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Areca nut extracts-activated secretion of leukotriene B_4, and phosphorylation of p38 mitogen-activated protein kinase and elevated intracellular calcium concentrations in human polymorphonuclear leukocytes

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Background and Objective: Polymorphonuclear leukocytes are the major source of leukotriene B_4, which is synthesized via the 5-lipoxygenase pathway. Activation of the 5-lipoxygenase pathway is regulated by intracellular calcium and the phosphorylation of p38 mitogen-activated protein kinase (MAPK). The impact of areca nut extracts on the biosynthesis of leukotriene B_4 by human polymorphonuclear leukocytes was evaluated, and some of the possible mechanisms underlying the responses were examined.

Material and Methods: Polymorphonuclear leukocytes were treated with various concentrations of areca nut extracts. The concentrations of leukotriene B_4 released into the supernatants were evaluated using enzyme immunoassay. The phosphorylation of p38 MAPK was monitored using immunoblotting, and the cytosolic calcium kinetics were assessed fluorometrically using Fura-2.

Results: Exposure of polymorphonuclear leukocytes to areca nut extracts led to a dose-dependent increase in the production of leukotriene B_4, with levels peaking at 30 min and decreasing thereafter. Areca nut extracts enhanced the phosphorylation of p38 MAPK, an enzyme known to activate 5-lipoxygenase. Incubation with areca nut extracts also resulted in a rapid elevation of intracellular calcium concentrations in polymorphonuclear leukocytes. The induction of leukotriene B_4 by areca nut extracts was suppressed with the p38 MAPK inhibitor, SB203580, or with the intracellular calcium chelator, BAPTA-AM.

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Conclusion: The interaction of areca nut extracts with polymorphonuclear leukocytes activated the arachidonic acid metabolic cascade. Incubation of polymorphonuclear leukocytes with areca nut extracts resulted in the activation of intracellular events, such as phosphorylation of p38 MAPK and Ca^{2+} mobilization, involved in the release of pro-inflammatory lipid mediators. The results of this study emphasize the potential importance of polymorphonuclear leukocytes as a source of leukotriene B_4, which may modulate the inflammatory response in areca chewers.

Areca (betel) quid chewing, the commonly addictive habit, is associated with an increased risk of oral squamous cell carcinoma and oral submucous fibrosis (1). Areca quid chewing also enhances gingival bleeding and increases the prevalence of periodontal diseases (2–5). The probability of subgingival infection with the periodontal pathogens, *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*, is also associated with areca chewing (4). Areca quid chewing causes constant aberrations of the oral surfaces. This, combined with poor oral hygiene in the areca chewers, provide ample opportunities for oral infections to occur, and provides the toxic and carcinogenic components in areca quid direct access to the cells.

Areca quid generally consists of areca nut (*Areca catechu*), *Piper betle* leaf, and slaked lime, with or without additives such as tobacco and *P. betle* inflorescence. Areca nut possesses genotoxic and carcinogenic potencies (1). Areca nut extracts inhibit the growth, attachment, and matrix protein synthesis of cultured gingival fibroblasts (6). Areca nut extracts enhance the production of prostaglandin and cyclooxygenase-2 of human oral epithelial cells (7) and may be associated with tissue inflammatory responses. Areca nut extracts also impair T-cell activation and induce tumor necrosis factor-α and interleukin-6 production in oral epithelial cells (8). Thus, areca nuts may be responsible for inflammatory damage to oral tissues in areca chewers. Areca nut extracts also have an effect on human osteoblasts in the down-regulation of alkaline phosphatase and in the up-regulation of receptor activator of nuclear factor-kappa B ligand (RANKL) (9), suggesting a possible mechanism by which areca nut can be involved in the development of periodontal diseases associated with areca chewing.

Polymorphonuclear leukocytes represent the first line of the host defense mechanism by their ingestion (phagocytosis) and killing of microorganisms. Polymorphonuclear leukocytes are recruited to the site of infection by sensing, and migrating towards, a gradient of chemotactic substances, and are capable of phagocytosing and killing a wide range of bacteria (10,11). Polymorphonuclear leukocytes within the gingival crevice are able to promote periodontal health by decreasing bacterial colonization, the bacterial growth rate, and the viability of periodontal pathogens (11,12). Impaired functions of polymorphonuclear leukocytes may lead to recurrent microbial infection and severe periodontal disease (13–16). Areca nut extracts interfere with the release of reactive oxygen species and inhibit the bactericidal and phagocytic activity of polymorphonuclear leukocytes (17–20). Alteration of the defensive functions of polymorphonuclear leukocytes by areca nut extracts may contribute to a less efficient elimination of bacteria from the periodontal environment.

