Ajou Medical Journal
Vol. 1, No. 2, 1996

Intracellular Signaling Mechanisms Involved in LPS-induced Microglial Activation

Hankyoung Pyo and Eunhye Joe*

Department of Pharmacology, Ajou University School of Medicine, Suwon, Korea 442-749

Microglia activated within the injured nervous system further aggravate injuries[1,2]. To study intracellular signal mechanisms underlying microglial activation, we cultured microglila obtained from 1~3-day-old rat brains and activated them with a bacterial endotoxin, 500 ng/ml of lipopolysaccharide (LPS) for 2 days.

The activated microglia underwent morphological changes from small and round or ramified cell bodies to large and flat ones. The activated microglia induced nitric oxide synthase (iNOS), resulting in nitric oxide (NO) release. However, both the morphological changes and iNOS induction were completely blocked if either 30 uM genestein, a tyrosine kinase inhibitor or 1 uM Go, a protein kinase C inhibitor was added concomitantly with LPS. These results suggest that tyrosine kinase and protein kinase C are involved in LPS-induced microglial activation.

Key Words: Microglia; Activation; Tyrosine Kinase; Protein Kinase C; Nitric oxide

INTRODUCTION

Microglia, known as brain's immune cells, migrate to sites of cell damage and are activated by pathological conditions such as trauma and ischemia[3,4]. Following activation, the typical round or ramified cell bodies become enlarged and flattened appearing like macrophages[4,5,6]. Furthermore, the activated microglia proliferate, show an enhanced phagocytosis[7] and secrete NO which is harmful to neuron[1,2]. However, it is not known how microglia are activated during brain injury.

The best known intracellular signaling pathways involved in proliferation and differentiation(or activation) of several cells are protein tyrosine phosphorylation and intracellular kinase cascade[8,9]. Generally, factors to induce proliferation and differentiation such as growth factors or cytokines bind to receptors and initiate protein phosphorylation[10,11,12,13,14]. Growth factor receptors, upon being tyrosine phosphorylated, activate either phosphatidyl inositol-3 kinase(PI-3K) or phospholipase C γ (PLC γ). PLC γ, in turn, activates protein kinase C(PKC) and mitogen-activated protein kinases (MAPK)[8,9,15]. Cytokine receptors which lack of intracellular domain recruit and activate an intracellular tyrosine kinase, janus kinase(Jak) which, in turn, phosphorylates STAT(signal transducer and activator of transcription). Studies on LPS signaling pathway(s) revealed that LPS induced tyrosine phosphorylation of MAPK, janus kinase 3(Jak3), and PKC in endothelial cells[16], monocytes[17], and MONO-MAC-6 cell lines, respectively. Although LPS has been widely used as an microglial activator, it is not known what kind of intracellular signaling pathways are involved in microglial activation. To answer this, we examined the effects of several kinase inhibitors on LPS-induced microglial activation, demonstrated by morphological changes and NO release.

Reprint requests to: *To whom correspondence should be addressed at Dept. of Pharmacology, Ajou University School of Medicine, Suwon, Korea 442-749.

MATERIALS AND METHODS

Culture of microglia

Microglia and astrocytes were cocultured from the cerebral cortices of 1-3-day-old Sprague Dawley rats as previously described[7] with some modifications. The cortices were triturated into single cells in minimal essential media(Gibco) containing 5% fetal bovine serum(Gibco). Cells were plated

into 24-well plates and cultured at 37°C and 5% CO_2. After 1~2 weeks, microglia were activated with 500 ng/ml LPS(Sigma). To test the involvement of tyrosine kinase and/or protein kinase C in microglial activation, microglia were treated with LPS in the presence of either 30 uM genestein or 1 uM Go.

Immunocytochemistry

Identification of microglia and detection of iNOS expression were performed by immunocytochemistry using antibodies against microglial surface antigen OX-42(Accurate) and iNOS (UBI), respectively, following described procedure[18]. Cells were washed with phosphate buffered- saline(PBS), fixed with

