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In vitro anti-inflammatory effects of arctigenin, a lignan from *Arctium lappa* L., through inhibition on iNOS pathway

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ABSTRACT

Ethnopharmacological relevance: Arctigenin, a bioactive constituent from dried seeds of *Arctium lappa* L. (Compositae) which has been widely used as a Traditional Chinese Medicine for dispelling wind and heat included in Chinese Pharmacophere, was found to exhibit anti-inflammatory activities but its molecular mechanism remains unknown yet.
Aim of the study: To investigate the anti-inflammatory mechanism of arctigenin.
Materials and methods: Cultured macrophage RAW 264.7 cells and THP-1 cells were used for the experiments. Griess assay was used to evaluate the inhibitory effect of arctigenin on the overproduction of nitric oxide (NO). ELISA was used to determine the level of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6). The inhibitory effect on the enzymatic activity of cyclooxygenase-2 (COX-2) was tested by colorimetric method. Western blot was used to detect the expression of inducible nitric oxide synthase (iNOS) and COX-2.
Results: Arctigenin suppressed lipopolysaccharide (LPS)-stimulated NO production and pro-inflammatory cytokines secretion, including TNF-α and IL-6 in a dose-dependent manner. Arctigenin also strongly inhibited the expression of iNOS and iNOS enzymatic activity, whereas the expression of COX-2 and COX-2 enzymatic activity were not affected by arctigenin.
Conclusions: These results indicated that potent inhibition on NO, TNF-α and IL-6, but not COX-2 expression and COX-2 activity, might constitute the anti-inflammatory mechanism of arctigenin. Arctigenin suppressed the overproduction of NO through down-regulation of iNOS expression and iNOS enzymatic activity in LPS-stimulated macrophage.

1. Introduction

Fructus Arctii is a common herbal medicinal preparation in China which has been used clinically as a therapeutic agent to treat inflammation, such as the affection of anemopyretic cold, swelling of throat, cough, measles and syphilis and so on. Arctigenin, one of the major bioactive component of Fructus Arctii, naturally occurs in *Bardanae fructus*, *Arctium lappa* L., *Saussurea medusa*, *Torreya nucifera* and *Ipomea cairica*. It has been reported to exhibit antioxidant, antitumor and anti-inflammatory activities as a phenylpropanoid dibenzylbutyrolactone lignan (Awale et al., 2006; Cho et al., 2004; Matsumoto et al., 2006). In the present study, we investigated the molecular mechanism underlying the anti-inflammatory properties of arctigenin in macrophages including a murine macrophage cell line RAW 264.7 and a human macrophage cell line THP-1.

Upon inflammatory stimulation, macrophages produce nitric oxide (NO) and pro-inflammatory cytokines such as tumor necrosis factor (TNF)-alpha, interleukin (IL)-6. Overproduction of these mediators is present in macrophage of many inflammatory diseases, including rheumatoid arthritis, atherosclerosis, and hepatitis (Isomaki and Punnonen, 1997; Libby et al., 2002; Tilg et al., 1992). NO, which plays as an important cellular second messenger, is produced via three types of nitric oxide synthase (NOS). Small amounts of NO produced by the constitutive NOS (cNOS) are essential for maintaining the cellular function. Inducible NOS (iNOS) can sustainedly produce a high output of NO, which is believed as one of the most important inflammatory reactions in activated macrophage (Pokharel et al., 2007). In addition, the inducible cyclooxygenase-2 (COX-2) is believed to be the target enzyme for the anti-inflammatory activity of nonsteriodal anti-inflammatory drugs. Many studies have demonstrated that some

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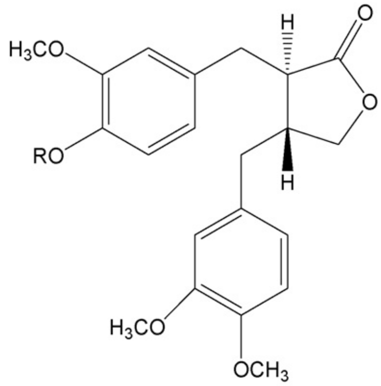

R = H Arctigenin
R = Glc Arctiin

Fig. 1. Chemical structures of arctiin and arctigenin.

inducible enzyme (COX and iNOS)/cytokines and their reaction products are involved in chronic inflammatory disease (Abd-El-Aleem et al., 2001; Bruch-Gerharz et al., 1996, 2001). Improper up-regulation of iNOS and/or COX-2 is associated to pathophysiology of certain types of cancers as well as inflammatory disorders (Yang et al., 2006).

