Metabolism of ginsenoside Rg1 by intestinal bacteria II. Immunological activity of ginsenoside Rg1 and Rh1

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KEY WORDS ginseng; saponins; cytokines; radioimmunoassay; reverse transcriptase polymerase chain reaction

INTRODUCTION
Ginsenoside Rg1 was converted into Rh1 and 20 (S) propanoanatriol (Ppt) by intestinal bacteria, and Rh1 was detected in serum and urine of both rat and human after oral administration of Rg1. The pharmacological activity of Rg1 possesses immune modulation and anti-aging activities. In order to reveal the genuine active component on oral administration of Rg1, comparison between Rg1 and Rh1 was carried out regarding the immunopharmacological aspects.

MATERIALS AND METHODS

Drugs Ginsenoside Rg1, ginsenoside Rh1 were purchased from Department of Natural Medical Chemistry of Noman Bethune University of Medical Sciences, China.

Cell lines Human myelomonocytic leukemic cell line, THP-1 (Monocyte, human, TIB202, ACTT, USA). Human peripheral blood mononuclear cells (PBMC).

Reagents Human recombinant cytokines (IL-1α, TNFα, IL-8) were kind gifts from Tsukuba Research Institute, Banyu Pharmaceutical Company (Tsukuba, Japan); All the rabbit polyclonal antibodies for above cytokines were prepared in our laboratory; 125I was purchased from China Institute of Atomic Energy; new born calf serum (NCS) was purchased from Dalian Biological Reagents Factory (Dalian, China); lipopolysaccharides (LPS, E coli serotype 0111: B4) and phorbol myristate acetate (PMA) were purchased from Sigma (USA). RPMI-1640 medium, trypsin blue and TRIZOL reagent were products of Gibco (NY USA); Primers for TNFα and β-actin cDNA amplification were purchased from Life Tech Oriental Company, Japan; diethyl pyrocarbonate

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(DEPC), Taq DNA polymerase and RNase were purchased from Sigma. RPMI-1640 medium contained 10 % heat-inactivated newborn bovine serum; 1 % L-glutamine 2 mmol/L; 1 % HEPES 10 mmol/L, penicillin 100 kU/L, and streptomycin 100 μg/L (pH 7.4).

**RIA buffer** Contained phosphate buffer 500 mL 0.5 mol/L 10 mL, BSA 10 % 12.5 mL, sodium azide 2 % 1.5 mL, NaCl 0.15 mol/L 465 mL.

**PEG** PEG (polyethylene glycol, MW:8000) solution contained PEG 6 % and goat anti-rabbit IgG 2 % in 10 mmol/L phosphate buffer.

**THP-1 cell culture and determination of optimal cell density and concentration of LPS and PMA** The cells were counted by hemocytometer and adjusted to cell densities ranging from 0.08 x 10^6 to 1.25 x 10^6 cells/L in RPMI-1640 medium containing 10 % NCS, then seeded in 96-well culture plates (180 μL/well), different doses of LPS (10 μg/mL) and various concentrations of PMA (10 μg/mL) were added into the THP-1 cell culture and then incubated at 37 °C, 4 % CO₂, for 20 h. Production of IL-1α, TNFα, and IL-8 was quantitated using RIA. It was proved that the production of these three cytokines was maximal at 0.32 x 10^6 cells/L, therefore, this cell density was used in this study. The highest amount of IL-1α and TNFα were produced by LPS 10 mg/L and PMA 200 or 400 nmol/L, while IL-8 production reached maximal stage in the presence of LPS 10 mg/L and PMA 200 nmol/L.

**Separation and incubation of human PBMC**

1. **Separation** Heparin 0.1 mL was taken into a 50 mL injection syringe. The blood was obtained from three healthy adult volunteers, 40 mL/person. PBMC were separated by Ficoll-Hypaque density gradient solution, then washed three times with 0.9 % sodium chloride.

2. **Incubation** PBMC were suspended in RPMI-1640 medium containing human AB serum 2 % and adjusted to 5 x 10^6 cells/L, then seeded into 96-well culture plate (90 μL/well). The cell suspensions were disturbed and non-adherent cells were discarded 1 h after incubation. The adherent cells were washed with saline 3 times, and RPMI-1640 medium containing human AB serum 2 % and ginsenoside samples (Rg1 and Rh1, 10 μg/mL) were added, followed by a 40-h incubation.

**Measurement of cell number** The adherent cells were detached by pipetting, stained by trypan blue, then counted under microscopic, magnified 100 times.

**Effect of Rg1 and Rh1 on secretion of cytokines from THP-1 cells** THP-1 cells were treated with different doses of Rg1 and Rh1 (10 μg/mL). After incubation for 1 h, LPS 10 or 100 mg/L and PMA 200 nmol/L were added to the cell culture, then the cell culture was continued for 20 h. The cells were collected into Eppendorf tubes after lysis by three freeze and thaw cycles, centrifuged at 1 200 x g for 10 min, then the concentration of IL-1α, IL-8, and TNFα were quantitated RIA.

