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Ginsenoside Rg1, a Novel Glucocorticoid Receptor Agonist of Plant Origin, Maintains Glucocorticoid Efficacy with Reduced Side Effects

Juan Du,*,1 Binbin Cheng,*,1 Xiaoyan Zhu,‡ and Changquan Ling*

Glucocorticoids (GCs) are widely used to treat inflammatory diseases. However, they cause debilitating side effects, which limit the use of these compounds. In the past decade, many researchers have attempted to find so-called dissociated GCs that have separate distinct transactivation and transrepression activities. Anti-inflammation of GCs is a result of glucocorticoid receptor (GR)-mediated transactivation and transrepression in some tissues, similar to their side effects; therefore, the goal to discover a compound that has anti-inflammatory properties, but lacks the negative side effects seen with GCs, has yet to be achieved. In the present study, we introduce a plant-derived compound, ginsenoside Rg1, which possesses GC and estrogen-like activities. In this study, we show that Rg1 downmodulates LPS-induced proinflammatory cytokine release and inhibits NF-κB nuclear translocation and DNA binding activity. The negative effects on NF-κB activation are due to a decrease in IκB phosphorylation and protein stabilization. Furthermore, the inhibitory effect of Rg1 on NF-κB is GR-dependent, as small interfering RNA knockdown of GR abrogated this function. Rg1 also displayed profound inhibitory effects on LPS-induced MAPK activation. Importantly, Rg1 did not impair proliferation or differentiation of mouse osteoblasts. Finally, we show that Rg1 can effectively inhibit acute and chronic inflammation in vivo, but it does not cause hyperglycemia or osteoporosis as seen with dexamethasone. These results suggest that ginsenoside Rg1 may serve as a novel anti-inflammatory agent and may exhibit a potential profile for therapeutic intervention in inflammatory diseases. The Journal of Immunology, 2011, 187: 942–950.

Glucocorticoids (GCs) are presently the most potent drugs for the treatment of acute and chronic inflammatory diseases. Unfortunately, the use of GCs is limited by unwanted severe side effects, such as osteoporosis, disorders of glucose and lipid metabolism, and hypertension. Therefore, there is a major need for the development of compounds with the anti-inflammatory potency of standard GCs but with reduced side effects (1–3).

The effects of GCs are mediated mainly by glucocorticoid receptor (GR), a member of the nuclear receptor superfamily. GR regulates its target genes in either a positive or negative way. Positive regulation, or transactivation, is mediated mainly by direct binding of ligand-activated homodimerized GR onto positive GC response elements. In contrast, the suppressive effects, or transrepression, are classically attributed to the repression of proinflammatory transcription factors, such as NF-κB and AP-1 (4–6). NF-κB is a heterodimeric transcription factor, composed of p65 and p50 subunits anchored to an inhibitor molecule, IκB. Upon stimulation of cells with LPS, TNF, irradiation, or viral infection, IκB is phosphorylated and subsequently degraded, allowing NF-κB nuclear translocation into the nucleus where it regulates transcription of its target genes (7).

The hypothesis that the active and repressive functions of GR are responsible for negative side effects and anti-inflammatory effects, respectively, has led to the discovery of a number of interesting compounds (2, 3, 8–14). However, accumulating evidence suggests that the transcriptional repression by GR alone does not fully account for the repression of inflammatory genes (15). There are a number of GC-inducible genes with anti-inflammatory effects that have been discovered, indicating that transactivation also contributes to the anti-inflammatory effects (16–24). As a result, novel compounds that lack capacity for steroid-inducible genes may in fact show lessened anti-inflammatory effects (9–11). Therefore, the goal has focused primarily on developing novel GR ligands that induce a just a subset of GR activities (25–27).

Ginseng, the root of Panax ginseng C.A. Meyer, has been a key component in traditional Chinese medicine for >1000 y. It is now one of the most extensively used alternative medicines throughout the world. The molecular components responsible for ginseng’s actions are ginsenosides (28, 29), which are triterpene saponins that have a rigid steroidal skeleton with sugar moieties (30, 31). Among the ginsenosides, Rg1 is the most abundant and is the active ingredient of P. ginseng (32, 33). Previous studies have shown Rg1 to be a functional ligand of GR (34–36). Furthermore, Rg1 inhibits LPS-stimulated cytokine production in vitro, indicating that Rg1 may exert anti-inflammatory effects via GR (37, 38). Interestingly, Rg1 also has estrogen-like activities, although direct interaction with the estrogen receptor was undetected (39). Estrogen has been shown to have a positive effect on glucose metabolism and bone formation. For example, estrogen protected osteocytes against dexamethasone (DEX)-induced
apoptosis and prevented olanzapine-induced hepatic glucose output increase (40–42). Based on these findings, we hypothesize that Rg1 may be used as a novel therapeutic modality for inflammation without the negative side effects seen with other more widely used steroid anti-inflammatory drugs.

