Protocatechuic Acid, a Metabolite of Anthocyanins, Inhibits Monocyte Adhesion and Reduces Atherosclerosis in Apolipoprotein E-Deficient Mice

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INTRODUCTION

Polyphenols, including anthocyanins, from various plant foods are effective in the prevention of atherosclerosis in animal and human studies. Protocatechuic acid (PCA), a major metabolite of anthocyanins, has been found to possess the anti-carcinogenic effect, whereas the in vivo effect of PCA as an anti-atherosclerotic agent remains unknown. We demonstrated herein that PCA inhibited monocyte adhesion to tumor necrosis factor-α (TNF-α)-activated mouse aortic endothelial cells, associated with the inhibition of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) expression. Furthermore, PCA inhibited the nuclear content of p65, a subunit of nuclear factor-κB (NF-κB), along with reduced NF-κB binding activity. Finally, PCA administration in the apolipoprotein E (ApoE)-deficient mouse model reduced aortic VCAM-1 and ICAM-1 expression, NF-κB activity, and plasma-soluble VCAM-1 and ICAM-1 levels, with inhibiting atherosclerosis development. We suggest that PCA possesses the anti-atherogenic effect at least partially via its anti-inflammatory activity.

KEYWORDS: Anthocyanins; protocatechuic acid; atherosclerosis; VCAM-1 and ICAM-1; NF-κB

Polyphenols represent a group of secondary metabolites that exist widely in fruits, vegetables, grains, wine, tea, olive oil, chocolate, and other plant products (1). Extensive epidemiological studies have shown that dietary polyphenol consumption is inversely associated with the incidence of atherosclerotic diseases (2). Experimental animal work has also shown the anti-atherogenic properties of polyphenols from various sources (3). Furthermore, both in vitro cell culture studies and experimental animal work have been used to explore mechanisms underlying the protective effect of these natural agents (4). Among the potential mechanisms revealed to date, the anti-inflammatory effect of polyphenols has drawn our attention intensively (5).

Anthocyanin, the flavonoid consumed by human beings the most (6), is abundant in various colorful fruits, vegetables, red wine, and grains (7, 8). Our previous studies have shown that the outer layer fraction of black rice reduces the severity of atherosclerosis in rabbits fed a high-fat diet (9). We have then demonstrated that the anthocyanin extract from black rice possesses the anti-atherogenic property in apolipoprotein E (ApoE)-deficient mice (10). We found that one of the anthocyanins, cyanidin-3-O-β-glucoside (Cy-3-G), not only stimulates cholesterol efflux from macrophages (11) but also attenuates CD40-mediated endothelial cell activation and apoptosis via inhibiting the CD40-induced mitogen-activated protein kinase (MAPK) signaling pathway (12). These along with the observations made by other groups (13, 14) indicated that the inhibition of inflammatory response is among the mechanisms underlying the protective effects of anthocyanins.

It should be pointed out that the bioavailability of polyphenols in humans and experimental animals remains largely unknown. The percentage of absorbed natural polyphenols is usually quite low (1). Instead, researchers have seen a large quantity of metabolites of polyphenols in forms of simple phenolic acids in the blood (15). Recently, several investigations have shown that protocatechuic acid (PCA), a major metabolite of anthocyanins (16), has appreciable anti-inflammatory and anti-oxidative properties (17, 18).

In the current study, we demonstrated the anti-inflammatory effect of PCA in a tumor necrosis factor-α (TNF-α)-induced monocyte adhesion test. More importantly, we show here for the first time that PCA supplementation attenuated the formation of early atherosclerosis in the ApoE-deficient mouse model, along with reducing vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) expression.

MATERIALS AND METHODS

Materials. RPMI 1640 medium, penicillin-streptomycin, L-glutamine, and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY). PCA (purity > 97%), bovine serum albumin (BSA), endothelial cell growth
supplement (ECGS), and 3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma–Aldrich (St. Louis, MO). Recombinant human TNF-α was from Cytoblast (Rehovot, Israel). Calcin acetoxy-methyl ester (calcein-AM) was purchased from Molecular Probes (Eugene, OR). The VCAM-1 and ICAM-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and Calbiochem (Merck KgA, Darmstadt, Germany), respectively. The p65 and β-actin antibodies were from Cell Signaling Technology (Danvers, MA). Trizol reagent, M-MLV reverse transcriptase, and PCR Master Mix were purchased from Invitrogen Life Technology (Carlsbad, CA).