**Cytokine quantitation by RIA** Cytokine quantitation was performed according to previous reports.

**Reverse transcription polymerase chain reaction (RT-PCR)** THP-1 cell suspensions (0.32 x 10^6 cells/L) were added to the 6-well culture plate (6 mL/well) and cultured as previously described. The cells were collected after incubation for 0, 1, 3, 5, and 8 h, centrifuged at 500 x g for 10 min, then the supernatants were discarded. Total RNA was extracted by using TRIZOL (1 mL/tube). According to our previous results, the optimal incubation time for the highest production of TNFα mRNA was observed at 3 h. Therefore, various doses of Rg1 and Rh1 were added and the cells were cultured for 3 h to determine the effects of the compounds on expression of mRNA for TNFα by RT-PCR method.

1. **Primers for TNFα** TNFα primers were synthesized as below:

   Primer 1: 5' ATGAGCCTGAAAGCATGATC
   Primer 2: 3' TCCAGGGCAATGATCCCAAGT-AGACCTGCC

2. **Reaction conditions:**

   24 cycles: 94 °C 45 s, 60 °C 45 s, 72°C 90 s
   30 cycles: 94 °C 45 s, 60 °C 45 s, 72°C 90 s

**RESULTS**

**Effect of Rg1 and Rh1 on proliferation of human PBMC** Rg1 and Rh1 had no obvious stimulatory or inhibitory effect on proliferation of human PBMC from three volunteers (data not shown).

**Effect of Rg1 and Rh1 on the production of cytokines from THP-1 cell** Under the stimulation of LPS 10 or 100 mg/L and PMA 200 nmol/L, Rg1 had no obvious effect on production of TNFα, on the other hand, Rh1 enhanced the production of TNFα stimulated by LPS 100 mg/L and PMA 200 nmol/L in a dose-dependent manner. However, Rh1 1 mg/L induced 6.5 times higher production of TNFα than control in the presence of LPS 10 mg/L and PMA 200 nmol/L (Tab 1).
Tab 1. Effect of Rh4 and Rh1 on TNFα production by THP-1 cells. n = 4 wells. x ± s. ①P < 0.01 vs control. ②P < 0.01 vs the same concentration of Rh4.

<table>
<thead>
<tr>
<th>TNFα production (ng/L)</th>
<th>+ LPS 100 (mg/L)</th>
<th>+ LPS 10 (mg/L)</th>
<th>Absence of stimulant</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ PMA 200 (nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>107 ± 58</td>
<td>17 ± 4</td>
<td>0.29 ± 0.13</td>
</tr>
<tr>
<td>Rh4 100 (mg/L)</td>
<td>30 ± 5①</td>
<td>7.6 ± 1.7</td>
<td>0.52 ± 0.21</td>
</tr>
<tr>
<td>10</td>
<td>26 ± 15①</td>
<td>7 ± 11</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>1</td>
<td>10 ± 8①</td>
<td>11 ± 7</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Rh1 100 (mg/L)</td>
<td>200 ± 21②</td>
<td>19 ± 4</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>10</td>
<td>151 ± 10③</td>
<td>23 ± 19</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>1</td>
<td>140 ± 55</td>
<td>110 ± 39④</td>
<td>8.5 ± 1.7</td>
</tr>
</tbody>
</table>

Rg1 and Rh1 had no obvious effect on the production of IL-1α at higher doses of LPS 100 mg/L and PMA 200 nmol/L, but Rh1 1 mg/L and Rh1 100 mg/L increased the production of IL-1α significantly at lower doses of LPS 10 mg/L and PMA 200 nmol/L (Tab 2).

Tab 2. Effect of Rh4 and Rh1 on IL-1α production by THP-1 cells. n = 4 wells. x ± s. ①P < 0.01 vs control. ②P < 0.01 vs the same concentration of Rh4.

<table>
<thead>
<tr>
<th>IL-1α production (ng/L)</th>
<th>+ LPS 100 (mg/L)</th>
<th>+ LPS 10 (mg/L)</th>
<th>Absence of stimulant</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ PMA 200 (nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.6 ± 1.2</td>
<td>2.3 ± 0.5</td>
<td>0.26 ± 0.14</td>
</tr>
<tr>
<td>Rh4 100 (mg/L)</td>
<td>3.3 ± 0.12</td>
<td>1.4 ± 0.3</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>6 ± 3</td>
<td>2.1 ± 0.5</td>
<td>0.18 ± 0.017</td>
</tr>
<tr>
<td>1</td>
<td>2.9 ± 0.5</td>
<td>8.3 ± 8③</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>Rh1 100 (mg/L)</td>
<td>2.5 ± 0.7</td>
<td>8.5 ± 2.0④</td>
<td>0.21 ± 0.08</td>
</tr>
<tr>
<td>10</td>
<td>3.3 ± 1.6</td>
<td>4.6 ± 2.5</td>
<td>0.4 ± 0.03</td>
</tr>
<tr>
<td>1</td>
<td>4.8 ± 2.6</td>
<td>2.5 ± 1.5</td>
<td>0.4 ± 0.4</td>
</tr>
</tbody>
</table>

Rh1 1 mg/L induced remarkably higher production of IL-8 than the control at LPS 10 mg/L and PMA 200 nmol/L (Tab 3).