In this study, we demonstrate that Rg1 is capable of efficiently inhibiting inflammation via GR but with minimal side effects on osteoblast proliferation and differentiation in vitro. Most interestingly, apart from being just as effective as DEX as an anti-inflammatory agent in both acute and chronic inflammatory in vivo models, Rg1 also displays a better side effect profile in vivo. That is, Rg1 does not induce osteoporosis, hyperglycemia, or immune organ weight loss. Taken together, these results may lead to the classification of this plant-derived compound as an effective anti-inflammatory agent with better therapeutic index than classic GCs.

Materials and Methods

Cytokines and reagents

LPS and DEX were purchased from Sigma-Aldrich (St. Louis, MO). Rg1 was purchased from Shanghai Dongfang Pharmaceutical (Shanghai, China). Phospho-IκB-α (Ser211), phospho-MAPK family Abs (total and phosphorylated: JNK, p38, and ERK), phospho-GR (Ser211), phospho-MAPK family Abs (total and phosphorylated: JNK, p38, and ERK), phospho-Isocitrate dehydrogenase, and NF-kB were purchased from Cell Signaling Technology (Danvers, MA). p-Phospho-IκB-α, p-ERK, p-JNK, p-p38, NF-kB, and p-RELA were purchased from Cell Signaling Technology (Danvers, MA). p-NF-kB-Luc and p-RELA luciferase-thymidine kinase were purchased from Promega (Madison, WI). Small interfering RNA against GR (siGR) and NF-κB were purchased from Shanghai RiboBio Co. Ltd. (Shanghai, China). Promega (Madison, WI). Small interfering RNA against GR (siGR) and NF-κB were purchased from Shanghai RiboBio Co. Ltd. (Shanghai, China).

Cell culture

RAW264.7 and A549 cell lines were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO2. The MC3T3 cell line was cultured described previously (46). Western blot analysis

Cells were seeded in six-well plates. Total protein and cytoplasmic and nuclear proteins were extracted as described previously (43, 44). The protein concentration was determined by the bicinchoninic acid method.

EMSA

NF-kB EMSA was performed as described in Matsasaka et al. (45).

NF-kB reporter gene assay

After A549 cells reached 90–95% confluence in 24-well dishes (5 × 104 cells/well), they were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 20 pM siCtrl or siGR, 0.48 μg/ml p-NF-κB-Luc, and 0.08 μg/ml p-RELA luciferase-thymidine kinase for normalization in serum-free medium. Four hours later, the medium was replaced with medium with 10% FBS. Forty-eight hours posttransfection, cells were pretreated for 6 h in serum-free DMEM containing 1 μM DEX or 10 μM Rg1, after which LPS (1 μg/ml) was added and the reporter gene activity was measured according to the recommendation of the manufacturer (Promega, Madison, WI). Results were normalized by thymidine kinase reporter activity.

Cell proliferation and alkaline phosphatase activity

Cell proliferation was measured by MTT colorimetric assay. This system is active only in metabolically active cells, and changes in MTT reductase activity are detectable even before membrane lysis; thus, the MTT assay is a reporter marker of cellular viability. MC3T3-E1 cells were cultured in 96-well plates (1 × 2000 cells/well) in differentiation medium with various concentrations (10–12–10–7 M) of DEX or Rg1 for 48 h at 37˚C, after which the absorbance at 570 nm was recorded. The OD of each well was quantified as a percentage compared with the control, and the data were expressed as the means in six duplicate wells from at least three independent experiments. To analyze alkaline phosphatase (ALP) activity, the cells were cultured in 12-well plates, and after the cells reached confluence, the medium was replaced with differentiation medium. After 2 d culture in differentiation medium, the cells were cultured for 10 d in the absence or presence of various doses of Rg1 or DEX. The medium was removed and the cell monolayer was gently washed twice with PBS. The cells were lysed with 0.1% Triton X-100, and the lysate was centrifuged at 14,000 × g for 5 min. Aliquots of supernatants were subjected to protein assay using a bicinchoninic acid-protein assay kit (Pierce Chemical, Rockford, IL), and ALP activity was determined as previously described (46).

