Effects of green tea, black tea and Rooibos tea on angiotensin-converting enzyme and nitric oxide in healthy volunteers

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Abstract

Objective: Tea has been reported to reduce cardiovascular mortality, but the underlying mechanisms are largely unknown. The aim of the current project was to investigate the effect of green tea (Japanese Sencha), black tea (Indian Assam B.O.P.) and Rooibos tea (South Africa) on angiotensin-converting enzyme (ACE) and nitric oxide (NO).

Design: Seventeen healthy volunteers received a single oral dose of 400 ml green tea, black tea or Rooibos tea in a randomized, three-phase, crossover study. ACE activity and NO concentration were measured (at 0, 30, 60 and 180 min) in all phases. ACE activity was analysed by means of a commercial radioenzymatic assay. Nitrite was analysed as a marker of NO concentration. In addition, *ACE* genotype was determined using a PCR method.

Results: Oral intake of a single dose of Rooibos tea significantly inhibited ACE activity after 30 min ($P < 0.01$) and after 60 min ($P < 0.05$). A significant inhibition of ACE activity was seen with green tea for the *ACE II* genotype 30 min after intake of the tea ($P < 0.05$) and for the *ACE ID* genotype 60 min after intake ($P < 0.05$). A significant inhibition of ACE activity was also seen with Rooibos tea for the *ACE II* genotype 60 min after intake ($P < 0.05$). No significant effect on NO concentration was seen.

Conclusions: These results suggest that green tea and Rooibos tea may have cardiovascular effects through inhibition of ACE activity.

Keywords
Tea
Angiotensin-converting enzyme
Nitric oxide

Green tea and black tea are derived from the leaves of *Camellia sinensis* L. (Theaceace). Green tea is produced by rapid heating of the leaves to preserve the colour and antioxidative compounds present in *C. sinensis*. Black tea is produced by a fermentation process, which is known to reduce the content of antioxidants[1]. Rooibos tea is produced from the leaves and stems of *Aspalathus linearis* Dahlg. (Leguminosae) and is a caffeine-free alternative to green and black tea. Tea is suggested to reduce mortality in CVD[2,3], such as acute myocardial infarction[3]. Several positive effects of tea on the cardiovascular system have been shown: reduction of atherosclerosis[4] and lipid peroxidation[4–7], anti-inflammatory properties[6,7] and improvement of endothelial function[8].

The effect of tea is suggested to be due to its high content of the flavonoid group catechins, i.e. epicatechin, epigallocatechin, epicatechingallate and epigallocatechingallate[9–11]. Epigallocatechingallate is the catechin most associated with the preventive effects of tea on CVD, due to its strong antioxidative properties[9,10]. In contrast to green and black tea, Rooibos tea contains dihydrochalcones, flavones and flavonols[12] but does not contain any catechins[11].

Angiotensin-converting enzyme (ACE) is a key enzyme in the renin–angiotensin aldosterone system (RAAS). ACE converts the inactive decapeptide angiotensin I, forming the active octapeptide angiotensin II. Angiotensin II is a main effector of the RAAS and seems to be involved in developing CVD. Angiotensin II binds to the angiotensin receptors AT_1 and AT_2. Activation of AT_1 receptors is associated with endothelial dysfunction[13], vasoconstriction[13], cell proliferation[14], platelet aggregation[15], inhibition of nitric oxide synthase[16], aldosterone release[17], cognitive and behavioural processes such as depression, anxiety, enhanced learning and memory[18–20], dementia[20] and increases in reactive oxygen species[21].

The *ACE* gene polymorphic sites are an insertion/deletion (I/D) consisting of three genotypes: *II* and *DD* homozygotes and *ID* heterozygote[22]. The I/D polymorphism of the *ACE* gene determines individual serum levels of ACE[22,23]. The levels of ACE are two- to three-fold higher in people with the *DD* genotype than in those with the *II* genotype, and people with the *ID* genotype have an intermediate level of ACE[22,24]. The *D* allele of the ACE gene is proposed to correlate with CVD[25,26].

