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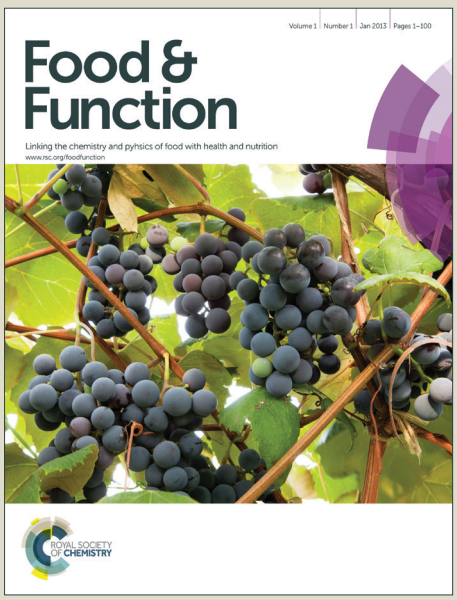

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ARTICLE

Modulation of nitric oxide by flavonoids

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One of the main mechanism by which dietary flavonoids are thought to influence cardiovascular disease is via protection of the bioactivity of the endothelium-derived nitric oxide (NO). Additionally, flavonoids may also interfere with the signalling cascades of inflammation and prevent overproduction of NO and its deleterious consequences in shock and ischemia-reperfusion injury. In the present paper we review the evidence of the effects of flavonoids on NO. Flavonoids exert complex actions on the synthesis and bioavailability of NO which may result both in enhanced or decreased NO levels: 1) In cell free systems, several flavonoids may scavenge NO via its pro-oxidant properties by increasing superoxide. However, under conditions of oxidative stress, flavonoids may also protect NO from superoxide-driven inactivation. 2) In intact healthy tissues, some flavonoids increase eNOS activity in endothelial cells. Paradoxically this effect involves a pro-oxidant effect which results in Ca^{2+}-dependent activation of eNOS. As inhibitors of PI3K, flavonoids may potentially inhibit the PI3K/Akt-dependent activation of eNOS. 3) Under conditions of inflammation and oxidative stress, flavonoids may prevent the inflammatory signalling cascades via inhibition of NFκB and thereby downregulate iNOS. On the other hand, they also prevent the overexpression ROS generating enzymes, reducing superoxide and peroxynitrite levels, hence preventing superoxide-induced NO inactivation and eNOS uncoupling. Therefore, the final effect of flavonoids on NO levels will depend on the flavonoid structure and the concentrations used, on the cell type under study and specially on the presence of inflammatory/oxidative conditions.

Introduction

Flavonoids comprise a large group of polyphenolic compounds widely distributed in dietary fruits, vegetables and beverages[1]. Numerous epidemiological reports show that dietary intake of the main classes of flavonoids is associated with a reduced incidence and mortality from coronary heart disease and/or of stroke[2, 3]. These beneficial effects are supported by a wide range of biological activities of these polyphenols including antioxidant and vasodilator effects, protective effects on endothelial function, platelet antiaggregant effects, inhibition of low-density lipoprotein (LDL) oxidation, reduction of adhesion molecules and other inflammatory markers and prevention of neuronal oxidative and inflammatory damage. In animal models of disease, some flavonoids produce antihypertensive and antiatherogenic effects, prevent endothelial dysfunction and protect the myocardium and the brain from ischemic damage and may also affect lipid and glucose metabolism[4, 5]. Therefore, flavonoids are currently thought to strongly contribute to the beneficial effects of diets rich in vegetables and fruits on cardiovascular health.

Nitric oxide (NO) is an ubiquitous signalling molecule playing a fundamental role in many physiological and pathological processes. NO is synthesized from L-arginine in several cell types by three NO synthases[6] as described below. It is an essential regulator of endothelial function and endothelial-derived NO activates soluble guanylyl cyclase in smooth muscle cells and platelets inducing vasodilatation and platelet antiaggregant effects although some effects may be cGMP independent. Several pathophysiologic conditions are characterized either by excess or deficit of NO production. Reduced NO bioavailability is the hallmark of endothelial dysfunction and it is considered the earliest step in the pathogenesis of atherosclerosis, hypertension and other cardiovascular diseases. In fact, endothelial dysfunction is inversely associated with future cardiovascular events independently of other cardiovascular risk factors[7, 8]. On the other hand, an excessive and sustained production of NO produced by the inducible form of NO synthase under inflammatory conditions may exert deleterious effects including hypotension, inhibition of mitochondrial respiration, apoptosis or necrosis[9].

One of the main mechanism by which dietary flavonoids are thought to influence cardiovascular disease is via protection of the bioactivity of the endothelium-derived NO. Additionally, flavonoids may also interfere with the signalling cascades of inflammation and prevent overproduction of NO and its deleterious consequences in shock and ischemia-reperfusion injury. In the present paper we review the evidence of the effects of flavonoids on NO. It will focus primarily on the most important dietary classes of flavonoids in a quantitative basis, flavonols (e.g. quercetin, isorhamnetin and kaempferol), flavones (e.g. luteolin and apigenin) and flavan-3-ols (e.g. catechin and epicatechin). Because most flavonoids circulate in plasma mainly as conjugated metabolites with glucuronic and/or sulfate[10], we are also reviewing the available literature on the effects

of metabolites. Nevertheless, it should be kept in mind that tissue deconjugation by glucuronidase and sulfatase may release the parent aglycone and the effects of the conjugates may be due to the deconjugated forms[11, 12].

Interaction of flavonoids with NO, superoxide and peroxynitrite

A number of studies have analysed the chemical interactions of flavonoids with NO and NO-derived nitrogen species in cell free systems.

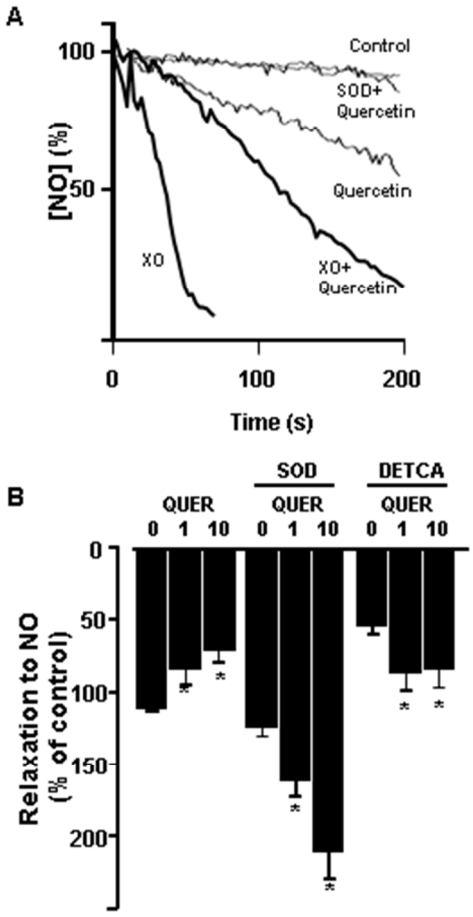

Figure 1. Effects of quercetin on NO in cell-free systems. (A) Quercetin accelerates the destruction of NO compared to control NO decay. The effect is prevented by superoxide dismutase, indicating that the effect is mediated by auto-oxidation of quercetin and generation of NO. Xanthine oxidase which generates superoxide induces a rapid destruction of NO which is partially prevented by quercetin. (B) The effects observed in panel A correlate with the biological activity of NO in an isolated artery. Exogenous NO was added to induce a relaxant response which was inhibited by quercetin. However when quercetin auto-oxidation is prevented by superoxide dismutase quercetin enhances the relaxant effect of NO. The increase in intracellular superoxide with DETCA (SOD inhibitor) results in reduced relaxation to NO which is prevented by quercetin. Modified from Lopez-Lopez et al.,2004[14].

