Panax notoginseng attenuates LPS-induced pro-inflammatory mediators in RAW264.7 cells

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Abstract

Herbals or dietary supplements are not regulated as drugs by the United States Food and Drug Administration (USFDA) although many may have associated therapeutic effects and toxicities. Therefore, the immunomodulatory effects of the herbal extract Panax notoginseng on cultured macrophages (RAW264.7 cells) were investigated to address potential therapeutic or toxic effects. Cells were stimulated with LPS (1 µg/ml) and treated with notoginseng at 5, 25 and 50 µg/ml. Notoginseng inhibited the LPS-induced production of TNF-α and IL-6 by the cultured macrophages in a concentration-dependent manner. The expression of COX-2 and IL-1β mRNA was also attenuated by notoginseng. TNF-α production was inhibited in samples treated with notoginseng 24 h before, or at the same time as LPS stimulation, but not in samples treated 8 h after LPS stimulation. Notoginseng reduced expression of the accessory molecules CD40 and CD86 on the RAW264.7 cells while CD14 and TLR4 expression remained unaffected. Furthermore, Rb1 and Rg1 ginsenosides also inhibited macrophage production of TNF-α, but to a lesser extent than did the whole notoginseng extract. Collectively, these results indicate that notoginseng inhibits LPS-induced activation of RAW264.7 macrophages and demonstrates that notoginseng possesses anti-inflammatory and immunosuppressive properties in vitro.

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1. Introduction

Over the last decade there has been a steady increase in the use of herbal and dietary supplements. The rise in popularity of natural products may be attributed to more aggressive sales tactics, enhanced Internet accessibility and dissatisfaction with conventional medicines (Barnes et al., 2004). There also exists a misconception that “natural” is synonymous with safe despite the fact that herbal products can cause medical problems if not taken correctly or if taken in excessive amounts (Klepsner and Klepsner, 1999). The United States Food and Drug Administration (USFDA) does not classify natural products as drugs despite associations with both therapeutic effects and toxicities. This means that, unlike conventional drugs, natural products are not required to meet rigorous standards to demonstrate safety, efficacy and mechanisms of action. Recent evidence demonstrates that natural products have the capacity to interact with conventional drugs via modulation of various xenobiotic metabolizing enzymes such as CYP2C9 and CYP3A4 (He and Edeki, 2004).

The rise in natural product usage and the potential for adverse or advantageous reactions has led to increased attention to their potential safety and efficacy.

Among these widely used natural products, ginseng was the second most frequently purchased herbal by the US adult population in 2002 (Barnes et al., 2004). Over thirteen species of ginseng have been identified, including Panax notoginseng (Burk.) F.H. Chen ex C.Y. Wu & K.M. Feng (Yun, 2001). The biologically active compounds of ginseng species are thought to be the saponins of which the ginsenosides Rb1, Rg1, Re1, Rh1 and the notoginsenoside R1 are considered to be the major components of Panax notoginseng (Yun, 2001; Li et al., 2005). The biological activity of Panax notoginseng is similar to the more widely known Panax ginseng plant, with differences in activity associated with higher levels of ginsenosides in the...
notoginseng species (Zhu et al., 2004; Chuang et al., 1995). Ginseng is highly regarded in China for its therapeutic ability to stop hemorrhages, influence circulation, act as a tonic, induce variable effects on systemic blood pressure, and generate analgesic and anti-inflammatory effects (Xu et al., 2003). Notoginseng’s cardiovascular effects occur via inhibition of calcium entry through receptor-mediated calcium channels without affecting voltage gated calcium channels or intracellular calcium release (Kwan, 1995). The ginsenosides Rb1 and Rg1 also have stimulatory effects on the central nervous system. They can improve memory, learning and confer neuroprotection in some instances (Attele et al., 1999).

The immunomodulatory effects of notoginseng have not been fully characterized. Several studies have described the effects of ginseng and ginsenosides on the immune system, but specific mechanisms of action have yet to be identified. Noted immunologic effects include anti-allergic and anti-inflammatory activities of Rb1 (Park et al., 2004), a reduction in TNF-α levels by Rb1 (Smolinska and Pestka, 2003), an increase in both humoral and cell-mediated immune responses by Rg1 (Kwan, 1995), and a decrease in phospholipase A2 activity and neutrophil numbers by Panax notoginseng extract (Li and Chu, 1999).

