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Simvastatin Inhibits LPS-induced Alveolar Bone Loss during Metabolic Syndrome

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APPENDIX

METHODS

Study Design

LPS from *Aggregatibacter actinomycetemcomitans* (strain Y4, serotype B) was extracted by the hot-phenol-water method as described (Yu *et al.*, 2011) and diluted in phosphate-buffered saline (PBS). Periodontal disease was induced by the injection of *A. actinomycetemcomitans* lipopolysaccharide (LPS) as described previously (Rogers *et al.*, 2007; Li *et al.*, 2011; Yu *et al*., 2011). Briefly, each rat was injected with 20 μg of LPS through the palatal gingiva between the maxillary 1st and 2nd molars 3 times *per* wk for 4 wk (n = 7). Rats injected with PBS were used as control animals (n = 7). To determine the effect of simvastatin on LPS-induced periodontal disease, rats with LPS injection as described above were treated with simvastatin (20 mg/kg/day) daily *via* gavage for 4 wk (n = 8). The LPS treatment and simvastatin administration were started on the same day.

Metabolic Measurements

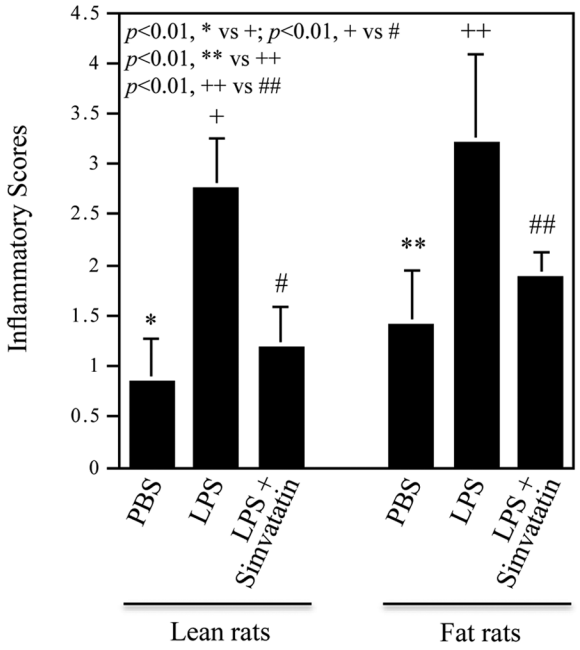
Blood samples were obtained from tail veins in the fasted condition, and glucose level was determined by means of a Precision QID glucometer (MediSense Inc., Bedford, MA, USA). Serum cholesterol and triglycerides were measured with the Cholestech LDX Lipid Monitoring System (Fisher Scientific, Pittsburgh, PA, USA). Serum free fatty acids (FFAs) were determined with the use of the EnzyChrom™ free fatty acid kit (BioAssay systems, Hayward, CA, USA). Serum fasting insulin was assayed with the Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem, Inc., Downers Grove, IL, USA). Fasting whole-body insulin sensitivity was estimated with the homeostasis model assessment of insulin resistance (HOMA-IR) according to the formula [fasting plasma glucose (mg/dL) × fasting plasma insulin (μU/mL)]/405 (Agil *et al*., 2012).

Micro-computed Tomography and Bone Volume Fraction Analysis

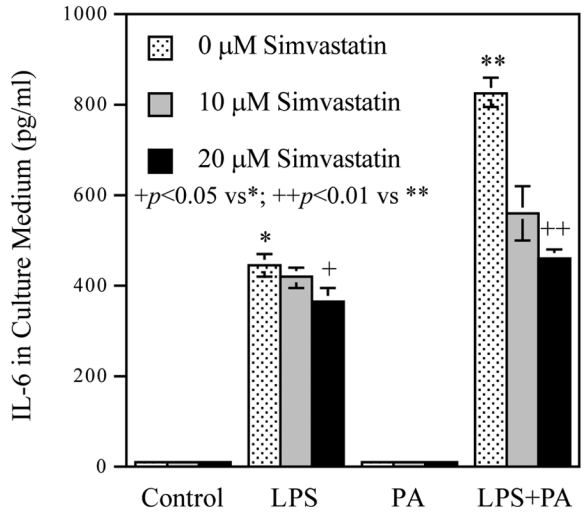
Non-demineralized rat maxillae were scanned in 70% ethanol by a cone-beam micro-computed tomography (μCT) system (GE Healthcare BioSciences, Chalfont St. Giles, UK). Each scan was reconstructed at a mesh size of 18 microns x 18 microns x 18 microns, and three-dimensional digitized images were generated for each specimen. Using GEHC MicroView software (version Viz+ 2.0 build 0029), we rotated the images into a standard orientation and threshold to distinguish between mineralized and non-mineralized tissue. Loss of bone volume was assessed by means of 3-D isoform displays as previously described (Kirkwood *et al*., 2007; Rogers *et al.*, 2007). Briefly, after proper image orientation, the region of interest (ROI) was determined. The width of the ROI was dictated by the height of contour of the molars at the cemento-enamel junction. Height of the ROI was measured from molar cusp tips to root apices. Depth was equal to the bucco-lingual size of the teeth plus 100 voxels (1.8 mm^3). After establishing the threshold, we calculated the bone volume fraction (BVF) as the percentage of bone within the ROI.