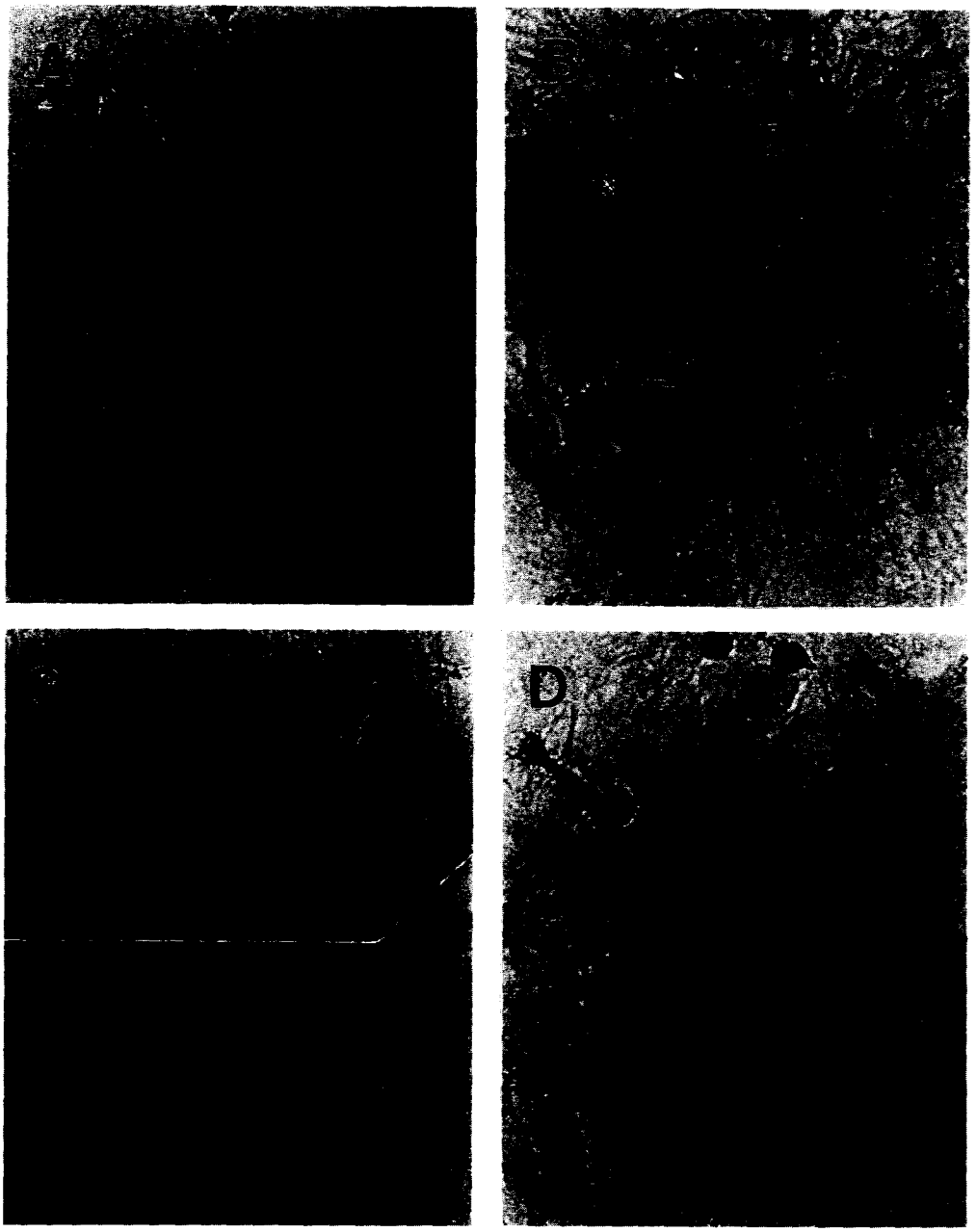

Fig. 1. Effect of Go and genestein on LPS-induced morphological changes of microglia. Microglia were identified using antibodies against OX-42. A, control; B, treated with 500 ng/ml LPS for 2 days; C and D treated with LPS in the presence of 1 uM Go and 30 uM genestein, repectively.

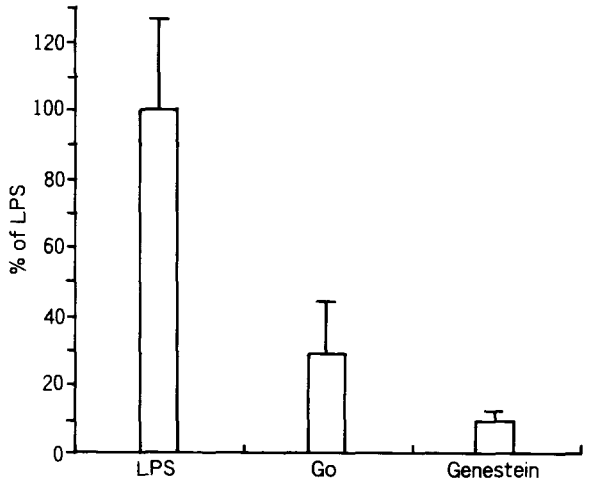

Fig. 2. Effect of Go and genestein on LPS-induced NO release. NO release was almost completely abolished by either 1 μM Go or 30 μM genestein. Values are mean±SEM and the number of samples for each condition is 8. The values are statistically significant ($p<0.05$ compared with LPS-induced NO release).

