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Research Paper

Huang-Lian-Jie-Du-Tang exerts anti-inflammatory effects in rats through inhibition of nitric oxide production and eicosanoid biosynthesis via the lipoxygenase pathway

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Abstract

Objectives Huang-Lian-Jie-Du-Tang (HLJDT) is a traditional Chinese medicine with a long history of anti-inflammatory use, but its pharmacological effects have not been thoroughly investigated. This study aimed to evaluate the anti-inflammatory activity of HLJDT *in vivo* and *in vitro*.
Methods The carrageenan rat air pouch model was used to investigate the anti-inflammatory action of HLJDT after oral administration. Moreover, we exploited a modified method based on liquid chromatography–tandem mass spectrometry (LC-MS/MS) technique to assay the effects of HLJDT on arachidonic acid metabolites.
Key findings Our data demonstrate that oral administration of HLJDT significantly inhibited the inflammatory responses in carrageenan-injected rat air pouches, and also significantly reduced the production of nitric oxide (NO) and leukotriene B_4 (LTB_4) *in vivo*, without any influence on biosynthesis of cyclooxygenase (COX)-derived eicosanoids. Similar behaviour of HLJDT was also observed by using calcium ionophore A23187-stimulated peritoneal macrophages, where HLJDT markedly inhibited eicosanoids derived from different lipoxygenases. The NO production and the mRNA expression of inducible nitric oxide synthase (iNOS) and chemotactic factors (CCL3, CCL4, CCL5 and CXCL2) were also inhibited by HLJDT in RAW 264.7 macrophages stimulated by lipopolysaccharide.
Conclusions Our data revealed, for the first time, that HLJDT could inhibit biosynthesis of eicosanoids derived from different lipoxygenases. Also, HLJDT may exert its anti-inflammatory effects by its suppression on eicosanoid generation, NO production and gene transcription of chemotactic factors, which supports its effectiveness in the treatment of inflammatory diseases.
Keywords chemotactic factors; Huang-Lian-Jie-Du-Tang (HLJDT); inducible nitric oxide synthase; lipoxygenase; nitric oxide

Introduction

Huang-Liang-Jie-Du-Tang (HLJDT, also known as Oren-gedoku-to in Japan) is a traditional Chinese medicine, which is an aqueous extract of four medicinal herbs (*Rhizoma coptidis*, *Radix scutellariae*, *Cortex phellodendri* and *Fructus gardeniae*). The record of HLJDT can be traced back to AD 752 in Wai Tai Mi Yao, an ancient classical Chinese medical book written by Tao Wang. Its efficacy and safety have been studied in humans and in several animal models.[1–4] Studying mechanisms of HLJDT remains difficult because HLJDT comes from four medicinal herbs and may exert its effects via multiple pathways. The fact that HLJDT has some advantages over each of the component herbs makes mechanism study more imperative.[5]

Most pharmacological studies of HLJDT have focused on its anti-inflammatory activity in acute inflammation. During this process, first, macrophages resident in tissue encounter different inflammatory stimuli and are activated. Macrophage activation and subsequent increased production of several pro-inflammatory molecules (e.g. arachidonic acid

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metabolites, chemokines, nitric oxide, etc.) lead to the local release of other mediators and leucocyte infiltration into sites of inflammation. All these processes play important roles in immune regulation and inflammation.[6] The macrophage represents an excellent therapeutic target for anti-inflammatory drugs.

Several authors have attributed the anti-inflammatory activity of HLJDT to its inhibition of the generation of chemokines and eicosanoids.[4,5,7–9] Moreover, the inhibition of baicalin, baicalein and wogonin (the main constituents of HLJDT) on arachidonic acid metabolism have also been reported.[10–12] In these studies researchers usually selected to observe only PGE_2 and LTB_4 as biomarkers of inflammation. However, the effect of HLJDT on the entire arachidonic acid metabolic process is still unclear.

This study was designed to determine the anti-inflammatory efficacy of HLJDT in the rat air pouch model and investigate whether HLJDT could modulate the production of nitric oxide (NO) and eicosanoids *in vivo* and *in vitro*. Moreover, we exploited a modified method based on liquid chromatography–tandem mass spectrometry (LC-MS/MS) technique to assay the effects of HLJDT on arachidonic acid metabolites. Its effect on mRNA expression of iNOS, and some chemokines, including CCL3/MIP-1α (macrophage inflammatory protein-1α), CCL4/MIP-1β (macrophage inflammatory protein-1β), CCL5/RANTES (regulated upon activation normal T-cell expressed and secreted) and CXCL2/MIP-2 (macrophage inflammatory protein-2), were also observed.

Materials and Methods

Chemicals

Arachidonic acid was purchased form Cayman Chemicals (Ann Arbor, USA). The calcium ionophore A23187, nordihydroguaiaretic acid (NDGA), sulfanilamide, carrageenan, indometacin, dexamethasone, aminoguanidine, lipopolysaccharide (LPS), thiazolyl blue tetrazolium bromide (MTT) and thioglycolate broth (Fluka product) were purchased from Sigma-Aldrich (St Louis, MO, USA). Other reagents were purchased from Sinopharm Chemical Reagent Company Ltd (Shanghai, China).

Arachidonic acid and the calcium ionophore A23187 were dissolved in ethanol. Carrageenan solution (1%, dissolved in saline) was prepared a day before the experiments. LPS, MTT and thioglycolate broth were prepared with water (or media) and sterilized. NDGA, indometacin, dexamethasone and aminoguanidine were used as control drugs; they were prepared as stock solution and diluted with sterile working media. All the solutions above were stored frozen before use.

Preparation of Huang-Lian-Jie-Du-Tang

All component decoction pieces in HLJDT, including *Rhizoma coptidis*, *Radix scutellariae*, *Cortex phellodendri* and *Fructus gardeniae*, were purchased from Bozhou (Anhui province, China) and authenticated by Professor HanMing Zhang (Second Military Medical University, Shanghai, China). The samples were stored in the school of Pharmacy of Second Military Medical University, Shanghai, China.

