J Korean Soc Appl Biol Chem (2012) 55, 803–807
DOI 10.1007/s13765-012-2156-8

SHORT COMMUNICATION

Resveratrol Inhibits Lipopolysaccharide-induced Phagocytotic Activity in BV2 cells

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Received: 12 July 2012 / Accepted: 26 September 2012 / Published Online: 31 December 2012

Abstract The inhibitory effects of resveratrol, a natural bioactive polyphenolic phytoalexin rich in grape, on lipopolysaccharide (LPS)-induced microglial activation and its beneficial effects on dopaminergic neurodegeneration were studied. Resveratrol dose-dependently suppressed LPS-induced nitric oxide production and the expression of inducible NO synthase (iNOS) in BV-2 microglial cells. Furthermore, resveratrol (1 μM) significantly blocked BV2 microglial phagocytosis induced by LPS (0.1 μg/mL). Although the conditioned media from LPS-stimulated BV2 cells caused the SN4741 dopaminergic neuronal cell death, that from resveratrol-pretreated BV2 cells did not diminish the viability of SN4741 cells. These results suggest that resveratrol can prevent neuronal death possibly through the modulation of phagocytosis and microglial activation.

Keywords conditioned media · dopaminergic neuron · microglia · phagocytosis · resveratrol

The number of activated microglia in the substantia nigra (SN) greatly increased in the Parkinson's disease (PD) patients (McGeer and McGeer, 2008). Activated microglia serve as a major source of proinflammatory cytokines, nitric oxide (NO), and other neurotoxic intermediates, which lead to dopaminergic neuronal damage in the SN (Orr et al., 2002). Thus, suppression of microglial activation process will become a promising therapeutic target for the amelioration of PD. Prenatal infection of bacteria, a source of lipopolysaccharide (LPS), caused microglial activation and promoted dopaminergic neurodegeneration. Several studies indicated that LPS is an effective initiator of SN dopaminergic neurodegeneration (Herrera et al., 2000; Kim et al., 2000). Additional studies have revealed that the SN has a higher sensitivity to LPS than other brain regions and LPS leads to selective damage to dopaminergic neurons, but was not harmful to GABAnergic or serotonergic neurons at the same dose treatment in SN (Herrera et al., 2000; Kim et al., 2000). Therefore, LPS-activated microglia could be a good cellular model to investigate the relationship between microglial activation and dopaminergic neuronal damage, as well as for screening the potential therapeutic compound to attenuate neuronal inflammation.

The non-flavonoid polyphenol resveratrol (trans-3,4',5-trihydroxy-stilbene) is a naturally occurring phytoalexin in various plant families such as grape, soy, peanuts, and knotweed (*Polygonum cuspidatum*) (Burns et al., 2002). Resveratrol has been known to have a wide range of biological and pharmacological activities such as cardioprotective, anti-carcinogenic, antioxidative, and anti-inflammatory effects (Baur and Sinclair, 2006). Recent studies have revealed that resveratrol is capable of crossing the blood-brain barrier and exerts protective effects against neuronal damages (Jin et al., 2008; Zhang et al., 2010). Other studies suggested that resveratrol suppressed the activation of microglia (Bi et al., 2005; Candelario-Jalil et al., 2007). However, the effects of resveratrol against the phagocytotic activity of microglia have not been proved. In the present study, the effects of resveratrol on LPS-induced phagocytotic activation of BV2 microglia were examined, and the consequences of resveratrol treatment in microglia for dopaminergic neurons viability were evaluated.

Resveratrol and other chemicals were purchased from Sigma (USA). Antibodies against iNOS and actin were obtained from Santa Cruz Biotechnology Inc. (USA). Resveratrol was initially prepared in ethanol as a stock solution (10 mM) and was diluted with phosphate buffered saline for experiments. The ethanol content in the final experimental conditions (resveratrol concentrations ranging from 0.25 to 1 μM) did not exceed 0.01% and was not toxic to cells. The murine microglial cell line BV2 was cultured

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in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/mL)-streptomycin (100 μg/mL) at 37°C in 5% CO_2. The dopaminergic neuronal SN4741 cells were cultured at 33°C as previously described and maintained to less than 70% confluence to minimize dedifferentiation (Chun et al., 2001). The culture medium was changed to fresh medium with low-serum (0.5% fetal bovine serum) before any treatments to reduce the serum effect.

