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REVIEWS: CURRENT TOPICS

Effects of resveratrol and other wine polyphenols on vascular function: an update

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Abstract

Several epidemiologic observations show that moderate wine drinking reduces cardiovascular morbidity and mortality. Wine contains several polyphenols, and among them, resveratrol in particular has been shown to exert a number of important biologic activities on the cardiovascular system that may contribute to the protective effects of wine. The mechanisms through which resveratrol and other wine polyphenols protect from ischemic cardiovascular events are many, but protection from oxidative stress and radical oxygen species production, a facilitating activity on nitric oxide production and activity and the ability to modulate the expression of adhesive molecules by blood cells and the vascular wall seem to be the most important. In this overview, the in vitro and in vivo evidence on the activity of resveratrol on vascular function and circulating blood cells, with a special emphasis on blood platelets, is thoroughly presented.

Keywords: Atherosclerosis; Ischemic cardiovascular disease; Platelets; Resveratrol; Wine

1. Introduction

The last 2 decades have seen renewed interest in the health benefits of wine, as documented by increasing research and several epidemiologic observations showing that moderate wine drinkers have lower mortality rates than heavy drinkers or teetotalers [1].

The so-called French paradox refers to the observation of a low prevalence of coronary heart disease-related deaths in the French population despite high intake of dietary cholesterol and saturated fat and was explained by the regular intake of wine. Most of the beneficial effects of wine on cardiovascular disease have been attributed to the presence in wine of resveratrol and other polyphenols [2,3]. Wines contain polyphenolic compounds that can be roughly classified in flavonoid and non flavonoid compounds. Flavonoids are the polyphenols (anthocyanins), and non flavonoids include hydroxycinnamic acid, benzoic acid, tannins and stilbenes. Both classes of compounds have been implicated in the protective effects of wine on the cardiovascular system; in particular, stilbenes, including resveratrol, display several biologic activities on the cardiovascular system.

The mechanisms through which resveratrol and other wine polyphenols exert their vascular protective action are not fully understood yet, but several experimental studies have shown an activity on some of the basic mechanisms involved in the provocation of and in the protection from ischemic cardiovascular disease, such as oxidative stress and reactive oxidant species (ROS) production, nitric oxide (NO) formation and activity and the expression of adhesive molecules by blood cells and the vascular wall.

We shall overview here the main mechanisms through which wine polyphenols and, in particular, resveratrol affect vascular function and circulating blood cells, with a special emphasis on blood platelets, thus providing a protective activity against ischemic cardiovascular events.

2. Resveratrol and other polyphenols in wine

Fruits, vegetables and wine contain several thousand structurally diverse phytochemicals, a large fraction of which are polyphenols [4]. Red wine in particular contains a variety of polyphenols derived from the skin of the grapes (Table 1) [5,6].

Resveratrol (*trans*-3,5,4′-trihydroxystilbene), a stilbene derivative, is one of the most biologically active polyphenols contained in wine. Initially characterized as a phytoalexin (a toxic compound produced by higher plants in response to infection or other stresses, such as nutrient deprivation), resveratrol attracted little interest until 1992 when it was postulated to explain some of the cardioprotective effects of red wine [2,3]. Since then, dozens of reports have shown that resveratrol can prevent or slow the progression of a wide variety of illnesses, including cancer, cardiovascular disease and ischemic injuries, enhance stress resistance and extend the lifespan of various organisms, from yeast to vertebrates [7,8].

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Table 1
Main polyphenols in wine

Flavonols
Quercetin
Myricetin
Flavanols
Catechin
Epi(gallo)cathechin
Phenolics
Gallic acid
Stylbenes
Resveratrol
Condensed tannins
Cathechin
Epicatechin polymers
Polymeric anthocyanins
Simple phenols
Tyrosol *
Hydroxytyrosol *

Modified from Howard et al [5].
* In white wine.

3. Effects of resveratrol on ROS and their role in the antithrombotic activity on blood cells and the vascular wall

3.1. ROS, vascular function and blood cells

ROS are molecules containing one or more unpaired electrons in atomic or molecular orbitals giving them a remarkable degree of reactivity [7].

Radicals derived from oxygen represent the most important class of ROS generated in living systems. The addition of one electron to molecular oxygen (dioxygen) forms the superoxide anion radical (O_2^-) [9]. Superoxide anion is considered the "primary" ROS, and it can further interact with other molecules, either directly or through enzyme- or metal-catalyzed processes, to generate "secondary" ROS, such as hydrogen peroxide or lipid peroxides [10] (Fig. 1).

Primary as well as secondary ROS are generated within the vasculature and in the blood stream, especially in pathologic conditions. Endothelial cells (EC), smooth muscle cells (SMC), neutrophils and platelets are sources and targets of ROS at the same time. Several enzymes, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, myeloperoxidases and xanthine-xanthine oxidases, are involved in ROS formation. However, among the cellular sources of ROS, NADPH oxidase is likely to play the most important role [11].

Enzymatic antioxidants include superoxide dismutase, glutathione peroxidase and catalase [12]. Nonenzymatic antioxidants are represented by ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, carotenoids, flavonoids and other molecules. An unbalance between the activities and the intracellular levels of these antioxidants is associated with an increase of ROS production.

ROS play a role in cardiovascular disease, such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure, by inducing oxidative stress [13].

3.2. Antioxidant properties of resveratrol (and other wine polyphenols)

The antioxidant capacity of a molecule depends upon its ability to react directly with ROS (quenching) or to interact with the enzymatic pathways involved in ROS formation [10]. Many polyphenols have direct antioxidant properties (i.e., they act as reducing agents) and may react with reactive chemical species forming products with much lower reactivity. Polyphenols may also affect indirectly the redox status by increasing the capacity of endogenous antioxidants or by inhibiting enzymatic systems involved in ROS formation [14].

3.2.1. In vitro models

Experiments performed in vitro suggest that polyphenols, in particular resveratrol, may behave as direct or indirect antioxidants. Resveratrol was shown to reduce in vitro the oxidation of human low-density lipoprotein (LDL) induced by incubation with metal ions, such as copper [15], or with peroxynitrite [16]. Various enzymatic systems, present in ECs or in macrophages, are implicated in the oxidation of LDL. Some of them, like NADPH oxidase, hypoxanthine/xanthine oxidase, 15-lipoxygenase and MPO, are negatively modulated by resveratrol [17]. The interaction between resveratrol and these enzymes reduces intracellular ROS formation in ECs [18] and inhibits leukocyte adhesion and activation [19,20].

