Molecular Aspects of Medicine 61 (2018) 31–40

Contents lists available at ScienceDirect

Molecular Aspects of Medicine

journal homepage: www.elsevier.com/locate/mam

Plant bioactives and redox signaling: (−)-Epicatechin as a paradigm

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ARTICLE INFO

Article history:
Received 4 January 2018
Received in revised form
25 January 2018
Accepted 25 January 2018
Available online 10 February 2018

Keywords:
Flavonoid
Oxygen radicals
Antioxidants
Polyphenols
Superoxide
Nitric oxide
NADPH-oxidase
NF-κB
Nrf2

ABSTRACT

Polyphenols are bioactives claimed to be responsible for some of the health benefits provided by fruit and vegetables. It is currently accepted that the bioactivities of polyphenols can be mostly ascribed to their interactions with proteins and lipids. Such interactions can affect cell oxidant production and cell signaling, and explain in part the ability of polyphenols to promote health. EC can modulate redox sensitive signaling by: i) defining the extent of oxidant levels that can modify cell signaling, function, and fate, e.g. regulating enzymes that generate superoxide, hydrogen peroxide and nitric oxide; or ii) regulating the activation of transcription factors sensible to oxidants. The latter includes the regulation of the nuclear factor E2-related factor 2 (Nfr2) pathway, which in turn can promote the synthesis of antioxidant defenses, and of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway, which mediates the expression of oxidants generating enzymes, as well as proteins not involved in redox reactions. In summary, a significant amount of data vindicates the participation of EC in redox regulated signaling pathways. Progress in the understanding of the molecular mechanisms involved in EC biological actions will help to define recommendations in terms of which fruit and vegetables are healthier and the amounts necessary to provide health effects.

1. Introduction

The human diet is highly based on foods of plant origin. Plants contain a number of nutrients that animals cannot synthesize and thus have to incorporate from the diet. Examples of these chemicals are vitamins such as vitamin A, vitamin E, and in certain mammals, vitamin C. Extending the list of chemicals providing benefits for animal biology are those called plant bioactives. The term bioactive, encompasses many different chemical species that are not essential nutrients but can target a plethora of animal cell components (Lupton et al., 2014). Following the observed association between the consumption of fruits and vegetables and human health (Liu, 2013; Aune et al., 2017; Fulton et al., 2016; Hartley et al., 2013; Appel et al., 1997), plant bioactives emerge as molecules that complement the actions of nutrients.

Polyphenols are bioactives claimed to be responsible for some of the health benefits of fruit and vegetables (Herrera et al., 2009; Hertog et al., 1993; McCullough et al., 2012; Buijsse et al., 2006; Curtis et al., 2012; Hooper et al., 2008). For decades, their positive effects on health were mostly explained considering polyphenol antioxidant capacity, based on free radical scavenging reactions. However, it is currently accepted that the bioactivities of polyphenols can be mostly ascribed to their interactions with proteins and lipids. Such interactions can affect cell oxidant production and cell signaling, and explain the ability of polyphenols to promote health.

For practical reasons, this review will focus on a single polyphenol, the flavonoid (−)-epicatechin (EC) (Fig. 1). EC and EC-containing foods were used in several clinical trials and have shown to mostly affect NO metabolism and protect from metabolic disorders (Dower et al., 2015; Rassaf et al., 2016; Gasper et al., 2014; Ramirez-Sanchez et al., 2013; Sansone et al., 2017). The effects of EC (as a parent compound), EC oligomers (procyanidins), and EC metabolites (ECm) will be discussed considering their potential sites of action. ECm are compounds derived from EC and procyanidins after ingestion and metabolism. The information available on galloylated derivatives of EC, e.g. (−)-epicatechin gallate, (-)-epigallocatechin, and (-)-epigallocatechin gallate will not be discussed in this paper.

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https://doi.org/10.1016/j.mam.2018.01.007

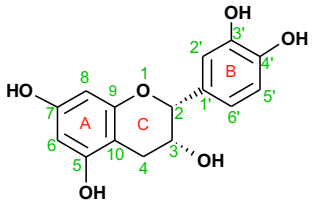

Fig. 1. Chemical structure of (-)-epicatechin.

