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Regulation of oxidative-stress responsive genes by arecoline in human keratinocytes

G. S. Thangjam, P. Kondaiah
Department of Molecular Reproduction Development and Genetics, Indian Institute of Science, Bangalore, India

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Background and Objective: Arecoline, an arecanut alkaloid present in the saliva of betel quid chewers, has been implicated in the pathogenesis of a variety of inflammatory oral diseases, including oral submucous fibrosis and periodontitis. To understand the molecular basis of arecoline action in epithelial changes associated with these diseases, we investigated the effects of arecoline on human keratinocytes with respect to cell growth regulation and the expression of stress-responsive genes.

Material and Methods: Human keratinocyte cells (of the HaCaT cell line) were treated with arecoline, following which cell viability was assessed using the Trypan Blue dye-exclusion assay, cell growth and proliferation were analyzed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and 5-bromo-2-deoxyuridine incorporation assays, cell cycle arrest and generation of reactive oxygen species were examined using flow cytometry, and gene expression changes were investigated using the reverse transcription–polymerase chain reaction technique. The role of oxidative stress, muscarinic acetylcholine receptor and mitogen-activated protein kinase (MAPK) pathways were studied using specific inhibitors. Western blot analysis was performed to study p38 MAPK activation.

Results: Arecoline induced the generation of reactive oxygen species and cell cycle arrest at the G1/G0 phase in HaCaT cells without affecting the expression of p21/Cip1. Arecoline-induced epithelial cell death at higher concentrations was caused by oxidative trauma without eliciting apoptosis. Sublethal concentrations of arecoline upregulated the expression of the following stress-responsive genes: heme oxygenase-1; ferritin light chain; glucose-6-phosphate dehydrogenase; glutamate-cysteine ligase catalytic subunit; and glutathione reductase. Additionally, there was a dose-dependent induction of interleukin-1alfa mRNA by arecoline via oxidative stress and p38 MAPK activation.

Conclusion: Our data highlight the role of oxidative stress in arecoline-mediated cell death, gene regulation and inflammatory processes in human keratinocytes.

Paturu Kondaiah, PhD, Department of Molecular Reproduction Development and Genetics, Indian Institute of Science, Bangalore 560 012, India
Tel: +91 80 22932688
Fax: +91 80 23600999
e-mail: paturu@mrdg.iisc.ernet.in

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Arecanut (*Areca catechu* Linn) chewing in the form of betel quid (the combination of arecanut, betel leaf and lime, with or without tobacco) is popular in southeast Asian countries and has been associated with cancer and several precancerous lesions of the oral cavity, including leukoplakia and oral submucous fibrosis (1). Arecanut chewers are also susceptible to several other periodontal inflammatory diseases (2–4), and some studies have

proposed the importance of arecoline, the major arecanut alkaloid, in the manifestation of these diseases (2,5,6). Epithelial changes are hallmarks of oral submucous fibrosis and are attributed to salivary arecoline derived from betel quid chewing (7,8). Although oral submucous fibrosis and other inflammatory diseases are basically diseases of fibroblasts, epithelial changes could be important in these processes.

Arecoline is a cholinomimetic agent and binds to M-1, M-2 and M-3 muscarinic acetylcholine receptors (9). Previously, it was demonstrated that atropine, a muscarinic acetylcholine receptor antagonist, blocks arecoline-mediated neurological functions in the human brain (10). The physiological effects of arecoline are mediated via M-1, M-2 and M-3 muscarinic acetylcholine receptors in experimental animals (11–13). Furthermore, muscarinic acetylcholine receptors are also expressed in extraneuronal and non-excitatory cells (14). HaCaT cells express M-3 muscarinic acetylcholine receptors (15). Stimulation of muscarinic acetylcholine receptors leads to either inhibition of cAMP production or elevation of intracellular Ca^{2+} levels, depending on the receptor subtype (16).

Arecoline causes depression of antioxidants (i.e. superoxide dismutase, catalase and glutathione-*S*-transferase) and hampers the neutralization of reactive oxygen species (5). Oxidative stress plays critical roles in cellular growth, apoptosis (17), gene expression (18) and cellular signaling (19). The cytotoxic effect of arecoline has been demonstrated in oral epithelial cells (20) and in fibroblast cells (2) and suggests the importance of cellular glutathione depletion in the process of cytotoxic effect (21,22).

The role of arecoline in the regulation of inflammatory processes has been demonstrated. The alkaloid has been found to regulate immunological functions in experimental animals (5,23). Moreover, it has also been suggested that arecoline might initiate epithelial inflammation by eliciting prostaglandin E2 and interleukin-6 production and inducing cyclooxygenase-2 expression (24,25). Interleukin-1 is important in the cellular response to stress, tissue injury and inflammation. It can elaborate both cellular and humoral immunity by inducing targets, including endothelial, fibroblast, chondrocyte, osteoclast and pituitary cells, to express key factors such as interleukin-4, interleukin-5, interleukin-12 and interleukin-18, as well as the secondary expression of additional interleukin-1, interleukin-6 and tumor necrosis factor (26). Interleukin-1alfa has been implicated in the development of oral submucous fibrosis (27). Furthermore, arecoline has been reported to induce interleukin-1 secretion in oral epidermoid carcinoma cells (28). However, the molecular mechanism underlying the role of arecoline in the regulation of gene expression and other cellular processes is not clearly understood.

The purpose of our study was to elucidate the basic molecular events elicited by arecoline in human normal keratinocytes that lead to changes in epithelial cell proliferation and gene expression which might be crucial for the manifestation of oral inflammatory diseases, including oral submucous fibrosis.