3.2.2. In vivo models

Ischemia-reperfusion damage is a pathologic phenomenon strongly mediated by ROS generation, as demonstrated by the observation that the modulation of ROS formation by antioxidants favourably

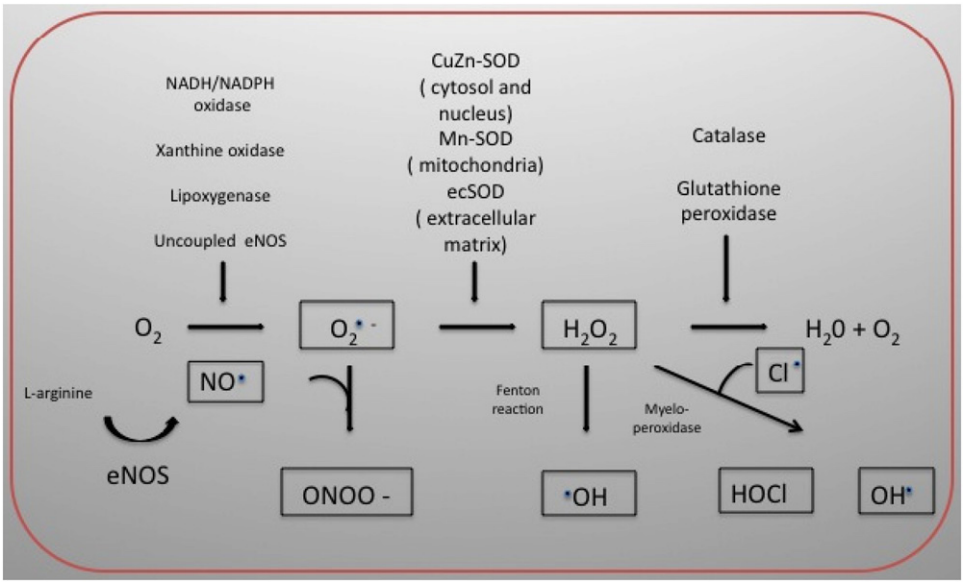

Fig. 1. Scheme of superoxide anion formation, interaction with enzymatic systems and neutralization.

influences organ outcome [21]. In a mouse model of anoxia/reoxygenation, resveratrol prevented superoxide-dependent proinflammatory reactions [22]. In a rat model of middle cerebral artery occlusion, animals given resveratrol showed lower levels of malondialdehyde, a marker of oxidant stress, in brain, coincidentally with a reduction of the brain infarction area [23].

The ability to prevent organ damage generated by ischemia–reperfusion is not a characteristic of resveratrol only but of other wine polyphenols as well. In a model of myocardial ischemia different groups of rats were given red wine (rich in polyphenols), white wine (poor in polyphenols), resveratrol, tyrosol, or hydroxytyrosol. Red wine and its constituent resveratrol, and white wine and its constituents tyrosol and hydroxytyrosol, all displayed some degree of cardioprotection, as shown by their ability to improve postischemic ventricular performance, to reduce myocardial infarction size and cardiomyocyte apoptosis and to reduce peroxide formation [6]. Moreover, other antioxidants contained in food or beverages were shown to reduce the infarct size following ischemia–reperfusion injury to the heart in rats: 1-month administration of white wine, which contains other flavonoids, such as hydroxycinnamic acids (caffeic acid) and monophenols (tyrosol), significantly reduced infarct size, cardiomyocyte and endothelial cell apoptosis induced by short-term occlusion of the anterior descending coronary artery [24]; in another study, long-term dietary supplementation with plant-derived flavonoids (anthocyanins) to rats made the myocardium less susceptible to ischemia–reperfusion injury ex vivo as well as in vivo, possibly through mechanisms related to improved antioxidant defenses of the heart [25]. There is also evidence that combining polyphenols may enhance their antioxidant effects [26].

3.2.3. Studies in humans

The effect of resveratrol and other wine polyphenols on oxidative stress has been scarcely explored in humans and only a few studies have analyzed the effects of wine supplementation on indexes of oxidation in vivo.

The antioxidant effect of proanthocyanidins, the main phenolic antioxidants of red wine, was investigated in two groups of healthy volunteers who consumed a lipid-rich meal without (control group) or with 300 mg of a proanthocyanidin-rich grape seeds extract (GSE). Lipid hydroperoxide (LPO) concentration and LDL resistance to oxidation were measured in postprandial plasma. GSE ingestion significantly reduced plasma LPO formation and LDL oxidation [27].

In another study, the effect of red and white wine on oxidative stress, as assessed by the measurement of urinary prostaglandin-F2α (PGF2α), was tested in 20 healthy volunteers. After 15 days of wine supplementation, a significant decrease of oxidative stress was observed in the subjects given red wine. The higher antioxidant effect of red wine observed in this study was ascribed to the higher polyphenol content of red wine compared to white wine (about 10-fold) [28]. Similarly, in another study, plasma levels of polyphenols were higher in subjects given red wine and inversely correlated with urinary PGF2α [29].

Wine polyphenols could also exert an antioxidant effect by influencing omega-3 fatty acids metabolism and, in turn, arachidonic acid-mediated ROS formation. In fact, epidemiological studies found a significant correlation between alcohol consumption and omega-3 fatty acids in plasma and red cells membrane in healthy subjects [30]. More specifically, the relationships between wine drinking and marine omega 3 fatty acid in plasma were evaluated in a cross-sectional study that analyzed 353 male patients with coronary heart disease demonstrating that moderate wine consumption was associated with higher marine ω3 concentrations in plasma than no alcohol use [31].

3.3. Effects of oxidant stress on the vessel wall and the protective properties of resveratrol and other wine polyphenols

The key vessel wall-derived regulators of vasomotor function are the vasodilator NO and the vasoconstrictor endothelin-1 (ET-1) [32]. Resveratrol is able to inhibit the production of endogenous vasoconstrictors and thereby to restore vasomotion, which is impaired in atherosclerosis.