2. (−)-Epicatechin metabolism in mammals: overview

EC has the C6-C3-C6 flavonoid chemical structure which consists of two aromatic rings (A and B) linked by a three-carbon chain, forming an oxygenated heterocycle (ring C) (Fig. 1). The absorption, distribution, metabolism, and excretion (ADME) of EC in mammals have been extensively described before (Crozier et al., 2010; Ottaviani et al., 2016; Beekmann et al., 2012; Redan et al., 2017; Borges et al., 2017; Ottaviani et al., 2015; Barnett et al., 2015; Rodriguez-Mateos et al., 2015; Actis-Goretta et al., 2012, 2013). Briefly, in terms of metabolism, once ingested EC can: i) exert effects at the gastrointestinal tract as the parent compound; ii) be absorbed and conjugated by phase II enzymes, e.g. to (−)-epicatechin-3′-O-glucuronide, 3′-O-methyl-(−)-epicatechin-5-sulfate and (−)-epicatechin-3′-sulfate; and iii) be metabolized by the microbiota to conjugated compounds or to smaller molecules (ring fission products, e.g. γ-valerolactone metabolites) that can be absorbed, enter the circulation and reach distal organs (Ottaviani et al., 2016; Williamson and Clifford, 2017).

Procyanidins, especially those larger than dimers, are not absorbed but can be metabolized in the intestine by the microbiota. In addition, EC, ECm and procyanidins can change microbiota profiles (Oteiza et al., 2018). In summary, the early interactions of EC at the gastrointestinal tract are mostly related to the parent compound; while in other tissues most effects are mainly ascribed to ECm, which bioactivity may or may not be different from that of EC.

3. Oxidants, antioxidants, and (−)-epicatechin

After the discovery of superoxide dismutase in the late sixties (McCord and Fridovich, 1969) and the subsequent findings of a relationship between oxygen radical production and disease, the relevance of molecules able of trap radicals, defined as antioxidants, grew steadily as potential agents for preventing or cure diseases. The “free radical field” evolved and now it is accepted that most of the oxidant/antioxidant reactions in cells define changes in redox signaling that ultimately trigger regulatory events (Fraga, 2007; Sies, 2017; Azzi, 2017). Only when the oxidant production is out of control, it can lead to irreversible cell damage. The changes in redox signaling involves reactions leading to phosphorylations and dephosphorylations, sulfide oxidation, and NADPH oxidation, among many others. This is different from the previous concept that oxidative stress (imbalance between oxidants and antioxidants) irreversibly damages cell components, and that protection by free radical trapping (direct) antioxidants, would be of benefit to preserve cell survival/function. Thus, cell oxidant production, depending on qualitative and quantitative conditions, can generate either reversible changes in cell functions (redox signaling), or irreversible cell damage (Sies, 2017).

Considering its chemical characteristics, EC, as many other plant phenolics, is able to trap oxygen-derived free radicals in thermodynamically-favored reactions (Fraga, 2007; Galleano et al., 2010b; Fraga et al., 2010). However, given the limited bioavailability and extensive metabolism in most organs and tissues (Rein et al., 2000; Wang et al., 2000; Holt et al., 2002), the direct antioxidant actions of EC and ECm are limited to tissues exposed to high amounts of EC, e.g. the gastrointestinal tract after EC ingestion (Galleano et al., 2010b). In this manner, both oxidants present in foods, and oxidants produced by intestinal epithelial cells, could be scavenged by EC, reducing the uptake of oxidized toxins and/or mitigating the oxidative damage/dysfunction of mucosal cells (Oteiza et al., 2018).

On the other hand, the presence of hydroxyl groups and double bonds in the EC molecule would allow interactions with proteins and lipids, mostly structural lipids forming membranes. These interactions can define the oxidant/antioxidant status of the cell and the activation of redox signaling (Fraga and Oteiza, 2011; Fraga, 2007; Fraga et al., 2010).

4. Redox cell signaling: overview

Regulation of redox cell signaling can be understood considering two different situations; i) the presence of high oxidant levels that can modify cell signaling, structure and function; or ii) stimuli that commit cells to generate oxidants both as a regulatory event and/or as a damaging (oxidizing) event (Winterbourn, 2015; Winterbourn and Hampton, 2008). Under certain conditions, cells can increase oxidant production, usually generating superoxide, which leads to lipid and sulfhydryl oxidation, and related redox events. This can trigger responses related or unrelated to free radical reactions. One example is the activation by oxidants of transcription nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) leading to a higher production of cytokines. On the other hand, many non-redox regulatory events, e.g. angiotensin II or insulin binding to their respective receptors, prompt the production of oxidants via NADPH-oxidase (NOX) activation (Nguyen Dinh Cat et al., 2013; Besse-Patin and Estall, 2014). While this is a regulatory mechanism, if out of control it can lead to cell damage.

In addition, the regulation of redox signaling can occur through: i) generic effects, e.g. alterations of membrane physical properties, which can in turn affect the activity of signaling proteins, or the regulation of oxidant production that secondarily modulates a number of redox sensitive responses; and/or ii) specific interactions, with particular components of a signaling cascade, e.g. receptors, transcription factors, and enzymes, that lead to the regulation of the pathway.

