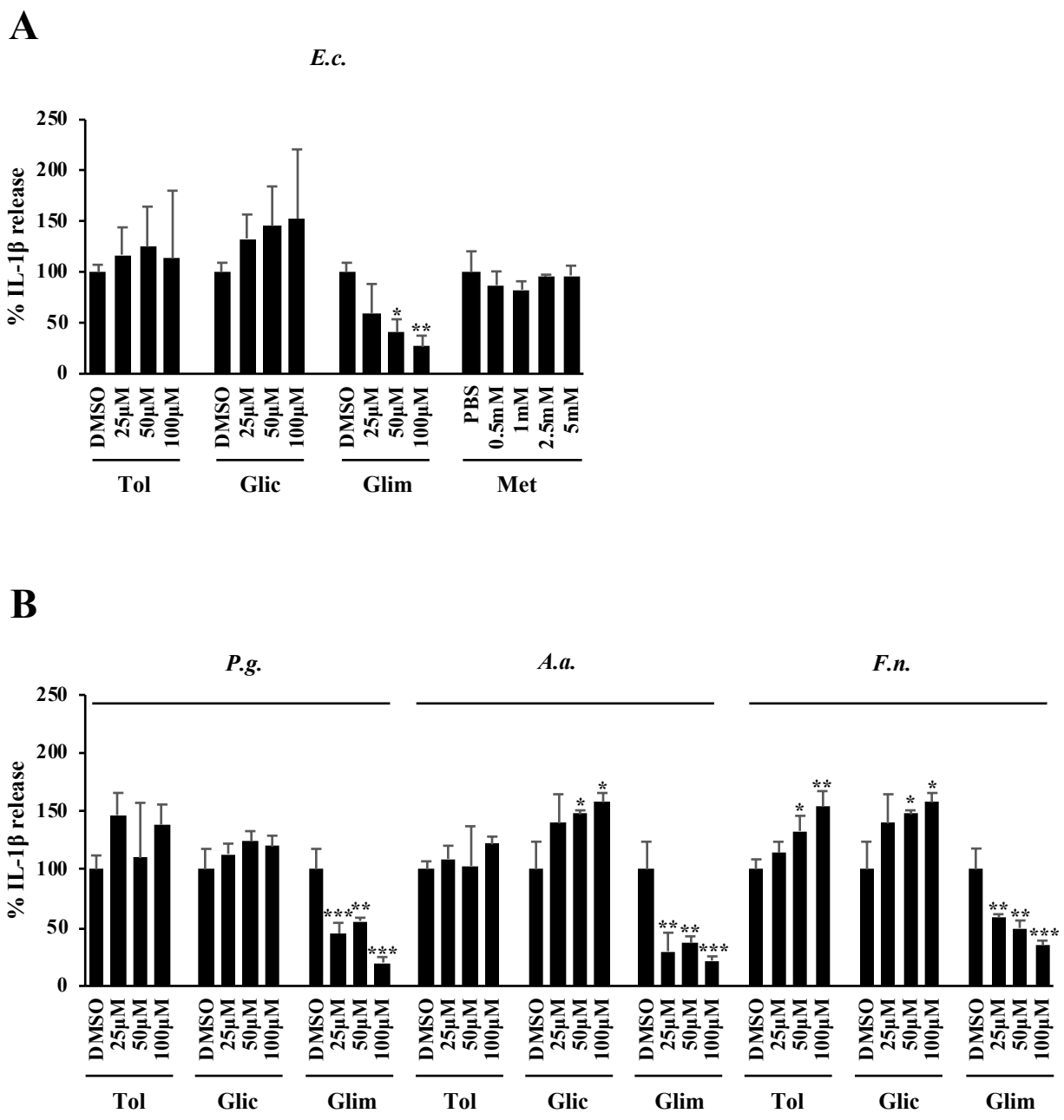
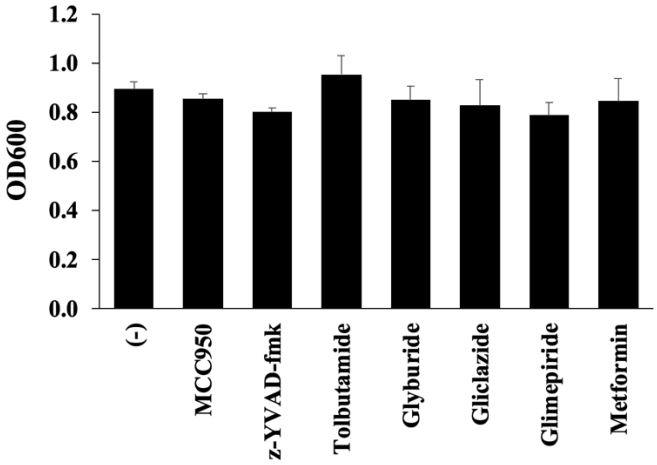


Fig. 4 Kawahara et al.

