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Linking biomarkers of oxidative stress and disease with flavonoid consumption: From experimental models to humans

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ABSTRACT

Identification of the links among flavonoid consumption, mitigation of oxidative stress and improvement of disease in humans has significantly advanced in the last decades. This review used (−)-epicatechin (EC) as an example of dietary flavonoids, and inflammation, endothelial dysfunction/hypertension and insulin resistance/diabetes as paradigms of human disease. In these pathologies, oxidative stress is part of their development and/or their perpetuation. Evidence from both, rodent studies and characterization of mechanisms in cell cultures are encouraging and mostly support indirect antioxidant actions of EC and EC metabolites in endothelial dysfunction and insulin resistance. Human studies also show beneficial effects of EC on these pathologies based on biomarkers of disease. However, there is limited available information on oxidative stress biomarkers and flavonoid consumption to allow establishing conclusive associations. The evolving discovery of metabolites that could serve as reliable markers of intake of specific flavonoids constitutes a powerful tool to link flavonoid consumption to disease and prevention of oxidative stress in human populations.

1. Introduction

Humans consume foods from animal and plant origin to obtain nutrients that are essential for development and to sustain life. Foods are also a source of a plethora of small molecules with different relevance for human (animal) biology. Among these molecules are those defined as bioactives, which overall importance for life is recognized but it is not fully understood [1,2]. Plant bioactives include thousands of chemical compounds. This represents an advantage for human health because bioactives can provide a wide spectrum of benefits. On the other hand, this complexity makes it difficult to establish robust and practical relationships among consumption, function, and health effect.

The explosion of research on bioactives as health promoters started in the 80's driven by the assumed relevance of generic "antioxidants" for human health. This explosion was followed by a second wave of research at the beginning of the XXI century, in which the epidemiological associations between fruit and vegetable consumption and health benefits drove great interest on plant bioactives [3]. Although extensive, this research failed in providing definitive associations among plant bioactives, antioxidant actions, and health. Major assumptions behind such failure were: i) to consider by default "antioxidants" as universal health promoters, and "oxidants" as health damaging agents; ii) to unify the concepts of bioactives and antioxidants, both at chemical and functional levels; and iii) to make ambitious and misleading extrapolations from *in vitro* and preclinical studies to human health benefits. During the last decade, research on the mechanisms responsible for flavonoids health benefits incorporated new views on feasible interactions with biological targets, bioactive chemical identity, and actual tissue concentrations [4]. In terms of redox biology, these new views also integrate with the current understanding of redox tone, redox signaling, and oxidative

Abbreviations: BP, blood pressure; DBP, diastolic blood pressure; EC, (−)-epicatechin; Daox, direct antioxidant; eNOS, endothelial nitric oxide synthase; FMD, flow mediated dilation; GI, gastrointestinal; Iaox, indirect antioxidant; MDA, malondialdehyde; NOX, NADPH-oxidase; NOS, nitric oxide synthase; RFM, ring fission metabolites; SREM, structurally-related (−)-epicatechin metabolites; SBP, systolic blood pressure; sNOX2-dp, soluble NADPH-oxidase-2 derived peptide; T2D, type-2 diabetes; VL, valerolactone; γVL, 5-(3′,4′ dihydroxyphenyl)-γ-valerolactone; γVA, 5-(3′,4′ -dihydroxyphenyl)-γ -hydroxyvaleric acid.

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stress [5,6].

In this review, we will discuss the relevance of flavonoids interfering with redox reactions that could define their potential bioactivities, and the links among biomarkers of oxidative stress, flavonoids (consumption) and health. Our discussion will center on (−)-epicatechin (EC), focusing on mechanisms that can also apply to other members of the large group of flavonoid compounds. We will analyze the current knowledge based on the effects of EC on redox biology, including antioxidant capabilities that can affect oxidative stress biomarkers in inflammation and two pathological conditions, i.e. hypertension and type 2 diabetes (T2D). EC (and flavonoids) effects will only be discussed for studies using: i) amounts provided through food consumption and rational supplementation in humans, and ii) concentrations compatible with those found in plasma and tissues when assayed in *in vitro* systems.

