Polyphenols, intracellular signalling and inflammation

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Summary. Excessive inflammation is considered as a critical factor in many human diseases, including cancer, obesity, type II diabetes, cardiovascular diseases, neurodegenerative diseases and aging. Compounds derived from botanic sources, such as phenolic compounds, have shown anti-inflammatory activity *in vitro* and *in vivo*. Recent data suggest that polyphenols can work as modifiers of signal transduction pathways to elicit their beneficial effects. These natural compounds express anti-inflammatory activity by modulation of pro-inflammatory gene expression such as cyclooxygenase, lipoxygenase, nitric oxide synthases and several pivotal cytokines, mainly by acting through nuclear factor-kappa B and mitogen-activated protein kinase signalling. This review will discuss recent data on the control of inflammatory signalling exerted by some dietary polyphenols contained in Mediterranean diet. A clear understanding of the molecular mechanisms of action of phenolic compounds is crucial in the valuation of these potent molecules as potential prophylactic and therapeutic agents.

Key words: polyphenols, inflammation, molecular mechanisms.

Riassunto *(Polifenoli* signalling *intracellulare e stato infiammatorio)*. Un aumento dello stato infiammatorio, è attualmente considerato una condizione critica in molte patologie umane quali obesità e diabete di tipo 2, malattie cardiovascolari, disordini neurodegenerativi e invecchiamento. Gli studi finora effettuati, sia *in vitro* che *in vivo*, hanno evidenziato che sostanze derivanti da vegetali, quali i polifenoli, possiedono attività antinfiammatoria. I polifenoli svolgono la loro azione protettiva interagendo con diversi *pathways* molecolari responsabili della trasduzione del segnale all'interno della cellula. Queste sostanze naturali agiscono modulando l'espressione di geni pro-infiammatori quali ciclossigenasi, lipossigenasi, sintetasi dell'ossido nitrico e diverse citochine, principalmente mediante l'interazione con il fattore di trascrizione *nuclear factor-kappa B* e le chinasi *mitogen-activated protein kinase*. Questa rassegna discuterà di dati recenti riguardanti il controllo del signalling infiammatorio da parte di polifenoli contenuti nella dieta Mediterranea. Una maggiore e più chiara conoscenza dei meccanismi molecolari mediante i quali i polifenoli agiscono è un punto cruciale nella valutazione di queste molecole come possibili agenti di prevenzione e profilassi.

Parole chiave: polifenoli, infiammazione, meccanismi molecolari.

INTRODUCTION

Worldwide morbidity and mortality from infectious diseases is being replaced by chronic diseases, such as cancer, obesity and type II diabetes, cardiovascular diseases, neurodegenerative diseases and aging [1, 2]. In addition, evidence is mounting regarding a range of a diet-chronic disease link. Thus, nutrition research has shifted from focusing exclusively on alleviating nutrient deficiencies to also stressing chronic disease prevention [2]. Polyphenols constitute one of the most numerous and ubiquitously distributed group of plant secondary metabolites, present in all plants that are commonly consumed in the Mediterranean diet including grains, legumes, fruits, vegetables, extra virgin olive oil (EVOO), red wine and tea [3]. Epidemiological studies show that populations consuming predominantly a Mediterranean diet exhibit lower incidence of coronary heart disease than those eating a Northern European or North American diet. This diet is rich in EVOO, which contains phenolic compounds, leading to the suggestion that the high consumption of this fat, at least in part, contribute to the health benefits [4-8]. Polyphenols have been described to have a wide range of biological activities and many reports, published during recent years, have highlighted the beneficial effects of phenolic compounds illustrating their promising role as therapeutic tools in several acute and chronic disorders [9-15]. They are extensively metabolised *in vivo* and many studies have been focusing attention

