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Effects of Naturally Occurring Flavonoids on Inflammatory Responses and Their Action Mechanisms

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Abstract – Flavonoids are natural polyphenolic compounds widely distributed in plant kingdom. Although many flavonoids were found to show anti-inflammatory activity *in vitro* and *in vivo*, the potency of anti-inflammatory activity was not enough for a clinical trial. Thus, a search for finding potential flavonoid molecules is continuing. In this review, *in vivo* anti-inflammatory activity of various flavonoid derivatives is summarized mainly based on the results obtained in authors' laboratories. Among them, several biflavonoids such as amentoflavone and ginkgetin were found to possess anti-inflammatory activity on animal models of acute/chronic inflammation comparable to nonsteroidal and steroidal anti-inflammatory drugs currently used. In respect of their action mechanisms, the effects on arachidonic acid metabolism and nitric oxide production were described. Some flavonoids directly inhibit cyclooxygenase and/or lipoxygenase. Biflavones such as ochnaflavone and ginkgetin are inhibitors of phospholipase A_2. In recent studies, certain flavonoids were also found to suppress cyclooxygenase-2 and inducible nitric oxide synthase expression induced by inflammatory stimuli. Therefore, it is suggested that anti-inflammatory activity of the certain flavonoids (mainly flavones, flavonols and biflavonoids) may be mediated by direct inhibition of arachidonic acid metabolizing enzymes as well as suppression of the enzyme expression involved in inflammatory responses.

Key words – Flavonoid, biflavonoid, flavone, inflammation, cyclooxygenase, lipoxygenase, phospholipase, nitric oxide synthase, lymphocyte proliferation

Flavonoids, one of abundant classes of plant constituents, are known to be nature's tender drugs showing various biological/pharmacological activities such as anticancer, antimicrobial, antiviral, anti-inflammatory, immunomodulatory, and antithrombogenic activities, etc. Among these activities, anti-inflammation of flavonoids is thought to be mediated, at least in part, by inhibition of cyclooxygenase (COX) /lipoxygenase (LO) as well as their antioxidative nature (Bauman *et al.*, 1980; Havsteen, 1983). Although there have been numerous reports to show anti-inflammatory activity of flavonoids from many medicinal plants as active principles (For review, Gabor, 1986; Lewis, 1989), a few studies were previously carried out to establish the structural-activity relationships. Therefore, it is meaningful to elucidate anti-inflammatory activity of various flavonoids not only for finding anti-inflammatory principles in the medicinal plants, but also for developing a new class of anti-inflammatory agents. In this review, *in vivo* anti-inflammatory activity of flavonoids and their cellular action mechanisms are discussed mainly based on the experimental results obtained in our laboratories.

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In Vivo Anti-inflammatory Activity of Flavonoids

For comparing *in vivo* anti-inflammatory activity of various flavonoid derivatives (Fig. 1), mouse ear edema bioassay, one of animal models of acute inflammation, was employed according to the previously published procedure of Kim *et al.* (1993). Ear edema was induced by applying croton-oil or arachidonic acid (AA). Cróton-oil and its major constituent, phorbol ester, are known to induce the prolonged swelling of ear by activation of protein kinase C pathway. In contrast, AA topically applied is converted to prostaglandins (PG) and leukotrienes (LT) by COX/LO from epidermal keratinocytes,