Macrophages are immune cells usually dispersed throughout the body. They are particularly important in innate immunity as they are among the first cells responding to microbial infection. They can kill pathogens directly by phagocytosis and indirectly via the secretion of pro-inflammatory cytokines such as the TNF-α, IL-1β, and IL-6. These cytokines lead to a variety of responses including the induction of cyclooxygenase-2 (COX-2) expression (Turini and D’Andrea, 2002), increased expression of adhesion molecules on vascular endothelial cells (Luscinskas and Gimbrone, 1996), the induction of acute-phase response of adhesion molecules on vascular endothelial cells (Luscinskas and Gimbrone, 1996), the induction of acute-phase response proteins by the liver (Diehl and Kincon, 2002), and the production of colony stimulating factors by activated endothelial cells which induce hematopoiesis (Watowich, 1996).

Macrophages also serve an important role as an interface between innate and adaptive immunity. They are responsible for processes such as antigen processing and presentation to antigen-specific T cells. Following activation, macrophages can modulate expression of accessory molecules commonly expressed on macrophages. Moreover, these results demonstrate that notoginseng attenuates the production of several pro-inflammatory mediators by macrophages following in vitro stimulation by LPS.

2. Materials and methods

2.1. Chemicals

Noto-G™ extracts from the plant Panax notoginseng (Burk.) F.H. Chen ex C.Y. Wu & K.M. Feng were kindly supplied by Technical Sourcing International, Inc. (TSI) (Missoula, MT). Notoginseng was extracted from the root of the plant using ethanol and standardized to contain Rb1 and Rg1 ginsenosides at 35 and 34% of the whole extract, respectively. The quantification of Rb1 and Rg1 in the notoginseng extract was determined by high-performance liquid chromatography analysis by TSI. Documentation by TSI also showed no detectable levels of Escherichia coli or Salmonella enterica in the notoginseng preparation (unpublished data). Certification of analyses were approved by Xia Ronglong (QA manager TSI). The extract was dissolved in complete media (see below) or culture-grade DMSO (Sigma–Aldrich, St. Louis, MO) and subsequently sterilized through a 0.22 μm Millipore membrane. The purified ginsenosides Rb1 and Rg1 were purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ). Lipopolysaccharide (LPS) from Escherichia coli (055:B5) was obtained from Sigma–Aldrich.

2.2. Cell Culture

RAW264.7 cells were obtained from ATCC (Manassas, VA). Cells were grown in RPMI (GibcoBRL, Grand Island, NY), supplemented with 10% FBS (HyClone, Logan, UT), 50 μg/ml mercaptoethanol, 20 mM HEPES, 10 mM sodium pyruvate and 25 μg/ml gentamicin (GibcoBRL, Grand Island, NY). RAW264.7 cells were maintained via weekly passage and cells were utilized for experimentation at 60–80% confluency.

2.3. Cell activation and treatment

RAW264.7 cells (5 × 10⁵ cells per well) were incubated overnight at 37 °C and 5% CO₂ in 6-well plates to facilitate attachment and spreading before experimentation. Cells were then stimulated with 1 μg/ml LPS and treated with 0, 5, 25 or 50 μg/ml notoginseng or Rb1 and Rg1 ginsenosides at concentrations equivalent to that in 50 μg/ml notoginseng. After an additional 24 h, supernatants were collected for evaluation by ELISA and cells were harvested for RT-PCR and FACS analysis.
2.4. Cytokine assays

The inhibitory effects of notoginseng on the production of IL-6 and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA) using supernatants collected from treated cells. Samples were analyzed per the manufacturer’s recommendations with mouse cytokine-specific BD OptEIA ELISA kits (BD PharMingen, San Diego, CA).

2.5. RT-PCR

RT-PCR for the detection of IL-1β and COX-2 mRNA was performed as previously described (Shepherd et al., 2001). Briefly, total RNA was isolated using TriZol™ and reverse transcribed into cDNA. COX-2 and IL-1β transcripts were identified using specific forward and reverse primers as per manufacturer’s instructions (Clontech, Palo Alto, CA). β-2 microglobulin expression was included as an internal, housekeeping gene control. Ethidium bromide-stained reaction products were separated by electrophoresis on a 2% agarose gel in TBE and visualized by UV transillumination. Images were captured by a Kodak EDAS 290 camera system (Kodak, Rochester, NY).

