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5-Caffeoylquinic acid and caffeic acid orally administered suppress P-selectin expression on mouse platelets

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Abstract

Caffeic acid and 5-caffeoylquinic acid are naturally occurring phenolic acid and its quinic acid ester found in plants. In this article, potential effects of 5-caffeoylquinic acid and caffeic acid on P-selectin expression were investigated due to its significant involvement in platelet activation. First, the effects of 5-caffeoylquinic acid and caffeic acid on cyclooxygenase (COX) enzymes were determined due to their profound involvement in regulating P-selectin expression on platelets. At the concentration of 0.05 μM, 5-caffeoylquinic acid and caffeic acid were both able to inhibit COX-I enzyme activity by 60% ($P<.013$) and 57% ($P<.017$), respectively. At the same concentration, 5-caffeoylquinic acid and caffeic acid were also able to inhibit COX-II enzyme activity by 59% ($P<.012$) and 56% ($P<.015$), respectively. As expected, 5-caffeoylquinic acid and caffeic acid were correspondingly able to inhibit P-selectin expression on the platelets by 33% ($P<.011$) and 35% ($P<.018$), at the concentration of 0.05 μM. In animal studies, 5-caffeoylquinic acid and caffeic acid orally administered to mice were detected as intact forms in the plasma. Also, P-selectin expression was respectively reduced by 21% ($P<.016$) and 44% ($P<.019$) in the plasma samples from mice orally administered 5-caffeoylquinic acid (400 μg per 30 g body weight) and caffeic acid (50 μg per 30 g body weight). These data suggest that both 5-caffeoylquinic acid and caffeic acid orally administered can be absorbed and suppress P-selectin expression on mouse platelets.
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Keywords: 5-Caffeoylquinic acid; Chlorogenic acid; Caffeic acid; COX inhibitor; P-selectin; Platelet activation; Mice

1. Introduction

Caffeic acid is a phenylpropenoic acid and 5-caffeoylquinic acid is a caffeic acid ester, also known as a chlorogenic acid. They are commonly found in numerous plants including fruits, vegetables and coffee [1–4]. In plants, 5-caffeoylquinic acid is produced via forming an ester bond between the carboxyl group of caffeic acid and the 5-hydroxyl group of quinic acid [3]. 5-Caffeoylquinic acid and caffeic acid have been reported to decrease the risk of chronic diseases such as inflammation, cardiovascular disease and cancer [4,5]. Also, several studies suggested beneficial effects of fruits, vegetables and coffee consumption on cardiovascular and other diseases [5–8]. However, the effects of 5-caffeoylquinic acid and caffeic acid on cardiovascular diseases and their underlying mechanism have not been fully elucidated.

P-selectin is a 140-kDa, type 1 transmembrane glycoprotein commonly used as a biomarker for platelet activation. P-selectin is involved in platelet–leukocyte interactions and platelet–endothelium interactions via binding to P-selectin ligand 1 (PSGL-1) on leukocytes and endothelium [9–14]. Those interactions are often implicated in pathophysiological progress of several cardiovascular diseases such as atherosclerosis, angina, acute myocardial infarction and ischemic cerebral stroke [15–18]. P-selectin expression is mainly regulated by cyclooxygenase (COX) enzymes, catalyzing the conversion of arachidonic acid to prostaglandin H2, which is the intermediate molecule for prostacyclin and thromboxane A2. Currently, little is known about the effects of 5-caffeoylquinic acid and caffeic acid on P-selectin expression on platelets. Therefore, potential effects of 5-caffeoylquinic acid and caffeic acid on COX enzymes and P-selectin expression were investigated in this study,

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using in vitro and in vivo models. Also, plasma concentrations of 5-caffeoylquinic acid and caffeic acid orally administered to mice were measured by high-performance liquid chromatography (HPLC) in order to validate the potential effects of 5-caffeoylquinic acid and caffeic acid on P-selectin expression in vivo.

2. Materials and methods

2.1. Materials

COX-I and COX-II enzymes, 5-caffeoylquinic acid, caffeic acid and other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA).

