INTRODUCTION

There are about 600 million people with the betel quid (BQ) chewing habit throughout the world (1). Studies showed that chewing BQ is associated with oral diseases, e.g. oral submucous fibrosis (OSF), oral leukoplakia (OL), and oral cancer (2-5). In addition, the alkaloid extracts from BQ have been characterized as carcinogenic (3-5), immunosuppressive, hepatotoxic (6), immunotoxic (7), genotoxic (8, 9), and teratogenic (9) materials. Also, arecoline, the major component in the BQ extracts, has been demonstrated to be mutagenic in mammalian cells (10, 11).

Cyclooxygenase (COX) catalyzes the synthesis of PGs from arachidonic acid. COX-2 acts in the course of inflammatory processes and tissue repair and it is enhanced was by many of diverse stimuli including hormones, growth factors, cytokines, chemokines, environmental stress factors (12). A potential role of the COX-2 promoter region in the development of betel-related oral cell carcinoma (OSCC) has been demonstrated.

Prostaglandins exert their effects via prostanooid specific Gs coupled receptors (13). In 2003, Jeng et al. demonstrated that BQ chewing contributes to the pathogenesis of cancer and oral cancer and OSF by T cell activation, induction of PGE2, tumor necrosis factor alpha (TNF-α) and IL-6 production, which affect oral mucosal inflammation and growth of oral fibroblast (OMF) and oral epithelial cells (14). Chang et al. also have shown that U0126 and PD98059 (50 µM) decreased aerca nut (AN) extract and arecoline associated PGE2 and IL-6 production in GK and KB cells (11). Arecoline inhibits the secretion of cytokine seem via decrease the expression of COX-2 and PGE2 and then cytokine secret (11).

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ARECOLINE INHIBITS INTERLEUKIN-2 SECRETION IN JURKAT CELLS BY DECREASING THE EXPRESSION OF ALPHA7-NICOTINIC ACETYLCHOLINE RECEPTORS AND PROSTAGLANDIN E2

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The purpose of the present study was to explore the effect of arecoline on phytohemagglutinin (PHA)-stimulated interleukin-2 (IL-2) secretion, the expression of alpha7-nicotinic acetylcholine receptors (α7-nAChRs), prostaglandin E2 (PGE2) protein, and IL-2 mRNA in human lymphocyte cells (Jurkat cell line). The IL-2 and PGE2 were determined by enzyme-linked immunosorbent assay (ELISA). The expressions of phosphorylated extracellular signal-regulated kinase (ERK) and α7-nAChRs were determined by Western blotting. The level of IL-2 mRNA was determined by reverse-transcriptase polymerase chain reaction (RT-PCR). Arecoline, in a dose-dependent manner, significantly decreased IL-2 and PGE2 secretion by Jurkat cells incubated with 0 or 5 µg/ml PHA. PGE2 also significantly inhibited IL-2 secretion by Jurkat cells in a dose-dependent manner. In addition, reduced expression of PHA-induced ERK phosphorylation was observed in Jurkat cells treated with arecoline. PHA-enhanced IL-2 mRNA expression was also inhibited by arecoline. These results imply that arecoline inhibits the release of PGE2 and PHA-induced IL-2 secretion by Jurkat cells and that these effects seem to occur, at least in part, either through the attenuation of ERK in conjunction with a decrease of PHA-induced IL-2 mRNA expression. These results imply that arecoline inhibits the protein expression of α7-nAChRs, the release of PGE2 and PHA-induced IL-2 secretion by Jurkat cells.