Material and methods

Cell culture and treatment

Spontaneously immortalized human normal dermal keratinocyte cells (of the HaCaT cell line) (provided by Dr Sudhir Krishna at NCBS Bangalore) were maintained in Dulbecco's modified Eagle's minimal essential medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated certified fetal bovine serum, 100 units/mL of penicillin and 100 μg/mL of streptomycin (Invitrogen Corporation, Carlsbad, CA, USA) at 37°C in a humidified chamber with 5% CO_2. The cells were serum starved for 24 h and treated with 0–50 μg/mL of arecoline hydrobromide (Sigma-Aldrich) in serum-free conditions for 48 h. To investigate the role of oxidative stress and muscarinic acetylcholine receptors in arecoline-mediated changes, the cells were pretreated for 1 h with 10 mM *N*-acetyl-L-cysteine (a glutathione precursor and reactive oxygen species scavenger) or 1 mM atropine (a non-selective muscarinic acetylcholine antagonist) (both from Sigma, St Louis, MO, USA) and then treated with or without 50 μg/mL of arecoline for 48 h. To study the influence of mitogen-activated protein kinase (MAPK) pathways, the cells were pretreated for 1 h with 10 μM extracellular signal-regulated kinase (ERK)1/2 inhibitor (PD 98059), 10 μM p38 MAPK inhibitor (SB 203580) and 1.0 μM L-type c-Jun N-terminal kinase inhibitor (Calbiochem, San Diego, CA, USA).

RNA extraction from cells and semiquantitative reverse transcription–polymerase chain reaction

Total RNA was extracted from HaCaT cells using TRI-reagent (Sigma-Aldrich), according to the manufacturer's protocol. Two micrograms of total RNA was reverse transcribed using the High Capacity Archive cDNA kit (Applied Biosystems, Foster, CA, USA) and cDNA equivalent to 20 ng of total RNA was used in each 20-μL polymerase chain reaction (PCR) amplification. All PCR amplifications were performed using DyNAZYME Master mix (Finnzyme, Espoo, Finland) with primers specific for the following genes: p21/Cip1; glucose-6-phosphate dehydrogenase; heme oxygenase-1; ferritin light chain; glutamate-cysteine ligase catalytic subunit; glutathione reductase; and interleukin-1alfa. RPL-35a expression (a ribosomal protein encoding RNA) was used as normalizing control. The PCR products were resolved on 2% agarose gels and the band intensities were determined using a gel-documentation system (Eastman Kodak, Rochester, NY, USA). Gene-specific primer sequences and PCR conditions are described in Table 1.

Cell cytotoxicity assay

Briefly, 5000 cells were plated in each well of 96-well tissue culture plates. After 4 h the cells were treated with 0–100 μg/mL of arecoline for 24–72 h in 200 μL of medium containing 10% serum. Three hours before the termination of culture, 20 μL of 5 mg/mL of

Table 1. Description of primers used in this study

Serial No.	Gene name	5′-3′ sequence	Description
1	Interleukin-1alfa (IL-1α)	TGTGACTGCCCAAGATGAAG CGCCTGGTTTTCCAGTATCT	238 bp, 50.0°C
2	Catalase	CGTGCTGAATGAGGAACAGA CAGATTTGCCTTCTCCCTTG	235 bp, 50°C
3	Glutathione reductase (GRD)	CCCGATGTATCACGCAGTTA GTAGGGTGAATGGCGACTGT	195 bp, 50°C
3	Glucose-6-P dehydrogenase (G6PD)	CCTCATCCTGGACGTCTTCT CCACTTGTAGGTGCCCTCAT	211 bp, 50°C
4	Glutamate-cysteine ligase catalytic subunit (GCLC)	TCCTGGACTGATCCCAATTC CCAAGTAACTCTGGGCATTCA	267 bp, 50°C
5	Ferritin light chain (FTL)	ACGAGCGTCTCCTGAAGATG CCCAGGGCATGAAGATCCAAA	165 bp, 50°C
6	Heme oxygenase-1 (HMOX-1)	ATGACACCAAGGACCAGAGC GTGTAAGGACCCATCGGAGA	158 bp, 58°C
7	p21/Cip1	CACCACTGGAGGGTGACTTC AAATCTGTCATGCTGGTCTGC	280 bp, 60.0°C
8	Rpl-35a	GAACCAAAGGGAGCACACAG CAATGGCCTTAGCAGGAAGA	236 bp, 58°C

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) solution was added to each well. At the end of the culture period, the medium was completely removed, 200 μL of dimethylsulfoxide was added to each well to dissolve the purple formazan crystals and the absorbance at 550 nm was determined using the BIO-RAD Model680 MICROPLATE READER. To investigate the role of oxidative stress in arecoline cytotoxicity, the MTT assay was performed in the presence of increasing concentrations of *N*-acetyl-L-cysteine, with or without 100 μg/mL of arecoline, for 48 h. All treatments were performed in triplicate and the results are presented as dose–response curves.

Cell viability determined by Trypan Blue dye exclusion

HaCaT cells were seeded at 1.0×10^5 cells in each well of six-well tissue-culture plates, cultured for 24 h, treated for 72 h with 0–100 μg/mL of arecoline in culture medium supplemented with 10% fetal bovine serum and then processed for the cell viability assay. Cells were harvested by trypsin digestion, and a 1:1 mixture of 0.4% Trypan Blue solution (Sigma-Aldrich) and cell suspension was prepared. The total cells, as well as the number of dead cells (stained blue), were counted using a Neubauer hemocytometer and the results are presented as percentage viability of untreated cells.

Cell proliferation assay

Cell proliferation was estimated using the 5-bromo-2-deoxyuridine incorporation assay (QIA58 Kit; Calbiochem) that was carried out according to the manufacturer's protocol(. Five-thousand HaCaT cells were plated in each well of a 96-well tissue-culture plate and treated with 0–100 μg/mL of arecoline in 0.2% serum-containing medium for 24 h before the assay was performed.