2. Flavonoids as antioxidants

Flavonoids are plant (poly)phenols with a basic chemical structure of two phenolic rings (A and B) and one heterocyclic ring (C) (EC is shown as a flavonoid example in Fig. 1). The basic structure is modified to define hundreds of compounds, which characteristics have been published elsewhere [7]. In terms of antioxidant reactions, hydroxyl groups in ring B make flavonoids excellent free radical scavengers [8]. Certainly, their reduction potential in biological milieus favors the abstraction of one electron from the hydroxyl group by an oxidant radical and the stabilization of the resulting free radical by resonance in the B phenolic ring and sometimes in the contiguous heterocyclic ring [8]. Flavonoid reduction potential ranges between −300 and −500 mV, values that are similar to those of other substances accepted to be physiological free radical scavengers able to act as direct antioxidants (Daox), i.e. alpha-tocopherol and ascorbate [9]. Then, based on thermodynamic analysis, flavonoids could act as Daox in biological systems. The same rationale, in terms of thermodynamic properties, applies for flavonoids as metal chelators preventing, for example, iron or copper-mediated hydroxyl radical production [10–12]. However, the concentration of Daox at the reaction site is what defines the rate at which the free radical scavenging reaction will occur, and consequently, the impact on the biological system. The high concentration of flavonoids occurring after food consumption makes feasible their activity as Daox at the gastrointestinal (GI) tract (Figs. 1 and 2). In contrast, in blood plasma the Daox reaction rate for flavonoids is more than one order of magnitude below that of ascorbic acid, making their *in vivo* Daox activity practically negligible [11–13]. Thus, changes observed in oxidative stress parameters measured in experimental animals and humans after the consumption of flavonoids cannot be in general explained by their capacity to act as Daox, but more likely as indirect antioxidant (Iaox). Being Iaox activities based on mechanisms more specific than Daox, the amount of compound necessary for an antioxidant action is lower, and often compatible with the minute concentrations reached by flavonoids (and other (poly)phenols) in organs/tissues other than the GI tract (Fig. 2).

These Iaox actions, which will be further discussed in sections 4 and 5, include interactions with: i) enzymes involved in oxidant production, e.g. NADPH oxidases (NOXs); ii) membrane receptors and/or membrane elements, e.g. lipid-rafts, involved in redox signaling; iii) intracellular receptors, transcription factors and/or enzymes not direct involved in oxidant production but in redox signaling; and iv) modulators of ions, e.g. calcium, and iron or substances, e.g. NAD(P)H and GSH, involved in maintaining the physiological redox tone.

3. Flavonoid metabolism

3.1. Flavonoid metabolism and its relevance to antioxidant mechanisms

Flavonoids consumed both, in plant-derived foods or in dietary supplements, are extensively metabolized before reaching the organs in

