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Effects of sirtuin 1 activation on nicotine and lipopolysaccharide-induced cytotoxicity and inflammatory cytokine production in human gingival fibroblasts

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Background and Objective: Although sirtuin 1 (SIRT1) over-expression and resveratrol exert anti-inflammatory and proinflammatory effects, their effects and the mechanism of action on human gingival fibroblast (HGF)-mediated inflammation are unknown. The aim of this study was to demonstrate the effects of activating SIRT1 using resveratrol and recombinant adenovirus encoding SIRT1 (Ad-SIRT1) on the expression of proinflammatory cytokines and to elucidate its mechanism of action of lipopolysaccharide (LPS) and nicotine stimulated-HGF.

Material and Methods: Cytotoxicity and the production of reactive oxygen species (ROS) were measured using the 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry, respectively. The amount of prostaglandin E_2 (PGE_2) released into the culture medium was measured by radioimmunoassay. mRNA and protein levels were analyzed using RT-PCR and western blotting, respectively.

Results: Nicotine and LPS up-regulated the expression of SIRT1 mRNA and SIRT1 protein in a time- and concentration-dependent manner. Resveratrol and Ad-SIRT1 decreased LPS and nicotine-induced cytotoxicity, ROS and PGE_2 production, and expression of cyclooxygenase-2 in HGFs. Resveratrol and Ad-SIRT1 inhibited nicotine and LPS-mediated protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), p38, ERK, JNK, MAPK and nuclear factor-kappa B (NF-κB) activation.

Conclusion: This study is the first to show that the anti-inflammatory and cytoprotective effects of SIRT1 activation in HGFs occur through the PKC, PI3K, MAPK and NF-κB pathways.

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Periodontitis is a chronic inflammatory disease generally caused by infection with gram-negative bacteria and characterized by gingival inflammation and alveolar bone resorption (1). Lipopolysaccharides (LPS) are cell-wall components of virtually all subgingival gram-negative organisms. *Porphyromonas gingivalis*, an oral, black-pigmented gram-negative bacterium, is mostly found in deep periodontal pockets and particularly in active disease sites (2). *P. gingivalis* LPS is an important pathogenic component for the initiation and development of periodontal disease because bacterial LPS is a potent simulator of inflammatory cytokine production and bone resorption (3). One target of LPS is gingival fibroblasts, which play an important role in periodontal soft-tissue remodeling (4). Gingival fibroblasts may also function as regulators of the cytokine network in periodontal tissues because they produce several types of cytokines when stimulated with inflammatory cytokines or bacterial cell components (5).

Cigarette smoking is one of the most important environmental risk factors for periodontitis (6), as more clinical attachment loss and bone loss have been observed in smokers than in non-smokers (7). Moreover, smoking may be responsible for a less favorable outcome after periodontal treatment and may more frequently cause disease progression despite a strict periodontal maintenance care program (8). Nicotine is the major constituent of the particulate phase of tobacco smoke and its most cytotoxic and vasoactive substance. Although the pathogenesis of tobacco-related periodontal disease is poorly understood, *in-vitro* studies have demonstrated that nicotine inhibits neutrophil/monocyte defensive functions (9), potentiates LPS-stimulated human peripheral blood monocyte secretion of prostaglandin E_2 (PGE_2) and has direct adverse effects on various functions of periodontal ligament cells (PDLCs) (10) and human gingival fibroblasts (HGFs) (11,12). We previously reported that induction of cellular antioxidants and phase II enzymes contribute to the cellular defense mechanisms against nicotine-induced cytotoxicity and osteoclastic differentiation in PDLCs (13). In addition, we demonstrated that endoplasmic reticulum stress contributes to nicotine-induced cell necrosis and periodontal connective tissue destruction via MAPK, phosphatidylinositol 3-kinase (PI3K) and nuclear factor-kappa B (NF-κB) pathways in PDLCs (14).

The combination of nicotine and LPS stimulates the formation of osteoclast-like cells by increasing macrophage colony-stimulating factor and PGE_2 production in osteoblasts (15); increasing PGE_2 and cyclooxygenase-2 (COX-2) expression in osteoblasts (16); affecting the expression of cytokines such as interleukin (IL)-7, IL-10, IL-15, regulated on activation, normal, T-cell expressed, and secreted (RANTES) and interferon gamma in HGFs (17); and synergistically up-regulating IL-6 production in HGFs (18). We recently reported that nicotine and LPS synergistically induce the production of nitric oxide and PGE_2 and increase inducible nitric oxide synthase and COX-2 expression in PDLCs (19). Furthermore, we showed that apigenin significantly inhibits nicotine- and LPS-induced production of nitric oxide, PGE_2, IL-1β, tumor necrosis factor alpha (TNF-α), IL-6, IL-12 and the up-regulation of inducible nitric oxide synthase and COX-2 in PDLCs (20).

