DOI 10.1002/mnfr.200500103 Mol. Nutr. Food Res. 2005, *49*, 1154 – 1159

Suppression of arachidonic acid metabolism and nitric oxide formation by kudzu isoflavones in murine macrophages

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Inhibitory effect of kudzu isoflavones on arachidonic acid metabolism and nitric oxide (NO) production in lipopolysaccharide activated RAW 264.7 macrophages were investigated. Isoflavone aglycones, such as daidzein, genistein, biochanin A, and formononetin significantly suppressed arachidonic acid release (50 μM). Biochanin A, which displayed the most active inhibition on arachidonic acid release in HT-29 human colon cancer cells, exhibited its most potent suppression in RAW 264.7 cell (by 86%) without showing cytotoxicity. However, isoflavone glucosides, puerarin and daidzin, showed lower inhibitory activities on the release of arachidonic acid and its metabolites. In NO formation, biochanin A showed marked inhibition, by 62% (50 μM), followed by genistein, daidzein, formononetin, and daidzin, 56, 39, 33, and 8%, respectively. 5,7-Dihydroxyl group in the A-ring of isoflavones could be a key functional group responsible for the strong inhibitory activity of biochanin A and genistein on NO production. These activities may contribute to the antiinflammatory and anticarcinogenic properties of kudzu isoflavones.

Keywords: Arachidonic acid / Isoflavones / Kudzu / Macrophage / Nitric oxide

Received: July 2, 2005; revised: September 13, 2005; accepted: September 17, 2005

1 Introduction

The root of Kudzu (*Pueraria lobata* Ohwi), along with soybean and other leguminous plants, is a rich source of isoflavones. It has been widely applied in Oriental medicine due to its beneficial effects such as antipyretic, antidiarrhetic, spasmolytic, diaphoretic, and antiemetic properties [1, 2]. Isoflavones have been of great interest for many years because of their substantial health benefits including reduction of osteoporosis, reducing blood cholesterol levels, lowering the risk of hormone related cancers, and improvement of menopausal symptoms [3–5].

Arachidonic acid is an unsaturated fatty acid (20:4, *n*-6) found at the lipid bilayer of cell membrane. In mammalian cells, phospholipase A_2 (PLA_2) enzymes have been implicated to catalyze cleavage of membrane-bound arachidonate at the *sn*-2 position and release arachidonic acid [6, 7]. The released arachidonic acid is further metabolized into various forms of eicosanoids including prostaglandin G2 (PGG_2) and H2 (PGH_2) by enzymes such as cyclooxygenase (COX), lipoxygenase (LOX), or cytochrome P-450 monooxygenase (CYP) [8, 9]. Arachidonic acid release and the resulting formation of eicosanoids are thought to play a critical role in many biological processes such as aging, inflammation, platelet aggregation, angiogenesis, atherosclerosis, and cancers. Many studies have indicated that the inhibition of arachidonic acid metabolism might have potential therapeutic value for cancer prevention [10–13].

Nitric oxide (NO) is a diatomic free radical generated from conversion of L-arginine to L-citrulline by NO synthases (NOSs). So far, three types of NOSs have been identified and named according to their activity or the tissue types in which they are present [14]. Endothelial NO synthase (eNOS) is mainly found in endothelium and responsible for the control of vascular tone. Neural NO synthase (nNOS) is in neurons and acts as a neurotransmitter [15, 16]. Both eNOS and nNOS are expressed constitutively (called cNOS collectively) and calcium-dependently. On the other hand, inducible NOS (iNOS) is calcium-independent and

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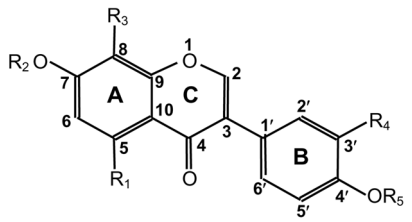
expressed in response to certain stimuli such as endotoxin or *Escherichia coli* lipopolysaccharide (LPS) and cytokines including tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), and interleukins (IL) [14]. It has been reported that NO generated from cNOS is beneficial in regulating homeostasis, while the NO produced by iNOS is responsible for cytotoxicity, tissue damage, and inflammation [17–19].