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on the interaction with specific proteins of intracellular signalling cascades vital to cellular function [16]. Particularly, epidemiological and experimental studies have been focused on the anti-inflammatory activity of dietary polyphenols [1, 17]. In the classic literature, inflammation is described as the principal response of the body invoked to deal with injuries and its hallmarks include swelling, redness, pain and fever (tumor, rubor, dolor and calor) [18]. Inflammation is a reaction of the microcirculation that is characterized by the movement of serum proteins and leukocytes (neutrophils, eosinophils and macrophages) from the blood to the extra-vascular tissue. There are many mediators, such as vasoactive amines: histamine and 5-hydroxytryptamin (5-HT); adhesion molecules: intercellular adhesion molecule 1 (ICAM 1), vascular adhesion molecule 1 (VCAM 1), selectins; lipid-derived eicosanoids: prostaglandin E_2 (PGE_2), prostaglandin I_2 (PGI_2), leukotriene B_4 (LTB_4), leukotriene C_4 (LTC_4); cytokines: tumour necrosis factor α (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10) and chemokines: interleukin-8 (IL-8), monocyte-chemoattractant protein-1 (MCP-1), macrophage inflammatory molecule 1α (MIP1α), that coordinate the events of acute inflammation, regulate vascular changes and inflammatory cell recruitment [1, 18-20]. The inflammatory response is a complex self-limiting process precisely regulated to prevent extensive damage to the host. When the self-limiting nature of this protective mechanism is inappropriately regulated, it is transformed to a detrimental, chronic state of inflammation. All chronic diseases are interrelated as they contain an element of increased inflammatory response, often observed long before the disease is clinically documented [1]. The increase in inflammatory *tonus* is mainly the result of lifestyle and nutritional habits, making the increase controllable [21]. During the past several decades, the incidence of obesity has significantly raised worldwide [22]. Obesity is associated with a state of chronic, low-grade inflammation, particularly in white adipose tissue [23] demonstrating a close link between metabolism and immunity. The integration of metabolism and immunity, under normal condition can be viewed as a central homeostatic mechanism, but whose dysfunction (described as meta-flammation) can lead to a cluster of chronic metabolic disorders, particularly obesity, type 2 diabetes and cardiovascular diseases [24, 25]. It is safe to suggest that the link between inflammatory and metabolic signalling is a delicate balance [24]. It is clear that chronic excess of nutrients engages common or overlapping pathways regulating both metabolic and immune functions through common key regulatory molecules and signalling systems. It has been shown that phenolic compounds can exert modulatory action in cell by interacting with a wide spectrum of molecular targets central to the cell signalling machinery The molecular mechanisms involved in the anti-inflammatory activities of polyphenols have also been suggested to include: i) the inhibition of pro-inflammatory enzymes, such as cyclooxygenase (COX-2), lipoxygenase (LOX) and inducible nitric oxide synthase (iNOS), through the activation of peroxisome proliferators-activated receptor gamma (PPARγ); ii) the inhibition of phosphoinositide 3-kinase (PI 3-kinase), tyrosine kinases, nuclear factor-kappa B (NF-κB), c-JUN and iii) the activation of phase II antioxidant detoxifying enzymes, mitogen-activated protein kinase (MAPK), protein kinase C (PKC), serin/threonin protein kinase Akt/PKB as well as iv) the modulation of several cell survival/cell-cycle genes [16, 17, 26, 27]. This review will discuss the anti-inflammatory activity and cell signalling modulation of the well known polyphenols contained in characteristic components of the Mediterranean diet including vegetables, fresh fruits and extra virgin olive oil.

POLYPHENOLS INHIBIT ARACHIDONIC ACID PATHWAY

One of the important anti-inflammatory mechanisms is the inhibition of eicosanoids generating enzymes including phospholipase A_2, cyclooxygenase, and lipoxygenase thereby reducing the concentration of prostanoids and leukotrienes [26]. Arachidonic acid (AA) is released by membrane phospholipids through phospholipase A_2 (PLA_2) cleavage; it can be metabolized by cyclooxygenase (COX) pathway into prostaglandins (PGs) and thromboxan A_2 (TXA_2), or by lipoxygenase (LOX) pathway to hydroperoxyeicosatetraenoic acids (HpETEs), hydroxyeicosatetraenoic acids (HETEs) and leukotrienes (LTs) [17]. Cyclooxygenase exists in two major isoforms (COX-1 and COX-2) and one variant (COX-3) [28]. COX-1 is constitutively expressed in many tissues, while COX-2 is known as an inducible enzyme that produce, in most cases, large amount of prostaglandins. COX-2 is highly expressed in the inflammation-related cell types including macrophages and mast cells after stimulation by pro-inflammatory cytokines and/or lypopolisaccharide (LPS) [29]. Lipoxygenases are the enzymes responsible for generating hydroxyl acid and leukotrienes from arachidonic acid. 5-, 8-, 12-, and 15- LOXs have been found in different cells/tissues. 5- and 12- LOXs produce 5-HETE and 12-HETE respectively, that induce inflammatory response [30]. Anti-inflammatory molecules, such as aspirin and its derivatives (and other non-steroidal anti-inflammatory drugs), at low therapeutic doses, irreversibly inhibit the activity of COX-1 and COX-2 and the subsequent formation of prostaglandins, mainly PGE_2 [31]. However, several synthetic drugs provide unknown side effects, consequently, there has been a need for new and safe anti-inflammatory agents. Dietary polyphenols have been found to inhibit cellular enzymes, such as PLA_2, COX and LOX, in order to reduce arachidonic acid, prostaglandins and leukotrienes production, thus exerting an important anti-inflammatory action [17, 32-35] *(Figure 1)*. Polyphenolic compounds extracted from red wine and black tea

