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# Inhibition of Angiotensin Converting Enzyme Activity by Flavanol-Rich Foods

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Angiotensin converting enzyme (ACE) activity was evaluated in the presence of flavanol-rich foods, i.e., wines, chocolates, and teas, and of purified flavonoids. All foods assayed inhibited ACE activity, red wines being more effective than white wine, and green tea more effective than black tea. The inhibition of ACE activity was associated with both phenolic and flavanol content in the foods. When isolated polyphenols were assayed, procyanidins (dimer and hexamer) and epigallocatechin significantly inhibited enzyme activity; similar concentrations of (+)-catechin, (−)-epicatechin, gallic acid, chlorogenic acid, caffeic acid, quercetin, kaempferol, and resveratrol were ineffective. When ACE activity was assayed in rat kidney membranes in the presence of chocolate extracts or purified procyanidins, it was observed that the inhibition depended on the chocolate content of flavanols and the number of flavanol units constituting the procyanidin. These experiments demonstrate that flavanols either isolated or present in foods could inhibit ACE activity. The occurrence of such inhibition in vivo needs to be determined, although is supported by the association between the consumption of flavanol-rich foods and reductions in blood pressure observed in several experimental models.

**KEYWORDS: Flavanol-rich foods; flavanols; procyanidins; angiotensin converting enzyme**

## INTRODUCTION

Epidemiological evidences indicate an inverse association between the consumption of diets rich in fruit and vegetables and the incidence of certain diseases, including cardiovascular disease (CVD), cancer, and neurodegenerative disorders (*1*−*3*). The health benefits ascribed to fruit and vegetable consumption may be explained by the biological effects of certain flavonoids and related phenolic compounds (*4*, *5*).

In terms of cardiovascular health one class of flavonoids, the flavanols (flavan-3-ols), is receiving increasing attention (*6*). Cocoa, tea, and grapes are examples of edible plants that are rich in flavanols. Dietary intervention studies in humans and animals indicate that flavanol-rich foods and beverages can exert cardioprotective effects with regard to vascular function and platelet reactivity (*7*−*10*). In addition, it has been recently shown that regular consumption of flavanol-rich foods including cocoa products (*11*, *12*), tea (*10*, *13*, *14*), and red wine (*15*) can be associated with decreases in blood pressure in humans.

The biological mechanisms through which these flavanols modulate vascular function and blood pressure appear to be associated with the action of nitric oxide (NO). Although the initial events leading to an increased production of NO are not totally identified, it is accepted that the regulation of the renin−angiotensin system in endothelial cells could be involved in the control of NO production (*16*).

Angiotensin converting enzyme (ACE) is crucial in the regulation of the renin−angiotensin system; it cleaves angiotensin-I to produce angiotensin-II and also hydrolyzes and inactivates the vasodilator peptide, bradykinin (*17*). ACE inhibition is an important therapeutic approach in the treatment of high blood pressure. The therapeutic administration of certain ACE inhibitors has also been associated with positive health effects beyond the regulation of blood pressure (*18*). The finding that the consumption of certain flavanol-rich foods can mediate a reduction in blood pressure opens up the possibility that flavanols may act as pharmacological ACE inhibitors.

We have already characterized the inhibition of ACE activity by purified flavanols and procyanidins in vitro (*19*). However, the evaluation of ACE inhibition by flavanol and procyanidin-rich foods remains important. The objective of this study was to investigate the inhibition of ACE activity by certain flavanol containing foods, i.e., cocoa, tea, and wine, and to determine if this inhibition is attributable to the presence of flavanols.

