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Effect of Hypothermia on Inflammatory Reaction in Glial Cells

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Background Since glial activation has been shown to play a key role in the pathogenesis of many brain disorders, understanding the mechanisms of glial activation is essential to make treatment strategies. In the brain, astrocytes and microglia exist as the neighborhood and interact with each other. In addition, mixed glial reaction is different from pure microglial reaction. Hypothermia is a robust neuroprotectant against the brain injury and modulates inflammation. In this study, our aim is to compare the hypothermic effect in the pure microglia and mixed glial cells.

Methods Primary mixed glial culture was prepared from postnatal Sprague-Dawley rat pups. BV2 cell, mouse microglial cell line, was also used. The cells were exposed to lipopolysaccharide (LPS, 10 μg/mL) under normothermia (37℃) or hypothermia (33℃). From the cultured cells, we isolated mRNA and protein. Culture media was taken to analyze nitric oxide (NO) production. Gene expression of some inflammation markers was measured. Activation of extracellular signal-regulated kinase (ERK) and p38 was also compared.

Results After treatment with LPS, we found increased NO production and gene expression of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, interleukin (IL)-1beta and IL-6 in the normothermia. Hypothermia attenuated increase of iNOS, had no effect on COX-2 and IL-1beta, and enhanced IL-6 level. Activation of ERK 1/2 and c-jun N-terminal kinase (JNK) was also observed by LPS treatment and hypothermia re-duced this activation.

Conclusions Our data suggest that hypothermic reduction of inflammation was observed in BV2 cells and mixed glial cells in a complicated pattern. **Vascular Neurology 2009;1:53-59**

Key Words Hypothermia, Inflammation, Lipopolysaccharide, Glia.

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Introduction

Cerebral ischemia or trauma triggers an inflammatory reaction that causes injury to the brain and progresses for several days. Data are accumulating which demonstrate that inflammation plays an important role in the development of secondary brain damage following stroke or trauma, and is associated with increased damage, neuronal loss and impaired clinical outcome.[1] Ischemia or trauma is accompanied by increased free radical generation, which induces expression of inflammatory cytokines and chemokines. Cytokines upregulate the expression of adhesion molecules on endothelial cells and leukocytes, and mediate the interaction between endothelial cells and leukocytes, leading to infiltration of leukocytes into the brain parenchyma. Cytokines also activate glial cells, whereas chemokines direct blood-borne cells toward their targets. Inflammatory cells contribute to brain damage by producing free radicals and other inflammation-related products that are toxic and lead to blood-brain barrier (BBB) dysfunction, edema and cell death.[2] Cerebral ischemia or trauma stimulates signal pathways that produce significant changes in gene expression and enzyme activation, thereby contributing to brain inflammation. Various factors have now been studied in cerebral ischemia or trauma models. Mitogen activated protein kinase (MAPK) is one of the most important kinase families in inflammatory and glial cells, which comprises three subfamilies: (i) extracellular signal-regulated kinase (ERK); (ii) c-jun N-terminal kinase (JNK) or stress activated protein kinase (SAPK); and (iii) p38.[3] If stroke or trauma victims receive no treatment, brain injury can start within a few minutes and there are many pathologic mechanisms responsible for brain damage. Thus, it is not only important to stop brain insult as fast as possible but to also overcome delayed cerebral injury. While pharmacologic strategies have been disappoint-

ing until now, hypothermia is considered as a promising therapeutic tool. It is well accepted that hypothermia affects the outcome of cerebral ischemia.[4]

Especially two clinical studies reported improved outcomes when hypothermia was induced in comatose survivors from cardiac arrest.[5,6]

Glial cells play active and vital roles in the central nervous system (CNS), including regulation of brain development, maintenance of cellular homeostasis and reparative responses to acute and chronic neurologic insults. However, in pathological conditions, glial function may be dysregulated resulting in enhanced neuroinflammation and further neurologic injury.[7] Astrocyte is the most numerous cell type in the CNS. They provide structural, trophic, and metabolic support to neurons and modulate synaptic activity. Accordingly, impairment in astrocytic functions during brain ischemia and other insults is critical. In addition, the death or survival of astrocytes themselves may affect the ultimate clinical outcome and rehabilitation through effects on neurogenesis and synaptic reorganization.[8] Microglial cells are currently accepted as immune cells in the CNS that respond to injury and brain disease. The main function of microglia is believed to be brain defense, as they are known to scavenge invading microorganisms and dead cells, and also to act as immune or immunoeffector cells. However, microglia are also thought to contribute to the onset of or to exacerbate neuronal degeneration and/or inflammation in many brain diseases by producing deleterious factors including superoxide anions, nitric oxide and inflammatory cytokines. Since the brain has many glial cell types and glial cells interact with each other, the prediction and interpretation of glial reaction is not easy task in the culture system. In this study, we intend to evaluate the effect of hypothermia on the inflammatory reaction in the BV2 cells and mixed primary glial cell culture system.

