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Inhibitory effect of a novel resveratrol derivative on nitric oxide production in lipopolysaccharide-activated microglia

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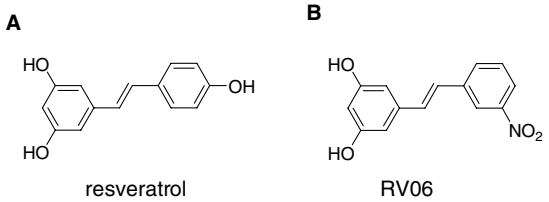
Excessive nitric oxide (NO) production by activated microglial cells has been implicated in various neurodegenerative diseases. In the present study, we found that a new resveratrol derivative, (*E*)-5-(3-nitrostyryl)benzene-1,3-diol (RV06), has a more potential inhibitory effect on the production of NO in LPS-activated N9 microglial cells, and the result was confirmed on primary rat microglial cells. Further studies showed that RV06 inhibited LPS-induced iNOS expression in N9 microglial cells, with no activity on direct scavenging nitric oxide radical in a cell-free environment. The results suggest that RV06 might be a potential anti-inflammatory agent or leading compound which can inhibit inflammatory responses of microglia.

1. Introduction

Microglia are the resident immune cells of the central nervous system (CNS). Excessive amount of inflammatory mediators produced by activated microglia, such as nitric oxide (NO), oxygen radicals, and pro-inflammatory cytokines, are involved in the pathogenesis of a different neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) (Dickson et al. 1993; Gonzalez-Scarano and Baltuch 1999; Mcgeer et al. 1993; Meda et al. 1995).

Resveratrol (*trans*-3,4′,5-trihydroxystilbene), a polyphenol present in relatively large amounts in grapes and red wine, possesses a variety of biological actions including anti-inflammatory, chemopreventive, neuroprotective, and cardioprotective properties (Anekonda 2006; Bradamante et al. 2004; Jang et al. 1997; Okawara et al. 2007; Wang et al. 2002). The inhibitory effect of resveratrol on microglial activation has been previously reported, and resveratrol was found to suppress the production of tumor necrosis factor-α (TNF-α) and NO induced by lipopolysaccharide (LPS) in microglia (Bi et al. 2005). The inhibitory effects on PGE_2 and IL-1β production, as well as radical and ROS scavenging properties of resveratrol in microglia have also been reported (Bureau et al. 2008; Candelario-Jalil et al. 2007; Kim et al. 2007; Lorenz et al. 2003). However, due to its photosensitivity and metabolic instability (Goldber et al. 1995; Jeandet et al. 1995; Trela et al. 1996), as well as the relatively high concentrations of resveratrol which are required to modulate microglial activation (Bi et al. 2005; Lorenz et al. 2003), chemical modifications of resveratrol to find new and stable derivatives which can more efficiently modulate microglial activation have been undertaken.

Thus, a series of resveratrol derivatives and analogues were synthesized and screened for their inhibitory activities on NO production from LPS-activated microglia. In this study, we found that a novel resveratrol derivative, (*E*)-5-(3-nitrostyryl)benzene-1,3-diol (RV06), performs fairly better than resveratrol on NO production in N9 microglia cell line and primary rat microglia. In order to understand the mechanism of action of RV06, we detected the NO-scavenging activity of RV06 in non-cellular systems and the inhibitory effect on iNOS induction in N9 microglia.



2. Investigations, results and discussion

It is well known that excessive pro-inflammatory cytokine and NO productions by activated microglia play an important role in neurodegenerative diseases. This is the first report for the potent inhibitory activity of RV06, a novel resveratrol derivative, on LPS-induced iNOS expression and NO release, reflecting the activation of microglial cells.

By comparing the chemical structure of RV06 with that of resveratrol, it is obvious that RV06 resembles resveratrol in terms of chemical structure but should be more stable than resveratrol. Thus, RV06 might be more stable to exert persistent biological effects.