Sirtuin 1 (SIRT1) is a nicotinamide adenine dinucleotide (NAD^+)-dependent class III protein deacetylase involved in cell growth, differentiation, stress resistance, reduction of oxidative damage and metabolism (21–23). Several recent studies have implicated SIRT1 in the regulation of inflammatory responses. SIRT1 over-expression leads to suppression of the inflammatory response, whereas deletion of the protein in hepatocytes results in increased local inflammation (24). SIRT1 over-expression and addition of the SIRT1 agonist, resveratrol, markedly reduces NF-κB signaling stimulated by amyloid beta and has strong neuroprotective effects on microglial cells (25) as a result of the presence of TNF-α. Furthermore, SIRT1 over-expression has cytoprotective effects against nonsmall-cell lung cancer (26) and damage induced by cigarette smoke, and anti-inflammatory effects in macrophages *in vitro* as well as in rat lung (27). In contrast, the SIRT1 inhibitor, sirtinol, attenuates antigen-induced airway inflammation and hyper-responsiveness (28). Similarly, SIRT1 small interfering RNA knockdown and treatment with sirtinol result in a reduction of proinflammatory cytokines in LPS-stimulated rheumatoid arthritis synovial fibroblasts (29) and in TNF-α- and IL-1β-stimulated human dermal microvascular endothelial cells (30). Resveratrol shows antimicrobial properties against periodontal pathogens *in vitro* (31), inhibits vascular endothelial growth factor production in periodontal pathogen-stimulated HGFs (32), inhibits *P. gingivalis* LPS-induced endothelial adhesion molecule expression in endothelial cells (33) and reverses osteogenesis inhibition by smoke-derived aryl hydrocarbons and bacterial LPS in rat bone-marrow cells (34). However, whether SIRT1 activation or inhibition exerts any anti-inflammatory or proinflammatory effects in HGFs is not fully understood. The present study investigated the effects of SIRT1 activation on nicotine and LPS-induced cytotoxicity and inflammatory mediator production and its possible signals in HGFs, which are the major constituents of gingival connective tissue that may directly interact with smoke, bacteria and bacterial products, including LPS, in periodontitis lesions (4).

Material and methods

Reagents

LPS (from *P. gingivalis*) and nicotine were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). A PGE_2 ELISA kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). TNF-α and an IL-1β OptELA set were obtained from eBioscience (San Diego, CA, USA). Affinity purified mouse SIRT-1 monoclonal antibody (mAb), mouse anti-COX-2 mAb, goat anti-mouse IgG horseradish peroxidase (HRP), and goat anti-rabbit IgG HRP Ab were purchased from Abcam (Cambridge, MA, USA) and Cayman

Chemical (Ann Arbor, MI, USA). Polyclonal antibodies against mouse inhibitor of NF-κB (IκB-α) and NF-κB p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-ERK, ERK, phospho-p38, p38, phospho-JNK and JNK were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

We used the immortalized HGF cell line by transfection with the E6/E7 open reading frames of human papillomavirus type 16, following a method described previously (35). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Primary cultured HGFs were obtained via biopsies of attached gingiva from sound premolar and permanent molar teeth of healthy subjects, as described previously (36). Informed consent, based on an appropriate protocol, was obtained from the donors. This protocol was reviewed and approved by the Institutional Review Board. Each sample of gingival tissue was cut into small pieces and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HGFs growing from the explanted tissue were subcultured. Primary HGFs from passages 4–6 were used.

Immortalized or primary HGFs were seeded in culture plates and cultured in Dulbecco's modified Eagle's medium containing fetal bovine serum for 3 d until 70% confluent. Then, the cells were treated with nicotine and LPS for the indicated time periods in the presence of serum. All experiments were conducted three times, with consistent results obtained each time.

Construction and preparation of the recombinant adenovirus

Wild-type SIRT1 or dominant-negative SIRT1-H363Y, expressing adenovirus and LacZ-bearing adenovirus, have been described previously (36). Recombinant adenoviruses were amplified in human embryonic kidney (HEK-293) cells and purified using the Vivapure AdenoPACK kit (Sartorius AG, Göttingen, Germany), according to the manufacturer's instructions.

Cell viability

Cytotoxicity was determined using the 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells seeded on 96-well microplates at 1×10^4 cells/well were incubated with nicotine and LPS for the indicated time period. Medium was removed and the cells were incubated with 100 μL of MTT assay solution for 4 h. Absorbance was measured in an ELISA reader at 595 nm. Data are presented as a percentage of the cell viability in control wells. Each set of data was collected from multiple replicate wells of each experimental group ($n = 5$).

Determination of reactive oxygen species production

Intracellular reactive oxygen species (ROS) were measured using dihydrodichlorofluorescein diacetate (Molecular Probes, Eugene, OR, USA). Trypsinized cells were suspended in phosphate-buffered saline containing 2% bovine serum albumin. Dihydrodichlorofluorescein diacetate was added (final concentration, 20 μM), and the cells were incubated for 30 min at 37°C in a CO_2 incubator. After centrifugation (500 *g*, 5 min), washing and resuspension in 2% bovine serum albumin/phosphate-buffered saline, the cells were analyzed using the FL-1H channel on a FACSCalibur cytometer (BD Biosciences, Sparks, MD, USA). The geometric mean of the peak was calculated using CellQuest Pro software (BD Biosciences) and corrected for cellular background fluorescence.

RNA isolation and RT-PCR

HGF total RNA was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. Then, 1 μg of RNA was reverse-transcribed for first-strand complementary DNA synthesis (Gibco-BRL, Rockville, MD, USA). The complementary DNA was amplified in a final volume of 20 μL containing 2.5 mM $MgCl_2$, 1.25 units of Ex Taq polymerase (Bioneer, Daejeon, Korea) and 1 μM specific primers. Amplification was carried out for 30 cycles in a DNA thermal cycler. Primer sequences for the differentiation markers are shown in Table 1. The PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

Western blot analysis

Cells (1×10^6) from each set of experiments were harvested and washed twice in cold Tris-buffered saline, then solubilized in ice-cold 1% Triton X-100 lysis buffer. After 30 min on ice, the lysates were clarified by centrifugation. Proteins (20 μg) were resolved by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel (10% acrylamide), transferred to nitrocellulose membranes and probed with specific antibodies (diluted 1 : 1000), followed by incubation with secondary horseradish peroxidase-conjugated antibody (diluted 1 : 5000). Proteins were detected with an enhanced chemiluminescence system, used according to the manufacturer's instructions, and then exposed to X-rays.

