

Suppression of inducible cyclooxygenase and nitric oxide synthase through activation of peroxisome proliferator-activated receptor- γ by flavonoids in mouse macrophages

Yu-Chih Liang^{a,c}, Shu-Huei Tsai^a, De-Cheng Tsai^a, Shoei-Yn Lin-Shiau^b, Jen-Kun Lin^{a,*}

^a*Institute of Biochemistry, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan*

^b*Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan*

^c*School of Medicine, Taipei Medical University, Taipei, Taiwan*

Received 11 January 2001; revised 13 March 2001; accepted 5 April 2001

First published online 18 April 2001

Edited by Masayuki Miyasaka

Abstract Peroxisome proliferator-activated receptor (PPAR) γ transcription factor has been implicated in anti-inflammatory response. Of the compounds tested, apigenin, chrysin, and kaempferol significantly stimulated PPAR γ transcriptional activity in a transient reporter assay. In addition, these three flavonoids strongly enhanced the inhibition of inducible cyclooxygenase and inducible nitric oxide synthase promoter activities in lipopolysaccharide-activated macrophages which contain the PPAR γ expression plasmids. However, these three flavonoids exhibited weak PPAR γ agonist activities in an in vitro competitive binding assay. Limited protease digestion of PPAR γ suggested these three flavonoids produced a conformational change in PPAR γ and the conformation differs in the receptor bound to BRL49653 versus these three flavonoids. These results suggested that these three flavonoids might act as allosteric effectors and were able to bind to PPAR γ and activate it, but its binding site might be different from the natural ligand BRL49653. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Peroxisome proliferator-activated receptor- γ ; Flavonoid; Inflammation; Cyclooxygenase; Nitric oxide synthase; Lipopolysaccharide

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor gene family [1]. PPARs bind to specific response elements as heterodimers with the retinoid X receptor and activate transcription in response to a variety of endogenous and exogenous ligands, including certain polyunsaturated fatty acids, arachidonic acid metabolites [2], and some anti-diabetic drugs [3] and non-steroidal anti-inflammatory drugs (NSAIDs) [4]. Currently, PPARs subfamily has been defined as PPAR α ,

PPAR β (also called PPAR δ and NUC1) and PPAR γ . Three PPAR isoforms differ in their tissue distribution and ligand specificity [5]. PPAR α is predominantly expressed in tissues exhibiting high catabolic rate of fatty acids (heart, liver, and kidney), whereas PPAR δ expression is ubiquitous, and its physiological role is unclear. PPAR γ is expressed predominantly in adipose tissue, the adrenal gland, spleen, large colon and the immune system [6–9]. Several lines of evidence indicated that PPAR γ plays an important role in regulating adipocyte differentiation and glucose homeostasis [10]. Both PPAR α and PPAR γ have been shown that also have anti-inflammatory actions through activating by arachidonic acid metabolites. PPAR α bind and be activated by leukotriene B₄ [11], and the levels are induced at the transcriptional level by anti-inflammatory glucocorticoids [12]. PPAR γ are activated by the prostaglandin D₂ metabolite 15-deoxy- $\Delta^{12,14}$ prostaglandin J₂ (15d-PGJ₂) and synthetic anti-diabetic thiazolidinedione drugs (e.g. BRL49653 and ciglitazone) and resulted in negatively regulating the expression of pro-inflammatory genes, and suppressing tumor cell growth [13–16]. Furthermore, both PPAR α and PPAR γ are activated by a number of NSAIDs, such as indomethacin [4]. Recently, the PPAR γ agonists have been considered to inhibit production of monocyte inflammatory cytokines and the expression of inducible nitric oxide synthase (iNOS) [17,18].

