Anti-Inflammatory Activity of Licochalcone A Isolated from *Glycyrrhiza inflata*

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Licochalcone A was isolated from the roots of *Glycyrrhiza inflata* and evaluated for its anti-inflammatory activity in xylene-induced mice ear edema and carrageenan-induced paw edema tests. At the same time, the inhibition of prostaglandin biosynthesis by licochalcone A was also studied in lipopolysaccharide (LPS)-induced mouse macrophage cells. At 5 mg/ear, licochalcone A showed remarkable effects against acute inflammation induced by xylene, and at the doses of 2.5, 5, 10 mg/kg (p. o.), licochalcone A reduced significantly paw edema induced by carrageenan compared to the control at the fourth hour. Both COX-2 activity and expression were significantly inhibited by licochalcone A at all the test doses. Therefore, licochalcone A could be a useful compound for the development of new anti-inflammatory agents.

Key words: Licochalcone A, *Glycyrrhiza inflata*, Anti-Inflammatory Activity

Introduction

Licorice, the underground material derived from the leguminous *Glycyrrhiza* plant species, has been employed for centuries as herbal drug in Western and Eastern medicine. Phytochemical investigations on licorice have demonstrated the presence of a wide variety of bioactive phenolic constituents, which have attracted attention as potential drug source (Shibata, 2000). *Glycyrrhiza inflata* is one of the main botanical sources of licorice, and is chemically characterized by the presence of retrochalcones, which are distinguished from ordinary chalcones by the absence of an oxygen functionality at the 2-position. Five retrochalcones, licochalcones A–D and echinatin, have been isolated from *G. inflata* roots and characterized (Haraguchi *et al.*, 1998); the content of licochalcone A (Fig. 1) was found to be very high among the licochalcones (Shibata, 2000).

Various biological activities of licochalcone A, such as antioxidative, antiprotozoal, antitumour promoting and antimicrobial, have been reported (Chen *et al.*, 2001; Rafi *et al.*, 2000). As a part of the systematic survey of botanical sources for anti-inflammatory activity, licochalcone A was isolated from the roots of *Glycyrrhiza inflata* and examined for its activity against xylene-induced mice ear edema and carrageenan-induced paw edema. The effect of licochalcone A on the concentration of prostaglandin E2 (PGE_2) (as an index of COX-2 activity) was also investigated for identifying the possible mechanisms of the anti-inflammatory effect.

Experimental

Plant material

The roots of *Glycyrrhiza inflata* were donated by Xinjiang Kunlunshenonong Co. Ltd. and authenticated by Ass. Prof. Ming-Zhang Ao, College of Life Science & Technology, Huazhong University of Science & Technology, Wuhan, China, where voucher specimens had been deposited for reference. The roots were dried at 30–40 °C for 48 h and pulverized.

Chemicals

Methanol (HPLC grade) was purchased from Fisher Scientific Worldwide Co., Ltd. Silica gel (200–300 mesh) and TLC plates were purchased from Sinopharm Group Chemical Reagent Co., Ltd., Shanghai, China. Carrageenan, indomethacin, arachidonic acid (AA), lipopolysaccharide (LPS) from *Escherichia coli* and PGE_2 assay kits were purchased from Sigma Chemical Co., USA. Other reagents were of analytical grade.

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Extraction and isolation of licochalcone A

The root powder (1 kg) was extracted by maceration in 10 L of acetone at room temperature for 48 h, and then filtered. This step was repeated twice, and the filtrates were mixed up and condensed to a viscous mass by a rotary evaporator under low pressure at 50 °C. Then the residue was lyophilized and weighed (32 g). One part of the extract (30 g) was dissolved in MeOH and absorbed on silica gel (30 g), and the mixture was concentrated *in vacuo*. The absorbed material was transferred to a silica gel column (48–75 μm, 4.5 × 100 cm, flow rate 2 mL/min) packed in $CHCl_3$. Elution was done with a step gradient of $CHCl_3$/MeOH 100:0 (1 L), 99:1 (2 L) and 96:4 (2 L). Fractions of 200 mL each were collected and monitored by TLC (silica gel) at 365 nm with the solvent system $CHCl_3$/MeOH (9:1, v/v); the R_f value was 0.48. Fractions 9–13 (1600–2600 mL, 5.8 g) were combined and subjected to a Sephadex LH-20 column (100 × 4.5 cm, flow rate 1 mL/min) eluted with MeOH/H_2O (1:1, v/v). 160 fractions (20 mL each) were collected and fractions 80–91 (1600–1820 mL, 2.2 g) were combined. Pure licochalcone A (1.2 g) was obtained by crystallization from MeOH/H_2O.