Leukotriene B_4, produced mainly from polymorphonuclear leukocytes, is a membrane-derived lipid mediator (21). Leukotriene B_4 is generated from the metabolism of arachidonic acid, via a 5-lipoxygenase enzymatic pathway, which may be regulated by changes in calcium concentration (22,23) and the phosphorylation of p38 mitogen-activated protein kinase (MAPK) (24,25). Leukotriene B_4 possesses a variety of biological functions during the inflammatory response (26,27), including enhancing the recruitment of polymorphonuclear leukocytes and activating the production of lysosomal enzyme and superoxide production (27), and also increases vascular permeability (28). The production of leukotriene B_4 is increased in the early stages of inflammation in the periodontium (29,30). Leukotriene B_4 may be involved in regulating inflammatory responses during the pathogenesis of periodontal disease (29–31). However, the impact of areca nut extracts on the production of the lipid mediators by polymorphonuclear leukocytes was not clear. The present study was carried out to investigate the effects of areca nut on the biosynthesis of leukotriene B_4 by human polymorphonuclear leukocytes. The effects of areca nut on the phosphorylation of p38 MAPK and possible changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were also determined.

Material and methods

Preparation of areca nut extracts

Areca nut extracts were extracted from dried ripe areca nuts, without husk, as previously described (32). Finely chopped dried nuts (10 g) were extracted with 250 mL of distilled water for 1 h. The filtrate was freeze-dried. The yield, after extraction, was ≈12%. Areca nut extracts were dissolved in dimethylsulfoxide (Sigma Chemical Co., St Louis, MO, USA) before being diluted in Hank's balanced salt solution (Gibco BRL Laboratories, Grand Island, NY, USA), supplemented with 1.6 mM $CaCl_2$ and 10 mM HEPES (Hank's balanced salt solution/Ca^{2+}). The final concentration of dimethylsulfoxide in each sample was less than 0.5%.

Preparation of polymorphonuclear leukocytes and incubation conditions

Polymorphonuclear leukocytes were freshly purified from human venous peripheral blood, from healthy volunteers, by dextran sedimentation followed by Ficoll density-gradient centrifugation, as described previously (17). The polymorphonuclear leukocyte/red blood cell pellet was transferred to a new tube and washed once with Hank's balanced salt solution. Residual red blood cells were eliminated by hypotonic lysis. Polymorphonuclear leukocytes were then washed and resuspended in Hank's balanced salt solution/Ca^{2+}. Freshly isolated polymorphonuclear leukocytes (2×10^6 cells/mL) were incubated with different concentrations of areca nut extracts (0, 3.125, 6.25, 12.5, or 25 μg/mL) in Hank's balanced salt solution/Ca^{2+} for 30 min at 37°C. Polymorphonuclear leukocytes were also treated with dimethylsulfoxide (0.125 or 0.5%) under similar conditions. For kinetics experiments, polymorphonuclear leukocytes were incubated with 0.5% dimethylsulfoxide, or with different concentrations of areca nut extracts (0, 3.125, 6.25, 12.5 μg/mL), in Hank's balanced salt solution/Ca^{2+}, for 2, 4, 8 or 20 h at 37°C. For experiments studying the effect of inhibitors, SB203580 (Molecular Probes, Junction City, OR, USA) and BAPTA-AM (Merck KGaA, Darmstadt, Germany) were first dissolved in dimethylsulfoxide as stock solutions and further diluted in Hank's balanced salt solution/Ca^{2+}. Polymorphonuclear leukocytes were pretreated with Hank's balanced salt solution/Ca^{2+} only, or with Hank's balanced salt solution/Ca^{2+} containing dimethylsulfoxide (0.5%), SB203580 (10, 20 μM) or BAPTA-AM (50 μM), for 30 min at 37°C. Polymorphonuclear leukocytes were further incubated with or without 25 μg/mL of areca nut extracts. Each inhibitor was present throughout the incubation. After each treatment, the supernatants were harvested by centrifugation (1000 *g*, 37°C, 10 min) and frozen in small aliquots at −80°C for future analysis of leukotriene B_4. The viability of the remaining cells was also analyzed.

