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ORIGINAL ARTICLE

Augmented heme oxygenase-1 expression in areca quid chewing-associated oral submucous fibrosis

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OBJECTIVES: Heme oxygenase-1 (HO-1) is known as a stress-inducible protein and functions as an antioxidant enzyme. HO-1 is consistently and dramatically upregulated in a variety of fibrotic diseases. The aim of this study was to compare HO-1 expression in normal human buccal mucosa and oral submucous fibrosis (OSF) specimens and further explore the potential mechanism that may lead to induce HO-1 expression.

METHODS: Twenty OSF specimens and 10 normal buccal mucosa were examined by immunohistochemistry. The mRNA levels of HO-1 from fibroblasts cultured from OSF and normal buccal mucosa fibroblasts (BMFs) were evaluated by reverse transcription polymerase chain reaction. The effect of arecoline, the major areca nut alkaloid, was added to explore the potential mechanism that may lead to induce HO-1 expression.

RESULTS: Heme oxygenase-1 expression was significantly higher in OSF specimens ($P < 0.05$) and expressed mainly by fibroblasts, endothelial cells, and inflammatory cells. OSF demonstrated significantly higher HO-1 mRNA expression than BMFs ($P < 0.05$). Arecoline was also found to elevate HO-1 mRNA and protein expression in a dose-dependent manner ($P < 0.05$).

CONCLUSIONS: Taken together, the data presented here demonstrated that HO-1 expression is significantly upregulated in OSF from areca quid chewers, and arecoline may be responsible for the enhanced HO-1 expression *in vivo*.

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Keywords: arecoline; buccal mucosa fibroblasts; heme oxygenase-1; oral submucous fibrosis

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Introduction

Oral submucous fibrosis (OSF) is characterized by the submucosal accumulation of dense fibrous connective tissue with inflammatory cell infiltration and epithelial atrophy (Warnakulasuriya *et al*, 1997). Although the etiology of OSF is not completely understood, there is strong epidemiological evidence suggesting the association of OSF with the areca quid chewing habit (Sinor *et al*, 1990; Maber *et al*, 1994). It was estimated that approximately 2 million people have the habit of areca quid chewing in Taiwan (Ko *et al*, 1992). The areca quid in Taiwan consists of two halves of a fresh areca nut sandwiched with a piece of inflorescence *Piper betle* and lime mixture, but no tobacco consisted in the quid.

Arecoline, a major areca nut alkaloid, was found to stimulate human buccal mucosa fibroblast (BMF) proliferation (Harvey *et al*, 1986; Chang *et al*, 1998) and collagen synthesis *in vitro* (Harvey *et al*, 1986). Recently, our studies have shown that upregulation of vimentim (Chang *et al*, 2002a), cyclooxygenase-2 (Tsai *et al*, 2003), tissue inhibitor metalloproteinase-1 (Chang *et al*, 2002b), plasminogen activator inhibitor-1 (Yang *et al*, 2003, 2007), interleukin-6 (Tsai *et al*, 2004), keratinocyte growth factor-1 (Tsai *et al*, 2005a), insulin-like growth factor-1 (Tsai *et al*, 2005b), nuclear factor-kappa B (Ni *et al*, 2007) cystatin C (Tsai *et al*, 2007), and heat shock protein 47 (Yang *et al*, 2008) may contribute to the extracellular components accumulation in OSF. However, very little is currently known the precise mechanisms about the biochemical/molecular biology of OSF.

Heme oxygenase-1 (HO-1), a 32-kDa microsomal enzyme (Choi and Alam, 1996), participates in maintaining the cellular homeostasis and plays an important protective role in the tissues due to reducing oxidative injury and attenuating the inflammatory response whose expression is upregulated by a variety of stimuli, including heme, oxidative stresses, heavy metals, and inflammatory cytokines (Maines and Gibbs, 2005; Ryter and Choi, 2005). HO-1 catalyzes the oxidative

degradation of heme to biliverdin, which in turn is reduced to bilirudin (Maines, 1988). HO-1 functions as an antioxidant enzyme because locally produced bilirudin works as an efficient scavenger of reactive oxygen species (ROS). HO-1 is consistently and dramatically upregulated in a variety of fibrotic diseases, such as benign prostatic hyperplasia (Maines and Abrahanamsson, 1996) and the cystic fibrosis of lung (Zhou *et al*, 2004).

