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Inhibition by licochalcone A, a novel flavonoid isolated from liquorice root, of IL-1β-induced PGE_2 production in human skin fibroblasts

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Abstract

Licochalcone A, a novel flavonoid isolated from the root of *Glycyrrhiza inflata*, has been reported to exhibit anti-inflammatory activity in animal models. In this study, we examined the effect of licochalcone A on the production of chemical mediators such as prostaglandin (PG)E_2 and cytokines by interleukin (IL)-1β in human skin fibroblasts. Licochalcone A (IC50 15.0 nM) inhibited PGE_2 production, but not IL-6 and IL-8 production, in response to IL-1β. NS-398 (IC50 1.6 nM), a COX-2 selective inhibitor, also suppressed the PGE_2 production. Furthermore, licochalcone A and NS-398 suppressed $PGF_{2\alpha}$ production by IL-1β. However, licochalcone A (1 μM) had no effect on increased levels of cyclooxygenase (COX)-2 mRNA and protein in cells. Dexamethasone (100 nM) not only inhibited PGE_2, $PGF_{2\alpha}$, IL-6 and IL-8 production but also strongly suppressed the expression of COX-2 mRNA and protein. Licochalcone A had no effect on COX-1-dependent PGE_2 production, whereas indometacin (100 nM), a dual inhibitor of COX-1 and COX-2, was very effective. These results suggest that licochalcone A induces an anti-inflammatory effect through the inhibition of COX-2-dependent PGE_2 production. Furthermore, it appears that the inhibitory effect of licochalcone A on PGE_2 production in response to IL-1β is quite different from that of the steroid.

Introduction

Liquorice root has been used as a traditional medicine in the East and West for the treatment of gastric ulcer, bronchial asthma and inflammation. Licochalcone A is a major and biogenetically characteristic chalcone (Saito & Shibata 1975) (Figure 1) isolated from the root of Xinjiang liquorice, *Glycyrrhiza inflata* Batalin (Leguminosae) (Hatano et al 1988). Licochalcone A is known to possess radical scavenging effects (Hatano et al 1988), anti-leishmanial activity (Chen et al 1994a), anti-malarial activity (Chen et al 1994b) and anti-microbial activity, such as inhibiting the growth of *Staphylococcus aureus*, *Bacillus subtilis* (Okada et al 1989) and the activity of *Helicobacter pylori* (Fukai et al 2002). We also reported that licochalcone A has anti-tumorigenic activity and an anti-inflammatory effect (Shibata et al 1991). In fact, this chalcone induced apoptosis in a tumour cell line through a decrease in the expression of anti-apoptotic protein bcl-2 (Rafi et al 2000). There is also evidence for the inhibition by licochalcone A of leukotriene synthesis in human polymorphonuclear neutrophils (Kimura & Okuda 1988). Licochalcone A has been reported to inhibit the proliferation of human mononuclear cells and production of cytokines, such as TNF-α (Barfod et al 2002). However, it is unclear whether licochalcone A is able to inhibit prostaglandin (PG)E_2 production in human cells.

Skin comprises several cell types such as keratinocytes, melanocytes and fibroblasts. Among them, fibroblasts, as well as macrophages and neutrophils, are involved in inflammatory responses (Goerig et al 1988) and migrate to a site of injury where they proliferate and regulate production of collagen (Kingsnorth & Slavin 1991). It has been established that chemical mediators such as prostanoids and cytokines are involved in skin diseases. Our previous study has shown that human skin fibroblasts induce chemical mediators, including PGE_2 and cytokines in response to interleukin (IL)-1β (Inoue et al 2001). Cultured skin fibroblasts have already been

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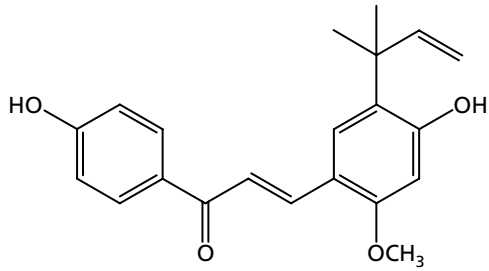

Licochalcone A

Figure 1 Structure of licochalcone A.

used to assess the effect of compounds on the IL-1β-induced PGE_2 production (Inoue et al 1997).

