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Wine polyphenols and ethanol do not significantly scavenge superoxide nor affect endothelial nitric oxide production

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Abstract

Epidemiological studies have shown that moderate intake of red wine reduces the risk of coronary heart disease. It has been proposed that the antiatherogenic effect be due to the scavenging of reactive oxygen species by polyphenols and ethanol or an effect on endothelial nitric oxide (NO) production. We have determined the reaction rates of superoxide with four different polyphenols and ethanol. The superoxide reaction rates were determined at 37°C and pH 7.4 using competitive spin trapping and electron paramagnetic resonance (EPR) spectroscopy. Ethanol did not scavenge superoxide. For the polyphenols catechin, epicatechin, gallic acid, and quercetin, we find rate constants of respectively $2.3*10^4$, $2.2*10^4$, $2.3*10^3$ and $1.9*10^4$(mole per second)$^{-1}$. Polyphenols can only exert a significant scavenging effect, if the plasma concentration reach sufficiently high levels. At concentrations found *in vivo* (low nanomolar range), the scavenging of superoxide by polyphenols and ethanol is negligible in comparison with endogenous protection against superoxide. Incubation of cultured endothelial cells with 5μmol/L of catechin, epicatechin, gallic acid, quercetin, or ethanol 0.05% (v/v) did not influence the maximal production of NO by these cells as measured by fluorescent nitric oxide cheletropic traps (FNOCT). The observed antiatherogenic effects must be caused by a mechanism other than direct scavenging of superoxide or influence on maximal endothelial NO production.

Keywords: Antioxidants; Atherosclerosis; Polyphenols; Superoxide rate-constant; Wine

1. Introduction

Epidemiological studies have shown that moderate intake of red wine correlates with reduced incidence of coronary heart disease [1−3]. Moderate consumption of red wine has been shown to reduce atherosclerosis and coronary mortality in both animal and human models [2,3]. Despite these observations, the mechanism by which red wine consumption exerts its antiatherogenic effect remains unclear. It is hypothesized that these antiatherogenic effects reside within the action of polyphenols and ethanol, the main constituents of red wine. Polyphenols are a class of compounds naturally found in many foods in the human diet. They can be found in considerable amounts in green vegetables, fruits, vegetable oils from olives, soybeans, chocolate, green tea, and red wine [4]. The polyphenols are well known for the beneficial effect on health. Polyphenols have been implicated in the dietary protection against coronary heart disease [4−6]. Many authors consider the antioxidant effect of the polyphenols as the major contributing antiatherogenic effect. For example, several studies claim that red wine protects human LDL against peroxidation *in vitro* as well as *in vivo* [7,8]. This finding has been disputed by others [9,10]. In the present study, we have addressed the pressing question as to which mechanism can account for the ameliorative effect of the polyphenols and ethanol on atherosclerosis. Several mechanisms have been proposed. One of these mechanisms is based on the assumption that polyphenols or ethanol are antioxidants acting as direct scavengers of the superoxide radical. An alternative mechanism assumes that polyphenols or ethanol may have antiatherosclerotic properties via enhancing endothelial nitric oxide (NO) production. Such enhancement of endothelial NO production is generally considered to be antiatherosclе-

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rotic. We have determined the reaction constants of superoxide and the influence on endothelial NO production with some polyphenols found in red wine. Data concerning the antioxidant capacity of several polyphenols in a lipid environment have been reported [11]. Data in an aqueous environment are scarce and consider nonphysiological conditions, so that the implications for human physiology remain unclear. In this work we have concentrated on the capacity to scavenge superoxide in aqueous physiological conditions. The reaction constants were determined in a xanthine oxidase assay using spin trapping and electron paramagnetic resonance (EPR) spectroscopy. From the reaction rates, we are able to estimate the total scavenging capacity for superoxide, which might be achieved in plasma by dietary uptake of polyphenols. Influence of polyphenols on maximal endothelial NO production measured with the help of fluorescent nitric oxide cheletropic traps (FNOCT) was determined after 24-hour incubation with different polyphenols and ethanol.

