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Inhibition of endothelial NADPH oxidase as target of flavonoids in nitric acid metabolism[2]

1. Introductory remarks: What is reason for the health benefit of dietary flavonoids? A paradigm shift

The foregoing article was focused on the modulation by dietary flavonoids of generation and biological role of plasma lipoproteins and the consequences as to the risk for cardiovascular diseases. The objective of the second article of this series is to gain deeper insight into the cellular and molecular action of flavonoids and other dietary polyphenols and their bioactive species, respectively.

A decade ago, the beneficial effects of flavonoids were believed to be attributable to free radical-scavenging properties, which are observed in numerous *in vitro* assays. Evidence for a direct antioxidant activity *in vivo*, however, is poor. By estimation of parameters *in vivo* or *ex vivo* believed to be appropriate, many efforts were undertaken to demonstrate an attenuation of free radical-mediated processes upon intake of dietary polyphenols. The outcome of such studies was throughout disappointing, i.e. the effects were either lacking or only marginal, even though some authors interpreted their data in an exaggerated manner as "positive". This tendency was promoted by the pressure of the investigators to achieve positive results for obtaining financial support, enabling them to sustain polyphenol research. Moreover, in many studies the selection of clinical parameters was far from reasonable. As an example, the widely used "thiobarbituric acid-reactive substance" (TBARS) assay provides unreliable data for several reasons, which will not be discussed in detail here. For detection of lipid peroxidation *in vivo* the plasma level of F_2 isoprostanes may be the parameter of choice, since these arachidonic acid oxygenation products actually originate from non-enzymatic free radical-mediated attack and can be unequivocally discriminated from eicosanoids generated by enzymes of the arachidonic acid cascade [Roberts & Fessel, 2004; Basu, 2004]. For this reason, we conducted in Magdeburg a clinical study on the effect of intake of a high-flavanol cocoa beverage on plasma F_2 isoprostanes [Wiswedel et al, 2004]. Although the volunteers were subjected to physical exercise by treadmill as a free radical-generating process, sizable lowering of the F_2 isoprostane level was not observed. By contrast, the same type of high-flavanol cocoa exerted clear-cut beneficial effects in other clinical trials (see Section 3). Detailed statistical analysis of the data from the Magdeburg study, however, revealed that the cocoa flavanols prevented the increase in F_2 isoprostanes induced by the low-flavanol control drink, an effect interpreted as postprandial oxidative stress [Sies et al, 2005a].

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[2] This article was subject of an invited lecture at The Rank Prize Funds Symposium on "Dietary Flavonoids: Mechanisms of Action and Human Health", Grasmere, UK, 19th to 22nd April 2010, but could not be delivered owing to an unforeseen transportation problem (Iceland volcano ash cloud). Part of the data was also presented at the Leibniz Sozietaet, Berlin, on 12th February 2009.

A number of clinical studies included the "total antioxidant capacity" (TAC) or closely related parameters in blood plasma for evaluation of antioxidant activity of dietary flavonoids *in vivo*. This approach is however absolutely useless for this purpose (for arguments, see [Sies, 2007] and refs. therein). Such assays quantify the total amount of reducing or radical-absorbing compounds in plasma rather than the antioxidant systems of the organism including enzymes that are really responsible for counteracting pro-oxidative insults. The plasma TAC is mainly determined by the plasma level of urate and, to a minor extent, of ascorbate, whereas the contribution of micronutrients such as dietary polyphenols and their metabolites is negligible. Increase in TAC occurring after consumption of tea or flavonoid-rich foods, however, as reported by some investigators, indicates most probably increase in urate and must be regarded – when solely restricted to this parameter – as deleterious rather than beneficial because high urate may cause gout and is also discussed as a risk factor for cardio- and cerebrovascular diseases.

The difficulties to verify the importance of free radical-scavenging activity by dietary polyphenols *in vivo* contrast the recent promising data of both epidemiologic studies and clinical trials showing beneficial effects on vascular endothelial function. In a number of studies, favorable modulation of nitric oxide metabolism and of cell signaling pathways was identified as targets of dietary polyphenols or their plasma metabolites, respectively. This led to a reappraisal of the role of these micronutrients. In **Table 1** the current view is opposed to the traditional view demonstrating that a paradigm shift has taken place. The various aspects of this overview will be commented in the next parts of this article. The inhibitions of lipoxygenases and of myeloperoxidase-initiated processes were already subject of the foregoing article.

Table 1: ***Paradigm shift in flavonoid research*** **(from [Schewe et al, 2008])**

Concept	**Current view**	**Traditional view**
Flavonoids as radical scavengers	Minor, if at all relevant *in vivo*	Major action
Flavonoids as antioxidants	Yes, but in an indirect sense (inhibition of prooxidant enzymes such as NADPH oxidases, lipoxygenases and myeloperoxidase)	Major action
Flavonoids as enzyme inhibitors	Few key enzymes, particularly NADPH oxidases	Many enzymes
Target in cell metabolism	•NO metabolism	Lipid peroxidation
Target in •NO metabolism	•NO loss ↓	NOS activity↑

Role of flavonoid glucuronides	Transport metabolites in blood plasma to target cells	Urinary excretion products

2. What is the message from epidemiological studies?

Epidemiological studies revealed an inverse correlation between dietary intake of flavanols and cardiovascular diseases including cardiac mortality. The outcome of the Zutphen Elderly Study in The Netherlands [Buijsse et al, 2006] and a similar study from U.S.A. [Mink et al, 2007] were mentioned in the foregoing article. Other epidemiological studies suggest such a correlation also for flavonoids other than cocoa flavanols [Knekt et al, 2002; Huxley & Neil, 2003; Maron, 2004]. It must be stressed, however, that this correlation is an association and does not prove cause-effect relationship. High intake of polyphenols may also reflect a more general lifestyle favorable for cardiovascular health. Thus, in the Mediterranean diet the high intake of oleic acid and of n–3-polyenoic fatty acids and a comparatively low intake of saturated fatty acids are likewise important for cardiovascular health. Furthermore, a high intake of cocoa products could be rather associated with non-smokers than with smokers. Therefore, clinical studies are required to identify flavonoids as bioactive health-promoting ingredients of foods. Moreover, the cellular and molecular targets of the flavonoids or their metabolic conversion products need to be identified.