2.2. COX inhibition assay

COX-I and COX-II activities were measured in a 96-well plate using a chemiluminescent COX kit (Assay Designs, Ann Arbor, MI, USA). Briefly, 50 μl of Tris-phenol buffer (100 μM Tris, 0.5 μM phenol buffer, pH=7.3) was added into the wells; 50 μl of hematin solution (hematin was dissolved in DMSO at 0.380 mg/ml, and diluted 5000-fold with 100 mM phosphate buffer, pH=7.5) and 50 μl COX-I (700 U) or COX-II (700 U) were added into the wells. The samples were incubated at room temperature for 5 min. After the incubation, 5-caffeoylquinic acid, caffeic acid or COX inhibitors were added. For an additional 10 min, the samples were incubated at room temperature (in the dark). Following the incubation, COX activity was measured using a luminometer, by injecting 50 μl of chemiluminescent COX substrate (4°C) and arachidonic acid, respectively. Relative light units output was measured to determine COX activity.

2.3. Measurement of P-selectin expression

Blood was collected in siliconized microfuge tubes containing 15% EDTA. The modified Tyrodes buffer [134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose and 0.35% (w/v) bovine serum albumin, pH 7.0] was added to bring the sample volume to 100 μl. From the diluted samples, aliquots were placed in 12×75 polypropylene tubes along with the appropriate antibody and the modified Tyrodes buffer in a final volume of 200 μl. 5-Caffeoylquinic acid and caffeic acid were dissolved in ethanol and added to diluted blood samples, where the final ethanol volume never exceeded 0.5% (v/v) in both control and test tubes. Samples were analyzed for P-selectin (CD62p) expression on platelets within 1 h of the collection.

2.4. Determination of 5-caffeoylquinic acid and caffeic acid in plasma

5-Caffeoylquinic acid and caffeic acid were determined by HPLC after extraction from the blood plasma. To extract 5-caffeoylquinic acid and caffeic acid from plasma samples, the plasma samples (60 μl) were precipitated with methanol (40 μl) and centrifuged at 14,000×*g* for 10 min. The supernatant was injected onto an HPLC column. Spherisorb ODS2 (octadecyl silica 2; 5 μm, 4.6×250 mm) was used as the stationary phase to analyze 5-caffeoylquinic acid and caffeic acid in plasma samples, and an isocratic buffer of 50 mM NaH_2PO_4 (pH 4.3) containing 20% methanol was used as the mobile phase for the HPLC analyses. Peaks were detected by an electrochemical detector with four electrode channels (CoulArray, ESA, Chelmsford, MA, USA) and quantified by its software (v.1.0). For optimal measurement of 5-caffeoylquinic acid; the four channels were set at 100, 300, 550 and 800 mV, and they were quantitatively determined by an external standard method. Both 5-caffeoylquinic acid and caffeic acid could be reliably measured up to 0.01 μM, with linear detector response up to 10 μM.

2.5. Animal study

Swiss Webster mice 4-6 weeks old were purchased from Charles River (Wilmington, MA). Mice were placed in standard cages and housed in the environmentally controlled Beltsville Human Nutrition Research Center Animal Facility. The animal room was maintained at 20°C and 55% relative humidity. On arrival, mice were fed AIN-76A purified diet that provides the recommended amount of all nutrients required for maintaining optimal health. After 8 weeks, mice were assigned and remained to 3 groups (n=5) for 10 weeks. Mice in the first group (control) were orally administered distilled water (100 μl) using a dosing needle; mice in the second group were orally administered distilled water (100 μl) containing 5-caffeoylquinic acid (400 μg) and mice in the third group were orally administered distilled water (100 μl) containing caffeic acid (50 μg). Blood was collected via tail bleeding technique after the oral administrations, and blood samples from each group were used for P-selectin assay.

2.6. Statistical analysis

Treatments effects on the parameters measured were compared by analyzing the means for differences using either analysis of variance (ANOVA) or ANOVA by ranks, as appropriate. Differences were considered to be significant when $P<.05$. Data points represent the mean±S.D. of three or more samples.