HLJDT was prepared as previously described.[13] Briefly, the four medicinal herbs were mixed in the ratio 3 : 2 : 2 : 3 and extracted by boiling water. The mixture was decocted twice by refluxing with water (1 : 10 and then 1 : 8 w/v) for 1 h. The extracted solution was filtered through five-layer gauze, and the solution obtained was concentrated to give a freeze-dried powder. The yield ratio was around 10%. For quality evaluation, high-performance liquid chromatography (HPLC) analysis was performed by examining the quantity of key constituents. For using *in vivo*, the HLJDT extract was dissolved in 0.5% carboxymethylcellulose-Na (CMC-Na) and given orally according to indication. For using *in vitro*, the HLJDT was dissolved in water at a concentration 10 mg/ml and the fluid was stirred at 37°C for 1 h. After centrifugation, the supernatant was sterilized by filtration through a 0.2-μm filter and stored at –20°C before use.

High-performance liquid chromatography-diode array detector analysis

HPLC-diode array detector (DAD) analysis of HLJDT was performed on a Shimadzu LC 2010AHT HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of a quaternary pump, a column oven, an autosampler and DAD (Shimadzu Co., Japan) coupled with a CLASS-VP workstation. The chromatographic separation was performed on a C_{18} XTerra column (4.6 mm × 250 mm, 5 μm; Waters, Milford, USA) with the column temperature set at 30°C. A linear gradient elution of A (0.5% formic acid) and B (acetonitrile) was used with the gradient elution as follows: 0 min, 95% A, 5% B; 45 min, 77% A, 23% B; 60 min, 65% A, 35% B; 70 min, 25% A, 75% B. Elution was performed at a solvent flow rate of 1.0 ml/min. Data acquisition was performed using a CLASS-VP workstation (Shimadzu Corporation, Kyoto, Japan).

Reference standards, including geniposide, jatrorrhizine, palmatine, berberine, baicalin, baicalein and wogonin, were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Coptisine, epiberberine and wogonoside were purchased from Maker Company (Tianjing, China).

Animals

Sprague–Dawley rats, 160–200 g, were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). The rats were housed in a temperature- and humidity-controlled room. Free access to food and water was provided. All animal treatments were strictly performed in accordance with the National Institutes of Health Guide of the Care and Use of Laboratory Animals (National Research Council, 1996). The experiments were carried out under the approval of the Committee of Experimental Animal Administration of the University.

Rat air pouch model

Air pouches were produced by subcutaneous injection of 20 ml sterile air into the intrascapular area of the back of

the rats on day 0. Pouches were developed for 6 days. Ten millilitres of air were injected into the pouch on day 2 and day 5. Rats were fasted with free access to water during the test. HLJDT was administered by oral gavage at a dose of 150 and 300 mg/kg in 1 ml of 0.5% CMC-Na once a day on day 4–6. Indometacin (5 mg/kg), as reference compound, was given orally in 1 ml of CMC-Na on day 4–6. One hour after the last treatment on day 6, carrageenan solution (2 ml, 1% dissolved in saline) was injected into the pouch. The rats in the saline group were injected with 2 ml saline. Six hours later, the rats were anaesthetized with ether, and 1 ml heparinized saline (10 U/ml) was injected into the pouch. Fluid was collected and volumes were measured. The leucocyte numbers in the exudates were counted. After centrifugation at 3000*g* for 10 min at 4°C, the supernatants were collected and stored in –20°C for analysis of NO and eicosanoids.

Cells

RAW 264.7 murine macrophages, a cell line well suitable for the study of inflammation, were used to test HLJDT *in vitro*, which reduced the number of test rats, and made it easy to maintain RNA integrity during real-time PCR. However, RAW macrophages are not suitable for testing a drug on the lipoxygenase pathway. They produced a much lesser amount of lipoxygenase metabolites than peritoneal macrophages did in our system (data not shown). Major differences between their actions on arachidonic acid metabolism have been reported.[14] Therefore, rat peritoneal macrophages were chosen to further the study of HLJDT on the lipoxygenase pathway.

RAW 264.7 murine macrophages were obtained form the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and maintained in media recommended by the suppliers, supplemented with 10% fetal bovine serum (Gibco, Paisley, UK), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37°C.

Rat elicited peritoneal macrophages were prepared as described.[15] Rats were injected intraperitoneally with 3 ml thioglycolate broth three days before use. Then, the rats were killed and 20 ml ice-cold phosphate-buffered saline (PBS; pH 7.4) was injected into the peritoneal cavity. The liquid containing the peritoneal cells was collected aseptically using a sterile Pasteur pipette. After centrifugation (1000*g* for 10 min, 4°C) and lavage, the macrophages were harvested (about 3×10^7 cells per rat). The cells were resuspended in media as indicated.

Cell viability test

RAW 264.7 macrophages and rat peritoneal macrophages seeded into 96-well plates (1×10^4 cells per well) were incubated with the compounds tested for 68 h, then MTT was added to give a final concentration of 0.5 mg/ml. Cells were incubated for another 4 h and 100 μl dimethyl sulfoxide was added per well. The amount of formazan accumulated in the growth medium was assessed using an absorbance microplate reader Elx800 (BioTek Instruments, Inc., Winooski, USA). Conditions were considered toxic if the cells' ability to metabolize MTT to formazan was lowered by more than 20% as compared with control.

Measurement of nitric oxide

The amount of NO was assessed by determining the nitrite concentration with Griess reagent. Briefly, in the experiment to assess NO in culture supernatants, RAW 264.7 macrophages were seeded into 48-well plates (2×10^4 cells per well). The cells were pretreated with HLJDT, aminoguanidine or vehicle solution for 20 min, then stimulated with LPS (1 μg/ml) for 18 h. Samples of supernatants (100 μl) were incubated with 50 μl 1% sulfanilamide, then 50 μl of 0.1% naphthylethylenediamine in 2.5% phosphoric acid solution. The absorbance at 570 nm was read and referred to a standard curve of sodium nitrite solution to determine the nitrite concentration.

