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The interaction of tea flavonoids with the NO-system: discrimination between good and bad NO

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Abstract

The effect of black and green tea on the NO system was tested. Beside a general effect i.e. NO scavenging, the teas interacted with good, i.e. relaxation of the aorta, and bad effects, i.e. peroxynitrite scavenging, of NO. Green tea was a better NO and peroxynitrite scavenger than black tea. Epigallocatechin gallate was the major identified contributor to both the peroxynitrite and NO scavenging. The theaflavins, only present in black tea, also had a substantial contribution to the NO scavenging of black tea. The teas were found to have only a minor and nonspecific effect on the NO mediated vasorelaxation. Based on these results it is concluded that tea discriminates between the good and bad effects of NO; tea is likely to prevent NO toxicity primarily.

Keywords: Flavonoid; Black tea; Green tea; NO scavenging; Peroxynitrite scavenging

1. Introduction

Nitric oxide (nitrogen monoxide, NO) plays an ambiguous role in physiology. On the one hand NO is involved in various regulatory processes as second messenger. For example, the acetylcholine-induced relaxation in aorta is NO-mediated. Upon stimulation of the acetylcholine-receptor, the constitutive NO-synthase (NOS) produces NO. NO activates guanylate cyclase, which leads to the production of cyclic GMP, and ultimately to vasorelaxation. On the other hand NO is toxic, predominantly due to the formation of peroxynitrite formed in the reaction with superoxide radicals (Radi, Beckman, Bush & Freeman, 1991; Rubbo, Darley-Usmar & Freeman, 1996).

Previously, it has been shown that flavonoids, i.e. a group of polyphenolic antioxidants (Miller, Castelluccio, Tijburg & Rice-Evans, 1996; Salah, Miller, Paganga, Tijburg, Bolwell & Rice-Evans, 1995), interact with the NO system. For example, it has been found that flavonoids are efficient scavengers of the nitric oxide radical (Haenen & Bast, 1999; Verhagen, Haenen & Bast, 1996). In their effect on the NO system, e.g. NO scavenging, the flavonoids do not distinguish between good and bad NO. It has been reported that flavonoids accumulate in vascular tissue between the endothelial layer and the vascular smooth muscle cells (Neumann, Carlsson & Brom, 1992). Interestingly at this site both beneficial (vasorelaxation) and detrimental (atherosclerosis) processes with NO as a protagonist take place.

A major source of flavonoids in the western diet is tea (Camellia sinensis). The most frequently consumed teas are black and green tea. In the production of green tea, steaming and drying of the tealeaves prevent oxidation of the polyphenols in the leaves. The major polyphenols of green tea are catechins, mainly epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG).

In the production of black tea fermentation, i.e. enzymatic, aerobic oxidation of leaf polyphenols, and subsequent condensation, takes place. Due to this process, the total catechin content is reduced to approximately 20% of the total catechin content of green tea, and new products, such as theaflavins, are formed. The theaflavins comprise approximately 1% of the total flavonoids of black tea. The major ones are theaflavin,

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theaflavin 3-gallate, theaflavin 3′-gallate and theaflavin 3,3′-digallate. The most important catechins and theaflavins are depicted in Fig. 1.

In the present study, the interaction of tea with the NO system was determined. Beside a general effect (NO-scavenging), the interaction with good (the acetylcholine-dependent relaxation of the aorta) and bad NO (peroxynitrite scavenging) was measured. Both black and green tea were investigated.

2. Materials and methods

2.1. Chemicals

The flavonoids and green and black tea powder were gifts from Lipton (Englewood Cliffs, New Jersey, USA). The composition of the tea powders is given in Table 1. The amount of solids obtained from tea is variable, depending e.g. on brewing time and agitation. The average amount of solids obtained from an average English cup is 2–5 g/l. For calculation of the activity of tea, 3 g solids per litre was used. Peroxynitrite was synthesised in a quenched flow reactor, as previously described by Radi et al. (1991). Excess hydrogen peroxide was removed by MnO_2 treatment. All other chemicals were of analytical purity grade.

