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p38 MAPK and NF-κB are Required for LPS-Induced RANTES Production in Immortalized Murine Microglia (BV-2)

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Using murine immortalized microglial cells (BV-2), we examined the regulation of RANTES production stimulated by lipopolysaccharide (LPS), focusing on the role of mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB. The result showed that RANTES (regulated upon activation of normal T cell expressed and secreted) was induced at the mRNA and protein levels in a dose- and time-dependent manner in response to LPS. From investigations of second messenger pathways involved in regulating the secretion of RANTES, we found that LPS induced phosphorylation of extracellular signal-regulated kinase (Erk), p38 MAPK and c-Jun-N-terminal kinase (JNK), and activated NF-κB. To determine whether this MAPK phosphorylation is involved in LPS-stimulated RANTES production, we used specific inhibitors for p38 MAPK and Erk, SB 203580 and PD 98059, respectively. LPS-induced RANTES production was reduced approximately 80% at 25 μM of SB 203580 treatment. But PD 98059 did not affect RANTES production. Pyrrolidine-dithiocarbamate (PDTC), NF-κB inhibitor, reduced RANTES secretion. These results suggest that LPS-induced RANTES production in microglial cells (BV-2) is mainly mediated by the coordination of p38 MAPK and NF-κB cascade.

Key Words: Microglia, BV-2, RANTES, LPS, MAPK, NF-κB

INTRODUCTION

Microglia is a macrophage-like cell in the CNS. In response to trauma, immune-mediated events and infections within the CNS, microglial cells become activated and the activated microglia enhances phagocytosis and produces several substances involved in inflammation. One of these substances is chemokine. Chemokine is a secreted protein that function as a chemoattractant mediating the recruitment of specific subsets of leukocytes to sites of tissue damage and immunological reactions. In the CNS, chemokines were found to be involved in the pathogenesis of many important neuroinflammatory diseases such as multiple sclerosis, stroke and HIV encephalopathy (McGeer & McGeer, 1995; Asensio et al, 1999).

Recent studies have demonstrated that activated microglial cells produce both α- and β-chemokines including RANTES, a member of the β-chemokine family (Hayashi et al, 1995; Peterson et al, 1997; Hausmann et al, 1998; Hua & Lee, 2000). RANTES has been proposed to play a role in recruiting immunocytes from the periphery into the CNS due to its potent chemoattractant properties (Schall et al, 1988; Schall et al, 1990; Bell et al, 1996; Howard et al, 1996).

Recently, several MAPK signaling pathways have been demonstrated to play a central role in mediating intracellular signal transduction from the cell surface to the nucleus. Many extracellular stimuli elicit specific biological responses through activation of MAPK cascades (Davis, 1994). At least three MAPK subfamilies are present in mammalian cells and form distinct signaling cascades. These include Erk, p38 MAPK and JNK. p38 MAPK is activated in response to stress signals, including hyperosmotic shock, heat shock, cold shock, UV irradiation and inflammatory cytokines, and play an important role in apoptosis and cytokine expression (Han et al, 1994; Rouse et al,

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1994; Raingeaud et al, 1995; Pandey et al, 1996). Whereas Erk is activated by mitogenic stimuli, and plays a central role in cell proliferation and differentiation (Cowley et al, 1994; Force & Bonventre, 1998). However, recent studies have shown that Erk and JNK also play important roles in signal cascades of induction of various inflammatory mediators including cytokines and chemical mediators (Bhat et al, 1998; Rawadi et al, 1998).

The transcriptional activator, NF-κB is also a key component controlling the synthesis of cytokines and many other immunoregulatory gene products (Baeuerle & Henkel, 1994; Schulze-Osthoff et al, 1995). It is triggered by a great variety of proinflammatory or pathogenic stimuli including inflammatory cytokines, phorbol ester and LPS, as well as ionizing and UV irradiation (Wulczyn et al, 1996; Li & Karin, 1998; Djavaheri-Mergny et al, 1999; Manna et al, 1999). NF-κB is a dimer consisting of two Rel family proteins (p50 and p65) that is activated in the cytoplasm as a result of the phosphorylation and consequent degradation of IκB-α. The subsequent translocation of NF-κB to the nucleus results in the activation of target genes (Henkel et al, 1993; Baeuerle & Baltimore, 1996).