The flavonoids are a diverse family of chemicals commonly found in fruits and vegetables. Flavonoids are plant polyphenolic compounds, which comprise several classes including flavonols, flavanones, flavanols and flavans. Epidemiological studies have shown that the consumption of vegetable, fruits and tea is associated with a decreased risk of cancer and cardio-vascular diseases, and flavonoids are believed to play an important role in preventing these diseases [19]. Numerous numbers of this family have anti-carcinogenic [20], anti-inflammatory [21], cytostatic [22], apoptotic [18], anti-oxidant [23], anti-angiogenic [24] and estrogenic [25] activities. Several reports have also shown that flavonoids are potent modulators of both the expression, and activities of specific cytochrome P450 genes/proteins [26]. These data indicate that certain flavonoids have attracted attention as possible chemoprotective or chemotherapeutic agents.

NSAIDs such as aspirin, sodium salicylate, and indomethacin exert their anti-inflammatory effects in part by inhibition of inhibitor κ B (I κ B) kinase- β (IKK- β), thereby preventing activation by nuclear factor- κ B (NF- κ B) of genes involved

*Corresponding author. Fax: (886)-2-23918944.
E-mail: jklin@ha.mc.ntu.edu.tw

Abbreviations: PPAR, peroxisome proliferator-activated receptor; NSAIDs, non-steroidal anti-inflammatory drugs; 15d-PGJ₂, 15-deoxy- $\Delta^{12,14}$ prostaglandin J₂; COX, cyclooxygenase; PGE₂, prostaglandin E₂; iNOS, inducible nitric oxide synthase; NO, nitric oxide; LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; I κ B, inhibitor κ B; IKK, I κ B kinase; IFN- γ , interferon- γ ; Gst, glutathione S-transferase

in the inflammatory response [27]. However, indomethacin and several other NSAIDs (fenoprofen, ibuprofen, and flufenamic acid) are also PPAR γ ligands and block production of inflammatory cytokines in human monocytes [17]. In addition, Ricote et al. [13] also demonstrated that treatment of peritoneal macrophages with 15d-PGJ₂ or several synthetic PPAR γ ligands reduce the expression of iNOS by interferon- γ (IFN- γ) and inhibited induction of gelatinase B and scavenger receptor A gene transcription in response to phorbol ester stimulation. Recently, we reported that apigenin and related flavonoids could suppress the transcriptional activity of cyclooxygenase (COX)-2 and iNOS in part through inhibition of IKK activity [28]. The current study was designed to determine whether the anti-inflammatory effects of flavonoids were correlative with their activation of PPAR γ .

2. Materials and methods

2.1. Chemicals

Lipopolysaccharide (LPS) (*Escherichia coli* 0127:B8), flavone, 5-methoxyflavone, 7,8-dihydroxyflavone, apigenin, 3-hydroxyflavone, kaempferol, morin, quercetin, myricetin, rutin, genistein, indomethacin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Chrysin, luteolin, tangeretin, galangin, fisetin, pinocembrin, naringenin, isosakuranetin, eriodictyol, hesperetin, naringin, and biochanin A were purchased from Extrasynthese Inc. (Genay, France). Mouse IFN- γ was purchased from R&D systems Inc. (Minneapolis, MN, USA). Two kinds of tea polyphenols, (–)-epigallocatechin-3-gallate and theaflavin-3,3'-digallate were purified as previously described [29].

2.2. Cell culture

The mouse macrophage cell lines RAW264.7 (ATCC, T1B71) were cultured as previously described [28]. Thioglycollate-elicited peritoneal macrophages were obtained from specific pathogen-free female Balb/c mice as previously described [30]. For all assays except the luciferase assay, cells were plated in 60 mm dishes at 5×10^6 cells/dish and allowed to grow for 18–24 h. Treatment with vehicle (0.1% DMSO), test compounds and/or LPS or IFN- γ were carried out under serum-free conditions.

2.3. Determination of prostaglandin E₂ (PGE₂) and nitrite

The cultured medium of control and treated cells were collected, centrifuged, and stored at -70°C , until tested. The level of PGE₂ released into culture media was quantified using a specific enzyme immunoassay according to the manufacturer's instructions (Amersham). The nitrite concentration in the cultured medium was measured as an indicator of NO production according to the Griess reaction [28].