## MATERIALS AND METHODS

**Materials.** Purified ACE from rabbit lung, hippuric acid, hippuryl-L-histidyl-L-leucine, (−)-epicatechin, (+)-catechin, epigallocatechin, gallic acid, chlorogenic acid, caffeic acid, resveratrol, kaempferol, and quercetin were from Sigma (St. Louis, MO). HPLC grade acetonitrile and acetic acid were from Merck KGaA (Darmstadt, Germany). Purified

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**Table 1.** Phenolic Content, Flavanol Content, (+)-Catechin, (–)-Epicatechin, Gallic Acid, Chlorogenic Acid, and Caffeic Acid Content in the Assayed Flavanol-Rich Foods[a]

| food | phenolic mM | flavanol mM | (+)-catechin μM | (–)-epicatechin μM | gallic acid μM | chlorogenic acid μM | caffeic acid μM |
|---|---|---|---|---|---|---|---|
| cabernet sauvignon wine | 12.9 ± 0.5 | 2.31 ± 0.05 | 120 ± 23 | 107 ± 14 | 98 ± 1 | nd | 45 ± 2 |
| malbec wine | 9.0 ± 0.4 | 2.13 ± 0.09 | 110 ± 17 | 48 ± 4 | 61 ± 9 | nd | 17 ± 1 |
| generic wine | 5.1 ± 0.3 | 1.42 ± 0.04 | 69 ± 5 | 22 ± 5 | 29 ± 2 | nd | 6 ± 1 |
| white wine | 1.6 ± 0.2 | 0.18 ± 0.06 | 10 ± 1 | 2 ± 1 | 36 ± 2 | nd | 11 ± 2 |
| black tea | 2.9 ± 0.2 | 0.94 ± 0.08 | 11 ± 1 | 21 ± 8 | 153 ± 36 | 23 ± 2 | nd |
| green tea | 2.9 ± 0.2 | 0.92 ± 0.13 | 84 ± 17 | 101 ± 15 | 95 ± 17 | 34 ± 3 | nd |
| high-procyanidin chocolate | 6.2 ± 0.5 | 2.22 ± 0.09 | 81 ± 19 | 101 ± 37 | 41 ± 6 | nd | nd |
| chocolate | 5.8 ± 0.5 | 2.08 ± 0.05 | 51 ± 1 | 72 ± 7 | 33 ± 3 | nd | nd |
| low-procyanidin chocolate | 4.2 ± 0.4 | 1.10 ± 0.11 | 36 ± 9 | 6 ± 1 | nd | nd | nd |

[a] Phenolic content is expressed as gallic acid equivalents and flavanol content as (+)-catechin equivalents. Values indicated mean ( SEM of at least three independent experiments.

procyanidins fractions, dimer, purity 98.8%, and hexamer, purity 86.2%, were isolated from cocoa and provided by Mars Incorporated (Hackettstown, NJ). The polyphenols were dissolved in ethanol 80% (1 mg in 50 μL). Then, solutions were diluted more than 1/100 with HPLC grade water.

**Food Sources and Preparation.** Argentinean cabernet sauvignon, malbec, generic, and white wines, chocolate, and black and green tea were obtained from commercial sources. High-procyanidin chocolate and low-procyanidin chocolate were provided by Mars Inc. (Hackettstown, NJ). For the experiments, solid foods (teas and chocolates) were prepared as extracts following normal household procedures. Black tea and green tea extracts were prepared using 2 g of tea in 250 mL of boiling water as infusion. High-procyanidin chocolate, low-procyanidin chocolate, and chocolate extracts were prepared using 25 g of chocolate (1 serving) in 250 mL of hot water.

**Determination of Phenolic and Flavanol Content.** The total content of phenolic substances (phenolic content) was evaluated using the Folin−Ciocalteau assay (*20*). Briefly, a mixture of 50 μL of extract, 250 μL of Folin−Ciocalteau reagent, 500 μL of sodium carbonate solution 20% (w/v) was incubated at room temperature for 30 min. The absorbance of the solutions was determined at 700 nm. The results are expressed as gallic acid equivalents. Flavanol content was determined by the vanillin assay (*21*, *22*). Essentially, a mixture of 50 μL of extracts and 600 μL of 1% vanillin (w/v) in HCl/methanol (4:96, v/v) was incubated at room temperature for 20 min. The absorbance of the solution was determined at 500 nm, and the results are expressed as (+)-catechin equivalents. Gallic acid, (−)-epicatechin, (+)-catechin, chlorogenic acid, and caffeic acid were evaluated by HPLC with electrochemical detection as previously described (*23*).