Key words: arecoline, interleukin-2, prostaglandin E2, α7-nicotinic acetylcholine receptors, Jurkat cells, cyclooxygenase
The nicotinic acetylcholine receptors (nAChRs) are ligand-regulated ion-channel complexes that can mediate neurotransmitters. The nAChRs can also act as second messenger in the nervous system (15-17). Recent studies have shown that in the neurons and the immune system, nicotine modulates multiple immune via the a7-nAChRs pathway (18). It seems that arecoline inhibited the secretion of IL-2 via a7-nAChRs pathway. It is well known that IL-2 is normally produced by the body during an immune response (19, 20). Recent review supports the unique role of IL-2 in the elimination of self-reactive T cells and the prevention of autoimmunity (21). In regarding the association between BQ chewing and many oral diseases, the primary risk factor is thought to be arecoline. Also, studies demonstrated the existence of an interaction between arecoline and immunity. Jurkat is a cell line derived from human lymphocytes, which has been extensive employed in many studies (22, 23). The purpose of the present study was to explore if the effect of arecoline on PHA-stimulated IL-2 production in T-lymphocytes is through the expression of IL-2 mRNA, phosphorylation of MAPK, nAChRs, and PGE2 secretion.

MATERIAL AND METHODS

Materials

Arecoline, PHA, L-glutamine, sodium pyruvate, and glucose were purchased from Sigma (St. Louis, MO, USA). The following materials were purchased from the companies indicated: RPMI 1640 medium (Gibco, Green Island, NY, USA), sodium bicarbonate (AppliChem, Denmark), HEPES (BioShop, Burlington, Canada), and fetal bovine serum (Biological Industries, CKibbutz Beit Haemek, Israel). IL-2 capture antibodies, detection antibody, and streptavidin horseradish peroxidase were obtained from R&D Systems (Minneapolis, MN, USA). Anti-ERK1/2 and secondary antibodies of anti-β-actin antibodies were obtained from Cell Signaling Technology Inc (Danvers, MA, USA). Anti a7-nAChRs antibody was obtained from Abcam PLC (Cambridge Science Park, United Kingdom). Goat anti-rabbit Ig-G were purchased from QED Bioimmunoassay kit (EIA) was obtained from Cayman Chemical (Ann Arbor, MI, USA). Anti-ERK1/2 and secondary antibodies of anti-β-actin antibodies were obtained from Cell Signaling Technology Inc (Danvers, MA, USA). Anti a7-nAChRs antibody was obtained from Abcam PLC (Cambridge Science Park, United Kingdom). Goat anti-rabbit Ig-G were purchased from QED Bioimmunoassay kit (EIA) was obtained from Cayman Chemical (Ann Arbor, MI, USA).

Cell culture

Jurkat cells, a type of lymphocytic cell line, were obtained from the Food Industry Research and Development Institute (Shin-Chu, Taiwan). Cells were cultured in RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, in 10% fetal bovine serum. These cells were cultured at 37°C and 5% CO2 and the cells doubling time was 48 hours. After six days, the cells were separated into 1x10^6/ml concentrations in a 24-well plate. The cultured cells were treated with arecoline for 1 hour and PHA for the next 24 hours. The collected media were stored at -20°C for the IL-2 assay.

Evaluation of cell proliferation/viability (WST-1 assay)

To test the toxic effect of arecoline on the proliferation of Jurkat cells, WST-1 cell proliferation assay kits (Bio Vision) were applied for the viability of cultured Jurkat cells. The results, which reflect the capacity of nicotinamide adenine dinucleotide (NAD) (NADH)-dependent mitochondrial dehydrogenases to reductively cleave WST-1 reagent, were expressed as the percentage of basal level (25).

ELISA of interleukin-2

Medium IL-2 concentrations were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (25) with some modification. Briefly, 0.1 ml of capture antibodies (R&D Systems, Minneapolis, MN, USA) was coated on the polystyrene microtitre plates (NUNC, U16 Maxisorp type, Denmark) and incubated at room temperature overnight. The plates were blocked next day for 1 hour. Then, 0.1 ml of standard/samples was added and incubated for 2 hours. After washing for 3 times, 0.1 ml of detection antibody (R&D Systems) was applied for 2 hours. The addition of 100 microliters of streptavidin horseradish peroxidase (R&D Systems) and 0.1 ml of tetramethylbenzidine substrate (Clinical Science Products Inc, Mansfield, MA, USA) followed this incubation. The reaction was stopped using 2 N sulphuric acid and the optical density (OD) was read at 450 nm (BioTek, Winooski, VT, USA). All samples were run in duplicate. The results were expressed as concentration of cytokines (pg/ml) and hormones (ng/ml) as obtained from the standard curve. The detection range, the sensitivity, and the intra-assay and the inter-assay coefficient of variation for the IL-2 ELISA were 31.5 to 2000 pg/ml, 7 pg, 6.4%, and 10.2%, respectively.

