Liquiritigenin enhances cyclic adenosine monophosphate production to mitigate inflammation in dendritic cells

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Abstract

Objective: This study aims to dissect the mechanism of traditional Chinese medicinal herbs against asthma; we chose to first focus on the main chemical components of licorice to investigate their contribution to asthmatic inflammation inhibition. Methods: Production of cellular nucleotide molecules such as cAMP, cGMP, and cGAMP was examined by using enzyme-linked immunosorbent assay (ELISA). Enzyme-encoding genes were tested *in vitro* using quantitative real-time PCR and protein level was detected by Western blotting analysis. In addition, co-culturing of murine dendritic cells together with T cells was conducted to examine the expression of cytokine genes and host immune response. Results: We found that one of the components within licorice, named liquiritigenin (LR), could efficiently enhance cAMP production in different cell lines. The augmentation of such molecules was linked to the high expression of cAMP synthesis genes and repressed expression of cAMP breaking down genes. In addition, the downstream immune response was also alleviated by the increase in cAMP levels by LR, suggesting the great potential of this molecule against inflammation. Subsequent immunological tests showed that LR could efficiently inhibit the expression of several cytokines and alter the NF-κB pathway and T cell polarization. Conclusion: Altogether, we have identified a promising antiasthmatic agent LR that could exhibit immunosuppressive function by elevating the cAMP level.

Keywords

liquiritigenin, licorice, cAMP, asthma

Introduction

Asthma is one of the chronic respiratory diseases that affect millions of people all over the world, which are characterized by airway obstruction, chronic inflammation, and airway remodeling.[1] It is known that the airway obstruction in asthma is reversible by bronchodilators and is linked to airway hyperresponsiveness.[2] Mechanistically, airway inflammation in asthma is associated with infiltration of eosinophils, mast cells, and CD4^{+} T-lymphocytes.[3]

Currently, the treatment of asthma relies mainly on inhaled corticosteroid or short-acting β2-adrenoreceptor (AR) agonist or long-acting β2-AR agonist.[4] In addition, oral roflumilast has been suggested as an alternative therapy for patients with moderate or severe asthma.[5,6] As a selective inhibitor of phosphodiesterase-4 (PDE-4), roflumilast mitigated the allergen-mediated bronchoconstriction (FEV1)

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among asthmatic patients and significantly reduced the allergen-mediated TNF-α production and airway inflammation.[7-9] Although those therapies have been proven to be effective in the suppression of eosinophilic inflammation (T2-high) through T2 inhibition, they might increase the accumulation of neutrophils in the airways.[10] Until now, there is still a lack of agents that could efficiently suppress neutrophilic inflammation (T2-low).[11]

The intracellular signaling molecule, cyclic adenosine monophosphate (cAMP), is implicated in the pathophysiology of asthma, which was shown to promote smooth muscle relaxation and inhibit airway inflammation.[12] cAMP is produced by adenylyl cyclases (ACs), and the breakdown of cAMP is achieved by various families of phosphodiesterases (PDEs).[13] In general, an increase in cAMP through either cAMP agonists or PDE inhibition has been suggested to markedly reduce inductive agent-related inflammatory responses, like lipopolysaccharide (LPS).[14] Based on this recognition, a great number of PDE inhibitors have been discovered and used for therapeutic purposes.[15]

It was reported that traditional medicinal herbs such as ginger and garlic possess anti-inflammation properties.[16,17] Increasing evidence suggests that aged ginger extract could inhibit platelet activation by increasing intracellular cAMP.[16] In addition, another medicinal herb, licorice, also displayed potential therapeutic efficacy against inflammation.[18-20] However, the modes of mechanism of this traditional Chinese medicine (TCM) are still missing.

In this study, we focus on the chemical basis of licorice and investigate the roles played by its main components. We have screened four of its main components (glycyrrhizic acid, GA; glabridin, GB; licochalcone, LC; and liquiritigenin, LR) and found that LR could efficiently increase the concentration of cAMP in different cell types especially in dendritic cells (DC), indicating that it could be used as a potential agent against allergic asthma.

Materials and methods

Cell lines, chemicals, and reagents

Each chemical and reagent utilized in the present work was analytically pure. The reference substances had the purity > 98%. cAMP and bovine serum albumin (BSA) were provided by Sigma-Aldrich (Shanghai). Cells lines (epithelial cell line BEAS-2B and macrophages cell line THP-1) were obtained from the American-type culture collection (ATCC) and cultured in a specific medium (DMEM and RPMI 1640 medium, Gibco) that contained 10% fetal bovine serum (FBS) with or without antibiotics in a humid incubator under 5% CO_2 and 37°C conditions.

Type and place of study

The type of this study belongs to basic research using cell lines to investigate the effect of TCM on inflammation. This study was mainly conducted in the Department of Pediatrics, Jinan Central Hospital, Cheeloo College of Medicine, Shandong University, Jinan, China.

