Biochanin A ameliorates the cytokine secretion profile of lipopolysaccharide-stimulated macrophages by a PPARγ-dependent pathway

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Received June 24, 2011; Accepted September 22, 2011

DOI: 10.3892/mmr.2011.599

Abstract. The role of peroxisome proliferator-activated receptors (PPARs) as anti-inflammatory mediators has been established, and the fact that some isoflavones are dual agonists of PPARα/γ indicates the involvement of PPARα and/or PPARγ in the anti-inflammatory action of certain isoflavones. However, the dependency of isoflavones on PPARs in their anti-inflammatory action has not been demonstrated. Here, we report the dependency of an isoflavone biochanin A and the independency of another isoflavone genistein in relation to PPARγ to ameliorate the cytokine secretion profile of lipopolysaccharide (LPS)-stimulated mouse RAW264.7 macrophages. A total amount of 10 μmol/l of biochanin A or genistein significantly suppressed the secretion of tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) in LPS-induced RAW264.7 cells, whereas another two isoflavones, formononectin and daidzein, only significantly suppressed the secretion of IL-6. Their anti-inflammatory efficiencies were not in correspondence with their PPARα/γ agonist activities. Inhibition of PPARγ activity by its antagonist GW9662 significantly reversed the anti-inflammatory effect of biochanin A but not genistein, which demonstrated the dependency of biochanin A and the independency of genistein on PPARγ in their anti-inflammatory actions. Meanwhile, the PPARγ-dependency of biochanin A was further confirmed by the result that the suppression of LPS-induced NF-κB activation by biochanin A was reversed following GW9662 co-treatment. Moreover, inhibition of PPARα activity by its antagonist MK886 did not significantly reverse the anti-inflammatory effects of biochanin A and genistein, indicating that their anti-inflammatory properties were PPARα-independent.

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Key words: isoflavone, anti-inflammatory, peroxisome proliferator-activated receptors γ, biochanin A, genistein

Introduction

Chronic inflammation has been shown to be increasingly involved in the onset and development of diseases such as arteriosclerosis, obesity, neurodegenerative diseases, diabetes and even cancer. Although steroidal anti-inflammatory drugs and NSAIDs are currently used to treat acute inflammation, these drugs have not been entirely successful in curing chronic inflammatory disorders due to their unexpected side effects. Therefore, there is an urgent requirement for safer anti-inflammatory compounds. Isoflavones are a group of polyphenolic compounds that are widely found in the plant kingdom and possess various biological activities including anti-inflammatory properties (1). The most studied isoflavones are genistein, daiazein, biochanin A and formononectin. *In vitro*, genistein was found to be capable of suppressing the secretion of proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) from lipopolysaccharide (LPS)-induced RAW264.7 cells (2) or bone marrow-derived mouse macrophages (3) while daidzein showed inconsistent effects in various reports (2,3). *In vivo*, intraperitoneally injected genistein was shown to protect rats from endotoxin-induced organ failure (4), and genistein was also found to reduce the degree of inflammation and joint destruction in collagen-induced arthritic mice (5). However, little is known regarding the anti-inflammatory property of biochanin A other than that it was reported to inhibit TNFα and IL-6 production in LPS-induced RAW264.7 cells (2,6).

Several mechanisms have been proposed to explain the anti-inflammatory actions of isoflavones, including anti-oxidant activity, inhibition of eicosanoid generating enzymes, modulation of the production of proinflammatory molecules or modulation of proinflammatory gene expression (1). The antioxidant and radical scavenging activities (7,8), the inhibition of COX-2 expression (9), the modulation of MAPK expression (10) and the modulation of NF-κB expression (11,12) were all implicated in the anti-inflammatory action of genistein. However, little was known regarding the mechanism behind the anti-inflammatory action of biochanin A until it was recently reported to block the activation of NF-κB (6).