Viability of polymorphonuclear leukocytes

The viability of the treated polymorphonuclear leukocytes was determined by detecting the influx of propidium iodide into polymorphonuclear leukocytes (33). This assay is based on the fact that propidium iodide stains nucleic acids in dead cells that have lost their membrane integrity (34). Polymorphonuclear leukocytes, fixed in 3% paraformaldehyde, served as the controls for dead cells. Treated polymorphonuclear leukocytes were washed and incubated in Hank's balanced salt solution, or Hank's balanced salt solution containing 4 μg/mL of propidium iodide (Sigma Chemical Co.), at 37°C for 15 min. After two washes with Hank's balanced salt solution, polymorphonuclear leukocytes were passed through a nylon filter (41 μm) (Spectrom®; Spectrum Laboratories, Inc., Laguna Hills, CA, USA) and analyzed by a flow cytometer (FACSort™; Becton Dickinson, San Jose, CA, USA) equipped with an argon laser operating at an excitation wavelength of 488 nm. The instrument settings were as follows: sideward scatter threshold set at 52; and detector set at E00, 381, and 582 for forward scatter, sideward scatter and fluorescence 2 (FL2, red fluorescence), respectively. Data were analyzed using the CELLQUEST® and WINMDI 2.8 software programs (Becton Dickinson). The fluorescence intensities of a total of 10,000 cells were measured. The viability of polymorphonuclear leukocytes in each sample was determined by the following formula: (number of unstained cells in experimental sample ÷ number of unstained cells in control polymorphonuclear leukocytes) × 100%.

Detection of leukotriene B_4

The culture supernatants were assayed for leukotriene B_4 using a commercial solid-phase competition enzyme immunoassay kit with acetylcholinesterase as the label (Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's protocol. The detection limit of the assay was 13 pg/mL. Fifty microliters of each sample was analyzed. The concentration (pg/mL) of each sample was calculated using the standard curve generated from purified leukotriene B_4 supplied with the kit. Hank's balanced salt solution/Ca^{2+} alone, or Hank's balanced salt solution containing 25 μg/mL of areca nut extracts, 0.5% dimethylsulfoxide, 20 μM SB203580 or 50 μM BAPTA-AM, were also examined for a background reaction with the kit.

Western blotting analysis

Polymorphonuclear leukocytes were incubated in Hank's balanced salt solution/Ca^{2+}, containing dimethylsulfoxide (0.5 or 0.125%) or areca nut extracts (0, 3.125, 6.25, 12.5 or 25 μg/mL), for 30 min at 37°C. Treated cells were lysed with lysis buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 100 mM NaF, 100 mM Na_3VO_4 and 1 × protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)]. Cell lysates were analyzed by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel. Proteins were transferred onto a poly(vinylidene difluoride) membrane (Pall Gelman Laboratory, Ann Arbor, MI, USA) and the membrane was immunoblotted with polyclonal antibodies against the phosphorylated p38 MAPK (0.2 μg/mL) (sc-17852-R), or the phosphorylated and nonphosphorylated p38 MAPK (0.2 μg/mL) (sc-535) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h and then incubated with goat antirabbit immunoglobulin G conjugated to horseradish peroxidase (0.1 μg/mL) (Amersham Pharmacia Biotech, Little Chalfont, UK). Between each step, the membranes were washed with phosphate-buffered saline containing 0.1% Tween-20. Proteins were revealed with the enhanced chemiluminescence detection system (Super signal®, West Pico Chemiluminescent substrate; Pierce Biotechnology Inc., Rockford, IL, USA).

Measurements of $[Ca^{2+}]_i$

Polymorphonuclear leukocytes were incubated at 37°C in the dark with 2 μM of the fluorescent probe, Fura-2-acetoxymethyl ester (Molecular Probes). After 30 min, cells were washed twice to remove the extracellular probe and finally resuspended at a concetration of 2×10^6/mL in 2.5 mL of loading buffer (152 mM NaCl, 1.2 mM $MgCl_2$, 1.6 mM $CaCl_2$, 5 mM KCl, 10 mM HEPES, 10 mM glucose, pH 7.4) and placed in a plastic cuvette equipped with a cuvette stirrer. The fluorescence of polymorphonuclear leukocytes was monitored at 37°C for 1 min with a dual-wavelength spectrofluorimeter (model CM1T11I; Spex Industries, Edison, NJ, USA). After the addition of 20 μL of agents to reach final concentrations of dimethylsulfoxide (0.125%) or areca nut extracts (0, 3.125, 6.25, 12.5 or 25 μg/mL), the fluorescence was further monitored for 8 min. Using excitation wavelengths of 340 and 380 nm, the fluorescence emission at 505 nm was measured and the $[Ca^{2+}]_i$ was determined from the Fura-2 fluorescence-ratio signal, using SPEX DM3000 software, according to the formula derived by Grynkiewicz (35). Fluorescence signals were calibrated by adding 20 μL of 2% digitonin for 30 s (maximum) followed by 500 μL of 500 mM EGTA (minimum). In each experiment, the variations in $[Ca^{2+}]_i$ induced by areca nut extracts were calculated as the mean 10-s pretreatment values (basal levels), the highest values reached after addition of the agent (peak levels), and the mean values from 300 to 400 s after addition of the agent (sustained levels). The lag time between the addition of areca nut extracts and peak attainment (time to reach peak) was also calculated. Data were expressed as nM. All tests were performed independently, four times, using polymorphonuclear leukocytes obtained from different donors.

