Effects of Anti-inflammatory Biflavonoid, Ginkgetin, on Chronic Skin Inflammation

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Ginkgetin, a biflavonoid from Ginkgo biloba leaves (Ginkgoaceae), was previously demonstrated to inhibit phospholipase A2 and to suppress proinflammatory gene expression such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase. In this study, the effects of ginkgetin were examined on an animal model of chronic skin inflammation and proinflammatory gene expression. When topically applied to ICR mouse ear, ginkgetin (20–80 µg/ear/treatment) inhibited ear edema (22.8–39.5%) and prostaglandin E2 production (30.2–31.1%) induced by multiple treatment of 12-O-tetradecanoylphorbol-13-acetate (TPA) for 7 consecutive days. By histological comparison, ginkgetin was also found to reduce epidermal hyperplasia. The expression of proinflammatory gene, interleukin-1β, was suppressed by ginkgetin. From the results, it is suggested that ginkgetin may be beneficial against chronic skin inflammatory disorders like atopic dermatitis.

Key words: ginkgetin; biflavonoid; skin inflammation; gene expression

Although the etiology of chronic skin inflammatory disorders including psoriasis and atopic dermatitis (AD) is not clearly understood, many studies have shown that various proinflammatory enzymes/cytokines play an important role in these inflammatory diseases. For example, phospholipase A2 (PLA2) was highly expressed in some psoriatic tissues.1) In addition to lipooxygenase products,2) cyclooxygenase (COX) and prostaglandins (PG) were involved in dermal inflammation and skin wound healing.3,4) Moreover, it was found that high concentrations of cytokines, such as tumor necrosis factor (TNF)-α, and adhesion molecules, including intercellular adhesion molecule (ICAM)-1, were deeply involved in the regional site of chronic skin inflammation.5,6) Recent studies have also indicated that inducible nitric oxide synthase (iNOS) may be associated with psoriasis, and its reaction product, NO, is involved in various skin disorders.7,8) Therefore, it is reasonably thought that an interference of activity and/or expression of these proinflammatory molecules may result in attenuation of chronic skin inflammatory syndromes, and the agents acting on these points may give beneficial effects.

Biflavonoids are flavonoid-flavonol dimers and some of them possess anti-inflammatory activity. For instance, several biflavonoids including amentoflavin, ginkgetin, and isoginkgetin inhibited histamine release.9) Amentoflavone and tetrahydrocarmeloneflavone were found to inhibit COX-1 and/or COX-2.10,11) Some biflavonoids were revealed to suppress the expression of proinflammatory molecules such as COX-2 and iNOS.12,13) These previous investigations have shown the potential of certain biflavonoids for anti-inflammatory agents. In particular, ginkgetin (Fig. 1) mainly found in Ginkgo biloba leaves (Ginkgoaceae) was previously demonstrated as an inhibitor of group IIa sPLA2 inhibitor14) and cPLA215) In addition, ginkgetin was reported to suppress COX-2 expression from lipopoly saccharide (LPS)-treated macrophages without affecting COX-2 activity, and in vivo study has also revealed that ginkgetin inhibited COX-2 expression and PGE2 production from mouse skin induced by 3-d treatment of 12-O-tetradecanoylphorbol-13-acetate (TPA).16) All these findings strongly suggest that ginkgetin may show an inhibition against inflammatory disorders, especially skin inflammation by topical application. Thus, for further characterization of the pharmacological property, the effects of ginkgetin were examined on an animal model of chronic skin inflammation and proinflammatory gene expression in the present investigation.

MATERIALS AND METHODS

Chemicals TPA and prednisolone were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Ginkgetin was isolated from the methanol extract of Ginkgo biloba leaves according to the previously described procedure.17) The purity was >95% based on HPLC analysis.