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Hence, it seems that the *DD* genotype could be a risk factor for CVD[24].

There are several interactions known between the RAAS and nitric oxide (NO). Angiotensin II and NO act as physiological antagonists concerning vascular tone and growth[27]. NO has also been proposed as a negative regulator of angiotensin receptors, and endothelial angiotensin receptors may stimulate NO release[28]. Furthermore, activation of the RAAS is believed to be involved in tolerance to organic nitrates[29]. NO is proposed to act as an ACE inhibitor in rats[30]. It has also been shown that exogenous and endogenous NO can inhibit ACE in pigs and man *in vitro* and this NO-mediated ACE inhibition is additive with traditional ACE inhibitors like captopril, and of functional importance concerning angiotensin I-induced vasoconstriction and platelet aggregation[31,32]. This implies that NO not only has direct inhibitory effects on atherogenesis, but also indirect effects by decreasing the amount of angiotensin II.

In previous studies we found that green tea and black tea can inhibit ACE activity in human endothelial cells and that green tea, black tea and Rooibos tea can increase NO concentration from the same cells[11]. Since there are no *in vivo* data supporting these effects, we wished to study the effect of green tea, black tea and Rooibos tea on ACE activity and NO concentration in healthy volunteers *in vivo*.

Materials and methods

Study subjects

The study protocol was approved by the regional Ethics Committee at Linköping University, Sweden (Dnr M56-07) and informed consent was obtained from the twenty non-nicotine-using healthy volunteers (ten males and ten females) who took part in the investigation. Their age ranged between 20 and 31 years with a mean value of 26 years. All subjects were healthy, as assessed by medical history and physical examination. Study participants did not use any kind of drugs (medical or herbal) two weeks prior to and during the entire sampling period. Intake of beverages or foods containing high amounts of catechins, the flavonol quercetin or anthocyanins (i.e. chokeberries, aubergine, blackberries, bilberries, elderberries, raspberries, strawberries, coffee, cacao/chocolate, cherries, onions, plums, pears, radishes, red and black currants, red cabbage, black beans, cranberries, tea, wine, grapes and apples) was not allowed 48 h before the experiment. A list of foods not allowed was distributed to the participants when they registered to the study. The participants also answered a questionnaire concerning everyday intake of certain foods, i.e. vegetables, fruits and berries, containing the flavonoids catechins, quercetin and anthocyanins. Three subjects were excluded (one male and two female); two because of practical difficulties when collecting blood and one due to oral use of tobacco.

Study design

A randomized three-phase crossover design was used and the phases were separated by a period of 1–4 weeks. Forty-eight hours prior to each phase, the participants were not allowed intake of food or beverages containing high amounts of certain flavonoids (as mentioned above). The study subjects received 400 ml of green tea (Japanese Sencha), black tea (Indian Assam B.O.P.) or Rooibos tea (South Africa) in each phase. All experiments started at 08.00 hours and the subjects were not fasting, to resemble ordinary intake of tea. The tea was freshly prepared and cooled so the participants could drink it within approximately 2 min. The tea was prepared from green tea, Japanese Sencha imported by Charabang, Stockholm, Sweden; black tea, Indian Assam B.O.P. imported by Norrköping Kolonial, Sweden; and Rooibos tea imported by Norrköping Kolonial, Sweden. The teas were bought at Tebladet, Linköping, Sweden. Infusions were made with 10 g tea in 400 ml fresh-boiled water for 5 min with the green tea and the black tea, and for 10 min with the Rooibos tea, using a tea filter (Agatha's Bester, Germany). The different boiling times were used to mimic the brewing of tea in households.