ELISA

The concentrations of TNF-α and IL-1β in culture supernatants were determined using an OptELA set, according to the manufacturer's procedure. The PGE_2 concentrations in culture supernatants were determined using an ELISA kit, according to the manufacturer's procedure. The plates were read at 450 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Differences among the groups were analyzed using one-way analysis of

Table 1. RT-PCR primers and conditions

Gene	Sequence (5′–3′)	Size(bp)	Tm(°C)
SIRT1	Forward: TCAGTGTCATGGTTCCTTTGC Reverse: AATCTGCTCCTTTGCCACTCT	*820*	*55*
COX2	Forward: ATTGACCAGAGCAGGCAGAT Reverse: CAGGATACAGCTCCACAGCA	163	55
IL17	Forward: CGATGACTCCTGGGAAGACCTC Reverse: GTGTGGGCTCCCCAGAGCTCTTA	820	60
TNFα	Forward: CTCTFFCCCAFFCAFTCAGA Reverse: CTCTFFCCCAFFCAFTCAGA′	519	60
IL1β	Forward: TGGAGATGACAGTTCAGAAG Reverse: GTACTGGTGCCGTTTATGC	288	60
IL6	Forward: ATGAACTCCTTCTCCACAAGC Reverse: CTACATTTGCCGAAGAGCCC	347	60
IL8	Forward: ATGACTTCCAAGCTGGCCGTGGCT Reverse: TCTCAGCCCTCTTCAAAAACTTCTC	289	62
β-actin	Forward: CATGGATGATGATATCGCCGCG Reverse: ACATGATCTGGGTCATCTTCTCG	371	55

COX2, cyclooxygenase-2; *IL*, interleukin; *SIRT1*, sirtuin 1; *TNFα*, tumor necrosis factor alpha; Tm, melting temperature.

variance combined with Duncan's multiple range test. A p-value of < 0.05 was considered significant.

Results

Effects of LPS and nicotine on cytotoxicity and SIRT1 expression

The effect of nicotine and LPS on the viability of immortalized HGFs was initially measured using the MTT assay (Fig. 1A and 1B). As maximal cytotoxicity was achieved with 1 μg/mL of LPS only (data not shown), 1 μg/mL of LPS and 0–10 mM nicotine were incubated with HGFs for 24 h. Combined treatment of cells with 1 μg/mL of LPS and nicotine increased cytotoxicity in a dose- and time-dependent manner, compared with that in the untreated-control, LPS-only and nicotine-only groups (Fig. 1A and 1B).

As SIRT1 is down-regulated by a cigarette smoke extract in macrophages (27), we next assessed whether nicotine and LPS could down-regulate or up-regulate SIRT1 expression. As shown in Fig. 1C and 1D, the expression of *SIRT1* mRNA and SIRT1 protein, induced by nicotine or LPS alone, was comparable with that in the control. In contrast, combining LPS with nicotine had a synergistic effect on SIRT1 expression in HGFs. Because maximal expression of *SIRT1* mRNA and SIRT1 protein was achieved in HGFs after 24 h of incubation with 1 μg/mL of LPS and 5 mM nicotine, 1 μg/mL of LPS and 5 mM nicotine was used in all subsequent experiments.

Effects of resveratrol and adenovirus-mediated SIRT1 overexpression on LPS- and nicotine-stimulated cytotoxicity, PGE_2 production and COX-2 expression

The SIRT1 inducer resveratrol was used to study the relationships among SIRT1 expression, LPS- and nicotine-induced cytotoxicity, and PGE_2 production. Resveratrol (25 mM) is largely nontoxic and was chosen for experiments in which HGFs were exposed to nicotine and LPS. The results of the MTT assay showed that resveratrol suppressed nicotine and LPS-induced cytotoxicity (Fig. 2A). Resveratrol inhibited LPS- and nicotine-induced expression of *COX2* mRNA and *COX-2* protein and PGE_2 production (Fig. 2B and 2C). As ROS are known to contribute to inflammatory

