FEBS Letters 513 (2002) 208–212 FEBS 25799

Induction of inducible nitric oxide synthase expression by 18β-glycyrrhetinic acid in macrophages

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Received 3 December 2001; revised 14 January 2002; accepted 14 January 2002

First published online 25 January 2002

Edited by Ned Mantei

Abstract Glycyrrhizin (GL), a triterpenoid saponin fraction of licorice, is reported to have anti-viral and anti-tumor activities and is metabolized to 18β-glycyrrhetinic acid (GA) in the intestine by intestinal bacteria. However, the mechanism underlying its effects is poorly understood. To further elucidate the mechanism of GA, the aglycone of GL, we investigated the effects of GA on the release of nitric oxide (NO) and at the level of inducible NO synthase (iNOS) gene expression in mouse macrophages. We found that GA elicited a dose-dependent increase in NO production and in the level of iNOS mRNA. Since iNOS transcription has been shown to be under the control of the transcription factor nuclear factor κB (NF-κB), the effects of GA on NF-κB activation were examined. Transient expression assays with NF-κB binding sites linked to the luciferase gene revealed that the increased level of iNOS mRNA, induced by GA, was mediated by the NF-κB transcription factor complex. By using DNA fragments containing the NF-κB binding sequence, GA was shown to activate the protein/DNA binding of NF-κB to its cognate site, as measured by electrophoretic mobility shift assay. These results demonstrate that GA stimulates NO production and is able to up-regulate iNOS expression through NF-κB transactivation in macrophages.

Key words: 18β-Glycyrrhetinic acid; Macrophage; Inducible nitric oxide synthase; Nuclear factor κB

1. Introduction

In immunocompetent hosts, the innate and adaptive arms of the immune system are relatively efficient at containing and killing microbial pathogens. Nitric oxide (NO) is a radical messenger molecule produced by the enzyme NO synthase (NOS) [1]. Three NOS isoforms have been characterized: the constitutively expressed neuronal NOS, endothelial NOS, and the inducible isoform of NOS (iNOS). iNOS expression is significantly induced by lipopolysaccharide (LPS) or cytokines in a variety of immune cells, including macrophages [1]. Moreover, NO has been identified as the major effector molecule involved in the destruction of microorganisms and tumor cells by activated macrophages during the non-specific host defense of the immune system [2–5]. In contrast, with these host protective actions, NO has also been implicated as a mediator of tissue injury. As a host defense molecule, NO also inhibits the proliferation of viruses, such as ectromelia virus, coxsackie virus B3, cytomegaloviruses, and hepatitis B virus [5–10]. In macrophages, nuclear factor κB (NF-κB) in cooperation with other transcription factors has been found to coordinate the expression of genes encoding iNOS. Moreover, NF-κB plays a critical role in the activation of immune cells by up-regulating the expression of many cytokines essential for the immune response [11].

Licorice (*Glycyrrhizza glabra* L.) and its main water-soluble constituent glycyrrhizin (GL), a pentacyclic triterpene derivative of the β-amyrin type (oleanane), have been widely used as an antidote, demulcent and as a folk medicine for generations in Asia and Europe, and it is currently used as a flavoring and sweetening agent in food products. After oral administration or intravenous injection, GL has been shown to be hydrolyzed by the glucuronidase in intestinal bacteria to its active principle aglycone, 18β-glycyrrhetinic acid (GA), which is then absorbed into the blood [12]. GL and GA have been shown to possess several beneficial pharmacological activities, which include an anti-ulcerative effect, anti-inflammatory activity, interferon (IFN)-γ induction, and anti-hepatotoxicity effect [13–15]. Moreover, GL has also been described as an anti-viral agent [16–18], and to have anti-tumor activity [19,20]. GL is extensively used in Japan and is being examined in Europe in patients with active and chronic hepatitis [21,22]. However, the details of its mechanism remain unclear.

It is well known that the inducible production of NO by macrophages inhibits the growth of many pathogens, including bacteria, fungi, viruses, and parasites. Thus, it is possible that GA-derived NO production may mediate the anti-viral and anti-tumor activities of GA. To test this hypothesis, we investigated the effects of GA on NO production and the molecular mechanisms underlying this effect.

