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Myeloperoxidase-mediated LDL oxidation and endothelial cell toxicity of oxidized LDL: attenuation by (−)-epicatechin

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Abstract
Recent data suggest an inverse epidemiological association between intake of flavanol-rich cocoa products and cardiac mortality. Potential beneficial effect of cocoa may be attributed to flavanol-mediated improvement of endothelial function, as well as to enhancement of bioavailability and bioactivity of nitric oxide *in vivo*. (−)-Epicatechin is one bioactive flavanol found in cocoa. This review deals with protective actions of (−)-epicatechin on two key processes in atherogenesis, oxidation of LDL and damage to endothelial cell by oxidized LDL (oxLDL), with emphasis on data from this laboratory. (−)-Epicatechin not only abrogates or attenuates LDL oxidation but also counteracts deleterious actions of oxLDL on vascular endothelial cells. These protective actions are only partially shared by other vasoprotective agents such as vitamins C and E or aspirin. Thus, (−)-epicatechin appears to be a pleiotropic protectant for both LDL and endothelial cells.

Keywords: *Endothelial cells, flavonoids, myeloperoxidase, NO synthase, oxLDL*

Abbreviations: *ASA, acetylsalicylic acid; BrdU, 5-bromo-2′-deoxyuridine; HUVEC, human umbilical vein endothelial cells; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; oxLDL, oxidatively modified low-density lipoprotein; MPO, myeloperoxidase; MTT, 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide; REM, relative electrophoretic mobility; ROS, reactive oxygen species*

Introduction

Epidemiological studies have accrued evidence for an inverse correlation between daily intake of polyphenols and incidence of coronary heart disease mortality as well as other chronic vascular diseases [1–4 and refs. therein]. 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. For cocoa flavanols, however, there is recent accumulating evidence for an improvement of endothelial function or alleviation of endothelial dysfunction *in vivo* [5–10]. Intake of high-flavanol cocoa drink or chocolate resulted in improved flow-mediated dilation (FMD) of the brachial artery or other vasodilatory actions with both healthy individuals [6,7] and patients at risk for cardiac diseases [5] as well as with smokers [9]. These actions: (i) were simulated by administration of pure (−)-epicatechin [10]; (ii) coincided with the peak of plasma (−)-epicatechin metabolites [7,9,10]; (iii) were suppressed by simultaneous administration of ˙NO synthase inhibitors such as L-NMMA or L-NAME [6,9]; and (iv) were paralleled by elevated plasma levels of nitroso-compounds (RNO) [5,9], known to originate mainly from oxidative protein nitrosation [11] and to contribute to the systemic vasodilatory effects of nitric oxide [12]. High-polyphenol chocolate but not polyphenol-

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free chocolate also caused pronounced lowering of blood pressure [13], even though this action appears to be of secondary epidemiological importance for cardiac mortality [4]. Together, these studies suggest that consumption of the cocoa flavanol (−)-epicatechin may protect the vascular endothelium via improvement of bioavailability and bioactivity of nitric oxide. The mechanism of this action, however, is still far from clear.

Previous assumptions, that dietary polyphenols would simply act primarily as antioxidants by virtue of their radical-scavenging properties, could not convincingly be substantiated so far. Today this paradigm has changed to the view that the beneficial effects of polyphenols are multifaceted and encompass antioxidant activities in both direct and extended sense as well as non-antioxidant activities. If antioxidative capacity would be solely decisive for the health benefit of polyphenols, other dietary antioxidants such as vitamins E and C should exert comparable actions. Another challenge to the concept that the flavanols in cocoa exert their vascular effects through their function as antioxidants is made by the fact that upon consumption, epicatechin from cocoa undergoes significant biotransformation including glucuronidation, methylation, and/or sulfation [14,15]. These metabolites, which make up the majority of (−)-epicatechin in plasma [9,10], have reduced hydrogen donating capacity, thus limiting the capacity of these metabolites to function as effective antioxidants *in vivo*.

Another vasoprotective agent is acetylsalicylic acid (ASA, aspirin; [16]). Some similarities between the actions of cocoa polyphenols and those of aspirin such as anti-platelet activity [17–21] prompted some authors to the view that these two types of intervention may substitute for each other, in other terms, polyphenol-rich dark chocolate might be an alternative to aspirin [19]. This opinion, however, was recently contested [21].

Endothelial dysfunction and atherogenesis are, to a sizeable part, initiated by interaction of endothelial cells with oxidized LDL (oxLDL) through a variety of mechanisms [22]. Therefore, both generation of oxLDL and its detrimental actions on vascular endothelial cells are potential sites of actions of vasoprotective agents.

In a recent review, we have treated the enzymatic properties of myeloperoxidase (MPO) with emphasis on its reactions with nitrite, flavonoids and LDL and their putative biological role in atherogenesis [23]. Main focus of present review is recent work from this laboratory on the cytotoxic action of oxLDL, generated by the MPO/H_2O_2/nitrite reaction system, on vascular endothelial cells and its possible role in endothelial dysfunction as well as protective actions of various vasoprotective agents.

Myeloperoxidase-mediated modifications of LDL

Oxidation of LDL can be brought about by several enzymatic and non-enzymatic reactions, among others by the MPO/H_2O_2/nitrite system [24–26]. We have demonstrated that (−)-epicatechin and other flavonoids protect LDL against oxidation by either MPO/H_2O_2/nitrite reaction system or Cu^{2+} catalysis [23,27,28]. Moreover, (−)-epicatechin protects endothelial cells against both cytotoxicity of oxLDL and oxLDL-mediated proteasomal degradation of endothelial NO synthase protein [29].