Recent studies have shown that MAPK and NF-κB are involved in the synthesis of cytokines in microglial cells (Bhat et al, 1998; Pyo et al, 1998; Lee et al, 2000). But, less is known about the signal cascade in the LPS-induced RANTES production of microglial cells. Therefore, in the present study we attempted to examine the role of MAPK and NF-κB in RANTES production in LPS-stimulated microglial cells (BV-2). BV-2 cell line used in this study was established by immortalization of mouse microglial cells using infection with the J2 retrovirus (Blasi et al, 1990) and confirmed that it has its own properties of microglia (Bocchini et al, 1992; Wood et al, 1994).

METHODS

Materials

LPS, SB 203580 and PDTC were obtained from Sigma (St. Louis, MO. USA). RANTES goat monoclonal antibody and RANTES were from R & D systems (Minneapolis, MN). Antibodies of p38 MAPK, phospho-p38 MAPK, Erk (p44/p42 MAPK), phospho-Erk (p44/p42 MAPK), phospho-SAPK/JNK and PD98059 were from New England Biolabs (Beverly, MA). IκB-α antibody and anti-goat IgG antibody were from Santa Cruz Biotechnology. Oligo (dT) 12~18 primer, dNTP mixture and SuperScript II RNase reverse transcriptase were from GIBCO-BRL (Gaithersburg, MD). BV-2 cells were a generous gift from Dr. Choi EJ (Korea University). All other chemicals used were of the highest grade available.

Cell culture

The murine microglial cell line (BV-2) was cultured in DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 45 mM sodium bicarbonate, 10 units/ml and 10 mg/ml of a mixture of penicillin/streptomycin. The cells were seeded to 60 mm^2 dishes in a density of 1.8×10^6/well. After 24 h incubation, experiments were conducted.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Reverse transcription of 1 μg of RNA was performed with Oligo (dT) 12~18 primer followed by the addition of a reaction mixture containing 5$\times$first strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$, 0.1 M DTT), SuperScript II RNase H-Reverse Transcriptase and 10 mM deoxynucleoside triphosphates mix (10 mM each of dATP, dGTP, dCTP and dTTP) in a final volume of 20 μl. The mixture was incubated at 42°C for 1 h followed by termination at 70°C for 15 min. Amplification of RANTES or β-actin cDNA was performed with an automatic thermocycler in a reaction mixture containing 400 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates mixture, 1 U of Taq DNA polymerase, 10 pM primer (sense and antisense), cDNA and H_2O. Amplification was set at 94°C for 30s, 60°C for 40s, and 72°C for 60s followed by a 6 min extension at 72°C. The RANTES PCR product (205 bp) and β-actin (435 bp) were amplified for 28 cycles and 20 cycles, respectively. Both PCR products were viewed under UV light after 2% agarose gel electrophoresis and staining in ethidium bromide. The RANTES primer sets were 5'-CCC CAT ATT CCT CGG ACA CCA CAC-3' (sense) and 5'-TCC TAG CTC ATC TCC AAA GAG TTG-3' (antisense). The β-actin primer sets were 5'-ATG GGT CAG AAG GAT TCC TAT GTG-3' (sense) and 5'-CTT CAT GAG GTA GTC

AGT CAG GTC-3' (antisense).

Western blot analysis

Western blot was performed for the analysis of p38 MAPK, Erk, JNK phosphorylation and NF-κB activation. Microglial cells were treated with 100 ng/ml LPS for 5 min to 3 h. Cells were washed with cold phosphate-buffered saline and lysed with a lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 0.25% sodium deoxycholate, 1% NP-40, 1 mM NaF and 1 mM $NaVO_3$), including protease inhibitors (1 mM PMSF, 1 μg/ml leu-peptin, pepstatin, aprotinine and 1 mM EDTA). Protein samples were separated by 12% SDS-PAGE and electrophoretically transferred to membrane. The membrane was incubated with specific antibodies to phosphorylated threonine and tyrosine of p38 MAPK, Erk, JNK and IκB-α antibody. Next, it was incubated with the horseradish peroxidase (HRP)-conjugated anti-rabbit antibody. Blots were incubated with enhanced chemiluminescence solution (LumiGLO) for 1 min and exposed on Hyperfilm ECL (Amersham LIFE SCIENCE).

Enzyme-linked immunosorbent assay (ELISA)

The secretion of RANTES in the culture supernatants was measured by an ELISA developed in our laboratory. Briefly, 96-well culture plates were coated with a standard series of diluted RANTES and supernatant samples. The plates were stored overnight at 4°C. The following day, the plates were washed with 0.05% PBS/Tween-20 (PBST) and nonspecific binding was blocked by treatment with 5% BSA for 30 min at room temperature. After washing, goat anti-RANTES antibody (1 : 1,000 in PBST) were added and incubated for 2 h at room temperature. After washing, anti-goat IgG horseradish peroxidase conjugate (1 : 1,000 in PBST) was added for 2 h at room temperature. After extensive washing with PBST, substrate was added for 30 min at room temperature for color development. OD was read at 450 nm and compared with standard values for quantification.