Histological Tissue Processing and Pathological Evaluation

After the maxillae were examined by μCT as described above, they were formalin-fixed and decalcified in a 10% EDTA solution for 2 wk at 4°C. The EDTA solution was changed 3 times *per* wk. The maxillae were paraffin-embedded, and sagittal tissue sections (7 μm) were cut and stained with hematoxylin and eosin. The mononuclear cells under the first molar were manually counted by a pathologist. Tissue inflammation and bone resorption were also evaluated according to the criteria for scoring tissue inflammation and bone resorption: 0 = within normal limits; 1 = focal, some leukocyte infiltration, no significant bone resorption; 2 = moderate leukocyte infiltration with mild bone



Appendix Figure 1. The images in Fig. 3A (main paper) were examined, and the inflammatory scores for leukocyte infiltration and alveolar bone resorption for rats treated with phosphate-buffered saline (PBS), lipopolysaccharide (LPS), or LPS plus simvastatin were made. The data are mean ± SD.



Appendix Figure 2. Simvastatin inhibited IL-6 secretion induced by lipopolysaccharide (LPS), palmitic acid (PA), or LPS plus PA. RAW246.7 macrophages were treated with 1 ng/mL of LPS, 100 μM of PA, or both LPS and PA in the absence or presence of simvastatin at 10 or 20 μM for 24 hr. After the treatment, IL-6 in culture medium was quantified by ELISA. The data are mean ± SD of 1 of 3 experiments with similar results.

resorption; 3 = severe leukocyte infiltration with moderate bone resorption; and 4 = severe leukocyte infiltration with extensive bone resorption.

RNA Isolation and Quantitative Real-time Polymerase Chain-reaction

RNA was extracted from gingival tissue removed from the injection sites with the RNeasy Mini Kit (Qiagen, Santa Clarita, CA, USA). First-strand complementary DNA (cDNA) was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) using 20 μL of reaction mixture containing 0.25 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 min at 25°C, 30 min at 42°C, and 5 min at 85°C by a PTC-200 DNA Engine (MJ Research, Waltham, MA, USA). The reverse transcription (RT) reaction mixture was then diluted 1:10 with nuclease-free water and used for polymerase chain-reaction (PCR) amplification of cDNA in the presence of primers. The Beacon designer software (PREMIER Biosoft International, Palo Alto, CA, USA) was used for primer designing (rat receptor activator of nuclear factor kappa-B ligand (RANKL): 5′ primer sequence, TGGGCCAAGATCTC TAACATGA; and 3′ primer sequence, TCATGATGCCTGAAG CAAATG. Rat colony-stimulating factor (CSF)2: 5′ primer sequence, GACCATGATAGCCAGCCACT; and 3′ primer sequence, TTCCAGCAGTCAAAAGGGATA). The primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). The primers for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Qiagen (catalog #: PPR06557B). The real-time PCR was performed in duplicate with 25 μL of reaction mixture containing 1.0 μL of RT mixture, 0.2 μM of both primers, and 12.5 μL of iQ™ SYBR Green Supermix (Bio-Rad Laboratories). Real-time PCR was run in the iCycler™ real-time detection system (Bio-Rad Laboratories) with a two-step method. The hot-start enzyme was activated (95°C for 3 min), and cDNA was then amplified for 40 cycles consisting of denaturation at 95°C for 10 sec and annealing/extension at 60°C for 60 sec. A melt-curve assay was then performed to detect the formation of primer-derived trimers and dimers. Data were analyzed with the iCycler iQ™ software (Bio-Rad Laboratories). 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.

Cell Culture

Mouse RAW264.7 macrophages were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in DMEM (ATCC) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT, USA). The cells were maintained in a 37°C, 90% relative humidity, 5% CO_2 environment and treated with 1 ng/mL of LPS and 100 μM of palmitic acid (PA) (Sigma, St. Louis, MO, USA) for 24 hr. The PA used in this study was bovine-serum-albumin-free as described previously (Schwartz *et al.*, 2010).

Enzyme-linked Immunosorbent Assay

Interleukin (IL)-6 in medium was quantified with the use of sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the protocol provided by the manufacturer (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis

GraphPad Instat statistical software (Version 3.1a) (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) was performed to determine the statistical significance of differences of gene expression levels among experimental groups, and data were presented as mean ± SD. Student's *t* test was used for comparison of means between 2 groups. A value of $p < .05$ was considered significant.

APPENDIX REFERENCES

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