Treatment with MPO and nitrite (50 μM) in the presence of an H_2O_2-generating system causes lipid peroxidation of LDL as evident from increase in absorbance at 234 nm indicating conjugated diene formation. As shown in Figure 1, (−)-epicatechin at a concentration as low as 2 μM strongly suppressed this reaction, mainly by extending the lag phase. Trolox, the water-soluble vitamin E analogue, was equally active, whereas comparable concentrations of ascorbate exhibited only a weak effect, and ASA (100 μM) was completely inactive. Since the free radical scavengers probucol and vitamin E inhibit this system only

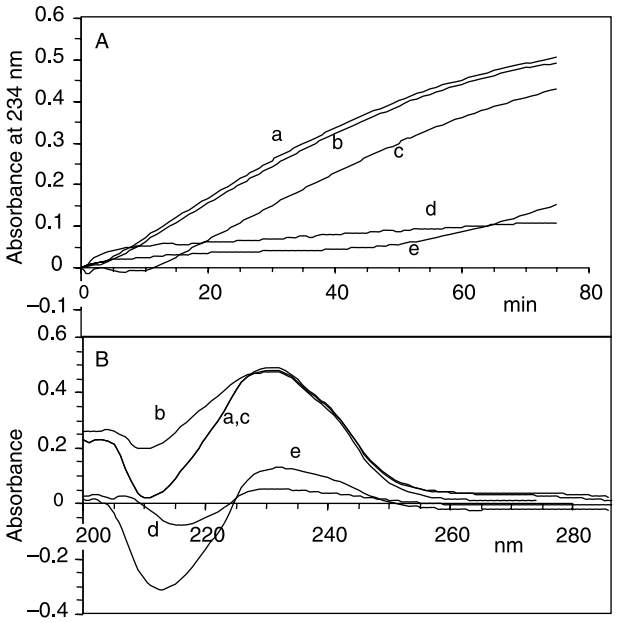

Figure 1. Effect of various antioxidants on MPO/H_2O_2/nitrite-mediated lipid peroxidation of LDL. Human LDL (0.1 μM) was prepared according to Kleinveld et al. [30] and exposed to the MPO/H_2O_2/nitrite reaction system (50 μM nitrite, 0.56 mM glucose, 1.19 nkat/ml glucose oxidase, 42.5 nM MPO and 0.1 mM diethylenetriamine-pentaacetic acid in 0.1 M phosphate buffer, pH 7.4 for 80 min at 37°C in the presence of the compounds indicated: (a) control; (b) 100 μM ASA; (c) 4 μM ascorbate; (d) 4 μM Trolox; (e) 2 μM (−)-epicatechin. (A) Time-course of conjugated diene formation. (B) UV difference spectra of lipid extracts from LDL after treatment *versus* control spectrum of untreated LDL. Representative examples of experiments at least in triplicate are shown.

partially at comparatively high concentrations (50 μM) [24], (−)-epicatechin proved to be one of the most efficient inhibitors of MPO/H_2O_2/nitrite-mediated lipid peroxidation, which may be plausibly explained with extraordinarily effective scavenging of NO_2 radicals generated by this system [31] (Section 5). The nature of the lag phase of the MPO/H_2O_2/nitrite-induced lipid peroxidation and its prolongation by (−)-epicatechin has not been addressed so far in mechanistic terms. It is tempting to speculate that the NO_2 radical destroys vitamin E, a major antioxidant in LDL, and (−)-epicatechin is protective by virtue of scavenging this detrimental radical.

MPO also modifies apoB-100, the apoprotein moiety of LDL. Protein tyrosine nitration, however, requires higher concentrations of nitrite (≥100 μM) than lipid peroxidation (5–50 μM) [28,32], which is supposedly a consequence of different intermediates in the catalytic cycle of peroxidases at low and high nitrite [33]. Here again, (−)-epicatechin protects more efficiently against tyrosine nitration of LDL than other antioxidants, and ASA is ineffective (Figure 2). Other modifications of apoB-100 by MPO such as oxidation of lysine residues are known to require the presence of chloride [32]. Lysine modification is reflected by increase in relative electrophoretic mobility (REM). As shown in Figure 3, the MPO reaction system containing chloride and nitrite caused a time-dependent increase in REM, which was dose-dependently abrogated by micromolar concentrations of (−)-epicatechin.

Taken together, (−)-epicatechin proves to be a potent universal protectant against MPO-mediated proatherogenic modifications of the lipid and protein moieties of LDL *in vitro*. This quality may be one way by which (−)-epicatechin or its metabolites could limit oxidative modification of LDL *in vivo*.

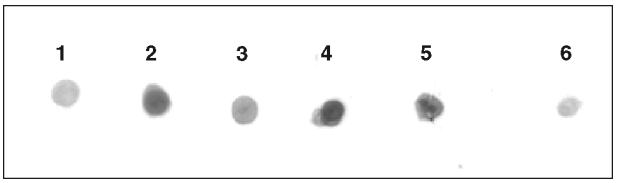

Figure 2. Effect of various antioxidants on MPO/H_2O_2/nitrite-mediated protein tyrosine nitration of LDL. Human LDL was treated with the MPO/H_2O_2/nitrite reaction system for 80 min as in Figure 1 with the exception that 1000 μM nitrite was present. Afterwards the samples were subjected to dot blot immunostaining. Dot blots were blocked in 1% fetal calf serum in 20 mM Tris/HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20 over night. The membranes were then probed with mouse monoclonal antibody against 3-nitrotyrosine (1:1000) at 4°C for 2 h. Following washing and incubation with a horseradish peroxidase-coupled anti-mouse immunoglobulin G antibody (1:2000) at 4°C for 2 h, the blots were washed again and developed using enhanced chemiluminescent detection kit. Dot blots were taken from an analysis representative for three independent experiments with qualitatively similar outcome. (1) Untreated LDL; (2) MPO/nitrite-treated LDL; (3) MPO/nitrite +4 μM Trolox; (4) MPO/nitrite +4 μM ascorbate; (5) MPO/nitrite +100 μM ASA; and (6) MPO/nitrite +2 μM (−)-epicatechin. Similar data were reported elsewhere [27].

Endothelial cell toxicity of myeloperoxidase-oxidized LDL

After having found that (−)-epicatechin is protective against *formation* of oxLDL, we examined whether (−)-epicatechin also protects against the cytotoxic *actions* of oxLDL towards endothelial cells. MPO-oxLDL, as generated under the conditions of the experiments in Figure 1, causes severe damage to human umbilical vein endothelial cells (HUVEC) as judged by both diminished reduction of 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) and elevated release of lactate dehydrogenase (LDH) (Figure 4). Since these parameters mirror mainly the mitochondrial energy conservation and the integrity of the plasma