In order to exclude the possibility that the cytotoxic action of RV06 caused inhibition of LPS-stimulated NO produc-

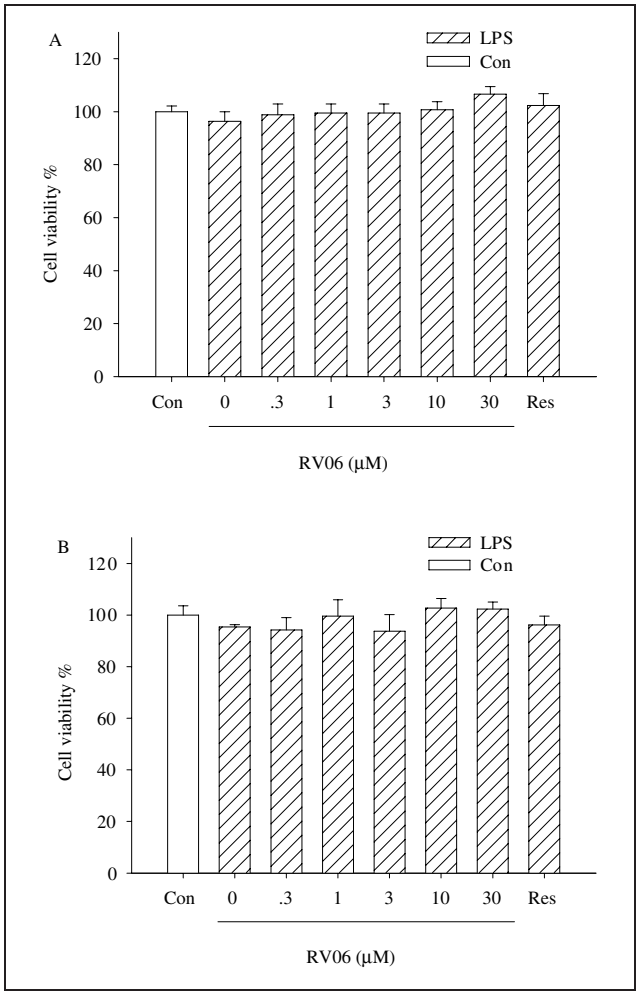


Fig. 1: Effect of RV06 on microglial cell viability. N9 cells (A) or primary microglial cells (B) were treated with RV06 (0.3–30 μM) or resveratrol (10 μM) in the presence of LPS (1 μg/ml) for 48 h. Cell viability was examined by MTT reduction assays and the results were expressed as percentage of surviving cells over control cells (cultured in medium alone). Data were represented as mean ± SEM of three separate experiments