Sample collection

Data collected at each treatment were blood pressure, heart rate and venous blood samples for analyses of ACE activity, NO concentration and *ACE* genotype. Blood pressure, heart rate and blood samples for analyses of ACE activity and NO concentration were taken before drinking tea and after 30 min, 60 min and 3 h. Blood pressure and heart rate were measured in both supine and standing position, while blood samples were collected in supine position. Vacutainer tubes without anticoagulant were used to collect blood for serum-ACE and serum-nitrite analyses. After 2 h, the tubes were centrifuged at 1000*g* for 20 min at 4°C. Serum was transferred to plastic tubes and ACE activity was analysed the same day, as described below. Remaining serum was frozen at −70°C until nitrite analysis. Vacutainer tubes with EDTA were used to collect blood for determination of polymorphism concerning the *ACE* genotype. The blood was transferred to sterile test tubes and frozen at −70°C until analysis.

Analytical methods

ACE activity was analysed using a commercial radioenzymatic assay (ACE-direct REA; Bühlmann Laboratories, Allschwil, Switzerland) with the following modifications. Blank and standard serum and the human serum were added to wells in a microtitre plate. The synthetic substrate [3H]hippuryl-glycyl-glycine was then added directly to all wells. The serum was incubated for 1 h at 37°C to allow for the ACE present to cleave the substrate to [3H]hippuric acid. Then serum and substrate was transferred from each well into scintillation vials containing 50 μl 1 M-HCl, to stop the enzymatic reaction.

Scintillation liquid was added and the samples were counted in a scintillation counter.

Nitrite was analysed as a marker of NO concentration as proposed by Lauer *et al.*[33]. Nitrite concentration in the serum was analysed with a commercially available nitrite/nitrate assay: nitric oxide (NO_2^-/NO_3^-) assay kit obtained from R&D Systems, Abingdon, UK. The principle for the colorimetric detection of nitrite is the Griess reaction. In short, diazonium ions are produced when acidified nitrite reacts with sulfanilic acid. These diazonium ions form chromophore agents when reacting with *N*-(1-naphthyl)-ethylenediamine. Optical density was determined with a Spectramax reader at 540 nm.

Genomic DNA was isolated from venous blood using QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany). The *ACE* gene contains a polymorphism based on the absence and/or presence of a 287 bp non-sense DNA domain in intron 16, which results in three different genotypes: deletion/deletion homozygote (*DD*), insertion/insertion homozygote (*II*) and insertion/deletion heterozygote (*ID*). Three primers, 5′-CTG CAG GTG TCT GCA GCA TGT GC-3′, 5′-GAT TAC AGG CGT GAT ACA GTC ACT TTT-3′ and 5′-GCC ATC ACA TTC GTC AGA TCT GGT AG-3′ (Invitrogen Ltd, Paisley, UK), were used according to the method of Cheon *et al.*[34].

For the PCR, a 'ready-to-use' reaction mixture (REDExtract-N-Amp Blood PCR ReadyMix; Sigma-Aldrich, St. Louis, MO, USA) was used in a total volume of 20 μl. Each primer was used at a concentration of 0.4 μM and for each reaction 2 μl DNA solution was added to the reaction mixture.

The PCR was performed in a Perkin Elmer Cetus DNA Thermal Cycler and started with 5 min of initial denaturation at 94°C. Then the samples underwent thirty cycles of amplification: 45 s of denaturation (94°C), 45 s of annealing (65°C) and 45 s of extension (72°C). The reaction was finished with 7 min of final extension (72°C) and hold at 4°C. The PCR products were separated by electrophoresis on a 2 % agarose gel, then stained with ethidium bromide and visualized with UV light. The PCR products detected with the primers are one 237 bp fragment for the deleted allele, and one 525 and one 155 bp fragment for the inserted allele.

Statistical analysis

Results are presented as means with their standard error. One unit (U) of ACE activity is defined as the amount of the enzyme required to release 1 μmole hippuric acid per minute and litre. Graph Pad Prism 5·0 (La Jolla, CA, USA) was used for statistical calculations. One-way ANOVA for repeated measures was performed followed by Dunnett's *post hoc* test or Bonferroni's *post hoc* test (questionnaire). Statistical significance was taken at $P < 0.05$.

