Introduction

Bronchial asthma is the result of smooth muscle dysfunction, airway inflammation, and airway remodeling[1]. Smooth muscle dysfunction is evidenced by exaggerated bronchoconstriction, bronchial hyper-responsiveness, hyperplasia, and hypertrophy of airway smooth muscle cells and the release of pro-inflammatory mediators. Beta2-adrenergic receptor (β2-AR) agonists and glucocorticoids are often used in combination in the treatment of bronchial asthma because they have distinct mechanisms of action and pathophysiological targets. When used in combination, their hypothesized interaction yields a broader activity profile than when they are used alone[2]. Glucocorticoids can increase β2-AR transcription in human lung in vitro[3] and protect pulmonary β2-ARs from β-agonist-induced down-regulation in vivo[4]. Glucocorticoids can also modulate the coupling efficiency between the β2-AR and Gs protein, thereby increasing adenylyl cyclase activity and cAMP accumulation[4]. On the other hand, β2-AR agonists can prime the glucocorticoid receptor for steroid-dependent activation, enhance glucocorticoid-dependent transcription and inhibit transcription of inflammatory factors such as NF-κB[5–7].

Glycyrrhizin (GL), a major and active constituent of licorice root, has many attributed pharmacological effects, including anti-inflammatory[8], anti-allergy[9], and anti-viral activities[10], which may be due to the steroid-like structure of 18β-glycyrrhetinic acid. Therefore, we hypothesized that GL could interact with β2-AR agonists at both the molecular and receptor level in a manner similar to glucocorticoids. Co-treatment with GL and a β2-AR agonist would yield synergistic anti-asthmatic effects, thereby increasing β2-AR agonist bronchodilation activity and yielding complementary anti-inflammatory effects. The potential of substituting GL for glucocorticoids in combination with β2-AR agonists in the treatment of asthma was investigated.

To address these hypotheses, the effect of GL on β2-ARs and β2-AR activity was evaluated by examining the effect of GL on β2-AR mRNA levels, salbutamol (SA)-induced cAMP accumulation, and the anti-inflammatory effects of GL and SA in vitro and in vivo.
accumulation and relaxation of guinea pig tracheal strips. Anti-inflammatory effects induced by GL and a β2-AR agonist were evaluated via TNF-α-stimulated NF-κB transcription factor activation levels and Western blotting analysis of NF-κB inhibitor (I-κBα) degradation. The inhibition of NF-κB-related inflammation was further investigated by testing interleukin-8 (IL-8) production in pulmonary cells. To further investigate inflammation was further investigated by testing interleukin-8 inhibitor (I-κBα) degradation. The inhibition of NF-κB-related were evaluated via TNF-α-stimulated NF-κB transcription factor.

Cell culture
Polyclonal anti-I-κBα, anti-actin, and secondary horse radish peroxidase-conjugated anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (San Diego, CA, USA).

Animals
Male Hartley guinea pigs (300–500 g) and Wistar rats (300–400 g) were provided by the Academy of Military Medical Science (Beijing, China). All experimental protocols were approved by the Committee of Animal Care of Nankai University (Tianjin, China). Animals were bred in an animal facility under controlled temperature (20 °C), relative humidity (50%) and light (14:10 h, light:dark cycle) conditions, with free access to food and water.

Real-time RT PCR of β2-AR mRNA in rat lung
Twelve rats were divided into four groups and exposed to inhaled atomized physiological saline, 0.1 μmol/L SA, 0.3 μmol/L GL, or 0.1 μmol/L SA and 0.3 μmol/L GL for 2 min twice a day for 7 d. Total cell RNA was isolated from rat lung using the SV Total RNA Isolation System (Promega, Madison, WI, USA) following the manufacturer’s protocols. Total RNA was reverse-transcribed using the Reverse Transcription System (Promega, Madison, WI, USA). Quantitative RT-PCR was performed for β2-AR mRNA with SYBRGreen (Toyobo Co, Ltd, Osaka, Japan). Results were normalized to the housekeeping gene GAPDH. Primers for β2-AR were: forward 5’-ACCTCCTTCTTGCTATCCA-3’ and reverse 5’-TAGGTGGTCGAAGAGACCG-3’. Primers for GAPDH were: forward 5’-CCACCCCATGGCAAATTCCATGGCA-3’ and reverse 5’-TCTAGACGCAGGTCAAGTCCAC-3’. GAPDH was used as an internal control. Beta2-AR mRNA levels were compared among drug-treated and negative controls. Gene expression was evaluated with threshold cycles using the 2^-ΔΔCT method[11]. The PCR product specificity was confirmed by a melting curve. Experiments were performed in triplicate.

