Induction of Nitric Oxide Synthase by Saponins of Heat-Processed Ginseng

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Total saponin of heat-processed ginseng (TSHG) stimulated the production of nitric oxide (NO) in interferon-γ (IFN-γ)-primed macrophages through the increased expression of inducible nitric oxide synthase (iNOS). However, TSHG by itself had a very weak effect on the NO synthesis without IFN-γ priming. The saponins of white ginseng inhibited the NO production in lipopolysaccharide (LPS)/IFN-γ activated macrophages rather than the stimulation of NO production found in IFN-γ primed macrophages. The NO production by TSHG-stimulated macrophages was inhibited by the NOS inhibitor (N⁵-monomethyl-L-arginine (L-NMMA)) and nuclear factor-kappaB inhibitor (pyrrolidine dithiocarbamate (PDTC)). TSHG showed different serum-dependence from LPS on the activation of IFN-γ primed macrophages. This property of TSHG may explain the intensified anti-tumor properties of heat-processed ginseng through its immunostimulating activity.

Key words: ginseng; heat processing; nitric oxide synthase; macrophage; saponin

Panax ginseng, commonly referred to “Korean ginseng”, has been used for thousands of years as a herbal medicine. Many pharmacological studies have been conducted to investigate the biological applications of ginseng. The biologically active components of ginseng are saponins which have diverse beneficial effects including anti-inflammatory, anti-oxidant, anti-tumor, anti-allergic, and anti-ulcer activity.¹–³ Heat-processed ginseng has recently been prepared in order to intensify the biological activities of ginseng which can mimic red ginseng.⁴ We have reported several new ginsenosides that can be produced by the heat-processing of ginseng.⁵ The promoted biological activities are endothelium-dependent relaxation,⁴ anti-oxidant and anti-tumor activity.⁵ In order to verify the possible mechanism for the intensified anti-tumor activity of the total saponin of heat-processed ginseng (TSHG), we evaluated the effect of TSHG on the nitric oxide (NO) production by macrophages that may mediate the immunostimulating property of TSHG. NO, an intracellular mediator, is synthesized by oxidation of the terminal guanidine nitrogen of L-arginine, and this reaction is catalyzed by three types of nitric oxide synthase (NOS) enzyme. The constitutive NOS (cNOS) found in neuronal tissues (type I) and in vascular endothelia (type III) is Ca²⁺-dependent and releases the small amount of NO required for physiological functions,⁷ whereas inducible NOS (iNOS, type II) can be induced by several stimuli and leads to a micromolar level of NO.⁸ NO produced by activated macrophages serves as an important signaling molecule in the immune system and may exert anti-viral and anti-tumor activity.⁹

There are many plant-derived constituents that can affect the NO signaling pathway.¹⁰ As an inducer of NO synthesis from macrophages, there have been many reports of polysaccharides that mimic LPS and terpenic acids such as glycyrrhetinic acid and ursolic acid.¹¹,¹² Ginseng polysaccharide has also shown NO-inducing activity through the activation of nuclear factor kappaB (NF-κB) in macrophages.¹³ As the ginseng saponin has a steroidal skeleton in its structure like ursolic acid, we attempted to identify the NO-inducing activity of the saponin of heat-processed ginseng which could explain the intensified anti-tumor activity.

Materials and Methods

Reagents and materials. Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Gibco Laboratories (Detroit, MI, U.S.A.). LPS (Escherichia coli, 0127: B8), bovine serum albumin, sodium nitrite, N-(1-naphthyl) ethylenediamine, N⁵-monomethyl-L-arginine (l-NMMA), and pyrrolidine dithiocarbamate (PDTC) were all obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). A polyvinylidene fluoride (PVDF) membrane was purchased from Bio Rad Laboratories (Hercules, CA, U.S.A.), and the anti-mouse iNOS polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY, U.S.A.).

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Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; TSHG, total saponin of heat-processed ginseng; TSWG, total saponin of white ginseng; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappaB; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; LBP, LPS binding protein; l-NMMA, N⁵-monomethyl-L-arginine; PDTC, pyrrolidine dithiocarbamate; PVDF, polyvinylidene fluoride
Preparation of saponin from white and heat-processed ginseng. Heat-processed ginseng was prepared from six-year-old fresh ginseng by steaming at 120°C for 3 hours. Fresh ginseng and heat-processed ginseng were extracted by methanol under reflux three times for 2 hours. The methanol extracts of ginseng were partitioned between ethyl ether and water to remove the lipid-soluble fraction. The water layer was further partitioned sequentially with EtOAc and BuOH with increasing polarity. The BuOH layer (total saponin of white ginseng, TSWG and total saponin of heat-processed ginseng, TSHG) was dissolved in dimethyl sulfoxide (DMSO) and applied to cells after appropriate dilution with a cell culture medium. The final concentration of DMSO in the culture medium was 0.1%, and this concentration did not have any effect on the assay system.

