Original Article

Diosgenin suppressed LPS-induced inflammation through inhibiting HSP90 induced phosphorylation of NF-κb p65

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Abstract: Asthma is a heterogeneous disease which affects millions of people’s life. Glucocorticoids (GCs) are able to relieve their symptoms through interacting with glucocorticoid receptors (GRs). Diosgenin, extracted from Dioscorea nipponica, was hypothesized to exert anti-inflammation effect through interaction with GRs as well. In this study, lipopolysaccharide (LPS)-inducted primary human bronchial epithelial cells (HBECs) were utilized as asthma models. ELISA was applied to measure the secretion of IL-1β and IL-6. Quantitative PCR was applied to evaluate the expression of IL-1β, IL-6 and GRα. Western blot was utilized to test the protein level of HSP90 and GRα and phosphorylation of NF-κb p65. As a result, our ELISA and quantitative PCR data demonstrated that diosgenin and prednisone acetate suppressed the secretion of IL-1β and IL-6 as well as their mRNA level. Our western blot data indicated that both diosgenin and prednisone acetate treatment enhanced the expression of GRα and inhibited the phosphorylation of NF-κb p65 without affecting the protein level of HSP90. After applied HSP90 inhibitor (17-AAG) to cells, we found that the secretion of IL-1β and IL-6 and the phosphorylation of NF-κb p65 were both suppressed. As a conclusion, prednisone acetate treatment and diosgenin treatment might suppress inflammation by inhibiting HSP90 induced phosphorylation of NF-κb p65 together with GR activation. These data revealed novel molecular mechanism of the suppression of diosgenin on inflammation.

Keywords: Diosgenin, glucocorticoid, glucocorticoid receptor, inflammation, HSP90, NF-κb

Introduction

Asthma is one type of airway inflammatory diseases strongly associated with defective structural and functional changes of epithelial cells in response to environmental insults [1, 2]. It damages about 300 million people’s life worldwide and causes 250000 deaths annually. Currently, although there is no way to cure this disease, the regular use of inhaled glucocorticoids (GCs) is capable to manage the symptoms by suppressing inflammation [3].

GCs are important chemicals broadly involved in cell survival, proliferation, differentiation and inflammation suppression through interacting with glucocorticoid receptors (GRs) in many cells and tissues [4, 5]. As reported, once interacted with GCs, GRs were activated and able to upregulate the transcription of anti-inflammatory genes and to repress the function of NF-κB and AP-1 [6, 7]. In order to become active and bind to their ligands, GRs needed to form complexes with heat shock protein 90 (Hsp90) [8]. HSP90 is a 90 kDa molecular chaperone required for activation of numerous clients including steroid receptors and transcription factors by guiding late-stage tertiary folding and maintaining the conformational integrity [9]. Meanwhile, the expression of HSP90 is also promoted at inflammatory status. It was reported that HSP90 could improve the expression of inflammatory cytokines such as IL-1β and IL-6 by facilitating the phosphorylation of NF-κb p65 [10]. Therefore, it might be possible that GC treatment inhibits HSP90 induced phosphorylation of NF-κb p65, which is not yet reported.

Diosgenin is a natural steroidal saponin extracted from Dioscorea nipponica, which was found
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Figure 1. Comparisons of chemical structural formula of Glucocorticoid and diosgenin.

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to suppress intestinal inflammation and treat asthma [11, 12]. As Figure 1 indicated, its structure is similar to GCs, thus it may function through interacting with GRs as well [13]. Our results indicated it could suppress the secretion of IL-1β and IL-6 and enhance the expression of GRs in lipopolysaccharide (LPS)-induced primary human bronchial epithelial cells (HBECs). Our data also demonstrated that diosgenin enhanced the expression of GRs and reduced the phosphorylation of NF-kb p65 in HBECs without any affection on the expression of HSP90. Meanwhile, as one type of GCs, prednisone acetate was found to act in the same way as well. We also found that HSP90 inhibition suppressed the secretion of IL-1β and IL-6 and inhibited the phosphorylation of NF-kb p65. Therefore, prednisone acetate treatment and diosgenin treatment might suppress inflammation by inhibiting HSP90 conducted phosphorylation of NF-kb p65 together with GR activation.