2. Methods and materials

2.1. Chemicals

Catechin, epicatechin, 5,5-dimethyl-1-pyrroline N-oxide (DMPO), gallic acid, hypoxanthine (HX), quercetin, L-arginine, Nω-nitro-L-arginine-methyl-ester (L-NAME) and xanthine oxidase (grade I, from buttermilk) (EC 1.1.3.22) (XO) were obtained from Sigma (St. Louis, MO). Calciumionophore A23187 was obtained from Calbiochem (La Jolla, CA), ethanol from Merck (Darmstadt, Germany), and 2-amino-2-(hydroxymethy l)-1,3,-propanediol (Tris) from Roche (Mannhein, Germany). The total wine polyphenol mixture (grape polyphenol WSP1) was obtained from Cybercolors (Cork, Ireland), and bovine serum albumin (BSA) from ICN (Aurora, IL). FNOCT, an absolutely specific trap for the detection of NO, was generously given by Dr. Hans Gert Korth (Department of Organic Chemistry, Essen University, Essen, Germany). Cell culture materials were obtained from Gibco (Paisley, UK).

2.2. EPRM measurements

The EPR spectra were recorded at 37°C on a modified Bruker ESP 300 spectrometer as described previously [12]. All EPR experiments were performed in triplicate in 10 mmol/L Tris buffer (pH 7.4, 37°C) containing 100 mmol/L DMPO in a total reaction volume of 100 μL. Of this reaction mixture 30 μL was used as sample volume for EPR assessment. Superoxide radicals were generated by 50 mU/mL XO in the presence of excess HX (0.5 mmol/L). In the HX-XO system, the spin trap adducts were formed by trapping of superoxide, as the formation of adducts could be prevented by the addition of superoxide dismutase.

2.3. NO detection with FNOCT

FNOCT is a molecular trap for NO, the reaction product being strongly fluorescent in the visible region. FNOCT fluorescence was measured in the supernatant using a fluorescence microtiter plate reader (Flex Station, Molecular Devices, Sunnyvale, CA). The NO-FNOCT adduct was excited at a wavelenght of 340 nm and the emission was set to the emission maximum (455 nm) of the adduct.

2.4. Determination of superoxide trapping rates by competitive superoxide trapping

In competitive superoxide trapping (CST), the trapping rates for superoxide were determined by comparing the trapping efficiency of ethanol, catechin, epicatechin, gallic acid, and quercetin with the known trapping efficiency of the spin trap DMPO. In a XO solution (10 mmol/L Tris buffer, pH 7.4, 37°C), excess HX substrate induces a steady and constant rate of superoxide production. In presence of a compound with the capacity for scavenging superoxide (such as ethanol, catechin, epicatechin, gallic acid, or quercetin), the generation of DMPO radical adducts is reduced by an amount that depends on the concentration and reaction rate of the compound. The EPR intensity in the HX/XO system starts from zero and increases with time toward a steady state asymptotic spin adduct concentration. The time dependence is given by a single-exponential with a time constant, $\tau = 7.0 \pm 0.5$ min at pH 7.4, 37°C. This time constant presents the average lifetime of a spin adduct in the solution, and proves that the adducts have a reasonable stability in this assay. Assuming that the rate of superoxide production is not affected by the presence of the scavenger (ethanol, catechin, epicatechin, gallic acid, or quercetin), the EPR intensity of the DMPO adduct is given by the following formula:

$$I(0)/I(X) = 1 + (k/k_d) \cdot X \quad (1)$$

where X = [scavenger]/[DMPO] is the ratio of the scavenger and DMPO concentrations, respectively. I(0) is the EPR intensity obtained in absence of the scavenger (X=0). The reaction rates with superoxide are given by k and k_d for the scavenger and DMPO respectively. There is considerable uncertainty in the literature with regard to the absolute value of the rate constant for superoxide trapping by DMPO (k_d). Certainly, this reaction rate depends sensitively on pH [13] and temperature. We are confident that a value of $k_d = 50(M.s)^{-1}$ is reliable. Certainly, the protonated form HO_2. is trapped by DMPO with a far higher rate than superoxide itself, so that pH is a very important parameter. At pH 7.4 we consider the DMPO trapping rate of 26 $(M.s)^{-1}$ (at 25°C) to be the most reliable [13]. As a temperature increase of 12° will increase the rate further by a factor of 2, k_{dmpo} is estimated to be 50 $(M.s)^{-1}$ for the trapping of superoxide by DMPO at pH 7.4 and 37°C. The value of k, that is, the reaction rate of the test compound with super-

oxide, may now be determined by plotting the ratio I(0)/I(X) as a function of the concentration ratio X. Before the EPR experiments, quercetin was firstly dissolved in 96% ethanol and subsequently diluted to 50% ethanol with 10 mmol/L Tris buffer pH 7.4.

2.5. XO and uricase activity

In eqn. [1] it is assumed that the test compound does not affect the rate of superoxide production by XO. In particular, the test compound should not act as an inhibitor of the enzyme. Therefore, we determined the enzymatic activity of XO independently from the formation of superoxide by measuring urate, using the uricase– hydrogen peroxide assay [14]. All experiments were performed in triplicate. The samples were measured at the Vitros-950 system (Ortho Clinical Diagnostics, Rochester, NY). The incubations were performed in a 10 mmol/L Tris buffer pH 7.4 containing 80 mU/mL XO, 7.5 mmol/L HX, and a concentration range of the test compound used in the superoxide trapping rates experiments. Urate production proceeded during 30 minutes of incubation at 37°C. The reaction was terminated by placing the test tubes on ice. The urate levels were determined immediately after termination of the incubation. In all cases, we checked carefully whether the scavenger interferes with this uricase hydrogenperoxide assay. This was done by a control experiment in which the same concentration series of the scavenger was added to a solution containing a known quantity of urate. This known quantity of urate was subsequently determined using the uricase–hydrogen peroxide assay. Of the polyphenols used by us, gallic acid interfered with the uricase assay: the quantity of urate was underestimated, showing that gallic acid acts as an inhibitor of the uricase enzyme. This phenomenon is reminiscent of the uricase inhibition by gentisic acid (a warning is found in the application note for the uricase assay of the supplier). The analysis of our data has been corrected for the uricase inhibition by gallic acid.

2.6. Cell culture

Immortalized murine microvascular endothelial bEND.3 [15,16] cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mmol/L L-glutamine, and 100 IU/mL penicillin, and 100 μg/mL streptomycin and split 1:4 to 1:8 upon reaching confluence. Culture media was replaced every 2 to 3 days. Cells were cultured at 37°C under humidified conditions containing 6% oxygen (6 kPa, normal physiological level) and 5% CO_2 (Sanyo MCO175M multigas incubator, Sanyo, Osaka, Japan). It has been reported that bEnd.3 cells express high levels of eNOS but do not express a detectable amount of iNOS [17]. For the incubation experiments, the cells were grown on 48-well plates (Costar, Acton, MA).

2.7. Influence of polyphenols on endothelial cell NO production

Confluent endothelial cells were incubated with either 5 μmol/LμM of catechin, epicatechin, gallic acid, or quercetin, 2mg/L polyphenol mixture, or 5 ‰ (v/v) ethanol in DMEM plus 10% FCS and penicillin/streptomycin during 24 hours. Each incubation experiment was performed 12 times. After 24 hours the medium was exchanged and cells were washed twice with a buffer solution containing 20 mmol/L Tris, 133 mmol/L NaCl, 6.5 mmol/L KCl, 1 mmol/L CaCl2, 1 mmol/L MgCl2, 5.5 mmol/L glucose, and 0.1 % (w/v) BSA (pH 7.4). To each well, 50μmol/L FNOCT [18−20] was added, and to one half of the wells 2.5 mmol/L of the NO synthase inhibitor L-NAME was also added. NO production was stimulated by the addition of 5 μmol/L calciumionophore A23187. Formation of NO-FNOCT adducts was measured by fluorescence 45 minutes after stimulation of the cells. NO production was determined as the difference in fluorescence intensity between cells incubated with and without L-NAME.