Since getting permission for clinical studies is sometimes difficult, studies with laboratory animals are an alternative. Unfortunately, an unfavorable study design limits the value of a number of animal studies reported. Thus, it is not wise to feed animals with quercetin. Although quercetin belongs to the most abundant flavonols in foods, it is never contained as free compound in sizable amounts. In relevant sources such as onions or buckwheat it is contained as glycosides, which are absorbed from the intestine via special pathways. For this reason, intake of onions and that of free quercetin lead to different profiles of quercetin metabolites. Moreover, free quercetin is quite more toxic than the corresponding flavan-3-ols, (–)-epicatechin and (+)-catechin, and must not be administered to humans. Another limitation is the species-dependence of flavonoid metabolism. While in humans a major (–)-epicatechin plasma metabolite is (–)-epicatechin-3'-glucuronide, the respective metabolite in rats is (–)-epicatechin-7-glucuronide [Natsume et al, 2004]. This structural difference has profound consequences for the chemical behavior of the two metabolites. The rat metabolite has retained the catechol structure responsible for radical-scavenging and metal-chelating properties, whereas these features are lost for the human metabolite.

3. High-flavanol cocoa drink ameliorates endothelial function in vivo through improved bioactivity and bioavailability of nitric oxide

A variety of cardio- and cerebrovascular diseases such as coronary heart disease and stroke are closely connected with arterial endothelial dysfunction, which is also a pre-stage of atherosclerosis. Stability and functionality of the vascular endothelium essentially depends on multiple functions of nitric oxide (•NO, formerly designated as "endothelium-derived relaxing factor"), which is formed mainly in endothelial cells and serves at the same time as mediator of cell signaling, e.g. for smooth muscle cells. It is responsible for widening of arterial vessels to permit sufficient blood flow and, hence, continuous oxygen supply for the aerobic energy metabolism of heart, brain, extremities and other organs. Although chemically being a relatively stable radical, •NO is capable of reacting with a number of dif-

ferent compounds such as thiols and amines as well as with $\bullet O_2^-$ forming nitrosothiols, nitrosoamines and peroxynitrite, respectively. For this reason, •NO is a short-lived mediator in living systems. The maintenance of a sufficiently high steady-state level of •NO is a prerequisite for preservation of endothelial function. The steady-state level of bioavailable •NO is determined by two processes, synthesis of •NO mainly from l-arginine, and elimination of •NO via several reactions. The aim of any dietary or pharmacologic intervention is to improve the beneficial bioactivity of •NO without enhancement or even with attenuation of the formation of •NO-derived deleterious compounds like peroxynitrite and $\bullet NO_2$.

This scenario prompted us to look at a presumed modulation of bioactivity and bioavailability of •NO by high-flavanol cocoa. A double-blind placebo-controlled crossover study was performed in Duesseldorf in collaboration with the cardiologists of the same university [Heiss et al, 2003]. 20 individuals (11 males, 9 females) at risk for cardiovascular diseases (assignment according to the presence of risk factors such as history of coronary artery disease, hypertension, hypercholesterolemia, diabetes mellitus or smoking) received either a high-flavanol cocoa drink or a low-flavanol control drink. Before and 2 h after intake of the beverage the flow-mediated dilation (FMD) of the brachial artery was non-invasively measured with high-resolution ultrasonography after 5 min forearm occlusion by means of a cuff. This method is a well established approach to assess the vascular endothelial function in cardiology. A low FMD value indicates diminished function or even endothelial dysfunction. Besides FMD, the plasma concentration of organo-nitroso compounds (RNO) was determined, which are known to originate mainly from oxidative protein nitrosation. The data are summarized in **Fig. 1**. Before administration of the cocoa drinks, the FMD values were lower than those of healthy subjects defining the test persons to be afflicted by endothelial dysfunction. Upon intake of the high-flavanol drink, but not of the low-flavanol control drink, there was a highly significant increase in FMD (from 3.4 ± 0.5% to 6.3 ± 0.6%; $P < 0.001$), which was dependent on the dose of high-flavanol cocoa and peaked after about 2 h, thus intermittently approximating the average value of healthy control individuals. The plasma RNO levels behaved quite similar. The maximums coincided with that of the plasma levels of (–)-epicatechin metabolites.

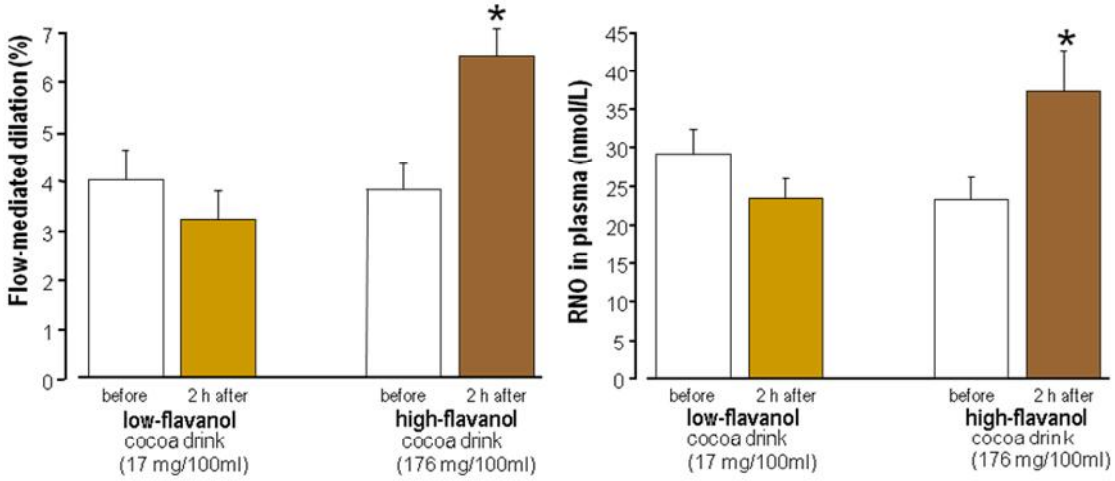


Fig. 1. Summary of the Duesseldorf double-blind randomized crossover study (n = 20) demonstrating for the first time that high-flavanol cocoa improves endothelial function through elevation of bioactivity and bioavailability of •NO. For details, see text as well as [Heiss et al, 2003] and [Sies et al, 2005b].

In a later study with smokers was shown that the increase in FMD upon intake of high-flavanol cocoa was abolished by administration of L-N(G)-monomethyl arginine (L-NMMA), an oral •NO synthase inhibitor [Heiss et al, 2005]. This observation indicates that the FMD mirrors the bioactivity of •NO in the arterial vascular endothelium. The dependence on •NO is also supported by the parallel increase in the plasma level of RNO. Although some authors believe that RNO would be an indirect source of bioactive •NO, it appears more likely that the RNO level is simply an indicator for •NO bioavailability, inasmuch as the generation of RNO requires a certain level of •NO.

