Ginsenoside Rg1 exerts synergistic anti-inflammatory effects with low doses of glucocorticoids in vitro

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DMSO (PubChem CID: 679)
MTT (PubChem CID: 64965)
Corticosterone (PubChem CID: 5753)
Ginsenoside Re (PubChem CID: 73149)
Ginsenoside Rg1 (PubChem CID: 441923)

Abstract

Glucocorticoids (GCs) are usually used to treat inflammatory diseases. However, they cause severe and irreversible side effects, which limit the use of these compounds. Ginsenoside Rg1 had been demonstrated to possess anti-inflammatory and anti-cancer effects. The present study was designed to investigate whether Rg1 exhibits synergistic anti-inflammatory effects when combined with glucocorticoids. After stimulated by lipopolysaccharide (LPS), murine macrophagic RAW264.7 cells were treated with Rg1, corticosterone (Cort) or Rg1 and Cort. Then nitric oxide (NO), tumor necrosis factor-α (TNF-α) and glucocorticoid receptor (GR) expression were measured. The results showed that Rg1 or Cort could reduce the production of NO and TNF-α, and Rg1 dose-dependently up-regulated GR expression, while Cort dose-dependently down-regulated GR expression. The combination of low concentrations of Rg1 with Cort, which alone could not markedly inhibit the release of inflammatory factors, inhibited the secretion of NO and TNF-α in LPS-stimulated RAW264.7 macrophage cells, and up-regulated the expression of GR. The findings suggested Rg1 can synergize with glucocorticoid to enhance its anti-inflammatory effect.

Keywords:
Glucocorticoids
Anti-inflammatory effect
Ginsenoside Rg1
Synergism

1. Introduction

Glucocorticoids (GCs) are the most potently used anti-inflammatory and immunosuppressive drugs presently. The anti-inflammatory and immunosuppressive effects of GCs are mediated predominantly by glucocorticoid receptor (GR), a member of the nuclear receptor superfamily. Unfortunately, long-term and/or high-dose glucocorticoid administration is commonly associated with side effects, such as hypertension, disorders of glucose and lipid metabolism, osteoporosis, and especially femoral head necrosis [1–3]. Of the methods of minimizing the undesirable side effects of corticotherapy is through their association with other pharmaceuticals, especially with other more specific anti-inflammatory or immunosuppressors, aiming at synergistic effects in order to avoid the use of glucocorticoids or to reduce the dosage and duration of corticotherapy [4].

Ginseng is a traditional Chinese medicine which consists of the dried roots of Asiatic ginseng, Panax ginseng C.A. Meyer [5], and has been used for more than 1000 years. It is now one of the most extensively used alternative medicines in the treatment of inflammation. Ginsenosides (GSS), the major active components of ginseng, which are triterpene saponins that have a rigid steroidal skeleton with sugar moieties and produce multiple pharmacological responses [6]. Based on their chemical structure, GSS are generally divided into 2 groups: protopanaxadiols (PD) and protopanaxatriols (PT). The sugar moieties in the PT group are attached to a 6-position...
of GSS including Re, Rf and Rg1, etc. However the sugar moieties of 6-position are not the same. Each ginsenoside may have different effects in pharmacology and mechanisms due to their different chemical structures. As to the anti-inflammatory effects of GSS, it has been showed that ginsenoside Rd could inhibit the expressions of iNOS and COX-2 by suppressing IRAK-1 activation—the key step of inflammation [7,8]. Many studies reported that Rg1 could inhibit LPS-induced cytokine production in vitro [9–13]. Furthermore, Rg1 showed the glucocorticoid-like effects, and to be a functional ligand of GR [14]. But it was not clear whether there is an interaction between Rg1 and glucocorticoids.

In this study, the aim was to investigate whether the ginsenoside Rg1 could exert synergistic anti-inflammatory effects with low concentrations of glucocorticoids in vitro and the mechanism of the synergistic effect.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS), DMSO, MTT, and corticosterone (Cort) were obtained from Sigma-Aldrich. Rg1 (Fig. 1) was purchased from Yantai Science & Biotechnology Co., Ltd. (HPLC ≥ 98%). Rabbit polyclonal antibody to GR and β-actin antibody were purchased from Santa Cruz Biotechnology, Inc.