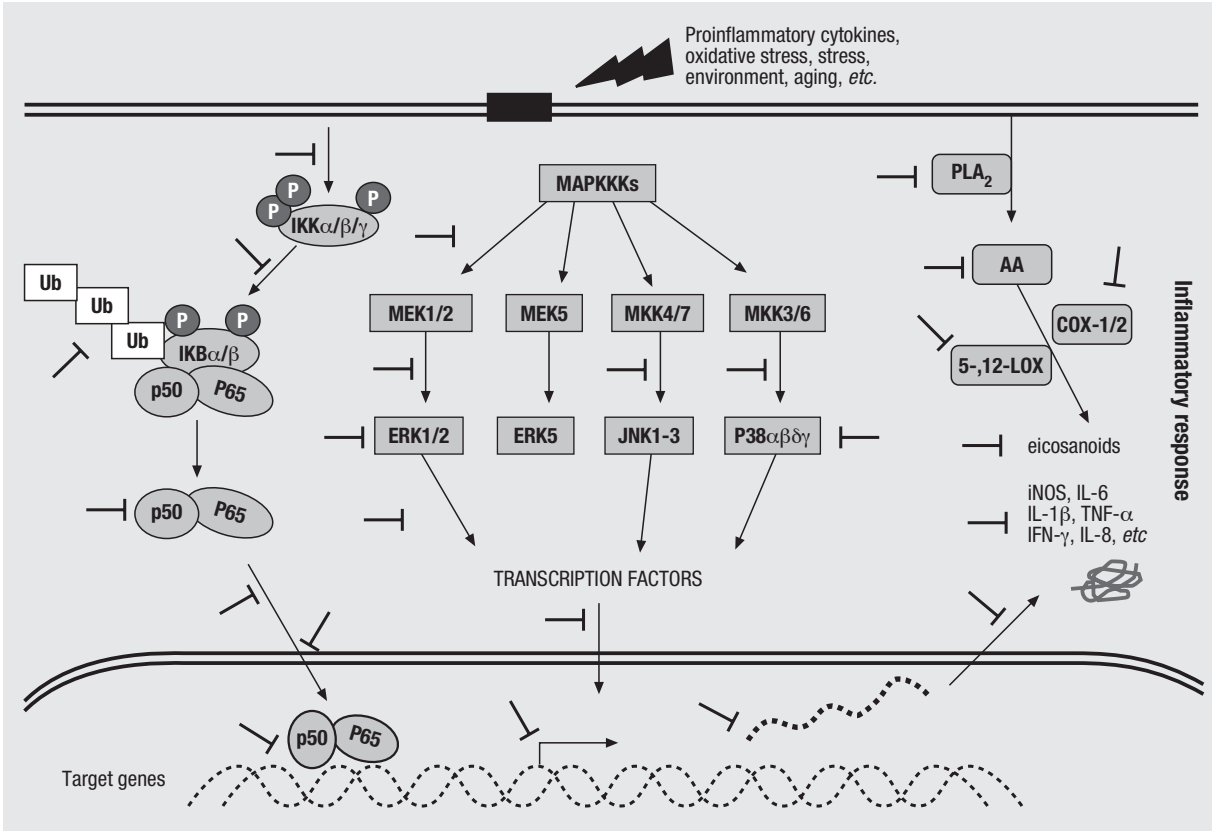

Fig. 1 | *Potential points of action of polyphenols (⊥) within inflammatory cascade. IKB, inhibitor kB, Ub, ubiquitin; IKK, IkB-kinase; ERK, extracellular signal-related kinases; JNK, c-Jun amino-terminal kinases; p38 (or p38-MAPK), p38-mitogen-activated protein kinase; MEK (or MKK), MAPK-kinase; MAPKKK, MAPK kinase kinase; IL-8, interleukin-8; IFNγ, interferon-γ; TNF-α, tumour necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; LOX, lipoxygenase; COX, ciclooxygenase; AA, arachidonic acid; PLA_2, phospholipase A_2.*

were able to modulate COX-2 activity and gene expression in different cell types [36, 37]. For instance, quercetin inhibited COX and LOX in leukocyte infiltration in mice [38], in rat peritoneal leukocyte [34] and in guinea-pig epidermis [39]. Recent reports, have shown that other green tea polyphenols, namely pro-delphinidin B-4 3'-O-gallate [40] and pro-delphinidin B2 3,3' di-O-gallate [41], suppressed mRNA and protein expression of COX-2 and the release of PGE_2 in a dose-dependent manner, in LPS-activated murine macrophage RAW264 cells. This inhibitory action occurred through the suppression of NF-κB and MAPK pathways, respectively; studies on structure-activity relationship using different proanthocyanidins revealed that the galloyl moiety of proanthocyanidins appeared important to their inhibitory actions [41]. Furthermore, (-)-epigallocatechin (EGC), (-)-gallocatechin (GC), (-)-epicatechin gallate (ECG), (-)-catechin gallate (CG), (-)-epigallocatechin gallate (EGCG), have been shown to have COX-1/COX-2 inhibitory activity in different human and mouse cell lines [42-45]. Likewise, kaempferol, a flavonoid present in various natural sources including apples, onions, leeks, citrus fruits, grapes, red wines and tea, significantly decreased the production of PGE_2 by LPS-stimulated human whole blood cells in culture [46]. Growing interest has been focused on the anti-inflammatory effects of phenolic components present in extra virgin olive oil (EVOO). *In vivo* studies have added further evidence to the hypothesis that consumption of EVOO with increasing phenolic content, contribute to the health benefits of Mediterranean diet [7, 8, 46-50]. EVOO is a source of at least 30 phenolic compounds and glycoside oleuropein, hydroxytyrosol and tyrosol are the phenolic compounds present in the highest concentration [51]. Some EVOO phenolics have been shown to inhibit eicosanoids production by animal and human cells *in vitro*, indicating their anti-inflammatory effects. Specifically, oleuropein glycoside, caffeic acid, and tyrosol were able to inhibit LTB_4 production by exerting selective inhibitory activity on 5-LOX pathway, in human activated leukocytes [52]. Tyrosol reduced the ROS-induced [^{3}H]AA release and the subsequent eicosanoids (PGE_2/LTB_4) production, in phorbol 12-myristate-13-acetate (PMA)-stimulated macrophages RAW 264.7 [53]. The same tyrosol, as well as lycopene and