Cell culture. The murine macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD, U.S.A.) was cultured in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/ml) and streptomycin (10 μg/ml). The RAW 264.7 cells were seeded at 2 × 10^5 cells/well in 24-well plates and treated with a sample. The supernatant was collected as the source of secreted NO.

Nitrite assay. NO released from macrophages was assessed by determining the NO_2^- concentration in the culture supernatant. A sample (100 μl) of the culture medium was incubated with 150 μl of the Griess reagent (1% sulfanilamide and 0.1% naphthylethylene diamine in a 2.5% phosphoric acid solution) at room temperature for 10 min in a 96-well microplate. The absorbance at 540 nm was read with an ELISA plate reader, and standard calibration curves were prepared by using sodium nitrite as the standard.

Western blot analysis of iNOS. The cells were rinsed with phosphate-buffered saline and lysed by boiling with a lysis buffer (1% SDS, 10 mM Tris at pH 7.4) for 5 min. Thirty μg of protein from the cell lysate was applied to 8% SDS–polyacrylamide gel and transferred to a PVDF membrane by the standard method. The membrane was probed with the polyclonal antibody for anti-mouse iNOS. The Western blot was visualized by using an enhanced chemiluminescence (ECL) detection kit (Amersham Bioscience, Piscataway, NJ, U.S.A.) according to the manufacturer’s instructions.

Statistics. Each result is expressed as the mean ± S.D. of three experiment. A statistical analysis was performed by Student’s t-test, a P value of <0.05 being considered to indicate a significant difference.

Results and Discussion

Macrophages can be converted from the resting to activated state by the priming signal of IFN-γ produced by T cells during an infection. This process mediates the production of pro-inflammatory cytokines and NO that exert anti-microbial and tumoricidal activity. To fully activate macrophages, IFN-γ priming and subsequent triggering by LPS or tumor necrosis factor-α (TNF-α) is required. Our preliminary experiment for macrophage activation enabled us to optimize the concentration of IFN-γ and LPS as 30 U/ml and 0.1 μg/ml, respectively. The incubation time for the maximal production of NO was 18 h (data not shown). Treatment by TSHG substantially increased the NO synthesis from IFN-γ primed macrophages (Fig. 1). The concentration of NO in the culture medium was 14.5 ± 0.12 μM at 100 μg/ml of TSHG. Without IFN-γ priming, the induced activity of TSHG was very weak (3.70 ± 0.65 μM). The ether-soluble and EtOAc-soluble fractions obtained from the methanol extract of heat-processed ginseng both showed a marginal effect on NO synthesis. This means that the induction of NO synthesis was mainly influenced by saponins that could be extracted from the methanol extract into the BuOH layer. The stimulation of NO synthesis by TSHG was dose dependent from 10 to 100 μg/ml as shown in Fig. 2(A). The level of iNOS expression was also increased by TSHG as determined by an immunoblotting analysis of the lysate of TSHG-induced macrophages (Fig. 2(B)).
As shown in Fig. 3, the saponins of heat-processed ginseng and white ginseng had an adverse effect on the NO synthesis in IFN-γ primed macrophages. A co-treatment by TSHG with LPS (0.1 μg/ml) slightly increased the level of NO (42.1 ± 1.7 μM) compared with the LPS control (38.2 ± 2.2 μM), although not significantly. The total saponin of white ginseng (TSWG, 100 μg/ml) did not stimulate NO synthesis by IFN-γ-primed macrophages. When LPS and TSWG were used together, the level of NO was less than that of the LPS control. This means that TSWG may have contained inhibitors of NO synthesis by IFN-γ/LPS-activated macrophages, while TSHG contained a stimulator. Ginseng saponins have been reported to regulate the NO production in different cell culture systems. Ginsenoside Rg1 reduced the dopamine-induced NO synthesis in pheochromocytoma (PC12) cells,15) and 20(S) protopanaxadiol inhibited the iNOS level in LPS-activated macrophages.16) Ginsenoside Rd completely inhibited NO production in TNF-α induced C6 glioma cells.17) Our results interestingly showed that TSWG inhibited the overproduction of NO by activated macrophages, while TSHG increased the production of NO when treating IFN-γ-primed macrophages. This difference might have been the result of structural modification of the saponins by the heat treatment of ginseng. Ginseng saponin generally underwent hydrolysis and dehydration by heating. The reported saponins found exclusively in heat-processed ginseng are ginsenosides F4, Rg5, Rg6, Rk1, Rk2, Rk3, Rs3, Rs4 and Rs5.4,5,18) These modified ginsenosides might have stimulated NO synthesis that indicated the anti-tumor activity of ginseng.6,19)

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PDTC is an NF-κB inhibitor that can suppress the expression of iNOS. The production of NO by TSHG-treated macrophages was inhibited by L-NMMA and PDTC in a dose-dependent manner as shown in Table 1. These results suggest that TSHG might induce the NO production in macrophages by activating the NF-κB pathway. The precise mechanism for iNOS induction by TSHG remains to be elucidated, and further study is also needed to identify the structure of the active principles of TSHG and their biological application.