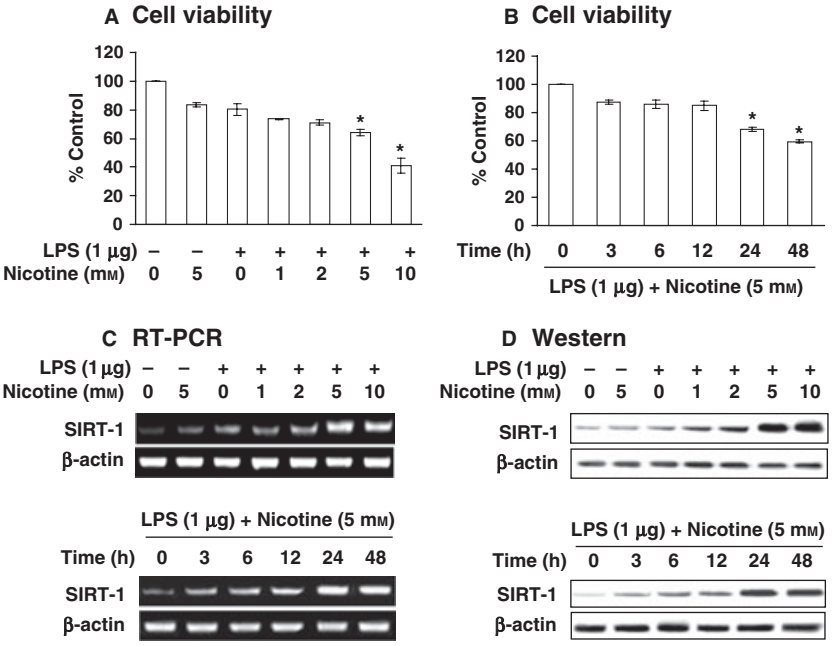

Fig. 1. Effects of nicotine and lipopolysaccharide (LPS) on cell viability (A, B) and on the expression of sirtuin 1 (SIRT*1*) mRNA (C) and SIRT1 protein (D) in human gingival fibroblasts (HGFs). Cells were treated for 24 h with the indicated concentrations of nicotine (A, D) and with a single concentration of nicotine (5 mM) for the indicated periods of time (B, D). Cell viability, mRNA expression and protein expression were determined using the 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, RT-PCR and western blot analyses, respectively. Data were obtained from three independent experiments. Values are mean ± standard deviation ($n = 5$). *Significant difference compared with the control group ($p < 0.05$). % Control = percentage of the cell viability in test cells compared with that of control cells (100%).

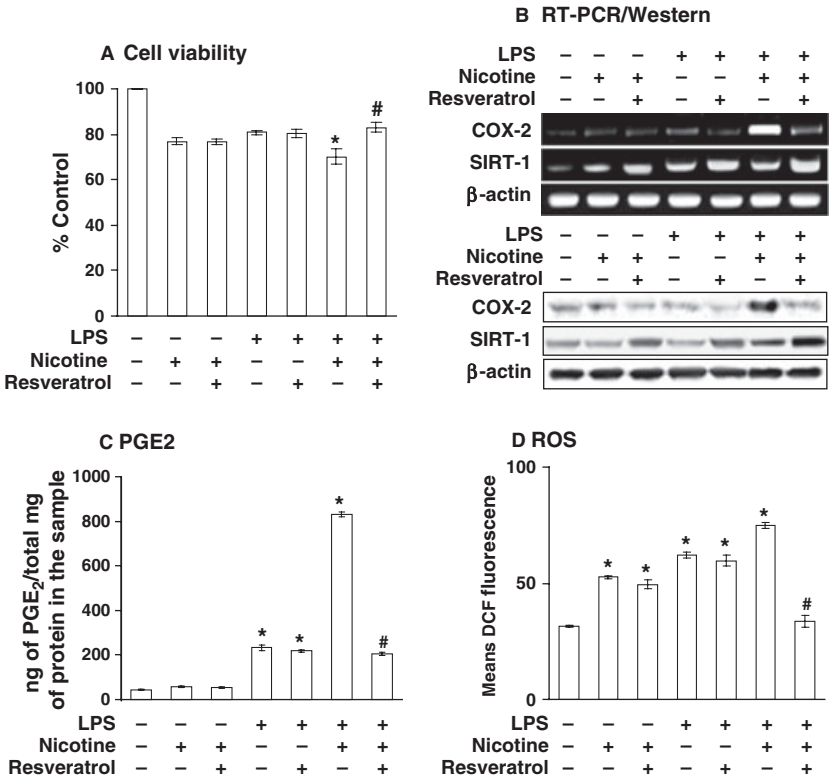

Fig. 2. Effect of resveratrol on lipopolysaccharide (LPS)- and nicotine-induced cytotoxicity (A), expression of cyclooxygenase-2 (COX-2), and sirtuin 1 (SIRT1) mRNA and protein (B), secretion of prostaglandin E_2 (PGE_2) (C), and the production of reactive oxygen species (ROS) (D). Cells were pretreated with 25 μM resveratrol (a SIRT1 activator) for 1 h and then stimulated with LPS and nicotine for an additional 24 h. Cytotoxicity, ROS production and expression of PGE_2 mRNA and protein were determined using the 3-(4, 5-;dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry analysis, RT-PCR, and western blotting and ELISA, respectively. A representative of three separate experiments is shown. *Significant difference compared with the control group ($p < 0.05$, $n = 4$); #significant difference compared with the LPS- and nicotine-treated group ($p < 0.05$, $n = 4$). % Control = percentage of the cell viability in test cells compared with that of control cells (100%).

reactions, changes in ROS levels were examined in HGFs. Pretreatment with resveratrol almost prevented the elevation in ROS level in LPS- and nicotine-stimulated HGFs (Fig. 2D).

HGFs were infected with LacZ- and wild-type-SIRT1-expressing adenoviruses and then treated with nicotine and LPS to determine the protective and anti-inflammatory roles of SIRT1 in nicotine- and LPS-induced cytotoxicity and PGE_2 production. As shown in Fig. 3A–C, the nicotine- and LPS-induced increases in cytotoxicity, PGE_2 production and levels of *COX2* mRNA and *COX-2* protein were inhibited by recombinant adenovirus encoding SIRT1 (Ad-SIRT1). However, cytotoxicity, PGE_2 production and expression of COX2 mRNA and COX-2 protein were not affected in LacZ over-expressing cells compared with those treated with nicotine and LPS (Fig. 3B and 3C). SIRT1 overexpression also inhibited LPS- and nicotine-induced ROS production, whereas expression of control LacZ had no such effect (Fig. 3D).

