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Suppression of lipopolysaccharide-induced expression of inducible nitric oxide synthase by brazilin in RAW 264.7 macrophage cells

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Abstract

Brazilin (7,11b-dihydrobenz[b]indeno[1,2-d]pyran-3,6a,9,10 (6H)-tetrol) isolated from *Caesalpinia sappan* has been known as a natural red pigment. Many studies suggest that inducible isoform of nitric oxide synthase (NOS) plays an important role in inflammation and carcinogenesis. On this line, we evaluated the inhibitory effect of brazilin on nitric oxide (NO) production and investigated its mechanism of action. As a result, brazilin exhibited the inhibitory effect on lipopolysaccharide (LPS)-stimulated NO production in a dose-dependent manner (IC_{50}=24.3 μM). In addition, brazilin suppressed LPS-induced iNOS protein and mRNA expression in RAW 264.7 macrophage cells, indicating that the inhibitory activity of brazilin possibly involved in the regulation of iNOS expression. To further investigate the mechanism responsible for the suppression of iNOS gene expression by brazilin, the effect of brazilin on LPS-induced transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) activation was examined. The DNA binding activity of NF-κB and AP-1 stimulated LPS was inhibited by treatment of brazilin in a dose-dependent manner, suggesting that brazilin-mediated inhibition of NO production might be associated with the regulation of transcription factors NF-κB and AP-1. Taken together, these findings suggest that the suppressive effect of iNOS gene expression by brazilin might provide one possible mechanism for its anti-inflammatory and cancer chemopreventive activity.

Keywords: Brazilin; NO (nitric oxide); iNOS (inducible nitric oxide synthase); NF-κB (nuclear factor-κB); AP-1 (activator protein-1); Anti-inflammatory

1. Introduction

Brazilin (7,11b-dihydrobenz[b]indeno[1,2-d]pyran-3,6a,9,10 (6H)-tetrol; Fig. 1) isolated from the heartwood of *Caesalpinia sappan* L. (Leguminosae) has been used as a natural red pigment for histological staining (Puchtler and Sweat, 1964; Puchtler et al., 1986). Previous studies also exhibited various biological activities including hypoglycemic activity (Moon et al., 1993; Kim et al., 1998), anti-hepatotoxicity (Moon et al., 1992), inhibition of protein kinase C activity (Kim et al., 1998), anti-platelet aggregation (Hwang et al., 1998), induction of immunological tolerance (Choi and Moon, 1997; Mok et al., 1998), and anti-inflammatory activity (Hikino et al., 1977). Recent studies revealed that brazilin caused vasorelaxation effect by activation of endothelial nitric oxide synthase (eNOS) (Hu et al., 2003). However, the effect of brazilin on other nitric oxide synthase (NOS) isoforms, neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS), has not been investigated yet. Since the plant extract has been used as an anti-inflammatory and analgesic agent in traditional medicines, we tried to extent whether the active principle brazilin is associated with the anti-inflammatory effect by modulation of target molecule iNOS.

Nitric oxide (NO) has been implicated in a variety of pathophysiological conditions including atherosclerosis, inflammation, and carcinogenesis (Mordan et al., 1993; Ohshima and Bartsch, 1994; Krönche et al., 1998). Since iNOS is responsible for the overproduction of NO in inflammation, it has become a new target to develop new

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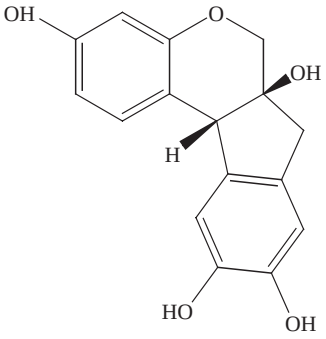

Fig. 1. Chemical structure of brazilin.

substances for the treatments of chronic inflammatory diseases (Hobbs et al., 1999). In addition, NO synthesized by iNOS has also been considered as an important mediator of carcinogenesis. NO can react with reactive oxygen species and then produce reactive nitrogen species contributed to DNA damage and mutagenesis (Wiseman and Halliwell, 1996). In addition, endogenous NO appeared to cause the neoplastic transformation of mouse fibroblasts (Mordan et al., 1993). Overexpressed iNOS has also been detected in several human cancerous tumors (Rosbe et al., 1995; Thomsen et al., 1995; Gallo et al., 1998).