Preparation of drug extract

Ultrapure water (500 mL) was utilized to extract GB, GC, LB, and LR powder (purity ≥ 98.0%) purchased from Tongrentang Group Co., Ltd (Beijing, China) for 24 h under 50°C. Thereafter, aqueous extracts were subjected to 60 min of centrifugation at 25,000 rpm. The supernatants were collected and concentrated to obtain aqueous extract (100 mL) under vacuum condition, followed by lyophilization. Afterward, the resultant powders were preserved under −50°C prior to use. 5% DMSO was used to dissolve the lyophilized extracts before use.

Ethics statement

The Ethics Committee of Jinan Central Hospital, Cheeloo College of Medicine, Shandong University (China) approved the protocol used in this work. Patients signed the consent form before this study.

In vitro production of peripheral blood mononuclear cell–derived DCs

DC cells were generated based on peripheral blood mononuclear cells (PMBCs) as described previously with modifications.[21] Specifically, negative selection was utilized to purify monocytes from PBMCs by an EasySep human monocyte isolation kit (Stem Cell Technology Inc). Briefly, we separated PBMCs in human whole peripheral blood collected from the normal subjects at the Jinan Central Hospital, Cheeloo College of Medicine, Shandong University (China) in line with specific protocols. Afterward, stem cell technology was adopted to treat PBMCs in line with specific instructions; then, the obtained monocytes (90% CD14+ CD45+) were processed and cultured within RPMI 1640 (Sigma-Aldrich) that contained 10% FCS, 1% nonessential amino acids, 100 mg/mL kanamycin (Sigma-Aldric), 1% sodium pyruvate, 250 U/ml rhIL-4 (Invitrogen Life Technologies), and 500 U/ml rhGM-CSF (Invitrogen Life Technologies). On the third day, we replaced the supplement-containing medium. We also prepared DCs derived from monocytes through Percoll gradient centrifugation; later, the adherent cells were cultured according to previous description.[21] Similar immature CD1a+CD14− DCs were prepared on the sixth day in the two protocols.

Induction of dendritic cell maturation with/without phosphodiesterase-4 inhibitors

Induction of DC maturation was done according to instructions from a previous study with modification.[22] On the sixth day, cells were treated with 25 ng/mL rhTNF-α or 1 μg/mL LPS for 2 days to induce CD1a+ CD14− DC maturation. To analyze cell phenotype and to carry out experiments on mixed lymphocytes, cells were treated with 1 μg/mL LPS with IFN (10^3 U/ml) to induce DC maturation.

Effect of liquiritigenin on cytokine contents

IL-12p70 contents within supernatants were measured through the specific solid-phase sandwich ELISA according to previous descriptions.[23] TNF-α was measured through ELISA by adopting specific recombinant cytokine standards and mAbs from Invitrogen Life Technologies.

Effect of liquiritigenin on cyclic adenosine monophosphate, cGMP, and cGAMP concentrations

10~50 μM licorice components (GA, GB, LC, and LR), or vehicle (5% DMSO) with/without prostaglandin E2 (PGE2; 0.5 μM, Invitrogen Life Technologies), or LPS (1 μg/mL) were used to treat cell lines ($2x10^6$ cells/ml HBSS) for 30 min. After cell homogenization, we measured the contents of cAMP, cGMP, and cGAMP through ELISA per specific protocols (Enzo Life Sciences) and normalized them to overall protein content.

Role of liquiritigenin in NF-κB induction

Human CD4 cells were isolated according to the previous method[24] and cultured within the serum-free RPMI-1640 for a period of 2 h. Thereafter, a 20 μM vehicle or LR was used to treat cells for 5 min and then treated with vehicle or anti–CD3/CD28-coated beads (bead-to-cell ratio, 1:1; Dynabeads Human T-Activator CD3/CD28, Thermo Fisher Scientific) for 15 min. Afterward, $2x10^6$ cells were adopted to obtain total cellular protein lysates, whereas the phosphorylated and total Ser536 p65 contents were measured through ELISA (NF-κB p65 (pS536) + Total NF-κB p65 SimpleStep ELISA Kit, Abcam, Cambridge, UK).

Effect of liquiritigenin on Treg polarization

We cultured CD4 cells with 5 ng/mL TGFβ (Invitrogen Life Technologies), anti–CD3/CD28-coated beads, and 20 μM LR, or vehicle (5% DMSO), and then cultured (at $1x10^6$ cells/ml) within the RPMI-1640 medium that contained 10%FBS, antibiotics/antimycotics (Thermo Fisher Scientific), as well as β-mercaptoethanol (50 μM,

Sigma-Aldrich) under 37°C and 5% CO2 conditions. On days 4 and 7, flow cytometry was adopted to analyze Treg polarization (CD4+CD25+FOXP3+) in cells.[25] In brief, after harvesting $1x10^6$ cells from the culture, cells were subjected to centrifugation at 300 g, rinsing by PBS, and 1 h of staining to measure CD4 (RM4-5, BD Bioscience) as well as CD25 (PC61.5, Thermo Fisher Scientific) expression under 4°C (antibody content, 1:100; $1x10^6$ cells/100 μL FACS buffer).