Previously, Chang *et al* (2004) have demonstrated the relationship between (GT)n microsatellite repeat in HO-1 promoter and areca quid chewing associated-oral submucous fibrosis. This suggests that HO-1 might play a role in the pathogenesis of areca quid chewing-associated OSF. However, the authors do not take into account the cellular source of the HO-1 in OSF as well as normal buccal mucosa. On the basis of these observations, the present work was undertaken to identify the *in situ* localization of HO-1 expression in normal buccal mucosa and OSF specimens. More specifically, we have therefore measured the relative levels of HO-1 in OSF compared with normal buccal mucosa and the effects of arecoline, a major areca nut alkaloid, on HO-1 in normal human BMFs *in vitro*.

Materials and methods

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of six normal buccal mucosa from non-areca quid chewers, and 25 OSF specimens from areca quid chewers, were drawn from the files of the Department of Pathology, Chung Shan Medical University Hospital. Diagnosis was based on histological examination of hematoxylin- and eosin-stained sections. Institutional Review Board permission at the Chung Shan Medical University Hospital was obtained for the use of discarded human tissues. Five micrometer sections were stained with the polyclonal anti-HO-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:100 dilution) using a standard avidin–biotin–peroxidase complex method (Lee *et al*, 2008). Diaminobenzidine (DAB; Zymed, South San Francisco, CA, USA) was then used as the substrate for localizing the antibody binding. Negative controls included serial sections from which either the primary or secondary antibodies were excluded. The preparations were counterstained with hematoxylin, mounted with Permount (Merck, Darmstadt, Germany) and examined by light microscopy.

Cell culture

Ten healthy individuals, without areca quid chewing habits, attending the Oral Medicine Center (Chung Shan Medical University Hospital, Taichung, Taiwan) were enrolled with informed consents for this study. Biopsy specimens were derived from histologically normal oral mucosa at the time of surgical third molar extraction. The OSF specimens were obtained from 25 male patients with areca quid chewing habits during surgical biopsy. Clinical diagnosis was confirmed by histopathological examination of the biopsy specimens. Fibroblast cultures were grown and maintained using procedures described previously (Tsai *et al*, 2003; Yang *et al*, 2008). Cell cultures between the third and eighth passages were used in this study.

Expression of HO-1 mRNA in OSF and BMF

Confluent cells were trypsinized, counted, and plated at a concentration of 1×10^5 cells in 60 mm culture dish and allowed to achieve confluence. Total RNA was prepared using TRIzol reagent (Gibco Laboratories, Grand Island, NY, USA) following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in a 15 μl reaction mixture containing 100 mg random hexamer and 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco Laboratories). The reaction mixture was diluted with 20 μl of water, and 3 μl of the diluted reaction mixture was used for the polymerase chain reaction (PCR). PCR reaction mixture contains 10 pmol of forward and reverse primers and 2 units of Taq DNA polymerase. Amplification was performed at 25 cycles for GAPDH and 30 cycles for HO-1 in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C. The sequences of primers used were as follows (Lee *et al*, 2008):

(A) GAPDH
Forward: 5′-TCCTCTGACTTCAACAGCGAC-ACC-3′
Reverse: 5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′
(B) HO-1
Forward: 5′-AAGGCTTTAAGCTGGTGATGG-3′
Reverse: 5′-AGCGGTGTCTGGGATGAACTA-3′

The PCR products were analyzed by agarose gel electrophoresis, and a 527 bp band for HO-1 was noted. When the band densities were measured and compared with the density of the band obtained for the house-keeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with GAPDH mRNA was quantified by the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA).

Effect of arecoline on HO-1 mRNA and protein in BMF

BMF derived from three strains were seeded 1×10^5 cells per well into 10 cm culture dish and incubated for 24 h. Cells arrested in G0 by serum deprivation (0.5% FCS; 48 h) were used in the experiments (Chang *et al*, 2005). Nearly confluent monolayers of cells were washed with serum free medium and immediately thereafter exposed at the various concentrations (0, 10, 20, 40, 80, and 160 μg ml^{-1}) of arecoline (Sigma, St Louis, MO, USA). Total RNA was isolated after 6-h incubation period for RT-PCR as described above. Cell lysates were collected at 24 h for Western blot analysis.

Western blot

For Western blot analysis, cell lyates were collected as described previously (Chang *et al*, 2005). Briefly, cells were solubilized with SDS-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% SDS-PAGE and immediately transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 3% BSA for 2 h, rinsed, and then incubated with primary antibodies anti-HO-1 (1:1000) in PBS 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 DAB. The intensities of the obtained bands were determined using a densitometer (AlphaImager 2000; Alpha Innotech). Each densitometric value was expressed as the mean ± s.d.

