J. Exp. Biomed. Sci. 10 (2004) 325-332

Protein Kinases as Pharmacological Targets for the Reduction of Interleukin-1 Expression in Lipopolysaccaride-Activated Primary Glial Cell

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Inflammatory factor such as Interleukin-1 play important roles in determining the fate of both acute and chronic neurological disorders. We investigated whether inhibitors of PKC or PTK can serve as pharmacological agents to reduce IL-1 production and the mechanisms underlying their pharmacological effects in a mixed population of glia. Inhibitors of PKC such as H7, Gö6976 and Ro31-8220 significantly reduced both the mRNA and protein levels of IL-1α and IL-β in lipopolysaccharide-activated primary glial cells. While the PTK inhibitor genistein also significantly reduced the production of these cytokines, it did not affect the expression of their mRNA. Taken together, inhibitors of PKC and PTK could serve as pharmacological agents to reduce IL-1 production. However, the mechanisms underlying their pharmacological effects are different. Our results provide evidence that inhibitors of protein kinases can serve as pharmacological agents to modulate IL-1 production in glial cell, and in turn, alleviate neuronal injury.

Key Words: Glial cell, Microglia, Interleukin-1, Neuroinflammation

INTRODUCTION

Inflammation in the central nervous system (CNS) has been demonstrated in both acute and chronic neurological disorders such as ischemic and traumatic brain injuries, Parkinson's disease and Alzheimer's disease (Rothwell, 1997; Rothwell et al., 1995). Of all the cell types in the CNS, microglia (brain macrophages) and astrocytes play important roles in cerebral inflammation (Beta et al., 1996; Dickson et al., 1993; Rothwell et al., 1995) and consequently, determine the fate of neurodegeneration. It has long been known that both microglia and astrocytes produce nitric oxide (NO) and various cytokines in microglia- and astrocyte-enriched cultures (Chung et al., 1990; Giulian et al., 1994; Lee et al., 1993; Liu et al., 1996; Sawada et al., 1989; Wood et al., 1994). It is not clear why inflammatory factors are produced by both types of glial cells in the CNS. Apparently, tight regulations between these two types of glial cells occur during the complex process of inflammation. In fact, conditioned medium from cultured astrocytes modulates the morphology and the channel properties of cultured microglia (Eder et al., 1998). It is also not clear of the effectiveness of pharmacological agent in reducing production of inflammatory factors when microglia and astrocytes are closely connected.

Among different inflammatory factors, interleukin 1 (IL-1) has been demonstrated to be an important cytokine in cerebral ischemia (Beta et al., 1996; Stroemer et al., 1998). Injection of IL-1β agumented neuronal injury in an animal model of ischemia (Stroemer et al., 1998). On the other hand, injection or over expression of IL-1 receptor antagonist reduce neuronal injury (Stroemer et al., 1997; Yang et al., 1997). Beside neuronal injury, blocking IL-1 activity with zinc protoporphyrin also reduced brain edema (Yamasaki et al., 1994). It have been shown that IL-1 combined with other cytokines could synergisitically increase neurtoxicity in a mixed neuron-glia culture model (Jeohn et al., 1998). Taking all these findings together, IL-1 can be regarded as a pathogenic factor in cerebral ischemia (Yamasaki et al., 1995).

*Received: November 7, 2004
Accepted after revision: December 10, 204
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We have been tried to study the signaling events for cytokines, such as IL-1, in glial cells, and whether these events can be targets for pharmacological agents. The other group have been demonstrated that the inhibitor of protein tyrosine kinase (PTK) genistein significantly reduced the production of NO and cytokines elicited by the bacterial endotoxin Lipopolysaccharide (LPS) in glia (Kong et al., 1996; LY et al., 1997). However, the mechanisms underlying its effects are still not clear. Besides PTK, protein kinase C (PKC) is also an important signaling pathway for the production of IL-1 (Fenton, 1992). Therefore, this study was designed to investigate whether inhibitors of PKC can serve as pharmacological agents to modulate production of IL-1. In addition, the underlying mechanisms of the PKC inhibitors were studied and compared with that of genistein.