Results

Angiotensin-converting enzyme activity

After oral intake of 400 ml green tea, black tea or Rooibos tea, significant inhibition of ACE activity was seen with the Rooibos tea after 30 min ($P < 0.01$) and after 60 min ($P < 0.05$). No significant inhibition was seen with the green tea or the black tea (Figs 1a, b and c). When the participants were grouped according to *ACE* genotype, a significant inhibition of ACE activity was seen with the green tea for the *II* genotype 30 min after intake of the tea
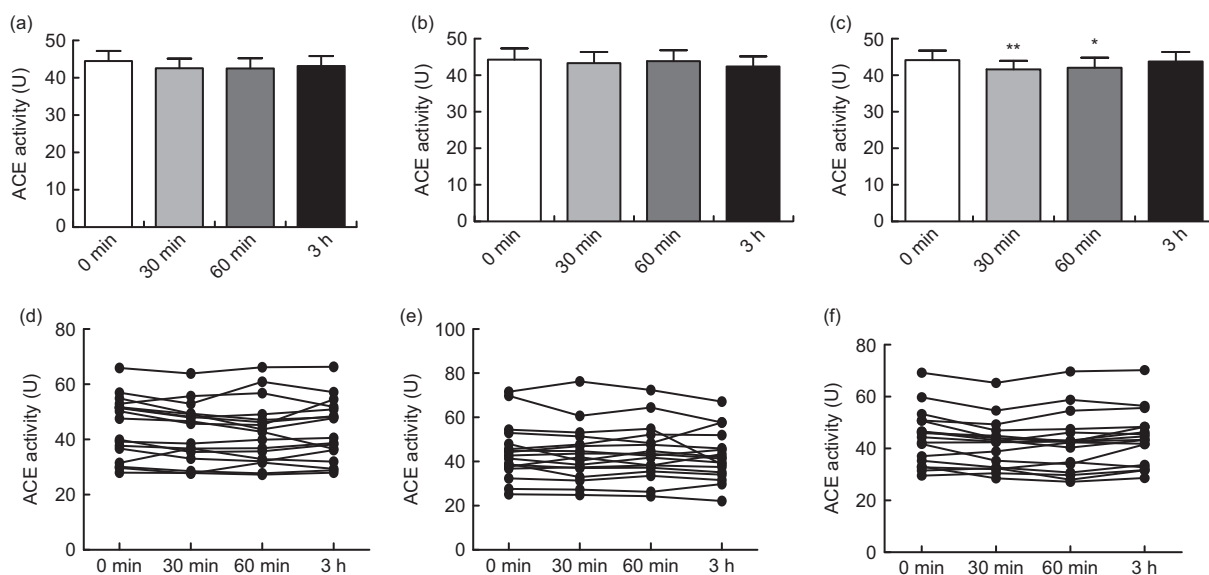

