Upregulation of Free Fatty Acid Receptors in Periodontal Tissues of Patients with Metabolic Syndrome and Periodontitis

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Abstract

Background and Objective: Metabolic syndrome (MetS) exacerbates periodontitis. Since saturated fatty acid (SFA) is increased in MetS and enhances lipopolysaccharide (LPS)-induced proinflammatory cytokine expression in macrophages, it has been considered to play a role in MetS-exacerbated periodontitis. However, it remains unknown how fatty acid receptors, which mediate the interaction of cells with SFA and uptake of SFA, are expressed and regulated in the periodontal tissue. In this study, we tested our hypothesis that the periodontal expression of fatty acid receptors GPR40 and CD36 is increased in patients with both MetS and periodontitis. We also determined the effect of SFA and LPS on GPR40 and CD36 expression *in vitro*.

Material and Methods: Periodontal tissue specimens were collected from 11 participants without MetS and periodontitis, 12 participants with MetS, 11 participants with periodontitis, and 14 participants with both MetS and periodontitis after surgeries. The tissues were processed, and GPR40 and CD36 were detected by immunohistochemistry. Furthermore, cultured macrophages and gingival fibroblasts were treated with LPS, palmitate, a major SFA, or LPS plus palmitate and the expression of GPR40 and CD36 was then quantified.

Results: Analysis of clinical data showed that age, smoker, gender and race/ethnicity were not significantly different among 4 groups. Immunohistochemistry showed that GPR40 and CD36 were expressed by epithelial cells, fibroblasts and immune cells. Quantitative data showed that GPR40 expression is increased in patients with periodontitis, MetS or both periodontitis and MetS while CD36 expression is increased only in patients with both periodontitis and MetS. The *in vitro* studies showed that the expression of GPR40 and CD36 in macrophages and fibroblasts was upregulated by the combination of LPS and palmitate.

Conclusion: Periodontal expression of GPR40 and CD36 was upregulated in patients with both MetS and periodontitis, and GPR40 and CD36 in macrophages and fibroblasts were upregulated *in vitro* by the combination of LPS and palmitate, suggesting that GPR40 and CD36 may be involved in MetS-exacerbated periodontitis.

1. INTRODUCTION

Periodontitis is a disease of the supporting structures of the teeth, characterized by tissue inflammation and destruction (1, 2). Periodontitis is caused by periodontal bacteria and exacerbated by systemic diseases such as diabetes and metabolic syndrome (MetS) (3–6). MetS is a cluster of cardiovascular risk factors including obesity, high triglycerides, low HDL, high blood pressure, insulin resistance and increased glucose (7, 8). The research finding that MetS is associated with periodontitis is of importance since 34% of the US population have MetS as contrasted to 9.3% of the US population having diabetes (9). Since MetS is considered as a pre-diabetic state (10), early treatment of periodontitis in patients with MetS may reduce the severity of periodontitis after pre-diabetes advances to diabetes. However, in contrast to the extensive studies on the mechanisms involved in diabetes-associated periodontitis (11), the investigation on the pathogenesis of MetS-related periodontitis is lacking.

It is known that MetS is associated with increased free fatty acid, in particular saturated fatty acid (SFA) (12), and dietary SFA is a key factor involved in the pathogenesis of MetS (13). Therefore, we hypothesized that SFA may boost host inflammatory response triggered by periodontal pathogen-derived virulence factors such as lipopolysaccharide (LPS). Interestingly, our *in vitro* study supported our hypothesis as it showed that palmitate, the most abundant SFA in animal and human (14), robustly amplified LPS-stimulated proinflammatory cytokine expression in macrophages (15).

Long-chain free fatty acids including SFA interact with cells through free fatty acid receptors such as GPR40 (16), GPR120 (17, 18) and CD36 (19). Studies have shown that GPR120 is a fatty acid receptor engaged preferentially by omega-3 (ω−3) polyunsaturated fatty acids (PUFAs) and mediates ω−3 PUFA-stimulated antiinflammatory actions (17, 18), but GPR40 and CD36 have proinflammatory properties (19, 20). Therefore, GPR40 and CD36 may contribute to MetS-associated periodontitis by mediating the proinflammatory effect of SFA. However, it is uninvestigated if GPR40 and CD36 are expressed on

periodontal tissue and if the periodontal expression of GPR40 and CD36 is regulated by periodontitis and MetS.

