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Anti-inflammatory properties of dietary flavonoids

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Abstract

Flavonoids are a group of natural substances that are located in sources of vegetal origin. More than 4,000 varieties of flavonoids have been identified. All of them are phenyl-benzopyrones of low molecular weight with a basic structure formed by two benzene rings united through a heterocyclic pyrane or pyrone. Besides their relevance in plants, flavonoids are important for human health. Their antioxidant capacity confers a therapeutic potential in cardiovascular diseases, gastric or duodenal ulcers, cancer or hepatic pathologies. Also important are their antiviral and anti-allergic actions, as well as their anti-thrombotic and anti-inflammatory properties. Prostaglandins and nitric oxide biosynthesis is involved in inflammation, and isoforms of inducible nitric oxide synthase (iNOS) and of cyclooxygenase (COX-2) are responsible for the production of a great amount of these mediators. It has been demonstrated that flavonoids are able to inhibit both enzymes, as well as other mediators of the inflammatory process such as reactive C protein or adhesion molecules. Modulation of the cascade of molecular events leading to the overexpression of those mediators include inhibition of transcription factors such as nuclear factor kappa B and AP-1, through the inhibition of protein kinases involved in signal transduction. Increased antioxidant defenses through activation of the NF-E2 related factor 2 (Nrf2) also contribute to the anti-inflammatory capacity of flavonoids.

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Key words: *Flavonoids. Inflammation. Oxidative stress. Nuclear factor kappa B. Nitric oxide.*

PROPIEDADES ANTIINFLAMATORIAS DE LOS FLAVONOIDES DE LA DIETA

Resumen

Los flavonoides son un grupo de las sustancias naturales que se encuentran en fuentes de origen vegetal, existiendo más de 4.000 variedades. Todos son fenilbenzopironas de peso molecular bajo con una estructura básica formada por dos anillos heterocíclicos de benceno unidos a través de un pirano o de una pirona. Además de su función en las plantas, los flavonoides son importantes para la salud humana. Su capacidad antioxidante confiere un potencial terapéutico en enfermedades cardiovasculares, úlceras gástricas o duodenales, cáncer o patologías hepáticas. También son importantes sus acciones antivirales y antialérgicas, así como sus características antitrombóticas y antiinflamatorias. La síntesis de prostaglandinas y de óxido nítrico está implicada en la inflamación, e isoformas de la óxido nítrico sintetasa (iNOS) y de la ciclooxigenasa (COX-2) son responsables de la producción de una gran cantidad de estos mediadores. Se ha demostrado que los flavonoides pueden inhibir ambas enzimas, así como otros mediadores del proceso inflamatorio tales como la proteína C reactiva o diversas moléculas de adhesión. La modulación de la cascada de los acontecimientos moleculares que conducen al aumento en la expresión de estos mediadores incluye la inhibición de factores de transcripción tales como el factor nuclear kappaB y el factor AP-1, a través de la inhibición de diferentes proteína quinasas. Otros factores, tales como el incremento de las defensas antioxidantes a través del factor Nrf2 pueden también contribuir a las propiedades antiinflamatorias de los flavonoides.

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Palabras clave: *Flavonoides. Inflamación. Estrés oxidativo. Factor nuclear kappa B. Òxido nítrico.*

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General characteristics of flavonoids

Flavonoids belong to a group of natural substances containing in their chemical structure a variable number of hydroxyl phenolic groups. They are located in sources of vegetal origin (fruits, seeds, roots, flowers, tea or wine). Their name derives from the Latin *flavus* (yellow) and many of these compounds are responsible for the coloration of flowers, yolks or leaves in autumn. Its function in plants seems to be of attracting pollinators or fruit-eating animals towards with the intention that they can disperse better the seeds. It is the case of the bromeliaceous, which develop their flowers on a species of stem formed by bracts that display an intense reddish colour before or during pollination, changing later to more greenish. In other cases flavonoids contribute to attract the prey. For example, carnivorous plants like the *Drosera*, use anthocyanins in their flowers to attract the insects that soon will devour. Sometimes, flavonoids constitute the adaptive answer of plants to the intense ultraviolet radiation. Effects such as defence against the infections, control of the breathing, participation in the photosynthesis or activation of implied bacterial genes in the fixation of nitrogen have been also described.[1,2]

