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Review

Vascular protection by dietary polyphenols

Jean-Claude Stoclet*, Thierry Chataigneau, Mamadou Ndiaye, Min-Ho Oak, Jasser El Bedoui, Marta Chataigneau, Valérie B. Schini-Kerth

Pharmacologie et Physico-Chimie des Interactions Cellulaires et Moléculaires, UMR CNRS 7034, Faculté de Pharmacie, Université Louis Pasteur de Strasbourg, B. P. 60024, 74 route du Rhin, F 67401 Illkirch, France

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Abstract

Consumption of polyphenol-rich foods, such as fruits and vegetables, and beverages derived from plants, such as cocoa, red wine and tea, may represent a beneficial diet in terms of cardiovascular protection. Indeed, epidemiological studies demonstrate a significant inverse correlation between polyphenol consumption and cardiovascular risk. Among the numerous plausible mechanisms by which polyphenols may confer cardiovascular protection, improvement of the endothelial function and inhibition of angiogenesis and cell migration and proliferation in blood vessels have been the focus of recent studies. These studies have indicated that, in addition to and independently from their antioxidant effects, plant polyphenols (1) enhance the production of vasodilating factors [nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin] and inhibit the synthesis of vasoconstrictor endothelin-1 in endothelial cells; and (2) inhibit the expression of two major pro-angiogenic factors, vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2) in smooth muscle cells. The mechanisms of these effects involve: (1) in endothelial cells, increased Ca^{2+} level and redox-sensitive activation of the phosphoinositide 3 (PI3)-kinase/Akt pathway (leading to rapid and sustained activation of nitric oxide synthase and formation of EDHF) and enhanced expression of nitric oxide synthase; and (2) in smooth muscle cells, both redox-sensitive inhibition of the p38 mitogen-activated protein kinase (p38 MAPK) pathway activation (leading to inhibition of platelet-derived growth factor (PDGF)-induced VEGF gene expression) and redox-insensitive mechanisms (leading to inhibition of thrombin-induced MMP-2 formation). The current evidence suggests that all these mechanisms are triggered by polyphenols with specific structures, although the structural requirements may be different from one effect to the other, and that they all contribute to the vasoprotective, anti-angiogenic, anti-atherogenic, vasorelaxant and anti-hypertensive effects of acute or chronic administration of plant polyphenols found in vivo in animals and in patients.

Keywords: Polyphenol; Wine; Tea; Chocolate; Vascular protection

Contents

* Corresponding author. Tel.: +33 03 90 24 42 46; fax: +33 03 90 24 43 13.
E-mail address: stoclet@aspirine.u-strasbg.fr (J.-C. Stoclet).

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1. Introduction

Phenolic substances, especially polyphenols containing several hydroxyl groups directly associated with a cyclic benzene ring, are widely distributed in plants. Growing epidemiological evidence suggests the existence of a negative correlation between consumption of polyphenol-rich foods (fruits, vegetables, cocoa contained in chocolate, etc.) or beverages (wine, especially red wine, grape juice, tea, etc.) and the incidence of cardiovascular disease (Miyagi et al., 1997; Nakachi et al., 2000; Osakabe et al., 2000; Rein et al., 2000; Renaud and de Lorgeril, 1992; Saint-Leger et al., 1979; Sasazuki et al., 2000) and stroke (Truelsen et al., 1998). The popular term "French paradox" has been coined by epidemiologists who pointed out the relatively low incidence of coronary heart disease in the French population, despite high saturated fat consumption, a well-known major risk factor for coronary disease (Artaud-Wild et al., 1993; Criqui and Ringel, 1994; Richard et al., 1981).

The conclusions of epidemiological studies are summarized below. They prompted investigations on the effects of plant polyphenols in animal models and human beings. A major difficulty with this approach is the extreme complexity of the polyphenolic content of food and beverages. There are several hundred plant phenolic compounds including non-flavonoids (phenolic acids, stilbenes like *trans*-resveratrol) and flavonoids, i.e., molecules possessing two phenols joined by a pyran carbon ring structure (flavanols, flavonols and anthocyanins). These molecules may be present under their free form or polymerized to other non-flavonoid or flavonoid or sugar molecules, or a combination of these, forming, for example, condensed or hydrolysable tannins or combined phenolic compounds. The phenolic composition of wine has received particular attention (see, for instance, German and Walzem, 2000; Soleas et al., 1997), because it is particularly high (0.01% in white and up to 0.2% in red wines) and because of the hypothesis that polyphenols may be involved in the "French paradox" (as red wine consumption is high in France). All classes of phenols are more abundant in red than in white wines, particularly anthocyanins (20 to 500 mg/l) which give the red colour. Chocolate containing cocoa and tea are also a rich source of flavonoids, particularly flavan-3-ols in chocolate (Wollgast et al., 2001) and catechins in tea (up to 30% of the dry weight in green tea (Arts et al., 2001; Harbowy and Balentine, 1997). Due to extreme complexity of the polyphenolic composition, crude preparations from dietary components have been used in experimental studies, and their effects have been compared to those of commercially available reference defined compounds. The exact composition of the extracts and the degree of polymerization of compounds in the used solutions are generally unknown.

Various chemical properties and biological effects of dietary polyphenols might be involved in protection against cardiovascular risk. An obvious hypothesis is that the antioxidant properties of polyphenols might protect blood vessels against the deleterious consequences of oxidant stress associated with many if not all cardiovascular risk factors. This hypothesis is briefly discussed below.

As early as 1990s, Fitzpatrick et al. (1993) reported that grape juices and grape skin extracts cause endothelium-dependent vasorelaxation. The same effect (although with different potencies) has later been found with aqueous extracts from a variety of vegetables and fruits and with different wine extracts (Fitzpatrick et al., 1995). A subsequent study has suggested that the endothelium-dependent vasorelaxant effect of wine polyphenols is not due to their superoxide scavenging effect but rather to increased formation of nitric oxide (NO) likely resulting from direct interaction with target(s) located on endothelial cells (Andriambeloson et al., 1997, 1998). These early findings prompted further studies, discussed below, on the effects of polyphenols on production and biological activity of endothelium-derived vasoactive factors.