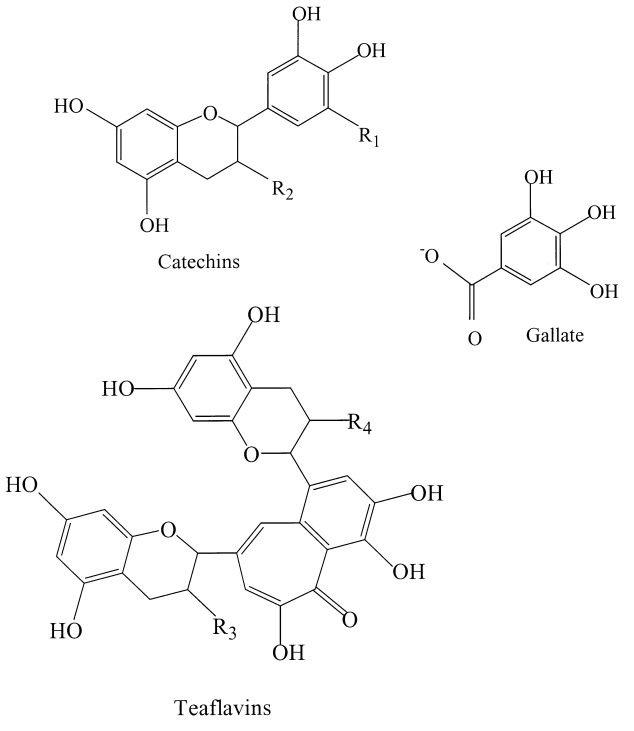

Fig. 1. Structures of the catechins and theaflavins. For the catechins: epicatechin $R_1 = H$ and $R_2 = OH$; for epigallocatechin $R_1 = OH$ and $R_2 = H$; for epicatechin gallate $R_1 = H$ and $R_2 =$ gallate; for epigallocatechin gallate $R_1 = OH$ and $R_2 =$ gallate. For the theaflavins: theaflavin, $R_3 = R_4 = OH$; for theaflavin-3-monogallate $R_3 =$ gallate and $R_4 = OH$; for theaflavin-3′-monogallate $R_3 = OH$ and $R_4 =$ gallate; for theaflavin 3,3′-digallate $R_3 = R_4 =$ gallate (Pannala et al., 1997).

2.2. NO scavenging

NO scavenging was determined according to Vriesman (Vriesman, Haenen, Westerveld, Paquay, Voss & Bast, 1997). In short, deoxygenated water was purged with NO gas for about 1 min. Two microliters of the NO spiked water was added to 20 ml of 50 mM phosphate buffer (pH 7.4) in a thermostatted test vessel (37°C). During measurements the test vessel was kept under a N_2 atmosphere. The NO concentration was monitored with an Iso-NO meter (World Precision Instruments, Sarasota, USA) which was coupled to both a MacLab™ interface (ML020 MacLab/8, ADInstruments, London, UK). The decrease in NO concentration was followed in time in the presence or absence of the test compound in solution. The stock solution of the test compound was incubated at 37°C for 10 min before being used. The second order reaction constant (k) was calculated.

2.3. Peroxynitrite scavenging

Peroxynitrite scavenging was measured by the oxidation of dihydrorhodamine 123, as described by Kooy, Royall, Ischiropoulos and Beckman (1994). Fluorescence measurements were performed on a Shimadzu RF-5001 PC fluorimeter with excitation and emission wavelengths of 500 and 536 nm respectively at 37°C. The effects are expressed as the concentration giving 50% inhibition of the oxidation of dihydrorhodamine 123 (IC_{50}).

Table 1
Composition of the teas[a]

	Green Tea	Black Tea
Catechins	*32.8*	*6.80*
(+)-catechin	1.3	0.32
(+)-Gallocatechin	1.4	0.28
(+)-Gallocatechin gallate	0.39	< LLOQ[b]
EC	4.2	0.79
EGC	7.9	1.2
ECG	5.8	1.4
EGCG	11.8	2.8
Theaflavins		*1.18*
Theaflavin	< LLOQ	0.36
Theaflavin-3-gallate	< LLOQ	0.36
Theaflavin-3′-gallate	< LLOQ	0.19
Theaflavin-3,3′-digallate	< LLOQ	0.27
Gallic acid	0.15	0.80
Caffeine	6.4	6.6
Theobromine	0.25	0.25
Unknown material	60.5	84.4

[a] Results are represented as percentage (w/w).
[b] < LLOQ = below the lower limit of quantification i.e. 0.1%.