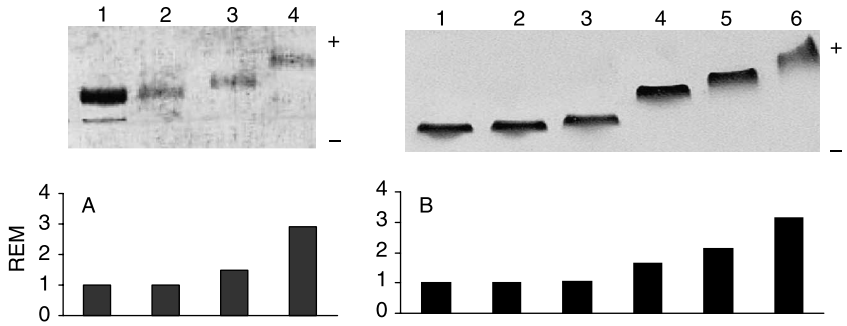

Figure 3. (−)-Epicatechin protects against MPO-mediated oxidative modification of the apoprotein moiety of LDL as judged by REM. Human LDL was treated with the MPO reaction system (Figure 1) in the presence of phosphate-buffered saline and 50 μM nitrite for the times indicated and subjected to electrophoresis on preformed 0.5% agarose gels as described [34]. (A) Dependence on reaction time: (1) untreated control; (2) 30 min; (3) 60 min; 4) 80 min; (B) dose-dependent suppression by (−)-epicatechin (EC): (1) untreated control; (2) MPO +6 μM EC; (3) MPO +4 μM EC; (4) MPO +2 μM EC; (5) MPO +1.5 μM EC; and (6) MPO control; reaction time: 80 min. Representative example from two similar series of experiments.

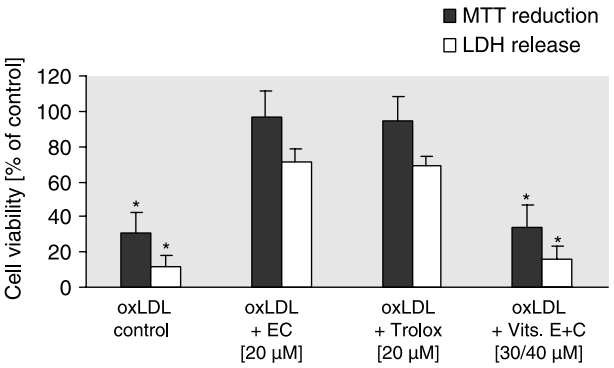

Figure 4. (−)-Epicatechin and Trolox, but not antioxidant vitamins protect HUVEC against MPO/nitrite-oxLDL-mediated cytotoxicity. Human LDL was treated with the MPO/H_2O_2/nitrite reaction system for 80 min as indicated in Figure 1, and the reaction was stopped by addition of catalase to inhibit MPO activity. HUVEC were treated with MPO/nitrite-oxLDL (150 $\mu g\,ml^{-1}$) for 24 h in the absence or presence of the compounds indicated. EC, (−)-epicatechin. After treatment, cell viability was assessed in parallel samples by the MTT reduction [35] and LDH release assays. The values are means ± SD from four independent experiments, each measured in triplicate. *Denotes significant cytotoxicity *versus* untreated control ($p < 0.05$).

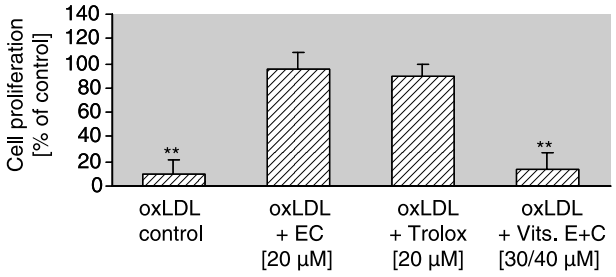

Figure 5. (−)-Epicatechin and Trolox, but not antioxidant vitamins protect HUVEC against MPO/nitrite-oxLDL-mediated inhibition of cell proliferation. 10^4 cells/well were plated in 96-well plates, cultured to confluence, and treated with MPO/nitrite-oxLDL (150 $\mu g\,ml^{-1}$) for 24 h. After treatment, the cells were incubated for 12 h with 10 μM BrdU diluted in growth medium. Then, cultures were fixed and incubated with peroxidase-labeled monoclonal anti-BrdU antibody. Following 3 h incubation, tetramethylbenzidine was added as substrate for peroxidase; after a 30 min interval time required for color development, absorbance was read at 450 nm. The values are means ± SD from four independent experiments, each measured in triplicate. **Denotes significant cytotoxicity *versus* untreated control ($p < 0.01$).

membrane, respectively, the oxLDL-mediated damage to cells seems to involve multiple cellular events. (−)-Epicatechin or Trolox (20 μM) abrogated these changes, whereas comparable concentrations of α-tocopherol and ascorbate—single or combined—did not protect. In the experiments of Figures 4–6, α-tocopherol was added as solution in vegetable oil; therefore poor delivery to the cells cannot be excluded as reason for lacking protection. Also, ASA at 100 μM did not protect under these conditions (not shown). Similar data were obtained with bovine aortic endothelial cells [29].

Quite similar observations were made for cell proliferation as assessed by the 5-bromo-2′-deoxyuridine (BrdU) assay (Figure 5). MPO/H_2O_2/nitrite-oxLDL inhibited cell proliferation, which was prevented by either (−)-epicatechin or Trolox, but not by vitamins E and C. Again, neither ASA nor its metabolite salicylate did protect (data not shown).

The strong inhibition of cell proliferation suggested damage to DNA, which was actually substantiated. Electrophoresis in 2% agarose gel after ethidium bromide staining revealed dose-dependent fragmentation of cellular DNA by oxLDL, which was completely prevented by (−)-epicatechin (20 μM) (Figure 6).

The multiple deleterious effects of LDL treated with the MPO/H_2O_2/nitrite reaction system on vascular endothelial cells suggest a common basic mechanism. Preliminary data argue in favor of generation of reactive oxygen species (ROS) *via* oxLDL-signaling, possibly involving activation of NADPH oxidase (Section 5). Treatment of endothelial cells with MPO/H_2O_2/nitrite–oxLDL gave rise to generation of ROS as evidenced from oxidation of the fluorescent dye precursors dihydrorhodamine 123 or 2′,7′-dichlorodihydrofluorescein diacetate [36], which was mitigated by (−)-epicatechin more effectively than by ascorbate, whereas ASA was ineffective (data not shown). Strong inhibition of dihydrorhodamine 123-oxidation by (−)-epicatechin has also been reported for HL-60 cells treated with the peroxynitrite-generating compound 3-morpholino-sydnonimine [37]. This fact suggests that protection against oxidative stress does not depend on the agent inducing it.

In line with these observations, MPO/H_2O_2/nitrite-oxLDL strongly lowered the cellular glutathione level in HUVEC, and (−)-epicatechin prevented this effect (Figure 7). Collectively, we conclude that MPO-oxLDL causes severe oxidative stress in endothelial cells, which is causally abrogated by (−)-epicatechin.