More than 4,000 varieties of flavonoids have been identified. All of them are phenyl-benzopyrones of low molecular weight with a basic structure formed by two benzene rings united through a heterocyclic pyrane or pyrone. They present differences in their chemical structure that can facilitate the interaction with certain receptor molecules and/or their respective pathways within the cells, such as apoptosis, cell activation to stress and cascades of protein signalling kinases.[3] Based on his molecular structure they are divided in four fundamental groups: flavones, flavanones, flavanoles and anthocyanins (fig. 1). Flavones are characterized by the presence of a double bound in the central aromatic ring. Quercetin, one of the better known flavonoids, is a member of this group along with kaempferol, and it is in abundance in apples, onions, Brussels sprouts or fruits of citruses. Flavanones, such as naringenin or hesperedin, are located fundamentally in citruses. Between flavanoles, catechins are mainly found in green and black tea or in red wine. Finally, anthocyanins appear in strawberries, grapes, wine or tea.[4]

Besides their relevance in plants, flavonoids are important for human health. Many of these compounds are found in the human diet, but unanimity of criteria concerning the average dietary intake do not exist, having been reported values of approximately 250 mg/day in Dutch population,[5] 128 mg/day in Australian women[6] or up to 1 g/day in the USA.[7] An exact estimation is difficult, because it depends on very diverse factors, such as the compound of reference used for the analysis, or the design and methodology of the study. In any case, important differences probably exist between diverse countries, and the Mediterranean

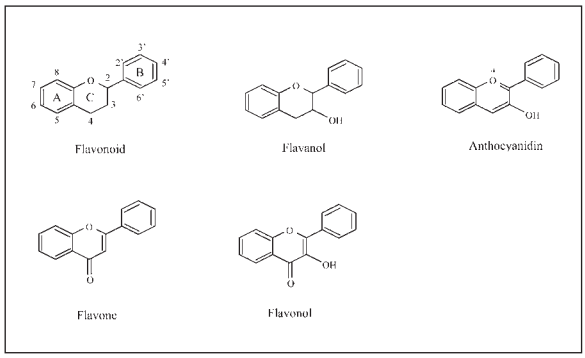

Fig. 1.—Flavonoids. Basic structure and groups.

diet, rich in fruit, olive oil of citruses or vegetables, may suppose an intake considerably greater than that in other surroundings.[3] Large differences also exist concerning content in foods, which depends, among others, on genetic factors, such as the vegetal species, light environmental conditions or the processing after the harvest.[8] Having trustworthy data on this content can have enormous importance to carry out studies on pharmacodynamic effects of flavonoids, and for a better knowledge of optimal consumption levels. Recently, the Department of Agriculture of the United States (USDA) has published the updated version of database from 2003, in which analytical values for 26 selected flavonoids in 393 foods are included.[9] The Laboratory of Data of Nutrients of USDA has released separated databases for the content in foods of isoflavones and proanthocyanidins that are equally available in the USDA Web page. Given the different biological actions derived from their chemical structure, some studies recommend the diversified consumption of flavonoids from diverse nutritional sources and, at the present time, the possibility of adding specific flavonoids to food which do not naturally contain them is being explored.[10]

Flavonoids seem to play an important role in human health and to possess beneficial effects in the prevention of human diseases. A fundamental property of these molecules, responsible for many of their beneficial effects, is the antioxidant capacity, linked to the presence of a series of structural characteristics that allow them, among others, to quelate ions of transition metals such as Fe^{2+}, Cu^{2+} or Zn^{2+}, to catalyze the electron transport, to scavenger reactive oxygen species (ROS) like the superoxide anion, oxygen singlet and lipidic peroxyradicals, or to stabilize free ROS by means of the hydrogenation or formation of complexes with oxidating species.[11] The antioxidant capacity of flavonoids confers a therapeutic potential in diseases between which cardiovascular diseases[12], gastric or duodenal ulcers,[13] cancer[14] or hepatic pathologies are included.[15] Also important are their antiviral and antiallergic actions, as well as their anti-thrombotic and anti-inflammatory properties.[4] This last one constitute

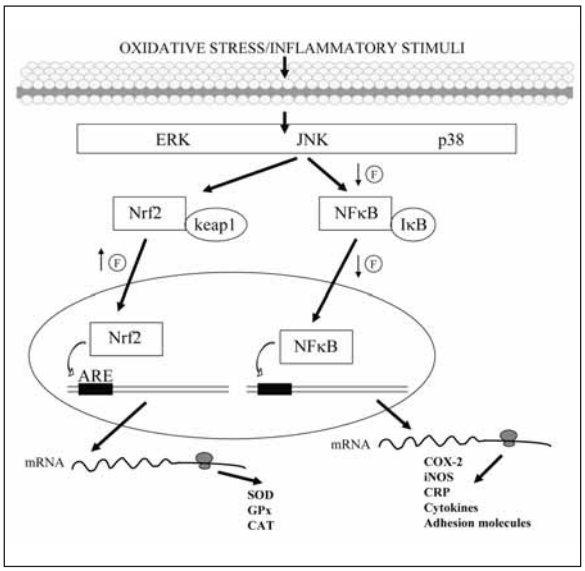

Fig. 2.—Schematic model for some anti-inflammatory effects of flavonoids.

an aspect used for a long time in the Chinese traditional medicine and the cosmetic industry under the form of plant extracts, but recently it has began to be explored in depth, in order to identify the mechanisms responsible and the possibility for use of flavonoids as anti-inflammatory agents.