Similar responses of FMD of the brachial artery were observed subsequently by [Engler et al, 2004], who administered high- and low-flavanol chocolates to healthy individuals over a two-week period. Vasodilation in healthy humans by high-flavanol cocoa drink has also been reported by Fisher et al, 2003. Here again, cocoa-augmented dilation was reversed by an inhibitor of •NO-synthases, L-NAME, which also led to increase in blood pressure. In another study, in elderly individuals afflicted with isolated systolic hypertension, dark chocolate (polyphenol-rich), but not white chocolate (polyphenol-free) caused pronounced lowering of blood pressure [Taubert et al, 2003]. A vasodilatory effect of dark, polyphenol-rich chocolate was also demonstrated for coronary arteries [Flammer et al, 2007], thus substantiating a role in cardiac protection. The multitude of clinical studies with high-flavanol cocoa products has been reviewed by [Ding et al, 2006] as well as by [Cooper et al, 2008].

Three major flavan-3-ols are contained in cocoa products, (–)-epicatechin, (+)-catechin and procyanidin B2 (formulas, see foregoing article). Since procyanidin B2 is very poorly absorbed from the intestine and (+)-catechin reaches lower plasma levels of its metabolites than (–)-epicatechin, the latter was the most probable candidate for being responsible for the biological actions on endothelial •NO. Final evidence came from a further clinical study performed in U.S.A. in collaboration with the Duesseldorf group [Schroeter et al, 2006]. In this study, (–)-epicatechin was isolated in pure form from high-flavanol cocoa and administered to the test subjects. At doses as low as 1 or 2 mg per kg body weight (–)-epicatechin produced comparable elevation of FMD as high-flavanol cocoa did [Schroeter et al, 2006]. In this context must be stressed that not every sort of chocolate is rich in polyphenols. Traditional processing of cocoa beans includes high temperature and alkali treatment, which destroys a large part of the polyphenols including the valuable (–)-epicatechin. More gentle procedures have meanwhile been developed, which preserve a high polyphenol content during processing.

Unfortunately, the rise in FMD upon single administration of high-flavanol cocoa is only transient; 6 h after intake the initial value is reached again. In a later study, the daily consumption of a flavanol-rich cocoa drink (3 x 306 mg flavanols/d) over 7 days was examined [Heiss et al, 2007]. Under these conditions FMD at baseline (after overnight fast and before flavanol ingestion) increased continuously from 3.7 ± 0.4% on day 1 to 6.6 ± 0.5% on day 8 and returned to 3.3 ± 0.3% after a washout week of cocoa-free diet (day 15). It was concluded from this study that the action of (–)-epicatechin on the bioactivity of •NO in the vascular endothelium involves two components: a short-term effect and a longer-term effect. **Table 2** describes the characteristics of these effects also considering the data from our *in vitro* investigations.

Taken together, from *in vivo* studies the following conclusions could be drawn:

(i) (–)-Epicatechin is a bioactive ingredient of cocoa polyphenols.
(ii) Vascular endothelium of conduit, capillary and coronary arteries is target of (–)-epicatechin.
(iii) Dietary (–)-epicatechin improves bioavailability and bioactivity of •NO by short-term and longer-term mechanisms.

Table 2: ***Dissection of a short-term effect from a longer-term effect of (–)-epicatechin***

- ‚Short-term' is the FMD response more or less online with the flavanol metabolite time-course in plasma (peaks at ca. 2 h, back to baseline at ca. 6 h)
- ‚Longer-term' is reflected by the baseline FMD as a consequence of repetitive flavanol intake over several days/weeks

Mechanisms:

Short-term:	Inhibition of endothelial NADPH oxidase activity
Longer-term:	Gene expression, e.g. eNOS protein level ↑, arginase ↓

Table 3: ***The vascular endothelium as target for polyphenol research***

- In man it comprises an area of up to 240 m^2
- Major site of •NO synthesis and action
- Borderline between plasma and vascular tissue
- Dysregulation of endothelial function may lead to cardiovascular diseases
- Endothelial cells accumulate (–)-epicatechin

4. Searching for modes of action of (–)-epicatechin

4.1 Why is the vascular endothelium a promising target for (–)-epicatechin?

The identification of (–)-epicatechin as bioactive ingredient of cocoa polyphenols as well as of the •NO metabolism of the vascular endothelium prompted us to conduct appropriate *in vitro* experiments to elucidate the mode of action of (–)-epicatechin. Cultured vascular endothelial cells were chosen. In Table 3 plausible arguments in favor of this choice are compiled. Both aortic endothelial cells of various species and human umbilical vein endothelial cells (HUVEC) were used.

4.2 How does epicatechin improve bioavailability of •NO in the vascular endothelium?

Other authors including some of our collaboration partner speculated from the *in vivo* observations that the cocoa flavanols would stimulate endothelial •NO synthase (eNOS). In fact, the dilatory effect in the FMD method is due to phosphorylation of Ser-1177 of eNOS protein leading to activation of this enzyme and, in turn, to increase in •NO synthesis. Therefore it appeared at first sight plausible that (–)-epicatechin could act at the same level. It must be stressed, however, that the steady-state concentration of a metabolite is not only determined by the rate of synthesis but also by the elimination rate. Hence it follows that there are two possibilities to explain the effect(s) of (–)-epicatechin: elevated production or diminished elimination of •NO in endothelial cells or even both. For this reason, our concept was addressed in two directions: (i) effects on •NO-generating enzymes (eNOS, iNOS), (ii) effects on •NO-diminishing enzymes (NADPH oxidases).

A careful study was performed by the group of R. Heller in Jena, to examine whether treatment of HUVEC with (–)-epicatechin or (+)-catechin leads to activation of eNOS by any mode of action. The following observations were achieved (data not published): (i) Epicatechin and catechin do not alter basal and shear stress-induced eNOS phosphorylation. (ii) Epicatechin does not activate either eNOS or soluble guanylyl cyclase in cell extracts. (iii) Epicatechin and catechin do not increase intracellular levels of tetrahydrobiopterin, a cofactor of eNOS. Thus, any effect at the level of •NO synthesis was excluded. Using aortic endothelial cells similar data were independently obtained by an Austrian group [Wippel et al, 2004].

Looking at the level of •NO elimination, we studied the effect on superoxide generation in HUVEC because $\bullet O_2^-$ reacts rapidly in a diffusion-controlled fashion with •NO to peroxynitrite that in turn isomerizes to nitrate. The $\bullet O_2^-$ release by endothelial cells was stimulated by either oxLDL or angiotensin II, which also belong to the physiologic agents to evoke atherogenesis and hypertension, respectively (see foregoing article). The data are shown in Fig. 2.