Since brazilin has been shown potential induction of vasorelaxation through activation of eNOS but not studied yet other NOS systems and described with anti-inflammatory activity, we investigated the effect of brazilin on the NO production and iNOS gene and protein expression, and attempted to clarify its mechanism of action in LPS-stimulated RAW 264.7 macrophage cells.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, antibiotics-antimycotics solution, and trypsin-EDTA were purchased from Invitrogen Co. (Grand Island, NY, USA). Lipopolysaccharide (LPS, *Escherichia coli* 0111: B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals otherwise indicated were from Sigma (St. Louis, MO, USA). Brazilin (Fig. 1) used in this study was isolated from the dried heartwood of *C. sappan* (3 kg) as described previously (Mar et al., 2003).

2.2. Cell culture

Mouse macrophage RAW 264.7 cells, obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were incubated at 37 °C, 5% CO_2 in a humidified atmosphere.

2.3. Nitrite assay

To evaluate the inhibitory activity of the test material on LPS-induced NO production, RAW 264.7 cells in 10% FBS-DMEM without phenol red were plated in 24 well plates (5×10^5 cells/ml), and incubated for 24 h. Cells were washed with phosphate-buffered saline (PBS), replaced with fresh media, and then incubated with 1 μg/ml LPS in a presence or absence of brazilin. After additional 20 h incubation, the media were collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reaction (Green et al., 1982). % Inhibition was expressed as $[1-(\text{NO level of test samples}/\text{NO levels of vehicle-treated control})] \times 100$. The IC_{50} value, the sample concentration resulting in 50% inhibition of NO production, was determined using non-linear regression analysis (% inhibition versus concentration).

2.4. MTT assay

After Griess reaction, MTT solution (final 500 μg/ml) was added to each well and further incubated for 4 h at 37 °C. Media were discarded, and dimethyl sulfoxide (DMSO) was added each well to dissolve generated formazan. The absorbance was measured at 570 nm and % survival was determined by comparison with control group.

2.5. Preparation of total cell lysates

RAW 264.7 cells (5×10^5 cells/ml in 60 mm dish) were incubated with or without various concentrations of brazilin and LPS (1 μg/ml) for the indicated time. To obtain total cell lysates, cells were washed with ice-cold PBS and lysed in boiling 2× sample loading buffer (250 mM Tris–HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromophenol blue, 50 mM sodium fluoride, 5 mM sodium orthovanadate, and 2% β-mercaptoethanol). Cell lysates were boiled for additional 20 min and stored at −20 °C. The protein content of cell lysates was determined by Bradford assay (Bradford, 1976).

2.6. Preparation of nuclear extracts

RAW 264.7 cells (5×10^5 cells/ml) were seeded in 100 mm dish and incubated for 24 h. After incubation, cells were pre-treated with various concentrations of brazilin for 1 h, and then added 1 μg/ml of LPS for additional 1 h. For preparing nuclear extracts, cells were washed three times with ice-cold PBS, scraped, and suspended in ice-cold PBS. After centrifugation at $1000 \times g$ for 5 min at 4 °C, cells were resuspended in ice-cold lysis buffer (10 mM Tris–HCl (pH 8.0), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.2% NP-40, 50 mM sodium fluoride, 5 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma)) on ice for 5 min. After centrifugation at $1000 \times g$ for 5 min at 4 °C, supernatant was removed

and then pellets were washed twice with ice-cold lysis buffer without NP-40. Cells were resuspended in hypertonic nuclear extract buffer (20 mM Tris–HCl (pH 8.0), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 50 mM sodium fluoride, and protease inhibitor cocktail) on ice for 5 min, and then centrifuged at 15,000 × *g* for 20 min at 4 °C. The supernatant containing nuclear extracts was collected and stored in aliquots at −70 °C. The protein content of cell lysates was determined using Bradford assay (Bradford, 1976).

2.7. Western blot analysis

Equal amounts of cell lysates (40–50 μg) were subjected to 8–12% sodium dodesyl sulfate-polyacrylamide gel electrophoresis and electro-transferred onto polyvinylidene difluoride membranes (Milipore, MA, USA). Membranes were blocked in PBST (PBS with 0.1% Tween-20) containing 5% non-fat dry milk for 1 h at room temperature. After washing three times with PBST, membranes were incubated with primary antibodies against iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (Sigma) for 3 h at room temperature or overnight at 4 °C. Membranes were washed three times with PBST and incubated with corresponding secondary antibodies (Santa Cruz) for 90 min at room temperature. The blots were washed three times with PBST, and visualized using enhanced chemiluminescence western blotting detection system (Amersham Biotech Inc., Piscataway, NJ, USA).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 cells were stimulated with 1 μg/ml LPS in a presence or absence of brazilin for 4 h. Total cellular RNA was extracted with TRI reagent (Sigma) according to the manufacturer's recommended procedure. About 1 μg of total RNA was reverse-transcribed using oligo-$(dT)_{15}$ primers and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). PCR was performed in a reaction mixture containing the obtained cDNA, 0.2 mM dNTP mixture (Promega), 10 pmol of target gene-specific primers, and 0.25 unit of Taq DNA polymerase (Promega) using GeneAmp PCR system 2400 (Applied Biosystems, Foster, CA, USA). Each of PCR steps was performed as follows: initial denaturation step for 4 min at 94 °C; 25–30 cycles of amplification step consisting denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, and elongation for 30 s at 72 °C; and final extension step for 5 min at 72 °C. PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR-Gold (Molecular Probes, Eugene, Oregon, USA), and visualized by UV transillumination. Forward and reverse primers used for this study were detailed in Table 1.