3.3.1. In vitro models

In cultured vascular SMCs oxidative stress increases ET-1 expression, which is an effector of endothelial dysfunction. Resveratrol inhibits ET-1 production and the activity of cytosolic phospholipase A_2 stimulated by oxidative stress [33]. Moreover, it increases the expression of mRNA for eNOS and decreases the H_2O_2-induced expression of mRNA for ET-1 in cultured bovine aortic endothelial cells and in human umbilical vein endothelial cells (HUVEC) [34,35]. Different red wine extracts were shown to reduce ET-1 synthesis by bovine aortic endothelial cells, the degree of inhibition correlating with their respective total polyphenol content.

3.3.2. In vivo models

Oxidative stress plays an important role in the endothelial dysfunction associated with Type 2 diabetes. In aortas from diabetic mice, resveratrol restored endothelial function improving ACh-induced vasorelaxation. This vascular protective activity was mediated by the inhibition of tumor necrosis factor α (TNFα)-induced activation of NADPH oxidase, thus lowering H_2O_2 formation and preserving eNOS function. Furthermore, resveratrol attenuated both mRNA and protein expression of TNFα [36].

3.4. Effect of resveratrol and other wine polyphenols on angiogenesis

EC migration and proliferation are essential events in angiogenesis and are involved in the neovascularisation processes that accompany atherosclerosis. Vascular endothelial growth factor (VEGF), a strong stimulator of neoangiogenesis, colocalizes with endothelial cells, macrophages and SMCs in atherosclerotic plaques [37]. Resveratrol inhibits VEGF-induced angiogenesis by abrogating VEGF-mediated tyrosine phosphorylation of vascular cadherin and its protein complex partner, β-catenin. The inhibition of VEGF-induced angiogenesis is mediated by the disruption of ROS-dependent Src-kinase activation and the consequent vascular endothelial cadherin tyrosine phosphorylation [38]. VEGF stimulates O_2^- production by activating NADPH oxidase [39]. Considering that the blockade of NADPH oxidase by specific antisense peptides fully prevents VEGF-induced endothelial cell signalling and angiogenesis [40], a role may be hypothesized for resveratrol in inhibiting NADPH oxidase in this model of angiogenesis.

3.5. Effect of resveratrol and other wine polyphenols on platelet function

Thrombosis depends upon the generation of several proaggregating substances able to activate circulating platelets, recruiting them to the site of thrombus growth. NADPH oxidase activation, through the formation of O_2^-, is the primary source of ROS involved in this critical phase of platelet activation, and superoxide anions are among the platelet activating substances formed during atherothrombotic phenomena [41].

Polyphenols are able to modulate several pathways involved in platelet activation and in the consequent thrombus growth. Flavonoids are metabolised in the intestine and liver into methylated, sulphated and glucuronidated counterparts, which inhibit platelet function. Platelets themselves take part in flavonoid metabolism: quercetin, and its plasmatic metabolite 4′-0-methyl quercetin

(tamarixetin), are internalized by platelets and further metabolized by the addition of sulphate or glucuronide groups. Thus, formed compounds inhibit platelet activation by antagonizing surface receptors (especially estrogen receptors and thromboxane A2 receptors) [42].

3.5.1. In vitro models

In vitro experiments showed that two red wine polyphenols, namely catechin and quercetin, are able to blunt synergistically ROS formation by activated human platelets, and consequently calcium mobilization and aggregation [43]. In an in vitro model of thrombus formation, this polyphenol combination was shown to modulate ROS formation, via the inhibition of NADPH oxidase activation and to inhibit collagen-induced CD40L expression on the platelet surface [44].

A mixture of polyphenols, at concentrations found in plasma from healthy subjects given moderate amounts of red wine, inhibited also platelet-dependent LDL oxidation and protein kinase C (PKC)-mediated NADPH oxidase activation, suggesting that a synergism between different polyphenols may account for the antioxidant effect of wine. The in vitro effect on platelet activation of concentrations of resveratrol achievable in plasma after chronic moderate wine intake was also evaluated. Resveratrol was able to enhance AKT activity and to inhibit the p38 mitogen-activated protein (MAP)-kinase (p38MAPK) phosphorylation pathway involved in NADPH-oxidase activation [45,46].

3.5.2. In vivo models

Only a few models described the effect of resveratrol and other wine polyphenols in vivo on platelet function. In a canine model of coronary thrombosis, the acute administration of red wine or grape juice, but not of white wine that has a lower polyphenolic content, inhibited platelet-mediated flow reductions in coronary blood flow [47]. Antithrombotic effect of proanthocyanidin, a highly purified ingredient of grape seed and the main phenolic antioxidant of red wine, was assessed by a shear-induced thrombosis test in vitro and by a laser-induced thrombosis test in a mouse carotid artery model. Intravenously (20-mg/kg body weight) or orally (2×200 mg/kg) administered proanthocyanidin significantly inhibited the laser-irradiation induced thrombus formation in the carotid artery. Moreover, after oral administration of proanthocyanidin, in vitro platelet reactivity to shear stress has been inhibited. This suggests that the in vivo antithrombotic effect of proanthocyanidin may be due to its direct inhibitory effect on platelet function [48]. Experiments were also performed to evaluate the direct effect of resveratrol on platelet activation. Resveratrol (5 mg/kg) significantly prolonged platelet plug formation in a mice model of microthrombus induced by irradiation of venules from a segment of the small intestine [49]. The authors also demonstrated that resveratrol inhibited two pathways involved in the redox status balance, namely, the p38MAPK-cPLA2-AA cascade and the NO activity. These lead them to postulate that the effect of resveratrol on platelet function could be due to a direct action of this molecule on oxidative stress balance. The role of resveratrol was also evaluated in a condition of platelet hyper reactivity such as hypercholesterolemia. Hypercholesterolemic rabbits showed enhanced adenosine diphosphate (ADP)-induced platelet aggregation that was significantly inhibited by resveratrol (4 mg/kg per day) [50]. In the same study, Wang et al also demonstrated that the inhibitory effect of resveratrol was even evident when animals received intragastrically Chinese red wine rich in resveratrol with or without alcohol (4 ml/kg per day).