In this study, we performed immunohistochemistry to detect GPR40 and CD36 expression in periodontal tissues and determine if the periodontal expression of GPR40 and CD36 is altered in patients with MetS and periodontitis. Furthermore, we also determined if the *in vitro* expression of GPR40 and CD36 is upregulated by LPS and palmitate, a major SFA, and if GPR40 or CD36 expression is involved in palmitate-enhanced proinflammatory cytokine expression.

2. MATERIAL & METHODS

2.1 Study Participants

Forty-eight participants, including 11 control participants without periodontitis and MetS (group 1), 12 with periodontitis alone (group 2), 11 with MetS alone (group 3) and 14 with both periodontitis and MetS (group 4), were enrolled in the study. Participants in group 1 who had oral surgeries such as crown lengthening, extractions, and periodontal plastic surgery served as controls. All the tissue specimens collected were periodontal tissues. The participants in groups 2 and 4 met the diagnostic criteria for chronic periodontitis: periodontal pocket depth (PPD) 6 mm in 2 or more teeth or clinical attachment loss (CAL) 5 mm in 2 or more teeth (21). Radiographic evidence of alveolar bone loss was apparent. The oral examination was conducted as previously described (22). The participants in groups 3 and 4 met the diagnostic criteria for MetS by International Diabetes Foundation (23): Elevated waist circumference (40 inches for men and 35 inches for women) with any two of the following - Elevated triglycerides (equal to or greater than 150 mg/dL); Reduced HDL cholesterol (less than 40 mg/dL for men and 50 mg/dL for women); Elevated blood pressure (equal to or greater than 130/85 mm Hg or use of medication for hypertension; Elevated fasting glucose (equal to or greater than 100 mg/dL or use of medication for hyperglycemia.

The exclusion criteria were: serum creatinine 1.6 mg/dl; abnormal hepatic function; hemoglobinopathy; unwillingness to sign the informed consent form or enter the study; aggressive periodontitis; any platelet and coagulation disorders; diabetes. The participants in groups 2 and 4 had periodontal surgery and specimens were obtained from the greatest PPD and/or CAL sites. All participants provided informed consents for the specimen collection. The study protocol and consent form were approved by the Medical University of South Carolina Institutional Review Board.

2.2 Immunohistochemical Analysis of GPR40 and CD36 Expression

Periodontal tissue samples were embedded in Tissue-Tek® OCT™ compound (EMS, Hatfied, PA) and frozen immediately after surgery and stored at −80°C. Using a cryostat, 6 μm sections were cut and fixed in 10% of buffered formalin for 10 minutes and then washed with 0.1 M PBS. The sections were incubated with 5% normal goat serum for 1 hour. The sections were then incubated with antibody against GPR40 (1:300) (Santa Cruz Biotechnology, Santa Cruz, CA) or CD36 (1:300) (Novus Biologicals, Littleton, CO) overnight at 4°C. The sections were incubated with secondary biotinylated antibody (1:250)

from the ABC Elite kit (Vector Laboratories, Burlingame, CA) for 1 h and then the ABC reagent (Vector Laboratories) for 30 min. Slides were rinsed in 0.01 M PBS, covered with diaminobenzidine peroxidase substrate solution from the Impack DAB kit (Vector Laboratories) for 2 min and then rinsed with water. Counterstaining was performed with hematoxylin solution, Gill No. 2 (Sigma–Aldrich, St. Louis, MO). Slides were then dehydrated using increasing concentrations of ethanol and xylenes and mounted. Staining with normal IgG was used as a negative control. Photomicrographs of tissue sections were taken using an Olympus BX53 digital microscope with Cellsens digital image software (Olympus American Inc., Center Valley, PA). The area with positive staining was quantified using a computer based morphometry software (*Image-Pro Plus 6*, Media Cybernetics, Bethesda, MD) as described previously (24).