Zymosan-induced inflamed paw model

Male C57BL/6j mice (6–8 wk old) were purchased from the Second Military Medical University Laboratory Animal Center (Shanghai, China). The experimental setup contained three groups with eight animals per group. Group 1 was treated i.p. with 500 μl 10% ethanol solution (vehicle for Rg1 and DEX) followed 30 min later by a s.c. injection of 20 μl zymosan solution (15 mg/ml in PBS, sterilized) in the right footpad and 20 μl PBS in the left footpad. Group 2 received 50 μg DEX i.p. (500 μl 0.1 mg/ml DEX in 10% ethanol) 30 min before zymosan-PBS treatment, and group 3 was treated with 250 μg Rg1 (500 μl 0.5 mg/ml Rg1 in 10% ethanol) followed 30 min later by a zymosan-PBS treatment. Twenty-four, 48, and 72 h after the zymosan-PBS treatment, the thickness of both footpads was measured using a caliper, and the difference between zymosan and PBS-injected footpads was compared for all three experimental groups. The study was performed in a double-blind fashion (10).

Collagen-induced arthritis mouse model

Male DBA/1 mice (6–8 wk old) were purchased from the Second Military Medical University Laboratory Animal Center (Shanghai, China). On day 1, mice were injected intradermally at the base of the tail with 1:1 emulsion of CFA (Ameresco, Solon, OH) and bovine type II collagen (Chondrex, Redmond, WA) (dissolved and stored in 0.01 M acetic acid at 2 mg/ml) (100 μl/mouse). The injection was repeated at day 21. From day 18, mice were monitored daily for clinical symptoms of arthritis. Once arthritis was evident, the animals were randomized in one of the following treatment groups: vehicle, Rg1, and DEX by i.p. injection for 14 d. There were three groups with eight mice per group. Mice were scored for clinical signs of arthritis and grades ranging from 0 to 4 (14, 47): grade 0, no visible abnormalities; grade 1, mild redness or swelling of the wrist or up to three inflamed digits; grade 2, more than three inflamed digits or moderate redness and swelling of ankle or wrist; grade 3, severe ankle and wrist inflammation; grade 4, extensive ankle and wrist inflammation, including all digits, or new bone formation with reduced motion. A maximal score of 16 could be achieved for each mouse. The data were analyzed by one-way ANOVA, followed by the Fisher least significant difference test. A P value <0.05 was considered to be statistically significant (14).

Blood glucose determination

Food was removed overnight. Six hours after i.p. injection of vehicle (10% ethanol), Rg1 (12.5 mg/kg), or DEX (2.5 mg/kg) (eight mice per group), blood samples were taken by caudal vein puncture under ethyl ether anesthesia. Blood glucose levels were determined by CareSens blood glucose monitoring system (i-SENS, Seoul, Korea) according to the manufacturer’s instructions.

Bone formation in vivo

Male ICR mice (6–8 wk old) were purchased from the Second Military Medical University Laboratory Animal Center (Shanghai, China). Mice were injected i.p. with 10% ethanol (vehicle for Rg1 and DEX), DEX, or Rg1 daily for 4 wk. Throughout the experiment, animals were maintained on a 12 h light/12 h dark cycle (lights on at 6:00 AM) at 22˚C with food and water available ad libitum. Treatment groups consisted of vehicle, DEX (2.5 mg/kg), and Rg1 (12.5 mg/kg). After 28 d treatment, animals were sacrificed and bones were harvested for analysis. Changes in bone mineral density (BMD) and total cross-sectional area (CSA) of the left tibia were analyzed by peripheral quantitative computed tomography (pQCT). For histomorphometric examination, paraffin-embedded histological sections were stained using H&E techniques. We measured the bone volume in a standard zone, situated at least 0.3 mm from the growth plate, excluding the primary spongiosa and trabeculae connected to the cortical bone using BioQuant software. The ALP activity in serum was determined as described previously (46).
**Thymus, spleen, and body weight**

To determine the effects on total body weight, animals were weighed before and after treatments. To determine the effects on immune organs, animals were treated for 4 wk, sacrificed, and the thymus and spleen were removed from animals and weighed. All procedures involving animals were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609 EEC) and approved by the Ethics Committee of Shanghai Hospital.