Materials and Methods

Cell culture

1) Mixed glial culture: Astrocyte cultures were prepared from postnatal (day 1−3) Sprague-Dawley rats as previously described.[9]

All procedures were carried out according to a protocol approved by the Animal Care and Use Committee of our University and in accordance with the NIH guide for the care and use of laboratory animals. Briefly, dissociated neocortical cells were plated in 15 mm Falcon 6-well plates at a density of 1−2 hemispheres per plate, in Eagle's minimal essential medium (Gibco, Gland Island, NY, USA) supplemented with 10% equine serum (Hyclone, Logan, Utah, USA), 10% fetal bovine serum (Hyclone), 21 mM (final concentration) glucose (Sigma, ST Louis, MD, USA) and 10 ng/mL epidermal growth factor (Sigma). The cultures were maintained in a 37℃ humidified incubator with a 5% CO_2 in room air atmosphere. Once confluent, we used the cells for the experiments. Immunocytochemical studies revealed that cells in our cultures stained for glial fibrillary acidic protein (GFAP, astrocyte marker), galactocerebroside (oligodendrocyte marker), or isolectin B4 (microglial marker). All experiments were performed at least three times on cultures from a minimum of three different dissections. Glia were activated by exposure to LPS (10 μg/ mL) (Sigma). Cultures were washed three times in LPS-containing media and then returned to the incubator. Control cultures were washed in only plating media. This dose of LPS was chosen because it was the lowest concentration in pilot experiments that consistently transformed resting glia into the activated form. To perform hypothermia, cells were kept in an incubator, with the temperature set at 33℃ during LPS treatment. To observe the toxic effect of LPS, cellular injury was estimated morphologically by phase-contrast light microscopy and these morphological changes correlated with loss of Trypan Blue exclusion. By performing tryphan blue exclusion test, we estimated the number of cell death after 24 hours following LPS (10 ug/mL) treatment.

2) BV2 cells: Cells were maintained in DMEM supplemented with 5% FBS under a humidified atmosphere of 5% CO2/ 95% air.

NO generation measurement

Nitric oxide (NO) production was evaluated measuring the nitrite, the stable metabolite of NO, from the culture media with the Griess reaction as previously described.[10]

Culture media were collected and centrifuged at 12,000 g for 1 h at 4℃. After centrifugation, supernatant was collected and kept at −80℃ until use. Duplicates of 100 ul of supernatant were added to 96-well microtiter plates and mixed with 100 μL of modified Griess reagent (Sigma). The plate was then read on a microtiter plate reader using a 540 nm filter. A standard curve with increasing concentrations of sodium nitrite was done in parallel and used for quantitation.

RNA isolation and reverse transcription polymerase chain reaction assay

Total RNA was isolated from the cultured cells using RNeasy Midi kit (Qiagen, Valencia, CA, USA). The concentration of total RNA isolated was quantified by ultraviolet spectrophotometry at 260/280 nm. Reverse transcription polymerase chain reaction (RT-PCR) assays were performed with ThermoScript RT-PCR system (Invitrogen, Paisley, Scotland, UK). One microgram of total RNA from each sample was reverse-transcribed into complementary DNA (cDNA),

and PCR was performed following the manufacturer's protocol. The following primers were used: TNF-alpha, sense: 5'-GCTGCCCCGACTATGTGCTCCTCA-3', antisense: 5'-GACGCCCCGGCCTTCCAAATAAAT-3'; beta-actin, sense: 5'-CCTTCCTGGGCATGGAGTCCTG-3', antisense: 5'-GGAGCAATGATCTTGATCTTC-3; iNOS, sense: 5 CTGCATGGAACAGTATAAGGCAA AC 3, antisense: 5'-CAGACAGTTT CTGGTCGATGTCATGA-3'. Amplification cycles were performed in a thermal cycler (Techne Inc, Princeton, NJ, USA). Before the cycle started, the sample was preheated at 94℃ for 2 minutes. Each cycle consisted of 29 cycles of 45 seconds at 94℃, 30 seconds at 63℃, 45 seconds at 72℃ (TNF-alpha), 26 cycles of 45 seconds at 94℃, 30 seconds at 55℃, 45 seconds at 72℃ (beta-actin), 30 cycles of 35 seconds at 93℃, 45 seconds at 63℃, and 45 seconds at 72℃ (iNOS) followed by 10 minutes at 72℃. Negative control reactions were performed with each batch of cDNA synthesis without reverse transcriptase. The PCR products were analyzed by electrophoresis on 1% agarose gels (Gibco) with 1 tris/borate/EDTA buffer (Gibco). The gels were stained with ethidium bromide and densitometric measurements were made from the film using a GS-700 imaging densitometer (Bio-Rad, California, USA) and then quantified using Multi-Analyst (Bio-Rad). The messenger RNA (mRNA) levels of constitutively expressed beta-actin were determined to control for differences in cDNA synthesis efficiency. Thus, cDNA levels were calculated by comparison with synthesis of beta-actin. To account for slight differences in RNA sample loading, all values obtained from the PCR products were normalized to the values obtained with the beta-actin.