Animals Male ICR mice (4 weeks, specific pathogen-free) were obtained from Orient Co. (Korea). Animals were fed with laboratory chow (Purina Korea) and water ad libitum. They were acclimatized in an animal facility (KNH) under the conditions of 20–22°C, 40–60% relative humidity and 12 h/12 h (light/dark) cycle at least 7 d prior to experiment.

Chronic Skin Inflammation On day of the experiment (day 1), TPA (1 µg/20 µl acetone) was applied to the inner and outer surfaces of mouse ear for inducing a chronic type of skin inflammation according to the original procedure10) with some modification. Test compounds dissolved in oil-based vehicle were topically applied to the same site (20 µl) at 1 and 12 h after TPA treatment. TPA-treated control group only received TPA and vehicle. On day 2–6, the same treat-

Fig. 1. Chemical Structure of Ginkgetin

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ment regimen was carried out with TPA and test compounds. On day 7, TPA was applied, and one hour later, test compounds were treated. Three randomly selected mice/group were sacrificed by cervical dislocation 3 h after final treatment of test compounds. Ears were removed and stored in RNA stabilization reagent (Qiagen, Germany) at -70°C for reverse transcription-polymerase chain reaction (RT-PCR) analysis. For determination of anti-inflammatory activity, ear thickness of the remaining five mice/group was measured using an engineering gauge (Mitutoyo, Japan) 2 h after final treatment of test compounds. Immediately after, in order to determine PGE$_2$ concentration and to prepare histological samples, ears were removed. For evaluation of the effects on normal skin, test compounds in the vehicle were treated topically to ears of mice using the same treatment schedule as above without TPA treatment. In this case, the control group received acetone and vehicle only. After 7 d, ear thickness of the test compound-treated groups was measured, but no noticeable difference was observed between the treated and the control groups.

**RT-PCR Analysis** After cutting into small pieces, ear samples were homogenized in RLT buffer containing 1% β-mercaptoethanol for 30 s using Polytron homogenizer. Total RNA was extracted using RNeasy mini kit (Qiagen) according to the supplier’s protocol. The concentration of RNA content was determined by measuring the absorbance at 260 and 280 nm. cDNAs were synthesized using RT reaction at 42°C, 50 min and 99°C, 5 min in Gene Cycler thermal cycler (Bio-Rad). Primers were synthesized on the basis of the repeated mRNA sequence for COX-1, COX-2, interleukin-1β (IL-1β), TNF-α, INOS, ICAM-1, fibrinectin and G3PDH. The primer sequences used for PCR were as follows: COX-1 sense, 5′-TGC ATG TGG CTG TGG ATG TCA TCA A-3′; antisense, 5′-CAC TAA GAC AGA CCC GTC ATC TCC A-3′; 450 bp; COX-2 sense, 5′-ACT CAC TCA GTT TGT TGA GTC ATT C-3′; antisense, 5′-TTT GAT TAG TAC TGT AGG GTT AAT G-3′; 583 bp; IL-1β sense, 5′-TGC AGA GGT CCC CAA CTC GGA CAT C-3′; antisense, 5′-GTC CTG CCT AAT GTC CCC CCT TTG CAT C-3′; 387 bp; TNF-α sense, 5′-ACA AGC TGG TAG CAG ACC ACG-3′; antisense, 5′-TCA AAA GAT GAC CCT CCG-3′; 428 bp; INOS sense, 5′-CCC TTC CGA CTA TTC TCG CAG CAC C-3′; antisense, 5′-GCA TTT GTG CAG ACC TTC GTC GCT TTG G-3′; 469 bp; ICAM-1 sense, 5′-TGC GAG GAT -CAC AAA CGA AGC-3′; antisense, 5′-AAC ATA AGA GGC TGC CAT CAC G-3′; 471 bp; fibrinectin sense, 5′-GGA ACG TGT TAT GAC GTG G-3′; antisense, 5′-CTA ACG GCA TGA AGC ACT CA-3′; 253 bp; G3PDH sense, 5′-TGA AGG TCG TGT TGA AGG TGG TGC GC-3′; antisense, 5′-CAT GTA GGC CAT GAC GAT TTC GCA CAC-3′; 983 bp. PCR was carried out for 25–30 cycles under saturation, in 25 μl reaction mixture. After amplification, 5 μl of reaction mixture was analyzed on 1.5% agarose gel electrophoresis. The bands were visualized by ethidium bromide staining for 10 min. The band density was quantified by densitometric scanning using SigmaGel (Version 1.0, Jandel Sci.). The signal intensities were normalized by comparison with that of G3PDH and represented as relative ratios.