2. Materials and methods

2.1. Chemicals

Chemicals and cell culture materials were obtained from the following sources: GA, *Escherichia coli* 0111:B4 LPS and polymyxin B sulfate from Sigma Co.; BAY 11-7082 from Biomol; MTT-based colorimetric assay kit from Roche Co.; LipofectAMINE Plus, RPMI 1640, fetal bovine serum, and penicillin–streptomycin solution from Life Technologies, Inc.; pGL3-4κB-Luc and the luciferase assay system from Promega; pCMV-β-gal from Clontech; and AmpliTaq® DNA polymerase from Perkin Elmer. Other chemicals were of the highest commercial grade available.

2.2. Animals

Specific pathogen-free BALB/C mice (female, 5–7 weeks old) were

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PII: S0014-5793(02)02311-6

obtained from KRIBB (South Korea). Animals were housed under normal laboratory conditions, i.e. at 21–24°C and 40–60% relative humidity under a 12 h light/dark cycle with free access to standard rodent food and water.

2.3. Preparation of peritoneal macrophages and cell cultures

Peritoneal macrophages were isolated from mice and cultured as described previously [23]. RAW 264.7 cells, a mouse macrophage cell line, were obtained from the American Type Culture Collection (Bethesda, MD, USA), and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator. GA was dissolved in dimethylsulfoxide and added directly to the culture media. Control cells were treated only with solvents, the final concentration of which never exceeded 0.1%, and this concentration did not show any effect on the assay systems.

2.4. Cell viability

Cell viability was assessed using a MTT-based colorimetric assay kit (Roche Co.), according to the manufacturer's instructions.

2.5. Nitrite assay

Peritoneal macrophages (2×10^5 cells/ml) or RAW 264.7 cells (5×10^5 cells/ml) were cultured in 48-well plates. After incubating for 24 h, NO synthesis was determined by assaying the culture supernatants for nitrite, the stable reaction product of NO with molecular oxygen, using Griess reagent as described previously [23].

2.6. Endotoxin assay

An E-Toxate test (*Limulus* amebocyte lysate; Sigma Chemical Co.) was used to assay GA for the presence of Gram-negative bacterial endotoxin (LPS), according to the manufacturer's instructions.

2.7. RNA preparation and iNOS mRNA analysis by reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 cells were cultured with GA at a density of 1×10^6 cells/ml for 6 h. Total cellular RNA was isolated by the acidic phenol extraction procedure of Chomczynski and Sacchi [24]. cDNA synthesis, semiquantitative RT-PCR for iNOS and β-actin mRNA, and the analysis of results were performed as described previously [23]. cDNA was synthesized from 2 μg of total RNA using an Omniscript RT-PCR kit as instructed. A cycle number was used that fell within the exponential range of response for iNOS (754 bp, 35 cycles) and β-actin (153 bp, 17 cycles). PCR reactions were electrophoresed through a 2.5% agarose gel and visualized by ethidium bromide staining and UV irradiation. Gel images were captured on a Gel Doc Image Analysis System (Kodak) and the yield of PCR products was normalized to β-actin after quantitative estimation using NIH Image software (Bethesda, MD, USA). The relative expression levels were arbitrarily set at 1.0 in the control group.

2.8. Transfection and luciferase and β-galactosidase assays

RAW 264.7 cells (5×10^5 cells/ml) were plated in each well of a 12-well plate, and 12 h later transiently co-transfected with the plasmids pGL3-4κB-Luc and pCMV-β-gal, using LipofectAMINE Plus according to the manufacturer's protocol. Briefly, the transfection mixture containing 0.5 μg of pGL3-4κB-Luc and 0.2 μg of pCMV-β-gal was mixed with the LipofectAMINE Plus reagent and added to cells. After 18 h, the cells were treated with GA or LPS for 12 h, and then lysed. Luciferase and β-galactosidase activities were determined as described previously [23]. Luciferase activity was normalized using β-galactosidase activity and was expressed relative to the activity of the control.

