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The up-regulation of heme oxygenase-1 expression in human gingival fibroblasts stimulated with nicotine

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Background: Cigarette smoking is a major risk factor in the development and further progression of periodontal diseases. Heme oxygenase-1 (HO-1) is known as a stress-inducible protein and functions as an antioxidant enzyme. There is limited information on the expression of HO-1 in smoking-associated periodontal disease.

Objectives: The aim of the present study was to investigate the effects of nicotine on the expression of HO-1 protein in cultured human gingival fibroblasts *in vitro* and further to compare HO-1 expression in gingival tissues obtained from cigarette smokers and non-smokers *in vivo*.

Methods: Western blot assay was used to investigate the effects on human gingival fibroblasts exposed to nicotine. In addition, antioxidants catalase, superoxide dismutase (SOD), and *N*-acetyl-L-cysteine (NAC) were added to test how they modulated the effects on nicotine-induced HO-1 expression. Gingival biopsies taken from the flap surgery of 20 male patients with periodontal disease (10 cigarette smokers and 10 non-smokers) were examined by immunohistochemistry.

Results: The exposure of quiescent human gingival fibroblasts to 10 mM nicotine resulted in the induction of HO-1 protein expression in a time-dependent manner ($p < 0.05$). The addition of glutathione (GSH) precursor NAC inhibited the nicotine-induced HO-1 protein expression ($p < 0.05$). However, SOD and catalase did not decrease the nicotine-induced HO-1 protein expression ($p > 0.05$). The results from immunohistochemistry demonstrated that HO-1 expression was significantly higher in cigarette smokers ($p < 0.05$). HO-1 was noted in the basal layers of epithelium, inflammatory cells, and fibroblasts in specimens from cigarette smoking.

Conclusions: Taken together, these results suggest that HO-1 expression is significantly up-regulated in gingival tissues from cigarette smokers, and nicotine may, among other constituents, be responsible for the enhanced HO-1 expression *in vivo*. The regulation of HO-1 expression induced by nicotine is critically dependent on the intracellular GSH concentration.

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Expression of stress-related genes are particularly induced following exposure to thiol-reactive compounds (1), reactive oxygen intermediates (2), heavy metal cations (3), and UV radiation (4). These stimuli all induce the expression of a 32 kDa protein identified as the microsomal inducible heme oxygenase-1 (HO-1), whose regulation

is related to heat-shock proteins in rodents, but not in human (5, 6). HO-1 catalyzes the oxidative degradation of heme to biliverdin, which in turn is reduced to bilirudin, an antioxidant. This mechanism, along with the induction of ferritin, mediates the cytoprotective antioxidant effects of HO-1 (7). HO-1 functions as an antioxidant enzyme because locally produced bilirubin works as an efficient scavenger of reactive oxygen species.

Cigarette smoking is a risk factor for periodontal disease (8–10). A more definitive study in 2004 described the causal relationship between smoking and periodontal disease (11). Much attention has been given to analyzing the effect of nicotine on cell function, irrespective of the 4000 chemical substances that exist in cigarette smoke (12). Previous studies have shown that nicotine is a cytotoxic agent to fibroblasts derived from periodontium by inhibiting cell viability, attachment, proliferation, and matrix protein synthesis (13–18). Recently, we found that the inhibitory effects of nicotine are associated with intracellular thiol levels (18, 19). However, the underlying mechanisms have not been completely identified.

The purpose of this study was to test whether expression of HO-1 can be triggered in human gingival fibroblasts by nicotine *in vitro*. In addition, we evaluated the distribution of HO-1 in gingival specimens of cigarette smokers and non-smoker periodontally diseased patients by using immunohistochemistry. Finally, given the relevance of oxidative stress in cigarette smoking mediated periodontal disease, we investigated the effects of the antioxidants catalase, superoxide dismutase (SOD), and *N*-acetyl-L-cysteine (NAC) on nicotine-induced HO-1 protein expression in human gingival fibroblasts.