Statistical analysis

Triplicate separate experiments were performed throughout this study. For testing of differences in the HO-1 between the BMF and OSF, the Wilcoxon–Mann–Whitney rank sum test was applied. The significance of the results obtained from control and treated groups was statistically analyzed by one-way analysis of variance (ANOVA). A *P*-value of < 0.05 was considered to be statistically significant.

Results

The HO-1 staining in OSF was stronger than normal buccal mucosa. Figure 1a represents normal buccal mucosa 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 OSF group, intensive staining for HO-1 expression was observed in the epithelial cells, inflammatory cells, and fibroblasts (Figure 1b).

RT-PCR assay was used to compare HO-1 mRNA gene expression of the fibroblasts cultured from BMF and OSF. As shown in Figure 2, OSF specimens exhibited significantly higher HO-1 mRNA expression than BMFs. From the AlphaImager 2000 (Figure 3), the intensity of HO-1 mRNA from OSF was elevated about 2.98-fold compared with BMFs ($P < 0.05$).

RT-PCR were used to verify whether arecoline could affect HO-1 mRNA gene expression by human BMFs. As shown in Figure 4a, arecoline was found to elevate HO-1 mRNA gene expression in a dose-dependent manner ($P < 0.05$). From the AlphaImager 2000, the amount of HO-1 mRNA was elevated about 1.1, 1.4, 1.5, 1.8, and 2.6 at concentrations of 10, 20, 40, 80, and 160 μg ml^{-1}, respectively, compared with control (Figure 4b).

In addition, the results of RT-PCR were confirmed by Western blot analysis. As shown in Figure 5a, arecoline was found to elevate HO-1 protein expression in a dose-dependent manner ($P < 0.05$). From the AlphaImager 2000, the amount of HO-1 protein was elevated about 1.5, 5.7, 7.8, 5.8, and 3.0 at concentrations of 10, 20, 40, 80, and 160 μg ml^{-1}, respectively, compared with control (Figure 5b).

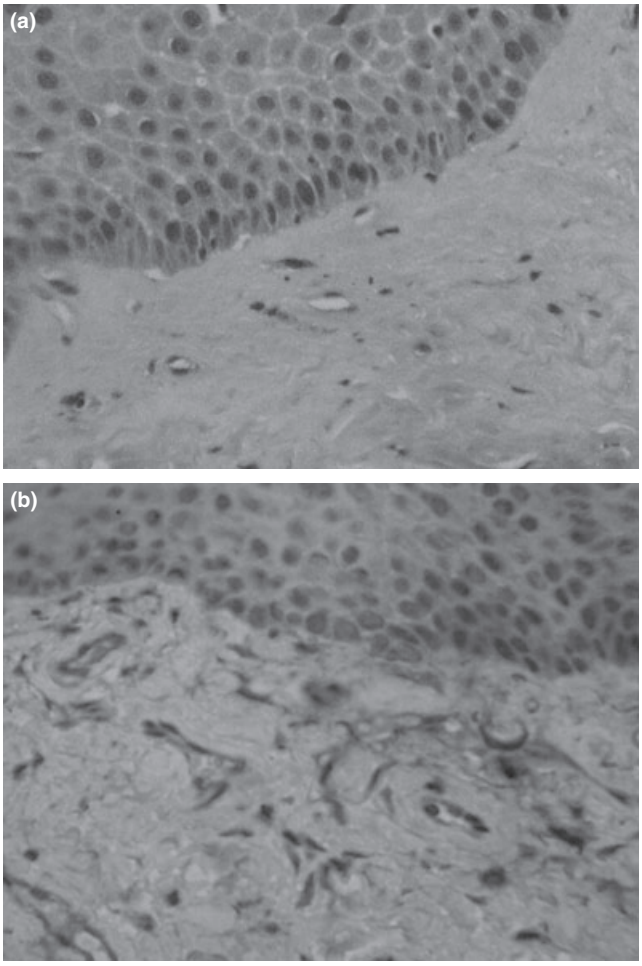

Figure 1 (**a**) Very faint immunoreactivity of heme oxygenase-1(HO-1) was observed in normal buccal mucosa and the strong signal was limited in the epithelial cells and endothelial. (200×) (**b**) In oral submucous fibrosis (OSF) group, HO-1 was evident as intense, diffuse brown coloring throughout the connective tissue and detected at relatively high levels in the epithelium (400×)