2.4. Plasmids

The PPAR γ expression plasmid and AOX-TK reporter plasmid were generously provided by Professor Christopher K. Glass (California University) [13].

The mouse iNOS promoter plasmid was generously provided by Professor Charles J. Lowenstein (Johns Hopkins University) [31]. The mouse COX-2 promoter plasmid containing a 1035 bp fragment, -966 to $+70$ relative to the transcription start, and constructed as previously described [28]. To generate the pGEX-2T-PPAR γ LBD chimeric receptor expression plasmid, cDNA encoding the ligand binding domains (LBD) of the mouse PPAR γ 1 (amino acids 174–475) were amplified by polymerase chain reaction and subcloned into the pGEX-2T expression plasmid. Transient cotransfection and luciferase activity assay using these plasmids were performed as described previously [28].

2.5. Ligand binding assay

The glutathione S-transferase (Gst)-PPAR γ LBD was expressed in JM109 *E. coli* [32] and the fusion proteins were bound to the glutathione Sepharose-4B beads according to the manufacturer's instructions (Pharmacia Biotech). For competition binding assay, 10 μl of glutathione Sepharose-4B beads containing 0.1 μg of Gst-PPAR γ

LBD chimeric protein were incubated with or without unlabeled flavonoids at 4°C for 12 h in buffer containing 10 mM Tris (pH 7.4), 50 mM KCl, 10 mM dithiothreitol and proteinase inhibitors, then added [³H]BRL49653 (specific activity, 60 Ci/mmol) for additional 8 h. Bound [³H]BRL49653 was precipitated from free radioactivity by centrifugation, and washed three times with PBS. The beads containing [³H]BRL49653 were collected and quantitated by liquid scintillation counting.

2.6. Limited protease digestion assay

The protease digestion assays were performed by the method of Allen et al. [33], with some modification. The PPAR γ expression plasmid [13] was used to synthesize ³⁵S-radiolabeled PPAR γ in a coupled transcription/translation system according to the protocol of the manufacturer (Promega). Approximately 5 μl of the transcription/translation reactions was preincubated with 1 μl of tested compounds for 20 min at 25°C . Trypsin was added and allowed to proceed for 10 min at 25°C , then terminated by the addition of SDS sample loading buffer and boiling for 8 min. The products of the digestion were separated by electrophoresis through a 12% SDS-polyacrylamide gel. Labeled PPAR γ was visualized by autoradiography.

3. Results

3.1. Apigenin, chrysin, and kaempferol activated PPAR γ in macrophages

A series of flavonoids including flavone, 5-methoxyflavone, 7,8-dihydroxyflavone, apigenin, 3-hydroxyflavone, kaempferol, morin, quercetin, myricetin, rutin, genistein, chrysin, luteolin, tangeretin, galangin, fisetin, pinocembrin, naringenin, isosakuranetin, eriodictyol, hesperetin, naringin, and biochanin A, tea polyphenols (Table 1), and indomethacin were first tested with regard to their activation effects on PPAR γ in RAW264.7 cells. As RAW264.7 cells express very low levels of PPAR γ [13] and required transfection of a PPAR γ expression plasmid. The cells therefore allowed a direct assessment of the role of PPAR γ in mediating the effects of these flavonoids on macrophage gene expression. The PPAR γ expression plasmid was cotransfected into RAW264.7 cells with a reporter construct containing three copies of the acyl CoA oxidase PPAR responsive element upstream of the thymidine kinase (TK) promoter driving luciferase gene expression. In the absence of a cotransfected PPAR γ expression plasmid, treatment of RAW264.7 macrophages with the tested flavonoids at 10 μM had little effect on activation of PPAR γ . However, when a PPAR γ expression plasmid was cotransfected into the cells, apigenin, chrysin, and kaempferol significantly induced the PPAR γ 8.13-, 5.60- and 7.66-fold, respectively ($P < 0.05$) (Fig. 1A). The positive control of indomethacin (100 μM) strongly induced the PPAR γ activity 13.34-fold which was compared with the mock experiment. Apigenin, chrysin, and kaempferol increased the PPAR γ activity in a dose-dependent manner, with the EC₅₀ of approximately 5 μM , 10 μM , and 10 μM , respectively (Fig. 1B). However, there was a cytotoxic effect in RAW264.7 cells with 20 μM of apigenin and a decrease in the activation of PPAR γ . Flavone, 7,8-dihydroxyflavone, 3-hydroxyflavone, luteolin, galangin, genistein, biochanin A also increased the PPAR γ activities when RAW264.7 cells were transfected with the expression plasmid of PPAR γ . However, the induction folds of these flavonoids showed no significant difference in activation of PPAR γ compared with control RAW264.7 cells that were transfected with PPAR γ expression plasmid (Figs. 1A and 2, 2.74-fold). The other tested flavonoids were unable to activate the PPAR γ , and the data were not shown in Fig. 1A.