MEHTODS AND MATERIALS

1. Materials

LPS (*E. coli* O111:B4) was purchased from List Biological Laboratories (Campbell, CA, USA). Cycloheximide and TRI-reagent™ were obtained from Sigma (St. Louis, MO, USA). Tissue culture 24 well plates were purchased from Costar (Cambridge, MA, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium with Ham's nutrient mixture F12 (DMEM/F12), genistein, and the Superscript™ Preamplication System were obtained from Life Technologies (Gaithersburg, MD). H7, N-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA1004), Gö6976, Ro31-8220, and actinomycin D were purchased from Calbiochem-Novabiochem International (La Jolla, CA, USA). The Gene Amp PCR kit with AmpliTaq DNA polymerase was purchased from Perkin Elmer (Foster City, CA, USA). The primers for IL-1α, IL-1β, iNOS, and β-actin and competitive DNA fragments for IL-1α, IL-1β, and iNOS were purchased from Clontech Laboratories (Palo Alto, CA, USA).

2. Glia cultures and stimulation of the glial cells

Primary cultures of murine mixed glia were prepared as described before (Jeohn et al., 1998). Briefly, mixed glial cultures were prepared from the whole brains of postnatal day 1 CD-1 mice. The brains were mechanically dissociated by trituration in a Ca^{2+}- and Mg^{2+}-free physiological buffer (145 mM NaCl, 5.4 mM KCl, 1 mM NaH_2PO_4, 15 mM HEPES, and 11 mM glucose at pH 7.4). One-half ml of the cell suspension (0.5×10^6 cells) was added into each well of 24-well tissue culture plates, and cells were grown in an incubator equipped with humidified 5% CO_2 / 95% air at 37℃. Glial cells were cultured with DME/F12 medium containing 10% heat- inactivated FBS. The DME/F12 medium was replenished one and four days after plating, and was changed every three days thereafter. Cells were used 15 days after plating. Cell composition was examined by immunocytochemical analysis of using antibodies against markers for microglia (Mac-1, Boehringer Mannheim Biochemicals, Indianapolis, IN, USA: anti-Mac-1, 1:10) and astrocytes (glial fibrillary acidic protein, GFAP: DAKO Corporation, Carpinteria, CA, USA: anti-GFAP, 1:1,000). Glial cells contained about 30% microglia and about 65% astrocytes (Jeohn et al., 2000). For the pharmacological studies, mixed glial cultures were incubated with the following agents at different concentrations (LPS at 1 μg/ml, cycloheximide at 5 μM, Actinomycin D at 2 μM, HA1004 at 25 μM, H7 at 2~50 μM, Gö6976 at 2 μM, Ro31-8220 at 5 μM, or genistein at 2~50 μM). All pharmacological agents were diluted with the culture medium.

3. RT-PCR analysis

Total RNA of glial cells was prepared using the TRI-reagent™ according to the method of Chomczynski and Sacchi (Chomcynski et al., 1987). One microgram of the total RNA from each treatment was used for the reverse transcription reaction in 20 μl with the Superscript™ Preamplification System. The level of target mRNA was examined by a modification of the previously described RT-PCR analysis of iNOS mRNA (Jeohn et al., 2000). On and one-half microliters of the reverse transcription reaction (corresponding to 0.075 μg of template RNA) were used for each PCR analysis using the Gene Amp PCR kit with AmpliTaq DNA polymerase. PCR reaction for IL-1α were performed with the forward primer 5'-AAGATGTCCAACTTCACCTTCAAGGAGAGCCG-3' and the reverse primer 5'-AGGTCGGTCTCACTACCTGTGATGAGTTTTGG-3', giving a product of 491 bp. For IL-1β, the forward and reverse primers giving a product of 563 bp were 5'-ATGGCAACTGTTCCTGAACTCAACT-3' and 5'-CAGGACAGGTATAGATTCTTTCCTTT-3' respectively. For iNOS, the forward and reverse primers giving a product of 496 bp were 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' and 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3' respectively.