3.7% formaldehyde and labeled with anti-OX-42 antibodies or anti-iNOS antibodies for 2 hours. Cells were treated with peroxidase-conjugated secondary antibodies followed by color-developing reagents(Vector) according to manufacturer's guidence and examined under a bright field microscope using a Nikon Diaphot 300.

NO release

NO release was measured as described[19]. The supernatant (50 ul from a total volume of 400 ul) from microglial cultures was mixed with an equal volume of Griess reagent(0.1% naphthylethylene diamine, 1% sulfanilamide, 2.5% H_3PO_4). After 10 minute incubation at room temperature, optical density at 540 nm was measured with a microplate reader (Molecular Dynamics). Data were analyzed by Student-Newman-Keuls' test.

RESULTS

Microglia cocultured with astrocytes were identified with antibodies against OX-42(Fig. 1A). Two different morphological Characterity were detected(Fig. 1A). One type was round(arrow), and the other type was irregular and ramified(arrow head). When cells were treated with LPS(500 ng/ml), large and flattened cells were detected instead(white arrows in Fig. 1B). This morphological transition to the activated form was markedly blocked if cells were treated with LPS in the presence of either 1 uM Go or 30 uM genestein(Fig. 1C and D, respectively).

NO release was measured from microglia treated with LPS for 2 days in the presence or absence of kinase inhibitors(Fig. 2). The contribution of astrocytes to No release from this culture was negligible because cultures of pure astrocytes failed to produce NO in the presence of LPS(data not shown), as reported previously[1,20,21]. LPS-treated microglia released about 1 nmoles of NO in 2 days. However, both 1 uM Go and 30 uM genestein completely blocked LPS- induced No release.

To test whether inhibition of NO release by Go and genestein is due to blocking of iNOS expression, microglia were stained with antibodies against iNOS(Fig. 3). iNOS expression in microglia but not in underneath astrocytes was markedly increased in the presence of LPS(Fig. 3B). However, this iNOS expression was not detected in the presence of either 1 uM Go or 30 uM genestein(Fig. 1C and D). Thus, Go and genestein rather blocked gene expression induced by LPS than blocking the iNOS activity.

DISCUSSION

Recent studies revealed that LPS-induced activation of monocytes and endothelial cells is initiated by binding of LPS to CD14, subseguently increasing tyrosine phosphorylation of several proteins of MAPK family[22]. Furthermore, tyrosine kinase inhibitors blocked LPS-induced toxicity[23]. In this study, we examined the possibility that similar pathways might be involved in LPS-induced activation of microglia, since microglia are the counterpart of macrophages in the brain[4,7].

The results described herein show that genestein, receptor tyrosine kinase inhibitor, almost completely blocked microglial activation, evidenced by morphological changes and NO release(Fig. 1, 2, 3). Particularly, NO release was inhibited via blocking of iNOS expression rather than via blocking of enzyme activity(Fig. 3). This suggests that as in macrophages, receptor tyrosine kinase(s) is(are) involved in microglial activation. In order to find out the downstream signaling molecules of receptor tyrosine kinase(s), Go, a protein kinase C inhibitor, was tested due to the fact that MAPK(s) involved in activation of macrophages is downstream signaling

