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Stimulation of nitric oxide synthesis by the aqueous extract of *Panax ginseng* root in RAW 264.7 cells

*,[1]Roswitha Friedl, [1]Thomas Moeslinger, [2]Brigitte Kopp & [1]Paul Gerhard Spieckermann

[1]Institute for Physiology, Schwarzspanierstrasse 17, 1090 Vienna, Austria and [2]Institute of Pharmacognosy, Center of Pharmacy, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

1 In this study, we investigated the effect of *Panax ginseng* root aqueous extracts upon inducible nitric oxide synthesis in RAW 264.7 cells. *Panax ginseng* root extract has been used in the Asian world for centuries as a traditional herb to enhance physical strength and resistance and is becoming more and more popular in Europe and North America.

2 Incubation of murine macrophages (RAW 264.7 cells) with increasing amounts of aqueous extracts of *Panax ginseng* ($0.05-0.8\ \mu g\ \mu l^{-1}$) showed a dose dependent stimulation of inducible nitric oxide synthesis.

3 Polysaccharides isolated from *Panax ginseng* showed strong stimulation of inducible nitric oxide synthesis, whereas a triterpene-enriched fraction from an aqueous extract of *Panax ginseng* did not show any stimulation.

4 Inducible nitric oxide synthase protein expression was enhanced in a dose dependent manner as revealed by immunoblotting when cells were incubated with increasing amounts of *Panax ginseng* extract. This was associated with an incline in inducible nitric oxide synthase mRNA-levels as determined by semiquantitative polymerase chain reaction and electromobility shift assay studies indicated enhanced nuclear factor-κB DNA binding activity.

5 As nitric oxide plays an important role in immune function, *Panax ginseng* treatment could modulate several aspects of host defense mechanisms due to stimulation of the inducible nitric oxide synthase.

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Keywords: Inducible nitric oxide synthase; *Panax ginseng*; ginsenosides; food additive; herbal medicine; cell culture

Abbreviations: AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; CTAB, cetyltrimethylammonium bromide; DIG, digoxigenin; DMEM, Dulbecco's Modified Eagle's Medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FCS, foetal calf serum; iNOS, inducible nitric oxide synthase; LAK, lymphokine-activated killer; LPS, lipopolysaccharide; MMLV-RT, murine leukaemia virus reverse transcriptase; NBT, nitro blue tetrazolium reagent; NF-κB, nuclear factor κB; NK, natural killer; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate buffered saline; Th1, T helper cells type 1; RT–PCR, reverse transcription- polymerase chain reaction

Introduction

In the Asian world, *Panax ginseng* (Korean Ginseng) root has been used for centuries as a traditional herb to enhance physical strength and resistance. As the use of traditional Chinese herbs as a food supply becomes more and more popular in the western world, sales of *Panax ginseng* are increasing in North America as well as in Europe. Several companies offering *Panax ginseng* extracts claim that their preparation exerts 'tonic effects'.

Major active components in *Panax ginseng* are the ginsenosides, a group of saponins with triterpenoid dammarane structure. A preparation of *Panax ginseng* root is characterized by the type and amount of ginsenosides. In *Panax ginseng*, the most abundant ginsenoside is the ginsenoside Rg1. It is assumed that the ginsenosides partly account for ginseng's medicinal effects, yet the mechanisms of ginseng actions remain unclear. Various studies have been published claiming effects on the central nervous system (memory, learning, and behaviour), neuroendocrine function, carbohydrate and lipid metabolism, immune function, and the cardiovascular system (reviewed in Gillis, 1997) and some of these effects point to a participation of NO (nitric oxide) (Kim *et al*., 1992; Han & Kim, 1996).

NO is synthesized from L-arginine by the L-arginine-nitric oxide pathway (Palmer *et al*., 1988) and is converted to nitrite and nitrate in oxygenated solutions (Marletta *et al*., 1988). A family of enzymes, termed the nitric oxide synthases (NOS), catalyze the formation of NO and citrulline from L-arginine, O_2, and NADPH (Marletta, 1993). The constitutive NOS isoforms (NOS-1 and NOS-3) produce low levels of NO as a consequence of increased intracellular Ca^{2+} (Nathan & Xie, 1994). By contrast, the inducible isoform of NOS (NOS-2 or iNOS; EC 1.14.13.39) generates large amounts of NO upon stimulation over a prolonged period of time through a Ca^{2+} independent pathway (Xie *et al*., 1992). Inducible NOS expression has been observed in many cells, including murine macrophages (Hibbs *et al*., 1988), endothelial (Gross *et al*., 1991), smooth muscle cells (Beasley *et al*., 1991), and cardiac myocytes (Schulz *et al*., 1992).

Human iNOS is most readily observed in monocytes or macrophages from patients with infectious or inflammatory

*Author for correspondence; E-mail: roswitha.friedl@univie.ac.at

diseases (MacMicking *et al*., 1997). Kröncke *et al*. (1998) concluded that in many human diseases with predominant proinflammatory T helper (Th)1 reactions, such as airway inflammations, activated macrophages that are capable of high-output NO-production, are involved. In these diseases, NO serves as a protective agent limiting bacterial invasion or down-regulating local inflammatory reactions by suppression of T cell proliferation. It has been shown previously that the nasal airways are a site of enhanced NO production as iNOS has been found to be continuously induced. Therefore, NO is highly likely to play an important role for pathogen defence at these sites (Guo *et al*., 1995).