Myeloperoxidase, modification of LDL, and endothelial dysfunction

MPO belongs to a variety of prooxidant enzymes that are supposedly involved in proatherogenic and proinflammatory processes in the subendothelial space of arterial vessel walls leading to endothelial dysfunction and atherosclerotic lesions. A number of experimental observations strongly argue in favor of involvement of MPO in these pathogenic processes (Table I). Moreover, MPO has been shown to evoke lipid peroxidation *in vivo* at sites of inflammation [39]. Nevertheless its biological role appears to be more complex than expected at first sight, since antiatherogenic actions were also described. Although an involvement of MPO in nitration of extracellular proteins was demonstrated *in vivo* [31], MPO

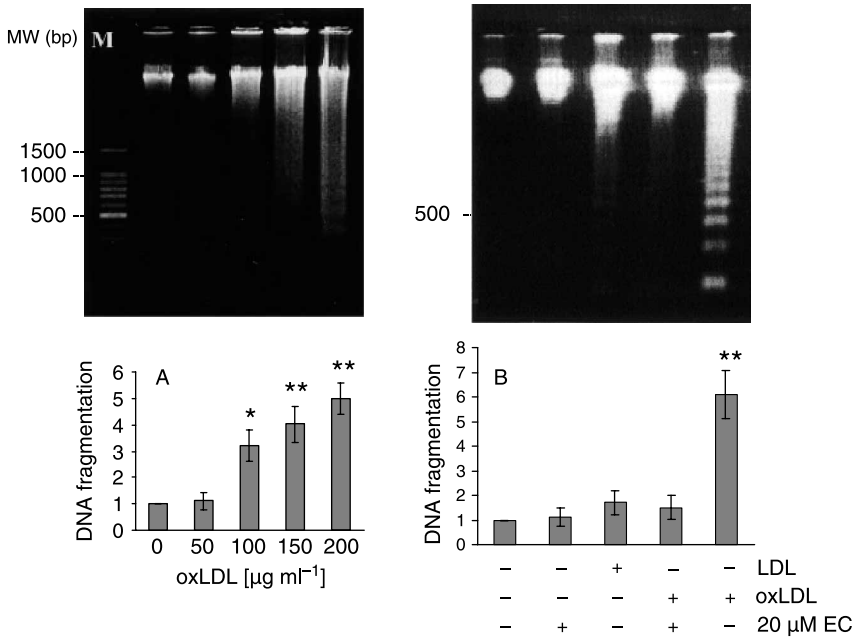

Figure 6. DNA fragmentation in HUVEC caused by MPO/H_2O_2/nitrite-oxLDL and protection by (−)-epicatechin. Confluent HUVEC previously cultured for 24 h in conventional medium were further incubated for 24 h with or without (−)-epicatechin (20 µM) where indicated and varying concentrations of oxLDL (150 µg ml^{-1} unless stated otherwise). Intranucleosomal DNA fragmentation was assessed by electrophoresis in 2% agarose gel and examined under ultraviolet light upon staining with ethidium bromide. (A) Dependence on oxLDL concentration. (B) Effect of (−)-epicatechin; 150 µg ml^{-1} oxLDL or untreated LDL, respectively. The column diagrams show densitometric estimations of the relative intensities of the DNA fragments visible on the electropherograms.

deficiencies in humans and mice give curiously rise to higher levels of 3-nitrotyrosine [40]. This paradox may be explained with a missing decomposition of 3-nitrotyrosine, formed by other pathways such as peroxynitrite, by MPO-derived hypochlorite [41]. MPO is capable of reacting with a large array of substrates and co-substrates including hydrogen peroxide, organic hydroperoxides, chloride and other halides or pseudohalides, nitric oxide and nitrite as well as phenolics. This large substrate heterogeneity may account for a complex network of MPO-mediated reactions, which include both adverse and beneficial routes and compete or cross talk with each other. Therefore, any pharmacological or dietary intervention into MPO-mediated metabolism must consider this fact.

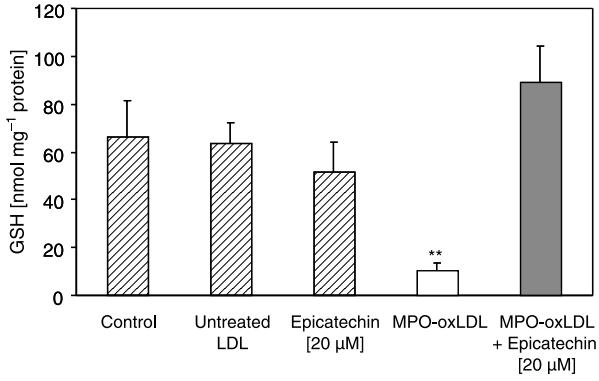

Figure 7. Lowering of glutathione level in HUVEC by MPO/H_2O_2/nitrite-oxLDL and prevention by (−)-epicatechin. Confluent cells were pretreated with (−)-epicatechin or solvent in six-well plates for 24 h. Thereafter the culture medium was changed to serum-free medium containing oxLDL or untreated LDL (100 µg ml^{-1}), respectively. The cells were cultured for another 24 h, and the intracellular GSH levels were determined according to Adams et al. [38]. Values are means ± SD ($n = 4$).

Although the contribution of MPO in proatherogenic modifications of LDL *in vivo* is unknown, the treatment of LDL with the MPO/H_2O_2/nitrite reaction system as well as the interaction of MPO/H_2O_2/nitrite-oxLDL with endothelial cells are an appropriate cohort of *in vitro* tandem models for atherogenesis and endothelial dysfunction. Considering the facts listed in Table I they promise to mirror *in vivo* oxidation of LDL more closely than, e.g. copper-catalyzed oxidation. Moreover, the reactions of MPO with nitric oxide and nitrite underline a close interconnection of this enzyme with the ·NO metabolism that is crucial for function and integrity of the vascular endothelium.

We adduce evidence that (−)-epicatechin is capable of protecting human LDL against various MPO-mediated modifications (Figures 1–3). Its high protective efficacy, which surpasses that of ascorbate, may be due to the following: (i) this and other flavonoids have been shown to be MPO substrates, possibly via reacting with MPO-compounds I and II [27]; (ii) a high scavenging capacity for $\cdot NO_2$ radicals

Table I. Indications for a role of MPO in atherogenesis and endothelial dysfunction.