2.9. Electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described [25]. Two double-stranded deoxyoligonucleotides containing the NF-κB binding site (5′-GGGGACTTTCC-3′) [11] were end-labeled with [γ-^{32}P]dATP. Nuclear extracts (5 μg) were incubated with 2 μg of poly(dI-dC) and the ^{32}P-labeled DNA probe in binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin and leupeptin) for 10 min on ice. DNA was separated from the free probe using a 4.8% polyacrylamide gel in 0.5×TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA). Following electrophoresis, the gel was dried and subjected to autoradiography.

2.10. Statistical analysis

All experiments were repeated at least three times. Student's *t*-test was used to assess the statistical significance of differences. A confidence level of <0.01 was considered significant.

3. Results and discussion

As a host defense molecule, the inducible production of NO by macrophages appears to be important in the elimination of viruses and tumors [2–10]. Moreover, since GL is known to have anti-viral and anti-tumor activity [16–20], we investigated the effects of GA on the NO production and its effects on the level of iNOS gene expression in mouse macrophages. Our results demonstrate that GA stimulates NO production and is able to up-regulate iNOS expression through NF-κB transactivation. GA-induced NO production was assessed after incubating for 24 h using the Griess reaction. The basal level of NO in medium from untreated peritoneal macrophages was found to be less than 2 μM (Fig. 1). However, upon GA stimulation, NO release by peritoneal macrophages increased in a dose-dependent manner in the range 1–20 μM, and showed a cytotoxic action upon macrophages at concentrations exceeding 30 μM (Fig. 1). The potent macrophage activator LPS (0.5 μg/ml), when used as immunostimulator, increased NO production compared to the control. Consistent

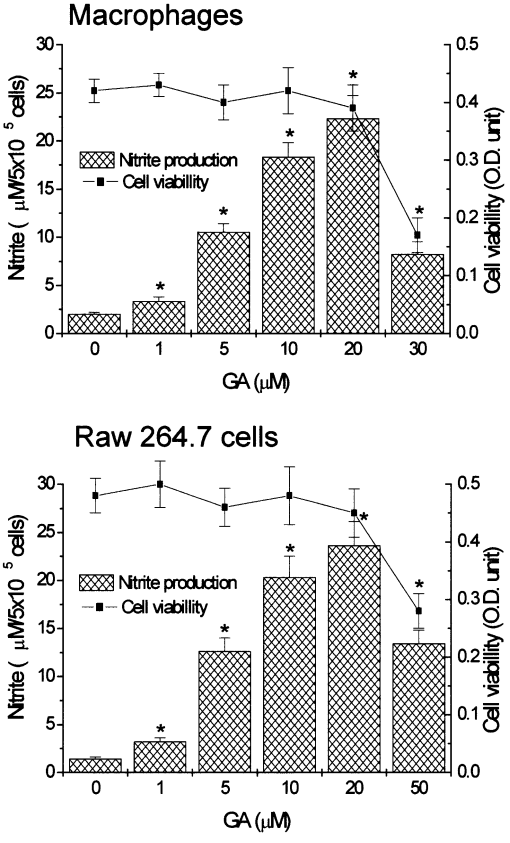

Fig. 1. Effects of GA on NO production. Murine peritoneal macrophages (2×10^5 cells/ml) or RAW 264.7 cells (5×10^5 cells/ml) were cultured for 24 h in the presence of media alone, with the indicated concentrations of GA. NO production was determined by measuring the accumulation of nitrite in the culture medium. Cell viability was assessed by MTT assay. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. $^*P<0.01$, significantly different from the control.