**Cell Culture and Treatments.** Human promyelocytic leukemia cell line HL-60 was obtained from American Type Culture Collection (Manassas, VA) and cultured per the instructions of the manufacturer. Mouse aortic endothelial cells (MAECs) from male BALB/c mice (Jackson Laboratories, Sacramento, CA) were isolated and cultured from mouse aorta as previously described (19). Previous studies have demonstrated that MAECs obtained from BALB/c mice are an ideal cell model for the aim of monocyte adherence to the endothelial cells (20, 21).

**Cell Viability Assay.** The MTT assay was conducted to evaluate the effect of PCA on cell viability, as described previously (22). Briefly, MAECs were seeded on a 96-well plate. After the treatment of the cells with or without PCA (0.05–40 μmol/L) for 24 h, MTT was added and incubated for 4 h for the formation of formazan. After the addition of dimethylsulfoxide (DMSO) to dissolve formazan crystals, absorbance of formazan was measured at \( \lambda = 550 \text{ nm} \).

**Endothelial Monocyte Adhesion Assay.** Monocyte adhesion assays were performed as previously described (23), with some minor modifications. Briefly, calcin-AM-labeled HL-60 cells were added to confluent MAECs (control, TNF-α-treated, or TNF-α-treated and PCA-pretreated) in 96-well plates and co-incubated at 37 °C for 30 min. After a washing procedure to remove unattached HL-60 cells, calcin-AM-labeled HL-60 cells bound to MAECs were dissolved in a lysis buffer. The fluorescence intensity was measured in a fluorescence plate reader (Infinite F200, TECAN, Männedorf, Switzerland) at 485 nm excitation and 535 nm emission.

**Nuclear Factor-κB (NF-κB) Activity Assay.** It is well-known that, after activation, NF-κB will be translocated to the nucleus and bind to DNA with its p65 subunit (24). To determine NF-κB activation, nuclear extracts from cultured cell lines or mouse tissues were prepared with the method previously documented (25), and the binding of p65 to DNA was measured with TransAM NF-κB P65 enzyme-linked immunosorbent assay (ELISA) kit (Active Motif, Carlsbad, CA), following the instructions of the manufacturer.

**Western Blotting.** Proteins (40 μg) from whole-cell lysates or nuclear extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The method for Western blotting has been previously described (25).

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).** Total mRNA levels for VCAM-1 and ICAM-1 in MAECs were quantified using a qRT-PCR assay as previously described (26). DNA sequences of the primers are listed in Table 1. β-Actin mRNA was used as the internal control.

**Mouse Studies.** ApoE-Deficient mice from Jackson Laboratories (Sacramento, CA) were housed under the conditions of constant temperature (22 °C) and a light/dark cycle of 12 h, with free access to food and water. Male, 5-week-old mice were fed with the AIN-93G diet plus PCA (0.03 g/kg of diet) for 20 weeks. At the end of the experimental period, blood samples, aortas, and the whole heart were taken (27). All procedures were conducted according to protocols and guidelines approved by the Animal Care and User Committee of Sun Yat-sen University.

**Plasma Biochemical Parameters.** The levels of plasma total cholesterol (TC), total triacylglycerols (TGs), and high-density lipoprotein cholesterol (HDL-C) after overnight fasting were determined using commercial kits (BioSino Biotechnology Company, Ltd., Beijing, China).