The anti-inflammatory properties of the nuclear hormone receptor family known as peroxisome proliferator-acti-

vated receptors (PPARs) have been established, despite originally being implicated with obesity, diabetes, and atherosclerosis (13,14). There are 3 known isotypes of PPAR: PPARα, PPARγ and PPARδ. It is of note that biochanin A, genistein and formononectin are PPARα/γ dual agonists (15). However, PPARα-specific agonist WY14643 and PPARγ agonist rosiglitozone were not shown to inhibit the production of TNFα and IL-6 in LPS-induced RAW264.7 macrophages (16), while pioglitozone, another PPARγ agonist, was capable of suppressing the secretion of TNFα in LPS-induced RAW264.7 macrophages (17). Therefore, it is necessary to determine whether or not isoflavones act as anti-inflammatory agents by activating PPARα and/or PPARγ. The present study aimed to investigate the dependence of the isoflavones on PPARα and/or γ to mediate inflammatory factors. Our data indicate that biochanin A ameliorates the cytokine secretion profile of LPS-stimulated macrophages through the activation of PPARγ, but not PPARα. Furthermore, the anti-inflammatory activity of genistein is PPARα- and PPARγ-independent.

Materials and methods

Reagents and plasmids. Biochanin A, genistein, formononectin, daidzein, GW9662, MK886 and lipopolysaccharides from *Escherichia coli* 0111:B4, were purchased from Sigma (St. Louis, MO, USA). ELISA kits for measuring IL-6 and TNFα cytokines were purchased from Bender MedSystems (Vienna, Austria). NF-κB p65 antibody was obtained from Cell Signaling (Beverly, MA, USA).

pcDNA-FLAG-PPARγ (18) and PPRE-tk-luc (19) with 3 PPAR response elements as described previously were obtained from the Addgene. pcDNA-FLAG-PPARα was a kind gift from Professor Yunqing Yang (Xiamen University, China). Plasmid pSV-β-galactosidase was from Promega.

Cell culture. RAW 264.7 and 293T cells were cultured in complete Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS in a 37˚C incubator with 5% CO_2/95% air.

PPAR transcriptional activities. 293T cells were plated on 24-well plates at the cell density of approximately $1.8x10^5$/well. On the next day, cells were co-transfected with pcDNA-FLAG-PPARα or pcDNA-FLAG-PPARγ, the PPRE-tk-Luc plasmid and pSV-β-galactosidase at a ratio of 1:3:1 using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were fed with fresh media with 10 μmol/l biochanin A, genistein, formononectin or daidzein, respectively, 6 hours after transfection. Cells were further incubated for an additional 36 h prior to luciferase reporter assays. Luciferase reporter activities were determined using a luciferase reporter gene assay system (Promega, Madison, WI, USA) as instructed. PPAR activities were expressed as relative luciferase activity corrected for transfection efficiency (β-galactosidase activity) and normalized to the controls (normalized RLU).

Determination of the modulation of cytokine secretion from LPS-stimulated macrophages cell culture. LPS-stimulated macrophages were used to study the anti-inflammatory activity of isoflavones. RAW 264.7 cells were seeded at a density of $1x10^6$ cells/well in 6-well plates. After 24 h, test substances in less than 0.1% dimethyl sulfoxide (DMSO) solution were added to the culture medium, and cells were incubated for another 3 h. LPS was added at a final concentration of 1 μg/ml, and cells were incubated for another 24 h. The cells were then centrifuged at 1,000 rpm, the supernatant was aliquoted and samples were stored at -20˚C until cytokines were measured. The concentrations of TNFα and IL-6 in cell supernatants were tested in ELISA assays according to the manufacturer's protocol.