Statistical analysis

The data were calculated to obtain the mean and standard error of the mean. The effects of areca nut extracts on viability or leukotriene B_4 production at different concentrations were assessed using a one-way analysis of variance and Tukey multiple comparison intervals, based on $\alpha = 0.05$. Analysis of variance and Tukey intervals were also used to compare basal level, peak level, and sustained level of $[Ca^{2+}]_i$.

Results

Leukotriene B_4 production by polymorphonuclear leukocytes after treatment with areca nut extracts

The viability of polymorphonuclear leukocytes, and the production of leukotriene B_4 from endogenous arachidonic acid by polymorphonuclear leukocytes after treatment with areca nut extracts, were evaluated. As areca nut extracts were first dissolved in dimethylsulfoxide, the effect of vehicle dimethylsulfoxide was also examined in the same experimental conditions. Treatment with 0.5% dimethylsulfoxide, or with 3.125 or 6.25 μg/mL of areca nut extracts, for 30 min did not affect the viability of polymorphonuclear leukocytes (Fig. 1A). However, the relative viability of polymorphonuclear leukocytes was 86.6 ± 1.4% or 71.0 ± 2.8% when 12.5 or 25 μg/mL of areca nut extracts was used, respectively. The concentration of leukotriene B_4 in the supernatant of control polymorphonuclear leukocytes

