行政院國家科學委員會專題研究計畫 成果報告

低氧誘導因子-1 alpha在人類正常頰黏膜及口腔黏膜下纖維化的表現
研究成果報告(精簡版)

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計畫主持人：楊順發
共同主持人：謝易修、張育超
計畫參與人員：碩士班研究生-兼任助理：楊嘉欣、揚惟恩

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The Upregulation of HIF-1α in Oral Submucous Fibrosis

Objectives: Hypoxia-inducible factor-1α (HIF-1 α) was identified from its role in regulating transcription of the erythropoietin gene which responds to changes in oxygen, providing cells with a master regulator. The potential for HIF-1 α to regulate physiological responses is clear. However, several studies found that HIF-1 α was involved in fibrotic diseases, including renalinterstitial fibrosis and pulmonary fibrosis. Currently, there is limited information about the expression of HIF-1 α expression in areca quid-associated oral submucous fibrosis (OSF). The aim of this study was to compare HIF-1 α expression in normal human buccal mucosa and OSF specimens and further to explore the potential mechanisms that may lead to induce HIF-1 α expression. **Methods**: Twenty OSF specimens were examined by immunohistochemistry. The expression of HIF-1 α from fibroblasts cultured from OSF and normal buccal mucosa fibroblasts (BMFs) were examined by RT-PCR. Furthermore, the effect of arecoline, the major areca nut alkaloid, was added to explore the potential mechanism that may lead to induce HIF-1 α expression. Furthermore, mitogen-activated protein kinase kinase (MEK) inhibitor U0126, phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 , P38 inhibitor SB203580, cyclooxygenase-2 (COX-2) inhibitor NS-398 and glutathione precursor

N-acetyl-L-cysteine (NAC) were added to find the possible mechanisms.

Results: HIF-1α expression was significantly higher in OSF specimens and expressed mainly by fibroblasts. OSF demonstrated significantly higher HIF-1α mRNA expression than BMFs by RT-PCR ($P<0.001$) and arecoline was also found to elevate HIF-1α expression in a dose-dependent manner ($P<0.05$). In addition, pretreatment with pharmacological agents markedly inhibited the arecoline-induced HSP47 expression ($p<0.05$).

Conclusion: The data presented here demonstrated that HIF-1α expression is significantly upregulated in OSF from areca quid chewers and arecoline may be responsible for the enhanced HIF-1α expression *in vivo*. This study was supported by NSC 95-2314-B-040-014.

Introduction

Areca quid chewing is one of the most worldwide addictive oral habits. Epidemiological studies have clearly associated that areca quid chewing is strongly implicated in oral leukoplasia, oral submucous fibrosis (OSF), and oral cancer. OSF is characterized by the submucosal accumulation of dense fibrous connective tissue with inflammatory cell infiltration and epithelial atrophy.

Our previous studies have demonstrated that upregulation of tissue inhibitor of metalloproteinase-1, vimentin, cyclooxygenase-2, Keratinocyte growth factor-1 and plasminogen activator inhibitor-1 may play important roles in the pathogenesis in OSF. However, the precise mechanism underlying OSF is not well known. Studies of fibrotic disorder in other tissues suggest that a number of growth factors may play an important part in the molecular pathogenesis of these diseases.

Hypoxia-inducible factor-1α (HIF-1 α) was identified from its role in regulating transcription of the erythropoietin gene which responds to changes in oxygen, providing cells with a master regulator. The potential for HIF-1 α to regulate physiological responses is clear. However, several studies found that HIF-1 α was involved in fibrotic diseases, including renalinterstitial fibrosis and pulmonary fibrosis. These finding may indicate that HIF-1 α is involved in the development of fibrosis. However, little is known about the expression and role of HIF-1 α in OSF. In this

study, we have therefore measured the relative levels of HIF-1 α in OSF compared with normal buccal mucosa and the effects of arecoline, the major areca nut alkaloid, on HIF-1 α in normal human buccal mucosa fibroblasts (BMFs) in vitro.