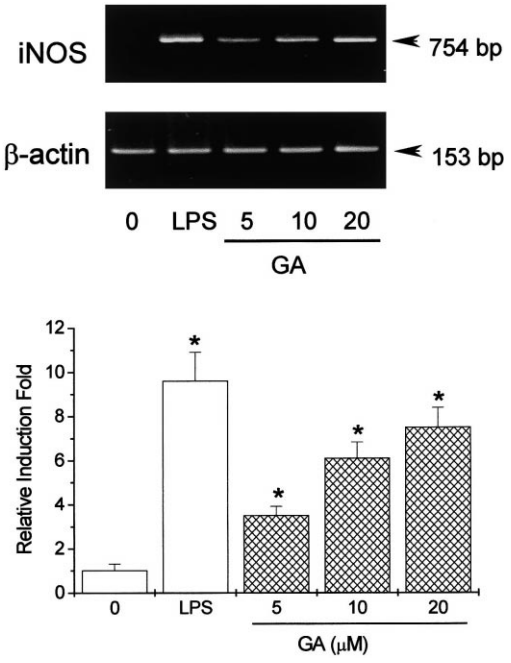

Fig. 2. Effects of GA on iNOS mRNA expression. RAW 264.7 cells (1×10^6 cells/ml) were cultured for 6 h in the presence of media alone, with the indicated concentrations of GA, or with LPS (0.5 μg/ml). Cells were lysed and total RNA was prepared for the RT-PCR analysis of gene expression. PCR amplification of the housekeeping gene, β-actin, was performed for each sample. The PCR amplification products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide. One of three representative experiments is shown. The ratio of the RT-PCR products of iNOS to β-actin was calculated. Induction-fold represents the mean ± S.D. of three separate experiments. $^*P<0.01$, significantly different from the control.

with these findings, GA also induced NO generation in a dose-dependent manner in RAW 264.7 cells (Fig. 1). Based on these results, and the relationship between NO and the anti-viral and anti-tumor functions of macrophages, we suggest that these effects of GA might be mediated in part through the activation of NO production.

Previous studies have shown that although GL alone did not induce NO from resting or unstimulated macrophages, NO production was enhanced in IFN-γ- or LPS-activated macrophages isolated from GL-treated mice [20,26], which suggested that GL has difficulty inducing NO production in the absence of some other stimulation. This may be because

Table 1
Effects of polymyxin B on NO secretion by GA and LPS

Treatment[a]	Nitrite (μM)[b]
Control	1.98 ± 0.23*,**
GA	19.62 ± 2.23*
GA+polymyxin B	20.24 ± 2.27*
LPS	42.38 ± 6.31**
LPS+polymyxin B	8.43 ± 0.93*,**

[a]RAW 264.7 cells (5×10^5 cells/ml) cultured with GA (10 μM) or LPS (0.5 μg/ml), in the presence or absence of polymyxin B (10 μg/ml).
[b]Supernatants were harvested after being cultured for 24 h and assayed for NO. Values are the means ± S.D. of three individual experiments, performed in triplicate.
$^*P<0.01$, significantly different from the LPS. $^{**}P<0.01$, significantly different from the GA.

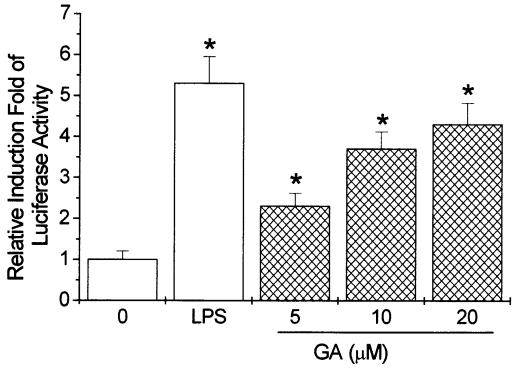

Fig. 3. Effects of GA on NF-κB-dependent luciferase gene expression. RAW 264.7 cells (5×10^5 cells/ml) were transiently co-transfected with pGL3-4κB-Luc and pCMV-β-gal. After 18 h, cells were treated with the indicated concentrations of GA or LPS (0.5 μg/ml) for 12 h. Cells were then harvested, and their luciferase and β-galactosidase activities determined. Luciferase activities are expressed relative to the control. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. $^*P<0.01$, significantly different from the control.

GL works synergistically with IFN-γ to induce NO production of macrophages or because of the low sensitivity of the assay. Moreover, it has been reported that GL enhances IFN-γ production in mice [14]. We also observed that GL slightly increased NO production in resting macrophages (data not shown). In the present study, however, GA, the aglycone of GL, significantly elicited a dose-dependent increase in NO production in the absence of any stimulator. Unlike as in previous studies [20,26], we did not need to co-stimulate with IFN-γ or LPS to generate NO with GA. Moreover, this GA-induced NO production was reversed when cells were treated with both GA and with *N*-nitro-L-arginine methyl ester, a competitive inhibitor of NOS (data not shown). Therefore, GA, unlike GL, has the ability to increase NO production alone in resting macrophages. The biological significance of the different effects of GA and GL on NO production in the resting and stimulated state needs to be determined.

