The Induction of Prostaglandin E₂ Production, Interleukin-6 Production, Cell Cycle Arrest, and Cytotoxicity in Primary Oral Keratinocytes and KB Cancer Cells by Areca Nut Ingredients Is Differentially Regulated by MEK/ERK Activation*

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There are about 200–600 million betel quid (BQ) chewers in the world. BQ chewing is one of the major risk factors of hepatocarcinoma, oropharyngeal, and esophageal cancers in Taiwan, India, and Southeast Asian countries. Thus, the precise molecular mechanisms deserve investigation. We used cultured primary keratinocytes and KB cells, RT-PCR, flow cytometry, Western blotting, and ELISA to evaluate whether alterations in early gene expression is crucial in the carcinogenic processes of BQ. We observed the induction of c-Fos mRNA expression in human gingival keratinocyte (GK) and KB carcinoma cells by areca nut (AN) extract and arecoline. A maximal increment in c-fos gene expression was shown at about 30 min after challenge. AN extract (100–800 μg/ml) and arecoline (0.1–0.8 mM) also stimulated ERK1/ERK2 phosphorylation with a maximal stimulation at 5–10 min of exposure. Pretreatment by U0126 (30 μm), a MEK inhibitor, markedly inhibited the c-Fos, cyclooxygenase-2 (COX-2), and IL-6 mRNA expression of the KB epithelial cells. In addition, U0126 and PD98059 (50 μm) also decreased AN extract- and arecoline-associated PGE₂ and IL-6 production in GK and KB cells. However, U0126 by itself arrested the cells in G₀/G₁ phase, but was not able to prevent AN- and arecoline-induced cell death or apoptosis. In contrast, U0126 enhanced the AN-induced apoptosis of KB cells. AN ingredients thus play a significant role in the pathogenesis of oropharyngeal cancer by activation of MEK/ERK/c-Fos pathway, which promotes keratinocyte inflammation, cell survival, and affects cell cycle progression.

Smoking, betel quid (BQ) chewing, and the consumption of alcohol, coffee, and tea are the five most popular oral habits in the world. There are about 200 to 600 million betel quid (BQ) chewers distributed across India, Sri Lanka, Pakistan, Taiwan, many other Southeast Asian countries, and South Africa (1, 2). In Taiwan, about one-tenth of the total population (about 2–2.8 million people) have a BQ chewing habit. Some even chew BQ all day with a consumption of more than 25 quids/day (3). Chewing BQ has long been considered to be a major risk factor for oral leukoplakia, oral submucous fibrosis, and oral cancer (1). Moreover, BQ chewing has been shown to increase the incidence of cancer in oral cavity (1, 2), oropharynx, pharynx (4, 5), esophagus (6–8), and liver (9–12). Recently, oropharyngeal cancer (9.7% of all cancer deaths, including cancer of the oral cavity, oropharynx, hypopharynx) and esophageal cancer (3%) has become the 4th and 9th popular cancer death in the male population of Taiwan, respectively. Epidemiological studies have indicated a multifactor etiology for these malignant diseases (14). Histological observation of upper end esophageal mucosa of BQ chewers by endoscopy showed marked submucous fibrosis (66%), atrophy of the squamous epithelium (52%), hyperkeratosis (52%), parakeratosis (30%), dyskeratosis (14%), acanthosis (14%), and mild dysplasia (2%) (15). Thus, the molecular pathogenesis of how BQ components contribute to carcinogenesis deserves further delineation.

BQ usually comprises a piece of areca nut (AN), influences Piper betle, and lime with or without Piper betle leaves. AN contains many polyphenols and several alkaloids such as arecoline, guvacoline, arecaidine, and guvacine. Many experiments have demonstrated that AN extract or its alkaloids possess cytotoxic or genotoxic effects on several kinds of cells in vitro (1, 2). Arecoline, a major AN alkaloid, is mutagenic to mammalian cells (16) and causes chromosomal aberration in Chinese hamster ovary cells in vitro or mouse bone marrow cells in vivo (17, 18). Sundqvist et al. (19) have shown that AN extract and nitroso-derivatives of AN alkaloids are cytotoxic and genotoxic to buccal epithelial cells. AN extract induces the differentiation, DNA strand breaks and DNA protein cross-links in cultured buccal mucosal epithelial cells (19, 20). We have demonstrated that AN extract can induce DNA strand breaks and is cytotoxic to oral mucosal fibroblasts (21). AN
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extract also induces unscheduled DNA synthesis and morphological alterations in cultured gingival keratinocytes (GK) (22). These results indicate that AN ingredients may be important to the pathogenesis of BQ chewing-related oral cancer.

