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Effect of rottlerin, a PKC-δ inhibitor, on TLR-4-dependent activation of murine microglia ☆

Dong-Chan Kim [a,b], Sun-Hee Kim [a], Min-Woo Jeong [a], Nam-in Baek [c], Kyong-Tai Kim [a,*]

[a] Division of Molecular and Life Science, SBD-NCRC, Pohang University of Science and Technology, Pohang, Republic of Korea
[b] Division of Research and Development, Neuronex, Inc., San31, Hyoja-dong, Nam-gu, Pohang 790-784, Republic of Korea
[c] Graduate School of Biotechnology and Plant Metabolism Research Center, Kyung Hee University, Suwon 449-701, Republic of Korea

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Abstract

In microglia, Toll-like receptors have been shown to recognize pathogen-associated molecular patterns and initiate innate immune responses upon interaction with infectious agents. The effect of rottlerin, a PKC-δ specific inhibitor, on TLR-4-mediated signaling was investigated in murine microglia stimulated with lipopolysaccharide and taxol. Pretreatment of microglia cells with rottlerin decreased LPS- and taxol-induced nitric oxide production in a concentration-dependent manner ($IC_{50} = 99.1 \pm 1.5$ nM). Through MTT and FACS analysis, we found that the inhibition effect of rottlerin was not due to microglial cell death. Rottlerin pretreatment also attenuated LPS-induced phosphorylation of IκB-α, nuclear translocation of NF-κB, and expression of type II nitric oxide synthase. In addition, microglial phagocytosis in response to TLR-4 activation was diminished in which rottlerin was pretreated. Together, these data raise the possibility that certain PKC-δ specific inhibitors can modulate TLR-4-derived signaling and inflammatory target gene expression, and can alter susceptibility to microbial infection and chronic inflammatory diseases in central nervous system.

Keywords: Rottlerin; Microglia; TLR-4; Nitric oxide; Phagocytosis

Microglia are the first non-neuronal cells that respond to central nervous system (CNS) injury [1]. Microglia have been appropriately called "sensors of pathology" because of their ability to react quickly to virtually all kinds of acute CNS injuries. The response of microglia to CNS injury is called microglial activation [2]. Although microglial activation is a key factor in the defence of the neural infectious diseases, excessive microglial activation can provoke severe neuronal damage by carrying destructive cascades [3]. In response to central nervous system injury, microglial cells are rapidly activated and migrate to the affected sites of neuronal damage where they secrete neurotrophic factors and phagocytose damaged cells [4].

TLR-4 serves as a specific receptor for lipopolysaccharide (LPS) and is localized on the surface of a microglial cells. LPS, also known as endotoxin, activates microglial cells to produce proinflammatory cytokines, proteases, eicosanoids, and reactive oxygen and nitrogen species [5]. Upon activation by LPS, TLR-4 signaling pathway activates IkappaB kinase (IKKs) which phosphorylates IkappaB (IκB). Phosphorylation of IκB leads to its ubiquitination and proteasomal degradation, thus allowing the anchored nuclear factor-kappaB (NF-κB) heterodimer to translocate to the nucleus and engage in transcription [6].

The protein kinase C (PKC) family mediates essential cellular signals required for activation, proliferation, differentiation, and survival [7]. There are at least 12 different PKC isoforms. Among the PKC family, three different

☆ *Abbreviations:* TLR-4, Toll-like receptor-4; NO, nitric oxide; IKK, IkappaB kinase; IκB, IkappaB; NF-κB, nuclear factor-kappaB; iNOS, type II nitric oxide synthase.

* Corresponding author. Fax: +82 54 279 2199.
E-mail address: ktk@postech.ac.kr (K.-T. Kim).

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general groupings can be characterized: (1) classical PKC isotypes (α, β, and γ) are dependent on both diacylglycerol and calcium, (2) novel PKC isotypes (δ, ε) are diacylglycerol dependent but calcium independent, and (3) atypical PKC isotypes (ζ, λ) are independent on both diacylglycerol and calcium [8]. Previous studies have shown that the classical PKC family controls nuclear translocation of NF-κB through activation of IKKs and degradation of IκB-α [9] and [10]. The involvement of novel isotype PKC-δ in TLR-4-induced BV-2 microglial cell activation and its phagocytic activity has not been examined. Here, we explored these issues and present that rottlerin, a well-known PKC-δ inhibitor, down-regulated phosphorylation of IκB and nuclear translocation of NF-κB in TLR-4-stimulated murine microglia.