The following sections will focus on different mechanisms by which EC and ECm can protect biological systems from alterations in redox signaling. Evidence will be presented showing that EC and ECm can either reduce oxidant production or inhibit non-oxidative pathways leading to oxidant production through generic or specific mechanisms.

5. (−)-Epicatechin and the production of superoxide and hydrogen peroxide

Superoxide and hydrogen peroxide are products of the partial reduction of oxygen, and their biological production is mainly the result of enzymatic reactions by oxidoreductases. Among these enzymes, NADPH oxidases (NOX) appear as the most sensitive to be activated and the ones able to respond to different stimuli generating amounts of oxidants that can reach levels toxic for cells (Sies, 2017). Additionally, several members of the mitochondrial respiratory chain are sources of superoxide. However, the tight regulation of mitochondria respiration, and the difficult access of many stimuli to mitochondria, would make such source quantitatively less important when cells are challenged.

Additionally, more than thirty enzymes are putative sources of

superoxide and hydrogen peroxide, being the physiologically most important xanthine oxidase and cytochrome P450 oxidases (Sies, 2017; Go et al., 2015). The associations between EC and ECm with these sources of superoxide and hydrogen peroxide will be discussed in the following sections.

5.1. NADPH-oxidases

NOXs catalyze the transfer of electrons from NADPH to oxygen to produce superoxide (Brandes et al., 2014; Schröder et al., 2017). Originally described as enzymes mediating neutrophil bactericidal actions, today it is accepted that NOXs are ubiquitous in most animal cells, and are activated upon the interaction of an ample and heterogeneous number of stimuli with their target receptors. The most important NOX isoforms which modulation was associated with EC consumption are NOX2, NOX1, and NOX4. For this reason, these, but not other isoforms, e.g. NOX3, NOX5, Duox1 and Duox2, will be discussed in this review.

NOX2 is inactive under non-stimulated (basal) conditions. To be active it needs to be assembled into a multimer composed by a transmembrane catalytic subunit (gp91phox), a membrane stabilizer subunit (p22phox), and several cytosolic subunits, i.e. p47phox, p40phox, and p60phox. The phosphorylation of p47phox triggers the recruitment of other cytosolic subunits and their translocation to the membrane. NOX1 is also a multimeric enzyme, homologue to NOX2, constituted by a transmembrane catalytic subunit (NOX1), the stabilizer subunit p22phox, and several cytosolic subunits, i.e. NOXO1 (that has a role similar to p47phox), and NOXA1 (Schröder et al., 2017). Both, NOX2 and NOX1 produce and release superoxide. By contrast, NOX4 is a constitutive dimeric enzyme that releases hydrogen peroxide. NOX4 is formed by a transmembrane catalytic subunit (NOX4) and a stabilizer subunit (p22phox), and its activity has been detected in intracellular membranes including mitochondria (Schröder et al., 2017; Nisimoto et al., 2014; Block et al., 2009).

Table 1 summarizes studies done in rodents supplemented with EC and subjected to different stress conditions, in which modulation of NOX activity and/or expression were studied. In conditions of hypertension, endotoxemia and diet-induced obesity, the administration of EC decreased the associated NOX activation. These effects were observed in different tissues and organs, and occurred through the modulation by EC of NOX activity and/or expression of different NOX subunits. In most cases, the expression of p47phox was pivotal, and in many the expression of NOX4. Importantly, in all these studies EC treatments reduced superoxide production, and risk factors associated with the corresponding experimental model, i.e. high blood pressure, proteinuria, increased fat deposition, and development of insulin resistance (see references in Table 1).

In terms of a specific inhibition of enzyme activity, elegant experiments showed that besides EC, specific ECm, i.e. 3'- and 4'-O-methyl-epicatechin, were effective NOX inhibitors (Steffen et al., 2007, 2008).

5.2. Oxidant production by mitochondria

During mitochondria oxidative phosphorylation to generate ATP oxygen is reduced to water. However, some redox centers in the chain, as complexes II and I, may leak electrons to oxygen generating superoxide or hydrogen peroxide (Boveris and Chance, 1973; Boveris, 1984; Yin and Cadenas, 2015).

Studies in which isolated mitochondria or submitochondrial particles were exposed to EC showed that different parameters of mitochondrial function and oxidant production, e.g. oxygen consumption, NADH oxidation, mitochondria membrane potential, and in few cases, hydrogen peroxide production, were only marginally affected (Moini et al., 1999; Dorta et al., 2005; Lagoa et al., 2011). A significant decrease in hydrogen peroxide production was observed when rat brain and heart mitochondria were exposed to 1 and 10 μM EC, respectively (Lagoa et al., 2011). In general, this type of studies have the same limitations of most *in vitro* experiments with flavonoids, i.e. the use of the parent compound (EC) and not of ECm, and that assays are done at non physiological high concentrations of EC (Fraga et al., 2014). It should be also noted that there is no experimental evidence that EC or ECm can reach mitochondria at biologically significant amounts, pass through the mitochondria membranes, and affect the respiratory chain and its components, as well as other mitochondrial structures.