Western blot analysis

Expression of MAPKs was determined semi-quantitatively by immunoblotting and densitometer scanning. Cells were solubilized (0.7% Trizma base, 10% glycerol, 2% sodium dodecyl sulphate at pH 6.8), and equal amounts of total protein were separated on a 12.5% gel, followed by electrotransfer to 0.2 um nitrocellulose membrane. After transfer, the nitrocellulose membrane was stained with Ponceau S to confirm equal loading and transfer of the protein samples. The membrane was blocked with 5% nonfat dry milk in phosphate buffered saline (PBS) containing 1% Tween 20 and probed with antibodies against phosphorylated ERK1/2, phosphorylated JNK, or phosphorylated p38 (Santa Cruz, Inc, Santa Cruze, CA, USA) respectively. The membranes were washed with 5% nonfat dry milk in PBS containing 1% Tween 20, incubated with horseradish peroxidase linked secondary antibody (Santa Cruz) for 1 h, and immunoreactive protein was visualized with enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, UK). Bands were quantitated by scanning densitometry using GS-700 imaging densitometer (Bio-Rad).

Statistics

Data are given as means±SEM. Comparisons between groups were performed using SigmaStat (SPSS, Chicago, IL, USA). The data were analyzed by one-way ANOVA followed by Tukey's test. Statistical significance was determined at the $p<0.05$ level.

Results

NO determination

The supernatants of cells incubated for different intervals with or without LPS under 37℃ or 33℃ were collected, centrifuged, and stored at −80℃ until tested. In the first experiment, BV2 cells received a single treatment of 10 ug/mL LPS and the induced productions of NO were measured in the supernatants from the cultures at 0, 1, 3, 6, and 24 hours. In the second experimental procedure, mixed glial cells were stimulated in the same manner as the first experiments. As shown in Fig. 1, the highest production of NO under normothermia was detected at 24 h and significantly reduced by the de-

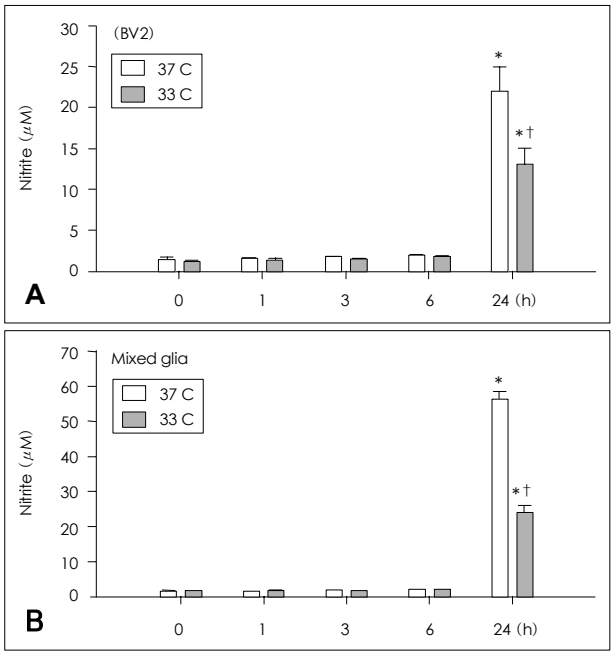

Figure 1. NO production. The supernatant from BV2 or mixed glial culture with or without lipopolysaccharide (LPS, 10 ug/mL) treatment under 37℃ or 33℃ was used to measure the amount of NO produced. BV2 cells received LPS and incubated for 0, 1, 3, 6, and 24 hours. NO production was peaked at 24 hour and attenuated by hypothermia (Fig. 1A). Mixed glial cells were stimulated in the same manner and the highest production was detected at 24 hour and significantly reduced by hypothermia (Fig. 1B). The peak level of NO production was higher in the mixed glia. *$p<0.05$ versus control (0 hour) and †$p<0.05$ versus normothermia (37C). LPS: lipopolysaccharide, NO: nitric oxide.