Later, the fixable viability dye was utilized to stain cells according to specific protocols (Thermo Fisher Scientific). After cell fixation and permeabilization (FOXP3/ Transcription Factor Staining Buffer Kit, Thermo Fisher Scientific), cells were subjected to staining to examine intracellular FOXP3 expression (antibody content: 1:100; $1x10^6$ cells/100 μL). Both FSC Express software and LSRII flow cytometer were utilized for analysis. Dead/non-singlet cells and debris were removed before analysis. CD4+CD25+FOXP3+ cells were defined as the Tregs.[25,26]

RT-PCR

Total mRNA was isolated using Kit RNAfast200 (TaKaRa Biotechnology, China). The mRNA quality was analyzed by the ND-2000 ultra-micro nucleic acid protein analyzer (NanoDrop, USA); later, RNA samples were preserved under −80°C prior to use. Briefly, in every RT-PCR process, PrimeScript™ RT reagent kit was utilized to prepare cDNA from total RNA (2 μg) by the use of gDNA Eraser (TaKaRa, Da Lian, China). Thereafter, relative mRNA contents were measured through qRT-PCR by the SYBR green reagent kit (Roche) conducted on the 7500 Fast Real-Time PCR System (Applied Biosystems). The primers were synthesized by Shenzhen BGI Biotechnology Co. Ltd and were shown as follows:

β-actin-Fw (5'-3'): TCTACAATGAGCTGCGTGTG,
β-actin -rev: GGTGAGGATCTTCATGAGGT;
Gαs-Fw: GTCCTTGCTGGGAAATCG,
Gαs-Rev: CGCAGGTGAAATGAGGGTAG;
Gαi-Fw: CAGCCAGGCTTGCAAATCAC,
Gαi-Rev: AACACACAAGGCAACTTTCAGCTTC;
Gαt-Fw: CGTCAGTGCTGCGACAGTC,
Gαt-Rev: CCAGCGTACTCCGACACACA.

Transcript relative changes because of LR treatment compared with control were determined by comparative CT approach,[27] with β-actin being the control gene.

PDE assay

Phosphodiesterase activity of CD4 cells were performed according to a previously described method.[28] At least six replicates were done for each independent assay.

Cell viability assay

Cell viability assay was done according to the instructions of CCK-8 kit (APExBIO). In brief, every well that contained 200 μL medium was added with CCK-8 reagent (20 μL) into the 96-well plate, followed by 4 h of incubation under 37°C. At last, we detected OD (450 nm) values for diverse groups (n = 3). Cell viability was considered to be 100% in control group (with no treatment) and that in other groups was determined on this basis.

Western blotting assay

Protein content was measured by the BCA protein detection kit (Sigma-Aldrich, Shanghai, China). Every well was loaded with an aliquot of protein (20 μg), and then 10% SDS-PAGE was adopted to separate proteins, and 5% skimmed milk (BD) within TBST was utilized to block proteins for 1 h, followed by 12 h of incubation using primary antibodies (1:1000; Abcam, USA) under 4°C. Thereafter, we rinsed membranes for 7 min thrice, followed by 1 hf of incubation using suitable secondary antibody conjugate (Abcam, USA) or HRP-labeled antibody (Sigma-Aldrich, Shanghai, China) under ambient temperature. Thereafter, we rinsed the membranes thrice and stained them by DAB HRP (Beyotime, Shanghai, China). Finally, the gel visualization (Tanon, Shanghai, China) was adopted to detect proteins. Protein levels were normalized to GAPDH and quantified via densitometry.

Statistical analysis

Statistical analysis was completed by Graphpad Prism software (version 7). All tests are shown in figure legends. Measurements were presented in a form of mean ± SD and compared by Student's t-test. $P < 0.05$ stood for statistical significance.

Results

Liquiritigenin augments cyclic adenosine monophosphate concentration in a lipopolysaccharide-dependent and independent way

It was reported that extracts of licorice could reduce LPS-induced inflammatory cells including neutrophils, macrophages, and lymphocyte accumulation in bronchoalveolar lavage fluid.[18] However, the detailed mechanisms underlying this anti-inflammation event remain unclear. To solve this, we adopted a chemical biology approach to identify its main effective components. We have used four main ingredients from licorice extract, namely, glycyrrhizic acid (GA), glabridin (GB), licochalcone (LC), and liquiritigenin (LR) to study their respective roles in anti-inflammation process. Since the cAMP level has been linked with several immune responses, we first attempted to examine the effects of these four components on the level of cAMP in different cell lines.

As can be seen from Figure 1a, when we used these four ingredients to treat human CD4 cells, we clearly observed that LR treatment could lead to a 2-fold increase in the production of cAMP. However, the remaining three components (GA, GB, and LC) did not show any sign of augmentation in cAMP levels. To confirm whether LR indeed enhanced the production of cAMP in cells, we further used different concentrations of LR (0, 10, 20, and 50 μM) and different cell types to verify the function of LR. It was shown that LR could efficiently elevate cAMP levels in a dose-dependent manner (Figure 1b), and this enhancement could be observed in dendritic cells (DC), T cells, and epithelial cells (BEAS-2B). As for macrophage cells, the augmentation was not significant (Figure 1c). Our results indicated that licorice component LR could act as a cAMP stimulator in different cell types and may function as a new therapeutic solution to inflammation associated with certain immune cells.