Material and methods

Nicotine, diethymaleate (DEM), catalase, SOD, and NAC were purchased from Sigma (St. Louis, MO, USA). Mouse anti-human HO-1 monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tissue culture biologicals were purchased from Gibco Laboratories (Grand Island, NY, USA).

Cell culture

Human gingival fibroblasts were cultured by using an explant technique as described previously (20). Three healthy individuals without cigarette smoking habits were selected from the crown lengthening procedure with the informed consent for this study. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of fungizone). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. Cell cultures between the third and eighth passages were used.

HO-1 expression analysis

Cells arrested in G_0 by serum deprivation (0.5% fetal calf serum; 48 h) were used in the experiments (19, 20). Nearly confluent monolayers of human gingival fibroblasts were washed with serum-free Dulbecco's modified Eagle's medium and immediately thereafter exposed at the indicated incubation times to 10 mM nicotine. Cell lysates were collected at 0, 0.5, 1, 2, 4, 8, and 24 h for western blot analysis. Cultures without fetal calf serum were used as negative control. Subsequently, various pharmacological agents without cytotoxic concentrations were also added to wells to test their regulation effects during an 8-h incubation period. The final concentrations of SOD, catalase, NAC, and DEM used in this study were 100 μg/ml, 50 μg/ml, 1 mM, and 0.5 mM, respectively.

Western blot

For western blot analysis, cell lyates were collected as described previously (20). Briefly, cells were solubilized with sodium dodecyl sulfate–solubilization buffer (5 mM EDTA, 1 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5 and 0.5% Trition X-100, 2 mM phenylmethysulfonyl fluoride, and 1 mM N-ethylmaleimide) for 30 min on ice. Then, cell lysates were centrifuged at 12,000 *g* at 4°C and the protein concentrations determined with Bradford reagent using bovine serum albumin as standards. Equivalent amounts of total protein per sample of cell extracts were run on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immediately transferred to nitrocellulose membranes. The membranes were blocked with phosphate-buffered saline containing 3% bovine serum albumin for 2 h, rinsed, and then incubated with primary antibodies anti-HO-1 (1 : 1000) in phosphate-buffered saline containing 0.05% Tween 20 for 2 h. After three washes with Tween 20 for 10 min, the membranes were incubated for 1 h with biotinylated secondary antibody diluted 1 : 1000 in the same buffer, washed again as described above and treated with 1 : 1000 streptavidin–peroxidase solution for 30 min. After a series of washing steps, the reactions were developed using diaminobenzidine (Zymed, South San Francisco, CA, USA). The intensities of the obtained bands were determined using a densitometer (AlphaImager 2000: Alpha Innotech, San Leandro, CA, USA). Each densitometric value was expressed as the mean ± SD.

Immunohistochemistry

Twenty periodontally diseased patients were selected from subjects referred to the Department of Periodontics, Chung Shan Medical University Hospital. All patients were duly informed of the nature and extent of the study, and their informed consent was obtained according to the Helsinki Declaration. Subjects displayed periodontal disease with moderate to severe bone loss. Selected subjects were those who had no other serious medical conditions, and had not taken any antibiotic medication in the past 6 months except in preparation for initial periodontal therapy. Ten cigarette smokers were defined as individuals who

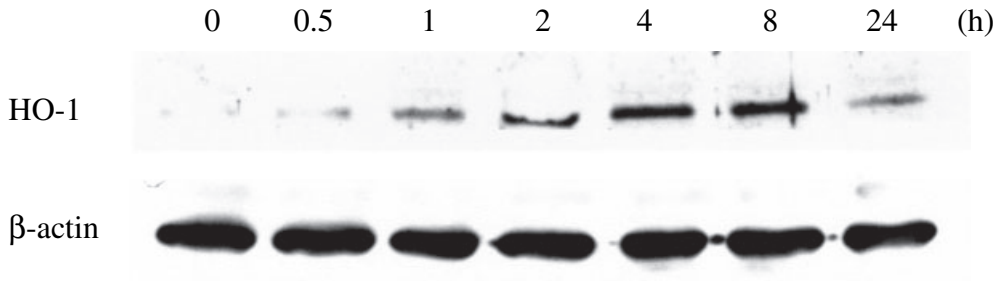

Fig. 1. Kinetics of heme oxygenase-1 (HO-1) protein expression in human gingival fibroblasts exposed to 10 mM nicotine for 0, 0.5, 1, 2, 4, 8, and 24 h, respectively. β-Actin was performed in order to monitor equal protein loading.

