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Highly oligomeric procyanidins from areca nut induce lymphocyte apoptosis via the depletion of intracellular thiols

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ABSTRACT

Procyanidins are plant-derived polyphenolic compounds possessing a variety of biological activities, such as immunomodulation, and induction of tumor cell apoptosis. We previously reported that total extract of areca nut exhibited a suppressive effect on the metabolic activity and cytokine expression in normal splenic lymphocytes. As areca nut contains a rich amount of polyphenols, the objective of the present study was to investigate the pro-apoptotic effect of polyphenol-enriched areca nut extract (PANE) and its fractionated oligomeric procyanidins in splenic lymphocytes. Our data showed that PANE markedly induced lymphocyte apoptosis in a concentration- and time-dependent manner. Notably, the fractionated oligomeric procyanidins from pentamers to decamers were active in inducing the apoptosis, whereas monomers to tetramers were inactive. In addition, a marked diminishment in the level of intracellular thiols was revealed in lymphocytes treated with pentamers to decamers. Pretreatment with *N*-acetyl-L-cysteine, a precursor of glutathione, resulted in significant attenuation of both apoptosis and thiol diminishment induced by areca procyanidins. Taken together, our results indicated that highly oligomeric procyanidins derived from areca nut exhibited a chain length-dependent pro-apoptotic effect in primary lymphocytes, which is mediated, at least in part, by the diminishment of intracellular thiols.

1. Introduction

Flavonoids are a group of polyphenolic compounds widely distributed in a variety of dietary plants, including apple, grape, cocoa, areca, among many others (Hertog et al., 1993). Areca nut (*Areca catechu*) has been shown to be a rich source of flavan-3-ol polyphenols containing monomers of (+)-catechin and (−)-epicatechin, and their polymerized oligomers (procyanidins; Fig. 1) (Wu et al., 2007). The procyanidins of areca is similar as those found in apple and grape seed, except that grape procyanidins are galloylated to some extent, whereas the others are not (Wu et al., 2007). In addition to polyphenols, areca nut contains alkaloids, polysaccharides, fats and crude fibers (Ranadive et al., 1976).

The potential of procyanidins as health-promoting phytochemicals has attracted a great deal of attention. Recent studies have shown a number of promising pharmacological effects exhibited by procyanidins, including antitumor, immunomodulatory, antiallergic, antioxidative, chemopreventive, and anti-inflammatory effects (Gosse et al., 2005; Ito et al., 2000; Kenny et al., 2007; Nakano et al., 2008; Neuwirt et al., 2008; Shibata et al., 2009; Yoshioka et al., 2008; Zhang et al., 2005). One of the mechanisms for procyanidin-mediated antitumor effect has been attributed to the induction of tumor cell apoptosis (Kaur et al., 2006; Neuwirt et al., 2008; Ramljak et al., 2005; Seeram et al., 2004). In contrast, limited evidence is available pertaining to the influence of procyanidins on apoptosis of primary cells. A previous report showed that grape procyanidins at a high concentration caused apoptosis in chick cardiomyocytes via an oxidative stress-dependent mechanism (Shao et al., 2006). In addition, we have demonstrated that a total extract of areca nut (ANE) markedly induced apoptosis in murine splenic lymphocytes (Wang et al., 2009).

Accumulating evidence describes the immunomodulatory effect of procyanidins on T cell-mediated immune reactions. Of interest, animal studies have shown that procyanidins administration

Abbreviations: ANE, areca nut extract; CMF-DA, 5-chloromethylfluorescein diacetate; DMSO, dimethyl sulfoxide; EC_{50}, 50% effective concentration; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; IFN, interferon; IL, interleukin; NA, naïve; NAC, *N*-acetyl-L-cysteine; PANE, polyphenol-enriched areca nut extract; PBS, phosphate-buffered saline; PI, propidium iodide; SE, standard error; UV, ultraviolet; VH, vehicle.

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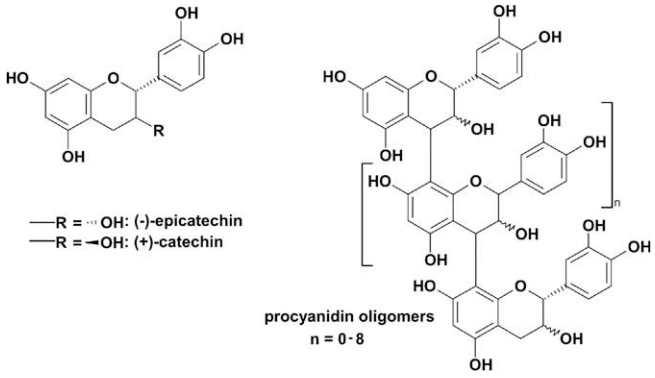

Fig. 1. Chemical structures of catechin monomers and areca-derived oligomeric procyanidins.

attenuated the development of food allergy, autoimmune encephalomyelitis and inflammatory bowel disease (Akiyama et al., 2005; Miyake et al., 2006; Yoshioka et al., 2008). The pathophysiology of these disease models involves T cell-mediated immune responses. In vitro studies further reported that the expression of cytokines, including interleukin (IL)-2, IL-4, and IL-5 by peripheral blood mononuclear cells stimulated with the T cell mitogen phytohemagglutinin was suppressed by procyanidins (Mao et al., 2002). In accordance with these results, we also recently reported that ANE markedly attenuated the metabolic activity and the production of IL-2 and interferon (IFN)-γ by splenic lymphocytes via an oxidative stress-dependent mechanism (Wang et al., 2007). As areca nut contains a considerable amount of procyanidins, we speculated that the effects may be mediated by its procyanidins.