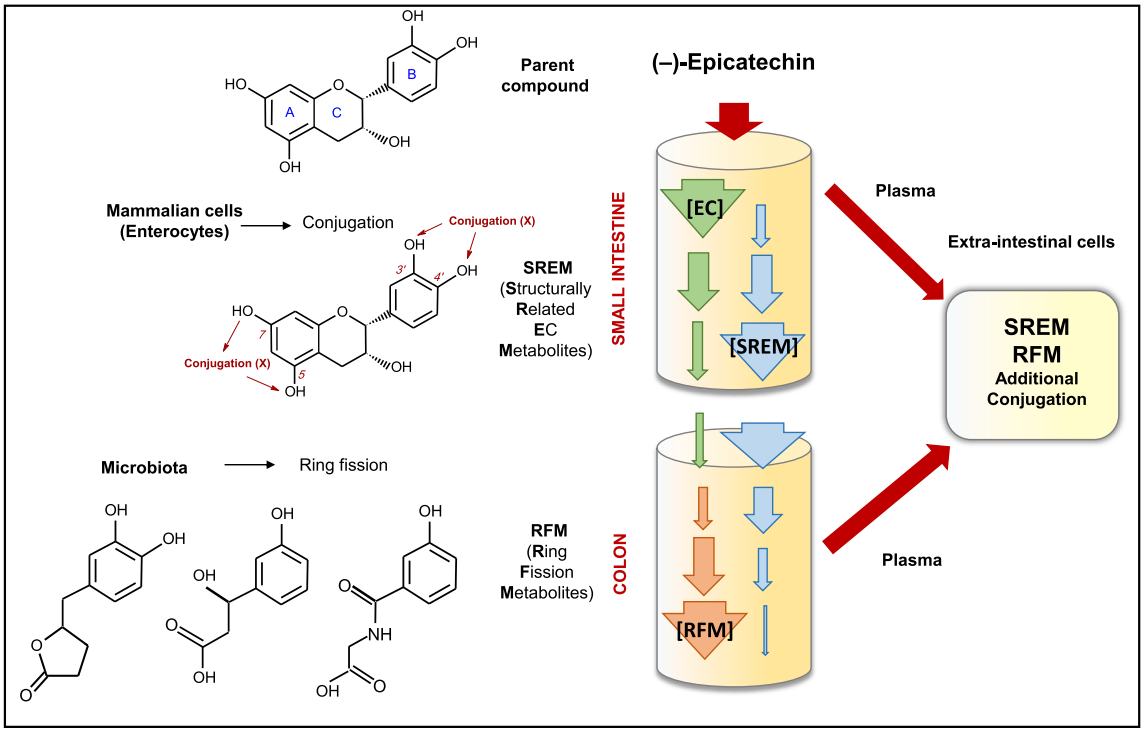

Fig. 1. (−)-Epicatechin metabolism. After ingestion (−)-epicatechin (EC) reaches the small intestine as the parent compound. At the enterocyte, EC is conjugated and either transported into the plasma or to the intestinal lumen. In this metabolic step, the 3-rings skeleton of the parent compound is maintained, producing structurally related EC metabolites (SREM). Subsequently, EC and SREM are transformed by colonic microbiota that through fission of the C ring produce ring fission metabolites (RFM). Once transported into the circulation, EC, SREM, and RFM can undergo additional conjugation in extra-intestinal tissues before exerting a biological action.

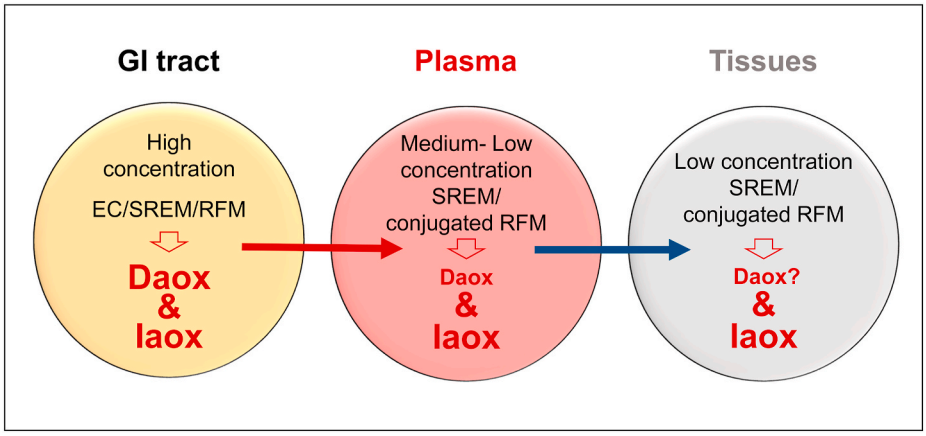

Fig. 2. Fate of flavonoids and its impact on their antioxidant actions in different body compartments. Metabolites derived from (−)-epicatechin (EC), as an example of the general fate of flavonoids upon ingestion, are dissimilarly distributed in the different body compartments (gastrointestinal -GI-tract, plasma, and tissues). The chemical characteristics and the concentration of a specific metabolite in each body compartment will define the mechanism of action as direct (Daox) or indirect (Iaox) antioxidant. Daox actions involve free radical scavenging and/or metal chelation, and Iaox actions include downregulation of oxidant production and/or upregulation of antioxidant defenses. Letter size indicates the probability of each mechanism to occur. SREM, structurally related EC metabolites, RFM, ring fission metabolites.

which they could exert their bioactivities. Thus, the parent compound is not usually the chemical structure interacting with the biological target. Identification of the compounds and quantities that would reach targets is critical to define Iaox or Daox activity.

Both flavonoids Daox and Iaox actions will be defined by the chemical structure interacting with the biological target. Focusing on EC, both parent EC molecule and EC-containing proanthocyanidins are present in large amounts at the GI tract after consumption of many fruits and vegetables [14,15]. EC metabolism in humans was recently reviewed [16]. Briefly, after consumption, EC is metabolized into structurally related EC metabolites (SREM) at proximal small intestine enterocytes and subsequently either transported into the plasma or to the intestinal lumen [17] (Fig. 1). The major SREM are phase II metabolites products, mainly sulfated, methylated and glucuronidated compounds which are well absorbed. Once in the circulation, EC and SREM are further metabolized in the liver by phase II enzymes. Maximum blood concentrations of EC and SREM are in the upper nM range and are reached within 2–3 h post EC-containing food intake [18,19], or 1 h post pure EC consumption [20].