The forward and reverse primers giving a product of 540 bp for β-actin were 5'-GTGGGCCGCTCTAGGCACCAA-3' and 5'-CTCTTTGATGTCACGCACGATTTC-3' respectively. Competitive PCR reactions were run with 1 attomole of a competitive DNA fragment (referred to as MIMIC) from the IL-1α gene (344 bp), 1 attomole of MIMIC from the IL-1β gene (397 bp), or 0.125 attomole of MIMIC from the iNOS gene (272 bp). For every PCR reaction, the concentration of the primer was 1 μM. The remperatures for the denaturing, annealing and extension reactions for PCR were as follows : first cycle : 3 min at 95℃ : followed by 1 min at 55℃ (IL-1α) and 65℃ (iNOS), 60℃ (IL-1β), or 60℃ (β-actin): and 1.5 min at 72℃. The second cycle was for 1 min at 95℃; followed by 1 min at 55℃ (IL-1α) and 65℃ (iNOS), 60℃ (IL-1β), or 60℃ (β-actin): and 1.5 min at 72℃. All subsequent cycles were 94℃ for 30 sec, followed by 30 sec at 55℃ (IL-1α) and 65℃ (iNOS), or 60℃ (IL-1β and β-actin), and for 45 sec at 72℃. The template was amplified for 23 cycles for IL-1α and IL-1β, 25 cycles for iNOS, or 21 cycles for β-actin. The amplified DNA fragments were quantified by densitometry using the MULTI-ANALYST program of Model GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA, USA).

4. Biochemical assay for the production of IL-1

Based on a previous study (Kong et al., 1997), the culture supernatants were collected at 48 h for the quantification of IL-1α and IL-1β with enzyme-linked immunosorbent assay (ELISA) kits (Genzyme, Cambridge, MA, USA). The antibodies used did not have any detectable cross-reactivity with other known cytokines.

5. Data analysis

The data were expressed as the mean ± SD. An analysis of variance (ANOVA) followed by the Bonferroni-Dunn multiple comparison test was used for statistical comparisons. A value of $P<0.05$ was considered significant.

RESULTS

1. Expression of mRNA for inflammatory factors in LPS-stimulated glia

In order to determine the experimental conditions suitable for the study of pharmacological agents against protein kinases, we examined the expression of the mRNAs for IL-1α and IL-1β in glia stimulated with LPS using competitive RT-PCR. Fig. 1A shows the results from different PCR cycle numbers for the detection of the mRNA transcripts for IL-1α and IL-1β. After stimulation with LPS for 3 h, cells expressed both forms of IL-1 and the optimal cycle number for PCR was 20-24. LPS stimulated mRNA expression of IL-1α and IL-1β in a time-dependent manner. The profile of IL-1 expression was distinct from that of iNOS for which the onset time was relatively late. Inhibition of transcription with actinomycin D markedly reduced the