**Results**

Ginsenoside Rg1 inhibits LPS-induced TNF-α and IL-6 production by means of negative interference with NF-κB pathways

It has been previously reported that Rg1 inhibits LPS-induced production of proinflammatory cytokines (37, 38). In agreement with these anti-inflammatory properties of Rg1, we found that ginsenoside Rg1 inhibits LPS-stimulated production of TNF-α and IL-6 by RAW264.7 cells in a manner similar to what we see when they are treated with DEX (Fig. 1A, 1B). To define the impact that Rg1 and DEX have on NF-κB activation, we first treated RAW264.7 cells with ginsenoside Rg1 or DEX and then stimulated the cells with LPS for 30 min. The cells were lysed and the nuclear and cytoplasmic fractions were separated and sub-

**FIGURE 1.** Effects of ginsenoside Rg1 and DEX on LPS-induced cytokine production and NF-κB activation. A and B, TNF-α and IL-6 ELISAs were performed with cell culture supernatant of RAW264.7 cells. Cells were treated with various concentrations of DEX (10⁻⁵–10⁻⁸ M) or ginsenoside Rg1 (10⁻⁵–10⁻⁸ M) for 2 h followed by 1 μg/ml LPS stimulation for 8 h. Data are shown as percentage inhibition. This experiment is representative of three independent experiments. C, After pretreatment with Rg1, LPS was added for 30 min and localization of p65 was determined by Western blot. In the presence of LPS, p65 was predominantly localized in the nucleus (lane 4). In the case of treatment with DEX or ginsenoside Rg1 following LPS stimulation, nuclear p65 was noticeably reduced (lanes 5 and 6, respectively). D, After pretreatment with Rg1 or DEX, LPS was added for 1 or 2 h (as indicated) and DNA-binding activity of NF-κB was determined by EMSA in nuclear extracts. E, After pretreatment with Rg1, LPS was added for the indicated time periods (15, 30, or 60 min). Western blot analysis with IκBα and phospho-IκBα Abs was performed on total cell extracts. β-actin was used as a loading control.

**FIGURE 2.** Attenuation of MAPK activation by ginsenoside Rg1. Cells were treated for 2 h with DEX (1 μM), Rg1 (10 μM), or vehicle, after which a kinetics experiment was performed. LPS (1 μg/ml) was added and left on the RAW264.7 cells for the indicated times (15, 30, or 60 min). Western blot analysis with phosphorylation-specific Abs that detect phosphorylated p38, ERK, and JNK was performed on total extracts. β-actin was used as a loading control.

**FIGURE 3.** Functional GR is required for inhibition of NF-κB activity. A, A549 cells were transiently transfected with 240 ng p-NF-κB-Luc, 40 ng p-RELLA luciferase-thymidine kinase Renilla control plasmid, and 20 pM siCtrl (left) or siGR (right) as indicated. Forty-eight hours later, cells were pretreated for 2 h with 1 μM DEX or 10 μM Rg1, and then LPS (1 μg/ml) was added where indicated. All inductions were allowed to continue for another 6 h. Relative luciferase activity was normalized by the thymidine kinase promoter activity. Data are presented as the means ± SD of at least three independent experiments. **p < 0.01 versus LPS stimulation. Statistical analysis was done using a one-way ANOVA followed by a Fisher least significant difference test. B, RAW264.7 (left) and A549 (right) cells were serum-starved in phenol red-free medium for 8 h before induction with vehicle, 1 μM DEX, or 10 μM ginsenoside Rg1 for 1 h. Western blot analysis with GR Abs was performed on nuclear and cytoplasm extracts. β-actin was used as a loading control.
greatly reduced when cells were treated with ginsenoside Rg1 or DEX prior to LPS stimulation (Fig. 1D).

To investigate the molecular basis of the altered subcellular localization and DNA binding of p65 after ginsenoside Rg1 treatment, we assessed phospho-IκBα and total IκBα protein levels in RAW264.7 cells (Fig. 1E). After stimulation of RAW264.7 cells with LPS for 15 min, phospho-IκBα protein expression levels increased (lane 4 versus lane 1). However, after 30 min LPS stimulation, total IκBα levels were dramatically decreased (lane 7 versus lane 1) and after 60 min of LPS treatment IκBα almost completely disappeared (lane 10 versus lane 1). LPS stimulation in combination with ginsenoside Rg1 or DEX treatment resulted in a decrease of phospho-IκBα (lanes 5 and 6 versus lane 4) and subsequent IκBα degradation (lanes 8 and 9 versus lane 7, and lanes 11 and 12 versus lane 10). It seems that the increase of phospho-IκBα protein expression by LPS at 30 and 60 min is rather small compared with LPS in combination with Rg1 or DEX, which may be explained by degradation of total IκBα protein after LPS stimulation at 30 and 60 min.

