Role of SIRT1 in Heat Stress- and Lipopolysaccharide-induced Immune and Defense Gene Expression in Human Dental Pulp Cells

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Abstract

Introduction: Although bacterial infection and heat stress are common causes of injury in human dental pulp cells (HDPCs), little is known about the potential defense mechanisms mediating their effects. This study examined the role of SIRT1 in mediating heat stress and lipopolysaccharide (LPS)-induced immune and defense gene expression in HDPCs. **Methods:** HDPCs were exposed to heat stress (42°C) for 30 minutes after stimulation with LPS (1 μg/mL) for 48 hours. The expression of defense genes was evaluated by reverse-transcriptase polymerase chain reaction, Western blotting, and enzyme-linked immunosorbent assay. **Results:** LPS and heat stress synergistically increased the expression of SIRT1 and immune and defense genes such as interleukin (IL)-8, hemeoxygenase-1 (HO-1), and human β-defensin 2 (hBD-2). Resveratrol enhanced LPS- and heat stress–induced expression of HO-1 and hBD-2 but reduced IL-8 messenger RNA levels. The stimulation of HO-1 and hBD-2 messenger RNA expression by LPS and heat stress was inhibited by sirtinol; SIRT1 small interfering RNA; and inhibitors of p38, ERK, JNK, and nuclear factor κB. **Conclusions:** These results show for the first time that SIRT1 mediates the induction of immune and defense gene expression in HDPCs by LPS and heat stress. SIRT1 may play a pivotal role in host immune defense system in HDPCS. *(J Endod 2011;37:1525–1530)*

Key Words

Defense system, human β-defensin 2, hemeoxygenase-1, human dental pulp cells, interleukin-8, SIRT-1

Under both physiological and pathological conditions, dental pulp is exposed to several sources of stress, including bacterial infection and heat (1). Bacteria that invade the dentin as well as their products that diffuse through dentinal tubules are involved in the pathogenesis of pulpitis (2, 3). Lipopolysaccharide (LPS) is a major cell wall component in gram-negative bacteria that has been shown to be a potent inducer of pulpitis (4). LPS has been shown to enhance the production of prostaglandins, cyclooxygenase-2, and proinflammatory cytokines in human dental pulp cells (HDPCs) (5, 6).

In dental pulp cells, heat stress has been shown to increase leukotriene B4 levels (7); induce apoptosis (8); and elevate levels of heat shock protein (HSP) 70 (9), alkaline phosphatase, HSP25 (10), HSP27, and heat shock transcription factor-1 (1). In other studies, the combination of LPS and heat stress up-regulated the expression of Toll-like receptor 2 and Toll-like receptor 4 in human monocytes (11) and increased plasma levels of interleukin (IL)-1β, tumor necrosis factor α (TNF-α), and IL-6 in rats (12).

The defense system in dental pulp operates through various immune mediators including cytokines and antimicrobial peptides (13). Notably, IL-1 and TNF-α play important roles in immune responses to infection (14). The results of recent studies suggest that LPS induces IL-1, TNF-α, IL-6, and IL-8 in HDPCs (15, 16). One host defense mechanism that involves activation of an innate immune response after exposure to the external environment is the production of defensin. Previously, we reported that TNF-α and IL-1α synergistically up-regulate human β-defensin-2 (hBD-2) expression and activity in HDPCs (17).

SIRT1 is a stress-activated nicotinamide adenine dinucleotide-dependent protein deacetylase (18). Previous studies showed that resveratrol, an activator of SIRT1, inhibited cigarette smoke–induced IL-8 release in a monocyte-macrophage cell line (19), TNF-α–induced nuclear factor-κB (NF-κB) activation in mouse embryonic fibroblasts (20), and TNF-α–induced monocyte chemoattractant protein-1 (MCP-1) secretion in adipocytes (21). Collectively, these observations suggest that SIRT1 has anti-inflammatory effects. However, another study showed that the SIRT1 inhibitor sirtinol significantly reduced bronchial inflammation and levels of IL-4, IL-5, and IL-13 in the lungs of ovalbumin-sensitized mice (22).

Recently, we showed that heat stress activates chemokines such as IL-8 and defense genes such as hemeoxygenase-1 (HO)-1 in HDPCs (23). However, the role of SIRT1 in the regulation of the defense responses to environmental stressors such as LPS and heat is not known. This study aimed to investigate the involvement of

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SIRT1 in LPS- and heat stress-induced expression of immune and defense genes in HDPCs.

Materials and Methods

Culture of HDPCs

The use of human primary pulp cells conformed to an informed consent protocol reviewed and approved by the Institutional Review Board of Wonkwang University Hospital (Iksan, Korea). The *Porphyromonas gingivalis* LPS used was a commercial preparation purified by the supplier (InvivoGen, San Diego, CA) according to the method described previously (24). HDPCs were subjected to LPS pretreatment and heat stress as described by Kitamura et al (1, 8). Briefly, cells were pretreated with LPS from *P. gingivalis* (1 μg/mL) for 48 hours and then exposed to heat stress at 42°C for 30 minutes. They were returned to a temperature of 37°C in a humidified incubator (0 hours). Samples were then harvested at specific time points (0, 1, 2, 3, 6, 12, 18, and 24 hour) after heat treatment.