3.5.3. Studies in humans

Studies on the effect of the acute administration of wine on platelet function in humans have provided contradictory results. Numminnen et al., who measured platelet aggregation before and immediately after moderate wine intake (60 g ethanol) by healthy subjects, reported an inhibition of shear-induced platelet aggregation [51]. In contrast, Kikura et al. did not find any change of platelet aggregation after the acute administration of red or white wine in a small group of healthy subjects [52]. Upon chronic intake of red or white wine, instead, a significant reduction of platelet function was observed in three different studies, independently from the type of wine [53–55]. Pignatelli et al. found a lower aggregation response to collagen of platelets from subjects who had taken red compared to those who had taken white wine [55]. These findings, however, are flawed by the lack of a control group. Conversely, Miceli et al. found no difference in platelet aggregation between light or moderate wine consumers as compared with life-long abstainers [56]. Therefore, the effects of wine intake on ex vivo platelet function in healthy humans are still controversial. Likely, part of the discrepancies derive from the different sensitivity of the techniques adopted to study platelet function in the different studies and from their inherent variability [57]. Most conceivably, a more clear inhibitory effect of wine intake may be observed in patients with cardiovascular disease and/or risk factors, in whom a platelet hyperreactivity is present, than in healthy volunteers.

4. Effects of resveratrol on nitric oxide formation and activity

4.1. NO and the cardiovascular system

NO is a cell signaling molecule formed from the amino acid L-arginine, via the action of three different isoforms of the enzyme NO synthase (NOS), that functions both as an intracellular and an extracellular messenger. The NOS enzyme itself requires five cofactors (reduced flavin mononucleotide [FMNH2], reduced flavin adenine dinucleotide [FADH], NADPH, calmodulin and tetrahydrobiopterin) and two cations (calcium and heme iron) (Fig. 2).

A neuronal (nNOS), an inducible (iNOS), and an endothelial (eNOS) isoform of NOS have been so far identified. Moreover, splice variants for all these isoforms have been found.

NO is formed by nNOS (also known as cNOS, bNOS, NOS-1, or Type I NOS) in the central nervous system, where it plays a role in information storage associated with learning and memory; by iNOS (also known as NOS-2 or Type II NOS) in inflammatory cells, where it participates in antimicrobial activity, cytotoxicity, and in inflammatory responses; and by eNOS (also known as NOS-3 or Type III NOS) in endothelial cells, but also in circulating blood cells, where is responsible for blood pressure regulation [58], platelet inhibition

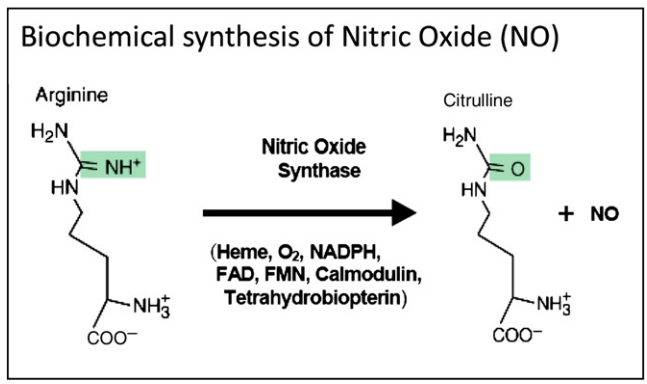

Fig. 2. Biochemical synthesis of NO. Free arginine is metabolized to NO and citrulline by NOS catalytic activity and involves a five-electron oxidation of the guanidine nitrogen of L-arginine with molecular oxygen to give the stoichiometric production of NO and L-citrulline. The NOS enzyme itself requires five cofactors (FMNH2, FADH, NADPH, calmodulin and tetrahydrobiopterin) and two divalent cations (calcium and heme iron).

and antiproliferative effects on smooth muscle cells [59]. Most cells and tissues possess one, and sometimes more, isoforms of NOS. The endothelial and neuronal NOS are constitutive isoforms and their activity is highly regulated by Ca^{2+} and calmodulin, while the inducible isoform of NOS is expressed after exposure to bacterial endotoxins and/or to several inflammatory cytokines. NO has been implicated in inflammation, neurotransmission, cytotoxicity, and vasodilation [59,60]. NO is a highly lipophilic and diffusible gas that permeates biological membranes, reaching targets outside the cellular compartment in which it was generated and diffusing to its primary cytosolic target in cells, i.e., soluble guanylycyclase (sGC). Indeed, most of the physiologic effects of NO are mediated by its ability to activate sGC, a heme-containing, heterodimeric cytosolic enzyme that converts guanosine triphosphate into the second messenger 3′,5′-cyclic GMP (cGMP) [61]. The increase in intracellular cGMP leads to the activation of protein kinase G (PKG) which, in turn, produces protein phosphorylation and many consequent biological processes, such as the regulation of smooth muscle tone, photo-transduction and fluid and electrolyte homeostasis, acting via downstream effectors, such as PKG kinases, cyclic nucleotide-gated channels, and cGMP-regulated phosphodiesterases [59,61–63]. cGMP is quickly removed by the action of the phosphodiesterase 5A enzyme [61,62]. In contrast, several of the cytotoxic and inflammatory effects of NO are independent of cGMP and may result from its interaction with metal ions, thiol groups and other free radicals that, in turn, mutate DNA and inhibit key enzymes involved in energy metabolism [64]. The NOS catalytic activity involves a five-electron oxidation of the guanidine nitrogen of L-arginine with molecular oxygen to give the stoichiometric production of NO and L-citrulline.

NO exerts important biologic effects on blood platelets, by inhibiting a wide range of platelet responses to stimuli, thus modulating thrombosis in vivo [59]. NO inhibits thrombin-induced phosphoinositide-3-kinase activation, a very basic signal transduction event in platelet activation [65]. Moreover, it inhibits the expression of activated glycoprotein IIb/IIIa (GPIIb/IIIa) on platelet membranes [66], thus impairing the binding of fibrinogen which forms the bridges that permit platelet aggregates formation. NO inhibits also the very first phases of platelet activation, by preventing von Willebrand factor-mediated platelet adhesion and spreading, through the regulation of GPIIb/IIIa and myosin light chain activation [67].