Flavonoids and inflammation

Inhibition of inflammation-related enzymes

Effects of flavonoids on a variety of inflammatory processes have been object of diverse reviews[16] and it has been demonstrated that they are able to inhibit a series of enzymes which are activated in the course of the inflammatory process.[17] Prostaglandins and nitric oxide biosynthesis is involved in inflammation, and isoforms of inducible nitric oxide synthase (iNOS) and of cyclooxygenase (COX-2) are responsible for the production of a great amount of these mediators. *In vitro* studies have confirmed that the flavonoid quercetin inhibits nitric oxide production and the expression of iNOS.[18] Differences between various flavonoids exist and, for example, quercetin and kaempferol show little differences in their inhibiting capacity of the expression of iNOS in RAW264.7 cells,[19] but the second inhibits to a greater extent than quercetin nitrite accumulation in culture medium of lipopolysaccharide (LPS)-stimulated J774.2 cells.[20] Although the inhibition of iNOS can contribute to the anti-inflammatory effect of flavonoids, the mechanism responsible for such effect is not known in depth. Thus, while in lung adenocarcinoma cell lines quercetin downregulates iNOS at translational level, inhibiting nitric oxide production and protein level,[21] transcriptional effects have been described in interleukin 1β-activated hepatocytes.[18] Conflicting results have also been reported concerning regulation of COX-2 expression. Thus, quercetin downregulates COX-2 expression in macrophages.[21] However, it increases COX-2 expression in human-derived colon cancer cells[23] or does not modify expression in human lung carcinoma cells.[19] Moreover, a unique dose of quercetin is able to reduce the expression of COX-2 in human lymphocytes *in vivo* but not *ex vivo*.[24] Our group has recently demonstrated that both quercetin and kaempferol reduce iNOS and COX-2 protein levels in hepatic cells of the Chang Liver line,[25] agreeing with similar descriptions of the effect of quercetin in RAW 264.7 macrophages[26] or kaempferol in mouse macrophages.[22]

Effects on transcription factors activation

There are several critical steps at which flavonoids can modulate the cascade of molecular events leading to the overexpression of iNOS or COX-2. These include inhibition of protein kinase C, phospholipase C or A2 and phosphodiesterases,[3] indirect modulation of iNOS by inhibition of the ciclooxygenase and/or lipooxygenase pathways,[27] inhibition of transcription factors AP-1 and C/EBPd,[28,29] or effects on the interferon regulatory factor (IRF)-1 and Akt signaling pathway.[30] In any case, pathways of induction of iNOS and COX-2 seem to converge in the activation of a transcription essential for the expression of proinflammatory genes, the nuclear factor kappa B (NF-kappaB).[31] The NF-kappaB is one of the main inducible transcription factors whose modulation triggers a cascade of molecular events, some of which can constitute potential key targets for the treatment of the inflammation. In alterations coursing with inflammation it exists an activation of cells, such as macrophages, which release cytokines (i.e. tumour necrosis factor alfa, TNFα), as well as ROS. The ROS can contribute to the appearance of oxidative stress, mainly in those cases in which an imbalance with enzymatic and nonenzymatic (glutathione, vitamins, and probably flavonoids) antioxidant defenses exist.[32,33] In situations that course with oxidative stress, this can be an important stimulus for the activation of NF-kappaB, which appears in latent form in the cytoplasm of nonstimulated cells, forming a complex with its inhibitors, the IkappaBs (IkappaBα and IkappaBβ).[34] When the cell is stimulated, NFkappaB factor activates by means of the phosphorylation and degradation of the IkappaB proteins and migrates to the nucleus, stimulating the expression of its target genes.[35] The phosphorylation of IkappaB involves two IkappaB kinases, IKKα and IKKβ. The degradation of IkappaBα results in rapid changes in the induction of NF-kappaB, whereas the degradation of IkappaBβ is associated with a prolonged activation of NF-kappaB.[36] Once activated, NFkappaB can stimulate the expression of iNOS, with an increase in the nitric oxide formation.[37] The reaction of this one with ROS,

such as the superoxide anion, produces the formation of peroxynitrite, which additionally contributes to cellular injury.[38] We have demonstrated in an experimental model of ischemia-reperfusion how antioxidant substances such as glycine can block in parallel oxidative stress, the activation of NF-kappaB and nitric oxide production.[38] Studies on models of inflammation in isolated hepatocytes also confirm that the protective effects of the immunosuppressant agents FK506 and rapamycin are also related to the inhibition of the commented factors.[39]