Cell cycle analysis

HaCaT cells were grown in six-well tissue culture plates to 30% confluence, then treated for 24 h with 0–100 μg/mL of arecoline in 10% fetal bovine serum. The cells were harvested and fixed in chilled 70% ethanol on ice for 30 min with frequent mixing. The cells were washed three times with ice-cold phosphate-buffered saline and resuspended in 500 μL of 0.8× phosphate-buffered saline containing 0.1% Triton X-100 and 0.1 mg/mL of DNase-free RNase for 5 h at 37°C. After the digestion was complete, 5 μL of a 2-μg/mL ethidium bromide solution was added and the cells were incubated for 1 h at 37°C. Then, the cells were washed twice with phosphate-buffered saline, a histogram of cell distribution was obtained using the FL2 channel (595 nm) of a flow cytometer (FACScan, BD Biosciences, Franklin Lakes, NJ, USA) and the distribution of cells in the various cell cycle phases was determined, using the CELL QUEST PRO software (BD Biosciences), from the histogram generated.

ROS generation

HaCaT cells were grown in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal bovine serum for 12 h and treated with 0–100 μg/mL of arecoline for 48 h. The cells were harvested and incubated with 1 μM 2′,7′-dichlorofluorescein diacetate (a fluorescent hydrogen peroxide probe) for 15 min and analyzed in the FL1 channel (590 nm) of a FACScan (Becton Dickinson), and the distribution of cells was determined, from the histogram generated, using the CELL QUEST PRO software (BD Biosciences).

Catalase assay

The catalase assay was performed as described previously (29). Briefly, HaCaT cells were cultured in six-well tissue culture plates to 70–75% confluence. The cells were serum starved for 24 h and then treated with 0–100 μg/mL (0–0.4 mM) of arecoline in serum-free conditions. After the incubation, the cells were washed once in chilled phosphate-buffered saline and lysed in 200 μL of lysis buffer containing 50 mM potassium phosphate buffer (pH 7.0 at 25°C), 10% glycerol and 1% Triton X-100. The protein content of the lysates was estimated using Lowry's method. An equal amount of total protein was taken for analysis in the catalase (EC 1.11.1.6) assay. In a 0.3 mL reaction mix, the final concentrations were 50 mM potassium phosphate (pH 7.0 at 25°C), 0.035% (w/w) H_2O_2 and 10 μL of cell lysate. The time taken, in minutes, for the reduction (from 0.45 to 0.40) in absorbance at 240 nm (ΔA_{240}) of the assay mix was measured using a spectrophotometer (BIO-RAD SmartSpec™ Plus, Biorad Laboratories, Hercules, CA, USA). The

enzyme activity was calculated according to the catalase assay manual (Sigma-Aldrich). All the reagents used in the assay were purchased from Sigma-Aldrich.

Protein extraction and western blot analysis

Arecoline-treated HaCaT cells were lysed in 25 mM HEPES buffer, pH 7.4, containing 175 mM NaCl, 10% glycerol, 5 mM EDTA, pH 8.0, 50 mM NaF, 10 mM Na_3VO_4, 1× protease cocktail III (Calbiochem, Merck, Darmstadt, Germany) and 1.0% Nonidet P-40 on ice for 30 min. The lysate was cleared by centrifugation for 30 min at 16000 ***g*** at 4°C. Total protein was estimated using Lowry's method. One-hundred micrograms of total protein was resolved by electrophoresis through a 10% sodium dodecyl sulfate–polyacrylamide gel and then transferred to a poly(vinylidene difluoride) membrane (Millipore, Bilterica, MA, USA). The membrane was blocked with 5% defatted milk and then probed with p38 or phospho-p38 antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membrane was reprobed with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and the bands of interest were detected using the ECL Plus kit (GE Life Sciences, Singapore, Singapore) on an X-ray film (Eastman Kodak Products).

Statistical analysis

Statistical significance was evaluated using the Student's *t*-test. The results were considered significant for a *p*-value of ≤ 0.05.

Results

Inhibition of HaCaT cell proliferation and induction of cell death by arecoline

Arecoline treatment of HaCaT cells resulted in cell death in a time- and dose-dependent manner, as revealed by MTT and Trypan Blue exclusion assays. As shown in Fig. 1A, treatment for 24, 48 and 72 h with arecoline resulted in a significant decrease in cell numbers at concentrations of arecoline higher than 50 μg/mL, as revealed by the MTT assay. At 48 and 72 h the effect was highly pronounced. Furthermore, at 42 h, there was a significant decrease in cell viability at concentration of arecoline higher than 50 μg/mL (Fig. 1B). In order to ascertain whether the decrease in cell numbers was caused by reduced proliferation following arecoline treatment, the 5-bromo-2-deoxyuridine incorporation assay was performed. The results of this assay showed a reduction, of approximately 50%, in HaCaT cell proliferation when the cells were incubated for 24 h with concentrations of arecoline higher than 50 μg/mL (Fig. 1C). This was confirmed by flow cytometric analysis. Arecoline induced HaCaT cell cycle arrest at the G1/G0 phase. There was an increase in the G1/G0 cell population from 17.58 ± 1.12% (mean ± standard error) in the untreated cells to 41.74 ± 0.94% in the cells treated with 100 μg/mL of arecoline. A negligible proportion of cells (< 4%) were in the sub-G1/G0 phase after 24 h of treatment, as shown in the histogram (Fig. 2). Interestingly, there was no induction of p21/Cip1 mRNA, which has been shown to be involved in growth arrest at the G1/G0 phase by a variety of treatments (data not shown). The DNA fragmentation assay and the Poly ADP ribose polymerase (PARP) cleavage assay revealed no evidence of apoptotic cell death of HaCaT cells as a result of treatment with arecoline (data not shown).