Materials and methods

Cell culture

Ten healthy individuals, without areca quid chewing habits, attending the Department of Oral and Maxillofacial Surgery (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 twenty 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. Cell cultures between the third and eighth passages were used in this study.

Expression of HIF-1 α 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 15μl reaction mixture containing 100 mg random hexamer and 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco Laboratories, Grand Island, NY, USA). 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 pmole of forward and reverse primers and 2 units of Tag DNA polymerase. Amplification was performed at 25 cycles for GAPDH and 30 cycles for HIF-1 α in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C. The sequences of primers used were as follows:

A) GAPDH Forward: 5'-TCCTCTGACTTCAACAGCGACACC-3'

Reverse: 5'-TCTCTCTTCCTCTTGTGCTCTTGG-3'

B) HIF-1 α Forward: 5'- GTCGGACAGCCTCACCAAACAGAGC-3'

Reverse: 5'- GTTAACTTGATCCAAAGCTCTGAG -3'

The PCR products were analyzed by agarose gel electrophoresis. When the band densities were measured and compared with the density of the band obtained for the housekeeping 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 and other different reagents on HIF-1 α mRNA in BMF

BMF derived from six strains were seeded 1×10^5 cells per well into 10 cm culture dish and incubated for 24 h. Then the medium was changed to a medium containing 10 % heated activated FCS and various concentrations of arecoline (Sigma, St. Louis, MO, USA) (0 to 80 μg/ml). Total RNA was isolated after 6 h incubation period for RT-PCR as described above.

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of six normal buccal mucosa from non-areca quid chewers, and twenty 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. Five μm sections were stained with the monoclonal anti- HIF-1 α antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:100 dilution) using a standard avidin-biotin-peroxidase complex method. AEC (DAKO, Carpinteria, 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.

Statistical analysis

Triplicate or more separate experiments were performed throughout this study. For testing of differences in the HIF-1 α 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 the Student *t*-test. A *p*-value of < 0.05 was considered to be statistically significant.

Conclusion

As far as we known, this is the first systematic attempt to evaluate the role of HIF-1 α expression in areca quid associated-OSF in human in vitro levels. We have demonstrated for the first time that HIF-1 α is elevated in OSF than normal buccal mucosa. Data from our in vitro experiments showed that arecoline was capable of stimulating HIF-1 α mRNA expression in human BMFs. This suggests that one of the pathogenic mechanisms of OSF in vivo may be the synthesis of HIF-1 α by resident cells in response to areca nut challenge. In addition, HIF-1 α was inhibited by U0126, LY294002, NAC and NS-398. Therefore, studying the signal transduction pathway involved in HIF-1 α expression may prove versatile. However, more detailed studies should be undertaken to clarify the agents that can regulate HIF-1 α in vitro and in vivo. Further studies will be of importance to address the contribution of various areca

nut ingredients in the areca quid-associated OSF.

Legends for figures

Fig. 1 Comparison of the HIF-1 α mRNA level from BMFs and OSFs using RT-PCR assay. GAPDH gene was performed in order to monitor equal RNA loading. OSF specimens are significantly upregulated HSP47 mRNA expression than BMFs.

Fig. 2 Expression of HIF-1 α mRNA gene in arecoline-treated human BMFs by RT-PCR assays. Cells were exposed for 6 h containing arecoline concentrations as indicated. M = DNA molecular size marker. * Significant differences from control values with $p<0.05$.

Fig. 3 Expression of mRNA gene in Effects of pharmacological agents on arecoline induced HIF-1 α mRNA expression in BMFs. Cells were preexposed with U0126, LY294002, SB203580, NAC and NS-398 for 1 h then were treated for 2 h in the presence arecoline. * Significant differences from arecoline-treated values with $p<0.05$.

Fig. 4 In OSF specimens, HIF-1 α was noted subepithelially and expressed in the cytoplasm of fibroblasts.