Topical application of licochalcone A results in the inhibition of TPA (12-*O*-tetradecanoylphorbol 13-acetate)- and arachidonic acid-induced mouse ear oedema (Shibata et al 1991). Increased production of PGE_2 contributes to the development of ear oedema in response to TPA and arachidonic acid (Young et at 1983; Carlson et al 1985). In this study, we extended our investigation of the anti-inflammatory properties of licochalcone A and examined the effect of licochalcone A on the production of PGE_2, $PGF_{2\alpha}$, IL-6 and IL-8 by IL-1β in human skin fibroblasts.

Materials and Methods

Reagents

Human recombinant IL-1β was purchased from BD Biosciences (Bedford, MA). NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide) and arachidonic acid were bought from Cayman Chemical (Ann Arbor, MI). Indometacin and dexamethasone were obtained from Sigma Aldrich Incorporated (St Louis, MO). Licochalcone A (4.8 g) was isolated from Xinjiang liquorice (500 g) according to our previous report (Shibata et al 1991).

Cell culture

Normal human dermal fibroblasts isolated from the foreskin were purchased from Kurabo (Osaka, Japan) and cultured in α-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA) containing heat-inactivated 10% fetal calf serum (FCS; Nippon Bio Supply, Tokyo, Japan) and 60 $\mu g\,mL^{-1}$ kanamycin sulfate (Invitrogen) at 37°C in a humidified atmosphere of 95% air–5% CO_2. The culture medium was replaced twice a week. The confluent cells were dispersed with trypsinization and then transferred to new plastic dishes in split ratio of 1:2 or 1:4. The cells at more than four passages were used for subsequent experiments.

Cells were placed onto 48-well plates at 2×10^4 cells mL^{-1}. When cells grew up to confluence, cells were kept in 0.25 mL of α-MEM containing 0.5% FCS for 48 h and then exposed to a concentration of 1 ng mL^{-1} IL-1β (Inoue et al 2001) in the presence or absence of test compounds dissolved in dimethyl sulfoxide (DMSO; < 0.1%) for 24 h.

Cell viability

The viability of the cells was examined by the WST-1 method (Ishiyama et al 1993), which is based on the cleavage of WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium salt) to formazan by mitochondrial dehydrogenase in viable cells. Cells were plated onto 96-well plates at 1×10^5 cells mL^{-1} and incubated for 48 h in 100 μL of α-MEM containing 10% FCS in the presence or absence of licochalcone A. After incubation, 10 μL of WST-1 dye solution (Cell Counting Kit; Dojindo Laboratories, Kumamoto, Japan) was added to the 96-well plates followed by incubation for 2 h in the CO_2 incubator. The absorbance of converted dye was measured at 450 nm with a reference wavelength at 650 nm using a microplate reader.