RESULTS

LPS-induced RANTES production

We investigated the RANTES production of microglial cells in terms of mRNA and secreted protein. The RANTES production was increased in a time-dependent manner following LPS (100 ng/ml) stimulation, which was plateau at the time of 12 h (67.1 $\pm$ 2.1 ng/ml; Fig. 1A). The expression of RANTES mRNA was not detected in cells per se. It was induced by LPS at 4 h and sustained to 16 h (Fig. 1B). The RANTES production was increased in a dose-dependent manner. It was secreted at the concentration of 10 ng/ml LPS and reached the plateau at 100 ng/ml (64.4 $\pm$ 1.9 ng/ml; Fig. 2A). RT-PCR product of RANTES was also detected in a dose dependent manner (Fig. 2B).

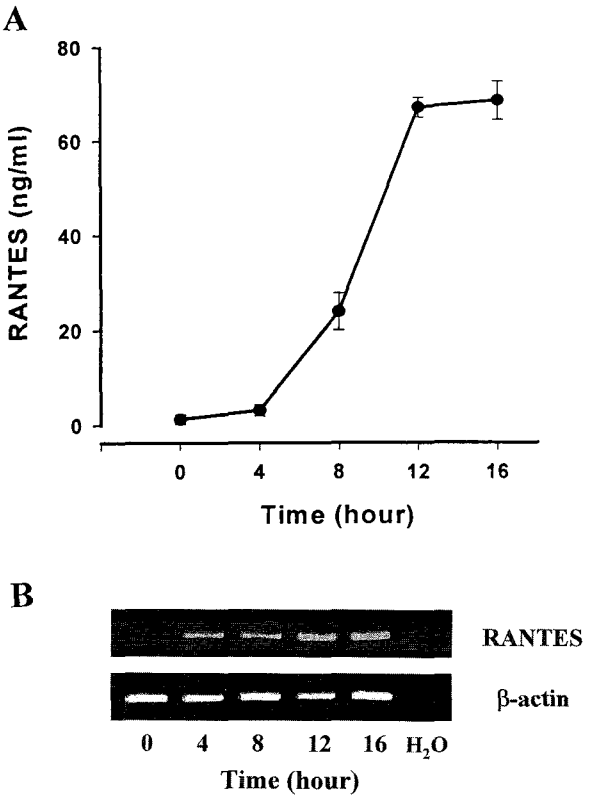

Fig. 1. Effects of LPS on RANTES expression in a time dependent manner. Cells were treated with a constant amount (100 ng/ml) of LPS at various times. (A) RANTES in the supernatant was quantified by ELISA technique. (B) Total RNA was then harvested from the cells after using the supernatant as shown in (A) for RT-PCR. β-actin primer was used as an internal control. The RT-PCR products shown were amplified for 28 cycles in RANTES primer sets and 20 cycles in β-actin primer sets. DATA are presented as mean $\pm$ SEM of three experiments.