**Determination of ACE Activity.** ACE activity was evaluated following the hydrolysis of hippuryl-L-histidyl-L-leucine to hippuric acid (*24*). Hippuric acid, thus formed, was separated and quantified by HPLC with UV detection (*25*). The chromatographic system consisted of a binary Perkin-Elmer ISS 250 pump and a Perkin-Elmer ISS 200 automatic injector equipped with a 150 mm × 4.6 mm i.d. 5 μm, Supelcosil LC-18-DB (15 cm × 4.6 mm × 5 μm) (Supelco, Bellefonte, PA) and a mobile phase composed of 0.1% (v/v) trifluoroacetic acid in $H_2O$/acetonitrile (75:25, v/v). The flow rate used to achieve a retention time of 2.9 min was 1 mL/min. The hippuric acid detection was carried out at 228 nm with a UV−vis detector (Jasco, Japan). Commercial hippuric acid was used as the standard.

**Inhibition of Purified ACE Activity by Flavanol-Rich Foods and Polyphenols.** A mixture of 100 μL of 0.9 μg/mL (6.3 nM) ACE in 50 mM HCl−TRIS, 300 mM NaCl (pH 8.3), and 100 μL of wine, food extracts, or polyphenol solutions (10−500 μM) was preincubated for 30 min at 37 °C. The above mixture was added with 100 μL of 3 mM hippuryl-L-histidyl-L-leucine (1 mM final concentration), and the mixture was incubated for 60 min at 37 °C. The reaction was stopped by addition of 100 μL of 12% (w/v) phosphoric acid, and the hippuric acid was determined as indicated above.

**Inhibition of ACE Activity in Rat Tissues.** Kidneys, lungs, and testes from 300 g male Sprague−Dawley rats were homogenized in 10 vol of 20 mM potassium phosphate (pH 8.3) with leupeptin (10 μg/mL), aprotinin (2 μg/mL), and pepstatin A (10 μg/mL). These homogenates were centrifuged at 600*g* for 10 min, washed, and the supernatants centrifuged at 40 000*g* for 20 min. Pellets were diluted to 0.2 g of tissue/mL in 100 mM potassium phosphate (pH 8.3). The resulting subcellular fractions (membrane suspensions) were used for experiments. To assay ACE activity, 50 μL of high-procyanidin chocolate, low-procyanidin chocolate, (−)-epicatechin, dimer, hexamer, and captopril were preincubated with 100 μL of membrane suspensions at 37 °C for 30 min. (−)-Epicatechin, dimer, hexamer, and captopril were evaluated at 100 μM. Further, 25 μL of 6.88 mM hippuryl-L-histidyl-L-leucine (1 mM final concentration) was added and incubated at 37 °C for 60 min (*26*). The reaction was stopped by addition of 200 μL of 12% (w/v) phosphoric acid. Then 900 μL of ethyl acetate were added to extract the hippuric acid (*26*). After a 2 min of centrifugation at 1000*g*, a 700 μL aliquot of the ethyl acetate layer was transferred to a clean tube and dried under a $N_2$ stream. The pellet was resuspended in 0.1% (v/v) trifluoroacetic acid in $H_2O$/acetonitrile (75:25, v/v), and the hippuric acid determined as described above.

**Data Analysis.** Data were analyzed for statistical significance using one-way ANOVA followed by Tukey test (Stat View 5, Mountain View, CA). Curves were adjusted using functions present in Sigma Plot 8.0. Inhibitory volume 50 ($IV_{50}$), the volume required to inhibit 50% of the ACE activity, was calculated using values obtained from assaying different volumes of each flavanol-rich food and then by adjusting the curves to two-parameter hyperbolic decay equations. ACE activity = $ACE_0 \times IV_{50}/(IV_{50} + [I])$, in which $ACE_0$ = ACE activity using 1 mM hippuryl-L-histidyl-L-leucine and $[I]$ = volume of wine or extract in μL. Inhibitory concentration 50 ($IC_{50}$), the concentration required to inhibit 50% of the ACE activity, was calculated using values obtained from assaying different amounts of each purified polyphenol and then by adjusting the curves to two-parameter hyperbolic decay equations. ACE activity = $ACE_0 \times IC_{50}/(IC_{50} + [P])$, in which $ACE_0$ = ACE activity using 1 mM hippuryl-L-histidyl-L-leucine and $[P]$ = concentration of polyphenol in μM. Correlations curves were adjusted using Sigma Plot 8.0.