Materials and methods

Cell culture. The immortalized murine BV-2 cell line that exhibits both the phenotypic and functional properties of reactive microglia cells [1] were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (Welgene, Korea), streptomycin, and penicillin as described previously [11]. Under a humidified 5% CO_2/95% air atmosphere and at 37 °C, cells were split twice a week and plated in 10 cm^2 Petri dishes (Corning, Acton, MA, USA) at a density of 5×10^5 cells for BV-2 cell line. For the experiments, cells were plated on 6-well dishes (2×10^6 cells/well).

NO production assay. To stimulate TLR-4, BV-2 cells were washed with phosphate-buffered saline (PBS) twice, replenished with a serum-free DMEM (Welgene, Korea) and LPS (Sigma, St. Louis, MO) was added to the culture medium. PKC inhibitors, GF109203X, RO318220, chelerythrine, and rottlerin (Calbiochem, San Diego, CA), were added to cells 10 min before LPS (100 ng/ml) treatment. In the studies with inhibitors, care was taken to ensure that cell viability was not altered under the concentrations of inhibitors used. NO produced by the BV-2 cells was determined by assaying the levels of NO_2^- using the Griess reagent (Sigma, St. Louis, MO).

Cell viability assay. Cell viability was assessed by using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl-tetrazolium bromide (MTT) assay [12]. Briefly, around 10,000 cells per well were plated in 96-well microtiter plates with 100 μl medium. Next day, the medium was changed and 10 μl MTT (5 mg/ml stock in PBS) was added to each well for 1 h at 37 °C. One hundred microliters of solubilization solution containing 20% SDS/50% DMF of pH 4.7 was added and absorption readings were performed at 540 nm with reference at 690 nm.

Flow cytometry analysis. Approximately 1×10^6 cells were washed with cold PBS before being resuspended in 200 μl cold 1× binding buffer. Ten microliters Annexin V and five microliters propidium iodide (PI) were added and incubated for 15 min at room temperature in the dark and washed twice with 1 ml FACS buffer (PBS containing 0.5% FBS and 0.09% sodium azide). Flow cytometric analysis was made immediately of at least 10,000 cells with a FACSCalibur instrument (Becton Dickinson Immunocytometry System).

Western blot analysis. Western blot analysis was performed as described previously [13]. In brief, BV-2 cells were cultured in 60-mm dishes and treated with LPS (100 ng/ml), in the presence or absence of rottlerin. After stimulation with LPS, cells were washed with chilled phosphate-buffered saline and then lysed with Triton lysis buffer for 45 min on ice. The lysates were clarified by centrifuging at 15,000*g* for 15 min, and the supernatants were stored at −80 °C. After quantifying the protein, total cell lysate containing the same amount of protein was loaded and separated by SDS–PAGE and transferred to nitrocellulose membranes. Protein immunoreactivity was visualized using a SUPEX kit (Neuronex, Korea), according to the manufacturer's instructions.

Immunocytochemistry. The translocation of NF-κB was visualized by immunocytochemistry. Cells multiplied in a chamber slide to 1×10^4 and, after a 15-h incubation, consisted of 60% monolayered cells. They were washed in PBS at 3 h after stimulation with LPS and fixed in 4% paraformaldehyde (PFA). After a 1-h fixation, cells were washed in PBS 3 times and then mouse anti-NF-κB p65 antibody (Santa Cruz, CA) was applied for 12 h. After 12 h of incubation, FITC-conjugated secondary antibody (Sigma, St. Louis, MO) was applied for 1 h at 37 °C. The stained preparations were washed with distilled water, floated onto microscope slides, and mounted with coverslips. Images were viewed with the fluorescence microscope (Carl Zeiss).