Measurement of PGE_2, $PGF_{2\alpha}$, IL-6 and IL-8

Concentrations of PGE_2 (detection limit 40 pg mL^{-1}; Cayman Chemical), $PGF_{2\alpha}$ (detection limit 8 pg mL^{-1}; Cayman Chemical), IL-6 (detection limit 2 pg mL^{-1}; Biosource International, Nivelles, Belgium) and IL-8 (detection limit 8 pg mL^{-1}; Assay Designs, Ann Arbor, MI) in the culture supernatant were measured directly by the enzyme-linked immunosorbent assay (ELISA) kits according to the manufacture's directions. Sample and standard dilutions were made with experimental medium.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Confluent cells were kept in 5 mL of α-MEM containing 0.5% FCS for 48 h at 37°C in a humidified atmosphere of 95% air–5% CO_2 and incubated for 4 h in the presence or absence of IL-1β (1 ng mL^{-1}) and test compounds. After washing twice with phosphate-buffered saline (PBS; pH 7.4), total RNA was isolated from cells (2×10^6 cells) by acid guanidine–phenol–chloroform extraction using ISOGEN (Nippon Gene, Toyama, Japan). About 1 μg of total RNA was subjected to RT using TaKaRa RNA PCR Kit (AMV) Ver.2.1 (Takara Bio, Shiga, Japan) with oligo dT primer for 1 h at 42°C. The reaction was terminated by incubation of the mixture at 99°C for 5 min. The resulting cDNA was used as a template for PCR amplification. PCR mixture contained 0.2 mM dNTP Mixture, 0.2 μM of each of two primers, and 1.25 U of TaKaRa rTaq in 1-reaction buffer. The following primers were used to amplify cyclooxygenase (COX)-1 and COX-2 cDNA: sense, 5′-TGC CCA GCT CCT GGC CCG CCG CTT-3′ and antisense, 5′-GTG CAT CAA CAC AGG CGC CTC TTC-3′ (303 bp products) for COX-1; sense, 5′-GGA ACA CAA CAG AGT ATG CG-3′ and antisense, 5′-GAC AGC CCT TCA CGT TAT TG-3′ (806 bp products) for COX-2. The amplification condition for COX-1 was 95°C for 5 min, followed by 33 cycles of 94°C for 60 s, 60°C for 60 s, 72°C for 60 s and elongation at 72°C for 7 min and that for COX-2 was 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 60 s and elongation at 72°C for 7 min.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as positive control. The primers were used as follows: sense, 5′-ACC ACA GTC CAT GCC ATC AC-3′ and antisense, 5′-CCA CCA CCC TGT TGC TGT AG-3′ (451 bp products). The amplification condition was 95°C for 5 min, followed by 20 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s and elongation at 72°C for 7 min. PCR products were electrophoresed on 2% agarose gels and stained with SYBR gold (Molecular Probes, Eugene, OR). No PCR product was amplified without RT.

Western blot

Confluent cells (2×10^6 cells) were kept in 5 mL of α-MEM containing 0.5% FCS for 48 h at 37°C in a humidified atmosphere of 95% air–5% CO_2, and incubated with test compounds in the presence or absence of IL-1β (1 ng mL^{-1}) for 6 h. After incubation, the cells were washed twice with PBS (pH 7.4), dipped in 300 μL of lysis buffer (Laemmli sample buffer; Bio-Rad Laboratories, Hercules, CA) and disrupted with a Ultrasonic Disrupter (UD 201, TOMY, Tokyo, Japan). Cell lysates were centrifuged at 13 000 g at 4°C for 15 min, and the supernatant fraction was boiled for 5 min. Samples of protein (10 μL, 30 μg) were subjected to electrophoresis on a 5–20% gradient SDS-PAGE gel and transferred to a PVDF membrane (ATTO, Tokyo, Japan). After blocking with 1% non-fat dry milk in 0.1% Tween 20 in PBS for 2 h at room temperature, membranes were incubated with rabbit anti-COX-1 (Oxford Biomedical Research, Oxford, MI) (1:100 dilution) or rabbit anti-COX-2 (Cayman Chemical) (1:1000 dilution) polyclonal antibodies overnight at 4°C. The blot was subsequently incubated with secondary antibody (horseradish peroxidase conjugated goat anti rabbit IgG at a 1:2000 dilution) for 1 h at room temperature. Reactive bands were visualized with an ECL plus detection system (Amersham Biosciences, Piscataway, NJ) and analysed with an ATTO Light Capture (ATTO).