2.3 Cell Culture Study

Human gingival fibroblasts were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in a 5% CO_2 atmosphere in RPMI 1640 medium (GIBCO, Invitrogen Cop. Carlsbad, CA) containing 10% fetal calf serum, 1% MEM non-essential amino acid solution, and 0.6 g/100 ml of HEPES. The medium was changed every 2–3 days. RAW264.7 macrophages were purchased from ATCC and grown in DMEM (ATCC, Manassas, VA) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT). The cells were maintained in a 37 °C, 90% relative humidity, 5% CO_2 environment. Fibroblasts and RAW264.7 macrophages were grown to 80% confluence before treatments with LPS, palmitate or LPS plus palmitate. LPS was isolated from *Porphyromonas gingivalis* (*P*g) (InvivoGen, San Diego, CA). Palmitate was prepared from palmitic acid (Sigma, St. Louis, MO). Palmitic acid was dissolved in 0.1 N NaOH and 70% ethanol at 70 °C to make 50 mM. The solution was kept at 55°C for 10 min, mixed, and brought to room temperature. GW1100, an antagonist of GPR40 (25) and Sulfo-N-succinimidyl oleate (SSO), an inhibitor of CD36 (26), were purchased from Sigma-Aldrich.

2.4 Real-time Polymerase Chain Reaction (PCR)

Total RNA was isolated from cells using the RNeasy minikit (Qiagen, Santa Clarita, CA). First-strand complementary DNA (cDNA) was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) using 20 μl of reaction mixture containing 1 μg of total RNA, 4 μl of 5x iScript reaction mixture, and 1 μl of iScript reverse transcriptase. The complete reaction was cycled for 5 minutes at 25 °C, 30 minutes at 42 °C and 5 minutes at 85°C using a PTC-200 DNA Engine (MJ Research, Waltham, MA). The reverse transcription (RT) reaction mixture was then diluted 1:10 with nuclease-free water and used for PCR amplification of cDNA in the presence of the primers. The human IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers for real time PCR were purchased from Qiagen [IL-6, catalog [#]: PPM03015A-200; GAPDH, catalog [#]: PPM02946E-200]. The Beacon designer software (PREMIER Biosoft International, Palo Alto, CA) was used for human GPR40 and mouse CD36 primers designing (human GPR40: 5' primer sequence, TCAGCCTCTCTCTCCTGCTC; 3' primer sequence, CGCACACACTGTCTTCAGGC; mouse CD36: 5' primer sequence, TGCTGGAGCTGTTATTGGTG; 3' primer sequence, CATGAGAATGCCTCCAAACA). Primers were synthesized (Integrated DNA Technologies, Inc., Coralville, IA) and real-time

PCR was performed in duplicate using 25 μl of reaction mixture containing 10 μl of RT mixture, 0.2 □M of both primers, and 12.5 μl of iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was run in the iCycler™ real-time detection system (Bio-Rad) with a two-step method. The hot-start enzyme was activated (95°C for 2 min) and cDNA was then amplified for 40 cycles consisting of denaturation at 95°C for 10 sec and annealing/extension at 52.5°C for 45 sec. A melt-curve assay was then performed (55°C for 1 min and then temperature was increased by 0.5°C every 10 sec) to detect the formation of primer-derived trimers and dimers. Data were analyzed with the iCycler iQ™ software. The average starting quantity (SQ) of fluorescence units was used for analysis. Quantification was calculated using the SQ of targeted cDNA relative to that of GAPDH cDNA in the same sample.

2.5 Statistical Analysis

The differences of demographics were assessed using Fisher's Exact Test. The GraphPad Instat 3 software (GraphPad Software, Inc., San Diego, CA) was used for statistical analysis. Nonparametric analyses test using the Mann-Whitney procedure were performed to detect significant differences in continuous variable between two groups based on the two-tailed p value. A value of $p < 0.05$ was considered significant.

3. RESULTS

3.1 Study Population

Demographic data of participants including age, smoker, gender and race/ethnicity are presented in Table 1. The average ages in four groups had no significant difference ($p > 0.05$), ranging from 57 to 60. The smoker, gender and race/ethnicity also had no significant difference for all groups ($p > 0.05$). Furthermore, the CAL for Group 2 (periodontitis) and Group 4 (periodontitis and MetS) was 5.86 ± 0.99 and 6.27 ± 1.10 mm, respectively. Although the CAL for Group 4 appeared higher than that for Group 2, the difference was not statistically significant ($p = 0.30$).