The aim of this study was to investigate the effect of aqueous extracts from commercially available root powder of *Panax ginseng* on the iNOS of murine macrophage-like cell line RAW 264.7 by means of nitrite level measurement as well as several analyses exploring ginseng-modulated iNOS expression. Our data should serve as a contribution to the discussion about the molecular mechanism of *Panax ginseng* action.

Methods

Materials

Reagents Panax ginseng root powder was obtained by Nature's Way (Springville, UT, U.S.A.) and claimed a ginsenoside content of 2%. An aqueous extract of ginseng was prepared by dispersing 20 mg ginseng root powder in 1 ml PBS and incubating the suspension for 1 h at 60°C in a water-bath. The soluble fraction of the extract was filtered through a 0.45 μM filter (Sartorius; Goettingen, Germany) to remove solid particles. A fractionation of this solution was done by n-butanol extraction. The obtained n-butanol solution was evaporated and the remaining water phase was lyophilized and reconstituted in PBS for experiments. The triterpene content of each fraction was determined by thin layer chromatography and comparison to a standardized extract of known composition. Concentrations are expressed as micrograms of the original ginseng root powder per μl solution. Rabbit anti-iNOS polyclonal antibody and iNOS protein was supplied by Calbiochem, San Diego, CA, U.S.A. Rabbit anti-p65-antibody was obtained by Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Mouse anti-rabbit antibody, alkaline phosphatase (AP) -conjugated, was obtained from Promega, Madison, Wisconsin (U.S.A.). Cell culture materials and all other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Polysaccharide isolation (Tomoda et al*., 1993)*

Six hundred mg ginseng root powder was extracted in 6 ml of hot water The aqueous fraction was separated from non-soluble compounds by centrifugation. To 6 ml supernatant, 66 μl 1% sodium sulphate and 990 μl 5% cetyltrimethylammonium bromide (CTAB) was added. After centrifugation, two volumes of ethanol were added to the supernatant. The obtained pellet was washed once with 80% ethanol, dried and dissolved in 6 ml water. For experiments, this solution was dissolved in PBS 1 : 5 corresponding to a polysaccharide content derived from 20 mg ginseng root powder per ml solution. Results are expressed as micrograms of the original ginseng root powder per μl solution.

Cell culture

The mouse monocyte/macrophage cell line RAW 264.7 (ATCC TIB 67) was cultured in DMEM (dulbeccos modified eagle's medium) supplemented with 10% FCS, 25 mM HEPES, 2 mM Glutamine, 100 IU penicillin ml^{-1} and 100 μg streptomycin ml^{-1} at 37°C, 5% CO_2, and 95% humidity. Cells were studied between passages 7–30. Cells were seeded in 24-well dishes at a density of 2×10^5 cells per well and incubated either with or without the indicated amount of *Panax ginseng* for 24 h.

Nitrite analysis

Nitrite was determined spectrophotometrically by using the Griess reagent (0.5% sulfanilic acid, 0,002% N-1-naphtyl-ethylenediamine dihydrochloride, 14% glacial acetic acid) in supernatants. Absorbance was measured at 550 nm with baseline correction at 650 nm and nitrite concentration was determined using sodium nitrite as a standard (Green *et al*., 1982). Medium alone was used to calculate the assay background level, and this was subtracted from all data.

Limulus amoebocyte lysate test for bacterial endotoxins

Aqueous extract of ginseng were assayed for endotoxin contamination by using a gel-clot Limulus amoebocyte lysate assay (E-TOXATE, Sigma, Chemical Co., St. Louis, MO, U.S.A.). One hundred μl of ginseng stock solution (20 $\mu g\ \mu l^{-1}$) was mixed with 100 μl E-Toxate working solution in a pyrogen-free glass vial and incubated at 37°C for 1 h. After the incubation, the vial was slowly inverted to observe for evidence of gelation. According to the manufacturer's protocol, the formation of a Hard Gel was considered positive.

Protein determination

Protein was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

Western blotting

Cells were lysed in ice-cold buffer containing 25 mM monosodium phosphate (pH 7.4), 75 mM NaCl, 5 mM EDTA, 1% Triton X-100, 100 $\mu g\ ml^{-1}$ phenylmethylsulphonylfluoride, 10 $\mu g\ ml^{-1}$ antipain, 10 $\mu g\ ml^{-1}$ leupeptin, 10 $\mu g\ ml^{-1}$ pepstatin, 20 $\mu g\ ml^{-1}$ aprotinin, and 10 $\mu g\ ml^{-1}$ trypsin inhibitor and centrifuged at 50,000 $\times$ *g* for 20 min at 4°C. The cytosolic proteins (20 μg per lane) were separated by 12% sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose filters, and then immunoblotted either with a rabbit anti-iNOS polyclonal antibody or with a rabbit anti-p65-polyclonal antibody. Control blots were done with anti-actin antibody. Anti-rabbit AP-conjugated antibody was used as a secondary antibody at a dilution of 1 : 7500. Finally, the blots were incubated with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) reagent (Promega) for 10–15 min.