**Effects of ginsenoside Rg1 on MAPK activation**

MAPK pathways are considered important for fine-tuning the activity of NF-κB in the nucleus. Therefore, we investigated the effect of DEX and ginsenoside Rg1 on the activation of several members in the MAPK family of proteins (Fig. 2). As expected, stimulation of RAW264.7 cells with LPS for 15 and 30 min resulted in a significant increase in JNK, ERK, and p38 activation, as measured by phosphorylation (lanes 4 and 7 versus lane 1). Importantly, treatment with ginsenoside Rg1 inhibited LPS-induced activation of JNK, ERK, and p38. Similar results were seen with DEX (lanes 5 and 6 versus lane 4, and lanes 8 and 9 versus lane 7).

**Anti-inflammatory effect of ginsenoside Rg1 is GR-dependent**

Previous studies have demonstrated that ginsenoside Rg1 is a functional GR ligand (34). The fact that the transcriptional activity of NF-κB can also be modulated by different kinases, and that Rg1 treatment resulted in an efficient downregulation of MAPK phosphorylation, led us to ask whether Rg1 possesses inhibitory effects on NF-κB in a GR-dependent manner or other kinases. To address this, A549 cells were transfected with siGR or siCtrl (Fig. 3A, right and left panels, respectively). Downregulation of GR protein was monitored by Western blot. A significant silencing efficiency was achieved with siGR (data not shown). Therefore, we used these cells to test whether the transrepressive effects of Rg1 on NF-κB require the presence of functional GR. Fig. 3A shows that LPS-activated p-NF-κB-Luc expression can be significantly repressed (p < 0.01) by either DEX or Rg1 and that this inhibition is dependent on the presence of GR.

Because GR nuclear translocation is a prerequisite for its transactivation or transrepressive functions, the localization of GR after Rg1 treatment was determined. We demonstrate in this study that incubation with DEX or ginsenoside Rg1 leads to a clear shift of endogenous GR from cytoplasm to nucleus in both RAW264.7 and A549 cells (Fig. 3B, left and right panels, respectively). These results suggest that Rg1 exerts anti-inflammatory effects in part by inducing GR nuclear translocation.

**The effects of ginsenoside Rg1 and DEX on osteoblast proliferation and differentiation in vitro**

Inhibition of osteoblast proliferation is one of the mechanisms of GC-induced osteoporosis, which is mediated by GR (48, 49). In this study, we show that Rg1 had no inhibitory effect on the proliferation of osteoblast MC3T3-E1 cells, even at high concentrations (Fig. 4A). In fact, we begin seeing an increase in proliferation at the highest dose. In contrast to Rg1, and as expected, DEX strongly inhibited osteoblast proliferation and does so in a concentration-dependent manner (Fig. 4A; p < 0.01).

In addition to proliferation studies, we also examined the effect of DEX and Rg1 on osteoblast differentiation. ALP is an osteoblast phenotypic marker and an essential enzyme for mineralization. ALP activity was measured to determine the effects of DEX and Rg1 on osteoblast cells that were in culture for 10 d (Fig. 4B). Control cells that were cultured only in differentiation medium had a robust increase in ALP activity. However, upon treatment with 10⁻⁹ M or 10⁻⁸ M DEX, ALP activity decreased significantly compared with positive control group and Rg1 group (p < 0.01). Importantly, we saw no difference in ALP activity between the Rg1 group and the control group (Fig. 4B; p > 0.05).

Ginsenoside Rg1 displays anti-inflammatory properties in vivo without inducing hyperglycemia or osteoporosis

**Zymosan-induced inflamed paw model**

To study the possible anti-inflammatory effects of ginsenoside Rg1 in vivo, we opted for the zymosan-induced inflamed paw model, which is an acute inflammation mouse model. Mice are injected s.c. with zymosan in the footpad, and swelling is determined at several time points after the injection. Pretreatment with ginsenoside Rg1 (i.p.) is performed...
to evaluate anti-inflammatory effects, and DEX (i.p.) was chosen as positive control. In Table I, we show the means of the differences between zymosan- and PBS-treated footpads for all three groups (see Materials and Methods), and we also indicate whether there are significant differences between the individual groups by statistical analysis using the Student t test. Both DEX and ginsenoside Rg1 pretreatment show clear anti-inflammatory activities as demonstrated by significantly less swelling than that of vehicle control group (p < 0.01). There were no significant differences between DEX and ginsenoside Rg1 groups.