NO spontaneously reacts with triplet oxygen ($^3O_2$), decomposes, and produces stable nitrite (NO_2^-) and nitrate (NO_3^-), which are indirect markers in evaluation of NO *in vitro* [20]. NO has been found to contribute to many diseases such as inflammation and cancer. In inflammation, macrophages simultaneously produce NO as well as superoxide anion (O^{2-}). The reaction of NO and O^{2-} generates peroxynitrite anion ($ONOO^-$), which can enhance the activity of COXs and therefore stimulates eicosanoids production [21, 22]. Furthermore, peroxynitrite has been found to be a highly reactive oxidant and able to cause DNA damage [23]. Therefore, suppression of NO production by certain phytochemicals might be one of the prominent strategies for the treatment of inflammation and cancer.

Several naturally occurring compounds have been investigated for their inhibitory roles on NO formation processes. Epigallocatechin gallate (EGCG), apigenin, theaflavin-3,3′-digallate, carnosol, citral, curcumin, quercetin, resveratrol, and procyanidins have been recently reported to inhibit the production of peroxynitrite radicals and nitrite [15, 16, 24–28]. Genistein has been shown to inhibit LPS-induced nitrite production probably through suppressing iNOS activity and/or iNOS gene expression [29, 30].

Previously, we reported the inhibitory activities of five major isoflavones (Fig. 1) from kudzu against free radicals and arachidonic acid release in HT-29 human colon cancer cells [31], which led to our great interest in biochanin A. The present study was designed to investigate the roles of kudzu isoflavones on arachidonic metabolism and NO production in RAW 264.7 cells.



	R_1	R_2	R_3	R_4	R_5
Puerarin	H	H	Glc	H	H
Daidzin	H	Glc	H	H	H
Daidzein	H	H	H	H	H
Genistein	OH	H	H	H	H
Biochanin A	OH	H	H	H	Me
Formononetin	H	H	H	H	Me

Figure 1. Structures of isoflavones from kudzu root. The structures of 6 isoflavones were identified previously [31].

2 Materials and methods

2.1 Chemicals and cell lines

Isoflavones (puerarin, daidzin, daidzein, genistein, biochanin A, and formononetin) were isolated from kudzu roots as described previously [31]. [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid was purchased from NEN Life Science (Boston, MA). McCoy's 5A media was purchased from Mediatech (Herndon, VA). Murine macrophage 264.7 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). RAW 264.7 cells were maintained in DMEM containing L-glutamine, glucose, and sodium bicarbonate supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, in 95% humidity and 5% CO_2 at 37°C. LPS (*E. coli*, serotype 055:B5) and all other chemicals were purchased from Sigma Chemical (St. Louis, MO).

2.2 Release of arachidonic acid and its metabolites in RAW 264.7 cells

RAW 264.7 murine macrophage cells were plated into a 24-well plate at approximately 3.0×10^5 cells in fresh growth media. After 24 h, the media was removed and replaced with 1 mL of serum free-DMEM media containing 0.1 μCi/mL [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid. The cells were incubated overnight resulting in >90% of arachidonic acid absorption by cells. Cells were then washed twice with PBS containing 0.1% BSA in order to remove unabsorbed free arachidonic acid. RAW cells were stimulated with 2 μg/mL LPS (from *E. coli*, serotype 055:b55) for 1 h, and 1 mL of media was then replaced with one containing isoflavones or vehicle (DMSO). After 18 h incubation (or at a certain time point), the extracellular media was taken and centrifuged for 10 min at 12 000 rpm. Radioactivity of the extracellular fluids (600 μL) was measured by a scintillation counter [32]. The supernatants were analyzed by an RP HPLC system as described previously [33].

2.3 Dose-dependent effects of biochanin A and genistein on arachidonic acid release in RAW 264.7 cells

Different concentrations of biochanin A (0.5, 1, 2.5, 5, 10, 20, and 50 μM) were incubated with LPS-activated RAW 246.7 macrophages. Genistein (2.5, 5, 10, 20, and 50 μM) was used as a comparison with biochanin A. Experimental procedure was the same as above except for treatment with

different doses of biochanin A and genistein. The results were expressed as percentage release of arachidonic acid and its metabolites.

