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Bioorganic & Medicinal Chemistry 13 (2005) 3289–3294
Bioorganic & Medicinal Chemistry

1′*S*-1′-Acetoxychavicol acetate as a new type inhibitor of interferon-β production in lipopolysaccharide-activated mouse peritoneal macrophages

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Received 11 January 2005; revised 10 February 2005; accepted 10 February 2005
Available online 22 March 2005

Abstract—1′*S*-1′-Acetoxychavicol acetate from the rhizomes of *Alpinia galanga* was known to show potent inhibitory effect on the production of nitric oxide (NO) in lipopolysaccharide-activated mouse peritoneal macrophages. To clarify its mechanism of action, the effects of 1′*S*-1′-acetoxychavicol acetate on the expression of interferon-β (IFN-β) mRNA and activation of nuclear factor-κB (NF-κB), both of which participate in the induction of inducible NO synthase, were examined in lipopolysaccharide-activated macrophages. The results were compared with those of two inhibitors of the NF-κB activation, costunolide and caffeic acid phenethyl ester. 1′*S*-1′-Acetoxychavicol acetate inhibited IFN-β mRNA expression as well as NF-κB activation, and two related compounds, (±)-1-acetoxy-1-(2-acetoxyphenyl)-2-propene and (±)-1-acetoxy-1-(4-acetoxyphenol)-3-butene, also inhibited IFN-β mRNA expression. In addition, 1′*S*-1′-acetoxychavicol acetate inhibited the production of NO stimulated by poly(I:C) via Toll-like receptor 3.

1. Introduction

The Zingiberaceae plant, *Alpinia galanga* SWARTZ (syn. *Languas galanga* STUNZ), is widely cultivated in South and Southeast Asian countries. The rhizomes of this plant are extensively used as a spice or ginger substitute for flavoring foods, and also as a stomatic in traditional Chinese medicine, or as carminative, anti-flatulent, anti-fungal, and anti-itching agents in traditional Thai medicine. We previously reported gastroprotective and anti-allergic constituents from the rhizomes of *A. galanga* and their structure–activity relationships,[1,2] and that 1′*S*-1′-acetoxychavicol acetate (**1**) showed inhibitory activity against production of nitric oxide (NO) with IC_{50} of 2.3 μM.[3,4]

In our continuing studies on this natural medicine, we investigated the mechanism of action of 1′*S*-1′-acetoxychavicol acetate (**1**), which had the most potent inhibitory effect on NO production among constituents isolated from an aqueous acetone extract from the rhizomes of *A. galanga*, in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages.[3]

The inorganic free radical nitric oxide (NO) has been implicated in physiological and pathological processes such as vasodilation, nonspecific host defense, ischemia-reperfusion injury, and chronic or acute inflammation. NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). In the NOS family, inducible NOS (iNOS) is in particular well known to be involved in the pathological overproduction of NO.[5] Previously, **1** was reported to inhibit production of NO by preventing the activation of nuclear factor-κB (NF-κB), which regulates iNOS in LPS or interferon (IFN)-γ-treated murine macrophage–like RAW264 cells.[6,7] However, recent studies revealed that three nuclear factors, signal transducer and activator of transcription-1 (STAT1) and interferon regulatory factor-1 (IRF1) as well as NF-κB, were essential to bind the gene promoter area for expression of iNOS mRNA, and iNOS gene expression is inhibited by the suppression of either of these nuclear factors.[8–10] IRF1 is regulated by NF-κB and STAT1, and STAT1 was activated by IFN-β or IFN-γ.[10]

Previously, **1** was reported to inhibit the activation of NF-κB by attenuating the degranulation of IκB and the inhibition of STAT1 by IFN-γ.[6,7] However, the effect of **1** on IFN-β was not clarified.

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doi:10.1016/j.bmc.2005.02.022

In the present study, we describe an inhibitory effect on the expression of IFN-β and IRF1 mRNA as an additional mechanism of action of **1** affecting NO production in LPS-activated mouse peritoneal macrophages.