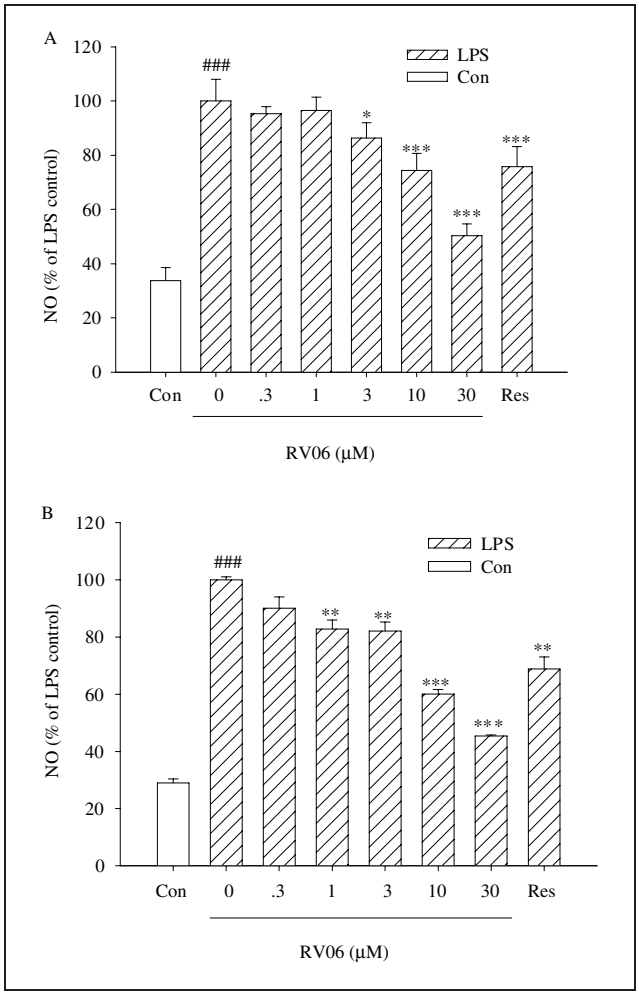


Fig. 2: Effect of RV06 on NO production induced by LPS in microglial cells. N9 cells (A) or primary microglial cells (B) were treated with RV06 (0.3–30 μM) or resveratrol (10 μM) in the presence of LPS (1 μg/ml). The results were expressed as the percentage values taking LPS treatment group as 100%. Data were represented as mean ± SEM of three separate experiments. $^{###}$ $P < 0.001$ as compared with the control group (cultured in medium alone), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with only LPS groups

tion, we investigated the effect of RV06 on the cell viability. Treatment of N9 or primary rat microglial cells with RV06 (0.3–30 μM) in the presence of LPS for 48 h did not provoke any cytotoxicity (Fig. 1A and B). LPS alone (1 μg/ml) did not cause significant loss of cell viability (data not shown). These data indicated that the effect of RV06 on NO production from LPS-activated microglia was not due to its cytotoxic action.

NO is synthesized from L-arginine by NOS in various cells and tissues. NOS is broadly classified into two subtypes: the constitutive "neuronal" and "endothelial" NOS (cNOS) and inducible NOS (iNOS) (Schroeder and Kuo 1995). iNOS is expressed only when the cells are exposed to LPS or cytokines such as interferon-γ and TNF-α. NO produced by iNOS is a key mediator and its production is a crucial step in microglia activation (Possel et al. 2000). Thus, we detected the effect of RV06 on LPS-induced NO production in microglial cells. The result showed that nitrite, a NO product, was accumulated in the medium after 48 h incubation with LPS. RV06 inhibited LPS-induced NO production in N9 and primary rat microglial cells in a concentration-dependent manner, with the IC_{50} being 15.1 and 9.0 μM, respectively. In the parallel experiments, resveratrol (10 μM) significantly reduced NO production (Fig. 2A and B). Most important is that RV06 has more potential inhibitory effect on NO production in N9 and primary rat microglial cells ($IC_{50} = 15.1$ and 9.0 μM, respectively) than that of resveratrol ($IC_{50} = 20.8$ and 15.5 μM, respectively). These results suggest that RV06 may act as a more efficiently NO inhibitor in microglial cells than resveratrol.

It is demonstrated that resveratrol has a strong NO-scavenging effect (Chan et al. 2000). To investigate the NO scavenging capacity of RV06, we used sodium nitroprusside (SNP) as a donor for NO in a cell-free assay. SNP is an unstable molecule that breaks down when exposed to light and NO is found as NO^+. SNP was used to test the NO-scavenging ability of various substances (Mirkov et al. 2004; Yen et al. 2001). In this study, resveratrol (10 μM) significantly reduced NO levels of SNP solution. However, no change was observed when RV06 (0.3–30 μM) was added to the SNP solution (Fig. 3). This result indicated that, unlike resveratrol, RV06 has no NO-scavenging activity. Therefore, this is not likely to be the main mechanism of action of RV06 to inhibit NO levels in medium of microglial cells.