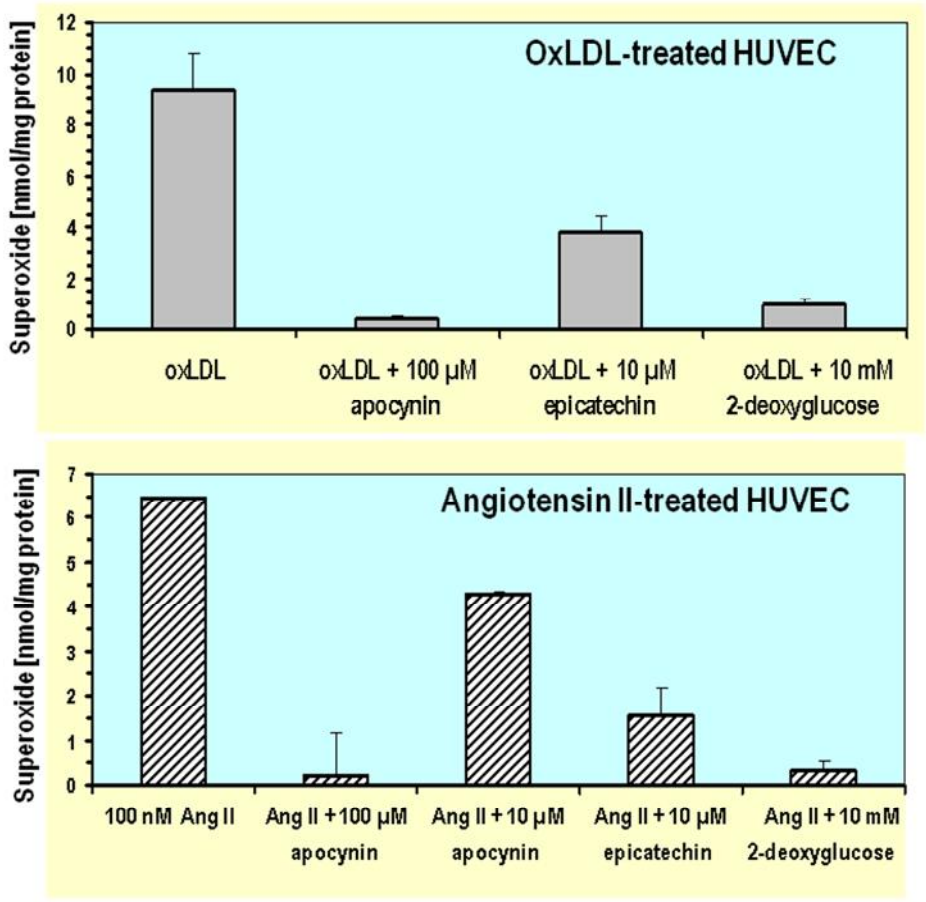


Fig. 2. Generation of $\bullet O_2^-$ in HUVEC elicited by myeloperoxidase/H_2O_2/nitrite-oxLDL and angiotensin II and the effects of (–)-epicatechin, apocynin and 2-deoxyglucose (from [Steffen et al, 2007a]).

In both systems pretreatment of the cells with (–)-epicatechin suppressed the superoxide release [Steffen et al, 2007a]. A similar effect was achieved with apocynin, a well established inhibitor on NADPH oxidase, but (–)-epicatechin was even active at a lower concentration. 2-Deoxyglucose, an anti-metabolite for glucose and metabolic inhibitor of the NADPH-generating oxidative pentose pathway, also strongly suppressed the superoxide release. These data clearly demonstrate that the $\bullet O_2^-$ release from endothelial cells requires both NADPH supply and active NADPH oxidase. In further experiments we have found that pretreatment of HUVEC with (–)-epicatechin protected the cells against angiotensin II-elicited protein carbonyl formation, whereas external superoxide dismutase did not protect, suggesting that $\bullet O_2^-$ generation, oxidative damage to proteins and protection by (–)-epicatechin occurred intracellularly, whereas the released portion of total $\bullet O_2^-$ was not involved [Steffen et al, 2008].

The protection against agonist-elicited $\bullet O_2^-$ generation and their pro-oxidative consequences required a pretreatment of the cells with (–)-epicatechin for several hours. Several possibilities had to be regarded to explain this time-dependence. We obtained evidence that intracellular metabolic conversion of the flavanol is implicated. (–)-Epicatechin is converted in HUVEC to 3'-*O*-methyl-epicatechin and 4'-*O*-methyl-epicatechin with the former predominating. These products were identified by LC/MS-MS and comparison with authentic standards [D. Sendker, unpublished data]. Moreover, catechol-O-methyltransferase (COMT) was identified as the enzyme involved. Two isoforms of COMT protein were found to be

expressed in HUVEC with the 30-kDa membrane-bound isoform (MB-COMT) predominating over the 25-kDa soluble isoform (S-COMT). Isolated COMT preparations converted (–)-epicatechin to the same product pattern as HUVEC did [E. Kravets, Master thesis, University of Duesseldorf, 2008]. This finding was surprising, since up to that time the formation of methylated flavonoid metabolites, which also belong to the abundant metabolites in blood plasma, was believed to be restricted to intestinal and liver cells, but not occurring in the vascular endothelium.

Next we addressed the issue whether the protection of endothelial cells by (–)-epicatechin and/or its B-ring methylethers elevates the steady-state level of •NO in these cells. For intracellular detection of •NO we applied the diaminofluorescein DAF-2 diacetate, which reacts after intracellular ester cleavage with •NO yielding a green-fluorescent triazolofluorescein [Nakatsubo et al, 1998]. Thus, the intensity of the green fluorescence within the cells correlates with the intracellular NO level. An increase in intracellular •NO was expected as being in line with the data from clinical studies using high-flavanol cocoa (see Section 3). As shown in **Fig. 3**, this was actually the case. Treatment of HUVEC with angiotensin II lowered the •NO level below the detection limit (control sample, left panel). Pretreatment with (–)-epicatechin, however, yielded a marked signal, which was attenuated when the COMT inhibitor 3,5-dinitrocatechol was present during preincubation. A similar effect was achieved with the established NADPH oxidase inhibitor apocynin without pre-incubation, and the strongest signal occurred with 3'-*O*-methyl-epicatechin, the authentic major (–)-epicatechin metabolite in HUVEC.