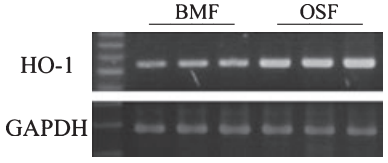

Figure 2 Comparison of the heme oxygenase-1(HO-1) mRNA level from buccal mucosa fibroblasts (BMFs) and oral submucous fibrosis (OSF) using RT-PCR assay. GAPDH gene was performed to monitor equal RNA loading. OSF specimens are significantly upregulated HO-1 mRNA expression than BMFs

Discussion

Heme oxygenase-1 is known as a stress-inducible protein. The immunohistochemical expression of HO-1 in hyperplasia tissue has been demonstrated in many

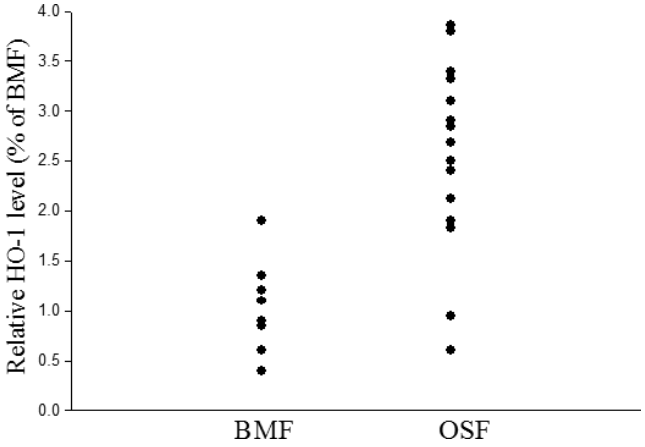

Figure 3 Densitometric analysis of heme oxygenase-1(HO-1) bands by AlphaImager 2000, the intensity of HO-1 mRNA from oral submucous fibrosis (OSF) was elevated about 2.98-fold compared with buccal mucosa fibroblasts ($P < 0.05$)

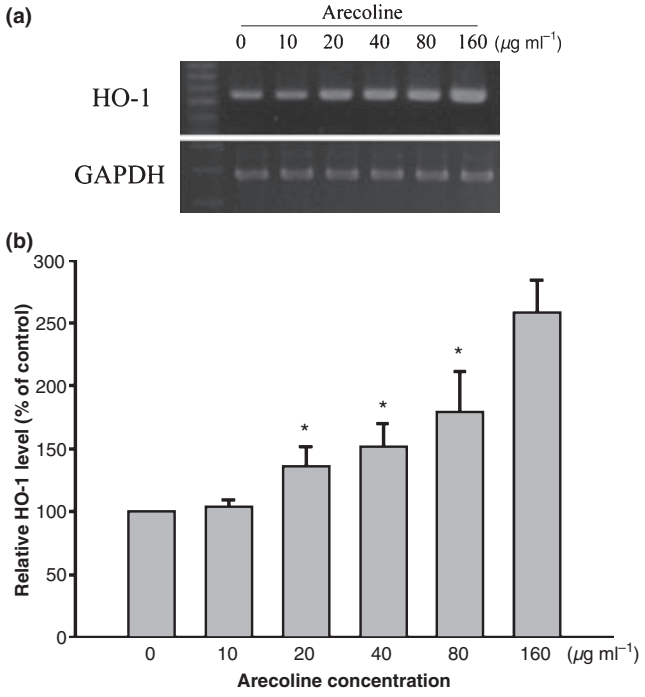

Figure 4 (**a**) Expression of heme oxygenase-1(HO-1) mRNA gene in arecoline-treated human buccal mucosa fibroblasts (BMFs) by RT-PCR assays. Cells were exposed for 6 h containing arecoline concentrations as indicated. M = DNA molecular size marker. (**b**) Levels of HO-1 mRNA gene treated with arecoline were measured by AlphaImager 2000. The relative level of HO-1 mRNA gene expression for each sample was normalized against GAPDH mRNA signal, and the control was set as 1.0. Optical density values represent the mean values of three different BMF ± s.d. *Significant differences from arecoline-treated values with $P < 0.05$

fibrotic disorders (Maines and Abrahanamsson, 1996; Zhou *et al*, 2004). In this study, HO-1 expression was first found to overexpress in areca quid chewing associated-OSF compared with normal buccal mucosa. This finding suggests that HO-1 may play an important role in the pathogenesis of OSF.