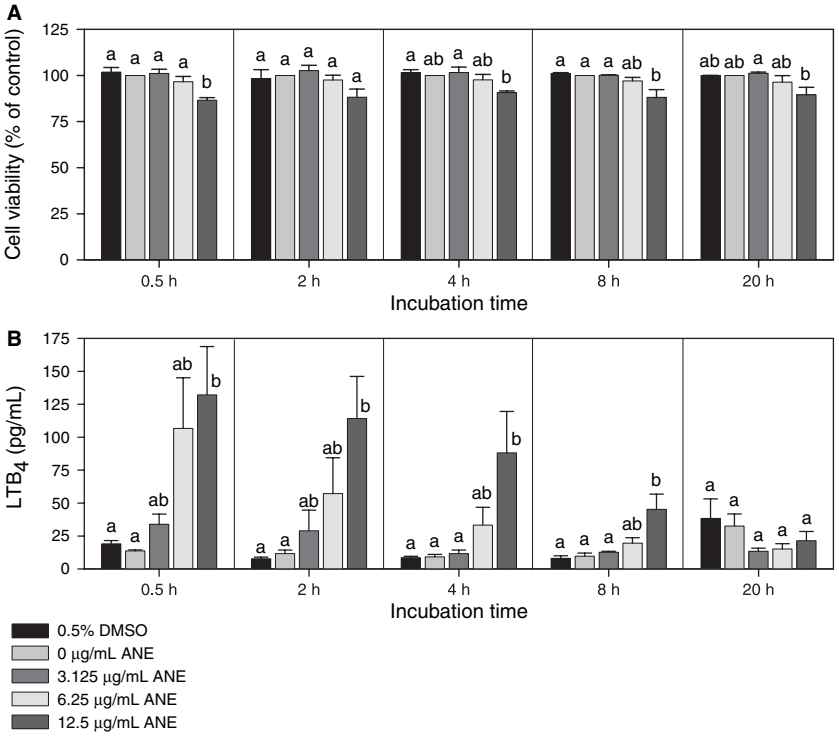

Fig. 1. Effects of areca nut extracts on the viability and production of leukotriene B_4 in polymorphonuclear leukocytes. Polymorphonuclear leukocytes were incubated with 0.5% dimethylsulfoxide or different concentrations of areca nut extracts for 30 min at 37°C ($n = 4$), or for the indicated times (2 to 20 h) ($n = 3$). (A) The relative cellular viability was determined using propidium iodide staining. At each indicated time-point, the viability of polymorphonuclear leukocytes treated with buffer only (0 μg/mL of areca nut extracts) was considered as 100%. (B) The concentration of leukotriene B_4 in the supernatants was measured using enzyme immunoassay. Values represent the mean ± standard error of the mean from at least three independent experiments performed with polymorphonuclear leukocytes obtained from different donors. Statistical differences among different leukotriene B_4 concentrations at the indicated time-points are shown by different lowercase letters (analysis of variance, Tukey, $\alpha = 0.05$). ANE, areca nut extracts; DMSO, dimethylsulfoxide; LTB_4, leukotriene B_4.

was measured at a low level of 13.6 ± 0.8 pg/mL (Fig. 1B). Incubation of polymorphonuclear leukocytes with areca nut extracts for 30 min induced a significant accumulation of leukotriene B_4 in a dose-dependent manner. Polymorphonuclear leukocytes exposed to 25 μg/mL of areca nut extracts produced a higher concentration of leukotriene B_4 (256.4 ± 48.6 pg/mL). With the isolated polymorphonuclear leukocytes, we clearly demonstrated that expression of leukotriene B_4 from polymorphonuclear leukocytes treated with 3.125–25 μg/mL of areca nut extracts for 30 min was increased from 2.5 times to 18.9 times in comparison with the control polymorphonuclear leukocytes.

The kinetics of cellular viability, and the synthesis of leukotriene B_4 induced by areca nut extracts, were further evaluated. The viability of polymorphonuclear leukocytes treated with 0.5% dimethylsulfoxide, or with 3.125 or 6.25 μg/mL of areca nut extracts, did not differ significantly from the control at the indicated time points (Fig. 1A). The relative cellular viability was at least 88% when treated with 12.5 μg/mL of areca nut extracts at various time points (2–20 h). At the 4, 8 and 20 h time points of incubation with 12.5 μg/mL of areca nut extracts, the difference of viability reached statistical significance (Fig. 1A). Incubation of areca nut extracts for 2, 4, or 8 h enhanced the production of leukotriene B_4 in a concentration-dependent manner. However, the concentration of leukotriene B_4 in the supernatants decreased over the incubation period.

Phosphorylation of p38 MAPK in polymorphonuclear leukocytes

The effects of areca nut extracts on the activation of p38 MAPK in polymorphonuclear leukocytes were determined using western blot analysis. The total amounts of p38 MAPK were not altered when incubated with areca nut extracts or dimethylsulfoxide for 30 min (Fig. 2). Activation of p38 MAPK was barely detectable in the polymorphonuclear leukocytes treated with buffer only (0 μg/mL of areca nut extracts) or with dimethylsulfoxide. However, phosphorylation of p38 MAPK was activated by areca nut extracts in a concentration-dependent manner.

Changes in $[Ca^{2+}]_i$

The ability of areca nut extracts to induce changes in the $[Ca^{2+}]_i$ of polymorphonuclear leukocytes was examined. The treatment of polymorphonuclear leukocytes with 3.125 μg/mL of areca nut extracts or vehicle dimethylsulfoxide (0.125%) did not affect the $[Ca^{2+}]_i$ (Fig. 3A). However, incubation with 6.25, 12.5 or 25 μg/mL of areca nut extracts elicited a rapid and dose-dependent response on $[Ca^{2+}]_i$ changes in polymorphonuclear leukocytes. The peaks of $[Ca^{2+}]_i$ in polymorphonuclear leukocytes after incubation with 6.25, 12.5 or 25 μg/mL of areca nut extracts was reached in 54.0 ± 5.3, 28.0 ± 4.1 or 15.4 ± 1.1 s, respectively. In addition, areca nut extracts elicited a dose-dependent sustained $[Ca^{2+}]_i$ increase that lasted for up to 8 min (Fig. 3A). The peak levels of $[Ca^{2+}]_i$ elicited by 6.25, 12.5 or 25 μg/mL of areca nut extracts were significantly higher than the basal conditions in polymorphonuclear leukocytes (Fig. 3B). The sustained level of $[Ca^{2+}]_i$ elicited by areca nut extracts (12.5 μg/mL) was also higher than the basal level. The areca nut extracts-induced $[Ca^{2+}]_i$ changes were blocked by addition of the calcium chelator, EGTA (5 mM), prior to stimulation with areca nut extracts (data not shown), indicating that the rise of $[Ca^{2+}]_i$ by areca nut extracts was dependent on calcium influx in polymorphonuclear leukocytes.

