Regulation of hypoxia-inducible factor-1α in human buccal mucosal fibroblasts stimulated with arecoline

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Introduction

Oral submucous fibrosis (OSF) is a chronic, insidious disease that affects the lamina propria of oral mucosa. Areca quid chewing is an important risk factor for the pathogenesis of OSF. Arecoline, a major areca nut alkaloid, was found to stimulate human normal buccal mucosal fibroblasts (BMFs) collagen synthesis,1 growth factor overexpression,2–3 and...
Regulation of HIF-1α in BMFs

epithelial-mesenchymal transition leading to OSF. Moreover, nutrition deficiencies such as vitamin B12 and folic acid were also noted in OSF patients.

Recently, hypoxia was found to play an important role in the areca quid chewing-associated OSF. Arecoline could upregulate hypoxia-inducible factor (HIF)-1α protein in normal BMFs. However, the possible regulatory mechanisms are still remain unclear. The aims of this study were to investigate the levels of HIF-1α mRNA in OSF BMFs relative to BMFs from normal controls (normal BMFs) and to assess the effects of arecoline on HIF-1α expression in normal BMFs in vitro. In addition, mitogen-activated protein 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 and their protective effects.

Materials and methods

Cell culture

After approved by the Ethics Review Board at the Chung Shan Medical University Hospital, normal BMFs derived from 10 normal controls and OSF BMFs from 15 OSF patients were cultured according to previous criteria and methods.

Expression of HIF-1α mRNA in OSF and normal BMFs

After each fibroblast achieved confluence, total RNA was prepared using TRIzol reagent (Gibco Laboratories, Grand Island, NY, USA) following the manufacturer’s instructions, as described previously. The sequences of primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH): Forward 5′-TCTCTGAGTTCAACAGGCACC-3′ Reverse 5′-TCCTCTTCTCTTCTTTGGG-3′; HIF-1α: Forward 5′-CCCCAGATTACAGATCAGACA-3′ Reverse: 5′-CATGTTTCATTTCGGC-3′. The polymerase chain reaction products were analyzed by agarose gel electrophoresis and a 704-bp band for HIF-1α was noted. The intensity of each band after normalization with GAPDH mRNA was quantified by the AlphaImager 2000, U0126, LY294002, SB203580, NAC, and NS398 were found to inhibit the arecoline-induced HIF-1α mRNA expression by lowering about 1.62, 1.54, 1.34, 1.31, and 1.18 fold, respectively (p<0.05).

Effect of arecoline on HIF-1α mRNA in normal BMFs

After cell confluence, normal BMFs were treated with various concentrations of arecoline (0–160 μg/ml) (Sigma, St. Louis, MO, USA). In addition, 80 μg/ml arecoline with or without various pharmacological agents 23μM U0126 (Promega, Madison, WI, USA), 163μM LY294002 (Promega, Madison, WI, USA), 26μM SB203580 (Promega, Madison, WI, USA), 5mM NAC (Sigma, St. Louis, MO, USA), and 10μM NS-398 (Sigma, St. Louis, MO, USA) was used to test their regulatory effects. Total RNA was isolated after 6 hours of incubation period for reverse-transcriptase polymerase chain reaction as described above. Cultures without fetal calf serum were used as negative control.

Statistical analysis

Triplicate or more separate experiments were performed throughout this study. For testing of differences in the HIF-1α between the normal BMFs and OSF BMFs, 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. A p-value of < 0.05 was considered to be statistically significant.

Results

Fibroblasts from OSF specimens exhibited significantly higher HIF-1α mRNA expression than normal BMFs (Figure 1A). From Alphalmager 2000, the intensity of HIF-1α mRNA from OSF BMFs was elevated about 1.96-fold compared with normal BMFs (p<0.05).

As shown in Figure 1B, arecoline was found to upregulate HIF-1α mRNA gene expression in a dose-dependent manner (p<0.05). From Alphalmager 2000, the amount of HIF-1α mRNA was elevated about 1.53, 2.04, 2.91, 3.54, and 3.60 fold at the concentrations of 10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml, and 160 μg/ml, respectively, compared with control.