The protective effect of dietary polyphenols against vascular risk, i.e., their ability to decrease the ischaemic obstruction events rate, might be attributed, in part, to

their ability to retard the progression of early atherosclerotic lesions to advanced plaques which are prone to rupture with superimposed thrombosis. In support of this hypothesis, it has been reported that polyphenolic compounds from red wine and green tea are able to prevent the progression of atherosclerosis in animal models (da Luz et al., 1999; Hayek et al., 1997; Miura et al., 2001; Vinson et al., 2001), although red wine is not any more able to reduce mature atherosclerosis once the plaques formed in apolipoprotein-deficient mice (Bentzon et al., 2001). The observation that the number of adventitial vasa vasorum is increased in advanced human and experimental atherosclerosis (Kamat et al., 1987; Williams and Tabas, 1998; Zamir and Silver, 1985) and that the severity of atherosclerotic plaques is correlated with the development of vasa vasorum (Kumamoto et al., 1995) has led to hypothesize that vasa vasorum contribute to the development and progression of atherosclerosis and possibly to complications such as intimal haemorrhage and plaque rupture. Therefore, recent investigations have examined the possibility that dietary polyphenols prevent the development of atherosclerosis by inhibiting the angiogenic process and proliferation and migration of endothelial and vascular smooth muscle cells. These studies will be discussed below.

The beneficial effects of dietary polyphenols on vascular ischaemic obstruction events might also be related to prevention of thrombosis resulting from inhibition of platelet activation (Sagesaka-Mitane et al., 1990; Wang et al., 2002; Wollny et al., 1999) or from decreased expression of pro-thrombotic and pro-atherosclerotic molecules such as tissue factor (Pendurthi et al., 1999b) and monocyte chemotactic protein-1 (Feng et al., 1999). The present short review will not discuss all these effects in a comprehensive manner. Rather, it will focus on investigations from this and other laboratories on two aspects of the effects of dietary polyphenols: improvement of endothelium function and anti-angiogenic properties.

2. The epidemiological evidence

Pioneer studies leading to the concept of "French paradox" were based on preexisting data from government agencies. More thorough and extensive epidemiological studies like the MONICA project, using identical criteria to determine the coronary-event rate in different populations, confirmed the existence of a decreasing gradient in coronary-event rate from northern to southern Europe, although not restricted to France (Kuulasmaa et al., 2000; Tunstall-Pedoe et al., 1999, 2000). Strikingly, the relatively low coronary-event rate found in these studies in France and other Mediterranean countries was associated with a global risk score (including factors such as blood pressure, low- and high-density lipoprotein (LDL and HDL, respectively) cholesterol, age, sex, smoking and glucose intolerance) comparable to the one found in populations from other developed countries in which the coronary-event rate was much higher. The reason(s) for this discrepancy remains an open question.

A strong negative correlation between moderate consumption of alcoholic beverages (one to three "drinks" or glasses of wine per day) and coronary heart disease or ischaemic stroke mortality has been recognised for many years, whereas higher alcohol intake increases the risk of mortality from various causes (Friedman and Kimball, 1986; Kiechl et al., 1998; Klatsky et al., 1992; Marmot et al., 1981; Renaud et al., 1998; Rimm et al., 1999; Sacco et al., 1999; Saint-Leger et al., 1979). The involvement of alcohol itself is supported by the finding that different alcoholic beverages decreased the risk in light drinkers. The beneficial effects of alcohol might be due to enhanced high-density lipoprotein (HDL) and decreased fibrinogen levels. However, the inverse association between mortality and moderate drinking was found stronger for wine than for beer and spirits in some studies (de Gaetano and Cerletti, 2001; Gronbaek, 2000; Renaud et al., 1998), suggesting the implication of other constituents of wine than alcohol. A major difficulty with such studies is that differences in consumption patterns and aspects of lifestyle associated with choice of a drink may interfere in the results.

3. The antioxidant hypothesis

The most popular hypothesis on the mechanism of beneficial effects of polyphenols is that the direct antioxidant effect of polyphenols, through their interactions with superoxide and other reactive oxygen species such as hydroxy and peroxy radicals (Hu et al., 1995; Nijveldt et al., 2001; Robak and Gryglewski, 1988; Torel and Cillard, 1986), may be implicated. For instance, it has been suggested that flavonoids might protect antithrombotic endothelial factors NO and prostacyclin from breakdown owing to their superoxide scavenging effect (Gryglewski et al., 1987a,b). Indeed, the peroxynitrite scavenging properties of procyanidins condensed tanins oligomers (arising from condensation of flavan-3-ol and flavan 3,4-diol) can protect endothelial cells from membrane lipid oxidation and cytotxicity (Aldini et al., 2003).

In addition it has been proposed that antioxidant properties of polyphenols might protect vascular endothelial function against the deleterious consequences of oxidation of low-density lipoproteins (LDLs), as oxidized LDL can impair endothelium-dependent vasorelaxation (Deckert et al., 1997). It is well established that oxidant stress, by causing oxidation of the NO synthase co-factor tetrahydrobiopterin, "uncouples" the enzyme and switches its activity from NO to superoxide production. This mechanism might

be involved in endothelial dysfunction associated with diabetes, obesity, hypercholesterolemia and atherosclerosis, and in smokers (Harrison, 1997; Li and Forstermann, 2000; Miller et al., 2000).

It has been reported that consumption of wine (Fuhrman et al., 1995), or polyphenols from red wine (Nigdikar et al., 1998), or purple grape juice (Stein et al., 1999) enhances the antioxidant capacity of plasma and reduces circulating LDL oxidability. After grape juice consumption, these effects were associated with restoration of endothelial function in patients with coronary artery disease. However, reduction of LDL oxidability after diet supplementation with wine polyphenols has not been found in all studies, despite enhanced antioxidant capacity of plasma (Carbonneau et al., 1997). In addition to delaying LDL oxidation, phenols like caffeic acid may act as cytoprotective agents against apoptosis of endothelial cells, by blocking signalling triggered by oxidized LDL (Vieira et al., 1998).

An objection against the antioxidant hypothesis of beneficial effects of polyphenols in vascular diseases is that compounds with identical antioxidant properties have differential effects. This is the case, for instance, of polyphenols producing endothelium-dependent vasorelaxation (Andriambeloson et al., 1998). However, stimulation of endogenous antioxidant enzymes (Nijveldt et al., 2001) and inhibition of xanthine oxidase and NAD(P)H oxidase (Nijveldt et al., 2001; Orallo et al., 2002), two enzymes generating large amounts of reactive oxygen species, may also participate in reduction of oxidant stress by some polyphenols (independently from their direct antioxidant properties). Thus, in vivo vasculoprotective effects of polyphenols may be produced either by direct inhibition of circulating LDL oxidation or by stimulation of endogenous defence mechanisms against oxidant stress, or both. Further investigations are required to elucidate the underlying mechanisms.