Effects of p38 MAPK inhibitor SB203580 and the intracellular calcium chelator BAPTA-AM

To determine whether the induction of leukotriene B_4 in polymorphonuclear leukocytes by areca nut extracts is dependent on the activation of p38 MAPK or changes in intracellular calcium, the p38 MAPK inhibitor (SB203580), or the intracellular calcium chelator (BAPTA-AM), were used, respectively. At the same concentration of areca nut extracts, viable numbers of polymorphonuclear leukocytes pretreated with or without 20 μM SB203580 or 50 μM BAPTA-AM were similar, suggesting that neither inhibitor affected the viability of polymorphonuclear leukocytes. The p38 MAPK inhibitor, SB203580, reduced the areca nut extracts-induced leukotriene B_4 formation (Fig. 4). In addition, depletion of intracellular Ca^{2+} by BAPTA-AM clearly suppressed the secretion of leukotriene B_4 in polymorphonuclear leukocytes treated with areca nut extracts (Fig. 5). Collectively, activation of p38 MAPK and changes of $[Ca^{2+}]_i$ appear to be involved in areca nut extracts-induced leukotriene B_4 production.

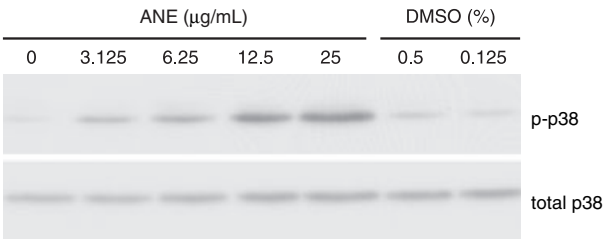

Fig. 2. Effects of areca nut extracts on the phosphorylation of p38 mitogen-activated protein kinase (MAPK). Polymorphonuclear leukocytes were incubated with different concentrations of areca nut extracts (0, 3.125, 6.25, 12.5, 25 μg/mL) or dimethylsulfoxide (0.5, 0.125%) for 30 min at 37°C. Cell lysates from each sample were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting using antiphosphorylated p38 MAPK (p-p38) or the phosphorylated and nonphosphorylated p38 MAPK (total p38). Results shown are representative of three independent experiments. ANE, areca nut extracts; DMSO, dimethylsulfoxide.

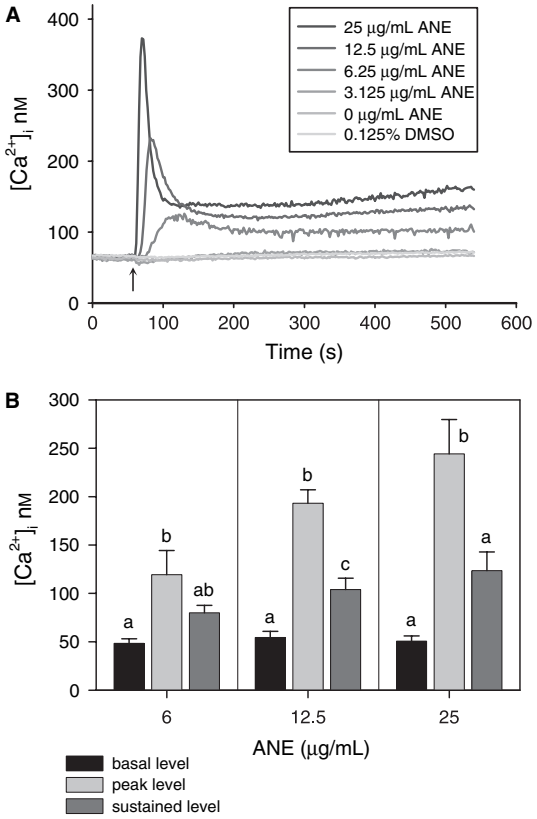

Fig. 3. Effects of areca nut extracts on intracellular calcium concentrations $[Ca^{2+}]_i$ in human polymorphonuclear leukocytes. (A) The arrow indicates the time of addition of the indicated agents. The results shown are representative of four independent experiments. (B) Concentration-response for $[Ca^{2+}]_i$ changes induced by areca nut extracts in human polymorphonuclear leukocytes. Results shown are the mean 10-s pretreatment values (basal level), the highest values reached after addition of the agent (peak level), and the mean values from 300 to 400 s after addition of the agent (sustained level). Each value is the mean ± standard error of the mean of four independent experiments. Statistical differences among basal level, peak level, and sustained level at the indicated concentrations are shown by different lowercase letters (analysis of variance, Tukey, $\alpha = 0.05$). ANE, areca nut extracts; $[Ca^{2+}]_i$, intracellular calcium concentration; DMSO, dimethylsulfoxide.