Reaction with NO

Most flavonoids effectively scavenge NO in aqueous buffers, an often ignored pro-oxidant property of these polyphenols[13]. This reaction is strongly dependent on oxygen concentrations and on the pH, with NO destruction increasing in alkaline conditions[14]. NO scavenging involves the auto-oxidation of the flavonoid and the formation of superoxide radical (O_2^{-}) as an intermediate, which rapidly reacts with NO, and can be prevented by O_2^- dismutase (Figure 1A). Among flavonoids, flavonols are most effective and auto-oxidation can be minimized by blocking the hydroxyl group in position 3. Thus, the glucuronidated metabolites of flavonols in position 3, which are the most abundant forms in plasma, lack the NO scavenging effect[15]. The biological consequences *in vivo* are not entirely determined. The NO scavenging effect correlated with a reduced relaxation induced by exogenous NO in isolated rat aortic rings (Figure 1B). However, the biological activity of endogenously generated NO in the endothelial cells, as induced by acetylcholine, was unaffected, probably as a consequence of the reduced diffusion pathway and by the presence of endogenous antioxidants[14].

Reaction with superoxide (O_2^-)

Reactive oxygen species (ROS) exert a fundamental influence on the bioavailability of NO. O_2^- is particularly important because it rapidly reacts and inactivates NO. In fact, excess O_2^- is thought to be the most important mechanism involved in endothelial dysfunction[16]. The direct scavenging effect of flavonoids on ROS has been widely studied. Flavonols, flavones and flava-3-ols are well-known O_2^- scavengers[17]. This effect is shared with their glucuronidated metabolites[10]. Therefore, by lowering O_2^- concentrations, flavonoids preserve NO and enhance its biological activity[14]. Protection of NO from O_2^--driven inactivation is opposed to its NO-scavenging effects. Competition studies indicate that, under conditions of increased O_2^-, quercetin is a better scavenger of O_2^- than of NO. Therefore, depending on the experimental conditions, flavonoids can show anti-oxidant or pro-oxidant effects which result in increased or reduced NO bioavailability. As shown in Figure 1, when authentic NO is applied to an organ bath, quercetin reduces the response to NO (pro-oxidant effect). If O_2^- dismutase is present in the extracellular medium, the auto-oxidation of quercetin is prevented and quercetin potentiates the response to NO. If endogenous O_2^- dismutase is inhibited using DETCA, the increased endogenous O_2^- results in reduced NO response and this can be prevented by quercetin (anti-oxidant effect).

Reaction with peroxynitrite ($ONOO^-$)

$ONOO^-$ is the product of the reaction of NO and O_2^- radicals which has a strong oxidizing and nitrating properties and interacts with most biological molecules. $ONOO^-$ modifies tyrosine in proteins to create nitrotyrosines, leaving a footprint detectable *in vivo*. Nitration of tyrosine residues can disrupt protein structure and function with major pathological consequences. Much of NO and O_2^- -dependent cytotoxicity resides on $ONOO^-$, affecting mitochondrial function and triggering cell death via oxidation and nitration reactions[18]. By scavenging O_2^-, flavonoids not only protect NO but also prevent the formation of $ONOO^-$. In addition, direct scavenging of $ONOO^-$ by dietary flavonoids, may represent one mechanism by which these compounds may exert their beneficial actions *in vivo*. Flavonoids may react with $ONOO^-$ either by nitration of their own structures or by deactivating $ONOO^-$ by electron donation[19]. As a consequence of both, flavonoids prevent $ONOO^-$ -induced nitration of tyrosine[19, 20]. Methylated and glucuronidated metabolites showed a reduced

reactivity with $ONOO^-$ but still physiological levels of quercetin-3'-*O*-sulphate (Q3'S) and quercetin-3-glucuronide (Q3GA) effectively prevented $ONOO^-$ -induced nitrotyrosine formation in human serum albumin in *in vitro* experiments[21]. As described above, the reaction of NO scavenging under certain conditions involves the generation of O_2^-, which leads to $ONOO^-$ formation[14]. To our knowledge, protein nitration induced by flavonoids has not been reported suggesting that this reaction is overcome by the antioxidant effect *in vivo*.

Cellular and molecular targets

Independently from their scavenging action on NO and $ONOO^-$ (described above), plant flavonoids can modulate NO bioavailability, at the cellular level, by acting on the expression and/or activity of NO-producing enzyme, nitric oxide synthase (NOS; EC 1.14.13.39). Three isoforms of NOS have been identified, namely neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3)[6]. They all utilize L-arginine and molecular oxygen as substrates, bind calmodulin and require the cofactors reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and (6R-)5,6,7,8-tetrahydrobiopterin (BH4). The isoforms nNOS and eNOS are constitutively expressed and are activated by an increase in the intracellular levels of Ca^{2+} (but Ca^{2+}-independent mechanisms may also play a role as described below for eNOS) while iNOS expression is induced by certain stimuli (e.g. lipopolysaccharide, LPS) and its activity is independent of intracellular Ca^{2+} levels[6]. A mitochondrial NOS isoform (mtNOS) has been under debate, however recent evidence supports the notion that mtNOS is mostly a variant of nNOS.

An excessive and sustained production of NO by iNOS has been associated to the pathogenesis of septic shock and inflammatory diseases[22, 23], while a decrease in the NO production by eNOS in the vasculature leads to endothelial dysfunction[6]. Therefore, modulation of inducible and constitutive NO production is an important pharmacological target. In this section, the effect of flavonols, flavones and flavan-3-ols on constitutive and inducible NOS isoforms will be described. Furthermore, NO bioavailability is regulated by oxidative stress and several intracellular signalling pathways; thus, its modulation by flavonoids is also briefly summarized below.

Neuronal nitric oxide synthase (nNOS; NOS1)

The neuronal isoform of NOS is constitutively expressed in specific neurons of the brain, in the spinal cord, sympathetic ganglia and adrenal glands, in peripheral nitrergic nerves, in epithelial cells of various organs, in kidney macula densa cells, pancreatic islet cells, and in vascular smooth muscle[6]. The activity of this enzyme has been implicated in several physiological functions like learning, memory, and neurogenesis. In the central nervous system (CNS), nNOS mediates long-term regulation of synaptic transmission and central regulation of blood pressure. In the periphery, the NO derived from nNOS in nitrergic nerves stimulates NO-sensitive guanylyl cyclase in its effector cells, thereby decreasing the vascular and gastrointestinal tone[24].

An overproduction of NO by nNOS is likely to contribute to neurodegenerative pathologies such as Alzheimer's and Parkinson's diseases, multiple sclerosis and excitotoxicity following stroke[25]. Epigallocatechin gallate (EGCG) and, to a lesser extent, apigenin were able to attenuate the nNOS activity and extracellular nitrite production in quinolinic acid-induced excitotoxicity on primary human neurons[26]. The mechanisms involved in excitotoxicity seem to involve $ONOO^-$-mediated oxidative stress, resulting in DNA damage, activation of poly(ADP-ribose) polymerase (PARP) and/or mitochondrial permeability transition together with energy depletion due to inhibition of glycolysis and mitochondrial respiration[25]. EGCG and, to a lesser extent, apigenin impaired the quinolinic acid-induced excitotoxicity in human neurons by counteracting the increase of intracellular Ca^{2+} levels, PARP activation and NAD+ depletion[25]. Furthermore, EGCG, but not catechin or epicatechin, inhibited the activity of rat nNOS protein purified from BL21 (DE3) *Escherichia coli* cells[27]. The activity of nNOS from mouse brain extract is also inhibited by 7-44% after addition of 100–600 μM EGCG[28].