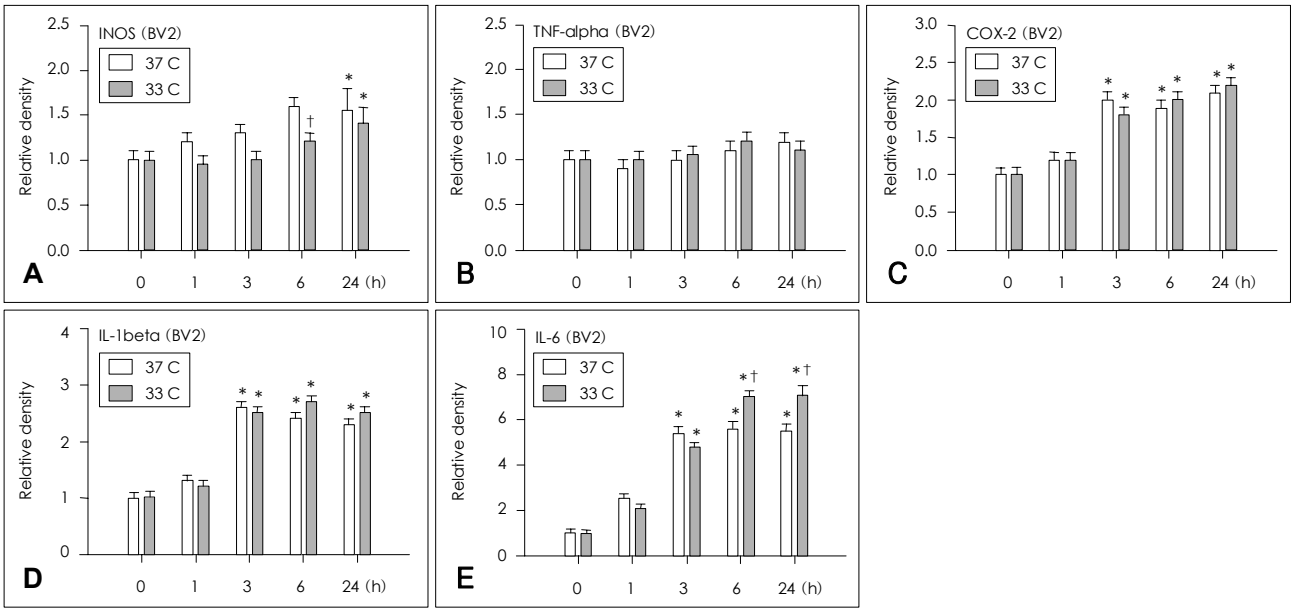

Figure 2. Gene expression of iNOS, TNF-alpha, COX-2, IL-1beta & IL-6 from BV2 cells. mRNA from the cultured cells at 0, 1, 3, 6, and 24 hours after LPS stimulation was analyzed using RT-PCR. Expression of these genes except TNF-alpha was increased after LPS treatment under normal temperature. While hypothermia significantly attenuates the LPS induced increase of iNOS expression, COX-2 and IL-1beta was not significantly affected by hypothermia. IL-6 was further increased by hypothermia. *p<0.05 versus control (0 hour) and †p<0.05 versus normothermia (37C). iNOS: inducible nitric oxide synthase, COX: cyclooxygenase, IL: interleukin, LPS: lipopolysaccharide, RT-PCR: reverse transcription polymerase chain reaction.

crease of temperature. When we compared the peak NO production, mixed glia (Fig. 1B) demonstrated higher level than BV2 cell (Fig. 1A).

Expression of inflammation related genes

Because iNOS, TNF-alpha, cyclooxygenase (COX)-2, interleukin (IL)-1beta & IL-6 are important inflammation-related genes, we examined whether hypothermia can alter the expression of those genes. mRNA levels from the cultured cells at 0, 1, 3, 6, and 24 hours after LPS stimulation were analyzed by RT-PCR. In the BV2 cells, expression of these genes except TNF-alpha was increased after LPS treatment with different time course and peak level depending on the genes under normal temperature (Fig. 2). While hypothermia significantly attenuates the LPS induced increase of iNOS expression, COX-2 and IL-1beta was not significantly affected by hypothermia. In contrast to other genes, IL-6 was further increased by hypothermia (Fig. 2). In the mixed glial culture, slightly different pattern was demonstrated. Expression of iNOS was reduced by hypothermia as in BV2 cells. This result is consistent with the nitrite production data in Fig. 1. TNF-alpha which showed no significant increase in BV2 cells was increased by LPS treatment. In addition, hypothermia attenuated increase of TNF-alpha (Fig. 3).