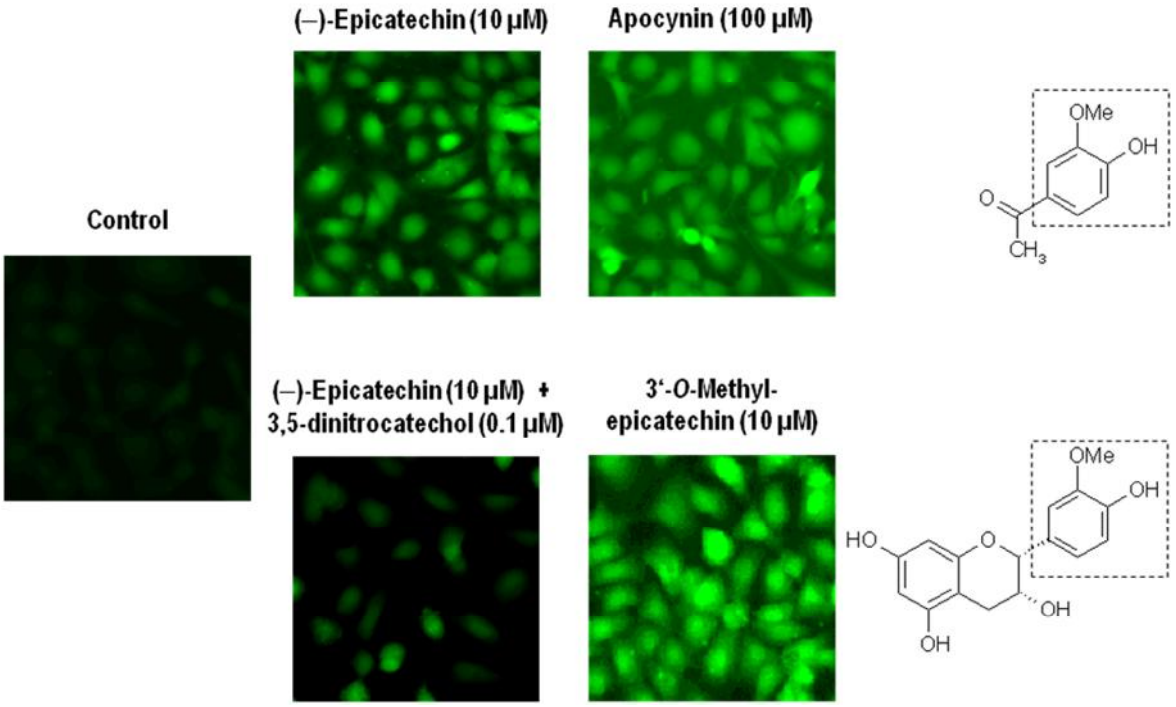


Fig. 3. Epicatechin elevates the •NO level in angiotensin II-stimulated HUVEC via conversion to an apocynin-like metabolite. Explanations see text. Note the structural similarity between apocynin and the epicatechin metabolite 3'-O-methyl-epicatechin (from [Steffen et al, 2007b]).

Although the data in **Fig. 3** suggest improvement of bioavailability of •NO by treatment with (–)-epicatechin, this observation does not compellingly mean that

the higher •NO level also implies higher bioactivity of this mediator. •NO signaling involves activation of guanylyl cyclase, which in turn generates cyclic GMP as second messenger that is also responsible for the vasodilatory effect of •NO. Hence, we also investigated the effect on the cyclic GMP level of the cells (**Fig. 4**). Here again, treatment of HUVEC with (–)-epicatechin (Epi) caused elevation like apocynin (Apo) did. The effect by (–)-epicatechin was abolished by the eNOS inhibitor l-NAME showing •NO dependence or by 3,5-dinitrocatechol (DNC) showing involvement of COMT.

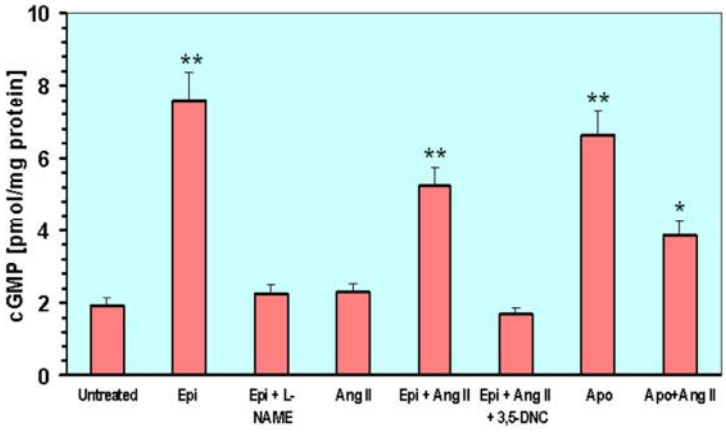


Fig. 4. Epicatechin elevates the cyclic GMP level in HUVEC. Explanations see text. Concentrations of agents were chosen as in Fig. 3. It must be considered that a basal value of ~2 pmol/mg protein is due to an •NO-independent portion of the cyclic GMP pool and does not reflect •NO response (from [Steffen et al, 2007b]).

Taken together, we have demonstrated that the improvement of bioavailability and bioactivity of •NO by high-flavanol cocoa is most likely due to (–)-epicatechin, which acts as a 'prodrug' for an inhibitor of endothelial NADPH oxidase that is endogenously generated in vascular endothelial cells.

4.3 Structural requirements for inhibition of NADPH oxidase by dietary polyphenols

It became evident from the foregoing data that endothelial NADPH oxidase activity must be the immediate target of the epicatechin metabolite. To gain deeper insight into the mechanism of action and structure-activity relations of the inhibitory effect, a reliable test system was needed. Most test systems from the literature are not suitable for this purpose. Many of them are based on quantification of $\bullet O_2^-$ generation. Applying such systems it would be impossible to discriminate between NADPH oxidase inhibition and $\bullet O_2^-$ scavenging, a property that is well known for a number of polyphenols, in particular of those having a catechol arrangement of the hydroxyl groups. Therefore we decided to measure the consumption of NADPH (Fig. 5). Such an assay is only possible in lysed cells because NADPH does not penetrate intact cells. Moreover, conditions must be chosen to achieve full enzymatic capacity. The NADPH oxidases constitute a family of multi-component complexes consisting of both membrane-bound and soluble protein components, and activation is achieved by binding of soluble components to the membrane complex (for review, see [Brown & Griendling, 2009]). For the isoform Nox2, activation is assumed to be initiated by phosphorylation of the cytosolic component p47phox via cell signaling followed by binding of the latter to

the membranous component p22phox and subsequent steps eventually leading to the membrane-associated enzymatically active multi-complex. The actiation routes of this or other NADPH oxidase isoforms can be short-circuited *in vitro* by the presence of 90 µM sodium dodecylsulfate, which acts under these conditions as anionic amphiphile to assemble the active complex [Park & Park, 1998].