2.4. The isolated aorta

Male Wistar rats (200–220 g, Harlan Olac, Horst, The Netherlands) were used. After decapitation, the aorta was rapidly excised, and small rings (approx. 1 mm thick) were mounted in a thermostated organ bath (37°C) containing Krebs buffer gassed with a mixture of 95% O_2 and 5% CO_2; pH 7.4 and fixed to a isotonic transducer. The composition of the Krebs buffer was (mM): NaCl (117.5), KCl (5.6), $MgSO_4$ (1,18), $CaCl_2$ (2.5), $NaHPO_4$ (1.28), $NaHCO_3$ (25) and glucose (5.5). The tension was adjusted to 0.1 g. The concentration and relaxation of the aorta was determined isotonically. To determine the acetylcholine-receptor mediated relaxation, the aorta rings were first pre-contracted using phenylephrine (PE) (Kenakin, 1987). A dose–response curve was constructed. Subsequently, the dose-dependent relaxation mediated by the acetylcholine-receptor was determined. Metacholine (MCh), a chemically more stable receptor agonist than acetylcholine, was used for this purpose.

Firstly, a reference curve of both PE and MCh was constructed in each organ. Subsequently, the organs were incubated for 30 min with tea at several concentrations. After this period, a second dose–response curve of PE and MCh was constructed. The pD_2 values (-log of the molar concentration that produces half maximal effect) of the compounds were calculated. The maximal effect produced by the compounds in the second dose-response curve was related to that of PE in the first dose–response curve. The reversibility of the effect of tea was examined by washing 3 times for 5 min with 20 ml of fresh Krebs buffer.

2.5. Statistics

The results were expressed as mean ± SEM and statistical analysis were performed with a non-parametric Mann–Whitney-*U*-test.

3. Results

The ability of tea to scavenge the NO radical was tested. Green tea (the rate constant, $k = 0.101 \pm 0.016$ ml mg^{-1} s^{-1}, $n = 3$) was more effective than black tea ($k = 0.019 \pm 0.009$ mg^{-1} s^{-1}, $n = 3$). The contribution of various polyphenols to the NO scavenging, calculated from their own activity and their content in tea, is given in Fig. 3. In this calculation it is assumed that only additive and not synergistic effects occur. The ability of tea to scavenge peroxynitrite was also tested. Again green tea ($IC_{50} = 0.380 \pm 0.037$ μg ml^{-1}) appeared to be a better scavenger than black tea, $P < 0.05$ ($IC_{50} = 0.882 \pm 0.036$ μg ml^{-1}). The contribution of the various polyphenols to the peroxynitrite scavenging is also quantified (Fig. 4).

The effect of tea on the NO mediated vasorelaxation was examined in isolated aorta rings. Addition of PE and MCh to the aorta rings resulted respectively in a contraction and relaxation (Fig. 2). The pD_2 values of PE and MCh in the reference curve were respectively 7.27 and 6.98 (Table 2). The maximal relaxation by MCh was 69% of the contraction induced by PE. Neither black nor green tea had an effect on the tonus of