Western blot analysis

The effects of arecoline and PHA on the expression of p-ERK, a7-nAChRs in Jurkat cells were evaluated by Western blot. Jurkat cells were pretreated with arecoline for 1 hour and then treated with PHA (0.1 µg/ml) for 15 (p-ERK) and 20 min (a7-nAChRs). Jurkat cell suspensions were washed twice with fresh PBS, then mixed with 100 µl lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Na2HPO4, 100 mM NaCl, 20 mM NaF, 0.2 mM PMSF, 1 mM DTT). The cell lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as previously described (26). After incubation, the proteins of the cells were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting (27). The first antibodies included anti-β-actin antibodies (1:10,000, mouse, Cell Signaling Technology Inc, Danvers, MA, USA, for loading control), anti-pERK antibodies (1:1000, rabbit) and anti-a7-nAChRs antibodies (1:1000, rabbit). The secondary antibody for p-ERK was anti-rabbit immunoglobulin (1:2000, Cell Signaling Technology Inc, Danvers, MA, USA). The secondary antibody of a7-nAChR was anti-rabbit (immunoglobulin 1:3000, Cell Signaling Technology Inc) and anti-β-actin was anti-mouse (1:10,000, Cell Signaling Technology Inc). The secondary antibody of anti-β-actin was anti-mouse (β-actin; 1:10,000, Cell Signaling Technology Inc). The specific protein bands were detected by chemiluminescence using the electrogenerated chemiluminescence (ECL) Western blotting detection reagents (Amersham International PLC, Buckinghamshire, UK) and exposure to X-ray film. The density of specific bands, such as p-ERK (42, 44 kDa), a7-nAChR (56 kDa) and β-actin (45 kDa), was scanned by a scanner (Personal Densitometer, Molecular Dynamics, Sunyvale, CA, USA). Quantification of the scanned images was performed according to the Image QuaNTTM program (Molecular Dynamics).
The real-time polymerase chain reaction (RT-PCR) was performed according to the method described elsewhere (28). The total RNA was isolated with TRIzol reagent, and cDNA was synthesized by using the superscript III pre-amplification system. The expression of multiple cytokine genes (TNF-α, IL-1β, IL-6, and IL-8) was determined by a multiplex polymerase chain reaction (MPCR) kit for human sepsis cytokines set (Maxim Biotech, San Francisco, CA, USA). Primers were used for the amplification of sequences specific to human IL-2: 5'-ACCTCAACTCCTGCCACAAT -3' (sense) and 5'-GCACTTCCTCCAGAGGTTTG -3' (anti-sense). The cDNA quality was verified by performing controlled reactions by using primers derived from GAPDH 5'-GAGTCAACGGATTTGCGT -3' (sense) and 5'-GACAAGCTTCCCGTTCTCAG -3' (anti-sense). The PCR reaction was performed in a thermal cycler (Thermolyne, Dubuque, IA, USA) and the parameters were as follows:

1. IL-2: 35 cycles of 94°C for 0.5 min, 52°C for 1 min, and 72°C for 1 min.
2. GAPDH: 30 cycles of 94°C for 0.5 min, 50°C for 1 min, and 72°C for 1 min.

The PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

**Statistical analysis**

All data were expressed as the mean ± standard error of the mean (S.E.M.). In some cases, the means of the treatment were tested for homogeneity by analysis of variance (ANOVA), and the difference between specific means was tested for significance by Duncan's multiple range test (29). In other cases, Student's t-test was employed. A difference between two means was considered statistically significant when P<0.05.