2.8. Statistical analysis

Data are reported as means ± SD. The Student *t* test was used for analysis.

3. Results

3.1. Effect on XO activity

Catechin, epicatechin, the polyphenol mixture, ethanol, and DMPO do not influence the superoxide production from XO in the concentration range used in the rate constant experiments (data not shown).

The situation is more complex with gallic acid, which acts as an inhibitor of the uricase enzyme. If not corrected for, this inhibition would easily be mistaken for reduction of XO activity. After careful calibration of the uricase assay at known urate concentrations, we find that gallic acid does not inhibit the superoxide production by XO. In contrast, quercetin does inhibit the XO enzyme with a half maximum inhibiting concentration IC_{50}= 200 ± 25 μmol/L (pH 7.4, 37°C) determined from a concentration series as shown in Fig. 1. This inhibition persists even in the presence of plasma protein, as shown by an additional experiment in which bovine serum albumin (BSA, 15 mg/mL) was added to the reaction mixture. This inhibition can be fully attributed to quercetin, as BSA itself does not affect the enzymatic activity of XO. We note that the value of IC_{50} is strongly dependent on the pH of the assay. At pH 7.4 and 37°C, we find that quercetin inhibits the urate production by XO with an IC_{50} of ~200 μmol/L. This value is an order of magnitude greater than inhibitions quoted by Robak et al. (IC_{50}= 10 μmol/L) [21] and by Chang et al. (IC_{50}= 7.23

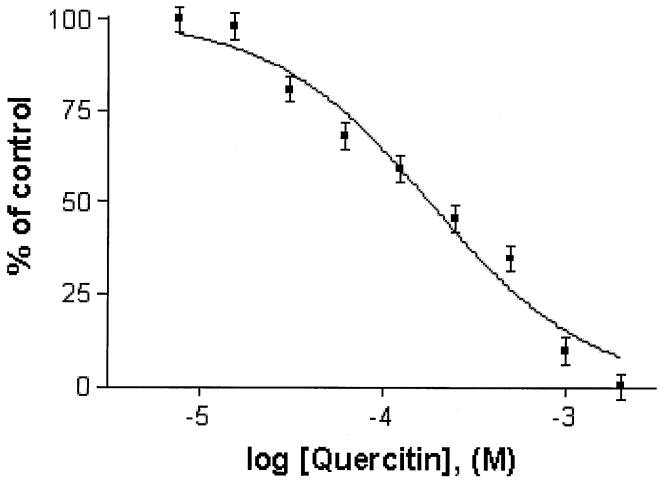

Fig. 1. Effect of quercetin on urate production by XO. Values are expressed as the percentage of urate forming activity in the control. The experiments are performed in triplicate.

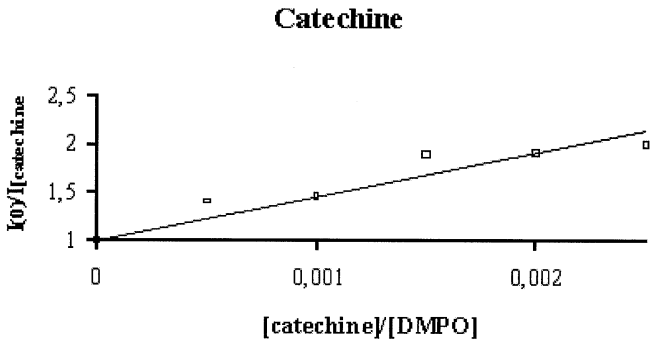

Fig. 2. Competitive spintrapping. Plot of the ratio I(X=0)/I(X) *versus* [X]/[DMPO], where I(X) is the steady state EPR intensity in presence of catechin and X is the concentration of catechin. A steep slope indicates a high reaction rate with superoxide. The experiments are performed in triplicate.