LC-MS analysis

LC-MS data were obtained with an Agilent 1100 series HPLC system consisting of an autosampler, high-pressure mixing pump, column oven and DAD detector connected to a Perkin Elmer API 165 single quadrupole instrument equipped with a PE Sciex Turbo ion probe.

HPLC conditions: Eclipse XDB-C18 column (5 μm, 4.6 mm × 150 mm; Agilent, Santa Clara, California, USA); solvent system, methanol/0.5% aqueous acetic acid (6:4, v/v); flow rate, 1 mL/min; injection volume, 20 μL; sample concentration, 10 mg/mL in MeOH.

ESI-MS conditions: Positive ion mode; split ratio, 1:4; scan range, 140–1000 amu; source temperature, 350 °C; ion spray voltage, 5 kV; focussing potential, 230 V; declustering potential, 20 V.

Licochalcone A

Yellow needles from MeOH/H_2O, m.p. 101 °C. – UV: λ_{max}(MeOH) = 256, 310, 376 nm. – ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.45 (6H, *s*, Me-4″ and Me-5″), 3.83 (3H, *s*, OMe), 5.35 (1H, *d*, *J* = 10.8 Hz, H-3″), 5.36 (1H, *d*, *J* = 18.0 Hz, H-3″), 6.19 (1H, *dd*, *J* = 10.4 and 10.4 Hz, H-2″), 6.45 (1H, *s*, H-3), 6.97 (2H, *d*, *J* = 8.4 Hz, H-3′ and H-5′), 7.49 (1H, *s*, H-6), 7.61 (1H, *d*, *J* = 15.6 Hz, H-α), 7.99 (2H, *d*, *J* = 9.2 Hz, H-2′ and H-6′), 8.03 (1H, *d*, *J* = 16.4 Hz, H-β). – ^{13}C NMR ($CDCl_3$, 400 MHz): δ = 189.3 (C=O), 160.8 (C-4′), 158.7 (C-4), 157.9 (C-2), 146.5 (C-2″), 139.6 (C-β), 129.4 (C-2′ and C-6′), 128.9 (C-1′), 127.5 (C-5), 125.8 (C-6), 116.7 (C-α), 113.7 (C-3′ and C-5′), 113.2 (C-3″), 108.0 (C-1), 98.3 (C-3), 53.4 (OCH_3), 38.4 (C-1″), 24.7 (C-4″ and C-5″). – EI-MS: *m/z* = 339 $[M + H]^+$. These data are in accordance with the reported literature ones (Wang *et al.*, 2004).

Animals

Kunming mice [20–25 g, SCXK (Hubei) 2006–0007] and Wistar rats (150–200 g) of either sex were obtained from a randomly bred colony maintained on special diet in the animal house of Testing Animal Center of Hubei Province, China. Animals were housed in a colony room under a light/dark (12h/12 h) cycle at (21 ± 1) °C and had free access to water and food. All experimental designs and procedures had received approval from the Animal Ethics Committee of Huazhong University of Science & Technology, Wuhan, China.

Anti-inflammatory study

The effect of licochalcone A on acute topical inflammation was evaluated by a modification method of Atta and Alkohafi (1998). The mice were divided into three groups of 8 animals each. Licochalcone A was dissolved in acetone and applied to the outer surface of the right ear of each mouse (5 mg/ear). Topical inflammation was instantly induced on the posterior surface of the same ear by application of xylene (0.05 mL). Control animals received either the vehicle (acetone) or indomethacin (5 mg/ear). 2 h later, the mice were killed by diethyl ether anaesthesia, and both ears were removed. Circular sections (7 mm diameter) of both the right (treated) and left (untreated) ears were punched out using a cork borer and weighed. Edematous response was quantified as the weight difference between the two earplugs.