Semiquantitative RT–PCR

Total RNA was isolated using the guanidinium thiocyanate method (Chomczynski & Sacchi, 1987). To determine the RNA concentration, the absorption at 260, 280, and 320 nm was measured photometrically (UV/VIS Spectrophotometer Lambda 2, Perkin-Elmer, Norwalk, CT, U.S.A.). Single-stranded cDNA synthesis was carried out on 2 μg of total RNA primed with oligo(dT)12–18 (Pharmacia, Freiburg, Germany) using murine leukaemia virus reverse transcriptase (MMLV-RT; MBI Fermentas, Vilnius, Lithuania) at 42°C for 60 min in a 20 μl reaction volume. Reactions were stopped by heating for 5 min at 70°C. cDNA was subjected to DNA amplification by PCR using 0.5 units of Taq DNA polymerase (MBI) and both oligonucleotide primer pairs complementary to murine iNOS cDNA and actin cDNA (MWG-Biotec, Ebersberg, Germany) at a final concentration of 0.25 μM for iNOS primer and 0.033 μM for actin primer. Reaction mixtures were subjected to the following conditions in a PE 2400 DNA thermal cycler (Perkin-Elmer): denaturing at 94°C for 30 s, annealing at 58°C for 35 s, and extension at 72°C for 35 s. After 30 cycles, the reaction mixture was cooled down to 4°C. The primers for iNOS were 5′-CTA AGA GTC ACC AAA ATG GCT CCC-3′ (sense) and 5′-ACC AGA GGC AGC ACA TCA AAG C-3′ (antisense).

The expected product length was 775 bp. The primers for the 'housekeeping gene' beta-actin were: 5′-ATG GTG GGA ATG GGT CAG AAG GAC-3′(sense) and 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′ (antisense). The expected product length was 513 bp. All PCR-reactions were in linear range. Final PCR products were separated on a 1% agarose gel and detected by ethidium bromide staining. Semiquantitative estimation was done by comparing mRNA expression of iNOS to beta-actin represented by the amount of the PCR product formed.

Preparation of nuclear extracts

RAW 264.7 cells (1×10^6) were seeded in 6 well plates and incubated with or without ginseng (0.8 μg μl^{-1}) or LPS (1 μg ml^{-1}) for 24 h. After that, cells were washed twice, scraped in ice cold PBS and pelleted at $450 \times g$ for 5 min at 4°C. Nuclear proteins were extracted using a kit obtained from Sigma (St. Louis, MO, U.S.A.) and following the manufacturer's manual. In short, cell pellets were resuspended in 200 μl lysis buffer (mM:) HEPES 10, pH 7.9; $MgCl_2$ 1.5, KCl 10, phenylmethylsulphonylfluoride 1, 1 μg ml^{-1} leupeptin, DTT 1 and incubated on ice for 15 min. Ten per cent IGEPAL CA-630 were added to the swollen cells to a final concentration of 0.6%. Nuclei were pelleted by centrifugation at $11{,}000 \times$ g. Nuclear proteins were extracted by resuspending the pellet in 30 μl of a buffer containing (in mM:) HEPES 20, pH 7.9, MgCl2 1.5, NaCl 0.42 M, EDTA 0.2, 25% (v v^{-1}) glycerol, phenylmethylsulphonylfluoride 1, 1 μg ml^{-1} leupeptin, DTT 1 and shaking for 30 min at room temperature. Nuclear proteins were collected by centrifugation at 20,000 $\times$ g for 5 min at 4°C. The protein content of the supernatant was determined by the Bradford method. The supernatants were kept as aliquots at −70°C until analysed.

EMSA

An overlapping double-stranded oligonucleotide containing a functional nuclear factor NF-κB binding element (*italics*) from the murine iNOS promoter (EMBL nucleotide sequence database accession no L09126) was used: for-primer: pos.1487–1522 of the EMBL-sequence: 5′-ACTTGCA-CACCCAACTG*GGGACTCTCC*CTTTGGGAA-3′; back-primer, pos. 1493–1528: 5′-TAACTGTTCCCAAAG*GGA-GAGTCCC*CAGTTGGGTGT-3′. 4 pmol of double-stranded oligonucleotide were 3′-end-labelled with digoxigenin (DIG)-11-ddUTP using a kit obtained by Roche Diagnostics (Mannheim, Germany) according to the manufacturer's manual. In a total reaction volume of 20 μl, 5 μg of nuclear extract were mixed with 4 μl 5$\times$ binding buffer (in mM:) HEPES 100, pH 7.6, EDTA 5, $(NH_4)_2SO_4$ 50, DTT 5, 10 w v^{-1}% Tween 20, KCl 150, 1 μg poly[d(I-C)], 0.1 μg poly-L-lysine, 30 fmol DIG-labelled oligonucleotide and incubated at room temperature for 25 min. For specific competition, a 200 fold molar excess of unlabelled oligo was added. Reaction mixtures were run on a 6% non-denaturing polyacrylamide gel at 70 V until the front dye reached the bottom of the gel. Nuclear protein-oligonucleotide complexes were transferred on positively charged nylon membranes (Immobilon Ny+, Millipore, Bedford, MA, U.S.A.) by electroblotting and fixed by u.v. crosslinking. Membranes were incubated with an AP-conjugated anti-DIG polyclonal antibody. Signal detection was obtained by incubating with BCIP/NBT reagent (Promega) for 5–6 h.