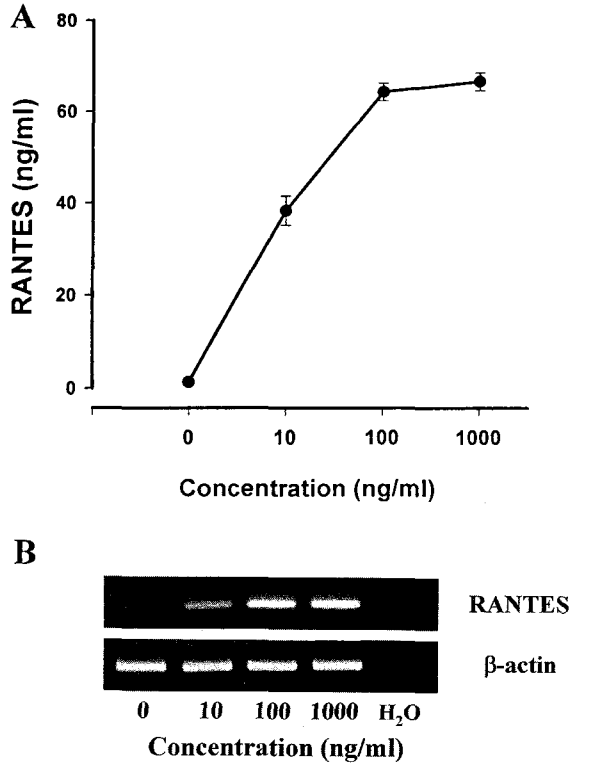

Fig. 2. Effects of LPS on RANTES expression of in a dose-dependent manner. Cells were incubated with various concentration of LPS for 12 h. (A) RANTES in the supernatant was quantified by ELISA technique. (B) Total RNA was then harvested from the cells after using the supernatant as shown in (A) for RT-PCR. β-actin primer was used as an internal control. The RT-PCR products shown were amplified for 28 cycles in RANTES primer sets and 20 cycles in β-actin primer sets. DATA are presented as mean$\pm$SEM of three experiments.

LPS-induced p38 MAPK, Erk and JNK phosphorylation

To determine whether LPS could induce the MAPK, cells were treated with LPS (100 ng/ml). Samples were collected at different time points and immunoblotted with antibodies for p38 MAPK phosphorylated at Thr180 and Tyr182, Erk phosphorylated at Thr202 and Tyr204 or SAPK/JNK phosphorylated at Thr183 and Tyr185. Phospho-p38 MAPK increased at 10 min, reached to the plateau at 2 h (Fig. 3A, upper panel). Phospho-Erk increased highly at 10 min and it was sustained until 3 h (Fig. 3B, upper panel). Phospho-JNK was detected at 10 min, increased to 30 min and then diminished (Fig. 3C). β-actin was blotted as an internal control (Fig. 4D).

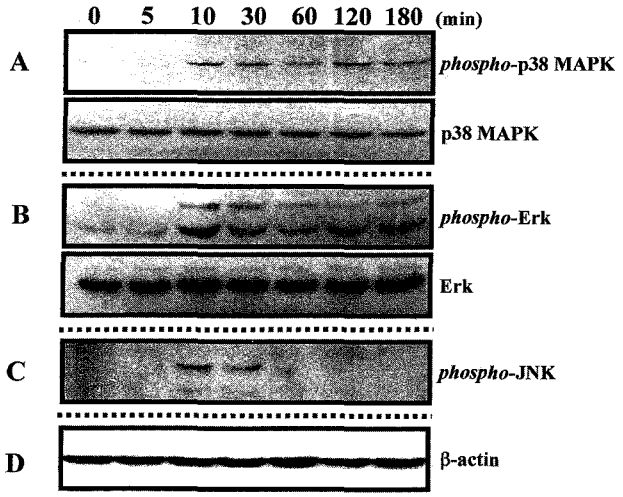

Fig. 3. LPS-induced threonine and tyrosine phosphorylation of p38 MAPK, Erk, and JNK. Cells were stimulated with LPS (100 ng/ml). Samples were collected at different time points and separated by 12% SDS-PAGE, transferred to membranes, and blotted with specific antibodies to phosphorylated threonine and tyrosine of p38 MAPK (upper panel of A), Erk (upper panel of B) and JNK (panel of C). Lower panels of A and B show the amounts of p38 MAPK and Erk blotted. β-actin was blotted as an internal control (panel D).

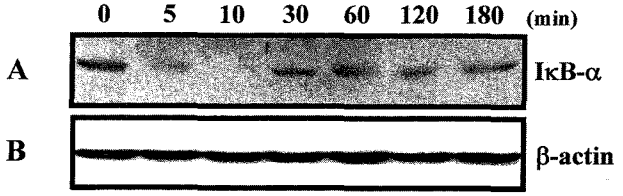

Fig. 4. LPS-induced NF-κB activation. Cells were stimulated with LPS (100 ng/ml). Samples were collected at different time points and separated by 12% SDS-PAGE, transferred to membranes, and blotted with a specific antibody to IκB-α (panel A). β-actin was blotted as an internal control (panel B).

LPS-induced NF-κB activation

To investigate whether LPS could induce NF-κB activation, we observed degradation of IκB-α, an inhibitor of the transcription factor NF-κB (Henkel et al, 1993). Samples treated with LPS (100 ng/ml) were collected at different time points and immunoblotted with antibody for IκB-α. The IκB-α was decreased at 5 min, disappeared at 10 min, and then returned to the basal levels at 30 min (Fig. 4A). β-actin was blotted as an internal control (Fig. 4B).

