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Endoplasmic reticulum stress modulates nicotine-induced extracellular matrix degradation in human periodontal ligament cells

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Background and Objective: Tobacco smoking is considered to be one of the major risk factors for periodontitis. For example, about half the risk of periodontitis can be attributable to smoking in the USA. It is evident that smokers have greater bone loss, greater attachment loss and deeper periodontal pockets than non-smoking patients. It has recently been reported that endoplasmic reticulum (ER) stress markers are upregulated in periodontitis patients; however, the direct effects of nicotine on ER stress in regard to extracellular matrix (ECM) degradation are unclear. The purpose of this study was to examine the effects of nicotine on cytotoxicity and expression of ER stress markers, selected ECM molecules and MMPs, and to identify the underlying mechanisms in human periodontal ligament cells. We also examined whether ER stress was responsible for the nicotine-induced cytotoxicity and ECM degradation.

Material and Methods: Cytotoxicity and cell death were measured by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide assay and flow cytometric annexin V and propidium iodide staining. The mRNA and protein expressions of MMPs and ER markers were examined by RT-PCR and western blot analysis.

Results: Treatment with nicotine reduced cell viability and increased the proportion of annexin V-negative, propidium iodide-positive cells, an indication of cell death. Nicotine induced ER stress, as evidenced by survival molecules, such as phosphorylated protein kinase-like ER-resident kinase, phosphorylated eukaryotic initiation factor-2α and glucose-regulated protein-78, and apoptotic molecules, such as CAAT/enhancer binding protein homologous protein (CHOP). Nicotine treatment led to the downregulation of ECM molecules, including collagen type I, elastin and fibronectin, and upregulation of MMPs (MMP-1, MMP-2, MMP-8 and MMP-9). Inhibition of ER stress by salubrinal and transfection of CHOP small interfering RNA attenuated the nicotine-induced cell death, ECM degradation and production of MMPs. Salubrinal and CHOP small interfering RNA inhibited the effects of nicotine on the activation of Akt, JNK and nuclear factor-κB.

Conclusion: These results indicate that nicotine-induced cell death is mediated by the ER stress pathway, involving ECM degradation by MMPs, in human periodontal ligament cells.

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Smoking is a major environmental risk factor for the development and progression of periodontal disease, including gingivitis and periodontitis. It is associated with increased pocket depths, a loss of periodontal attachment and alveolar bone and a higher rate of tooth loss (1). Tobacco smoke contains over 4000 chemicals, which include nicotine, various nitrosamines, hydrogen cyanide, carbone monoxide, trace elements and a variety of poorly characterized substances (2). Nicotine is the major toxic component of cigarette smoke. Previous reports demonstrated that nicotine could be detected on the root surfaces of teeth, in the saliva (3) and in the gingival crevicular fluid of smokers (4). Furthermore, nicotine is a cytotoxic agent to gingival fibroblasts and periodontal ligament cells, inhibiting their viability, attachment, proliferation and matrix protein synthesis (5,6). We previously reported that the induction of cellular antioxidants and phase II enzymes contributes to the cellular defense mechanisms against nicotine-induced cytotoxicity and osteoclastic differentiation in periodontal ligament cells (7). Furthermore, we demonstrated that both nicotine- and lipopolysaccharide-induced inflammatory effects on periodontal ligament cells are mediated through the action of heme oxygenase-1 (8).

In periodontitis, a number of proteases degrade collagen and extracellular matrix (ECM). Matrix metalloproteinases (MMP) are the major group of enzymes responsible for degradation of ECM (9). The MMPs include the collagenases (MMP-1, MMP-9 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3 and MMP-10), membrane-associated MMPs (MMP-14, MMP-15, MMP-16 and MMP-17) and other MMPs (10,11). The expression and activity of MMPs in the noninflamed periodontium is low, but it is drastically enhanced to pathologically elevated levels in the presence of dental plaque and infection-induced periodontal inflammation (10,12). Suppression of MMPs has been proposed as a potential alternative therapeutic target for the treatment of periodontal tissue destruction in periodontitis (9,10). Cigarette smoke condensate increased the concentrations of MMP-2 and MMP-14 in gingival fibroblasts (13) and of MMP-1, MMP-8 and MMP-9 in human vascular endothelial cells (14). Nicotine treatment induced expression of MMP-1, MMP-2 and MMP-3 in normal human osteoblasts (15), MMP-14 and MMP-2 in human gingival fibroblasts (16) and MMP-1, MMP-2, MMP-3 and MMP-13 in human osteoblastic Saos-2 cells (17). However, the direct influence of nicotine on production of MMPs in periodontal ligament cells is not completely understood.

Endoplasmic reticulum (ER) stress is an essential adaptive cellular response to the accumulation of misfolded proteins and is induced by the quality-control system that ensures the transit of correctly folded proteins to the Golgi (18). Endoplasmic reticulum stress leads to the activation of genes possessing an unfolded protein response, which either improves local protein folding or results in cell death (19). Glucose-regulated protein 78/immunoglobulin heavy chain binding protein (GRP78/BiP) acts as the principal regulator of three ER-resident transmembrane proteins, PERK (double-stranded RNA-activated protein kinase-like ER kinase), IRE-1 (inositol requiring-1) and ATF6 (activating transcription factor 6), which are primary sensors and transducers of unfolded protein response (19). Endoplasmic reticulum stress is thought to be involved in the physiopathology of cardiovascular diseases, neurodegenerative diseases, diabetes mellitus, obesity and liver diseases (19). Recently, ER stress has been associated with the pathogenesis of periodontal disease, as the expression of unfolded protein response-related molecules was significantly higher in periodontitis compared with gingivitis (20). However, lipopolysaccharide from *P. gingivalis*, but not *E. coli* or interferon-γ, fails to upregulate ER stress gene expression in macrophages (20).