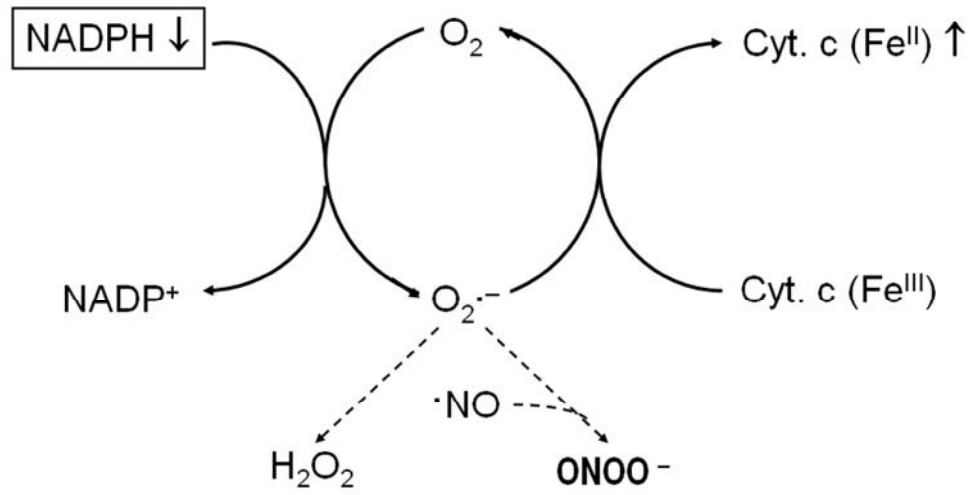


Fig. 5. Principle of measurement of NADPH oxidase activity in homogenates of endothelial cells for inhibitor studies without disturbance by superoxide-scavenging activities. Unlike with measurement of $\bullet O_2^-$ release from intact cells (Fig. 2), ferricytochrome c serves here as $\bullet O_2^-$ scavenger preventing accumulation of this dioxygen radical followed by conversion to deleterious species such as hydrogen peroxide and peroxynitrite.

Applying the NADPH oxidase assay outlined in Fig. 5, we tested (–)-epicatechin and its mono-methyl ethers for both NADPH oxidase inhibitory and $\bullet O_2^-$ scavenging activities. As shown in **Table 4**, (–)-epicatechin proved to be an $\bullet O_2^-$ scavenger as reported before by other authors, but did not inhibit NADPH oxidase activity, whereas the converse pattern was observed for the endothelial cell and plasma metabolites 3' and 4'-*O*-methyl epicatechin.

Table 4: ***Anti $\bullet O_2^-$ activities of epicatechin and its methyl ethers (from [Steffen et al, 2008][3])***

Compound	**NADPH oxidation** (disintegrated HUVEC) IC_{50} (µM)	**$\bullet O_2^-$ scavenging** (xanthine oxidase) IC_{50} (µM)
(–)-Epicatechin	> 100	5.1 ± 2.1
3'-*O*-Methyl epicatechin	7.9 ± 2.6	> 100
4'-*O*-Methyl epicatechin	20.7 ± 3.7	> 100
Apocynin	50.0 ± 9.1	> 100

[3] This original paper was awarded by Elsevier Publisher as "Top Cited Article 2008 – 2010" in *Arch. Biochem. Biophys.*

Furthermore, 45 phenolics were tested, most of them being of dietary importance. From these data, an apocynin-like mode of NADPH oxidase inhibition has been concluded [Steffen et al, 2008].
The structural requirements are compiled in **Table 5**. It should be noted that there exist both selective $\bullet O_2^-$ scavengers and selective NADPH oxidase inhibitors, but also compounds that combine both activities such as the (–)-epicatechin dimer procyanidin B2 or the gallic-type flavan-3-ols. Taken together, it can be concluded, that anti $\bullet O_2^-$ activities are widely distributed among food micronutrients.

Table 5: ***Structure-activity relationship of NADPH oxidase inhibition and $\bullet O_2^-$-scavenging by* polyphenols (from [Schewe et al, 2008])**

Structural feature	**Examples**	**NADPH oxidase inhibitor**	**$O_2^{\cdot -}$ scavenger**
3-Methoxy-4-hydroxyphenyl	Apocynin, ferulic acid, vanillic acid,	+	–
Flavonoids with unsubstituted catechol arrangement at the B ring	Epicatechin, catechin, quercetin, fisetin, luteolin	–	+
Mono-*O*-methylation of the catechol at the B ring	Isorhamnetin, diosmetin, hesperitin, 3`- and 4`-*O*-methyl-epicatechin,	+	–
Flavonoids of non-catechol type with a single 4`-OH group at the B ring	Apigenin, kaempferol, naringenin	+	–
Hydrogenation of the C2=C3-double bond at the C ring	Taxifolin, dihydrokaempferol, dihydrotamarixetin, naringenin	+	–/+
Flavan-3-ols of gallic type	Epicatechin gallate, epigallocatechin, epigallocatechin gallate	+	+
Procyanidin-like flavan-3-ol dimers	Procyanidin B2, myeloperoxidase-generated epicatechin dimer	+	+

4.3 Longer-term effect of (–)-epicatechin includes suppression of proteasomal breakdown of eNOS protein

As mentioned in Section 3, (–)-epicatechin exerts *in vivo* both short-term and longer-term effects (see Table 2). The longer-term effects are believed to occur at the level of gene expression. With respect to endothelial •NO metabolism the expression level of eNOS protein is relevant. Although other authors have reported up-regulation of eNOS protein synthesis by flavanols, we have observed that the latter effect requires very long pre-incubations for at least 36 h. By contrast, attenuation of eNOS protein breakdown was already detectable after 12 h. We have provided evidence that the breakdown of eNOS protein in endothelial cells occurs via proteasomal degradation when the cells are subjected to oxidative stress by treatment with oxLDL [Steffen et al, 2005; Steffen et al, 2007a]. As mentioned in the foregoing article, oxLDL gives rise to oxidative and nitrating modification of endothelial proteins. Modified proteins are known to be preferably degraded by the 20S proteasome, and eNOS protein was found to be both carbonylated and nitrated upon exposure to oxLDL [Steffen et al, 2007a]. As shown in **Table 6**, exposure of bovine aortic endothelial cells to oxLDL caused sizable loss of eNOS protein, which was prevented by two structurally different proteasome inhibitors, MG132 and lactacystin. Treatment of the cells with (–)-epicatechin prevented the eNOS loss as well, although it does not inhibit proteasomal activity. Notably, the water-soluble vitamin E-like free radical scavenger trolox failed to prevent the eNOS loss. These observations are in line with the assumption that (–)-epicatechin after methylation prevents modifications of eNOS protein through inhibition of NADPH oxidase, thus eliminating the signal for proteasomal degradation.

Table 6: ***Loss of eNOS protein is prevented by two different inhibitors of the chymotrypsin-like activity of 20S proteasomes as well as by (–)-epicatechin (from [Steffen et al, 2007a])***

Exposure of cells to	**eNOS level** (% of control)
Untreated LDL	97 ± 6
oxLDL	34 ± 4 **
oxLDL + MG132 (10 µM)	101 ± 7
oxLDL + lactacystin (100 nM)	99 ± 4
oxLDL + epicatechin (30 µM)	102 ± 6
oxLDL + trolox (30 µM)	40 ± 7 **

5. Serum albumin modulates the biological activity of dietary polyphenols and their metabolites. Reappraisal of the role of flavonoid glucuronides

As described above, we conducted many experiments at the cellular level with (–)-epicatechin. Even though relevant results were achieved, this approach proved to be an artificial system because these experiments were conducted in the complete absence of serum or serum albumin, which does not mirror the *in vivo* conditions. Thus, we have observed that 1 mM serum albumin prevented the protection of HUVEC against protein modification elicited by angiotensin II or oxLDL. By contrast, epicatechin glucuronide protected the cells both in the absence and in the presence of serum albumin. Apparently, strong binding of the flavanol to serum albumin prevents the entry into the cells, whereas the albumin binding of the glucuronide seems to be weaker, thus allowing uptake by cells [Steffen et al, 2008]. This assumption was confirmed in subsequent experiments, in which the metabolism of (–)-epicatechin and epicatechin glucuronide was studied in the absence and presence of serum albumin (**Fig. 6**).