Phagocytosis assay. The phagocytotic ability of cells was determined by following the uptake of latex beads. Latex beads (particle diameter = 1.09 μm; 1 μl beads/ml; Sigma) were added to wells containing cultured cells. After an overnight incubation, cultures were rinsed several times with PBS. The phagocytosis of latex beads by the cells was examined under a microscope.

Statistical analysis. Results were expressed as means ± SEM. Statistical significance was determined by a modified *t* test. A P value less than 0.05 was considered statistically significant.

Results

Rottlerin inhibits LPS-induced NO production

The endotoxin LPS is the best-known target of innate recognition and induces a robust inflammatory response by microglial cells and induces stimulation of the NF-κB signaling pathways and NO synthesis [5]. To assess the role of PKC-δ in TLR-4-induced microglial activation, BV-2 cells were incubated with LPS in the absence or presence of distinct PKC inhibitors, GF109203X, RO318220, chelerythrine, and rottlerin (Fig. 1A). Only rottlerin significantly reduced LPS-induced NO production in a concentration-dependent manner (Figs. 1A and B), indicating that PKC-δ mediates TLR-4 signaling. Taxol also induces the production of NO in a Toll-like receptor-4 (TLR-4)-dependent manner in addition to its anti-tumor effects [14]. Taxol-induced NO production was also blocked by PKC-δ inhibitor (Fig. 1B).

Rottlerin treatment and cell viability

The PKC-δ inhibitor rottlerin itself may induce apoptosis that has been demonstrated in several cell lines including macrophage through mitochondrial membrane depolarization and caspases' cascade [15]. So, we investigated the effect of rottlerin on BV-2 cell viability. As shown in Figs. 2A and B, rottlerin treatment did not cause any significant cell injury as determined by MTT assay and FACS measurement, indicating that the inhibition effect of rottlerin on TLR-4-mediated NO production is not due to its cellular toxicity.

Rottlerin effects on TLR-4 signaling cascade and iNOS expression

TLR-4 signaling cascade includes MyD88/IRAKs/TRAF6 complex and this complex triggers phosphorylation of downstream components of the IKK (IκB kinase

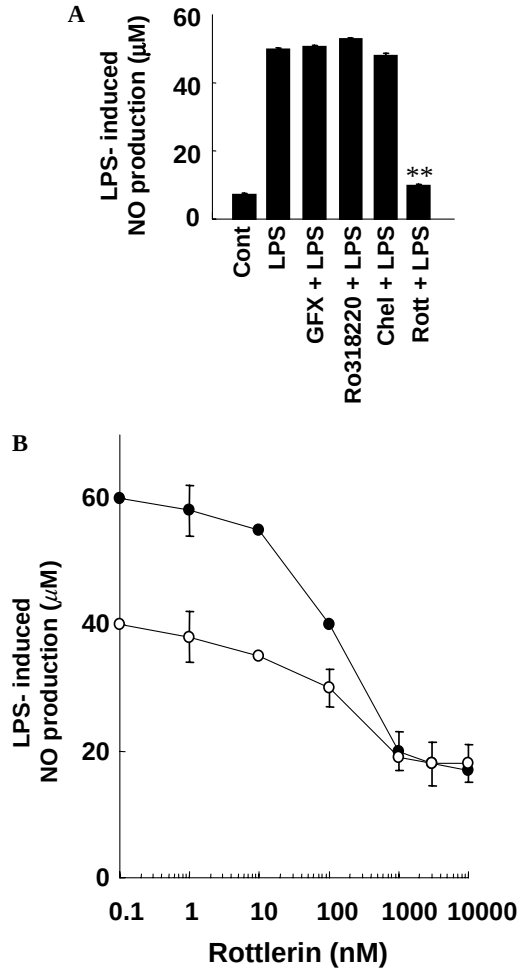

Fig. 1. Effect of rottlerin on TLR-4-mediated NO release. (A) BV-2 cells were pretreated with vehicle or 1 μM GF109203X, RO318220, chelerythrine, and rottlerin for 10 min, and stimulated with LPS (100 ng/ml) for 24 h. (B) BV-2 cells were pretreated with indicated concentrations of rottlerin and stimulated with 100 ng/ml LPS (closed circle) or 10 μM Taxol (open circle) for 24 h. Data represent means ± SEM of three independent experiments. Significant differences between LPS and Rott + LPS are presented. $^{**}P < 0.005$.