Collagen-induced arthritis mouse model. A chronic inflammation model is the collagen-induced arthritis (CIA) model. CIA is an animal model that shares certain clinical and pathological features with rheumatoid arthritis and has been used to examine the mechanism and progression of the disease. Dosing begins when soft tissue injury (edema, synovitis, and joint separation) is maximal, but before the onset of changes in the bone. On day 37, animals were scored for clinical signs of arthritis. Fig. 5 compares the inhibitory activity of ginsenoside Rg1 and DEX in the CIA model. Ginsenoside Rg1 and DEX are equally effective at decreasing the clinical signs of arthritis, with i.p. injection of 12.5 and 2.5 mg/kg, respectively (p < 0.01). This potency difference corresponds well with the slightly less potent activity detected in several in vitro assays.

Side effect induction. The transactivation-mediated increase in blood glucose after GC treatment reflects the risk for induction of diabetes mellitus. After treatment of mice with DEX, there was a significant increase in blood glucose levels (p < 0.01) compared with that of vehicle-treated mice (Fig. 6). In contrast, ginsenoside Rg1 did not affect blood glucose level.

Additionally, total body weight, thymus, and spleen weight were tested after systemic administration of DEX, Rg1, or vehicle. ICR mice treated with DEX for 28 d exhibited a number of steroid-related side effects, including decreased body weight (Fig. 7A), thymus weight (Fig. 7B), and spleen weight (Fig. 7C) compared with both vehicle- and Rg1-treated mice (p < 0.01). In contrast, ginsenoside Rg1 did not affect the body weight, thymus weight, or spleen weight (Fig. 7). Even though the efficacy of ginsenoside Rg1 and DEX were equivalent in the paw measurement parameter, these non-quantitative assessments suggest that ginsenoside efficacy was actually better than DEX.

The most severe side effect of long-term steroid use is osteoporosis and an increased risk of bone fracture. Our finding that ginsenoside Rg1 does not repress osteoblast proliferation or differentiation prompted us to test the BMD of ICR mice treated i.p. with vehicle, DEX at 2.5 mg/kg, or ginsenoside Rg1 at 12.5 mg/kg for 28 d. The changes of BMD and total CSA of the bones were analyzed by pQCT (Fig. 8A). After treatment with DEX we saw significantly decreased cortical bone thickness (Fig. 8C; p < 0.05) and area of tibiae compared with the vehicle control group (Fig. 8D; p < 0.05). Instead of thinning the bone cortex, which is a main feature of osteoporosis, ginsenoside Rg1 did not affect the tibia cortical thickness compared with vehicle control (Fig. 8C; p > 0.05). Furthermore, the bone content of the DEX treatment group was also significantly less than that of ginsenoside Rg1-treated (Fig. 8B; p < 0.05) and vehicle groups (Fig. 8B; p < 0.01). Because the bone content (Fig. 8B) and the bone area (Fig. 8D) of the DEX-treated group were both significantly reduced, no significant differences in BMD were observed among DEX, ginsenoside Rg1, and vehicle-treated groups (Fig. 8E; p > 0.05).

To further investigate the effects of Rg1 and DEX on bone formation in vivo, a histomorphometric analysis was done. Our results reveal that there was less trabecular bone volume/total volume in the DEX-treated group compared with vehicle control group or to the ginsenoside Rg1-treated group (Fig. 9B; p < 0.05). There was also a decrease in trabecular number in the DEX group compared with vehicle- and Rg1-treated mice (Fig. 9C; p < 0.01) as well as reduced trabecular thickness compared with vehicle- and Rg1-treated mice (Fig. 9D; p < 0.05). We saw a concomitant increase in trabecular separation in the DEX group compared with the vehicle and Rg1 groups (Fig. 9E; p < 0.01). Finally, we also looked at ALP activity, which as mentioned above, is a major feature of normal of bone formation, and we found that ALP activity in the serum of DEX-treated mice was significantly lower than that of vehicle and ginsenoside Rg1 groups (Fig. 9F; p < 0.01).