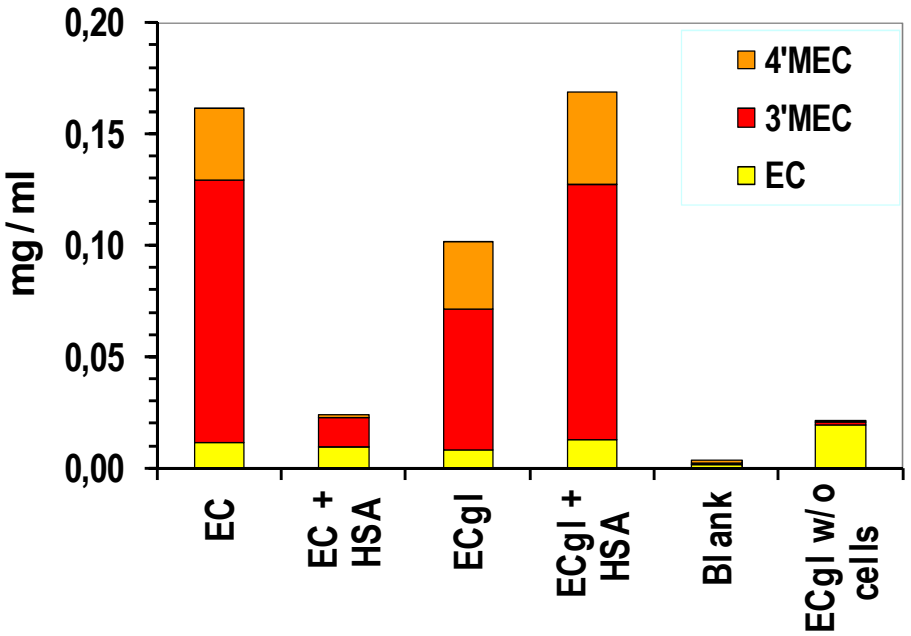
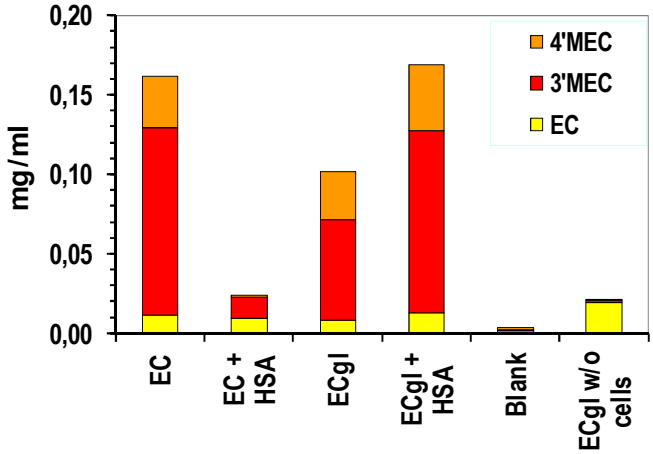


Fig. 6. Metabolism of (–)-epicatechin (EC) and (–)-epicatechin glucuronide (ECgl) in the absence and presence of 1 mM human serum albumin (HSA). The products were identified as 3'-O-methyl epicatechin (3'MEC) and 4'-O-methyl epicatechin (4'MEC). (Unpublished data, 2008).

From these data the following conclusions are drawn:

- Uptake of (–)-epicatechin glucuronides by HUVEC involves cleavage by endothelial β-glucuronidase.
- Glucuronides of (–)-epicatechin and related flavonoids may serve as transport metabolites in plasma obviating strong binding to serum albumin.
- Intestinal metabolism of (–)-epicatechin, in particular glucuronidation, may be assigned for targeting to the vascular endothelium.

Thus, flavonoid glucuronides must not regarded any longer as common urinary excretion products. Instead, sulfate esters appear to fulfill the latter role.



6. *Synopsis: How does (–)-epicatechin preserve endothelial •NO?*

In **Fig. 7** the scenario of the mode of action of dietary (–)-epicatechin in endothelial cells is illustrated based of the data described in this article.