There is some controversy about the presence of functional NOS-3 in platelets and the pathophysiologic role of platelet-derived NO [68–70]. However, several experimental data confirm a role of platelet NOS-3 in regulating platelet activation in vivo [71–73]. NO plays several other important activities within the cardiovascular system. Since the discovery of an endothelium-derived relaxing factor [74], later identified as NO [75,76], it has become clear that NO plays a central role in the regulation of vascular tone [58]. Moreover, it plays an important role in preventing atherogenesis and related adverse outcomes. In fact, NO inhibits nuclear factor κB (NF-κB)-dependent expression of various chemo-attractant and adhesion molecules which mediate the recruitment of leukocytes to the endothelium, one of the initial events leading to atherosclerotic plaque development. In addition, NO decreases the oxidation of low-density lipoprotein (LDL) and tissue-factor expression by monocyte/macrophages. NO also suppresses the abnormal proliferation of vascular smooth muscle cells, a process contributing to lumen narrowing in atherosclerotic vessels or to restenosis. Decreased availability of NO in the vasculature may promote the progression of vascular disease.

Risk factors for cardiovascular disease have a preeminent role in generating oxidative stress, and cause a disruption in the balance between NO and ROS, with a resulting decrease in bioavailable NO. This leads to endothelial and vascular smooth muscle cell dysfunction thus creating a prothrombotic and proinflammatory condition that leads to the formation, progression and, ultimately, destabilization of atherosclerotic plaques resulting in myocardial infarction, stroke and cardiovascular death.

4.1.1. Resveratrol and other wine polyphenols and NO

Several data suggest that resveratrol increases NO production and protects NO from inactivation, favouring its biologic activities. Resveratrol has been shown to up-regulate the expression of eNOS in mouse aorta and to down-regulate the expression of NAD(P)H oxidase, an enzyme involved in the production of ROS, suggesting that the improvement of vascular function generated by resveratrol may be related both to the increase of NO formation and to the decrease of ROS production [77,78].

The molecular mechanism of action through which resveratrol increases NO production is not entirely clear, but some evidence suggests that resveratrol acts via membrane-bound structures, like estrogen receptors, as well as via intracellular targets, like the cyclic adenosine 3'-5' monophosphate (cAMP)-activated protein kinase that leads to eNOS activation *via* the phosphorylation on Ser 1177, or SIRT1 that, in turn, activates the dimethylarginine dimethylaminohydrolase II and decreases oxidative stress, thus reducing intracellular asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS activity. In addition, resveratrol reduces eNOS acetylation, leading to eNOS activation [79–81]. Resveratrol also activates PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1α), that owns mitochondrial biogenesis-, antioxidation, growth factor signaling regulation and angiogenetic activities [82].

4.1.2. In vitro studies

Incubation with resveratrol has been shown to increase the production of NO by human blood platelets, via PI3K-dependent AKT phosphorylation and the subsequent phosphorylation and activation of eNOS. The NO thus produced induces in turn cGMP-dependent effects, such as vasodilator-stimulated phosphoprotein (VASP) phosphorylation and inhibition of p38MAPK phosphorylation, with the consequent inhibition of platelet activation and of ROS production. These effects can be obtained using in vitro concentrations of resveratrol that are very similar to those obtained after the ingestion of a moderate quantity of red wine [46].

Moreover, the cGMP-mediated activation of PKG promotes the refilling of intraplatelet Ca^{2+} stores, thereby decreasing the intracytoplasmic levels of Ca^{2+}. cGMP inhibits also inositol-1,4,5-trisphosphate-stimulated Ca^{2+} release from the sarcoplasmic reticulum, contributing to further decrease cytosolic Ca^{2+} [83,84]. PKG also phosphorylates the TxA_2 receptor, an important platelet activating receptor, thus inhibiting its function [85]. In addition, cGMP increases intracellular cAMP by inhibiting phosphodiesterase Type 3 [86], and cGMP and cAMP synergistically inhibit platelet activation [86,87]. Finally, resveratrol inhibits ROS production by stimulated platelets [44–46].

Cultured HUVEC incubated with resveratrol and quercetin showed an increased expression of mRNA for eNOS and VEGF and a decreased secretion of endothelin, effects that would be expected to result in vasodilation and blood pressure regulation [88].

In vitro, with cultured human SMCs, resveratrol markedly enhanced cGMP formation and stimulated kinase-G activity. cGMP formation by resveratrol was significantly attenuated by estrogen receptor blockers, linking resveratrol with pGC/kinase-G activation downstream to oestrogen receptors in the vasculature [89]. Moreover, in endothelium-denuded rat aorta, resveratrol-enhanced, agonist-stimulated [Ca^{2+}] influx and an intracellular Ca^{2+} increase may trigger NO synthesis by endothelial cells [90].

Resveratrol showed significant dose-dependent inhibitory effect on the generation of superoxide anion [O_2^-)] by polymorphonuclear leukocytes in whole blood and hypochlorous acid and NO production by isolated cells [91].

Resveratrol may also increase the number of circulating endothelial progenitor cells (EPC) and their function through an enhancement of NO bioavailability. Resveratrol in vitro attenuated TNFα-induced EPC senescence modifying some functions of EPC, including promotion of EPC adhesion, migration, and tube formation. These data suggest that resveratrol may alter the biology of EPC, and this may contribute to its unique cardiovascular-protective effects [92].

4.1.3. In vivo models

Improved endothelial function upon treatment with resveratrol has been reported in several studies in animal models of atherosclerosis and metabolic diseases, and in most cases, this was associated with a decreased production of superoxide ion [93–98].

In a model of metabolic syndrome in obese Zucker rats, chronic intake of quercetin [98] or resveratrol [94] enhanced eNOS expression and down-regulated iNOS expression in visceral adipose tissue and aorta. Moreover, dyslipidemia, hypertension and hyperinsulinemia were improved by the chronic intake of quercetin or resveratrol, producing anti-inflammatory effects and a reduction in body weight gain [94–98]. Similar effects were also found upon supplementation with resveratrol in an experimental model of streptozotocin-induced diabetes in rats [95]. In a model of hypercholesterolemia in rabbits, the administration of red wine, dealcoholized red wine or resveratrol decreased plasma levels of ET-1 in parallel with a significant elevation in NO levels. Moreover, endothelial function was improved [99]. These data indicate that resveratrol and other wine polyphenols exert a protective effect on vascular function by restoring NO formation. In estrogen receptor alpha (ERα)-deficient mice, the administration of a red wine polyphenols extract (Provinols) or of delphinidin, an anthocyanin, induced vasorelaxion via ERα receptor that activates molecular pathways, like the phosphorylation of Src, ERK1/2 and eNOS Ser 1177 that lead to endothelial NO production, confirming the importance of estrogen receptors in vivo in the NO biosynthesis-stimulatory activity of resveratrol [100].