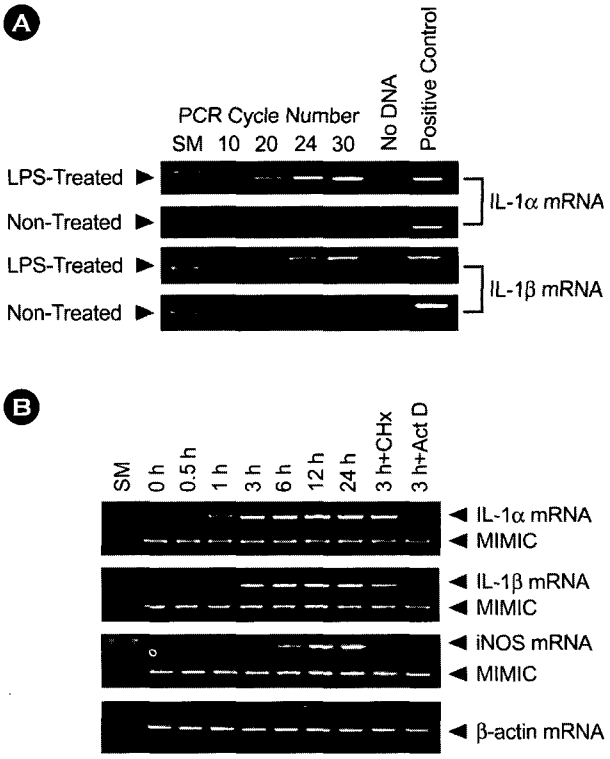

Fig. 1. Effect of LPS on the expression of IL-α and IL-β in mouse primary mixed glia cultures. **A.** RT-PCR amplification of LPS-induced expression of mRNAs for IL-1α and IL-β. Primary mixed glial cells were exposed to LPS (1 μg/ml) for 3 h. One microgram of the total RNA from each treatment was used for the reverse transcription reaction, and 1.5 (l of the reaction product was used for PCR in order to determine the optimal PCR-cycle number. Each amplified cDNA fragment was consistent with the calculated size between the two sense and antisense primers. The induction by LPS and RT-PCR analysis of each target gene was repeated 3 times and one representative gel is shown. SM, size marker (100 bp DNA ladder). **B.** The time-course of LPS-induced expression of IL-1α, IL-1β and iNOS mRNAs; and the effects of cycloheximide and actinomycin D on the LPS-induced stimulation of the proinflammatory cytokines. Glial cells were stimulated with LPS, and total RNA was extracted at the different indicated time points. For the study of transcription and protein synthesis for IL-1, cells were pretreated with culture media containing 5 μM cycloheximide or 2 μM actinomycin D for 1 h before stimulation with 1 μg/ml LPS. SM, size marker (100 bp DNA ladder).

transcription of LPS-stimulated IL-1α and IL-1β by about 95%. While LPS-stimulated IL-1α transcription was not reduced by the protein synthesis inhibitor cycloheximide, IL-1β transcription was reduced by about 50% (Fig. 1B). The expression of the mRNA for the housekeeping gene β-actin remained unchanged at all time points examined (Fig. 1B).

2. Effects of inhibitors for serine/threonine kinases on mRNA expression for IL-1

After studying the experimental conditions and time-course of mRNA expression for IL-1, the inhibitors for serine/threonine kinases were examined to determine whether they could reduce transcription of IL-1 in LPS-stimulated glial cells. The concentrations of inhibitors employed were based on previous studies (Kong et al., 1997; Chung et al., 1992). Glial cells were pretreated with different inhibitors for 1 h prior to the stimulation with 1 mg/ml LPS. Afterwards, the expression of the mRNAs for IL-1α and IL-1β was examined by using RT-PCR at 3 h after stimulation with LPS. The mRNAs for IL-1α and IL-1β and β-actin were not induced when glial cells were treated with the inhibitors alone for 3 h (data not shown). The levels of β-actin mRNA remained constant under all conditions examined. A non-specific inhibitor of serine/threonine kinases [i.e., a protein kinase A (PKA), PKC and protein kinase G (PKG) inhibitor] H7 at 25 μM reduced the levels of LPS-induced IL-1α and IL-1β-mRNA. An inhibitor for PKA and PKG HA1004 at 25 μM did not show any significant effect on the LPS-induced IL-1 mRNA expression (Fig. 2). Other potent PKC inhibitors, Gö6976 at 2 μM and Ro31-8220 at 5 μM, also inhibited the expression of the mRNAs for IL1α and IL-1β, an effect similar to that of H7 (Fig. 2).