Analysis of NF-κB activation. NF-κB activation was determined by analysis of NF-κB p65 translocation by Western blotting. RAW 264.7 cells were seeded at a density of $1x10^6$ cells/well in 6-well plates. After 24 h, test substances in <0.1% DMSO solution were added to the culture medium, and cells were incubated for another 3 h. LPS was added at a final concentration of 1 μg/ml, and cells were incubated for another 30 min. After incubation, the total cellular and nuclear proteins were extracted according to instructions of nuclear and cytoplasmic extraction reagents kit (Beyotime, Jiangsu, China). The protein concentrations of the extracts were measured using a bicinchoninic acid protein assay kit (Pierce). A total amount of 40 μg protein of each sample was loaded and separated on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Blotted membranes were then incubated with anti-p65 (1:1000) in TBS-0.1% Tween-20 with 5% non-fat milk at 4˚C overnight. After several washes, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000) in TBS-0.1% Tween-20 with 5% non-fat milk. The detection was achieved using the Supersignal chemiluminescent substrate kit (Pierce).

Statistical analysis. All data were processed and analyzed by GraphPad software (Prism 4.0) and expressed as the mean ± SEM. One-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test was used for multi-group analyses. Two-way ANOVA with post test was used to analyze the effect of PPARα and PPARγ antagonist treatment. P-values <0.05 were considered to be statistically significant; P-values <0.01 more so.

Results

The anti-inflammatory activities of biochanin A, genistein, formononetin and daidzein did not correspond with their PPARα/γ agonist activities. Inflammation is characterized by a sequential release of proinflammatory cytokines, including TNFα and IL-6. To determine the anti-inflammatory properties of the isoflavones, we assayed the secretion levels of TNFα and IL-6 from LPS-stimulated mouse RAW264.7 macrophages following isoflavone treatment. In a previous study (15), biochanin A, formononetin and genistein were reported to activate PPARα and PPARγ activities; for PPARα, the EC_{50} was 1, 3.7 and 9.5 μmol/l, respectively and for PPARγ, 1, 4.3 and 12 μmol/l, respectively. Thus, we chose the concentration of 10 μmol/l for each isoflavone to compare their anti-inflammatory activities. As shown in Fig. 1A, biochanin A and genistein significantly inhibited the secretion of TNFα by 37.8% (P<0.05)

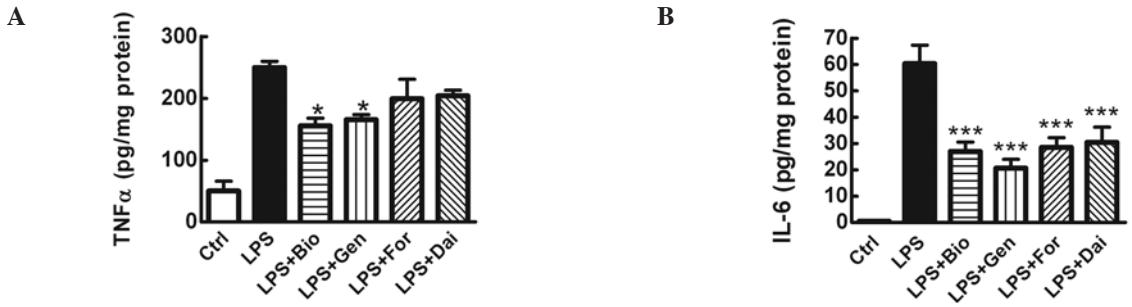

Figure 1. Levels of cytokine secretion of (A) TNFα and (B) IL-6 in RAW264.7 macrophages following incubation with lipopolysaccharide (LPS) and isoflavones biochanin A, genistein, formononectin and daidzein. The measured cytokine levels were normalized to total protein concentrations to reduce variations caused by cell density differences. Cells were pretreated with 10 μmol/l isoflavones for 3 h followed by incubation with 1 μg/ml LPS for another 24 h. All experiments were performed in triplicate. Values are expressed as the mean ± SEM. ***P<0.001; *P<0.05 vs. LPS treated cells. Bio, biochanin A; Gen, genistein; For, formononectin; Dai, daidzein.