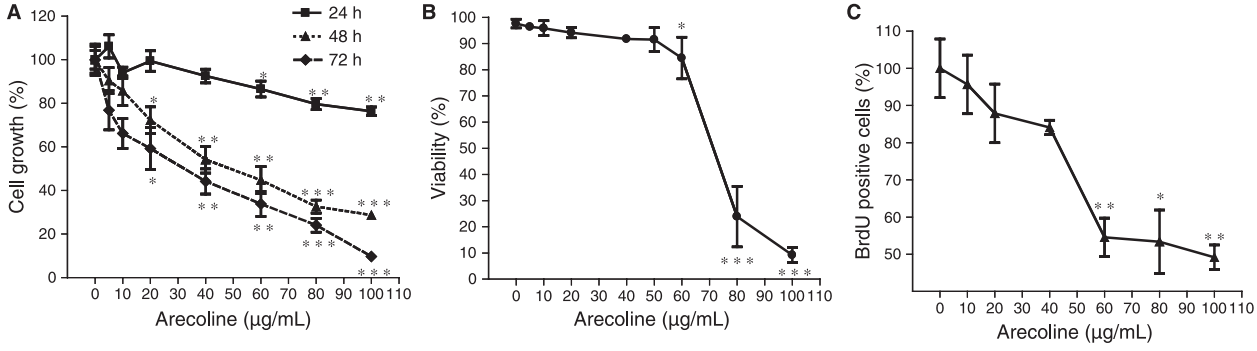

Fig. 1. Inhibition of HaCaT cell proliferation and induction of cell death by arecoline. (A) MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay showing the numbers of arecoline-treated HaCaT cells at 24, 48 and 72 h under serum-free conditions. The graphs represent dose–response curves of the results obtained of triplicate experiments at each time-point, and the bars represent mean ± standard error; *$p < 0.05$, **$p < 0.002$, and ***$p < 0.001$. The experiment was repeated twice. (B) Cell viability of HaCaT cells following treatment for 72 h with various concentrations of arecoline in 10% fetal bovine serum. The graph represents the percentage of viable cells relative to the untreated control. Cell death became significant beyond ~ 60 μg/mL of arecoline ($p < 0.05$). The bars represent the mean ± standard error of four readings; *$p < 0.05$ and ***$p < 0.001$. The experiment was repeated twice. (C) Cell-proliferation assay measuring 5-bromo-2-deoxyuridine incorporation following treatment of HaCaT cells with arecoline for 24 h in the presence of 0.2% serum-free conditions. The graph represents the percentage of 5-bromo-2-deoxyuridine-positive cells relative to the untreated control, and the bars represent the mean ± standard error of triplicate readings; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

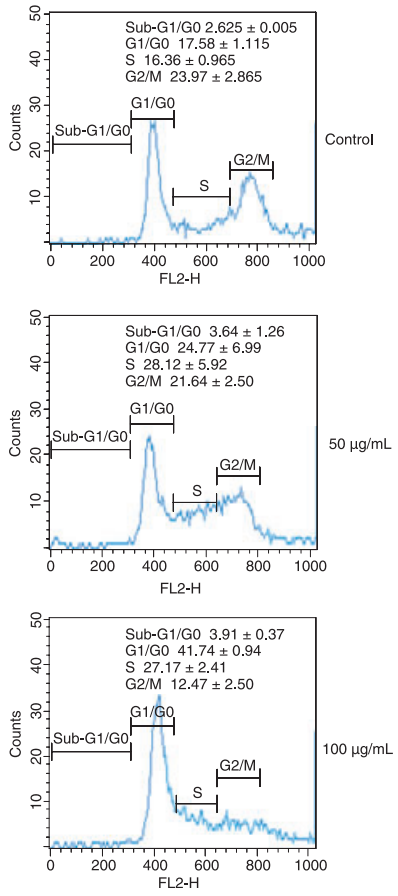

Fig. 2. Induction of HaCaT cell cycle arrest at the G1/G0 phase by arecoline. The results of flow cytometric analyses of HaCaT cells treated with 0, 50 and 100 µg/mL of arecoline for 24 h in 10% serum are shown as histograms. The percentage distribution in various cell cycle phases of control HaCaT cells, and of HaCaT cells treated with 50 and 100 µg/mL of arecoline, is indicated. The data represent the mean of two independent experiments, ± standard error.

Generation of reactive oxygen species and induction of stress response genes by arecoline in HaCaT cells