The primers used in these experiments were designed to span introns thereby allowing differentiation between amplified genomic DNA and cDNA PCR products. Primer sequences used were β-2 microglobulin, 5′-ATGGCTCGGTGA-CCCT and 3′-TCATGATGCTTGATCACATG; IL-1β, 5′-ATGGCAACTGTTCCTGAACTCAACT and 3′-CAGGACAGG-TATAGATTCTTTCCTTT; and COX-2, 5′-AACACAGCTAC-GAAAACC and 3′-CACAGTATGATGTAACAGTCC.

2.6. Flow cytometry

The detection of accessory molecule expression on RAW264.7 cells by fluorescent activated cell sorting (FACS) analysis was performed as previously described (Shepherd et al., 2001). Briefly, RAW264.7 cells were harvested and washed with PBS (1% bovine serum albumin and 0.1% sodium azide in PBS). Cells were blocked with 50 μl of 600 μg/ml purified rat and/or hamster IgG (Jackson ImmunoResearch, West Grove, PA) for 10 min to inhibit non-specific staining, and then stained with optimal concentrations of fluorochrome-conjugated antibodies for an additional 10 min. The antibodies used in these experiments were CD86-APC, CD40-PE, TLR4-PE, CD14-FITC, and their corresponding isotype controls (BDPharmingen, San Diego, CA, except for the anti-TLR4 eBiosciences, San Diego, CA). One hundred thousand viable cells per treatment (as determined by light scatter profiles and propidium iodide staining) were analyzed using a BD FACSaria flow cytometer and FACS Diva software (BD Biosciences, San Jose, CA).

2.7. Statistics

All statistical analyses were performed using GraphPad Prism 4.0a for the Macintosh (GraphPad Software, San Diego, CA). Data signify the mean plus or minus of three samples and are representative of three independent experiments. Differences between two means were analyzed by Student’s t-test. Data sets with multiple comparisons were evaluated by one-way analysis of variance (ANOVA) with Dunnett’s post test. Values of p < 0.05 were determined to be significant.

3. Results

3.1. Notoginseng suppresses the LPS-induced production of TNF-α and IL-6 by RAW264.7 cells

RAW264.7 cells are an immortalized monocyte/macrophage murine cell line that has been used extensively to evaluate monocyte and macrophage fate and function in vitro. To evaluate the potential effects of notoginseng on macrophage function, RAW264.7 cells were stimulated with 1 μg/ml LPS and con-

![Fig. 1. Notoginseng inhibits the LPS-induced TNF-α and IL-6 production by RAW264.7 cells. Cells were treated simultaneously with 1 μg/ml LPS and 5, 25 or 50 μg/ml of notoginseng. Supernatants were collected after 24 h and assayed for TNF-α (A) and IL-6 (B) production as described in Section 2. Data represents mean ± S.E.M. of three samples. Hash (#) indicates significant difference between stimulated and unstimulated cells; asterisk (*) indicates significant differences between the LPS-stimulated control- and notoginseng-treated samples (p < 0.05). Data are representative of three independent experiments.](image-url)
concomitantly treated with notoginseng. Unstimulated RAW264.7 cells secrete a basal level of TNF-α but barely detectable amounts of IL-6 (Fig. 1A and B, respectively). At the highest concentration tested, notoginseng did not evoke TNF-α or IL-6 release in the absence of LPS stimulation. The addition of LPS resulted in a 9- and 120-fold increase in TNF-α and IL-6 protein levels, respectively. Notoginseng significantly inhibited the production of both TNF-α and IL-6 in a concentration-dependent manner. At the highest concentration of notoginseng tested, TNF-α production was reduced approximately 3-fold while IL-6 production was reduced 7-fold. Importantly, no cytotoxicity was observed at any of the concentrations of notoginseng examined, as assessed by trypan blue exclusion (data not shown). Also, no significant differences were observed in cell recoveries between notoginseng- and control-treated cells.