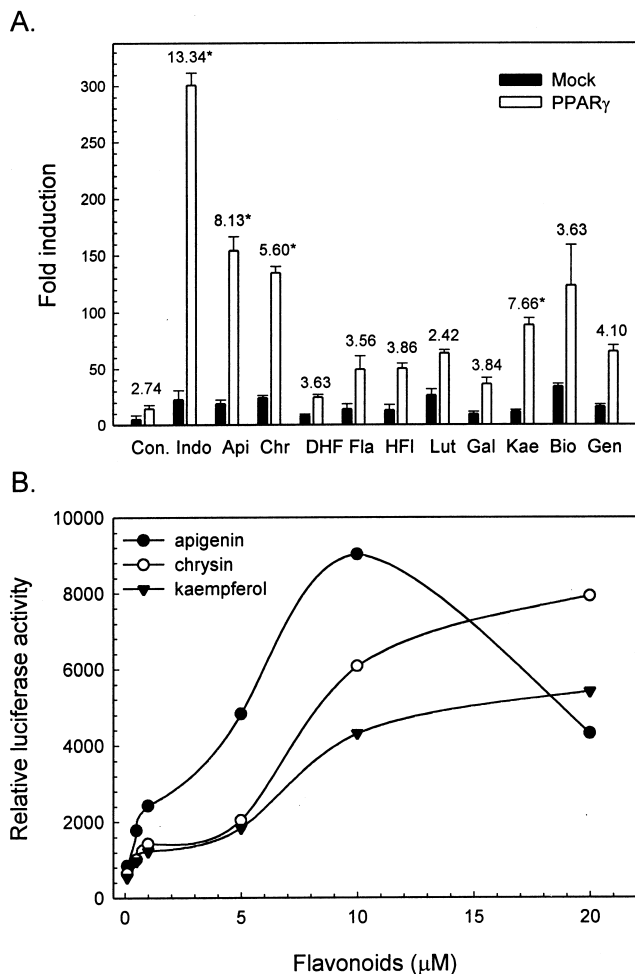


Fig. 1. Effects of apigenin, chrysin, and kaempferol on the activation of PPAR γ in cells. RAW264.7 cells were cotransfected with the AOX-TK reporter plasmid and PPAR γ expression plasmid (open squares) or PPAR γ mock plasmid (closed squares) and β -galactosidase expression vector (pcDNA lacZ) as internal control. A: Transfected cells were treated with 10 μ M of flavonoids, or 100 μ M of indomethacin for 18 h, and cell extracts subsequently assayed for luciferase and β -galactosidase activities as described in Section 2. The values were represented as means \pm S.E.M. of triplicate tests. The numbers were obtained from ratios of the induction fold of transfected PPAR γ expression to mock expression and indicated in the figure. * P < 0.05 (Student's t -test) versus control (2.74). B: Transfected cells were treated with various concentrations of apigenin (closed circles), chrysin (open circles), and kaempferol (closed triangles) for 18 h, and cells extracts assayed for luciferase and β -galactosidase activities as described above. Con., control; Indo, indomethacin; Api, apigenin; Chr, chrysin; DHF, 7,8-dihydroxyflavone; Fla, flavone; HFI, 3-hydroxyflavone; Lut, luteolin; Gal, galangin; Kae, kaempferol; Bio, biochanin A; Gen, genistein.