Macrophages can be induced to produce NO by LPS or cytokines [11]. To confirm that the observed ability of GA to induce NO was not due to LPS contamination, the GA was tested for the presence of contaminating LPS by using the *Limulus* amebocyte lysate test. The level of LPS in GA was found to be below the detection limit, which is typically below 12.5 pg/ml (data not shown). Polymyxin B sulfate has been used previously as an LPS inhibitor in macrophage cultures [27], and although GA contained no detectable activity in the *Limulus* amebocyte lysate assay, we rechecked for possible LPS contamination in GA by adding polymyxin B (10 μg/ml) to cell cultures treated with GA (10 μM). As shown in Table 1, polymyxin B effectively inhibited the NO production induced by LPS, but had no effect on the induction by GA, which demonstrated that the production of NO by GA was unlikely to have resulted from LPS contaminating the GA.

As stated above, GA induced macrophage secretion of NO. In order to determine whether GA regulates NO production at the mRNA level, an RT-PCR assay was conducted. LPS was used as a positive control. Consistent with the results obtained from the NO assay, iNOS mRNA levels were markedly increased by GA treatment (Fig. 2). This result indicates

that GA up-regulates NO accumulation in macrophages in a dose-dependent manner. Therefore, we believe that increased NO production by GA is regulated through transcriptional activation.

Activated macrophages have the capacity to produce relatively large quantities of NO and NO-derived species, such as $NO_2^{\bullet}$, NO_2^-, N_2O_3, N_2O_4, *S*-nitrosothiols, and peroxynitrite ($ONOO^-$). Moreover, DNA and proteins are targets of reactive nitrogen intermediates. In addition, nitrogen intermediates and reactive oxygen intermediates can synergistically interact through the formation of peroxynitrites [28]. The reactive nitrogen intermediates formed by NO play a significant role in tumoricidal and microbiocidal activities [3]. Cysteine proteases are critical for virulence or replication of many viruses, bacteria, and parasites, and *S*-nitrosylation of pathogen cysteine proteases may be a general mechanism of the antimicrobial host defenses [29]. In addition, NO has been reported to interfere with specific stages in the life cycles of viruses. For example, NO inhibits DNA synthesis of the vaccinia virus and herpes simplex virus type 1, late protein translation, and virion assembly [30,31]. One specific viral target of NO has been identified; NO can inhibit the function and expression of the Epstein–Barr virus immediate early transactivator Zta [32]. Since NO can inhibit a variety of viruses, it is possible that NO also inhibits the cellular processes necessary for viral replication.

NF-κB is a member of the Rel family, and is a common regulatory element in the promoter region of many cytokines. In activated macrophages, NF-κB, in synergy with other transcriptional activators, plays a central role in coordinating the expression of genes encoding iNOS, tumor necrosis factor-α, and interleukin (IL)-1 [11]. To further investigate the role of GA on iNOS gene expression, we assessed the effect of GA on NF-κB-dependent gene expression by using the luciferase reporter gene assay. RAW 264.7 cells were transiently transfected with a plasmid containing four copies of the NF-κB binding sites, and the luciferase activities were measured. LPS, an immunostimulatory agent, was used as a positive control. When cells were stimulated with LPS a near five-fold increase in luciferase activity was observed versus the unstimulated control cells. Consistent with NO production and iNOS mRNA expression, GA also significantly increased NF-κB-dependent luciferase activities in a dose-dependent manner (Fig. 3). To further investigate the putative mechanism by which GA activates iNOS, the effect of GA on the activation of a family of transcription factors was monitored by electrophoretic mobility gel shift assay. NF-κB binding activity was examined in the light of its critical role in the regulation of iNOS. The results demonstrated that GA induced a marked increase in NF-κB binding at its conserved site, which was visualized as a distinct band (Fig. 4). Recently, it has been reported that GL treatment augmented IL-12 p40 mRNA expression in mice and that this effect may be associated with NF-κB activation [33]. To further confirm the role of NF-κB in iNOS expression by GA, we used BAY 11-7082, an inhibitor of IκBα kinase, which specifically inhibits NF-κB activation by inhibiting the phosphorylation and the subsequent degradation of IκBα, the endogenous inhibitor of NF-κB [34]. As shown in Fig. 5, pretreated RAW 264.7 cells with BAY 11-7082 effectively inhibited the NO production induced by GA. Although we demonstrated the up-regulatory effect of GA on iNOS gene expression through NF-κB transactivation in macrophages, the mechanism by which GA activates NF-κB is unknown, such as the activation of Raf-1 and mitogen-activated protein kinases [35]. Additional studies are needed to answer these questions and further elucidate the mechanisms involved.