Inducible nitric oxide synthase (iNOS; NOS2)

The inducible isoform of NOS (iNOS) is expressed in cells after stimulation with bacterial LPS, cytokines, and other agents[29]. Although primarily identified in macrophages, iNOS can be expressed in any cell or tissue, provided that the appropriate inducing agents have been identified[6]. Once expressed, iNOS is always active and not regulated by intracellular Ca^{2+} levels[29]. In macrophages, the high output of inducible NO can directly interfere with the DNA of target cells, causing strand breaks and fragmentation[30], or inhibit iron-containing enzymes (including mitochondrial complexes I and II, ribonucleotide reductase and cis-aconitase)[31]. The combination of these effects is likely to be responsible for the cytotoxic action of NO on parasitic microorganisms and certain tumour cells. Additionally, non-immune cells can also produce NO and affect the neighbouring cells. For example, cytokine-activated endothelial cells have been shown to lyse tumour cells[32], while hepatocytes can use NO to kill malaria sporozoites[33]. Besides its beneficial role on host protection against undesired microbes, parasites, or tumour cells, the high levels of NO may also harm healthy cells. Cell and tissue damage by NO radical itself or $ONOO^-$ contributes to the pathogenesis of septic shock and inflammatory-related diseases including Alzheimer, cardiovascular diseases, diabetes and cancer[22, 23]. Compounds able to reduce iNOS-derived NO may be thus attractive anti-inflammatory agents. For this reason, the effects of plant flavonoids on iNOS expression and/or activity have been extensively studied, using *in vitro* models of inflammation. Noteworthy, some caution should be taken on the interpretation of the results because many *in vitro* studies often used concentrations (10–50 μM) far exceeding those that might be considered physiological[34].

Evaluating the structure-activity relationships of naturally occurring flavonoids on NO production in the LPS-activated RAW 264.7 macrophages, it was found that apigenin, luteolin, and quercetin inhibited NO production, having IC_{50} values of 23, 27 and 107 μM, respectively[35]. The results indicated that a C-2,3 double bond might be important, and that the potency of inhibition depends upon the substitution patterns of the flavonoid molecules. Furthermore, in this study, it was found that the inhibitory activity of apigenin and quercetin on NO was not due to a direct inhibition of iNOS enzyme activity because they did not inhibit iNOS activity, as measured by [^{3}H]citrulline formation from [^{3}H]arginine, which may be even increased. Instead, a reduction on iNOS expression by flavonoids has been reported in several cell lines[14, 35, 36].

In particular, quercetin inhibited the production of NO through reduction of iNOS mRNA and protein levels in RAW 264.7 macrophages stimulated with interferon (IFN)-γ plus gliadin[37] or with LPS[38] (Figure 2). Furthermore, quercetin concentration-

dependently suppressed LPS-induced NO production in RAW 264.7 macrophages and primary peritoneal macrophages (obtained from thioglycollate-treated Balb/c mice), associated with a decrease in iNOS protein expression in both cells[39]. Quercetin can also inhibit iNOS expression and activity in other cell types. In BV-2 microglia, quercetin suppressed LPS- and IFN-γ-induced NO production and iNOS gene transcription[40, 41]. Kaempferol and quercetin show little differences in their inhibiting capacity of iNOS expression in RAW 264.7 cells[42, 43] or in Chang Liver hepatic cells[44] while kaempferol inhibits to a greater extent than quercetin the LPS-induced NO production in J774.2 cells[45]. The kaempferol-3-*O*-glucoside astragalin was able to significantly reduce LPS-induced iNOS expression and NO production in J774A.1 mouse macrophages[46].

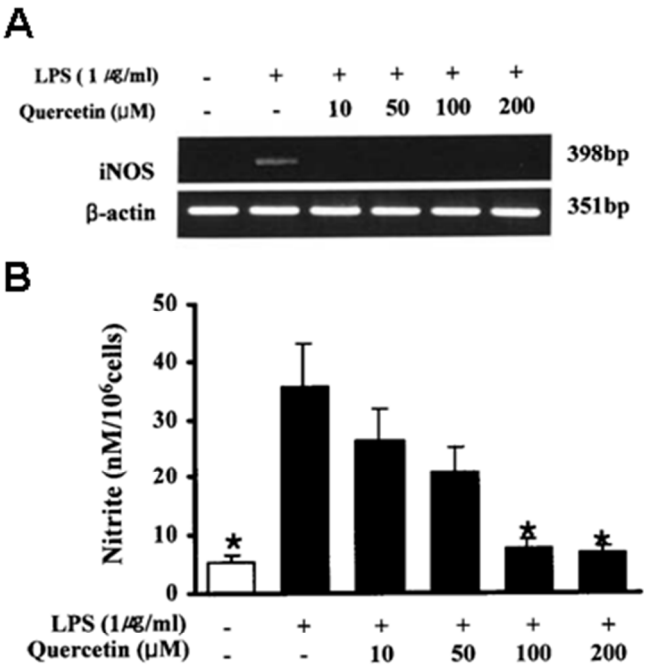

Figure 2. Quercetin inhibits the induction of NO and the production of NO measured by the Griess reaction (adapted from Cho et al., 2003[38]).

Apigenin significantly blocked transcriptional activation of iNOS, with IC_{50}<15 μM[27] in LPS-stimulated RAW 264.7 cells[43]. However, its two *C*-glycosylated derivatives, vitexin and isovitexin were inactive[47]. Lin and co-workers found opposed results to isovitexin. In particular, it was reported that isovitexin reduced LPS-stimulated NO production with an IC_{50} of 58.5 μM through inhibition of IKK kinase activity and prevention of the IκBα degradation, in activated RAW 264.7 cells[48]. Meeteren and co-workers found that both apigenin and luteolin inhibited NO production and reduced the iNOS protein levels in LPS-stimulated NR8383 macrophages, pointing them potential anti-inflammatory agents targeting neurodegenerative and neuroinflammatory diseases[49]. Luteolin also has been described to attenuate iNOS gene expression in LPS-stimulated RAW 264 264.7[50] and activated microglia[51-54]. Luteolin and kaempferol demonstrated inhibitory activity on LPS-induced NO production in RAW 264.7, with IC_{50} values of 10.41+/-0.02 μM and 10.61+/-0.44 μM, respectively[55]. These effects were related to suppression of iNOS mRNA expression[55]. At a non-toxic concentration, luteolin 5-*O*-glucoside and luteolin-7-*O*-glucoside (concentrations lower than 20 μM), were able to inhibit the LPS-induced iNOS expression and NO production in RAW 264.7 macrophages[56, 57]. Considering the structure of flavones, it was found that the optimal chemical structures to inhibit the NO production in LPS-stimulated RAW 264.7 cells were A-ring 5,7-dihydroxyflavones having the B-ring 2',3'-dihydroxy or 3',4'-dihydroxy or 3',4'-hydroxy/methoxy (methoxy/hydroxy) groups[58]. The strongest inhibition was found for 2',3',5,7-tetrahydroxyflavone and 3',4',5,7-tetrahydroxyflavone with IC_{50} of 19.7 and 17.1 μM, respectively[58].

Regarding to flavanols, there is also evidence that inhibition of iNOS may be a mechanism behind its anti-inflammatory effects. EGCG and other flavanols inhibit the induction of iNOS mRNA and activity after treatment with LPS, IFN-γ[28, 59, 60], interleukin (IL)-1 and tumour necrosis factor (TNF)-α[61] *in vitro*. In addition, EGCG (50–750 μM) inhibited, in a concentration-dependent manner, the enzyme activity of iNOS, to 85–14%[28]. Additionally, EGCG impaired LPS-induced NO release by microglia[62].