In addition to the genotoxic stress, tissue inflammation, and release of inflammatory mediators such as prostanooids, interleukin-1α (IL-1α), IL-6, and TNF-α have been suggested to be a key factor for carcinogenesis of gastrointestinal systems including colorectum, stomach, esophagus, liver, and pancreas (23–27). Elevation of cyclooxygenase-2 expression in the squamous cell carcinoma of the hypopharynx, esophagus, and oral cavity has also been reported (28, 29). Recently, BQ components have been shown to induce keratinocyte inflammation by stimulation the prostanooids, interleukin-6 and TNF-α production of gingival keratinocytes (GK), and KB cancer cells (28, 30, 31). However, the precise mechanisms responsible for induction of prostanooids and IL-6 production are not fully clear. Induction of COX-2 and IL-6 gene expression has been associated with the MEKI/ERK-1/2/c-Fos/AP-1 activation in colon carcinoma cells, intestinal epithelial cells, and other kind of cells (32–34). Alterations in the expression of MAPK, several proto-oncogenes or tumor suppressor genes have been identified in cancer tissues from different sites and are considered to be important in the sequential stages of chemical carcinogenesis (35–37). In this study, we used cultured primary GK and KB cancer cells to test whether BQ components stimulate cell cycle dysregulation, prostanooids, and IL-6 production in epithelial cells via MEKI/ERK-1/2/c-Fos signal transduction pathways.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum, penicillin/streptomycin, keratinocyte growth medium (KGM-SFM), pituitary gland extract, and epidermal growth factors etc. were obtained from Invitrogen Life Technologies, Inc. ELISA kits for IL-6 measurement were from BIOSOURCE (BIOSOURCE International, Inc.). Arecoline, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), calfskin type I collagen and bovine plasma fibronectin were obtained from Sigma. PGE2, ELISA kits were purchased from Cayman Chemical Company (Ann Arbor, MI). Ethidium bromide, aprotinin, and kits for reverse transcription (RT) and PCR were purchased from Transduction Laboratories (Lexington, KY). Mouse antihuman-c-Fos, IL-6, and COX-2 antibodies were from Santa Cruz, CA. AN extract was prepared and weighed as previously described (30, 31). Total RNA isolation kits were purchased from HT Inc. Total RNA isolation kits were from Qiagen Inc. (Santa Clara, CA). AN extract or arecoline was obtained from Bio-Rad. Flow cytometric assay kits were obtained from BD Biosciences. KB carcinoma cells were obtained from ATCC, CA. AN extract or arecoline (100–800 μg/ml) or arecoline (0.4–0.8 μM) for 24 h were used for this study. KB carcinoma cells were cultured in DMEM containing 10% fetal calf serum and penicillin/streptomycin.

Chemical Exposure and DNA Isolation—Confluent GK were incubated in fresh KGM-SFM containing AN extract (100–800 μg/ml) or arecoline (0.1–1.2 μM) for 0.5, 1, 2, 4, and 24 h. Total RNA was isolated using Qiagen RNA isolation kits. The KB carcinoma cells were serum withdrawn and then treated with AN extract or arecoline for 0.5–24 h. Total RNA was isolated at each time point using Qiagen RNA isolation kits. In some experiments, KB cells or GK were exposed to different concentrations of AN extract (100–800 μg/ml) or arecoline for 30 min or 24 h for evaluation of c-Fos, COX-2, and IL-6 mRNA expression.