2.4 Morphological changes of RAW 264.7 cells by kudzu isoflavones treatments

RAW 264.7 cells were distributed into six-well plates (at approximately 4.0×10^5 cells) in growth media and incubated in serum-free DMEM media overnight. Cells were stimulated with 2 μg/mL LPS for 1 h, and 1 mL of the media was then replaced with media containing isoflavones (50 μM) or vehicle (DMSO). After 18 h, cells were washed twice with ice-cold PBS and then fixed with 4% formalin-PBS for 10 min. Morphology was then analyzed by microscope ($\times 50$, $\times 100$, $\times 200$) and cell pictures were taken (Nikon, model dxm 1200).

2.5 NO formation in LPS-stimulated RAW 264.7 cells

RAW 264.7 cells were plated with growth media into a 24-well plate at approximately 3.0×10^5 cells and incubated in serum-free DMEM overnight. Cells were stimulated with 2 μg/mL LPS for 1 h, and then 1 mL of media was replaced with one containing isoflavones (50 μM) or vehicle (DMSO). Fifty-microliter of extracellular media were collected at each time point (1, 10, 14, 18, 22, and 32 h). Nitrite concentration was used as an indication of NO production. Based on Griess reaction, a general index for measuring NO formation, nitrite in the culture medium was measured [34]. In brief, 50 mL of 0.5% sulfanilamide and 50 μL of 1 mg/mL naphthylethylenediamine were added to 50 μL of culture medium. After 30 min incubation at room temperature, absorbance was measured at 560 nm by a microplate reader. A standard nitrite curve was generated by using $NaNO_2$. The inhibitory activities of tested compounds were expressed in both fold and the percentage decrease of NO formation as follows:

$$PI\ (\text{Percentage Inhibition}, \%) = 100 - (NO)^a/(NO)^b \times 100$$

where $(NO)^a$ represents the NO concentration of macrophages exposed to both LPS and test isoflavones and $(NO)^b$ represents the NO concentration of LPS-stimulated control macrophages without treatment with test isoflavones.

2.6 Statistical analysis

All experiments were performed in triplicate. For each experiment, data are expressed as the means ± SE ($N = 3$). Differences were considered significant when the P values were <0.05.

3 Results and discussion

3.1 Effect of kudzu isoflavones on the release of arachidonic acid and its metabolites

LPS stimulation led to an increase of prelabeled arachidonic acid and its metabolites release over three times in RAW 264.7 murine macrophage. The released radioactivity in the media accounted for 3–5% of total labeled arachidonic acid during 18-h incubation. The HPLC profiles in previous study demonstrated that most of the released radioactivity (>95%) was from arachidonic acid metabolites rather than arachidonic acid itself [32]. Therefore, the measured radioactivity in the present study is also considered as the metabolites of the released arachidonic acid. Arachidonic acid release is mainly mediated by the activation of $cPLA_2$. Previous study has demonstrated that blocking phosphorylation of $cPLA_2$ almost completely inhibits the release of arachidonic acid in RAW264.7 cells [32]. Release of arachidonic acid from phospholipids by cPLA2 is known to be a rate-limiting step for further arachidonic acid metabolism, accordingly the overall arachidonic acid metabolism seems to be effectively controlled by modulating this cPLA2 action.

Inhibitory effects of kudzu isoflavones on release of arachidonic acid and its metabolites in RAW 264.7 cells are shown in Fig. 2. All isoflavone aglycones, such as daidzein, genistein, biochanin A, and formonontein, significantly suppressed the release of arachidonic acid and its metabolites in RAW 264.7 cells below the basal level during 18-h-incubation. Inhibitory effects of isoflavones on arachidonic acid release in RAW 264.7 cells were significantly reduced when glucosylated. For example, puerarin, a *C*-glucoside

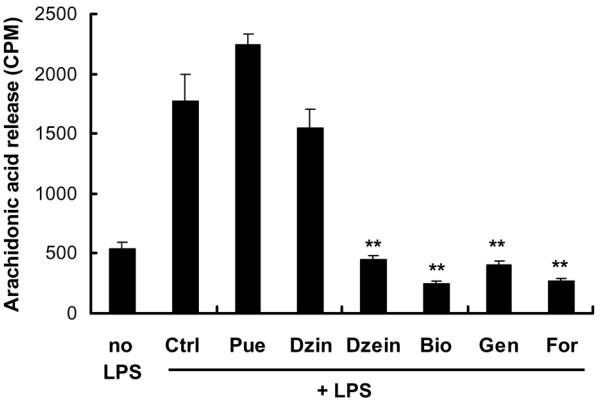


Figure 2. Effects of kudzu isoflavones on the release of arachidonic acid and its metabolites in murine macrophage RAW 246.7 cells after 18 h incubation. Pue, puerarin; Dai, daidzin; Daie, daidzein; Bio, biochanin A; Gen, genistein; For, formononetin. All data represent the means ± SD of three experimental determinations. ** Significantly different from control ($P < 0.01$).