The central mechanisms controlling iNOS expression seems to be the activation of nuclear factor (NF)-κB pathway and janus kinase/signal transducer and activator of transcription (JAK/STAT). Quercetin inhibited iNOS expression by preventing the nuclear translocation of p50 and p65 subunits of NF-κB, and the activation of STAT-α and IRF-1, in IFN-γ and gliadin-stimulated RAW 264.7 cells[37]. Quercetin, and to a lower extent kaempferol, reduced the activation of NF-κB, by preventing the degradation of its inhibitor IκB, as well as the activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinases (MAPK) in LPS-stimulated RAW 264.7 macrophages[38, 63]. In BV-2 microglia, quercetin reduced the activation of LPS-induced IKK, NF-κB and AP-1 activation, and IFN-γ-induced NF-κB, STAT1 and IRF-1 leading to suppression of iNOS gene transcription and NO production[40]. These effects are accompanied by the down-regulation of ERK, c-Jun N-terminal kinase (JNK), p38 MAPK, Akt, Src, Janus kinase-1, Tyk2, and probably other serine/threonine and tyrosine phosphatase activities[41]. Kaempferol impairs the LPS-induced iNOS expression through inhibition of NF-κB activation and p38 MAPK, JNK and Akt phosphorylation in BV-2 microglia[64]. In the human hepatocyte-derived Chang Liver cell line incubated with a cytokine mixture, the inhibition of iNOS mRNA expression by quercetin and kaempferol was associated with a decreased concentration of phosphorylated IκB-α protein and IKK-α and consequent inhibition of NF-κB activation[44]. In LPS-stimulated J774A.1 mouse macrophages, astragalin also inhibited LPS-induced NF-κB activation by inhibition of IκB-α degradation, nuclear translocation of NF-κB, and NF-κB dependent gene reporter assay with consequent reduction on NO production[46].

Recently, an extensive study on the anti-inflammatory mechanism of quercetin in LPS-induced RAW 264.7 was performed. It was found that quercetin inhibited LPS-induced NO and iNOS while it promoted heme oxygenase (HO)-1 induction in a dose- and time-dependent manner[65]. It also suppressed IκB-phosphorylation, NF-κB translocation, AP-1 and NF-κB-DNA-binding and reporter gene transcription, as well as p38 MAPK and JNK1/2, but not ERK1/2 activation[65]. Quercetin further arrested Src and Syk tyrosine phosphorylations and their kinase activities followed by inhibition of PI3K tyrosine phosphorylation[65]. Apigenin (50 μM) was able to counteracted the TNF-α-induced expression of eNOS and activation of Akt, p38 MAPK and JNK signalling in EAhy926 endothelial cells[66]. This effect is thought to be derived from its ability to bind to oestrogen receptors.

Apigenin inhibited NF-kB activation through the prevention of both IKK activity and IκB degradation, which might be associated with

the suppression of iNOS transcriptional activation (with consequent decrease in mRNA and protein levels) in LPS-stimulated RAW 264.7 cells[43]. This flavone also suppressed p38 MAPK and JNK activation without affecting ERK in BV-2 microglia[67]. Luteolin did not reduce MAPK activation neither NF-κB transcriptional activity at the level of IκB-α degradation[50]. However, this flavone down-regulates NF-κB signalling through inhibition of nuclear translocation and DNA binding activity of NF-κB p50 subunit[50]. Furthermore, luteolin suppressed STAT1 signalling.

Concerning to catechins, it was found that EGCG inhibited iNOS expression in LPS-stimulated macrophages also by prevention of IκB degradation[60]. The (+)-catechin inhibited LPS-induced NO production in LPS-stimulated murine peritoneal macrophages, probably through c-Jun N-terminal kinase and p38 MAPK signalling pathways[68].

MicroRNAs (miRNAs), small noncoding RNAs that modulate translation and/or degradation of target messenger RNAs, have been involved in major pathologies such as cancer[69], metabolic[70] and autoimmune diseases[71], and endotoxic shock[72]. Among them, miR-155 regulates iNOS expression in LPS-stimulated macrophages[73]. Transfection of miR-155 attenuated the expression of suppressor of cytokine signal (SOCS)-1, with the subsequent activation of signal transducer and activator of transcription (STAT)-1 and -3, and the increase in iNOS protein. This proinflammatory miR-155 was down-regulated by quercetin and isorhamnetin, but not by Q3GA in murine RAW264.7 macrophages stimulated with LPS[74]. However, no specific link has been established between miR155 down-regulation and iNOS expression inhibition induced by these flavonoids in macrophages.

Endothelial nitric oxide synthase (eNOS; NOS3)

Endothelial NOS is typically expressed in endothelial cells. However, this NOS isoform has also been found in cardiac myocytes, platelets, certain neurons of the brain, LLC-PK1 kidney tubular epithelial cells and in syncytio-trophoblasts of the human placenta[6]. NO derived from eNOS is an important homeostatic regulator of essential cardiovascular functions[75]. In vascular smooth muscle cells, NO directly exerts vasodilator effects via activation of soluble guanylyl cyclase and increase in cyclic GMP[76], playing a major role in the regulation of vascular tone. NO may also exert cGMP-independent effects, interacting with other heme containing enzymes or via S-nitrosylation. The NO released in vasculature also protects against thrombosis and atherosclerosis. NO decreases oxidation of low-density lipoprotein (LDL) and antagonizes platelet aggregation by inhibiting platelet activation and tissue-factor expression[75]. Moreover, NO inhibited the expression of chemoattractant and adhesion molecules, which mediate recruitment of leukocytes to the endothelium, and suppresses abnormal proliferation of vascular smooth muscle cells. The number of protective roles of NO in the vasculature indicates that decreased bioavailability of this mediator may promote the progression of vascular diseases[6]. Understanding the regulation of endothelial NO production and how dietary flavonoids positively influence eNOS activity could provide valuable clues for prevention or treatment of cardiovascular diseases.

NO has a short half-life and so, tight temporal and spatial regulation of its production is critical. Several highly organised levels of regulation exist, controlling the eNOS gene expression and mostly the enzyme activity (for extensive review see[75, 77]). Post-translational modifications of the enzyme (phosphorylation, acetylation and S-nitrosylation) regulate its activity. In human eNOS, phosphorylation at Ser^{1117} increases electron flux within the enzyme and the Ca^{2+} sensitivity, representing an additional and independent mechanism of eNOS activation. Phosphorylation could be catalyzed by Akt, AMP-activated protein kinase (AMPK), Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) and activating protein kinase A (PKA), among others. Thr^{495} phosphorylation attenuates eNOS activity by interfering with calmodulin binding. Phosphorylation of Ser^{633} seems to be important for the maintenance of NO synthesis after activation of eNOS at Ser^{1177}. Deacetylation at Lys^{496} and Lys^{506} (bovine sequence) by sirtuin 1 can enhance eNOS activity, and S-nitrosylation leads to catalytic inhibition of eNOS and subcellular redistribution. Protein–protein interactions also play a meaningful role in the regulation of eNOS activity. Binding to calmodulin (CaM) is brought about by a rapid increase in intracellular Ca^{2+} and enhances the enzyme activity. Heat shock protein 90 (Hsp90) is an eNOS allosteric modulator that increases the affinity of enzyme towards CaM and provides a dynamic scaffold for eNOS activation by Akt and other regulatory proteins. The caveolae scaffolding protein caveolin-1 (Cav-1) is an important negative regulator of eNOS activity in endothelial cells. Among others, eNOS also co-localizes with proteins of the cytoskeleton and the cationic amino acid transporter-1 (CAT-1), which is responsible for the uptake of the eNOS substrate L-arginine. The concentration of substrate L-arginine and asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS, are also important factors for NO production[78].