Fig-1

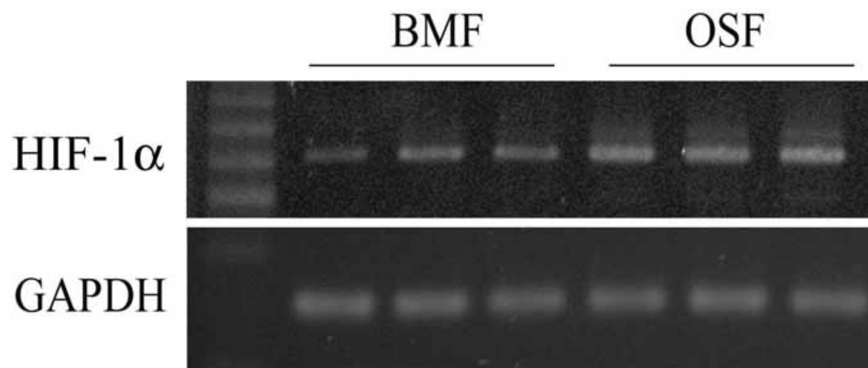


Fig. 2

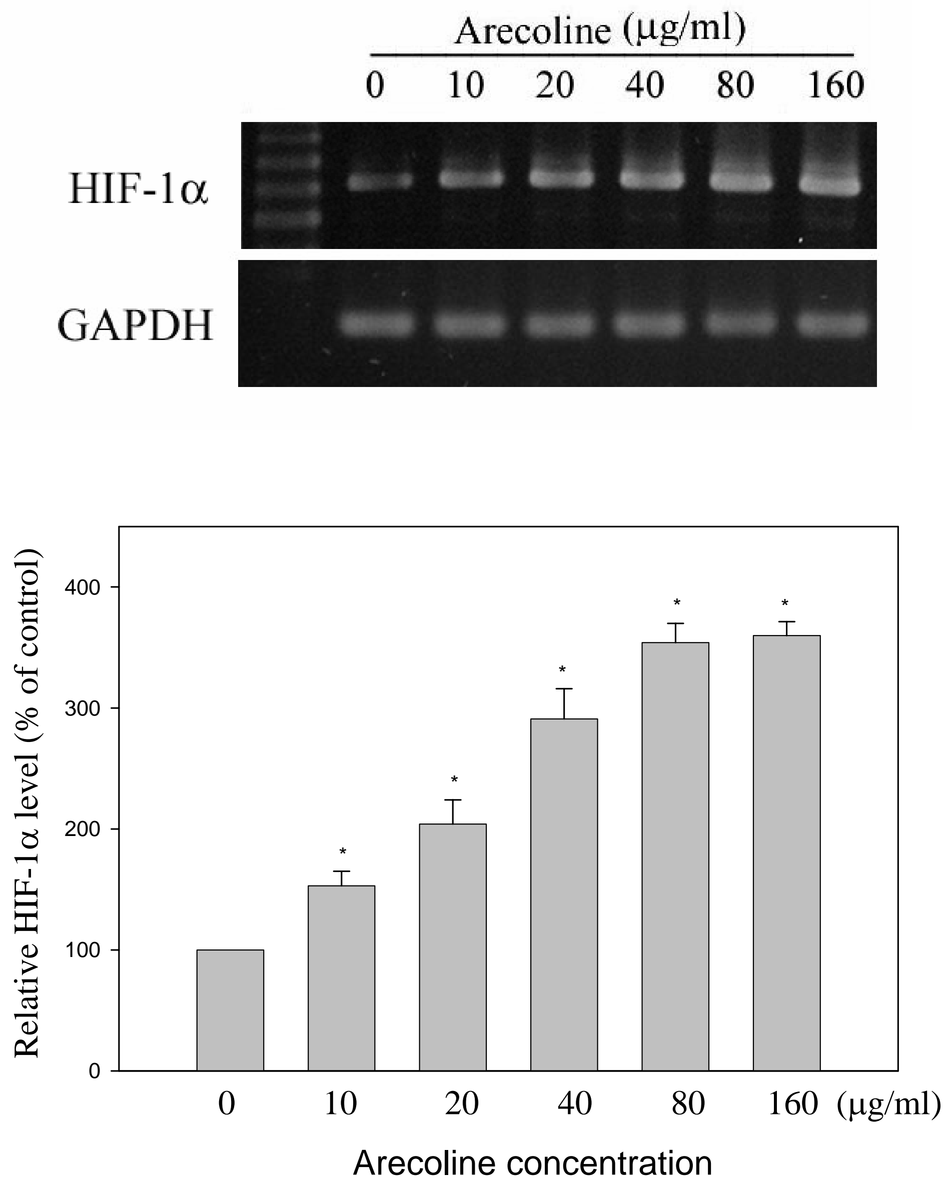
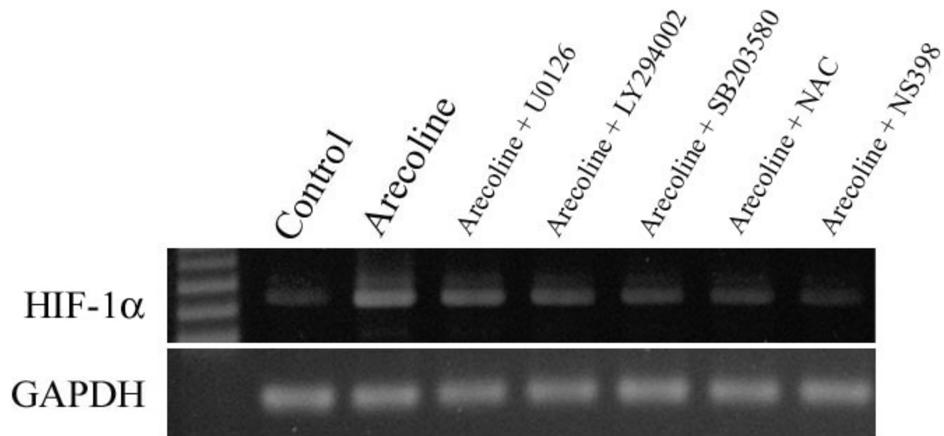