In the other experiment to determine the NO concentration of exudates from rat air pouches, the exudates (50 μl) were incubated with nitrate reductase solution (200 μl; Jiancheng Bioengineering Institute, Nanjing, China) at 37°C for 1 h. Nitrate was converted into nitrite. After centrifugation, the nitrite concentration in the cell-free supernatants was assessed with Griess reagent as described above.

Stimulation of rat peritoneal macrophages by A23187

The rat peritoneal macrophages were suspended in PBS (pH 7.4, 2 mM $CaCl_2$ and 0.5 mM $MgCl_2$) at a concentration of 2×10^7 cells/ml. After pretreatment with HLJDT, indometacin, NDGA or vehicle solution for 20 min, 1-ml volumes were incubated with arachidonic acid (50 μM) and A23187 (5 μM) for 15 min. The reaction was stopped by adding 2 ml cold alcohol and stored frozen for LC-MS/MS analysis.

Measurement of arachidonic acid metabolites by liquid chromatography–mass spectroscopy/mass spectroscopy

Eicosanoids formed in samples (exudate or cell supernatant) were determined by LC-MS/MS. Briefly, prostaglandin B_2 (PGB_2; 200 ng) was added into each sample as an internal standard. Then, the eicosanoids thus formed were extracted by solid-phase extraction (Sep-Pak C_{18} 200 mg; Waters Corporation, Milford, MA, USA). An improved assay for arachidonic acid metabolites using HPLC tandem mass spectrometry has been developed.[16,17] An Agilent 6410A triple quadrupole LC-MS system (Agilent Corporation, Andover, MA, USA) equipped with G1311A quaternary pump, G1322A vacuum degasser, G1329A autosampler and G1316A thermo column compartments was used for all analyses. The system was controlled by MassHunter software (Agilent Corporation). The separation was performed by a ZORBAX SB-C_{18} column (3.5 μm, 2.1 × 100 mm; Agilent Corporation) and a C_{18} guard column (5 μm, 4.0 × 2.0 mm; Phenomenex, Torrance, CA, USA). The arachidonic acid metabolites were identified by comparing their retention time and ion pairs (the parent and specific fragment ions) with those of authentic standards and quantified by the peak area. The relative amount was

calculated by comparing the peak area of analyte to the peak area of internal standard PGB_2, and expressed as ratio of analyte versus PGB_2.

The authentic standards used were: arachidonic acid, 5-hydroxyeicosatetraenoic acid (5-HETE), 8-hydroxyeicosatetraenoic acid (8-HETE), 11-hydroxyeicosatetraenoic acid (11-HETE), 12-hydroxyeicosatetraenoic acid (12-HETE), 15-hydroxyeicosatetraenoic acid (15-HETE), 12-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT), prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), prostaglandin B_2 (PGB_2) and leukotriene B_4 (LTB_4) and were purchased form Cayman Chemicals (Ann Arbor, MI, USA). They were dissolved in ethanol and stored at −80°C before use.

RNA extraction and reverse transcription real-time quantitative polymerase chain reaction

The cell lines were seeded in six-well plates (2×10^5 cells per well) for RNA extraction. Cells grown to confluence were exposed to fresh culture medium containing HLJDT, dexamethasone or vehicle solution, and cultured for 3 h, then stimulated with LPS 1 μg/ml. After 24 h incubation, the cell pellets were washed twice with cold PBS. Thereafter, RNA was extracted using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. The total RNA concentration was determined spectrophotometrically. Total RNA (500 ng) was reverse-transcribed to cDNA in a volume of 10 μl at 37°C for 30 min and inactivated at 86°C for 1 min. cDNA obtained from the RT reaction (10 ng of total RNA) was subjected to real-time PCR using fluorescent dye SYBR Green and Thermal Cycler Dice Real time System TP800 (TaKaRa Bio Inc., Kyoto, Japan). The reagents used above were from SYBR PrimeScript RT-PCR Kit (TaKaRa Bio Inc., Kyoto, Japan). The primers were designed according to the corresponding reference sequences in the NCBI data base and were shown in Table 1. PCR thermocycler conditions comprised an initial holding at 95°C for 15 s and subsequently a two-step PCR programme consisting of 95°C for 15 s and 60°C for 1 min for 40 cycles. Each sample was determined in duplicate. The relative changes of iNOS and chemokine mRNA between test and control samples were quantified using the $2^{-\Delta\Delta Ct}$ method.[18] The data were normalized with β-actin and expressed as the fold change in gene expression relative to the untreated cell group.

Statistical analyses

Results are expressed as mean ± SEM. Statistical significance of the results was calculated by analysis of variance followed by Dunnett's multiple comparison test. Differences were considered significant when $P < 0.05$.

Results

Quality evaluation of Huang-Lian-Jie-Du-Tang

Using the proposed method, HPLC-DAD chromatograms were acquired from HLJDT. Based on comparison analysis of ultraviolet spectrum and retention time with the corresponding reference compounds, three groups of the major bioactive constituents can be identified: alkaloids (berberine, palmatine, coptisine, epiberberine and jatrorrhizine), flavonoids (baicalin, baicalein, wogonoside and wogonin) and iridoid (geniposide) (Figure 1).

Cell viability

The effects of HLJDT on macrophage viability was determined by an MTT assay. HLJDT did not show any cytotoxicity at concentrations up to 300 μg/ml compared with control (data not shown).

Effect of Huang-Lian-Jie-Du-Tang on inflammatory responses induced by carrageenan in air pouch rats

Injection of carrageenan into the air pouches of rats led to a robust inflammatory response in rats (influx of leucocytes, leakage of plasma and release of arachidonic acid, LTB_4,

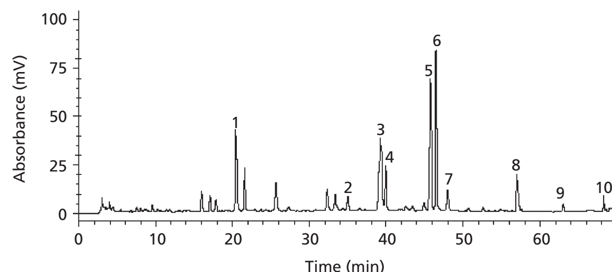

Figure 1 High-performance liquid chromatography analysis of Huang-Lian-Jie-Du-Tang in methanol at 254 nm. 1, geniposide; 2, coptisine; 3, epiberberine; 4, jatrorrhizine; 5, palmatine; 6, berberine; 7, baicalin; 8, wogonoside; 9, baicalein; 10, wogonin.