## RESULTS

**Food Characterization. Table 1** presents the phenolic, flavanol, (+)-catechin, (−)-epicatechin, gallic acid, gallic acid, chlorogenic acid, and caffeic acid contents of the different foods. Among the studied wines, cabernet sauvignon wines presented the highest content of phenolics, flavanols, (+)-catechin, and (−)-epicatechin. Although both phenolic and flavanol content were found to be similar in black and green tea, (+)-catechin and (−)-epicatechin were about 8 and 5 times higher in green than in black tea. The content of flavanols, (+)-catechin, and (−)-epicatechin was significantly lower in low-procyanidins chocolates than in high-procyanidins chocolate as it was informed by the manufacturer. Chlorogenic acid was only detected in teas and caffeic acid only in wines. The ratio of

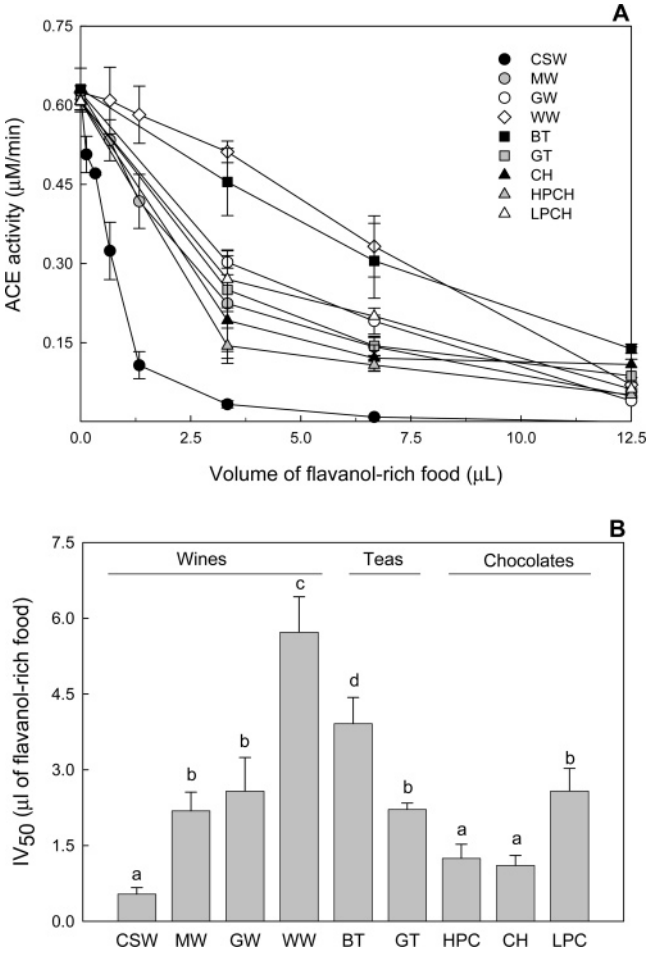


**Figure 1.** (**A**) Effect of flavanol-rich foods on ACE activity. Values are mean ± SEM of at least three independent experiments. (**B**) Inhibitory volume 50 ($IV_{50}$) calculated for the inhibition of ACE activity by wines and food extracts. Values are mean ± SEM of at least three independent experiments. (CSW) cabernet sauvignon wine, (MW) malbec wine, (GW) generic wine, (WW) white wine, (BT) black tea, (GT) green tea, (CH) chocolate, (HPCH) high-procyanidin chocolate, and (LPCH) low-procyanidin chocolate. Significant differences ($p < 0.05$) between a, b, c, and d groups.