**Measurement of PGE$_2$ Concentration** As an index of skin PLA$_2$ and/or COX activity, PGE$_2$ concentration was measured essentially following the previously described procedure. In brief, the biopsies were obtained by 4 mm punch from four randomly selected ear samples/group. Then, they were homogenized in 100 mm phosphate buffer (pH 7.4) containing 1 nm EDTA and 10 μM indomethacin. After centrifugation at 1500 g for 10 min, 50 nm of citrate buffer (pH 3.5) was added to the supernatant. The mixture was centrifuged again at 2500 g for 10 min. The resulting supernatant was applied to a 6 ml Sep-Pak C$_{18}$ cartridge (Waters Associate, U.S.A.) and eluted with 5 ml ethyl acetate containing 1% methanol. The eluent was dried under N$_2$ stream and PGE$_2$ concentration was measured with an ELISA kit (Coye Chem.) according to the manufacturer’s instruction.

**Histology** Ear samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) based on the standard procedures.

**Statistical Analysis** All results were represented as arithmetic mean±S.D. One-way ANOVA and Student’s t-tests were used for evaluation of a statistical significance.

**RESULTS**

Seven day multiple treatment of TPA on ICR mouse ear induced a chronic type of skin inflammation, characterized by edema, epidermal hyperplasia and infiltration of inflammatory cells (Figs. 2a, b). By histological comparison, ginkgetin was found to considerably reduce these responses (Fig. 2c). Prednisolone used as a reference drug reduced these responses more profoundly (Fig. 2d). When the ear thickness was measured, more than twice increase was observed compared with those of non-treated mice (0.475±0.031 mm from 0.206±0.005 mm (Fig. 3). By topical application, ginkgetin significantly inhibited ear edema (22.8, 30.5% inhibition at 20, 80 μg/ear/treatment, respectively) (Fig. 3a). Prednisolone (10 μg/ear/treatment) showed potent inhibition of ear edema (66.3%). Using the ear biopsies, PG levels were checked. As expected, TPA treatment drastically increased PGE$_2$ concentration in the lesion (24.8±2.1 ng/biopsy) from the basal level (3.4±0.3 ng/biopsy). Ginkgetin moderately reduced PGE$_2$ concentration (30.2—31.1%), while prednisolone showed a slightly higher inhibition of PGE$_2$ production (36.5%).

For elucidating the effects on proinflammatory gene expression, RT-PCR was employed using the ear biopsies. TPA treatment considerably induced the expression of COX-2 and IL-1β genes (Fig. 4). Other inducible genes of ICAM-1 and TNF-α were very weakly induced whereas INOS mRNA was not detected. The constitutive genes, COX-1 and fibrinectin, were constantly expressed as expected, but there was some increase of mRNA expression by 7-d TPA treatment. Under this condition, ginkgetin dose-dependently inhibited IL-1β expression among the inducible genes tested, but not statistically significant (16.6, 50.9% inhibition at low and high dose treatment, respectively). COX-2 expression was weakly reduced only by high dose treatment of ginkgetin (13.7% inhibition). The changes of ICAM-1 and TNF-α gene expression by ginkgetin were not meaningful since expression levels of these two genes were too low. On the other hand, prednisolone potently inhibited COX-2 and IL-1β expression (75.7, 95.7% inhibition, respectively).

For toxicity evaluation, ginkgetin was applied to the dorsal