3.2. Apigenin, chrysin and kaempferol enhanced the inhibition of COX-2 and iNOS promoters' activities in a PPAR γ -dependent manner

RAW264.7 cells was transiently transfected with the reporter plasmids of COX-2 or iNOS and both promoters' activities were markedly increased when RAW264.7 cells were treated with LPS (Fig. 2). Both promoters' activities were inhibited by concurrent treatment of the cells with apigenin, chrysin and kaempferol. Moreover, transfection of PPAR γ expression plasmid enhanced the inhibitory effects of these three flavonoids (Fig. 2A,B). The results suggested that apigenin, chrysin

and kaempferol inhibited the promoters' activities of COX-2 and iNOS genes partially through PPAR γ pathways.

3.3. Apigenin, chrysin, and kaempferol bound with PPAR γ and induced conformational change in PPAR γ

We next sought to determine whether these three flavonoids activated PPAR γ through direct interaction with the PPAR γ receptor. The abilities of these flavonoids to bind to PPAR γ were assessed in a competition binding assay using [3 H]BRL49653 and the glutathione Sepharose beads containing Gst-PPAR γ LBD fusion protein. As shown in Fig. 3, [3 H]BRL49653 bound specifically and saturably to Gst-PPAR γ LBD beads with a K_d of 8 nM (Fig. 3A,B). No binding was detected in control Gst-PPAR γ LBD beads (data not shown). Apigenin, chrysin, and kaempferol competed with [3 H]BRL49653 for binding to the PPAR γ LBD in a dose-dependent manner, with an IC_{50} of approximately 50 μ M. Pro-

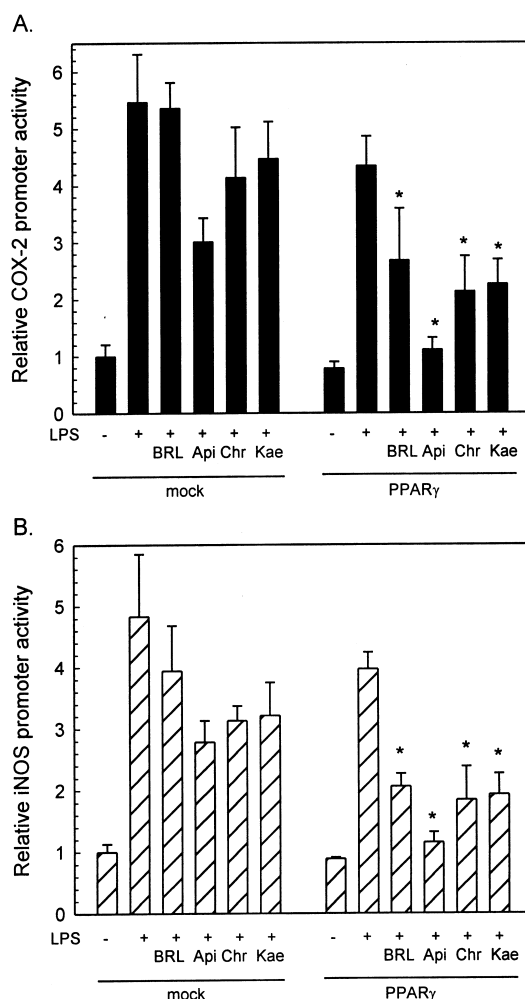


Fig. 2. Apigenin, chrysin and kaempferol enhanced the inhibition of the promoter activities of COX-2 and iNOS in a PPAR γ -dependent manner. RAW264.7 cells were cotransfected with a COX-2 (A) or iNOS (B) promoter-luciferase reporter plasmid, pcDNA lacZ internal control vector, and either a PPAR γ expression plasmid or mock plasmid as indicated. Cells were treated with LPS (50 ng/ml) and BRL49653 (20 μ M), or various flavonoids (10 μ M) for 18 h, and extracts subsequently assayed for luciferase and β -galactosidase activities as described in Section 2. The values were represented as means \pm S.E.M. of triplicate tests. * P < 0.05, compared with the individual test of mock expression (Student's t -test).