quercetin, inhibited COX-2 and iNOS gene expression in RAW 264.7 macrophages stimulated by gliadin in association with interferon-γ (IFNγ), probably through NFκB pathway. These data suggest that these compounds may represent a non toxic agents for the control of pro-inflammatory genes involved in celiac disease [54]. Moreover, hydroxytyrosol, one of the major phenolic constituent in EVOO, inhibited in a dose-related manner the production of LTB_4 by calcium ionophore-stimulated human leukocytes [55] and blocked the production and accumulation of TXB_2 and 12-HETE leading to reduced platelet aggregation, in human platelet rich plasma [56, 57]. It is presumed that hydroxytyrosol penetrates in cell membranes and, consequently, can effectively inhibit the production of LTB_4 from endogenous arachidonic acid [58]. In addition, hydroxytyrosol was able to inhibit COX-2 and iNOS gene expression, in LPS-stimulated J774 murine macrophages [59]. These findings are consistent with an *in vivo* study which demonstrated a significant decrease in inflammatory markers, namely TXB_2 and LTB_4, and a concomitant increase of serum antioxidant capacity, in healthy men after EVOO consumption [60]. Furthermore, it has been observed that consumption of hydroxytyrosol decreased TXB_2 production in the serum of type 1 diabetic subjects [61]. Another olive oil-phenolic compound, namely oleocanthal, acts as natural anti-inflammatory agent structurally related to the anti-inflammatory drug ibuprofen. In fact, oleocanthal, similarly to ibuprofen, caused dose-dependent inhibition of COX-1 and COX-2 activity. This finding rises the possibility that long term consumption of oleocanthal may help to protect against some diseases [62], although it is very likely that the entire battery of structurally-related phenolic compounds present in olive oil enhances the anti-inflammatory action of oleochantal [63].

POLYPHENOLS MODULATE NITRIC OXIDE SYNTHASE FAMILY

Nitric oxide (NO) is one of the cellular mediators of physiological and pathological process. NO is synthesized from L-arginine by nitric oxide synthase (NOS) family, which include endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoforms. The former are constitutively expressed in the body, whereas the latter type is an inducible enzyme highly expressed by inflammatory stimuli [64]. While a small amount of NO, synthesized by eNOS and nNOS, is essential to maintain normal body function (homeostasis), a significant increase of NO synthesized by iNOS participates in provoking inflammatory process and acts synergistically with other inflammatory mediators [65]. Compounds able to reduce NO production by iNOS may be thus attractive as anti-inflammatory agents and, for this reason, the effects of polyphenols on iNOS activity have been intensively studied. Catechin, EGC, naringenin, and fisetin repressed NO production in RAW 264.7 macrophages and human peripheral blood mononuclear cell LPS/PMA stimulated [66]. Results, so far obtained, suggest that polyphenols inhibit NO release by suppressing NOS enzymes expression and/or NOS activity [26, 67] *(Figure 1)*. In particular, a varieties of flavonoids, including apigenin, luteolin, kaempferol, myricetin and genistein, down-regulate NO production and/or iNOS enzyme expression and activity, in RAW 264.7 macrophage cells [68, 69]. Quercetin has been demonstrated to inhibit NO production in LPS/cytokine-treated macrophages or macrophages-like cells by regulating iNOS protein expression [70, 71] and mRNA transcription [72]. The anti-inflammatory effects of tea catechins could be exerted by differential modulation of the three different NOS isoforms. In fact, EGCG and other catechins inhibited the induction of iNOS mRNA and activity in rodent cell lines after treatment with LPS or IFNγ [73, 74]. The inhibition of iNOS transcription seems to occur by preventing binding of NFκB to the promoter of the iNOS gene thereby inactivating it [75]. Interestingly, EGCG exerted its effect on iNOS expression and activity reducing their activity by competitively inhibiting the binding of arginine and tetrahydrobiopterin, and it has been demonstrated that the gallate structure of this catechin is important for its action [74]. On the other hand, administration of EGCG to rat aortic rings, induced a dose-dependent vasorelaxation occurring simultaneously with the induction of eNOS activity in endothelial cells. It has been proposed that EGCG induced eNOS to produce NO, which, in turn, activated guanylate cyclase to produce cyclic guanosine monophosphate and caused vasorelaxation by PI3K, protein kinase A and Akt-dependent signalling pathways [76]. In addition, also cyanidin-3-glucoside (Cy3G) induced eNOS expression and escalated NO production via an Src and extracellular signal-regulated kinase 1/2 - Sp1 (Src-ERK1/2-Sp1) signalling pathway in bovine artery endothelial cells [77]. Increased eNOS expression may help to ameliorate endothelial dysfunction, harmonize blood pressure, and prevent atherosclerosis as long-term beneficial effects of flavonoids. Moreover, EGCG is a potent antioxidant and neuroprotective agent against ischemia-induced (HI) brain damage. Wistar rats, administered with EGCG before HI induction, significantly showed a reduced iNOS activity and protein expression and, in contrast, a significant increase of eNOS and nNOS proteins. These data demonstrated that the neuroprotective effects of EGCG are, in part, due to the modulation of the different NOS isoforms [78]. Furthermore, a study carried out in a transgenic mouse model of Amyotrophic Lateral Sclerosis (ALS), provided further evidence that EGCG has multifunctional therapeutic effects. In fact, EGCG showed neuroprotective effects which increased the number of motor neurons, diminished microglial activation and reduced protein level of iNOS and NFκB in the spinal cords [79]. Flavonoids appear to be a potential therapeutic agents against type 1 [80]