Non-absorbed EC and SREM reach the colon where they are catabolized by the microbiota to ring fission metabolites (RFM) (Fig. 1). The major resulting RFM are 5C-ring fission valerolactones (VL), mainly 5-(3′,4′ dihydroxyphenyl)-[gamma]-valerolactone (γVL) and 5-(3′,4′ -dihydroxyphenyl)-γ -hydroxyvaleric acid (γVA). These catabolites are absorbed and further metabolized by phase II enzymes in the liver yielding sulfated, methylated and/or glucuronidated VL conjugates. Both, non-conjugated and conjugated RFM peak in plasma 6 h post EC consumption [20]. It was reported that 82% of the EC ingested is absorbed, both in rodents and humans, either in the upper intestine or in the colon [20]. In the case of proanthocyanidins, those larger than dimers are not absorbed but are metabolized by the colonic microbiota to small phenolic compounds, including γVL [20–22].]. It is to note that EC-containing proanthocyanidins are not broken to monomers in the GI lumen, as demonstrated through findings that they do not contribute to the pool of circulating EC and SREM [21,23]. Being VL catabolites of different flavonoids they can reach blood concentrations in the micromolar range, amplifying their possibilities for chemical interactions. In terms of biological activity, the question is if this panoply of compounds, from parent compound to VL conjugates, can have common or specific bioactivities. Regarding antioxidant capabilities, if chemical modifications to the parent molecule could disrupt their potential capacity to act as Daox and/or Iaox. Very few studies characterized the bioactivities of SREM and RFM *in vivo* [24] or *in vitro* [25–32]. Of note, the use of metabolites in *in vivo* experiments is limited because of the still difficult access to synthetic or isolated compounds in the amounts necessary to run experiments in animals or humans.

It will be relevant to determine if flavonoids modified by oxidants could constitute biomarkers of oxidant production and/or of oxidative stress. So far, the fact that these products, e.g flavonoid radicals, and flavonoid quinones, were only detected after *in vitro* oxidation of biological or chemical systems [33–37] restricts their value as biomarkers of oxidative stress, especially in humans.

3.2. *Flavonoids metabolism and assessment of human consumption*

In addition to provide information about the possible molecules interacting with the biological targets, the generation of extensive knowledge on flavonoid metabolism has opened the possibility to advance in other challenging areas. One is the concrete evaluation of human flavonoid intake, which is an important point to be considered to assess the impact of flavonoids on health/disease especially in epidemiological studies [38,39]. This evaluation implies the use reliable methods and biomarkers. Major limitations for flavonoid intake assessment are given by: i) constraints in the self-report tools currently available to assess human consumption [40]; ii) the variability in flavonoid content for a particular plant or food which is affected by multiple factors including, breed/cultivar, location/climate of plant growth, agricultural practices, post-harvest storage and processing, etc. [41], and iii) the current limited understanding of individual's metabolism and nutrigenetics.

Rigorous studies were carried out looking for biomarkers of flavonoid intake using strict validation criteria established by regarded agencies, e.g. International Agency for Research on Cancer, and Institute of Medicine (U.S.), including the reliability of the analytical method used to measure the flavonoid/metabolite, correlations between food intake and urinary/blood metabolite concentration, and applicability in large cohort studies. Thus, SREM in urine showed to be a reliable biomarker for EC intake [42] and urinary γVL proved to be a consistent biomarker for the assessment of flavan-3-ols dietary consumption in humans [22]. For example, evaluation of these biomarkers allowed identifying, from a mixture of ingested monomers and EC-containing proanthocyanidins, that EC and SREM, but not VL or the proanthocyanidins, were involved in the beneficial effects of the mixture on parameters of cardiovascular function [21].

4. Flavonoids and redox biology in the pathophysiology of disease: evidence from rodent models and cells

4.1. *(−)-Epicatechin and inflammation*

Inflammation is a physiological response of the immune system to pathogens and cell stress signals [43]. In terms of redox biology, immune cells generate oxidants as part of the physiological mechanism of body's inflammatory protective responses [44]. In addition, most animal cells can activate redox-sensitive pathways, e.g. the activation of NF-κB and/or mitogen activated kinases (MAPKs) to signal for immune

cells recruitment and/or activation [45]. These signals promote the transcription of proinflammatory cytokines, which amplify the inflammatory response, and of proteins involved in oxidant production, e.g. NOXs, and nitric oxide synthases (NOSs). While chronic inflammation is associated to oxidative stress, chronic oxidative stress can also lead to inflammation; however, most studies do not allow establishing the order in which these events occur. Many flavonoids have been shown to decrease both oxidative stress and inflammation [4,46,47]. EC exert anti-inflammatory actions in humans and experimental animals through the regulation of oxidant production and modulation of redox-sensitive signaling pathways [4,48]. These activities and the associated changes in oxidative stress biomarkers are described in sections 4.2 and 4.3, in the context of EC capacity to regulate endothelial function and improve insulin sensitivity in rodents and/or cell cultures.