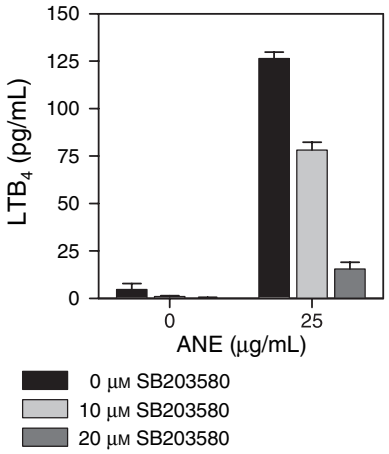

Fig. 4. Effects of SB203580 on the production of leukotriene B_4. Polymorphonuclear leukocytes were pre-incubated with 0, 10 or 20 μM SB203580 for 30 min at 37°C. Areca nut extracts (0 or 25 μg/mL) were then added and incubated for 30 min at 37°C. The concentration of leukotriene B_4 in the supernatants was determined. Results are given as mean ± standard error of the mean of two independent experiments. ANE, areca nut extracts; LTB_4, leukotriene B_4.

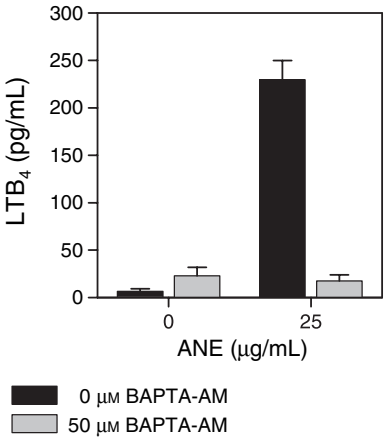

Fig. 5. Effects of BAPTA-AM on leukotriene B_4 production. Polymorphonuclear leukocytes were pre-incubated with 0 or 50 μM BAPTA-AM for 30 min at 37°C. Areca nut extracts (0 or 25 μg/mL) were added and incubated for 30 min at 37°C. The leukotriene B_4 concentration in the supernatants was determined. Results are given as mean ± standard error of the mean of three independent experiments. ANE, areca nut extracts; LTB_4, leukotriene B_4.

Discussion

Activation of the arachidonic acid cascade that leads to the synthesis of arachidonic acid metabolites with potent pro-inflammatory properties is one important mechanism involved in inflammation and tissue destruction (36,37). 5-Lipoxygenase is the key enzyme in the metabolism of arachidonic acid to produce leukotriene B_4, which is present in the inflammatory response. Polymorphonuclear leukocytes are recognized as a major source of leukotriene B_4. The present study showed that areca nut extracts stimulated the synthesis of leukotriene B_4 in human polymorphonuclear leukocytes. The tyrosine-phosphorylated substrate, p38 MAPK, in polymorphonuclear leukocytes was also stimulated by areca nut extracts. Moreover, incubation with areca nut extracts resulted in a rapid elevation of $[Ca^{2+}]_i$ levels in resting polymorphonuclear leukocytes. Both the p38 MAPK inhibitor (SB203580) and the intracellular calcium chelator (BAPTA-AM) greatly inhibited the induction of leukotriene B_4 by areca nut extracts. Activation of 5-lipoxygenase is associated with the phosphorylation of p38 MAPK and the intracellular concentration of calcium (38). Thus, the results of this study suggested that areca nut extracts might play a role in regulating the 5-lipoxygenase pathway.

New production of leukotriene B_4 might play an important role in tissue damage by areca nut. Leukotriene B_4,

a potent lipid mediator, can activate chemotaxis, aggregation and provoke adhesion of polymorphonuclear leukocytes to endothelial cells; and can stimulate lysosomal enzyme release and superoxide production (27,39). In addition, leukotriene B_4 can modulate functions of lymphocytes by stimulating production of interferon gamma and interleukin-2 from T cells, and increasing the synthesis of interleukin-1 by monocytes (27). Leukotriene B_4 also activates the proliferation of osteoclasts and stimulates bone resorption (40,41). All of these leukotriene B_4-dependent mechanisms may contribute to the inflammation and destruction of the bone and connective tissue in periodontal disease.