kinase) [16]. Activated IKKs phosphorylate IκB (inhibitor of NF-κB), which leads to its ubiquitination and proteasomal degradation, thus allowing the anchored NF-κB heterodimer to translocate to the nucleus and engage in transcription [17]. Rottlerin pretreatment markedly reduced the LPS-induced phosphorylation of IκB and the expression of iNOS (Fig. 3A). The activation of NF-κB was based on the detection of its translocation into cell nuclei from its initial location in the cytoplasm, where it exists in an inactive form [18]. As shown in Fig. 3B, NF-κB was primarily detected in the cytoplasm of BV-2 cells. Cells stimulated with LPS showed translocation of NF-κB into the nucleus, which was apparent from 1 h after stimulation (data not shown), and this became more evident 3 h after stimulation (Figs. 3B and C). However, the rottlerin-pretreated cells did not show LPS-induced NF-κB nuclear translocation (Figs. 3B and C).

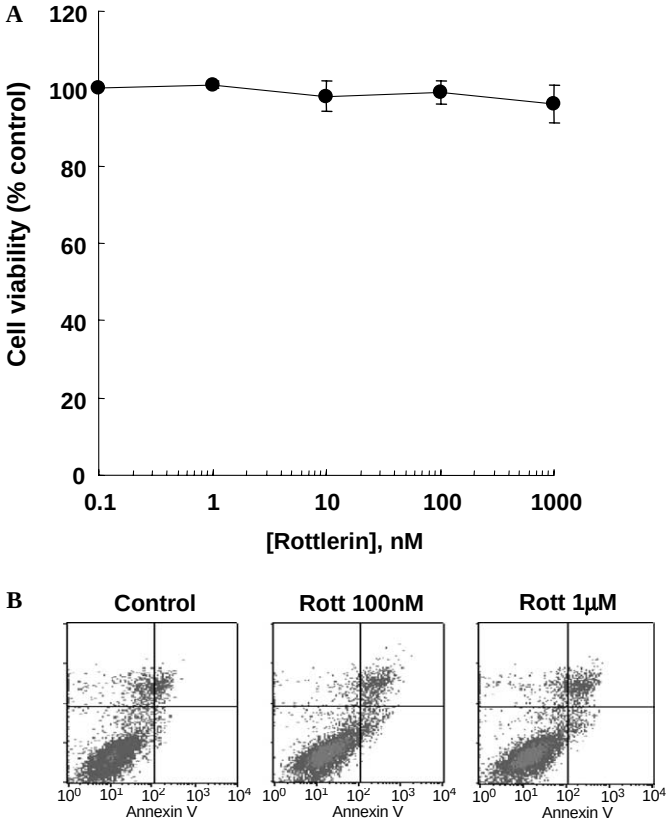

Fig. 2. Rottlerin effect on cell viability. MTT assay (A) and FACS analysis (B) in BV-2 microglial cells after 24 h exposure to indicated concentration of rottlerin. Results are means ± SD for 3–7 experiments.

Rottlerin effect on LPS-induced microglial phagocytosis

As the resident macrophage-type cells of the CNS, one of the physiological functions of microglia is the phagocytosis of large particles. Microglia can be activated to cytocidal status by stimulation with agents such as LPS [19,20]. To examine the involvement of PKC-δ on the phagocytotic activity of the BV-2 cells, we performed a phagocytosis assay using latex beads. Phagocytosed beads were counted using phase contrast microscopy (Fig. 4B). The LPS-stimulated BV-2 cells showed an increase in the number of phagocytosed bead particles in response to LPS (Figs. 4A and B). However, the rottlerin pretreatment dramatically reduced the LPS-induced phagocytosis (Figs. 4A and B). Together, our results strongly suggest that PKC-δ plays a critical role in the regulation of TLR-4-mediated NF-κB cascade and microglial activation.