form of daidzein, displayed a stimulation of the LPS-induced arachidonic acid release rather than inhibition. Daidzin, the *O*-glucoside form of daidzein, slightly decreased (12%) the LPS-induced arachidonic acid release at the concentration of 50 μM. The decreased inhibitory activities of isoflavone glucosides are probably due to higher molecular weight and hydrophilicity and/or steric hindrance by additional sugar moieties compared to the aglycones, which might decrease the cellular uptake level [15, 28, 31]. An earlier study showed that the affinity of isoflavones to the liposomal membrane was increased in the order of genistin = daidzin < daidzein < genistein in caco-2 cell monolayers of human intestinal epithelium [35]. According to the authors, the transport of isoflavone glucosides, such as genistin and daidzin, through Caco-2 monolayers was less than one-tenth that of the aglycones, genistein, and daizein, respectively.

Biochanin A, which has a methoxyl group in B-ring and displayed the most potent inhibitory effect on arachidonic acid release in HT-29 human colon cancer cells [31], exhibited a potent inhibition (by 86%) in murine RAW 264.7 macrophage cells as well (50 μM). Formononetin, with the same methoxyl group but one less hydroxyl group than biochanin A, possessed a similar inhibitory potency as that of biochanin A (by 85%). Genistein and daidzein decreased the release of arachidonic acid and its metabolites in RAW 264.7 cells by 77 and 75%, respectively. Biochanin A, which has one more hydroxyl group (5,7-dihydroxyl groups in the A-ring) than formononetin, had better suppression on arachidonic acid release than formononetin. The same results were also found with genistein and daidzein. Genistein, with one more hydroxyl group (5,7-dihydroxyl groups in the A-ring) than daidzein, exhibited stronger arachidonic acid release inhibition than daidzein. These observations suggest that the 5,7-dihydroxyl groups in the A-ring of both biochanin A and genistein together with the 4′-methoxyl group in B-ring of biochanin A and formononetin might be key functional groups of isoflavones in suppressing arachidonic acid release in RAW 264.7 cells.

Since biochanin A displayed the most potent inhibitory effect on arachidonic acid metabolism in both HT-29 and RAW 264.7 cells, the concentration-dependent/time-dependent inhibitions by biochanin A were further analyzed (Figs. 3, 4). The inhibitory effects of biochanin A and genistein on release of arachidonic acid and its metabolites in RAW 264.7 macrophages were dose-dependent. Arachidonic acid release decreased with the increase in the concentration of biochanin A and genistein. Biochanin A inhibited the arachidonic acid release even at 1 μM.

Morphological changes after treatment with kudzu isoflavones in the LPS-stimulated RAW 264.7 macrophages are illustrated in Fig. 5. Macrophages became largely elongated

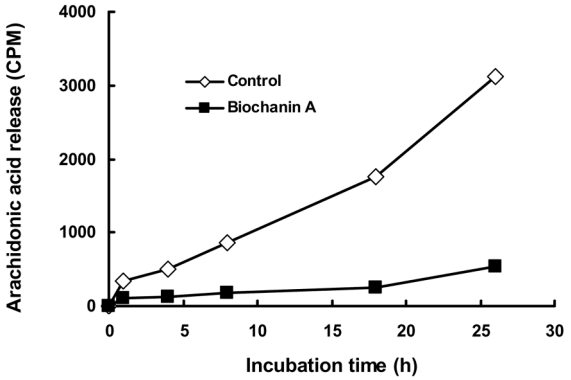


Figure 3. Time dependent effect of biochanin A (50 μM) on the release of arachidonic acid and its metabolites in RAW 264.7 macrophage cells. ** Significantly different from control ($P < 0.01$). (Error bars are not visible due to the small values).