Table 1
Sequences of gene specific primers used in the PCR

Genes		Sequences
Mouse iNOS	Sense	5′-ATGTCCGAAGCAAACATCAC-3′
	Antisense	5′-TAATGTCCAGGAAGTAGGTG-3′
Mouse β-actin	Sense	5′-TGTGATGGTGGGAATGGGTCAG-3′
	Antisense	5′-TTTGATGTCACGCACGATTTCC-3′

2.9. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described above. About 5 μg of each extracts was incubated with 2 μg of poly (dI-dC) and 100,000 cpm of [^{32}P]-labeled NF-êB and AP-1 consensus oligonucleotide (Promega) in a binding buffer consisting of 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1 mM DTT, and 10% glycerol for 25 min at room temperature. The DNA-protein complexes were separated by 5% native polyacryamide gel electrophoresis. The gels were dried and exposed to X-ray film at −70 °C.

2.10. Statistics

All experiments were repeated at least three times. Data were presented as means ± S.E.M. for the indicated number of independently performed experiments. Student's t-test was used for the determination of statistical significance. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of brazilin on NO production

The effect of brazilin on LPS-induced NO production was first examined in RAW 264.7 macrophage cells. Nitrite, the stable metabolite of NO and used as an indicator for NO production, was monitored in cultured LPS-stimulated RAW 264.7 cells. LPS (1 μg/ml) markedly increased the production of NO from the basal level of 0.7 ± 0.5 μM to 49.5 ± 2.5 μM for 20 h incubation. In this assay system, L-NMMA, a positive control of a non-selective inhibitor of NOS (Leiper and Vallance, 1999), exhibited an IC_{50} of 26.8 μM (Fig. 2A). When the cells were simultaneously treated with various concentrations of brazilin and LPS, NO production was significantly inhibited in a concentration-dependent manner with an IC_{50} value of 24.4 μM (Fig. 2B). Over 95% inhibition of NO production was shown at 40 μM brazilin. No significant effect on cell viability was observed at a test concentration up to 40 μM brazilin as determined by MTT assay (>85% cell survival) (Fig. 2B).

3.2. Suppression of iNOS protein and mRNA expression

In order to elucidate the mechanism of action of brazilin on the inhibition of NO production, further study

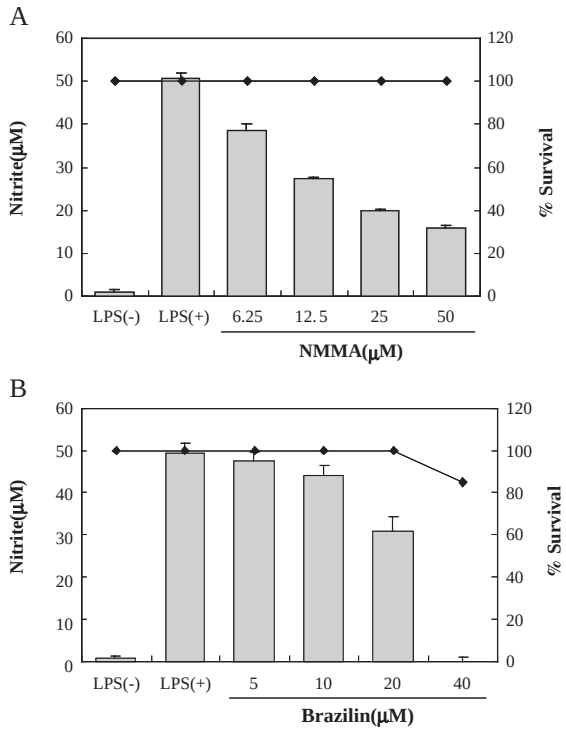

Fig. 2. Brazilin dose-dependently inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO) production. (A) Effect of L-NMMA, a positive control, on NO production and cytotoxicity in LPS-stimulated RAW 264.7 cells. (B) Effect of brazilin on NO production and cytotoxicity in LPS-stimulated RAW 264.7 cells. The data were expressed as mean ± S.E.M. of triplicate tests (■, NO production; ●, cell viability).