Assay for COX-1-dependent PGE_2 production

Confluent cells in 48-well plates were pre-incubated in 0.25 mL of α-MEM containing 0.5% FCS and dexamethasone (100 nM) for 24 h at 37°C. After washing three times with medium, the cells were exposed to test compounds for 30 min and then washed, and incubated with arachidonic acid (100 μM) for 30 min. The PGE_2 concentration in the supernatant was measured directly by an ELISA kit.

Statistical analysis

All data were expressed as means ± s.e.m. Statistically significant differences between control and test groups were tested by Kruskal–Wallis test (non-parametric one-way analysis of variance) followed by Dunn's multiple comparison test.

Results

Effects of licochalcone A on IL-1β-induced PGE_2, $PGF_{2\alpha}$, IL-6 and IL-8 production in skin fibroblasts

The production of PGE_2, $PGF_{2\alpha}$, IL-6 and IL-8 was induced for up to 24 h after IL-1β (1 ng mL^{-1}) treatment (data not shown). Basal levels of PGE_2, $PGF_{2\alpha}$, IL-6 and IL-8 were 0.07 ± 0.02 ng mL^{-1}, 12 ± 2 pg mL^{-1}, 0.17 ± 0.03 ng mL^{-1} and 0.25 ± 0.08 ng mL^{-1} (n = 5), whereas IL-1β increased their levels to 17.34 ± 0.65 ng mL^{-1}, 191 ± 16 pg mL^{-1}, 354.20 ± 22.75 ng mL^{-1} and 357.40 ± 23.58 ng mL^{-1} (n = 5), respectively. Licochalcone A at a concentration of 100 nM significantly ($P < 0.001$) inhibited PGE_2 and $PGF_{2\alpha}$ production but had little effect on IL-6 and IL-8 production. NS-398 (100 nM), a COX-2-specific inhibitor (Futaki et al 1994), was also very effective in inhibiting the IL-1β-induced PGE_2 and $PGF_{2\alpha}$ production but did not exhibit significant inhibition of cytokine production. In contrast, dexamethasone (100 nM) significantly ($P < 0.001$) suppressed the production of prostanoids and cytokines (Table 1). Licochalcone A exhibited the inhibition of IL-1β-induced PGE_2 production in a dose-dependent manner with a 50% inhibitory (IC50) value of 15.0 nM. The IC50 values of NS-398 and dexamethasone were 1.6 nM and 0.9 nM, respectively (Figure 2). Licochalcone A at a concentration of 1 μM had no effect on the cell viability (105.2 ± 4.1% of the control, n = 6).

Effects of licochalcone A on the IL-1β-induced expression of COX-2 mRNA and protein in skin fibroblasts

Expression of COX-2 mRNA was up-regulated at 4 h after IL-1β (1 ng mL^{-1}) treatment whereas COX-1

Table 1 Effects of licochalcone A on PGE_2, $PGF_{2\alpha}$, IL-6 and IL-8 production in human skin fibroblasts

Compound		Inhibition (%)			
		PGE_2	$PGF_{2\alpha}$	IL-6	IL-8
Licochalcone A	10 nM	43.4 ± 13.0	46.2 ± 7.2	21.0 ± 5.4	24.8 ± 4.8
	100 nM	80.0 ± 2.5***	98.7 ± 0.6***	28.3 ± 2.9	23.6 ± 3.3
NS-398	100 nM	100***	99.9 ± 0.1***	9.4 ± 4.4	−2.6 ± 7.9
Dexamethasone	100 nM	99.1 ± 0.1***	100	88.4 ± 0.4***	57.3 ± 2.2***

Cells were treated with IL-1β in the presence or absence of licochalcone A or NS-398 for 24 h. Values are means ± s.e.m. of 5 samples. ***$P < 0.001$ vs control (Dunn's test).