Different studies have shown that flavonoids can modulate the NF-kappaB signalling pathway during inflammation and modify through this the expression of genes involved in the inflammatory process (fig. 2).[40,41] Reports concerning the relationship between flavonoids and the NF-kappaB pathway are, however, conflicting. Thus, it has been reported that quercetin does not reduce NF-kappaB activation in the renal cortex of rats with established chronic glomerular disease,[42] and that while diverse flavonoids downregulate iNOS expression in RAW264.7 cells, they do not suppress DNA binding activities of NF-kappaB.[19] It also appears that only some flavonoids such as kaempferol suppress diverse tyrosine kinase-mediated signalling pathways in prostate cancer cells[43] or inhibit TNFα production and TNFα-induced NF-kappaB traslocation in osteoblasts.[44] On the contrary, both quercetin and kaempferol diminish the degradation of IkappaB through the regulation of members of the IKK complex.[25] It has also been demonstrated that quercetin prevents LPS-induced IkappaB phosphorylation in bone marrow macrophages,[41] inhibits the activation of NF-kappaB induced by interleukin 1β in murine fibroblasts[45] and reduces IkappaBα and IkappaBβ phosphorylation in peripheral blood mononuclear cells.[16] Moreover, quercetin inhibits iNOS gene transcription induced by LPS in mouse BV-2 microglia through an effect mediated by attenuation of IkappaB phosphorylation[46] or abrogates in parallel iNOS overexpression and the activation of NF-kappaB in rat hepatocytes activates by interleukin 1β.[18] Curcumin reduces hepatic inflammation in mice with experimental steatohepatitis in parallel to an inhibition of NF-kappaB activation and the expression of proinflammatory genes,[47] and recently a relation between changes in NF-kappaB activation induced by coca flavonoids and the expression of COX-2 has been shown in mouse skin cells.[48] It has been also reported that targeting of NF-kappaB can contribute to the inhibition COX-2 expression by anthocyanidins in RAW 264 cells[29] or by amenthoflavone in A549 cells.[49]

Reactive C protein (CRP) is an acute phase reactant whose elevation in serum is considered as indicator of chronic inflammation, and whose interaction with endothelial cells may be the mechanistic link with atherosclerosis.[50] It has been documented that CRP concentration has a predictive value in cardiovascular disease, induces adhesion molecules expression[51] and presents another series of proatherogenic effects in endothelial cells.[52] Although it had been indicated that plasma concentration of CRP is not related to flavonoid intake,[53] recent data demonstrate that diverse flavonoides may reduce CRP protein level in hepatic cells and that the effect is dose-dependent (fig. 2).[25] It is known that IL-6 induces CRP by means of a mechanism that involves NF-kappaB activation,[54,55] and that alterations in the intracellular state redox cause an inhibition of the nuclear NF-kappaB traslocation and of CRP synthesis in hepatocytes.[56] Moreover, overexpressed NF-kappaB can participate in the induction of CRP in HepG2 cells by enhancing the effect of C/EBPbeta and the activator of transcription-3.[57] It is therefore probable that effects of flavonoids on CRP expression could be mediated, at least partly, by the modulation of the NF-kappaB-dependent pathway.

Effects on adhesion molecules

Endothelial dysfunction has an enormous importance in the inflammatory processes, and involves changes in the expression of endothelial adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin, among others,[58,59] able to trigger the inflammatory process by stimulating the migration and adhesion of leukocytes and originating tissue damage.[60] NF-kappaB activation is a necessary step in the transcripcional induction of adhesion molecules,[61] as confirms the fact that blockers of NFkappaB also inhibit VCAM-1 expression in endothelial cell stimulated by the TNFα.[62] In addition, activated endothelial cells release IL-6 that stimulate hepatocyte fibrinogen and CRP production,[63] which contributes to the exacerbation of endothelial dysfunction. Flavonoids, in addition to his capacity to inhibit *in vitro* lipoprotein oxidation and to their antithrombotic effects, seem to also exert their beneficial action in cardiovascular diseases by modulating monocyte adhesion during the atherosclerotic inflammatory.[64] Although all the mechanisms involved in this effect are not known with exactitude, it has been indicated that they could inhibit the expression of inflammatory mediators such as ICAM-1, by acting on NFkappaB activation.[65,66] Nevertheless, it has also been shown that some flavonoids inhibit the expression of adhesion molecules in activated endothelial cells by process independent from NF-kappaB.[67]