2. Chemicals

1′*S*-1′-Acetoxychavicol acetate (**1**) and costunolide (**2**) were isolated from the rhizomes of *A. galanga* and the roots of *Sausurea lappa*, respectively, and (±)-1-acetoxy-1-(2-acetoxyphenyl)-2-propene (**4**) and (±)-1-acetoxy-1-(4-acetoxyphenyl)-3-butene (**5**) were synthesized as described previously[3,4,11] (Fig. 1). Caffeic acid phenethyl ester (CAPE) (**3**) was obtained from Calbiochem Co.; LPS (from *Salmonella enteritidis*) and RPMI 1640 medium from Sigma-Aldrich Co.; 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl tetrazolium bromide (MTT) from Dojindo Laboratories; protease inhibitor cocktail (Complete Mini) from Roche Diagnostics GmbH; fetal calf serum (FCS) from Equitech-bio, Inc.; anti-mouse iNOS antibody (monoclonal) from BD Biosciences; anti-mouse IgG antibody conjugated to horseradish peroxidase, and the enhanced chemiluminescense (ECL) kit, γ-[^{33}P]-ATP, RT-PCR kit (Ready-to-Go RT-PCR Beads), and polyriboinosinic: polyribocytidylic acid [poly(I:C)] from Amersham Biosciences Co.; thioglycolate (TGC) medium from Nissui Pharmaceutical Co., Ltd; NE-PER® nuclear and cytoplasmic extraction reagents from Pierce Biotechnology Inc.; NF-κB consensus oligonucleotide and T4 polynucleotide kinase (Gel Shift Assay Systems) from Promega Co. (Madison, WI, USA); Nitrocellulose membranes (0.25 μm) from Bio-Rad Laboratories, Inc.; the total RNA extraction kit (RNeasy™Mini) from Quiagen, Inc.; and 96-well microplates and 6-well culture plates (3.4 cm, id) from Sumitomo Bakelite Co., Ltd. Other reagents were from Wako Pure Chemical Industries, Ltd.

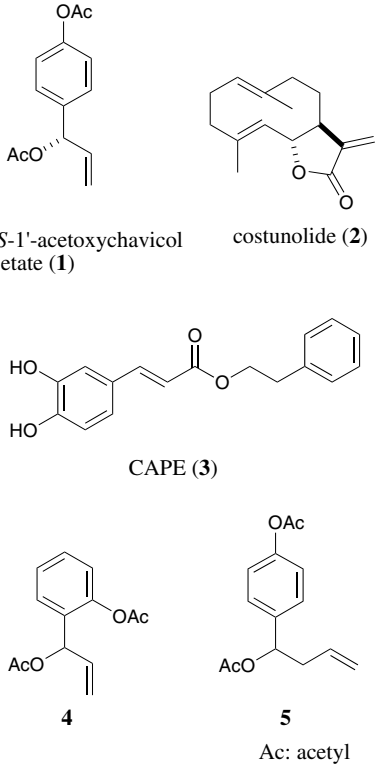

Figure 1. Chemical structures of **1**–**5**.

3. Bioassay methods

3.1. NO production

NO production by LPS via Toll-like receptor (TLR) 4 was examined as described previously.[12–14] Briefly, peritoneal exudate cells (5×10^5 cells/well) were collected from the peritoneal cavities of male ddY mice that had been injected (ip) with 4% TGC four days before, and were suspended in 100 μL of RPMI 1640 medium supplemented with 5% FCS, penicillin (100 units/mL) and streptomycin (100 μg/mL), and pre-cultured in 96-well microplates at 37 °C in 5% CO_2 in air for 1 h. Nonadherent cells were removed by washing with PBS, and the adherent cells were cultured in 200 μL of fresh medium containing 10 μg/mL LPS and various concentrations of test compound for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite (NO_2^-) in the culture medium using Griess reagent. NO production by poly(I:C)[15] via TLR3 was examined as described above using 125 μg/mL poly(I:C) instead of LPS.