4.2. (−)-Epicatechin: redox signaling and oxidative stress in endothelial dysfunction and hypertension

Nitric oxide (NO) plays key functions in the vasculature, not only through the increase of vasodilation, but also through the promotion of angiogenesis, and the inhibition of thrombosis [49–51]. NO is generated from L-arginine in a reaction catalyzed by NOSs, and specifically by the endothelial isoform (eNOS) in the vascular endothelium [52]. Sufficient NO bioavailability is associated with normal vasodilation and consequently, normal blood pressure (BP), while decreased NO generation or accelerated NO consumption lead to hypertension [53,54]. To regulate BP, NO reacts with guanylate cyclase at smooth muscle cells, activating cGMP-dependent vasorelaxation. Additionally, the reaction between NO and $O_2^{\cdot-}$ has a very high reaction constant (near diffusion-controlled rate) generating peroxynitrite ($ONOO^-$), with the consequent reduction of NO bioavailability [55]. Thus, an increased production of $O_2^{\cdot-}$ through either the upregulation of NOXs, the most important source of $O_2^{\cdot-}$ in the vascular environment, and/or through the uncoupling of eNOS or mitochondrial dysfunction, can compromise NO bioavailability [56]. Thus, while NO is essential in sustaining normal BP, increased oxidant production contributes to the pathogenesis and/or maintenance of endothelial dysfunction and hypertension.

Studies in animal models of hypertension have provided relevant mechanistic information on the capacity of EC to restore adequate NO bioavailability through eNOS upregulation [57–63] and/or NOXs downregulation in the vascular wall [57–60]. These effects were associated with a decrease of oxidative stress biomarkers including: i) MDA [57,58], GSSG/GSH ratio [57], and nitrotyrosine [61] in plasma/serum; ii) F_2-isoprostanes in urine [58]; and iii) Nrf2 mRNA and protein expression in aorta [58].

Additional studies in animals and in cultured cells, allowed exploring the mechanisms involved in EC modulation of NO bioavailability. Regarding NO production, EC exerted several effects at a post-translational level by: i) increasing the activating eNOS phosphorylation at Ser-1177 and Ser-633; ii) decreasing the inhibitory eNOS dephosphorylation at Thr-495 [64]; and iii) downregulating the eNOS inhibitor protein caveolin-1 [60,64]. Additionally, EC can favor NO production by inhibiting the enzyme arginase, which activity competes with eNOS for L-arginine [30,65,66]. In terms of normalizing hypertension-dependent $O_2^{\cdot-}$ overproduction, EC and/or select EC metabolites could act: i) mitigating the overexpression of different NOX subunits, as observed in hypertensive rodents [57,58,60], ii) inhibiting NOX activity through a mechanism that does not involve a direct scavenging reaction of EC with $O_2^{\cdot-}$ [31,32] and iii) decreasing mitochondrial $O_2^{\cdot-}$ production in vascular endothelial cells [67]. Additionally, EC was shown to inhibit other pathways associated with oxidant production, e.g. those regulated by angiotensin II [68].

Overall, EC regulates endothelial function in experimental hypertension rodent models through its capacity to modulate NO bioavailability via the regulation of oxidant production, which ultimately reflects in improvements of biomarkers of oxidative stress. Other flavonoids have shown actions modulating NO bioavailability in cultured cells, ex-vivo experiments on aorta isolated rings, or in experimental models of hypertension in animals, particularly quercetin [69–73].

4.3. (−)-Epicatechin: redox signaling and oxidative stress in insulin resistance and type 2 diabetes

Inflammation, oxidative stress and deregulation of redox-sensitive signaling are major mechanisms involved in the pathogenesis of insulin resistance and T2D [74,75]. Mechanistically, a pro-oxidant environment leads to the activation of redox-sensitive kinases, e.g. c-Jun N-terminal kinase 1/2 (JNK) [76,77], inhibitor of nuclear factor κB (IκB) kinase (IKK) [78]), and protein kinase C (PKC) [74,79]. All these kinases act inhibiting the insulin cascade by phosphorylating insulin receptor 1 (IRS-1) at inhibitory serine residues. IKK/NF-κB activation also increases the expression, among other proteins, of: i) protein tyrosine phosphatase 1B (PTP1B), which cleaves activating tyrosine phosphate groups in both the insulin receptor and insulin receptor substrate 1, resulting in the inhibition of the insulin pathway [80]; ii) $O_2^{\cdot-}$- [81] and NO- [82] generating enzymes, and iii) pro-inflammatory cytokines that fuel inflammation, oxidative stress, and insulin resistance. Within the context of overnutrition and obesity, mitochondria dysfunction and endoplasmic reticulum stress further contribute to oxidative stress leading to insulin resistance and T2D [83].