and type 2 diabetes progression [81]. In particular quercetin [82], epicatechin and EGCG [83] have been shown to exert protective effect on β-cells by different mechanisms, including blocking the streptozotocin-induced NO production and counteracting the IL-1β- and IFNγ-mediated cytotoxicity likely by inhibiting iNOS gene expression. Procyanidin extract, a mixture of polyphenols obtained from grape seeds, significantly inhibited, in a dose- and time-dependent manner, the overproduction of NO, by diminishing iNOS mRNA and protein amount in RAW 264.7 macrophages stimulated with LPS plus INF-γ. It is worth of note that trimeric and longer oligomeric-rich procyanidin fractions from the extract inhibited iNOS expression, while the monomeric forms catechin and epicatechin did not, showing that the degree of polymerization plays an important role in determining procyanidin effects [84]. In the same vein several reports have demonstrated that EVOO phenolics, such as oleuropein, hydroxytyrosol, caffeic acid and tyrosol, can differently modulate the production of nitric oxide in activated cell lines [69] depending on the concentration and chemical structure [85]. An interesting *in vivo* study was carried out by treating mice with an hydroxytyrosol-rich extract prepared from olive mill wastewater. Results demonstrated that hydroxytyrosol enhanced the resistance to oxidative stress and attenuate NO-induced cytotoxicity in dissociated brain cells. These data reinforce the promising biological effects exerted by EVOO suggesting that the neuroprotective effects of oral hydroxytyrosol intake might contribute to the lower incidence of neurodegenerative diseases, as observed in the Mediterranean area [86].

POLYPHENOLS ACT ON CYTOKINE SYSTEM

Cytokines are the major mediators of local, intercellular communications required for an integrated response to a variety of stimuli in immune and inflammatory processes [26]. Numerous cytokines have been identified in tissues across a range of immuno-mediated inflammatory diseases. Moreover, a "balance" between the effects of pro-inflammatory (*i.e.* IL-1β, IL-2, TNFα, Il-6, IL-8 and IFN-γ) and anti-inflammatory cytokines (*i.e.* IL-10, IL-4, TGFβ) is thought to determine the outcome of disease, whether in the short or long term [87-89]. Because of this, the cytokine system constitutes a very interesting target for the development of clinically relevant anti-inflammatory drugs. Identification of plant-derived compounds, such as phenolic compounds, able to selectively interfere with the production and/or function of cytokines could offer an important alternative for the treatment of many inflammatory diseases [90, 91] *(Figure 1)*. To this end, it has been observed that several flavonoids are able to decrease the expression of different pro-inflammatory cytokines/chemokins, among which TNFα, IL-1β, IL-6, IL-8, MCP-1, in many cell types such as LPS-activated mouse primary macrophages, PMA or phytohemagglutinin (PHA) stimulated human peripheral blood mononuclear cell [91, 92], activated human astrocytes [93], human synovial cells [94], activated human mast cell line HMC-1 [95], nasal mucosal fibroblasts and A549 bronchial epithelial cells [96]. These studies strongly support the idea that flavonoids have the capacity to modulate the immune response and have a potential anti-inflammatory activity [66]. However, the effects on the balance between pro- and anti-inflammatory cytokine expression have been shown to be specific for specific cytokines and influenced by polyphenol structures highlighting the complex action exerted by these compounds. In fact, polyphenols, such as quercetin and catechins, coupled their inhibitory action on TNFα and IL-1β to the enhancement of IL-10 release [91, 97]. Phenolic compounds from EVOO have been shown to modulate the expression of several cytokines [4, 46]. In fact, in activated human whole blood cultures, oleuropein glycoside and caffeic acid decreased the production of IL-1β without affecting IL-6 concentration [46] while kaempferol decreased the production of IFNγ [98]. A dose-dependent inhibition of IFN-γ production by kaempferol has been observed also in murine spleen cells and T cell lines [99]. In a model system of inflammation, the LPS-treated BALB/c mice, as well as in the human monocyte cell line THP-1, the treatment with olive vegetation water, especially rich in polyphenols, decreased the production of TNFα [100]. Finally, a recent clinical trial, carried out in stable coronary hearth disease patients, provided interesting evidence that consumption of polyphenol-enriched extra virgin olive oil is associated to decreased IL-6 and C-reactive protein expression [8].