cAMP-regulated reporter gene assay
The CHO-β2-AR-CRE-EGFP cell line was constructed and cultured as previously described[12]. Briefly, this cell line expressed exogenous β2-AR and contains a cAMP-regulated EGFP reporter gene. When the cells were stimulated by β2-AR agonists, the increase of intracellular cAMP induced the expression of EGFP. The cells were treated with different drug combinations in 12-well plates. Drug-treated cells were trypsinized, washed and resuspended in PBS (0.01 mol/L) containing 2% FBS. The number of green fluorescent protein(GFP)-positive cells per ten thousand cells was measured using a FACs Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), equipped and analyzed using CellQuest software (Becton Dickinson). All experiments were performed in triplicate, and results were expressed as mean±SEM and analyzed with the GraphPad Prism 4.0 program (GraphPad Software, San Diego, CA, USA).

The isolated guinea pig tracheal strips relaxation model
Hartley strain guinea pigs were sacrificed with an overdose of pentobarbital (75 mg/kg, ip). Tracheal strips were removed and manipulated as previously described[12]. Acetylcholine (ACh) was added to the organ bath to induce the contraction of smooth muscle. SA, GL, and DEX were added to the organ bath 10 min before the addition of ACh. PRO was added 10 min before the addition of SA, GL, and DEX. The peak contractile response was designated as 100%. Tension was expressed as a percentage of the peak contractile response.

Transient transfection and luciferase assay
The NF-κB-responsive luciferase reporter constructed with Igx2-IFN-LUC was established as previously described[13]. Transient transfection with Igx2-IFN-LUC was performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s recommendations. HEK 293 cells were treated for 6 h with drug combinations 24 h after transfection and harvested for the detection of luciferase activity using a Lumat LB9507 luminometer (EG&G Berthold, Oak Ridge, TN, USA) and Dual Luciferase Assay kits (Promega, San Luis Obisco, CA, USA). Co-transfection with pRL-TK (Promega), which expresses Renilla luciferase, served to normalize the data and account for transfection efficiency.
Western blotting of I-κBα

NIH-3T3 cells were lysed in lysate buffer (20 mmol/L Tris-HCl, pH 7.5, 1% Triton X-100, 137 mmol/L sodium chloride, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 25 mmol/L β-glycerophosphate, 2 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulphonyl fluoride and 1 mg/mL leupeptin). A polyclonal I-κBα antibody (1:500) was used to assess I-κBα levels, and proteins of interest were visualized using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, San Diego, CA, USA). Equal protein loading was verified by actin immunoblot. A minimum of three separate experiments were performed.

Enzyme-linked immunosorbent assay (ELISA) of IL-8

A549 cells and hBSMCs were pre-incubated with the tested drugs for 1 h and continuously incubated with TNF-α for 6 h. IL-8 levels in the culture medium were quantified by ELISA using an anti-IL-8 antibody according to the manufacture’s instructions (R&D Systems, Minneapolis, MN, USA).

In vivo bronchial hyperreactivity test

Thirty-five male Hartley strain guinea pigs were divided into five treatment groups. During the experiment, animals were manipulated as previously described[12]. Briefly, the animals were placed in an inhalation cage, and each animal was exposed to atomized 0.1% histamine solutions for 20 s at the rate of 0.8 mL/min. The animals were withdrawn from the inhalation cage, and the time until the appearance of asphyxial convulsions was measured as the latent period of asthma. Two days after the first treatment, animals were exposed a second time using this identical procedure. Asthma latency period of each animal was calculated as the ratio of increased latency time to the original latency time by Vogel’s method[14]. Normal saline served as a vehicle control.