Effect of Rh4 and Rh1 on the mRNA expression for TNFα by THP-1 cells. Under the coherence of β-actin, effects of Rh4 and Rh1 on the production of TNFα mRNA produced by THP-1 cells were compared. After incubation for 0, 1, 3, 5, and 8 h, the highest production of TNFα mRNA was detected at 3 h. According to the result of RIA, the effects of Rh4 and Rh1 on the TNFα mRNA expression were compared, and several groups which had obvious influence on cytokine production from control were selected. Two concentrations of Rh4 (100 and 1 mg/L) and Rh1 (100 mg/L) were selected under the stimulation of LPS (100 mg/L) plus PMA (200 nmol/L). Rh1 (1 mg/L) was also selected under the stimulation of LPS (10 mg/L) plus PMA (200 nmol/L) (Fig 1).

At 24 cycles of RT-PCR, there was no obvious DNA band on lane C or lane H, and on lane I a light DNA band appeared. At 30 cycles, DNA bands appeared in lane C and lane H, in lane I, cDNA amplification had reached a plateau stage. Therefore, we could compare the effect of Rh4 and Rh1 on original amount of cDNA at 24 cycles, where original mRNA amount for the cytokine was reflected.

According to the results of RIA, when the production of TNFα protein was inhibited by Rh4 (Tab 1 LPS 100 mg/L), the production of TNFα mRNA (Fig 1 Lane A, B) was almost the same as that of control (Lane J). Rh1 100 mg/L improved the production of TNFα protein under the stimulation of LPS 100 mg/L (Tab 1), but the amount of mRNA was reduced (Lane C). Rh1 1 mg/L increased both TNFα protein production and its mRNA expression (Lane D).

From results mentioned above, we could find that there was no direct relation between mRNA amount and protein production at higher concentration of LPS 100 mg/L. On the other hand, there was a close relationship at low dose of LPS 10 mg/L. The ginsenoside sample control did not induce obvious expression of mRNA (lane E-H).
Fig 1. Effect of Rg₁ and Rh₁ on TNFα mRNA expression of THP-1 cells by semi-quantitative RT-PCR.
Lane A: Rg₁ 100 μg/L + LPS 100 μg/L + PMA 200 nmol/L. Lane B: Rg₁ 1 mg/L + LPS 100 μg/L + PMA 200 nmol/L. Lane C: Rh₁ 100 μg/L + LPS 100 μg/L + PMA 200 nmol/L. Lane D: Rh₁ 1 mg/L + LPS 10 μg/L + PMA 200 nmol/L. Lane E: Rg₁ 1 mg/L. Lane F: Rh₁ 1 μg/L. Lane G: Rh₁ 100 μg/L. Lane H: Rh₁ 1 mg/L. Lane I: LPS 10 μg/L + PMA 200 nmol/L. Lane J: LPS 100 μg/L + PMA 200 nmol/L. Lane K: cell control

DISCUSSION

It has been reported that Rg₁ can enhance the immune function and anti-aging activity[2-4]. Proved by our previous study, Rg₁ was metabolized by rat and human intestinal bacteria. Among the metabolites, Rh₁ was detected in the blood and urine of humans[1].

THP-1 cell line has a kind of human myelomonocytic leukemia cell origin and it has the similar reaction as the human macrophage to a variety of stimulation[5,6]. Therefore, we observed the effect of ginsenosides on cytokine production by THP-1 cells. It was found that Rg₁ and Rh₁ had no toxic effect on PBMC culture at concentrations of 0.1, 1, 10, or 100 μg/mL.

It has been reported that some drugs, especially traditional Chinese medicine (TCM), have bi-directional regulation on cytokine production. Low dose of TCM can enhance the release of some cytokines, while at higher dose they reduce the production.

Protein kinase C (PKC) and P38 protein kinase, mitogen-activated protein kinases (MAPK), play a pivotal role in a variety of cellular signal transduction pathways, especially, of induction of proinflammatory (TNFα and IL-1) cytokine production. The mechanism of the bi-directional regulation of Rh₁ on the production of the proinflammatory cytokines might be attributed to the activities of these two kinases[10-12]. The relationship between them remains to be elucidated.

In this study, it was proved that Rh₁ 1 mg/L could stimulate THP-1 cell to produce TNFα on the stimulation with low dose LPS. However, Rh₁ at high concentration 100 μg/L could inhibit the proliferation of three tumor cell lines (data not shown). Therefore, whether there is a direct relationship between TNF production and reduced tumor cell proliferation waits further studies. According to our results, Rh₁ 1 mg/L can increase the production of IL-8 induced by LPS 10 μg/L. However, Rg₁ 1, 10, and 100 μg/mL and Rh₁ 10, 100 μg/mL have no significant effect on the production of IL-8.

The results revealed that the prodrug Rg₁ and metabolite Rh₁ absorbed into serum showed different immuno-pharmacological activities in vitro, attributed to their different chemical structures. Rh₁ at suitable concentration can induce production of some proinflammatory cytokines on stimulation while Rg₁ shows no significant effect. Whether the results can be consistent with in vivo research still need to be further studied.
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