was performed. Since the accumulation of NO by LPS treatment in macrophages is highly correlated to the enhanced expression of iNOS protein and mRNA (Lyons et al., 1992; Schmidt et al., 1992; Geller et al., 1993), the effects of brazilin on the iNOS protein and gene expression were determined. As shown in Fig. 3, the treatment of LPS (1 μg/ml) for 16 h markedly enhanced expression of iNOS protein, and this effect was abolished by the co-treatment of brazilin (10–40 μM) in a dose-dependent manner, indicating the specific inhibition of iNOS protein expression by brazilin. To further investigate the effect of brazilin on LPS-induced enhancement of iNOS mRNA, steady-state levels of iNOS mRNA was determined using RT-PCR analysis. As illustrated in Fig. 4, brazilin significantly decreased iNOS mRNA levels induced by LPS in a dose-dependent manner, suggesting that the inhibitory activity of iNOS by brazilin might be correlated to the suppression of iNOS gene and protein expression.

3.3. Effect of brazilin on LPS-induced NF-κB and AP-1 binding activity

It is well known that NF-κB is one of major transcription factors for the induction of iNOS gene by LPS (Xie et al., 1994). To further investigate whether the

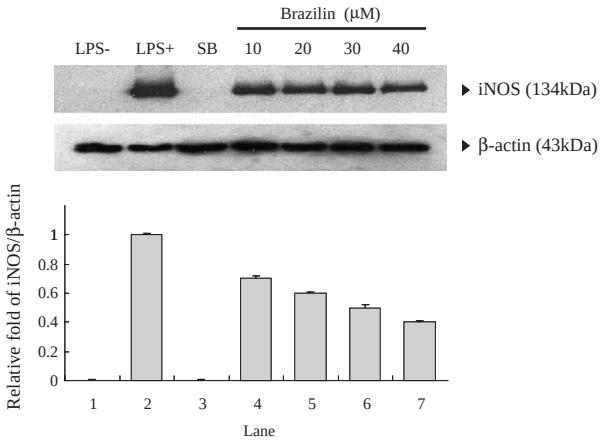

Fig. 3. Expression of inducible nitric oxide synthase (iNOS) protein in RAW 264.7 cells stimulated with lipopolysaccharide (LPS) (1 μg/ml) or in the presence of brazilin (10–40 μM) for 16 h. Western blot analysis was subjected for the iNOS protein expression, and bands were quantified by densitometry and expressed by iNOS/β-actin ratio. Data were representative of three separated experiments. SB (sample blank) indicates treatment of 40 μM brazilin without LPS.

transcription factor NF-κB is an important target for the action of brazilin in LPS-induced RAW 264.7 cells, electrophoretic mobility shift assay (EMSA) was conducted. LPS (1 μg/ml) treatment for 1 h markedly enhanced the binding activity of NF-κB in the nuclear extracts, and this effect was suppressed by the co-treatment of brazilin in a dose-dependent manner as shown in Fig. 5A. In addition, AP-1 is also considered another important transcriptional factor in the induction of iNOS gene expression (Aktan, 2004). When treated with LPS the binding activity of AP-1 was also increased, but the treatment of brazilin simultaneously inhibited the binding

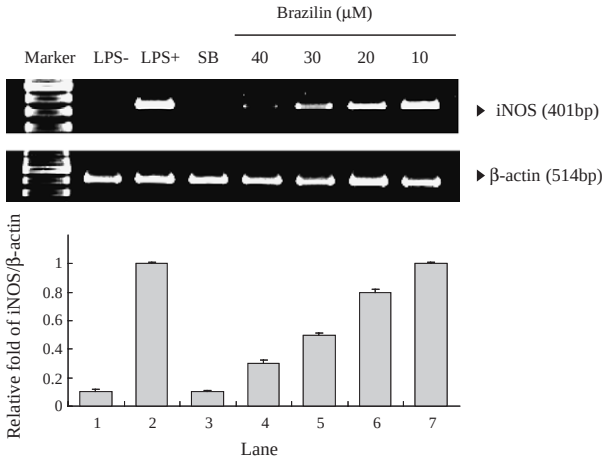

Fig. 4. Expression of inducible nitric oxide synthase (iNOS) mRNA in RAW 264.7 cells stimulated with lipopolysaccharide (LPS) (1 μg/ml) or in the presence of brazilin (10–40 μM) for 4 h. iNOS mRNA expression was determined by RT-PCR analysis, and bands were quantified by densitometry and expressed by iNOS/β-actin ratio. Data were representative of three separated experiments. SB (sample blank) indicates treatment of 40 μM brazilin without LPS.