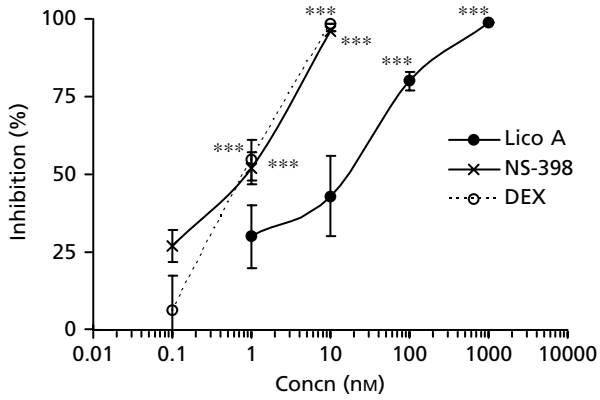

Figure 2 Inhibition by test compounds of IL-1β-induced PGE_2 production in human skin fibroblasts. Confluent cells were treated with IL-1β (1 ng mL^{-1}) in the presence or absence of test compounds at various concentrations for 24 h. Values are the mean ± s.e.m. of 5 samples. ***$P < 0.001$ vs control (Dunn's test). Lico A, licochalcone A; DEX, dexamethasone.

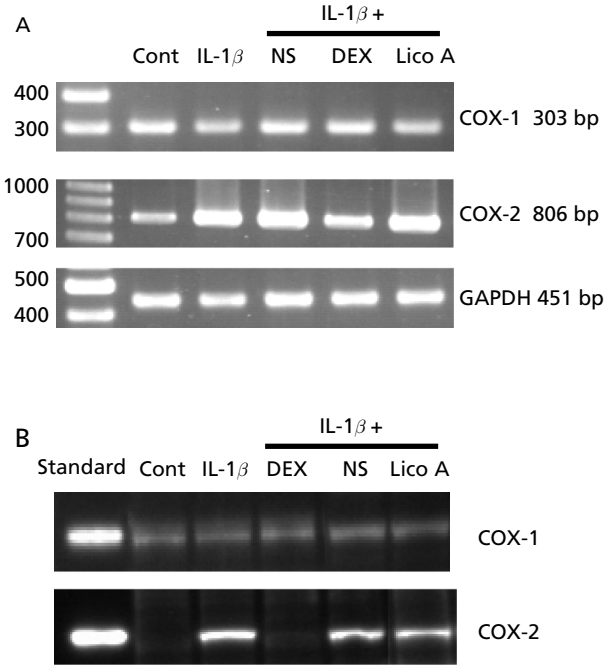

Figure 3 Effect of licochalcone A on expression of COX-2 mRNA and protein in human skin fibroblasts treated with IL-1β. Expression of mRNA for COX-1 and -2 was examined by RT-PCR analysis (A), and that of COX-1 and COX-2 protein was determined by Western blot analysis (B). Confluent cells were treated with IL-1β (1 ng mL^{-1}) in the presence or absence of licochalcone A (Lico A, 1 μM), NS-398 (NS, 1 μM) or dexamethasone (DEX, 100 nM) for 4 h (RT-PCR analysis) and 6 h (Western blot analysis), respectively. The cultures without IL-1β treatment served as controls. The electrophoresis pattern illustrated was representative of that in three separate experiments.

mRNA expression did not change in skin fibroblasts (Figure 3A). Licochalcone A (1 μM) had no effect on levels of COX-1 or COX-2 mRNAs. Similarly, NS-398 (1 μM) also did not inhibit the expression of mRNA for COX-1 and COX-2. Dexamethasone (100 nM) had no effect on COX-1 mRNA expression but suppressed increased level of COX-2 mRNA in response to IL-1β.

Licochalcone A and NS-398 showed no inhibition of COX-1 and COX-2 protein expression at 6 h after IL-1β treatment (Figure 3B). In contrast, dexamethasone completely suppressed COX-2 protein expression but had no effect on COX-1 protein.