Table 1 Sequences of real-time polymerase chain reaction primers for mouse

	Forward primer	Reverse primer
β-actin	5′TGTCCACCTTCCAGCAGATGT3′	5′AGCTCAGTAACAGTCCGCCTAGA3′
iNOS	5′GCCACCAACAATGGCAACA3′	5′CGTACCGGATGAGCTGTGAA3′
CCL3/MIP-1α	5′TTGCTCCCAGCCAGGTGT3′	5′GCATTCAGTTCCAGGTCA3′
CCL4/MIP-1β	5′TGTGCTCCAGGGTTCTCA3′	5′ACTGCTGGTCTCATAGTAATC3′
CCL5/RANTES	5′TGCTCCAATCTTGCAGTC3′	5′AACCCTCTATCCTAGCTCAT3′
CXCL2/MIP-2	5′CGCCCAGACAGAAGTCATA3′	5′GTTAGCCTTGCCTTTGTTC3′

iNOS, inducible nitric oxide synthase; CCL3/MIP-1α, macrophage inflammatory protein-1α; CCL4/MIP-1β, macrophage inflammatory protein-1β; CCL5/RANTES, regulated upon activation normal T-cell expressed and secreted; CXCL2/MIP-2, macrophage inflammatory protein-2.

Table 2 Effects of Huang-Lian-Jie-Du-Tang on leucocyte accumulation and exudate volume in the rat air pouches

	Dose	Exudate volume (ml)	Leucocyte influx 10^6 cells (total)
Saline		$1.22 \pm 0.23^{**}$	$3.8 \pm 2.5^{*}$
Carrageenan[a]	1% 2 ml	3.85 ± 0.43	69.4 ± 16.9
Indometacin[b]	5 mg/kg	$1.90 \pm 0.68^{*}$	$48.3 \pm 5.3^{*}$
HLJDT	300 mg/kg	$2.86 \pm 0.54^{*}$	$44.3 \pm 7.5^{*}$
	150 mg/kg	$3.00 \pm 0.55^{*}$	$51.1 \pm 8.9^{*}$

[a]Responses in rats treated with 1% carrageenan (2 ml, s.c.) were set as the negative control; [b]responses in rats pretreated with indometacin (5 mg/kg, p.o.) were set as the positive control. Each value represents the mean ± SEM of 8–10 rats. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with carrageenan control.

12-HHT and PGE_2). HLJDT significantly relieved the inflammatory responses induced by carrageenan. As shown in Table 2, exudate volume and leucocyte influx were significantly reduced by HLJDT at two of the doses used (150 mg/kg and 300 mg/kg, p.o.), with the inhibition ratio for exudate volume being 22.1% and 25.7%, and for leucocyte influx 26.4% and 36.2%, respectively. Indometacin (5 mg/kg, p.o.) significantly inhibited the exudate volume and leucocyte influx, with inhibition ratios of 50% and 30.4%, respectively.

PGE_2, LTB_4 and NO are known to be important pro-inflammatory mediators. They were assayed in exudates of rat air pouches, and 12-HHT was also evaluated as a biomarker of the COX pathway, since it is abundant *in vivo* and easy to monitor by HPLC, but the biological role of 12-HHT is still uncertain. As shown in Figure 2, the formation of lipoxygenase metabolite LTB_4 was strongly inhibited by HLJDT at the doses assayed, whereas no inhibition of the formations of COX metabolites (PGE_2 and 12-HHT) was observed, and the level of endogenous arachidonic acid was also unaffected. Indometacin, a COX inhibitor, markedly inhibited the production of 12-HHT and PGE_2. No effect of indometacin on LTB_4 and endogenous arachidonic acid was observed.

After stimulation with carrageenan, the mean NO concentration in the control group (92.1 ± 9.6 μM) was higher than that in the saline group (26.2 ± 9.9 μM). Oral administration of HLJDT (150 and 300 mg/kg) significantly reduced the NO level in exudates, 75.6 ± 13.4 μM ($P < 0.05$) and 66.4 ± 11.6 μM ($P < 0.01$), respectively, in the indometacin group; the mean NO concentration was decreased as expected (70.6 ± 12.1 μM, $P < 0.05$).

Activity of Huang-Lian-Jie-Du-Tang on arachidonic acid metabolites in A23187-stimulated peritoneal macrophages

Intact cells were harvested from the rat peritoneal cavity after thioglycolate broth injection. Compared with arachidonic acid metabolites in exudates, the corresponding compounds in cell samples could be isolated more efficiently, because of the lesser interference with LC-MS/MS. The conversion of arachidonic acid by cells into products of the COX and lipoxygenase pathways were studied. The determination of products of other lipoxygenase isoforms was also followed. As Figure 3 shows, HLJDT (10, 100 and 300 μg/ml) had no effect on PGE_2, PGD_2, 12-HHT or 11-HETE, whereas it markedly inhibited the biosynthesis of LTB_4 and 5-HETE (IC50 = 23.4 and 21.6 μg/ml, respectively) and showed moderate inhibition on 8-HETE, 12-HETE and 15-HETE (IC50 = 51.7, 82.7 and 59.5 μg/ml, respectively). The effect of indometacin and NDGA, two positive control drugs with distinct mechanisms, was also observed on the cells. Indometacin (20 μM), a COX inhibitor, showed potent inhibition on 12-HHT, PGE_2, PGD_2 and 11-HETE, moderate or weak inhibition on LTB_4, 15-HETE and 5-HETE and no inhibition on 8-HETE and 12-HETE, respectively. Different from indometacin, NDGA (10 μM), a lipoxygenase inhibitor, showed potent inhibition on 5-HETE, 8-HETE, 12-HETE, 15-HETE and LTB_4, moderate or weak inhibition on PGE_2, PGD_2 and 11-HETE, and no inhibition on 12-HHT, respectively.