RPMI 1640 medium and fetal bovine serum were purchased from Invitrogen. Penicillin-streptomycin stock solution, mouse TNF-α ELISA kit, and Bradford protein assay kit were purchased from Yantai Science & Biotechnology Co., Ltd.

2.2. Cell culture

The mouse monocyte-macrophage cell line RAW 264.7 was purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were kept at 37 °C with 5% CO2 in a fully humid atmosphere. The medium was routinely changed every day.

2.3. Cell viability assay

Cells were treated with Re, Rg1 or Cort at the concentrations of 1–100 μM, respectively. The control group received an equal amount of DMSO, which resulted in a final concentration of 0.2% DMSO in the culture medium. The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan was used to measure cell respiration as an indicator of cell viability [15]. Briefly, after 24 h incubation, an MTT solution (final concentration is 200 μg/ml) was added, and the cells were incubated for another 4 h at 37 ºC. After removing the supernatant, 150 μl of DMSO were added to the cells to dissolve the formazan. The absorbance of each group was measured by using a microplate reader at a wavelength of 570 nm. The control group, consisting of untreated cells, was considered as having 100% of viable cells. Results are expressed as percentage of viable cells when compared with the control group.

2.4. Analysis of NO

RAW 264.7 cells were treated by LPS (1 μg/ml) with or without drugs (1–25 μM) for 24 h. The concentration of nitrite in the cell culture supernatant corresponds to the production of NO and was measured using the Griess reagent. Briefly, 100 μl supernatant of medium was mixed with an equal volume of Griess reagent (1% sulphanilamide in 5% H3PO4 and 0.1% naphthylethylene diamine dihydrochloride). The absorbance at 540 nm was measured, and concentrations of nitrite were calculated in accordance to the standard curve obtained from sodium nitrite [16].

2.5. Measurement of TNF-α

Cells were treated by LPS (1 μg/ml) with or without drugs (10–50 μM) for 6 h. 100 μl of the culture supernatant were taken out to determine the level of TNF-α using ELISA assay kits according to the manufacturer’s instructions. The ELISA data representing mean values ± SD were obtained in duplicate from at least three independent experiments.

2.6. Western blot of GR

After the treatment with LPS (1 μg/ml) and respective drugs (0.1–100 μM) for 24 h, RAW 264.7 cells were washed with cold PBS and lysed in ice-cold lysis buffer (Beyotime, China) plus 1:100 volume of phenylmethylsulfonyl fluoride (PMSF). Cell debris was removed after centrifugation (12,000 g, 4 ºC, 10 min). After the protein concentration for each aliquot was determined by the Bradford method, suspensions were boiled in SDS-PAGE loading buffer. 30 μg of the proteins obtained were separated using 10% SDS-PAGE gels. Blots were transferred to nitrocellulose membranes and incubated in blocking buffer (5% nonfat dried milk in Tris-buffered saline with Tween 20) at room temperature for 2 h, and then incubated with rabbit polyclonal antibody against GR in diluent buffer (Beyotime, China) overnight at 4 ºC (1:1000 dilution) and anti-β-actin antibody respectively. The membranes were washed with TBS-T and incubated with HRP-conjugated secondary antibody solution for 1 h at room temperature. The blots were washed thrice in TBS-T.
and detected by using enhanced chemiluminescence reagent (ECL) and exposed to photographic films (Kodak). Images were collected and the bands corresponding to GR and \( \beta \)-actin protein were quantitated by densitometric analysis using the DigDoc100 program (Alpha Ease FC software). Data of GR were normalized on the basis of \( \beta \)-actin levels.

2.7. Evaluation of drug interactions

The interaction between Rg1 and Cort was analyzed using the Berenbaum’s method to determine whether the combination was synergistic, which is performed based on the following equation: 
\[
E(da,db) = E(da) + E(db) - E(da,db),
\]
where E is the observed effect, da and db are the doses of agents a and b. Synergism is indicated when the total effect of a combination is greater than expected from the sum of its effects [17].

Fig. 2. Rg1 and Cort dose-dependently inhibit LPS-induced NO release. (A) The effects of Cort on LPS-induced nitric oxide secretion. (B) The effects of Re on LPS-induced nitric oxide secretion. (C) The effects of Rg1 on LPS-induced nitric oxide secretion. Values are means ± SEM of three independent experiments. **p < 0.01 compared with the untreated group, *p < 0.05, **p < 0.01 compared with the LPS alone treated group.