As we previously reviewed [83,84], consumption of EC or EC-rich foods is associated with improvements in glucose tolerance and insulin sensitivity. While these effects have been observed in humans and rodents, the potential mechanisms of EC actions were mostly studied in rodents with diet (high fat and/or high fructose)-induced insulin resistance and T2D [85–90]. The capacity of EC to improve insulin sensitivity in these rodent models was associated to its capacity to mitigate tissue inflammation, oxidative and endoplasmic reticulum stress. Thus, in rats fed a high fructose diet [85] or mice fed a high fat diet [87,91], EC improved liver and adipose tissue responses to insulin. These effects were associated with EC-mediated NOX downregulation and decreases in biomarkers of tissue oxidative stress, i.e. protein carbonylation and 4-hydroxynonenal-protein adducts. As a proof of concept, *in vitro* studies showed that both EC and SREM, at biologically relevant concentrations, inhibited palmitate-induced inflammation, oxidant production, NOX increased expression and activation, protein/lipid oxidation (protein carbonyls, 4-hydroxynonenal-protein adducts), JNK and IKK activation and insulin resistance in HepG2 cells [28]. Furthermore, mitochondria dysfunction, often cause of increased oxidant production, is involved in the pathogenesis of insulin resistance [92,93]. Several studies in rodents have shown that EC improves the function and promotes the biogenesis of mitochondria in different tissues [86,90,94,95].

Overall, EC and its metabolites have beneficial effects on insulin resistance and T2D, which is frequently associated with the modulation of redox-sensitive signaling and improvements in biomarkers of oxidative stress. While there is evidence on a potential capacity of other flavonoids to improve insulin resistance in rodents and cell models (reviewed in Refs. [96,97]), very few studies have linked this action to changes in redox tone and improvements in biomarkers of oxidative stress [98–102].

4.4. Conclusions from studies in rodents and cells in culture

As described above, a large body of evidence supports the anti-inflammatory activity of EC and other flavonoids [4,46,47], which is highly relevant given the multiple diseases that have inflammation as a pathogenic factor. The intrinsic association of inflammation with oxidative stress suggest a direct link between flavonoid anti-inflammatory and mostly Iaox activities. Findings in rodents and in *in vitro* models provide indication of an improvement in biomarkers of oxidative damage to proteins and lipids by flavonoids and/or their

Table 1
Disease biomarkers of vascular function and glucose homeostasis and oxidative stress biomarkers after dietary consumption of EC or EC-rich foods and beverages.

EC source[a]	Individuals	Disease biomarkers	Oxidative stress biomarkers	References
Vascular function				
Chocolate[b]	Healthy	↑FMD=BP	= LDL oxidation resistance, plasma F_2-isoprostanes	[103]
Chocolate[c]	Healthy	↓diastolic and mean BP	= MDA, oxo[h]dG, vitamin E, urate, lycopene, coenzyme Q10	[106]
Chocolate[d]	Healthy	↑FMD	= MDA	[105]
Chocolate[e]	Healthy	↑FMD	↓serum F_2-isoprostanes	[104]
Cocoa beverage[f]	Smokers	↑FMD	= MDA, ascorbate, urate	[108]
Chocolate[g]	Heart transplant recipients	↑coronary vascular function	↓serum F_2-isoprostanes	[112]
Chocolate[h]	Pre-hypertensive/hypertensive stage 1	↓diastolic and systolic BP	= plasma F_2-isoprostanes	[111]
Chocolate[i]	Smokers	↑FMD	↓urinary F_2-isoprostanes, sNOX2dp	[107]
Chocolate[i]	Peripheral artery disease	↑FMD	↓serum F_2-isoprostanes, sNOX2dp	[109]
Chocolate[i]	NASH	↑FMD	↓serum F_2-isoprostanes, sNOX2dp	[110]
Glucose homeostasis				
Chocolate + cocoa beverage[j]	T2D or heart failure	= Hba1c	↓muscle protein carbonyls, nitrotyrosines, GSH	[117]
Cocoa beverage[k]	Obese (35% insulin resistant)	= OGTT, QUICKI, ISI	↓plasma F_2-isoprostanes	[118]
Cocoa beverage[l]	Healthy	↓glycaemia	↓MDA, serum protein carbonyls	[119]
Pure compound[m]	Metabolic syndrome	= glycaemia, HOMA-IR	= oxidized LDL, vitamin C, vitamin E	[120]