3. Results

3.1. Effects of 5-caffeoylquinic acid and caffeic acid on COX-I enzyme

COX-I enzyme is constitutively expressed in numerous cells including platelets, and the COX-I enzyme is involved in prostaglandin homeostasis. The inhibition of COX-I enzyme is known to inhibit platelet activation via several mechanisms including the inhibition of P-selectin expression. COX-I inhibitors such as ibuprofen and

aspirin are believed to suppress P-selectin expression via inhibiting platelet COX-I, converting arachidonic acid to the potent platelet agonist thromboxane A2. Therefore, the effects of 5-caffeoylquinic acid and its precursor (caffeic acid and quinic acid) on COX-I enzyme were investigated to elucidate the mechanism of the P-selectin inhibition. As shown in Fig. 1, 5-caffeoylquinic acid and caffeic acid were potent compounds able to inhibit COX-I enzyme respectively by 60% ($P<.013$) and 57% ($P<.017$) at the concentration of 0.05 μM. The decreasing order of the inhibitory activity was 5-caffeoylquinic acid≥caffeic acid>quinic acid. The inhibition was positively correlated to the concentrations of 5-caffeoylquinic acid and caffeic acid (Fig. 1). On comparison to a well-known COX-I inhibitor (ibuprofen), 5-caffeoylquinic acid and caffeic acid were able to inhibit COX-I to a greater extent than ibuprofen. Surprisingly, quinic acid did not inhibit COX-I at all, even though it is a chemical moiety of 5-caffeoylquinic acid. However, caffeic acid did inhibit COX-I to the extent that 5-caffeoylquinic acid did, suggesting that the phenylpropenoic acid moiety may be more critically involved in inhibiting COX-I enzyme than the quinic acid moiety.

3.2. Effects of 5-caffeoylquinic acid and caffeic acid on COX-II enzyme

Although the inhibition of COX-I enzyme is primarily involved in suppressing platelet activation, the effects of 5-caffeoylquinic acid and caffeic acid on COX-II enzyme were also investigated in this article because the COX-II enzyme is likely to be involved in many important physiological processes. As shown in Fig. 2, the decreasing order of the inhibitory activity was 5-caffeoylquinic acid≥caffeic acid>quinic acid. Similar to the COX-I inhibition, at the concentration of 0.05 μM, 5-caffeoylquinic acid and caffeic acid were also very potent compounds inhibiting COX-II enzyme by 59% ($P<.012$) and 56% ($P<.015$), respectively. The inhibition was positively correlated to the concentrations of 5-caffeoylquinic acid and caffeic acid, as shown in Fig. 2. Compared to NS-398 (a COX-II specific inhibitor), 5-caffeoylquinic acid and caffeic acid were able to inhibit COX-II to a greater extent than NS-398 (Fig. 2). Like the COX-I inhibition, quinic acid did not have inhibitory effect on COX-II. The data suggest that 5-caffeoylquinic acid and caffeic acid may be potent compounds able to inhibit both COX-I and COX-II enzymes, probably through the moiety of caffeic acid.

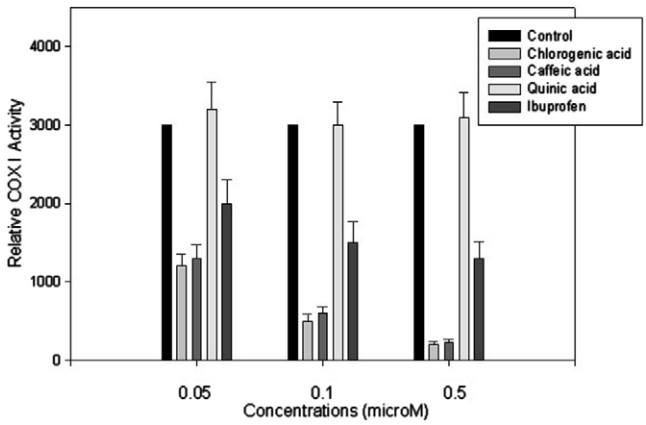

Fig. 1. Effects of 5-caffeoylquinic acid, caffeic acid and quinic acid on COX-I. 5-Caffeoylquinic acid, caffeic acid, quinic acid and inhibitors (A group: 0.05 μM, B group: 0.1 μM and C group: 0.5 μM) were added to the samples, and the reaction mixtures were incubated at room temperature (in the dark) for 10 min. Following the incubation, COX-I activity was measured according to the kit's protocol using a luminometer.