3.2 Periodontal Expression of GPR40 and CD36 in Participants with Periodontitis, MetS or both Periodontitis and MetS

Expression of GPR40 and CD36 protein in periodontal tissue specimens was detected by immunohistochemistry. Results showed that GPR40 and CD36 proteins were expressed by gingival epithelial cells, fibroblasts and immune cells in periodontal tissue (Fig. 1A and Fig. 2A). Quantification of GPR40 and CD36 showed that GPR40 expression is increased in patients with periodontitis, MetS or both periodontitis and MetS (Fig. 1B) while CD36 expression is increased in patients with both periodontitis and MetS (Fig. 2B).

3.3 GPR40 and CD36 Are Upregulated by LPS and Palmitate, and Involved in the Stimulation of Proinflammatory Gene Expression by LPS and Palmitate

To understand the mechanisms involved in the upregulation of GPR40 and CD36 in periodontal tissues of patients with periodontitis and MetS, we hypothesized that the pathological factors associated with periodontitis or MetS may play a role in the upregulation of GPR40 and CD36. Since it is known that LPS is associated with

periodontitis (27, 28) and SFA is associated with MetS (13), we determined the effect of LPS and palmitate, a major SFA, on GPR40 and CD36 expression in fibroblasts and macrophages *in vitro*. Results showed that GPR40 was upregulated by the combination of LPS and palmitate in gingival fibroblasts (Fig. 3A) and CD36 was upregulated by LPS, palmitate and the combination of LPS and palmitate in RAW264.7 macrophages (Fig. 3B). Furthermore, results showed that the GPR40 antagonist GW1100 or CD36 inhibitor SSO effectively inhibited the expression of IL-6 mRNA in gingival fibroblasts stimulated with LPS, palmitate or LPS plus palmitate (Fig. 3C and D), suggesting that GPR40 and CD36 play an essential role in the stimulation of IL-6 expression by LPS and palmitate in gingival fibroblasts.

4. DISCUSSION

Our previous studies have shown that SFA augments LPS-induced proinflammatory gene expression in macrophages and vascular endothelial cells by amplifying TLR4-mediated inflammatory signaling such as mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NFκB) cascades (15, 25). These findings strongly suggest that free fatty acid receptors such as GPR40 and CD36 are involved in SFA-enhanced proinflammatory gene expression since SFA engages GPR40 and CD36, which mediate MAPK and NFκB signaling activation (29, 30). The role of GPR40 and CD36 in the upregulation of proinflammatory cytokine expression was further supported by our findings that inhibition of GPR40 or CD36 attenuated proinflammatory cytokine expression in gingival fibroblasts stimulated by palmitate and LPS (Fig. 3). Consistently, studies from other laboratories have also demonstrated a vital role of free fatty acid receptors GPR40 and CD36 in SFA-promoted inflammation (31, 32).

Regulation of surface receptor expression is a well-known cell function to control cellular response to ligands or uptake of ligands (33, 34). Under certain conditions, cells either upregulate or downregulate the receptor expression, resulting in increased or decreased cell response to ligands and ligand uptake, respectively. In our current study, we found that GPR40 and CD36 were upregulated in cells in periodontal tissues of patients with both periodontitis and MetS, suggesting a potentially increased interaction between the cells in periodontal tissue and SFA, leading to an enhanced inflammatory response to SFA and SFA uptake.

It has been shown that GPR40 expression is upregulated by mild hyperlipidemia associated with obesity-prone diabetes (35). Furthermore, it has been also reported that GPR40 expression is upregulated by peroxisome proliferator-activated receptor-γ, a nuclear receptor associated with fatty acids and glucose (36), in pancreatic β-cells (37). These studies indicate that GPR40 expression is upregulated by hyperlipidemia and hyperglycemia-related factors. Given that the participants in groups 3 and 4 had MetS that is associated with hyperlipidemia and hyperglycemia, it is plausible that the hyperlipidemia and hyperglycemia in patients in groups 3 and 4 may promote GPR40 upregulation in periodontal tissue. Moreover, as differing from the upregulation of GPR40 by hyperlipidemia and hyperglycemia, it has been reported that CD36 expression is upregulated by proinflammatory cytokines such as IL-1β, macrophage colony-stimulating factor and

granulocyte-macrophage colony-stimulating factor (38). Since proinflammatory cytokines in periodontal tissue are increased in patients with MetS (39), it is possible that CD36 is upregulated in periodontal tissues by increased proinflammatory cytokines. Our *in vitro* study showed that GPR40 and CD36 were upregulated by the palmitate and LPS, supporting the roles of dyslipidemia and inflammation in the upregulation of GPR40 and CD36 expression.