Materials and methods

Reagents and antibodies

Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific, Waltham, MA, USA. Rabbit anti-human GRα, HSP90, phosphorylated NF-kb p65 (p-p65) and β-actin were bought from Santa Cruz Biotechnology, Dallas, Texas, USA. Goat anti-Rabbit IgG/HRP were get from KPL, Inc, Gaithersburg, Maryland, USA. All primers of GRα, IL-1β, IL-6 and GAPDH were synthesized by GenePharma, shanghai, China. HBECs were provided by Promo Cell Heidelberg Germany. ELISA kit of human IL-1β and IL-6 were purchased from abnova, Taipei, Taiwan.

Treatment of human bronchial epithelial cells

Human bronchial epithelial cells (HBECs) were one type of the main cells that form the barrier against allergens, pollutants and infectious agent [14]. These cells highly express GRs and hold critical role in inflammation related signaling [5, 15]. They are maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (50 µg/ml). Inflammation was induced by LPS treatment at the concentration of 100 ng/ml and 1 μg/ml for 24 hrs. And 1 hr before induction, cells were treated with different drugs including (1) 10 μM diosgenin; (2) 100 nM prednisone acetate; (3) 100 nM 17-AAG (HSP90 inhibitor). After all treatments, cell medium and cells were collected separately for different tests. Cell medium and cells with no treatments were used as control. Cells medium was utilized for ELISA tests. Cells were tested with quantitative PCR and western blotting.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-1β and IL-6 in cell culture medium was measured by ELISA Kit. Cells were planted in 6-well culture plate. 24 hr after each treatment, culture supernatant was collected and kept at -80°C before measurement. All steps were done according to the instructions.

Quantitative PCR

Total RNA was extracted from cells using TRIzol Reagent and used for cDNA synthesis. For mRNA reverse transcription, total RNA was reverse transcribed with Superscript Reverse Transcription kit (Thermo fisher). Quantitative real-time PCR was performed using SYBR Green master Mix (Bio-rad). The following primers were used: GRα-forward: 5’-ACACAGGCTTCAGGTATCCTTTGCAAG-3’; GRα-reverse: 5’-ACTGCTTCTGGCATCAG-3’; IL-1β-forward: 5’-GAATGCCCACCTTTGACAGT-3’; IL-1β-forward: 5’-GAAACGCCAACC-3’; IL-1β-forward: 5’-CTCATCAGGAGACAG-3’; IL-6-forward: 5’-CTGCAAG-
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AGACTTCCATCCAG-3', IL-6-reverse: 5'-AGTGGTAGTATAGACAGGTCTGTTGG-3'; GAPDH-forward: 5'-CCAGGTGGTCTCCTCTGA-3', GAPDH-reverse: 5'-GCTGTAGCAATCGTTGT-3'. The mRNA expression values were normalized to GAPDH. Relative expression levels of mRNA were analyzed using the ABI 7500 (Applied Biosystems by Life Technologies).

Western blot

Whole-cell protein was extracted with protein lysis solution. Protease inhibitors were added into cellular lysate. Protein concentration was measured with bicinchoninic acid assay. Western analysis was performed using the following antibodies: Rabbit anti-human GRα, HSP90, p-p65, and β-actin antibody. Briefly, equal amounts of protein (50 ng) were separated by SDS-PAGE and transferred to PVDF membranes. HRP-conjugated goat-anti-rabbit IgG was used as secondary antibody. Bound fragments were detected with ECL chemiluminescent kit (Pierce) and exposed on x-film.

Statistical analysis

All ELISA data and quantitative PCR data were analyzed through SPSS 10.0. The Student's t test was applied to analyze statistical differences between two groups. P<0.05 was considered to be statistically significant. Each test data from independent experiments were repeated at least three times.