The rats were divided into five groups of eight animals each and pretreated as follows: vehicle control group (propyleneglycol, 5 mL/kg, p.o.), licochalcone A groups (2.5, 5 and 10 mg/kg, p.o.), indomethacin group (10 mg/kg, i.g.) (the doses of drug administration were determined by pre-experiments; the data are not shown). The compounds were given once daily for 3 d. The paw

volumes of the animals were determined using a plethysmometer and 0.1 ml freshly prepared carrageenan was injected into the right hind paw of each animal 1 h after the last dose. The change in paw volumes was detected at 1 and 4 h following the carrageenan administration. The anti-inflammatory potency of licochalcone A was determined via comparison with the results obtained from animals that received indomethacin and propyleneglycol.

Measurements of PGE_2 accumulation by COX-2 in cultured LPS-induced macrophage cells

Purified mouse peritoneal macrophages were harvested by peritoneal lavage after i.p. 4 mL D-hank's (a balanced salt solution without Ca^{2+} and Mg^{2+}). Cells were re-suspended in RPMI-1640 (a liquid medium which is applied to cell the culture) medium supplemented with penicillin-streptomycin and 10% FBS in a 96-well culture plate and incubated at 37 °C in humidified air with 5% CO_2 for 4 h. The supernatant was removed, washed three times with fresh medium, and then incubated in the fresh medium with 1 μg/mL of lipopolysaccharide (except for the blank). Test materials were simultaneously added to each well (except for the control and blank). After additional 6 h of incubation, 2 μg/mL AA were added into each well and incubated for 40 min. PGE_2 synthesis was determined by a radioimmunoassay using a commercial PGE_2 kit (Shu *et al.*, 2006).

Mouse peritoneal macrophages were prepared and treated as above. The cells were incubated in fresh medium with 1 μg/mL of LPS. After 6 h of incubation, test materials were added to each well for 40 min of incubation. AA (2 μg/mL) was then added into each well and incubated for additional 40 min. PGE_2 synthesis was determined by a radioimmunoassay using a commercial PGE_2 kit (Shu *et al.*, 2006).

Statistical analysis

Data were expressed as mean ± S.E. and the significance was evaluated by Student's *t*-test. $p < 0.05$ was considered as significant.

Results

Anti-inflammatory effect of licochalcone A on xylene-induced ear edema

Licochalcone A was used in the xylene-induced ear edema test to evaluate the topical anti-inflammatory effect. As shown in Table I, licochalcone A (5 mg/kg) decreased the ear edema rate by 30.3% and the edema rate was smaller than that of indomethacin (41.2%). Both licochalcone A and indomethacin inhibited markedly the ear edema compared to the control.

Anti-inflammatory effect of licochalcone A on carrageenan-induced paw edema

The results presented in Table II demonstrate that licochalcone A exhibited significant anti-inflammatory activity in the later phase of the carrageenan-induced paw edema test. The paw volume in the control group prominently increased after intraplantar injection of carrageenan. At doses of 2.5, 5 and 10 mg/kg body weight, licochalcone A caused a potent and dose-dependent inhibition of the inflammation and reduced the paw edema rate by 41.3, 39.7 and 30.7%, respectively, at the fourth hour after administration. As a standard drug, indomethacin (10 mg/kg body weight, i.g.) reduced the edema rate by 21.6%.

Effect of licochalcone A on the synthesis of COX-2 in cultured LPS-induced macrophage cells

The effect of licochalcone A on inhibiting PGE_2 accumulation by suppression of the COX-2 synthesis was studied. As shown in Table III, treatment of the cells with 1 μg/mL LPS produced 14.5 pg/mL of PGE_2, causing a 2.35-fold increase of the PGE_2 production compared with the vehicle alone. When cells were treated with indomethacin (0.5 μg/mL), a selective cyclooxygenase inhibitor, the cells produced a lower concentration of PGE_2, causing 73.5% decrease of the PGE_2 production compared to the control. These results suggested that LPS treatment might cause over-synthesis of COX-2. When the cells were pretreated with 0.1,

Treatment	Dose [mg/kg]	Weight of the left ear [mg]	Weight of the right ear [mg]	Edema rate (%)
Control		0.76 ± 0.05	1.43 ± 0.21	88.2
Indomethacin	5.0	0.77 ± 0.08	1.19 ± 0.12	41.2*
Licochalcone A	5.0	0.76 ± 0.09	0.99 ± 0.14	30.3*

Table I. Effect of licochalcone A on xylene-induced acute topical edema in the mouse ear.

Values are means ± S.E.
* $p < 0.01$ compared to control.

Table II. Effect of licochalcone A on carrageenan-induced paw edema in rats.