4.1.4. Studies in humans

Endothelial dysfunction (defined as an impairment of endothelium-dependent relaxation, largely dependent upon NO production from endothelial cells) is an early event in the development of atherosclerosis and is present even before structural changes occur in the vasculature [101].

In healthy individuals under a high-fat diet, the intake of 240 ml of red wine daily effectively counteracted the diet-induced endothelial dysfunction [102]. Moreover, the administration of a red grape polyphenol extract acutely improved endothelial function in patients with coronary heart disease [103]. Oral supplementation with purple grape juice, rich in flavonoids, decreased platelet aggregation, increased platelet-derived NO release and decreased superoxide production in healthy subjects [104]. Finally, in healthy human volunteers, a moderate wine consumption (300 ml/d for 2 weeks) was able to increase the release of nitrite plus nitrate, NO metabolites, by stimulated platelets [46].

5. Effects of resveratrol on adhesion molecules, and the interactions between blood cells and the vessel wall

5.1. Cell–cell interactions in atherosclerosis and thrombosis

The initial stage of cell-mediated thrombosis involves platelet and white blood cells adhesion to a thrombogenic endothelial surface and between themselves, through a signalling cascade initiated and amplified by expression and activation of adhesive molecules, such as P-selectin on platelets and beta-2 integrins on leukocytes [101,102]. Table 2 lists the main adhesion molecules involved in endothelium, platelet and leukocyte interactions, their cellular expression, specific ligands and main functions. The main platelet adhesive molecules expressed upon activation, such as P-selectin and CD40-ligand, besides intercellular adhesion, trigger other leukocyte functions, such as expression of tissue factor, chemokine and cytokine secretion, up-regulation of adhesive receptors, protease activation and monocyte differentiation into macrophages [102–106]. Increased circulating levels of cell-derived adhesive molecules, such as P-selectin and CD40L from platelets, L-selectin from leukocytes, and inter-cellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) from the endothelium [107,108], or of platelet-leukocyte conjugates [109,110] have been associated with inflammatory conditions in cardiovascular disease: whether these are epiphenomena reflecting the ongoing inflammatory and thrombotic processes, or pathogenetic mechanisms contributing to acute coronary events remains to be established.

Arachidonic acid metabolites, such as leukotrienes, lipoxins or thromboxane, are also active products of cell–cell interactions and their plasma or urinary levels represent biomarkers of potential interest [111].

The epidemiologic evidence of a significant association between leukocyte count and the incidence of coronary heart disease is

Table 2
Adhesion molecules involved in endothelium-, platelet- and leukocyte-interactions

Molecules	Cell expression	Ligand	Main function
Selectins			
P-selectin	Stored in EC and platelet granules; expressed on cell surface on stimulation and released	PSGL-1	Rolling of leukocytes on EC and platelets and of platelets on EC
E-selectin	Induced by cytokines on EC	PSGL-1, ESL-1, CD44	Rolling of leukocytes on EC
L-selectin	Expressed on leukocytes	PSGL-1	Secondary leukocyte recruitment
Integrins			
β2-integrins	Expressed on leukocytes; require activation	ICAMs, VCAM fibrinogen	Firm adhesion to EC and platelets
β3-integrins	Expressed on platelets (αIIbβ3 or GPIIb/IIIa) and on neutrophils/EC (αVβ3); require activation	Fibrinogen; extracellular matrix molecules	Firm cell adhesion
Immunoglobulins			
ICAM-1	Up-regulated by cytokines on EC and leukocytes	β2-integrins	Firm adhesion and transmigration of leukocytes
ICAM-2	Constitutive on EC and platelets	β2-integrins	Firm adhesion and transmigration of leukocytes; platelet adhesion to leukocytes
VCAM-1	Up-regulated by cytokines on EC	α4-integrins	Firm adhesion and transmigration of leukocytes
PECAM-1	Constitutive on EC, platelets and leukocytes	PECAM-1	Transmigration
Tumor necrosis factor family			
CD40	Constitutive and expressed on EC, leukocyte and platelet surface	CD40L	Activates different EC, leukocyte and platelet function
CD40L		CD40; αIIb β_3 on platelets	

Abbreviations: PSGL-1, P-selectin glycoprotein ligand-1; ESL-1, E-selectin-ligand-1 ; PECAM-1, platelet/endothelial cell adhesion molecule-1.

supported by numerous studies providing biological plausibility to this association, although treatments reducing leukocyte function or number have not been so far shown to prevent ischemic events in patients at risk [112,113].

5.2. Effect of resveratrol and other wine polyphenols on cell–cell interactions

Wine polyphenols modulate several cell functions involved in thrombosis [114], such as the endothelial production of prostacyclin [115] and of endothelin-1 [35], arachidonic acid metabolism in platelets and leukocytes, platelet aggregation or the expression and activation of genes that regulate adhesive functions and tissue factor activity in endothelial cells or leukocytes [116,117].

5.2.1. In vitro studies

Inhibition by resveratrol of endothelial cell interactions with blood cells and of leukocyte activation have been reported in several in vitro models. Table 3 summarizes these effects [20,118–124].

Similar inhibitory effects have been reported for other wine polyphenols, including flavonols such as quercetin and its metabolites and kaempferol, flavones such as apigenin and luteolin and catechin metabolites [116–118,125–131]. 4-*O*-Methylgallic acid, a major metabolite of gallic acid abundant in most red wines, was also shown to directly inhibit DNA binding of NF-κB [127]. Native catechin and epigallocatechin gallate, on the other hand, were ineffective [129]. The observation of a critical structure-activity relationship for the inhibition of adhesive molecule expression by different wine polyphenols allows to speculate that selected polyphenols may have specific molecular interactions with enzymes, such as kinases, phospholipases, lipoxygenases or cyclo-oxygenases, or with receptor target(s) such as the P-selectin receptor. For instance, gallic acid markedly and dose-dependently decreased the adherence of monocytic HL60 cells to P-selectin-transfected Chinese hamster ovary cells under flow conditions and increased the velocity of P-selectin-dependent rolling of human leukocytes on a platelet monolayer. It also inhibited rolling and tumbling of platelet-leukocyte complexes over endothelium of normal or hypercholesterolemic mice, without affecting the deposition of isolated platelets [132].