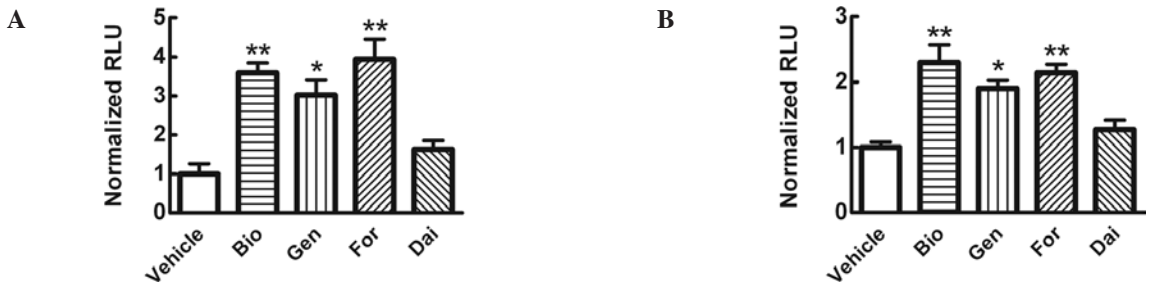

Figure 2. Effects of isoflavones biochanin A, genistein, formononectin and daidzein on peroxisome proliferator-activated receptor (PPAR)α/γ transcripitional activities. Biochanin A, genistein and formononectin (10 μmol/l, respectively) significantly activated (A) PPARα and (B) PPARγ. PPARα/γ activity (normalized RLU) was assayed as described in Materials and methods (n=3). Values are expressed as the mean ± SEM. **P<0.01; *P<0.05 vs. vehicle. Bio, biochanin A; Gen, genistein; For, formononectin; Dai, daidzein.

and 33.8% (P<0.05), respectively when compared to that of the vehicle-treated LPS-induced RAW264.7 cells, whereas formononectin and daidzein slightly but not significantly suppressed the secretion of TNFα. Meanwhile, biochanin A, genistein, formononectin and daidzein suppressed the secretion of IL-6 by 55.5% (P<0.001), 65.9% (P<0.001), 52.8% (P<0.001) and 49.7% (P<0.001), respectively when compared to that of the vehicle-treated LPS-induced RAW264.7 cells (Fig. 1B).

The data in Fig. 1 show that genistein possessed a more efficient anti-inflammatory property than biochanin A and formononectin at the dose of 10 μmol/l. However, in a previous report, biochanin A and formononectin were found to be more active agonists of PPARα and PPARγ compared to genistein (15). Therefore, to confirm and compare the effect of these isoflavones on PPARα and PPARγ transcriptional activities at the concentration of 10 μmol/l, we treated the 293T cells with 10 μmol/l of these isoflavones and assayed the PPARα and PPARγ transcriptional activities using a reporter gene assay system as decribed in Materials and methods. As shown in Fig. 2A and B, biochanin A increased PPARα and PPARγ transcriptional activities by approximately 3.6-fold (P<0.01) and 2.3-fold (P<0.01), respectively, whereas genistein exhibited less efficiency; it increased PPARα and PPARγ transcriptional activities approximately 3.0-fold (P<0.05) and 1.9-fold (P<0.05), respectively. Meanwhile, formononectin increased PPARα and PPARγ transcriptional activities 3.9-fold (P<0.01) and 2.1-fold (P<0.01), respectively. However, daidzein was not capable of activating PPARα and PPARγ.

Biochanin A ameliorated the cytokine secretion profile of LPS-stimulated RAW264.7 cells by activating PPARγ but not PPARα, whereas the anti-inflammatory action of genistein was PPARα/γ-independent. The above results indicated that the anti-inflammatory efficiencies of these isoflavones were not in correspondence with their efficiency as agonists of PPARα and PPARγ. In addition, only biochanin A and genistein suppressed both TNFα and IL-6 secretion in LPS-induced RAW264.7 cells. To further investigate the dependence or independence of biochanin A and genistein on PPARα when they inhibit the release of inflammatory factors, PPARα antagonist MK886 was added to suppress the activity of PPARα at the same time as the LPS-induced RAW264.7 cells were treated with biochanin A or genistein, and TNFα and IL-6 levels were assayed. MK886 did not significantly reverse the suppression of TNFα and IL-6 secretion caused by biochanin A or genistein treatment (Fig. 3A and B). Our data indicate that neither biochanin A nor genistein is dependent on PPARα when they inhibit the secretion of inflammatory factors from LPS-induced RAW264.7 cells.