Treatment	Dose [mg/kg]	Paw volume before inflammation [mL]	Paw volume 1 h after inflammation [mL]	Paw volume 4 h after inflammation [mL]	Edema rate (%)	
					1 h	4 h
Control	–	0.77 ± 0.06	1.16 ± 0.05	1.22 ± 0.05	50.7	58.8
Indomethacin	10	0.78 ± 0.04	0.93 ± 0.06	0.95 ± 0.03	19.2**	21.6**
Licochalcone A	2.5	0.78 ± 0.03	1.15 ± 0.04	1.10 ± 0.03	47.5	41.3*
	5	0.79 ± 0.09	1.15 ± 0.07	1.10 ± 0.07	45.5	39.7*
	10	0.77 ± 0.11	1.08 ± 0.10	1.01 ± 0.12	40.2*	30.7**

Values are means ± S.E.
* $p < 0.01$ and ** $p < 0.001$ compared to control.

Treatment	Concentration [μg/mL]	PGE_2 concentration [pg/mL]	Inhibition of COX-2 (%)
Blank		4.1 ± 0.7	–
Control		17.3 ± 1.2	–
Licochalcone A	0.1	13.2 ± 0.8*	31.1
	0.5	9.6 ± 0.7*	58.3
	1	6.7 ± 0.3*	80.3
Indomethacin	0.5	7.6 ± 0.4*	73.5

Table III. Inhibition of COX-2 protein synthesis in LPS-induced macrophages by licochalcone A.

Values are means ± S.E.
* $p < 0.001$ compared to control.

Treatment	Concentration [μg/mL]	PGE_2 concentration [pg/mL]	Inhibition of COX-2 (%)
Blank		4.8 ± 0.8	–
Control		28.7 ± 2.7	–
Licochalcone A	0.1	21.3 ± 1.9*	31.0
	0.5	18.1 ± 3.1*	44.8
	1	14.5 ± 1.2*	59.4
Indomethacin	0.5	25.6 ± 0.6	13.0

Table IV. Inhibition of COX-2 activity after COX-2 protein synthesis in cultured LPS-induced macrophages by licochalcone A.

Values are means ± S.E.
* $p < 0.01$ compared to control.

0.5 and 1 μg/mL of licochalcone A before LPS treatment, LPS-induced production of PGE_2 was decreased by 31.1, 58.3 and 80.3%, respectively. At a concentration of 1 μg/mL of licochalcone A, the inhibitory effects on LPS-induced PGE_2 production were more effective than those of 0.5 μg/mL of indomethacin.

Effect of licochalcone A on COX-2 activity after COX-2 synthesis

The effect of licochalcone A on PGE_2 production by inhibiting the COX-2 activity after COX-2 synthesis in cultured LPS-induced macrophage cells was studied. As shown in Table IV, indomethacin treatment only caused 13.0% reduction of PGE_2 accumulation compared to the control. But when the cells were treated with 0.1, 0.5 and 1 pg/mL of licochalcone A, PGE_2 accumulation was significantly decreased in a dose-dependent manner (31.0, 44.8 and 59.4%, respectively).

Discussion

Ear edema induced by xylene can be mediated by a variety of agents, such as leukocytes and prostanoids (Atta and Alkohafi, 1998). The presence of a hydrophobic prenyl moiety (C_5 units) in the B-ring of licochalcone A (as shown in Fig. 1) would be important, since it would increase the lipophilic character of a molecule penetrating the cell membrane and exert antiphlogistic action.

Carrageenan-induced paw edema is widely used for determining the acute phase of inflammation. The inflammation induced by carrageenan consists of two phases. In the early phase, histamine and bradykinine have been shown to be the first detectable mediators (Portanova *et al.*, 1996), and

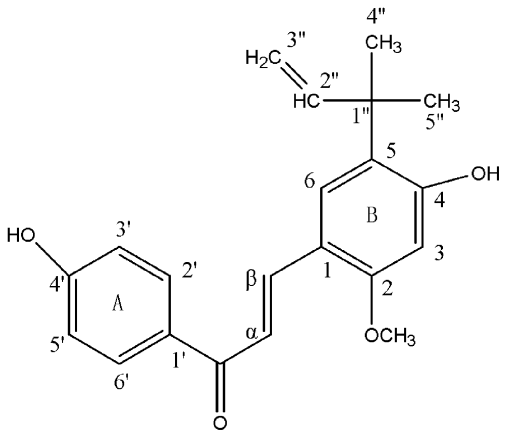

Fig. 1. Structure of licochalcone A.

prostaglandins (PGs) are found to have a role in the late phase of inflammation (Ialenti *et al.*, 1992). In our experiment, licochalcone A caused a potent inhibition of the inflammation at the fourth hour. Therefore, it may inhibit the synthesis of prostaglandins in the late phase of inflammation.