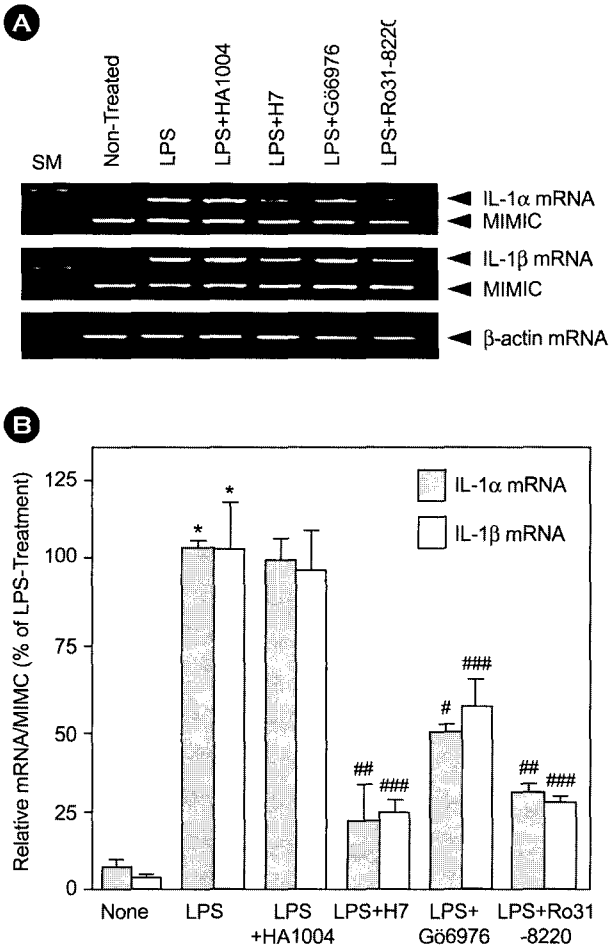

Fig. 2. Comparison for the effects of inhibitor for PKC and PTK on the induction of IL-1α and IL-1β by LPS in primary mixed glial cultures. **A.** RT-PCR analysis of the LPS-induced expression of proinflammatory cytokine mRNAs. **B.** The analysis of the optical density of the PCR-amplified cDNA bands from panel A. Glia cultures were pretreated with various inhibitors, such as HA1004, H7, Gö6976 and Ro31-8220 for 1 h prior to stimulation with LPS. The cells were then stimulated with 1 μg/ml LPS for 3 h. The induction by LPS and RT-PCR analysis of each target gene was repeated twice and one representative gel is shown. SM, size marker (100 bp DNA ladder). **P*<0.0001 compared to non-treatment; #*P*<0.01, ##*P*<0.001 and ###*P*<0.0001 compared to the corresponding LPS-treated group.

3. Differential effects of genistein and H7 on LPS-induced IL-1α and IL-1β

Among all the serine/threonine kinases examined above, the non-specific PKC inhibitor H-7 exerted potent inhibitory effects on the mRNA expression for both IL-1α and IL-1β. Therefore, the pharmacological effect of H7 on the production of proinflammatory cytokines was further studied. Glial cells were treated with different concentrations of H7 from 2 to 50 μM followed by the stimulation with LPS. H7 at 25 μM significantly reduced the expression of mRNAs for IL-1α and IL-1β (Fig. 3). H7 did not induce either the proinflammatory cytokine mRNAs or the release of the cytokines into the (data not shown). The inhibitor for PTK genistein reduced the production of proinflammatory cytokines (Kong et al., 1997), suggesting that PTK pathways can also be the targets for pharmacological agents to reduce the induction of IL-1. Therefore, the effect of H7 on LPS-induced IL-1 mRNA expression was compared with that of genistein (Fig. 3). Although the expression of the mRNA for IL-1α and IL-1β was markedly reduced by H7 at 25