In this regard, the effect of arctigenin on the LPS-stimulated pro-inflammatory mediator, such as NO, TNF-α and IL-6 in macrophage was tested. And the effect of arctigenin on the expression of iNOS and COX-2 was investigated. In addition, author further described the inhibition on iNOS enzymatic activity in LPS-stimulated RAW 264.7 cells treated with arctigenin.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium, penicillin, streptomycin and fatal bovine serum were purchased from Invitrogen (NY, USA). Lipopolysaccharide (LPS), hydrocortisone, dimethyl sulfoxide (DMSO) and MTT were obtained from Sigma. Mouse TNF-α ELISA kit, Human TNF-α ELISA kit and mouse IL-6 ELISA kit were purchased from R&D. Colorimetric COX (ovine) inhibitor screening assay kit and anti-murine iNOS polyclonal antibody were obtained from Cayman. Bradford protein assay kit, anti-phosphol-COX-2 antibody, anti-β-actin antibody and nitric oxide synthase assay kit were purchased from Beyotime Biochemical Co. (Haimen, China).

2.2. Isolation of arctiin and preparation of arctigenin

Arctiin was isolated from dried seeds of *Arctium lappa* L. by using reported methods (Han et al., 1994; Takasaki et al., 2000). Briefly, dried seeds of *Arctium lappa* (100 g) were extracted with 80% ethanol under reflux (1 L, 2 h, twice). The extract solutions were filtered and then evaporated at 40 °C under reduced pressure. The ethanol extract was resuspended in 500 ml water and then partitioned with the same volume of *n*-hexane, AcOEt, *n*-BuOH to give an *n*-hexane fr., AcOEt fr., *n*-BuOH fr. and H_2O fr., respectively. The AcOEt fr. was subjected to a silica gel column chromatography and eluted in a stepwise manner with $CHCl_3$–MeOH (1:0; 19:1; 9:1; 4:1; 1:1; 0:1) mixtures to obtain six fractions. Arctiin was obtained by recrystalization from the second fraction. Arctigenin was prepared by enzymolysis of arctiin with snail hydrolase (chemical structures of arctiin and arctigenin are shown in Fig. 1). The yield rate of arctigenin by enzymolysis was up to 75.8%. The optimum enzymolysis condition was achieved in a pH 5.0 sterilized solution of water or normal saline for 36–48 h with snail hydrolase at 1/5 amount of the substrate arctiin. Arctigenin was isolated and purified by silica gel column chromatography (Hu et al., 2004). Its chemical structure was confirmed by physiochemical properties and NMR spectral data (Liu et al., 2003). The purity of arctigenin was determined to be >98% by high performance liquid chromatography. Arctigenin was dissolved in cell culture grade DMSO and stocked frozen until use.

2.3. Cell culture

RAW 264.7 mouse monocyte-macrophage (ATCC TIB-71) and THP-1 human monocyte-macrophage (ATCC TIB-202) were maintained in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat inactivated fetal bovine serum at 37 °C in a humidified incubator with 5% CO_2 and 95% air. The medium was routinely changed every two days. RAW 264.7 and THP-1 cells were passaged by trypsinization until they attained confluence.