Activity of Huang-Lian-Jie-Du-Tang on nitric oxide production and inducible nitric oxide synthase mRNA expression in RAW 264.7 macrophages stimulated by lipopolysaccharide

HLJDT (50–200 μg/ml) significantly inhibited LPS-induced NO overproduction in a concentration-dependent manner. HLJDT at a concentration of 200 μg/ml caused a significant reduction of NO (27.5%) without cytotoxicity (Table 3). Production of NO via iNOS pathways can be regulated at the transcriptional level.[19] Real-time PCR was applied to assay the effect of HLJDT on the mRNA expression of iNOS in macrophages stimulated by LPS. HLJDT (100, 200 μg/ml) significantly inhibited the iNOS mRNA expression, with maximal inhibition percentage of 39.7 % (Figure 4). Aminoguanidine (an iNOS inhibitor) and dexamethasone (an inhibitor of the induction of iNOS) were used as control drugs. Aminoguanidine (50 μM) significantly inhibited the NO production with an inhibition ratio of 68.1%. Dexamethasone (1 μM) significantly inhibited the gene expression of iNOS with an inhibition ratio of 39.2%.

Effect of Huang-Lian-Jie-Du-Tang on CCL3, CCL4, CCL5 and CXCL2 mRNA expression

To further study the effect of HLJDT on leucocyte infiltration, the activity of HLJDT on several chemokines (including CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES and CXCL2/MIP-2) at the transcriptional level were also observed in RAW 264.7 macrophages. No significant effect of HLJDT (100 μg/ml) was observed on the mRNA expression level of those genes above in unstimulated cells (data not shown). In the cells stimulated by LPS, the gene expression of these chemokines increased to various degrees upon LPS stimulation. The mRNA of CXCL2 increased greatly (about 5.8 fold). At a dose of 100 μg/ml, HLJDT could block the expression of these chemokines (Figure 5).

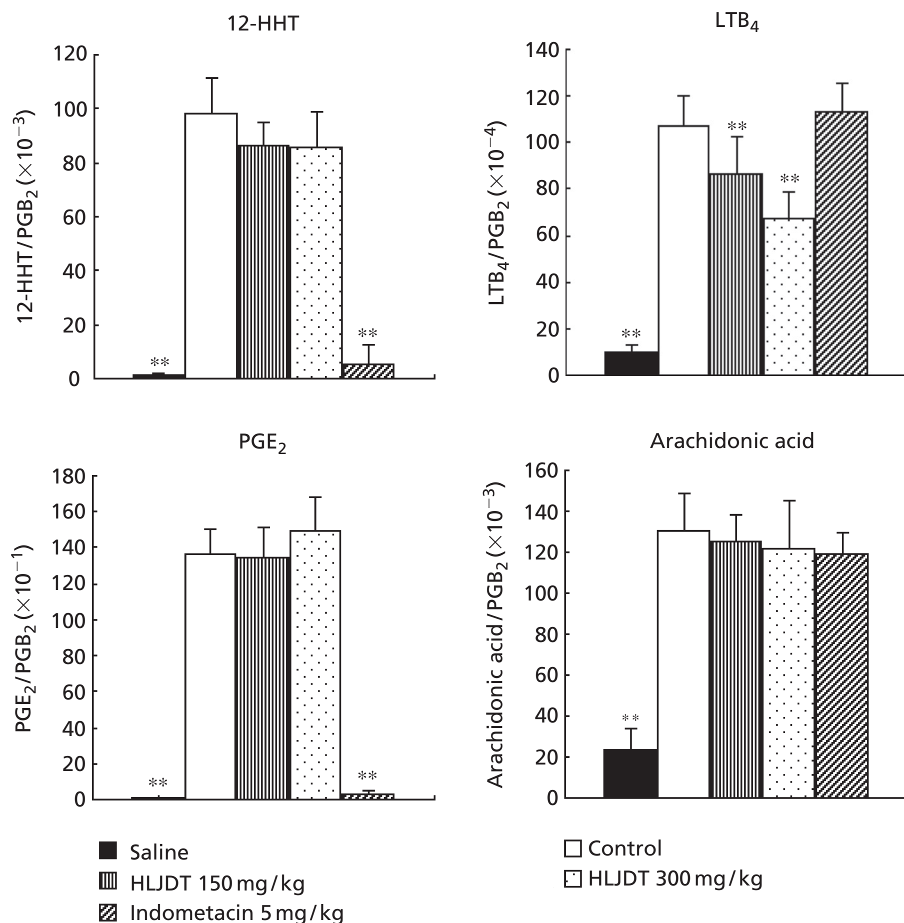

Figure 2 Effect of Huang-Lian-Jie-Du-Tang on eicosanoid formation in exudates of rat air pouches. Responses in rats treated with 1% carrageenan (2 ml, s.c.) were set as the negative control; responses in rats pre-treated with indometacin (5 mg/kg, p.o.) were set as the positive control. Each value represents the mean ± SEM of 8–10 rats. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the carrageenan control.

Discussion

HLJDT, as reported, is able to reduce rat paw oedema and increase dye permeability[2,7] and inhibit leucocyte infiltration into the sites of inflammation.[8,20,21]

Our results are consistent with those reports and suggest oral administration of HLJDT produces anti-inflammatory effects in the rat air pouch model and inhibits the increased migration of leucocytes, which is an index of the early stage of inflammation, through interference with arachidonic acid metabolism and production of NO.

Further study demonstrated the inhibition of HLJDT on the biosynthesis of lipoxygenase metabolites in carrageenan-injected rat air pouch and A23187-stimulated peritoneal macrophages; interestingly, no inhibition of HLJDT on COX-derived eicosanoids was observed. HLJDT inhibited the biosynthesis of lipoxygenase-derived eicosanoids but not COX-derived eicosanoids, which is similar to NDGA (a lipoxygenase inhibitor), but different to indometacin (a COX inhibitor). Furthermore, the release of endogenous arachidonic acid was unaffected in HLJDT reated rats, which excluded an inhibitory effect of HLJDT on the release of arachidonic acid.