Data analysis

Each experimental result as shown in the figures is the mean $\pm$ s.d. for at least three measurements. When s.d. is not displayed, it is smaller than the size of the symbol. Statistical analyses were performed by use of ANOVA followed by Student's t-tests for unpaired data. Statistical significance was defined as $P < 0.05$.

Results

Stimulation of inducible NO synthesis by Panax ginseng *aqueous extract*

RAW 264.7 macrophages were incubated with increasing amounts (0.05–0.8 μg μl^{-1}) of *Panax ginseng* aqueous extracts. Culture media were collected and assayed for nitrite after 24 h as described in the Methods section. Nitrite levels were related to protein levels determined by the Bradford assay. Incubation of RAW 264.7 cells with increasing amounts of *Panax ginseng* was associated with a dose dependent increase in NO production (Figure 1A). Stimulation of nitrite release after 24 h became significant at 0.05 μg μl^{-1} of *Panax ginseng*. To exclude that *Panax ginseng* interferes with detection of nitrite by the Griess reaction, we incubated the NO donor linsidomine (0.5 mM) in the presence or absence of *Panax ginseng* (0.05–0.8 μ μl^{-1}) for 24 h at room temperature. *Panax ginseng* had no statistically significant effect on the measured nitrite levels. We conclude therefore that *Panax ginseng* does not interfere with the detection of nitrite by the Griess reaction. To exclude

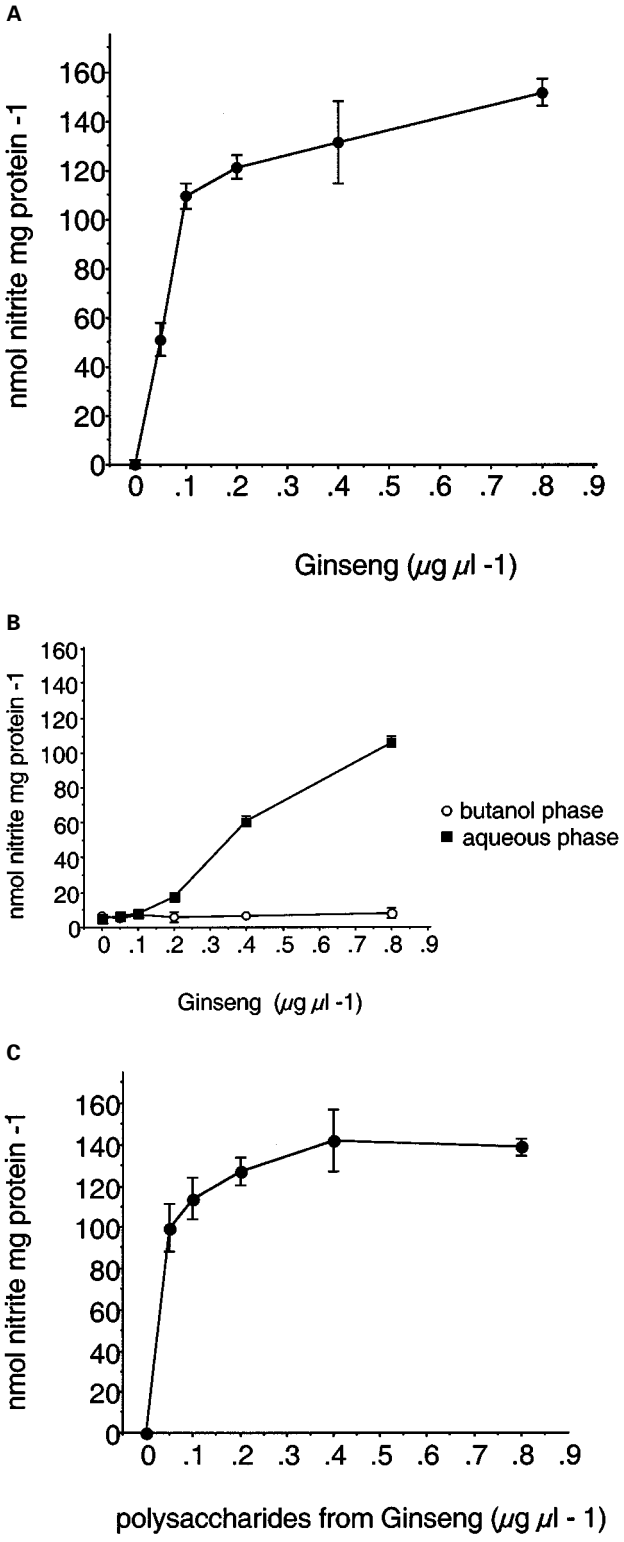

Figure 1 Line charts showing dose-dependent stimulation of inducible NO production in RAW 264.7 cells. Cells were incubated with increasing amounts of (A) total aqueous extract of *Panax ginseng* (B) components of the two phases after a butanol extraction of the aqueous extract of *Panax ginseng* (C) polysaccharides isolated from the aqueous extract of *Panax ginseng* as indicated. NO production was determined as nitrite accumulation in the medium after 24 h as described in the Methods section. Each dot shows the mean of triplicate measurements.

unspecific iNOS stimulation due to bacterial contamination of the ginseng root powder, a Limulus amoebocyte lysate test was performed. The negative result at a detection limit of 0.05–0.1 endotoxin units ml^{-1} indicated that iNOS stimulation is ginseng specific.