Effects of resveratrol and adenovirus-mediated SIRT1 overexpression on LPS- and nicotine-stimulated proinflammatory mediators in HGFs

RT-PCR and ELISA analyses were performed to determine the inhibitory effects of SIRT1 activation on proinflammatory mediators such as TNF-α, IL-1β, IL-6, IL-8 and IL-17, which play important roles in the pathogenesis of periodontitis by causing inflammation and destruction of periodontal tissue and resorption of alveolar bone (37,38). Pretreatment of HGFs with resveratrol and Ad-SIRT1 inhibited nicotine- and LPS-induced expression and secretion of *TNFα* and *IL1β* mRNAs (Fig. 4A and 4B). Furthermore, pretreatment with resveratrol and Ad-SIRT1 abolished nicotine- and LPS-mediated induction of *IL6*, *IL18* and *IL17* mRNAs (Fig. 4B). In addition, these osteoclastic cytokines were blocked in resveratrol- and Ad-SIRT1-treated HGFs compared with those in the nicotine-only and LPS-only groups.

Effects of SIRT1 activation on LPS- and nicotine-stimulated cytotoxicity, PGE_2 production and COX-2 expression in primary cultured HGFs

We examined the effects of SIRT1 activation by resveratrol and Ad-SIRT1 in primary cultured HGFs to confirm that HGF transformation with the human papillomavirus constructs did not cause a nonphysiological response. SIRT1 induction by resveratrol and Ad-SIRT1 in primary cultured HGFs inhibited LPS- and nicotine-induced cytotoxicity, expression of *COX2* mRNA and COX-2 protein, PGE_2 secretion and ROS production (Fig. 5A–D).

Effects of resveratrol and adenovirus-mediated SIRT1 overexpression on LPS- and nicotine-stimulated HGFs

To elucidate the pathway underlying the effects of SIRT1 activation by resveratrol and Ad-SIRT1, activation of NF-κB, a known SIRT1 target, was determined by western blotting. As shown in Fig. 6A, IκB-α was degraded after treatment with LPS and nicotine for 1 h, and this degradation was markedly inhibited by pretreatment with resveratrol and Ad-SIRT1. Consistently, resveratrol and Ad-SIRT1 had significant inhibitory

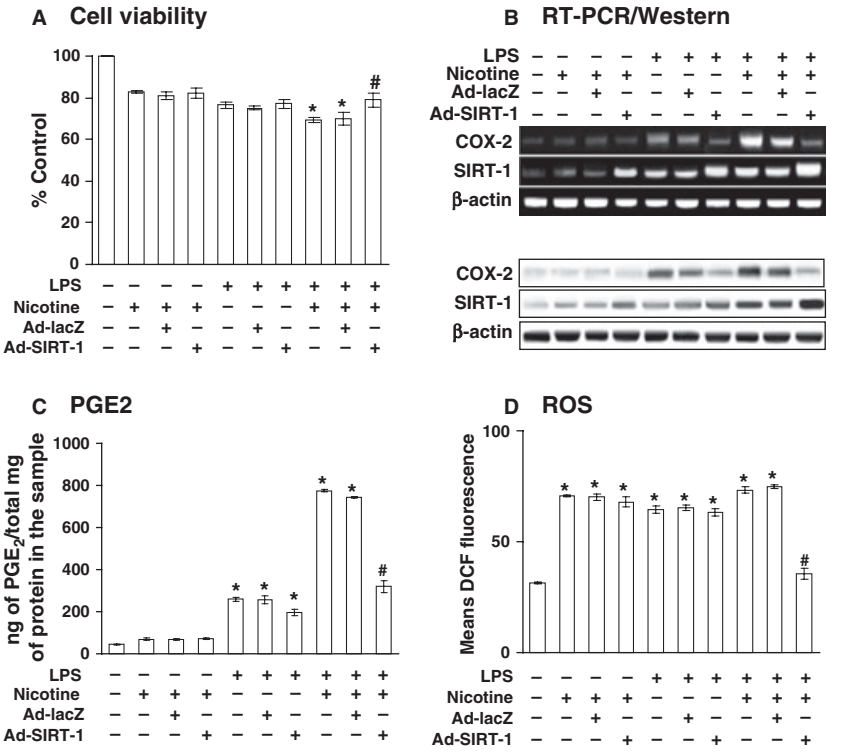

Fig. 3. Effect of sirtuin 1 (SIRT1) over-expression on lipopolysaccharide (LPS)- and nicotine-induced cytotoxicity (A), expression of cyclooxygenase-2 (COX-2) and SIRT1 mRNA and protein (B), prostaglandin E_2 (PGE_2) secretion (C) and reactive oxygen species (ROS) production (D). Cells were infected with 1×10^9 plaque-forming units of SIRT1 or lacZ adenovirus (Ad-SIRT1 and Ad-lacZ, respectively) for 12 h and then exposed to LPS and nicotine for 24 h. *Significant difference compared with the control group ($p < 0.05$, $n = 4$); #significant difference compared with the LPS and nicotine group ($p < 0.05$, $n = 4$). Representative PCR results and immunoblots of three independent experiments with similar results are shown. % Control = percentage of the cell viability in test cells compared with that of control cells (100%). Dichlorodihydrofluorescein (DCF) .

effects on LPS- and nicotine-induced nuclear NF-κB p65.

To explore, more closely, the upstream signaling pathway involved in SIRT1 activation-mediated NF-κB inhibition, activation of protein kinase C (PKC), PI3K and MAPKs were examined in HGFs. Nicotine and LPS also significantly stimulated PKC, PI3K, p38 MAPK, ERK and JNK phosphorylation or activation, which was inhibited by pretreatment with resveratrol and Ad-SIRT1 but not by Ad-LacZ (Fig. 6B). Furthermore, PKC, PI3K and MAPK activation by nicotine and LPS only was decreased by SIRT1 activation.