Fig. 1 Angiotensin-converting enzyme (ACE) activity in serum before oral intake and 30, 60 min and 3 h after intake of 400 ml green tea (a, d), black tea (b, e) or Rooibos tea (c, f) in healthy volunteers (mean age 26 years, range 20–31 years), Sweden. The upper row (a, b, c) shows mean values with their standard errors represented by vertical bars; the lower row (d, e, f) shows values from each individual. Mean values were significantly different from those at baseline (one-way ANOVA for repeated measures, *n* 17): *$P < 0.05$, **$P < 0.01$
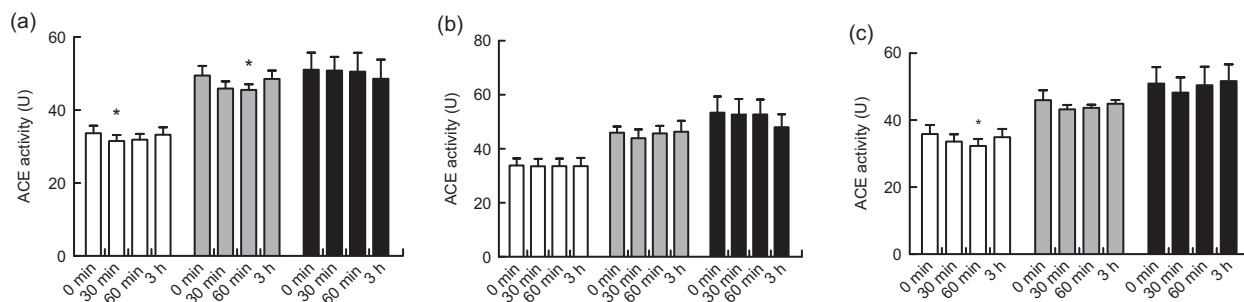
Fig. 2 Angiotensin-converting enzyme (ACE) activity in serum before oral intake and 30, 60 min and 3 h after intake of 400 ml green tea (a), black tea (b) or Rooibos tea (c) according to *ACE* genotype (*II*, □; *ID*, ▨; *DD*, ■) in seventeen healthy volunteers (mean age 26 years, range 20–31 years), Sweden. Values are means with their standard errors represented by vertical bars. Mean values were significantly different from those at baseline (one-way ANOVA for repeated measures; *II*, n 6; *ID*, n 5; *DD*, n 6): *$P < 0.05$

($P < 0.05$) and for the *ID* genotype 60 min after intake of the tea ($P < 0.05$; Fig. 2a). A significant inhibition of ACE activity was also seen with the Rooibos tea for the *II* genotype 60 min after intake of the tea ($P < 0.05$; Fig. 2c). No significant effect was seen with the black tea (Fig. 2b). No significant effect was seen with any of the teas for the *DD* genotype (Figs 2a to c). The basal level of ACE activity was significantly higher ($P < 0.001$) for the participants with the *ID* and *DD* genotypes compared with those with the *II* genotype (Fig. 3). No difference was seen between males and females in any of the groups.

Blood pressure and heart rate

Basal blood pressure (supine, standing; mmHg) was 121 (SE 1·2)/71 (SE 1·1), 122 (SE 1·5)/74 (SE 1·1). Blood pressure (supine, standing; mmHg) was 118 (SE 1·4)/71 (SE 1·1), 121 (SE 1·8)/74 (SE 1·3) after 30 min; 117 (SE 1·5)/72 (SE 1·0), 120 (SE 1·8)/75 (SE 1·3) after 60 min; and 116 (SE 1·5)/71 (SE 0·9), 119 (SE 2·0)/74 (SE 1·3) after 3 h. No significant change in blood pressure was seen after intake of any of the teas.

Basal heart rate (supine, standing; beats/min) was 75 (SE 2), 83 (SE 2). Heart rate (supine, standing; beats/min) was 65 (SE 2), 71 (SE 2) after 30 min; 63 (SE 2), 71 (SE 2) after 60 min; and 60 (SE 2), 70 (SE 2) after 3 h. No significant change in heart rate was seen after intake of any of the teas.

Nitric oxide concentration

After oral intake of 400 ml green tea, black tea or Rooibos tea, no significant effect on the NO concentration was seen (Fig. 4). After grouping the participants according to *ACE* genotype *II*, *ID* or *DD*, no significant effect was seen on NO concentration (data not shown).

The questionnaire

Average daily intake of green tea, black tea, Rooibos tea, coffee, vegetables and fruits is shown in Table 1. A significant ($P < 0.01$) difference was seen in intake of fruits and vegetables plus fruits between females and males (Table 1). The most consumed fruit were apples and bananas, and the most consumed vegetables were
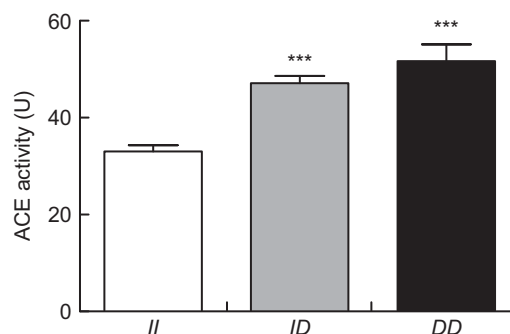