4. Improvement of endothelium function by plant polyphenols

Since the initial demonstration that the endothelium not only functions as a permeability barrier but also as an authentic organ implied in the control of vascular homeostasis (Furchgott and Zawadzki, 1980), several endothelium-derived relaxing factors have been identified. These factors include NO, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF; Feletou and Vanhoutte, 1988; Furchgott and Zawadzki, 1980; Ignarro et al., 1987; Moncada and Vane, 1978; Palmer et al., 1987). In addition to vasodilatory and anti-aggregant properties shared by both NO and prostacyclin, anti-thrombotic and anti-proliferative properties have been attributed to NO. The chemical structure of EDHF is not entirely elucidated; it is probably not identical in different vessel types (Feletou and Vanhoutte, 1988). EDHF is involved in the regulation of vascular tone and this participation increases as the vessel size decreases (Shimokawa et al., 1996). However, the active role of EDHF in the control of arterial blood pressure remains to be fully demonstrated (Feletou and Vanhoutte, 2004).

The vascular endothelium can also produce potent contracting factors such as endothelin-1. In addition, endothelin-1 is pro-inflammatory and promotes fibrosis, arterial remodeling and vascular injury. If endogenously generated endothelin-1 appears to play a modest role in the healthy organism, increased plasma levels of the vasoconstrictor endothelin-1 are associated with some forms of hypertension or heart failure, corroborating a major role of the peptide in the ethiology of these pathologies (Moe et al., 2003; Touyz and Schiffrin, 2003).

Thus, endothelium-derived factors play not only a major role in the control of vascular tone but also in other important aspects of vascular biology. In particular, endothelial NO exerts vasoprotective effects. Its deficiency favours the development of atherosclerosis and is associated with increased cardiovascular risk in pathological situations such as diabetes, the metabolic syndrome, hypertension and atherosclerosis (for reviews, see Busse and Fleming, 1996; Gewaltig and Kojda, 2002). Restoration of the equilibrium between endothelium-derived factors might be involved in vasoprotective effects of polyphenols.

4.1. Plant polyphenols induced increase of endothelial NO synthase activity

In addition to their antioxidant effects which are assumed to increase the biodisponibility of NO, polyphenols have been shown to increase the formation of NO by endothelial NO synthase. After the pioneer studies of Fitzpatrick et al. (1993), performed in the rat aorta with various grape products, similar conclusions were drawn from studies performed in various animal and human isolated vessels (Aldini et al., 2003; Cishek et al., 1997; Fitzpatrick et al., 2000; Flesch et al., 1998; Mendes et al., 2003; Ndiaye et al., 2003a,b; Soares De Moura et al., 2002), using plant polyphenols from various sources such as different wines, cocoa, tea, hawthorn, maritime pine bark, honey and propolis (Andriambeloson et al., 1997; Chen et al., 1998; Duarte et al., 2001; Fitzpatrick et al., 1995; Flesch et al., 1998; Karim et al., 2000; Kim et al., 2000; Lemos et al., 1999; Lorenz et al., 2004; Taubert et al., 2002). In all studies, plant polyphenols produced endothelium-dependent vasorelaxation that was generally associated with increased cyclic GMP formation and blunted by inhibitors of NO synthase, indicating that it was mediated by the NO-cyclic GMP pathway. Furthermore, the relaxant effect was strongly correlated with the concentration of polyphenols in wine (Burns et al., 2000). The effect of plant extracts containing polyphenols and derived from Chinese medicinal herbs on the NO/cGMP pathway in vascular cells has been recently reviewed (Achike and Kwan, 2003) and will not be exposed

in this review. The demonstration of an increased formation of NO by intact segments of rat aorta and porcine coronary artery has been performed using electron paramagnetic resonance spectroscopy and a NO-selective microsensor, respectively (Andriambeloson et al., 1997; Stoclet et al., 1999; Taubert et al., 2002). It should be noted that, in addition to endothelium-dependent vasorelaxation, many of these plant polyphenols can cause endothelium-independent vasorelaxation. However, the latter effect generally takes place at much higher concentrations than the former.

Analysis of structure–activity (endothelial NO release and relaxation of isolated vessels) relationships for polyphenols of different classes has also been extensively examined (Ajay et al., 2003; Chan et al., 2000; Stoclet et al., 2000; Taubert et al., 2002). In red wine, the most active fractions have been found in flavanol-3-ol enriched oligomeric condensed tanins enriched fractions, especially dimers and trimers, and the most active monomer was the anthocyanin delphinidin (Andriambeloson et al., 1998). Interestingly, other anthocyanins with closely related structures, such as malvidin and cyanidin, were found inactive. Substitution of the flavan moiety with free hydroxyl residues at precise positions has been found important for induction of endothelial nitric oxide release (Taubert et al., 2002). Altogether, these studies indicate the structural specificity of the effect of polyphenols on NO release from endothelial cells. However, the molecular target(s) is(are) still unknown.

In endothelium, NO is formed from L-arginine by the constitutive endothelial NO synthase (eNOS) in response to shear stress, circulating hormones, local autacoids, substances released by platelets, by the coagulation cascade and by the autonomic nervous system (Mombouli and Vanhoutte, 1999). The mechanisms of NO synthase activation in response to the above enumerated stimuli, except shear stress, involve intracellular Ca^{2+}. By contrast, in response to shear stress, activation of the phosphoinositide 3 (PI3)-kinase/Akt pathway by blood flow causes rapid $[Ca^{2+}]_i$-independent eNOS stimulation through its phosphorylation at Ser1177 (Dimmeler et al., 1999). Studies have been performed on the implication of these two mechanisms in polyphenol-induced activation of NO synthase activity.

4.1.1. Role of intracellular Ca^{2+}

The involvement of Ca^{2+} is supported by the findings that relaxation of the rat aorta (Andriambeloson et al., 1999) and the increase in $[Ca^{2+}]_i$ in bovine aortic endothelial cells (Stoclet et al., 1999) caused by an alcohol-free red wine polyphenolic extract and by delphinidin were both Ca^{2+}-dependent and were both produced at the same concentrations. In addition the increase in $[Ca^{2+}]_i$ was associated with NO release, as shown in a cascade bioassay (Martin et al., 2002). Interestingly, wine polyphenols were unable to induce any rise in $[Ca^{2+}]_i$ in smooth muscle cells (Stoclet et al., 1999), showing the cell selectivity of this effect.