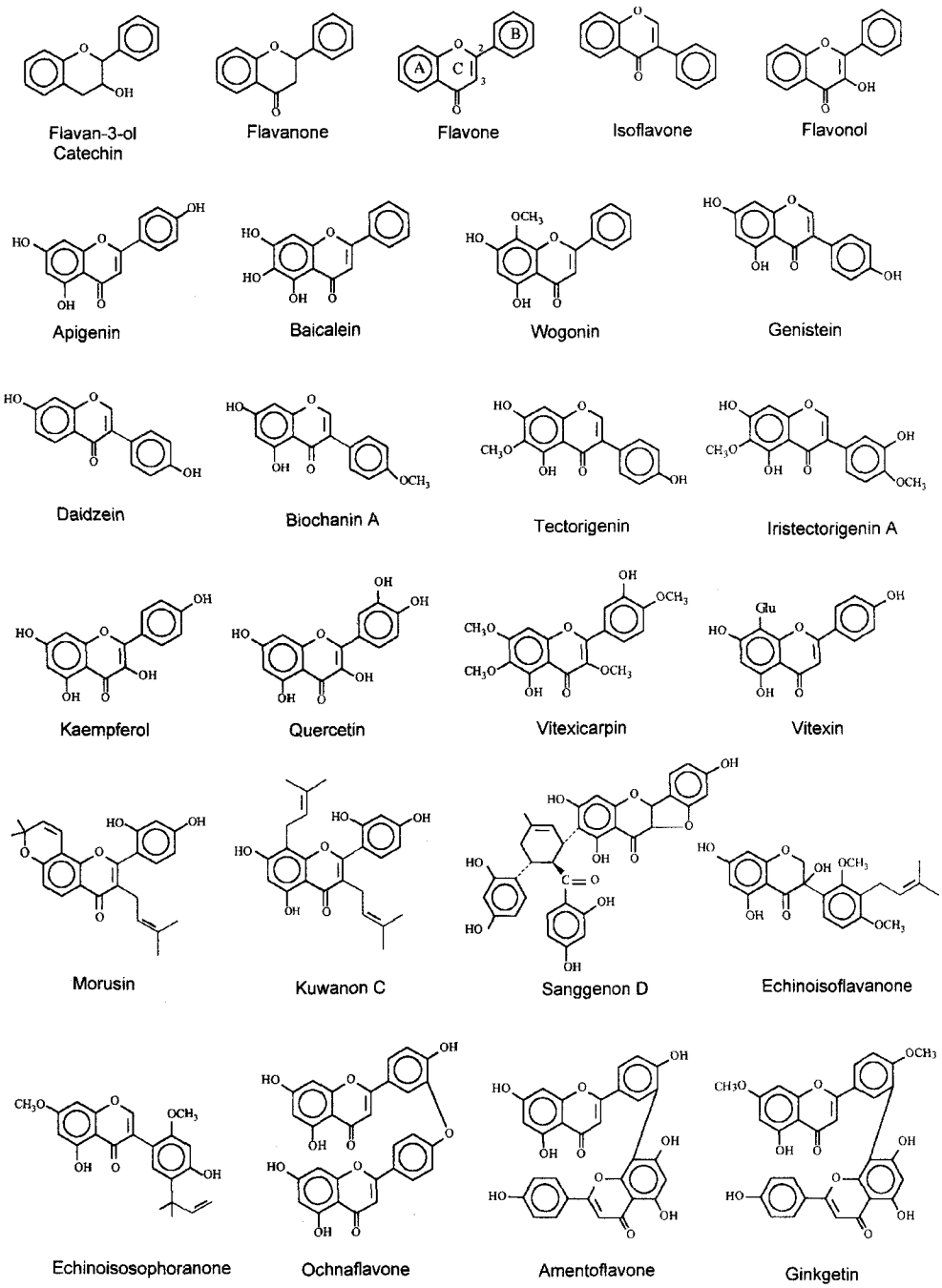

Fig. 1. Chemical structure of some representative flavonoids.

dermal fibroblasts and neutrophils infiltrated. These eicosanoids provoke erythema and edema (classical features of acute inflammation) peaking around 1 h after AA application, while edema induced by croton-oil was increased up to 5 h after an application of inflammagen and maintained for approximately 24 h.