Effect of fractions obtained from Panax ginseng *aqueous extract on nitrite release*

To determine which components present in the aqueous extract are responsible for the effects described in this study, a fractionation of the aqueous extract was done by butanol extraction yielding two fractions of different hydrophobicity. The residues of both phases were reconstituted with PBS to the initial concentration of 20 mg ml^{-1} ginseng powder. DC analysis revealed that the components of the butanol phase contained all the ginsenosides, whereas among the components of the aqueous phase, no triterpenoids were detectable. Cells were stimulated with a PBS solution of both phases. Cells stimulated with the water phase-derived solution showed a dose dependent increase in NO production after 24 h, whereas no stimulation was seen with the butanol phase-derived solution containing the ginsenosides (Figure 1B). These findings were consistent with earlier results showing that the commercially available purified ginsenoside Rg1 has no effect on NO production in untreated RAW 264.7 cells. In addition, polysaccharides were isolated from *Panax ginseng* aqueous extract. RAW 264.7 cells stimulated with increasing amounts of polysaccharides obtained from *Panax ginseng* aqueous extract showed an increase in NO production after 24 h (Figure 1C). To demonstrate specificity of the ginseng polysaccharide stimulation, cells were treated with the ubiquitous polysaccharide arabinogalactan, which did not cause any NO release.

Effect of Panax ginseng *aqueous extract on iNOS protein expression*

Figure 2 shows the Western blot analysis of iNOS in RAW 264.7 cells. Unstimulated macrophages were cultured with increasing amounts of *Panax ginseng* (0.05–0.8 $\mu g\ \mu l^{-1}$) for

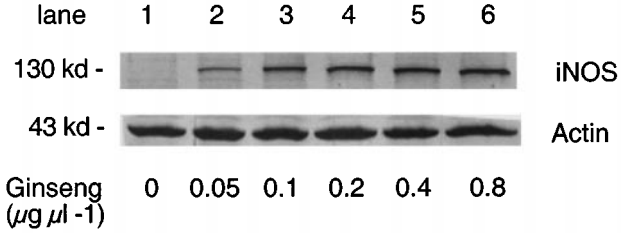

Figure 2 Immunoblotting against iNOS. Unstimulated RAW 264.7 mouse macrophages were cultured with increasing amounts of *Panax ginseng* (0–0.8 $\mu g\ \mu l^{-1}$) for 24 h. Western blotting was performed as described in the Methods section. Lane 1: unstimulated RAW 264.7 cells; lanes 2–6: incubations containing 0.05–0.8 $\mu g\ \mu l^{-1}$. *Panax ginseng*. Immunoblotting identified a band with an estimated molecular mass of 130 kD in stimulated RAW 264.7 mouse macrophages. iNOS protein was markedly increased in cells incubated with *Panax ginseng* (Lane 2–6). The constitutively expressed control protein actin (43 kD) was not increased, indicating that *Panax ginseng* induced iNOS stimulation is not associated with a generalized increase in protein expression.

24 h. Immunoblotting shows a band with an estimated molecular mass of 130 kD (the known molecular mass of iNOS) in stimulated RAW 264.7 mouse macrophages. An identical molecular mass was determined by blotting against purified iNOS protein. iNOS protein was increased in a dose-dependent manner by increasing amounts of *Panax ginseng* ($0.05-0.8\ \mu g\ \mu l^{-1}$). In contrast, beta-actin (43 kD) levels remained unchanged during incubations with *Panax ginseng*. This shows that *Panax ginseng* induced iNOS stimulation is not associated with a generalized increase in protein expression.

Effect of Panax ginseng *aqueous extract on iNOS mRNA expression*

Figure 3 shows the RT–PCR analysis of iNOS mRNA in RAW 264.7 cells. Unstimulated mouse macrophages were incubated with $0.05-0.8\ \mu g\ \mu l^{-1}$ *Panax ginseng* aqueous extract for 24 h. A dose-dependent increase of iNOS-mRNA was seen (Figure 3 A,B). Actin mRNA levels remained unchanged. Experiments were repeated with polysaccharides isolated from *Panax ginseng* aqueous extract. Cells were incubated with polysaccharides isolated from $0.05-0.8\ \mu g\ \mu l^{-1}$ aqueous extract of *Panax ginseng* for 24 h. A dose-dependent increase of iNOS-mRNA was seen up to $0.2\ \mu g\ \mu l^{-1}$ whereas for 0.4 and $0.8\ \mu g\ \mu l^{-1}$, a decline from maximum stimulation could be observed.