The underlying mechanisms for procyanidin-mediated biological effects remain largely unclear. Recent studies suggest that oligomer chain length may be a critical factor determining the antioxidant, pro-apoptotic and immunomodulatory effects of procyanidins (Kenny et al., 2007; Lotito et al., 2000; Pierini et al., 2008; Ugartondo et al., 2007). In the present study, we hypothesized that procyanidins might be the active principle in areca nut contributing to the immunotoxic effects on lymphocytes. We investigated the pro-apoptotic effect of polyphenol-enriched ANE (PANE) in splenic lymphocytes, and compared the activity of catechin monomers and the fractionated oligomeric procyanidins. Our data demonstrated the pro-apoptotic effect of areca-derived procyanidins in lymphocytes, and that the pro-apoptotic activity required at least five degrees of polymerization. In addition, we identified that the diminishment of intracellular thiols was one of the biochemical mechanisms underlying the pro-apoptotic effect.

2. Materials and methods

2.1. Reagents and areca nut extract

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. Fetal bovine serum (FBS) and cell culture medium were purchased from Hyclone (Logan, UT). Cell permeable caspase-6 and -3 inhibitors (Z-VEID-FNK and Z-DEVD-FMK, respectively) were purchased from BioVision (Mountain View, CA). A polyphenol-enriched areca nut extract (PANE) was prepared as previously described (Wang and Lee, 1996). Briefly, nuts were extracted three times with 80% aqueous acetone (1:10, w/v) and filtrated. The filtrate was evaporated to remove acetone, partitioned with *n*-hexane and ethyl-ether to remove lipids and then freeze-dried that consisted of 90% condensed tannins (data not shown). The fractionation of PANE was performed by normal-phase HPLC (Gu et al., 2006). In brief, PANE was dissolved in 30% aqueous methanol for application to a 250 × 4.6 mm Phenomenex Luna Silica column (Phenomenex, Torrance, CA). The mobile phase consisted of (A) dichloromethane:methanol:50% acetic acid = 82:14:4, and (B) methanol:50% acetic acid = 96:4. The gradient was programmed at: 0–20 min, 14–23.6% B; 20–50 min, 23.6–35% B; 50–55 min, 35–86% B; 55–65 min, 86% B; 65–70 min, 86–14% B with 1 mL/min flow rate and monitored at 280 nm with UV detector. The chromatographic distribution of monomeric fraction was confirmed with authentic standards, catechin and epicatechin. To measure the degree of polymerization, each fraction was thiolyzed and analyzed by reverse phase-HPLC using a LichroCART C_{18} 250 × 4.6 mm column (Merck & Co., Inc., Germany). The mobile phase consisted of (A) 0.1% acetic acid and (B) 0.1% acetic acid in acetonitrile. The gradient was as follows: 0–8 min, 8% B; 8–40 min, 8–24% B; 40–50 min, 24–80% B; 50–55 min, 80–8% B and the flow rate was 1 mL/min (Monagas et al., 2003). All signals were detected at 280 nm with UV detector (Gu et al., 2006). Our PANE contained dimers (6.5%), trimers (5.2%), tetramers (5.8%), pentamers (3.8%), hexamers (3.4%), heptamers (5.0%), octomers (3.6%), nonamers (2.9%) and decamers (3.7%). The chromatography was repeated three times.

2.2. Animals and cell culture

Male BALB/c mice, 5–6 weeks of age were obtained from the Animal Breeding Center of the National Taiwan University Hospital (Taipei, Taiwan). The mice were given standard laboratory food and water ad libitum and quarantined at least for 1 week before experimentation. The animal room was maintained at a temperature of 22 ± 2 °C and a relative humidity of 60 ± 20%, with a 12-h light/dark cycle. Their spleens were isolated aseptically and made into single cell suspensions as described previously (Wang et al., 2007). The erythrocytes in splenocyte cultures were lysed using a hypotonic buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4). The cells were then incubated in dishes for 2 h to remove adherent cells. The splenic lymphocytes were cultured in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 5% heat inactivated FBS, and cultured at 37 °C in 5% CO_2.

2.3. Cell cycle analysis of splenic lymphocytes

Cell cycle distribution of lymphocytes was determined by flow cytometry using propidium iodide (PI) staining as previously described (Nicoletti et al., 1991). DNA fluorescence of 5000 cells for each sample was measured using a flow cytometer at emission of 575 nm (FL2). The data were analyzed using the software Flowjo 8.0. The apoptotic cells were defined as sub-G_0/G_1-phase cells with hypodiploid DNA content (Nicoletti et al., 1991).

2.4. Hoechst staining of apoptotic cells

Splenic lymphocytes were harvested and spun onto the glass slide by a cytospin centrifuge, and fixed in 100% acetone at −20 °C for 20 min. The fixed cells were washed with PBS, and then stained with Hoechst 33258 (1 μg/mL) for 5 min at room temperature in the dark. The cells were examined and photographed under a fluorescence microscope (Optiphoto II, Nikon, Tokyo, Japan) using an excitation wavelength of 330–380 nm.

2.5. Measurement of intracellular thiols using flow cytometry

After treatment, cells were stained with 5-chloromethylfluorescein diacetate (CMF-DA; 25 μM) for 25 min. The single cell fluorescence of 10,000 cells for each sample was measured using a flow