5.3. Xanthine oxidase and cytochrome P450

Xanthine oxidase and cytochrome P450 are enzymes that

Table 1
Effects of EC on superoxide production and NOX subunit expression in experimental models of oxidative stress in rodents.

Experimental model (rodent)	EC treatment	Tissue	EC effects		Reference
			Superoxide-production	NOX subunits expression	
Hypertension (rats)					
DOCA-salt	10 mg/kg/d; 4w	Aorta	↓	↓ p47phox, ↓ p22phox	Gómez-Guzmán et al., 2012
L-NAME	~300 mg/kg/d; 4d	Aorta	↓	↓ p47phox	Litterio et al., 2012
		Heart	↓	↓ p47phox	Piotrkowski et al., 2015
L-NAME	10 mg/kg/d; 5w	Aorta	↓	↓ p22phox*	Gómez-Guzmán et al., 2011
Fructose-fed	20 mg/kg/d; 8w	Aorta	↓	↓ p47phox, ↓ p22phox, ↓NOX4	Litterio et al., 2015
		Heart	↓	↓ p47phox, ↓ NOX4	Calabro et al., 2016
		Kidney	↓	↓ p47phox, = gp91phox	Prince et al., 2016
		Liver	nd	↓ p47phox, ↓ gp91phox, ↓ NOX4	Bettaieb et al., 2014
		Fat	nd	↓ p47phox, ↓ gp91phox, ↓ NOX4	Bettaieb et al., 2014
Endotoxemia (rats)					
LPS	80 mg/kg/d; 4d	Kidney	↓	↓ p47phox, = gp91phox, ↓NOX4	Prince et al., 2017
Obesity (mice)					
High fat diet	20 mg/kg/d; 15w	Liver	↓	↓ p47phox, ↓ gp91phox*, ↓NOX4*	Bettaieb et al., 2016
		Fat	nd	.↓ p47phox, ↓ gp91phox*, ↓NOX4*	Bettaieb et al., 2016
		Ileum	↓	↓ NOX4 ↓ NOX1	Cremonini et al., 2018

In these experimental models, increases in superoxide production and/or NOX subunit expression relative to non-treated rodents were observed. In the EC treatment column, EC was administered orally and amounts are expressed per kg of body weight, being EC administered simultaneously with the different stimuli, except for the LPS model, in which EC was administered prior to the stimulus. Symbols (↓ and =) indicate relative changes associated to EC treatments; *a decrease in mRNA was also observed; nd, parameter not determined; DOCA, deoxycorticosterone acetate; L-NAME, N(G)-nitro-L-arginine methyl ester; LPS, bacterial lipopolysaccharide.

incorporate oxygen to different substrates generating as byproducts both, superoxide and hydrogen peroxide (Harrison, 2002; Cederbaum, 2015). Both enzymes have been claimed as targets of EC and ECm. However, structure-function studies using purified enzymes show that EC is not a potent inhibitor of xanthine oxidase (Beiler and Martin, 1951; Nagao et al., 1999; Cos et al., 1998) or cytochrome P450 (Muto et al., 2001; Anger et al., 2005; Satoh et al., 2016; Dong et al., 2016) compared to other flavonoids. There are no reports on *in vivo* effects of EC on these enzymes.

6. (−)-Epicatechin and the production of nitric oxide

In mammalian cells, nitric oxide (NO) is a key molecule in cell signaling and it has been suggested that many EC effects are mediated through the modulation of NO bioavailability, i.e. NO production and degradation rates. NO is produced both, enzymatically (Knowles and Moncada, 1994) and non-enzymatically (Rocha et al., 2012). Enzymatically, NO is produced by NO synthases (NOS) using L-arginine, NADPH, and oxygen as substrates, and flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin, and calmodulin as cofactors. There are several NO synthases isoforms widely distributed in mammalian organs and tissues: two constitutive NOS, endothelial (eNOS), and neuronal (nNOS); and one inducible isoform (iNOS) (Knowles and Moncada, 1994). NO is consumed through reactions with different targets. These reactions include coordination with metal centers, e.g. with the ferrous heme of soluble guanylate cyclase, formation of S-nitrosothiols, and reaction with superoxide to produce peroxynitrite that results in the nitration of different biomolecules (Heinrich et al., 2013). In addition, this reaction with superoxide would be also responsible for diminishing NO bioavailability, especially under conditions of high superoxide production.