To further investigate the dependence or independence of biochanin A and genistein in relation to PPARγ, PPARγ antagonist GW9662 was added simultaneously with biochanin A or genistein to treat the LPS-induced RAW264.7 cells, and the secretion of TNFα and IL-6 was assayed. GW9662 significantly reversed the suppression of TNFα and IL-6 secretion caused by biochanin A treatment, whereas it had no effect on the suppression of TNFα and IL-6 secretion caused by genistein

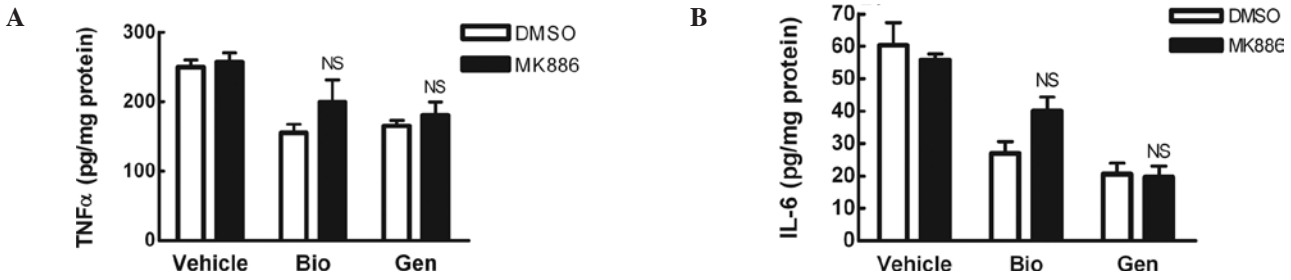

Figure 3. Effects of PPARα inhibition on (A) TNFα and (B) IL-6 secretion in LPS-stimulated RAW264.7 macrophages following treatment with biochanin A and genistein. Measured cytokine levels were normalized to total protein concentrations to reduce variations caused by cell density differences. Cells were pretreated with 10 μmol/l biochanin A and genistein in the presence of 20 μmol/l of PPARα antagonist MK886 for 3 h followed by incubation with 1 μg/ml LPS for another 24 h. Inhibition of PPARα by MK886 did not reverse the suppression of TNFα and IL-6 secretion caused by biochanin A and genistein. Experiments were performed in triplicate. Values are expressed as the mean ± SEM. NS, not significant vs. DMSO. Bio, Biochanin A; Gen, genistein.