Cytotoxicity was determined by the MTT colorimetric assay. Briefly, after 20 h incubation with the test compounds, MTT (10 μL, 5 mg/mL in PBS) solution was added to the wells. After a further 4 h of culture, the medium was removed, and isopropanol containing 0.04 M HCl was added to dissolve the formazan produced in the cells. The optical density (OD) of the formazan solution was measured with a microplate reader at 570 nm (reference: 655 nm). When the OD of the sample-treated group reduced and dropped below 80% of that in the vehicle-treated group, the test compound was considered to exhibit a cytotoxic effect. Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration, 0.5%). Inhibition (%) was calculated with the following formula and the IC_{50} was determined graphically ($N = 4$).

$$\text{Inhibition}(\%) = \frac{A - B}{A - C} \times 100$$

$$A - C : \text{NO}_2^- \text{ concentration}(\mu\text{M})$$

$$[A : \text{LPS}(+),\ \text{sample}(-); B : \text{LPS}(+),\ \text{sample}(+);$$

$$C : \text{LPS}(-), \text{sample}(-)]$$

3.2. Reverse transcription-coupled PCR (RT-PCR) analysis

Peritoneal exudate cells (3×10^6 cells/2 mL/well) obtained as described above were pre-cultured in 6-well culture plates for 1 h, and washed. The culture medium was then exchanged for the fresh medium containing 5% FCS, 10 μg/mL LPS, and test compound for 1 or 4 h.

Total RNA was extracted from cells using RNeasy™Mini (Quiagen) following the manufacturer's directions. RT-PCR was performed using Ready-to-Go RT-PCR Beads. Equal amounts of total RNA (0.6 μg) corresponding to each priming dose were reverse transcribed using oligo(dT)$_{12–18}$ (0.5 μg/μL) as a first-strand primer. The following specific primers (from Invitogen) were used: iNOS mRNA, sense 5′ CACCACAAGGCCACATCGGATT 3′, and antisense 5′ CCGACCTGATGTTGCCATTGTT 3′; IFN-β mRNA 5′ CTCCAGCTCCAAGAAAGGACG 3′, antisense 5′ GAAGTTTCTGGTAAGTCTTCG 3′; IRF-1 mRNA 5′ CACACGGTGACAGTGCTGG 3′, antisense 5′ CAGAGGAAAGAGAGAAAGRCC 3′; G3PDH 5′ ACCACAGTCCATGCCATCAC 3′, and antisense 5′ TCCACCACCCTGTTGCTGTA 3′. Reverse transcription was performed at 42 °C for 30 min. Thermocycling parameters were as follows: denaturation at 95 °C for 5 min and 27 cycles for IFN-β, 26 cycles for iNOS mRNA, 19 cycles for IRF1, and 23 cycles for G3PDH, consisting of incubations at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. After PCR, 15 μL of the reaction mixture was subjected to electrophoresis on a 2% agarose gel, and visualized by ethidium bromide staining.

3.3. Detection of iNOS

Detection of iNOS was performed as described in a previous report[11] with slight modification. TGC-induced peritoneal exudate cells (7.5×10^6 cells/2 mL/well) were pre-cultured in 6-well culture plates for 1 h, and washed. The culture medium was then exchanged for fresh medium containing 5% FCS, 10 μg/mL LPS, and test sample for 2–20 h. Cells were collected in lysis buffer [100 mM NaCl, 10 mM Tris, Complete Mini (1 tab/10 mL), 0.1% Triton X-100, and 2 mM EGTA] and sonicated. After the determination of the protein concentration of each suspension, the suspensions were boiled in Laemmli buffer. For SDS-PAGE, aliquots of 50 μg of protein of each sample were subjected to electrophoresis in 7.5% polyacrylamide gels. Following electrophoresis, the proteins were electrophoretically transferred onto nitrocellulose membranes, which were incubated with 5% nonfat dried milk in Tris-buffered saline (T-TBS, 100 mM NaCl, 10 mM Tris, and 0.1% Tween 20, pH 7.4) at 4 °C overnight. After a wash with T-TBS, the primary antibody solution, mouse monoclonal IgG against iNOS at a dilution of 1:1000, was applied for 1 h at room temperature. Membranes were washed with T-TBS, and the secondary antibody solution, anti-mouse IgG antibody conjugated to horseradish peroxidase at a dilution of 1:5000, was then applied for 1 h at room temperature. The blots were washed with TBS (without Tween 20), and incubated in ECL reagent and exposed to photographic film (Hyper Film, Amersham).