**Discussion**

The effort to find effective fully dissociated anti-inflammatory drugs has been ongoing for more than a decade (3). Despite extensive research, the development of a compound capable of se-

**Table I.** In vivo anti-inflammatory effects of ginsenoside Rg1 in a zymosan-induced inflamed paw mouse model (mean ± SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment</th>
<th>Difference with Zymosan-PBS</th>
<th>p Value Compared with Vehicle Group</th>
<th>p Value Compared with DEX Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Ethanol</td>
<td>1.31 ± 0.24</td>
<td><strong>0.01</strong></td>
<td></td>
</tr>
<tr>
<td>DEX</td>
<td>DEX</td>
<td>0.84 ± 0.23***</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>G-Rg1</td>
<td>G-Rg1</td>
<td>0.93 ± 0.27**</td>
<td>0.015</td>
<td>0.506</td>
</tr>
</tbody>
</table>

Mice were injected s.c. with zymosan in the footpad, and swelling was determined at 24 h after the injection. Pretreatment with ginsenoside Rg1, DEX, or solvent was for 30 min. Means ± SD were compared with a Student t test. G-Rg1, ginsenoside Rg1.

**p < 0.01 versus vehicle group.

**Figure 5.** Anti-inflammatory effects of ginsenoside Rg1 in a CIA mouse model. The impact of treatment by vehicle, ginsenoside Rg1, or DEX on arthritis disease score in a mouse model of arthritis is shown. Animals injected with adjuvant and collagen develop severe disease (vehicle), whereas untreated animals (normal) do not. DEX (2.5 mg/kg) and ginsenoside Rg1 (12.5 mg/kg) are capable of blocking existing disease in mice. **p < 0.01 versus vehicle. Statistical analysis used a one-way ANOVA followed by a Fischer least significant difference test.

**Figure 6.** Effect of Rg1 and DEX on blood glucose levels. Mice were treated with either vehicle, 2.5 mg/kg DEX, or 12.5 mg/kg ginsenoside Rg1. The blood glucose concentration was determined 6 h after treatment (mice were fasted 18 h before the blood samples were taken). Values are expressed as means ± SD. **p < 0.01 versus control group; ***p < 0.01 versus ginsenoside Rg1 group.
paring detrimental side effects from anti-inflammatory activity is still an unachieved pharmaceutical goal. This is likely because the anti-inflammatory and harmful side effects (e.g., hyperglycemia, weight loss) are both due to activated and repressed GR target genes in some tissues. Ginsenoside Rg1, as a kind of GR agonist, simultaneously possessing another steroid hormone activity, may be an alternative strategy to achieve the desired transcriptional output.

Our findings demonstrate that Rg1 dramatically represses the secretion of both TNF-α and IL-6 by RAW264.7 cells when stimulated with LPS in vitro. Inhibiting the production of IL-6 and TNF-α represents a potential mechanism for some aspects of the anti-inflammatory effect of GCs, possibly by the transrepression of NF-κB (50), suggesting that Rg1 also has anti-inflammatory activity. Furthermore, we set out to unravel how Rg1 imposes its inhibitory effect on NF-κB activation. We observed that Rg1 attenuates NF-κB cytoplasmic-to-nuclear translocation and DNA binding of p65. This observation was reflected in the decreased amount of p65 found in the nucleus of RAW264.7 cells treated with Rg1. The decrease in nuclear p65 correlated with a decrease

![FIGURE 7. Effect of ginsenoside Rg1 on mouse body weight, thymus weight, and spleen weight. A, Body weight was determined before and after treatment. B and C, Thymus and spleen weights were examined 4 wk after i.p. 2.5 mg/kg DEX or 12.5 mg/kg ginsenoside Rg1. D and E, The relative weights of the thymus and spleen (g/kg body wt). Values are expressed as means ± SD. *p < 0.05, **p < 0.01 versus vehicle group; ***p < 0.01 versus Rg1 group.](image)

![FIGURE 8. Effect of Rg1 on BMD of mouse tibia bone. A, The change of BMD and total CSA of the bone in vehicle, Rg1, and DEX treatment groups were analyzed by pQCT. The bone contents (B), bone cortex thickness (C), area of the cortex (D), and BMD (E) of vehicle-, DEX-, and Rg1-treated mice are shown. Values are expressed as means ± SD. *p < 0.05, **p < 0.01 versus vehicle group; ***p < 0.01 versus Rg1 group.](image)
in p65 transcriptional activity as measured by p65 binding to target DNA. The mechanism of Rg1-mediated p65 cytoplasmic retention was investigated in more detail, which revealed that Rg1 interfered with the NF-κB pathway in the cytoplasm of the cell. That is, we demonstrated that Rg1 decreased the phosphorylation of IkBα and attenuated IkBα degradation, and thereby hampered NF-κB translocation to the nucleus. This activity on NF-κB may be the major mechanism of Rg1-mediated anti-inflammation.