In our previous studies, we evaluated the inhibition of resveratrol on iNOS expression in LPS-activated microglia

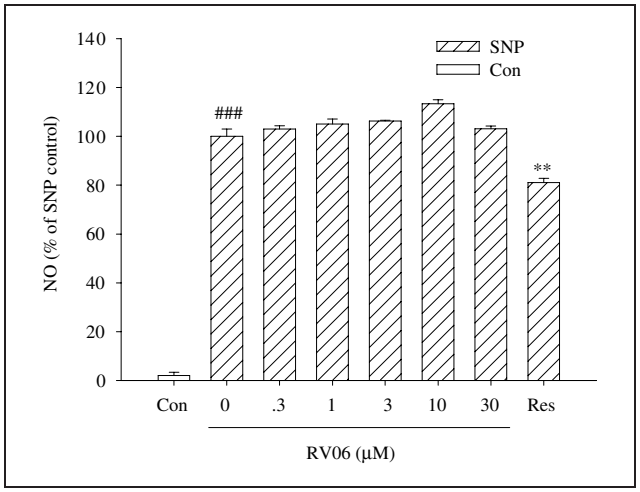


Fig. 3: Effect of RV06 on NO levels in SNP solution. SNP solution (2.5 mM) in PBS was incubated alone or with RV06 (0.3–30 μM) or resveratrol (10 μM) in light at room temperature for 60 min. Data were represented as mean ± SEM of three separate experiments. ### $P < 0.001$ compared to the control group (PBS alone), ** $P < 0.01$ as compared with the SNP group

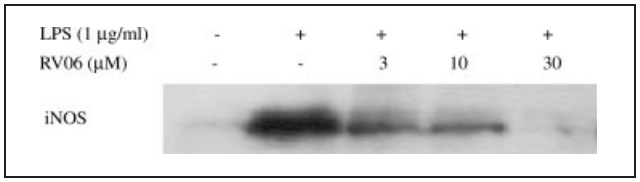


Fig. 4: Effect of RV06 on iNOS expression induced by LPS in N9 microglial cells. N9 cells were treated with RV06 (3–30 μM) in the presence of LPS (1 μg/ml) for 24 h. The expression of iNOS protein was detected by Western blot. The experiments were repeated three times and similar results were obtained

(Bi et al. 2005). Further experiments were carried out to examine whether the inhibitory effect of RV06 on NO production was associated with decreased expression of iNOS in LPS-stimulated microglia. The result of Western blot analysis showed that unstimulated cells expressed very low levels of iNOS protein whereas treatment of N9 microglia with LPS (1 μg/ml) for 24 h led to significant increases in iNOS protein. LPS-stimulated increase of iNOS was suppressed in the presence of RV06 (3–30 μM) (Fig. 4). Thus, the inhibitory activity of NO production by RV06 may result from the inhibition of the enzyme expression. Moreover, we previously demonstrated that, by blocking IκBα degradation and p38 mitogen-activated protein kinases (MAPKs) phosphorylation, resveratrol acts to reduced LPS-induced NO production in N9 microglial cells (Bi et al. 2005). Whether these mechanisms contribute to the inhibition of NO production of RV06 in LPS-activated microglia needs to be further investigated. Moreover, further studies are required to understand whether it might be a leading compound for future drug development. These include assessment of bioavailability, efficacy, and safety *in vivo*.

In summary, the data presented here demonstrated for the first time that RV06, a novel resveratrol derivative, exerted a more potential anti-inflammatory effect by inhibiting LPS-induced production of NO in microglial cells. Furthermore, RV06 reduced LPS-induced protein expression of iNOS, but did not display direct NO-scavenging activity up to 30 μM in cell-free system. Though the exact mechanism of this suppression is not clearly understood at present stage, it can be assumed that RV06 might be a potential anti-inflammatory agent or leading compound which can inhibit inflammatory responses of microglia.