↓,↑, = , indicates changes respect to control individuals or pre-treatment patients.
EC: (−)-epicatechin, NASH: non-alcoholic steatohepatitis, T2D: type 2 diabetes, FMD: flow mediated dilation, BP: blood pressure, Hba1c: glycosylated hemoglobin, OGTT: oral glucose tolerance test, QUICKI: quantitative insulin sensitivity check index, ISI: insulin sensitivity index, HOMA-IR: homeostasis model assessment of insulin resistance, LDL: low density lipoprotein, Oxo[h]dG: (8-oxo-7,8-dihydro-2′-deoxyguanosine, sNOX2-dp: soluble NOX2 derived peptide, GSH: reduced glutathione, MDA: malondiadehyde; E-PAC: EC related proanthocyanidins.
[a] Detailed quantities correspond to the single or daily amount administered.
[b] 46 mg EC + 215 mg E-PAC.
[c] 39 mg EC + catechin, and 126 mg E-PAC.
[d] 540 mg EC + EC dimers, and 760 mg others E-PAC.
[e] 447 mg EC + 59 mg catechin, and 14 mg quercetin.
[f] 177 mg EC + 45 mg catechin, and 696 mg E-PAC.
[g] 36 mg EC + 10.8 mg catechin.
[h] 5.1 mg EC +1.7 mg catechin, and 21.2 mg E-PAC.
[i] Amount present in 40 g, >85% cocoa.
[j] 100 mg EC.
[k] 34/72/184 mg EC + 10/24/72 mg catechin, and 130/260/676 mg E-PAC.
[l] 25 mg EC + 154 mg E-PAC.
[m] 25 mg EC.

metabolites. While measurements of oxidant production in cells and tissues have significant intrinsic limitations, the literature is consistent in showing that flavonoids affect biomarkers of oxidative stress, decreasing both oxidation of cell components and generation of oxidants. Very importantly in terms of a physiologically relevant extrapolation of rodent/*in vitro* results to humans, most of the findings described in this review for parent EC or SREM were obtained using concentrations compatible with those that can be reached in tissues. This makes the described results of great value as hypothesis-generating sources.

5. Flavonoids and redox biology in the pathophysiology of disease: evidence from human studies

Numerous human studies have provided experimental evidence supporting the beneficial effects of EC or EC-rich foods/beverages improving endothelial dysfunction and/or hypertension. On the other hand, the characterization of the effects of EC and EC-rich foods/beverages on insulin resistance and T2D is less robust. In sections 5.1 and 5.2, we will discuss the links among those pathologies, biomarkers of oxidative stressand EC consumption in humans.

5.1. (−)-Epicatechin and endothelial dysfunction/hypertension: human studies

Table 1 depicts human studies that investigated the relationships between consumption of EC or EC-rich foods/beverages and changes in biomarkers of oxidative stress and endothelial dysfunction or BP regulation. This information was analyzed separating the trials according to the study groups, i.e. healthy individuals or individuals at risk of or with established endothelial dysfunction. In healthy individuals, EC consumption increased flow-mediated dilation (FMD) regardless the amount of EC, and the duration of the treatment [103–105]. In parallel, daily intake for 2 w resulted in a decrease in diastolic and mean BP [106]. In these studies, a variety of oxidative stress biomarkers was measured: LDL oxidation resistance, F_2-isoprostanes, malondialdehyde (MDA), vitamin E, ascorbate, urate, lycopene, and coenzyme Q10. No robust correlations were observed between the beneficial effects of EC/EC-rich foods/beverages on endothelial dysfunction or BP endpoints, and biomarkers of oxidative stress, with exception for a decrease in serum F_2-isoprostanes in one study [104].

In different conditions associated with impaired FMD, normalization of dilation values was consistently observed upon EC supplementation. In smokers, both acute [107] and 3-d [108] EC administration as cocoa drinks improved FMD when measured 2 h post intake. FMD was also normalized in subjects with peripheral artery disease after acute EC administration [109], and in subjects with non-alcoholic steatohepatitis after 2 w of daily supplementation [110]. In pre-hypertensive and hypertensive stage 1 patients, consumption of EC daily for 18 w resulted in decreased SBP and DBP [111]. Finally, heart transplant recipients subjected to a single oral administration of EC showed an improvement in coronary vasomotion 2 h post-consumption [112]. In the above studies, a variety of oxidative stress biomarkers were assayedi.e., MDA, ascorbate, urate, F_2-isoprostanes, and a soluble NOX2 derived peptide (sNOX2-dp). In 4 of the described 6 studies [107,109,110,112], changes in oxidative stress biomarkers positively correlated with changes in vascular parameters. Overall, the concentration of plasma/urine

F_2-isoprostanes showed consistent associations with EC-mediated improvements of endothelial function. Blood levels of soluble sNOX2-dp also showed positive associations with health parameters, however this is a determination that has not been yet validated as an oxidative stress biomarker. Importantly, plasma total antioxidant capacity, measured in studies using different strategies, was not considered in our analysis given that it presents major limitations to be a reliable biomarker of oxidative stress [113,114].