The effect of nicotine or cigarette smoke on ER stress remains unclear. Jorgensen *et al.* (21) reported that cigarette smoke induces ER stress and unfolded protein response in normal and malignant human lung cells. In contrast, nicotine was shown to inhibit the tunicamycin-induced expression of GRP78 during ER stress-mediated apoptosis in PC12 cells (22). Although these studies do not demonstrate uniform results, it is certain that ER stress is affected by nicotine. Therefore, the aim of this study was to investigate the effects of nicotine on ER stress markers, ECM and MMPs expression, in periodontal ligament cells and to examine the possible signaling pathways and molecular mechanisms involved in ER stress-regulated cell death, ECM and MMPs expression, as contributing to the phenotype of periodontitis.

This study aimed to investigate whether nicotine-treated human periodontal ligament cells could induce ER-stress-mediated cell death. We further examined whether ER stress could regulate nicotine-induced ECM degradation and production of MMPs, characteristics observed in periodontal disease.

Material and methods

Reagents

The eukaryotic initiation factor 2 α (eIF2α) inhibitor (salubrinal) was purchased from Calbiochem (San Diego, CA, USA). Lipofectamine 2000 was obtained from Invitrogen Life Technology (Grand Island, NY, USA). The small interfering RNA (siRNA) against CHOP, antibodies to phospho (p)-PERK, PERK, phosphorylated eukaryotic initiation factor 2, eukaryotic initiation factor 2, CHOP, GRP78, p-AKT, AKT, MMP-1, MMP-8, inhibitory κB-α (IκB-α), p65 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against MMP-2, MMP-9, p-ERK, ERK, p-p38, p38, p-JNK and JNK were purchased from Cell Signaling Technology (Beverly, MA, USA). Nicotine and other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Cell culture

We used the human periodontal ligament cell line immortalized by transfection with telomerase catalytic

subunit *hTERT* gene (23). Cells were cultured in α-minimal essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere of air supplemented with 5% CO_2 at 37°C.

Cell viability assay

The cytotoxicity of nicotine was determined by the 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells seeded on 96-well microplates at 1×10^4 cells per well were incubated with nicotine for the indicated time period. Medium was removed and then incubated with 100 μL MTT assay solution for 4 h. Absorbance was measured in an ELISA reader at 595 nm.

Flow cytometric analysis

Cells were analysed by annexin V (AV)–propidium iodide (PI) double staining. Briefly, treated cells were trypsinized and collected in fluorescence activated cell sorting (FACS) analysis tubes. Cells were stained with AV labeled to fluorescein isothiocyanate in combination with PI. For each experiment, untreated cells served as a negative control. The FACS results were analysed by CellQuest software (Becton-Dickinson, Franklin Lakes, NJ, USA).

RT-PCR

The total RNA of cells was extracted using the TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. Reverse transcription of RNA was performed using AccuPower RT PreMix (Bioneer, Daejeon, Korea). Thereafter, the RT-generated DNA (2–5 μL) was amplified using AccuPower PCR PreMix (Bioneer). Primer sequences are detailed in Table 1. The PCR products were subjected to electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

Transfection with CHOP siRNA

Small interfering RNA was used for transient gene knockdown studies. Human CHOP siRNA (catalog no. SC-35437) and control scrambled siRNA (catalog no. SC-37007) were purchased from Santa Cruz Biotechnology. The periodontal ligament cells were transfected with double-stranded siRNAs (100 nmol/mL) for 24 h using Lipofectamine 2000 (Gibco; Invitrogen Ltd, Paisley, UK) according to the manufacturer's instructions.

Western blotting

Cells were solubilized in ice-cold 1% Triton X-100 lysis buffer. After 30 min on ice, the lysates were clarified by centrifugation. Protein content was quantified using the Bradford method. Proteins (30 μg) were mixed with an equal volume of 2 × sodium dodecyl sulphate sample buffer, boiled for 5 min, and then resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (12% acrylamide) and transferred to nitrocellulose membranes. The membranes were blocked in 5% skimmed milk (1 h), rinsed, and probed with specific antibodies (diluted 1:1000) in Tris-buffered saline overnight at 4°C. Primary antibody was then removed by washing the membranes four times in Tris-buffered saline, and labeled by incubating with peroxidase-labeled secondary horseradish peroxidase-conjugated antibodies (diluted 1:2000) against mouse and rabbit for 1 h. Following three washes in Tris-buffered saline, protein bands were visualized by an enhanced chemiluminescence system according to the manufacturer's instructions (Amersham Corp., Arlington Heights, IL, USA) and exposed to X-ray film.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from cells as described previously (24). Oligonucleotides containing nuclear factor-κB (NF-κB) sequences (Promega, Madison, WI, USA) were 5′-end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Subsequent binding