Discussion

Nicotine is absorbed rapidly through the skin and oral mucous membranes and is present on the root surface of periodontally diseased teeth in smokers (39). Nicotine levels of 0.43–9.6 mM are found in the saliva of smokeless tobacco users (40). Cotinine is the first-stage metabolite of nicotine and is a toxic alkaloid that stimulates autonomic ganglia and the central nervous system in humans (41). The choice of nicotine and not its metabolite, continine, was based on the observation that 70–80% of nicotine is converted to continine in the liver (41). Furthermore, nicotine can directly reach HGFs in an inflamed periodontium because the epithelial barrier of the inflamed gingiva may be more disorganized and may have widened intercellular spaces (42).

Immortal human cell lines have been produced by transformation with telomerase or viral oncogenes, such as human papillomavirus 16, to overcome the limited lifespan of primary cultured human cells (35). In this study, we used a human immortalized HGF cell line, which had a normal cellular phenotype and is a valuable tool for studying the biological characterization, repair and regeneration of human dental and periodontal tissues (43).

The Sir2 family of proteins (sirtuins) comprises NAD^+-dependent histone/protein deacetylases that tightly couple the cleavage of NAD^+ and the deacetylation of protein substrates to form nicotinamide, the deacetylated product, and a novel metabolite, 2′-*O*-acetyl-ADP-ribose (44). SIRT1, the mammalian ortholog of Sir2, is a nuclear protein that occupies a privileged position in the cell and governs critical metabolic and physiological processes (21–23). SIRT1 helps cells to be more resistant to oxidative and radiation-induced stress (45), promotes fat mobilization from white adipose tissue, which contributes to extension of the lifespan (46), and mediates the metabolism of energy sources in metabolically active tissues. SIRT1 enzymatic activity preferentially targets histone H3 at Lys9 and Lys14 and histone H4 at Lys16 in chromatin (44). In addition, many nonhistone proteins, including p53, forkhead box protein O3 (FOXO3), peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) and liver X receptor (LXR), are regulated by SIRT1-mediated deacetylation, emphasizing the pivotal function that this regulator plays in cellular control and responses (39,47).

SIRT1 is down-regulated by cigarette smoke in macrophages (27) and by cytokines in pancreatic beta-cells (48). In contrast, SIRT1 is activated in response to oxidative stress, DNA damage, nitric oxide, cyclic guanosine monophosphate, adiponectin and ionomycin (49). Moreover, resveratrol-induced SIRT1 activation and adenoviral-mediated SIRT1 over-expression block the expression and release of proinflammatory cytokines in response to environmental stressors (24,27), suggesting that SIRT1 plays a critical role in protecting the host

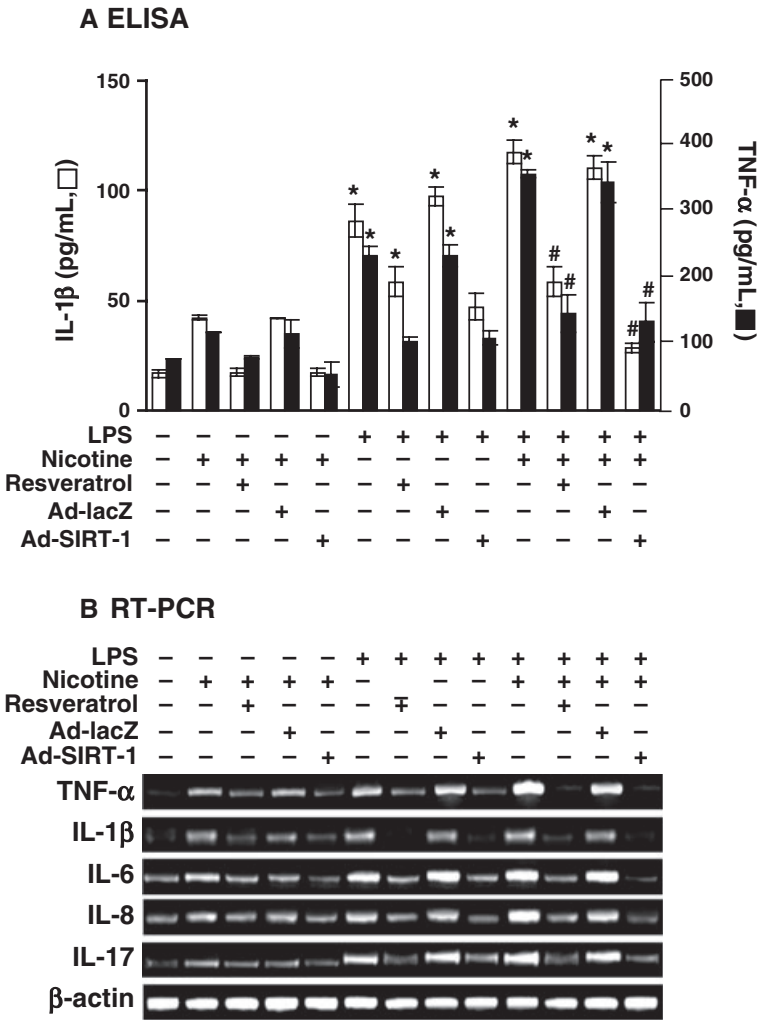

Fig. 4. Effect of sirtuin 1 (SIRT1) activation by resveratrol and SIRT1 adenovirus infection on nicotine and lipopolysaccharide (LPS)-induced proinflammatory mediators. Immortalized human gingival fibroblasts (HGFs) were pretreated with resveratrol for 1 h and with 1×10^9 plaque-forming units of SIRT1 or lacZ adenovirus (Ad-SIRT1 and Ad-lacZ, respectively) for 12 h and then exposed to LPS plus nicotine for 24 h. Cytokines were quantified by ELISA (A) and by RT-PCR) (B). Representative PCR data of three independent experiments with similar results are shown; *significant difference compared with the control group ($p < 0.05$, $n = 4$); #significant difference compared with the LPS and nicotine groups ($p < 0.05$, $n = 4$). IL, interleukin; TNF-α, tumor necrosis factor alpha.