Serum from healthy volunteers who had ingested 5 ml/kg body weight of red wine, added to endothelial cell culture medium down-regulates the expression of genes for VCAM-1 and ICAM-1, but not for monocyte chemoattractant protein-1 (MCP-1) [133]. In this study, however, the serum phenol composition was not investigated and the addition in vitro of the same wine sample to endothelial cells, at relatively low alcohol concentrations, comparable to those measured in serum after red wine drinking, produced a different effect, with an up-regulation of VCAM-1, ICAM-1 and MCP-1. These experiments suggest that great caution should be used when transferring in vitro observations to in vivo conclusions. Indeed, the identification and measurement of the polyphenol conjugates in blood, as well as the clarification of their biological activities, are a key prerequisite for studies aimed at understanding the role of wine polyphenols in vascular function. NF-κB-dependent endothelial cell activation induced by CD40 [with interleukin (IL)-6, IL-8 and MCP-1 production] was inhibited by cyanidine-3-glucoside and delphinidin-3-glucoside, two anthocyanins, through the inhibition of TNF receptor-associated factor 2 (TRAF2) traslocation to lipid rafts and the reduction of their cholesterol content [134].

Finally, polyphenols extracted from red wine inhibited the number of smooth muscle cells migrated across the edge regions of an experimentally-induced wound as well as endothelial cell migration in the Boyden chamber assay: the signalling mechanisms underlying these effects involve the PI3 kinase and p38MAPK pathways [135].

5.2.2. In vivo models

Long-term resveratrol administration to apolipoprotein E-deficient mice attenuated the development of atherosclerotic lesions and of peri-arterial fat deposition concomitantly with a reduced expression of ICAM-1 and VCAM-1 in atherosclerotic vessels [136].

In rats, the administration of either red wine or alcohol-free red wine, but not of white wine, caused a prolongation of the bleeding time, inhibition of ex vivo platelet adhesion to fibrillar collagen and prevention of venous thrombosis. The higher polyphenol content of red as compared with white wine and the increased antioxidant activity found in plasma from rats receiving red wine strongly support a role for polyphenols in these effects [137].

In a rat model mimicking the "French paradox," the increased arterial thrombosis tendency of animal fed a cholesterol-rich diet was almost totally prevented when the diet was simultaneously supplemented with alcohol-free red wine, independently from the several-fold increase of blood cholesterol levels produced by the high-fat diet [138]. In line with these findings, red wine extracts or purified catechin added to drinking water of hypercholesterolemic apolipoprotein-E-deficient mice significantly inhibited thrombosis in a perfusion chamber model [139]. These effects of red wine, independent of its alcohol content, are mainly attributable to inhibition of platelet function by polyphenols.

A 12-week administration of a polyphenol-enriched red wine extract to rats prevented the increase of collagen Type I and the decrease of collagen Type III induced in the aorta wall by chronic oxidative stress (administration of tetrachloromethane), likely by

Table 3
Cellular effects of resveratrol on in vitro models of endothelial cell interactions with blood cells and of leukocyte activation

Cellular effect	Reference
↓ Leukocyte adhesion to EC in different conditions (rotating, laminar or non uniform shear stress, activated)	[116–119]
↓ Expression of adhesive molecules (such as VCAM-1, ICAM-1, MCP-1, E-selectin) on ECs activated by different agonists (TNFα, IL-1β, LPS/TNFα) EC types: human saphenous, human aortic endothelial cells, HUVEC, aortic endothelium	[116–119]
↓ Transcription of adhesion molecules;	[117–119]
↓ NF-κB activation and activator protein-1 involvement;	
↓ I-κB phosphorylation and nuclear translocation of p65 subunit of NF-kB	
↓ Neutrophil respiratory burst induced by thrombin-activated platelets;	[120]
↓ Neutrophil migration towards thrombin-activated platelet supernatant. Both effects are mediated by inhibition of nucleotide secretion from platelets	
↓ PMN adhesion to thrombin-activated platelets in a dynamic system;	[32]
↓ Expression of the activated β2-integrin Mac-1 on PMN stimulated by fMLP, C5a or the calcium ionophore A23187;	
↓ fMLP-induced tyrosine kinase phosphorylation in PMN	
↓ Tumor cell (human fibrosarcoma HT1080) adhesion to ECs	[121]
↓ ICAM-1 expression and NF-kB activation on tumor cells, stimulated by PMA	

↓, inhibition; PMN, polymorphonuclear leukocytes; LPS, lipopolysaccharide of bacterial origin; fMLP, formyl methionyl leucyl phenylalanine; PMA, phorbol 12-myristate 13-acetate; MCP-1, monocyte chemoattractant protein-1; I-κB: inhibitor of NF-κB.

influencing matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinases (TIMP) activities [140].

In a rat model of ischemic stroke, chronic supplementation with red wine polyphenols significantly enhanced residual cerebral blood flow compared to untreated rats, as a result of arterial vasodilation. Proteomic analysis indicated that red wine polyphenols restored a normal expression of proteins involved in brain metabolism, such as enolase, during ischemia [141].

Moreover, resveratrol prolonged skin allograft survival in rats and reduced the leukocyte-endothelial cell adhesive interactions induced by ischemia-reperfusion injury [142].

In a dog model of coronary stenosis, platelet-mediated thrombus formation was eliminated by the intravenous administration of red wine or grape juice, but not of white wine. Ex vivo platelet aggregation in response to collagen in whole blood was also decreased. Polyphenol concentration was much higher in red wine as compared with white wine and was suggestive for an alcohol-independent antithrombotic activity of polyphenol compounds [47].