Discussion

PKC is a phospholipid-dependent serine/threonine kinase family consisting of over 12 closely related isoforms [21]. The different PKC isoforms play important roles in signal transduction pathways, and the exact significance of each isoform is not well known so far. However, it is clear that the PKC isoforms show high heterogeneity with respect to substrate specificity, sensitivity to various

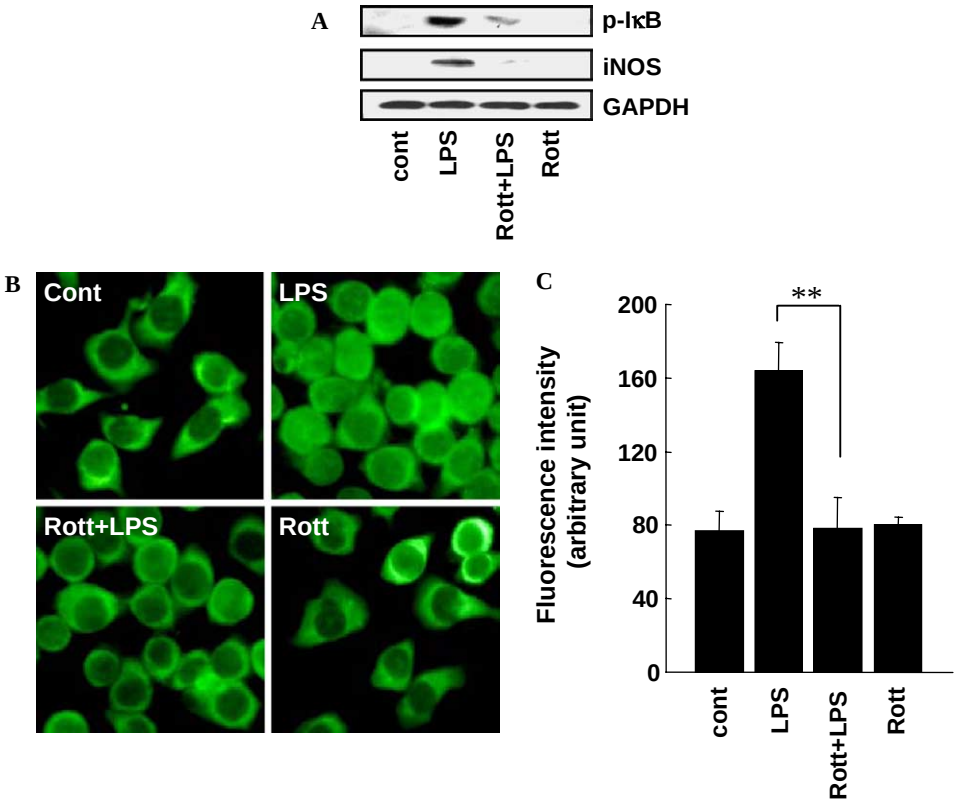

Fig. 3. Effect of rottlerin on LPS-induced IκB/NF-κB/iNOS signaling cascade. (A) Western blot shows that 1 μM rottlerin inhibited the 100 ng/ml LPS-induced phosphorylation of IκB-α and expression of iNOS proteins in BV-2 cells. Equal loading was ascertained by GAPDH. (B) The photomicrography of immunocytochemical staining of NF-κB. Control cells (Cont), the expression of NF-κB was detected in the cytoplasm. In cells stimulated with 100 ng/ml LPS (LPS), NF-κB was translocated into the nuclei. However, in rottlerin-pretreated cells (Rott + LPS) or rottlerin alone (Rott), NF-κB was not translocated into the nucleus. (C) Quantitative analysis showing fluorescent intensity of NF-κB (p65) protein in the BV-2 cell nucleus. Significant differences between LPS and Rott + LPS are presented. $^{**}P < 0.005$. Data represent means ± SEM of three independent experiments.

stimuli, lipid metabolites, and tumor-promoting phorbol ester binding activity [22]. Furthermore, the pattern of PKC isoform expression and subcellular localization varies in different cell types and in different stages of development [23], suggesting that individual PKC isoforms may regulate different functions within cells. In our present work, rottlerin pretreatment reduced LPS-induced NO production in murine microglia. Nitric oxide (NO) has been implicated in a number of important brain functions, such as long-term potentiation (LTP) and long-term depression (LTD) [24], and in events associated with neurodegeneration and neuroprotection [25]. In response to brain injury or disease, NO production is increased by an inducible enzyme (iNOS), which is only expressed under these conditions. Activated microglia are a major cellular source of iNOS in brain. Due to the important role of iNOS in brain injury and disease, a detailed understanding of intracellular mechanisms triggering the expression of iNOS in microglia would facilitate pharmacotherapeutic approaches.