When we checked the inhibitory activity of flavonoid aglycones commercially available and flavonoid glycosides isolated from various plant

materials via oral or topical administration, flavonoids having a C-2,3-double bond (planer ring system) generally inhibited ear edema at 100 mg/kg, p.o. or 2 mg/ear, topical (Kim *et al.*, 1993; Lee *et al.*, 1993). But the potencies of inhibition by flavonoids were much less than that of reference compounds, indomethacin and hydrocortisone. It should be emphasized that most flavones/flavonols/isoflavones tested exhibited relatively potent inhibition (40-72% inhibition) especially in AA-induced ear edema when topically applied at 2 mg/ear. The potent inhibition of the topically applied flavones/flavonols/isoflavones against AA-induced mouse ear edema suggest that they actually behave as COX/LO inhibitors *in vivo* as well as *in vitro*. From these studies, a C-ring 2,3-double bond is found to be important for *in vivo* anti-inflammatory activity and their potencies were depending on the patterns and numbers of hydroxylation(s) on A/B-ring. 5,7-Hydroxylations on A-ring and 4'-hydroxylation on B-ring were favorable. Flavonoid glycosides tested showed the comparable activity with their aglycones when administered orally. However, no structural activity relationship was found depending on the positions or types of sugar substitutions in flavonoid glycosides. Since previous publications demonstrated that flavonoid glycosides showed much less inhibitory activity *in vitro* against COX/LO (Ferrandiz *et al.*, 1990; Laughton *et al.*, 1991), it is reasonable to think that these glycoside derivatives would be hydrolyzed to their aglycones or other metabolic compounds before exhibiting anti-inflammatory activity. Thus, the differences of *in vivo* anti-inflammatory activities of flavonoid glycosides may be due to the differences of their bioavailability and/or metabolism, but not due to the differences of their intrinsic activity. And it is worth to mention that C-glycosides of apigenin (flavone), vitexin and isovitexin, also showed the comparable activity *in vivo* with O-glycosides of flavones/flavonols (Kim BJ *et al.*, 1998).

For comparing *in vivo* anti-inflammatory activity of isoflavonoids, 7 isoflavones having a C-2,3-double bond isolated from Pueraria radix and 6 7-O-alkylated biochanin A derivatives chemically synthesized were tested using the same animal model described above. Isoflavones including daidzein and puerarin were revealed to show anti-inflammatory activity although less active than flavones/flavonols (Lee *et al.*, 1994). When 7-O-alkylated derivatives of biochanin A were topically applied in AA-induced edema, 7-O-ethyl and 7-O-isopropylbiochanin A showed the inhibitory activity (52-54% inhibition at 2 mg/ear) compared with biochanin A (51%), while 7-O-isobutyl and 7-O-allylbiochanin A showed much less activity. A certain size limitation of the substituents at C-7 would be existed and 7-O-substitutions with bulky molecules may not be favorable for expressing *in vivo* anti-inflammatory activity.

All of our previous findings clearly demonstrated that the certain flavonoids including flavonoid glycosides possessed *in vivo* anti-inflammatory activity against acute inflammation and a C-2,3-double bond might be important. Their intrinsic activities were depending on the patterns and numbers of hydroxylation/methoxylation. In flavonoid glycosides, *in vivo* anti-inflammatory activity seems to be depending on their bioavailability/metabolism, not depending on their intrinsic activity. However, flavonoids tested above may not be suitable for a clinical trial mainly because of their weak activities compared with the currently used anti-inflammatory drugs, nonsteroidal and steroidal drugs such as indomethacin and prednisolone.

In Vivo Anti-inflammatory Activity of Biflavonoids

As stated below, ochnaflavone and several other biflavones were found to be phospholipase A_2 (PLA_2) inhibitors (Chang *et al.*, 1994) and they inhibited arachidonic acid release from the activated macrophages (Lee *et al.*, 1997). In addition, amentoflavone was demonstrated to be a COX-1 inhibitor (Kim HP *et al.*, 1998). Some of them were also revealed to inhibit lymphocyte proliferation (Lee *et al.*, 1995). These findings strongly suggested their anti-inflammatory potentials especially on chronic inflammation.