This study demonstrated direct evidence that areca nut extracts evoked a rapid rise of $[Ca^{2+}]_i$. This areca nut extracts-induced increase of $[Ca^{2+}]_i$ in polymorphonuclear leukocytes was abolished by Ca^{2+} chelation, suggesting that areca nut extracts could increase the plasma membrane permeability for Ca^{2+}. Induction of leukotriene B_4 by areca nut extracts was also abrogated using the intracellular calcium chelator, BAPTA-AM. Ca^{2+} plays a crucial role as a second messenger in human polymorphonuclear leukocytes (42). Changes in $[Ca^{2+}]_i$ are associated with the activation of polymorphonuclear leukocytes (43). An increase of $[Ca^{2+}]_i$ activates protein kinase C (44). Components of areca quid induce production of protein kinase C in the mouse skin model (45). Prolonged activation of protein kinase C is believed to be associated with cell growth, differentiation, migration, and tumorigenesis (46). Activation of intracellular calcium may activate all the calcium-dependent signal pathways. Thus, further experiments are needed to examine whether other calcium-dependent signal pathways are affected by areca nut extracts.

Increasing amounts of phosphorylated p38 MAPK were detected in polymorphonuclear leukocytes after treatment with areca nut extracts. Induction of leukotriene B_4 by areca nut extracts was inhibited using the p38 MAPK inhibitor, suggesting that induction of p38 MAPK may play a role in the synthesis of leukotriene B_4 induced by areca nut extracts in human polymorphonuclear leukocytes. Areca nut extracts strongly enhanced the phosphorylation of p38 MAPK, an enzyme known to phosphorylate cytosolic phospholipase A_2 in human polymorphonuclear leukocytes. Whether areca nut extracts affect the activity of phospholipase A_2 and the synthesis of a phospholipase A_2-activating protein requires further analysis. Activation of p38 MAPK has also been observed when oral squamous cell carcinoma cells (OECM-1) were incubated with 20 μg/mL of areca nut extracts for 18 h (47). In contrast, areca nut extracts repress the p38 MAPK activity in another oral squamous cell carcinoma cell line (SAS) (47). The discrepancy may be a result of differences in cell types, culture media, and culture conditions evaluated. Besides p38 MAPK, areca nut extracts can also activate Rac-1, a Ras-like small GTPase family member (48), nuclear factor-κB and c-Jun N-terminal kinase in OECM-1 cells (47).

Arecoline is one of the major alkaloids in the areca nut. The concentration of arecoline in areca nut extracts was found to be ≈9.1 μg/mg (Liu T-Y, unpublished data). The treatment of polymorphonuclear leukocytes with arecoline for 30 min had no apparent effect on the viability and production of leukotriene B_4 (Hung S-L, unpublished data), suggesting that arecoline could not be used to explain the effect of areca nut extracts on leukotriene B_4 production of polymorphonuclear leukocytes. *A. actinomycetemcomitans* induces the production of leukotriene B_4 in keratinocytes (49). Whether there is an additive effect of areca nut and bacterial infection remains to be examined. In addition, the concentrations of leukotriene B_4 in the serum of smokers were found to be nearly 60-fold greater than those in nonsmokers (50). However, in a rat model, chronic inhalation of nicotine in tobacco smoke was found to decrease the production of leukotriene B_4 from alveolar macrophages (51). Nicotine inhibits the production of leukotriene B_4 in polymorphonuclear leukocytes *in vitro* (52). Ethanol also inhibits the production of leukotriene B_4 by polymorphonuclear leukocytes *in vitro* (53). The majority of areca chewers also smoke and drink alcohol (54). It will be of interest to determine the levels of leukotriene B_4 in gingival crevicular fluid or gingival tissues in areca chewers.

In conclusion, we present novel evidence demonstrating that areca nut extracts significantly increased the intracellular calcium concentration, and promoted the extracellular production of leukotriene B_4 in human polymorphonuclear leukocytes. Areca nut extracts also activated p38 MAPK in polymorphonuclear leukocytes. Induction of p38 MAPK and mobilization of calcium may play roles in the synthesis of leukotriene B_4 induced by areca nut extracts in human polymorphonuclear leukocytes. Activation of signal transduction may also affect other functions of polymorphonuclear leukocytes. Leukotriene B_4 may function either directly or synergistically with other inflammatory mediators, thereby being involved in the amplification and continuation of the inflammatory response and in the ensuing tissue destruction. Thus, polymorphonuclear leukocyte-derived leukotriene B_4 may contribute to the amplification of areca nut extracts-induced inflammation and may be important in regulating the inflammatory response in the periodontal tissues of areca chewers.

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