3. Experimental

3.1. Materials

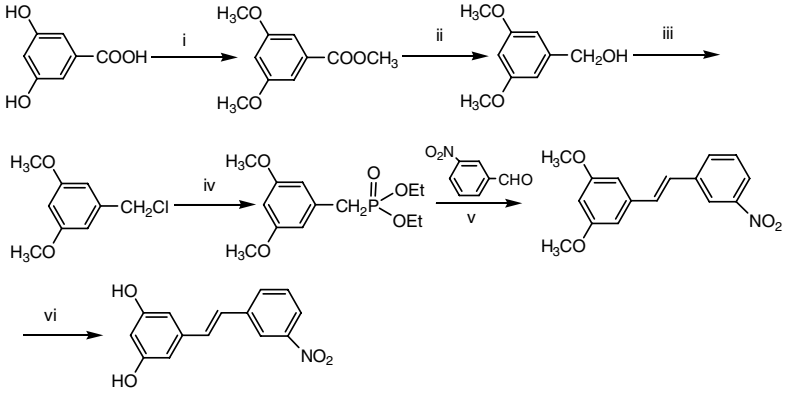
Fetal bovine serum (FBS) and Iscove's modified Dulbecco's medium (IMDM) were purchased from Gibco BRL (Grand Island, NY, USA). LPS (E5: 055) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Monoclonal antibody MRC OX-42 to rat CD11b/c was from ImmunoTools, Friesoythe, Germany. Thiazolyl blue (MTT) was from Sino-American Biotechnology (Beijing, China). iNOS antibody was from BD Biosciences (San Diego, CA).

3.2. Synthesis of resveratrol and RV06

All chemical reagents for synthesizing the compounds were purchased from a local supplier and were analytical grade. Melting points were determined with a Yanaco micro melting point apparatus and were not corrected. NMR spectra were recorded at 300 MHz using Bruker ARX-300 instrument with TMS as internal reference. Mass spectra (ESI-MS) were measured on Agilent 1100 Series MSD Trap (SL).

Resveratrol and RV06 were synthesized as described previously (Chen et al. 2005). The route of the synthesis of RV06 is shown in the Scheme. Starting from 3,5-dihydroxybenzoic acid, methyl 3,5-dimethoxybenzate was prepared in good yield and followed by reduction with $LiAlH_4$. Subsequent treatment with $SOCl_2$ afforded 3,5-dimethoxylbenzyl chloride with a yield of about 94%. Using the Wittig-Horner reaction, only the *trans* isomer was obtained. BBr_3 was used for deprotection of methyl groups in CH_2Cl_2 to afforded RV06, which is yellow solid, m.p. 196–197 °C.

Scheme Reagents and conditions: i. Me_2SO_4, K_2CO_3, CH_3COCH_3, reflux 3 h, 93.6%; ii. $LiAlH_4$, EtOEt, reflux 4 h, 92.3%; iii. $SOCl_2$, EtOEt, rt 3 h, 94.4%; iv. $P(OEt)_3$, reflux 3 h; v. NaOEt, RCHO, DMF, rt 3 h, 67% (for iv, v steps); vi. BBr_3, CH_2Cl_2, −10 °C 2 h, 90%



^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 6.01 (1 H, s), 6.50 (2 H, s), 7.20 (1 H, d, J = 16.4 Hz, −CH=CH−), 7.30 (1 H, d, J = 16.2 Hz, −CH=CH−), 7.65 (1 H, t), 8.08 (1 H, t), 8.42 (1 H, s), 9.33 (2 H, s, −OH). ESI-MS: 257.9 $[M + H]$, 279.9 $[M + Na]^+$, 255.7 $[M - H]^-$, 291.7 $[M + Cl]^-$.

3.3. Cell culture

The murine microglial cell line N9 was a kind gift from Dr. P. Ricciardi-Castagnoli (Universita Degli Studi di Milano-Bicocca, Milan, Italy). The cells were grown in IMDM supplemented with 5% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol.