The production of NO was measured by the accumulation of nitrite (NO^-_2) in the culture medium with the Griess reagent (Sigma, USA) (Chun and Low, 2012). After 24 h LPS treatment, 50 μL of culture supernatants were mixed with the same volume of Griess reagent, and the absorbance was read at 550 nm after 10 min incubation. Sodium nitrite was used as standard to calculate nitrite concentration. For Western blot analysis with whole cell lysates, BV2 cells treated with various experimental conditions were lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/mL phenylmethylsulfonyl fluoride, 30 mg/mL aprotinin, and 1 mM sodium orthovanadate in PBS). Equal amounts of protein (20 μg) were separated on 8% SDS-polyacrylamide gel elctrophoresis (PAGE) and blotted onto polyvinylidene difluoride membrane. The membrane was incubated with primary antibody for rabbit anti-iNOS (Santa Cruz Biotechnology sc-651, 1:1,000 dilution) or goat anti-actin (Santa Cruz Biotechnology sc-1616, 1:4,000 dilution), and then reacted with horseradish peroxidase-conjugated secondary antibody at 1:2,000 dilution. The specific protein expression was detected by enhanced chemiluminescence (ECL) reagent (GE Healthcare, USA). Microglial phagocytosis was analyzed by measuring the ingested latex beads. BV2 cells grown under various experimental conditions were treated with latex beads (3 mm diameter; 1 mL beads/mL; Sigma) for 6 h, and then extensively washed four times with PBS to remove uningested surface-bound beads. After methanol was fixed for 5 min, cells were stained with Giemsa dye for 10 min, followed by two times rinsing with PBS. The numbers of phagocytosed beads per cell were examined under a microscope. To detect sirtuin 1 (SIRT1) protein in BV2 cells, nuclear extracts were prepared with CelLytic NuClear Extraction kit (Sigma) following the manufacturer's protocol. Because the protease inhibitors and detergents can interfere with the activity of SIRT1, nuclear proteins were isolated without the use of detergents or protease inhibitors during the process of nuclear protein extraction. SIRT1 activity was measured by a SIRT1 Direct Fluorescent assay kit (Cayman Chemical Co., USA). Briefly, the assays were performed by incubation with nuclear extracts and SIRT1 peptide substrate (acetylated amino acids of human p53; Arg-His-Lys-Lys(ε-acetyl)-AMC) with NAD^+ as a co-substrate at room temperature for 45 min, as indicated in the manufacturer's instructions. After the reaction was stopped with Stop/Developing solution, SIRT1 activity was measured at 360 and 465 nm (excitation and emission, respectively) using a fluorescence multimode microplate reader (Infinite 200; Tecan, Austria). For conditioned medium treatment, BV2 cells were cultured under various experimental conditions for 24 h. The resulting cultured-supernatants were collected as conditioned media and centrifuged at 170 × g for 5 min to remove cell debris. SN4741 dopaminergic neuronal cells were incubated for 24 h in their own low-serum medium or with conditioned medium from unstimulated or chemical-treated BV2 cells. Neuronal cell viability was measured by XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay as previously described by Chun et al. (2001). In brief, 50 μL of XTT labeling mixture (50:1 mixture of 1 mg/mL XTT and 0.383 mg/mL N-methyl dibenzopyrazine methyl sulfate) was directly added to 100 μL cell culture in 96 well plate. After 4 h incubation, the resulting formazan product was measured at 490 nm using a microtiter plate (ELISA) reader. Data were expressed as the mean ± SEM. Statistical significance was analyzed by one-way factorial analysis of variance (ANOVA) followed by Student's t-test (SPSS 13.0 software, SPSS, USA). $p < 0.05$ and $p < 0.01$ were considered as statistically significant.