Fig. 3. Rg1 and Cort dose-dependently inhibit LPS-induced TNF-\( \alpha \) release. (A) The effects of Cort on LPS-induced TNF-\( \alpha \) secretion in macrophages. (B) The effects of Re on LPS-induced TNF-\( \alpha \) secretion in macrophages. (C) The effects of Rg1 on LPS-induced TNF-\( \alpha \) secretion in macrophages. Values are means ± SEM of three independent experiments. **p < 0.01 compared with the untreated group, *p < 0.05, **p < 0.01 compared with the LPS alone treated group.
2.8. Statistical analysis

All results were expressed as means ± SD. Statistical comparison was conducted using Student’s t-test after ANOVA. The results were considered to be significant when $p < 0.05$.

3. Results

3.1. The effects of ginsenosides Re, Rg1 or Cort on LPS-induced inflammatory factor secretion in macrophages

RAW 264.7 cells were treated with various concentrations of Re, Rg1 or Cort for 24 h, and the cell viability was tested. Re, Rg1 or Cort did not exhibit cytotoxicity at the range of 1–100 μM against RAW 264.7 cells (data not shown). This concentration range was used for treatment of Re, Rg1 or Cort in the following experiments. The results implied that the inhibition of inflammatory factor released by Rg1 or Cort was not due to cell death.

Stimulation of cells with 1 μg/ml LPS for 24 h induced significant increase in nitrite production compared to the basal level. In these experiments, Rg1 or Cort did not exhibit concentration-dependent inhibition of nitrite release, but Re did not change the level of NO (Fig. 2).

Following LPS stimulation, Rg1 (50 μM) or Cort (25 μM) intervention for 6 h significantly reduced the secretion of TNF-α (Fig. 3) compared to a single LPS stimulation ($p < 0.05$). However, Rg1 (12.5 μM) or Cort (12.5 μM), showed no significant inhibitory effect on the release of TNF-α in LPS-activated RAW 264.7 cells. Any concentration of Re had no effect on the secretion of TNF-α.

3.2. The effects of Rg1 combined with Cort on LPS-induced inflammatory factor secretion in macrophages

Based on the above experimental results, Rg1 having anti-inflammatory effect, not Re, was selected to investigate the synergistic anti-inflammatory effects with low concentrations of glucocorticoids. Rg1 (12.5 μM) or Cort (1 μM) alone did not significantly inhibit the secretion of nitric oxide induced by LPS. However, combination of Rg1 and Cort at these concentrations significantly inhibited nitric oxide secretion (57.6%; $p < 0.05$, Table 1). These results were also confirmed for the secretion of TNF-α, which was inhibited by 24.3% (Table 2). These were again confirmed as a synergistic effect of Rg1 and Cort, and demonstrated that they can be combined to inhibit inflammation.

3.3. The effect of Rg1 combined with Cort on GR expression

The effect of Rg1 combined with Cort on the expression of GR was examined by the Western blot method. As shown in Fig. 4A, compared with untreated group, Cort significantly down-regulated the expression of GR in a dose-dependent manner. As shown in Fig. 4B, compared with untreated group, Rg1 (10 μM) or Rg1 (10 μM) with Cort (1 μM)

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>LPS Concentration (μM)</th>
<th>NO (μM)</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>–</td>
<td>2.9 ± 0.03</td>
<td>–</td>
</tr>
<tr>
<td>LPS</td>
<td>1.0</td>
<td>6.5 ± 0.19$^a$</td>
<td>–</td>
</tr>
<tr>
<td>Rg1</td>
<td>1.0</td>
<td>6.3 ± 0.38</td>
<td>6.9</td>
</tr>
<tr>
<td>Cort</td>
<td>1.0</td>
<td>6.0 ± 0.10</td>
<td>15.3</td>
</tr>
<tr>
<td>Rg1 + Cort</td>
<td>1.0</td>
<td>4.4 ± 0.15$^b$</td>
<td>57.6$^c$</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SEM of three independent experiments.

$^a p < 0.01$ compared with the untreated group.

$^b p < 0.05$ compared with the LPS alone treated group.