Primary rat microglia were prepared from the cortex of newborn SD rats (1 day) (Department of Laboratory Animal Science, Shenyang Pharmaceutical University) (Barger and Harmon 1997). Briefly, the meninges and blood vessels of the rat cortex were removed. Tissues were dissociated with 0.25% trypsin at 37 °C for 10 min, and then the cell suspension was filtered through a 50-μm diameter nylon mesh. The cells were collected by centrifugation at 1000 rpm/min for 10 min, re-suspended in IMDM and then plated in culture flasks. Cells were incubated at 37 °C in a humidified atmosphere (5% CO_2). After 9–11 days, the flasks were shaken on a rotary shaker at 240 rev/min for 1 h. The resulting cell suspension, rich in microglia, was placed in culture dishes in which the cells adhered after 30 min at 37 °C. The purity of cells obtained was > 95% as verified by immunocytochemistry using the monoclonal antibody MRC OX-42 to rat CD11b/c (Bi et al. 2005).

3.4. Drug treatment

For experiments, resveratrol and RV06 (purity > 99%) were initially dissolved in dimethyl sulfoxide (DMSO) and were diluted with phosphate-buffered saline (PBS). DMSO at the highest possibly concentration under the experimental conditions (0.1%) was not toxic to the cells.

3.5. Cell viability

Cell viability was evaluated by the MTT reduction assay (Chang et al. 1998). Microglial cells at 5×10^4 cells/well were seeded into 96-well microtiter plates. RV06 (0.3–30 μM) in the presence LPS (1 μg/ml) were added to the culture medium of N9 microglia or primary rat microglia for 48 h. The cells were incubated with MTT (0.25 mg/ml) for 3 h at 37 °C. The formazan crystals in the cells were solubilized with DMSO. The level of MTT formazan was determined by measuring its absorbance at 490 nm with a SPECTRA (shell) Reader (TECAN, Grödig, Austria).

3.6. Nitrite assay

Accumulation of nitrite (NO_2^-), an indicator of NO synthase activity, in culture supernatant fluids was measured by the Griess reaction (Barger and Harmon 1997). Microglial cells at 5×10^4 cells/well were plated into 96-well microtiter plates and treated with RV06 (3–30 μM) or resveratrol (10 μM) in the presence of LPS (1 μg/ml) for 48 h. Fifty microliter culture supernatant fluids were mixed with 50 μl Griess reagent (part I: 1% sulfanilamide; part II: 0.1% naphthylethylene diamide dihydrochloride and 2% phosphoric acid) at room temperature. Fifteen minutes later, the absorbance was determined at 540 nm using the SPECTRA (shell) reader. Nitrite concentrations were calculated with reference to a standard curve of sodium nitrite generated with known concentrations.

3.7. NO-scavenging activity

The scavenging effect of RV06 (0.3–30 μM) on NO was measured according to Marcocci et al. (1994). Sodium nitroprusside (SNP) is an inorganic complex where NO is found as NO^+ and light irradiation is necessary for the release of NO. SNP (2.5 mM) was incubated alone or in combination with different concentrations of the compounds in light at room temperature for 60 min, and nitrite levels were determined by Griess reaction.

3.8. Western blotting

Western blot analysis was performed as previously described (Tzeng and Huang 2003) with slight modifications. N9 microglial cells were treated with RV06 (3–30 μM) in the presence of LPS (1 μg/ml) for 24 h. Cells were washed with ice-cold PBS and lysed for 10 min with RIPA lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% NP-40, 1% sodium-deoxycholate, 1 mM phenylmethyl-sulfonylfluoride, 1 mM EDTA, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The protein concentration in the supernatant fluid of the lysate was measured by the BCA protein assay (Pierce, Rockfold, IL). Equal amounts (60 μg) of protein were separated electrophoretically using 12% SDS-PAGE and then the gel was transferred to 0.45 μm polyvinylidene fluoride membrane (PVDF: Millipore, Bedford, MA). The membranes were soaked in blocking buffer (5% skimmed milk) and then incubated overnight with iNOS antibody, followed by horseradish peroxidase conjugated secondary antibody. The protein of interest was detected by enhanced chemiluminescence reagents.

3.9. Statistical analysis

Results were expressed as mean ± SEM of three experiments performed in triplicate. One-way ANOVA followed by Dunnett's t-test was used for statistical analysis (SPSS 13.0 software, SPSS, USA).

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