2.4. Cell viability assay

Cells in the exponential growth phase were seeded in a 96-well plate at a density of 5×10^5 cell/ml. Arctigenin was added at indicated concentrations. Control group received an equal amount of DMSO, which resulted in a final concentration of 0.2% DMSO in the culture medium. The mitochondrial-dependent reduction of 3-(4,5-dimethylthizaol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan was used to measure cell respiration as an indicator of cell viability (Denizot and Lang, 1986). Briefly, after 24 h incubation with or without arctigenin (1–100 μM), a MTT solution (final concentration is 200 μg/ml) was added and the cells were incubated for another 4 h at 37 °C. After removing the supernatant, 100 μl of DMSO was added to the cells to dissolve the formazan. The absorbance of each group was measured by using a microplate reader at wavelength of 570 nm. The control group consisted of untreated cells was considered as 100% of viable cells. Results are expressed as percentage of viable cells when compared with control groups.

2.5. Nitric oxide analysis

Nitric oxide was determined by measuring the amount of nitrite in the cell culture supernatant, using Griess reagent (mixture of equal amount of A and B. A:1% sulphanilamide, B:0.1% naphthylethylene diamine dihydrochloride in 5% H_3PO_4). RAW 264.7 cells were treated by LPS (1 μg/ml) with or without arctigenin (1–100 μM) for 24 h, then briefly centrifuged. 100 μl of the cell culture supernatant was mixed with 100 μl of Griess reagent, followed by incubation for 10 min at room temperature. The absorbance at 540 nm was measured and the inhibitory rates were calculated by using a standard calibration curve prepared from different concentrations of sodium nitrite (Ishihara et al., 2000).

2.6. Measurement of cytokines

Cells were treated by LPS (1 μg/ml) with or without arctigenin (1–100 μM) for 6 h. 100 μl of the culture supernatant was taken out to determine the level of TNF-α and IL-6 using respective enzyme-linked immunosorbent assay kit according to the manufacturer's recommendations.

2.7. Assay of COX-2 enzymatic activity

COX-2 activity was determined by using a colorimetric COX inhibitor screening assay kit in a cell-free system according to the manufacturer's instructions. Briefly, 160 μl of assay buffer and 10 μl of heme were added to the background well. 150 μl of assay buffer, 10 μl of heme and 10 μl of COX-2 enzyme were added to the 100% initial activity well. 10 μl of arctigenin (the final concentration is 100 μM and 50 μM) was added to the sample wells and 10 μl of DMSO was added to the background wells. The plate was carefully shaken for a few seconds and incubated for 5 min at 25 °C. 20 μl of the colorimetric substrate solution and then 20 μl of arachidonic acid was added to all the wells. The plate was carefully shaken for a few seconds and incubated for 5 min at 25 °C. The absorbance at 590 nm was read by using a microplate reader and the inhibition ratio on COX-2 enzymatic activity was calculated according to the manufacturer's instructions.

2.8. Detection of iNOS and COX-2 expression

After the treatment with LPS (1 μg/ml) and arctigenin (3–30 μM) for 24 h, RAW 264.7 cells were washed with cold PBS and lysated in a cold lysis buffer (10% NP-40, 150 mM NaCl, 10 mM Tris, 2 mM PMSF, 5 μM Leupeptin, pH 7.6). Cell debris was removed after centrifugation (12,000 × g, 4 °C, 5 min). After the protein concentration for each aliquot was determined by the Bradford method, suspensions were boiled in SDS-PAGE loading buffer. 25 μg of total protein from each sample were subjected to gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk in Tris buffered saline-Tween (TBS-T, 10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) at room temperature for 1 h. After being washed, the membranes were incubated in respective primary antibody solution (anti-iNOS, anti-COX-2, or anti-β-actin antibodies) overnight at 4 °C. The membranes were washed with TBS-T and incubated with HRP-conjugated secondary antibody solution for 1 h at room temperature. The blots were washed thrice in TBS-T and detected by using enhanced chemiluminescence reagent (ECL) and exposed to photographic films (Kodak). Images were collected and the bands corresponding to iNOS, COX-2 and β-actin protein were quantitated by densitometric analysis using DigDoc 100 program (Alpha Ease FC software). Data of iNOS and COX-2 were normalized on the basis of β-actin levels.