**RESULTS**

**Phytohemagglutinin stimulates the interleukin-2 secretion from Jurkat cells in a dose- and time-dependent manner**

Cultured Jurkat cells were treated with phytohemagglutinin (PHA) (1, 2, 5 µg) for 6, 12, or 24 hours. The secretion of IL-2 was not altered by the treatment of PHA for 6 hours (**Fig. 1**, upper panel). Treatment of PHA at 2 µg/ml for 12 hours increased IL-2 secretion from Jurkat cells (**Fig. 1**, central panel). Incubation of Jurkat cells with PHA at 1, 2, 5 µg/ml for 24 hours resulted in a dose-dependent increase of IL-2 secretion (P<0.01, **Fig. 1**, bottom panel).

**Arecoline inhibits the phytohemagglutinin-induced interleukin-2 secretion**

In the basal condition, the secretion of IL-2 was not altered by the treatment of arecoline (**Fig. 2**, panel A). In the presence of PHA=5 µg/ml, administration of 10⁻⁴~10⁻⁶ arecoline inhibited the PHA-evoked secretion of IL-2 by a dose-dependent manner (**Fig. 2**, panel B).

**Effects of arecoline on the phosphorylation of extracellular signal-regulated kinase**

Significant increase of phosphorylated ERK1/2 was observed in Jurkat cells treated with 1 µg/ml PHA for 15 min (P<0.01) (**Fig. 3**). In contrast decreased phosphorylation of ERK1 was found in Jurkat cells treated with PHA and 10 µM arecoline. Also, phosphorylated ERK2 was inhibited significantly in Jurkat cells with 10, 20, 50, and 100 µM arecoline and PHA (**Fig. 3**). These results showed arecoline inhibits the ERK2 phosphorylation induced by PHA in Jurkat cells.

**Arecoline reduces the secretion of prostaglandin E2 from Jurkat cells**

The secretion of PGE₂ was significantly reduced by incubation of Jurkat cells with 10, 20, 50, and 100 µM arecoline for 24 hours (P<0.01) (**Fig. 4**). These results indicated that high concentrations of arecoline decrease the PGE2 secretion may be through the decreased COX-2 expression (data not shown).

**Prostaglandin E₂ stimulates the interleukin-2 secretion from Jurkat cells**

The secretion of IL-2 was significantly enhanced by the incubation of Jurkat cells with 100 (P<0.01), 200 (P<0.05), 500 (P<0.05) and 1000 (P<0.05) pg/ml PGE₂ after co-incubation with PHA 1 µg/ml for 24 hours (**Fig. 5**). These results indicated that arecoline down-regulated the expression PGE₂ and then decreased the secretion of IL-2.

Fig. 1. Dose effects of PHA on the secretion of IL-2 in Jurkat cells after incubation for 6, 12, and 24 hours.

**P<0.01 compared to PHA = 0 µg/ml. Each value represents the mean ± S.E.M.
Arecoline inhibits the expression of α7-nAChRs proteins

The incubation of Jurkat cells with PHA alone enhanced the expression of α7-nAChRs by 24% (Fig. 6). High dose (50 µM) arecoline significantly reduced the expression of α7-nAChRs after 20 min of incubation (Fig. 6). The expression of α3-nAChRs, however, was not affected by arecoline treatment (data not shown). These results correlated with the IL-2 production.
levels. It seems that the administration of arecoline down-regulated the expression of α7-nAChRs and then decreased the secretion of IL-2.

*Arecoline attenuates the interleukin-2 mRNA expression*

The incubation of Jurkat cells with PHA alone enhanced the expression of IL-2 mRNA (Fig. 7). Arecoline (10–100 µM) decreased 40–83% of the expression of IL-2 mRNA evoked by PHA (Fig. 7). These results correlated with the production level of IL-2. It seems that administration of arecoline down-regulated the expression of IL-2 mRNA and therefore decreased the secretion of IL-2.

*Arecoline enhanced the cell proliferation of Jurkat cells treated with phytohemagglutinin*

WST-1 assay was applied to exam Jurkat cell proliferation after arecoline treatment in the presence or absence of PHA. Application of PHA did not alter the proliferation of Jurkat cells (Fig. 8). Arecoline alone (10, 50, and 100 µM) did not affect the cell proliferation except 20 µM arecoline (Fig. 8, upper panel).
However, arecoline significantly increased the proliferation rate of Jurkat cells in the presence of PHA (Fig. 8, lower panel). It seems that the decrease of IL-2 secretion is not related to the toxic effect of arecoline on the proliferation of Jurkat cells.