All the above mentioned mechanisms that are implicated in the regulation of eNOS expression and activity are potential targets to modulate NO bioavailability. Evaluation of the effectiveness of different polyphenols to enhance eNOS mRNA expression at 100 μM in a hybrid human endothelial cell line EA.hy926 revealed a significant stimulatory effect of quercetin, epicatechin-gallate, and EGCG[79]. Quercetin was shown to enhance eNOS activity in endothelial cells rapidly via increases in intracellular concentration of Ca^{2+} [80], by activation of K^+ channels or inhibition of Ca^{2+}-ATPases of the endoplasmic reticulum in endothelial cells[81]. Furthermore, in the absence of oxidative stress, quercetin has been reported to increase NO when measured by an amperometric electrode in endothelial cells[82] and to increase cytosolic Ca^{2+} measured by fura2 via a pro-oxidant mechanism[83]. However, interferences with the measuring systems cannot be ruled out due to the redox and fluorescent properties of quercetin[84]. Controversially, other *in vitro* studies showed no or even a negative effect of quercetin on eNOS expression and/or endothelial NO production[85, 86]. When NO production was measured in endothelial cells by electron paramagnetic resonance spectroscopy, quercetin failed to increase NO[87], and, in bovine endothelial cells, quercetin inhibited eNOS activity[85]. Thus, either increases or decreases in endothelial NO can be observed with quercetin. Notably, quercetin in the cell culture media can promote generation of H_2O_2[88]. Since H_2O_2 can, depending on its concentration, either activate eNOS or injure endothelial cells[89], this may lead to *in vitro* artefacts that may possibly explain the high variation on quercetin effects. Additionally, the metabolic transformation of quercetin prevents it from causing a potentially deleterious reduction in eNOS[90]. Quercetin aglycone reduced eNOS expression in TNF-α-stimulated HUVEC, whereas its metabolites were without effect in either TNF-α-stimulated or unstimulated cells[90].

Incubation with the flavan-3-ols (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechingallate and (-)-EGCG, increased NO production in cultured endothelial cells from human umbilical veins in a concentration-dependent way[91]. (-)-Epicatechin can stimulate NO synthesis under Ca^{2+}-free conditions, i.e. independent of calmodulin

binding to eNOS, because (-)-epicatechin induced eNOS phosphorylation at serine 615, 633, and 1177 with consequent enzyme activation[92] and promotes the formation of an active complex between eNOS, Akt, and Hsp90[93]. EGCG was able to induce NO liberation in cultured coronary artery endothelial cells via the redox-sensitive Src/PI3K/Akt-dependent phosphorylation of eNOS[94]. The hydroxyl group at the 3" position of the gallate ring and, also, to some extent, the two hydroxyl groups at positions 3' and 4' are essential to EGCG-induced eNOS activation[94]. Curiously, EGCG competitively inhibited binding of arginine and tetrahydrobiopterin, and the gallate structure is important for this action[28]. Evaluating green tea polyphenols, it was verified a down-regulation of Cav-1 expression, in a time- and dose- dependent manner, via activation of ERK1/2 and inhibition of p38MAPK signaling[95].

Under oxidative stress conditions, eNOS can generate itself O_2^- instead of NO, a phenomenon known as eNOS "uncoupling". Becoming a dysfunctional O_2^--generating enzyme, eNOS contributes to decreased NO bioavailability and further increase in vascular oxidative stress, both associated with endothelial dysfunction and cardiovascular pathologies[75]. Therefore, inhibition of ROS production by flavonoids can enhance the bioavailability of NO in the vasculature (discussed below).

Reactive oxygen species (ROS)

As mentioned above, bioavailability of NO is greatly influenced by oxidative stress[96]. When the production of O_2^- exceeds detoxification via superoxide dismutase (SOD), a kinetically preferred non-enzymatic reaction between O_2^- and NO occurs. The resulting $ONOO^-$ anion is a very deleterious oxidant species[97]. $ONOO^-$ production has been indicated as an important contributor to vascular diseases, ischaemia–reperfusion (I/R) injury, inflammation, circulatory shock, pain and neurodegeneration[97]. In the vasculature, oxidative stress and production of $ONOO^-$ is associated with eNOS "uncoupling". This is directly related to oxidation and inactivation of cofactor BH4, increase of ADMA levels triggered by ROS and lowered bioavailability of L-arginine. More recently, S-glutathionylation of eNOS promoted by oxidative stress has been proposed as another mechanism underlying eNOS uncoupling[6].

There are several enzyme systems that generate and degrade ROS. Enzymes implicated in the formation of O_2^- from molecular oxygen and an impaired electron include NADPH oxidase, xanthine oxidase, a dysfunctional eNOS (in which oxygen reduction is uncoupled from NO synthesis), and enzymes of the mitochondrial respiratory chain. Generated O_2^- could be converted to hydrogen peroxide (H_2O_2) by O_2^- dismutase (SOD), which can be detoxified via glutathione peroxidase (GPx), catalase, or thioredoxin (Trx) peroxidase to H_2O and O_2. Other enzymes with antioxidant properties are heme oxygenase (HO) and perhaps also paraoxonases (PON)[6].

Quercetin can inhibit the enzymatic sources of O_2^-, i.e. xanthine oxidase and NADPH oxidase[98], besides its direct scavenging activity on O_2^- as described above. In BV-2 microglia, quercetin was able to induce the gene expression of HO-1 through involvement of tyrosine kinase and MAPK activation[40], as well as NF-κB and NF-E2 related factor 2 (Nrf2)[99]. Luteolin suppressed TPA-triggered membrane translocation of p47(phox) (a NADPH oxidase subunit) in THP-1 monocytes[100]. Aucamp and co-workers had analysed the inhibition of xanthine oxidase by tea catechins[101]. The Ki values (μM) and types of inhibition were catechin, Ki=303.95 (uncompetitive), epicatechin, Ki=20.48 (mixed), epigallocatechin, Ki=10.66 (mixed), epicatechin gallate, Ki=2.86 (mixed) and EGCG, Ki=0.76 (competitive)[101].

The expression of antioxidant enzymes is highly dependent of Nrf2 transcription factor. Nrf2 is a redox-sensitive transcription factor of cap'n'collar subfamily containing the basic leucine zipper region, that binds to the antioxidant response elements (ARE) in the promoter regions of antioxidant enzymes[101]. The flavan-3-ol EGCG induced nuclear translocation of Nrf2 and HO-1 expression in B-lymphoblasts, at 30μM[102] Furthermore, it has been evidenced that Nrf2 is a negative regulator of iNOS expression and the overproduction of NO, being inhibition of NF-κB activation probably involved[103]. EGCG was described as Nrf2-activating agent which inhibit activation of NF-κB pathway[104].

Flavonoids, NO bioactivity and endothelial function: *in vitro* evidence.

The effects of flavonoids on arterial function and the role of NO have been widely studied and we refer the reader to our previous reviews on this topic for both flavonols[5, 105] and flavanols[106]. Briefly, most flavonoids exert vasodilator effects in isolated arteries albeit with different potency[107]. Some flavonoids, in contrast, may also exert vasoconstrictor effects such as the flavonol myricetin[108] or EGCG in the aged spontaneously hypertensive rat aorta[109] which are mediated by an activation of cyclooxygenase and the subsequent production of vasoconstrictor prostanoids. The mechanism for the relaxant effects is not completely elucidated but it seems that all flavonoids do not share the same mechanism, particularly regarding the role of endothelium and NO. The most potent flavonols, quercetin and isorhamnetin relax healthy vessels while the glucuronidated and sulphated metabolites are ineffective[110]. This effect is endothelium-independent and it is unaffected by inhibition of NO synthesis[111, 112] even when some studies have also reported a weak inhibition of the relaxant response by NOS inhibitors[83, 113]. This is consistent with the lack of a direct effect of these flavonols on eNOS (as described above). On the other hand, endothelium- and NO-dependent relaxation has been reported for several other isolated flavonoids such as the anthocyanin delphinidin[114] and the flavone chrysin[115]. Both endothelium-dependent and independent effects of the flavanols catechin, epicatechin and EGCG have been reported[87, 116-118]. Paradoxically, the endothelium- and NO-dependent effects are related to a pro-oxidant effect, i.e. generation of O_2^-, because it can be inhibited by O_2^- dismutase and catalase[117].

Besides the direct effects on endothelial NO synthase (eNOS), inhibitory effects on pathways that may negatively affect NO including NADPH oxidase, angiotensin-converting enzyme (ACE), asymmetrical dimethylarginine, and endothelin-1 (ET-1) have been proposed for several flavonoids[5, 105, 119]. Angiotensin-2 and endothelin-1 are potent stimulus for the induction of NADPH oxidase, a major source of oxidative stress in the vessel wall. Flavonoids may interfere with the synthesis of angiotensin II by interfering with ACE[120] or with the signalling pathways of both angiotensin II or endothelin-1[121, 122]. Reduction of oxidative stress indirectly also protects eNOS from the oxidative attack of O_2^- and $ONOO^-$ preventing eNOS uncoupling[121]. Some flavonoids may also reduce the endogenous inhibitors of NOS methylarginine[123, 124].