BV2 is a unique murine cell line exhibiting morphological, phenotypical, and functional properties of microglia (Blasi et al., 1990). This cell line has been extensively used for studying the mechanisms underlying microglial activation in neurodegeneration. Thus, in the present study, BV2 cells were used as a model system to investigate the role of resveratrol (Fig. 1A) against LPS-mediated microglial cell activation. Initial study was performed to examine the effect of resveratrol on NO production in LPS-stimulated BV2 microglia. Resveratrol (0.25–1 μM) dose-dependently reduced LPS-induced NO production (Fig. 1B). Pretreatment of resveratrol reduced the NO production by 5 (0.25 μM resveratrol), 44 (0.5 μM resveratrol), and 65% (1 μM resveratrol). Cell viability assay verified that neither cell proliferation nor cytotoxicity was affected by treatments at concentrations of LPS or resveratrol used in the present study (data not shown). Incubation of cells with resveratrol alone did not affect the basal level of nitrite in BV2 cells (Fig. 1B). Previous study suggested that LPS induces NO production in microglial cells by *de novo* synthesis of iNOS (Corradin et al., 1993). Therefore, we examined the effect of resveratrol on LPS-induced iNOS expression. Pretreatment of resveratrol led to a significant attenuation of LPS-induced iNOS protein expression dose-dependently (0.5–1 μM) (Fig. 1C). These results suggest that resveratrol inhibits NO production by regulating iNOS expression. In addition, pretreatment of resveratrol (1 μM) reduced LPS (0.1 μg/mL)-induced TNF-α production by 57% and IL-1β production by 89% (data not shown).

To investigate the effects of resveratrol on the phagocytotic activity of microglia, BV2 cells were cultured with latex beads (3 ìm) for ingestion. Representative micrographs showed that bead engulfment was potently induced by LPS treatment but was effectively blocked by resveratrol pretreatment (Fig. 2A). For quantification, phagocytotic ability of BV2 cells was evaluated by the number of cells ingesting at least one bead. In addition, phagocytotic capacity was counted as the mean number of beads in each BV2 cells in a random count of 200 cells that had ingested

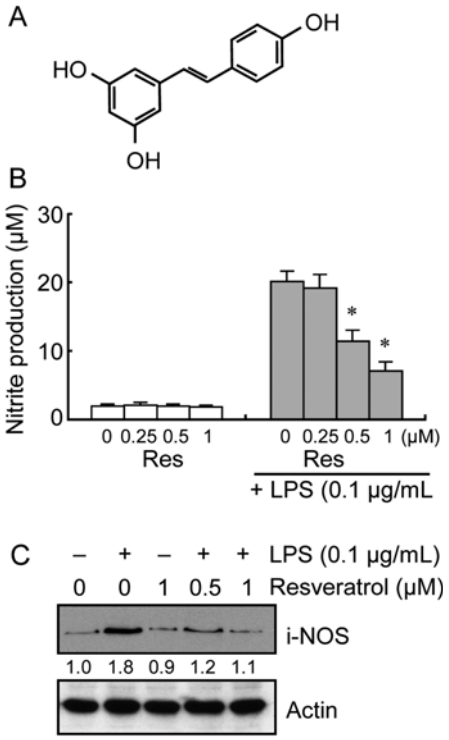

Fig. 1 Effects of resveratrol on NO production and iNOS expression in LPS-stimulated BV2 microglia. (A) Chemical structure of resveratrol. (B) Cells were pretreated with various concentrations of resveratrol (Res; 0.25, 0.5 and 1 μM) for 1 h followed by treatment with LPS (0.1 μg/mL) for 24 h. NO production was evaluated by measuring nitrite levels in the culture medium. (C) The expression levels of iNOS protein were determined in LPS-stimulated cells for 18 h by Western blot analysis. Band intensities were measured by densitometric analysis, and the normalized quantification values against actin were written below each band. All values are the mean ± SEM from three independent experiments. *, p <0.05 compared with LPS alone.