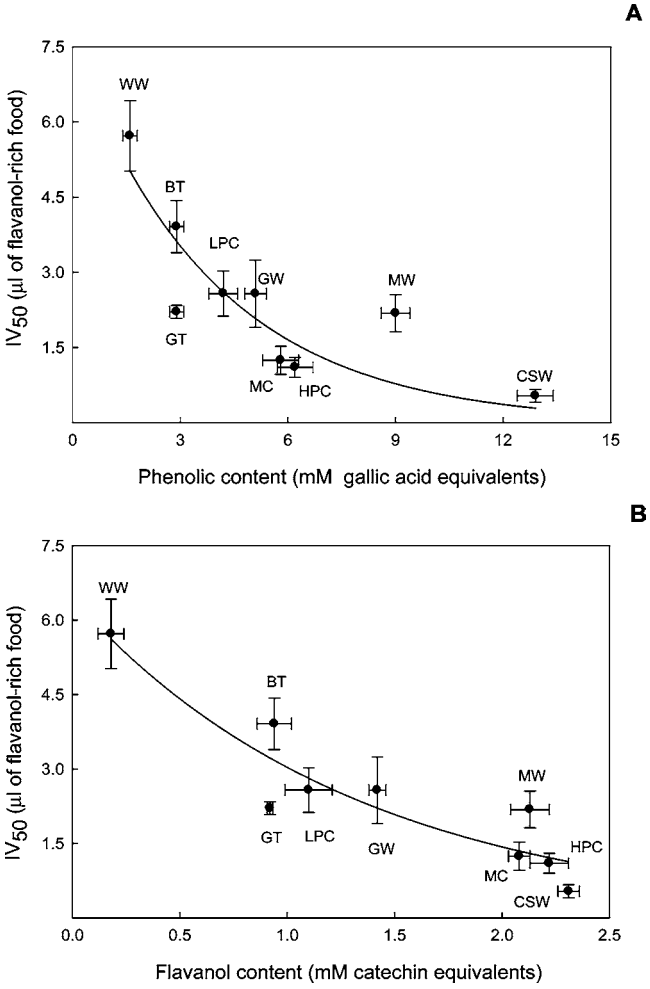


**Figure 2.** Logarithmic correlations between $IV_{50}$ and phenolic content (**A**) or flavanol content (**B**). $IV_{50}$ values were taken from **Figure 1B** and phenolic and flavanol content from **Table 1**. Values are mean ± SEM of at least three independent experiments. Abbreviations as for **Figure 1**.

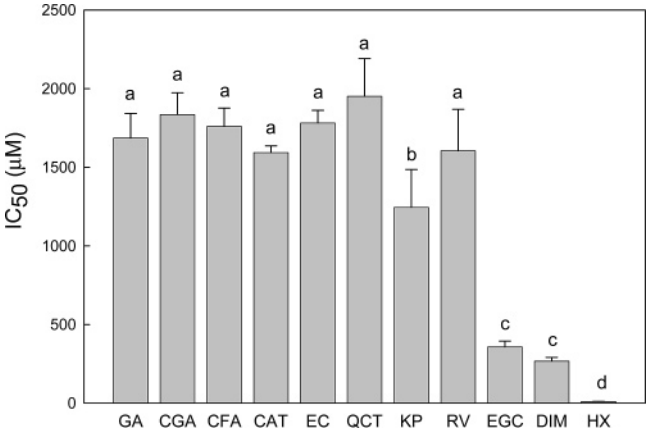


**Figure 3.** Inhibitory concentration 50 ($IC_{50}$) calculated for the inhibition of ACE activity by polyphenols. Values are mean ± SEM of at least three independent experiments. (GA) gallic acid, (CGA) chlorogenic acid, (CFA) caffeic acid, (CAT) (+)-catechin, (EC) (–)-epicatechin, (QCT) quercetin, (KP) kaempferol, (RV) resveratrol, (EGC) epigallocatechin, (DIM) dimer, and (HX) hexamer. Significant differences ($p < 0.05$) between a, b, c, and d groups.

flavanol to phenolics ranged from 11% (white wine) to 36% (high-procyanidins chocolate and chocolate).

