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Porphyromonas gingivalis lipopolysaccharide induces pro-inflammatory adipokine secretion and oxidative stress by regulating Toll-like receptor-mediated signaling pathways and redox enzymes in adipocytes

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ABSTRACT

Gut microbiota LPS contributes to obesity-related chronic inflammation and oxidative stress, promoting insulin resistance. Periodontal disease also represents a risk factor for type 2 diabetes and is associated with obesity. This study compared the effect of LPS from *P. gingivalis* periodontopathogen and *E. coli* enterobacteria on inflammatory adipokine secretion and redox status of 3T3-L1 adipocytes. We found that both LPS activated TLR2- and TLR4-mediated signaling pathways involving MyD88 adaptor and NFκB transcription factor, leading to an increased secretion of leptin, resistin, IL-6 and MCP-1. These effects were partly blocked by inhibitors targeting p38 MAPK, JNK and ERK. Moreover, *P. gingivalis* LPS reduced adiponectin secretion. Both LPS also enhanced ROS production and the expression of NOX2, NOX4 and iNOS genes. *P. gingivalis* LPS altered catalase gene expression. Collectively, these results showed that LPS of periodontal bacteria induced pro-inflammatory adipokine secretory profile and oxidative stress in adipocytes which may participate to obesity-related insulin resistance.

1. Introduction

During obesity, excessive fat storage within the adipose tissue alters adipose cell secretory functions. Indeed, obesity induces an overproduction of pro-inflammatory adipokines which mediate a chronic low grade inflammation in adipocytes (Ouchi et al., 2011). Obesity also promotes an imbalance between an excessive production of reactive oxygen species (ROS) and a lack of endogenous antioxidant capacity, leading to oxidative stress in adipose cells (Houstis et al., 2006; Furukawa et al., 2004). These disorders participate in the development of insulin resistance, type 2 diabetes and vascular complications (Houstis et al., 2006; de Ferranti and Mozaffarian, 2008).

Several exogenous stimuli comprising bacterial components may contribute to obesity-related chronic inflammation and oxidative stress. Moreover, a link between gut microbiota composition, adipose tissue dysfunction and obesity has been reported (Backhed et al., 2004). The lipopolysaccharide (LPS), defined as a major non-protein component of the external membrane of Gram-negative bacteria such as *Escherichia coli*, binds innate immunity Toll-like receptors (TLRs) which activate signaling cascades involving nuclear factor kappa B (NFκB) transcription factor and mitogen activated protein kinases (MAPKs) in adipocytes. This leads to a decreased production of key anti-inflammatory adipokines such as adiponectin and an enhanced secretion of major pro-inflammatory molecules including leptin, resistin, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and monocyte

Abbreviations: DCFH-DA, 2′,7′-Dichlorofluorescein-diacetate; ERK, Extracellular signal-regulated kinase; FAS, Fatty acid synthase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; IL-6, Interleukin-6; iNOS, inducible Nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPS, Lipopolysaccharide; MAPK, Mitogen activated protein kinase; MCP-1, Monocyte chemoattractant protein-1; MyD88, Myeloid differentiation primary response 88; NFκB, Nuclear factor kappa B; NOX, NADPH Oxidase; Nrf2, Nuclear factor erythroid 2-related factor 2; PBS, phosphate buffer saline; PPARγ, Peroxisome proliferator-activated receptor gamma; ROS, Reactive oxygen species; RT-qPCR, Reverse transcription-quantitative polymerase chain reaction; TLR, Toll-like receptor; TNF-α, Tumor necrosis factor-alpha.

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chemoattractant protein-1 (MCP-1) involved in insulin resistance onset (Ouchi et al., 2011; Houstis et al., 2006; Borst and Bagby, 2004). Metabolic endotoxemia, characterized by increased plasma levels of LPS, is induced by high fat diet. It stimulates the proliferation of adipocyte precursors (Luche et al., 2013) and the inflammatory process in the adipose tissue, contributing to obesity and insulin resistance (Cani et al., 2007).

Evidence was also provided for a positive correlation between a high body mass index and an increased number of periodontal pockets (Saxlin et al., 2011), suggesting an association between periodontal diseases and obesity (Saito et al., 2001; Chaffee and Weston, 2010). Periodontitis, characterized by a gum infection damaging the tooth supporting tissue including the bone, is commonly found in people with obesity (Amar and Leeman, 2013) and represents a risk factor for type 2 diabetes (Gurav and Jadhav, 2011). Interestingly, results from meta-analysis demonstrate that periodontal therapy providing antibiotics reduces circulating concentrations of C reactive protein and TNF-α in people with type 2 diabetes when compared to control (Artese et al., 2015). Additionally, a periodontal treatment based on oral hygiene instructions or surgical procedures improves glycemic control in type 2 diabetic patients (Teeuw et al., 2010). In this regard, a particular attention has been paid to *Porphyromonas gingivalis* recognized as a representative periodontopathogen (Mysak et al., 2014). Arimatsu et al. (2014) reported that oral administration of *P. gingivalis* in mice induces metabolic changes associated with insulin resistance and systemic inflammation. Furthermore, our recent data showed that *P. gingivalis* might participate in the pathogenesis of human abdominal aortic aneurysm by neutrophil activation (Delbosc et al., 2011). We detected the presence of periodontal bacteria DNA in human abdominal aortic aneurysm and hemorrhagic atherosclerotic carotid plaques, and positively correlated it with markers of neutrophil activation (Range et al., 2014). Despite the link established between periodontal diseases, obesity and insulin resistance, there is still a lack of data regarding the effect of LPS from periodontal bacteria on adipocyte biology.