It has been reported that in endothelial cell cultures, EC activates eNOS by increasing both, the phosphorylation of its activation sites (Ser-615, Ser-633, Ser-1177), and the dephosphorylation of Thr-495 (Ramirez-Sanchez et al., 2010). Increased eNOS phosphorylation in Ser-1177 was also observed in aorta from animals supplemented with EC (Gómez-Guzmán et al., 2011, 2012). The activation of nNOS in Ser-1417 was observed in femoris muscle of mice treated with EC (Nogueira et al., 2011).

Vascular effects of EC have been associated to restoration of NO bioavailability which favors vasorelaxation, and consequently, a reduction of blood pressure (Karim et al., 2000; Schroeter et al., 2006; Schewe et al., 2008; Fraga et al., 2011; Galleano et al., 2010, 2013). The actions of EC on NO bioavailability can be due to the activation of NOS, leading to a higher NO production, and/or to a decrease in NO reaction with superoxide. The latter occurs as a consequence of EC-mediated diminution of NOX-derived superoxide production (section 5.1). Optimization of NO levels by EC seems to be operative not only in the vasculature but in other organs/tissues, as heart and kidney (Piotrkowski et al., 2015; Calabró et al., 2016; Prince et al., 2016).

Contrary to the effects of EC on eNOS (and nNOS), EC does not favor the production of NO by iNOS preventing or attenuating the expression of this isoenzyme after different inflammatory stimuli (Kim et al., 2004; Kluknavsky et al., 2016; Prince et al., 2016, 2017). These effects were associated to the attenuation of NF-κB activation in the context of the anti-inflammatory actions of EC (Bettaieb et al., 2014). Through this downregulation of iNOS, EC would modulate an uncontrolled immune response.

Regarding non-enzymatic reduction of nitrite to NO that is favored at low pH, it has been demonstrated that EC facilitates NO formation in the stomach lumen which can potentially cause muscle relaxation in the stomach wall (Rocha et al., 2009).

7. (−)-Epicatechin and the regulation of cell signaling

Cells sense extracellular and intracellular stimuli, which trigger different pathways involved in cell functions and fate. Membranes and subcellular structures are major sensing sites for these signals. In this section, it will be revised how different cell components can receive, translate, and terminate molecular events involved in redox signaling and the influence of EC.

7.1. Cell membrane

The modulation of cell signaling by EC and ECm initiated at the membrane surface underlies the responses of cells to exogenous stimuli (Verstraeten et al., 2005, 2015; Oteiza et al., 2005; Fraga and Oteiza, 2011). These membrane actions can occur through different mechanisms: i) physical and chemical interactions that result in changes in membrane physical properties, which can lead to the activation/inactivation of receptors and other signaling molecules associated to the membrane; ii) the regulation of the structure and/or function of specialized domains in membranes, e.g. lipid rafts; and/or iii) a direct interaction with proteins or lipids present in cell membranes that are involved in signaling.

In terms of physical interactions of EC and related phenolic compounds with membranes, it was described that their capacity of maintaining lipid order in liposomes exposed to detergents was determined by the hydrophilicity of the phenolic, the overall number of hydroxyl groups, and the number of EC units forming procyanidins (Erlejman et al., 2004). The chemical-induced changes in lipids led to changes in liposome susceptibility to oxidation, which were prevented by EC and procyanidins (Verstraeten et al., 2003). An important aspect of membrane physiology is the regulation of calcium homeostasis that is a major player in cell signaling. At nanomolar concentrations, EC, dimeric and trimeric procyanidins regulated calcium fluxes at the cell membrane preventing the unwanted activation of NOX, protein kinase C, and NFAT (nuclear factor of activated T cells) in Jurkat cells (Verstraeten et al., 2008). Given the very limited possibility of procyanidins to enter cells, all these effects would be physiologically relevant at the gastrointestinal tract, and of less or no relevance in other cells/tissues.

The interaction of EC with lipid rafts can result in the modulation of signals that are initiated in these membrane domains (Verstraeten et al., 2015). We have studied the capacity of hexameric procyanidins composed of six units of EC (Hex) to modulate signals that are initiated at lipid rafts in intestinal cells. We observed that Hex: i) inhibited NF-κB activation initiated by TNFα (Erlejman et al., 2008); and ii) modulated the activation of NF-κB and other pro-oncogenic signals (ERK1/2, p38 and Akt) triggered by deoxycholic acid (Da Silva et al., 2012) (Erlejman et al., 2006). Importantly, Hex interacted with cholesterol in lipid raft–like liposomes and Caco-2 cell lipid rafts (Verstraeten et al., 2013). These interactions of procyanidins with lipid rafts can explain in part Hex capacity to modulate redox sensitive signals and promote apoptosis of colorectal cancer cells (Choy et al., 2016; Da Silva et al., 2012).