As we already know that an increase in cAMP levels through either cAMP agonist or PDE inhibition has been suggested to apparently reduce LPS stimulation-induced inflammatory response,[14] we aim to understand whether LR could function in response to LPS stimulation. Figure 1d showed that LR could significantly increase the cAMP level in LPS-treated DC cells by at least 3-fold as compared to non-stimulant controls.

Altogether, our results demonstrate that LR could efficiently augment the cAMP levels in cells, and this increase in cAMP is both LPS-dependent and independent conditions.

The production of cGMP or cGMP-AMP is not affected by the main components of licorice

The nitric oxide–soluble guanylate cyclase-cGMP (NO-sGC-cGMP) pathway has been recognized to be a major signaling pathway related to the relaxation of vascular smooth muscles[29] and airway smooth muscle.[30] We wonder whether the main components of licorice could affect the production of cGMP. As shown in Figure 2a, no ingredients could elevate the production of cGMP, indicating that the NO-sGC-cGMP pathway is not the target of licorice extract.

In addition, it was demonstrated that cGAMP is also involved in the DNA-associated microbial infection and acts as a central player in the cGAS-cGAMP-STING pathway.[31] We again questioned whether the four main components of licorice extract could target the production
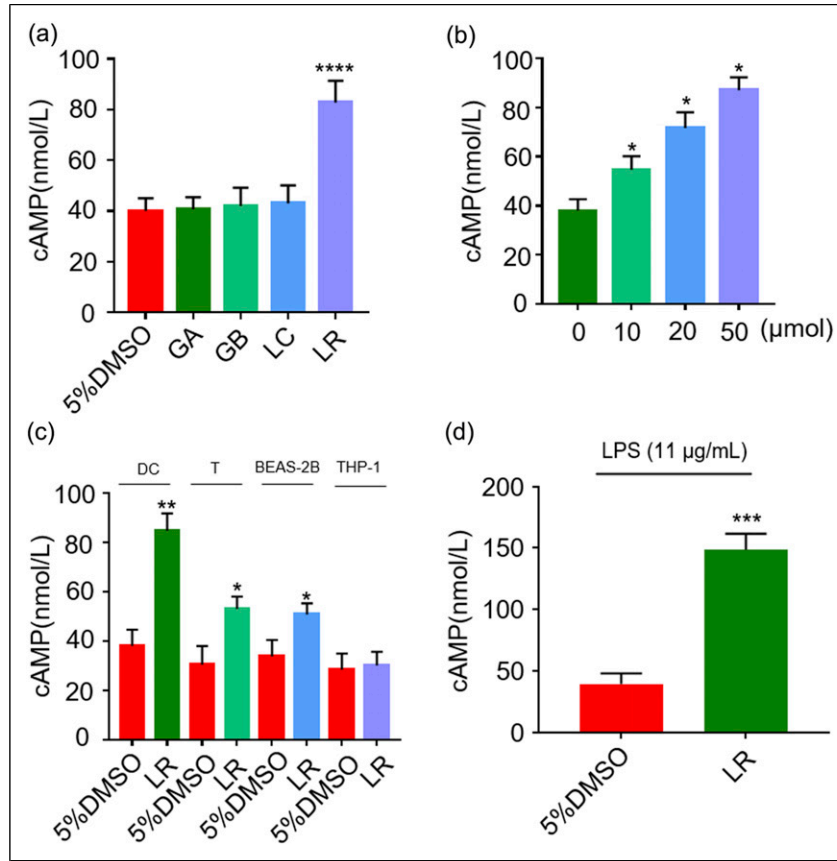
Figure 1. LR augments cAMP concentration in a LPS-dependent and independent way. *(a)*. cAMP levels in DC cells treated with vehicle (5%DMSO), GA, GB, LC, and LR (20 μM each). The cAMP level was quantified as nmol/L using an ELISA kit (See *Materials and methods*). Results are presented in the form of mean ± SD (n = 3). P < .05 stood for statistical significance. ***, P<.001. *(b)*. cAMP levels in DC cells treated with LR in different doses (0, 10, 20, and 50 μM). The cAMP level was quantified as nmol/L using an ELISA kit (See *Materials and methods*). Results are presented in the form of mean ± SD (n = 3). *, P < .05. *(c)*. cAMP contents within DC exposed to vehicle (5% DMSO) and LR (20 μM) in different cell lines (DC, T, BEAS-2B, and macrophages). The cAMP level was quantified as nmol/L using an ELISA kit (See *Materials and methods*). Results are presented in the form of mean ± SD (n = 3). ***, P < .001. *(d)*. cAMP levels in LPS-stimulated DC cells treated with LR. The cAMP level was quantified as nmol/L using an ELISA kit (See *Materials and methods*). Results are presented in the form of mean ± SD (n = 3). ***, P < .001.

of this particular molecule. As can be seen from Figure 2b, no significant change of the cGAMP level could be detected under our tested conditions.