Fig. 3 Basal angiotensin-converting enzyme (ACE) activity in serum according to *ACE* genotype in healthy volunteers (mean age 26 years, range 20–31 years), Sweden. Values are means with their standard errors represented by vertical bars. Mean values were significantly different from those of the *II* genotype (one-way ANOVA; *II*, n 6; *ID*, n 5; *DD*, n 6): ***$P < 0.001$

tomatoes. No differences in intake of apples, bananas and tomatoes were seen between males and females.

Discussion

The current *in vivo* study showed a significant inhibition of ACE activity at 30 and 60 min after oral intake of Rooibos tea, while green tea and black tea showed no significant inhibition of ACE activity for the total group of participants (Figs 1a, b and c).

It was previously shown that green tea and black tea significantly inhibit ACE activity in human endothelial cells *in vitro* while Rooibos tea did not inhibit ACE activity *in vitro*[11]. This discrepancy between the *in vitro* and *in vivo* studies may be due to either the content of the flavonoids or/and the metabolism of the components in the different teas, as there are differences in pharmaco-kinetics/metabolism between different flavonoids[35]. The discrepancy between the studies could also be due to the bioavailability or/and to a possible different mechanism of action of the Rooibos tea compared with the green tea and the black tea.
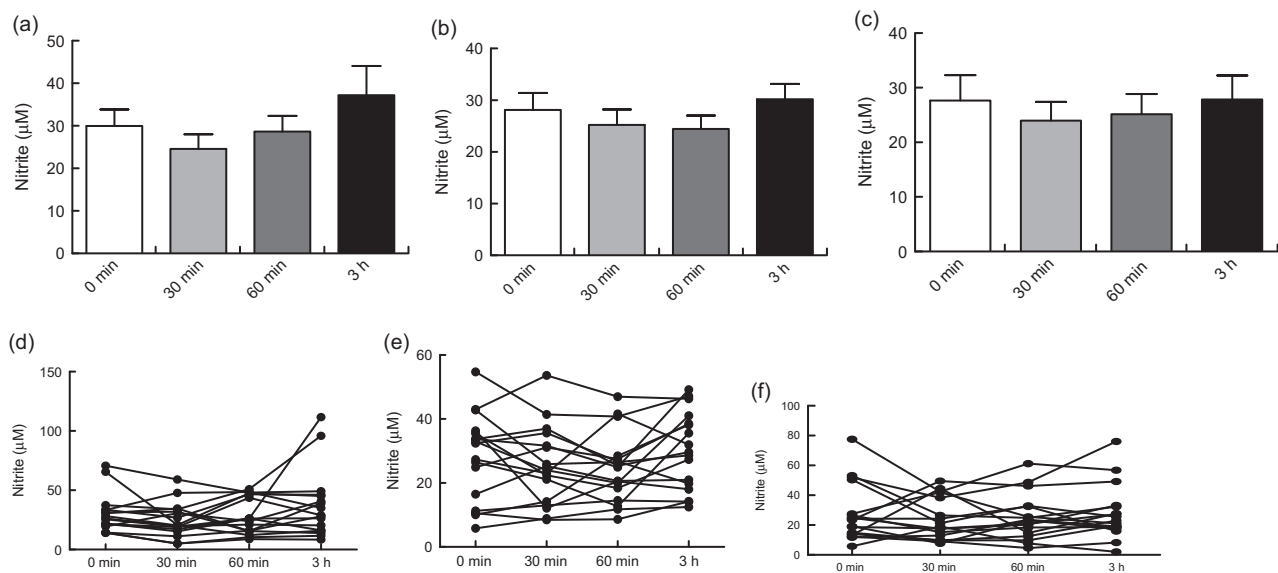
Fig. 4 Nitric oxide (NO) production (nitrite was analysed as a marker of NO concentration) in serum before oral intake and 30, 60 min and 3 h after intake of green tea (a, d), black tea (b, e) or Rooibos tea (c, f) in healthy volunteers (mean age 26 years, range 20–31 years), Sweden. The upper row (a, b, c) shows mean values with their standard errors represented by vertical bars; the lower row (d, e, f) shows values from each individual. There were no significant differences in NO production compared with baseline (one-way ANOVA for repeated measures, *n* 17)