Fig. 3



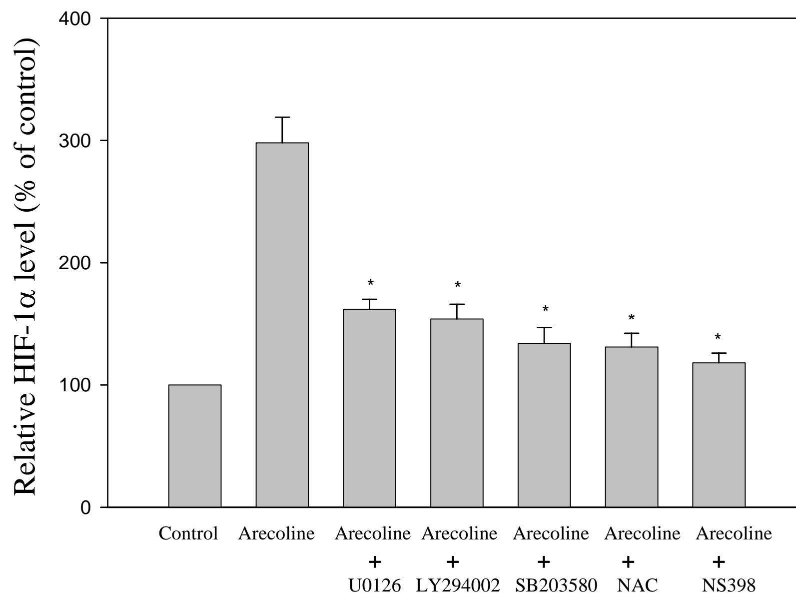


Fig. 4