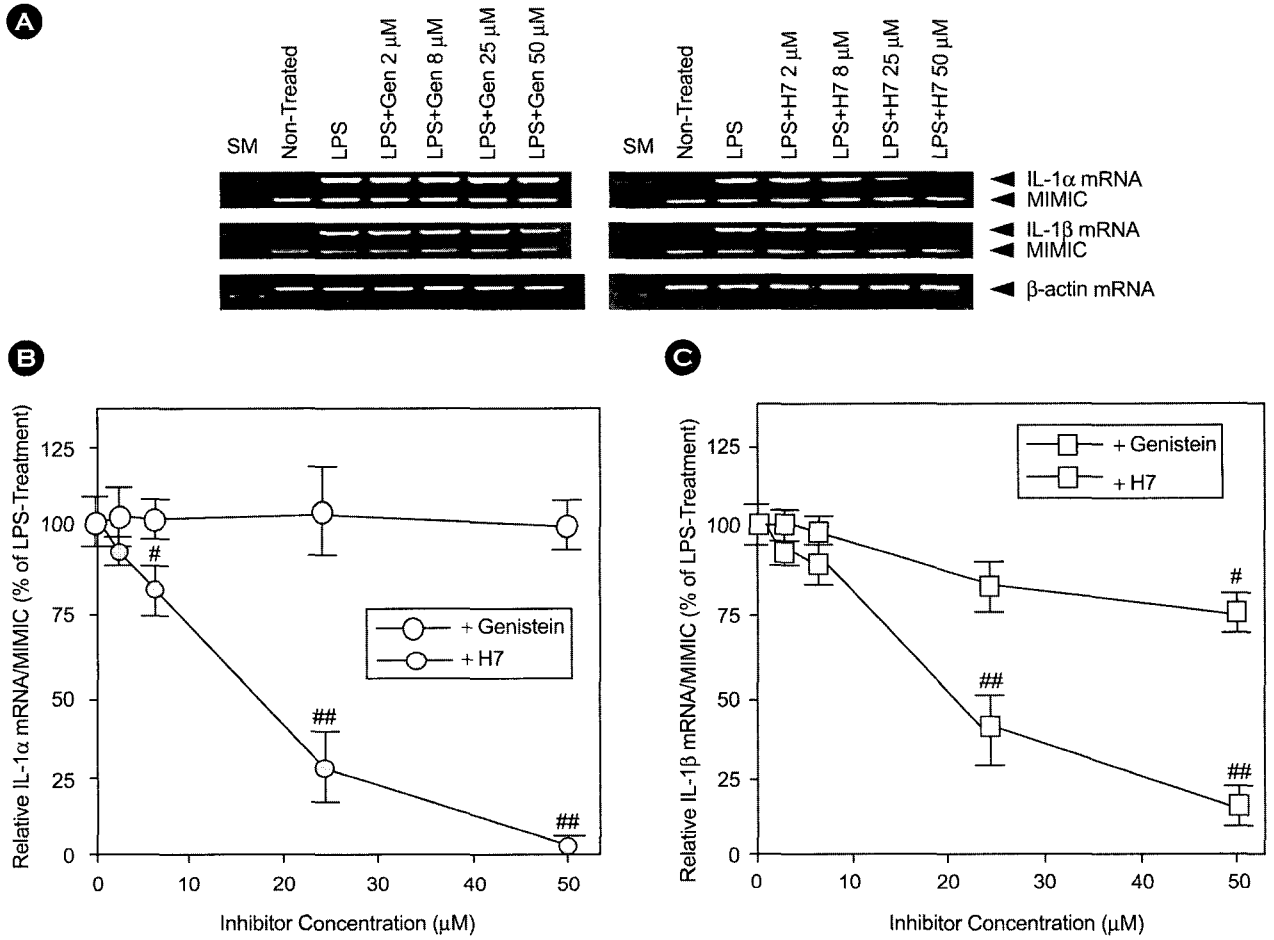

Fig. 3. Comparison of the effects of genistein and H7 on the induction of IL-1α and IL-1β in primary mixed glia cultures. **A.** RT-PCR analysis of the concentration-dependent effects of genistein and H7 on LPS-induced expression of IL-1α and IL-1β mRNAs. **B-C.** The analysis of the optical density of the PCR-amplified cDNA bands from panel A. Glia cultures were pretreated with 2~50 μM genistein or H7 for 1h prior to stimulation with 1 μg/ml LPS. The cells were then activated with 1 μg/ml LPS for 3 h for the analysis of IL-1α and IL-1β mRNAs. Each experiment was repeated three times and one representative gel is shown. SM, size marker (100 bp DNA ladder). #P<0.05, ##P<0.001 compared to the corresponding LPS-treated group.