GPR40 is a G protein-coupled receptor and mediates the proinflammatory effect of SFA (31). Previous studies have shown that SFA has a higher affinity than unsaturated fatty acid to bind GPR40 (40). It has been shown that GPR40 mediate palmitate-enhanced amyloid-β production by activating inflammatory signaling pathways in neuronal cells (29). It has been also shown that GPR40 mediated thiazolidinediones-activated proinflammatory signaling pathways (20). Furthermore, we have shown that GPR40 mediates palmitate-enhanced TLR4 inflammatory signaling in vascular endothelial cells (25). However, a number of studies have shown that GPR40 also had antiinflammatory properties. Interestingly, it was shown that GPR40 activation by GPR40 agonist GW9506 suppressed chemokine expression in keratinocytes and attenuated cutaneous immune inflammation (41). To understand why GPR40 has both proinflammatory and antiinflammatory effects, it is important to know that while GPR40 is coupled to Gq/11, the specific G protein coupled by GPR40 receptor, it is also functionally linked to a β-arrestin 2-mediated signaling cascade that is G protein-independent (42). Since different GPR40 natural ligands such as saturated and unsaturated fatty acids as well as synthetic ligands have different effects on Gq/11 and/or β-arrestin-mediated signaling pathways, they can exert either proinflammatory or antiinflammatory actions.

CD36 is present on the surface of a number of cells including monocytes, macrophages, endothelial cells, smooth muscle cells and adipocytes, and is a scavenger receptor for oxidized LDL, oxidized phospholipids, collagen, thrombospondin and parasitized erythrocytes (19). CD36 has been shown to play a pivotal role in mediating inflammation, insulin resistance and atherosclerosis through signaling activation, transport of fatty acids and uptake of oxidized lipids (30). Studies have also shown that CD36 signaling pathways lead to activation of N-terminal kinase (JNK), a signaling cascade involved in the cell growth, differentiation, survival, apoptosis, and inflammation (30). CD36 also interacts with TLRs, which contribute to proinflammatory cytokine expression in monocytes and macrophages (30).

In conclusion, we demonstrated in this study that the periodontal expression of GPR40 and CD36 is upregulated in patients with periodontitis and MetS. Moreover, we also demonstrated that the expression of GPR40 and CD36 expression in macrophages and gingival fibroblast cultured *in vitro* is upregulated by LPS and palmitate, and involved in the stimulation of proinflammatory cytokine expression by LPS and palmitate. All these findings suggest that GPR40 and CD36 are potential targets for MetS-associated periodontitis.