Statistical analysis

Data are presented as mean±SEM. Statistical analysis of the data for multiple comparisons was carried out by ANOVA followed by the Bonferroni post hoc test. For single comparisons, significance differences between means were determined by Student’s t-test. Statistical significance was set at P<0.01.

Results

GL modulates β2-AR function by increasing β2-AR mRNA levels and SA-stimulated cAMP accumulation

The effects of GL on β2-AR and β2-AR activity were evaluated by examining the effects of GL on β2-AR mRNA levels and SA-stimulated cAMP accumulation. As shown in Figure 1, inhaled 0.3 µmol/L GL significantly increased β2-AR mRNA levels (47±0.27% of untreated control), while 0.1 µmol/L SA treatment alone reduced β2-AR mRNA levels (56±0.12% of untreated control). Despite a modest decrease (12±0.08% of untreated control), combined treatment of SA and GL did not significantly alter β2-AR mRNA levels in rat lungs. However, the combination resulted in a significant decrease compared to the 0.3 µmol/L GL dose alone.

CHO-β2-AR-CRE-EGFP cells were treated with different concentrations of GL and SA alone or a combination of 0.3 µmol/L GL and SA in various concentrations. As shown in the dose-response curve displayed in Figure 2A, the EC50 of SA was 5.1±0.9 nmol/L. GL was unable to stimulate reporter gene expression, but it shifted the curve left when administered in combination with SA. The EC50 was 0.73±0.12 nmol/L and was significantly reduced compared to SA alone. In another experiment, the time course of SA- and GL-stimulated reporter gene expression in CHO-β2-AR-CRE-EGFP cells was
evaluated. As shown in Figure 2B, when activated by 0.01 µmol/L SA alone, intensity of EGFP peaked at approximately 9 h. However, the activity was significantly increased when administered in combination with GL (0.3 µmol/L) for 9–15 h. Because reporter gene expression level was indicative of intracellular cAMP, the results suggest that GL increased SA-stimulated adenylate cyclase activity and cAMP accumulation.

**Glycyrrhizin enhances the relaxing effect of SA on isolated guinea pig tracheal strips**

The ability of the SA and GL combination to enhance the relaxing effect on Ach-induced contraction of guinea pig tracheae was compared with that of the effect of the positive control drug, SA, alone. As shown in Figure 3, SA administration resulted in a concentration-dependent relaxation of Ach-induced contracted tracheal muscle, with 0.001, 0.01, and 0.1 µmol/L SA eliciting EC\textsubscript{50} values of 7.303±0.015, 16.28±0.21, and 66.61±0.33 µmol/L, respectively (Table 1). GL alone failed to yield a significant anti-Ach effect. However, addition of 0.003 µmol/L GL in combination with 0.001 µmol/L SA resulted in a significantly higher relaxation effect, approximately as high as that of 100-fold concentrated SA alone. In other words, the combination of 0.001 µmol/L SA and 0.003 µmol/L GL showed a potency similar to 0.1 µmol/L SA. PRO is a non-selective β-AR antagonist that has been shown to block the activation of β\textsubscript{2}-AR. As shown in Table 1, PRO blocked the tracheal muscle relaxation stimulated by SA. When the β\textsubscript{2}-AR was blocked, the ability of GL to enhance SA was also blocked. Effects observed with the positive control, 0.001 µmol/L DEX, were similar to those observed with GL (Table 1).

**Combined GL and SA treatment promotes anti-inflammatory activity**

The anti-inflammatory activity of the combination of GL and SA was examined using ELISA and Western blotting. HEK293 cells transfected with Igκ2-IFN-LUC significantly inhibited NF-κB promoter activation by 35%±3.5% and 40%±4.8%, respectively. Combined treatment had an additive inhibitive effect of 70%±7.4%.