Modulation of proinflammatory gene expression

Effects of flavonoids on the binding capacity of transcription factors such as NF-kappaB or AP-1 may be regulated through the inhibition of protein kinases involved in signal transduction, such as protein kinase C (PKC) and mitogen activated protein kinase (MAPK) (fig. 2). In macrophages and other cell types, LPS activates three kinds of MAPs, extracellular signal related kinase (ERK), Jun N-terminal kinase/stress activated

protein kinase (JNK/SAPK) and p38 kinase.[68] It has been reported that quercetin inhibits iNOS expression through inhibition of p38 MAPK[69] and that bocks AP-1 binding in LPS-induced RAW cells by inhibiting JNK/SAPK.[70] Data from LPS-activates macrophages also show that quercetin is able to suppress proinflammatory cytokines and NF-kappaB activation through ERK and p38 MAPK.[40] In RAW 264.7 cells, luteolin also inhibits LPS-stimulated pathways through inhibition of some MAP kinases such as ERK and p38 MAPK.[71]

Modulation of redox state

An additional mechanism which could contribute to the anti-inflammatory properties of flavonoids is the modulation of the redox state by increases in the endogenous antioxidant defense potential (fig. 2). Organisms have developed a variety of antioxidant defense systems as protection from ROS. The major endogenous antioxidant systems include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). SOD catalyzes the dismutation of the superoxide radical anion, and there is a Mn SOD localized in the mitochondria and a Cu/Zn SOD mainly localized in the cytosol. CAT and GPx convert H_2O_2 to H_2O, and GR recycles oxidised glutathione back to reduced glutathione.[72] The NF-E2 related factor 2 (Nrf2), a member of the cap'n'collar family of basic leucine zipper transcription factors, is a redox-sensitive factor whose nuclear translocation and binding to the antioxidant response elements (ARE) in their promoter regions may result in induction of antioxidant enzymes.[73] Thus, binding of Nrf2 to AREs of GPx2 in lungs[74] or Cu/Zn SOD[75] in liver have been reported, and Nrf2 is also involved in the regulation of catalase and other antioxidant enzymes in the fibroblasts.[76] Although changes in Nrf2 signaling in inflammatory diseases is not yet fully addressed,[77] it is known that polyphenols such as curcumin or epigallocathechin-3-gallate are Nrf2-ARE activators.[78,79]

Limitations

In spite to the described beneficial effects, it is necessary to be cautious when analyzing potential beneficial effects of flavonoid administration, because these molecules may act as prooxidants at high doses. Thus, quercetin and other flavonoids such as myricetin increase hydroxyl radical production and DNA damage at concentration > 30 µM,[80] quercetin at concentrations exceeding 75 µM has some intrinsic citotoxicity in cultured renal tubular cells,[81] and quercetin at high dose may present genotoxic effects.[82] Moreover, it has been recently reported that quercetin at concentrations > 50 µM is able to participate in the oxidation of NADPH in liver cells, shifting the cellular conditions to a more oxidized state.[83] This prooxidant character may explain why exposure of rat aortic smooth muscle cells to quercetin concentrations > 100 µM increases NFkappaB activation[84] or the fact that similar quercetin doses increase iNOS and COX-2 expression in parallel to the stimulation of the NF-kappa B-dependent pathway in liver cells.[25]

An additional aspect to stand out is the limited bioavailability of flavonoids, due to its low absorption and rapid elimination. Aglycons and glucosides are absorbed in the small intestine, but they are transformed quickly into methylated, sulfated or glucuronic acid-conjugated derivative. Bacteria of the colon play an important role in the metabolism and absorption of flavonoids and the derivatives do not necessarily present the same biological activity than the original compounds.[85] Consequently, although the numerous studies published with *in vitro* approaches allow to identify molecular mechanisms of flavonoid effects, data generated must be validated in humans and it is necessary to be very careful when extrapolating results of *in vitro* experiments with purified compounds to *in vivo* situations. Whatever it is the case, the data nowadays available make clear the potential utility that have dietary flavonoids or new flavonoid-based agents for the possible treatment of inflammatory diseases.

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