Figure 2. The secretion of IL-1β and IL-6 were increased with NF-κB phosphorylation and HSP90 expression in LPS treated HBECs. A and B. ELISA tests of IL-1β and IL-6; C. Quantative PCR test of IL-1β and IL-6; D. Quantative PCR test of GRα; E. Western blot test of p65 phosphorylation (p-p65), HSP90 and GRα. There were three different groups such as control, 100 ng/ml LPS and 1 μg/ml LPS. Values are means ± SD (n=3). ***P<0.05, significant differences between LPS-treated groups and control group.
Results

The expression of IL-1β, IL-6 and HSP90 as well as NF-κB phosphorylation were increased in LPS treated HBECs

As shown in Figure 2, in order to evaluate the effects of LPS treatment on HBECs, we first tested the concentration of IL-1β and IL-6 in cell medium of each group. The secretion of these cytokines was significantly raised in LPS treated group in a dose-dependent manner (Figure 2A and 2B). And then afterwards, similar data were collected with mRNA changes of IL-1β and IL-6 by quantitative PCR test (Figure 2C). We also measured the expression level of GRα with quantitative PCR, which was dramatically dropped as LPS concentration increased (Figure 2D). Finally, western blot was conducted to compare the phosphorylation of p65 and the changes of HSP90 and GRα at protein level. As expected, p65 phosphorylation and HSP90 expression were increased with LPS treatment, while GRα expression was down-regulated (Figure 2E). As previous reported, at inflammatory status, HSP90 was tend to phosphorylate p65 to further up-regulate NF-κB mediated transcription of pro-inflammatory cytokines.

Figure 3. HSP90 inhibitor treatment attenuated the effects of LPS treatment on HBECs. A and B. ELISA tests of IL-1β and IL-6; C. Quantitative PCR test of IL-1β and IL-6; D. Quantitative PCR test of GRα; E. Western blotting test of p65 phosphorylation (p-p65), HSP90 and GRα. There were three different groups such as control, 1 μg/ml LPS and 1 μg/ml LPS + 100 nM 17-AAG. Values are means ± SD (n=3). ***P<0.05, significant differences between LPS-treated groups and control group. ***P<0.05, significant differences between LPS-treated groups and drug-treated group too.
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such as IL-1β and IL-6 [10]. Taken together, LPS treatment might promote the expression and secretion of IL-1β and IL-6 while it enhanced the expression of HSP90 and inhibited the expression of GRα.

Suppressing HSP90 attenuated the effects of LPS treatment on HBECs

To investigate whether HSP90 played an essential role in LPS-induced inflammation, 17-AAG, one type of HSP90 inhibitors, was added. As shown in Figure 3A and 3B, the secretion of IL-1β and IL-6 was greatly reduced in 1 μg/ml LPS plus 100 nM 17-AAG-treated group, compared with 1 μg/ml LPS-treated group. And then afterwards, similar data were collected with mRNA changes of IL-1β and IL-6 by quantitative PCR test (Figure 3C). We also measured the expression level of GRα with quantitative PCR. It indicated that 17-AAG treatment rescued the inhibitory effect of LPS on the mRNA expression of GRα (Figure 3D). Finally, western blot was conducted to compare the phosphorylation of p65 and the changes of HSP90 and GRα at protein level. Compared with LPS treated group, 17-AAG addition attenuated p65 phosphorylation in the protein level of HSP90 and GRα (Figure 3E).

Diosgenin and prednisone acetate attenuated LPS-induced inflammation by reducing p65 phosphorylation

Although diosgenin and GC were reported to activate GR and facilitate its function in cell
nucleus, whether diosgenin and GC treatment affect p65 phosphorylation was not reported yet. Prednisone acetate was used as an representative of GCs in this study. As shown in Figure 4A and 4B, the concentrations of IL-1β and IL-6 in cell culture medium decreased with the addition of diosgenin and prednisone acetate. As Figure 4C indicated, diosgenin treatment significantly reduced the expression of IL-1β and IL-6 in HBECs and prednisone acetate exhibited similar effects. The mRNA level and protein level of GRα were both increased by diosgenin and prednisone acetate, respectively (Figure 4D and 4E). We also found that p65 phosphorylation was down-regulated with the addition of diosgenin, while the expression of HSP90 was not changed. Similarly, prednisone acetate treatment dephosphorylated p65 without change the expression of HSP90. Therefore our data indicated that diosgenin and prednisone acetate might suppress inflammation by functioning as HSP90 inhibitors to block the stimulative effect of HSP90 on phosphorylation of p65 together with GR activation.