3.4. Electrophoretic mobility shift assay

The electrophoretic mobility shift assay was performed as described previously[11] with slight modification. TGC-induced peritoneal macrophages (3×10^6 cells/2 mL/well) prepared as described above were cultured in RPMI 1640 medium supplemented with 5% FCS, penicillin (100 units/mL), streptomycin (100 μg/mL), LPS (10 μg/mL), and test compound for 1 h. Cells were collected in ice-cold PBS. Nuclear extracts were prepared using NE-PER® nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's instructions. The protein content of each supernatant was determined, and equal amounts of protein (3 μg) and ^{33}P-labeled NF-κB consensus oligonucleotide were added to reaction mixtures. The oligonucleotide–protein complex was separated by nondenaturing polyacrylamide gel electrophoresis (Gel Shift Assay Systems, Promega), and autoradiography was performed using an imaging analyzer (BAS 5000, Fuji Film). ^{33}P-Labeled NF-κB consensus oligonucleotide was prepared using γ-[^{33}P]-ATP (3000 Ci/mmol) and T4 polynucleotide kinase following the manufacture's directions.

4. Results and discussion

4.1. Effects on activation of NF-κB, expression of IFN-β, IRF1, and iNOS mRNA, and induction of iNOS protein

The IFN-β, IRF1, and iNOS mRNA expressions induced by LPS in mouse peritoneal macrophages were examined using RT-PCR. As shown in Figure 2, INF-β and IRF1 mRNAs were detected 1 h after the treatment with LPS. However, iNOS mRNA was detected 4 h, but not 1 h, and iNOS protein was detected 5 h by Western blotting.

To clarify the mechanism of action of 1′*S*-1′-acetoxychavicol acetate (**1**), effects of **1** on the activation of

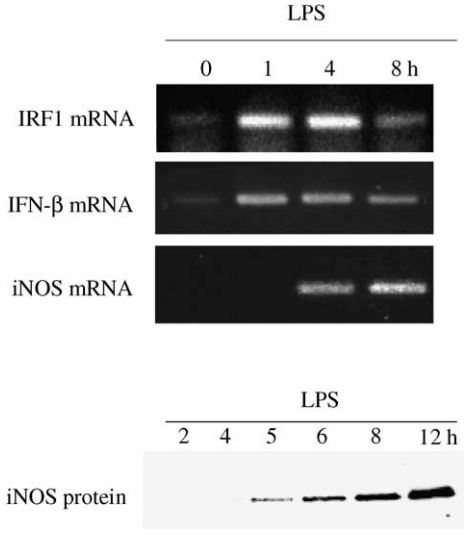

Figure 2. Time-dependent expression of IRF1, IFN-β, and iNOS mRNA, and production of iNOS protein in LPS-activated mouse peritoneal macrophages. IRF1, IFN-β, and iNOS mRNA: the cells were incubated with LPS and the mRNAs of IRF1, IFN-β, and iNOS were detected using RT-PCR. The expression of a housekeeping gene, G3PDH mRNA, was unchanged by each treatment (data not shown). iNOS protein: the cells were incubated with LPS and iNOS protein was detected using SDS-PAGE and Western blotting.