habitually smoked at least 10 cigarettes per day at the time of the initial examination. Ten non-smokers consisted of individuals who did not smoke and had never smoked. If, after 3 months, pockets were still present with probing depth > 5 mm, flap surgery was carried out. The surgically removed gingival tissues were fixed with 10% buffered formalin overnight, the specimens were dehydrated in an ascending series of graded alcohols and embedded in paraffin. Then 5-μm sections were stained with the monoclonal anti-HO-1 antibody (1 : 100 dilution) using a standard avidin–biotin–peroxidase complex method (21). 3-Amino-9-ethylcarbazole (Dako, Carpinteria, CA, USA) was then used as the substrate for localizing the antibody binding. The preparations were counterstained with hematoxylin, mounted with Permount and examined by light microscopy.

Statistical analysis

Three replicates of each concentration were performed in each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was by one-way analysis of variance (ANOVA). Tests of differences of the treatments were analyzed by Duncan's test.

Results

Expression of HO-1 in human gingival fibroblasts challenged with nicotine was directly assessed in cell lysates using western blot analysis. As shown in Fig. 1, 10 mM nicotine was found to up-regulate HO-1 protein expression by human gingival fibroblasts in a time-dependent manner ($p < 0.05$). The kinetics of this response showed that HO-1 protein was first detectable in cell lysates at 0.5 h post nicotine challenge and remained elevated throughout the 24-h incubation period (Fig. 1). The quantitative measurement of HO-1 protein was made by the densitometer (Fig. 2). The levels of the HO-1 protein increased about 1.7 and 3.0-fold after exposure to nicotine for 1 and 4 h ($p < 0.05$), respectively.

Three antioxidants were added to search for the possible regulation mechanisms on nicotine-induced HO-1 protein expression (Fig. 3). Treatment with NAC led to a decrease in HO-1 protein expression by nicotine ($p < 0.05$). However, treatment with SOD and catalase showed no change in nicotine-induced HO-1 protein levels ($p > 0.05$). From the densitometer, nicotine induced about 3.2-fold HO-1 protein over the 8-h incubation period (Fig. 4). Coincubation of nicotine and NAC induced about 1.7-fold HO-1 protein as compared with control. NAC was found to significantly inhibit the nicotine-induced HO-1 protein expression about 47% ($p < 0.05$). To further elucidate the roles of glutathione (GSH) in nicotine-induced HO-1 protein expression, DEM was used to deplete the intracellular GSH level. From the densitometer, addition of DEM induced about 8.7-fold HO-1

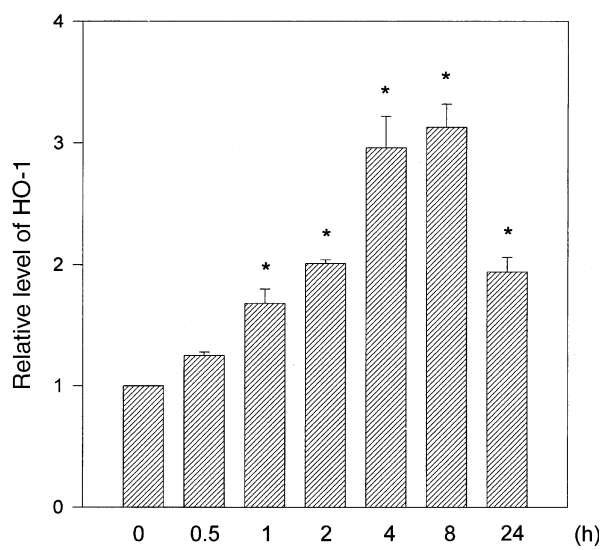

Fig. 2. Levels of heme oxygenase-1 (HO-1) protein treated with nicotine were measured by densitometer. The relative level of HO-1 protein expression was normalized against β-actin signal and the control was set as 1.0. Optical density values represent the mean ± SD. *Represents significant difference from control values with $p < 0.05$.