Our present results strongly suggest that rottlerin regulates not only in the TLR-4-mediated production of iNOS protein but also in NF-κB signaling in BV-2 cells. Furthermore, rottlerin also reduced LPS-induced phosphorylation of IκB. The results implicate the possibility that PKC-δ may contribute to kinase activity of IKK in response to LPS stimulation, although the molecular mechanism of interaction between PKC-δ and IKK remains to be elucidated.

Microglial activation occurs in response to infection, inflammation, and neurological disorders including Alzheimer's disease, Parkinson's disease, and multiple sclerosis [26]. The markers for the activated microglia are morphological change [27], migration [28], and phagocytosis [20]. Especially, microglial phagocytosis is tightly related with neurodegeneration [29]. Bacterial endotoxin, LPS, has been used as an activator of microglial phagocytosis [19,20]. Our data demonstrate that PKC-δ may be involved in LPS-induced microglial phagocytosis. Furthermore, rottlerin or its derivatives can be used as critical compounds for regulating microglial activation. Although the effect of rottlerin on TLR-4 signaling exhibited very clearly, there are still many points to be elucidated. Since PKC-independent roles of rottlerin also have been reported [30], we have to elaborate the different pathways regulated by rottlerin which is involved in TLR-4-dependent activation of murine microglia.

In summary, our study clearly shows the inhibition effect of rottlerin on the signaling pathways for the IκB/NF-κB cascade and induction of iNOS by LPS in murine microglial cells. Elucidation of novel and critical signaling regulator for the induction of iNOS and phagocytosis by TLR-4

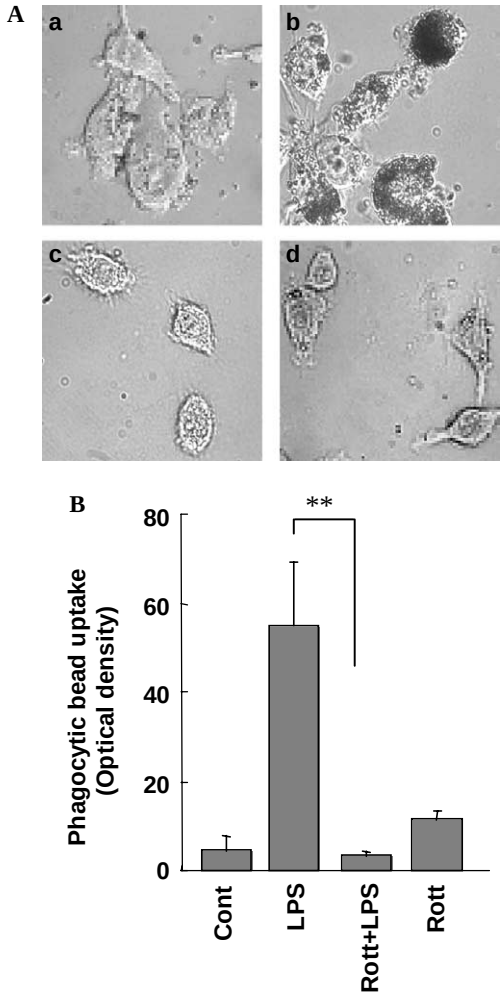

Fig. 4. Effect on rottlerin on phagocytotic activity of BV-2 cells. (A) LPS-induced phagocytosed beads were observed by phase contrast microscopy. BV-2 cells were stimulated with LPS (100 ng/ml) in the absence (b) or presence (c) of 1 μM rottlerin for 6 h. Picture (a) is vehicle-treated cells and (d) is rottlerin-treated one. The cells were then incubated with latex beads for 12 h. (B) The bead density was counted. Data represent means ± SEM of three independent experiments. Significant differences between LPS and Rott + LPS are presented. $^{**}P < 0.005$.

is an important step toward understanding the mechanisms underlying microglial activation in neurodegeneration and injury in the CNS.

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