Observation	Authors
MPO is present and enzymatically active in atherosclerotic lesions, but not in uninvolved intima	Daugherty et al. [42]
Reaction products of MPO (3-chloro-tyrosine, 3-nitro-tyrosine, *o,o′*-dityrosine from tyrosyl radicals are found in atherosclerotic lesions	Leeuwenburgh et al. [43,44]
MPO binds to LDL as well as to endothelial cells	Carr et al. [45]; Zouaoui Boudjeltia et al. [46]
MPO renders LDL atherogenic *in vitro*	Podrez et al. [47]
Plasma levels of MPO are elevated in subjects at risk for cardiac events	Brennan et al. [48]
MPO acts as ˙NO sink by virtue of intrinsic ˙NO oxidase activity	Abu-Soud and Hazen [49]

formed by the MPO/nitrite reaction system; and (iii) a favorable *n*-octanol/water distribution coefficient that permits sufficient solubility in both hydrophilic and, more importantly, lipophilic environments [50]. On the contrary, ascorbate, which also possesses high affinity to $\dot{N}O_2$ radicals, may have limited accessibility to $\dot{N}O_2$ dissolved in the lipid core of LDL where lipid peroxidation takes place. Besides scavenging of $\dot{N}O_2$, thus inhibiting lipid peroxidation, (−)-epicatechin at micromolar concentrations also protects against MPO- or peroxynitrite-mediated tyrosine nitration [28,37]. Notably, (−)-epicatechin is seemingly only a poor MPO inhibitor *per se*, since an IC_{50} as high as 77 μM with HL-60 cells was reported [37]; rather it may act as favorable modulator for the divergent array of MPO-catalyzed primary and secondary reactions.

Cocoa flavanols as protectants against oxidative stress in endothelial cells

(−)-Epicatechin occurs in cocoa both as monomer and to a large part as oligomers, the procyanidins. While procyanidins are poorly absorbed in non-cleaved form, monomeric flavanols [mainly (−)-epicatechin and (+)-catechin)] are satisfactorily absorbed as judged by the occurrence of respective metabolites in plasma upon intake of flavanol-rich cocoa [10]. Whether some depolymerization of procyanidins contributes to (−)-epicatechin formation, as reported for perfusion experiments with isolated small intestine [51], is currently disputed [52]. Together it follows that outside the gastrointestinal tract, (−)-epicatechin and related monomeric flavanols or their metabolites rather than procyanidins are the substantial bioactive compounds from cocoa. Although procyanidin dimers were found in human plasma after consumption of a flavanol-rich cocoa [53], the reported concentration range was two orders of magnitude lower than that of (−)-epicatechin metabolites; therefore, it appears not very likely that the dimers contribute a vascular benefit. Phenolic acids arising from microbial metabolism of procyanidins in the colon may only contribute secondarily if any to the reported *in vivo* effects of high-flavanol cocoa, since their formation occurs later than the biological activity on endothelial function.

As mentioned in Section 1, cocoa flavanols improve endothelial function of human arteries *in vivo*. This beneficial action may involve several mechanisms including elevation of bioavailability and bioactivity of nitric oxide, a major endothelium-derived mediator. Here we demonstrate that (−)-epicatechin abrogates MPO/H_2O_2/nitrite-mediated damage to endothelial cells as evidenced from counteraction of lowering of cell viability, diminished cell proliferation and fragmentation of DNA (Figures 4–6). Common cause for these detrimental actions of MPO/H_2O_2/nitrite-oxLDL is most probably oxidative stress. It is reasonable to assume that oxLDL elevates superoxide production in endothelial cells. Among various possible sources for superoxide in endothelial cells (NADPH oxidase, xanthine oxidase, uncoupled endothelial ˙NO synthase, mitochondrial electron transfer system), activation of NADPH oxidase may be the most probable site of action for both oxLDL [22] and (−)-epicatechin. Inhibition of leukocyte NADPH oxidase by flavonoids has been reported [54,55]. Further work is necessary to substantiate a possible inhibition of endothelial NADPH oxidase by (−)-epicatechin or its metabolites.

Suppression by (−)-epicatechin of superoxide formation in endothelial could be reason for the elevation of bioavailability of nitric oxide *in vivo*. The steady-state level of ˙NO in cells is determined by the rates of formation and breakdown. Withdrawal of superoxide and, in turn, suppressed peroxynitrite formation may slow down the breakdown of ˙NO, thus increasing its steady-state level, even though the rate of ˙NO generation would be unchanged. Such a principle has also been proposed for the enhancement of ˙NO bioavailability by cerivastatin [56]. In recent work, we have shown that MPO/H_2O_2/nitrite-oxLDL also induces a proteasomal breakdown of endothelial ˙NO synthase protein in endothelial cells, which is prevented by long-term pretreatment of the cells with (−)-epicatechin but not with Trolox [29]. Although the latter action, which may be classified as non-antioxidant activity of (−)-epicatechin, cannot contribute to the short-term *in vivo* effects of cocoa

flavanols, it may constitute a further ·NO-preserving pharmacological quality in addition to counteraction of endothelial oxidative stress.

Protection by oxLDL-mediated oxidative stress in endothelial cells required higher concentrations of (−)-epicatechin than suppression of LDL oxidation. One possible reason is an endothelial cell-mediated slow conversion to another bioactive metabolite; the more so as long-term incubations of endothelial cells were necessary in these experiments. In case metabolites are involved, they appear to be immediately formed in the arterial endothelium, since (−)-epicatechin has been reported to directly relax isolated rat mesenteric artery rings [57].

Are cocoa flavanols substitute or alternative to aspirin?

Epidemiological studies and *in vivo* investigations in humans have identified cocoa flavanols as protectants against vascular diseases. This fact provokes comparing cocoa flavanols with other vasoprotective agents. It has been reported that ingestion of dark chocolate by thirty healthy volunteers significantly inhibited collagen-induced platelet aggregation *ex vivo* [19], supporting earlier findings [17]. These authors interpreted their findings as an "aspirin-like action" and suggested, "eating a bar of dark chocolate may provide as much protection as taking an aspirin" [19]. From another study with a combination of flavanol-rich cocoa beverage and aspirin on platelet function, it was concluded that the effect of cocoa is qualitatively similar to aspirin although weaker [18]. Indeed, inhibition of platelet aggregation by other agents, most effectively by aspirin, is associated with significant reduction in the risk of myocardial infarction and stroke [16].