POLYPHENOLS MODULATE NF*K*B PATHWAY

Since their discovery, NFκB/Rel transcription factors have been suspected to play a key role in chronic and acute inflammatory diseases. In fact, NFκB plays a pivotal role in immune, inflammatory, stress, proliferative and apoptotic responses of a cell to a very large number of different stimuli [101]. NFκB coordinate the induction of a wide range of genes encoding pro-inflammatory cytokines (*e.g.*, IL-1, IL-2, IL-6, and TNFα), chemokines (*e.g.*, IL-8, MIP-1α and MCP-1), adhesion molecules (*e.g.*, ICAM, VCAM, and E-selectin), acute-phase proteins, immuno-receptors, growth factors, and inducible enzymes such as vascular endothelial growth factor (VEGF), COX-2, matrix metalloproteinases (MMPs), iNOS, all molecules involved in inflammation other then in angiogenesis, cell proliferation, adhesion, migration, and invasion [102]. The inhibition of NFκB is generally thought a useful strategy for treatment of inflammatory disorders [103] and this pathway represents an important and very attractive therapeutic target for compounds that selectively interfere with it. Recent data suggested that

dietary polyphenols can work as modifiers of signal transduction pathways to elicit their beneficial effects [104]. The NFκB/Rel family consists of five members: p65 (RelA), RelB, c-Rel, p50/p105 (NFκB1), and p52/p100 (NFκB2) composed of members of the Rel family of DNA-binding proteins that recognize a common sequence motif. NFκB is a dimer which classically consists of a p50 subunit and a trans-activating subunit p65 (or relA) but others variants also occur [105]. In un-stimulated cells, NFκB is sequestered in the cytoplasm as an inactive non-DNA-binding form, associated with the inhibitor κB proteins (IκBs), comprising IκBα, IκBβ, IκBγ, IκBε, Bcl-3, precursors p100 and p105 [106]. Upon cell stimulation with various NFκB inducers, IκB proteins are rapidly phosphorylated by IκB kinase (IKK) complex on two serine residues, which targets the inhibitor proteins for ubiquitination and subsequent degradation by the ubiquitin-proteasome pathway. The IKK contains two catalytic subunits, IKKα, and IKKβ, and the regulatory sub-unit NFκB essential modifier (NEMO, also known as IKKγ). The released NFκB dimer can then translocate into the nucleus and induces the expression of various genes [106]. The activated transcription of NFκB is maintained by continuous degradation of IκB, which is sustained by an extracellular stimulus, suggesting that the accumulation/degradation of IκB is a mechanism allowing the regulation of NFκB [107]. A variety of other signalling events, including the phosphorylation of NFκB, the hyper-phosphorylation of IKK, and the processing of NFκB precursors, provide additional mechanisms that modulate the level and duration of NFκB activity [101, 108]. Polyphenols have been shown to exert their anti-inflammatory activity by modulating NFκB activation and acting at multiple steps of the activation process [104, 109] *(Figure 1)*. In particular, the influence of EGCG on NFκB pathway has been extensively studied demonstrating its inhibitory effects on NFκB obtained by counteracting the activation of IKK and the degradation of IκBα [110, 111]. An interesting *in vivo* study carried out in rat showed that EGCG markedly attenuated the myocardial injury after ischemia and reperfusion. This cardio-protection was associated with decreased IL-6, reduced activation of IKK, reduced degradation of IκB-α and decrease of activated NFκB with consequent inhibition of the inflammatory process at the early event of the transcription mediated by the NFκB pathway [112]. Moreover, EGCG, by inhibiting IκBα degradation and by blocking DNA binding of NFκB, abolished IL-12p40 production [112] and iNOS expression [75] in LPS-activated murine macrophages. EGCG inhibited the phosphorylation of IκBα by TNFα-induced IKK in fetal rat intestinal epithelial cell line. This may occur as a direct effect on IKK or by interfering with the interaction of IKK with IκBα. Importantly, the gallate group was functionally necessary for inhibition of IKK activity, and the presence of the catechin structure dramatically enhanced this effect. Actually, IKK appears to be a key control point for NFκB activation and may be considered a suitable target for modulating NFκB-mediated cellular responses [113]. In activated RAW 264.7 macrophages, the expression of iNOS mRNA and protein was strongly inhibited by a mixture of polyphenols obtained from grape seeds, likely through the reduction of nuclear NFκB(p65) and of IκBα mRNA production [84]. Furthermore, pre-treatment of PMA-induced Jurkat T cells with epicatechin and catechin decreased NFκB activity. This effect was likely obtained by inhibiting the phosphorylation of IKKβ, the subsequent degradation of IκBα and, consequently, the binding of NFκB to its DNA consensus sequence. Thus, the modulation of the NFκB activation cascade by flavonoids can occur at early (regulation of oxidant levels, IKK activation) as well as late (binding of NF-κB to DNA) stages [114]. Worth of note are the results obtained in RAW 264.7 treated with IFNγ and gliadin to induce the inflammatory process. In these cells, quercetin, tyrosol and lycopene inhibited iNOS, COX-2 expression and the pro-inflammatory related genes by preventing the nuclear translocation of p50 and p65 subunits of NFκB, and the activation of signal transduction and activator of transcription -1α (STAT-1α) and interferon regulatory factor-1(IRF-1). Although further studies would need to evaluate the possibility to prevent/counteract gliadin citotoxicity by dietary intake, these results suggest that lycopene, quercetin and tyrosol may represent potential non toxic agents for the control of intestinal inflammation in celiac disease by preventing the activation of important signalling transduction pathways [54]. Moreover, the beneficial anti-inflammatory effects exerted by quercetin, both *in vitro* and *in vivo*, studies seemed to be due to the inhibition of IκBα protein phosphorylation which, by blocking the activation of the NFκB pathway, consequently counteract the expression of cytokines and inducible nitric oxide synthase [115]. Similarly, in activated human mast cell line, quercetin decreased the expression of pro-inflammatory cytokines TNFα, IL-1β, IL-6 and IL-8, by inhibiting the degradation of IκBα and the nuclear translocation of p65, thus blocking NFκB activation [95]. Moreover, a recent ex vivo study demonstrated that quercetin inhibited TNFα-induced expression of the pro-inflammatory cytokines interferon-inducible protein 10 (IP-10) and macrophage-inflammatory protein-2 (MIP-2) in primary murine small intestinal epithelial cell. Quercetin exerted this effect by inhibiting the recruitment of the NFκB co-factor CBP/p300 (hystone acetil transferase) to the IP-10 and MIP-2 gene promoters, suggesting that quercetin may specifically affect chromatin remodelling at native gene promoters [116]. In LPS- and IFN-γ-treated BV-2 microglia, quercetin suppressed NO production and inducible nitric oxide synthase (iNOS) gene transcription by reducing activation of IKK, NFκB, activating protein–1 (AP-1), STAT1