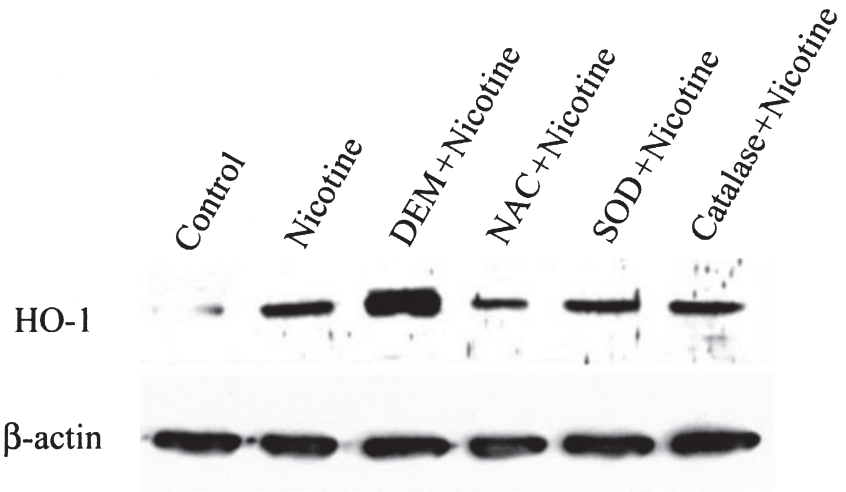

Fig. 3. Effects of antioxidants on nicotine-induced heme oxygenase-1 (HO-1) protein expression in human gingival fibroblasts. Cells were pre-exposed with diethymaleate (DEM), *N*-acetyl-L-cysteine (NAC), superoxide dismutase (SOD), or catalase for 1 h then were treated for 8 h in the presence of 10 mM nicotine. β-Actin was performed in order to monitor equal protein loading.

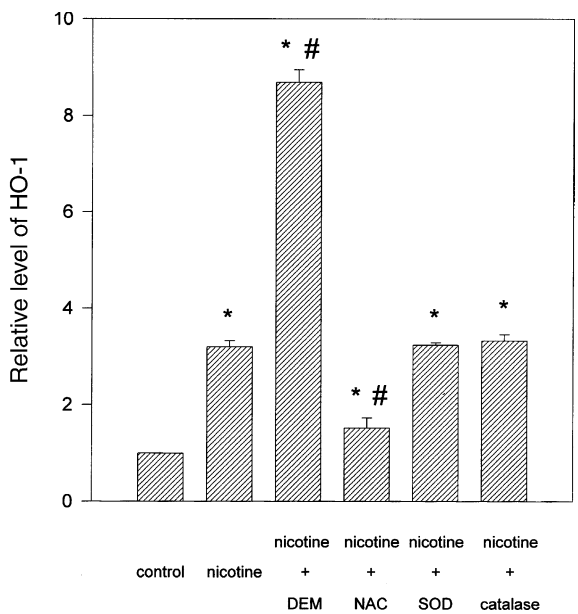

Fig. 4. Levels of heme oxygenase-1 (HO-1) protein in human gingival fibroblasts after coincubation with antioxidants and 10 mM nicotine. Quantization was achieved by densitometer as described in Fig. 2. *Represents significant difference from control values with $p < 0.05$. #Represents statistically significant between nicotine alone and nicotine with pharmacological agents; $p < 0.05$.DEM, diethylmaleate; NAC, *N*-acetyl-L-cysteine; SOD, superoxide dismutase.

protein as compared with control (Fig. 4). DEM was found to significantly up-regulate the nicotine-induced HO-1 protein expression about 5.5-fold ($p < 0.05$).

The HO-1 staining in gingival tissue was stronger in the cigarette smoker group than the non-smoker group. Figure 5 represents gingival tissue obtained from the non-smoker group with faint HO-1 expression. The strongest signal was seen in the epithelial cells and endothelial cells, with almost no staining in the lamina propria. In the cigarette smoker group, intensive staining for HO-1 expression was observed in the epithelial cells, inflammatory cells, and fibroblasts (Fig. 6).