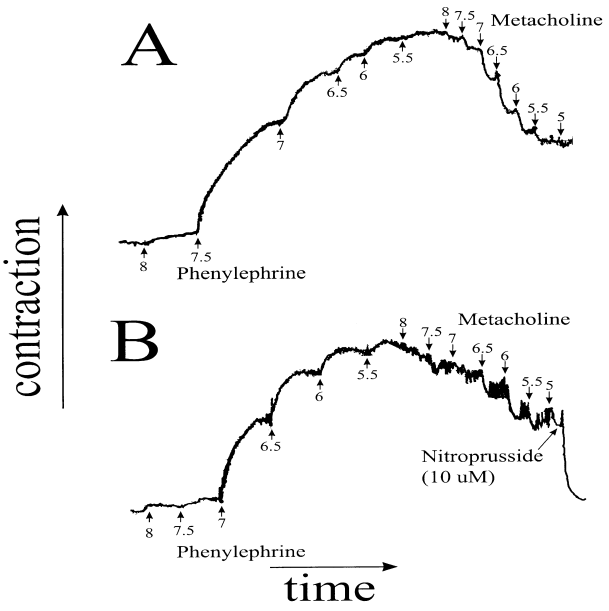

Fig. 2. Typical traces of the dose–response curves of PE and MCh in a control aorta ring (trace A) and an aorta incubated with 0.1 mg/ml green tea (trace B). The concentrations of PE and MCh are depicted as-log of the molar concentration.

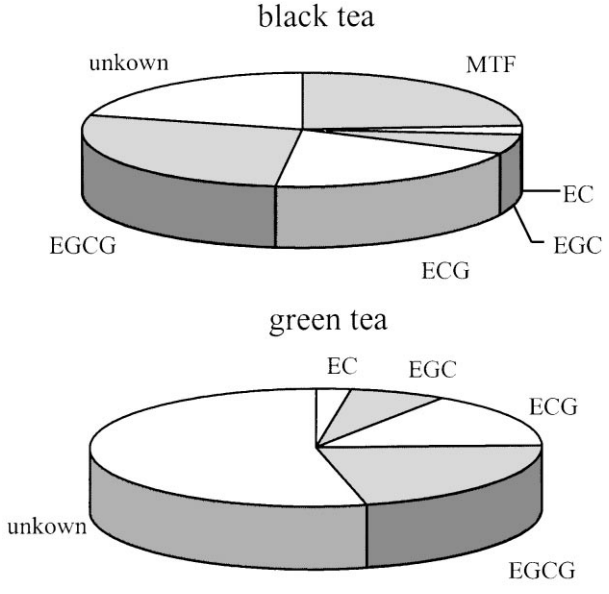

Fig. 3. Relative contribution of mixed theaflavins (MTF), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) to the NO scavenging by black and green tea.

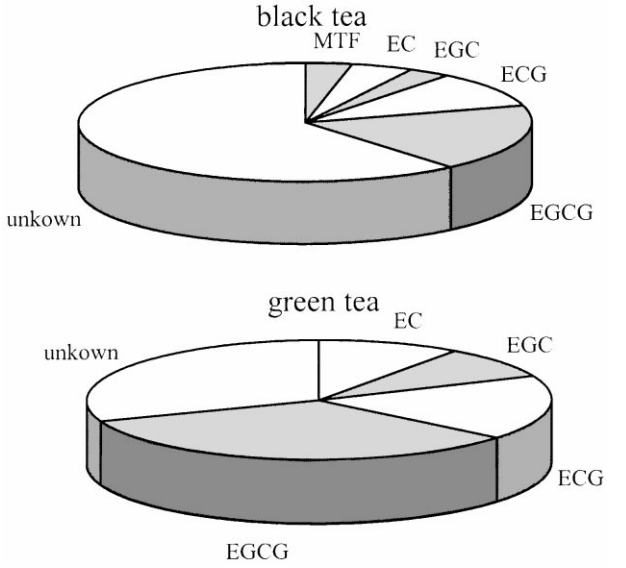

Fig. 4. Relative contribution of mixed theaflavins (MTF), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) to the peroxynitrite scavenging by black and green tea.

Table 2
The effect of black and green tea on the dose–response curves of PE and MCh in aorta rings. The aorta ring was incubated 30 min with the tea in the given concentration before the dose–response curves were constructed

		Control ($n=16$)	Treatment	
			Black tea	Green tea
			100 μg/ml ($n=4$)	100 μg/ml ($n=4$)
PE	pD_2	7.24±0.15	7.32± 0.19	6.63± 0.17
	E_{max} (%)	100[a]	87± 7	79± 9
MCh	pD_2	6.98 + 0.13	N.R.[b]	N.R.[b]
	E_{max} (%)	69± 12	0	0

[a] The maximal effect (E_{max}) of PE in the control curve was used as reference and set at 100%.
[b] N.R., no response.