Inhibition of LPS-induced RANTES production by MAPK inhibitors

To define the involvement of MAPK pathways in LPS-induced RANTES production, we used two specific inhibitors: PD 98059, Erk inhibitor (Dudley et al, 1995) and SB 203580, p38 MAPK inhibitor (Lee et al, 1994). Cells were preincubated for 1 h with various concentrations of SB 203580 and PD 98059 followed by treatment with LPS (100 ng/ml) for 12 h. The RANTES production was determined in the cell supernatants and compared to the supernatant treated with only LPS and the solvent of inhibitors (0.25% DMEM). As shown in Fig. 5A, p38 MAPK inhibitor significantly reduced the RANTES production in a dose-dependent manner. No cell toxicity was observed when cells were treated with SB 203580 at the highest used concentration of 25 μM (data not shown). The Erk inhibitor, PD 98059, did not inhibit the LPS-mediated RANTES production (Fig. 5B).

Inhibition of LPS-induced RANTES production by NF-κB inhibitor

To investigate the involvement of NF-κB activation in RANTES production induced by LPS, we used the recently reported specific NF-κB inhibitor, PDTC (Liu SF et al, 1999). Cells were preincubated for 1 h with various concentrations of PDTC before being challenged with LPS. RANTES production was determined in cell supernatants after 12 h of stimulation. As shown in Fig. 6, PDTC inhibited the RANTES production in a concentration-dependent manner, which underscores the involvement of NF-κB activation in RANTES production of microglial cells in response to LPS. No cell toxicity was observed with 100 μM PDTC.

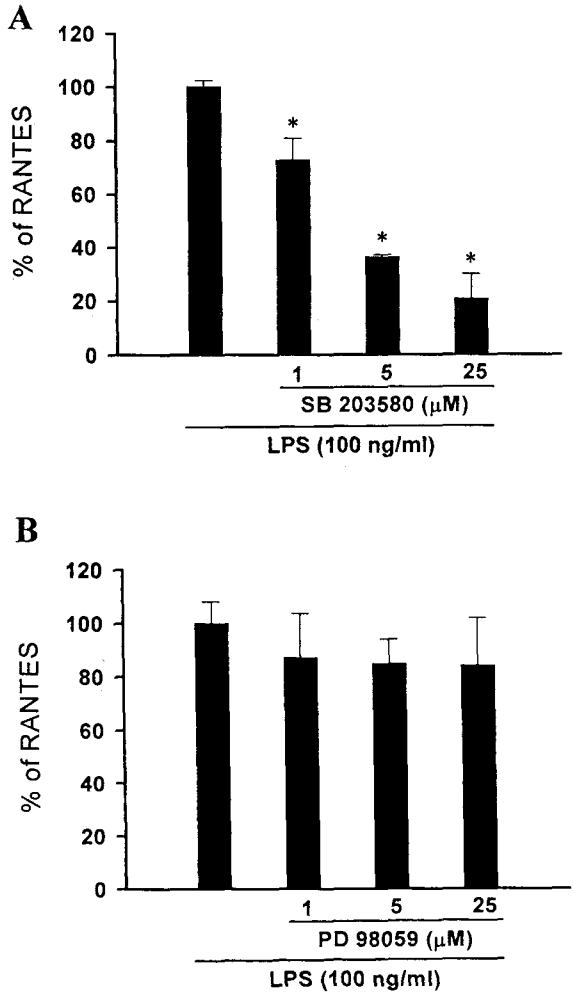

Fig. 5. Effect of p38 MAPK and Erkspecific inhibitors on RANTES production. Cells were preincubated with various concentrations of SB 203580 (A) and PD 98059 (B) for 1 h and then stimulated with LPS (100 ng/ml). The concentration of RANTES in the culture supernatants were measured at 12 h after cultivation by ELISA. DATA are presented as mean ± SEM of three experiments. *P < 0.05 vs. without SB 203580.

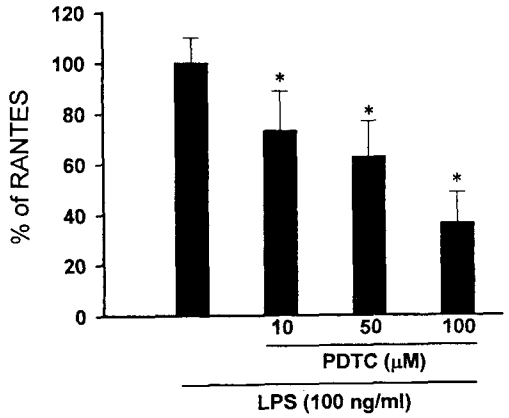

Fig. 6. Effect of NF-κB specific inhibitor on RANTES production. Cells were preincubated with various concentrations of PDTC for 1 h and then stimulated with LPS (100 ng/ml). The concentration of RANTES in the culture supernatants were measured at 12 h after cultivation by ELISA. DATA are presented as mean ± SEM of three experiments. *P < 0.05 vs. without PDTC.