Discussion

HO was originally identified as an enzyme that catalyzes the initial reaction in heme catabolism: the oxidative cleavage of the α-meso carbon bridge of b-type heme molecules to yield equimolar quantities of biliverdin IXa, carbon monoxide, and iron (22). The stress-inducible isoform HO-1 is localized to chromosome 22q12 (23). HO-1 expression is very sensitive to stress, and is induced by many stimuli. In this study, HO-1 was first found to be up-regulated in human gingival fibroblasts by nicotine. Thus, HO-1 expression might be one signal transduction pathway linked to the induction of stress response protein by cigarette smoking.

Previous studies have shown that exposure of Swiss 3T3 cells to cigarette smoke trapped in phosphate-buffered saline solutions resulted in the expression of HO-1 (24, 25). Tobacco smoke was also found to induce HO-1 expression in human monocytes (26), vascular endothelial cells (26), and premonocytic cell line U937 cells (27). Taken together, these studies suggest that the induction of HO-1 expression by tobacco products is not cell type-specific. In addition, cigarette smoking could persistently activate HO-1 expression.

Accumulated evidence indicates a role for HO protein in cellular or tissue damage and suggests that HO-1 induction is a protective response against oxidative stress (28). We previously demonstrated that nicotine significantly depleted intracellular GSH in periodontal ligament fibroblasts (20). To test the contribution of reactive oxygen species to the induction of stress responses by nicotine in human gingival fibroblasts, we treated human gingival fibroblasts with various antioxidants before and during exposure to nicotine. NAC is a more stable cysteine derivative than is GSH, and it protects cells against oxidative

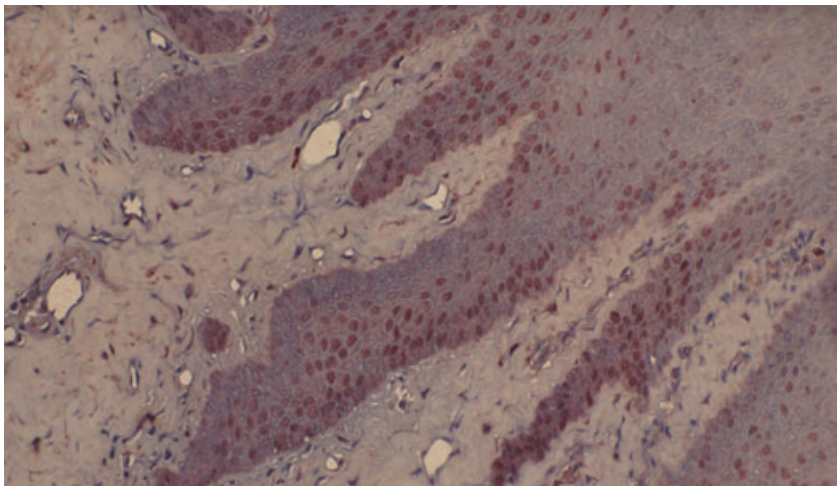

Fig. 5. In the non-smoker group, faint immunoreactivity of heme oxygenase-1 was observed in gingival connective tissue and the strong signal was seen in the epithelial cells and endothelial cells (200 ×).