Table 2 Effect of licochalcone A on COX-1-dependent PGE_2 production in human skin fibroblasts

Compound		Inhibition (%)
Licochalcone A	1 μM	15.9 ± 11.6
	10 μM	2.7 ± 2.3
Indometacin	100 nM	88.9 ± 1.0***
NS-398	100 nM	−1.1 ± 2.0

Cells were pre-incubated in medium containing 0.5% FCS and dexamethasone (100 nM) for 24 h. After washing 3 times, the cells were treated with or without licochalcone A, indometacin or NS-398 for 30 min and then stimulated with arachidonic acid (100 μM) for 30 min. Values are means ± s.e.m. of 5 samples. ***$P < 0.001$ vs control (Dunn's test).

Effects of licochalcone A on COX-1-dependent PGE_2 production in skin fibroblasts

PGE_2 production was markedly increased by stimulating cells with arachidonic acid for 30 min, and its value was 8.7 ± 0.9 ng mL^{-1} (n = 5). Licochalcone A did not inhibit the arachidonic acid-induced PGE_2 production even at a high concentration of 10 μM (Table 2). NS-398 (100 nM) also had no effect on PGE_2 production in response to arachidonic acid. However, treatment with indometacin (100 nM), a dual inhibitor of COX-1 and COX-2 (Vane & Botting 1995), caused about 90% inhibition of PGE_2 production.

Discussion

This study demonstrates that licochalcone A, a reversely constructed chalcone isolated from the root of *Glycyrrhiza inflata*, inhibited the PGE_2 and $PGF_{2\alpha}$ production in response to IL-1β in human skin fibroblasts. This inhibition did not contribute to cytotoxicity, as assessed by WST-1 assay and GAPDH gene expression. NS-398, a COX-2-specific inhibitor, also suppressed IL-1β-induced PGE_2 and $PGF_{2\alpha}$ production. Dexamethasone inhibited not only the PGE_2 and $PGF_{2\alpha}$ production but also an increase in COX-2 mRNA and protein expression, indicating that the inhibitory effect of dexamethasone is due to the suppression of COX-2 mRNA expression, as others previously reported (Angel et al 1994; Szczepanski et al 1994). In contrast, licochalcone A had no effect on the increased levels of COX-2 mRNA and protein by IL-1β. Thus, it seems unlikely that this compound prevents the

signalling pathways after IL-1β stimulation, leading to increases in COX-2 mRNA expression and protein on the production of PGE_2 and $PGF_{2\alpha}$. This indicates that the inhibitory effect of licochalcone A is quite different to that of dexamethasone. Furthermore, licochalcone A seems to inhibit the PGE_2 production as a COX-2 inhibitor in response to IL-1β. In fact, we confirmed that licochalcone A (IC50 15.4 μM) inhibits ovine COX-2 activity in a cell-free system (whereas the IC50 value of NS-398 was 6.2 μM) (data not shown). However, we cannot exclude the possibility that this compound might inhibit phospholipase A_2 activity in this model.

Cultured skin fibroblasts produce many soluble mediators, such as prostanoids, cytokines and growth factors, after stimulation with IL-1β (Inoue et al 2001). In this study, dexamethasone suppressed the production of cytokines, as well as PGE_2 and $PGF_{2\alpha}$, by IL-1β. However, licochalcone A failed to inhibit the IL-6 and IL-8 production. This finding indicates that licochalcone A has no effect on intracellular signal pathways leading to the production of these mediators after stimulation with IL-1β, and suggests that the anti-inflammatory profile of licochalcone A is different to that of steroids.

Indometacin strongly inhibited the arachidonic acid-induced PGE_2 production in skin fibroblasts. In contrast to this, NS-398 had no effect on the PGE_2 production. Licochalcone A, as well as NS-398, did not inhibit the COX-1-dependent PGE_2 production in skin fibroblasts. Hence, it would be possible that licochalcone A acts as a human COX-2-selective inhibitor in the inflammatory response.