2.9. Assay of iNOS enzymatic activity

After treated with LPS (1 μg/ml) and arctigenin (1–100 μM) for 2 h at 37 °C, the culture supernatant was removed and 100 μl of NOS assay buffer (1×) were added to each well. Then 100 μl of NOS assay reaction solution (50% NOS assay buffer, 39.8% MilliQ water, 5% L-Arginine solution, 5% 0.1 mM NADPH, 0.2% DAF-FMDA) was added to each well and incubated for 2 h at 37 °C. Fluorescence was measured with a fluorescence plate reader (Biotek) at excitation of 485 nm and emission of 528 nm.

2.10. Statistical analysis

All results are expressed as means ± SD. Statistical comparison was conducted using Student's *t*-test after ANOVA. The results are considered to be significant when $p < 0.05$.

3. Results

3.1. Arctigenin did not exhibit cytotoxicity against RAW 264.7 and THP-1 cells

RAW 264.7 and THP-1 cells were treated with various concentrations of arctigenin for 24 h and the cell viability was tested by MTT assay as described in Section 2. As shown in Fig. 2, arctigenin did not exhibit cytotoxicity at the range of 1–100 μM against both RAW 264.7 and THP-1 cells. This dose range was used for treatment of arctigenin in the following experiments.

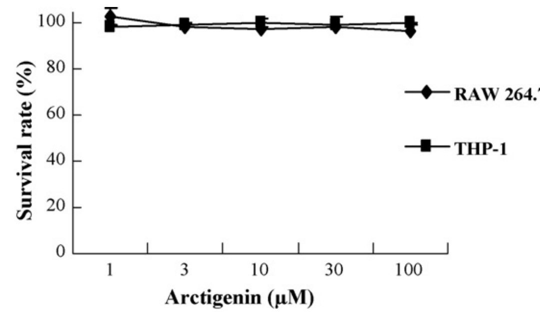

Fig. 2. Macrophages were treated with 1, 3, 10, 30, 100 μM of arctigenin for 24 h. Survival rates were tested with MTT assay in both RAW 264.7 and THP-1 cells.

3.2. Arctigenin blocked LPS-induced pro-inflammatory mediators in both RAW 264.7 and THP-1 cells

RAW 264.7 cells were treated with 1 μg/ml of LPS with or without indicated concentrations of arctigenin or hydrocotisone. The concentration of nitrite was monitored as the NO production after 24 h. As shown in Fig. 3, arctigenin (IC_{50} value is 8.4 μM) significantly suppressed the LPS-induced production of NO in a dose-dependent manner. Its inhibitory activity is even stronger than the positive control, a commonly used anti-inflammatory drug, hydrocortisone. Then RAW 264.7 cells and THP-1 cells were treated with 1 μg/ml of LPS with or without indicated concentrations of arctigenin or hydrocortisone for 6 h. The level of pro-inflammatory cytokines TNF-α and IL-6 in the supernatant was determined by ELISA assay according to the manufacturer's instructions. As shown in Table 1 and Table 2, LPS-induced TNF-α and IL-6 release were significantly blocked by arctigenin in a dose-dependent manner. Arctigenin inhibited the release of TNF-α in LPS-activated RAW 264.7 and THP-1 cells with IC_{50} values of 19.6 μM and 25.0 μM, respectively. It also suppressed the release of IL-6 with IC_{50} value of 29.2 μM in RAW 264.7 cells. Hydorcortisone also inhibited the release of TNF-α (IC_{50}: 65.6 μM) and IL-6 (IC_{50}: 43.9 μM). These results demonstrated that arctigenin significantly blocked LPS-induced pro-inflammatory mediators such as NO, TNF-*α* and IL-6 in macrophages, which might be responsible for its anti-inflammatory usage.