**DISCUSSION**

The present study demonstrated that arecoline inhibited the PHA-induced secretion of IL-2 by Jurkat cells. Also, PHA-induced phosphorylation of ERK1/2 proteins was decreased by arecoline. In addition, we found that the secretion of IL-2 was enhanced by PGE$_2$. Moreover, arecoline inhibited PGE$_2$ secretion. The expression of α7-nAChRs was attenuated by arecoline. Finally, we found that the PHA-induced increase in IL-2 mRNA expression was inhibited by arecoline.

Selvan *et al.* demonstrated that arecoline causes a dose-dependent and time-dependent suppression of IL-2 production by murine spleen cells *in vitro* (7). It has also been shown that arecoline suppresses interleukin-6 (IL-6) production by GK and keratinocytes (13). Therefore, based on our results and the above observations, it seems that arecoline might suppress cytokine secretion of immune cells via the suppression of ERK phosphorylation and thus may have an effect on the ERK pathway.

ERK is a promiscuous kinase and can phosphorylate many different substrates. Activation of ERK is able to affect a range of cellular functions including proliferation, survival, apoptosis, motility, transcription, metabolism and differentiation (30-32). Chang *et al.* demonstrated that ANE or arecoline is able to stimulate ERK1/ERK2 phosphorylation in human GK and human epidermoid carcinoma KB cells (11). It has also been shown that the ERK inhibitors U0126 and PD98059 are able to
decrease PGE2 and IL-6 production in GK and KB cells treated with ANE or arecoline (33). Deng et al. showed that arecoline decreases PGE2 and IL-6 production in GK and KB cells treated with ANE or arecoline (11). Deng et al. showed that arecoline stimulates connective tissue growth factor (CTGF) synthesis in buccal mucosal fibroblasts in a dose- and time-dependent manner (33). They also observed that pretreatment with inhibitors of nuclear factor kappaB (NF-kB), c-Jun N-terminal kinase (JNK), and p38 MAPK and with N-acetyl-L-cysteine, but not with an ERK inhibitor, are able to significantly suppress arecoline-induced CTGF synthesis (33). Singh et al. have shown that increased phosphorylation of ERK predisposes towards autoimmunity and prevents disease (34). Menschikowski et al. have shown that the effects of TNF-α and IL-1β on endothelial protein C receptor (EPCR) shedding in prostate cancer cells (e.g., DU-145) are mediated by various signaling cascades, namely MEK/ERK 1/2, JNK, and p38 MAPK. However, down-regulation of the MEK/ERK 1/2 pathway and incubation of PC-3 cells with cytokines does not enhance the phosphorylation of ERK-1/2 in the DU-145 cells (35). They also demonstrated that IL-1β and TNF-α regulate the shedding of EPCR in human umbilical endothelial cells (HUVEC), as well as the expression of downstream genes and various metalloproteinases, which occurs via the MAP kinase signaling pathway (36). In mesenchymal stem cells (MSCs), IL-6 stimulates MSC VEGF production, and this effect is additive with that of TGF-α via a mechanism involving ERK, JNK, and PI3K (37). The above results are similar to our present findings and suggest that arecoline may affect the ERK signaling pathway in relation to immunoactivity and carcinogenesis.

It has been demonstrated that enhancement of IL-2 secretion by Jurkat cells occurs via the binding of M1 muscarinic receptors and the action of the transcription factor AP-1 via MAPK and JNK pathways, but is independent of the p38MAPK pathway (38). These results are confirmed by the present findings. Pretreatment with arecoline inhibits IL-2 secretion via phosphorylation of ERK1/2 pathways which is independent of JNK1/2 and p38 pathway (data not shown).