Therefore, we drew the conclusion that licorice mainly functioned through elevating cAMP levels in cells to regulate the downstream signaling pathways.

Liquiritigenin functions as both an activator of adenylyl cyclases and an inhibitor of PDEs

To understand mechanisms underlying the potential stimulatory properties of LR on cAMP level in cells, we first examined its synthesis at the level of mRNA level. To confirm that LR could elevate the gene expression related to cAMP synthesis, we extracted the total RNA and performed qRT-PCR analysis to examine the influence of LR

on their transcription level in a time-series manner. Interestingly, the expression of the selected AC gene (Gαs) showed a dramatic increase in transcription level, indicating LR could upregulate the expression of cAMP synthesis-related genes (Figure 3a). Furthermore, we extracted the total proteins of LR-treated cells (after 48 h treatment) and compared them with those of nontreatment control through WB analysis. As a result, Gαs protein expression was greatly upregulated in the LR-treated group compared with that of the nontreatment control (DMSO, Figure 3b). Quantification of band intensity showed a more than 2-fold increase in expression level after LR treatment.

Given that LR could activate the expression of AC, we wonder whether LR could specifically target PDEs to further boost the level of cAMP. To our surprise, we examined the expression of two PDEs (Gαi and Gαt) in CD4
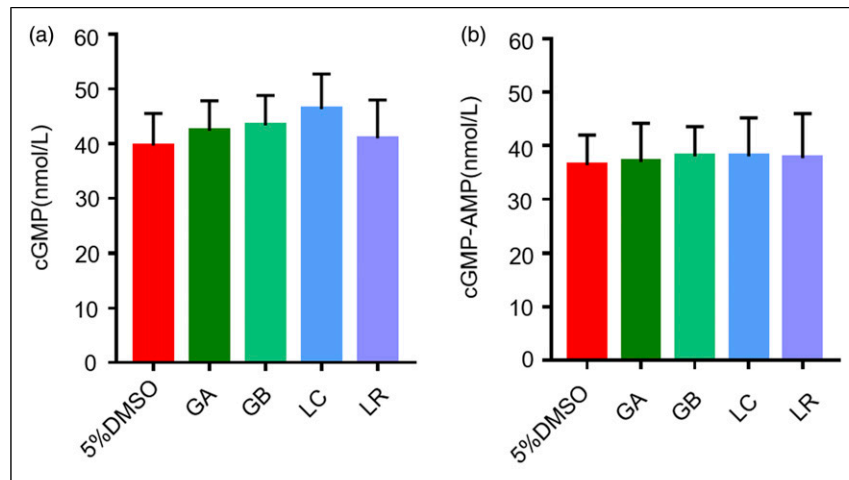
Figure 2. The cGMP or cGMP-AMP level is not affected by main components of licorice. *(a)*. cGMP levels in DC cells treated with vehicle (5%DMSO), GA, GB, LC, and LR (20 μM each). The cGMP level was quantified as nmol/L using an ELISA kit (See *Materials and methods*). Results are presented in the form of mean ± SD (*n* = 3). *(b)*. cGAMP levels in DC cells treated with vehicle (5%DMSO), GA, GB, LC, and LR (20 μM each). The cGAMP level was quantified as nmol/L using an ELISA kit (See *Materials and methods*). Results are presented in the form of mean ± SD (*n* = 3).

cells and found that LR could greatly attenuate the expression of both genes at mRNA levels (Figure 3c). Furthermore, we have proved that LR could suppress the expression of Gαi and Gαt at protein levels (Figure 3d). To confirm whether LR has an inhibition on PDE activity, we performed an enzymatic assay to examine the effect of LR on PDE activity. As can be seen in Figure 3e, PDE activity in whole-cell lysate was reduced compared to nontreatment control.

In sum, we have confirmed that LR could target both AC and PDE to coordinate the augmentation of cAMP in cells.

Liquiritigenin alters downstream cyclic adenosine monophosphate–mediated immune responses in cells

cAMP is known for its direct regulation of functions of effector T (Teff) cells in a negative manner and the mediation of Treg-related Teff functional inhibition.[32] This study questioned whether LR has effects on DC cytokine production during LPS stimulation. As can be seen from Figure 4a, adding 20 μM LR into the culture medium resulted in significantly decreased IL-12 and TNF-α contents in supernatants compared to that of LPS activated non-LR treatment control.

It was also reported that the NF-κB signal transduction pathway plays an important role in mediating the activation of CD4 cells and the contents of proinflammatory cytokines.[33] Therefore, we sought to investigate whether LR could affect this pathway and thus regulate the downstream

inflammation responses. To this aim, we first performed activation of the NF-κB pathway by using anti-CD3/CD28 co-ligation; subsequent examination of phosphorylation of p65 demonstrated a dramatic inhibition of the induction of NF-κB by LR (Figure 4b).