Effects of sulfonylureas on periodontopathic bacteria-induced inflammation

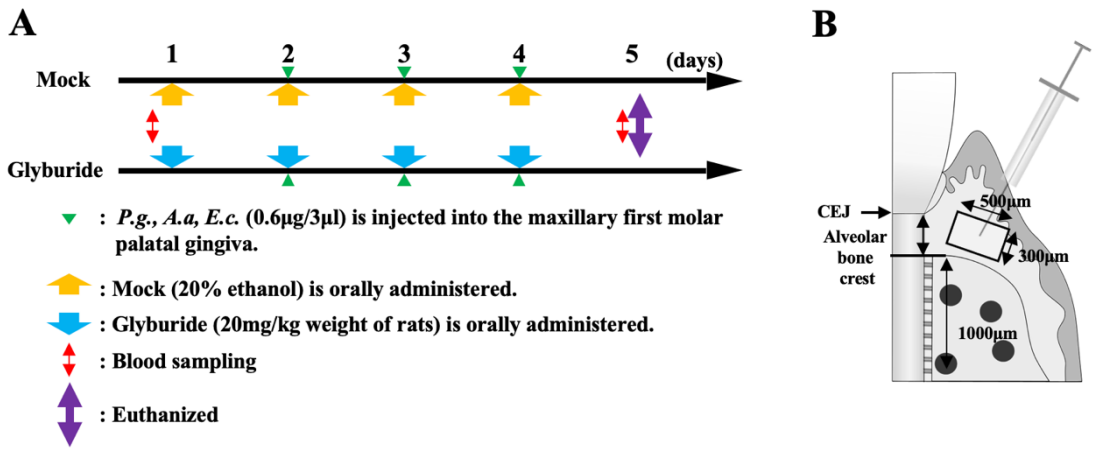
Yuri Kawahara, Takashi Kaneko, Yasunori Yoshinaga, Youichi Arita, Keiko Nakamura, Chihiro Koga, Atsutoshi Yoshimura, Ryuji Sakagami