PGs are hormone-like endogenous mediators of inflammation and formed from arachidonic acid by COX-1 and the inducible form COX-2 (Simon, 1999). COX-2 has been reported to play an important role in inflammation. Decreasing activity and expression of COX-2 can result in an anti-inflammatory effect in localized and systemic conditions (Subbaramaiah *et al.*, 1996). Therefore, those agents that could suppress the synthesis or activity of COX-2 are likely valuable medicine for anti-inflammation. In order to further provide insight into the molecular mechanism of the anti-inflammatory effect of licochalcone A, its effect on COX-2 activity was studied. In our test, the results clearly showed that licochalcone A inhibited the COX-2 synthesis and activity after COX-2 synthesis in LPS-induced macrophage cells, which suggested that licochalcone A could act as a naturally occurring COX-2 inhibitor. Some other chalcones, such as 3,4-dihydroxychalcones and 2′,5′-dihydroxychalcone, have been reported to possess COX inhibitory activity (Lin *et al.*, 1997). Thus, the inhibition of COX-2 expression and activity may be responsible for the anti-inflammatory activity of licochalcone A.

In conclusion, the results of this study demonstrate that the anti-inflammatory activity of licochalcone A may be mediated through inhibition of COX-2 synthesis and activity after COX-2 synthesis.

Atta A.-H. and Alkohafi A. (1998), Antinociceptive and anti-inflammatory effects of some Jordanian medicinal plants extracts. J. Ethnopharmacol. **60**, 117–124.

Chen M., Zhai L., Christensen S.-B., Theander T.-G., and Kharazmi A. (2001), Inhibition of fumarate reductase in *Leishmania major* and *L. donovani* by chalcones. Antimicrob. Agents Chemother. **45**, 2023–2029.

Haraguchi H., Ishikawa H., Mizutani K., Tamura Y., and Kinoshita T. (1998), Antioxidative and superoxide scavenging activities of retrochalcones in *Glycyrrhiza inflata*. Bioorg. Med. Chem. **6**, 339–347.

Ialenti A., Ianora A., Moncada S., and Di Rosa M. (1992), Modulation of acute inflammation by endogenous nitric oxide. Eur. J. Pharmacol. **211**, 177–182.

Lin C.-N., Lee T.-H., Hsu M.-F., Wang J.-P., Ko F.-N., and Teng C.-M. (1997), 2′,5′-Dihydroxychalcone as a potent chemical mediator and cyclooxygenase inhibitor. J. Pharm. Pharmacol. **49**, 530–536.

Portanova P.-J., Zhang Y., and Anderson G.-D. (1996), Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia and interleukin 6 production *in vivo*. J. Exp. Med. **184**, 833–891.

Rafi M.-M., Rosen R.-T., Vassil A., Ho C.-T., Zhang H., Ghai G., Lambert G., and DiPaola R. S. (2000), Modulation of bcl-2 and cytotoxicity by licochalcone-A, a novel estrogenic flavonoid. Anticancer Res. **20**, 2653–2658.

Shibata S. (2000), A drug over the millennia: pharmacognosy, chemistry, and pharmacology of licorice. Yakugaku Zasshi **120**, 849–862.

Shu X.-S., Gao Z.-H., and Yang X.-L. (2006), Anti-inflammatory and anti-nociceptive activities of *Smilax china* L. aqueous extract. J. Ethnopharmacol. **103**, 327–332.

Simon L. S. (1999), Role of regulation of cyclooxygenase-2 during inflammation. Am. J. Med. **106**, 37–42.

Subbaramaiah K., Telang N., Ramonetti J.-T., Araki R., Devito B., Weksker B.-B., and Dannenberg A.-J. (1996), Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. Cancer Res. **56**, 4424–4429.

Wang Q.-E., Lee S.-C., and Wang X. R. (2004), Isolation and purification of inflacoumarin A and licochalcone A from licorice by high-speed counter-current chromatography. J. Chromatogr. A **1048**, 51–57.