Furthermore, as can be seen from Figure 4c, LR could augment the transition of TGFβ-mediated naïve CD4 cell phenotype into Treg one, which were referred to as CD4+CD25+FOXP3+ cells in a time-dependent manner, suggesting suppression of inflammation by LR *via* Treg polarization.

Discussion

Cyclic nucleotides represent the common second messengers associated with the differential regulation of several cellular processes by the various downstream effectors.[34] For example, the involvement of cyclic nucleotide molecules such as cAMP, cGMP, and cGAMP in asthma has been recognized recently,[30,35,36] suggesting that interference with these signaling pathways would provide insights and benefit for the improvement of therapies such as airway muscle relaxation and airway inflammation. Such recognition induces considerable attention to the use of PDE4 inhibitors as the therapeutic agent.[6] However, these early compounds do not have consistent therapeutic effects and may cause adverse reactions, which has hindered their application compared with inhaled steroids in asthma.[6] This has urged the development of improved safety and efficacy of PDE inhibitors or AC agonists for the treatment of asthma.
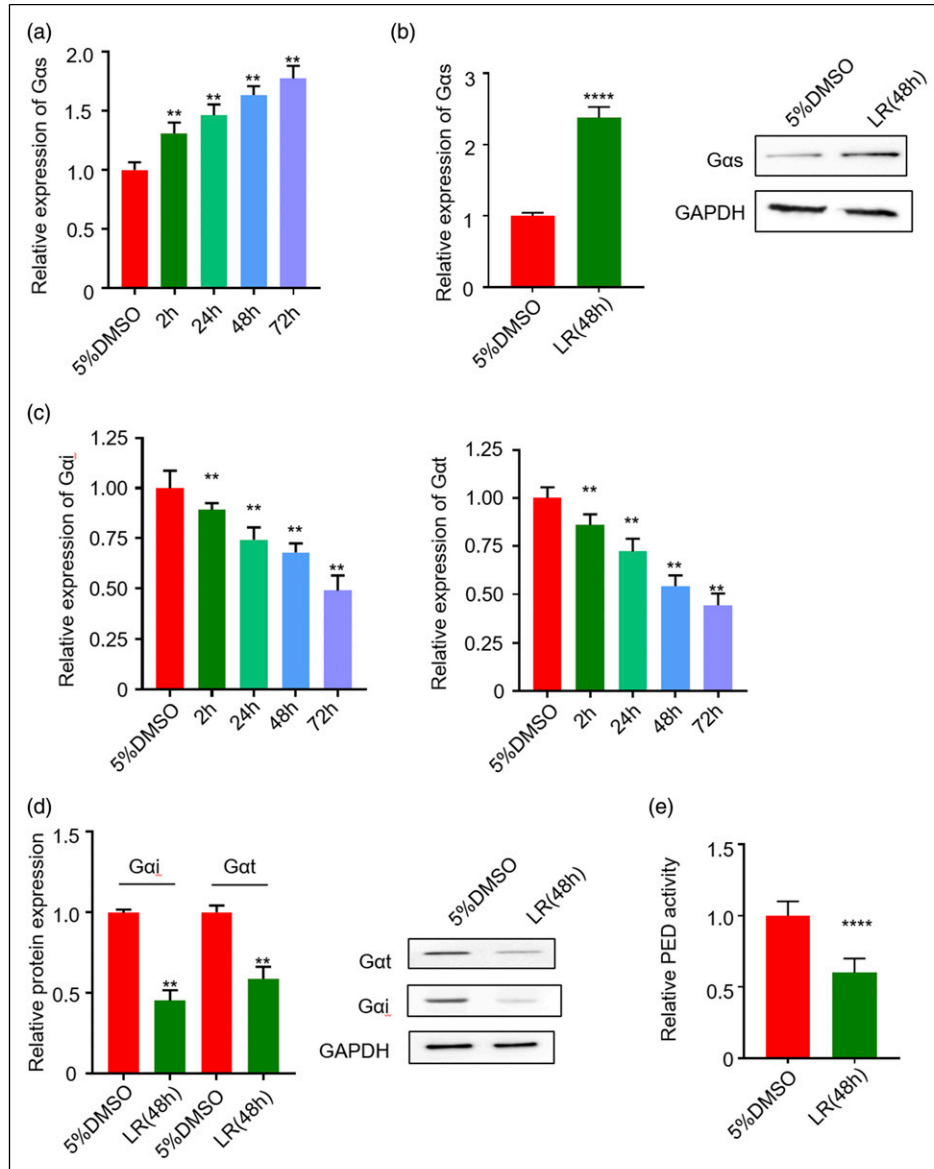
Figure 3. LR functions as both an activator of AC and an inhibitor of PDEs. *(a)*. The relative expression of *Gαs* at different time points post treatment using qRT-PCR. Results are presented in the form of mean ± SD (*n* = 3). **, *P* < .01. *(b)*. Cells were collected at 48 h post LR treatment and evaluated by Western blotting analysis for Gαs protein. GAPDH served as the housekeeping gene. Relative levels of immunoblots were determined alongside. Results are presented in the form of mean ± SD (*n* = 3). ****, *P* < .0001. *(c)*. Relative expression of *Gαt* and *Gαi* at different time points post treatment using qRT-PCR. Results are presented in the form of mean ± SD (*n* = 3). **, *P* < .01. *(d)*. Cells were collected at 48 h post LR treatment and evaluated by Western blotting analysis for *Gαt* and *Gαi* proteins. GAPDH served as the housekeeping gene. Relative levels of immunoblots were determined alongside. Results are presented in the form of mean ± SD (*n* = 3). **, *P* < .01. *(e)*. Relative PDE levels in LPS-stimulated DC cells treated with LR or DMSO. Results were expressed as mean ± SD (*n* = 3). **, *P* < .01.