Table 1. Primers and conditions for RT-PCR

Genes	Primer sequence (5′–3′)	Annealing temperature (°C)	Cycle number	Product size (bp)
MMP-1	Forward: 5′-GAAGGTGATGAAGCAGCCCAGATGT-3′ Reverse: 5′-CAGTTGTGGCCAGAAAACAGAAATG-3′	56	28	716
MMP-2	Forward: 5′-GCTGCTTATGAAGATTTTGACAGAG-3′ Reverse: 5′-ACAGCCACATTTGATTTTGCTTCAG-3′	56	28	374
MMP-8	Forward: 5′-ATCCGTGGTGAGATCTTCTTCTT-3′ Reverse: 5′-CAGGATACAGCTCCACAGCA-3′	60	35	366
MMP-9	Forward: 5′-TGCCAGTTTCCATTCATCTTCCAA-3′ Reverse: 5′-CTGCGGTGTGGTGGTGGTT-3′	56	32	519
Collagen I	Forward: 5′-GGACACAATGGATTGCAAGG-3′ Reverse: 5′-TAACCACTGCTCCACTCTGG-3′	54	30	461
Elastin	Forward: 5′-GTTGGTGTCGGCGTCCCTGG-3′ Reverse: 5′-AGCGGCTGCAGCTGGAGGTA-3′	60	35	345
Fibronectin	Forward: 5′-AGAAGTGGTCCCTCGGCCCC-3′ Reverse: 5′-GAGTCCCGGACCGTGTGGGT-3′	60	35	481
β-Actin	Forward: 5′-CATGGATGATGATATCGCCGCG-3′ Reverse: 5′-ACATGATCTGGGTCATCTTCTCG-3′	55	35	471

reactions were performed on ice for 15 min in a buffer consisting of 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 10% glycerol, 0.05% NP-40 and 2 μg/mL poly(dI-dC). Assay mixtures were incubated with the radiolabeled oligonucleotides for 30 min at room temperature. Following the addition of 6 × dye solution (0.1% bromophenol blue and 30% glycerol), mixtures were immediately electrophoresed on nondenaturing 6% polyacrylamide gels using 0.25 × Tris-Borate-EDTA. The gels were then dried in a vacuum drier at 80°C for 1 h and autoradiographed on Fuji RX X-ray films.

Statistical analysis

The data are expressed as means ± SD. Differences between groups were assessed by one-way ANOVA. A value of $p < 0.05$ was considered statically significant.

Results

Effects of nicotine on cytotoxicity and death in human periodontal ligament cells

We first assessed the effects of nicotine on periodontal ligament cell viability via MTT assay. Periodontal ligament cells exposed to different concentrations of nicotine for various lengths of time showed a concentration- and time-dependent reduction in cell viability compared with control cells (Fig. 1A). To elucidate whether nicotine inhibits the proliferation of periodontal ligament cells through the induction of apoptosis or cell death, we examined AV and PI dual staining by flow cytometry. The cells were exposed to 10 mM nicotine for 1 d; 10 mM represents the maximal physiological level in saliva (3,4). Intact viable (AV−/PI−), early apoptotic (AV+/PI−), late apoptotic (AV+/PI+) and necrotic cells (AV−/PI+) were distinguished based on double labeling for AV–fluorescein isothiocyanate and PI, a membrane-impermeable DNA stain, as previously described (25). As shown in Fig. 1B, nicotine treatment (10 mM) for 24 h increased the percentage of necrotic periodontal ligament cells (AV−/PI+) from 8.14% in untreated cells to 23.16% in treated cells. These data show that nicotine induces periodontal ligament cell death.

Effects of nicotine on the expression of ER stress pathway genes

To determine whether nicotine-induced cell death occurs as a result of ER stress, we assessed the phosphorylation patterns of protein kinase-like ER-resident kinase (PERK) and eIF2α, because PERK, an ER-resident transmembrane kinase, is known to autophosphorylate the cytoplasmic kinase domain in response to accumulated unfolded