the aorta in the concentrations studied (1 μg/ml–0.1 mg/ml) (data not shown). To test the effect of tea on the NO mediated relaxation of the aorta, the aorta was incubated 30 min with tea. Then dose–response curves for PE and MCh were constructed. The pD_2 of PE was not affected by a 30 min incubation period with 0.1 mg/ml black tea. Green tea (0.1 mg/ml) induced a small reduction of the pD_2 value. Incubation with either black or green tea in a concentration of 0.1 mg/ml reduced the maximal effect induced by PE slightly. The effect of green tea on the maximal PE induced contraction was absent when a concentration of 1 μg/ml was applied (data not shown). After incubation with either black or green tea at a concentration of 0.1 mg/ml, no MCh response was obtained. In contrast, when nitroprusside (10 μ M), a direct NO-donor, was added to the organ bath containing either black or green tea extract a full relaxation of the aorta was observed. Incubation with either black or green tea in a concentration of 0.1 mg/ml and subsequent washing, restored the MCh response from 0% to approximately 50% of the MCh response of the reference curve.

In an additional experiment, the aorta rings were contracted with PE, and MCh was added to give maximal relaxation. Subsequently, i.e. after the MCh induced relaxation, tea was added. It was found that tea in a concentration of 0.1 mg/ml induced a slow contraction of the aorta. Surprisingly, the effect was small compared to the complete inhibition of the MCh response when tea was added 30 min before the MCh was added.

4. Discussion

The effect of tea on the NO system was determined in the present study. It was found that tea is a scavenger of NO. Green tea was found to be a more efficient scavenger of NO than black tea; green tea powder was approximately five times more potent than black tea powder. Taking an average tea concentration as 3 g/l tea solids, it can be calculated that green tea has an activity comparable to white wine and is approximately 50 times less active than red wine (Verhagen et al., 1996).

The ability of flavonoids to protect against NO toxicity was monitored by the peroxynitrite scavenging activity. Previously, it has been shown that the catechins and theaflavins present in tea are peroxynitrite scavengers (Pannala, Rice-Evans, Halliwell & Singh, 1997). In the present study it appeared that green tea was the better peroxynitrite scavenger; green tea powder has more than twice the activity of black tea powder. It was calculated that green tea had a slightly higher activity than red wine, and was approximately 20 times more potent than white wine.

As shown in Figs. 3 and 4, a substantial part of the NO-scavenging activity as well as the peroxynitrite scavenging resides in the flavonoid fraction of both green and black tea. In black tea, theaflavins — that comprise only 1.2% (w/w) of the weight — are responsible for 25% of the NO scavenging of black tea powder. EGCG also accounts for 25% of the NO scavenging, while its content is more than twice that of the theaflavins. The other major contributor (20%) to the NO-scavenging of black tea is ECG (1.4% w/w). In green tea ECG and EGCG are the two major contributors that are identified. Theaflavins are not present in green tea.

Theaflavins appear to be better NO scavengers than peroxynitrite scavengers. The major reason for the lower peroxynitrite scavenging of black tea compared to

green tea can be found in the lower catechin content, due to the enzymic oxidation process in the production of black tea. ECG and EGCG were found to be the major identified contributors in both black and green tea. In green tea EGCG (11.8% w/w) and ECG (5.8% w/w) are responsible for respectively 33 and 18% of the total peroxynitrite scavenging.

The effect of tea on a major physiological role of NO was tested in vitro, i.e. the NO mediated relaxation of the aorta. It was found that tea is able to block the MCh-induced relaxation of aorta rings at a concentration that only slightly affects the PE response. These results are consistent with the observed NO scavenging activity of tea. Moreover, they are in line with the reduction of the acetylcholine response in rabbit aorta by red wine (Cishek, Galloway, Karim, German & Kappagoda, 1997). The effect of tea may be explained by a reduction of NO production, by NO scavenging or by reducing the NO response. Since tea did not affect the nitroprusside response, NO scavenging and reduction of the NO response seem less likely. The NO scavenging effect of black tea was 20% of that of green tea. The effect on the MCh response of 30 μg/ml green tea (data not shown) was less than that of 100 μg/ml black tea. This indicates that NO-scavenging is at its most only partly involved in the effect of black tea.