μmol/L) [22]. Both groups of investigators derived their values at pH 7.8 and ambient temperature. To account for the different conditions, we have studied the pH and temperature dependence of the XO activity as well as carefully calibrating the uricase assay. First, urate production was independently quantified via the photoabsorption by urate at a wavelength of 295nm (molar extinction ϵ = 9600)[23], and full agreement with the uricase assay was found in absence as well as presence of quercetin. Having verified the uricase assay, the effect of pH and temperature was studied on the urate production by 80 mU/mL XO in 0.1 mol/L phosphate buffer (Table 1). A reduction in temperature gives a modest reduction in XO activity, whereas the small increase in pH from 7.4 to 7.8 increases the turnover rate by a factor of ~1.5. These data show that the enzymatic activity of XO depends very sensitively on the ambient acidity. This sensitivity is also reflected in the inhibitory effect of quercetin: We find that IC_{50} is ~200 μmol/L at pH = 7.4 and independent of the temperature. The increase in pH to 7.8, however, enhances the inhibition by quercetin enormously, resulting in a reduction of IC_{50} to only 25±8 μmol/L (ambient temperature). This number is in reasonable agreement with the values reported by Robak et al. and Chang et al. Nonetheless, it is clear that quercetin has an only modest capacity to inhibit XO at the physiological pH 7.4 of plasma.

Table 1
Effect of pH and temperature on the urate production by 80 mU/mL XO in 0.1 mol/L phosphate buffer

	Ambient Temperature	37°C
pH 7.8	162 μmol/L	190 μmol/L
pH 7.4	101 μmol/L	146 μmol/L

All experiments were performed in duplicate.

3.2. Superoxide trapping rates

First, we observed that the addition of ethanol did not affect the formation of DMPO adducts (data not shown), even at very high concentrations (up to 2.2 mol/L). We conclude that ethanol has no significant scavenging capacity for superoxide. It means that the reaction rate must be smaller than a limiting value defined by the detection threshold of our EPR experiment. We find that that $k_{(Ethanol)} < 2.3\ (M.s)^{-1}$, and it may be even zero. For catechin, epicatechin, gallic acid, and quercetin however, we do find significant superoxide scavenging. Our experimental data are well described by the linear relationship given by eqn [1]. An example is shown for catechin in Fig. 2. From the slopes, and using $k_d = 50(M.s)^{-1}$, we have estimated the rate constants for scavenging of superoxide (valid for a temperature of 37°C and pH of 7.4) (Table 2). We now address the question of whether the scavenging capacity of the complete polyphenol extract can be explained by the scavenging capacity of its main four components. For the polyphenol extract, we also find significant superoxide scavenging: addition of 100 mg/L polyphenol-mixture reduces the number of DMPO adducts by 25%. This scavenging capacity is equivalent to that of a vitamin C concentration of ~5 μmol/L (for comparison of the various compounds we now define a convenient measure for scavenging as VCE, the vitamin C equivalent. At 100 mg/L, the polyphenol extract has a VCE = 5 μmol/L). The extract consists of a mixture of many compounds with different molecular weights (Table 2). In such a mixture of 100 mg of polyphenols per liter the four main constituents contribute a combined scavenging equivalent of VCE = 0.68 μmol/L. Therefore, these four constituents account for approximately 14% of the total scavenging capacity of the mixture. We conclude that a substantial degree of scavenging is due to other, yet unidentified compounds in the mixture.