Amentoflavone, a biflavone isolated from Sellaginella species or Ginkgo leaves, showed potent anti-inflammatory activity *in vivo* (Kim HK *et al.*, 1998). It possessed approximately 1/2-1/5 anti-inflammatory activities against several animal models of acute inflammation compared to indomethacin or prednisolone. Amentoflavone also possessed potent analgesic activity by intraperitoneal injection comparable to indomethacin. Amentoflavone, however, did not significantly reduce adjuvant-induced arthritis (AIA) in rats despite of potent inhibition against COX-1 (Kim HP *et al.*, 1998). In contrast, ginkgetin reduced the arthritic inflammation (secondary inflammation) dose-dependently at 5-20 mg/kg/day by intraperitoneal

injection in AIA-induced arthritis in rats, a chronic inflammatory animal model (Kim *et al.*, 1999b). As far as our knowledge, this finding might be the first to demonstrate the antiarthritic potential of biflavonoid. The inhibitory activity of ginkgetin against AIA was also confirmed by histologic observation of the affected paws, in which there were much less inflammatory cells infiltrated and almost no signs of inflammation at synovial membrane. Potency of inhibition by ginkgetin was 1/2-1/3 compared to that of prednisolone. No severe side effect was found during the experiment for 25 days. While prednisolone showed potent thymus and spleen atrophy because of pituitary-adrenal axis suppression, ginkgetin did not reduce thymus and spleen weights in adjuvant-treated rats. Ginkgetin also possessed analgesic activity comparable to indomethacin (IC_{50} for ginkgetin = 8.7 mg/kg, IC_{50} for indomethacin = 3.8 mg/kg). In view of weak *in vivo* activity of conventional flavonoids such as quercetin and kaempferol, the potent *in vivo* activity of biflavonoids may be strong enough for a clinical trial. Our finding is significant since many investigators including Pelzer *et al.* (1998) have claimed that flavonoids possess anti-inflammatory activity against acute inflammation, but they are not suitable on chronic inflammation. If oral or topical bioavailability of biflavonoids is improved, our study may lead to developing new type of anti-inflammatory drug from natural products.

Cellular Action Mechanisms of Flavonoids

Effects on arachidonic acid metabolism – Cyclooxygenase (COX) produces PGs from AA, while lipoxygenase (LO), 5-, 12- and 15-LO, are the enzymes responsible for generating hydroxy acids and LTs. Effects of several classes of flavonoids on COX, 5,-12-, and 15-LO enzyme activities were described previously (Bauman *et al.*, 1980; Havsteen, 1983). These inhibitory activities could explain, at least in part, anti-inflammatory and/or antiallergic activities of flavonoids. Certain flavones/flavonols such as 3-hydroxyflavone, kaempferol, fisetin and quercetin strongly inhibited 5-LO (Ferrandiz *et al.*, 1990; Laughton *et al.*, 1991). Flavone was known as a COX inhibitor (Welton *et al.*, 1988). Baicalein, 5,6,7-trihydroxyflavone isolated from Scutellaria radix, was reported as a selective and potent inhibitor of 12-LO (Sekiya *et al.*, 1982). Prenylated flavonoids such as morusin and artonin E were demonstrated as potent 5-LO inhibitors (Reddy *et al.*, 1991). Recently, our investigation has shown that isoflavones such as daidzein and tectorigenin isolated from Pueraria radix and Belamcamda radix possessed the inhibitory activity on COX from human platelets at 50-100 uM, although not very potent (You *et al.*, 1999). When biflavonoids were examined, amentoflavone was revealed to be a selective and potent inhibitor of COX without affecting LO activity from guinea-pig epidermal homogenate. IC_{50}'s for amentoflavone and indomethacin were 3 and 1 uM, respectively. However, a structurally similar biflavone, ginkgetin, did not show the inhibitory activity significantly against COX or LO activity from guinea-pig epidermis (Kim HP *et al.*, 1998) and human platelets (Kim HP, unpublished results).