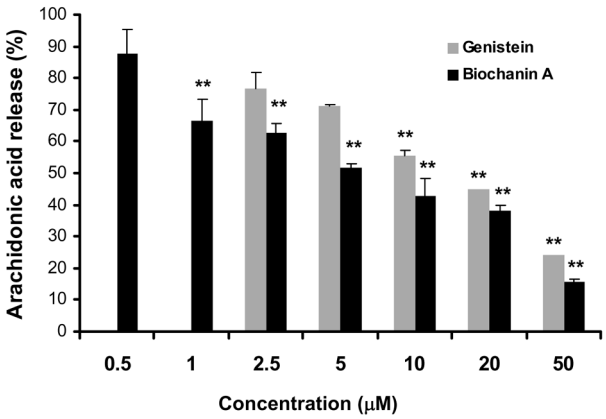


Figure 4. Dose dependent effects of biochanin A and genistein on arachidonic acid release in RAW 264.7 murine macrophage cells. Black bars represent biochanin A and gray bars represent genistein. All data represent the means ± SD of three experimental determinations. ** Significantly different from control (P<0.01)

with stimulation by LPS, a typical proinflammatory stimulator of macrophages. It is reported that LPS by itself constitutes signals for differentiation, increases cell size and, change the morphology of macrophage [36]. RAW 264.7 cells treated with isoflavone glucoside (puerarin and daidzin) after LPS stimulation exhibited same elongated morphology as control induced by LPS (data not shown). On the other hand, when cells were treated with isoflavone aglycones, in particular biochanin A, after LPS stimulation, the morphology of the cells was similar to that of LPS-untreated control cells, indicating suppression of LPS-mediated morphological changes by these aglycones. Biochanin A was also reported to have antimutagenic activity [37]. Exposure of hamster embryo cell cultures to biochanin A at a dose of 25 μg/mL resulted in a 37–50% inhibition in the binding of benzo(*a*)pyrene [B(*a*)P] to DNA.

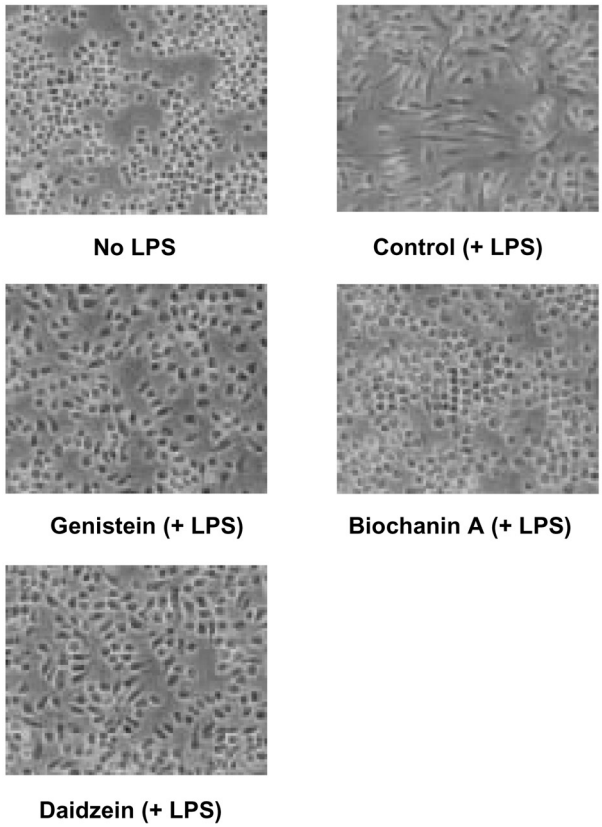


Figure 5. Modulation of kudzu isoflavones in murine macrophage RAW 264.7 cells. Cells were stimulated with LPS for 1 h and treated with kudzu isoflavones for 18 h.

Although biochanin A suppressed the growth of various cancer cell lines, it neither affected normal stomach fibroblasts at the same concentration nor inhibited the growth of HSC-41E6 tumor cells [35, 36]. Therefore, biochanin A might possess a specific inhibitory action, depending on cell type, which can be used for therapeutic purposes.