3.2. COX-2 and IL-1β mRNA levels are decreased in LPS-stimulated RAW264.7 cells following exposure to notoginseng

Because of the prominent role of COX-2 and IL-1β in the inflammatory response, the potential for notoginseng to alter the expression of these mediators was investigated. COX-2 and IL-1β mRNA levels in RAW264.7 cells were evaluated by RT-PCR. The mRNA expression of the housekeeping gene β-2 microglobulin was used to normalize samples. Untreated RAW264.7 cells expressed detectable levels of both COX-2 and IL-1β mRNA that increased following LPS stimulation (Fig. 2). Notoginseng inhibited LPS-induced IL-1β mRNA expression in a concentration-dependent manner and reduced the expression of COX-2 mRNA in LPS-stimulated macrophages at the higher concentrations examined (Fig. 2). Interestingly, mRNA expression levels of both pro-inflammatory genes were decreased in RAW264.7 cells that were not stimulated with LPS, but treated with the highest concentration of notoginseng only.

3.3. Notoginseng selectively modulates the expression of key accessory molecules

The expression levels of accessory/costimulatory molecules on macrophages can dually affect their inflammatory responsiveness and capacity to function as antigen presenting cells. To determine if notoginseng modulates accessory molecule expression on activated macrophages, RAW264.7 cells were treated concomitantly with LPS and 50 μg/ml of notoginseng and the fluorescent intensity of CD40, CD86, CD14 and TLR4 measured by flow cytometry. Only the highest concentration of notoginseng was evaluated in this experiment since significant inhibition of other inflammatory mediators had been demonstrated at this concentration (Figs. 1 and 2). Stimulation of RAW264.7 cells with LPS increased the expression of CD40, CD86 and CD14 (Table 1). Conversely, LPS treatment resulted in down-regulation of TLR4 expression on RAW264.7 cells as has been previously reported (Akashi et al., 2000). Notoginseng decreased the LPS-induced expression of CD40 by almost 20% (Fig. 3A) and CD86 by 30% (Fig. 3B) on RAW264.7 cells. In contrast, cell surface expression of TLR4 and CD14 remained unchanged following notoginseng exposure (Table 1).

3.4. TNF-α production is affected by notoginseng in a time-dependent manner

To assess if the duration of notoginseng exposure results in differential effects on TNF-α production, RAW264.7 cells were either pretreated with notoginseng for 24 h prior to LPS addition (~24 h timepoint), treated with notoginseng and LPS simul-
Fig. 3. Notoginseng treatment of RAW264.7 cells selectively modulates cell surface expression of accessory molecules. RAW264.7 cells were unstimulated (white histogram), stimulated with LPS (black histogram) or stimulated with LPS and concomitantly treated with 50 μg/ml notoginseng (grey histogram) for 24 h. Representative histograms demonstrating the cell surface expression of CD40 (A) and CD86 expression (B) on viable RAW264.7 cells receiving various treatments were determined by FACS analysis. Values in histograms correspond to specific mean channel fluorescence (MCF) values for each treatment. Data are representative of three independent experiments.

Consistently with the results presented in Fig. 1, concomitant treatment of RAW264.7 macrophages with LPS and notoginseng significantly suppressed the production of TNF-α after 24 h of culture by more than 50% (Fig. 4). Pretreatment of RAW264.7 cells with notoginseng for 24 h resulted in a 17% decrease in TNF-α levels. It should be noted that for these cultures, the herbal extract was not removed from the medium after 24 h but remained for the entire culture period of 48 h. In contrast, delaying the addition of notoginseng by 8 h relative to LPS treatment,
resulted in no significant reduction of TNF-α although a trend towards lesser levels was observed.

3.5. The purified ginsenosides Rb1 and Rg1 modulate the production of TNF-α by RAW264.7 cells

The whole notoginseng extract used in the preceding experiments is comprised of several ginsenosides although primarily of Rb1 (35%) and Rg1 (34%). Because ginsenosides are believed to comprise much of the biological activity of notoginseng, we sought to define the potential inhibitory effects of the purified ginsenosides Rb1 and Rg1 on LPS-induced TNF-α production by RAW 264.7 cells. To more closely mimic the approximate concentration of ginsenosides present in the whole extract, Rb1 and Rg1 were standardized to approximate concentrations found in 50 μg/ml of the intact notoginseng extract. Following LPS activation of RAW264.7 cells, both Rb1 and Rg1 and a combination of the Rb1/Rg1 purified compounds significantly inhibited TNF-α production (Fig. 5A). However, neither ginsenoside alone or in combination attenuated TNF-α production to the extent that was observed following exposure to the whole notoginseng extract. In addition, the whole notoginseng extract and ginsenoside Rg1 caused significant inhibition of LPS-induced IL-6 by RAW 264.7 cells (76% and 24% of LPS-stimulated controls, respectively). Conversely, no significant suppression was detected after the addition of Rb1 to these cultures (Fig. 5B).