COX is now known as COX-1 (constitutive) and COX-2 (inducible). Although various flavonoids inhibited COX-1 activity as described above, the flavonoids directly inhibiting COX-2 activity were rarely reported. One previous investigation has demonstrated that several flavan-3-ols weakly inhibited COX-2 at pharmacologically unobtainable concentrations, being more active on COX-1 (Noreen *et al.*, 1998). When the various flavonoids were checked for their inhibitory activity on COX-1 and COX-2 in order to find selective COX-2 inhibitor, quercetin and several prenylated flavonoids moderately inhibit COX-2, but the selectivity over COX-1 was generally low (Kim HP, unpublished results). In particular, wogonin isolated from Scutellaria radix selectively inhibits COX-2 activity from the homogenate of LPS-induced RAW 264.7 cells without inhibiting COX-1 activity from human platelet homogenate (Chi *et al.*, 2000). Further investigation concerning effects of flavonoids on COX-2 is definitely needed to understand cellular mechanism of anti-inflammatory activity *in vivo*. Concerning the modulation of COX-2 expression, several recent studies have shown that the flavonoid derivatives such as apigenin, tectorigenin and ginkgetin suppressed the induction of COX-2 (Liang *et al.*, 1999; Kim YP *et al.*, 1999; Baek *et al.*, 1999). Therefore, it is suggested that the down-regulation of COX-2 induction seems to be one of anti-inflammatory mechanisms of certain flavonoids.

Phospholipase A_2 (PLA_2) is a hydrolyzing enzyme liberating sn-2 fatty acid from glycerophospholipid family. The reaction products are lysophospholipid that may be metabolized to platelet activating factor,

and fatty acid, mostly arachidonic acid further converting to eicosanoids by COX/LO described above. Several different PLA_2 were found in cells including low molecular weight secretory PLA_2 ($sPLA_2$) and high molecular weight cytosolic PLA_2 ($cPLA_2$). And these PLA_2's are known to be coupled with COX depending on types of stimulation and to involve in inflammatory responses (Dennis, 1997; Naraba *et al.*, 1998). Therefore, a modulation of PLA_2 activity is important to control inflammatory processes. Among flavonoids, quercetin was reported to inhibit PLA_2 (Lindahl and Tagesson, 1993). And several polyhydroxylated flavonoids from Scutellaria radix were also shown to possess weak inhibitory activity of human recombinant PLA_2-IIA (Gil *et al.*, 1994). During our experiment, ochnaflavone (biflavone) was found to be a relatively selective inhibitor of $sPLA_2$ (PLA_2-IIA) from rat platelets (Chang *et al.*, 1994). Other biflavones tested such as amentoflavone and ginkgetin showed similar inhibitory activity. The PLA_2 inhibitory activity of these biflavones was also proved in rat peritoneal macrophages. They concentration-dependently inhibited AA release from the activated macrophages in culture (Lee *et al.*, 1997). The following study by Gil *et al.*, (1997) that morelloflavone, another biflavonoid, possessed the inhibitory activity of PLA_2 supported our initial finding. Recently, bilobetin and ginkgetin were again found to be PLA_2 inhibitors from several different sources (Kim YP *et al.*, 1999). These findings indicate that certain biflavonoids are PLA_2 inhibitors. Therefore, PLA_2 inhibition may also contribute to anti-inflammatory activity of biflavonoids.