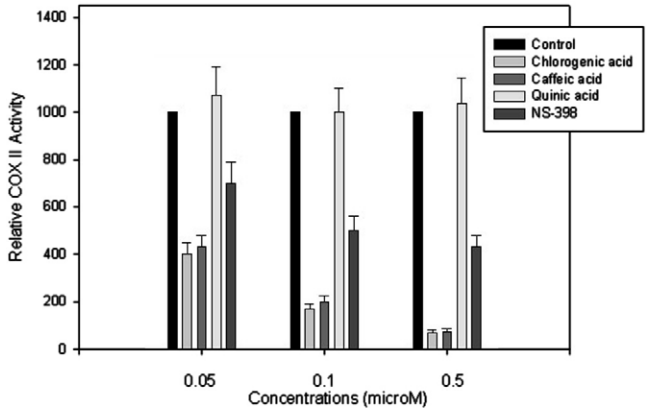

Fig. 2. Effects of 5-caffeoylquinic acid, caffeic acid and quinic acid on COX-II. 5-Caffeoylquinic acid, caffeic acid, quinic acid and inhibitors (A group: 0.05 μM, B group: 0.1 μM and C group: 0.5 μM) were added to the samples, and the reaction mixtures were incubated at room temperature (in the dark) for 10 min. Following the incubation, COX-II activity was measured according to the kit's protocol using a luminometer.

3.3. Effects of 5-caffeoylquinic acid and caffeic acid on P-selectin expression

P-selectin (CD62p) protein is a transmembrane glycoprotein, commonly used as a reliable biomarker for determining the level of platelet activation. The expression of the protein has been reported to be greatly modulated by COX enzyme activity [19–21]. In this study, 5-caffeoylquinic acid and caffeic acid are found to be potent compounds able to inhibit COX enzymes. Therefore, the effects of 5-caffeoylquinic acid and caffeic acid on P-selectin expression on platelets were determined as a means to evaluate the level of platelet activation. As shown in Fig. 3, 5-caffeoylquinic acid and caffeic acid were respectively able to suppress P-selectin expression on platelets by 33% ($P<.011$) and 35% ($P<.018$) at the concentration of 0.05 μM. The inhibition of P-selectin expression was correlated positively to COX inhibiting activity, suggesting that the inhibition of COX enzymes may be a main contributing factor to suppressing P-selectin expression. As expected, quinic acid did not suppress P-selectin expression, probably due to the lack of its ability to inhibit COX enzymes.

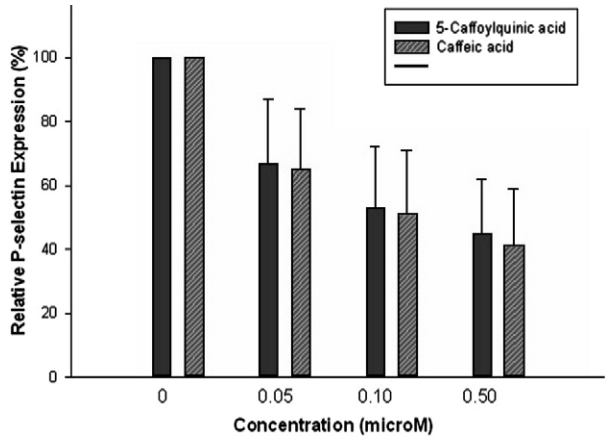

Fig. 3. Effects of 5-caffeoylquinic acid and caffeic acid on P-selectin expression. Platelets were incubated with 5-caffeoylquinic acid or caffeic acid for 10 min. P-selectin expression was determined as described in Materials and Methods. Data points represent the mean±S.D. of five samples.