While such statements suggest that aspirin and cocoa flavanols exert their effects on platelet function through the same mechanism, it is in fact likely that the mode of action of cocoa flavanols is distinct from that of aspirin. Aspirin blocks the synthesis of pro-aggregatory thromboxane A_2 (TXA_2) from arachidonic acid through inhibiting cyclooxygenase COX-1 *in vitro* ($IC_{50} \sim 10\,\mu M$) as well as *in vivo*. By contrast, flavanols such as (+)-catechin inhibit platelet thromboxane formation *in vitro* only at very high concentrations ($IC_{50} = 200 \pm 120\,\mu M$ [58]), which exceed flavanol plasma levels by two orders of magnitude. Inhibition of collagen-stimulated platelet aggregation *in vitro* requires similarly high concentrations of (+)-catechin [59]. Although corresponding data are not available for either (−)-epicatechin or its plasma metabolites, no evidence has been adduced that flavanols are in fact cyclooxygenase inhibitors.

Nevertheless, anti-platelet activity of cocoa flavanols *in vivo* as evidenced from *ex vivo* investigations is well substantiated [20,21]. Its mode of action, however, remains to be yet defined. Again, a close relation to the ·NO-preserving property seems to be self-evident. Nitric oxide possesses own anti-platelet activity [60,61]. Modulation of ·NO metabolism in platelets may be mediated via the ·NO/cGMP signaling pathway leading to strong inhibition of platelet activity [62]. ·NO-promoting action of flavonoids has also been demonstrated in animal models [63]. The following mechanisms of the anti-platelet activity of cocoa flavanols are conceivable: (i) increase in NO production in platelets with concomitant decrease in superoxide production [60]; and (ii) protection of the vascular endothelium against oxidative stress-mediated damage, thereby counteracting the adhesion of platelets to damaged endothelium.

Recent reports revealed that besides inhibition of thromboxane synthesis, non-cyclooxygenase actions related to nitric oxide metabolism might also contribute to the vasoprotective action spectrum of aspirin. Aspirin was shown to improve ·NO formation in platelets [64], to protect endothelial cells from hydrogen peroxide-mediated toxicity through the ·NO-cGMP pathway [65] and to induce nitric oxide release from vascular endothelium presumably *via* modification of endothelial ·NO synthase protein [66]. Thus, the action spectrum of cocoa flavanols and of aspirin proved to be similar, albeit not identical. Nevertheless, while a simple substitution for each other is likely not sufficiently justified on the basis of available data, the data do support the view that cocoa flavanols can both acutely and chronically modulate platelet reactivity and thus may provide a complementary approach to aspirin in the prevention of damaging clot formation. Moreover, we found a number of protective actions of (−)-epicatechin, which do aspirin not share.

The obvious advantage of flavanol-rich food or cocoa tablets over aspirin would be the absence of gastrointestinal and hemorrhagic complications occasionally produced by aspirin [16]. Moreover, flavanols might be indicated therapeutically in non-responders to aspirin; aspirin resistance occurs in 6–24% of patients with stable coronary artery disease, possibly because of an inability to inhibit COX-1 in platelets [67].

Conclusions and outlook

We demonstrated that the major cocoa flavanol (−)-epicatechin protects against MPO-mediated modifications of LDL as well as against oxidative stress-related damage to endothelial cells induced by oxLDL. The protection was detectable at several levels making (−)-epicatechin to a pleiotropic protectant for the vascular endothelium. The spectrum of protective actions observed was not shared by aspirin and only partially by antioxidative vitamins or

the synthetic antioxidant Trolox. Therefore substitution of various vasoprotective agents for each other may be justified at present only to a limited extent. Further studies are needed to explore their mutual interactions. Favorable modulation of nitric oxide metabolism in endothelial cells and possibly also in platelets is obviously a more important target for (−)-epicatechin or their metabolites than unspecific scavenging of free radicals.

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References

[1] Knekt P, Kumpulainen J, Järvinen R, Rissanen H, Heliövaara M, Reunanen A, Hakulinen T, Aromaa A. Flavonoid intake and risk of chronic diseases. Am J Clin Nutr 2002; 76:560–568.

[2] Huxley RR, Neil HAW. The relation between dietary flavonol intake and coronary heart disease mortality: A meta-analysis of prospective cohort studies. Eur J Clin Nutr 2003;57:904–908.

[3] Maron DJ. Flavonoids for reduction of atherosclerotic risk. Curr Atheroscler Rep 2004;6:73–78.

[4] Buijsse B, Feskens EJM, Kok FJ, Kromhout D. Cocoa intake, blood pressure, and cardiovascular mortality: The Zutphen elderly study. Arch Intern Med 2006;166:411–417.

[5] Heiss C, Dejam A, Kleinbongard P, Schewe T, Sies H, Kelm M. Vascular effects of cocoa rich in flavan-3-ols. JAMA 2003;290:1030–1031.

[6] Fisher NDL, Hughes M, Gerhard-Herman M, Hollenberg NK. Flavanol-rich cocoa induces nitric-oxide-dependent vasodilation in healthy humans. J Hypertens 2003;21:2281–2286.

[7] Engler MB, Engler MM, Chen CY, Malloy MJ, Browne A, Chiu EY, Kwak HK, Milbury P, Paul SM, Blumberg J, Mietus-Snyder ML. Flavonoid-rich dark chocolate improves endothelial function and increases plasma epicatechin concentrations in healthy adults. J Am Coll Nutr 2004;23:197–204.

[8] Sies H, Schewe T, Heiss C, Kelm M. Cocoa polyphenols and inflammatory mediators. Am J Clin Nutr 2005;81:304–312.

[9] Heiss C, Kleinbongard P, Dejam A, Perre S, Schroeter H, Sies H, Kelm M. Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. J Am Coll Cardiol 2005;46:1276–1283.

[10] Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Sies H, Kwik-Uribe C, Schmitz HH, Kelm M. (−)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. Proc Natl Acad Sci USA 2006;103:1024–1029.

[11] Zhang Y, Hogg N. *S*-nitrosothiols: Cellular formation and transport. Free Radic Biol Med 2005;38:831–838.

[12] Rassaf T, Kleinbongard P, Preik M, Dejam A, Gharini P, Lauer T, Erckenbrecht J, Duschin A, Schulz R, Heusch G, Feelisch M, Kelm M. Plasma nitrosothiols contribute to the systemic vasodilator effects of intravenously applied NO: Experimental and clinical Study on the fate of NO in human blood. Circ Res 2002;91:470–477.

[13] Taubert D, Berkels R, Roesen R, Klaus W. Chocolate and blood pressure in elderly individuals with isolated systolic hypertension. JAMA 2003;290:1029–1030.