**Inhibition of Purified ACE by Flavanol-Rich Foods.** Incubation of purified ACE in the presence of flavanol-rich foods resulted in a volume-dependent inhibition of the enzyme activity (**Figure 1A**). Cabernet sauvignon wine, high-procyanidins chocolate, and chocolate showed the lowest $IV_{50}$ values (**Figure 1B**). The $IV_{50}$ values for each food were significantly logarithmically correlated with both the phenolic content (**Figure 2A**; $R^2 = 0.73$, $p < 0.003$) and the flavanol content (**Figure 2B**; $R^2 = 0.85$, $p < 0.001$). When the $IV_{50}$ values of ACE inhibition for each food were plotted against the content of procyanidins [flavanol content − (catechin + epicatechin)], the correlation between inhibitory activity of ACE and procyanidin content remained significant ($R^2 = 0.80$, $p < 0.001$). $R^2$ for the relationship between the $IV_{50}$ value for ACE and the content of monomeric flavanols (catechin + epicatechin) was 0.78 ($p < 0.001$).

**Effect of Polyphenols on Purified ACE Activity.** When ACE was incubated in the presence of different polyphenol compounds, a differential inhibition of ACE was observed (**Figure 3**). Only the procyanidins and epigallocatechin generated an inhibition of the enzyme with $IC_{50}$ in the micromolar range. Polyphenol determined in the assayed food and other constituents present at relevant concentrations in wine (resvera-

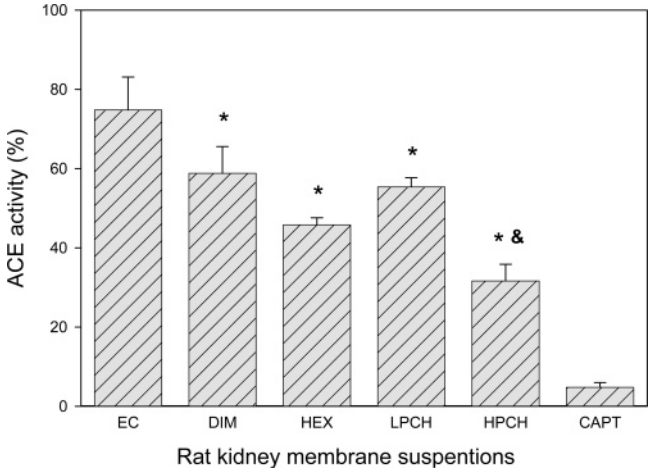


**Figure 4.** Effect of (–)-epicatechin (EC), dimer (DIM), hexamer (HEX), high-procyanidin chocolate (HPCH), and low-procyanidin chocolate (LPCH) on ACE activity in rat kidney membranes. Values are mean ± SEM of three independent experiments. ∗, $p < 0.001$ vs control; &, $p < 0.001$ vs LPCH.

trol) and tea (quercetin and kaempferol) did not generate ACE inhibition comparable to that of procyanidins.

**Effect of Flavanol-Rich Foods and Flavanols on ACE Activity in Membrane Suspensions.** To evaluate ACE activity inhibition in conditions closer to physiological, membrane suspensions isolated from rat kidney were incubated in the presence of captopril (positive control) or (−)-epicatechin, dimer, hexamer, high-procyanidin chocolate, and low-procyanidin chocolate (**Figure 4**). As observed for the purified enzyme, the ACE activity in kidney membrane suspensions was inhibited by 100 $\mu$M of dimer ($p < 0.001$) or hexamer ($p < 0.001$) but not by (−)-epicatechin. The use of equal volumes of high-procyanidin chocolate (634 $\mu$M (+)-catechin equivalents) and low-procyanidin chocolate (314 $\mu$M (+)-catechin equivalents) inhibited ACE activity by 70% and 45% ($p < 0.001$), respectively. The inhibition of ACE in tissue membrane suspensions was also observed in rat testes and lungs. Low-procyanidin chocolate and high-procyanidin chocolate inhibited 53% and 56% of ACE activity in testes and 19% and 30% of ACE activity in lung, respectively.

## DISCUSSION

We have previously reported that purified flavanols and related procyanidins can competitively inhibit ACE activity using synthetic substrates (*19*). In this study we extend these observations by characterizing the inhibitory effect of three of the most commonly consumed flavanol-rich foods, i.e., chocolate, wine, and tea, on purified and membrane-bound ACE.