Arecoline is known to induce oxidative stress in buccal keratinocytes (22) and in oral fibroblast cells (21) as a result of the depletion of cellular glutathione. Thus, arecoline might induce the generation of reactive oxygen species in the cells. In order to evaluate the generation of reactive oxygen species following treatment of HaCaT cells with arecoline, a flow cytometric analysis of 2′, 7′-dichlorofluorescein diacetate (DCF/DA)-loaded HaCaT cells was performed. Figure 3A shows that arecoline induced reactive oxygen species generation in HaCaT cells in a dose-dependent manner, with > 50% of the cells showing high-intensity fluorescence at 100 µg/mL of arecoline. Fluorescence microscopy of 2′, 7′- dichlorofluorescein diacetate (DCF/DA)-stained HaCaT cells demonstrated that the arecoline-stimulated production of reactive oxygen species in HaCaT cells was reversed by *N*-acetyl-L-cysteine, a glutathione precursor and antioxidant (data not shown), suggesting that perhaps cellular glutathione depletion is important in the process. Furthermore, arecoline treatment might lead to HaCaT cell death as a result of oxidative trauma. To test this, HaCaT cells were treated with 100 µg/mL of arecoline, with or without *N*-acetyl-L-cysteine, for 48 h. As shown in Fig. 3B, *N*-acetyl-L-cysteine reversed arecoline-induced HaCaT cell death in a dose-dependent manner with complete reversal of cell death at 1 mM *N*-acetyl-L-cysteine, highlighting the role of oxidative stress in this process. This was also verified by studying the expression of several oxidative stress-responsive genes (18) following arecoline treatment. Figure 3C shows the induction of ferritin light chain, heme oxygenase-1, glucose-6-phosphate dehydrogenase, glutamate-cysteine ligase catalytic subunit and glutathione reductase in HaCaT cells after treatment with arecoline. The induction of these genes was reversed by the addition of *N*-acetyl-L-cysteine (Fig. 3C, lane 4), but the presence of atropine had no effect (Fig. 3C, lane 6), suggesting a role for oxidative stress in the regulation of these genes by arecoline. It has been proposed that p38 and c-Jun N-terminal kinase MAPK pathways are crucial for cellular response to stress (30). Hence, the regulation of heme oxygenase-1 and ferritin light chain induction by arecoline was studied with or without p38 and c-Jun N-terminal kinase and ERK inhibitors. The induction by arecoline of heme oxygenase-1 and ferritin light chain in HaCaT cells was found to be reversed by p38 and c-Jun N-terminal kinase MAPK inhibitors respectively (Fig. 4A, lane 6; Fig. 4B, lane 8). As discussed, arecoline induces reactive oxygen species generation in HaCaT cells. Inhibition of catalase and superoxide dismutase activities might contribute to reactive oxygen species generation by arecoline in HaCaT cells. Hence, we studied the expression of catalase and superoxide dismutase 1 and 2 in HaCaT cells following treatment with arecoline. Surprisingly, treatment with arecoline for 48 h did not alter the expression of catalase, or of superoxide dismutase 1 and 2, in HaCaT cells (data not shown). On the other hand, after 48 h of treatment, arecoline suppressed catalase activity in HaCaT cells (Fig. 5A). Interestingly, the inhibition of catalase activity by arecoline was found to be dependent on heme oxygenase-1 activity because the heme oxygenase-1 inhibitor, zinc-protoporphyrin IX, recovered the reduction of catalase activity, mediated by 24 h of incubation with 50 µg/mL of arecoline, from approximately 64 to 89% in HaCaT cells (Fig. 5B). Furthermore, the regulation of heme oxygenase-1 was not dependent on the suppression of catalase activity, as the addition of exogenous bovine liver catalase (500 units/mL) could not block heme oxygenase-1 induction by arecoline, whereas the induction of heme oxygenase-1 expression by 300 µM H_2O_2 in 12 h was reversed by catalase in HaCaT cells (data not shown), suggesting that arecoline-mediated heme oxygenase-1 induction could be independent of catalase inhibition. Taken together, our data demonstrated that arecoline could induce reactive oxygen species generation in HaCaT cells and that the consequent oxidative stress contributed to gene expression and cell death in normal human keratinocytes.

Regulation of interleukin-1alfa by arecoline in HaCaT cells

Interleukin-1 has been reported to be induced under cellular stress in several cell lines (26). Hence, we studied the induction of interleukin-1alfa by reverse transcription–PCR analysis. Figure 6A shows the dose-dependent induction of interleukin-1alfa mRNA in HaCaT cells upon arecoline treatment, with approximately 1.33-,