during periodontal inflammation. The PI3K, PKC, MAPK and NF-κB pathways regulate cytokine production in various cell types (20,23). These signaling pathways in turn activate a variety of transcription factors, such as NF-κB, which are implicated in the induction of SIRT1 expression (25,27). Therefore, we hypothesized that SIRT1 activation would exert anti-inflammatory effects in HGFs and that these effects would enhance cytoprotection and antioxidation. Consistent with this idea, the present study used two different approaches (using an SIRT1 activator and Ad-SIRT1) to reveal a novel role for SIRT1 in the regulation of intracellular proinflammatory responses and in diminishing cytotoxicity in HGFs.

Among the inflammatory mediators involved in periodontitis, PGE_2 is associated with periodontitis as a potent stimulator of bone resorption, and increased PGE_2 levels have been reported in gingival tissue and gingival fluid from patients with periodontitis (50,51). Moreover, administration of nonsteroidal anti-inflammatory drugs, which are PGE_2 inhibitors, reduces the progression of alveolar bone resorption in patients with periodontitis, suggesting that PGE_2 is a key mediator in the pathogenesis of periodontal disease (52). In addition, TNF-α, IL-1β, IL-6, IL-9 and IL-17 are also involved in the regulation of inflammation and periodontal disease (37,38). These findings suggest that local inhibition of proinflammatory enzymes and cytokines may be a successful approach to reduce bone resorption in patients with periodontitis.

In the present study, LPS and nicotine induced the production of PGE_2 and the expression levels of COX-2 in HGFs, which is in agreement with our previous reports on PDLCs (19,20). Moreover, the present study is the first to demonstrate that the expression of *SIRT1* mRNA and SIRT1 protein increases with both nicotine and LPS in HGFs. However, because up-regulation of SIRT1 genes occurred in a time-dependent manner, which peaked after 24 h of incubation, we can rule out the possibility that this response was caused by chronic stress, such as serum deprivation. As serum starvation or stimulation can affect the levels of SIRT1 protein or *SIRT1* mRNA (53,54), we did not use a serum-starvation condition in our experimental models.

The involvement of SIRT1 in the antioxidant activity of SIRT1 activation was confirmed by resveratrol- and Ad-SIRT1-mediated over-expression, which significantly blocked nicotine- and LPS-induced cell death and ROS generation. Our findings strongly indicate that the observed cytoprotective effects of SIRT1 activation are caused by SIRT1 expression. Furthermore, SIRT1 activation by resveratrol and Ad-SIRT1 suppressed COX-2 expression in LPS- and nicotine-stimulated HGFs, thereby inhibiting COX-2-derived production of PGE_2. In addition, SIRT1 activation inhibited

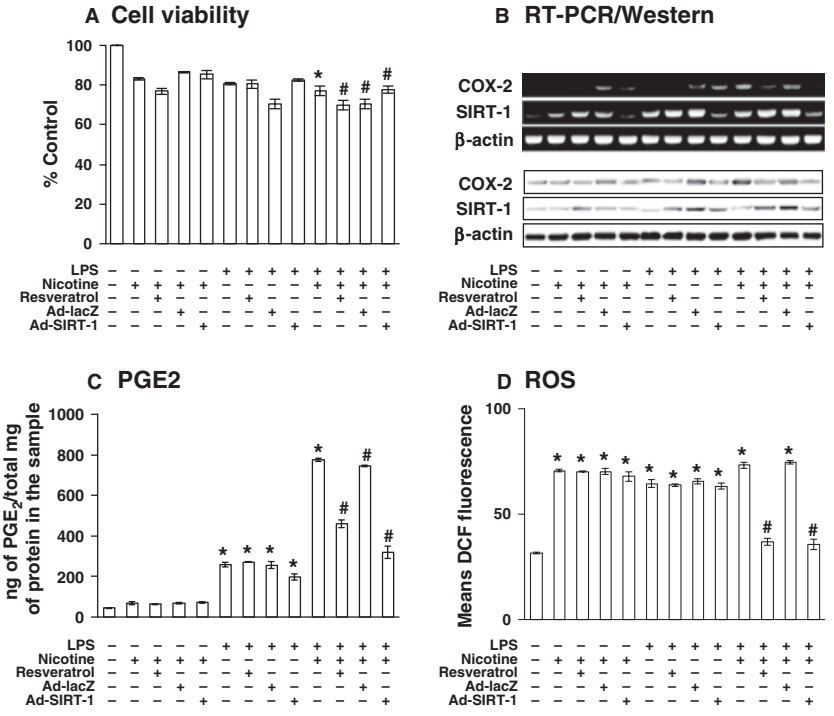

Fig. 5. Effect of sirtuin 1 (SIRT1) activation by resveratrol and recombinant adenovirus encoding SIRT1 (Ad-SIRT1) or lacZ (Ad-LacZ) on nicotine- and lipopolysaccharide (LPS)-induced cytotoxicity (A), expression of cyclooxygenase-2 (COX-2) and SIRT1 mRNA and protein (B), prostaglandin E_2 (PGE_2) secretion (C) and reactive oxygen species (ROS) production (D) in primary cultured human gingival fibroblasts (HGFs). Representative PCR and western blot data of three independent experiments with similar results are shown. *Significant difference compared with the control group ($p < 0.05$, $n = 4$); #significant difference compared with the LPS and nicotine groups ($p < 0.05$, $n = 4$). % Control = percentage of the cell viability in test cells compared with that of control cells (100%). DCF.