5.2. (−)-Epicatechin and insulin resistance and type-2 diabetes: human studies

As recently reviewed [83], consumption of EC or EC-containing foods/beverages improves parameters of insulin sensitivity and glucose homeostasis in humans. While several studies used cocoa as a source of EC, only a few investigated the effects of pure EC [115,116,120]. Additionally, even fewer studies linked EC consumption with parameters of oxidative stress and insulin sensitivity in humans (Table 1).

A small study characterized the effects of dark chocolate supplementation (100 mg EC/day) for 3 mo in five individuals with T2D or heart failure [117]. In muscle, these subjects showed high levels of protein carbonyl and nitrotyrosine residues and low GSH. After cocoa supplementation, these three biomarkers improved, while no changes in glycosylated hemoglobin (Hba1c) were observed. Given that, not all participants were diabetic and that, they all continued with their medications during the study, it is not possible to extrapolate a change in biomarkers of oxidative stress with T2D status. In a short-term (5 d) supplementation of obese individuals with cocoa containing 30–400 mg EC/d, the highest dose significantly decreased plasma F_2-isoprostanes levels during a glucose tolerance test [118]. Only 35% of the studied individuals showed evidence of insulin resistance which was not improved by cocoa consumption. Daily supplementation of older individuals with an EC-rich beverage for 12 w was associated with improvements of glycemia and lower plasma carbonyls and MDA [119]. On the other hand, a crossover study including individuals with metabolic syndrome and supplemented for 2 w with either placebo or a low amount (25 mg) of daily pure EC, did not show changes in parameters of glucose control. Although biomarkers of oxidative stress were not specifically evaluated, plasma levels of oxidized LDL and levels of vitamins C and E remained unchanged [120].

Overall, insulin resistance and T2D are characterized by altered tissue redox regulation and increases in biomarkers of oxidative stress. However, the limited evidence on the effects of EC and EC-rich foods on parameters of oxidative stress and improvements in glucose homeostasis does not allow establishing a potential link between them.

5.3. Human studies on flavonoids and oxidative stress markers: the current scenario

A robust identification of the links among flavonoids, oxidative stress, and disease is difficult. Human studies described above are very different in multiple aspects, e.g. characteristics of participating subjects, EC sources and amounts, and study duration. Additionally, the redox biology field lacks consensus on the reliability and definitive value of the oxidative stress biomarkers currently used. Even when the analysis of those concepts are beyond the scope of this review, it is worth to note that they are under in-depth discussion [121–124]. Progress in this area of research is essential for the comprehension of the links between biomarkers of oxidative stress and disease in association with the consumption of flavonoids and other bioactives.

6. Conclusions

Research is steadily advancing at establishing links among flavonoid consumption, reduction of oxidative stress, and mitigation of disease. This review used EC and EC-rich foods as an example of dietary flavonoids, and endothelial dysfunction/hypertension and insulin resistance/T2D as paradigms of diseases in which oxidative stress contribute to their development and/or perpetuation. Establishing links among flavonoid consumption, mitigation of oxidative stress and improvement of disease faces numerous challenges. Firstly, oxidative stress is not always evaluated with reliable and well-accepted biomarkers. Secondly, while studies in rodents and mechanistic studies in cells are encouraging and support Iaox actions of flavonoids/metabolites in endothelial dysfunction and insulin resistance, results from human studies are less conclusive. Thirdly, understanding the association between consumed flavonoids and their health effects is complex: i) foods are sources of multiple flavonoids, which can affect a biological target either as individual bioactives, or as family of compounds; and ii) flavonoid extensive metabolism leads to multiple potentially active molecules. On a very promising aspect, the evolving discovery of metabolites that could serve as reliable markers of intake of specific flavonoids constitutes a powerful tool to link the consumption of flavonoid to a better health, through the positive maintenance of redox tone, and canceling undesirable oxidative stress.

Author contributions

All authors participated in writing and editing the review.

Declaration of competing interest

Authors have no conflict of interest to declare.

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