Effect of Panax ginseng *aqueous extract on NF-κB binding activity*

The effect of *Panax ginseng* aqueous extract on the DNA-binding activity of NF-κB to a murine iNOS promoter sequence in RAW 264.7 cells was tested by EMSA. Cells were treated with $0.8\ \mu g\ \mu l^{-1}$ of *Panax ginseng* extract. Cells treated with LPS ($1\ \mu g\ ml^{-1}$), an established activating agent of iNOS (Marletta *et al*., 1988), were used as a reference. The amounts of LPS and *Panax ginseng* were based on previous experiments showing no further increase in iNOS expression with higher amounts of stimulating agent. The ratio of nitrite levels as a result of treatment with $0.8\ \mu g\ \mu l^{-1}$ ginseng compared to treatment with $1\ \mu g\ ml^{-1}$ LPS was approximately 1 : 1.5. A low basal level of NF-κB DNA binding activity was detected in

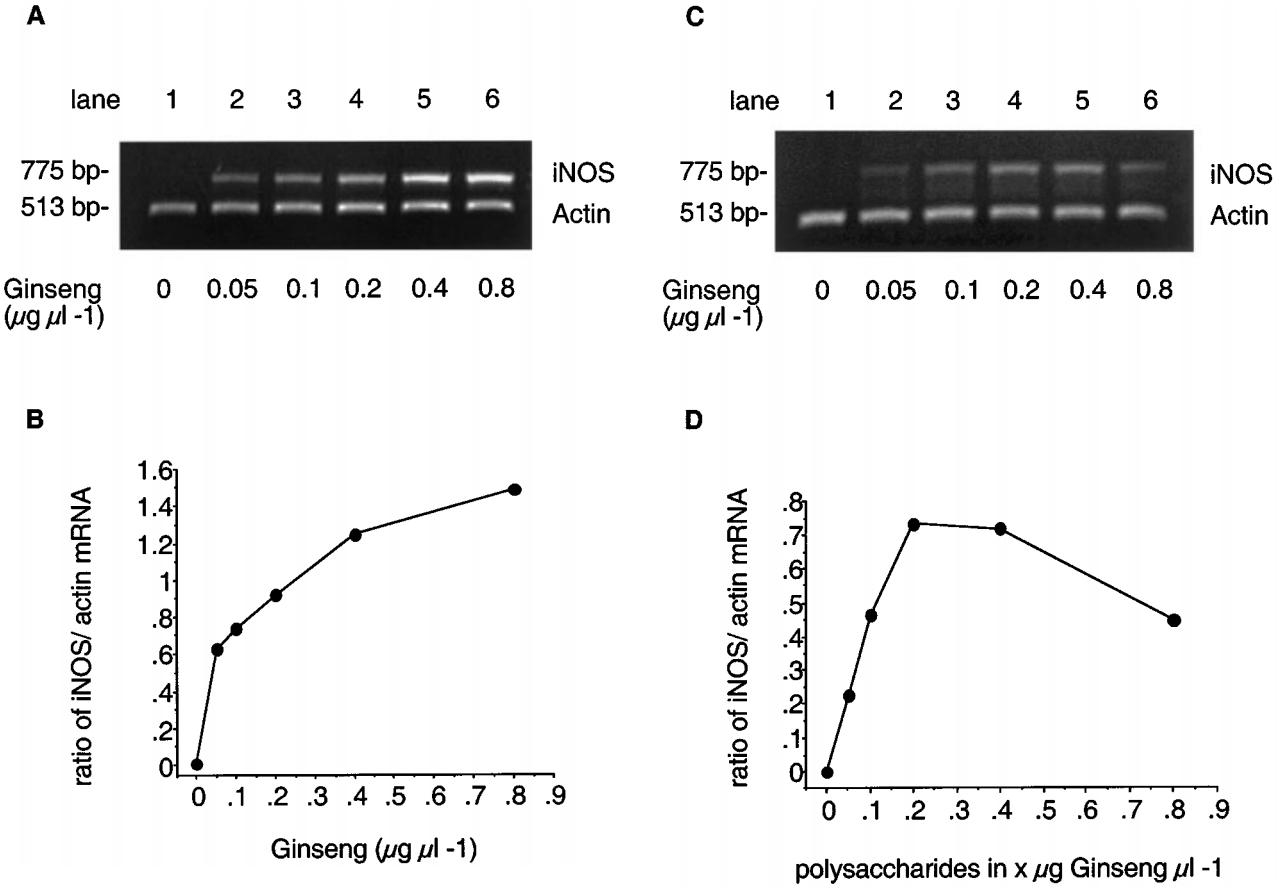

Figure 3 Effects of *Panax ginseng* and its constituents on iNOS mRNA expression determined by semiquantitative RT–PCR. RAW 264.7 mouse macrophages were incubated with either (A) $0-0.8\ \mu g\ \mu l^{-1}$ *Panax ginseng* total aqueous extract or (C) increasing amounts of polysaccharides isolated from the aqueous extract of *Panax ginseng* for 24 h. RNA was extracted and analysed as described in the Methods section. Single-stranded cDNA synthesis was performed, and DNA was amplified by PCR using specific primers for iNOS (775 bp product) and beta-actin (513 bp product). Semiquantitative estimation was done by comparing mRNA expression of iNOS to beta-actin represented by the amount of PCR product formed. lane 1: unstimulated RAW 264.7 cells; lanes 2–6: incubations with (A) $0.05-0.8\ \mu g\ \mu l^{-1}$ *Panax ginseng* or (C) polysaccharides isolated from $0.05-0.8\ \mu g\ \mu l^{-1}$ aqueous extract of *Panax ginseng*. iNOS mRNA levels were increased in RAW 264.7 cells incubated with increasing *Panax ginseng* concentrations compared to untreated cells. Actin mRNA levels remained unchanged. (B) and (D): Quantification of band intensities was done by densitometry. Three independent experiments were performed. A representative image is shown.

nuclear proteins from unstimulated macrophages. Intense signals appeared for nuclear extracts derived from the ginseng- as well as from the LPS-treated cells (Figure 4A). Specificity of the NF-κB-DNA binding complex was demonstrated by addition of excess unlabelled oligonucleotide which resulted in complete displacement of the NF-κB-DNA binding. Western blotting of the nuclear extract with antibody specific for p65 demonstrated an increase of the p65 band in comparison to untreated cells (Figure 4B). Densitometric analysis revealed a 2.2 fold increase of the p65 band in ginseng-treated cells versus a 1.9 fold increase in LPS-treated cells compared to untreated cells.