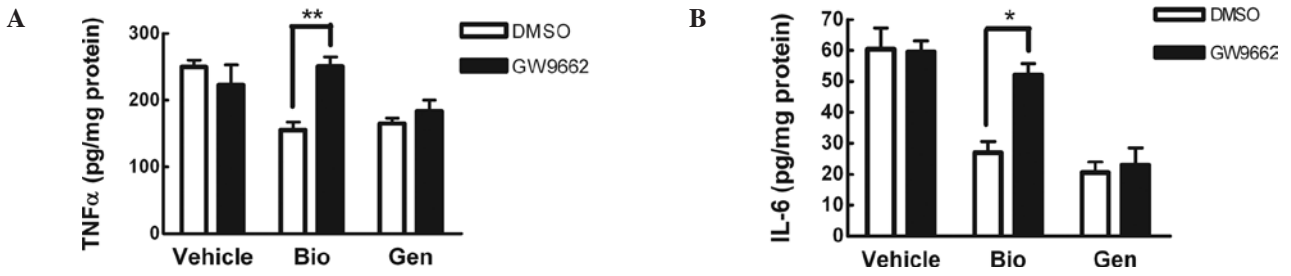

Figure 4. Effects of PPARγ inhibition on (A) TNFα and (B) IL-6 secretion in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages following treatment with biochanin A and genistein. Measured cytokine levels were normalized to total protein concentrations to reduce variations caused by cell density differences. Cells were pretreated with 10 μmol/l biochanin A and genistein in the presence of 10 μmol/l of PPARγ antagonist GW9662 for 3 h followed by incubation with 1 μg/ml LPS for another 24 h. Inhibition of PPARγ by GW9662 significantly reversed the suppression effect of biochanin A on TNFα and IL-6 secretion from LPS-induced macrophages. Experiments were performed in triplicate. Values are expressed as the mean ± SEM. $^{**}P<0.01$; $^{*}P<0.05$. Bio, Biochanin A; Gen, genistein.

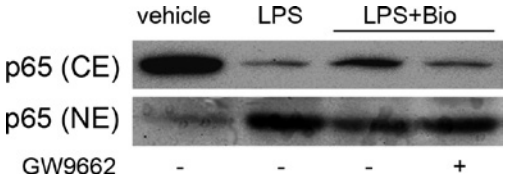

Figure 5. Effects of biochanin A on lipopolysaccharide (LPS)-induced NF-κB activation determined as nuclear translocation of NF-κB. RAW264.7 cells were pretreated with 10 μmol/l biochanin A in the presence or absence of 10 μmol/l of PPARγ antagonist GW9662 for 3 h followed by incubation with 1 μg/ml LPS for another 30 min, and cytoplasm and nuclear proteins were extracted. Western blotting was used to detect the p65 subunits of NF-κB in the cytoplasm and nuclear extracts. Bio, biochanin A; CE, cytoplasm extract; NE, nuclear extract.

(Fig. 4A and B). Our data indicate that the anti-inflammatory activity of biochanin A is PPARγ-dependent, whereas the anti-inflammatory activity of genistein is PPARγ-independent.

Biochanin A inactivated NF-κB via a PPARγ-dependent pathway. Activation of NF-κB is thought to play a key role in the LPS-induced release of TNFα, IL-6 and certain other cytokines (20). To monitor activation of NF-κB, we assessed translocation of the p65 subunit from the cytosol to the nucleus by Western blotting. As shown in Fig. 5, LPS caused translocation of p65 from the cytoplasm to the nuclei after 30 min of LPS challenge. The LPS-induced nuclear localization of p65 was prevented by pretreating the cells with biochanin A and the prevention was dismissed by co-treatment with PPARγ antagonist GW9662, indicating that biochanin A-induced PPARγ activation acts upstream to nuclear translocation of NF-κB.

Discussion

Certain isoflavones have been demonstrated to have anti-inflammatory properties *in vitro* and *in vivo*. Among these isoflavones, genistein is the most studied, and whose anti-inflammatory activities have been well documented in animals, humans and cell lines (1). Few data, however, are available regarding the anti-inflammatory effect of biochanin A and formononectin. In the present study, we first demonstrated using RAW264.7 cells that biochanin A was anti-inflammatory, which is consistent with a previous study by Mueller *et al* (2). We also demonstrated using LPS-induced RAW264.7 cells that formononectin suppressed IL-6 secretion at the concentration of 10 μmol/l, whereas 100 nmol/l of formononectin was previously reported to be inefficient in suppressing IL-6 secretion (2). Moreover, we compared the anti-inflammatory efficiency of genistein, biochanin A, formononectin and daidzein. The potency of inhibition was in the order of biochanin A > genistein > formononectin > daidzein in the case of TNFα secretion and genistein > biochanin A > formononectin > daidzein in the case of IL-6 secretion. However, the PPARα agonist activities of these isoflavones was in the order of formononectin > biochanin A > genistein >

daidzein and the PPARγ-agonist activities of these isoflavones was in the order of biochanin A > formononectin > genistein > daidzein. We therefore concluded that the anti-inflammatory efficiencies of these isoflavones were not in correspondence with their efficiencies as agonists of PPARα and PPARγ.