Discussion

Bronchial inflammation is the central feature of asthma, and involves increased activation of NF-κB signaling pathway [2, 3]. Diosgenin and GCs are both effective in asthma treatment, since they activate GRs and promote their anti-inflammatory effects in cell nucleus. However, whether Diosgenin and GCs affect p65 phosphorylation were not reported so far. Here in this study, we found that diosgenin and GCs enhanced the expression of GRα and triggered GR-mediated anti-inflammatory effects by inhibiting HSP90 induced p65 phosphorylation. At first, in this present study, LPS-induced HBECs were used for cell model. Through the in vitro study, we found that HSP90 were highly expressed under LPS-induced inflammatory condition and that it could promote p65 phosphorylation. Our data were consistent with previous reports. HSP90 was reported to be a key factor in LPS-induced inflammation by enhancing the phosphorylation of NF-kB p65 and further affecting the expression of IL-1β and IL-6 [10, 16]. However, 17-AAG treatment affected the mRNA and protein level of GRα in an opposite way. As reported, heat shock protein 70 (HSP70) and HSP90 work together to form a chaperone complex with GRα. While Hsp90 protects GRα from aggregation and enhances its ligand affinity, HSP70 facilitates GRα aggregation and leads it to degradation [8]. Therefore, 17-AAG treatment damaged the function of HSP90, GRα formed complex with HSP70 and underwent the degradation pathway. However, 17-AAG treatment rescued cells from LPS-induced inflammation; the mRNA level of GRα was up-regulated.

Moreover, in this study, we also found that diosgenin and GCs inhibited the secretion of pro-inflammatory cytokines such as IL-1β and IL-6, which was regulated by NF-kB and GRs. We also found that both of diosgenin and GCs could enhance the expression of GRs to activate it. Activated GRs were reported to translocate into cell nucleus and directly bind to cAMP response element binding protein (CREB) binding protein (CBP) to repress NF-kB mediated expression of pro-inflammatory cytokines [7, 17]. However, in this study, we found that diosgenin and GCs treatment reduced p65 phosphorylation, which would also lead to suppression of NF-kB mediated expression of pro-inflammatory cytokines. This is the first time to report that diosgenin and GCs treatment regulated NF-kB signaling pathway by affecting p65 phosphorylation. What is interesting, the expression of HSP90 was not changed. As reported, diosgenin and GCs are capable to activate GRs and facilitate their translocation into cell nucleus [18, 19]. The initial equilibrium between unactivated and activated GRs in cytoplasm might be broken after diosgenin and GCs treatment, which might favor the complex formation between HSP90 and GRs and block the enhancement of HSP90 on p65 phosphorylation. More work need to be done to reveal how this happen in the future.

Conclusion

As a conclusion, our data demonstrated that diosgenin suppressed the secretion of IL-1β and IL-6 by enhancing the expression of GRα and inhibiting the phosphorylation of NF-kB p65 without affecting the protein level of HSP90. Meanwhile, GCs were found to act in the same way as well. We also found that HSP90 inhibition suppressed the secretion of IL-1β and IL-6 by inhibiting the phosphorylation of NF-kB p65. Therefore, GCs treatment and
Diosgenin treatment might suppress inflammation by inhibiting HSP90 induced phosphorylation of NF-κb p65 together with GR activation. These data revealed novel molecular mechanism of the suppression of diosgenin on inflammation, which would facilitate its clinical applications.

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Disclosure of conflict of interest

None.

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