[14] Manach C, Donovan JL. Pharmacokinetics and metabolism of dietary flavonoids in humans. Free Radic Res 2004; 38:771–785.

[15] Williamson G, Barron D, Shimoi K, Terao J. *In vitro* biological properties of flavonoid conjugates found *in vivo*. Free Radic Res 2005;39:457–469.

[16] Mehta P. Aspirin in the prophylaxis of coronary artery disease. Curr Opin Cardiol 2002;17:552–558.

[17] Rein D, Paglieroni TG, Wun T, Pearson DA, Schmitz HH, Gosselin R, Keen CL. Cocoa inhibits platelet activation and function. Am J Clin Nutr 2000;72:30–35.

[18] Pearson DA, Paglieroni TG, Rein D, Wun T, Schramm DD, Wang JF, Holt RR, Gosselin R, Schmitz HH, Keen CL. The effects of flavanol-rich cocoa and aspirin on *ex vivo* platelet function. Thromb Res 2002;106:191–197.

[19] Innes AJ, Kennedy G, McLaren M, Bancroft AJ, Belch JJF. Dark chocolate inhibits platelet aggregation in healthy volunteers. Platelets 2003;14:325–327.

[20] Murphy KJ, Chronopoulos AK, Singh I, Francis MA, Moriarty H, Pike MJ, Turner AH, Mann NJ, Sinclair AJ. Dietary flavanols and procyanidin oligomers from cocoa (*Theobroma cacao*) inhibit platelet function. Am J Clin Nutr 2003;77:1466–1473.

[21] Holt RR, Pearson DA, Schmitz HH, Kwik-Uribe CL, Keen CC. Cocoa flavanols and platelet function: An alternative or adjunct to daily aspirin?. In: Vorster HH, Blaauw R, Dhansay MA, Kuzwayo PMN, Moeng L, Wentzel-Viljoen E, editors. Proceedings of the 18th International Congress of Nutrition. Nutrition Safari for Innovative Solutions. Durban, South Africa. Basel, Switzerland: Sept 19–23; S. Karger Medical and Scientific Publishers, Article 187; 2005.

[22] Galle J, Hansen-Hagge T, Wanner C, Seibold S. Impact of oxidized low density lipoprotein on vascular cells. Atherosclerosis 2006;185:219–226.

[23] Schewe T, Sies H. Myeloperoxidase-induced lipid peroxidation of LDL in the presence of nitrite. Protection by cocoa flavanols. Biofactors 2005;24:49–58.

[24] Byun J, Mueller DM, Fabjan JS, Heinecke JW. Nitrogen dioxide radical generated by the myeloperoxidase-hydrogen peroxide-nitrite system promotes lipid peroxidation of low density lipoprotein. FEBS Lett 1999;455:243–246.

[25] Schmitt D, Shen Z, Zhang R, Colles SM, Wu W, Salomon RG, Chen Y, Chisolm GM, Hazen SL. Leukocytes utilize myeloperoxidase-generated nitrating intermediates as physiological catalysts for the generation of biologically active oxidized lipids and sterols in serum. Biochemistry 1999;38:16904–16915.

[26] Carr AC, McCall MR, Frei B. Oxidation of LDL by myeloperoxidase and reactive nitrogen species: Reaction pathways and antioxidant protection. Arterioscler Thromb Vasc Biol 2000;20:1716–1723.

[27] Kostyuk VA, Kraemer T, Sies H, Schewe T. Myeloperoxidase/nitrite-mediated lipid peroxidation of low-density lipoprotein as modulated by flavonoids. FEBS Lett 2003;537:146–150.

[28] Kraemer T, Prakosay I, Date RA, Sies H, Schewe T. Oxidative modification of low-density lipoprotein: Lipid peroxidation by myeloperoxidase in the presence of nitrite. Biol Chem 2004;385:809–818.

[29] Steffen Y, Schewe T, Sies H. Epicatechin protects endothelial cells against oxidized LDL and maintains NO synthase. Biochem Biophys Res Commun 2005;331:1277–1283.

[30] Kleinveld HA, Hak-Lemmers HL, Stalenhoef AF, Demacker PN. Improved measurement of low-density-lipoprotein susceptibility to copper-induced oxidation: Application of a short procedure for isolating low-density lipoprotein. Clin Chem 1992;38:2066–2072.

[31] Brennan ML, Wu W, Fu X, Shen Z, Song W, Frost H, Vadseth C, Narine L, Lenkiewicz E, Borchers MT, Lusis AJ, Lee JJ,

Lee NA, Abu-Soud HM, Ischiropoulos H, Hazen SL. A tale of two controversies: Defining both the role of peroxidases in nitrotyrosine formation *in vivo* using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. J Biol Chem 2002;277:17415–17427.

[32] Carr AC, Frei B. The nitric oxide congener nitrite inhibits myeloperoxidase/H_2O_2/Cl^--mediated modification of low density lipoprotein. J Biol Chem 2001;276:1822–1828.

[33] Monzani E, Roncone R, Galliano M, Koppenol WH, Casella L. Mechanistic insight into the peroxidase catalyzed nitration of tyrosine derivatives by nitrite and hydrogen peroxide. Eur J Biochem 2004;271:895–906.

[34] Sparks DL, Phillips MC. Quantitative measurement of lipoprotein surface charge by agarose gel electrophoresis. J Lipid Res 1992;33:123–130.

[35] Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.

[36] Royall JA, Ischiropoulos H. Evaluation of 2′,7′-dichlorofluorescein and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch Biochem Biophys 1993;302:348–355.

[37] Wippel R, Rehn M, Gorren AC, Schmidt K, Mayer B. Interference of the polyphenol epicatechin with the biological chemistry of nitric oxide- and peroxynitrite-mediated reactions. Biochem Pharmacol 2004;67:1285–1295.

[38] Adams JD Jr, Lauterburg BH, Mitchell JR. Plasma glutathione and glutathione disulfide in the rat: Regulation and response to oxidative stress. J Pharmacol Exp Ther 1983;227:749–754.

[39] Zhang R, Brennan ML, Shen Z, MacPherson JC, Schmitt D, Molenda CE, Hazen SL. Myeloperoxidase functions as a major enzymatic catalyst for initiation of lipid peroxidation at sites of inflammation. J Biol Chem 2002;277:46116–46122.

[40] Ichimori K, Fukuyama N, Nakazawa H, Aratani Y, Koyama H, Takizawa S, Kameoka Y, Ishida-Okawara A, Kohi F, Suzuki K. Myeloperoxidase has directly-opposed effects on nitration reaction—study on myeloperoxidase-deficient patient and myeloperoxidase-knockout mice. Free Radic Res 2003;37:481–489.