The present study aimed at comparing the effect of *P. gingivalis* LPS and *E. coli* LPS used as a positive control, on the metabolic and secretory profile of 3T3-L1 adipocytes, by exploring their impact on insulin-mediated lipid accumulation, the secretion of adipokines as well as the production of innate immunity TLRs and key factors involved in inflammatory signaling pathways and redox homeostasis.

2. Materials and methods

2.1. Adipose cell culture

3T3-L1 mouse preadipocytes were obtained from the American Type Culture Collection. The culture medium Dulbecco's modified Eagle's medium (Pan Biotech) contained 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum (Pan Biotech), 5 mM L-glutamin (Pan Biotech), 2 μg/mL streptomycin and 50 μU/mL penicillin (Pan Biotech). The cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C. For cell differentiation assay, preadipocytes were plated in 6-well plates at a density of 125 × 10^3 cells/well and allowed to grow until confluence. Two days after confluence (day 0), preadipocytes were exposed to the culture medium supplemented with insulin (1 μg/mL, Sigma-Aldrich), isobutyl-1-methylxanthine (500 μM, Sigma-Aldrich) and dexamethasone (0.25 μM, Sigma-Aldrich) until day 2. Then, insulin-containing medium was replaced every two days. Two experimental conditions were performed. First, at day 7, mature adipocytes were exposed to 10 μg/mL of *P. gingivalis* LPS (Invivogen) or *E. coli* LPS (Sigma-Aldrich) during 24 h. Second, at day 7, mature adipocytes were pretreated during 30 min with Anti-TLR2 or Anti-TLR4 antibodies (Sigma-Aldrich) used at 20 μg/mL according to our previous study (Bes-Houtmann et al., 2007), or one of the following inhibitors used at 10 μM in accordance with literature data (Aouadi et al., 2007). Inhibitors were SB203580 for p38 MAPK, SP600125 for c-Jun N-terminal kinase (JNK) and U0126 for extracellular signal-regulated kinase (ERK, Sigma-Aldrich). Then, cells were treated during 24 h with *P. gingivalis* or *E. coli* LPS (10 μg/mL), and cell culture media, RNA and proteins were collected at day 8.

2.2. Evaluation of cell viability

To evaluate cell viability, MTT assay was performed as we previously described (Hatia et al., 2014). Briefly, 3T3-L1 preadipose cells were plated in 96-well plates (8 × 10^3 cells/well) and exposed to LPS at concentrations ranging 1–10 μg/mL for 24 h. Five hours before the end of the experiment, 20 μL of sterile filtered 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (5 mg/mL, Sigma-Aldrich) in phosphate buffered saline (pH 7.4) were added to each well and plates were incubated at 37 °C. Then, the unreacted dye was removed by centrifugation, the insoluble formazan crystals were dissolved in 200 μL dimethyl sulfoxide/well, and the absorbance was measured at 560 nm (FLUOstar Optima, Bmg Labtech).

2.3. Evaluation of lipid droplet accumulation

To estimate lipid droplet accumulation in differentiated adipocytes at day 8, cells were washed with phosphate buffer saline (PBS), fixed with 4% formaldehyde (Acros) for 20 min, washed three times with water and then stained with Oil red O dye (Sigma-Aldrich). Then, adipocytes were photographed at magnification 40 × (Olympus microscope). The developed chromogens were dissolved with isopropanol (Carlo Erba Reagent) and the optical density determined at 490 nm (FLUOstar Optima, Bmg Labtech).

2.4. Quantification of adipokine secretion

Two experimental conditions were performed. First, to evaluate the dose-dependent effects of LPS on adipokine secretion, 3T3-L1 cells were exposed to LPS at concentrations ranging 1–10 μg/mL during 24 h. Second, to determine LPS effects on adipokine secretion in differentiated adipocytes, cells were exposed to 10 μg/mL of LPS during 24 h. Then, cell culture media were collected and analyzed using Mouse IL-6 and MCP-1 ELISA kits (eBioscience) as well as Adiponectin (BosterBio), Leptin (Ray Biotech) and Resistin (BosterBio) ELISA kits. Absolute values were normalized according to total cellular protein content assessed by Bradford test after protein extraction (Bradford, 1976).

2.5. Determination of ROS production

Intracellular ROS levels were measured via the oxidation of 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) as previously described (Wang and Joseph, 1999). Briefly, cells were cultured in 96-well black plates (5 × 10^3 cells/well) for 24 h. Then, the medium was replaced by PBS containing 10 μM of DCFH-DA (Sigma-Aldrich) for 45 min at 37 °C and 5% CO_2. Next, cells were exposed to 10 μg/mL of *P. gingivalis* or *E. coli* LPS for 1 h. The fluorescence was measured at an excitation wavelength of 492 nm and an emission wavelength of 520 nm (FLUOstar Optima, Bmg Labtech).

2.6. Evaluation of gene expression

Total RNA was isolated from adipocytes with TRIzol (Ambion,