The molecular mechanisms underlying the anti-inflammatory effect of biochanin A and genistein, which exhibited the most potent anti-inflammatory activity, were further studied. Genistein has been reported to possess antioxidant and radical scavenging activities (7,8), to inhibit COX-2 expression (9) and to modulate MAPK (10) and NF-κB expression (11,12), and these actions are considered to contribute to its anti-inflammatory property. The fact that genistein and biochanin A are potent PPARα/γ dual agonists indicated the involvement of the nuclear receptors in isoflavone anti-inflammatory action, since PPARα/γ are extensively studied inflammatory mediators. However, our data demonstrated that only biochanin A ameliorated the cytokine secretion profile of LPS-stimulated RAW264.7 cells by activating PPARγ but not PPARα, whereas the anti-inflammatory action of genistein was PPARα/γ-independent, although it activated PPARα/γ. The PPARγ ligand pioglitagone consistently suppresses the production of TNFα by LPS-stimulated RAW264.7 cells, while another PPARγ ligand, rosiglitagone, lacks this property (16,17). An additional complication in studies of PPAR ligands is that certain ligands act via PPAR-dependent and PPAR-independent pathways. For example, 15d-PGJ2, a natural ligand for PPARγ, inhibits NF-κB-mediated transcriptional activation by a PPARγ-dependent mechanism (21) and two PPARγ-independent mechanisms, inhibition of IκB kinase activity and inhibition of NF-κB DNA binding (22). Mounting evidence indicates that interaction of nuclear receptors with coactivators may occur in a differential manner. For example, 15d-PGJ2 was capable of inducing ligand-activated receptor interaction with SRC-1, SRC-2 and p300, whereas troglitazone does not have the same effect (23). The preferential interaction of cofactors with nuclear receptors activated by specific ligands may partially explain these observations regarding the anti-inflammatory activity variance of various PPARα/γ ligands. However, further elucidation is required.

Ligands for PPARα had therapeutic activity in several rodent models of inflammatory diseases (24-28). These data demonstrated the mediator role of PPARα in inflammatory diseases. Several studies *in vitro* demonstrated repression of IFNγ by PPARα ligands in activated T cells. The PPARα ligand fenofibrate repressed IFNγ expression in anti-CD3-treated cultured human $CD4^+$ lymphocytes (29) and another PPARα ligand, WY14643, repressed IFNγ in activated mouse splenocytes (30). Moreover, PPARα ligands repressed TNF-elicited chemokine production by colonic epithelial cell lines (26). However, WY14643 was found to be unable to suppress the TNFα and IL-6 secretion from LPS-stimulated RAW264.7 macrophages (16). Our current data demonstrated that biochanin A and genistein, two agonists of PPARα, suppressed TNFα and IL-6 secretion in LPS-stimulated RAW264.7 cells via a PPARα-independent pathway. The fact that PPARα is expressed in T, B (31) and intestinal epithelial cells (26), and is not detectable in RAW264.7 macrophages may explain these observations. Therefore, further investigations in different cell lines or tissues are required to study the dependency of biochanin A and genistein on PPARα with regard to their anti-inflammatory properties.

To the best of our knowledge, the present study is the first to demonstrate the PPARγ-dependency of biochanin A and PPARγ-independency of genistein regarding their anti-inflammatory properties in mouse RAW264.7 macrophages. Moreover, PPARα is not involved in the anti-inflammatory action of biochanin A and genistein in RAW264.7 macrophages. Herein, we demonstrated the anti-inflammatory property and elucidated a new anti-inflammatory mechanism of biochanin A. Moreover, the present study evaluated the link between PPARα/γ agonist activities and their anti-inflammatory activities. Our data indicate the ambiguity of PPARα/γ agonists as anti-inflammatory drugs.

Acknowledgements

This study was supported by the Science and Technology Planning Project of Fujian Province, China (no. 2010N0023).

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