Semi-quantitative RT-PCR—In brief, 3 μg of denatured total RNA was reverse-transcribed in a total volume of 44.5-μl reaction mixture containing 4 μl of random primer (500 μg/ml), 8 μl of dNTP (2.5 μM), 4.5 μl of 10× RT buffer, 1 μl of RNase inhibitor (40 units/μl), and 0.5 μl of RT (21 units/μl) at 42 °C for 90 min. Four microliters of cDNA were then used for PCR amplification in a reaction volume of 50 μl containing 5 μl of 10× Super Taq buffer, 4 μl of dNTP (2.5 μM), 1 μl of specific primer, and 0.2 μl of Super Taq enzyme (2 units/μl). The reaction mixture was initially heated to 94 °C for 5 min in the first cycle, then the reaction was amplified for 15–35 cycles of 94 °C for 1 min, 55 °C for 1 min and then 72 °C for 2 min with a thermal cycler (Perkin Elmer 4800, PE Applied Biosystems, Foster City, CA). Finally, the reaction was set at 72 °C for 10 min. The primer pairs used in this study were: MESGAGAGGTCTTCTTACCCAC and TCTGGCTACTGGCTTCACCAAG (280 bp) (39), and β-actin, AAGAGGAGGACTCCACCATCG and TACATGCCTGGGGTGTTGAA (218 bp) (40). COX-2 is 5′-TCTAAAAAGGGTTGGAAAAATGCTC-3′ and 5′-AGATCTCTCCTGAGTATCCTT-3′. The PCR primer sequence for IL-6 was ACTGTTGTGCTTGCTCAGAC and CAGGGAGAATTCACAGAT (30, 31, 40). The amplified PCR products are 305 base pairs (bp) for COX-2, 218 bp for β-actin (BAC) and 459 bp for IL-6. The amplified DNA products were loaded onto a 1.8% agarose gel in 1× TBE buffer for electrophoresis. Gels were then exposed and developed with ethidium bromide, and photographs were taken. The radio amplified DNA product that was linear in relation to the input RNA was used for data presentation. Amplification of the BAC gene was used for a control.

Effects of AN Extract on AN Extract and Arecoline-induced IL-6 and COX-2 mRNA Expression of GK and KB Cells—KB cells were serum-starved for 24 h and then exposed to AN extract or arecoline for 2.5, 5, 10, 30, 60 min, 4 h, and 24 h. Cell lysates were prepared as described previously using freshly prepared lysis buffer (10 mM Tris-HCl, pH 7.4, 140 mM sodium chloride, 3 mM magnesium chloride, 0.5% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 1% aprotinin, and 5 mM dithiothreitol) (30, 31). The protein concentration of the cell lysates was measured with a Bio-Rad assay kit. Equal amounts of protein (50 μg/lane) were separated by 12% SDS-polyacrylamide gel electrophoresis (Scie-Plas) and transferred to polyvinylidene difluoride membrane by electroblotting. The membrane was blocked for 30 min at room temperature in a blocking reagent (20 mM Tris, pH 7.4, 125 mM NaCl, 0.2% Tween 20, 5% nonfat dry milk, and 0.1% sodium azide) and then incubated for 2 h with mouse anti-human-c-Fos, p-ERK, COX-2 monoclonal antibody (1:500) and then incubated with horseradish peroxidase-labeled goat anti-mouse secondary antibody for 1 h. The membrane was then washed four times with TBST. Finally the immunoreactive bands were developed by enhanced chemiluminescence (ECL) reagent and visualized on Fuji x-ray film.

Effects of MEKI Inhibitors on AN Extract and Arecoline-induced c-Fos, IL-6, and COX-2 mRNA Expression of GK and KB Cells—For elucidation of whether modulation of c-fos, IL-6, and COX-2 gene expression by AN extract and arecoline was mediated by the MEKI/ERK-1/2 pathway, GK and KB cells were prepared as described above and pretreated with U0126 (30 μM) for 15 min and then exposed to AN extract or arecoline for 24 h. Total RNA was isolated from GK and KB cells and reverse transcribed with Qiagen RNA isolation kits. The expression of c-fos, COX-2, and IL-6 mRNA was measured by semiquantitative RT-PCR as described above (30, 31).