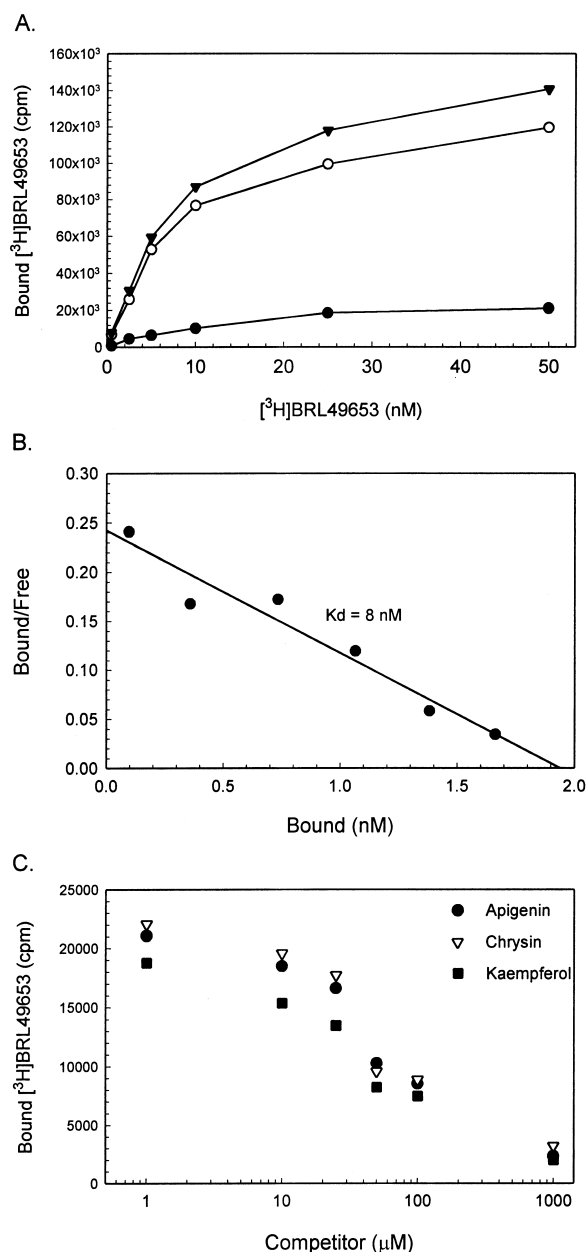


Fig. 3. The binding capacity of apigenin, chrysin, and kaempferol to PPAR γ in vitro. A: Glutathione Sepharose beads containing the Gst-PPAR γ LBD fusion protein were incubated with increasing concentrations of [^3H]BRL49653. Non-specific binding was determined using 500-fold excess of unlabeled BRL49653. Closed triangles, total binding; closed circles, non-specific binding; open circles, specific binding. B: Scatchard analysis of specific binding data from A. Linear regression yielded a K_d of 8 nM. Similar results were obtained in three independent experiments performed in duplicate. C: Competition binding analysis was performed with Sepharose-GST-PPAR γ LBD beads and 5 nM [^3H]BRL49653 in the presence of various concentrations of apigenin (closed circles), chrysin (open triangles), or kaempferol (closed squares) as unlabeled competitors. Similar results were obtained in two independent experiments performed in duplicate.

teolytic analysis have been used for several experiments to demonstrate that ligands of nuclear receptor can, upon binding, specifically alter the conformation of the receptor [33,34]. This conformational change was reflected by the increased resistance of the receptor to partial digestion by proteases.