NF-κB and expression of iNOS mRNA were examined using an electromobility shift assay and RT-PCR 1 h after the stimulation. As shown in Figure 3, **1** concentration-dependently inhibited the activation of NF-κB, expression of iNOS mRNA, and production of iNOS protein consistent with previous report using RAW264 cells.[6]

Recently, production of IFN-β by macrophages was revealed to be important for the expression of iNOS mRNA.[10,16] However, only a synthetic compound which inhibits the production of IFN-β was reported.[17] Therefore, the effect of **1** on the expression of IFN-β mRNA 1 h after stimulation by LPS in mouse peritoneal macrophages was examined. As a result, **1** inhibited the expression of IFN-β mRNA without affecting that of G3PDH mRNA, as a housekeeping gene (Fig. 3). On the other hand, costunolide (**2**) and CAPE (**3**), known inhibitors of NF-κB activation,[11,18] did not have any effect on the expression of IFN-β mRNA. In addition, compound **1** completely inhibited the expression of IRF1 mRNA 1 h after the stimulation.

Sesquiterpene lactones including costunolide (**2**), dehydrocostus lactone, and parthenolide are considered to mainly inhibit the phosphorylation of IκB and consequently inhibit the activation of NF-κB as a mechanism of action,[19–21] and CAPE (**3**) also selectively inhibits the activation of NF-κB,[18] although detailed mechanisms of action of **3** is not clarified yet. In previous studies, the activation of NF-κB by LPS was considered to mediate the transcription of IFN-β.[16,22–24] However, recent studies revealed that transcription of IFN-β by double-stranded RNA (dsRNA) via TLR3 was independent of the activation of NF-κB,[25,26] and transcription of IFN-β by LPS via TLR4 is also possibly independent of the activation of NF-κB. In the present study, the selective inhibitors (**2** and **3**) for NF-κB activation did not inhibit the expression of IFN-β mRNA (Fig. 3), suggesting that the transcription of IFN-β is independent of the activation of NF-κB by LPS in the macrophages.

These findings suggested that inhibition of IFN-β production as well as inhibition of NF-κB activation are part of the mechanism of action of **1**, different from those of costunolide (**2**) and CAPE (**3**). In addition,

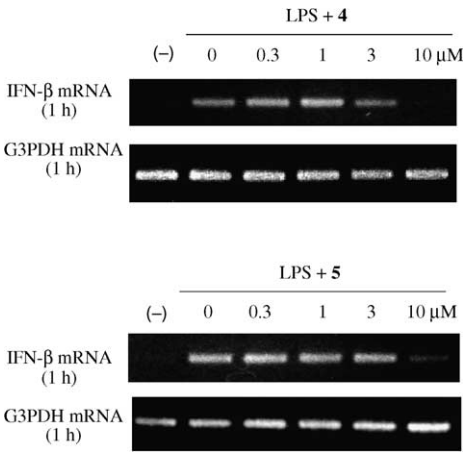

Figure 4. Effects of compounds **4** and **5** on expression of IFN-β mRNA in LPS-activated mouse peritoneal macrophages. IFN-β mRNA: the cells were incubated with or without LPS and a test sample for 1 h for the detection of IFN-β mRNA using RT-PCR.