Effects of MEKI Inhibitors on AN Extract and Arecoline-induced IL-6 and PGE2 Production of GK and KB Cells and the Concomitant Cytoxicity—KB cells (5 × 105 cells) were inoculated into each well of a 6-well culture plate. GK was used when growth was near confluence. Cells were first incubated in serum-free DMEM (for KB cells) or KGM-SFM without supplement (for GK) for 24 h. The medium (1 ml) was changed and then U0126 (30 and 50 μM) and PD98059 (50 μM) were added for 15 min. Various concentrations of AN extract and arecoline were added and the cells were further incubated for 24 h. Morphological changes in the GK and KB cells were photographed. The culture medium was collected for measurement of the IL-6 and PGE2 levels. The cell layers were washed three times to avoid any interference by the AN component on the measurement of cytoxicity. Finally, cytoxicity was measured by an MTT assay as described previously (30, 31). Briefly, cell layers were incubated with fresh medium containing 0.5 mg/ml of MTT for 2 h. The produced formazan was dissolved in 2 ml of dimethyl sulfoxide and read against a solvent blank (Me,SO) with a Datas Microwave Plate Reader (Dynatech Medical Products Ltd.) at an optical density of 540 nm.

Effects of MEKI Inhibitors on AN Extract and Arecoline-induced Cell Cycle Kinetics of KB Cells—KB cells were treated with U0126 (30 or 50 μM) and then exposed to AN extract (400 and 800 μg/ml) or arecoline (0.4 and 0.8 μM) for 24 h. Both floating cells and attached cells were collected for flow cytometric analysis as described previously (31, 42). Briefly, cells
were resuspended and fixed in 70% ice-cold ethanol containing 2 mg/ml RNase for 30 min. They were washed twice with phosphate-buffered saline and eventually stained with propidium iodide (PI) (400 μg/ml) for 10 min at room temperature. The PI fluorescence of individual KB cell was analyzed by a FACS Calibur Flow Cytometer (Becton Dickinson, Worldwide Inc., San Jose, CA) using an Argon ion laser. The wavelength of laser excitation was set at 488 nm and emission collected at >590 nm. In total, 20,000 cells each were analyzed for the control and the experimental samples. The percentage of cells in sub-G1, G0/G1, phase, S phase and G2/M phase were determined using standard ModFit software and the CellQuest programs.

Effects of MEK1 Inhibitors on AN Extract and Arecoline-induced COX-2 Protein Production of GK and KB Cells—For elucidation of whether MEK1/ERK-1/2 activation mediates the AN and arecoline associated PGE2 production via COX-2 protein expression, KB cells were serum-starved for 24 h, pretreated with Me2SO (control) or PD98059 (12.5, 25, and 50 μM) for 15 min and then exposed to AN extract for 24 h. Cell lysates were prepared as described above and subjected to 12% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membrane was blocked for 30 min at room temperature in a blocking reagent (20 mM Tris, pH 7.4, 125 mM NaCl, 0.2% Tween 20, 5% nonfat dry milk, and 0.1% sodium azide), incubated with a primary antibody (COX-2) (1:500) and GAPDH antibodies. After final membrane washing, the immunoreactive bands were visualized on Fuji x-ray film after developed by ECL reagents. Statistical Analysis—Four or more separate experiments were performed. Results were analyzed by a paired Student’s t test. A p value < 0.05 was considered to show a significant difference between groups.

RESULTS

Up-regulation of c-Fos mRNA Expression in GK and KB Cells by AN Extract and Arecoline—An increase of the c-Fos mRNA expression in KB cells was evident following exposure to AN extract (400 μg/ml) for 30 min. Elevated c-Fos expression was still noted after 1 h of exposure, but the level of c-Fos then decreased gradually to the basal level after 2 h of treatment (Fig. 1a). Arecoline (0.4 mM), the major alkaloid in AN extract, also stimulated the expression of c-Fos. The kinetics of its stimulatory effect on c-Fos was very similar to that of AN extract. This stimulatory effect was a transient phenomenon and became less evident after 1 h of exposure (Fig. 1b).

We then studied the effect of different doses of AN extract (100–800 μg/ml) or arecoline (0.1–0.8 mM) on c-Fos mRNA expression in KB cells. The induction of c-Fos mRNA expression in KB cells was noted after exposure to different concentrations of AN extract (100–800 μg/ml) for 30 min. However, at concentrations higher than 800 μg/ml, the stimulatory effect of AN on c-Fos mRNA expression became less apparent (Fig. 2a). Elevated expression of c-Fos mRNA was also noted after exposure to over 0.1 mM of arecoline for 30 min. However, the stimulatory effect became less evident after exposure to 0.8 mM arecoline (Fig. 2b). A similar effect of AN extract and arecoline on c-Fos expression in GK was also noted (data not shown).