4.1.2. Role of the PI3-kinase/Akt pathway

Even if an increase in ($[Ca^{2+}]_i$) in endothelial cells constitutes an important mechanism leading to eNOS activation by polyphenols, this does not preclude a possible implication of the PI3-kinase/Akt pathway. It has been recently shown that NO-mediated relaxations induced by the red wine polyphenolic extract that produced an elevation in $[Ca^{2+}]_i$ in bovine aorta endothelial cells, were reduced by inhibitors of PI3-kinase [wortmannin and 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one, LY294002] in porcine coronary arteries (Ndiaye et al., 2003a, b). In addition, the formation of NO caused by red wine polyphenolic compounds (RWPCs), as assessed by electron spin resonance spectroscopy, and the formation of cyclic GMP in coronary artery endothelial cells, were reduced by inhibitors of PI3-kinase. These polyphenols induced a sustained phosphorylation of Akt and eNOS at Ser1177 in endothelial cells, which was abolished by inhibitors of PI3-kinase (Ndiaye et al., 2003a,b). Altogether, these data demonstrate that RWPCs induced the activation of the PI3-kinase/Akt pathway in porcine coronary artery endothelial cells which, in turn, caused phosphorylation of eNOS resulting in an increased formation of NO.

Implication of this alternative mechanism to Ca^{2+} activation of eNOS has been confirmed by a study showing that epigallocatechin-3-gallate, a flavonoid from the 3-flavanol subclass found in green tea, also induced the phosphorylation of eNOS at Ser1179 in bovine aortic endothelial cells (Lorenz et al., 2004). A feature of this mechanism is that it leads to a rapid and long-lasting activation of eNOS (Lorenz et al., 2004; Ndiaye et al., 2003a,b). The respective implication of Ca^{2+} and PI3-kinase/Akt in stimulation of eNOS by polyphenols in the endothelial cells of different vascular beds deserves future investigations.

4.2. Plant polyphenols induced increase of eNOS expression

Long-term incubation of endothelial cells with red wine increases eNOS expression (Wallerath et al., 2002, 2003). However, this effect was achieved with large volumes of red wine (1 % v/v in culture medium for 10 days exposure, 3 % v/v for 24-h exposure and 10 % v/v for 12 h; Wallerath et al., 2003). The stimulatory effect was attributed to polyphenols given that ethanol alone had no effect on eNOS expression (Wallerath et al., 2003), whereas an increase was observed in response to a red wine extract without alcohol (Leikert et al., 2002). Nevertheless, the latter effect was also obtained with a relatively high concentration of the extract (400 mg/l).

Resveratrol, a polyphenolic phytoestrogen found in some grapes and wines, also stimulated eNOS expression in concentrations ranging from 10 to 100 μM (Hsieh et al., 1999; Wallerath et al., 2002). The upregulation of eNOS mRNA was associated to an enhanced expression in eNOS protein and enzymatic activity. Resveratrol increased the activity of the eNOS promoter (transcriptional effect) and

stabilized eNOS mRNA (posttranscriptional effect; Wallerath et al., 2002).

4.3. Plant polyphenols induced EDHF-mediated relaxation and hyperpolarization

In most arteries, there is a component of endothelium-dependent relaxation which is resistant to inhibitors of NO synthase and cyclooxygenase. These relaxations are associated to endothelium-dependent hyperpolarization of smooth muscle cells, also resistant to inhibitors of NO and prostanoid formation. These endothelium-dependent responses are abolished by the combination of two toxins, charybdotoxin (an inhibitor of intermediate and large conductance Ca^{2+}-activated K^+ channels, IK_{Ca} and BK_{Ca}, respectively and certain voltage-dependent K^+ channels) and apamin (a selective inhibitor of small conductance Ca^{2+}-activated K^+ channels, SK_{Ca}). They are attributed to EDHF (for review, see Vanhoutte, 2004). In fact, these two toxins abolish the endothelial hyperpolarization which is normally transmitted to the underlying smooth muscle cells and therefore, suppress the resulting EDHF-mediated relaxation of vascular smooth muscle cells (for review, see Busse et al., 2002; Griffith, 2004).

The first demonstration of a participation of EDHF to the mechanisms of vascular relaxation induced by plant polyphenols has been performed in isolated porcine coronary arteries (Ndiaye et al., 2003a,b). In this study, it was shown that RWPCs, at concentrations ranging from 1 to 100 mg/l, causes concentration-dependent relaxation and hyperpolarization of smooth muscle cells with a maximal effect reached at 100 mg/l.

The participation of EDHF to the endothelium-dependent relaxations induced by polyphenols has also been observed in response to the extract of Eucommia bark, a traditional Chinese medicinal herb containing polyphenols, in the rat mesenteric artery (Deyama et al., 2001; Kwan et al., 2004). The stilbene derivative, resveratrol, activated IK_{Ca} channels (24 pS) in endothelial cells through an increase in their open probability (Li et al., 2000). This effect could partially explain the stimulatory effect of polyphenols on EDHF-mediated response but other detailed mechanisms have been recently proposed.

4.3.1. A pro-oxidant effect of polyphenols in endothelial cells: involvement of superoxide anions in EDHF-mediated relaxation

Surprisingly, it has been observed that EDHF-mediated relaxation and hyperpolarization to RWPCs are critically dependent on a redox-sensitive mechanism involving the formation of superoxide anions by a flavin-dependent enzyme in porcine coronary endothelial cells (Ndiaye et al., 2003a,b). Indeed, these responses were reduced by antioxidants, such as *N*-acetylcysteine, and membrane permeant analogues of superoxide dismutase (SOD), such as Mn(III)-tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) and polyethylene glycol-SOD (PEG-SOD). However, they are unaffected by native SOD, native catalase or PEG-catalase. Furthermore, diphenylene iodonium, an inhibitor of flavin-dependent enzymes, reduced the EDHF-mediated relaxation. In addition, RWPCs induced the MnTMPyP-sensitive formation of superoxide in cultured endothelial cells, as shown by confocal microscopy experiments using the oxidative fluorescent dye hydroethidine (Ndiaye et al., 2003a,b).

4.3.2. The PI3-kinase/Akt pathway as a key pathway in EDHF-madiated relaxation induced by polyphenols

It was consecutively attempted to determine the role of redox-sensitive protein kinases, including p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2) and PI3-kinase/Akt, in red wine polyphenol-induced responses in porcine coronary arteries (Ndiaye et al., 2004). The EDHF-mediated relaxations were significantly reduced by wortmannin and LY294002, two inhibitors of PI3-kinase, and not affected by 2′ -amino-3′ -methoxyflavone (PD98059, an inhibitor of ERK1/2 kinase kinase) and 4-(4 fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H imidazole (SB203580, an inhibitor of p38 MAPK). In addition, red wine polyphenols elicited within min a sustained and concentration-dependent phosphorylation of p38 MAPK, ERK1/2 and Akt in endothelial cells. The phosphorylation of Akt in response to RWPCs was abolished by wortmannin and LY294002 as well as by MnTMPyP. These findings demonstrate that red wine polyphenol-induced EDHF-mediated relaxations of coronary arteries rely on the redox-sensitive activation of the PI3-kinase/Akt pathway in endothelial cells (Ndiaye et al., 2004).

