These results suggested that NO/iNOS expression in BV2 microglia.

Microglia are major inflammatory cells of the brain. It has been known that interferon-gamma (IFN-\(\gamma\)) induces nitric oxide (NO)/inducible nitric oxide synthase (iNOS) in microglia, and that protein kinase C (PKC) mediates the action of IFN-\(\gamma\). In this study, we investigated isoforms of PKC that are involved in IFN-\(\gamma\)-induced expression of microglia using BV2 murine microglial cells. NO release/iNOS expression in IFN-\(\gamma\)-treated BV2 cells was reduced in the presence of PKC inhibitors (Go 6976 and BIM), and by long-term pre-treatment (48 h) of cells with phorbol-12-myristate-13-acetate (PMA) or thymeleatoxin. PMA depleted \(\alpha\), \(\beta\), \(\delta\), and \(\varepsilon\) isoforms, and thymeleatoxin depleted \(\alpha\), \(\beta\), and \(\varepsilon\) isoforms although \(\gamma\), \(\eta\), \(\iota\), \(\lambda\), \(\theta\), \(\mu\), and \(\zeta\) were also detected in these cells. Furthermore, IFN-\(\gamma\) phosphorylated \(\alpha\) and \(\varepsilon\) on their tyrosine residues. These results suggested that \(\alpha\) and \(\varepsilon\) could be the major PKC isoforms involved in signaling pathways of IFN-\(\gamma\) to induce NO/iNOS expression in BV2 microglia. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Microglia; Protein kinase C; Interferon-gamma

Brain inflammation has been suggested as a risk factor of neurodegenerative disease [6,9]. Microglia, brain inflammatory cells, are activated in injured brain and released inflammatory mediators such as nitric oxide (NO), tumor necrosis factor-alpha (TNF-\(\alpha\)), and prostaglandins [4,8,12]. The inflammatory mediators could protect tissues from bacterial infection but also could potentiate the damage of neurons [5,13].

Interferon-gamma (IFN-\(\gamma\)) has been considered as a microglial activator. IFN-\(\gamma\) alone could induce microglial activation and also enhance microglial activation induced by beta-amyloid [1,2,9,10]. Although the intracellular signaling molecules involved in microglial activation have not been fully understood, protein kinase C (PKC) has been considered as an important mediator of microglial activation [15,17]. PKC inhibitors reduced NO release from lipopolysaccharide (LPS)-treated microglia and beta-amyloid-induced chemotaxis of these cells [15,17]. PKC is also an important mediator of IFN-\(\gamma\) to induce activation of macrophages [3].

Until recently, 12 isoforms of PKC have been identified. \(\alpha\), \(\beta\), \(\beta I\), \(\gamma\), \(\delta\), \(\epsilon\), \(\eta\), \(\iota\), \(\gamma\), \(\lambda\), \(\theta\), \(\mu\), and \(\zeta\). PKC isoforms are classified into three groups depending on factors, which are required for their activation. Classical PKCs (\(\alpha\), \(\beta I\), \(\beta II\), \(\gamma\)) require calcium, diacylglycerol, and phosphatidyl serine, novel PKCs (\(\delta\), \(\epsilon\), \(\eta\)) require diacylglycerol and phosphatidyl serine, whereas atypical PKCs (\(\iota\), \(\gamma\), \(\lambda\), \(\theta\), \(\zeta\)) and PKC \(\mu\) require phosphatidyl serine only. Thus, each isoform of PKCs could be regulated by differential stimuli and regulate differential downstream signaling molecules [11,14]. In this study, we examined the isoforms of PKCs involved in IFN-\(\gamma\)-induced microglial activation.

BV2 immortalized murine microglial cell line was provided by Dr. EJ Choi in Korea University. BV2 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY) containing 5% fetal bovine serum (Hyclone, Logan, UT). To measure NO release, cells (5 x 10^5 cells/well) were plated into 24-well plates and stimulated with 10 U/ml IFN-\(\gamma\) (Genzyme, Cambridge, MA) in the absence or presence of PKC inhibitors, 1 \(\mu\)M Gö 6976 (Calbiochem, La Jolla, CA) and 1 \(\mu\)M bisindolylmaleimide (BIM, Calbiochem) for 2 days. The amount of nitrite converted from NO released into the culture medium was determined by Griess method. The culture medium was
mixed with an equal volume of Griess reagent (0.1% naphthylethylene diamine, 1% sulfanilamide, 2.5% H₃PO₄). After 10 min incubation at room temperature, optical density at 540 nm was measured.