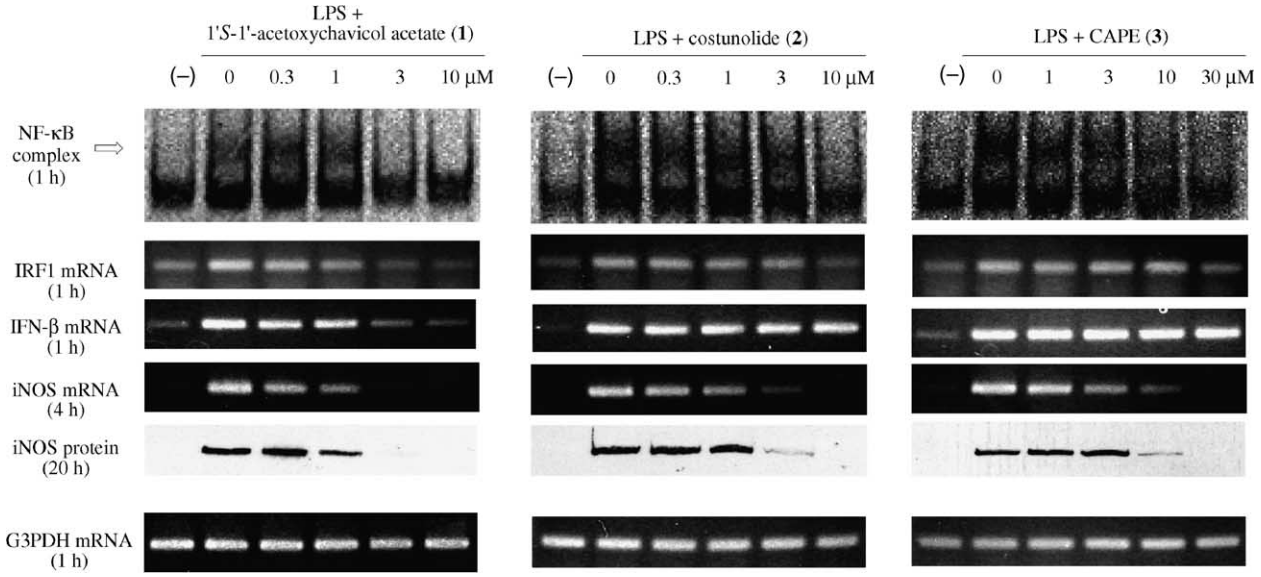

Figure 3. Effects of 1′*S*-1′-acetoxychavicol acetate (**1**), costunolide (**2**), and CAPE (**3**) on the activation of NF-κB, expression of IRF1, IFN-β, and iNOS mRNA, and production of iNOS protein in LPS-activated mouse peritoneal macrophages. NF-κB activation: the cells were incubated with or without LPS and a test sample for 1 h and activated NF-κB in the nuclei was detected by the electrophoretic mobility shift assay using ^{33}P-labeled NF-κB consensus oligonucleotide. IRF1, IFN-β, and iNOS mRNA: the cells were incubated with or without LPS and a test sample for 1 h for the detection of IRF1 and IFN-β mRNA or 4 h for the detection of iNOS mRNA using RT-PCR. The expression of a housekeeping gene, G3PDH mRNA, is shown at 1 h after the stimulation. iNOS protein: the cells were incubated with or without LPS and a test sample for 20 h for the detection of iNOS protein using SDS-PAGE and Western blotting.

Table 1. Inhibitory effects of 1′*S*-1′-acetoxychavicol acetate (**1**), costunolide (**2**), and CAPE (**3**) on NO_2^- accumulation in the medium stimulated by LPS or poly(I:C)

Treatments	IC_{50} values (μM)	
	LPS (10 μg/mL)	poly(I:C) (125 μg/mL)
1′*S*-1′-Acetoxychavicol acetate (**1**)	2.3	2.2
Costunolide (**2**)	3.7	2.7
CAPE (**3**)	11.4	10.2

two related synthetic inhibitors of NO production, **4** (IC_{50} = 3.2 μM for NO production) and **5** (IC_{50} = 9.2 μM),[4] also inhibited IFN-β mRNA expression similar to **1** (Fig. 4).

4.2. Effects on NO production stimulated by poly(I:C) via TLR3

Next, effects of **1**–**3** on NO production in mouse peritoneal macrophages stimulated by poly(I:C) via TLR3 were examined. As shown in Table 1, compounds **1**–**3** inhibited NO_2^- accumulation in medium stimulated by poly(I:C) with similar IC_{50} values to those obtained for LPS. This result suggested that **1**, **2**, and **3** regulated a signal pathway in the cells after the stimulation of TLRs, but did not act directly on TLRs on the cell surface. Figure 5 shows a model for the induction of iNOS by LPS in mouse peritoneal macrophages and possible site of action of **1**.

In conclusion, 1′*S*-1′-acetoxychavicol acetate (**1**) from the rhizomes of *A. galanga* inhibited the production of IFN-β as well as activation of NF-κB, both of which participate in the induction of iNOS, in the LPS-activated macrophages. The detail mechanisms of action of **1** including effects on the activation of IRF3[27] that regulate the transcription of IFN-β and on target molecules (e.g., TIR domain-containing molecule, TRIF[25]) in the upstream of IRF3 and NF-κB need to be studied further.