Table 1 Participants' daily intake of flavonoid-containing foods from the questionnaire†: seventeen healthy volunteers (mean age 26 years, range 20–31 years), Sweden

	Total (*n* 17)		Male (*n* 9)		Female (*n* 8)		*II* genotype (*n* 6)		*ID* genotype (*n* 5)		*DD* genotype (*n* 6)	
	Mean	Min, max	Mean	Min, max	Mean	Min, max	Mean	Min, max	Mean	Min, max	Mean	Min, max
Green tea	0·12	0, 0·57	0·09	0, 0·43	0·13	0, 0·57	0·20	0, 0·57	0·11	0, 0·43	0·00	–
Black tea	0·33	0, 2	0·16	0, 0·57	0·49	0, 2	0·08	0, 0·28	0·60	0, 2	0·30	0, 0·71
Rooibos tea	0·15	0, 1	0·13	0, 1	0·18	0, 1	0·27	0, 0·96	0·23	0, 1	0·01	0, 0·07
Coffee	0·89	0, 4	0·92	0, 4	0·87	0, 4	1·26	0, 4	0·26	0, 1·28	0·76	0, 4
Vegetables	1·36	0·57, 2	1·31	0·57, 2	1·40	0·57, 2	1·60	0·86, 2	1·51	0·71, 2	1·19	0·57, 2
Fruits	1·80	0·28, 4	0·95	0·57, 2	2·50**	0·71, 4	1·40	0·28, 3	1·71	0·28, 4	2·21	0·57, 4
Vegetables and fruits	3·16	1·14, 6	2·26	1·14, 4	3·97**	1·28, 6	3·00	1·57, 5	3·23	1·28, 6	3·40	1·14, 6

Min, minimum; max, maximum.
†Intake based on answers to the following questions: (i) 'How many cups of tea do you drink per day?' (1 cup of tea is 250 ml); (ii) 'How many cups of coffee do you drink per day?' (1 cup of coffee is 150 ml); (iii) 'How many times per day do you eat vegetables?'; and (iv) 'How many times per day do you eat fruit?' Mean values were significantly different from those of males: **$P < 0.01$.

The I/D polymorphism of the *ACE* gene is proposed to be important concerning serum levels of ACE[22,23]. As shown in Fig. 3, basal ACE activity of the participants showed a significant difference between the *ACE II* genotype and the *ID* and *DD* genotypes in accordance with the results of Rigat *et al.*[22]. The *D* allele of the *ACE* gene was previously proposed to be correlated with CVD[26,36]. According to Fig. 2, the *ACE II* and *ID* genotypes seem to be more sensitive to ACE inhibition by the different teas than the *DD* genotype. A significant inhibition of ACE activity was seen for the *II* and *ID* genotypes with green tea and for the *II* genotype with Rooibos tea. The same pattern in ACE inhibitory action depending on *ACE* genotype has previously been shown with glyceryl trinitrate[37]. Hence, it seems that the *DD* genotype could be a

risk factor for CVD, as previously proposed[24]. The difference between genotypes may also be a question of dosage. *ID* and even more the *DD* genotype might need higher amounts of flavonoids to affect ACE activity than the *II* genotype[38].