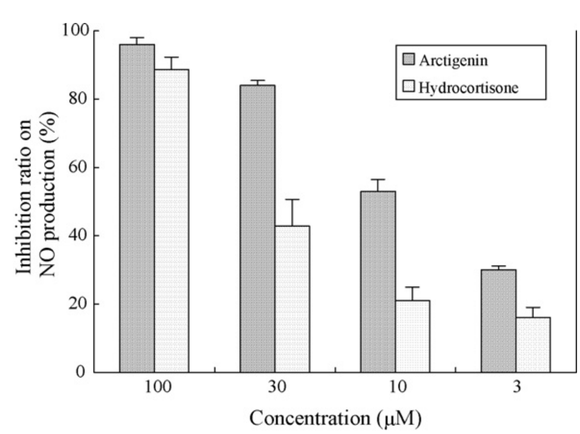

Fig. 3. RAW 264.7 cells were treated with 1 μg/ml of LPS with or without 3, 10, 30, 100 μM of arctigenin or hydrocortisone for 24 h. NO production was measured in triplicate.

Table 1
Inhibitory effect of arctigenin on IL-6 release in RAW 264.7 cells.

Treatment	Concentration (μM)	IL-6 (pg/ml)	Inhibitory rate (%)
Untreated	0	12.9 ± 0.1	
1 μg/ml LPS	0	301.8 ± 1.5###	
LPS + arctigenin	100	16.1 ± 0.4***	98.9
	30	155.9 ± 7.5**	50.5
	10	190.9 ± 9.6**	38.4
	3	214.1 ± 5.8**	30.4
LPS + hydrocortisone	100	49.3 ± 3.6***	87.4
	30	182.8 ± 8.8**	41.2
	10	231.0 ± 7.2*	24.5
	3	291.3 ± 6.3	3.62

The experiment was performed in triplicate, and the results are expressed as means ± SD of IL-6 level.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ versus LPS treatment group.
$p < 0.001$ versus untreated group.

3.3. Arctigenin inhibited LPS-induced iNOS expression, but did not inhibit COX-2 activity and COX-2 expression

The effects of arctigenin and hydrocortisone on COX-2 enzymatic activity were evaluated. The inhibitory ratios by 1 mM and 0.5 mM of arctigenin were calculated to be −12.5% and −20.1%, respectively whereas hydrocortisone inhibited COX-2 enzymatic activity with IC_{50} value of 366.7 μM, which demonstrated that arctigenin could not inhibit the COX-2 enzymatic activity (original data are not shown). Arctigenin has already been shown to block the NO production induced by LPS in a dose-dependent manner. Because the NO overproduction is always associated with the up-regulation of inducible iNOS expression or the activation of iNOS enzyme, then the iNOS expression together with COX-2 expression were examined by using Western blot. As shown in Fig. 4A, treatment with 30 μM and 10 μM of arctigenin completely inhibited LPS-induced expression of iNOS, but not the overexpression of COX-2. The density of bands corresponding to the iNOS and COX-2 proteins was normalized on the basis of β-actin and shown in Fig. 4B and Fig. 4C, respectively.

3.4. Arctigenin inhibited iNOS enzymatic activity

Furthermore, the inhibitory effect of arctigenin on the activity of iNOS enzyme was examined. RAW 264.7 cells were treated with LPS (1 μg/ml) with or without indicated concentrations of arctigenin (1–100 μM). As shown in Fig. 5, LPS treatment caused about six-fold increasement of iNOS enzymatic activity within 120 min. Arctigenin strongly inhibited the iNOS enzymatic activation in RAW 264.7 cells and showed good dose dependency.

4. Discussion

Macrophages produce NO and pro-inflammatory cytokines such as TNF-α and IL-6, etc. when upon inflammatory stimulations. Overproduction of these mediators is present in macrophage of many inflammatory diseases, including rheumatoid arthritis, atherosclerosis, and hepatitis (Isomaki and Punnonen, 1997; Libby et al., 2002; Tilg et al., 1992). Detection of high levels of these pro-inflammatory cytokines is considered to be essential for clinical diagnosis. Large amount of NO produced by iNOS is believed as one of the most important inflammatory reactions in activated macrophage (Pokharel et al., 2007). iNOS is one of three key enzymes generating NO from the amino acid L-arginine (Bogdan, 2001a). iNOS gene expression and subsequent mRNA translation are controlled by various agonists, especially pro-inflammatory mediators. The most prominent cytokines involved in iNOS stimulation are TNF-α, IL-1β, IL-6 and IFN-γ (Bogdan, 2001b). The expression of iNOS is regulated by transcription factors including NF-κB, activator protein 1, signal transducer and activator of transcription, 1α interferon regulatory protein 1, nuclear factor interleukin-6, and high-motility group I (Y) protein (Nathan, 1992). iNOS has been implicated in different stages of cellular changes that lead to malignancy: transformation of normal cells; growth of transformed cells;