Therefore, from all the previous studies, it can be concluded that plant polyphenols are potent inducers of endothelium-dependent vasorelaxations which involve both NO and EDHF (Fig. 1). Current evidence suggests that the signalling pathways leading to polyphenol-induced NO and EDHF formation are at least partially common.

4.4. Plant polyphenols induced increased prostacyclin release from endothelial cells

Procyanidins, oligomers of 3-flavanols (a subclass of flavonoids), from *Vitis vinifera* L. seeds induce concentration-dependent and endothelium-dependent relaxation in isolated human internal mammary artery, with a maximal vasorelaxant effect at 50 μM. This effect was significantly reduced (by almost 50%) following preincubation of arterial rings with indomethacin, a cyclooxygenase inhibitor, indicating the involvement of a prostanoid. Indeed, in these vessels, procyanidins stimulated, in a concentration-dependent fashion with a maximal effect between 25 and 50 μM, the prostacyclin release measured by evaluating the concentration of 6-keto-prostaglandin-$F_{1\alpha}$ in the bathing medium (Aldini et al., 2003). Both in vitro (cultured human aortic endothelial cells treated with the procyanidin extract

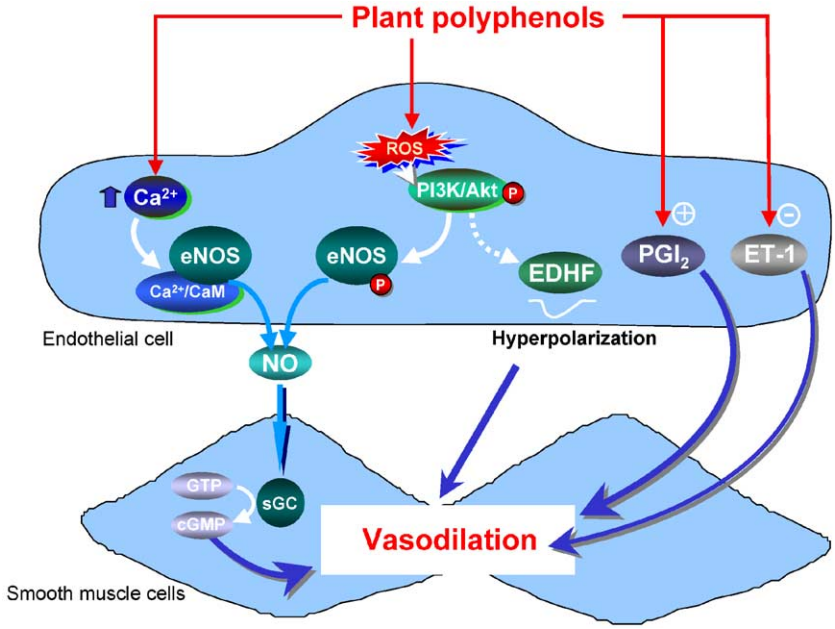

Fig. 1. Acute endothelium-dependent effects of plant polyphenols. Plant polyphenols induce nitric oxide (NO)-mediated endothelium-dependent relaxations in isolated arteries. The activation of endothelial NO synthase (eNOS) is due to two distinct mechanisms: (a) an increase in $[Ca^{2+}]_i$ and (b) a phosphorylation of eNOS by the PI3-kinase/Akt pathway. In addition, plant polyphenols cause endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxations of isolated arteries consecutively to a localized and controlled formation of superoxide anions leading to the activation of the PI3-kinase/Akt pathway. Polyphenols also increase endothelial prostacyclin release and inhibit the synthesis and the effects of endothelin-1. All these mechanisms might contribute to explain the vasodilatory, vasoprotective and anti-hypertensive effects of polyphenols in vivo. Long-term treatment of endothelial cells with polyphenols can also increase endothelial NO synthase expression (not shown). Ca^{2+}/CaM: Ca^{2+}/calmodulin complex; sGC: soluble guanylyl cyclase.

of cocoa, 2 mg/l) and in vivo (after an overnight fast, 10 healthy subjects consumed 37 g low-procyanidin, 0.09 mg/g high procyanidin and 4.0 mg/g chocolate), cocoa procyanidins decreased leukotriene/prostacyclin ratios by 58% and 52%, respectively (Schramm et al., 2001). On the other hand, if catechins from the 3-flavanol subclass increased the prostacyclin production by bovine aortic endothelial cells (Mizugaki et al., 2000), quercetin, another flavonoid but from the flavonol subclass, had opposite effects. Indeed, the prostacyclin release from cultured human umbilical vein endothelial cells, treated with activated platelets, was inhibited in a concentration-dependent manner by quercetin (1, 5 and 20 μM; Zhao et al., 1999).

Thus, it seems that some plant polyphenols, at least from the 3-flavanol subclass, could, by favourably altering eicosanoid synthesis, inhibit platelet activation and inflammatory processes that contribute to diseases. Because endothelium-dependent vasorelaxation produced by procyanidins could be completely suppressed by inhibition of the NO-cyclic GMP pathway, it has been suggested that NO was involved in the activation of cyclooxygenase (Aldini et al., 2003).

4.5. Plant polyphenols induced inhibition of the endothelial synthesis of endothelin-1

Red wine extracts strongly inhibit endothelin-1 release and transcription of the prepro-endothelin-1 gene in bovine aortic endothelial cells, with a maximal effect at concentrations lower than 50 mg/l (Corder et al., 2001; Khan et al., 2002). This effect is likely to be due to modifications of tyrosine kinase signaling (Khan et al., 2002). Resveratrol also potently inhibits strain-induced endothelin-1 secretion, endothelin-1 mRNA level and endothelin-1 promoter activity in human umbilical vein endothelial cells, partially by interfering with the ERK1/2 pathway through attenuation of reactive oxygen species formation (Liu et al., 2003). In addition, pretreatment of cultured human umbilical vein endothelial cells with quercetin, at 5 or 50 μM, reduces thrombin-induced endothelin-1 release (Zhao et al., 1999).

Hence, inhibition of endothelin-1 synthesis may represent one of the mechanisms by which plant polyphenols may restore the balance between endothelium-derived vasoconstrictor and vaodilating factors when pathologically impaired (Fig. 1), and prevent the development of hypertension or heart failure.