GL has been widely and effectively prescribed as a therapy for chronic hepatitis [21,22]. Recently, it was reported that the long-term treatment with GL for chronic hepatitis C effectively inhibited liver carcinogenesis [36]. Cellular immune response has recently been shown to play an important role in patients who have recovered from hepatitis C virus infection

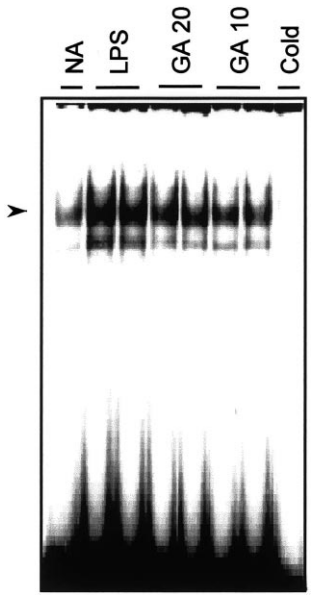

Fig. 4. Effects of GA on NF-κB binding. RAW 264.7 cells were treated with LPS (0.5 μg/ml) or GA (10, 20 μM) for 1 h. Nuclear extracts were isolated and used in an electrophoretic mobility shift assay with ^{32}P-labeled NF-κB oligonucleotide as a probe, as described in Section 2. The arrow indicates the NF-κB binding complex. Cold: 200-fold molar excess of non-labeled NF-κB probe. One of three representative experiments is shown.

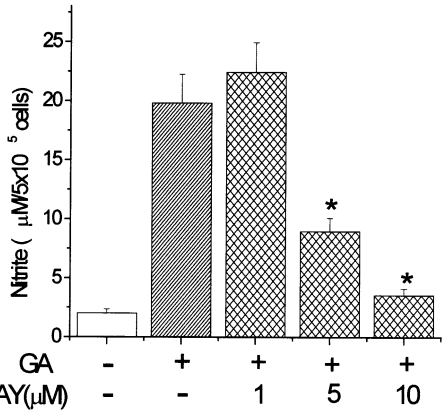

Fig. 5. Effects of NF-κB inhibition on NO production. RAW 264.7 cells (5×10^5 cells/ml) were pretreated with BAY 11-7082 for 1 h and then cultured for 24 h in the presence of media alone, with the indicated concentrations of GA. NO production was determined by measuring the accumulation of nitrite in the incubation medium. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. $*P<0.01$, significantly different from the control.

[37]. Therefore, GL may activate certain immune functions, and for this reason, it is important to elucidate the mechanisms associated with the immunomodulatory activities of GL. NO was investigated in the current study to confirm the possibility that GA might be an immunostimulator, and as a result, GA was found to elicit NO production. This result supports the notion that NO induction by GA may contribute in vivo to the immunomodulatory, anti-viral and anti-tumor activity of GA. Biological response modifiers are widely used in immunochemotherapy to potentiate therapeutic efficacy or to alleviate the toxicity of cytotoxic anti-cancer agents. Further studies on GA are needed to prove its immunochemotherapeutic usefulness and its exact mechanism.

In summary, our results show for the first time that GA stimulates macrophage-derived NO production, and is able to up-regulate iNOS expression through NF-κB transactivation in murine macrophages. These actions may provide a mechanistic basis for the anti-viral and anti-tumor properties of GA.

Acknowledgements: This work was supported by Korea Research Foundation Grant (KRF-2000-041-F00314).

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