μM, genistein from 2 to 50 μM did not significantly affect the expression of the mRNAs for these cytokines (Fig. 3). The study of mRNA expression for IL-1 showed that genistein and H7 exerted differential effects. Therefore, the secretion of IL-1 into culture medium 48 h after stimulation with LPS was further examined. Fig. 4 shows that secretion of IL-1α and IL-1β was significantly reduced by H7 at 25 μM by 39% and 80%, respectively. While genistein 25 μM did not affect LPS-induced expression of IL-1 mRNA, it significantly reduced the secretion of IL-1α and IL-1β into the culture medium by 62% and 73%, respectively (Fig. 4).

DISCUSSION

The present results show that protein kinases, such as PTK and PKC, can serve as pharmacological targets for reducing the production of IL-1. Even though we used in a mixed glial population in which IL-1 is produced by both microglia and astrocytes to different extents, inhibitors of PTK and PKC were still effective in reducing IL-1 production. Our laboratory and others have shown that the inhibitor of the PTK pathway genistein reduced LPS-stimulated IL-1 production by either microglia or macrophages (Kong et al., 1997; Shapira et al., 1994). However, further study of the effect of genistein on mRNA expression for IL-1α and IL-1β demonstrated that the inhibitor does not exert its effect on the transcription for these two cytokines. The results suggest that PTK pathways affect the production of IL-1 at post-transcriptional levels. Biochemical processes such as post-transcriptional modification, activation by

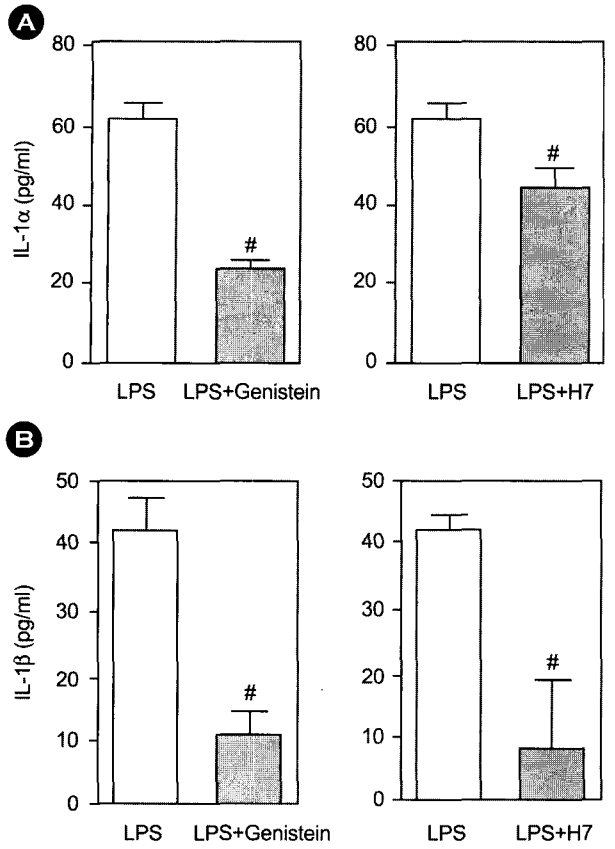

Fig. 4. Comparison for the effects of genistein and H7 on the production of IL-1α and IL-1β in primary mixed glia cultures. **A.** Effects of genistein and H7 on LPS-induced increase of IL-1α production. **B.** Effects of genistein and H7 on LPS-induced increase of IL-1β production. Glia cultures were treated with culture media containing 25 μM genistein or H7 for 1 h. Afterwards, the cells were stimulated with 1 μg/ml LPS. The culture supernatants were collected at 48 h after LPS stimulation for the determination of IL-1α and IL-1β. The quantity of IL-1α or IL-1β secreted into the medium was assayed with an ELISA. Each group consisted of 4 well from 24-well plates. The untreated group or the group treated with genistein alone did not release any detectable quantities of the cytokines into the media. #$P<0.0001$ compared to the group treated with LPS.

phosphorylation, or maturation to an active form from the precursor may be involved in the post-transcriptional control mechanism. Besides the PTK pathway, serine/threonine protein kinases such as PKC have also been shown to affect the production of IL-1. The results indicate that the inhibitor for serine/threonine kinases H7 affects both transcriptional and post-transcriptional levels.