4.6. Vasodilatory and anti-hypertensive effects of polyphenols in vivo and ex vivo

The clinical relevance of endothelium-dependent effects of plant polyphenols will be dependent upon their systemic availability. Therefore, intestinal absorption and metabolism of the plant polyphenols are a rate-limiting step for the protective effects of this class of compounds. Thus, in vitro

effects should always be confronted to in vivo experiments and even clinical trials before it can be clearly stated that they underlie the protective properties assigned to plant polyphenols in the cardiovascular system. An intake of 100 ml of red wine by healthy volunteers causes an increase of about 3 mg/l after 30 min of plasma total phenol content (Duthie et al., 1998). Therefore, the vasorelaxant effects of plant polyphenols in isolated arteries are compatible with the concentrations reached in human blood.

Studies have been performed in patients and in animals on haemodynamic effects of both acute and chronic administration of various preparations from polyphenol-rich foods or beverages, especially grape extracts, wine, tea or chocolate.

In healthy volunteers, the coronary flow-velocity reserve was increased 30 min after drinking red wine (1 g/kg ethanol), but not after drinking the same quantity of alcohol in white wine or vodka (Shimada et al., 1999). The endothelium-dependent vasodilation was also improved after acute intake of 500 ml of red wine or red wine without alcohol in men, as determined by ultrasonography of the brachial artery (Hashimoto et al., 2001).

Relatively short-term (14 days) ingestion of purple grape juice (7.7±1.2 ml/kg/day) was also associated with a significant improvement of the endothelium-dependent vasodilation in men (Stein et al., 1999). Furthermore, a regular ingestion of 5 cups/day of black tea for 4 weeks by 21 subjects has been shown to result in a significant increase in endothelium-dependent vasodilatation (Hodgson et al., 2002).

Consumption of wine polyphenol-, quercetin- or catechin-enriched diets increased aortic NO production in rats (Benito et al., 2002). Oral administration of an alcohol-free hydroalcoholic grape skin extract (from vinifera grape, *Vitis labrusca*) significantly reduced systolic, mean and diastolic arterial pressure in two distinct models of hypertensive Wistar rats (Soares De Moura et al., 2002). Intragastric administration of resveratrol (3 mg/kg/day), red wine (4 ml/kg/day) or even dealcoholized red wine (4 ml/kg/day) for 12 weeks to hypercholesterolemic rabbits improved the endothelial function, reduced plasma endothelin-1 levels and induced a significant elevation in NO levels (Zou et al., 2003).

In patients with mild isolated systolic hypertension, intake of 14 consecutive daily doses of 100 g dark polyphenol-rich chocolate (500 mg of polyphenols), but not polyphenol-free chocolate, decreased both diastolic and systolic blood pressure within 10 days (Taubert et al., 2003). Interestingly, it has been observed that in healthy humans, a regular intake of flavanol-rich cocoa for 4 days (821 mg of flavanols/day) induced a prominent peripheral vasodilation via activation of the NO pathway (Fisher et al., 2003). In addition, ingestion of 100 ml of cocoa drink containing high amounts of 3-flavanols (176 mg constituted by 70 mg of epicatechin plus catechin and 106 mg of procyanidins) increased the endothelium-dependent vasodilation, with a maximal effect at 2 h, in patients with at least one cardiovascular risk factor (history of coronary artery disease, hypertension, hyperlipidemia, diabetes or tobacco use) in comparison to controls (ingestion of 100 ml of cocoa drink containing less than 10 mg of 3-flavanols). This effect was correlated to an increase in plasma NO pool (Heiss et al., 2003).

All these studies strongly support the view that polyphenol-rich diet can improve endothelium function and that the mechanisms of this beneficial effect found in above discussed in vitro studies (especially increased NO and deceased endothelin formation) are also involved in vivo, both in patients and in animals.

5. Anti-angiogenic effects of polyphenols

Angiogenesis is a complex process characterized by the early degradation of extracellular matrix predominantly by matrix metalloproteinases followed by migration and proliferation of endothelial cells and the maturation of the new blood vessel in response to local pro-angiogenic factors such as vascular endothelial growth factor (VEGF, Fig. 2).

5.1. Polyphenols inhibit MMP-2 activation

The gelatinases, matrix metalloproteinases (MMP)-2 and MMP-9, have been identified as major MMPs expressed in blood vessels and atherosclerotic plaques that contribute to the turnover of most types of collagens (Galis and Khatri, 2002; Pasterkamp et al., 2000). MMP-2 is continuously secreted from vascular cells as an inactive precursor pro-MMP-2, which is converted to the active form MMP-2 at the cell surface by a membrane type-MMP and, in particular, by membrane type 1 (MT1)-MMP (Fig. 3). Potential physiological activators of MMP-2 include thrombin and plasmin. Recent studies have indicated that both red wine polyphenols and green tea polyphenols prevent effectively the thrombin-induced activation of MMP-2 in vascular smooth muscle cells (El Bedoui et al., 2004; Oak et al., 2003c). This effect is related to their ability to directly inhibit MT1-MMP activity in a reversible manner (El Bedoui et al., 2004; Oak et al., 2004). The inhibitory effect is observed at concentrations as low as 3 mg/l red wine polyphenols and green tea polyphenols. Among the green tea polyphenols, epigallocatechin-3-gallate and epicatechin-3-gallate mimicked the inhibitory effect on MMP-2 activation and MT1-MMP activity, whereas epigallocatechin was much less active and catechin and epicatechin did not have such an effect (El Bedoui et al., 2004). In contrast, catechin prevented MT1-MMP-dependent activation of MMP-2 in cancer cells (Annabi et al., 2002). The ability of red wine polyphenols and green tea polyphenols to prevent matrix degradation has also been demonstrated in a cell invasion assay, and this effect was as pronounced as that observed with the broad-spectrum MMP inhibitor *N*-[(2*R*)-2-(hydrox-