Figure 1C revealed that all pharmacological agents without cytotoxic concentrations were found to inhibit the arecoline-induced HIF-1α mRNA expression (p<0.05). From Alphalmager 2000, U0126, LY294002, SB203580, NAC, and NS398 were found to inhibit the arecoline-induced HIF-1α mRNA expression by lowering about 1.62, 1.54, 1.34, 1.31, and 1.18 fold, respectively (p<0.05).

Discussion

Our results show that OSF BMFs demonstrated significantly higher HIF-1α mRNA expression than normal BMFs. Similar results were found by Tilakaratne et al who reported that OSF tissues significantly augmented HIF-1α mRNA expression compared with normal buccal mucosa. Consistently, OSF BMFs were reported to demonstrate significantly higher HIF-1α transcript than normal BMFs. These findings indicate that the state of hypoxia may exist in OSF. Moreover, our results demonstrated that arecoline could upregulate of HIF-1α mRNA expression in normal BMFs. Therefore, increased expression of HIF-1α in normal BMFs may support hypoxia as a pivot in the progression of areca quid associated-OSF.

In this study, pharmacological agents were added to search the possible regulation mechanisms on arecoline-induced HIF-1α expression. Both U0126 and SB203580 were found to reduce HIF-1α expression. Consistently, Chang et al reported that U0126 decreased areca nut extract-associated cytokine production in human oral gingival keratinocytes and KB cells. Recently, Lin et al reported that areca nut extract affected interactive mitogen-activated protein kinases (MAPKs), including extracellular signal-related kinases (ERK), c-Jun N-terminal protein kinase, and p38 in oral keratinocytes. These data...
could deplete glutathione levels in normal BMFs. \(^{13}\) Taken expression. Our previous study has shown that arecoline regulated by the PI3K pathway in normal BMFs. \(^{11}\) This indicates that arecoline-induced HIF-1\(\alpha\) can be regulated by the PI3K pathway in normal BMFs.

LY294002, SB203580, NAC, and NS398 on 80 \(\mu\)g/mL arecoline augmented HIF-1\(\alpha\) mRNA expression in normal BMFs. Arecoline augmented HIF-1\(\alpha\) mRNA expression in a dose-dependent manner (\(p < 0.05\)). \(^{12}\) The regulatory effects of U0126, LY294002, SB203580, NAC, and NS398 on 80 \(\mu\)g/mL arecoline-induced HIF-1\(\alpha\) mRNA expression in normal BMFs. Levels of HIF-1\(\alpha\) mRNA in normal BMFs after co-incubation with various pharmacological agents at 80 \(\mu\)g/mL arecoline. These pharmacological agents were found to inhibit the arecoline-induced HIF-1\(\alpha\) mRNA expression (\(p < 0.05\)) in normal BMFs. BMP = buccal mucosal fibroblasts; GAPDH = glyceraldehydes-3-phosphate dehydrogenase; HIF-1\(\alpha\) = hypoxia-inducible factor-1\(\alpha\); NAC = N-acetyl-L-cysteine; OSF = oral submucosal fibrosis; RT-PCR = reverse-transcriptase polymerase chain reaction.

In the presented study, arecoline-induced HIF-1\(\alpha\) expression was significantly inhibited by LY294002. \(^{13}\) This indicates that arecoline-induced HIF-1\(\alpha\) activation could be regulated by the PI3K pathway in normal BMFs.

NAC was found to inhibit arecoline-stimulated HIF-1\(\alpha\) expression. Our previous study has shown that arecoline could deplete glutathione levels in normal BMFs. \(^{13}\) Taken together, arecoline-stimulated HIF-1\(\alpha\) expression may be partially related to glutathione levels.

In this study, NS-398 was found to inhibit arecoline-stimulated HIF-1\(\alpha\) expression. Previously, the inhibitory effect of NS398 on HIF-1\(\alpha\) transcriptional activity was found in PC3 cells and HCT116 cells. \(^{14}\) Therefore, COX-2 signal transduction pathway may be involved in the arecoline-stimulated HIF-1\(\alpha\) expression.

In conclusion, hypoxia through HIF-1\(\alpha\) expression may promote fibrogenesis in the progression/pathogenesis of areca quid chewing-associated OSF. In addition, HIF-1\(\alpha\) was inhibited by U0126, SB203580, LY294002, NAC, and NS-398. These pharmacological agents may be further used as chemoprevention agents for areca quid chewing-associated OSF.

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**References**