After exposure of glial cells to LPS, IL-1α and IL-1β mRNAs were induced to a detectable level in 30 min, suggesting the possibility that the intial gene transcription for these cytokines occurs rapidly after stimulation with LPS. The LPS-induced increases of both IL-1α and IL-1β mRNAs were inhibited by the transcriptional inhibitor actinomycin D. While the inhibitor for protein synthesis cycloheximide did not inhibit LPS-induced mRNA expression for IL-1α, the expression of the mRNA for IL-1β stimulated by LPS, iNOS mRNA transcription was suppressed by more than 90%. The results suggest that transcription for IL-1β might be induced by other newly synthesized genes.

The present results show that the inhibitor for PKA, PKG, and PKC H7 significantly reduces the induction and production of IL-1α and IL-1β in LPS-stimulated glia at both transcriptional and post-transcriptional levels. These results are consistent with previous studies using astrocyte-enriched cultures, microglia, or monocytes (Chung et al., 1992; Shapira et al., 1994; Wood, 1994).

Although H7 is not a specific inhibitor for PKC (Hidaka et al., 1984), a study using HA1004 to inhibit PKA and PKG did not show any significant effect on the transcription of either IL-1α or IL-1β. Therefore, it is most likely that PKC is inhibited by H7 and is involved in the signaling for both IL-1α and IL-1β. In fact, other potent inhibitors of PKC, Gö6976 and Ro31-8220, significantly reduced the expression of the mRNA for IL-1, further suggesting that PKC is involved in IL-1 production in glia stimulated by LPS. Also, it has been reported that elevation of intracellular cyclic AMP levels, which can in turn activate PKA, can significantly inhibit the production of proinflammatory cytokines (Tannenbaum et al., 1989). These results suggest that activation but not inhibition of PKA plays important roles in modulating IL-1 production. These result also explain why there was no significant effect of HA1004 in our study. Taking all these findings together, PKC may function as the primary mediator for LPS to induce the expression of IL-1α and IL-1β in glia. Subsequently, phosphorylation or activation of the down-stream pathways such as the Raf/MAP kinase network will in turn activate the expression of specific transcription factors for the expression of IL-1α and IL-1β (Reimann et al., 1994; Weinstein et al., 1992).

Besides PKC, PTK pathways have been reported to play significant roles in LPS-induced mRNA expression for proinflammatory cytokines in blood monocytes (Beaty et al., 1994; Geng et al., 1993; Weinstein et al., 1992). Activation of macrophages by LPS has also been shown to stimulate tyrosine phosphorylation (Weinstein et al., 1991). Our stu-

dies show that the inhibitor for PTK genistein can also down-regulate the production of IL-1 in glia, but only post-transcriptionally.

Taken together, the present results suggest that the induction of IL-1α and IL-1β occurs rapidly in brain glial cells after the stimulation with LPS. Inhibitors of PTK and PKC can significantly reduce either the expression of mRNA for IL-1 or the production of IL-1. IL-1 has been implicated as an important proinflammatory cytokine after cerebral ischemia and brain trauma. Reduction of IL-1 levels in these two acute pathological conditions reduces neuronal injury (Stroemer et al., 1997; Yang et al., 1997). Our results provide evidence that signaling pathways of IL-1 can serve as pharmacological targets even in a mixed population of glia. Studies along this direction may provide new avenues for therapeutic interventions against inflammation-related neurological diseases.

Acknowledgements

This work was supported The Ministry of Health and Welfare of Korea; Grant number: 02-PJ2-PG1-CH12-0002.

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