NIH-3T3 cells were treated prior to TNF-α stimulation for 1 h with 0.01 µmol/L GL, 0.01 µmol/L SA, or a combination of both drugs, and were assessed in a time-course experiment. As shown in Figure 4B, I-κBα was fully degraded after a 20 min incubation with TNF-α but gradually regenerated over a 30 min period in negative controls. Pretreatment with SA, GL,

| Concentration (µmol/L) | EC\textsubscript{50} (µmol/L) |
|------------------------|-----------------------------|
| Control                | 7.40±0.021                  |
| SA                     | 7.30±0.015                  |
| 0.01                   | 16.28±0.21\textsuperscript{a}|
| 0.1                    | 66.61±0.33\textsuperscript{a}|
| PRO+SA                 | 8.21±0.007                  |
| GL                     | 9.95±0.090                  |
| 0.003                  | 63.99±0.42\textsuperscript{d}|
| 0.1                    | 10.28±0.92                  |
| DEX+SA                 | 9.03±0.200                  |
| GL+SA                  | 60.45±0.11\textsuperscript{c}|
| PRO+GL+SA              | 7.369±0.032                 |

SA, salbutamol; GL, glycyrrhizin; DEX, dexamethasone; PRO, propranolol.

**Figure 3.** Effects of GL on enhancing the relaxing effect of β\textsubscript{2}-AR agonists in isolated guinea pig tracheal strips. Tension is expressed as a percentage of the peak contractile response, which was assigned as 100%. Mean±SEM. n=3.

**Figure 4.** Effects of GL and SA on inhibiting TNF-α-induced NF-κB activation. (A) After transfected with Igκ2-IFN-LUC and pRLTK plasmids, HEK293 cells in different groups were treated with 10 ng/mL TNF-α, 0.01 µmol/L SA or 0.01 µmol/L GL for 6 h. The luciferase activity was expressed as the ratio of firefly to renilla luminescence. Mean±SEM. n=3. \textsuperscript{c}P<0.01 vs control without any stimulation. \textsuperscript{d}P<0.01 vs the TNF-α-activated group. (B) NIH-3T3 cells, treated with 0.01 µmol/L GL, 0.01 µmol/L SA or the combination, were then stimulated by 10 ng/mL TNF-α. The time course of I-κBα protein degradation was analyzed by Western blot. Three separate experiments confirmed observed changes.
or a combination suppressed I-κBα protein degradation, and at 30 min the combination treatment showed a more pronounced effect than the other treatment groups. In other words, the regeneration rate of the combination group is faster than that of either the GL- or SA-alone group; thus, the combination treatment displays an additive effect.

IL-8 protein levels in spent culture media of A549 cells and hBSMCs treated with TNF-α were measured by ELISA to assess the effect of combined SA and GL treatment. As shown in Figure 5A and 5B, IL-8 levels in TNF-α-treated medium were significantly greater compared to the negative control. Pretreatment with 0.1 mmol/L SA, 0.01 mmol/L DEX or a combination of both markedly blocked TNF-α-mediated IL-8 release. GL treatment (0.1 mmol/L) alone or in combination with SA also blocked the increase in IL-8 release. GL and SA co-treatment resulted in the most pronounced inhibition compared to the TNF-α-activated group and significantly differed from the GL-alone group.

SA and GL synergy in the in vivo bronchial hyper-reactivity test
To confirm the synergistic effect of SA and GL in an in vivo model, guinea pigs inhaled atomized physiological saline, 0.1 µmol/L SA, 1.0 µmol/LSA, 0.3 µmol/L GL, or 0.1 µmol/LSA and 0.3 µmol/L GL two days after initial exposure to histamine. Animals were then exposed to histamine a second time. As shown in Figure 6, the latent period increased (1.1±0.32)-fold in the 0.1 µmol/L SA-treated group. GL alone failed to elicit a significant effect in the asthma model. However, when 0.3 µmol/L GL was administered in concert with 0.1 µmol/L SA, a significant increase (2.2±0.35)-fold was observed similar to that observed with a 10-fold higher SA concentration [1.0 µmol/L, (2.1±0.42) fold]. These data provide in vivo evidence for SA and GL synergy.