3.4. Measurement of 5-caffeoylquinic acid and caffeic acid in mouse plasma following oral administration

Because 5-caffeoylquinic acid and caffeic acid demonstrated great efficacy in inhibiting COX enzymes and P-selectin in vitro, bioavailability of 5-caffeoylquinic acid and caffeic acid were determined to verify their purported biological activities in vivo [22–24]. Bioavailability is the physiological availability of a compound at a given amount, and it is dependent mainly on initial administered amount, absorption, metabolism, tissue distribution and excretion. Studies are often performed using several different doses and different routes to determine bioavailability of an administered compound. Initially, several doses of 5-caffeoylquinic acid and caffeic acid (50, 100, 200, 400, 800 μg/30 g body weight) were orally administered to mice in order to find out which doses could provide plasma concentrations of each acid at 20–100 μM. Based on the data, 5-caffeoylquinic acid (400 μg/30 g body weight) and caffeic acid (50 μg/30 g body weight) were chosen for oral administrations (data not shown). In animal study, mice were divided into three groups (n=5); the control, 5-caffeoylquinic acid and caffeic acid groups. In the second and third groups, mice were orally administered 5-caffeoylquinic acid (400 μg/30 g body weight) and caffeic acid (50 μg/30 g body weight), respectively. After the oral administration of 5-caffeoylquinic acid (400 μg/30 g body weight), twenty plasma samples (n=5) were collected for 0–60 min with 3 min interval. 5-Caffeoylquinic acid (0.02 μM) was detected in mice plasma at 15 min (Fig. 4A). In the third group, the mice were orally administered of caffeic acid (50 μg/30 g body weight). After the oral administration, twenty plasma samples (n=5) were collected for 0–60 min with 3-min interval. As shown in Fig. 4B, 5-caffeic acid could be detected around 6 min after the oral administration, and the highest amount of caffeic acid (0.09 μM) was detected at around 9 min. After 15 min, plasma concentrations began to decrease rapidly, suggesting that caffeic acid taken orally may be metabolized quickly. The data indicate that the absorption of caffeic acid is likely faster and better than that of 5-caffeoylquinic acid in mice, if administered orally in the same amount.

3.5. Effects of 5-caffeoylquinic acid and caffeic acid on mouse P-selectin expression in vivo

Because 5-caffeoylquinic acid and caffeic acid are able to inhibit P-selectin expression on platelets in vitro, and because 5-caffeoylquinic acid and caffeic acid orally administered to mice can be detected as intact forms in the plasma, animal experiments were performed using mice in order to confirm the inhibitory effects of 5-caffeoylquinic acid and caffeic acid in vivo. As demonstrated above, plasma concentrations of 5-caffeoylquinic acid (400 μg/30 g body weight) orally administered to mice were between 0-0.02 μM and the highest peak in the plasma was approximately 15 min. Therefore, blood samples (n=5) were collected at 0–30