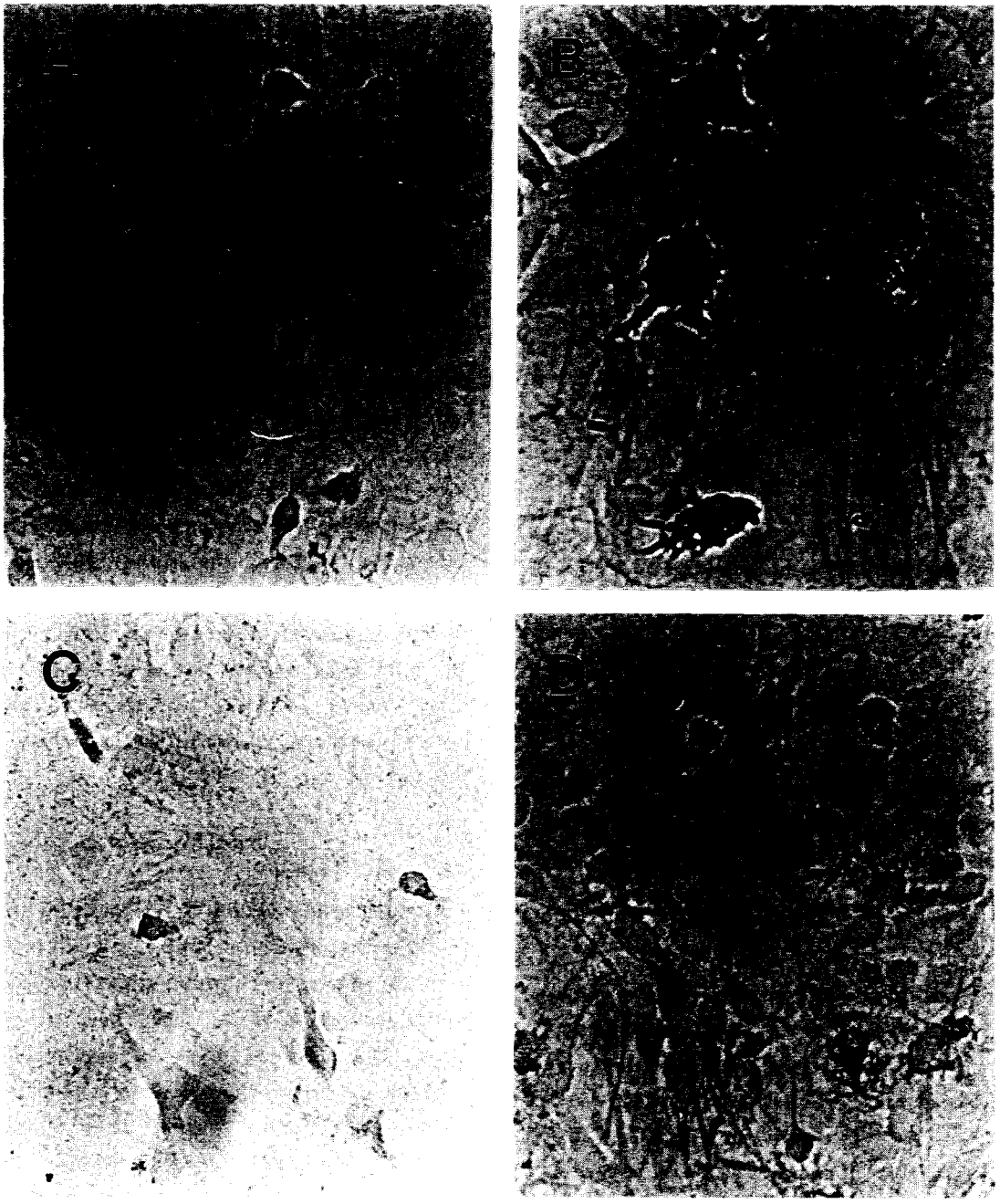

Fig. 3. Both Go and genestein blocked iNOS expression. Microglia were treated as in figure 1. A, untreated; B, treated with LPS; C, treated with LPS and Go; D, treated with LPS and genestein.

molecule(s) of protein kinase C[24,25,26]. Go also blocked both morphological changes and NO release/iNOS expression(Fig. 1, 2, 3). This means that receptor tyrosine kinase(s) and protein kinase C mediate microglial activation, and that both morphological changes and NO release may occur through the same signaling pathway.

Several studies on LPS-induced signaling pathways revealed the involvement of Jaks and STATs. Because Jaks are also tyrosine kinases which can be blocked by genestein, we can not exclude the possibility that Jaks and STATs are involved in LPS-induced microglial activation. Thus, further studies will be focused on elucidating the major pathway

involved in microglial activation between two main pathways, MAPKs and Jaks, which are known to be involved in proliferation and differention of many cells.