For Western blot analysis, cells (1 × 10⁶ cells/100 mm dish) were washed with phosphate-buffered saline (PBS) and lysed using modified RIPA buffer (150 mM NaCl, 10 mM Na₂HPO₄, pH 7.2, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 mM NaVO₃, 2 mM PMSF, 100 µg/ml leupeptin, 2 mM EDTA, 10 µg/ml pepstatin). Proteins were separated on a 10% SDS polyacrylamide gel and transferred onto PVDF (Millipore, Bedford, MA, USA) membrane. The membrane was incubated with 7.5% non-fat dry milk, and then with antibodies against iNOS (UBI, Lake Placid, NY) or PKC. Antibodies against PKC β, δ, ε, θ, υ, ζ and µ isotypes were from Transduction Laboratories (Lexington, KY), α and γ from Santa Cruz (Santa Cruz, CA), and η from Biomol (Plymouth Meeting, PA). The expression of iNOS and PKCs was detected using enhanced chemiluminescense (Amersham, England). PKC inhibitors, Gö 6976 (1 µM) and BIM (1 µM), were used to block PKC activity. To deplete PKC, cells were pretreated with 1 µM phorbol-12-myristate-13-acetate (PMA, Sigma, St. Louise, MO) or 20 nM thymeleatoxin (Thy, Sigma) for 48 h.

To determine tyrosine phosphorylation of PKC isoforms, cells were treated with 10 U/ml IFN-γ for 30 min. Cells were lysed using modified RIPA buffer and PKCs were immunoprecipitated using isofom-specific antibodies and protein G-sepharose (Santa Cruz, CA) at 4°C. After being separated on an 8% SDS polyacrylamide gel, Western blot analysis was carried out using phosphorytosine-specific antibodies (UBI, Lake placid, NY). Same membranes were stripped with 0.2 N NaOH for 20 min and used for Western blot analysis using isofom-specific antibodies.

To examine the effect of PKC on IFN-γ-induced NO release and iNOS expression, PKC was inhibited using its inhibitors or depleted by long-term treatment of cells with phorbol-12-myristate-13-acetate (PMA) or thymeleatoxin (Thy). Inhibition of PKC significantly abated IFN-γ-induced NO release and iNOS expression in BV2 microglia (Fig. 1). In the absence of any inhibitor, cells treated with 10 U/ml IFN-γ produced 9.7 ± 0.3 µM nitrite while untreated cells produced 1.4 ± 0.3 µM. However, in the presence of 1 µM Gö 6976 and 1 µM BIM, nitrite produced from cells treated with 10 U/ml IFN-γ was 5.5 ± 0.1 µM and 6.0 ± 0.3 µM, respectively (Fig. 1A). In Western blot analysis, similar results were obtained: IFN-γ-induced iNOS expression was significantly reduced by both 1 µM Gö 6976 and 1 µM BIM (Fig. 1B, upper panel). Pre-treatment of cells with 1 µM PMA and 20 nM Thy for 48 h also inhibited iNOS expression (Fig. 1B, lower panel).

Since pre-treatment of cells with PMA and Thy reduced iNOS expression, we examined which isoforms of PKCs were depleted by PMA- and Thy-treatment. All 12 isoforms of PKCs were detected in untreated cells. Cells treated with 1 µM PMA showed significant depletion of α, β, δ, and ε, while cells treated with 20 nM Thy showed significant depletion of ε and relatively weak depletion of α and β (Fig. 2). Both PMA and Thy had little effect on the level of η, υ, Λ, µ, θ, and ζ isoforms. Next, we examined PKC isoforms that could be phosphorylated on their tyrosine residues when cells were treated with IFN-γ since tyrosine phosphorylation could be considered as activation of PKCs [7,16]. Upon IFN-γ treatment, tyrosine phosphorylation of α and ε was increased while that of β and δ was not changed (Fig. 3).

Brain damage, such as human demyelinating disorder multiple sclerosis (MS), usually accompanies breakdown of the blood-brain barrier. Thus, T cells and natural killer cells could infiltrate into the brain and produce IFN-γ, which further stimulates microglia to produce NO to activate PKCs which can then further generate NO. This positive feedback loop limited the accessibility of NO from peripheral blood cells to the brain by increasing the local production of NO.
resulting in microglial activation [1]. The results of this study suggested PKC α and ε as the probable isoforms that mediate IFN-γ-induced microglial NO/iNOS expression. The PKC inhibitor BIM has high specificity for α, β, γ, δ and ε, and Gö 6976 shows specificity for α and β. Both BIM and Gö 6976 reduced NO/iNOS expression (Fig. 1A). Thus, PKC isoforms inhibited by these inhibitors, such as α, β, γ, δ and ε, could be involved in the action of IFN-γ. In PKC depletion experiments, α, β, and γ were depleted by both PMA and Thy, and δ was additionally depleted by PMA (Fig. 2). Furthermore, IFN-γ induced tyrosine phosphorylation of α and ε (Fig. 3). The results from three independent experiments led us to expect PKC α and ε as probable PKC isoforms involved in the action of IFN-γ. Nakai et al. [16] suggested the involvement of α and ε in microglial activation induced by beta-amyloid although the involvement of δ was also suggested. Although we still could not exclude the possible involvement of other isoforms such as β and δ in the action of IFN-γ, α and ε could be common PKC isoforms that are involved in microglial activation induced by IFN-γ as well as other stimulators such as beta-amyloid.

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[8] Lee, S.C., Liu, W., Dickson, D.W., Brosnan, C.F. and Berman, J.W., Cytokine production by human fetal microglia and


