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Metabolic Syndrome Exacerbates Inflammation and Bone Loss in Periodontitis

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Appendix

Methods

Metabolic Measurements

Blood samples were obtained under the fasted condition, and glucose level was determined through a Precision QID glucometer (MediSense Inc., Bedford, MA, USA). Serum cholesterol and triglycerides were measured via the Cholestech LDX Lipid Monitoring System (Fisher Scientific, Pittsburgh, PA, USA). Serum free fatty acids were determined through the EnzyChrom free fatty acid kit (BioAssay Systems, Hayward, CA, USA). Serum fasting insulin was assayed with the Ultra Sensitive 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 through the following formula: [fasting plasma glucose (mg/dL) × fasting plasma insulin (μU/mL)] / 405 (Agil et al. 2012).

Micro–computed Tomography and Bone Volume Fraction Analysis

Nondemineralized rat maxillae were scanned in 70% ethanol by a cone-beam micro–computed tomography system (GE Healthcare BioSciences, Chalfont St. Giles, UK). Each scan was reconstructed at a mesh size of 18 × 18 × 18 μm, and 3-dimensional digitized images were generated for each specimen. With GEHC MicroView software (version Viz+ 2.0, build 0029), the images were rotated into a standard orientation and threshold to distinguish between mineralized and nonmineralized tissue. Loss of bone volume was assessed by means of 3-dimensional 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 ROI width was dictated by the contour height of the molars at the cementoenamel junction. The ROI height was measured from molar cusp tips to root apices. Depth was equal to the buccolingual size of the teeth plus 100 voxels (1.8 mm^3). After the threshold was established, the bone volume fraction was calculated as the percentage of bone within the ROI.

RNA Isolation and Quantitative Real-time Polymerase Chain Reaction

RNA was extracted from gingival tissue that was removed from the injection sites via 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) with 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 with a PTC-200 DNA Engine (MJ Research, Waltham, MA, USA). The reverse transcription reaction mixture was then diluted 1:10 with nuclease-free water and used for polymerase chain reaction amplification of cDNA in the presence of primers (see Appendix Table 1). The primers for receptor activator of nuclear factor kappa-B ligand (RANKL) were purchased from Qiagen. The real-time polymerase chain reaction was performed in duplicate with 25 μL of reaction mixture containing 1.0 μL of reverse transcription mixture, 0.2μM of both primers, and 12.5 μL of iQ SYBR Green Supermix (Bio-Rad Laboratories) in the iCycler real-time detection system (Bio-Rad Laboratories) with a 2-step method. The hot-start enzyme was activated (95°C for 3 min), and cDNA was then amplified for 40 cycles consisting of denaturation

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Appendix Table 1. Sequences of the Primers Used in Real-Time Polymerase Chain Reaction.

Genes	Forward Primer	Reverse Primer
IL-6	ATGAAGTTCCTCTCTGCAAGAGACT	CACTAGGTTTGCCGAGTAGATCTC
M-CSF	CATCCACGCTGCGTGAAG	GGGATTCGGTGTCGCAATAT
MCP-1	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTGGGATCA
GAPDH	GCCTTCCGTGTTCCTACC	GCCTGCTTCACCACCTTC

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-6, interleukin 6; MCP-1, monocyte-chemotactic protein 1; M-CSF, macrophage colony-stimulating factor.

Appendix Table 2. Metabolic Data of Mice Fed Different Diets.

Metabolic Parameters	Regular Chow	High-Fat Diet
Weight, g	31 ± 2	50 ± 2[a]
Glucose, mg/dL	234 ± 19	267 ± 14
Insulin, ng/mL	0.79 ± 0.29	5.78 ± 1.02[a]
HOMA-IR	12.1 ± 4.6	96.6 ± 20.4[a]
Cholesterol, mg/dL	141 ± 11	235 ± 17[a]
Triglycerides, mg/dL	61 ± 4	86 ± 10
Free fatty acids, mg/dL	451 ± 29	622 ± 92[a]

Data are mean ± SD (n = 7).
HOMA-IR, homeostasis model assessment of insulin resistance.
[a]$P < 0.01$ vs mice fed regular chow.

Appendix Table 3. Enhancement of LPS-Stimulated Genes by PA in Bone Marrow–Derived Macrophages.

	Cycle Threshold in . . .				Fold of Control by . . .		
	Control Cells	LPS-Treated Cells	PA-Treated Cells	LPS- + PA-Treated Cells	LPS	PA	LPS + PA
IL-1α	33.56	27.16	31.95	24.37	85.0	3.1	584.5
CXCL10	34.50	29.75	32.37	27.20	26.9	4.4	158.4
IL-1β	32.01	25.76	31.56	25.50	75.7	1.4	90.9
CD86	30.86	29.52	28.56	27.78	2.5	4.9	8.4
CSF2	34.70	36.35	34.62	31.93	0.3	1.1	6.8
MCP-1	31.11	30.56	31.32	28.54	1.5	0.9	5.9
TLR2	31.84	29.66	31.32	29.33	4.5	1.6	5.7
TNFα	32.24	31.69	31.99	30.47	1.5	1.2	3.4
CD14	25.41	25.34	24.62	23.84	1.1	1.7	3.0
IL-6	32.93	33.28	32.93	31.63	0.8	1.0	2.5

Bone marrow–derived macrophages were treated with 1 ng/mL of LPS, 100 μM of PA, or both for 24 h. After treatment, cells were harvested and subjected to RNA isolation and polymerase chain reaction array as described in the Methods section. All gene expressions were normalized to GAPDH expression. Data presented were the averages of duplicate samples from 1 of 2 experiments with similar results.
CXCL10, C-X-C motif chemokine 10; CSF2, granulocyte-macrophage colony-stimulating factor; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; PA, palmitic acid; TLR2, Toll-like receptor 2; TNFα, tumor necrosis factor alpha.

at 95°C for 10 s and annealing/extension at 60°C for 60 s. A melt-curve assay was then performed to detect the formation of primer-derived trimers and dimmers. Data were analyzed with the iCycler iQ software (Bio-Rad Laboratories). The average starting quantity of fluorescence units was used for analysis. Quantification was calculated with the starting quantity of targeted cDNA relative to that of glyceraldehyde-3-phosphate dehydrogenase cDNA in the same sample.

Culture of Bone Marrow–derived Macrophages

Bone marrow–derived macrophages were isolated and cultured as described previously (Itoh et al. 2003). Briefly,

bone marrow cells were obtained from tibiae and femora of 4- to 6-wk-old C57BL/6 mice and cultured with α-MEM containing 10% fetal bovine serum in a humidified incubator (5% CO_2) at 37°C. After 24 h, nonadherent cells were incubated for 4 d in the presence of 50 ng/mL of macrophage colony stimulating factor (Sigma-Aldrich, Atlanta, GA, USA). It has been shown that almost all the adherent cells expressed macrophage-specific antigens, such as Mac-1, Moma-2, and F4/80 (Kobayashi et al. 2000).

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