REFERENCES

1. Chao CC, Hu S, Molitor TW, Shaskan EG and Peterson PK: Activated microglia mediated neuronal cell injury via a nitric oxide machanism. J Immunol 149: 2730-2741, 1992
2. Meda L, Cassatella MA, Szendrei GI, Otvos Jr L, Baron P, Billalva M, Ferrari D and Rossi F: Activation of microglial cells by β-amyloid protein and interferon-γ. Nature 374: 647-650, 1995
3. Giulian D: Ameboid microglia as effectors of inflammation in the central nervous system. J Neurosci Res 18: 155-171, 1987
4. Rieske E, Graeber MB, Tetzlaff W, Czlonkowska A, Streit WJ and Kreuberg GW: Microglia and Microglia-derived brain macrophages in culture; generation from axotomized rat facial nuclei, identification and characterization in vitro. Brain Res. 492: 1-14.
5. Streit WJ, Graeber MB and Kreutzberg GW: Functional plasticity of microgilla: a review. Glia 1: 310-307, 1988
6. Bignami A: Glial cells in the central nervous system. Discussion in Neuroscience 8: 11-45, 1991
7. Giulian D and Baker TJ: Characterization of ameboid microglia isolated from developing mammalian brain. J Neurosci 6: 2163-2178, 1986
8. Vetter ML, Martin-Zanca D, Parada LF, Bishop JM and Kaplan DR: Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C by a kinase activity associated with the product of the trk protooncogene. Proc Natl Acad Sci 88: 5650-5654, 1991
9. Ohmichi M, Decker S and Saltiel A: Activation of phosphatidylinositol 3-kinase by nerve growth factor involves indirected coupling of the trk protooncogene with src homology 2 domain. Neuron 9: 769-777, 1992
10. Barbacid M, lamballe F, Pulido D and Klein R: The trk family of tyrosine protein kinase receptors. Biochem. Biophys. Acta 1072: 115-127, 1991
11. Bothwell M: Keeping track of neurotrophin receptors. Cell 65: 915-18, 1991
12. Bothwell M: Functional interactions of neurotrophins and neurotrophin receptors. Annu Rev neurosci 18: 223-253
13. Chao M: Neutrophin receptors: a window into neuronal differentiation. Neuron 9: 583-593, 1992
14. Stahl N, Boulton T, Farruggella T, Ip N and Daviz S: Association and activation of JAK-Trk kinases by CNTF-LIF-OSM-IL-6β receptor components. Science 263: 92-95, 1994
15. Yao R and Cooper G: Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 267: 2003-2006, 1995
16. Arditi M, Zhou J, Torres M, Durden DL, Stins M and Kim KS: Lipopolysaccharide stimulates the tyrosine phophorylation of mitogen-activated protein kinases p44, p42, and p41 in vascular endothelial cells in a soluble CD14-dependent manner. Role of protein tyrosine phosphroylation in lipopolysaccharide-induced stimulation of endothelial cells. J Immunol 155: 3994-4003, 1995
17. Musso T, Johnston JA, Linnekin D, Varesio L, Rowe TK, O'Shea JJ and McVicar DW: Regulation of JAK3 expression in human monocytes: phosphorylation in response to interleukins 2, 4, and 7. J Exp Med 181: 1425-1431, 1995
18. Springer TA and Ho MK: Macrophage differentiation antigens: Markers of macrophage subpopulations and tissue localization. In hybridomas in Cancer Diagnosis and Treatment. M.S. Mitchell and H.F. Oettgen, eds., Raven, New York, pp35-46, 1982
19. Ding AH, Nathan CF and Stuehr DJ: Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. J Immunol 141: 2407-2412, 1988
20. Zielasek J, Tausch M, Toka KV and Hartung HP: Production of nitite by neonatal rat microglial cells/Brain Macrophages. Cell Immunol 141: 11-120, 1992
21. Galea E, Feinstein DL and Reis DJ: Induction of calcium-independent nitric oxide synthase activity in primary cultures of rat astrocytes. Proc Natl Acad Sci USa 82: 10945-10949, 1992
22. Ulevitch RJ and Tobias PS: Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu Rev Immunol 13: 437-457, 1995
23. Novogrodsky A, Vanichkin A, Payya M, Gazit A, Osherov N and levitzki A: Prevention of lipopolysaccharide-induced lithal toxicity by tyrosine kinase inhibitors. Science 264: 1319-1322, 1994
24. Hambleton J, McMahon M and DeFranco AL: Activation of Raf-1 and mitogen-activated protein kinase in murine macrophages partialy mimics lipopolysaccharide-induced signaling events. J Exp Med 182: 147-154, 1995
25. Buscher D, Hipskind RA, Krautwald S, Reimann T and Baccarini M: Ras-dependent and -indepedndent pathways target the mitogen-activated protein kinase network in macrophages. Mol Cell Biol 15: 466-475, 1995
26. Liu MK, Herrera Velit P, Brownsey RW and Reiner NE: CD14-dependent activation of protein kinase C and mitogen-activated protein kinases(p42 and p44) in human monocytes treated with bacterial lipopolysaccharide. J Immunol 153: 2642-2652, 1994