Activation of ERK-1/2 Phosphorylation of KB Cells by AN Extract and Arecoline—AN extract (400 μg/ml) rapidly induced ERK-1/2 phosphorylation of KB cells after 2.5 min of exposure and was sustained for 10 min. The phosphorylation of ERK-1/2 rapidly declined to below the basal level after 30 min of exposure (Fig. 3a). Similarly, arecoline (0.4 mM) also stimulated ERK-1/2 phosphorylation of KB cells after 5 min of exposure with a peak induction at 10 min of exposure (Fig. 3b). AN extract stimulated the ERK phosphorylation in a dose-dependent manner with maximal effect at a concentration of 800 μg/ml (Fig. 3c). Arecoline also induced the phosphorylation of ERK-1/2 at concentrations over 0.1 mM (Fig. 3d).

Inhibition of c-Fos mRNA Expression of KB Cells by U0126—The stimulatory effect of AN extract and arecoline on c-Fos mRNA expression of KB cells was inhibited by U0126 (30 μM), a MEK1 inhibitor. As shown in Fig. 4a, AN extract (400 μg/ml) and arecoline (0.4 and 0.8 mM) stimulated c-Fos mRNA expression of KB cells after 30 min of exposure (lanes 4, 6, and 8). Pretreatment with U0126 for 15 min markedly suppressed AN- and arecoline-induced c-fos gene expression (Fig. 4a, lanes 5, 7, and 9). Quantitatively, AN extract induced the c-Fos expression by 2.74-fold compared with the control, whereas arecoline (0.4 mM) elevate the c-Fos expression by 2.58-fold compared with the control, as revealed by densitometry analysis of multiple blots. Pretreatment with U0126 resulted in a marked attenuation of the AN extract and arecoline-induced c-Fos mRNA expression (Fig. 4b).

Modulation of AN- and Arecoline-associated PGE2 and IL-6 Production in GK and KB Cells by U0126 and PD98059—As shown in Table I, AN extract stimulated PGE2 and IL-6 production by KB cells. In the absence of AN extract, U0126 (30 μM) inhibited the basal level production of PGE2 but not IL-6 production in KB cells. However, U0126 almost completely prevented the AN-extract-induced production of both PGE2 and IL-6 in KB cells (Table I). Similarly, PD98059 (50 μM), another...
MEK1 inhibitor, also inhibited AN extract-induced PGE₂ and IL-6 production by KB cells (data not shown). Exposure of KB cells to AN extract (400–800 µg/ml) showed mild cytotoxicity (11–19%). Pretreatment with 30 µM of U0126 resulted in little protective effect with respect to AN cytotoxicity at this concentration. Similar results were observed when using GK (data not shown).

In contrast, arecoline inhibited IL-6 production, but stimulated PGE₂ production in KB cells (Table II). U0126 pretreatment suppressed arecoline-induced PGE₂ production, but potentiated the arecoline-associated decrease in IL-6 production by KB cells. U0126 showed little effect on arecoline cytotoxicity at this concentration (Table II).

Morphological Alterations of KB Cells Induced by AN Extract and Arecoline with or without U0126—Changes in inflammatory mediator release were accompanied with evident morphological alterations. The KB cells could be seen to have a cuboidal or triangular appearance with a clear intercellular space (Fig. 3a). After exposure to 50 µM U0126 for 24 h, most of the cells retained their original shape, but some cells became retracted, rounded and showed even cell surface blebbing (Fig. 5a).

**TABLE I**

| Chemicals | IL-6 (n = 5) | PGE₂ (n = 7) | MTT (n = 4) |
|-----------|-------------|-------------|-------------|
| AN 400    | 433 ± 65    | 721 ± 136   | 100         |
| AN 800    | 807 ± 69    | 1668 ± 263  | 89.3 ± 4.7  |
| U0126 30 µM | 422 ± 35   | 200 ± 38    | 87.8 ± 4.4  |
| U0126 + AN 400 | 438 ± 21    | 200 ± 39    | 89.8 ± 6.3  |
| U0126 + AN 800 | 1140 ± 161  | 2673 ± 486  | 82.0 ± 4.5  |

* Denotes marked difference (p < 0.05) when compared with untreated control.