Table 2
Rate constants for polyphenols with superoxide and VCE (vitamin C equivalent) for polyphenols

	Mw	Rate Constant $(M.s)^{-1}$*	Conc. (mg/L)†	Conc. (μmol/L)†	VCE (μmol/L)†	%
Gallic acid	170.1	$2.3\ 10^3$	0.35	2.06	0.02	0.4
Catechin	290.3	$2.3\ 10^4$	1.1	3.8	0.38	7.6
Epicatechin	290.3	$2.2\ 10^4$	0.74	2.5	0.25	5.0
Quercetin‡	338.3	$1.9\ 10^4$	0.13	0.38	0.026	0.5
Other§					4.32	86.5
				Sum:	5	100

* Rate constants for polyphenols with superoxide (at pH 7.4 and T = 37°C). Rate constants are experimentally determined in competitive spintrapping experiment.

† Concentrations and VCE for polyphenols in solution containing 100-mg polymixture/L. VCE are computed from rate constants and concentrations. Experiments are performed in triplicate. Data on composition of the mixture are provided by Dr M. van de Westelaken (personal communication).

‡ Corrected for the inhibiting effect on XO.

§ Unidentified compounds.

Conc = concentration.

3.3. Effect of polyphenol incubation on NO production by endothelial cells

Incubation of cultured endothelial cells during 24 hours with either 5 μmol/L catechin, epicatechin, gallic acid, or quercetin, 2 mg/L polyphenol mixture or ethanol 0.5‰ (v/v) does not significantly influence the maximal NO production by these cells stimulated by 5 μmol/L calciumionophore A23187 as determined by the FNOCT fluorescence (Fig. 3).

4. Discussion

In this study, we determined the rate constant of four polyphenols and ethanol in the reaction with superoxide at 37°C and pH 7.4 using EPR spectroscopy in combination with the competitive spin trapping technique and the effect of these polyphenols on endothelial NO production.

To estimate whether our polyphenols can provide significant protection against superoxide *in vivo*, it is necessary to see them in perspective of their respective plasma concentrations and a known antioxidant such as vitamin C. Information about plasma levels of the different polyphenols after consumption of red wine is scarce. The bioavailability of the polyphenols is poor [24,25], for wine it is estimated to be ~0.8% [24]. After consumption of wine, a maximum catechin plasma concentration of ~75 nmol/L is reported [26]. Far higher plasma concentrations of ~2μmol/L have been reported after consumption of a complex Mediterranean meal [27]. For quercetin consumption of wine results in a maximum plasma concentration of 26 nmol/L [24]. Again higher plasma levels of ~370 nmol/L are achieved by consumption of a complex meal [28], consumption of a single dose of 20 mg quercetin results in a maximal plasma concentration of approximately 150 nmol/L [29]. For gallic acid and epicatechin no data are reported for the plasma concentrations after consumption of wine. However, based on data from studies on other foods and beverages, plasma levels are estimated in the low micromolar range [25,30]. For ethanol, the legal upper plasma concentration limit for driving a motor vehicle in the Netherlands is 0.5‰ (v/v). This corresponds to [ethanol] ~10 mmol/L. In comparison, vitamin C has a rate constant of $2.7\ 10^5\ (M.s)^{-1}$ [31] and a plasma concentration of approximately 40–70μmol/L [32], intracellular vitamin C concentrations are reported to be in a range of a few mmol/L [31]. For example, intracellular vitamin C concentrations are as high as 4–6 mmol/L in human white blood cells [33]. Because a major part of the biological processes take place intracellularly, it is important to relate the biological effect of polyphenols to their intracellular concentration. However, information concerning intracellular polyphenol concentrations is scarce. To our knowledge, only data concerning intracellular and tissue concentrations of quercetin exist, obtained from *in vitro* and animal studies. *In vitro* studies show that quercetin is readily taken up by erythrocytes and accumulates to tenfold concentrations in the cytosol [34,35]. A study in rats showed that quercetin can be recovered in various tissues [36]. When rats were fed with a diet containing very high amounts of quercetin (5 g/kg), kidney concentrations of