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**Table 1. Sequences of Primers for qRT-PCR**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Primers (5' → 3')</th>
<th>Reverse Primers (5' → 3')</th>
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<tr>
<td>VCAM-1</td>
<td>TGAACCCCAACTGAGCCAGAGT</td>
<td>GGTATCCCCATACCTGTAGCAGG</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>CAAATTTCTCAGGCGACAGCAG</td>
<td>AGCTGAAAGTAGCAGAAGTCGG</td>
</tr>
<tr>
<td>β-Actin</td>
<td>AGTGTGAAGTTGACATCGCTGA</td>
<td>GCCAGACGCTAATCTCCTCTTCT</td>
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**RESULTS**

**PCA Inhibited Monocyte Adhesion to TNF-α-Activated MAECs.** Anthocyanins, especially Cy-3-G, have been shown to attenuate inflammation response (12). To determine the protective effect of PCA on monocyte adhesion to aortic endothelia cells, we used the inflammatory stimulator TNF-α to activate MAECs. As shown in Figure 1A, TNF-α treatment (6 h) led to a 3.9-fold increase of the adhesion of HL-60 cells to MAECs. We then pretreated MAECs with different dosages of PCA for 24 h. The cells then received 6 h of TNF-α treatment. At the dosages of 10 μmol/L or less, PCA did not show a significant protective effect. When the dosage of PCA was increased to 20 and 40 μmol/L, the pretreatment significantly attenuated the stimulatory effect.
of TNF-α on monocyte adhesion. The degrees of the protection were 44 and 62% for 20 and 40 μmol/L PCA treatment, respectively. Figure 1B shows that PCA pretreatment did not significantly affect the viability of MAECs (evaluated by the MTT assay), suggesting that the attenuation of HL-60 adhesion by PCA was not due to the damage of MAECs. Indeed, after PCA pretreatment, there was no obvious morphological change in the appearance of MAECs (data not shown).

PCA Decreased TNF-α-Induced NF-κB Activation in MAECs. It is well-known that adhesion molecules play a critical role in the attachment of monocytes to aortic endothelial cells (28). We found that TNF-α significantly induced the expression of both VCAM-1 and ICAM-1 proteins in MAECs (Figure 2A). PCA pretreatment at 20 and 40 μmol/L but not at 10 μmol/L was shown to partially block the stimulatory effect of TNF-α (Figure 2A). A recent study has demonstrated that anthocyanins from black soybean seed coats preferentially inhibit TNF-α-mediated induction of VCAM-1 over ICAM-1 in vitro, involving the transcription factors of the GATA family (29). We have therefore examined the effect of PCA on VCAM-1 and ICAM-1 expression at the mRNA level using the qRT-PCR approach. As shown in panels B and C of Figure 2, PCA at 20 and 40 μmol/L but not at 10 μmol/L significantly inhibited both VCAM-1 and ICAM-1 mRNA expression.

Figure 2. Effect of PCA on VCAM-1 and ICAM-1 expression in TNF-α-activated MAECs. MAECs were pre-incubated with indicated concentrations of PCA for 24 h before being treated with or without TNF-α (10 μg/L) for 6 h. The effect of PCA on VCAM-1 and ICAM-1 expression was assessed by (A) Western blotting and (B and C) qRT-PCR. Panel A is a representative image of three independent experiments. For panels B and C, results are mean ± SEM (n = 6 per group). (*p < 0.05).

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Figure 3. Effect of PCA on NF-κB activation in TNF-α-activated MAECs. MAECs were pre-incubated with indicated concentrations of PCA for 24 h before being treated with or without TNF-α (10 μg/L) for 6 h. Nuclear extracts were prepared for Western blotting of the p65 subunit of (A) NF-κB or (B) NF-κB binding activity assay. Panel A is a representative image of three independent experiments. For panel B, results are mean ± SEM (n = 6 per group). (*p < 0.05). (C) MAECs were treated with TNF-α for 6 h followed by nuclear protein extraction. Before the NF-κB p65 binding assay was conducted, nuclear proteins were incubated with PCA or DMSO (vehicle) for 10 min. Results are mean ± SEM (n = 3).