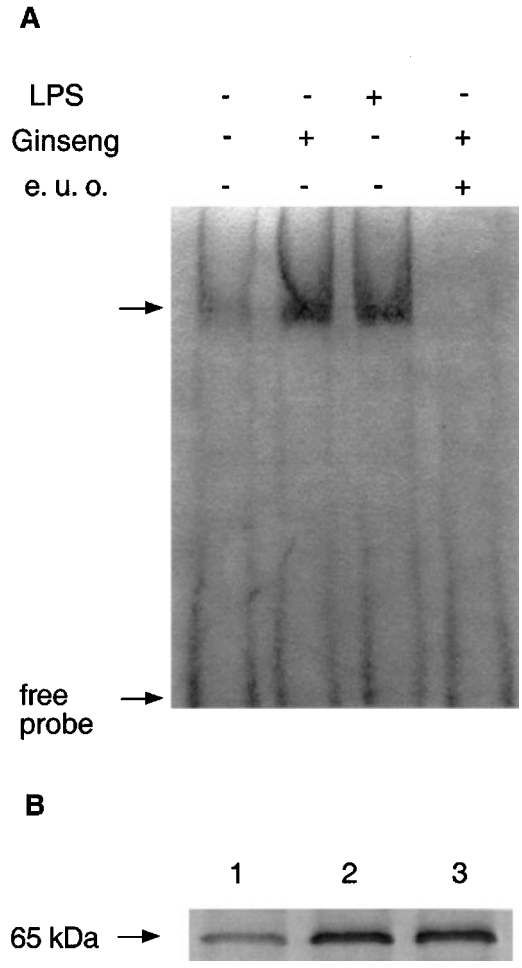

Figure 4 Effect of ginseng on NF-κB-DNA binding activity to murine iNOS promoter oligonucleotide. (A) RAW 264.7 cells were treated either with total aqueous extract of ginseng (0.8 $\mu g\ \mu l^{-1}$) or with LPS (1 $\mu g\ ml^{-1}$) and the effects on NF-κB binding were compared to untreated cells. Nuclear extracts were collected 24 h after treatment and incubated with a DIG-labelled oligonucleotide containing a murine iNOS promoter binding site for NF-κB. Resulting complexes were resolved by electromobility shift assay. An arrow indicates the position of the NF-κB-DNA complex. Ginseng (0.8 $\mu g\ \mu l^{-1}$) induced NF-κB-DNA binding activity when compared to untreated cells and this effect was comparable to NF-κB-DNA activation by LPS. Specificity was shown by addition of excess unlabelled oligonucleotide (=e.u.o.), which resulted in complete displacement of the NF-κB-DNA binding. (B) Effect of ginseng vs. LPS on nuclear p65 protein level. RAW 264.7 cells were either untreated (lane 1) or treated with ginseng (lane 2) or LPS (lane 3). Nuclear proteins were collected 24 h after treatment followed by Western immunoblotting as described in the Methods section. Densitometry revealed a 2.2 fold increase of the p65 band in ginseng-treated cells vs a 1.9 fold increase in LPS-treated cells compared to untreated cells. The blot shown is representative of two similar experiments.

Discussion

Ginseng attains growing popularity as a herbal remedy in western Europe and in northern America and a number of beneficial effects on health are attributed to ginseng root extract. It has been a well-known observation for a long time that ginseng 'increases the nonspecific resistance' (Brekhman & Dardymov, 1969).

Several immunomodulatory effects of ginseng extracts have been described. An aqueous extract of *Panax ginseng* C. A. Meyer administered orally to mice or added to cultures of mouse spleen cells enhanced antibody formation and natural killer (NK) cell activity (Jie *et al*., 1984). During a double-blind investigation, a standardized extract of ginseng root was given orally to volunteers and resulted in a reduced susceptibility for infectious respiratory tract diseases (Scaglione *et al*., 1996). Kim *et al*. demonstrated protection against infection with *Listeria monocytogenes*, associated with enhanced NK cell activity by *Panax ginseng* in mice (Kim *et al*., 1990). Some studies investigated the effects of defined components of *Panax ginseng*. Polysaccharides derived from aqueous extracts induced the production of interferon-gamma and of TNF-α in mouse spleen lymphocytes and peritoneal macrophages (Gao *et al*., 1996), two acidic polysaccharides, ginsenan S-IA and ginsenan S-IIA, showed anti-complementary activity (Tomoda *et al*., 1993), ginsenan S-IIA induced IL-8 production from THP-1 cells (Sonoda *et al*., 1998), another acidic polysaccharide, ginsan, induced cytokine production from Th1 and macrophages and generated lymphokine-activated killer (LAK) cells synergistically with rIL-2 (Kim *et al*., 1998). Ginsenoside Rg1 increased the number of Th cells and induced an augmentation of the production of IL-1 by macrophages (Kenarova *et al*., 1990; Kim *et al*., 1998) and enhanced the production of NO from IFN-γ activated RAW cells (Fan *et al*., 1995). In contrast, ginsenosides Rh1 and Rh2 have shown to inhibit NO production in LPS/IFNγ treated macrophages (Park *et al*., 1996).