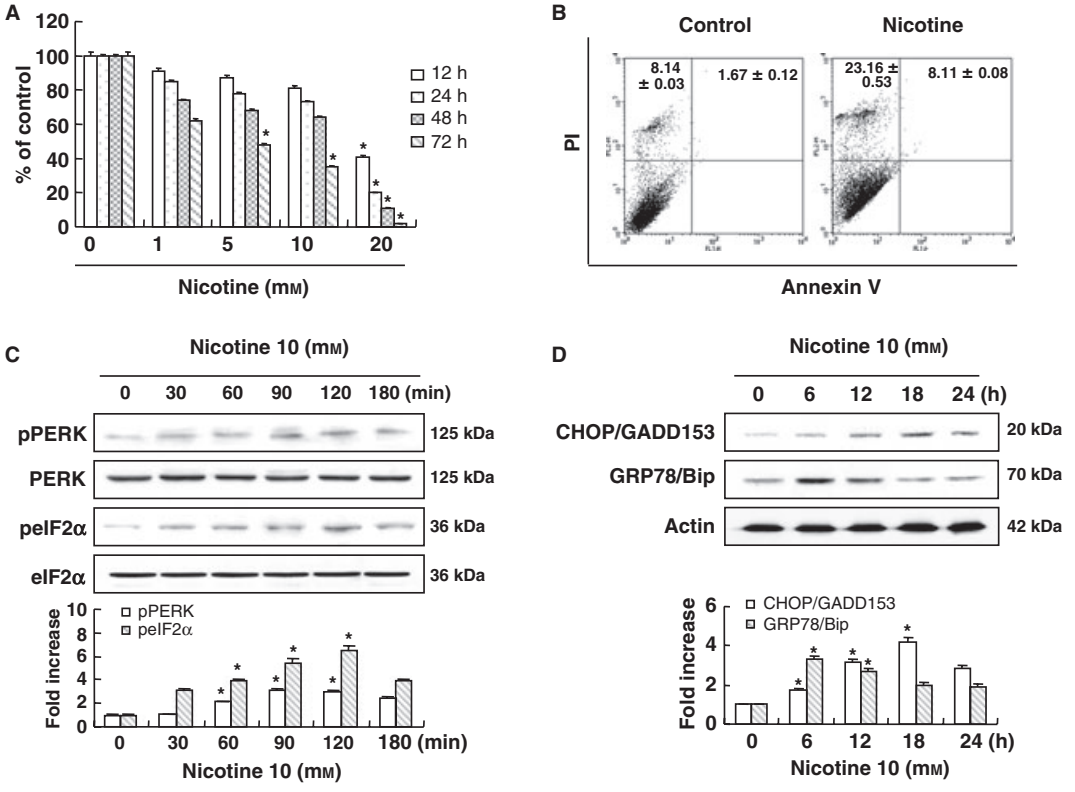

Fig. 1. Cytotoxicity (A), cell death (B) and ER stress markers in response to nicotine in periodontal ligament cells. Cells were incubated with 10 mM nicotine for the indicated time periods (A and C–F) and for 24 h (B). Cell proliferation and viability were measured by MTT assay (A) and flow cytometry (B), respectively. The mRNA and protein expression was determined by RT-PCR (C and D) and western blot analysis (E and F). Data are representative of three independent experiments. The bar graphs show the fold increase of protein expression compared with control cells. Columns show mean values of triplicate samples and error bars the standard deviation. * Statistically significant differences compared with the control, $p < 0.05$.

proteins in the ER lumen, and activated PERK is capable of phosphorylating several cytosolic proteins, including eIF2α (26). In this study, western blot analysis revealed that increased PERK phosphorylation and its downstream substrate, eIF2α, were evident 2 h after nicotine stimulation (Fig. 1C). Next, we examined the expression of GRP78/Bip, which serves as a gatekeeper for the activation of ER stress transducers, and C/EBP homologous protein/growth arrest and DNA damage-inducible gene 153 (CHOP/GADD135), which is a transcription factor induced by ER stress (26). As shown in Fig. 1D, nicotine-induced GRP78/Bip expression increased in a time-dependent manner, with the maximal induction occurring after 18 h of incubation.

Effects of nicotine on mRNA expression of ECM turnover in human periodontal ligament cells

We also examined the effect of nicotine on the mRNA expression of ECM molecules, including type I collagen, elastin and fibonectin, in periodontal ligament cells. Figure 2A and 2B demonstrates that the mRNA expression of these markers decreased in a dose- and time-dependant manner following nicotine exposure. The maximal breakdown of ECM molecules occurred after 24 h in the presence of 10 mM nicotine. As MMPs are implicated in tissue degradation and reduced ECM synthesis, we next examined the mRNA transcription levels of MMP-1, MMP-2, MMP-8 and MMP-9. As expected, the addition of nicotine caused a significant dose- and time-dependent increase in MMP-1, MMP-2, MMP-8 and MMP-9 mRNA and protein expression (Fig. 2C–F).

Role of ER stress in nicotine-induced cell death and ECM turnover

We next determined whether ER stress is involved in nicotine-induced upregulation of MMPs and ECM degradation in periodontal ligament cells. We tested the effects of the ER stress inhibitor salubrinal and CHOP/GADD153 siRNA on growth inhibition, cell death and the expression of MMPs and ECM molecules induced by 10 mM nicotine. As shown in Fig. 3A, both CHOP siRNA and salubrinal successfully knocked down CHOP/GADD135 and GRP78/Bip expression in periodontal ligament cells. Incubation of cells with CHOP siRNA and salubrinal resulted in significant protection against nicotine-induced cytotoxicity in periodontal ligament cells (Fig. 3B). Results of flow cytometry analysis showed that an increase in necrotic (AV−/PI+) cells induced by nicotine was blocked by siRNA and salubrinal addition (Fig. 3C). Moreover, CHOP knockdown and ER stress inhibitors blocked the nicotine-induced expression of MMP-1, MMP-2, MMP-8 and MMP-9 mRNA and protein, and the downregulation of ECM molecules, including type I collagen, elastin and fibronectin (Fig. 3D–F).