PCA Decreased TNF-α-Induced NF-κB Activation in MAECs. The transcription of both VCAM-1 and ICAM-1 can be upregulated by TNF-κB (24). To further explore the mechanism underlying the downregulation of VCAM-1 and ICAM-1 expression by PCA, we have investigated the effect of PCA on TNF-α-stimulated NF-κB expression. As shown in Figure 3A, TNF-α stimulated the appearance of subunit p65 of NF-κB (24). PCA at the concentrations of 20 and 40 μmol/L but not at 10 μmol/L attenuated the stimulatory effect of TNF-α (Figure 3A). We then assessed the binding activity of nuclear extract from MAECs to the consensus NF-κB binding motif, using a commercial kit. As shown in Figure 3B, when MAECs were treated with TNF-α for 6 h, nuclear extract showed an approximately 4.2-fold increase in the activity of binding of p65 to the consensus NF-κB binding motif compared to untreated control cells. PCA pretreatment at concentrations of 20 and 40 μmol/L but not at 10 μmol/L significantly attenuated the effect of TNF-α (Figure 3B). We then treated MAECs with TNF-α for 6 h, prepared nuclear extract, and divided the nuclear proteins equally into two parts. One part was treated with PCA for 10 min, while the other part was treated with the vehicle DMSO. The binding activity of these two samples to the consensus NF-κB binding motif was comparable (Figure 3C), indicating that that PCA did not enhance the binding
ability of the p65 subunit of NF-κB. Instead, it increases the expression level of the p65 subunit of NF-κB in MAECs (Figure 3A).

PCA Attenuated Aortic Adhesion Molecule Expression and NF-κB Activation in ApoE-Deficient Mice. To further explore the anti-inflammatory effect of PCA, we chose ApoE-deficient mice, a well-established animal model of atherosclerosis, for the in vivo investigations. Supplementation of the AIN-93G diet with PCA (0.003%, w/w) in ApoE-deficient mice for 20 weeks was shown to reduce the expression level of VCAM-1 and ICAM-1 in the aorta (Figure 4A), associated with significantly reduced sVCAM-1 and sICAM-1 levels (panels B and C of Figure 4). Nuclear extracts from isolated aorta were prepared for the NF-κB binding activity assay. As shown in Figure 4D, nuclear proteins from the PCA group demonstrated significantly reduced binding activity to the NF-κB consensus binding motif.

PCA Supplementation Delayed the Development of Atherosclerosis in ApoE-Deficient Mice. Finally, we investigated whether the anti-inflammatory effect of PCA is associated with the reduction of atherosclerosis in ApoE-deficient mice. At the end of the 20 week experimental period, ApoE-deficient mice fed with the AIN-93G diet were found to develop significant atherosclerotic lesions in the aortic sinus. The top panel of Figure 5A shows the representative Oil Red O staining of the aortic sinus of control and PCA-treated mice. The bottom panel of Figure 5A shows that PCA supplementation reduced the aortic sinus plaque area by 42%. The aortic cholesterol level has been developed as another index for the severity of atherosclerosis (42%). The aortic cholesterol level has been developed as another index for the severity of atherosclerosis (42%). The aortic cholesterol level has been developed as another index for the severity of atherosclerosis (42%). The aortic cholesterol level has been developed as another index for the severity of atherosclerosis (42%). The aortic cholesterol level has been developed as another index for the severity of atherosclerosis (42%). The aortic cholesterol level has been developed as another index for the severity of atherosclerosis (42%). The aortic cholesterol level has been developed as another index for the severity of atherosclerosis (42%).

In the present study, we found that PCA was able to alleviate the development of atherosclerosis in the ApoE-deficient mouse model. We suggest that this is at least partially mediated via the anti-inflammatory effect of PCA, as we have observed the anti-monocyte adhesion effect of PCA in vitro and the inhibition of the expression of intimal VCAM-1 and ICAM-1 both in vitro and in vivo by PCA treatment or consumption.