7.2. Plasma membrane receptors

EC intake has been empirically associated to the regulation of several membrane receptors, mostly without proof of direct interactions with the receptors. Only few reports have investigated in detail such interactions. EC and EC bound to dextran (that blocks EC internalization) triggers similar effects in human endothelial cells suggesting a mechanism mediated by a plasma membrane receptor (Moreno-Ulloa et al., 2014). The same research group proposed a G-protein-coupled estrogen receptor (GPER) as a target for EC based on *in silico* binding studies and experiments in endothelial cells

using GPER agonists, selective blockers, and siRNA (Moreno-Ulloa et al., 2015). In a different set of studies, a role for specific serotoninergic and opioid receptors was proposed to be involved in the action of EC on anti-nociception in rats (Quiñonez-Bastidas et al., 2013, 2017). The δ-opiod receptor appears to be specifically involved in the cardiac protection by EC in mice (Panneerselvam et al., 2010, 2013).

7.3. Mitochondria

Extending the associations between EC and mitochondria production of oxidants (section 5.2.), the actions of EC and ECm on mitochondria are relevant to the involvement of these organelles controlling energetic aspects and mitochondria participation in cell signaling.

Studies in rodents have analyzed the effects of EC oral administration on several aspects of mitochondrial physiology. Results can be summarized as follows: i) EC administration protects mitochondria from the damage associated to deleterious conditions including ischemia-reperfusion (Yamazaki et al., 2014; Ortiz-Vilchis et al., 2014), cisplatin nephropathy (Tanabe et al., 2012), and isoproterenol-induced myocardial infarction (Stanely Mainzen Prince, 2013); and ii) EC administration increases biogenesis that results in increased respiratory capacity and enhances exercise tolerance (Nogueira et al., 2011; Lee et al., 2015, 2016; Hüttemann et al., 2012; Hüttemann et al., 2013). One explanation of those protective effects was drawn from a study in high fat-fed rats in which eNOS activation is a key event in the action of EC on mitochondria (Ramírez-Sánchez et al., 2016). This interpretation was backed by data obtained in cultures of coronary artery endothelial cells (Moreno-Ulloa et al., 2013; Ramírez-Sánchez et al., 2016). To note, EC administration (100 mg/d as dark chocolate and a cocoa beverage for 3 months) to subjects with type-2 diabetes mellitus and heart failure led to modifications in mitochondria-associated parameters in skeletal muscle biopsies. This study showed that EC promotes increased expression of markers of mitochondria function (complexes I and V) and structure (proteins porin and mitofilin). In addition, mitochondria volume and cristae abundance, measured by electron microscopy, and some mitochondria biogenesis markers were increased respect to pretreatment values (Taub et al., 2012).

7.4. Endoplasmic reticulum

Beyond having enzymes that generate superoxide and hydrogen peroxide, the endoplasmic reticulum (ER) can participate in redox signaling via the unfolded protein response (UPR) system which controls for the adequate folding of proteins (Walter and Ron, 2011). The UPR is constituted by three branches: the PKR-like ER-regulated kinase (PERK), the inositol requiring protein 1α (IRE1α), and the activating transcription factor 6 (ATF6). Among other conditions, excess nutrient availability, e.g. high carbohydrate or high fat consumption, can cause ER stress. In rodents, chronic consumption of diets rich in fructose or fat causes ER stress in liver and adipose tissue (Bettaieb et al., 2014). Supplementation with EC attenuates the activation of the IRE1α branch in adipose tissue and of IRE1α and PERK branches in the liver of high fructose-fed rats. Consistently, supplementation with EC also mitigates the activation of the PERK and IRE1α pathways, but not that of ATF6, in mice fed a high fat diet (Bettaieb et al., 2016). In both, high fructose-fed rats and high fat diet-fed mice, ER stress occurred in parallel with oxidative stress, which was also attenuated by EC supplementation.

Different mechanisms are proposed to be involved in the crosstalk between the ER and oxidative stress. The PERK/ATF4/CCAAT/enhancer binding protein homologous protein (CHOP)-mediated induction of ER oxidase ERO1α, leads to the opening of calcium channels in the ER which causes the CaMKII-mediated activation of NOX2 (Li et al., 2010). While EC supplementation did not prevent high fat diet-mediated ERO1α upregulation, it mitigated the upregulation of NOX2 and NOX4 and consequent oxidative stress in adipose tissue (Cremonini et al., 2016). Inhibition of oxidant production by EC, could stop the cycle of oxidative stress, ER stress, NF-κB activation, and inflammation (Nakajima and Kitamura, 2013), and explain the modulation of the UPR by EC. Nevertheless, further research is necessary to understand why EC exert selective inhibition of PERK and IRE1α UPR branches, but does not affect the ATF-6 branch. Other mechanism of ER/oxidative stress crosstalk is through the regulation of the transcription factor Nrf2 by PERK (Cullinan et al., 2003). Nrf2 phosphorylation by PERK leads to dissociation of Nrf2/Keap1 and subsequent transport of Nrf2 to the nucleus. EC affects Nrf2 activation (see section 8.1), thus, the modulation of PERK could be another level of regulation of redox signaling by EC at the ER level.