and IRF-1. In addition, quercetin inhibited DNA binding activity of NFκB in a dose-dependent manner [117]. These results suggest that quercetin should provide therapeutic benefits for suppression of inflammatory-related neuronal injury in neurodegenerative diseases. In the human hepatocyte-derived cell line Chang Liver incubated with a cytokine mixture, the inhibition of mRNA expression of iNOS, COX-2, and CRP, induced by quercetin and kaempferol, was associated with a decreased concentration of phosphorylated IκBα protein and IKKα and the inhibition of NFκB activation [118]. The anti-inflammatory activity exerted by hydroxytyrosol in LPS-induced murine macrophages was determined by preventing NFκB, STAT-1α, IRF-1 activation, inhibiting, consequently, iNOS and COX-2 gene expression [59]. Tyrosol by decreasing NFκB activation, elicited similar effects on NO release and COX-2 expression, in PMA-activated RAW 264.7 macrophages [53]. Moreover, in LPS-stimulated human umbilical vein endothelial (HUVEC) cells, hydroxytyrosol, oleuropein and resveratrol, through inhibition of NFκB activation, suppressed the expression of VCAM-1 mRNA and protein, in a concentration-dependent fashion. In addition, reporter gene assays, performed with deletional VCAM-1 promoter constructs, indicated that additional transcription factors, such as AP-1 and GATA, could participate to the transcriptional regulation of VCAM [119]. In summary, all these findings strongly suggest that inhibition of the NFκB pathways, along one or several steps in their activation cascade, could be an important part of the mechanisms responsible for the potential benefit of these dietary natural agents.