DISCUSSION

In the present study, we investigated the intracellular signaling mechanisms of RANTES production in microglial cells (BV-2). The results showed that the well known microglial activator, LPS, induced RANTES production as mentioned in other studies in rat and human microglial cells (Peterson et al, 1997; McManus et al, 1998; Hu et al, 1999; Hua & Lee, 2000). Analysis of signal transduction pathway regulating RANTES production showed that LPS induced the phosphorylation of Erk, p38 MAPK and JNK but RANTES production was suppressed by only p38 MAPK inhibitor, SB 203580 (Fig. 3 & Fig. 5). In LPS-stimulated microgial cells, 25 μM of SB 203580 caused approximately 80% inhibition of RANTES production. In contrast to SB 203580, 25 μM of PD 98059 almost did not affect the inhibition of RANTES production, which implies that p38 MAPK may be the main pathway to produce RANTES in LPS-stimulated microglial cells.

Although we could not investigate the role of JNK because the specific inhibitor was not available yet, it might be one of the possible pathway in the regulation of RANTES production because JNK was also phosphorylated by LPS (Fig. 3C). Some reports have already shown that the JNK pathway is involved in cytokine expression (Pyo et al, 1998; Rawadi et al, 1998; Zhang et al, 1998). However, further study utilizing a specific inhibitor for JNK is required to determine the role of JNK in the regulation of RANTES production.

Our findings in murine microglial cells where SB 203580 reduced the production of RANTES protein are consistent with studies using other cell types and stimuli, which show that p38 MAPK plays an important role in RANTES production (Hashimoto et al, 2000). In addition, a recent study demonstrated that p38 MAPK is required for LPS-induced TNF-α in human microglial cells (Lee et al, 2000). It was reported that Erk and JNK pathways as well as p38 MAPK are involved in RANTES production in other cell type or stimuli (Kujime et al, 2000; Maruoka et al, 2000), and LPS-induced TNF-α production in rat microglia (Bhat et al, 1998; Pyo et al, 1998). However, based on our result in Fig. 5B, the Erk pathway did not induce RANTES production in microglial cells.

We also showed the role of NF-κB activation in LPS-induced RANTES production. In this study LPS induced NF-κB activation in microglial cells (Fig. 4) as other studies have shown (Bonaiuto et al, 1997; Hartlage-Rubsamen et al, 1999; Heyen et al, 2000). And 100 μM of PDTC caused a nearly 64% inhibition of RANTES production (Fig. 6). It has also been shown that NF-κB potently up-regulates the promoter activity of RANTES in human T cells and bronchial epithelial cells (Manni et al, 1996; Moriuchi et al, 1997). Moreover, Ehrlich et al (1998) reported that NF-κB is involved in cytokine expression in microglial cells. In this result, the inhibition of NF-κB activation partially suppressed RANTES production. Therefore, it could be assumed that other transcription factors also might regulate LPS-stimulated RANTES production in microglial cells as shown in other groups (Boehlk et al, 2000; Lakics et al, 2000).

There are several reports that SB 203580 prevents the expression of a NF-κB controlled reporter gene in response to external stimuli. These authors suggest that p38 MAPK affects NF-κB activation through activation of co-activators or intermediate kinases (Beyaert et al, 1996; Wesselborg et al, 1997). Although it has not been yet determined whether the activation of members of the MAPK family is involved in RANTES transcription in microglial cells some reports showed that SB 203580 reduced transcription of other cytokine genes such as IL-6 and IFN-γ in other cell types (Beyaer et al, 1996; Rincon et al, 1998). Based on these results, we suggest that NF-κB can be activated via p38 MAPK pathway. Further studies on the activation of NF-κB by the p38 MAPK pathway will help us to understand the mechanism of RANTES production in murine microglial cells.

In summary, our results show that the RANTES production is regulated by MAPK, at least p38 MAPK, and NF-κB in microglial cells (BV-2) stimulated by LPS. And also, we suggest that the RANTES production is modulated by the coordination of the p38 MAPK and NF-κB cascade.

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