4. Discussion and conclusions

In the current study, the potential for notoginseng to decrease the LPS-induced production or expression of cytokines, TNF-α, IL-6, and IL-1β, was tested because of their significance in inflammatory conditions. The production of these inflammatory molecules by RAW264.7 cells can be induced in response to LPS stimulation (Beutler, 2003; Rice and Bernard, 2005). TNF-α is primarily produced by monocytes, macrophages and T cells (Bondeson, 1997) and has various pro-inflammatory effects on many cell types. It is a potent activator of macrophages, can stimulate the production or expression of IL-1β, IL-6, prostaglandin E2, collagenase, type I and III collagens, adhesion molecules and is a growth factor for both B and T lymphocytes (Bondeson, 1997). Both TNF-α and IL-1β can lead to degranulation and bone resorption and are important cytokines in chronic inflammatory diseases such as rheumatoid arthritis (Bondeson, 1997; Dayer, 2004). IL-6 has also been shown to be important in a variety of inflammatory conditions and is particularly important in the production of acute phase proteins (Dielh and Rincon, 2002). Although the present study models predominately an acute inflammatory condition, our results provide valuable information on the effects of notoginseng on macrophages, cells critical in the process of chronic inflammation (LeKowitz et al., 1995). The results presented herein show that notoginseng can decrease the LPS-induced production of TNF-α and IL-6 and reduce the mRNA expression of IL-1β and COX-2 in LPS-stimulated RAW264.7 cells (Figs. 1 and 2). Cyclooxygenase is a key enzyme involved in the conversion of arachidonic acid into prostaglandins (Turini and DuBois, 2002). It has been demonstrated to be a critical pro-inflammatory enzyme contributing to the development of many chronic inflammatory diseases such as cardiovascular disease, cancer and rheumatoid arthritis (Rocca and FitzGerald, 2002). IL-1β is a cytokine that is produced early in the generation of an inflammatory response (Stylianou and Saklatvala, 1998). It is produced in increasing amounts in diseases such as rheumatoid arthritis and atherosclerosis (Bondeson, 1997; Andreau et al., 2004). Our results demonstrate that notoginseng effectively inhibits the generation of cytokines and enzymes in this monocyte/macrophage cell line that are paramount in the generation of an inflammatory response.