Overall, from the current evidence, it is possible to propose some associations between EC consumption and ER stress, but it is still potential to establish causality or to identify the target/s of EC actions and its cross talk with the production of oxidants by the ER.

8. (−)-Epicatechin and activation of redox sensitive transcription factors

It is well established that cells have an efficient system to transduce signals associated to the activation of transcription factors and many of them can be affected by nutrients and bioactives. In terms of redox sensitive transcription factors, Nrf2, and NF-kB, are those for which the effects of EC and ECm appear to have the most significant physiological importance.

8.1. Nrf2

Nrf2 is a transcription factor considered as a master regulator of antioxidant responses and xenobiotic metabolism. Nrf2 is activated by a variety of stimuli, including increases of endogenous substances, oxidants, radiation, environmental chemicals, and food xenobiotic as dietary polyphenols, (Huang et al., 2015; Houghton et al., 2016; Siow and Mann, 2010). As a result of Nrf2 activation, there is an increase in the expression of a group of antioxidant and phase II detoxifying enzymes, mediated by the specific enhancer ARE (antioxidant response element) (Rushmore and Pickett, 1990; Nguyen et al., 2003). Examples of these enzymes are glutathione S-transferases, heme oxygenase-1, quinone oxidoreductases, UDP-glucuronosyl transferase, epoxide hydrase, γ-glutamylcysteine synthetase, and peroxiredoxin 1 (Rushmore and Pickett, 1990; Nguyen et al., 2003; de Vries et al., 2008).

Mechanisms involved in Nrf2 activation by bioactives are multiple (Huang et al., 2015), and have been studied for some flavonoids, such as genistein and (-)-epigallocatechin gallate (EGCG), but not for EC. However, some observational studies, essentially focused on the brain, have shown that EC could activate Nrf2. This opens the possibility that EC and/or ECm could directly activate Nrf2 given the electrophilic characteristics of EC quinone formed as product of EC oxidation with free radicals (Forman et al., 2014). Cultures of primary cortical cells treated with EC showed the activation of Nrf2 in astrocytes, but not in neurons. Astrocytes treated with EC showed increased glutathione levels, consistent with an up-regulation of γ-glutamylcysteine synthetase expression (Bahia et al., 2008). EC administration *in vivo* showed protective effects in the brain of mice subjected to middle cerebral artery occlusion, in young as well as in old animals (Shah et al., 2010; Leonardo et al., 2015), in focal ischemia (Leonardo et al., 2013), intracerebral

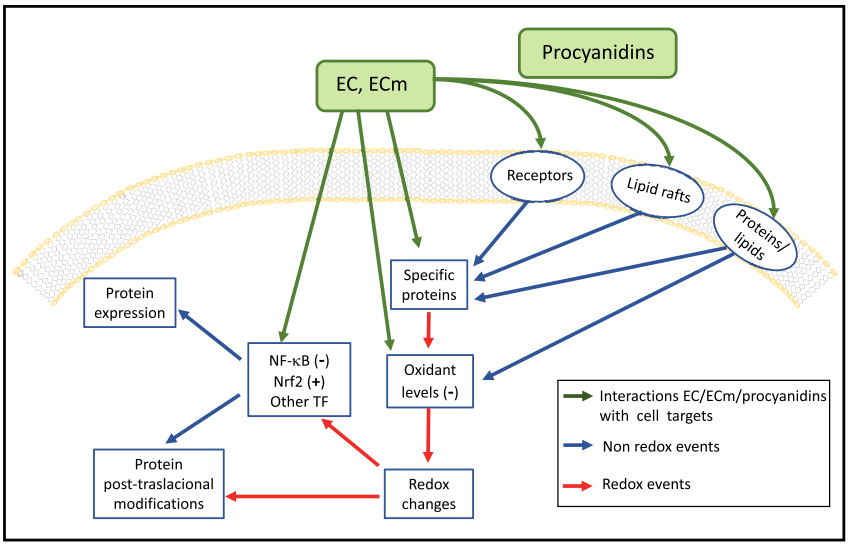

Fig. 2. (-)-Epicatechin in the regulation of redox signaling. Signs between parenthesis indicate activation (+), or inhibition (-). TF = transcription factors.

hemorrhage (Cheng et al., 2014) and traumatic brain injury (Cheng et al., 2016). These actions were associated to Nrf2 activation and/or increased expression of Nrf2-dependent proteins. EC was not effective when the experiments were reproduced in knockout animals for Nrf2 or in knockout animals for hemoxygenase-1.