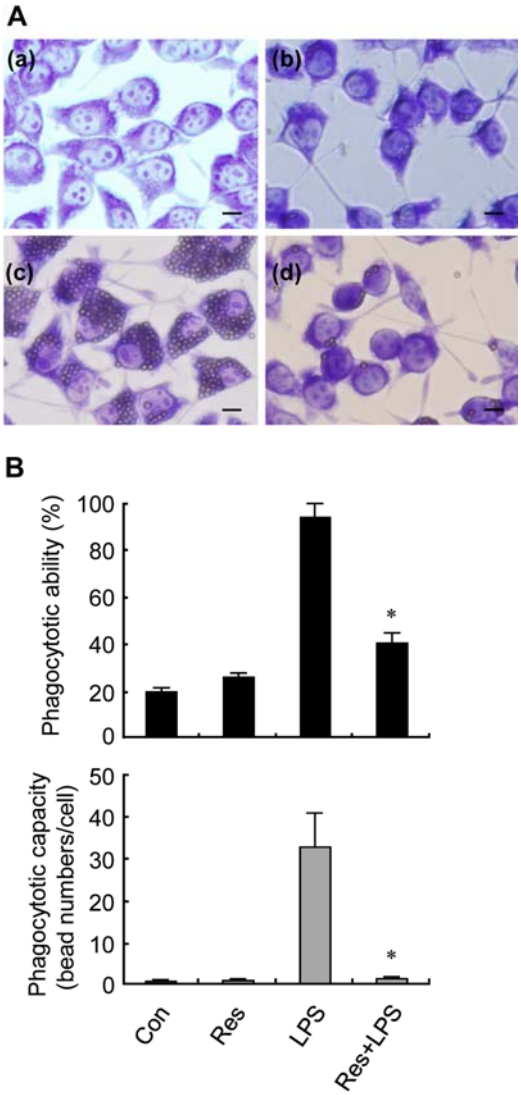

Fig. 2 Effect of resveratrol on phagocytotic activity. A. Micrographs indicate that BV2 cells exhibit phagocytotic activity by engulfment of latex beads. BV2 cells treated with vehicle (a), 1 μM resveratrol (b), 0.1 μg/mL LPS (c) or 1 μM resveratrol + 0.1 μg/mL LPS (d) for 18 h were incubated with latex beads for 6 h and then stained with Giemsa dye. The optical bead density was evaluated after counting 10 fields corresponding to at least 200 cells. B. Phagocytotic ability was evaluated by calculating the mean percentage cells engulfing at least one bead. The phagocytotic capacity was evaluated by estimating the mean number of engulfed beads/cell. Results are expressed as mean ± SEM of three independent experiments. *p <0.05, compared with LPS alone. Scale bars indicate 10 μm.

beads. Resveratrol attenuated LPS-induced phagocytotic ability by 57% and phagocytotic capacity by 96% (Fig. 2B). These results strongly suggest that resveratrol plays a critical role in the attenuation of microglia activation and microglial phagocytotic activity.

It has been observed that activated microglia mediate dopaminergic neuronal degeneration in progress of Parkinson's disease (Orr et al., 2002; McGeer and McGeer, 2008). Previous studies demonstrated that SIRT1 (the class III histone deacetylase) is activated by resveratrol in microglia and mediates the protective effects against microglia-dependent neurotoxicity (Borra et al., 2005; Chen et al., 2005). In accordance with previous studies, resveratrol *per se* increased SIRT1 activity (1.81-fold of control) in BV2 microglia. The decrease in SIRT1 activity by LPS (0.45-fold of control) was restored by pretreatment with resveratrol (0.95-fold of control) (Fig. 3A). To elucidate the inhibitory effects of resveratrol on microglial activation related to neurodegeneration, we examined the subsequent effects of the culture supernatants obtained from LPS-stimulated BV2 cells with or without resveratrol pretreatment on the dopaminergic SN4741 neuronal cells. As shown in Fig. 3B, exposure of SN4741 cells to LPS-stimulated BV2 conditioned medium caused a significant loss of viability (decreased by 40% compared to untreated control SN4741 cells). However, treatment with the conditioned media from the resveratrol-pretreated BV2 culture highly reduced neurotoxic effect (Fig. 3B). Interestingly, the beneficial effect of resveratrol