Although it has been described that GCs interfere with MAPK signaling pathways, thereby hampering full-blown NF-κB activity, this effect seems to be cell type-dependent (5). In this study we found that Rg1 treatment resulted in a strikingly efficient down-regulation of MAPK phosphorylation, including p38, ERK, and JNK, which was similar to the effects of DEX. This is in accordance with what has been described by Toh et al. (51), who demonstrated a clear suppressive effect of DEX on the amounts of phosphorylated p38, JNK, and ERK. A dual pathway, partially dependent on NF-κB and partially dependent on MAPK, may therefore explain the gene repression effects of Rg1 for inflammation inhibition.

It is widely accepted that the anti-inflammatory potential of steroidal ligands primarily resides in their ability to negatively modulate transcription factors, such as NF-κB, via GR. With a similar structure to GCs, we hypothesized that the biological effects of Rg1 are also likely mediated by GR. In line with this reasoning, we found that the presence of functional GR was needed for the effect of Rg1. The gene-repressive effect of Rg1 on LPS-induced NF-κB activation was negatively affected when GR was knocked down. This result indicates that Rg1 partially interferes with p65 activation in a GR-dependent manner. GR nuclear translocation is required for the physiological and pharmacological functions of GCs. Although no direct evidence for Rg1 binding to GR was reported in this study, our results clearly demonstrate that Rg1 induces nuclear translocation of GR in RAW264.7 and A549 cells, in accordance with previous studies that report Rg1 to be a functional ligand of GR in which a reporter gene and competitive binding methods were used (34–36).

Even though Rg1 was reported as a functional ligand of GR (34–36) and demonstrated to drive GR into nucleus, it seems that Rg1 exhibits an altered gene regulation profile, able to affect only a subset of the genes normally regulated by GCs. Our results in this study demonstrate that Rg1 does not impair proliferation or differentiation of osteoblasts, as is the case with DEX. It has been reported that Rg1 has estrogen-like activities (39) and, furthermore, estrogen has been shown to protect bone from DEX-induced apoptosis (40, 41). Whether the mechanism underlying the selectivity of Rg1 is related to the estrogen receptor is unknown at this time, but the issue deserves further study.

To confirm our in vitro results, we examined the role of Rg1 in acute and chronic mouse models: the zymosan-induced inflamed paw model and the CIA model. Both models have been used to test the anti-inflammatory activities of many compounds (10, 14, 52, 53). Our data clearly show an anti-inflammatory function of Rg1 in both the acute and chronic inflammatory models. Meanwhile, Rg1 had no unwanted effects on blood glucose or thymus and spleen weight. It is well known that GC-mediated anti-inflammatory activity comes with side effects, such as hyperglycemia and immunosuppression, that are related to GR-activated genes. For example, GCs activate hepatic gluconeogenesis, an important process in the adjustment of the blood glucose level, and GCs thus induce pathological changes in the liver (54). Thymus involution is another major negative side effect of GCs that is related to GR-activated genes in thymocytes (55). Our data show that Rg1 has significantly fewer negative effects on blood glucose and thymus and spleen weights compared with DEX, indicating fewer to no side effects related to the transactivation of GR induced by Rg1 in liver and immune organs.

Osteoporosis and increased risk of fracture and associated morbidity are the most debilitating side effects of long-term GC use (9), which are associated with both transcriptional repression and activation of GR (56). Rg1 did not repress osteoblast proliferation or differentiation in vitro, and therefore we examined the BMD and histomorphology of tibia in ICR mice following 4 wk administration of Rg1 or DEX. Although the total bone density and cortex bone density in the DEX-treated group was not significantly decreased, the cortex area, thickness, and bone mineral content were all decreased compared with those of the Rg1 and vehicle groups. Consistent with these results, there were greater trabecular bone volume/total volume, a higher trabecular number, and greater trabecular thickness with a concomitant reduction in trabecular separation in the Rg1-treated group, compared with those in the DEX group. Furthermore, ALP activity, which is one of the characteristic markers of bone formation, in serum of Rg1-treated mice was higher than that in DEX-treated mice. These in vivo data further suggest that Rg1 would not induce osteoporosis since it exerts few effects on osteoblast proliferation and differentiation at the anti-inflammatory dosage.

In summary, ginsenoside Rg1, which has been demonstrated to be a functional ligand of GR, shows a better therapeutic index in comparison with DEX in vitro and in vivo. We do not expect an advantage of Rg1 in comparison with standard GCs here, but ginsenoside Rg1 represents a novel GR agonist that exhibits tissue- or gene-specific activity. An extensive study of Rg1 on the re-
A relationship between its GCs and estrogen-like activity may offer more insights to fully understand its functional profiles.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