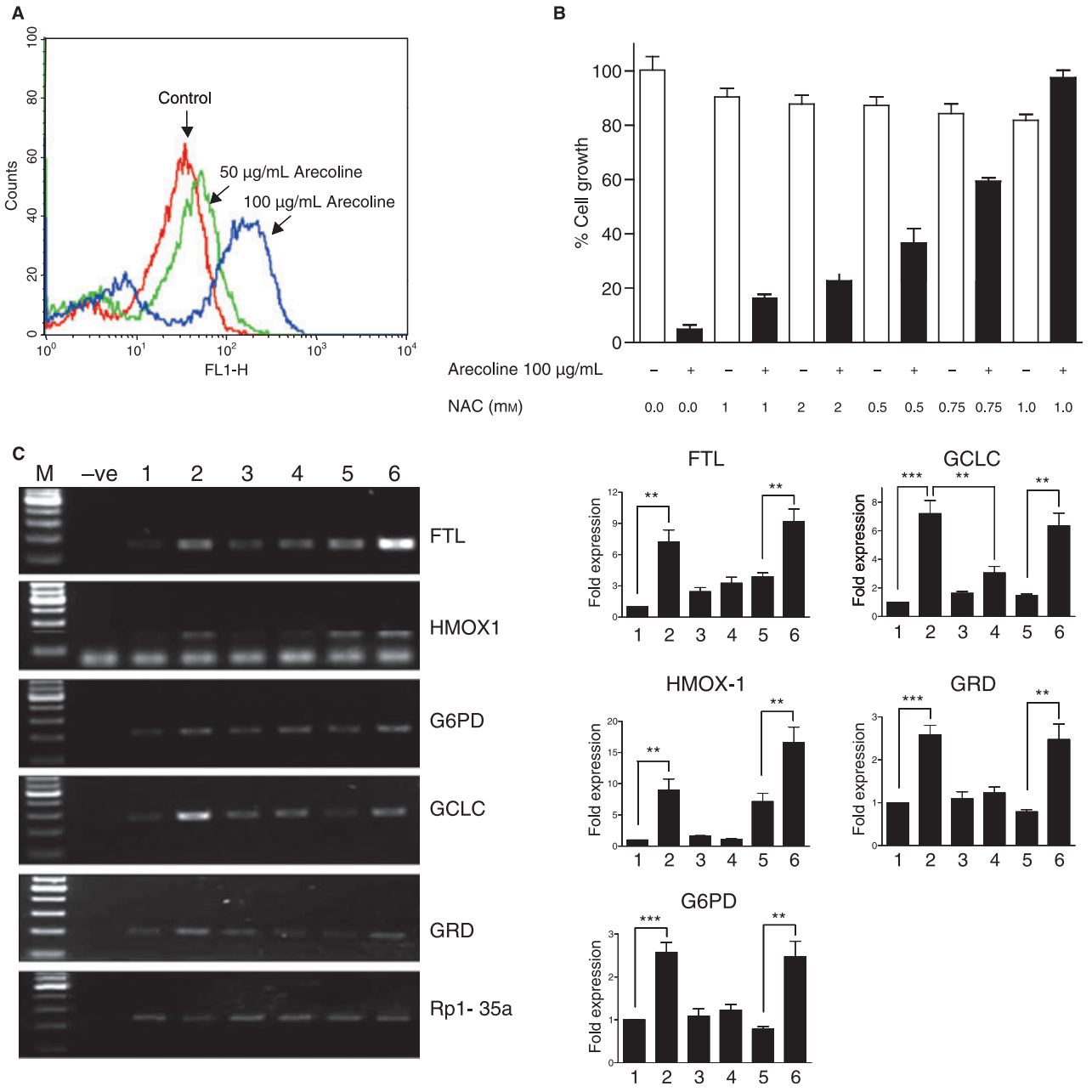

Fig. 3. Generation of reactive oxygen species and induction of stress-response genes by arecoline in HaCaT cells. (A) Flow cytometric analysis of HaCaT cells treated with the indicated concentrations of arecoline for 48 h and incubated for 15–20 min with 1 µM 2′,7′-dichlorofluorescein diacetate, showing the reactive oxygen species. The histogram is representative of three experiments. (B) HaCaT cell death caused by exposure to arecoline, and reversal (in a dose-dependent manner) by *N*-acetyl-L-cysteine. The experiment was performed in triplicate, and carried out twice. (C) Induction of stress-responsive genes by arecoline in HaCaT cells, and reversal by *N*-acetyl-L-cysteine. HaCaT cells were treated with 50 µg/mL of arecoline in the presence or absence of 10 mM *N*-acetyl-L-cysteine or 1 mM atropine for 48 h under serum-free conditions and the expression levels of ferritin light chain, heme oxygenase-1, glucose-6-phosphate dehydrogenase, glutamate-cysteine ligase catalytic subunit and glutathione reductase were assessed by reverse transcription–polymerase chain reaction analysis. Rpl-35a was used for normalization of the gene expression. The figure shows the ethidium bromide-stained agarose gel. Lanes: 1, control; 2, arecoline treated; 3, *N*-acetyl-L-cysteine treated; 4, *N*-acetyl-L-cysteine + arecoline treated; 5, atropine treated; and 6, atropine + arecoline treated. The bars on the right show quantification of the expression of respective genes, presented as the mean ± standard error (*$p < 0.005$; **$p < 0.002$; ***$p < 0.001$) of fold expression with respect to the untreated control of two independent experiments. FTL, ferritin light chain; G6PD, glucose-6-phosphate dehydrogenase; GCLD, glutamate-cysteine ligase; GRD, glutathione reductase; HMOX-1, heme oxygenase-1; NAC, *N*-acetyl-L-cysteine.

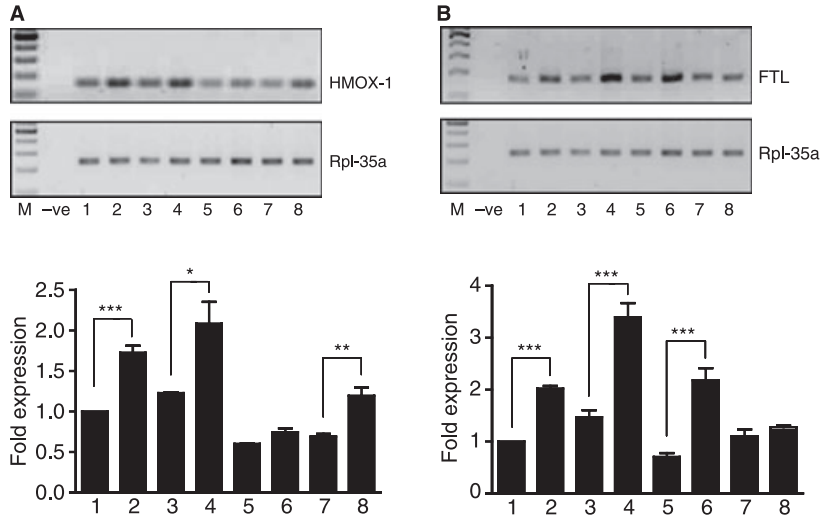

Fig. 4. Effect of mitogen-activated protein kinase (MAPK) pathway inhibitors on the induction of heme oxygenase-1 (HMOX-1) and ferritin light chain (FTL) by arecoline. HaCaT cells were treated with 50 μg/mL of arecoline in the presence or absence of 10 μM of the extracellular signal-regulated kinase (ERK)1/2 MAPK inhibitor (PD 98059), 10 μM of the p38 MAPK inhibitor (SB 203580) or 1 μM of the c-Jun N-terminal kinase MAPK inhibitor (L-form) for 48 h under serum-free conditions. A semiquantitative reverse transcription–polymerase chain reaction analysis was performed to evaluate the expression of heme oxygenase-1 (A) and ferritin light chain (B) mRNAs. The top panels show the ethidium bromide-stained agarose gels of the reverse transcription–polymerase chain reaction products of respective genes, and the bottom panels show the densitometric quantification of the DNA bands as fold changes with respect to the untreated control. Lanes: 1, control; 2, arecoline treated; 3, PD 98059 treated; 4, PD 98059 + arecoline treated; 5, SB 203580 treated; 6, SB 203580 + arecoline treated; 7, c-Jun N-terminal kinase inhibitor treated; 8, c-Jun N-terminal kinase inhibitor + arecoline treated. The data represent the mean ± standard error of two independent experiments. $*p < 0.05$; $**p < 0.002$; $***p < 0.001$. FTL, ferritin light chain; HMOX-1, heme oxygenase-1.

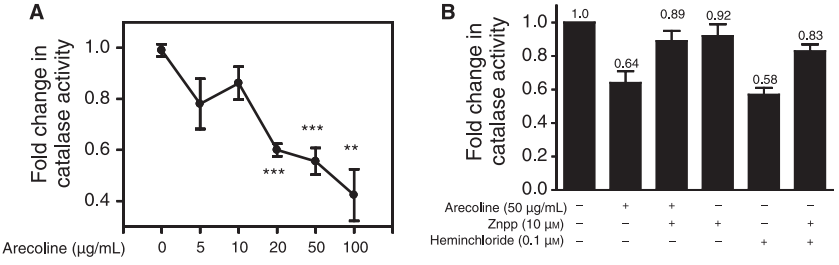

Fig. 5. Effect of arecoline on catalase activity in HaCaT cells. (A) HaCaT cells were treated with 0–100 μg/mL of arecoline for 48 h under serum-free conditions and a catalase assay was performed as described in the Material and methods. The results are expressed as fold change in enzyme activity and shown as a dose-response curve. The data represent the mean ± standard error of two independent assays. $**p < 0.01$; $***p < 0.001$. (B) Effect of zinc-protoporphyrin IX (Znpp) on arecoline-regulated catalase activity in HaCaT cells. The bars represent the mean ± standard error of fold change of two independent experiments, and the mean value of fold change in catalase activity is indicated.