Arecoline interferes with the immune system by targeting the muncrine muscarinic acetylcholine receptor (39). De Rosa et al. have demonstrated that the expression of α7- nAChRs increases after PHA stimulation. After PHA challenge, the activation of peripheral lymphocytes increased the α7 subunit mRNA expression (40). It has been shown that constant stimulation of α7 and α5 nAChRs can control the activity of T cell (41). These results are similar to our finding that showed increased α7- nAChRs in Jurkat cells treated with PHA and arecoline through these receptors, which inhibits IL-2 secretion. However, the expression of α3- nAChRs is not influenced by arecoline treatment.

It has been shown that arecoline enhances IL-6 expression in human buccal mucosal fibroblasts and that this is related to the intracellular glutathione concentration (42). Recent studies have indicated that there is a dose dependent induction of IL-1α mRNA in human keratinocytes by arecoline via oxidative stress and p38 MAPK activation (43). It has also been shown that arecoline can down-regulate the expression of collagens 1A1 and 3A1 in human primary gingival fibroblasts (44). Furthermore, cytokine secretion and mRNA expression in human oral mucous cells are suppressed by arecoline (43). These results are supported by our observations. Our results demonstrated that arecoline decreases the mRNA expression in Jurkat cells and then down regulate the secretion of IL-2.

Prostaglandin is one of the main inflammatory mediators and its production is controlled by various enzymes such as phospholipase A2 and COX-1/2. Recent studies have shown that GSK exposed to ANE show increased PGE2 and PGE2 production (1). COX-2 expression is significantly up-regulated in the OSF of areca quid chewers and arecoline may be responsible for this enhanced COX-2 expression in vivo (45). Brewer et al. have demonstrated that T-cell glucocorticoid receptor suppression of COX-2 is important for curtailing lethal immune activation (46). Recent study indicated that curcumin regulates prostanooid homeostasis in human coronary artery endothelial cells (HCAEC) by modulating multiple steps including the expression of COX-1, COX-2 and the synthesis of prostaglandins (47). Another study showed that heat shock protein 47 (HSP47) is significantly increased in the OSF of areca quid chewers, and that the arecoline induced expression of HSP47 in fibroblasts might be mediated by COX-2 signal transduction pathways (48). Lee et al. have shown that HSP47 expression is significantly enhanced in areca quid chewing-associated OSCCs (49). They also found that HSP47 could be used as a marker for lymph node metastasis of oral carcinogenesis. Arecoline induced HSP47 expression can be downregulated by a COX-2 inhibitor (NS-398) and other inhibitors (48). Peng et al. have demonstrated that endothelin-1 (ET-1) increases the expression of COX-2 and PGE2 production in A549 cells. ET-1 also increases IL-8 production through a COX-2 and PGE2 dependent pathway (50). In our studies, we found the expression of COX-2 was significantly reduced by incubation of Jurkat cells with 20~100 µM arecoline alone for 20 min (data have not shown). The COX2 enhanced the PGE2 production and then increased the IL-2 secretion. Thus, our results have shown that arecoline reduced the expression of COX-2, inhibited the PGE2 production and finally to descend the secretion of IL-2.
In conclusion, our study have demonstrated that the inhibitory effect of arecoline on IL-2 secretion by Jurkat cells would seem, at least in part, to occur via decreased IL-2 mRNA expression and lower ERK1/2 phosphorylation. The inhibitory effect of arecoline on IL-2 secretion is independent of cell proliferation. Furthermore, our study is the first report to demonstrate that arecoline inhibits IL-2 production through a decrease in α7-nAChRs expression and PGE2 production.

Acknowledgements: PS. Wang and S.W. Wang contributed equally to this work. This study was supported by grants NSC94-2320-B-182-022, NSC96-2413-H-182-003, CMRDP150281, CMRDP150282, CMRDP190011, and EZRPF380271. Thanks to Dr. Horng Heng Jiang for his RT-PCR technical assistance. Thanks to Miss Ya-Wen Cheng for her data collection and technical assistance. Thanks to Dr. Ralph Kirby for his English editing.

Conflict of interests: None declared.

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Received: January 14, 2013
Accepted: September 15, 2013

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