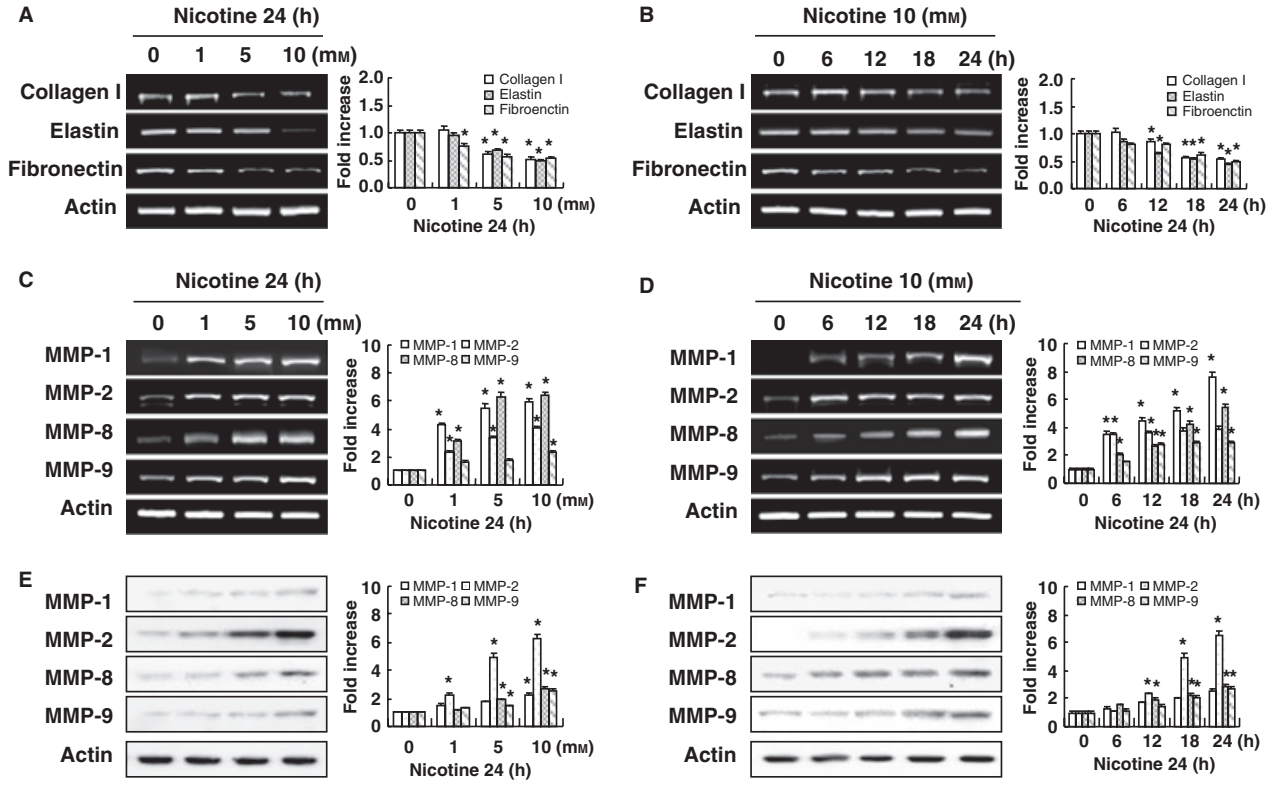

Fig. 2. Effects of nicotine on the expression of ECM molecules (A) and MMPs (C–F). The expressions of mRNAs and proteins were examined by RT-PCR (A–D) and western blot analysis (E and F), respectively. Data are representative of three independent experiments. The bar graphs show the fold increase of protein expression compared with control cells. Columns show mean values of triplicate samples and error bars the standard deviation. * Statistically significant differences compared with the control, $p < 0.05$.

Effects of actinomycin D and cycloheximide on nicotine-induced ECM turnover

To investigate the mechanism of nicotine-induced upregulation of MMPs and downregulation of ECM molecules, we pretreated periodontal ligament cells with the transcriptional inhibitor actinomycin D (ActD) or the protein-synthesis inhibitor cycloheximide (CHX). When periodontal ligament cells were pretreated with ActD prior to the addition of nicotine, the nicotine-induced MMP-1, MMP-2, MMP-8 and MMP-9 upregulation and nicotine-induced type I collagen, elastin and fibronectin downregulation were reversed (Fig. 4A, 4B and 4C). These results suggest that the effects of nicotine on MMP and ECM molecule expression are controlled at the transcription level. These data were confirmed using 10 mM CHX, a specific protein-translation inhibitor, which abolished MMP induction and recovered ECM molecule expression following nicotine treatment.

Effects of ER stress inhibition by salubrinal and CHOP siRNA on nicotine-induced signal cascade

To examine whether the MAPKs, Akt and NF-κB pathways are involved in ER stress inhibition, the effects of salubrinal and CHOP/GADD153 siRNA on these signaling cascades was investigated following nicotine incubation. Nicotine stimulated time-dependent phosphorylation of Akt, ERK, p38 and JNK, with a maximal response within 30 min in periodontal ligament