In our study, we have focused on the Chinese traditional medicines and aimed to determine the chemical mechanism of licorice, one of the promising and long-recognized herbs used for the treatment of asthma in China. We used four of its main components to test for their involvement in inflammation suppression using cAMP, cGMP, and cGAMP as targeted molecules.

Interestingly, we have found that one of the four components, named LR, could greatly increase the level of cAMP by increasing the expression of AC and repression of PDE in different cell lines including dendritic cells and T cells but not that much in macrophages. In addition, we have found that by using LR treatment of DC cells, the increase in cAMP levels is stimulation independent,
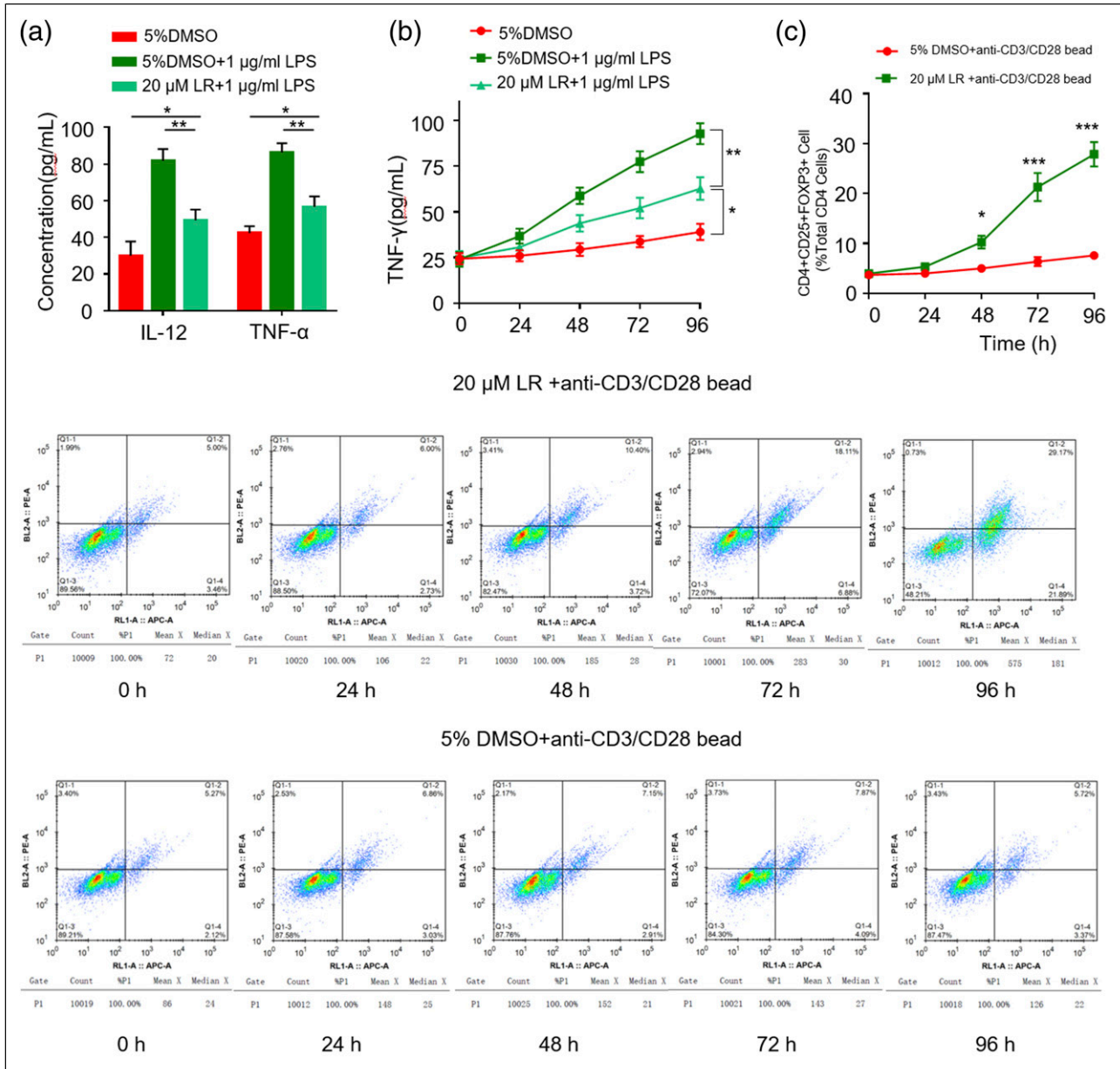
Figure 4. LR alters downstream cAMP-mediated immune responses in cells. *(a)*. Cytokines (IL-12 and TNF-α) concentration in LPS-stimulated DC cells. The cytokines levels were quantified as pg/mL using an ELISA kit (See *Materials and methods*). Results are presented in the form of mean ± SD ($n = 3$). *, $P < .05$; **, $P < .01$. *(b)*. Effect of LR on NF-κB pathway activation. ImageJ software was utilized to quantify p65 total ratio/nuclear location in every group. Results are presented in the form of mean ± SD ($n = 3$. **, $P < .01$. *(c)*. Effect of LR on T cell polarization at different time points. We cultured naïve CD4 cells with T cell receptor stimulation (activation of CD3 and CD28) for 7 days with 20 μM LR, 5 ng/mL TGFβ, both LR and TGFβ, or vehicle (5% DMSO). TGFβ is the well-recognized Treg polarization driver.[41] Results are presented in the form of mean ± SD ($n = 4$). *$P < 0.05$, ***$P < .001$.