Effects on nitric oxide (NO) production – NO is one of the cellular mediators of physiological and pathological processes (Moncada *et al.*, 1991; Nathan, 1992). NO is biochemically synthesized from L-arginine by nitric oxide synthase (NOS). Among iso-forms so far being identified, iNOS (inducible NOS or NOS type 2) is an inducible enzyme from various cell types when activated by bacterial lipopolysaccharide (LPS) and/or cytokines. iNOS produces micromolar concentration of NO for prolonged period, which affects inflammatory process. Therefore, a modulation of NO production by iNOS may be a new therapeutic target in association with inflammatory diseases. Previously, flavone and several other aminosubstituted flavones were reported to inhibit NO production (Krol *et al.*, 1995). Epigallocatechin gallate was also found to weakly inhibit NO production and reduced mRNA expression of iNOS from RAW 264.7 cells (Chan *et al.*, 1997). Genistein, a tyrosine kinase inhibitor, also inhibited NO production (Sadowska-Krowicka *et al.*, 1998). Several flavonoid derivatives including quercetin, apigenin and morin were revealed to inhibit NO production from LPS-activated C6-astrocytes (Soliman and Mazzio, 1998). Although these results have shown that some flavonoid derivatives inhibited NO production from several cell types, structural-activity relationships of flavonoids were not clearly established. Moreover, it was not clear whether flavonoids directly inhibit iNOS activity or not. Therefore, we have initially evaluated effects of 26 flavonoid derivatives including flavonoid glycosides on NO production from LPS-activated RAW 264.7 cells, and it was found that a C-2,3-double bond in flavonoid molecule was a prerequisite for inhibition of NO production by flavonoids (Kim HK *et al.*, 1999a). Flavones such as apigenin, luteolin and wogonin showed the most potent inhibition of NO production (IC_{50}'s were 17-23 uM). Isoflavones including tectorigenin and flavonols such as quercetin and myricetin, but not morin, also inhibited NO production, being less potent than the flavone derivatives. Flavonoid glycosides were inactive regardless of types of sugar substitutions as well as types of linkages (C-C or C-O-C). The active flavonoids such as wogonin and quercetin did not reasonably inhibit iNOS enzyme activity directly. In contrast, these flavonoids reduced iNOS expression revealed by western blotting. Therefore, the cellular mechanism of the reduced production of NO by flavonoids may be down-regulation of iNOS, not direct inhibition of iNOS activity. When other types of flavonoids were tested, biflavonoids including ginkgetin and several prenylated flavonoids including sanggenon D reduced NO production by down-regulating iNOS expression without affecting enzyme activity (Cheon *et al.*, 2000). The only exception among the flavonoids so far being tested is echinoisoflavanone that suppresses iNOS expression and directly inhibits iNOS activity. Taken together, the certain flavonoids inhibit NO production by down-regulating iNOS expression. The suppression of iNOS as well as COX-2 expression may also contribute to the anti-inflammatory activity of flavonoids *in vivo*.

Effects of flavonoids on other types of NOS are not fully understood yet. Apigenin and quercetin were previously reported to directly inhibit NADPH

diaphorase from mouse brain, probably a neuronal form of NOS (Tamura *et al.*, 1994). This study suggests that flavonoids may affect isoforms of NOS differently. This point will be unveiled by further study.