ACKNOWLEDGEMENT

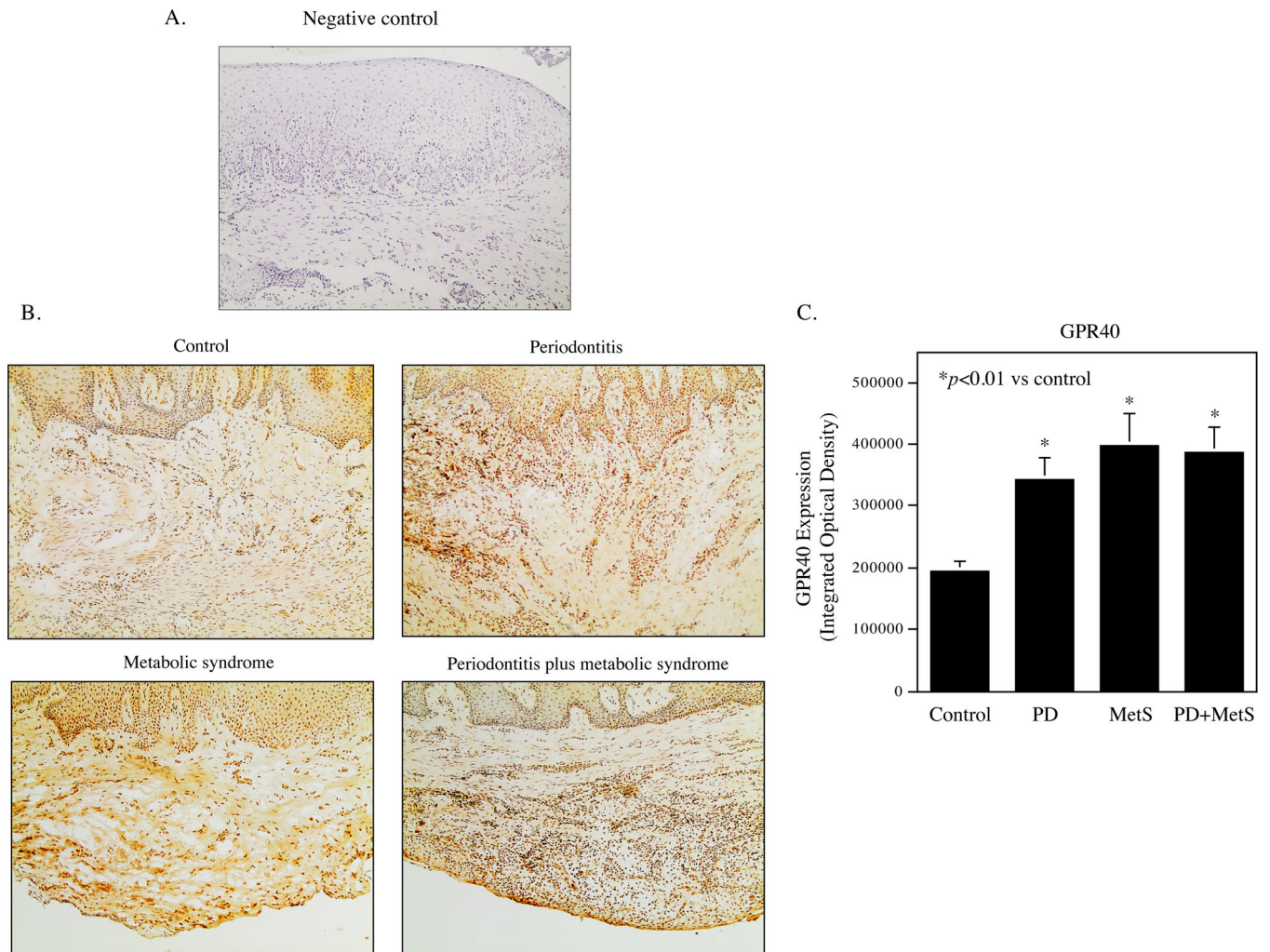## REFERENCES

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A. Negative control

B.

Control

Periodontitis

Metabolic syndrome

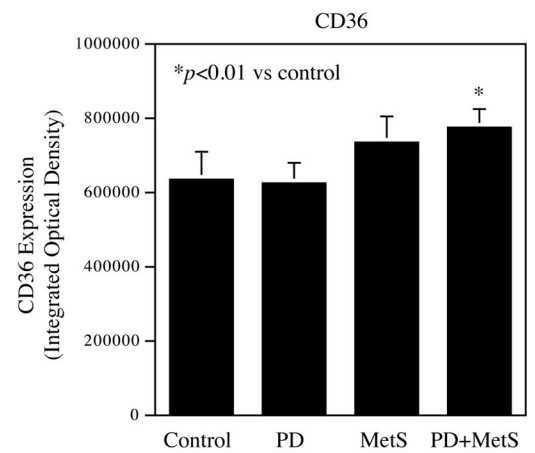Periodontitis plus metabolic syndrome

C.

GPR40

Figure 1.

The expression of GPR40 in periodontal tissues of participants without periodontitis and MetS, patients with periodontitis, MetS or both periodontitis and MetS. A. The negative control for immunohistochemical staining of GPR40 and CD36 in periodontal tissues. The tissue section incubated with normal human IgG as primary antibody was used as negative control. B. The GPR40 expression in periodontal tissues of control participants, patients with periodontitis, MetS, or both periodontitis and MetS. C. Quantification of GPR40 expression in periodontal tissues of control participants, patients with periodontitis (PD), MetS, or both PD and MetS.