$^c E(da,db) > E(da) + E(db)$.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>LPS Concentration (μM)</th>
<th>TNF-α (pg/ml)</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>–</td>
<td>318.32 ± 21.21</td>
<td>–</td>
</tr>
<tr>
<td>LPS</td>
<td>1.0</td>
<td>1013.33 ± 37.71$^a$</td>
<td>–</td>
</tr>
<tr>
<td>Rg1</td>
<td>1.0</td>
<td>1086.67 ± 47.14</td>
<td>– 13.27</td>
</tr>
<tr>
<td>Cort</td>
<td>1.0</td>
<td>943.33 ± 23.57</td>
<td>7.86</td>
</tr>
<tr>
<td>Rg1 + Cort</td>
<td>1.0</td>
<td>831.67 ± 25.93$^b$</td>
<td>24.3$^c$</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SEM of three independent experiments.

$^a p < 0.01$ compared with the untreated group.

$^b p < 0.05$ compared with the LPS alone treated group.

$^c E(da,db) > E(da) + E(db)$.
The adaptogenic activity of ginseng may relate to the corticosteroid-like activity of ginsenosides. Rg1 showed the glucocorticoid-like effects, and to be glucocorticoid receptor antagonism [18].

In our study, anti-inflammatory effects of the combination of ginsenoside Rg1 with glucocorticoid (low concentration) were observed. The results showed that the co-administration of Rg1 with glucocorticoid synergistically inhibited inflammation induced by LPS in vitro. LPS is a major component of the outer membranes of most Gram-negative bacteria. When stimulated by LPS, macrophages release NO, prostaglandinE2, TNF-α, IL-1, IL-6 and other proinflammatory cytokines [19].

In this study, NO and TNF-α levels in LPS-treated group significantly increased. Cort or Rg1 markedly suppressed the elevation of NO and TNF-α levels, but Re did not, which may be related to the substituents. Additionally, Cort significantly down-regulated the expression of GR at high concentration. In order to investigate how to avoid the severe side effects of high dose of Cort, the combination of low concentrations of Rg1 and Cort was applied in this experiment.

Based on the experimental results, we chose the low concentrations of Rg1 or Cort which did not affect the levels of NO and TNF-α, Rg1 (50 μM) or Cort (1 μM) (n = 3). *p < 0.05, **p < 0.01, compared with the untreated group.

The adaptogenic activity of ginseng may relate to the corticosteroid-like activity of ginsenosides. Rg1 showed the glucocorticoid-like effects, and to be glucocorticoid receptor down the development of drug resistance, decreasing the dosage but increasing or maintaining the same efficacy to avoid toxicity, and providing selective synergism against target (or efficacy synergism) versus host (or toxicity antagonism) [18].
agonist [36]. It is well-known that glucocorticoid, for example corticosterone, is a strong agonist for receptor. It is most possible that the ginsenosides exhibit partial agonist, as an antagonist to attenuate the effect of glucocorticoid when people have normal or high concentrations of glucocorticoid, but as an agonist or up-regulating the expression of GR to promote the effect of glucocorticoid when the body tissue becomes resistant to treatment by the development of fewer GR or with low affinity receptors [37–40]. These results provide the evidences for ginseng to be used to both normal people and patients.

The results suggested that ginsenoside Rg1 and glucocorticoid have synergistic anti-inflammatory effect. Glucocorticoid, including corticosterone, could down-regulate the GR [14]. The possible mechanism on synergistic anti-inflammatory effect of Rg1 and glucocorticoid is related to the following aspects: on one hand, Rg1 as partial agonist attenuates the down-regulating effect of corticosterone on GR, on the other hand, Rg1 itself up-regulates the expression of GR, with ginsenoside Rg1, combining the growing GR to achieve the synergistic anti-inflammation. The combination of Rg1 with glucocorticoids provides a beneficial method in the treatment of inflammatory diseases and the detailed mechanism required further study.

Conflict of interest
The authors have no conflict of interest.

Disclosure statement
The authors have nothing to disclose.

Acknowledgments
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References
[6] Ling C, Li Y, Zhu X, Zhang C, Li M. Ginsenosides may reverse the inflammatory effect of Rg1 and glucocorticoid is related to GR or with low affinity receptors [37–40].