On the other hand, quercetin has also been shown to counteract the development of tolerance *in vitro* to the NO donor glyceryl trinitrate, an effect shared with its metabolite Q3'S[111], which is probably mediated by its antioxidant effect[125].

Effects of flavonoids on NO in animal models

The effects of flavonoids rich food or supplements, especially those of red wine, cocoa, and tea polyphenols, on endothelial function and NO have been also analyzed in animal models of cardiovascular disease including hypertension, metabolic syndrome, stress and aging[105]. In Zucker rats, an experimental model of obesity and related metabolic syndrome, red wine polyphenols improved endothelial dysfunction by increasing NO- and EDHF-mediated relaxations associated with a reduced O_2^- release via decreased expression of the NADPH oxidase membrane subunit Nox1[126]. Red wine polyphenols also improved the impaired endothelial function, the oxidative stress and the overexpression of angiotensin receptors AT1 and AT2 associated to aging and also improved the aging-related decline in physical exercise[127]. The inhibition of the release of microparticles by polyphenols may contribute to prevent endothelial dysfunction.[128] Paradoxically, in chronically stressed rats which show increased NO compared to controls, red wine polyphenols prevented the elevated release of NO[129]. The information about the effects of pure flavonoids on endothelial function in animal models, besides the hypertensive animals is limited. However, there are several studies about the effects of flavonoids in inflammatory diseases mediated by iNOS inhibition.

Effects of flavonoids in animal models of hypertension

Hypertensive animals, as well as human essential hypertensive patients, develop reduced endothelium derived NO-dependent vasodilatation. In different experimental rat models of hypertension (spontaneously hypertensive rats [SHR], deoxycortocosterone acetate [DOCA]-salt and Goldblatt rats), chronic quercetin restored the impaired endothelial vasodilator function as measured by the relaxant response to acetylcholine and reduced blood pressure (Figure 3)[130-133]. Increased urinary NOx (nitrites + nitrates, main NO metabolites) was also found. All these models were associated with increased plasma, vascular and hepatic oxidative status as measured by plasma, tissue and urinary levels of either malonyldialdehyde or isoprostanes, and quercetin consistently reduced these parameters[130-133]. Altogether these results suggest a role for reduced O_2^--driven NO inactivation. Furthermore, in SHR, which show upregulated eNOS but with a paradoxical reduction in NOS activity compared to their normotensive WKY controls, quercetin normalized both parameters[131]. In addition, in SHR quercetin can prevent the upregulation of $p47^{phox}$ [131]. Moreover, chronic quercetin also reduced several markers of endothelial dysfunction in ApoE knockout mice [134, 135] and in rats treated with a high-fat high-sucrose diet[136].

In contrast to the endothelium-dependent vasodilatation to acetylcholine, the endothelium- and NO-dependent relaxations to insulin, which are also impaired in SHR, were unaffected after chronic treatment with quercetin[137]. The different profile of quercetin against the relaxations induced by these two endothelial NO-releasing agents, despite its protective effect on O_2^--driven NO inactivation, might be related to the different pathways of acetylcholine and insulin to activate eNOS. Acetylcholine is a classic cholinergic agonist that activates eNOS by a calcium-dependent mechanism. However, insulin has calcium-independent vasodilator actions that are mediated by a PI3K dependent mechanism involving phosphorylation of eNOS by Akt[138]. Insulin-stimulated Akt and eNOS phosphorylations were reduced in aortic rings from SHR and WKY rats treated with quercetin. This effect might be related to a direct inhibitory effect of quercetin on PI3K[139]. The effects of quercetin on hypochlorite-induced endothelial dysfunction have been attributed to its effect on HO-1[135].

Animal studies using flavanol rich foods and purified epicatechin are a valid alternative to advance on the comprehension of the mechanisms underlying their blood pressure lowering effects. Several polyphenolic extracts containing mainly flavanols (e.g. red wine polyphenols, grape skin extract, cocoa extract and black or green tea) reduced blood pressure in several rat models of experimental hypertension[140-143]. This effect was related to a combination of vasodilator and antioxidant actions. Other proposed mechanisms for the antihypertensive effects of flavanols include the ability to lower the activity of arginase-2, which is an enzyme that competes with eNOS for L-arginine[144] and inhibitory activity on angiotensin converting enzyme (ACE) *in vitro*[120].

Only a few studies analysed the effects of the purified flavanols in experimental models of hypertension (reviewed in[106]). The effects of pure epicatechin in SHR[118], and in the DOCA-salt[145], in the L-NAME models[146] of hypertension have been analysed (Figure 3). In SHR, (-)-epicatechin (3 g/kg diet) can modulate blood pressure in hypertension by increasing NO levels in the vasculature (Figure 3). The DOCA-salt hypertensive rat is a model with a markedly depressed plasma renin activity because of sodium retention. Patients with low renin (i.e. salt-sensitive hypertension) represent approximately the 30% of the essential hypertensives and show a poor therapeutic response to angiotensin-converting enzyme inhibitors and angiotensin receptor blockers. (-)-Epicatechin (2 or 10 mg/kg/day) improved the impaired endothelium-dependent relaxation response to acetylcholine and increased the phosphorylation of both Akt and eNOS in aortic rings from DOCA-salt rats, showing increased eNOS activity[147]. These *in vivo* results are consistent with previous *in vitro* observations showing epicatechin-induced eNOS activation via PI3K/AKT-mediated phosphorylation in human endothelial cells[92]. Interestingly, changes in this pathway are not dependent of the presence of epicatechin in plasma, since they were obtained after 48 h of depriving epicatechin, suggesting the involvement of *in vivo* mechanisms, which maintain phosphorylation. Moreover, most of the current research implies actions of epicatechin that depend on its actual presence. This discrepancy indicates new potential mechanism(s) for epicatechin action.

In addition, epicatechin increased both Nrf2 and Nrf2/ARE target genes, such as NADPH:quinone oxidoreductase 1 (NQO1), HO-1, and γ-glutamylcysteine synthetase, in aorta from both control and DOCA-salt rats[147]. Recently, Kim et al[148] demonstrated that NQO1 activation improves eNOS coupling through AMPK-mediated preservation of GTP cyclohydrolase-1, the rate-limiting step in the novo synthesis of the eNOS cofactor BH4. However, whether this mechanism is involved on the increased eNOS activity induced by epicatechin is unknown.