In this study, we examined the inhibitory effect of licochalcone A on PGE_2 production in human intact cell assays containing predominantly either COX-1 or COX-2. Previous studies on COX selectivity have been performed using purified (Mitchell et al 1993; Yamazaki et al 1997) or recombinant (Laneuville et al 1994; Glaser et al 1995) enzymes. However, it appears that these results are likely to be influenced by assay systems and conditions. Others have suggested that assays using intact cells are the most inclusive approach to screening for COX selectivity (Laneuville et al 1994). In addition, as COX is an integral membrane protein of the endoplasmic reticulum, the intact cell assays have the advantage of testing compounds in the normal cellular environment by leaving the three-dimensional structure of the enzyme unaffected (Otto & Smith 1994). It is known that COX-1 functions predominantly in the endoplasmic reticulum whereas COX-2 may function in the endoplasmic reticulum and the nuclear envelope (Morita et al 1995). Accordingly, it seems that licochalcone A is capable of crossing the cellular membrane of skin fibroblasts and surviving possible degradational pathways within the cell on its way to the action site.

Others have reported that the inhibition of COXs in the intact cell assay reflects cellular pharmacological and pharmacokinetic parameters, and could be predictive for enzyme inhibition of inflammatory animal models (Chulada & Langenbach 1997). In fact, licochalcone A inhibits the oedema formation in response to TPA (Shibata et al 1991). It has been suggested that COX-2 induction is correlated with the time-course of TPA-induced oedema (Sanchez & Moreno 1999). In addition, COX-2-selective inhibitors suppress the mouse ear oedema induced by TPA (Puignero & Queralt 1997). Taken together, our results suggest that a possible mechanism by which licochalcone A inhibits the development of TPA-induced mouse ear oedema may be the inhibition of PGE_2 production. Furthermore, anti-tumour promoting action of licochalcone A has been observed for mouse skin papilloma initiated by dimethylbenz[α]anthracence (DMBA) and promoted by TPA (Shibata et al 1991). COX-2 has been identified as a major target for the anti-tumour activity of nonsteroidal anti-inflammatory drugs (Dannenberg et al 2001). It has been reported that the specific COX-2 inhibitor celecoxib inhibits tumour formation by interfering with the promotion stage, but not the initiation phase, of skin papilloma induced by DMBA and TPA, indicating that COX-2 is involved in the promotion of skin tumour (Muller-Decker et al 2002). In addition, COX-2 is expressed only in papillomas but not in the tumour-free epidermis. Thus, it is conceivable that licochalcone A suppresses mouse skin tumour through the modulation of PGE_2 production in papillomas. Moreover, the results of this study support that this compound may be of value for the treatment of inflammatory skin diseases and skin tumour.

A number of studies on the biological activity and pharmacological effects of compounds isolated from liquorice root have been reported. Glycyrrhizin, the major component of liquorice extracts, is known to inhibit PGE_2 production by activated rat macrophages (Ohuchi et al 1981). A recent report has shown that isoliquiritigenin, a flavonoid contained in liquorice root, also reduces PGE_2 production in a mouse macrophage cell-line (Takahashi et al 2004). Taken together, our results imply that, in addition to glycyrrhizin and isoliquiritigenin, licochalcone A may play a role in the anti-inflammatory effect of Xinjiang liquorice through the inhibition of COX-2-dependent PGE_2 production.

Conclusion

In this study, we demonstrate for the first time that licochalcone A, a reversely constructed chalcone isolated from the root of liquorice, inhibits IL-1β-induced PGE_2 and $PGF_{2\alpha}$ production in human skin fibroblasts. The mechanism was not due to the inhibition of increases in COX-2 mRNA and protein expression in response to IL-1β. Licochalcone A had no effect on the COX-1-dependent PGE_2 production. Our data indicate that licochalcone A is a human potent inhibitor of the COX-2-dependent PGE_2 production, and suggest that it may contribute to an anti-inflammatory action of Xinjiang liquorice.

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