Denotes marked difference (p < 0.05) when compared with relative AN-treated groups.
5b). Exposure to 0.4 mM arecoline also induced retraction and rounding in numerous cells. Some KB cells showed intracellular vacuoles (Fig. 5c). More evident rounding and retraction of KB cells were noted after exposure to U0126 with arecoline, but most of these cells were still attached on the culture wells (Fig. 5d). Exposure of KB cells to AN extract (800 μg/ml) also led to evident cell retraction (Fig. 5e). Almost all of the KB cells became rounded, floating and had a shell-like appearance after exposure to U0126 (50 μM) and AN extract (800 μg/ml) for 24 h. Some cells with marked cell surface blebbing were observed (Fig. 5f).

**Effect of U0126 on AN Extract- and Arecoline-induced Cell Cycle Alterations, Apoptosis, and Cell Death of KB Cells**—When further checking whether U0126 may affect cell cycle progression, we interestingly found that exposure of KB cells to U0126 (50 μM) led to G_{2}/G_{1} arrest (Fig. 6a), whereas exposure to AN extract (400 μg/ml) alone induced late S and G_{2}/M cell cycle arrest. Pretreatment with U0126 and then co-incubation with AN extract resulted in more cell apoptosis as indicated by an increase in the sub-G_{2}/G_{1} population. Similarly, in Fig. 6b, arecoline (0.4 mM) also induced evident late S and G_{2}/M arrest of KB cells. Preincubation with U0126 led to more cells being arrested in G_{2}/G_{1} phase, but no enhancement of apoptosis was noted.

Quantitatively, the cytotoxicity was measured by an MTT assay. As shown in Fig. 6c, either U0126 or AN extract alone resulted in mild cytotoxicity with KB cells, namely, a 9 and 11% decrease in cells, respectively. However, pretreatment with U0126 and then co-incubation with AN extract (400 or 800 μg/ml) potentiated the cytotoxic effect on KB cells, as reflected by a decrease in cell numbers of 21 and 14% compared with the control. Arecoline also decreased cell numbers by 3 and 57% at concentrations of 0.4 and 1.2 mM, respectively (Fig. 6d). Elevation of the arecoline concentration to 1.2 mM was used to evaluate whether U0126 showed any preventive effect, but, preincubation with U0126 (50 μM) showed little effect on the arecoline cytotoxicity toward KB epithelial cells.

**Effect of MEK1 Inhibitor on AN Extract- and Arecoline-associated COX-2 and IL-6 Gene Expression of KB Cells**—As shown in Fig. 7, the basal levels of COX-2 and IL-6 gene expression of KB cells were generally low. AN extract (400 μg/ml) stimulated COX-2 and IL-6 mRNA expression in KB cells. Pretreatment of KB cells with the MEK1 inhibitor U0126 prior to the addition of AN extract markedly suppressed AN extract-induced COX-2 and IL-6 gene expression. Arecoline (0.4 and 0.8 mM) and U0126 showed little stimulatory effect on the arecoline cytotoxicity toward KB epithelial cells.

**Effect of MEK1 Inhibitor on AN Extract-induced COX-2 Protein Expression of KB Cells**—To further check whether MEK1 mediates AN-induced PGE2 production via induction of the COX-2 protein, KB cells were pretreated with different concentrations of PD98059, a MEK1 inhibitor, prior to exposure to AN extract for 24 h. As shown in Fig. 8, PD98059 (>25 μM) markedly inhibited AN extract (400 μg/ml)-induced COX-2 protein expression by KB cells.

**DISCUSSION**

BQ chewing is the main etiological factor of oral cancer and oral submucous fibrosis. Recently, epidemiological studies also support that BQ chewing increases the risk of cancer in the oral cavity, oropharynx, esophagus, and liver (4–12). BQ components exhibit genotoxicity, mutagenicity, and induce tumors in various organs of experimental animals (1, 2). However, the actual molecular mechanisms whereby the BQ ingredients induce cancers deserve further investigation. Chemical-induced inflammation has been linked to alimentary carcinogenesis (23–27). In the present study, AN components are shown to induce PGE2 and IL-6 production by epithelial cells. AN components also induced IL-6 mRNA, COX-2 mRNA, and protein expression in these cells at the transcriptional and translational levels (28, 30, 31). However, arecoline inhibited IL-6 production by GR and KB cells. Arecoline elevated PGE2 production by KB cells, but markedly less than by AN extract. This suggests that AN contains other components that are responsible for inflammatory mediator production. Wang et al. (43) have detected the expression of IL-6 in cancer cells of biopsy samples from 80 esophageal cancer patients by immunohistochemistry and RT-PCR. Elevated serum level of IL-6 is a bio-