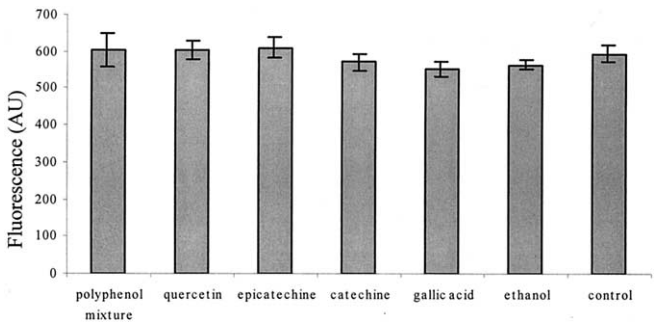

Fig. 3. NO production by cultured endothelial cells. Endothelial cells were incubated for 24 hours with a polyphenol, a polyphenol mixture or ethanol. Each value is the mean (±SD) of the difference between six different wells stimulated with calciumionophore (5 μmol/L) and six wells stimulated with calciumionophore (5 μmol/L) in presence of the NO synthase inhibitor L-NAME (2.5 mmol/L).

quercetin reach 15.4 nmol/mg protein, and plasma levels reach 15.3 nmol/L. We note that the uptake and distribution of a particular substance into the membranes and the cell is strongly related to its lipophilicity, which is expressed as the partition coefficient. Liao et al. have determined the partition coefficients of the polyphenols involved in our study [11]. This study showed that quercetin was the most lipophilic compound (partition coefficient: 3.84) followed by epicatechin (0.94), catechin (0.60), and gallic acid (0.45). Judging from these data, it seems unlikely that epicatechin, catechin, or gallic acid reach higher intracellular concentrations than quercetin. In fact, it is unknown whether these polyphenols accumulate intracellularly at all.

Combining our rate constants and the intracellular or plasma levels of the individual polyphenols, we find that the *in vivo* protection against superoxide by catechin, epicatechin, gallic acid, and quercetin is negligible (<1%) in comparison to that of vitamin C under physiological conditions and concentrations. The rate constant of ethanol with superoxide is virtually zero. Therefore, a superoxide scavenging effect of ethanol can be ruled out. There have been some previous reports on rate constants of polyphenols in reaction with superoxide [37,38]. However, these experiments were performed at unphysiological pH and temperature and are therefore the relevance for human physiology remains uncertain.

Some authors have suggested that polyphenols might enhance endothelial NO production. Reports on the effect of polyphenols on isolated vessels indicate that polyphenols enhance vasodilation via a NO mediated mechanism [39−41]. In contrast, our data show that polyphenols and ethanol do not directly enhance the stimulated NO production by cultured endothelial cells. Some authors suggest that polyphenols enhance endothelial NO production indirectly by raising intracellular Ca^{2+} levels [42,43]. This would be compatible with our observations: with calciumionophore as our stimulus, the endothelial NO production is independent of intracellular Ca^{2+} levels. Therefore, our data do not exclude enhancement of endothelial NO production via an effect on intracellular Ca^{2+} levels.

In conclusion, our data show that polyphenols do have some capacity to scavenge superoxide but insufficient to make an impact for human physiology. Neither the polyphenols nor the ethanol have significant scavenging rates for superoxide at physiological concentrations. In addition, polyphenols nor ethanol affect stimulated NO production from cultured endothelial cells. Therefore, the antiatherogenic effects of red wine cannot be explained by direct scavenging of superoxide or a direct effect on maximal endothelial NO production by the polyphenols.

Our data also show that inhibition of xanthine oxidase by polyphenols is unlikely to have any significance *in vivo* as previously suggested [22,44]. The undisputed beneficial effect of red wine consumption on coronary heart disease and atherosclerosis must be explained by mechanisms other than direct scavenging of superoxide.

Acknowledgments

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