HETEs, the important lipoxygenase metabolites, induce the recruitment and activation of leucocytes.[22] They are supposed to be biosynthesized via corresponding lipoxygenase isoforms.[23] In this study, HLJDT dose dependently inhibited the biosynthesis of LTB_4, 5-HETE, 8-HETE, 12-HETE and 15-HETE in rat peritoneal macrophages stimulated by A23187. Our data suggest that HLJDT presents a higher potency on 5-lipoxygenase inhibition than on the others. Interestingly, indometacin, but not NDGA or HLJDT, markedly inhibited the biosynthesis of 11-HETE. Similar findings have previously been reported with its inhibition on the biosynthesis of 11-HETE.[24] Several reports suggest that 11-HETE may be produced by the COX pathway.[25,26] Up to now, no definitive reports of a mammalian 11-lipoxygenase is available and it remains unclear by which pathway 11-HETE is produced. Our data strongly supported an important role for COX in the biosynthesis of 11-HETE. We demonstrated for the first time that HLJDT interferes with arachidonic acid metabolism via the lipoxygenase pathway. It is an over-simplification to state that HLJTD only acts as a lipoxygenase inhibitor, because the lack of inhibition of HLJDT on COX in our experiment may reflect its specificity on COX2. Although COX2 is rapidly expressed in the presence of tissue damage

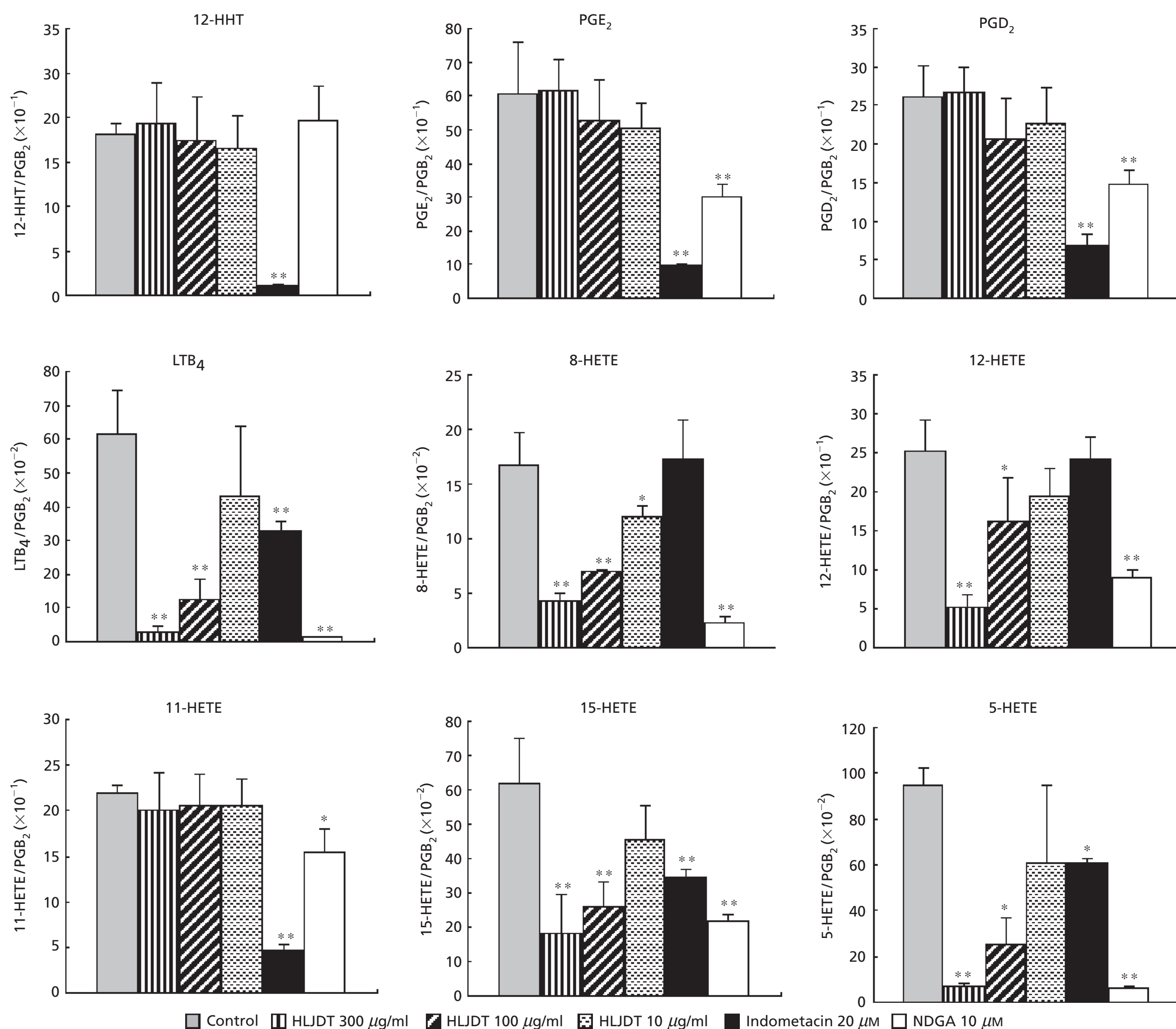

Figure 3 Effect of Huang-Lian-Jie-Du-Tang on eicosanoid formation in rat elicited peritoneal macrophages. Macrophages (2×10^7 cells) pretreated with HLJDT, indometacin, nordihydroguaiaretic acid (NDGA) or vehicle solution for 20 min were incubated with arachidonic acid (50 μM) and A23187 (5 μM) for 15 min. The reactions were stopped by adding 2 ml cold alcohol and extracted by solid phase extraction. LC-MS/MS was used to determine the amount of arachidonic acid metabolites. Responses in cells stimulated with A23187 were set as the controls; indometacin is a nonspecific inhibitor of cyclooxygenase, NDGA is a nonspecific inhibitor of lipoxygenase. Each value represents the mean ± SEM, $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the corresponding control.

or inflammation, COX1-derived prostaglandins still contribute to the inflammatory response,[14] which is supported by the finding that the extent of paw oedema induced by carrageenan in COX2-deficient mice was similar to that in wild type mice.[27]

Besides arachidonic acid metabolites, both macrophage-derived chemokines and NO are very important mediators involved in cell recruitment and damage to host tissues during initiation and maintenance of the inflammatory response.[28–30] Downregulating the gene expression of macrophage-derived chemokines and blocking NO production via iNOS shows therapeutic effects in some inflammatory diseases.[31] The activity of HLJDT on chemokines and NO also contributes to its anti-inflammatory effects.