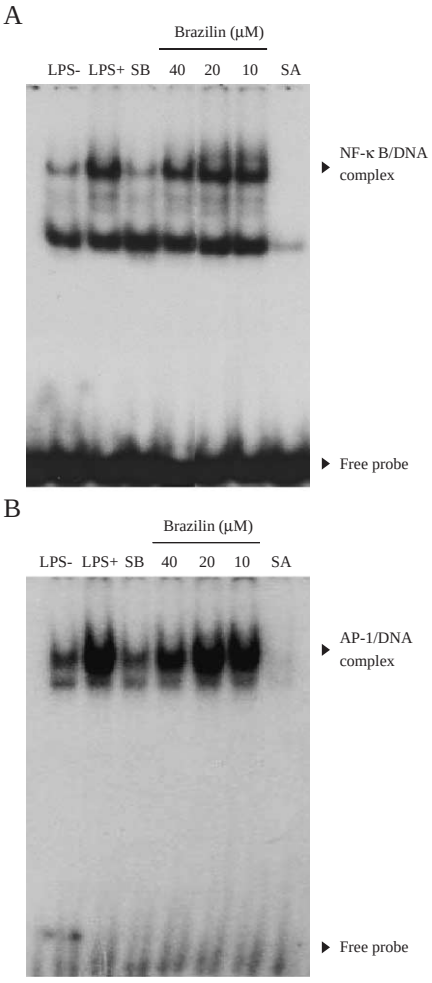

Fig. 5. Effect of brazilin on NF-κB (A) and AP-1 (B) activation in a macrophage cell line. RAW 264.7 cells were pre-treated with various concentrations of brazilin for 1 h, and then stimulated with lipopolysaccharide (LPS) (1 μg/ml) for additional 1 h, and nuclear extracts were then prepared for EMSA as described in Section 2. SA (specific binding activity) was demonstrated by competition of labeled probe with excess unlabeled specific probe. SB (sample blank) indicates treatment of 40 μM brazilin without LPS.

activity stimulated by LPS in a dose-dependent manner (Fig. 5B).

4. Discussion

Overproduction of NO is highly associated with inflammation (Lala and Chakraborty, 2001), and the levels of inducible nitric oxide synthase (iNOS) are playing a major role of production of NO in activated macrophages. Therefore, the regulation of NO production or iNOS expression levels might be an important target in the treatment of inflammation. In the course of searching for NO inhibitors or suppressors of iNOS as potential antiinflammatory agents from natural products, we investigated brazilin, an active principle isolated from *C. sappan*, for its potential therapeutic activity by modulating NO production and iNOS expression in activated macrophage cells.

The present results demonstrate that brazilin inhibits the production of NO in LPS-activated RAW 264.7 macrophage cells in a dose-dependent manner (Fig. 2 B), and this effect was shown without cytotoxic activity (Fig. 2B). Subsequent study revealed that the inhibitory effect of brazilin on NO production was highly related to the suppression of iNOS protein and gene expression as assessed by Western and RT-PCR analysis (Figs. 3 and 4). Both NF-κB and AP-1 binding sites have been identified on the murine iNOS promoter and play an important role in LPS-stimulated iNOS induction in RAW 264.7 macrophage cells (Aktan, 2004). Accordingly, when treated LPS (1 μg/ml) for 1 h, the DNA-binding activity of NF-κB was enhanced, and co-treatment of brazilin inhibited the binding activity, indicating that NF-κB might be one of major contributing transcription factor for the regulation of iNOS gene expression by brazilin. Additional experiments were conducted to assess whether brazilin modulated the nuclear binding activity of AP-1 stimulated by LPS. LPS also increased the nuclear binding activity of AP-1, and the enhanced binding activity was suppressed by the treatment of brazilin, suggesting that the inhibition of LPS-stimulated AP-1 activation by brazilin also contributes to the inhibition of iNOS expression.

In summary, this study suggests that brazilin from *C. sappan* inhibits NO production in LPS-stimulated RAW 264.7 macrophage cells through the suppression of iNOS protein and mRNA expression. Furthermore, the suppression of mRNA expression of iNOS is, at least in part, associated with the inhibition of transcription factors NF-κB and AP-1 activation. Finally, the anti-inflammaroty effect of the extract of *C. sappan* and brazilin might be explained by the modulation of NO production and iNOS expression as one of contributable mechanisms of action.

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