The adhesion of circulating monocytes to the intimal endothelial cell monolayer is among the earliest events of the formation of atherosclerosis (33). This process depends upon complex interactions between multiple adhesion molecules and their counter-receptor expression of adhesion by both vascular endothelial cells and circulating monocytes, such as selectins, VCAM-1, ICAM-1, as well as β1- and β2-integrins. Thus, the inhibition of expression of adhesion molecules, such as VCAM-1 and ICAM-1, may prevent the formation of atherosclerosis or attenuate its progression. Early studies showed that that knockdown of VCAM-1 (34) or ICAM-1 (35) led to the reduction of atherosclerosis formation in mice. Furthermore, Huo et al. reported that blockage of the function of VCAM-1 with a monoclonal antibody resulted in a reduction of monocyte adhesion to endothelial cells in carotid arteries from the atherosclerosis-prone ApoE-deficient mouse model (36). A monoclonal antibody-based functional knockdown
of ICAM-1 also led to a reduction of monocyte adhesion in both a hypercholesterolemic rat model (37) and ApoE-deficient mouse model (38). Studies have shown that dietary modification could lead to reduced atherosclerosis, associated with reduced VCAM-1 and ICAM-1 expression (39, 40). In the current study, we have shown for the first time that PCA inhibits VCAM-1 and ICAM-1 expression in vitro using the TNF-α-activated MAEC cell model. Furthermore, in the ApoE-deficient mouse model, we found that PCA consumption for 20 weeks reduced the expression of VCAM-1 and ICAM-1 in aortas. A previous study by Xia et al. demonstrated that the administration of the outer layer fraction of black rice in ApoE-deficient mice for 16 weeks inhibited the formation of atherosclerosis by 46% (10), which is comparable to the efficiency of 20 weeks of PCA administration in the current study (42%). It should be pointed out that the concentration of PCA used in current study is approximately 5 mg (kg of body weight)−1 day−1, much lower than the estimated concentration of anthocyanins [100 mg (kg of body weight)−1 day−1] used in the study using the outer layer fraction of black rice (10). We therefore suggest that PCA could be the major contributor of anthocyanins in attenuating the formation of atherosclerosis in the ApoE-deficient mouse model. However, previous studies indicated that anthocyanins are metabolized by gut microflora in humans (41). Because gut microflora is different between humans and mice, it is necessary to make clear whether anthocyanins can be metabolized to PCA in mice. Additionally, to verify our suggestion, the plasma level of PCA should also be compared between mice administered with the outer layer fraction of black rice and with PCA. Therefore, further investigations are needed to make a conclusive answer to those questions.

A few studies have shown that another phenolic acid, caffeic acid phenethyl ester, is also able to inhibit monocyte adhesion to endothelial cells in vitro (42) and to suppress atherosclerosis development in vivo (43). These investigations have demonstrated that caffeic acid phenethyl ester can block the activity of NF-κB, a crucial transcriptional regulator of VCAM-1 and ICAM-1. We found that PCA inhibits TNF-α-induced NF-κB activation in MAECs. However, mechanisms underlying this inhibition need to be further explored. It is well-accepted that reactive oxygen species (ROS) can activate NF-κB (44). Thus, PCA may suppress NF-κB activity by its well-recognized antioxidant activity in vitro (18). Studies have also demonstrated that certain polyphenols possess the antioxidant activity. For example, caffeic acid phenethyl ester (42) and resveratrol (45) could inhibit NF-κB activity in TNF-α-activated endothelial cells. Certain other polyphenols, such as hesperetin and naringenin, however, could not inhibit NF-κB activity in the TNF-α-activated endothelial cells (23). Controversial observations were also made on the effect of PCA in regulating NF-κB activity. In a recent study by Min et al., PCA was shown to suppress NF-κB activity through the inhibition of I-κBα degradation in RAW 264.7 cells activated by lipopolysaccharide (17). However, an early study showed that PCA activated instead of reduced NF-κB activity in human umbilical vein endothelial cells (46). The discrepancy could be due to the dosages of PCA and the cell type used in these two studies.

Most, if not all, polyphenols can be absorbed from the intestinal tract and rapidly transformed into these metabolites in humans. Therefore, we hypothesized that the metabolites of polyphenols facilitate the interpretation of observed protective effects on cardiovascular health in humans. Indeed, equal, a metabolite of isoflavone, has been demonstrated to be a critical factor for the clinical effectiveness of soy protein in cardiovascular health (47). Observations made in this study suggest that PCA, a metabolite of anthocyanins, may be an important contributor of anthocyanins in reducing the severity of atherosclerosis development in humans.

In conclusion, we demonstrated in this study that PCA reduces monocyte adhesion and NF-κB activation in vitro, decreases VCAM-1 and ICAM-1 in vitro and in vivo, and inhibits the formation of early atherosclerotic lesions in the ApoE-deficient mouse model. Whether PCA can exert the anti-atherosclerotic effect in humans deserves further intensive investigations.

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