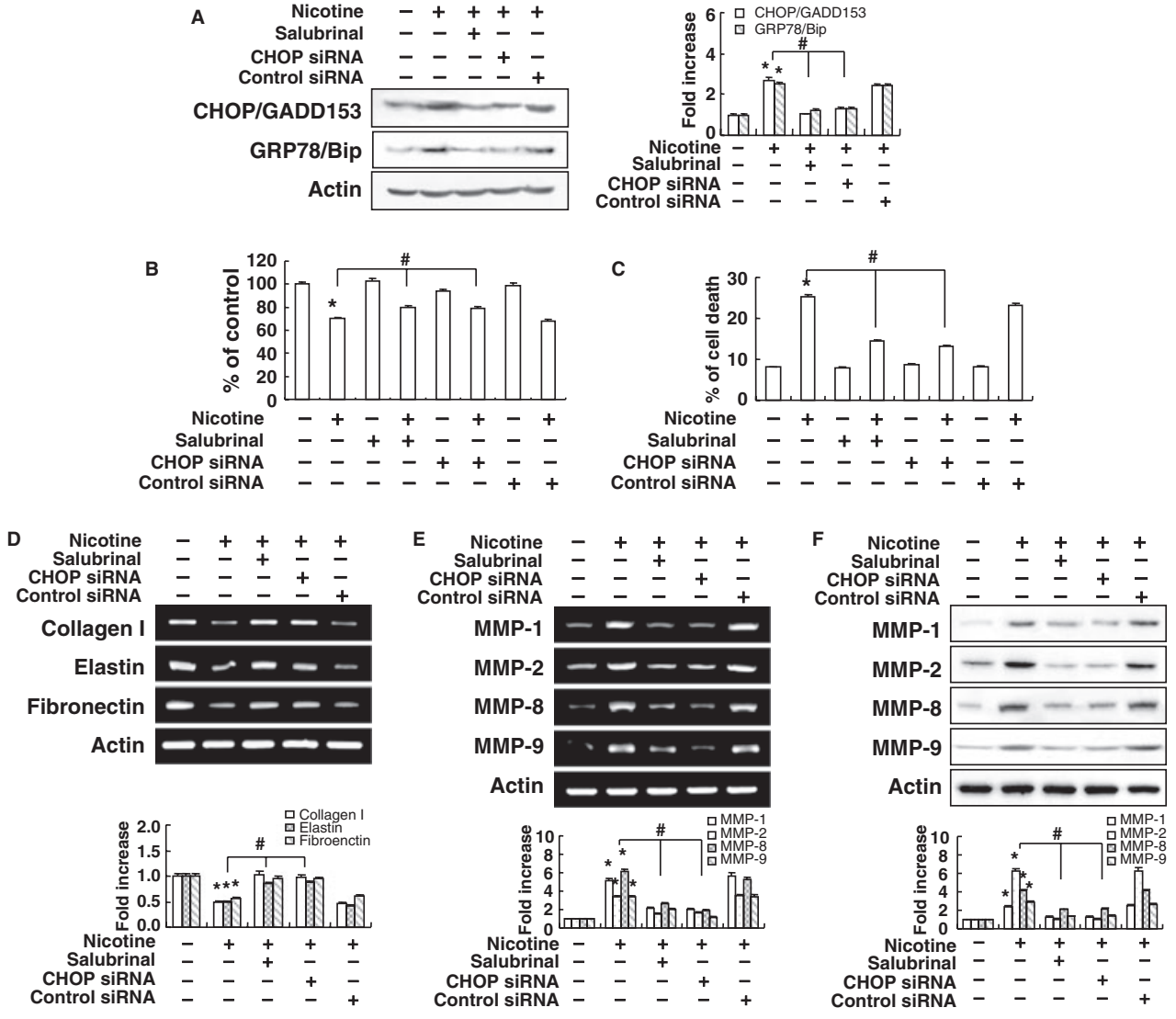

Fig. 3. Effects of ER stress inhibition by salubrinal and CHOP siRNA on nicotine-induced cytotoxicity (A), cell death (AV−/PI+cells; B), expression of ER stress markers (C), ECM (D) and MMPs (E and F). Cells were pretreated for 1 h with 10 μM salubrinal, followed by nicotine treatment for 24 h (A–E). Cells were transfected with 80 pmol of CHOP siRNA and control siRNA for 12 h and incubated with 10 mM nicotine for 24 h (A–F). Data are representative of three independent experiments. The bar graphs show the fold increase of protein expression compared with control cells. Columns show mean values of triplicate samples and error bars the standard deviation. * Statistically significant differences compared with the control, $p < 0.05$. Statistically significant difference compared with the nicotine-treated group, $p < 0.05$.

cells (data not shown). Figure 5A shows that the nicotine-stimulated Akt, ERK, p38 and JNK phosphorylation were inhibited by salubrinal. The CHOP siRNA significantly inhibited nicotine activation of Akt and JNK, whereas CHOP siRNA did not significantly alter nicotine activation of p38 and ERK compared with nicotine alone.

To examine whether ER stress inhibition affects nicotine-induced NF-κB activation, IκB-α degradation and phosphorylation, EMSA assays were employed to examine NF-κB DNA-binding capacity. We demonstrated that nicotine-induced IκB-α degradation and phosphorylation was inhibited by salubrinal and CHOP siRNA. Furthermore, the induction of the specific NF-κB binding activity in response to nicotine was markedly inhibited by salubrinal and CHOP siRNA (Fig. 5B). The specificity of binding inhibition was confirmed using cold consensus, demonstrating the failure to bind nuclear extracts from the nicotine-treated samples.

Discussion

As the major constituent of the particulate phase of tobacco smoke, nicotine is a major contributor to periodontitis (1). Although previous studies reported that nicotine causes injury to periodontal ligament cells via multiple mechanisms, including nuclear factor-erythroid 2 p45-related factor 2, heme oxygenase-1 and oxidative stress (7,8), the molecular mechanisms of these effects have not been fully elucidated. In particular, the role of ER stress, a major mechanism mediating cell death, has not been reported in periodontal ligament cells. In the present study, the role of ER stress in nicotine-induced periodontal ligament cell necrosis and ECM degradation was examined. We chose human periodontal ligament cells because they play an important role in periodontal remodeling during orthodontic tooth movement and in periodontitis.