Acknowledgements

A part of this work was supported by the 21st COE Program and Academic Frontier Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References and notes

1. Matsuda, H.; Pongpiriyadacha, Y.; Morikawa, T.; Ochi, M.; Yoshikawa, M. *Eur. J. Pharmacol.* **2003**, *471*, 59–67.
2. Matsuda, H.; Morikawa, T.; Managi, H.; Yoshikawa, M. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3197–3202.
3. Morikawa, T.; Ando, S.; Matsuda, H.; Kataoka, S.; Muraoka, O.; Yoshikawa, M. *Chem. Pharm. Bull.*, 2005, 53, in press.
4. Matsuda, H.; Ando, S.; Morikawa, T.; Kataoka, S.; Yoshikawa, M. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1949–1953.

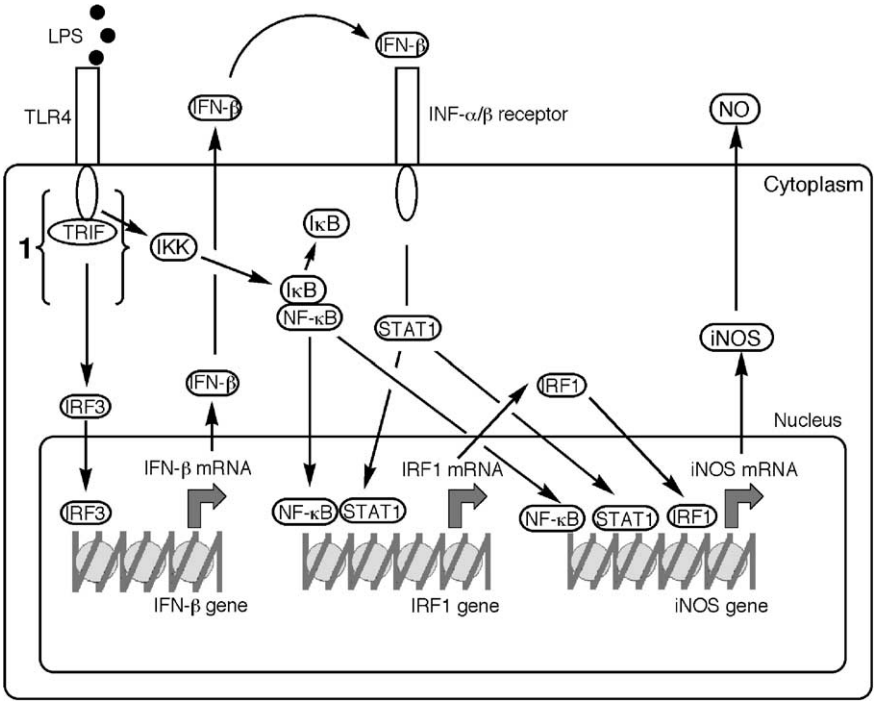

Figure 5. A model for the induction of iNOS by LPS in mouse peritoneal macrophages. This model is based on previous reports and this report (see text for appropriate references). LPS activates TLR4, which causes the activation of IRF3 and NF-κB. The gene encoding IFN-β is transcribed and translocated, and the secreted IFN-β binds to the IFN receptor, which results in the activation of STAT1. Activated NF-κB or STAT1 promotes transcription of the IRF1 gene. NF-κB, STAT1, and IRF1 act in concert to promote the expression of the iNOS gene and production of NO. {}: Possible site of action of 1′*S*-1′-acetoxychavicol acetate (**1**).