2.65- and 2.62-fold induction at 10, 20 and 50 μg/mL of arecoline respectively. This induction was blocked by 10 mM *N*-acetyl-L-cysteine. Interestingly, the addition of 1 mM atropine induced the basal level of expression of interleukin-1alfa without further induction by the addition of arecoline (Fig. 6B). This suggests the involvement of oxidative stress in the regulation of interleukin-1alfa by arecoline. Furthermore, we showed that this induction is mediated by p38 MAPK through the fact that a p38 inhibitor (SB 203580) blocked interleukin-1alfa induction, while ERK and c-Jun N-terminal kinase MAPK pathway inhibitors were ineffective (Fig. 6C). The induction of phospho-p38 by arecoline was demonstrated by Western blot analysis using specific phosphor antibodies (Fig. 6D). Hence, interleukin-1alfa regulation by arecoline in HaCaT cells is dependent on oxidative stress and p38 MAPK activation.

Discussion

The betel quid chewing habit has been proposed as one of the etiological causes for several oral inflammatory diseases, such as oral submucous fibrosis, gingivitis and other periodontal conditions. Although not unequivocally established, arecoline, an alkaloid present in the areca nut, has been strongly advocated as the causative agent for these conditions. Another interesting feature of these diseases is the epithelial changes, and, in the most severe cases, atrophic epithelium is observed. Arecoline in the saliva of betel quid chewers could be responsible for the epithelial changes seen in oral submucous fibrosis (7,8) and periodontal diseases (31). As the molecular mechanism underlying the action of arecoline on the epithelial cells of these diseases is not clearly understood, we studied the effects of arecoline on human keratinocytes, more specifically HaCaT cells. We chose HaCaT cells for the following reasons: they have been extensively used as normal epithelial cells to study carcinogenesis (32); they have been used as a substitute for oral keratinocytes in some studies (33); and although some immortalized oral keratinocyte cell lines, such as HOK-16B are available, these cell lines are immortalized with papilloma virus and have been reported to be different in certain responses, especially with respect to the c-Jun N-terminal kinase pathway (34). Therefore, for these reasons, we chose to study the effect of