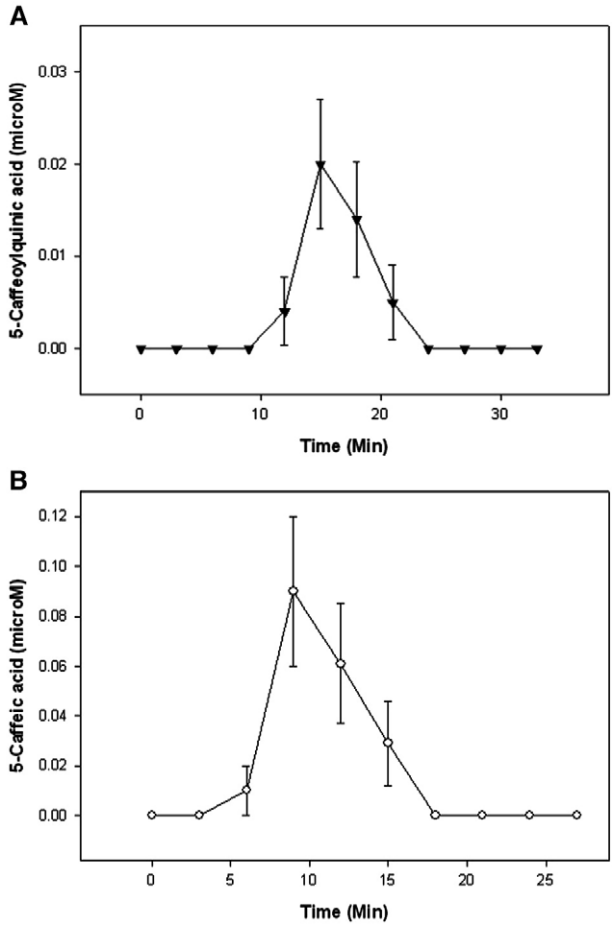

Fig. 4. Determination of 5-caffeoylquinic acid and caffeic acid in mouse plasma following oral administration. 5-Caffeoylquinic acid (400 μg/30 g body weight) (A) and caffeic acid (50 μg/30 g body weight) (B) were orally administered to the mice. The concentrations of 5-caffeoylquinic acid and caffeic acid in plasma samples were determined as described in Material and Methods.

min with 3 min intervals, and analyzed to determine the levels of P-selectin expression on platelets, as described in Materials and Methods. The plasma samples (15 min) demonstrated moderate reduction in the P-selectin expression by 21% ($P<.016$). Meanwhile the plasma sample collected at 0 min (plasma control) did not show any reduction in P-selectin expression on platelets (Fig. 5A). In mice, caffeic acid (50 μg/30 g body weight) orally administered was absorbed better than 5-caffeoylquinic acid (400 μg/30 g body weight), and detected higher in the plasma, whose concentrations are between 0-0.09 μM and the highest peak in the plasma was approximately 9 min. Therefore, blood samples (n=5) were collected at 0–30 min with 3 min intervals, and analyzed to determine levels of P-selectin expression on platelets, as described above. The plasma samples (9 min) demonstrated great reduction in the P-selectin expression by 44% ($P<.019$) (Fig. 5B). In vitro, approximately 50% P-selectin inhibition was observed at the concentration of caffeic acid (0.1 μM), which is compatible to the in vivo data described herein. These data suggest clearly that caffeic acid and 5-caffeoylquinic acid orally administered can be absorbed and inhibit P-selectin expression on mouse platelets.