To determine if there were conformational differences in PPAR γ bound to these three flavonoids, a limited trypsin digestion on a [^{35}S]methionine-labeled PPAR γ was performed. As shown in Fig. 4, incubation of PPAR γ with increasing concentrations of trypsin in the absence of ligand led to the complete digestion of PPAR γ . In contrast, BRL49653 induced a stronger protection of the 22, 29 and 30 kDa fragments. These three flavonoids binding yielded 29 and 30 kDa protected fragments, especially a 30 kDa band. These results indicated that these three flavonoids were able to bind to PPAR γ and flavonoid-bound PPAR γ had a distinct trypsin digestion pattern compared to the BRL49653-bound receptor.

4. Discussion

Flavonoids are naturally occurring plant polyphenols found in abundance in the diets rich in fruit, vegetables and plant-derived beverages such as tea. The PPAR γ ligands share certain structural characteristics including a lipophilic backbone and an acid moiety, usually a carboxylate. Although flavonoids only have a similar lipophilic backbone, several flavonoids also bind to PPAR γ in vitro (Fig. 4). Several reports have shown that treatment of various fibroblast and mesenchymal stem cell lines with PPAR γ ligands, including 15d-PGJ₂, the anti-diabetic drugs, and several NSAIDs, promotes their efficient conversion to adipocytes [3,4,35–37]. However, treatment of C3H10T1/2 stem cells with 10 μM of apigenin, chrysin, and kaempferol failed to promote adipocytes differentiation as indicated by Oil Red O staining (data not shown). Northern blot analysis indicated that PPAR γ expression levels were not induced in response to treatment with these three flavonoids in fibroblast cells (data not shown). As we know, adipocyte differentiation required forced expression of PPAR γ and was significantly enhanced in the presence of PPAR γ activators [38]. For example, treatment of C3H10T1/2 cells with BRL49653 increased PPAR γ expression levels approximately three-fold, and resulted in efficient adipocyte differentiation [3]. In addition, while flavonoids were potent inhibitors of several kinases involved in signal transduction, mainly protein kinase C [39] and tyrosine kinase [40,41]. However, adipocyte differentiation is characterized by a coordinate increase in adipocyte-specific gene expression through activating of gene transcription. Therefore, they were insufficient to initiate the adipogenic signaling cascade in a mesenchymal stem cell line. Devchand et al. [11] proposed that leukotriene B₄, an agonist of the related receptor PPAR α , has anti-inflammatory activity. The flavonoids might also activate the PPAR α and have anti-inflammatory effects, but PPAR α is not expressed in activated macrophages.

Of the tested flavonoids (Table 1), the groups of flavanones and flavan-3-ol were inefficient on activation of PPAR γ . This result suggested that the C2–C3 double bond of C ring was essential for their activation of PPAR γ . Some flavonoids of flavones, flavonols and isoflavones groups were able to activate PPAR γ . The activation of PPAR γ seems to be dependent on the number and the position of hydroxyl residues. The hydroxyl residues of the 5 and 7 positions of the A ring and 4' position of the B ring were important factors for activation of PPAR γ , such as in apigenin, chrysin and kaempferol. However, the additional hydroxyl residue of the 3' position of the B ring resulted in a decrease of the activation of PPAR γ , such as in luteolin and quercetin.