LPS- and nicotine-induced TNF-α and IL-1β production as well as the expression of *TNFα*, *IL1β*, *IL6*, *IL9* and *IL17* mRNAs. These findings suggest that SIRT1 activation in LPS- and nicotine-stimulated HGFs exerts anti-inflammatory and cytoprotective effects by limiting proinflammatory enzyme expression and secretion of proinflammatory cytokines. These results agree with those of a previous report that resveratrol and SIRT1 adenovirus have cytoprotective and anti-inflammatory effects on intestinal cells (55), microglial cells (25), macrophages *in vitro*, rat lung (27) and 3T3-L1 cells (56).

In the present study, nicotine or LPS alone did not significantly affect cytotoxicity, COX-2-derived PGE_2 production or SIRT1 expression in HGFs. However, the combination of nicotine and LPS synergistically up-regulated the production of cytokines and PGE_2 and the expression of SIRT1. These results could be explained by different culture systems, cell types, concentrations and sources of LPS or nicotine. We expected that activation of SIRT1 by resveratrol and Ad-SIRT would result in anti-inflammatory and cytoprotective effects. However, these results were obtained after the incubation of HGFs with the combination of nicotine and LPS but not after incubation with nicotine or LPS alone. The reasons for the difference in low cytotoxicity and basal release of cytokines by nicotine or LPS alone are unclear but may reflect a differential SIRT1 activation response on cytoprotective and anti-inflammatory effects by HGFs.

Whether resveratrol possesses therapeutic value for treating a variety of inflammatory diseases is unknown (24–27). Given the need for new periodontal disease drugs and in view of the data presented here, SIRT1 activation may be considered for the development of periodontal therapy. However, SIRT1 activity consumes NAD^+, and as such has the potential to deplete cellular energy. SIRT1 activity may be beneficial or detrimental, depending on the magnitude of SIRT1 activity and the cellular energy state (57). Further research and more clinical studies are necessary to ensure the safety of resveratrol and to ascertain the optimum doses for preventing and treating periodontal disease.

The NF-κB signal-transduction pathway regulates SIRT1 expression and activity (25,27). In the present study, resveratrol and SIRT1 over-expression blocked LPS- and nicotine-induced PI3K, PKC, p38, ERK, JNK, MAPK and NF-κB activation, suggesting that PI3K, PKC, MAPK and NF-κB are involved in these responses. However, the precise mechanisms by which SIRT1 expression modulates these activities need further elucidation.

In conclusion, this study is the first to demonstrate that SIRT1 activation and over-expression has potent cytoprotective and anti-inflammatory effects against nicotine- and LPS-induced cytotoxicity, ROS generation, and proinflammatory cytokine production in HGFs, possibly through the PI3K, PKC, MAPK, and NF-κB pathways. These results suggest that SIRT1 activation may be useful for inhibiting inflammation in periodontal disease.

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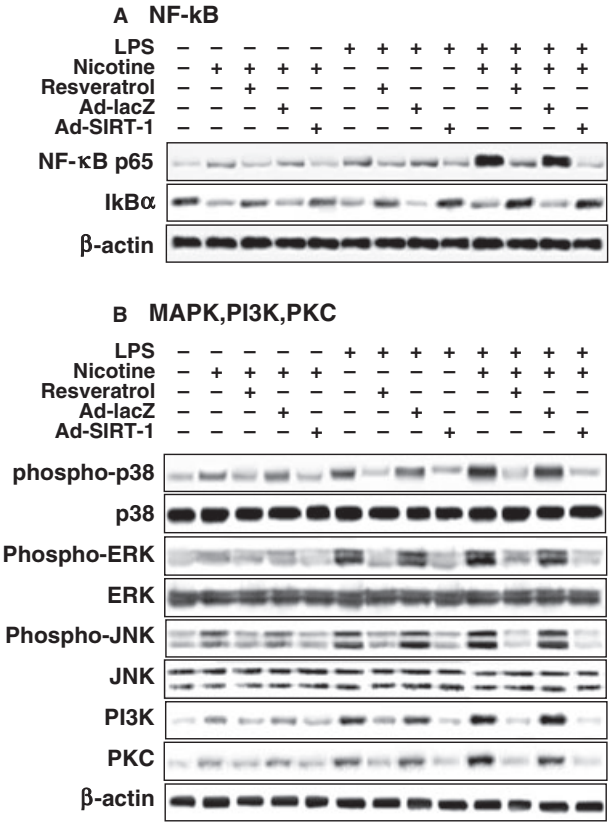

Fig. 6. Effect of sirtuin 1 (SIRT1) activation by resveratrol and adenovirus encoding SIRT1 (Ad-SIRT-1) and lacZ (Ad-lacZ) on nicotine- and lipopolysaccharide (LPS)-induced nuclear factor-kappaB (NF-κB) and IκBα (A), and on MAPK, phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) activation (B). Cells were pretreated with the proinflammatory mediators. Immortalized human gingival fibroblasts (HGFs) were pretreated with resveratrol for 1 h and with 1×10^9 plaque-forming units of SIRT1 or lacZ adenovirus for 12 h and then exposed to LPS and nicotine for 60 min. Representative immunoblots of three independent experiments with similar results are shown.

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