Measurement of Reactive Oxygen Species

Intracellular reactive oxygen species (ROS) generation was measured using the 5, 6-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) probe (Molecular Probes, Eugene, OR). Cells were subjected to LPS and heat stress treatment and then incubated with CM-H2DCFDA (10 μmol/L) for 20 minutes at 37°C in the dark. Mean CM-H2DCFDA fluorescence at 530 nm (bandwidth 30 nm) was recorded after excitation at 488 nm using a 15-mW argon laser.

Enzyme-linked Immunosorbent Assay

Concentrations of IL-8 in culture supernatants were determined using an enzyme-linked immunosorbent assay kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol. The lower limit of sensitivity of the assay was below 8 pg/mL. Absorbances at 450 nm were measured using a microplate reader (Molecular Devices, Sunnyvale, CA).

RNA Isolation and Reverse-transcriptase Polymerase Chain Reaction

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. It was then reverse transcribed using AccuPower RT PreMix (Bioneer, Daejon, Korea). The primers and thermal conditions used in the polymerase chain reactions (PCRs) are detailed in Table 1. PCR products were separated in 1.5% agarose gels and then stained with ethidium bromide.

Western Blotting

Western blotting was performed according to a standard protocol using the Mini-Protean II system (Bio-Rad, Hercules, CA). Sample protein concentrations were determined using a Bio-Rad protein assay kit. Antibodies against hemeoxygenase-1 (HO-1), hBD-2, SIRT1, mitogen-activated protein kinase (MAPK), phospho-MAPK, NF-κB, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Beverly, MA). Protein bands were isualized using ECL (Amersham Biosciences, Buckinghamshire, UK).

SIRT1 Small Interfering RNA Transfection

HDPCs were transfected with small interfering RNA (siRNA) encoding specific SIRT1 sequences (Invitrogen) or a control siRNA (to test for nonspecific effects). Briefly, cells in the exponential growth phase were plated to 6-well plates at a density of 5×10^5 cells/well, grown for 20 hours, and then transfected with SIRT1 siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Statistical Analysis

Differences between groups were identified by one-way analysis of variance.

Results

Effects of LPS and Heat Stress on Immune Gene Expression and ROS Production

To determine whether LPS influences the expression of cytokine and immune genes in pulp cells, cells were exposed to several concentrations of LPS (0, 0.1, 0.5, 1, 2, and 10 μg/mL) for 12, 24, 48, and 72 hours. After incubation with LPS, expression levels of the proinflammatory cytokines TNF-α and IL-8 were up-regulated in a dose- and time-dependent manner (Fig. 1*A* and *B*). In contrast, the expression of IL-6 and IL-1β messenger RNA remained unchanged. Expression levels of the defense genes HO-1 and hBD-2 at the messenger RNA and protein levels were increased by LPS in a concentration-dependent manner up to a concentration of 1 μg/mL (Fig. 1*A*). As shown in Figure 1*B*, a time-course experiment revealed that 1 μg/mL LPS increased HO-1 and hBD-2 messenger RNA and protein expression in pulp cells compared with

TABLE 1. RT-PCR Primers and Conditions

Genes	Primer sequence (5′-3′)	Annealing temp (°C)	Cycle number	Product size (bp)
TNF-α	F: 5′-CTCTFFCCCAFFCAFTCAGA-3′ R: 5′-GGCGTTTGGGAAGGTTGGAT-3′	60	35	519
IL-1β	F: 5′-TGGAGATGACAGTTCAGAAG-3′ R: 5′-GTACTGGTGCCGTTTAtGC-3′	60	35	288
IL-6	F: 5′-TAFCCGCCCCACACAGACAG-3′ R: 5′-GGCTGGCATTTGTGGTTGGG-3′	60	35	408
IL-8	F : 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′ R : 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′	62	25	289
HO-1	F: 5′-AAGATTGCCCAGAAAGCCCTGGAC-3′ R: 5′-AACTGTCGCCACCAGAAAGCTGAG-3′	55	30	399
hBD-2	F: 5′-CATGAGGGTCTTGTATCTCCTCT-3′ R: 5′-CCTCCTCATGGCTTTTTGCAGC-3′	55	35	201
SIRT1	F: 5′-TCAGTGTCATGGTTCCTTTGC-3′ R: 5′-AATCTGCTCCTTTGCCACTCT-3′	57	35	820
GAPDH	F: 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ R: 5′-CGGAGTCAACGGATTTGGTCGTAT-3′	62	25	306

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.