Our *in vitro* studies demonstrated that nicotine treatment of periodontal ligament cells induced growth inhibition and cell necrosis in a dose-dependent manner. This growth inhibition was consistent with our previous reports demonstrating that nicotine treatment leads to cytotoxicity in periodontal ligament cells (5,7,8). Nicotine is reported to induce apoptosis in Leydig cells, cardiomyocytes, gingival fibroblasts, hippocampal neural cells and oral cancer cells (27–29). In contrast, nicotine has been shown to prevent or delay apoptosis of normal and transformed human and mouse cells (30,31). Our results demonstrate that nicotine induces cell necrosis, but not apoptosis, in periodontal ligament cells, as evidenced by AV−/PI+ staining.

In this study, we focused on the involvement of ER stress in nicotine-induced periodontal ligament cell necrosis and ECM destruction. Our data show that nicotine induced the upregulation of GRP78/BiP, a classic marker of the ER stress response, and CHOP/GADD153, an ER stress-induced cell death modulator (26), indicating its ability to induce ER stress. Both pPERK and peIF2α, additional ER-stress genes that regulate cell death (26), were activated in nicotine-stimulated periodontal ligament cells. Consistent with our observations, ER stress mediates cigarette smoke-induced apoptosis of human bronchial epithelial cells (32). Based on these findings, we can confirm that nicotine is responsible for the induction of ER stress in periodontal ligament cells.

Periodontal disease is an inflammatory process, with increased MMP activity in the periodontal tissues, resulting in a loss of collagen in the periodontal structures even at the very early stages of the disease (9,10). MMP-1 is the major proteolytic enzyme that can cleave the native interstitial colla-

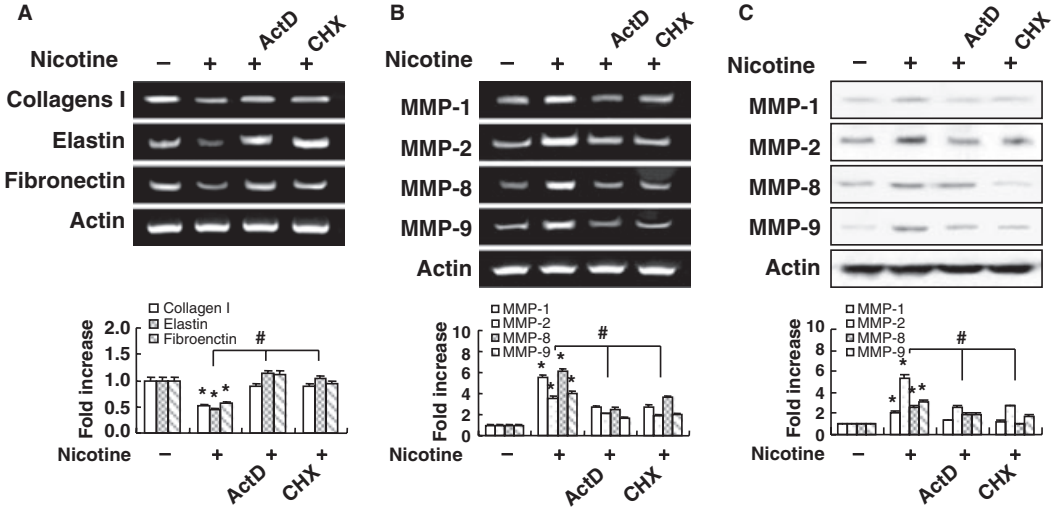

Fig. 4. Effects of actinomycin D (AcD) and cycloheximide (CHX) on nicotine-induced ECM molecules (A) and MMPs (B and C). Cells were pretreated with actinomycin D (5 μg/mL) or cycloheximide (10 mM) for 1 h and treated with nicotine (10 mM) for 24 h. The results represent the means of three independent experiments. The bar graphs show the fold increase of protein expression compared with control cells. Columns show mean values of triplicate samples and error bars the standard deviation. * Statistically significant differences compared with the control, $p < 0.05$. Statistically significant difference compared with the nicotine-treated group, $p < 0.05$.

