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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.
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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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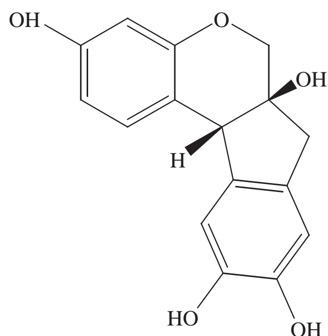

Fig. 1. Chemical structure of brazilin.

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 − (NO level of test samples / NO levels of vehicle-treated control)] × 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 × *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 × *g* for 5 min at 4 °C, supernatant was removed

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