5.2.3. Studies in healthy humans

A single administration of dealcoholized red wine (providing about 2 mM gallic acid equivalents) to healthy individuals did not modify ex vivo platelet P-selectin expression or GPIIb/IIIa activation in whole blood in response to either epinephrine or ADP; in contrast, a procyanidine-enriched cocoa beverage (providing about one gram of total epicatechins and oligomeric procyanidins) resulted in a significant reduction of platelet activation [143]. In this study, however, plasma polyphenol concentrations were not determined.

A prospective, randomized, cross-over study in eight healthy subjects evaluated the effect of a 4-week daily intake of two alcoholic beverages, red wine or gin, with the same alcoholic content (30 g ethanol/day), but with a high or low polyphenol content, on monocyte adhesion to endothelium. Both red wine and gin decreased ex vivo monocyte adhesion to TNFα-stimulated endothelial cells, although red wine to a significantly greater extent than gin. Plasma levels of epicathechin gallate, a marker of polyphenol constituents, increased significantly after red wine, but not after gin, intake [144]. In a similar study, involving 40 healthy males, the same group confirmed the effect of red wine, but not of gin, on the expression of monocyte adhesion molecules, such as Mac-1 and MCP-1, and observed a significant reduction of soluble endothelial adhesion molecules (sICAM-1 and sVCAM-1) and of monocyte adhesion to endothelial cells after red wine intake [145].

In a randomized, crossover study involving 35 healthy women, serum concentrations of sVCAM-1 and E-selectin were decreased after 4 weeks of red or white wine intake (20 g ethanol in 100 ml at lunch and dinner) with a greater effect for red wine. Soluble ICAM-1 and CD40L were reduced only after red wine consumption, while the expression of Mac-1, very late antigen-4 (VLA-4), MCP-1 and CD40 on monocytes was reduced after the intake of both red and white wine. However, monocyte adhesion to endothelial cells was significantly more inhibited after red wine than after white wine intake [146].

Reduction of monocyte activation markers and of circulating markers of atherosclerosis was observed in another cross-over study in healthy men, receiving either gin or a sparkling wine (cava) with medium-level of polyphenol content. High-sensitivity C reactive protein, ICAM-1, IL-6, MCP-1 and CD40L were reduced after cava, but not after gin, intake; surface expression of CD40L, lymphocyte function-associated antigen-1 (LFA-1) and VLA-4 on circulating monocytes was significantly reduced after the intake of both beverages, but to a greater extent after cava [147].

Thirty-two patients on hemodialysis were randomly assigned to receive for 2 weeks a concentrated red grape juice, as a source of polyphenols, vitamin E, or both: neutrophil NAPDH-oxidase activity and plasma levels of oxidized LDL were reduced by both treatments and by their combination. Among the plasma inflammatory biomarkers measured, only MCP-1 was significantly reduced by red grape juice supplementation [148].

5.3. Interaction of resveratrol and other wine polyphenols with aspirin

5.3.1. In vitro studies

Aspirin acts as an antiplatelet agent by irreversibly acetylating the platelet enzyme cyclo-oxygenase-1 (COX-1). Thus, it suppresses platelet activation mediated by agonists acting through arachidonic acid metabolism and thromboxane biosynthesis but only marginally interferes with platelet aggregation induced by agonists acting independently from COX-1, such as thrombin or cathepsin G, a neutrophil-derived protease. Resveratrol was able to inhibit the aggregation of aspirin-treated washed human platelets, suggesting an additional activity to aspirin, namely, on thromboxane-independent platelet aggregation [149].

The molecular structure of gallic acid is similar to that of salicylic acid, a hydroxybenzoic acid that is the main aspirin metabolite. Gallic acid, similar to salicylic acid, prevents the inhibitory effect of aspirin on COX-1 but also interferes with the platelet inhibitory actions of resveratrol and quercetin [150]. Given that all these polyphenols similarly suppress ROS generation by activated platelets, inhibition of radical oxygen production cannot be the main mechanism by which they affect platelet function. Other mechanisms, such as the modulation of platelet COX-1 activity, might play a role. Indeed, molecular modelling studies performed by in silico docking experiments showed that, like salicylate, gallic acid, resveratrol and quercetin form stable complexes into the COX-1 channel but have slightly different, but functionally relevant, interaction geometries with the enzyme. Interestingly, a mixture of resveratrol, quercetin and gallic acid, at relative concentrations similar to those found in most red wines, while not inhibiting platelet aggregation, potentiated the antiplatelet action of subinhibitory concentrations of aspirin [150]. Therefore, polyphenol-aspirin interactions may produce opposite effects depending on the polyphenols considered and their concentration. Such a possibility, therefore, should be carefully considered in future human studies.

5.3.2. Experimental in vivo models

Following administration to mice, gallic acid prevented the inhibition of TxB_2 biosynthesis induced by aspirin, resveratrol or quercetin, confirming in vivo the potential relevance of the gallic acid interaction with aspirin and other wine polyphenols observed in vitro [150].

5.3.3. Studies in patients with coronary artery disease

Resveratrol added in vitro to platelet-rich plasma from high-risk cardiac patients under chronic aspirin treatment inhibited residual platelet aggregation induced by collagen or epinephrine [151]. In a double blind, cross-over study in aspirin-treated patients with coronary artery disease, the consumption of purple grape juice for 14 days, although not providing additive antiplatelet effects, suppressed platelet-dependent inflammatory indices, such as plasma sCD40L levels, linked to cardiovascular disease [152].

6. Conclusions

Moderate wine intake reduces cardiovascular risk [1]. Although it is well known that alcohol favourably modifies the lipid pattern by decreasing total plasma cholesterol, in particular, LDL, and by increasing high-density lipoprotein cholesterol, cardiovascular risk reduction seems to be linked largely to the effect of non-alcoholic components, mainly resveratrol and other polyphenols, on the

vascular wall and blood cells. Although a great part of the beneficial effects of resveratrol on vascular function are due to its antioxidant effects, its positive modulation of endothelial- and platelet-derived NO biosynthesis and biologic activity, its ability to modulate cell–cell interactions involved in atherosclerosis and thrombosis and its inhibitory action on platelet COX-1 cannot be disregarded. The complete understanding of the relative importance of the antioxidant properties, NO stimulating activity and down-regulation of cell adhesive mechanisms in the reduction of cardiovascular risk associated with moderate wine consumption requires further studies.

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