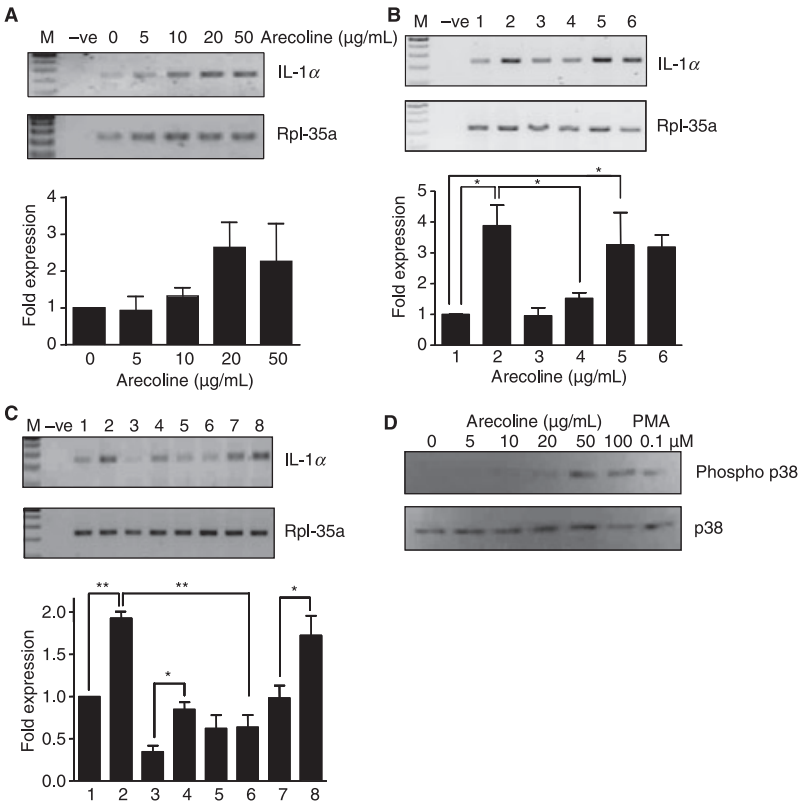

Fig. 6. Arecoline induces interleukin-1alfa expression in HaCaT cells. Expression of interleukin-1alfa in HaCaT cells treated with 0–50 μg/mL of arecoline for 48 h under serum free-conditions was measured in the absence or presence of various inhibitors. In all panels except for (D), the top panel shows the ethidium bromide-stained gel of the reverse transcription–polymerase chain reaction products and the bottom panel shows the densitometric quantification of the DNA bands as fold change compared with the control and are presented as the mean ± standard error of two independent experiments. In (B) and (C), $*p < 0.05$; $**p < 0.01$. (A) Induction of interleukin-1alfa by arecoline. (B) Effect of *N*-acetyl-L-cysteine or atropine on interleukin-1alfa regulation by arecoline. Lanes: 1, control; 2, arecoline treated; 3, *N*-acetyl-L-cysteine treated; 4, *N*-acetyl-L-cysteine + arecoline treated; 5, atropine treated; and 6, atropine + arecoline treated. (C) Effect of mitogen-activated protein kinase (MAPK) inhibitors in the presence or absence of 10 μM of the extracellular signal-regulated kinase (ERK)1/2 MAPK inhibitor (PD 98059), 10 μM of the p38 MAPK inhibitor (SB 203580) or 1 μM of the c-Jun N-terminal kinase MAPK inhibitor (L-form). Lanes: 1, control; 2, arecoline treated; 3, PD 98059 treated; 4, PD 98059 + arecoline treated; 5, SB 203580 treated; 6, SB 203580 + arecoline treated; 7, c-Jun N-terminal kinase inhibitor treated; 8, c-Jun N-terminal kinase inhibitor + arecoline treated. (D) Effect of arecoline on the activation of p38 MAPK. HaCaT cells were serum starved for 24 h and treated with 0–100 μg/mL of arecoline for 1 h, following which western blot analysis for phospho p38 and total p38 was performed. HaCaT cell lysate obtained for 1 h following stimulation with 0.1 μM 4β-phorbol 12-myristate 13-acetate (PMA) was used as the positive control for p38 MAPK activation. IL-1α, interleukin-1alfa.

arecoline in HaCaT cells, which may be reflective of effects in general of cells of epithelial origin. Arecoline has been suggested to induce oxidative stress as a result of depletion of cellular glutathione in cell culture (21,22) and depression of antioxidants (i.e. superoxide dismutase, catalase and glutathione-*S*-transferase (5). In addition to the induction of oxidative trauma, the alkaloid may activate redox-responsive signaling molecules and influence cell adhesion, migration, proliferation and apoptosis. The activation of stress-activated MAPKs, c-Jun N-terminal kinase and p38 MAPK are crucial for the cellular response to stress (30). We also observed that oxidative stress and MAPK pathway activation are important for arecoline-induced cellular changes in the HaCaT cells.

It has been demonstrated that arecoline induces cell cycle arrest and cell death in a variety of cell types. However, the action of arecoline might be cell-type and tissue-type specific because the mechanism seemed to be fairly diverse. In oral carcinoma cells (KB cells) and human umbilical vein endothelial cells, treatment with arecoline resulted in growth arrest at the G2/M phase of the cell cycle (20,24,35), whereas rat hepatocytes were reported to be arrested at the G1/G0 phase (36). KB cell cycle arrest occurred as a consequence of the generation of reactive oxygen species and was dependent on p21/Cip1 induction (20). The rat hepatocyte cell cycle was also arrested via p21/Cip1 induction in a p53-dependent and transforming growth factor-beta-dependent manner (36). Interestingly, HaCaT cell growth inhibition is independent of the p21/Cip1 pathway. HaCaT is a spontaneously immortalized human normal dermal keratinocyte cell line (37) having a mutated p53 (38). The Cycline dependent kinase (CDK) inhibitor, p21/Cip1 binds to and inhibits CDK-2 and CDK-4 complexes and regulates the G1/S phase transition of the cell cycle (39,40). The expression of IL-1α is tightly controlled by p53 and is involved in p53-dependent G1/G0 arrest in response to a variety of stress stimuli (40). Hence, the effect of arecoline on cell cycle arrest in p53 mutant cells could be independent of p21/Cip1. The lack of wild-type p53 in HaCaT cells was also probably reflected by the inability to undergo apoptotic cell death under the influence of arecoline. Oxidative stress is known to induce apoptotic cell death via p53 in several cells, including rat astrocytes and ovarian cancer cells (41,42). HaCaT cells do not show apoptotic cell death upon treatment with arecoline, as confirmed by a lack of the DNA laddering pattern and PARP cleavage, although the apoptotic machinery seemed to be

intact. However, the possible role of wild-type p53 in arecoline-mediated apoptosis has not been established.

One receptor pathway mediating the action of arecoline has been shown to be that of muscarinic acetylcholinergic receptors (11–13). Blocking muscarinic acetylcholine receptors with atropine could not reverse HaCaT cell death (data not shown) or induction of the mRNAs of the stress-response genes heme oxygenase-1, ferritin light chain, glucose-6-phosphate dehydrogenase, glutamate-cysteine ligase catalytic subunit, glutathione reductase and interleukin-1alfa. However, the addition of *N*-acetyl-L-cysteine, an antioxidant, reversed the induction of expression of these stress-response genes, suggesting that cellular stress induced by arecoline might be independent of activation of muscarinic acetylcholine receptors. Interestingly, the involvement of inhibition of catalase (a heme-dependent reactive oxygen species-scavenging enzyme) was also not involved in the regulation of stress-response genes by arecoline. Our data established MAPK pathway activation as an important step in the regulation of stress-response genes by arecoline. So far, arecoline has been shown to be involved in ERK activation, but not in p38 and c-Jun N-terminal kinase activation (24). It remains to be established how arecoline activates p38 and c-Jun N-terminal kinase MAPK pathways. Our data demonstrated that oxidative stress induced by arecoline not only results in oxidative damage to human epithelial cells but also involves the induction of expression of stress-responsive genes and initiation of the inflammatory reaction, as evidenced by the induction of the interleukin-1alfa gene, which may be important for oral submucous fibrosis pathogenesis.

To summarize, very high arecoline concentrations (equivalent to those present in the saliva of betel quid chewers) are perhaps responsible for inhibiting the proliferation and death of epithelial cells, leading to an atrophic epithelium. Acute cellular trauma caused by arecoline-induced oxidative stress is an important factor in epithelial cell death. In addition, sublethal concentrations of arecoline might lead to the induction of important inflammatory cytokines via oxidative stress and activation of MAPK pathways. It should be noted that these studies were carried out in HaCaT cells, which may not reflect true physiology compared with normal oral epithelial cells. However, we believe that the effects of arecoline on HaCaT cells may be similar to the effects on other epithelial cells, including those of oral origin. Thus, our study provides evidence for the involvement of stress-responsive signaling pathways that could be a possible mechanism of arecoline action leading to inflammatory oral diseases, including oral submucous fibrosis.

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