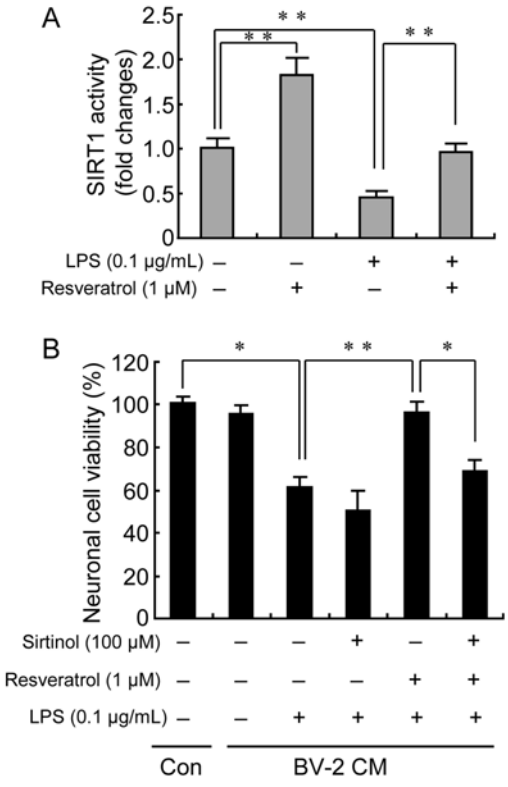

Fig. 3 Resveratrol activates SIRT1 in BV2 microglia and protects dopaminergic neurons against LPS-stimulated microgia. (A) Changes of SIRT1 activity in BV2 cells. After exposure of the cells to LPS (0.1 μg/mL) for 18 h with or without resveratrol (1 μM) pretreatment. SIRT1 level was determined using SIRT1 Direct Fluorescence Assay kit (Cayman Chemical) and assessed as the ratio against the value of untreated control. (B) Effect of resveratrol in LPS-stimulated BV2 microglial conditioned medium on neuronal cell viability. SN4741 cells, a murine dopaminergic neuronal cell line, were treated with different BV2 conditioned media. BV2 cells were pretreated with resveratrol (1 μM) with or without sirtinol (100 μM), a Sirt1 inhibitor, for 1 h and then stimulated with LPS (0.1 μg/mL) for 24 h. The conditioned media harvested from BV2 cells were transferred to SN4741 cells for further 24 h stimulation. The viability of neurons cultured with BV2 conditioned media was assessed by XTT assay. Con: untreated SN4741 control; BV2 CM: BV2 cells conditioned medium. All values represent mean ± SEM of three independent experiments. *p <0.05 and **p <0.01.

was almost completely blocked by sirtinol, an inhibitor of SIRT1, pretreatment. Sirtinol alone did not exert any toxicity on either BV2 cells or SN4741 cells (data not shown). When the SN4741 cells were treated with the conditioned media from BV2 cells sequentially treated with sirtinol (100 μM)/resveratrol (1 μM)/LPS (0.1 μg/mL), the viability of SN4741 cells was reduced to the similar level (viability 68%) to that caused by LPS-activated BV2 conditioned media (viability 60%) (Fig. 3B).