Appendix



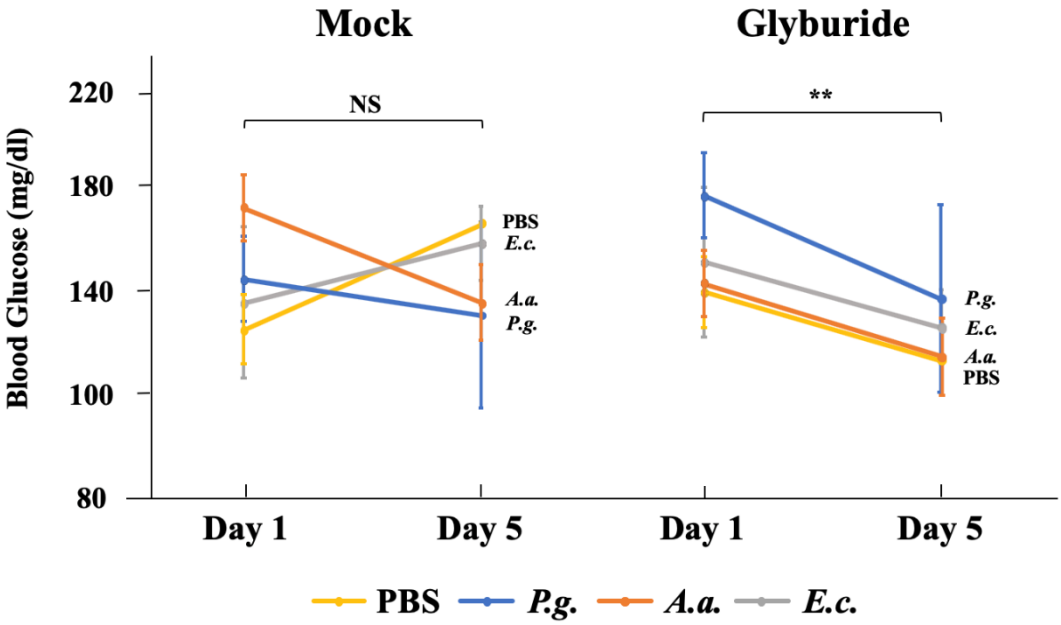
Appendix Figure 1

Effects of inhibitors and hypoglycemic drugs on growth of THP-1 macrophage-like cells. THP-1 macrophage-like cells (1×10^5 cells/100 µL/well in 96-well plates) were stimulated with MCC950 (10 µM), z-YVAD-fmk (2 µM), tolbutamide (100 µM), glyburide (100 µM), gliclazide (100 µM), glimepiride (100 µM), or metformin (5 µM). Twenty-four hours after incubation, 10 µL of the MTT reagent (5 mg/mL, Sigma-Aldrich) was added into the well and incubated for an additional 4 h. After adding 100 µL of the solubilizing solution (10% SDS in 10 mM HCl) overnight, optical density (OD) was measured colorimetrically at 600 nm. There were no statistically significant differences between samples and DMSO by one-factor ANOVA, followed by the Tukey's test.



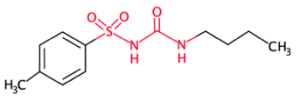
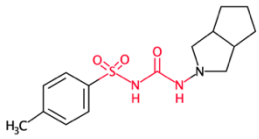
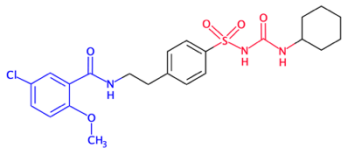
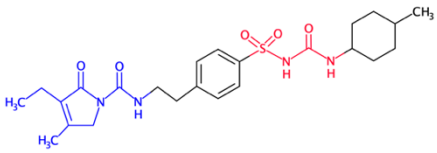
Appendix Figure 2

(A) Schedule of *in vivo* experiments. Periodontitis in rats was generated by injection of *P. gingivalis*, *A. actinomycetemcomitans*, or *E. coli* into the mesial gingiva in the upper first molar. Rats were divided into two groups: glyburide group (20 mg/kg weight of rats via oral administration with a tube every 24 h) and the mock group (20% Ethanol). Rats were euthanized 24 h after the third bacterial injection, and histological specimens of the periodontal tissues were stained by hematoxylin and eosin (H&E). Tartrate-resistant acid phosphatase (TRAP) was visualized enzymatically to identify osteoclasts. Whole blood was collected from rats on days 1 and 5. (B) Schema of histological analyses. The number of inflammatory cells in 500 μm × 300 μm of connective tissue above the bone crest. The distance between the cement-enamel junction (CEJ) to the alveolar bone crest. TRAP-positive cells on the bone surface of the periodontal ligament between the bone crest and the point 1,000 μm away from the bone crest.



Appendix Figure 3

Changes in blood glucose levels of glyburide-administered rats. Glyburide in 20% ethanol (20 mg/kg) or 20% ethanol (mock) was orally administered to each group (8 rats / group) every 24 h using a tube. Each bacterial sample was injected into the gingiva of rats as described in the Materials & Methods. Blood samples were collected from the retro‐orbital venous plexus of the rats at baseline (Day 1) and before sacrifice (Day 5). Serum was prepared from the collected blood. Using 10 μL of the serum, the blood glucose level was measured using a Glutestace Ace R (GlucocardTM G Black, Arkray, Kyoto, Japan). Statistical analysis was performed using the Wilcoxon-Mann-Whitney sign-ranked test. *P. g.*, *P. gingivalis*; *A. a.*, *A. actinomycetemcomitans*; *E. c.*, *E. coli*. $^{**}P < 0.01$. NS: not significant.

Generation	Sulfonylureas	Inhibition of IL-1 β release	Chemical structure
1	Tolbutamide	(-)	
2	Gliclazide	(-)	
	Glyburide	(+)	
3	Glimepiride	(+)	

Appendix Table 1

Chemical structures of sulfonylureas used in this study. Glyburide and glimepiride, but neither tolbutamide nor gliclazide, could suppress IL-1β release from THP-1 macrophage-like cells stimulated with periodontal bacteria. Sulfonylurea, which is an essential structure for binding ATP-sensitive potassium channels (resulting in insulin release), is shown in red. Benzamide and pyrrolamide groups in glyburide and glimepiride are shown in blue.