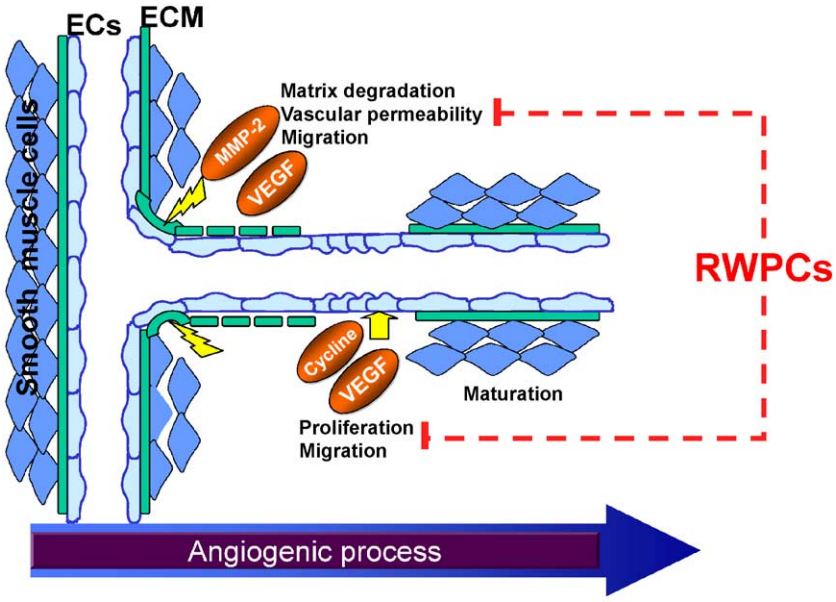

Fig. 2. The angiogenic process is initiated by the degradation of extracellular matrix by matrix metalloproteinases (MMP), such as MMP-2, and by an increase in vascular permeability. These effects promote endothelial cell migration and proliferation and, in turn, the maturation of the new blood vessel in response to local pro-angiogenic factors such as vascular endothelial growth factor (VEGF). Red wine polyphenols have potent anti-angiogenic properties by preventing MMP-2 activation, VEGF expression and the migration and proliferation of vascular cells. RWPCs, red wine polyphenolic compounds.

amidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide, GM6001 (El Bedoui et al., 2004; Oak et al., 2004).

5.2. Polyphenols prevent VEGF expression

VEGF is a major pro-angiogenic factor that stimulates endothelial cell migration and proliferation and also the formation of new blood vessels in both in vitro and in vivo experiments (Ferrara and Davis-Smyth, 1997). Abundant VEGF expression is observed in human atherosclerotic plaques, predominantly by vascular smooth muscle cells and foamy macrophages (Chen et al., 1999; Couffinhal et al., 1997). Potential physiological activators of VEGF expression in vascular smooth muscle cells are growth factors such as platelet-derived growth factor$_{AB}$ (PDGF$_{AB}$), transforming growth factor-β (TGF-β), thrombin- and platelet-derived products (Bassus et al., 2001; Kronemann

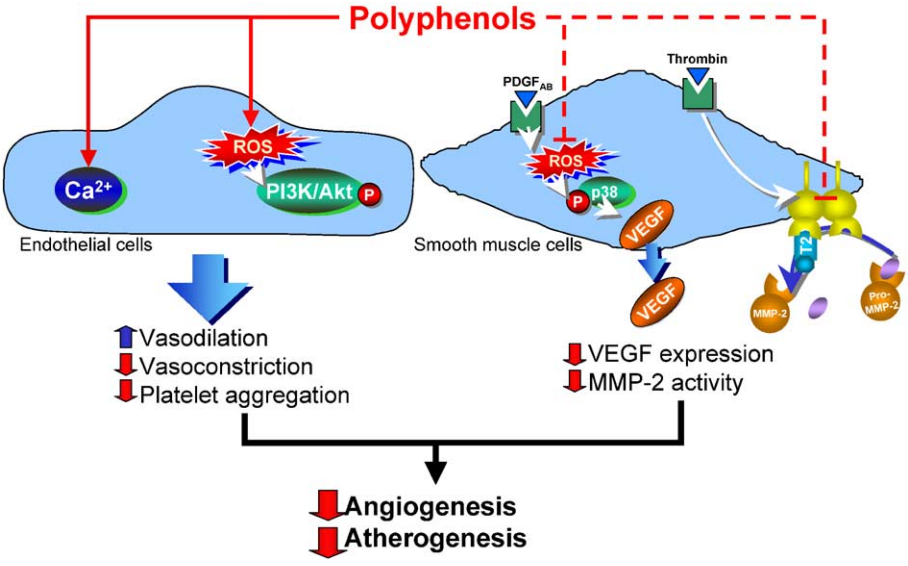

Fig. 3. Effects of dietary polyphenols on vascular endothelial and smooth muscle cells. Increased formation of vasodilating, anti-proliferative and anti-thrombotic factors and decreased formation of vasoconstrictor and proliferative factors produced by endothelial cells (see Fig. 1), together with inhibition of pro-angiogenic factors elicited by PDGF$_{AB}$ and thrombin (VEGF and MMP-2, respectively) may contribute to the anti-atherogenic and vasculoprotective actions of polyphenols. In the two cell types, these different effects imply different redox-sensitive and redox-insensitive signalling pathways that might be triggered by interaction with different sites.

et al., 1999). These activators increase VEGF gene expression, via the NADPH oxidase-dependent formation of reactive oxygen species with subsequent activation of redox-sensitive kinases such as p38 MAPK, resulting in turn in the activation of the transcription factor hypoxia-inducible factor-1 (Bassus et al., 2001; Gorlach et al., 2001; Oak et al., 2003a; Fig. 3). VEGF, once released, acts in a paracrine manner to stimulate angiogenesis by activating VEGF receptors located on endothelial cells. Recent investigations have indicated that VEGF expression and its release are prevented by red wine polyphenols at concentrations as low as 3 mg/l (Oak et al., 2003a). The inhibitory effect has been explained by the prevention of the growth factor-induced redox-sensitive activation of the p38 MAPK pathway leading to VEGF gene expression (Fig. 3). Moreover, the effect of red wine polyphenols is mimicked by the anthocyanins delphinidin and cyanidin, but not peonidin and malvidin, and also not by resveratrol, epicatechin and caffeic acid (Oak et al., 2003b). In addition, VEGF expression is also prevented by green tea polyphenols and epigallocatechin-3-gallate in several types of cancer cells by inhibiting epidermal growth factor receptor (EGFR)-related pathways of signal transduction such as the constitutive activation of Stat3 and nuclear factor (NF)-kappa B (Masuda et al., 2002; Sartippour et al., 2002).