A.
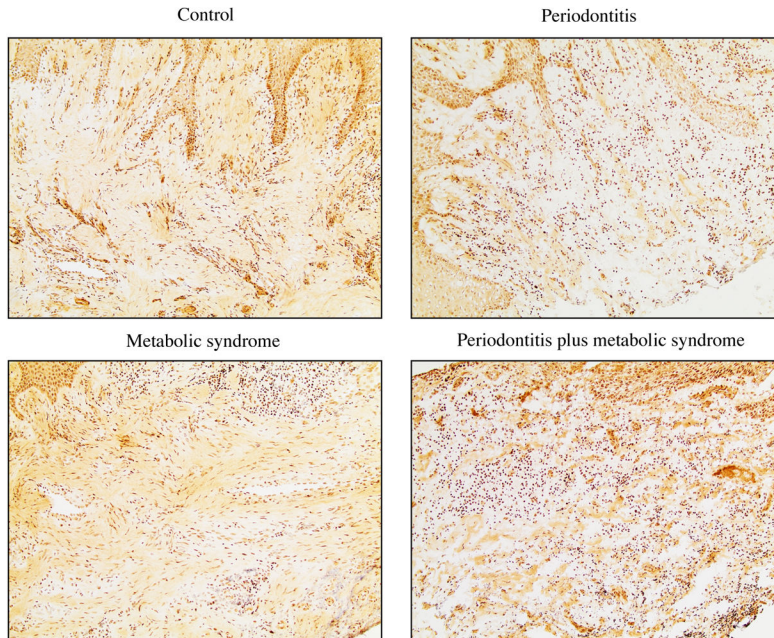
Figure 2.

The expression of CD36 in periodontal tissues of participants without MetS and periodontitis (control), patients with periodontitis, MetS or both periodontitis and MetS. A. The CD36 expression in periodontal tissues of control participants, patients with periodontitis, MetS, or both periodontitis and MetS. B. Quantification of CD36 expression in periodontal tissues of control participants, patients with periodontitis (PD), MetS, or both PD and MetS.

A.

B.

C.