suggesting that LR could be used as an anti-inflammatory agent or as an immunosuppressive agent. Finally, we have elucidated that LR could modulate the downstream immune responses such as cytokine production in T cells, after contact with DC cells, and furthermore, the Treg phenotype was augmented in the presence of LR. Based on these evidences, we drew the conclusion that LR could

be used as a potential drug therapy for the treatment of asthmatic inflammation.

Although we have observed that LR could be used as a drug lead, we still lack mechanical understanding on the potentiation of cAMP in different cell lines. It would be of great benefit that we perform large-scale RNA sequencing and proteomic analysis to identify the downstream

pathways that are responsible for the authentic increased cAMP levels in cells as well as the inhibition of downstream immune responses. As we have already shown in our study that LR could improve the expression of AC and downregulate the expression of PDE. This reciprocal regulation of cAMP production is interesting and provides a layered regulation of cAMP levels in the cell by TCM herbal ingredients. Previously, only one mode of action, either potentiation of AC[37] or inhibition of PDE,[34] has been observed. Therefore, it would be interesting to further elucidate the boosting effect of cAMP in cells by LR.

LR is a flavanone that was shown to act as an estrogenic compound with an effect on ER agonism by binding to its receptor at sufficient concentration.[38] In addition, LG showed great anti-inflammatory activity within the LPS-exposed microglial cells as well as in the hydrogen peroxide–exposed mouse liver.[39] Besides, it was shown that LR protected against the high glucose-mediated inflammation by suppressing the NF-κB and nod-like receptor protein 3 (NLRP3) inflammasome signaling pathway.[40] All those facts demonstrate that LR could be used as a good candidate as an anti-inflammatory agent. In our study, we have shown that LR could suppress the production of TNF-α and IL-12 cytokines as well as NF-κB signaling pathway. Previous studies suggested that cAMP would regulate the immune response in a negative way,[14] indicating LR could orchestrate the cAMP signaling pathway to downregulate immune responses. Altogether, we have identified LR as a promising candidate as an immune suppressive agent.

However, there are limitations in our study. First, the investigation of healthy donor cells may not ideally reflect the immune response to LR, and this would be better resolved by using donor cells from pathogenic subjects. Second, we have not completely ruled out the possibility that the contaminants of LR would affect our conclusion, and we will address this by further isolating LR with high-performance liquid chromatography. Third, the in-depth understanding of the elevation of cAMP will be further reinforced by applying multiple omics analyses since the current analysis was solely based on the information from structural genes but not from the regulatory point of view. Therefore, we will perform more comprehensive investigation on these topics.

In addition, it would be beneficial that LR could be used to treat different animal models as well as different cell lines. It is noticed that we have found LR could not increase the cAMP level in macrophages, suggesting that different biological processes occurred in that cell type. Recently, cAMP is suggested to have a certain impact on macrophages. Researchers have found that without factors that activate macrophages such as LPS, cAMP could modulate the differentiation of monocytes into

macrophages and elevate the protein and mRNA expression of proinflammatory chemokines such as CXCL and CCL depending on PDE4,[34] highlighting the link between cAMP and macrophages. The gap of LR in this link should be further uncovered.

Conclusion

We have found that LR could greatly increase the level of cAMP by increasing the expression of AC and repression of PDE in different cell lines including dendritic cells and T cells but not that much in macrophages. In addition, we have found that by using LR treatment of DC cells, the increase in cAMP levels is stimulation independent, suggesting that LR could be used as an anti-inflammatory agent or as an immunosuppressive agent. Finally, we have elucidated that LR could modulate the downstream immune responses such as cytokine production in T cells after contact with DC cells, and furthermore, the Treg phenotype was augmented in the presence of LR. Altogether, LR could be a potential antiasthmatic agent.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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