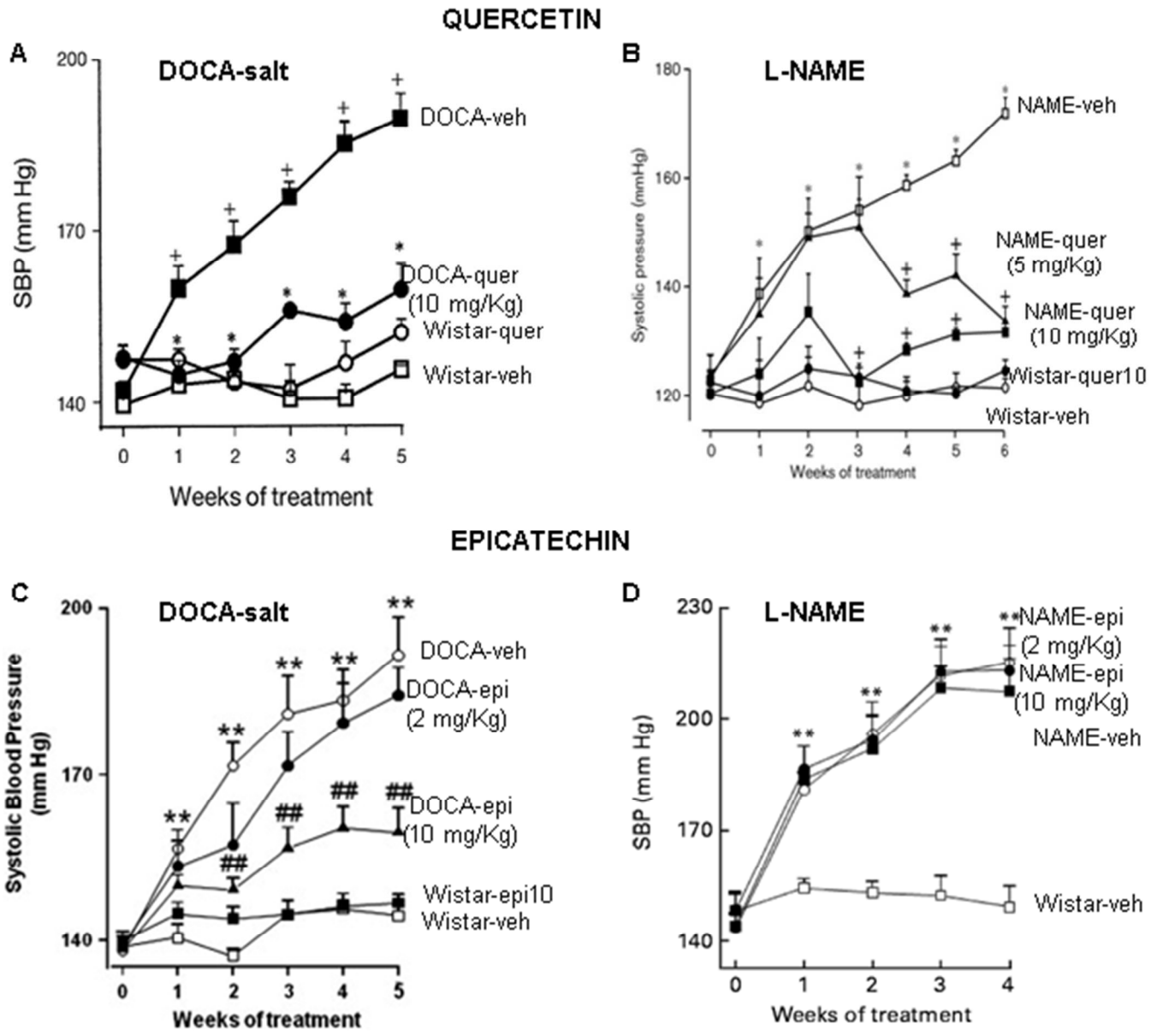

Figure 3. The flavonol quercetin (quer, A) and the flavanol epicatechin (epi, C) prevent the development of renin-dependent hypertension in the rat model of deoxicorticosterone-salt (DOCA). The antihypertensive effect of quercetin (B) is mantained in rats made hypertensive by inhibition of NO (with the NOS inhibitor L-NAME) while epicatechin fails to lower blood pressure in this model, suggesting that the effects of epicatechin are dependent on NO. Reprinted from Galisteo *et al.*[130] (A), Duarte *et al.*[187] (B), Gómez-Guzmán *et al*[143] (C) Gómez-Guzmán *et al*[144] (D).

ET-1 is involved in the development of oxidative stress and hypertension in DOCA-salt rats, since ET_A receptor blockade reduced arterial O_2^- levels with a concomitant decrease in systolic blood pressure. Recently, it has been described that epicatechin reduced plasma ET-1 levels in the apolipoprotein E (ApoE)(-/-) gene-knockout mouse[149] and in DOCA-salt hypertensive rats[145]. This inhibitory effect might be mediated via Akt-regulation of the ET-1 promoter, as previously suggested in *in vitro* experiments[150]. Epicatechin also induced a reduction in ET-1 release, systemic and vascular oxidative stress, and inhibition of NADPH oxidase activity in DOCA-salt rats. The alleviation of oxidative stress by epicatechin[145] diminished ROS-mediated NO inactivation and raised the bioavailability of NO, leading to an enhancement of the NO-mediated vasodilatory tone, which could account for the observed amelioration of hypertension. In fact, when the effects of epicatechin were studied in rats after chronic inhibition of NO synthesis with N^G-nitro-L-arginine methyl ester (L-NAME), chronic epicatechin treatment did not modify the development of hypertension[146] (figure 3D) despite of the reduction of systemic markers of ROS, demonstrating the key role of NO in the antihypertensive effects of this flavanol. Higher doses of epicatechin (>100 mg/kg) are required to restore NO levels and to reduce blood pressure in this model of chronic NO synthesis inhibition[151].

Effects of flavonoids in animal models of inflammatory diseases

An overproduction of NO from iNOS has been associated to the pathogenesis of septic shock and inflammatory diseases[22, 23].

Neuroinflammation. Sustained neuroinflammation processes may contribute to the cascade of events culminating in the progressive neuronal damage observed in many neurodegenerative disorders, most notably Parkinson's disease and Alzheimer's disease, and also with neuronal injury associated with stroke. Flavonoids may modulate neuroinflammation via their potential to modulate signalling pathways controlling the activation of glial cells. An uncontrolled activation of iNOS in glial cells constitutes a critical event in inflammatory-mediated neurodegeneration[152]. Flavonoids possess a potential to prevent neuroinflammation in *in vitro* models, related, at least in part, by iNOS down-regulation[153]. However, only few studies in animal models have been published to demonstrate their activity *in vivo*. Quercetin improved spatial memory performance in old, high-cholesterol-fed mice[154], increased Basso, Beattie and Bresnahan scores and inclined plane test scores in acute spinal injury (SCI) rats[155], improved neuronal count in I/R induced neuronal damage in young and aged rats[156], and reduced dopaminergic neuron damage induced by 6-hydroxydopamine in zebrafish[157]. The neuroprotective effect of rutin on head injury-induced neuronal behavioural, biochemical, and molecular alterations was potentiated by NOS inhibitors and prevented by L-arginine[158] suggesting a role for inhibition of iNOS. Luteolin reduced the severity of trauma induced by compression and improved the motor activity in SCI mice, reducing iNOS expression and restoring nNOS expression[159]. The flavone baicalein reduced cerebral infarct area after permanent focal cerebral ischemia[160]. Epicatechin inhibited iNOS expression in brain tissues after doxorubicin-induced brain toxicity[161]. Finally, intrathecal EGCG could produce an antiallodynic effect against spinal nerve ligation-induced neuropathic pain, mediated by blockade of nNOS protein expression and inhibition of the pronociceptive effects of NO[162]. In addition, Kim et al.[103] showed that inhibition of iNOS may be an important mechanism underlying the prevention of MPTP toxicity induced by oral EGCG, which may potentially be a neuroprotective agent against Parkinson's disease.

Sepsis syndromes. The oral administration of polyphenols protects rodents from endotoxemia and microbial sepsis. Under these circumstances, polyphenols attenuate microvascular hypermeability, tissue infiltration by leukocytes, oxidative and nitrosative stress, tissue injury, organ dysfunction, shock and vasoplegia, lactate production, and mortality[163]. The inhibition of NFκB activation and subsequent expression of iNOS by polyphenols is operative in ameliorating the sequelae of sepsis. Of the greater relevance are publications reporting superior survival rates in endotoxemic animals treated with flavonoids (naringin, luteolin, quercetin, baicalein, EGCG). In the majority of investigations, endotoxemia was induced by administrating to rodents a bolus of LPS. Other studies focused the effects of flavonoids in target tissues. For example, oral quercetin preserved vascular function and blood pressure in LPS-treated mice. These protective effects were associated with upregulation of eNOS and down-regulation of iNOS in aorta[164]. As described above, oral quercetin[165], baicalin[166], or naringin[167] treatment in rodents inhibited *ex vivo* iNOS expression induced by LPS in macrophages. Moreover, acute lung injury induced by LPS in mice was reduced by oral hesperidin[168], naringin[169], or icariin[170], which was accompanied by reduced iNOS expression in the lung. Similarly, liver injury induced by endotoxin was improved by oral catechin, quercetin[171], or baicalein[172], by reducing NFκB/iNOS pathway.