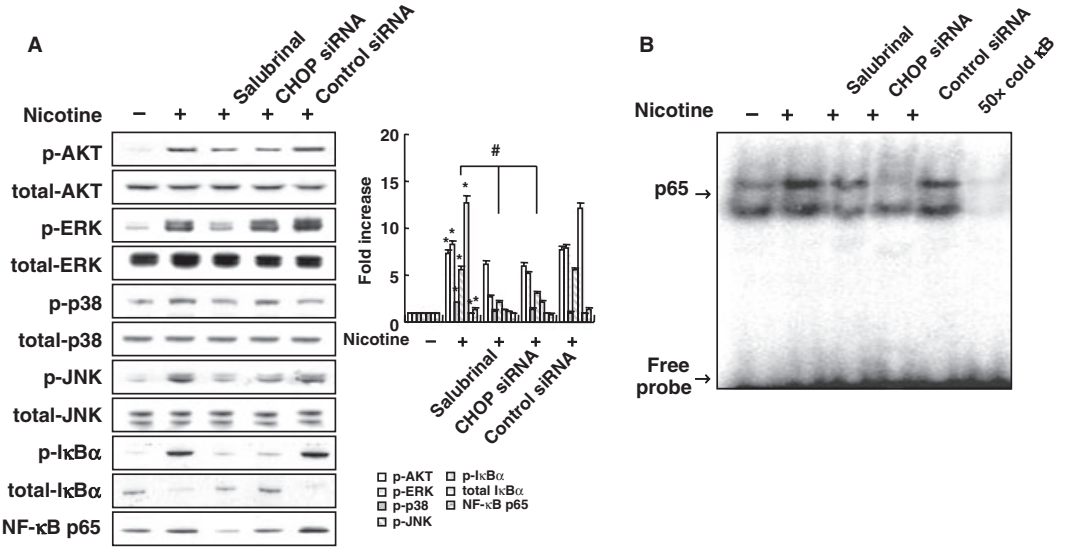

Fig. 5. Effects of salubrinal and CHOP siRNA on nicotine-induced Akt, MAPK and NF-κB signaling. Periodontal ligament cells were pretreated with CHOP siRNA for 24 h and slaubrinal for 1 h following incubation with 10 mM nicotine for 30 min. Nuclear factor-κB activation was examined by western blot (A) and electrophoretic mobility shift assays (EMSA; B). The notation '50X cold κB' denotes a 50-fold excess of unlabeled oligonucleotide relative to the ^{32}P-labeled probe; this was added to the binding assay for competition with the unlabeled oligonucleotide. Representative results from three independent experiments are shown. The bar graph shows the fold increase of protein expression compared with control cells. Columns show mean values of triplicate samples and error bars the standard deviation. * Statistically significant differences compared with the control, $p < 0.05$. Statistically significant difference compared with the nicotine-treated group, $p < 0.05$.

gens, types I and III, the most abundant protein components of the ECM in the periodontium. Enhanced protein and mRNA expression of MMP-1 has been demonstrated in inflammatory diseases, including periodontitis (33). MMP-2, MMP-3, MMP-8 and MMP-9 are also known to be increased in inflamed gingival tissue (34). Increased MMP-8 and MMP-9 have been described in gingival crevicular fluid during the active stages of periodontal disease (35). We therefore chose to examine the levels of MMP-1, MMP-2, MMP-8 and MMP-9, as well as ECM molecules, because MMP-mediated ECM degradation plays an important role in periodontal destruction (36–38). Our data demonstrated that nicotine treatment concomitantly downregulated the expression of the ECM markers type I collagen, elastin and fibronectin and upregulated the expression of MMP-1, MMP-2, MMP-8 and MMP-9 in periodontal ligament cells. These results are consistent with previous studies demonstrating that nicotine increases ECM degradation and MMP activity in gingival fibroblasts and osteoblasts (13,15). Collectivelly, nicotine stimulated downregulation of ECM molecules, upregulation of MMPs and cytotixicity or a decrease in cell viability of periodontal ligament cells. Therefore, this suggested that nicotine is responsible for enhanced deleterious cellular events, including cell death and cytotoxicity, and its byproduct, ECM-degrading ability of the periodontitis phenotypes.

The role of ER stress in periodontal disease has recently been reported (20). Even though a plausible implication of the unfolded protein response in periodontal tissue destruction was proposed, its role in the pathogenesis of periodontal disease has, to date, not been clarified. We found that the inhibition of ER stress by salubrinal and CHOP/GADD153 siRNA reduced growth inhibition, cell necrosis, ECM degradation and MMP production in nicotine-treated periodontal ligament cells (Fig. 3). These results strongly suggest that the ER stress pathway plays a central role in nicotine-induced periodontal ECM destruction.

The PI3K/Akt signaling pathway is a prominent pathway in regard to the transmission of anti-apoptotic signals in cell survival (39). The MAPKs, including the ERK1/2, JNK and p38 MAPK subgroups, are additionally known to play a fundamental role in cell survival and death (40). As CHOP is a downstream marker of ER stress, we tested whether the phosphorylation of MAPKs and Akt were influenced by ER stress. Salubrinal is known to protect cells from ER stress-induced apoptosis selectively by inhibiting the dephosphorylation of eIF2α, which is phosphorylated by PERK (41). In the present results, salubrinal partly blocked the activation of Akt, ERK, JNK and p38 MAPK. Knockdown of CHOP partly blocked nicotine-induced phosphorylation of Akt and JNK MAPK. In mouse and human T cells, ceramide induces apoptosis mediated by ER stress in a p38 MAPK- and JNK-dependent fashion (42). Moreover, fenretinide treatment activated JNK in ovarian cancer cells, and the phosphorylation was dependent on ER stress, because it was abrogated by addition of salubrinal (43). It appears that the role of ERK, JNK and p38 MAPK activation in ER-stress-induced cell death varies in different cell

types, as well as varying based on the nature of the ER stress inducer.

One of the major signal transduction pathways activated in response to oxidative stress is the NF-κB pathway (44). Its activation either promotes or blocks cell death, depending on the cell type and the nature of the oxidative stimuli (45). Our results showed that the phosphorylation and degradation of IκB-α and the NF-κB binding activity of nicotine were attenuated by salubrinal and CHOP siRNA. This suggests involvement of ER stress in nicotine-induced NF-κB activation.

In summary, our results demonstrate that ER stress contributes to nicotine-induced cell necrosis and periodontal connective tissue destruction via Akt, ERK, p38, JNK MAPK and NF-κB pathways in periodontal ligament cells. Thus, therapies that modulate ER stress may present a novel intervention for the prevention and treatment of periodontal disease.

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