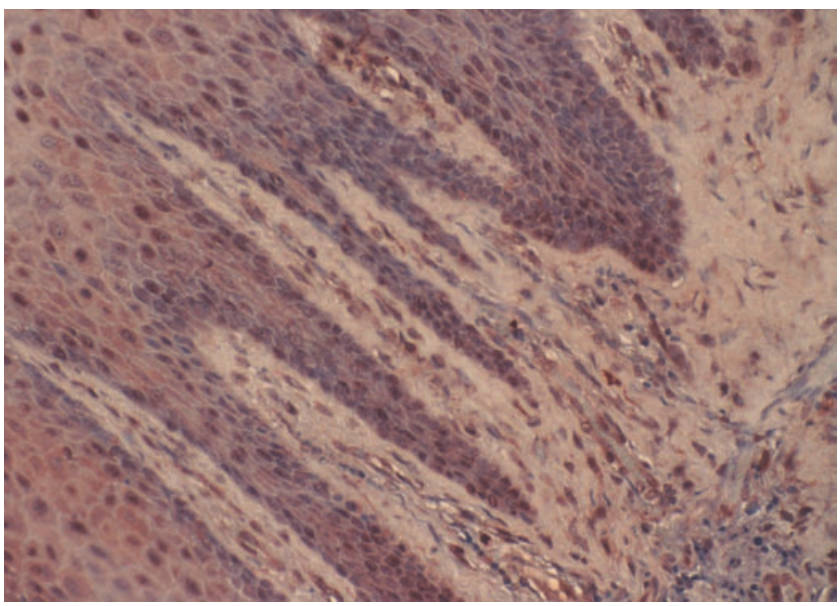

Fig. 6. In the cigarette smoker group, heme oxygenase-1 was evident as intense, diffuse red-brown coloring throughout the connective tissue and detected at relatively high levels in the epithelium (400 ×).

stress in different ways. NAC is easily deacetylated inside the cells and provides cysteine for cellular GSH synthesis and thus stimulates the cellular GSH system (29). DEM (a cellular GSH depleting agent) can directly form a complex with GSH by the GSH *S*-transferase-mediated reaction and lead to cellular GSH depletion (30). In the present study, addition of NAC leads to a decrease in the induction of HO-1 expression by nicotine. Our results are in agreement with Vayssier-Taussat *et al.* (26), who found that NAC can inhibit tobacco smoke and benzo[*a*]pyrene induced HO-1 expression in human endotheliel cells and monocytes, and Favatier & Polla (27), who demonstrated that pretreatment with NAC suppressed HO-1 expression by tobacco smoke in U937 cells. A similar result was found by Muller & Gebel (24), who reported that addition of cysteine inhibits the cigarette smoke trapped in phosphate-buffered saline induced HO-1 expression in Swiss 3T3 cells. In addition, our data also showed that pretreatment with DEM can up-regulate nicotine-induced HO-1 protein expression. Taken together, tobacco products-induced HO-1 expression is influenced by intracellular GSH levels.

In this study, the nicotine-induced HO-1 expression could be prevented by addition of extracellular GSH, but not catalase (scavenger of H_2O_2) and SOD (an extracellular superoxide free radical scavenger). This indicates that GSH depletion but not the attack of oxygen free radicals could be the mechanism for nicotine-induced HO-1 expression. In agreement with our data, Muller & Gebel (24) found that neither catalase nor *o*-phenanthroline was able to suppress or even reduce the enhanced of the HO-1 expression in smoke-bubbled phosphate-buffered saline-treated cells.

From immunohistochemtistry studies, HO-1 was noted in the basal layers of epithelium and fibroblasts in smoking specimens. In addition, HO-1 expression was significantly elevated for cigarette smoking specimens compared to non-smoker gingival tissue. A similar result was found that increased expression of HO-1 in alveolar spaces of cigarette smokers (31). These data suggest that oxidative stress due to cigarette smoking may increase tissue expressing HO-1. Consistently, the recent detection of high levels of metallothionein in the gingiva of smokers with advanced periodontitis would indicate an attempt to defend against free radicals in the gingiva of smokers (32). Taken together, the strong expression of the antioxidant proteins may generally reflect the pro-oxidative quality of cigarette smoking-associated periodontal disease.

This study represents, to the best of our knowledge, the first systemic attempt to evaluate the role of HO-1 expression in cigarette smoking associated-periodontal disease in human both *in vitro* and *in vivo*. We demonstrated for the first time that HO-1 is elevated in

gingival specimens obtained from a cigarette smoker group. Nicotine-induced HO-1 expression suggests that cigarette smoking may contribute the pathogenesis of periodontal diseases via HO-1 expression. In addition, the regulation of HO-1 expression induced by nicotine is critically dependent on the intracellular GSH concentration.

Acknowledgements

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