Tea in a concentration of 0.1 mg/ml that was added to the aorta 30 min before MCh was added completely blocked the MCh response. Surprisingly, tea (0.1 mg/ml) added after MCh, only slightly reduced the MCh response. This indicates that the onset of the effect of tea is relatively slow. Since NO-scavenging is expected to give a rapid onset of the effect, this again indicates that the effect of tea is not mediated by NO-scavenging. Duarte (Duarte, Perez-Vizcaino, Utrilla, Jiménez. Tamargo & Zarzuelo, 1993) reported that the vasodilatory effect of various flavonoids might be due to protein kinase C inhibition, phosphodiesterase inhibition or decreased calcium uptake. Chiesi and Schwaller (1995) found that the polyphenols tannin and quercetin are efficient inhibitors of the endothelial NOS (eNOS). Possibly, these effects of flavonoids are involved in the effect of tea on the MCh response reported in the present study. However, according to (Cishek et al., 1997) catechin and epicatechin had no effect on the response to acetylcholine. Girard, Sercombe, Sercombe, LeLem, Seylam and Potier (1995) reported that a new synthetic flavonoid induced vasorelaxation by scavenging superoxide. Superoxide was supposed to inactivate NO. Scavenging of superoxide by the flavonoid should prolong the half-life of NO, resulting in the observed relaxation. Given the NO scavenging capacity of tea, preservation of NO by tea does not seem probable.

Various studies report on a vasorelaxing activity of flavonoids and flavonoid containing products like tea and wine. Although these studies are partly contradictory and non-conclusive, most of them seem to point at an enhanced NO production induced by the flavonoids. This is, however, not in line with the reported inhibition of eNOS (Chiesi & Schwaller, 1995), the reduction of the acetylcholine receptor-mediated vasorelaxation by wine (Cishek et al., 1997) and tea (this study), and the observation that mechanical removal of the endothelium did not modify the relaxant effect of the flavonoids. The simultaneous induction of vasorelaxing and contracting factors by a single compound (Russel & Rohrbach 1989) may hamper a straightforward interpretation of the results. This becomes even more complicated when products that contain a mixture of flavonoids, such as wine and tea, are studied. This may be one of the reasons for the conflicting results and variation in the molecular mechanisms that have been proposed. In addition, it should be kept in mind that the flavonoids comprise approximately 5000 different compounds of natural origin. The chemical heterogeneity is even increased by the processing of flavonoid containing products, like the fermentation of tea. Due to this immense heterogeneity, a uniform mechanism for all flavonoids cannot be expected. The interaction of different flavonoids with the NO system may be different or even opposite. The relative high concentration needed to reduce the NO-mediated relaxation points at a rather unspecific and less relevant effect of tea on the beneficial effect of NO.

A beneficial effect of high flavonoid intake on the occurrence of cardiovascular heart diseases has been demonstrated in various studies (Frankel, Kanner, German, Parks & Kinsella, 1993; Hertog, Feskens, Hollman, Katan & Kromhout, 1993). Previously, it has been speculated that the molecular mechanism for this effect may be found in the protection by the flavonoids against NO toxicity. Tea is a major dietary source of flavonoids. However, the exact knowledge on digestion, absorption and metabolism is scarce. In the present in vitro study the potency of green tea relative to black tea to reduce either the good or bad effects of NO is higher. The NO scavenging of the teas studied is of limited importance; it is at its most only partly involved in the nonspecific inhibition by the teas of the MCh induced relaxation of the aorta. The scavenging activity of the undesirable form of NO, peroxynitrite, by both black and green tea is superior to the effect the teas have on the beneficial vasorelaxation effect of NO. Besides peroxynitrite scavenging, tea might prevent NO toxicity also in other ways. It has been reported that EGCG is able to inhibit the formation of bad NO by inhibition of the inducible form of NOS (iNOS) and suppression of the expression of iNOS. Apparently, in its effect on the NO system, tea discriminates between good and bad NO; tea is likely to prevent NO toxicity primarily.

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