The assayed foods presented a significant range of phenolic and flavanol content. Although the relative amounts of phenolics and flavanol could vary significantly depending on the sample selected for each food, the contents of (+)-catechin, (−)-epicatechin, gallic acid, chlorogenic acid, and caffeic acid determined here are similar to those reported in red wines (*27*−*33*), white wines (*32*−*35*), black tea (*29*, *32*, *36*−*38*), green tea (*29*, *36*−*38*), and chocolates (*29*, *39*−*41*).

All assayed foods inhibited ACE activity, and although the inhibition was associated to the phenolic content of the foods ($R^2 = 0.73$), the flavanol content demonstrated a stronger correlation ($R^2 = 0.85$), when compared using logarithmic associations. The same associations were obtained using linear correlation between the inhibition of ACE and phenolic content or flavanol content ($R^2 = 0.54$ vs $R^2 = 0.80$). Moreover, the ACE inhibition was better associated with the food procyanidin content than with the (−)-epicatechin plus (+)-catechin content ($R^2 = 0.80$ vs $R^2 = 0.78$). These relationships, considered together with the results obtained using purified compounds (*19*), support the concept that higher molecular weight procyanidins are more effective at inhibiting ACE activity than monomers. In addition, the significant inhibition observed for epigallocatechin would support the idea that the extent of ACE inhibition could be also associated with the number of hydroxyl groups in the flavanol/procyanidin available to establish hydrogen bonds with the ACE protein.

The nonflavanol polyphenol compounds, such as hydroxycinnamic acids (chlorogenic acid and caffeic acid), benzoic acids (gallic acid), stilbenes (resveratrol), or flavonols (quercetin and kaempherol), failed to inhibit ACE activity. This lack of effect on ACE activity does not allow the establishment of any structure−activity relationship, like that observed for membrane interaction (*42*) and antioxidant capacity (*43*).

The inhibition of ACE activity by flavanol-rich foods and procyanidins determined with the purified enzyme was also evident in membrane-bound ACE as indicated by the results observed in three different tissues. Two of these tissues contain the somatic form (kidneys and lungs) and one contains the germinal form of the enzyme (testes) (*44*).

With regard to the physiological relevance of the ACE inhibition by flavanols and procyanidins, it is important to consider the actual concentration of these compounds and the possibility of ACE inhibition in vivo. The presence of (+)-catechin, (−)-epicatechin, and dimer has been determined in human plasma at low-micromolar and nanomolar concentrations (*45*), but there are no studies addressing the presence of larger procyanidins in blood or in other tissues. However, studies in humans demonstrated that chocolate procyanidins are stable during the gastric transit and do not degrade to monomers and smaller oligomers (*46*) allowing the presence of high concentrations of these procyanidins in the intestine. ACE being a membrane-bound enzyme and flavanols present in foods being able to adsorb to a lipid−water interfaces (*42*), it is feasible that a local enrichment of these compounds on the membrane surface could lead to an enhanced interaction with the enzyme. If this accumulation could take place on the membranes in vascular endothelial cells, which are thought to be responsible for regulating blood pressure (*47*), the inhibition of membrane-bound enzymes as ACE becomes possible. In the digestive tract, biochemical and immunocytochemical studies have demonstrated the existence of ACE activity in the intestinal mucosa (*48*−*50*). Then, the presence of high concentrations of large procyanidins might generate a significant enzyme inhibition at the intestinal level. The relevance of such inhibition needs to be confirmed, since the physiological role of ACE in the digestive tract is still unclear.

In conclusion, our experiments show that, in addition to isolated procyanidins, flavanol-rich foods also inhibit the ACE activity. The occurrence of such inhibition in vivo needs to be determined; however, the association between the consumption of flavanol-rich foods and reductions in blood pressure provides an important rationale supporting this hypothesis.

## ABBREVIATIONS USED

ACE, angiotensin converting enzyme; $IV_{50}$, inhibitory volume 50; $IC_{50}$, inhibitory concentration 50.

## ACKNOWLEDGMENT

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