Macrophages constitute the first line of host defenses in conferring immunity against infections. Macrophages are known to produce NO, and one of the most prominent functions of NO is its participation in antimicrobial and antiviral defense (Nathan & Hibbs, 1991; Kröncke *et al*., 1998). Sustained NO production from macrophages endows them with cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, helminths, and tumour cells (Kwon *et al*., 1991; Lowenstein *et al*., 1996; MacMicking *et al*., 1997). According to several *in vivo* studies, iNOS inhibitors have been shown to exacerbate infectious diseases (Evans *et al*., 1993; Chan *et al*., 1995; Stenger *et al*., 1996). On the other hand, the enhancement of bacterial clearance in *Pseudomonas aeruginosa*-infected athymic rats by ginseng extract treatment has been shown (Song *et al*., 1997). Our data could provide the molecular basis for this result, suggesting that iNOS stimulation by *Panax ginseng* might contribute to a decreased susceptibility to infections due to an enhanced NO release.

There is evidence that human macrophages are capable of high-output NO production *in vivo*, e.g. when involved in

airway inflammation (reviewed in MacMicking *et al*., 1997; Kröncke *et al*., 1998) or in Mycobacterium tuberculosis infection (Rich *et al*., 1997). Hence, using a human macrophage cell line such as THP-1, usually only minute NO levels can be detected upon LPS and IFNγ stimulation (Perez-Perez *et al*., 1995), although iNOS mRNA production can be verified (Reiling *et al*., 1994). Therefore, the use of the murine macrophage model system is generally preferred when studying iNOS (Nathan & Hibbs, 1991).

In our study, we demonstrated a dose-dependent increase of NO release (expressed in terms of measured nitrite levels) from unstimulated murine macrophage-like RAW 264.7 cells by increasing amounts ($0.05-0.8\ \mu g\ \mu l^{-1}$) of *Panax ginseng* in aqueous solution after 24 h. Stimulation of nitrite release became significant at a *Panax ginseng* concentration of $0.05\ \mu g\ \mu l^{-1}$. When cells were incubated with *Panax ginseng* up to $0.8\ \mu g\ \mu l^{-1}$, nitrite levels inclined from zero (untreated cells) to 152 ± 5.35 nmol nitrite mg^{-1} protein. This result was associated with an incline in iNOS mRNA and iNOS protein. Immunoblotting experiments showed a distinct enhancement of iNOS protein in cells incubated with increasing amounts of *Panax ginseng*. At concentrations ranging from $0.05-0.8\ \mu g\ \mu l^{-1}$, a steady incline in iNOS protein but not beta-actin protein was seen, indicating that *Panax ginseng* induced iNOS stimulation was not associated with a generalized increase in protein expression.

It has been demonstrated that NF-κB is a transcription factor essential in the regulation of iNOS expression after LPS challenge (Xie *et al*., 1994). In the light of these findings, we investigated the effect of *Panax ginseng* aqueous extracts on NF-κB binding activity in comparison to the LPS-induced effect. EMSA experiments show that *Panax ginseng* upregulated NF-κB-DNA-binding activity to the same extent as LPS. These results were reinforced by Western blotting experiments showing that *Panax ginseng* also enhanced the translocation of NF-κB subunit p65 into the nuclear fraction. Therefore we conclude that aqueous extracts of *Panax ginseng* root directly enhance transcriptional activity of the iNOS promoter *via* NF-κB, leading to increased iNOS mRNA and protein levels as shown by semiquantitative PCR and Western blotting.

Fractionation of the aqueous extract of *Panax ginseng* root powder have shown that the stimulating activity is completely attributed to the polysaccharide-containing fraction. The fraction containing triterpenic ginsenosides did not display any stimulation of NO release. Experiments with polysaccharides isolated from the aqueous extracts confirmed the stimulating activity of these substances on the inducible NO synthase. The effect of very high NO output at the highest concentration (polysaccharide content in $0.4-0.8\ \mu g$ ginseng μl^{-1}) induced a phenomenon of negative feedback control of iNOS mRNA levels (Colasanti *et al*., 1995; Goldring *et al*., 1995).

In the light of our results, it seems questionable to qualify commercially available ginseng preparations solely *via* their ginsenoside content, as this will provide no definite predication about its overall effects. Cell culture systems as used in our experiments providing activity based tests could serve as a simple and effective means to overcome this need for information.

Our results are in accordance with the studies cited above claiming enhancement of host resistance due to ginseng treatment, as they indicate that *Panax ginseng* might display modulatory effects on the innate immune defense by stimulation of the inducible NO synthesis in macrophages. Polysaccharide components from ginseng could be determined to be responsible for this stimulation. We demonstrated a clear effect of ginseng on stimulation of iNOS expression *via* NF-κB, thus providing a valuable contribution to the discussion about the molecular basis of action from *Panax ginseng*.

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