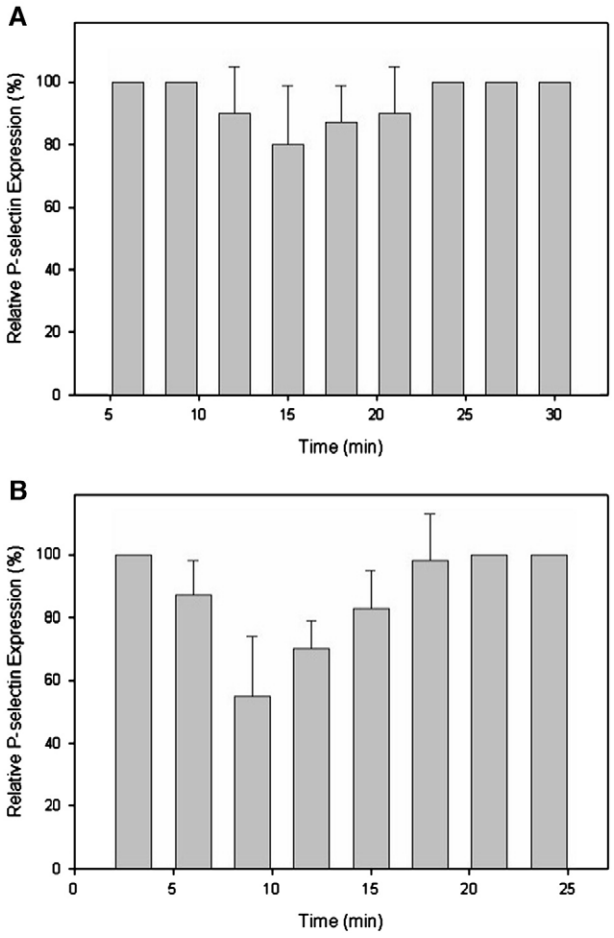

Fig. 5. In vivo effects of 5-caffeoylquinic acid and caffeic acid on P-selectin expression in mice. Blood samples were collected from mice orally administered with 5-caffeoylquinic acid (400 μg/30 g body weight) (A) and caffeic acid (50 μg/30 g body weight) (B), via tail blood collections. P-selectin expression was determined as described in Materials and Methods. Data points represent the mean±S.D. of five samples.

4. Discussion

Chlorogenic acids and caffeic acid are phytochemicals found abundantly in plants including coffee, fruits and vegetables [2,3]. Particularly, chlorogenic acids have been considered as main precursors of coffee flavor and pigments during roasting and often used as compounds assessing the quality of coffee [25–27]. Coffee is probably one of the most popular and widely consumed drinks worldwide, and the consumption of coffee has been reported to be positively correlated to reducing risk of human chronic diseases such as inflammation, diabetes and cardiovascular disease [1–4]. Because coffee contains high levels of chlorogenic acids, daily intake of chlorogenic acids are estimated about 100–500 mg/80 kg coffee drinkers [28–31]. Interestingly, chlorogenic acids and caffeic acid found in coffee and other plant sources have been reported to have antioxidant, anti-inflammatory and antimutagenic potentials [22–24].

For chlorogenic acids and caffeic acid to exert the purported biological effects in humans, they should be absorbed and enter into the blood circulation. Therefore, the information about absorption and bioavailability of chlorogenic acids and caffeic acid are vital in evaluating their purported health effects in vivo. Regarding the absorption of caffeic acid, it is generally accepted that most of caffeic acid ingested is absorbed relatively fast in the gut and caffeic acid can be detected in plasma samples, although several metabolites are also detected in the samples [28,32,33]. However, there is considerable disparity regarding the absorption of chlorogenic acids, particularly the hydrolysis of the ester bond of chlorogenic acids. Some reports suggest that the bioavailability of chlorogenic acids is largely dependent on its metabolism by microorganisms in the gut, and most of the ingested chlorogenic acids might be absorbed/metabolized and found as its metabolites in plasma [28,30,31]. Meanwhile, there is also a report indicating that chlorogenic acids ingested in rats can be absorbed and found as intact forms (100–170 μg/L) in plasma, even though chlorogenic acids may be metabolized/hydrolyzed in the gut [29]. In this study, 5-caffeoylquinic acid and caffeic acid orally administered to mice were both detected in mice blood by HPLC, indicating that 5-caffeoylquinic acid can be absorbed and detected as itself in plasma. However, efficiency in their absorption is greatly different, and the plasma concentrations of caffeic acid may be at least five times higher than that of chlorogenic acid, if the same amount is administered orally. Also, T_{max} for 5-caffeoylquinic acid is slower than that of caffeic acid if orally administered. During the experiments, it was also noticed that 5-caffeoylquinic acid was quickly metabolized in

plasma incubated at room temperatures. Therefore, it is likely that 5-caffeoylquinic acid may be metabolized heavily in the plasma, besides the gut and liver, and its metabolism may have profound effect on the absorption and bioavailability of 5-caffeoylquinic acid, although the acid can be detected as an intact form in plasma.

5. Conclusion

In summary, both 5-caffeoylquinic acid and caffeic acid orally administered are demonstrated to be absorbed and able to suppress P-selectin expression on platelets via inhibiting COX enzymes. Currently, five or more servings of fruits and vegetables are recommended daily in the United States for maintaining health and/or reducing the risk of chronic diseases. In fact, 5-caffeoylquinic acid and caffeic acid are abundantly found in fruits and vegetables. Therefore, high consumption of fruits and vegetables may have beneficial effects on cardiovascular diseases via suppressing P-selectin expression on platelets.

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