It is well known that serum and LPS-binding protein (LBP) enhance the LPS-stimulated activation of macrophages. Serum provides LBP, and the LPS–LBP complex facilitates the recognition of LPS by the LPS receptor on a cell surface such as CD14.20 As shown in Fig. 4, LPS and TSHG showed different serum dependence on NO induction by macrophages. The production of NO by LPS/IFN-γ-activated macrophages was decreased by removing fetal bovine serum from the culture medium, while the production of NO by TSHG-induced macrophages was increased by removing the serum. TSHG might have partially different mechanism from that of LPS for activating macrophages. One possibility is that TSHG can bind directly to the receptor on the cell surface for activation without the aid of serum.

There are several reports dealing with the immunostimulating effects of ginseng13 and the intensified anti-tumor activity through the heat treatment of ginseng.6,19 The induction activity of NO may explain these biological activities of ginseng through immunostimulation by TSHG. There is substantial evidence that the induction of NO synthesis provides the therapeutic possibility is that TSHG can bind directly to the receptor on a cell surface such as CD14.20 As shown in Fig. 4, LPS and TSHG showed different serum dependence on NO induction by macrophages. The production of NO by LPS/IFN-γ-activated macrophages was decreased by removing fetal bovine serum from the culture medium, while the production of NO by TSHG-induced macrophages was increased by removing the serum. TSHG might have partially different mechanism from that of LPS for activating macrophages. One possibility is that TSHG can bind directly to the receptor on the cell surface for activation without the aid of serum.

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Table 1. Effects of L-NMMA and PDTC on IFN-γ- and TSHG-Induced NO Production by Macrophages

<table>
<thead>
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<th>L-NMMA or PDTC (μM)</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-NMMA</td>
<td>17.4 ± 0.8</td>
<td>15.8 ± 0.7</td>
<td>8.4 ± 0.6*</td>
<td>6.0 ± 0.2*</td>
</tr>
<tr>
<td>PDTC</td>
<td>17.4 ± 0.8</td>
<td>15.5 ± 0.8</td>
<td>14.6 ± 0.4*</td>
<td>3.5 ± 0.3*</td>
</tr>
<tr>
<td>control</td>
<td>17.4 ± 0.8</td>
<td>15.5 ± 0.8</td>
<td>14.6 ± 0.4*</td>
<td>3.5 ± 0.3*</td>
</tr>
</tbody>
</table>

RAW 264.7 cells (2 × 10⁵/well) were stimulated for 18 h with IFN-γ (30 U/ml) and TSHG (100 μg/ml) with various concentrations of L-NMMA or PDTC. NO released into the cell culture medium was measured by the Griess reagent in the nitrite form. Each data value is presented as the mean ± SD of three independent treatments. Values with an asterisk represent a significant (p < 0.05) decrease of NO production compared with the control.

Fig. 4. Effects of Serum on the NO Synthesis by LPS- or TSHG-Induced Macrophages with or without IFN-γ Priming.

RAW 264.7 cells (2 × 10⁵/well) were treated for 18 h with a combination of LPS/IFN-γ and TSHG/IFN-γ with or without 10% serum in the culture medium (LPS, 10 ng/ml; TSHG, 100 μg/ml; IFN-γ, 30 U/ml). NO released into the cell culture medium was measured by the Griess reagent in the nitrite form. Each data value is presented as the mean ± SD of three independent treatments. Values with an asterisk represent significant (p < 0.05) difference in NO production compared with the 10% serum condition within each treatment.

expressing cells to tumor sites.24 The antitumor effect of NO has also been elucidated by the enhanced expression of iNOS resulting from immunization with IFN-γ-producing glioma cells.25

In conclusion, our results demonstrate that total saponin of heat-processed ginseng stimulated the production of NO in IFN-γ primed macrophages with the increased expression of iNOS protein. The production of NO by TSHG-induced macrophages was inhibited by a NOS inhibitor (L-NMMA) and NF-κB inhibitor (PDTC). The mechanism for macrophage activation by TSHG was different from that for LPS with respect to the serum dependence. The NO-inducing property of TSHG might explain the intensified anti-tumor activity of heat-processed ginseng that contains specific constituents different from those in white ginseng. Studies to identify the active principle of TSHG are in progress. The inducer of macrophage activation from TSHG would be an useful therapeutic agent for tumors.

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References

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