Table 2
Inhibitory effect of arctigenin on TNF-α release in RAW 264.7 and THP-1 cells.

Treatment	Concentration (μM)	TNF-α (pg/ml)	
		RAW 264.7 cells	THP-1 cells
Untreated	0	80.8 ± 0.7	1.5 ± 3.3
1 μg/ml LPS	0	1264.3 ± 103.0##	34.9 ± 1.1##
LPS + arctigenin	100	62.8 ± 23.5***	2.4 ± 0.6***
	30	459.5 ± 29.1**	15.0 ± 0.7***
	10	869.3 ± 37.4*	27.8 ± 2.4*
	3	1205.2 ± 0.2	31.2 ± 2.8
LPS + hydrocortisone	100	338.8 ± 18.7***	5.3 ± 4.6***
	30	874.9 ± 40.1*	20.6 ± 3.4**
	10	993.3 ± 55.3*	27.3 ± 3.2*
	3	1235.1 ± 97.6	32.8 ± 1.9

The experiment was performed in triplicate, and the results are expressed as means ± SD of TNF-α level.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ versus LPS treatment group.
$p < 0.01$ versus untreated group.

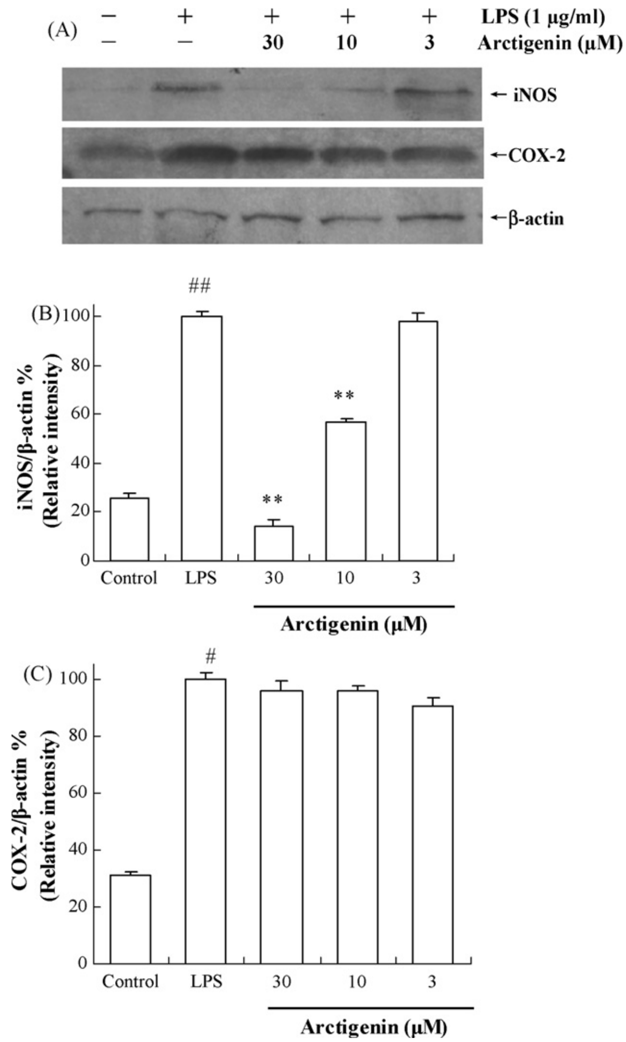

Fig. 4. RAW 264.7 cells were treated with LPS (1 μg/ml) with or without arctigenin (3, 10, 30 μM) for 24 h, and then the expression of iNOS protein and COX-2 protein were assessed by Western blot analysis (A). Detection of β-actin was carried out to confirm the equal loading of proteins. Densitometric analysis of iNOS protein (B) and COX-2 protein (C) expression represent the mean from three separate experiments. Data were normalized on the basis of β-actin levels. $^{**}p < 0.01$ versus LPS group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus control group.