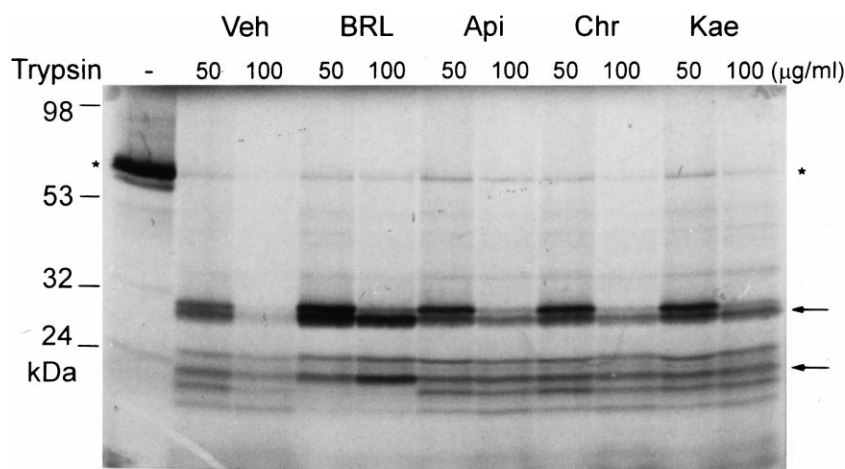


Fig. 4. Apigenin, chrysin and kaempferol induced a protease-resistant conformation of PPAR γ . [35 S]PPAR γ was synthesized in vitro in a coupled transcription/translation system. The receptor was preincubated with ethanol (Veh), apigenin (Api), chrysin (Chr) or kaempferol (Kae), then added to dH₂O or increasing concentration of trypsin. Digestion products were analyzed by SDS-PAGE followed by autoradiography. Asterisks and arrows indicate the full-length PPAR γ and trypsin-resistant fragment of PPAR γ , respectively.

These three flavonoids were able to activate PPAR γ in a transient reporter assay, with an EC₅₀ of approximately 5–10 μ M. However, they needed a higher concentration to bind to Gst-PPAR γ in an in vitro competitive binding assay (Fig. 3). The high concentration of IC₅₀ (50 μ M) suggested that these three flavonoids might not directly bind to PPAR γ or bind to PPAR γ in the other sites. A limited protease digestion assay (Fig. 4) indicated that PPAR γ has a conformational difference in the receptor bound to the three flavonoids versus natural ligand BRL49653. These results suggested that these three flavonoids might act as allosteric effectors, and were able to bind to PPAR γ and activate it, but its binding site might be different from the natural ligand BRL49653. Based on the different binding kinetics, it may interpret the fact that these three flavonoids did not promote differentiation of C3H10T1/2 stem cells to adipocyte. Our previous studies have shown that apigenin was able to inhibit IKK activity and prevent the activation of NF- κ B, and then suppress the promoter activities of COX-2 and iNOS [28]. Therefore, apigenin was a more stronger inhibitor of COX-2 and iNOS promoters activities than BRL49653 in the absence and presence of transfected PPAR γ expression plasmid (Fig. 2). However apigenin was more effective in the presence of PPAR γ than absence of PPAR γ in inhibition of COX-2 and iNOS promoters. These results indicated that these three flavonoids inhibited the expression of COX-2 and iNOS partially through activating PPAR γ . In RAW264.7 cells, the base level of PPAR γ protein was very low, so BRL49653 could not activate it to suppress the activities of COX-2 and iNOS promoters in the absence of transfected PPAR γ expression plasmid.

Our results suggested that certain flavonoids could activate PPAR γ to inhibit the protein expression of COX-2. Indomethacin was able to inhibit COX activity without affecting the protein levels of PPARs at lower concentration [42]. At a higher concentration, we thought that anti-inflammatory activity of indomethacin might be also mediated through activation of PPAR γ followed by inhibition of COX expression, since indomethacin also acts as a PPAR γ agonist, promoting adipocyte differentiation.

Our previous studies demonstrated that the anti-inflammatory properties of apigenin might be mediated through inhi-

bition of IKK activity. In this study, we showed that apigenin was also an efficacious activator of PPAR γ which regulated inflammatory responses. These results suggested that apigenin, chrysin, and kaempferol were possible activators of PPAR γ , and might have therapeutic applications in inflammatory diseases, such as atherosclerosis and rheumatoid arthritis. These findings also provide a significant molecular basis for explaining how dietary flavonoids are active in preventing cancer and inflammation.

Acknowledgements: We thank Prof. Charles J. Lowenstein for providing the pGL-iNOS plasmid, Prof. Christopher K. Glass for providing the PPAR γ and AOX-TK plasmids. This work was supported by the Grants of the National Research Institute of Chinese Medicine (NRICM-89102-2), the Department of National Health Research (NHRI-GT-EX89B913L), the National Science Council (NSC 89-EPA-Z-002-004, NSC 89-2320-B-038-075) and the Ministry of Education (89-B-FA01-1-4).

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