**TABLE II**

| Chemicals               | IL-6 (n = 5) | PGE2 (n = 7) | MTT (n = 4) |
|-------------------------|--------------|--------------|-------------|
|                         | pg/ml        | pg/ml        | % control   |
| Control                 | 533 ± 95     | 601 ± 116    | 100         |
| Arecoline 0.4 mM        | 339 ± 39     | 802 ± 164    | 89.3 ± 3.9  |
| U0126 30 μM             | 461 ± 63     | 158 ± 29     | 90.3 ± 1.4  |
| U0126 + arecoline 0.4 mM| 220 ± 10     | 206 ± 42     | 89.0 ± 2.2  |
| Arecoline 0.8 mM        | 285 ± 49     | 970 ± 184    | 83.0 ± 4.3  |
| U0126 + arecoline 0.8 mM| 150 ± 26     | 184 ± 38     | 86.3 ± 3.2  |

* Denotes marked difference (p < 0.05) when compared with untreated control.

* Denotes marked difference (p < 0.05) when compared with relative AN-treated groups.
A marker of poor prognosis for esophageal cancer. However, in this study the medical history in terms of oral habits is not given (43). Peng et al. (28) observed overexpression of COX-2 in 23 biopsy specimens of hypopharyngeal squamous cell carcinoma, all being BQ chewers. Zimmermann et al. (29) also found COX-2 overexpression in most of esophageal squamous cell carcinoma and adenocarcinoma. Selective COX inhibition induces apoptosis and reduces the proliferation of esophageal cancer cells, supporting the use of COX-2 inhibitors in the prevention of upper aerodigestive tract cancer (27, 44). Prostanoids are important for the initiation, promotion and progression in multistep chemical carcinogenesis. Prostanoids can modulate the humoral and cellular immune responses involved in the killing of malignant cancer cells (45). In contrast, IL-6 regulates the behavior of dermal keratinocytes, leading to skin inflammation, keratinocyte growth and carcinogenesis (46). IL-6 is involved in a variety of biological processes, including the immune response, inflammation and carcinogenesis and does this by regulating the growth, survival and differentiation of target cells (47). Alterations in PGE₂ and IL-6 production and their related gene expression in oropharyngeal epithelial cell by AN components may thus be crucial for the carcinogenesis of upper aerodigestive tract in BQ chewers.

Various genotoxic chemicals may induce the release of inflammatory mediators via MAPK activation (32–34). The Fos and Jun families of transcription factors play important roles in the tumor promotion and progression (48, 49). Induction of c-Fos and c-Jun also occurs during keratinocyte differentiation during in vivo and

![Effect of U0126 on AN extract- and arecoline-induced cell cycle alterations, apoptosis, and cell growth in KB epithelial cells.](image)

KB cells were serum-starved and pretreated with U0126 (50 μM) for 15 min. Next, AN extract (400 μg/ml) (a) or arecoline (0.4 mM) (b) were added. These cells were then incubated for 24 h and collected for flow cytometric analysis of cell cycle distribution. KB cells were treated with AN extracts with or without U0126 (n = 6) (c), or arecoline with/without U0126 (n = 4) (d) similarly for 24 h. An MTT assay was conducted to assay cytotoxicity. * denotes marked difference (p < 0.05) when compared with relative AN-treated group, respectively.
in vitro cell culture (50, 51). We interestingly found that AN extract and arecoline also induced c-fos mRNA expression by human primary GK and KB epithelial cells within 30 min of exposure. Although c-Fos is an early responsive gene mediating the mitogenic effects, c-fos gene is considered to be important in the differentiation of cultured normal neonatal foreskin keratinocytes (51). Sustained expression of c-fos also precedes cell death. It has been shown that DNA-damaging agents and cytostatic agents can induce c-fos gene expression (52). Consistently, exposure of GK to AN (200 µg/ml) for 6 h increased the planar cell surface area of GK, which is a marker of keratinocyte differentiation (22). Sundqvist and Grafstrom (20) also elucidated that exposure of oral mucosal epithelial cells to AN extract for 3 h increased the expression of involucrin, a marker of epithelial differentiation (20). This indicates that AN extract possibly can induce the differentiation of GK through the induction of c-fos. AN ingredients can induce DNA strand breaks, DNA-protein cross-links and unscheduled DNA synthesis in oral keratinocytes (19, 20, 22). Since c-Fos activation is often regulated by upstream MEK/ERK-1/2, we further found that AN extract and arecoline induced ERK1/2 phosphorylation in human epithelial cells within 5–10 min of exposure. Inhibition of c-Fos mRNA expression occurred on exposure to U0126. This indicates that induction of c-fos gene expression by AN components is mediated by the MEK1/ERK-1/2 pathway in GK and KB epithelial cells. This agrees with the elevated expression of MAPK and several proto-oncogenes that have been observed in cancer tissues from different sites during multistage chemical carcinogenesis (35–37). Alterations of these proto-oncogenes by AN ingredients may possibly explain why BQ chewers are more susceptible to the occurrence of cancer in upper aerodigestive tract.