D.
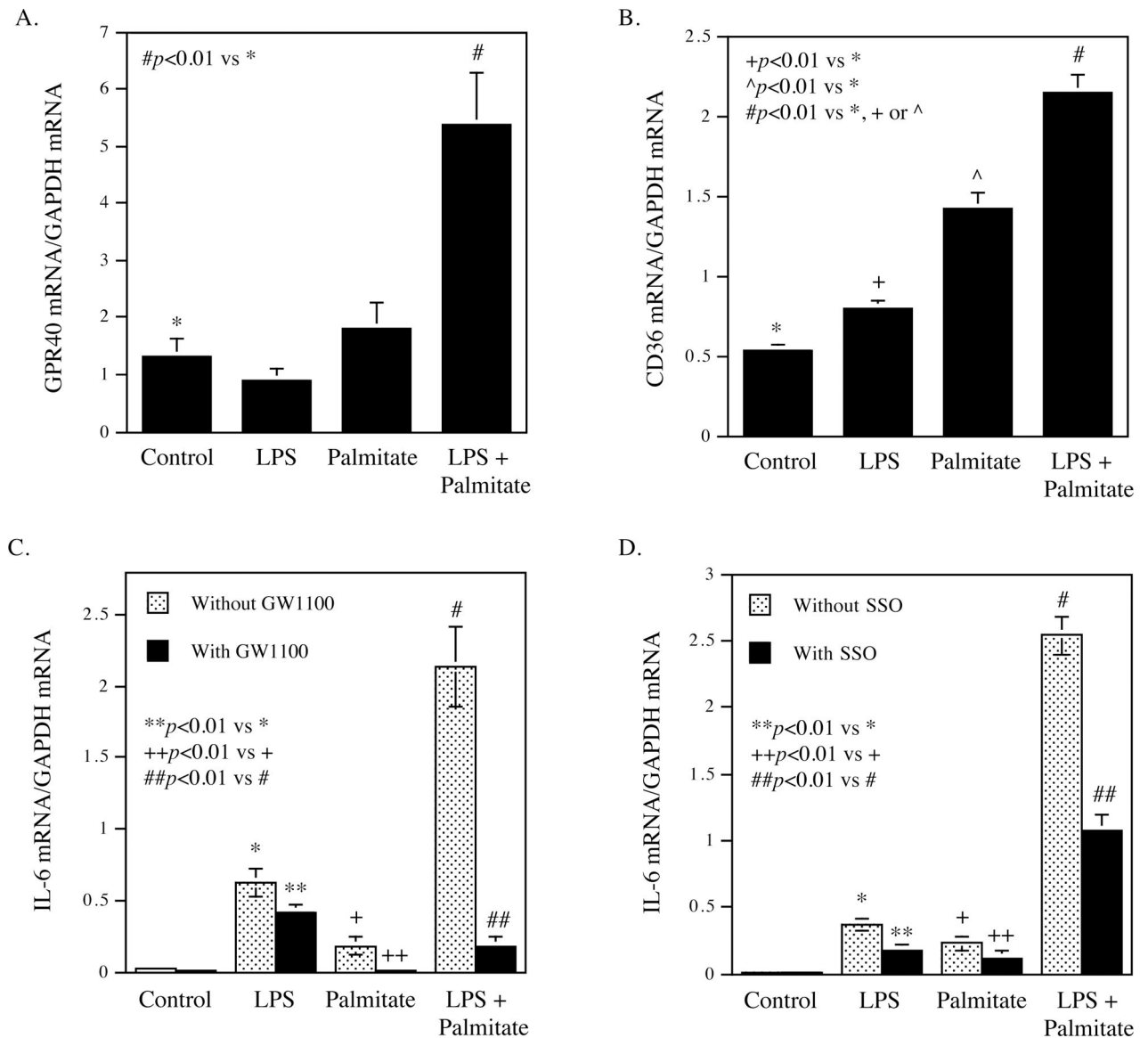
Figure 3.
Upregulation of GPR40 and CD36 mRNA expression by LPS and palmitate, and the involvement of GPR40 and CD36 in the stimulation of IL-6 expression by LPS and palmitate. A. Human gingival fibroblasts were treated with 1 μg/ml of *P*g LPS, 100 μM of palmitate or both 1 μg/ml of *P*g LPS and 100 μM of palmitate for 24 h. After the treatment, GPR40 mRNA was quantified using real-time PCR. B. RAW264.7 macrophages were treated with 1 μg/ml of *P*g LPS, 100 μM of palmitate or both 1 μg/ml of *P*g LPS and 100 μM of palmitate for 24 h. After the treatment, CD36 mRNA was quantified using real-time PCR. C and D. Human gingival fibroblasts were treated with 1 μg/ml of *P*g LPS, 100 μM of palmitate or both 1 μg/ml of *P*g LPS and 100 μM of palmitate in the absence or presence of 5 μM of GW1100 (C) or 100 μM of SSO (D) for 24 h. After the treatment, IL-6 mRNA was quantified using real-time PCR. The data (mean ± SD) presented is from representative of three experiments with similar results.

Table 1.

Demographic Data for the Study Population

	Group 1	Group 2	Group 3	Group 4	*P* value
	Non-periodontitis, non-MetS	**Periodontitis**	**MetS**	**Periodontitis and MetS**	
Total	11	12	11	14	
Age	57 ± 6	59 ± 4	58 ± 3	60 ± 3	*P*=0.5096
Smoker	2 (18.2%)	1 (8.3%)	2 (18.2%)	2 (14.3%)	*P*=0.9013
Gender (M/F)	2/9 (22%)	3/9 (33%)	6/5 (120%)	5/9 (55%)	*P*=0.3183
Race/Ethnicity					
White (Non-Hispanic)	41 (77.4%)	21 (75%)	5 (45.5%)	11 (44%)	*P*=0.3892
Black (Non-Hispanic)	12 (22.7%)	6 (21.4%)	6 (54.6%)	14 (56%)	
Hispanic	0 (0%)	1 (3.6%)	0 (0%)	0 (0%)	

The age was presented as mean ± SD. Other parameters were presented as percentile.