POLYPHENOLS INTERACT WITH MAPK PATHWAY

Despite the central role of NFκB in the inflammation associated genes expression, this transcription factor requires assistance from other sequence-specific transcription factors among which the mitogen-activated protein kinases (MAPK) [120-122]. MAPK are a family of Ser/Thr kinases that regulate important cellular processes including cell growth, proliferation, death and differentiation by modulating gene transcription in response to changes in the cellular environment and constitute upstream regulators of transcription factor activities. The MAPK signalling pathway is a three-tiered cascade. Mammals express at least four distinctly regulated groups of MAPKs: extracellular signal-related kinases (ERK)-1/2, c-Jun amino-terminal kinases (JNK1/2/3), p38-MAP kinase (α, β, δ, and γ) and ERK5, that are activated by specific MAP kinase kinases (MAPKK) such as MEK1/2 for ERK1/2, MKK3/6 for p38, MKK4/7 (JNKK1/2) for JNKs, and MEK5 for ERK5. Each MAPKK, however, can be activated, in turn, by more than one MAPKK kinases (MAPKKK), increasing the complexity and diversity of MAPK signalling [123]. The signalling specificity is also controlled by regulation of scaffolding proteins, which can sequester and insulate signalling components and direct them to specific sub-cellular localizations, enhancing the signal flux and mediating cross-talk with other pathways [124, 125]. Among the MAPK family members, mitogens and growth factors frequently activate ERK1/2 route, while stress and inflammation constitute main triggers for the JNK and p38 cascade, sometimes referred as "stress activated protein kinases" [126]. Increasing activity of MAPKs and their involvement in the regulation of the synthesis of inflammation mediators, at the level of both transcription and translation, make them potential targets for novel anti-inflammatory therapeutics. To this end, preliminary preclinical data suggest that inhibitors that target JNK and p38 cascades, as well as IKKβ, exhibit anti-inflammatory activity, indicating a complex interaction between MAPK and NFκB in the regulation of inflammatory response [121, 127]. Recently phenolic compounds have been shown to modulate MAPK pathway by acting on several steps of the activation cascade and consequently on downstream effectors [128] *(Figure 1)*. Polyphenols such as kaempferol, chrysin, apigenin and luteolin by inhibiting all three mitogen-activated protein kinase, ERK, JNK and p38, activities have been shown to be active inhibitors of TNFα-stimulated ICAM-1 expression in respiratory epithelial cells [129]. On the other hand, in LPS-activated mouse macrophages the pre-treatment with luteolin blocked the TNFα release by inhibiting ERK1/2 and p38, but not JNK1/2 phosphorylation, suggesting a specificity of the polyphenol activity likely depending on cell types, phenolic chemical structure and concentration [130]. Consistently with this hypothesis, in activated THP-1 human monocytes cell line, quercetin and catechin reduced oxidative stress and inhibited a wide range of pro-inflammatory genes by exerting different regulatory abilities on MAPK pathways. Specifically, quercetin showed an inhibitory effect on ERK, JNK and their phosphorylated forms while catechin inhibited p38, JNK and their phosphorylated forms [131]. Moreover, in LPS-treated murine macrophages, quercetin suppressed the transcription of TNF-α, by inhibiting the phosphorylation and the activation of JNK/SAPK, while blocked the production of TNF-α protein through the inhibition of ERK1/2 phosphorylation and p38 MAPK activity [132]. An *in vitro* study demonstrated that cyanidin-3-O-glucoside inhibited, in a concentration-dependent manner, both ERK-1/2 activation and IκBα degradation and, therefore, iNOS expression. Furthermore, the study gave evidence that cyanidin-3-O-glucoside could exert its inhibitory effect by attenuating the degradation of IκBα via ERK-1/2, or by inhibiting directly ERK-1/2 activation or by both mechanisms at the same time [133]. Delphinidin and cyanidin were able to block VEGF release stimulated by the platelet derived growth factor(AB) (PDGF(AB)), by preventing activation

of p38 and JNK MAPKs in human aortic vascular smooth muscle cells [134]. The extensively studied EGCG has been shown to elicit an anti-MAPK activity able to suppress the production of several pro-inflammatory cytokines in different cell types [135, 136]. In LPS-activated murine macrophages, EGCG prevented the IL-12 production, by inhibiting phosphorylation of p38 MAPK, augmenting phosphorylation of p44/p42 ERK and nuclear protein binding to NFκB site [112]. In osteoblast-like MC3T3-E1 cells as well as in primary cultured mouse osteoblasts, EGCG significantly reduced the endothelin1-induced synthesis of IL-6 by suppressing p44/p42 MAP kinase and MEK1/2 phosphorylation [137]. Moreover, through specific phosphorylation of the p38 MAPK, EGCG protected normal human salivary acinar cells from TNFα-induced cytotoxicity. EGCG may also provide a degree of protection, partially mediated through the activation of MAPK elements, against autoimmune-induced tissue damage in Sjogren's syndrome, a lymphocytic infiltration of the salivary and lachrymal glands associated with the destruction of the secretory functions [138]. Finally, an interesting *in vivo* study, carried out in mice, demonstrated that administration of EGCG inhibited the expression of COX-2, induced by the tumor promotor 12-O-tetradecanoylphorbol-13-acetate in the skin, by blocking the activation of p38 MAPK and the DNA binding of NFκB [139]. Both catechin and quercetin, participate to the repression of plasminogen activator inhibitor 1 (PAI-1) gene expression by activating the MAPKs, p38, ERK1/2 and JNK, in a time- and dose-dependent manner, in human coronary artery endothelial cells [140]. Generally, the modulator effects of polyphenols on signalling pathways are influenced by their concentration as demonstrated for quercetin able to inhibit the release of newly synthesized IL-6 by reducing p38 and PKC-θ phosphorylation in a dose-dependent manner in IL-1 stimulated human leukemic mast cells and human umbilical cord blood-derived cultured mast cells [95, 141]. In conclusion phenolic compounds able to inhibit MAPK pathways could be considered as potential therapeutic agents against inflammatory processes.

CONCLUSIONS

The bulk of published data illustrated the emerging and promising role of polyphenolic compounds as therapeutic tools in inflammatory diseases including obesity and type II diabetes, cardiovascular diseases, neurodegenerative diseases, cancer and aging. Polyphenols appear to be important metabolic modulators by virtue of their ability to influence several cellular pathways and molecules, that have been reported as potential targets for polyphenolic compounds. However, open questions hamper the clinical use of these natural compounds. It must be noted that interactions between intracellular signalling pathways and polyphenols could have unpredictable outcomes depending on the cell type, the disease studied, and the stimulus applied. An additional crucial point concerns the consequences of the interaction or the synergistic effects between different polyphenols compounds as they could have on various intracellular targets. Further work will need to fully elucidate the molecular mechanisms of action of polyphenols in several physiological processes in order to yield important insights into their prophylactic and therapeutic uses.

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