**Figure 5.** Effects of GL and SA on inhibiting TNF-α mediated IL-8 production. A549 cells (A) or human bronchial smooth muscle cells (HBSMC). (B) were incubated with 0.1 mmol/L SA, 0.1 mmol/L GL, 0.01 mmol/L dexamethasone (DEX) or their combinations for 1 h, in concert with 10 ng/mL TNF-α incubation for 6 h. IL-8 levels in culture medium were assessed by ELISA. Each bar represents the mean±SEM. n=3. *P<0.01 vs control without any stimulation. **P<0.01 vs the TNF-α-activated group. ***P<0.01 vs the 0.1 mmol/L GL group.

**Figure 6.** Synergistic anti-asthmatic effects of salbutamol (SA) and glycyrrhizin (GL) in the atomized histamine asthma induced guinea pig model. Animals were exposed to atomized 0.1% histamine solutions for 20 s. After a two day interval exposure to 0.1 µmol/L SA, 1.0 µmol/L SA, 0.3 µmol/L GL, or 0.3 µmol/L GL and 0.1 µmol/L SA occurred immediately prior to the second 0.1% histamine solution exposure. Asthma latency period increasing rate of each animal was presented as the ratio of increased time to the original latency time. Each bar represents the mean±SEM. n=7. *P<0.01 vs control.

**Discussion**

Glcyrrhizin (GL) is a triterpene glycoside that consists of a glycyrrhetic acid molecule and two glucuronic acid molecules located at the C-3 position. GL itself has a glucocorticoid-like action and holds promise as an alternative to steroid treatment with a similar potency when used in combination with a β2-AR agonist.

Corticosteroids modulate β2-AR via many different mechanisms, including stimulating the transcription of the β2-AR and increasing the efficiency of coupling between β2-AR and Gα.[4] In the present study, we hypothesized that GL could have a function similar to dexamethasone, which has been shown to increase β2-AR mRNA and protein levels in lung.[3]

As observed in β2-AR mRNA quantification assays and cAMP-regulated reporter gene experiments, GL is capable of increasing β2-AR mRNA levels and SA-induced cAMP accumulation. These results support the hypothesis that the combination of SA and GL could have a synergistic effect on the activity of β2-ARs. In the same assays, we identified that, in addition to SA, other β2-AR agonists, such as epinephrine, isoproterenol...
nor is it teratogenic in rats [24]. Despite their effectiveness, the use of glucocorticoids in lung tumorigenicity with long-term daily administration in mice [23], pound, does not provide any evidence of chronic toxicity or term without adverse effects is imperative. GL, a natural com recovery of non-steroidal drugs that can be administered long- term without adverse effects is imperative. GL, a natural com

GL on TNF-α-induced activation of NF-κB is attributable to inhibited I-κB kinase activity. Inhibition of IL-8 production in pulmonary cells was significantly greater when the two drugs were combined. Thus, the interaction among SA and GL may result in complementary anti-inflammatory effects in the airway.

Because of the chronic nature of asthma, long-term medications are required for asthma therapy. The combination of β2-AR agonists and glucocorticoids is more efficacious for asthma than either alone or in other combination therapies [31]. Despite their effectiveness, the use of glucocorticoids in lung diseases is limited due to severe side effects [22]. Therefore, discovery of non-steroidal drugs that can be administered long-term without adverse effects is imperative. GL, a natural compound, does not provide any evidence of chronic toxicity or tumorigenicity with long-term daily administration in mice [23], nor is it teratogenic in rats [24]. The acceptable daily GL intake is reported to be 0.015–0.229 mg/kg body weight per day [25]. It has been reported that GL has the ability to prevent nitrogen species-mediated lung cell damage [26]. Considering the therapeutic potential of GL, the results reported here demonstrate the important pharmacology of GL in combination with a β2-AR agonist, offering the possibility of therapeutic application in the treatment of asthma.

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Author contribution

Gang BAI designed the research; Yang YANG, Qian SHI, Ze LIU, Ruo-jie LI and Peng-wei PAN performed the research; Wan-ge LU contributed the new analytical tools and reagents; Yuan-yuan HOU analyzed the data.

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