A few studies using other cells or organs showed Nrf2 activation associated with EC treatment, including HepG2 cells (Granado-Serrano et al., 2010) and aorta isolated from DOCA-salt hypertensive rats (Gomez Guzman, 2012).

8.2. NF-κB

Extensive research has shown that EC modulates the expression of numerous NF-κB-regulated genes involved in inflammation, metabolic diseases, and carcinogenesis, in different cell types and *in vivo* rodent models of disease (Fraga and Oteiza, 2011). The regulation of the redox sensitive NF-κB pathway can be associated to: i) the modulation of cell oxidant/antioxidant status; ii) the inhibition of receptor-mediated NF-κB activation; and iii) the inhibition of specific steps in the NF-κB activation cascade.

Besides being a redox sensitive transcription factor, NF-κB specifically regulates the expression of oxidant-generating enzymes, i.e. NOX1 and NOX4 (Manea et al., 2010). This can generate a self-feeding cycle of NF-κB activation and increased oxidant production, which can be disrupted by EC. In this regard, EC inhibits NF-κB activation in association with the reduction of oxidant production in liver, brain and ileum from mice/rats fed high fat or high fructose diets (Bettaieb et al., 2014; Cremonini et al., 2018). In addition, this action of EC on both NF-κB and NOX activation, was observed in intestinal cells challenged with oxysterols (Guina et al., 2015) or TNFα (Cremonini et al., 2018). In these inflammatory models, the inactivation of the NF-κB and decreased oxidant production led to a reduction of inflammation (Guina et al., 2015) and prevention of Caco-2 monolayer permeabilization (Cremonini et al., 2018). On the other hand, the modulation of TLR-4 expression (Prince et al., 2017) via redox-independent pathways can also define the actions of EC on NF-κB activation.

Finally, in terms of EC affecting specific steps in NF-κB activation, we have demonstrated that EC and its B2 dimer can inhibit NF-κB activation pathway at multiple levels in Jurkat T (Mackenzie et al., 2004) and in Hodgkin's lymphoma (Mackenzie et al., 2008) cells. Functional evidence supported by a putative molecular model suggests that B2 could interact with NF-κB proteins and prevent the binding of NF-κB to the DNA κB sites (Mackenzie et al., 2009). The relevance of phenolic conformation agree with the finding that rotationally constrained variants of caffeic acid have less ability to inhibit NF-κB-DNA binding than the parent molecule (Natarajan et al., 1996). In the case of EC dimers, stacked rings B and A of dimers B1 and B2 lie very close to the positions occupied by the two guanine rings in the NF-κB DNA consensus sequence. In addition, the oxygen atoms of B1 and B2 are favorably placed to give rise to a similar hydrogen-bonding pattern to that observed in the complex with DNA. Differences between the spatial disposition of A and B dimers series can determine the differential inhibitory effects of these dimers on NF-κB binding to its DNA consensus sequence. This is one example on how specific polyphenol-protein interactions, can be driven by the structural and conformational characteristics of polyphenols.

9. Conclusions

EC participates in the modulation of cell signaling through its participation in redox reactions and redox sensitive pathways (Fig. 2). Once ingested, EC either as the monomeric parent molecule or forming procyanidins, will have bioactivities restricted to the gastrointestinal tract. In other organs and tissues, EC bioactivities will be mostly mediated by ECm. Integrating current knowledge, it is possible to conclude that both superoxide and NO, and consequently NOXs and NOSs, are relevant targets involved in EC biological effects. Additionally, EC affects redox sensitive transcription factors, especially Nrf2 and NF-κB.

Further research is necessary focusing on the mechanisms responsible for the phenomenological associations between EC and the reduction of pathologies and health optimization. Only with a full understanding of the molecular mechanisms mediating the effects of plant bioactives on health, it will be possible to define which fruit and vegetables, and in which amounts they should be part of an optimal diet or pharmacological supplementation. Finally, such mechanistic understanding is also important given that the biological effects observed for EC could be extrapolated to other flavonoids, polyphenols, and other plant bioactives.

Acknowledgements

Supported by grants from the Universidad de Buenos Aires UBACyT 20020130100760BA (MG) and 20020160100132BA (CGF); the Packer-Wentz endowment 2017 (CGF); and NIFA-USDA (CA-D*-XXX-7244-H) (PIO).

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