Because AN components and arecoline are shown to modulate a number of cellular events, such as PGE2, IL-6 production, TNF-α production, cell cycle dysregulation, and apoptosis in GK and KB epithelial cells (30, 31), we therefore evaluated whether modulation of these events are mediated by MEK1/ERK pathway. We found that AN-induced PGE2 production, COX-2 mRNA, IL-6 mRNA, and IL-6 protein expression were markedly inhibited by U0126 and PD98059, indicating the crucial role of the MEK1/ERK pathway in regulation of COX-2 and IL-6 at both the transcriptional and translational level. U0126 showed only a mild inhibition of the basal level of IL-6 production. Since arecoline stimulated ERK phosphorylation and c-fos gene expression, but inhibited the IL-6 production, this suggested that activation of ERK1/2/c-fos was not the only pathway responsible for IL-6 generation. However, U0126 markedly inhibited basal and AN-induced PGE2 production. Possibly, both COX-2 and COX-2 genes are regulated mainly by MEK1/ERK1/2 pathways in epithelial cells.

In the present study, exposure to U0126 arrested KB cells in G0/G1 phase. Pretreatment of KB cells with U0126 then co-incubation with AN extract or arecoline increased arrest of KB cells in G0/G1 instead of in late S and G2/M phase. Similarly, exposure of non-small cell lung cancer cells to U0126 and PD98059 also induce cell cycle arrest in G0/G1 phase (53). In addition, we found that inclusion of U0126 enhanced AN induced apoptosis and cell death, as analyzed by the MTT assay and flow cytometry. This is in agreement with the results from transfection of dominant negative mutants of ERK2, which potentiates paclitaxel-induced apoptosis of non-small lung cancer cells (53). Induction of Ras/ERK pathways by EGF also attenuates interferon-induced apoptosis of KB carcinoma cells (54). This indicates that the MEK1/ERK pathway is a survival factor for KB cancer cells and their induction by AN extract promotes cell survival with damaged DNA. Although IL-6 has been shown to be an anti-apoptotic factor in human esophageal carcinoma cells (13), promotion of cell survival by the MEK1/ERK-1/2 pathway seems to be mediated by a novel pathway other than IL-6 production, because inclusion of indomethacin and specific neutralization antibodies against IL-6 and TNF-α is not able to prevent AN cytotoxicity (31). Induction of cell death and apoptosis of GK and KB epithelial cells by AN components is thus mediated by pathways other than MEK1/ERK1/2 activation.

In summary, various proto-oncogenes, such as MEK1, ERK-1/2, and c-Fos, play a key role in the control of keratinocyte proliferation, differentiation, inflammatory mediator release and cell death. Dysregulation of the expression of these proto-oncogenes in keratinocytes by AN ingredients may therefore be important in the pathogenesis of carcinogenesis. Further studies are needed to clarify the signal transduction systems responsible for AN-induced cell cycle arrest and apoptosis in aerodigestive tract epithelial cells. In the future, MEK1/ERK and cyclooxygenase inhibitors or other anti-inflammatory agents need to be developed and tested in order to determine if they have a preventive effect on BQ chewing-related diseases.

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