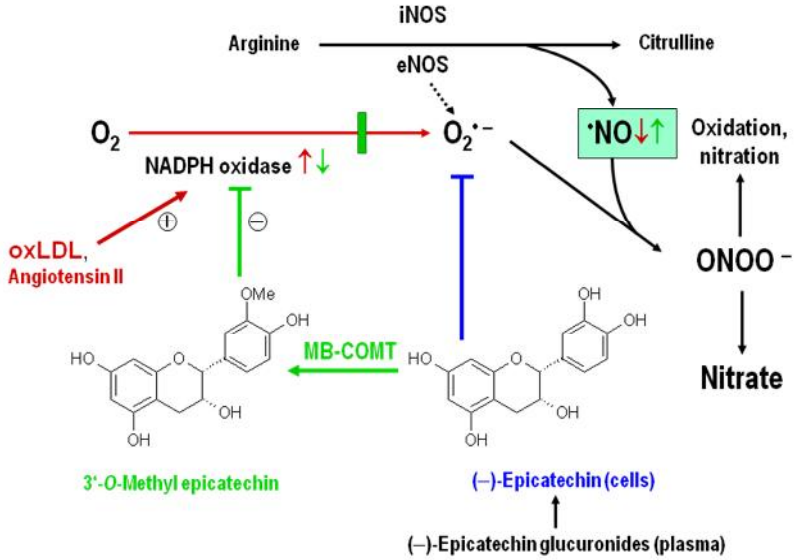


Fig. 7. •NO metabolism of endothelial cells and its interaction with (–)-epicatechin. •NO is generated by endothelial •NO synthase (eNOS) or upon proinflammatory stimulation also by inducible •NO synthase (iNOS). Receptor-mediated stimulation of the cell by either oxLDL or angiotensin II (red route) leads to assembling of the NADPH oxidase multi-component complex via cell signaling, thus activating it. The active NADPH oxidase dehydrogenates NADPH supplied by the oxidative pentose pathway of glucose metabolism, thereby reducing dioxygen to superoxide anion radical ($•O_2^-$). This species, which can also be formed by uncoupled eNOS reaction if arginine supply is limited, is capable of rapidly reacting with •NO in a diffusion-controlled reaction to peroxynitrite, thus leading to lowering of the intracellular steady-state level of •NO and, consequently, of the bioavailability and bioactivity of •NO in the vascular endothelium. Peroxynitrite either isomerizes to nitrate or modifies intracellular proteins including eNOS, the proteasomal degradation of which is accelerated under these conditions. The diminished eNOS level further contributes to the lowered •NO level. The cells take up (–)-epicatechin from epicatechin glucuronide circulating in plasma two hours after intake of a flavanol-rich meal. The uptake of epicatechin glucuronide proceeds under cleavage of the glucuronate moiety, so that (–)-epicatechin accumulates in endothelial cells. Epicatechin is capable of scavenging part of the $•O_2^-$ (blue route). A more effective protection is achieved, however, via methylation by membrane-bound catechol-O-methyltransferase (MB-COMT) generating 3'-O-methyl epicatechin, an NADPH oxidase inhibitor (green route). By this manner, the deleterious O_2^- generation is attenuated, which in turn preserves a higher •NO level.

7. Outlook: Open issues to be investigated

Although our work has enlarged the present knowledge on the beneficial role of dietary polyphenols, a number of new questions have arisen, which need to be addressed in future studies:

- Molecular mechanism of inhibition of NADPH oxidase activity by 3‘-*O*-methyl epicatechin and related polyphenols,
- Elucidation of oxidative bioconversions of (–)-epicatechin induced by oxidative stress,
- Regulation of bioconversions of flavanols in endothelial and other target cells,
- Reaction of myeloperoxidase with (–)-epicatechin and other flavonoids,
- Influx and efflux mechanisms of flavonoid transport in target cells.

In future flavonoid research some general rules should be observed:

(i) Administration of flavonoids or flavonoid-rich food to human subjects or laboratory animals should never be done without identification of the profile of plasma metabolites and pursuit of the time-course of their plasma levels. Application of labeled compounds may facilitate the methodological approach. Metabolite analyses should be conducted not only in plasma but also in cells and tissues, in particular in those that are the putative target of an assumed biologic action.

(ii) Plasma levels must never be overrated. Except for rare cases, plasma is not the space, in which the biologic action of flavonoids or their metabolites takes place. The intracellular concentrations, however, do not only depend on the plasma levels, but also on transport mechanisms, accumulation in cells via binding to intracellular proteins as well as intracellular metabolism. Caution is advised as to comparisons of ED_{50} or IC_{50}values of *in vitro* assays with plasma levels, since the conditions of an *in vitro* assay never correctly mirror the interactions of cells with plasma. Moreover, in cellular *in vitro* assays the doses should be related to a defined number of cells rather than expressed in µM or another concentration unit. It should be emphasized that *in vivo* a continuous supply of a compound from plasma to cells is possible even if the concentration is low, whereas *in vitro* such conditions are difficult to mimic. As an example, we have observed that 10 additions of 0.3 µM of a polyphenol every 10 min produced approximately the same effect as a single incubation with 3 µM of this polyphenol for 100 min.

(iii) Many flavonoids bind to serum albumin, but the binding affinities of flavonoids and their metabolites may vary and are determinants whether or not these compounds are taken up by cells. For this reason, *in vitro* assays should be largely conducted in the presence of 1 mM serum albumin, approximating the physiologic concentration in plasma.

(iv) A number of flavonoids such as procyanidins and anthocyanins are not or very poorly absorbed. Nevertheless, these compound classes seem to yield a health benefit, which occurs at two levels: First, during the gastrointestinal passage of food they reduce organic hydroperoxides and chelate toxic heavy metal ions owing to innate chemical properties. By this fashion, postprandial oxidative stress is counteracted. Second, they are nutrients for the colonic microflora, thus generating other phenolics, which are ab-

sorbed and metabolized and may exert beneficial biologic effects [Del Rio et al, 2010].

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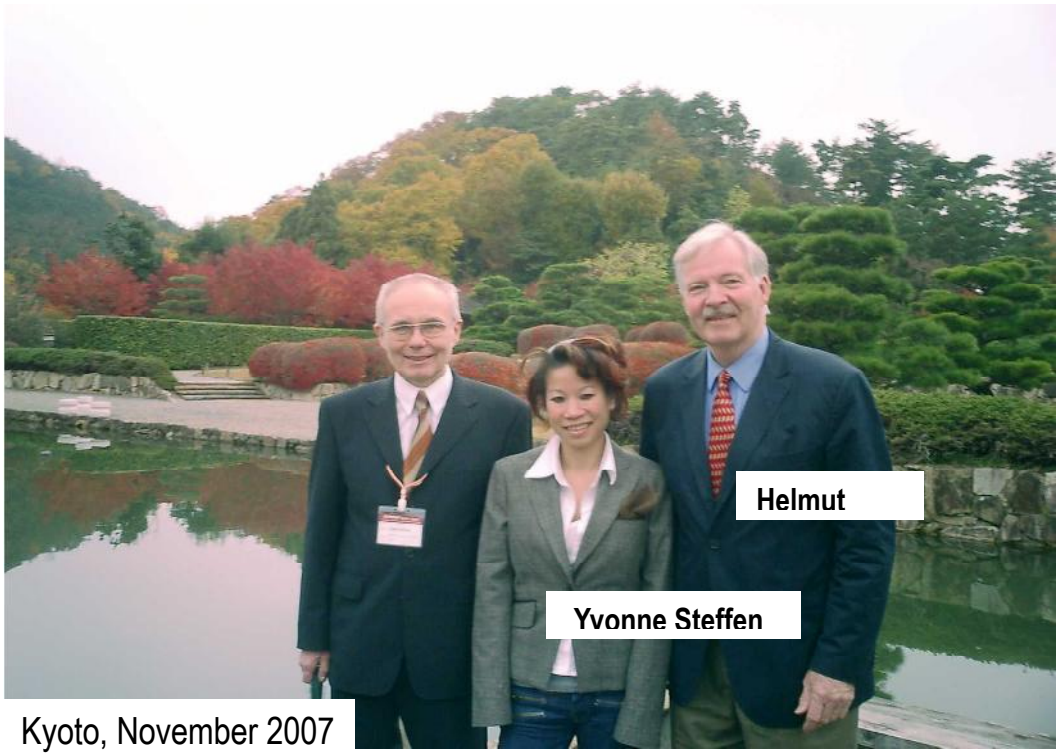

Kyoto, November 2007

This photo shows the author of the two articles together with his graduate student Yvonne Steffen and the former head of the Institute of Biochemistry and Molecular Biology I, Heinrich Heine University Duesseldorf, Professor Helmut Sies, during the 3rd International Conference on Polyphenols and Health in Kyoto, Japan, November 2007.

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