[41] Whiteman M, Halliwell B. Loss of 3-nitrotyrosine on exposure to hypochlorous acid: Implications for the use of 3-nitrotyrosine as a bio-marker *in vivo*. Biochem Biophys Res Commun 1999;258:168–172.

[42] Daugherty A, Dunn JL, Rateri DL, Heinecke JW. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. J Clin Invest 1994;94:437–444.

[43] Leeuwenburgh C, Hardy MM, Hazen SL, Wagner P, Oh-ishi S, Steinbrecher UP, Heinecke JW. Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. J Biol Chem 1997;272:1433–1436.

[44] Leeuwenburgh C, Rasmussen JE, Hsu FF, Mueller DM, Pennathur S, Heinecke JW. Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques. J Biol Chem 1997; 272:3520–3526.

[45] Carr AC, Myzak MC, Stocker R, McCall MR, Frei B. Myeloperoxidase binds to low-density lipoprotein: Potential implications for atherosclerosis. FEBS Lett 2000;487: 176–180.

[46] Zouaoui BK, Moguilevsky N, Legssyer I, Babar S, Guillaume M, Delree P, Vanhaeverbeek M, Brohee D, Ducobu J, Remacle C. Oxidation of low density lipoproteins by myeloperoxidase at the surface of endothelial cells: An additional mechanism to subendothelium oxidation. Biochem Biophys Res Commun 2004;325:434–438.

[47] Podrez EA, Schmitt D, Hoff HF, Hazen SL. Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form *in vitro*. J Clin Invest 1999;103:1547–1560.

[48] Brennan ML, Penn MS, Van Lente F, Nambi V, Shishehbor MH, Aviles RJ, Goormastic M, Pepoy ML, McErlean ES, Topol EJ, Nissen SE, Hazen SL. Prognostic value of myeloperoxidase in patients with chest pain. N Engl J Med 2003;349:1595–1604.

[49] Abu-Soud HM, Hazen SL. Nitric oxide is a physiological substrate for mammalian peroxidases. J Biol Chem 2000;275:37524–37532.

[50] Schroeder P, Klotz LO, Sies H. Amphiphilic properties of (−)-epicatechin and their significance for protection of cells against peroxynitrite. Biochem Biophys Res Commun 2003; 307:69–73.

[51] Spencer JP, Schroeter H, Shenoy B, Srai SK, Debnam ES, Rice-Evans C. Epicatechin is the primary bioavailable form of the procyanidin dimers B2 and B5 after transfer across the small intestine. Biochem Biophys Res Commun 2001; 285:588–593.

[52] Tsang C, Auger C, Mullen W, Bornet A, Rouanet JM, Crozier A, Teissedre PL. The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. Br J Nutr 2005;94:170–181.

[53] Holt RR, Lazarus SA, Sullards MC, Zhu QY, Schramm DD, Hammerstone JF, Fraga CG, Schmitz HH, Keen CL. Procyanidin dimer B2 [epicatechin-(4beta-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. Am J Clin Nutr 2002;76:798–804.

[54] Tauber AI, Fay JR, Marletta MA. Flavonoid inhibition of the human neutrophil NADPH-oxidase. Biochem Pharmacol 1984;33:1367–1369.

[55] Fuhrman B, Aviram M. Flavonoids protect LDL from oxidation and attenuate atherosclerosis. Curr Opin Lipidol 2001;12:41–48.

[56] Berkels R, Nouri SK, Taubert D, Bartels H, Roesen P, Roesen R, Klaus W. The HMG-CoA reductase inhibitor cerivastatin enhances the nitric oxide bioavailability of the endothelium. J Cardiovasc Pharmacol 2003;42:356–363.

[57] Chen ZY, Yao XQ, Chan FL, Lau CW, Huang Y. (−)-Epicatechin induces and modulates endothelium-dependent relaxation in isolated rat mesenteric artery rings. Acta Pharmacol Sin 2002;23:1188–1192.

[58] Corvazier E, Maclouf J. Interference of some flavonoids and non-steroidal anti-inflammatory drugs with oxidative metabolism of arachidonic acid by human platelets and neutrophils. Biochim Biophys Acta 1985;835:315–321.

[59] Pignatelli P, Pulcinelli FM, Celestini A, Lenti L, Ghiselli A, Gazzaniga PP, Violi F. The flavonoids quercetin and catechin synergistically inhibit platelet function by antagonizing the intracellular production of hydrogen peroxide. Am J Clin Nutr 2000;72:1150–1155.

[60] Freedman JE, Parker C III, Li L, Perlman JA, Frei B, Ivanov V, Deak LR, Iafrati MD, Folts JD. Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. Circulation 2001;103:2792–2798.

[61] Gries A, Herr A, Motsch J, Holzmann A, Weimann J, Taut F, Erbe N, Bode C, Martin E. Randomized, placebo-controlled, blinded and cross-matched study on the antiplatelet effect of inhaled nitric oxide in healthy volunteers. Thromb Haemost 2000;83:309–315.

[62] Danielewski O, Schultess J, Smolenski A. The NO/cGMP pathway inhibits Rap 1 activation in human platelets via cGMP-dependent protein kinase I. Thromb Haemost 2005;93:319–325.

[63] Benito S, Lopez D, Sáiz MP, Buxaderas S, Sánchez J, Puig-Parellada P, Mitjavila MT. A flavonoid-rich diet increases

nitric oxide production in rat aorta. Br J Pharmacol 2002;135:910–916.

[64] Madajka M, Korda M, White J, Malinski T. Effect of aspirin on constitutive nitric oxide synthase and the biovailability of NO. Thromb Res 2003;110:317–321.

[65] Grosser N, Schröder H. Aspirin protects endothelial cells from oxidant damage via the nitric oxide-cGMP pathway. Arterioscler Thromb Vasc Biol 2003;23:1345–1351.

[66] Taubert D, Berkels R, Grosser N, Schroder H, Grundemann D, Schomig E. Aspirin induces nitric oxide release from vascular endothelium: A novel mechanism of action. Br J Pharmacol 2004;143:159–165.

[67] Zimmermann N, Wenk A, Kim U, Kienzle P, Weber AA, Gams E, Schrör K, Hohlfeld T. Functional and biochemical evaluation of platelet aspirin resistance after coronary artery bypass surgery. Circulation 2003;108:542–547.