Among the major bioactive constituents of HLJDT, flavonoids possess the most potent anti-inflammatory activity. Some flavonoids showed significant inhibition on pro-inflammatory mediators.[32–35] Flavonoids may be partially responsible for the anti-inflammatory activity of HLJDT.

Conclusions

Our results demonstrate that HLJDT has anti-inflammatory activity in the rat air pouch model. Study of the mechanism suggests that the effects are on arachidonic acid metabolism via the lipoxygenase pathway, NO production via iNOS, and gene expression of chemokines may participate in the anti-inflammatory effect of HLJDT. The results support the

Table 3 Effect of Huang-Lian-Jie-Du-Tang on nitric oxide production in lipopolysaccharide-activated RAW 264.7 macrophages

	Dose	NO (μmol/l)	Inhibition (%)
Model	LPS 1 μg/ml	4.73 ± 0.15	n.a.
Aminoguanidine	50 μM	1.51 ± 0.12**	68.1
HLJDT	200 μg/ml	3.43 ± 0.29**	27.5
	100 μg/ml	3.61 ± 0.02*	23.7
	50 μg/ml	4.18 ± 0.15	11.6
	25 μg/ml	4.98 ± 0.09	0

RAW 264.7 macrophages (2×10^4 cells per well, 48-well plate) were co-incubated with HLJDT, aminoguanidine or vehicle solution for 20 min, then stimulated with LPS (1 μg/ml) for 18 h. The amount of NO was assessed with Griess reagent. Model group was set up as negative control; aminoguanidine group was set up as positive control. n.a., not applicable. Data are mean ± SEM, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

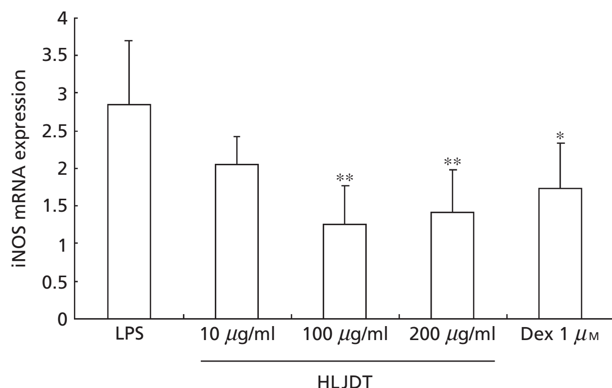

Figure 4 Effect of Huang-Lian-Jie-Du-Tang on inducible nitric oxide synthase mRNA accumulation induced by lipopolysaccharide in RAW 264.7 macrophages. RAW 264.7 macrophages (2×10^5 cells) were pretreated with HLJDT, dexamethasone (Dex) or vehicle solution for 3 h, then stimulated with lipopolysaccharide (LPS) (1 μg/ml) for 24 h. RNA was extracted with TRIzol reagent, and inducible nitric oxide synthase (iNOS) and β-actin mRNA were measured by real-time PCR. iNOS mRNA levels were normalized against β-actin. mRNA levels were expressed as folds of that of untreated cells. Data are means ± SEM, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the LPS group.

ethnomedical uses of HLJDT. Additional pharmacological studies on the main constituents of HLJDT are in progress.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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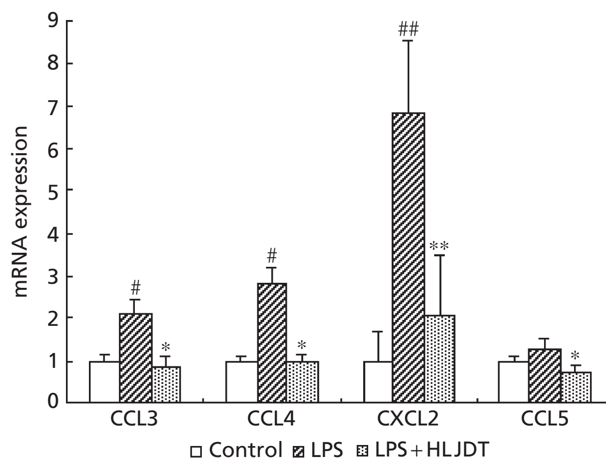

Figure 5 Suppression of chemokine expression by Huang-Lian-Jie-Du-Tang. RAW 264.7 macrophages were treated with HLJDT 100 μg/ml or vehicle solution 3 h before stimulation with lipopolysaccharide (LPS) 1 μg/ml. RNA was extracted after 24 h incubation and chemokine and β-actin mRNA were measured by real-time PCR. mRNA levels were normalized against β-actin. mRNA levels were expressed as folds of those of cells untreated. Data are means ± SEM, $n = 6$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the untreated cell group (control); $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the LPS group.