Resveratrol has received broad attention since 1992, when its presence in red wine was explained as the French Paradox. Although numerous effects of resveratrol have been suggested, no studies have yet proved that resveratrol prevents phagocytosis during the activation of microglia. In the present study, we demonstrated that resveratrol generally inhibits the induction of LPS-mediated microglial activation, and potently inhibits phagocytosis of LPS-stimulated BV2 microglia. It has been suggested that the effective concentration of resveratrol for neuroprotection is quite low and physiological concentration of resveratrol in total tissues and plasma after administration of red wine does not exceed 1 μM (Bhat et al., 2001). Although most previous studies used high concentrations (10–100 μM) of resveratrol with *in vitro* models, we used less than 1 μM of resveratrol to prevent nonspecific effects of high dose resveratrol such as general cytotoxicity and alterations of signal transduction. Resveratrol has been proposed to interact with various cell-signaling molecules and reduce the production of pro-inflammatory mediators in microglia (Bhat et al., 2001; Bi et al., 2005; Candelario-Jalil et al., 2007). Consistent with these studies, resveratrol effectively suppressed the expression of iNOS, as well as NO production from LPS-stimulated BV2 microglial cell cultures (Fig. 1).

Phagocytosis is an essential physiological function of activated microglia contributing to both normal and pathological conditions. Although microglia removes damaged neurons as part of a general repair process, inappropriately activated microglia cause early phagocytosis of normal neurons, and play the role as a significant risk factor associated with the process of neurodegenerative diseases (Kim and Joh, 2006). Previous studies suggested that activated microglia cluster around damaged dopaminergic neurons in the SN and perform phagocytotic clearance of those neurons at early stage of neuronal apoptosis (Banati et al., 1998; Sugama et al., 2003). Considering the inhibitory effects of resveratrol on microglial activation, we investigated whether resveratrol have a regulatory role in microglial phagocytosis (Fig. 2). Currently, there are no studies on the role of resveratrol in microglial phagocytosis. Our results clearly demonstrated for the first time that resveratrol effectively attenuated the LPS-induced phagocytosis in BV2 microglial cells. It has been known that microglia change their morphology from ramified resting form to enlarged amoeboid form during phagocytosis (Laurenzi et al., 2001). In accordance with previous study by Laurenzi et al (2001), our results showed that the cell body becomes enlarged into irregular amoeboidal shape by LPS treatment, but markedly reverted to regular unstimulated shape by resveratrol pretreatment (Fig. 2).

Because resveratrol markedly attenuated LPS-stimulated inflammatory and phagocytotic activation of microglia, we examined whether resveratrol pretreatment could prevent the harmful effects of LPS-stimulated microglia on dopaminergic neuronal cells (Fig. 3). As expected, the conditioned media taken from resveratrol pretreated microglia did not cause dopaminergic cell death. However, when BV2 cells were pretreated with SIRT1 inhibitor sirtinol, prior to resveratrol and following LPS treatment, the effects of resveratrol were reverted to basal level (Fig. 3). These results suggest that resveratrol may exert its inhibitory effects on microglial activation and subsequent dopaminergic damage through SIRT1 pathway. Recent studies suggested that

SIRT1 and SIRT1 agonist resveratrols are potent inhibitors of nuclear factor-kappaB transcription inducing the expression of several pro-inflammatory mediators such as iNOS, tumor necrosis factor (TNF)-α and interleukin (IL)-1β in microglia (Chen et al., 2005; Abraham and Johnson, 2009). In accordance with these studies, we found that resveratrol suppressed LPS-induced NF-κB activation, whereas pretreatment with the SIRT1 inhibitor, sirtinol, prior to resveratrol treatment retained the effect of LPS which elevates NF-κB activation (data not shown). Although the role of resveratrol on SIRT1 activation is controversial, recent studies showed that resveratrol could exhibit neuroprotection through the activation of SIRT1 (Bora et al., 2005; Chung et al., 2010). The present study provides first demonstration that resveratrol can inhibit phagocytosis of LPS-stimulated microglia. In addition, resveratrol prevented the dopaminergic cell death caused by LPS-stimulated microglia through SIRT1 pathway. Our results show that resveratrol could be a promising therapeutic compound for PD and other neurodegenerative diseases accompanied by microglial activation.

Acknowledgment This study was supported by research fund from Chosun University, 2012.

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