Effects on lymphocyte proliferation–Lymphocytes are one of the main cells participating in inflammatory reaction, especially in chronic inflammatory disorders such as rheumatoid arthritis. Since various flavonoid derivatives showed the immunosuppressive activity *in vivo* such as allergic response, it may be valuable to find effects of flavonoids on lymphocyte proliferation. Previously, flavonoids including quercetin, tangeretin, kaempferol and isoflavones showed the suppression of Con A or PHA-induced lymphocyte proliferation (Mookerjee *et al.*, 1986; Pignol *et al.*, 1988; Hirano *et al.*, 1989), indicating that some flavonoid derivatives suppressed lymphocyte proliferation. However, it was far from clear to establish the structural-activity relationships of flavonoids and specificity of suppression. We have examined about 70 flavonoid derivatives and demonstrated their suppressive effects depending on chemical structures of flavonoids and types of stimulation *in vitro* (Namgoong *et al.*, 1994a and 1994b). From the results, flavones/flavonols/isoflavones having a C-2,3-double bond were found to suppress mainly T-cell proliferation (Con A-induced and mixed lymphocyte reaction) at 1-10 uM, but not B-cell proliferation induced by LPS at the same concentration ranges. The suppressive activity was reversible and dependent on time of addition. Flavonoids affected initial inductive phase of lymphocyte activation. In contrast, flavonoids tested did not affect the spontaneous proliferation of lymphocytes. These results and other findings led to the conclusion that most flavonoids may affect preferentially stimulated and/or activated cells, but not resting normal cells as suggested by Middleton (1998). During our study, vitexicarpin (flavonol) isolated from *Vitex rotundifolia* (fruits) was found to be the most potent inhibitor of lymphocyte proliferation (You *et al.*, 1998). Vitexicarpin strongly inhibited T-cell as well as B-cell proliferation (IC_{50} for both = 0.7 uM), while it showed about 10-fold less activity in mixed lymphocyte reaction (IC_{50} = 6 uM). When biflavonoids were tested, biflavones such as ochnaflavone, ginkgetin and isocryptomerin were active (IC_{50} = 0.1-10 uM), whereas amentoflavone and sciadopitysin were not (Lee *et al.*, 1995). The active biflavones were found to be general inhibitors of lymphocyte proliferation, i.e. T-, B-cell proliferation and mixed lymphocyte reaction, compared to flavones/flavonols mainly effecting T-cells as stated above. Ginkgetin also inhibited immunoglobulin M secretion by LPS-activated human PMMC at less than 10 uM (Kim HK and Kim HP, unpublished result). In respect of participation of lymphocytes in inflammatory diseases, inhibition of lymphocyte proliferation by flavonoids may also contribute to their anti-inflammatory and antiallergic activity *in vivo*.

Effects of flavonoids on other cellular signal transduction and enzymes involved – Inflammatory process consists of various cellular and molecular aspects in many different tissues and cells. In inflammatory region, cells are activated/stimulated by different agonistic signals. After initial activation/stimulation, cellular signal transduction is followed leading to the final outcome (synthesis of inducible enzymes, synthesis and secretion of cytokines, eicosanoid generation, etc.). Thus, modulations of signal transduction pathways by flavonoids contribute to their anti-inflammatory activity *in vivo*. Flavonoids were demonstrated to inhibit protein kinase C (Ferriola *et al.*, 1989). Some flavonoids inhibit other kinases such as protein tyrosine kinase (Chang and Geahlen, 1992). Inhibition of rat brain phosphatidylinositol-phosphate kinase by morin was also described (Cheng, 1997). Recently, apigenin was reported to inhibit IκB kinase activity to prevent activation of nuclear transcription activating factor (NF-κB) (Liang *et al.*, 1999). These effects of flavonoids are related with down-regulation of COX-2 and iNOS expression, thereby affecting the inflammatory process.

Perspectives of Future Research

As noted above, the certain flavonoids showed inhibitions of COX/LO, PLA_2, NO production and/or lymphocyte proliferation. They also modulate COX-2 and iNOS enzymes at transcriptional level. Some derivatives also affected signal transduction pathways. However, any single mechanism could not explain all of their anti-inflammatory activity *in vivo*. It is thought that flavonoids have different action mechanism(s) depending on their chemical structures. They probably have multiple cellular mechanisms acting on multiple sites. And there may be other cellular action mechanisms to be elucidated. The possible new targets for flavonoid research in this field could be potential inhibitions of various types

of PLA_2, signal transduction elements such as transcription activating factors and interaction with various cytokines. Through modern techniques of biochemistry/pharmacology new insights of action mechanisms of flavonoids will be unveiled in near future. By the knowledge obtained, a new class of anti-inflammatory agents based on the flavonoid molecules will be developed.

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