In addition to the effects on cytokines production, notoginseng also significantly affects the expression of key accessory molecules expressed on macrophages. Bacterial products are potent inducers of many pro-inflammatory genes including accessory/costimulatory molecules such as CD40 and CD86. CD40 and CD86 subsequently promote sustained interactions between APCs and T cells (Grewal and Flavell, 1998). When stimulated with LPS, the cell surface expression of CD86 and CD40 is increased on RAW264.7 cells (Fig. 3 and Table 1). The upregulation of these molecules is typically observed during the course of an inflammatory response and functions to facilitate the recruitment and activation of leukocytes (Lenschow et al., 1996; Grewal and Flavell, 1998; Li and Stark, 2002). Notoginseng effectively decreased the expression of CD40 and CD86 molecules (Fig. 3). Because CD40 and CD86 are required for productive interactions between T cells and antigen presenting cells, decreasing their expression would be expected to attenuate conditions such as chronic inflammatory disease and autoimmunity (Lenschow et al., 1996; Quezida et al., 2004). In our experiments, LPS was used as the prototypical inflammatory stimulus because of its ability to initiate a range of pro-inflammatory mediators (Abreu and Arditi, 2004). LPS signaling occurs when LPS binding protein catalyzes the transfer of LPS to membrane or soluble CD14, which then mediates recognition of LPS via TLR4 signaling (Dunzendorfer et al., 2006). LPS (as well as TNF-α and IL-1β) is an effective activator of NF-κB (Karin and Ben-Neriah, 2000; Hanada and Yoshimura, 2002). NF-κB activation induces the expression of many inflammatory cytokine genes (including TNF-α and IL-1β) and accessory molecules. In our studies, notoginseng did not affect the expression of TLR4 or CD14 on RAW264.7 cells suggesting that this herbal extract does not decrease LPS uptake or TLR 4 signaling in these cells. However, although TLR4 and CD14 expression was unaffected by notoginseng, LPS signaling could potentially be interrupted in its downstream signaling pathway as has been previously reported (Lee et al., 2002). In these studies, disruption of NF-κB activation was demonstrated in both mouse skin cells and the human pro-myelomonocytic cell line, HL-60, by purified ginsenosides (Lee et al., 2002; Keum et al., 2003). Furthermore, Oh et al. (2004) recently reported that 20(S)-protopanaxatriol, a ginsenoside metabolite, inhibits iNOS and COX-2 expression via the inactivation of NF-κB in LPS-stimulated RAW264.7 cells. Currently, studies in our laboratory are underway to further characterize the effects of notoginseng on the activation of NF-κB.
Varying the duration of notoginseng exposure results in differential effects on the inflammatory responsiveness of our cultured macrophages. In studying the time-dependent effects of notoginseng on TNF-α production, a trend was observed towards decreasing LPS-induced production of TNF-α by cells stimulated 8 h prior to notoginseng addition. However, significant suppression of this cytokine was only noted for both the pretreated and concomitant-treated samples (Fig. 4). Similar results in our laboratory have also been observed with the dendritic cell line, DC2.4 (manuscript in preparation). Reduced effects following delayed notoginseng treatment may be attributed to an inability of notoginseng to disrupt LPS-stimulated signaling cascades following an initial activation period as notoginseng may affect initial events in the activation of RAW264.7 cells. Alternatively, LPS signaling may also reduce the uptake of notoginseng into cells ultimately decreasing its effectiveness. Thus, it is possible that notoginseng and LPS may bind to a similar receptor, thereby antagonizing the uptake of the other, if not added concurrently. This possibility is currently being investigated in our laboratory.

Ginsenosides are believed to be the primary bioactive compounds in notoginseng. Ginsenoside composition of plants can vary widely depending on factors such as time of harvest, location and seasonal variations (Lenschow et al., 1996). These parameters can result in the “yin and yang” in ginseng with various extracts containing significantly different activities based on variable ginsenoside profiles (Sengupta et al., 2004). Therefore, from a clinical perspective it is important to determine if a particular ginsenoside accounted for the bioactivity of notoginseng. To address this possibility, purified samples of ginsenosides Rb1 and Rg1 were obtained and their concentration matched to that present in our whole notoginseng extract. Although these purified ginsenosides suppressed TNF-α production, the degree of suppression was not as great as that observed in our original notoginseng samples even when both ginsenosides were combined (Fig. 5). This result could be because although Rb1 and Rg1 accounted for approximately 70% of our extract, the ginsenoside Re1 and notoginsenoside R1 (each comprising approximately 4% of notoginseng) may also be contributing to the activity of the whole extract. Indeed, studies have shown that notoginsenoside R1 can significantly antagonize the endotoxin-induced activation of endothelial cells in vitro and endotoxin-induced lethality in mice (Zhang et al., 1997). Furthermore, our whole notoginseng extract was obtained from a different commercial source than our purified ginsenosides. Thus, even if these compounds are similar in concentration, stereoisomerism may still exist. Stereosomism of natural and synthetic compounds has been shown to contribute to different activities in vivo and in vitro (Mullerheim et al., 2001; Smith et al., 2005). In vitro studies with different enantiomers of lipoic acid showed that the S isomer had more activity than the R isomer. Similar mechanisms could explain the reduced suppressive effects observed when the RAW264.7 cells were treated with the purified ginsenoside compounds but not the whole extract of notoginseng.

In conclusion, the current study demonstrated that notoginseng treatment of RAW264.7 cells results in a decreased production of the inflammatory cytokines TNF-α and IL-6, mRNA expression of COX-2 and IL-1β, and cell surface expression of CD40 and CD86 following LPS stimulation. These results establish that notoginseng has potent anti-inflammatory effects and may hold great promise for use in the treatment of acute and chronic inflammatory diseases in humans.

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