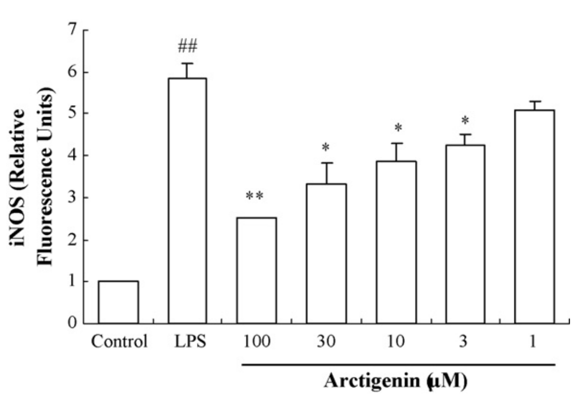

Fig. 5. RAW 264.7 cells were treated with LPS (1 μg/ml) with or without arctigenin (1, 3, 10, 30, 100 μM) for 2 h, and then the fluorescence was measured at excitation of 485 nm and emission of 528 nm. The levels of iNOS enzymatic activity were plotted as relative fluorescence units. The experiment was repeated twice and the same result was obtained. $^{*}p < 0.05$; $^{**}p < 0.01$ versus LPS group. $^{\#\#}$ $p < 0.01$ versus control group.

angiogenesis triggered by angiogenic factors released from tumor cells or from the surrounding tissue; and metastasis of malignant cells (Geller and Billiar, 1998). In a variety of human malignant tumors, e.g. breast, lung, prostate, bladder, colorectal cancer, and malignant melanoma, expression of iNOS can be observed (Lirk et al., 2002). Further studies are required to determine the role of the NO/iNOS pathway in tumorigenesis and to establish the utility of iNOS inhibitors as chemoprevention agents.

Previous studies have indicated that arctigenin could regulate immune responses in activated macrophages (Cho et al., 1999). Studies (Cho et al., 2004) showed that arctigenin potently suppressed I-κB alpha phosphorylation and nuclear translocation of p65, and also inhibited activation of MAP kinases including ERK1/2, p38 kinase and JNK through the inhibition of MKK activities, leading to AP-1 inactivation, which might, at least in part, contribute to the inhibition of TNF-α production. In the present study, the possible anti-inflammatory molecular mechanism of arctigenin in macrophages murine RAW 264.7 cells and human THP-1 cells has been further examined. Arctigenin blocked LPS-induced various responses of macrophage including the NO overproduction and the release of pro-inflammatory cytokines TNF-α and IL-6. The inhibitory effect of arctigenin on TNF-α release has been proved with respect to the inhibition of MAP kinases activation including ERK1/2, p38 kinase and JNK through the inhibition of MKK activities, leading to AP-1 inactivation (Cho et al., 2004). In the present study, the results suggested that arctigenin suppressed the NO production through down-regulation of iNOS expression and inhibition on iNOS enzymatic activity in LPS-activated macrophages. Furthermore, cytotoxicity was not detected in both RAW 264.7 and THP-1 cells. It is expected that arctigenin and the medicinal plants contain arctigenin and its analogues will have potent potency in the treatment on inflammatory diseases.

5. Conclusion

In conclusion, arctigenin exhibited potent inhibitory effects on the production of NO and the release of TNF-α and IL-6 in LPS-activated macrophages RAW 264.7 and THP-1. The mechanism of inhibition on NO production seems to be due to down-regulation of iNOS protein expression and inhibition on the iNOS enzymatic activity.

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