Other diseases. As in brain I/R processes were related with iNOS expression and tissues damage. Quercetin reduced myocardial injury induced I/R in rabbit[173], and rutin also protected kidney from I/R injury[174]. Colitis was also associated with increased iNOS expression in colon mucosa and intestinal damage. Naringenin[175], and quercitrin[176] exerted protective effects in DSS-induced murine colitis, reducing iNOS expression.

Effects of flavonoids on NO in humans

Evidence from epidemiological studies and randomised controlled trials support a potential role for some flavonoids in the reduction of risk of cardiovascular disease[2, 5]. Intervention studies on plant-derived food products are particularly complex to interpret, with many limitations: i) inadequate assessment of flavonoid composition of food, ii) limited dose response analysis, and iii) inconsistencies in biomarkers measured across studies. The cardiovascular effects of flavonoids have been evaluated based on a single flavonoid subclass within a food, such as flavan-3-ols in chocolate, anthocyanins in wine and quercetin in onions. However, this approach does not take into account the complex array of flavonoid compounds present within any given food product. Therefore, dietary interventions are often misclassified as providing one flavonoid source over another. Effects on endothelial function, inflammation, platelet function, angiotensin-converting enzyme activity and glucose transport have been involved as potential mechanism in the beneficial effects of flavonoids on cardiovascular risk. However, the relative impact of these mechanisms *in vivo* remains unclear.

Reduced NO bioavailability may contribute to the onset and progression of endothelial dysfunction, including impaired vasodilation and increased adhesive properties, leading to atherosclerosis[8]. Flow-mediated dilatation (FMD) is the standard technique to measure endothelial-derived NO bioavailability in humans. Recently, the relative impact of flavonoid composition, dose and structure on vascular function has been analysed in a systemic review of randomized controlled trials of flavonoid-rich food products[177]. Meta-analyses of combined subclasses showed significant improvements in FMD in both chronic (0.73%) and acute (2.33%) studies. Similar benefits were observed for flavan-3-ol, catechol flavonoids (catechins, quercetin, cyanidin, etc), procyanidins, epicatechin and catechin subgroups. Moreover, this flavonoid bioactivity does not follow a classical linear dose-response association[177].

Additionally, endothelium-derived NO may act to regulate arterial stiffness and wave reflection[178]. In fact, impaired endothelium-dependent vasodilation in human arteries increases pulse wave reflections, and decrease aortic distensibility. However, only few studies have analysed the effects of flavonoids-rich food in these parameters. The acute effect of black and green tea (6 g) on aortic stiffness and wave reflections was assessed in 29 healthy volunteers in a randomized, single-blind, sham-procedure controlled, cross-over design. Both black and green tea increases acutely wave reflections and only black tea increases aortic stiffness[179]. Botden et al.[180] investigated whether polyphenols extracted from red wine (280 and 560 mg) reduce peripheral and central blood pressure in subjects with high-normal blood pressure or grade 1 hypertension, in a double-blind, placebo-controlled three-period crossover trial. After 4-week intervention period neither dose of polyphenol treatment changed office or central blood pressure, aortic augmentation index or pulse wave reflection index. Therefore, the favourable effects of red wine polyphenols on central hemodynamics may only appear in

cardiovascular compromised subjects, such as smokers[181] or coronary heart disease patients[182]. Furthermore, the acute beneficial effects of red wine polyphenols on central hemodynamics may fade away after chronic intake, possibly by counter-regulatory mechanisms. Grassi et al[183] investigated the effects of flavanol-rich dark chocolate (100 g/d) administration for 3 days on FMD and wave reflections after oral glucose tolerance test (OGTT). OGTT causes acute, transient impairment of endothelial function (decreased FMD and increased wave reflection) and oxidative stress, which is attenuated by flavanol-rich dark chocolate. These results suggest cocoa flavanols may contribute to vascular health by reducing the postprandial impairment of arterial function associated with the pathogenesis of atherosclerosis.

Flavonoids are presumed to be the active constituents of flavonoid-rich food. However, to date, there is little direct evidence that flavonoids are the bioactive molecules responsible. For example, Schroeter et al.[184] reported that flavanol-rich cocoa improved FMD in conduit arteries and in microcirculation strongly correlated with the kinetic of increased NO species and (-)-epicatechin and its metabolites in plasma. They also found that oral administration of pure (-)-epicatechin closely emulated the acute vascular effects of flavanol-rich cocoa. The effects of pure (-)-epicatechin on FMD were abolished by NOS inhibition, involving NO in these protective effects. Similarly, flavonoid-rich (184 mg of quercetin, as quercetin glycosides, and 180 mg of (-)-epicatechin, as the aglycone) apple augmented NO status and improve endothelial function in healthy humans[185]. However, Larson et al[186, 187] found that FMD of hypertensive subjects was unaffected by acute quercetin aglycone (1095 mg), despite significant blood pressure reduction, suggesting that this flavonoid does not exert its acute effects by improving endothelial function. In contrast, Loke et al[188] have shown that acute oral administration of pure quercetin (200 mg) augmented NO status (elevation of circulating S-nitrosothiol and nitrite concentration) in healthy men, but not direct measures of vascular reactivity were assessed in that study. Human studies do not clarify if the increased NO status induced by some flavonoids, mainly (-)epicatechin, is the result of increased eNOS activity or enhanced the bioavailability of endothelium-derived NO. In addition, longer duration studies relaxation in healthy human and patients with endothelial dysfunction.

Conclusions

Flavonoids exert complex actions on the synthesis and bioavailability of NO which may result both in enhanced or decreased NO levels (Figure 4): 1) In cell free systems, several flavonoids may scavenge NO via its pro-oxidant properties by increasing O_2^-. However, under conditions of oxidative stress, flavonoids may also protect NO from O_2^- -driven inactivation. 2) In intact healthy tissues, some flavonoids increase eNOS activity in endothelial cells. Paradoxically this effect involves a pro-oxidant effect which results in Ca^{2+}-dependent activation of eNOS. As inhibitors of PI3K, flavonoids may potentially inhibit the PI3K/Akt-dependent activation of eNOS. 3) Under conditions of inflammation and oxidative stress, flavonoids may prevent the inflammatory signalling cascades via inhibition of NFκB and thereby downregulate iNOS. On the other hand, they also prevent the overexpression of ROS generating enzymes, reducing O_2^- and $ONOO^-$ levels, thus preventing O_2^--induced NO inactivation and eNOS uncoupling. It should be noted that many in vitro studies in the past have used very high concentrations of flavonoids that may not be physiologically relevant.

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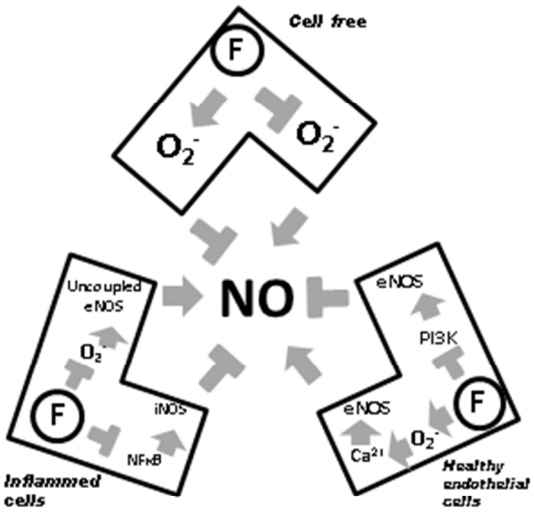

Figure 4. Summary of the effects of flavonoids (F) on NO. See text (conclusions) for an explanation.

Notes and references

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