References

1. Dai Y *et al*. Suppression of neuropeptides' mRNA expression by herbal medicines in a rat model of peripheral inflammation. *Life Sci* 2000; 66: 19–29.
2. Gao XK *et al*. Kampo medicines for mite antigen-induced allergic dermatitis in NC/Nga mice. *Evid Based Complement Alternat Med* 2005; 2: 191–199.
3. Arakawa K *et al*. Improvement of accessory symptoms of hypertension by TSUMURA Orengedokuto Extract, a four herbal drugs containing Kampo-Medicine Granules for ethical use: a double-blind, placebo-controlled study. *Phytomedicine* 2006; 13: 1–10.
4. Miura N *et al*. An herbal medicine orengedokuto prevents indomethacin-induced enteropathy. *Biol Pharm Bull* 2007; 30: 495–501.
5. Fukutake M *et al*. Suppressive effect of the herbal medicine Oren-gedoku-to on cyclooxygenase-2 activity and azoxymethane-induced aberrant crypt foci development in rats. *Cancer Lett* 2000; 157: 9–14.
6. Gordon S. The role of the macrophage in immune regulation. *Res Immunol* 1998; 149: 685–688.
7. Wang LM, Mineshita S. Preventive effects of unsei-in and oren-gedoku-to, Chinese traditional medicines, against rat paw oedema and abdominal constriction in mice. *J Pharm Pharmacol* 1996; 48: 327–331.
8. Zhou H, Mineshita S. The effect of Oren-gedoku-to on experimental colitis in rats. *J Pharm Pharmacol* 1999; 51: 1065–1074.
9. Wang LM *et al*. Effects of oren-gedoku-to and unsei-in, Chinese traditional medicines, on interleukin-8 and superoxide dismutase in rats. *J Pharm Pharmacol* 1997; 49: 102–104.

10. You KM *et al*. Inhibition of cyclooxygenase/lipoxygenase from human platelets by polyhydroxylated/methoxylated flavonoids isolated from medicinal plants. *Arch Pharm Res* 1999; 22: 18–24.
11. Burnett BP *et al*. A medicinal extract of Scutellaria baicalensis and Acacia catechu acts as a dual inhibitor of cyclooxygenase and 5-lipoxygenase to reduce inflammation. *J Med Food* 2007; 10: 442–451.
12. Somjen D *et al*. Lipoxygenase metabolites are mediators of PTH-dependent human osteoblast growth. *Bone* 2008; 42: 491–497.
13. Dou SS *et al.* Chromatographic fingerprinting and quantitative analysis for the quality evaluation of Huang-Lian-Jie-Du-Tang by HPLC-DAD and HPLC-ESI-MS. *Chromatographia* (DOI: 10.1365/s10337-008-0945-3 2009)
14. Rouzer CA *et al*. RAW264.7 cells lack prostaglandin-dependent autoregulation of tumor necrosis factor-α secretion. *J Lipid Res* 2005; 46: 1027–1037.
15. Ishida-Fujii K *et al*. Prevention of pathogenic Escherichia coli infection in mice and stimulation of macrophage activation in rats by an oral administration of probiotic Lactobacillus casei I-5. *Biosci Biotechnol Biochem* 2007; 71: 866–873.
16. Margalit A *et al*. Rapid quantitation of a large scope of eicosanoids in two models of inflammation: development of an electrospray and tandem mass spectrometry method and application to biological studies. *Anal Biochem* 1996; 235: 73–81.
17. Yang K *et al*. Finding multiple target optimal intervention in disease-related molecular network. *Mol Syst Biol* 2008; 4: 228.
18. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 2001; 25: 402–408.
19. Korhonen R *et al*. Nitric oxide production and signaling in inflammation. *Curr Drug Targets Inflamm Allergy* 2005; 4: 471–479.
20. Hwang YS *et al*. Hwangryun-Hae-Dok-tang (Huanglian-Jie-Du-Tang) extract and its constituents reduce ischemia-reperfusion brain injury and neutrophil infiltration in rats. *Life Sci* 2002; 71: 2105–2017.
21. Ohta Y *et al*. Preventive effect of Oren-gedoku-to (Huanglian-Jie-Du-Tang) extract on the development of stress-induced acute gastric mucosal lesions in rats. *J Ethnopharmacol* 1999; 67: 377–384.
22. Spector AA *et al*. Hydroxyeicosatetraenoic acids (HETEs). *Prog Lipid Res* 1988; 27: 271–323.
23. Yang XW *et al*. Anti-inflammatory and anti-tumour effects of Abies georgei extracts. *J Pharm Pharmacol* 2008; 60: 937–941.
24. Myers RF, Siegel MI. Differential effects of anti-inflammatory drugs on lipoxygenase and cyclo-oxygenase activities of neutrophils from a reverse passive Arthus reaction. *Biochem Biophys Res Commun* 1983; 112: 586–594.
25. Bailey JM *et al*. Characterization of 11-HETE and 15-HETE, together with prostacyclin, as major products of the cyclooxygenase pathway in cultured rat aorta smooth muscle cells. *J Lipid Res* 1983; 24: 1419–1428.
26. Kozak KR *et al*. Oxygenation of the endocannabinoid, 2-arachidonylglycerol, to glyceryl prostaglandins by cyclooxygenase-2. *J Biol Chem* 2000; 275: 33744–33749.
27. Wallace JL *et al*. Cyclooxygenase 1 contributes to inflammatory responses in rats and mice: implications for gastrointestinal toxicity. *Gastroenterology* 1998; 115: 101–109.
28. Salvemini D *et al*. Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br J Pharmacol* 1996; 118: 829–838.
29. Luster AD. Chemokines–chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998; 338: 436–445.
30. Viola A, Luster AD. Chemokines and their receptors: drug targets in immunity and inflammation. *Annu Rev Pharmacol Toxicol* 2008; 48: 171–197.
31. Weinberg JB. Nitric oxide synthase 2 and cyclooxygenase 2 interactions in inflammation. *Immunol Res* 2000; 22: 319–341.
32. Wakabayashi I. Inhibitory effects of baicalein and wogonin on lipopolysaccharide-induced nitric oxide production in macrophages. *Pharmacol Toxicol* 1999; 84: 288–291.
33. Nakajima T *et al*. Inhibitory effect of baicalein, a flavonoid in scutellaria root, on eotaxin production by human dermal fibroblasts. *Planta Med* 2001; 67: 132–135.
34. Chang YL *et al*. Chinese herbal remedy wogonin inhibits monocyte chemotactic protein-1 gene expression in human endothelial cells. *Mol Pharmacol* 2001; 60: 507–513.
35. Chen YC *et al*. Wogonin, baicalin, and baicalein inhibition of inducible nitric oxide synthase and cyclooxygenase-2 gene expressions induced by nitric oxide synthase inhibitors and lipopolysaccharide. *Biochem Pharmacol* 2001; 61: 1417–1427.