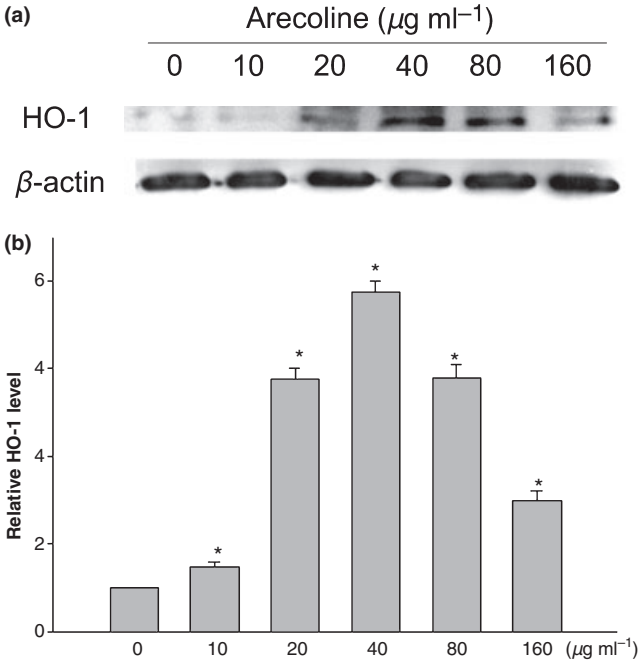

Figure 5 (**a**) Expression of heme oxygenase-1(HO-1) protein in arecoline-treated human buccal mucosa fibroblasts (BMFs) by Western blot. Cell lysates were exposed for 24 h containing arecoline concentrations as indicated. (**b**) Levels of HO-1 protein treated with nicotine were measured by densitometer. 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 values of three different BMF ± s.d. *Significant difference from control values with $P < 0.05$

The mechanism responsible for the HO-1 expression in areca quid chewing-associated OSF might be explained as follows. HO-1 is upregulated by oxidative stress and a variety of heavy metals, such as iron (Maines, 1988). Earlier studies have demonstrated that areca nut contains high iron content (IARC 1986) and generates ROS during chewing (Nair *et al*, 1992; Chen *et al*, 2002). It is therefore feasible to suggest that high levels of iron released and ROS generated may at least in part be responsible for the upregulation of HO-1 in areca quid chewing-associated OSF.

It is noteworthy that HO-1 is the only heat shock protein for which a catalytically based mechanism of action of cellular proliferation or transformation might be proposed in relation to iron and transferring (Vile and Tyrell, 1993). The following line of reasoning is use to propose a role for increase in HO-1 expression in fibroblasts proliferation within OSF, which is based on the function of the heme oxygenase system in the catalysis of the heme molecule and release of iron from areca quid.

The etiology of OSF is well known that an association exists with the habit of areca quid chewing. In this study, we first reported the upregulation of HO-1 mRNA and protein expression in BMF stimulated by arecoline. This suggests that one of the pathogenetic mechanisms of OSF may be the synthesis of HO-1 expression by BMF in response to areca quid challenge.

Numerous models have been proposed to explain the pathogenesis of OSF, many of which are related to the areca nut and its components. OSF may process from the connective tissue compartment where toxic substances released from the areca nut by chewing precipitate a change in gene expression in mesenchymal cells. In this study, we demonstrate that arecoline activates HO-1 expression in BMFs. This could however be the result of the presence of a subtype of fibroblast, which is more susceptible to external stimulation or gene modulation. Elevated HO-1 expression might offer a new clue for establishing the pathogenesis of OSF for patients habituated to the areca quid.

This study represents, to the best of our knowledge, the first systemic attempt to evaluate the role of HO-1 expression in areca quid chewing-associated OSF in human both *in vitro* and *in vivo*. We demonstrated that HO-1 is elevated in OSF specimens obtained from areca quid chewers. Arecoline-induced HO-1 expression suggests that areca quid chewing may contribute the pathogenesis of OSF by HO-1 expression. These results may advance our understanding of the role of HO-1 expression in pathogenesis of areca quid chewing-associated OSF. Further research is required, however, including the knockout experiments about HO-1, such as si-RNA, specifically whether OSF evolves solely as a result of increased/altered *de novo* synthesis and deposition of HO-1 by areca nut constitutes.

Author contributions

CH Tsai and YC Chang designed the study, analyzed the data and prepared the manuscript. SF Yang and SS Lee performed the plan of the study and data analysis.

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