The effects of green and black tea on CVD are suggested to be due to their high content of catechins, i.e. epicatechin, epigallocatechin, epicatechingallate and epigallocatechingallate[11], while Rooibos tea does not contain any catechins[11] but dihydrochalcones, flavones and flavonols[12]. The catechins especially epigallocatechingallate showed strong inhibitory effects on ACE activity *in vitro*[11]. The slight inhibition of ACE activity seen by Rooibos tea *in vitro* might depend on its quercetin (flavonol) content, since quercetin exhibits an ACE

inhibitory action *in vitro*[39]. The two active sites of ACE contain Zn^{2+}, and ability to bind the Zn^{2+} ion will inhibit ACE activity[40]. ACE inhibition leads to less angiotensin II, and an increase of NO leads to down-regulation of AT_1 receptors. Our previous *in vitro* study showed an increased NO concentration by green tea, black tea and Rooibos tea[11], but the current *in vivo* study did not show any significant effect on NO concentration after oral intake of any of the teas (Figs 4a, b and c). A possible explanation may be that we determined nitrite as a marker of NO, and nitrite is also a product of protein metabolism. As shown in Figs 1d to f and Figs 4d to f, the participants exhibited substantial individual variations in ACE activity as well as NO concentration and this fact may also influence the results. It must be taken into consideration that our *in vivo* study examined the effect on ACE activity and NO concentration after a single dose (one cup) of tea. The average inhibition of ACE activity 30 min after intake of Rooibos tea was 6 %, compared with 16 % inhibition of ACE activity after chronic intake of enalapril 10 mg[41]. In the current study, no effect on blood pressure was seen after intake of tea. Since the RAAS is involved in long-term regulation of homeostasis, such an effect could not be expected. One previous study with 1 week's intake of black tea showed a significant reduction in blood pressure and increase in flow-mediated vasodilation in healthy volunteers[42]. However, in a meta-analysis including the cardiovascular effects of black and green tea, no reduction in blood pressure was seen[43]. None of these studies included ACE activity among the investigated parameters. Further studies investigating regular long-term intake of tea remain to be performed.

Diet is of great importance to human health[44–47]. The prevention of CVD is attributed to a diversity of effects such as antioxidative[48], antithrombotic and anti-inflammatory properties, activation of endothelial nitric oxide synthase and inhibition of LDL oxidation[49–53]. Flavonoids, e.g. catechins found in green tea and black tea and flavonols found in Rooibos tea, are plant-derived antioxidants with alleged positive effect on the cardiovascular system[49,54]. However, the positive effect of fruit, vegetables and tea on CVD cannot always be solely explained by their antioxidative action. The present study shows an additional mechanism, flavonoids as ACE inhibitors.

The average intake of vegetables and fruit by the participants was approximately three times daily (Table 1). A significant difference was seen in frequency of intake of fruits and thereby in intake of vegetables plus fruits between females and males. Thus, according to this questionnaire, females consumed significantly more fruits than males. Recommendation from the Swedish National Food Administration is five times daily (or 500 g/d) for fruits and vegetables. According to the questionnaire, the most consumed fruits are apple and banana, and the most consumed vegetable is tomato. Apples, bananas and tomatoes also contain flavonoids, but at a much lower content than tea. Flavonoids, e.g. catechins found in tea, vegetables and fruits, are well known for their protective effect against CVD. Considering that, the intake of flavonoids among the subjects of the experiment seems to be generally low. Average daily intake of flavonoids is difficult to assess, it depends on season, availability, soil, climate and dietary habits.

Conclusion

In conclusion, oral intake of a single dose (400 ml) of Rooibos tea significantly inhibited ACE activity in healthy volunteers. When the participants were divided into subgroups according to *ACE* genotype, those with genotypes *II* and *ID* showed a significant inhibition of ACE activity after intake of green tea and Rooibos tea, while participants with *ACE* genotype *DD* seemed to be more difficult to affect with a single dose of tea. ACE inhibitors are the first-line treatment of hypertension and thus a common drug used for CVD. Intake of high amounts of flavonoids, i.e. tea, vegetables and fruits, might contribute to the prevention of CVD in the population and thereby prevent CVD mortality. These results suggest that green tea and Rooibos tea may have cardiovascular effects through inhibition of ACE activity. Additional experimental and clinical studies are needed in order to further elucidate these effects.

Acknowledgements

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