5.3. Polyphenols prevent migration and proliferation of vascular cells

Several studies have indicated that natural polyphenols can inhibit migration and proliferation of vascular cells (Fotsis et al., 1997; Iijima et al., 2000, 2002; Paper, 1998). Resveratrol prevented the progression of endothelial cells through S and G2 phases of the cell cycle by increasing the expression of the tumor suppressor gene protein p53 and the cyclin-dependent kinase inhibitor p21 (Hsieh et al., 1999). Endothelial cell migration and proliferation are also prevented by delphinidin through the involvement of cyclin D1- and A-dependent pathways (Favot et al., 2003; Martin et al., 2003). Green tea polyphenols retained endothelial cells in the G1 phase of the cell cycle (Kojima-Yuasa et al., 2003), and epigallocatechin-3-gallate inhibited endothelial cell migration and proliferation by inducing apoptosis (Yoo et al., 2002). Red wine polyphenols also decreased vascular smooth cell migration and proliferation through the downregulation of cyclin A expression and inhibition of p38 MAPK and PI3-kinase pathways (Iijima et al., 2002).

5.4. Anti-angiogenic effects of polyphenols in vivo

Although numerous studies have indicated that natural polyphenols have anti-angiogenic properties in vitro, only few studies have investigated their effects in vivo. Application of either red wine polyphenols, green tea polyphenols or epigallocatechin-3-gallate to the chick chorioallantoic membrane reduced its vascularization (Cao and Cao, 1999; Maiti et al., 2003). In addition, intake of resveratrol or tea prevented the corneal neovascularization induced by pro-angiogenic factors such as VEGF (Brakenhielm et al., 2001).

6. Conclusion

The current evidence suggests that the protection against cardiovascular diseases associated with polypphenol-rich diets results from the addition of a variety of effects produced by different mechanisms and, in some cases, different compounds. Besides antioxidant effects (either direct or indirect by stimulating endogenous defence mechanisms) that can decrease circulating LDL and membrane lipids oxidation and their deleterious consequences in endothelial cells, increasing evidence suggests that polyphenols with specific structures can, independently from their antioxidant properties, improve the endothelium function (by increasing NO, EDHF and prostacyclin and by decreasing endothelin-1 formation) and inhibit angiogenesis and migration and proliferation of vascular cells. As illustrated in Fig. 3, the improvement of endothelial function, especially the increase in production of anti-proliferative and anti-atherogenic NO, and the inhibition of angiogenesis (which might inhibit formation of vasa vasorum) may both contribute to the anti-atherogenic properties of polyphenols. In addition, the enhanced NO release may participate in their antithrombotic effects (Wollny et al., 1999), together with inhibition of tissue factor expression (Pendurthi et al., 1999a). Interestingly, in addition to participating in vascular protection, the anti-angiogenic effects of polyphenols might be involved in the protective effect of dietary polyphenols against cancer suggested by epidemiological and animal studies on tea (Hollman et al., 1999; Nakachi et al., 2000; Yang et al., 2002) and wine (Gronbaek, 2000; Hollman et al., 1999; Renaud et al., 1998).

Importantly, studies with reference compounds of known structure discussed above have shown structural specificity of the vascular effects of polyphenols (see Section 4.1 for NO formation and Sections 5.1. and 5.2 for inhibition of VEGF and MMP-2 expression and angiogenesis). For instance, the gallate moiety is necessary for the vascular effects of epigallocatechin-3-gallate on angiogenesis, which are not produced by epigallocatechin. In addition, differential structure–effect relationships have been found, depending of the effect. For instance, among structurally close anthocyanins, delphinidin, but neither cyanidin nor malvidin, are able to induce NO formation in endothelial cells. By contrast, cyanidin like delphinidin (but not mavidin) are able to inhibit $PDGF_{AB}$-induced VEGF formation in smooth muscle cells. These findings suggest that different recognition sites, with differential structural

requirements, associated with different signalling mechanisms, may be involved in these different effects of polyphenols. Indeed, epigallocatechin-3-gallate is able to bind to the 67 kDa laminin receptor, which triggers its anticancer activity (Tachibana et al., 2004). To the best of our knowledge, this is the first receptor for a dietary polyphenol to be identified. Whether the same receptor is present in vascular cells and able to mediate the anti-angiogenic and NO synthase-activating properties of epigallocatechin-3-gallate in these cells remains to be elucidated. The hypothesis of the existence in blood vessels of receptors for polyphenols with specific structures as well as identification in plant extracts of polyphenols acting on these receptors deserve further investigations.

One unexpected finding is involvement of reactive oxygen species in signaling mechanisms triggered by polyphenols. Formation of superoxide is implicated in the signalling leading to activation of the PI3-kinase/Akt pathway in endothelial cells induced by some polyphenols like delphinidin but not cyanidin (Table 1), despite their antioxidant properties. This is consistent with the hypothesis that the polyphenols exert their antioxidant properties outside cells, whereas they might interact with plasma membrane receptors triggering the release of superoxide within cells. In this case, the mechanism of superoxide production remains to be determined. Vice versa, other polyphenols, also including delphinidin but also cyanidin, inhibit the reactive oxygen species-dependent $PDGF_{AB}$-induced activation of p38 MAP kinase (Fig. 3), although other polyphenols with strong anti-oxidant properties in vascular smooth muscle cells, such as resveratrol and quercetin, fail to produce this effect. The mechanisms of interactions of polyphenols with the reactive oxygen species-dependent cell-signaling pathways deserve further investigations in vascular endothelial and smooth muscle cells.

Little is known on biodisponibility of dietary polyphenols, which are exposed to gastrointestinal metabolization especially by the human intestinal flora before being absorbed in the gut. Which compounds are finally released in the circulation after ingestion of foods or beverages with complex polyphenolic composition require further investigations. However, the findings that these nutriments produce an increase in the anti-oxidant activity of blood plasma and vasoprotective effects in humans and animals suggest that at least some active compounds are absorbed in the gut and reach blood vessels.

Finally, it is striking that ingestion of different polyphenol-rich foods and beverages, such as grape products, cocoa or tea, all produce beneficial effects with comparable patterns including improved endothelial function, anti-thrombotic, anti-atherogenic and anti-hypertensive effects. The possible mechanisms of these effects are multiple and may be different depending on the active polyphenols provided by each food or beverage. However, all the resulting in vivo effects oppose cardiovascular risk factors hyperlipidemia, smoking and hypertension. Their beneficial effects are such that polyphenols appear as a promising class of compounds for cardiovascular protection. Elucidation of their mechanisms of action should provide new insight in cardiovascular biology and pathophysiology, and perhaps new targets for future vasculoprotective drugs or nutritional adjuvants.

Table 1

	Endothelial cells		Vascular smooth muscle cells	
	Increase Ca^{2+}	Activate PI3K/Akt	Inhibit VEGF	Inhibit MMP-2
Delphinidin	+	+	+	?
Cyanidin	?	–	+	+
EGCG	+	+	+	+

EGCG: Epigallocatechin gallate.

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