5. Salerno, L.; Sorrenti, V.; Di Giacomo, C.; Romeo, G.; Siracusa, M. A. *Curr. Pharm. Des.* **2000**, *8*, 177–200.
6. Ohata, T.; Fukuda, K.; Murakami, A.; Ohigashi, H.; Sugimura, T.; Wakabayashi, K. *Carcinogenesis* **1998**, *19*, 1007–1012.
7. Murakami, A.; Matsumoto, K.; Koshimizu, K.; Ohigashi, H. *Cancer Lett.* **2003**, *195*, 17–25.
8. Xie, Q. W.; Kashiwabara, Y.; Nathan, C. *J. Biol. Chem.* **1994**, *269*, 4705–4708.
9. Kamijo, R.; Harada, H.; Matsuyama, T.; Bosland, M.; Gerecitano, J.; Shapiro, D.; Le, J.; Koh, S. I.; Kimura, T.; Green, S. J.; Mak, T. W.; Taniguchi, T.; Vilcek, J. *Science* **1994**, *263*, 1612–1618.
10. Ohmori, Y.; Hamilton, T. A. *J. Leukocyte Biol.* **2001**, *69*, 598–604.
11. Matsuda, H.; Toguchida, I.; Ninomiya, K.; Kageura, T.; Morikawa, T.; Yoshikawa, M. *Bioorg. Med. Chem.* **2003**, *11*, 709–715.
12. Morikawa, T.; Tao, J.; Ando, S.; Matsuda, H.; Yoshikawa, M. *J. Nat. Prod.* **2003**, *66*, 638–645.
13. Tao, J.; Morikawa, T.; Ando, S.; Matsuda, H.; Yoshikawa, M. *Chem. Pharm. Bull.* **2003**, *51*, 654–662.
14. Abdel-Halim, O. B.; Morikawa, T.; Ando, S.; Matsuda, H.; Yoshikawa, M. *J. Nat. Prod.* **2004**, *67*, 1119–1124.
15. Punturieri, A.; Alviani, R. S.; Polak, T.; Copper, P.; Sonstein, J.; Curtis, J. L. *J. Immnol.* **2004**, *173*, 1033–1042.
16. Jacobs, A. T.; Ignarro, L. J. *J. Biol. Chem.* **2001**, *51*, 47950–47957.
17. Inoue, T.; Hamada, Y.; Takeshita, K.; Fukushima, K.; Higaki, M. *J. Rheumatol.* **2001**, *28*, 1229–1237.
18. Natarajan, K.; Singh, S.; Burke, T. R., Jr.; Grunberger, D.; Aggarwal, B. B. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 9090–9095.
19. Hehner, S. P.; Heinrich, M.; Bork, P. M.; Vogt, M.; Ratter, F.; Lehmann, V.; Schulze-Osthoff, K.; Droge, W.; Schmitz, M. L. *J. Biol. Chem.* **1988**, *273*, 1288–1297.
20. Koo, T. H.; Lee, J. H.; Park, Y. J.; Hong, Y. S.; Kim, H. S.; Kim, K. W.; Lee, J. *J. Planta Med.* **2001**, *67*, 103–107.
21. Kwok, B. H.; Koh, B.; Ndubuisi, M. I.; Elofsson, M.; Crews, C. M. *Chem. Biol.* **2001**, *9*, 759–766.
22. Hiscott, J.; Alper, D.; Cohen, L.; Leblanc, J. F.; Sportza, L.; Wong, A.; Xanthoudakis, S. *J. Virol.* **1989**, *63*, 2557–2566.
23. Dirks, W.; Mittnacht, S.; Rentrop, M.; Hauser, H. *J. Interferon Res.* **1989**, *9*, 125–133.
24. Lenardo, M. J.; Fan, C. M.; Maniatis, T.; Baltimore, D. *Cell* **1989**, *57*, 287–294.
25. Yamamoto, M.; Sato, S.; Mori, K.; Hoshino, K.; Takeuchi, O.; Takeda, K.; Akira, S. *J. Immunol.* **2002**, *169*, 6668–6672.
26. Peters, K. L.; Smith, H. L.; Stark, G. R.; Sen, G. C. *PNAS* **2002**, *99*, 6322–6327.
27. Kawai, T.; Takeuchi, O.; Fujita, T.; Inoue, J.; Mühlradt, P. F.; Sato, S.; Hoshino, K.; Akira, S. *J. Immunol.* **2001**, *167*, 5887–5894.