3.2 Inhibition of NO production in LPS-stimulated RAW 264.7 macrophage cells by Kudzu isoflavones

The time-dependent inhibitory effects of Kudzu isoflavones on NO production in RAW 264.7 cells are shown in Fig. 6. After 10 h stimulation of cells with LPS, NO production increased over three-fold and gradually increased over 18-fold during 32-h LPS stimulation. Almost all isoflavones inhibited NO formation from LPS-activated RAW 264.7 cells (Fig. 7). Biochanin A and genistein displayed marked inhibitions on NO production in RAW 264.7 cells at 50 μM (by 62 and 56%, respectively). Inhibitory activity of biochanin A against NO production increased with incubation time, reached maximum inhibition at 14 h, and then gradually decreased. Puerarin and daidzin stimulated NO production after 22- and 32-h incubation, respectively and exhibited lower inhibition activity than aglycones in NO formation. Formononetin, which possessed similar inhibition activity with biochanin A in arachidonic acid metabolism, showed somewhat less inhibition in NO production (by 39%). Daidzein and daidzin showed approximately 46 and 33% inhibition, respectively, at 22-h incubation.

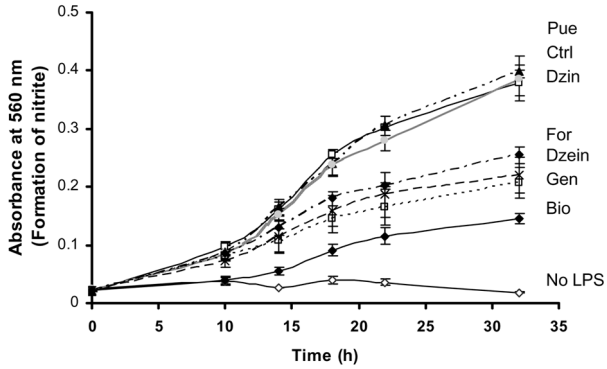


Figure 6. Time-dependent inhibitory effect of kudzu isoflavones on nitric oxide formation in RAW264.7 macrophage cells. The lines from the bottom: no LPS (cells not exposed to LPS nor isoflavones) < biochanin A < genistein < daidzein < formononetin < daidzin < control (cells induced by LPS but not treated with isoflavones) < puerarin. All data represent the means ± SD of three experimental determinations.

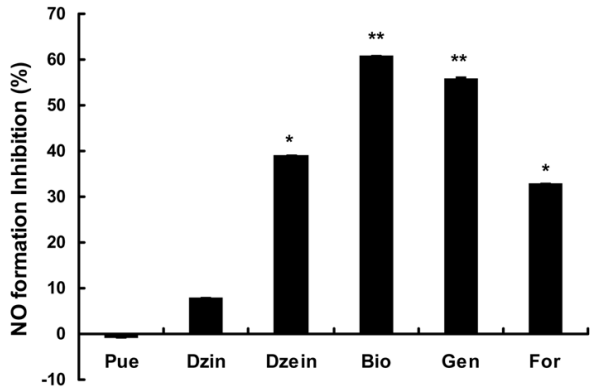


Figure 7. Inhibitory effect of kudzu isoflavones on the NO formation. Macrophages were incubated for 22 h with LPS in the presence of the kudzu isoflavones. Pue, puerarin; Dzin, daidzin; Dzein, daidzein; Bio, biochanin A; Gen, genistein; For, formononetin. All data represent the means ± SD of three experimental determinations. (Error bars are not visible due to small values). *, ** Significantly different from control ($P < 0.05$ and $P < 0.01$, respectively).

Our results with the inhibition of NO production by genistein and daidzein in macrophages agree with those in the previous studies [15, 28, 30]. Genistein with 5,7-dihydroxyl groups in the A-ring showed better inhibition activity than daidzein on NO formation. The stronger activity of genistein over daidzein on NO formation in LPS-activated RAW

264.7 cells suggests that the hydroxyl residues in flavonoids account for the inhibition of iNOS activity [30, 38]. The mode of action of genistein might be its direct inhibition of the iNOS gene expression, the enzyme activity and/or its scavenging activity, against NO radicals [30, 39]. In this case, the 5,7-dihydroxyl groups in the A-ring of isoflavone might be responsible for the potent inhibitory activity of biochanin A on NO production.

4 Concluding remarks

Much attention has been paid to genistein in most previous isoflavones research. According to our study, however, biochanin A has been found to be the most promising isoflavone at least in modulating arachidonic acid metabolism in both HT-29 and RAW 264.7 cells as well as in controlling NO production in RAW 264.7 cells. Further studies on the mode of action elicited by these isoflavones are needed to further elucidate the underlying molecular mechanisms involved.

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