MAPK activation

Activation or phosphorylation of two MAPKs, ERK1/2 and

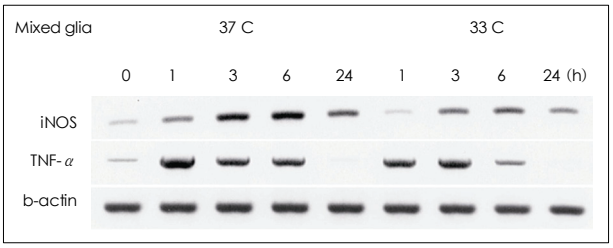

Figure 3. Representative image after gel electrophoresis of RT-PCR products of iNOS and TNF-alpha from the mixed glial culture. Expression of iNOS and TNF-alpha was stimulated by LPS and attenuated by hypothermia. iNOS: inducible nitric oxide synthase, LPS: lipopolysaccharide, RT-PCR: reverse transcription polymerase chain reaction.

JNK, was observed using Western blot analysis. In the BV2 cell, phosphorylated ERK is hardly detectable in the control group. Under normothermia (37℃), the amount of phosphorylated ERK was increased and showed peaks at 1 hour following LPS treatment. Hypothermia (33℃) attenuated the increase of ERK activation throughout the time course of the experiments (Fig. 4A). Phosphorylated JNK was increased at 1 hour after LPS treatment under normothermia but this was not statistically significant. In hypothermic groups, slight reduction of phosphorylated JNK was observed (Fig. 4B). In the mixed ERK1/2 phosphorylation showed 2 peaks at 1 and 24 hour. Hypothermia reduced the activation of ERK1/2 (Fig. 5A). Phosphorylation of JNK was peaked at 1 hour after LPS treatment. Hypothermia attenuated JNK activation (Fig. 5B).

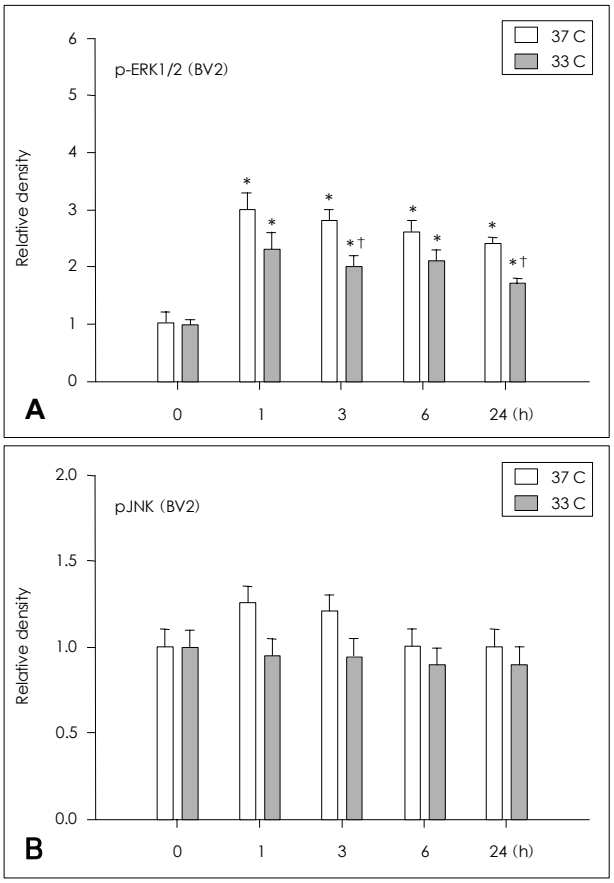

Figure 4. Phosphorylation of ERK1/2 and JNK was observed using Western blot analysis in the BV2 cell. Under normothermia (37℃), the amount of phosphorylated ERK was increased and showed peaks at 1 hour following LPS treatment. Hypothermia (33℃) attenuated the increase of ERK activation throughout the time course of the experiments (Fig. 4A). Phosphorylated JNK was increased at 1 hour after LPS treatment under normothermia but this was not statistically significant. In hypothermic groups, slight reduction of phosphorylated JNK was observed (Fig. 4B). *p<0.05 versus control (0 hour) and †p<0.05 versus normothermia (37℃). ERK: extracellular signal-regulated kinase, JNK: c-jun N-terminal kinase, LPS: lipopolysaccharide.

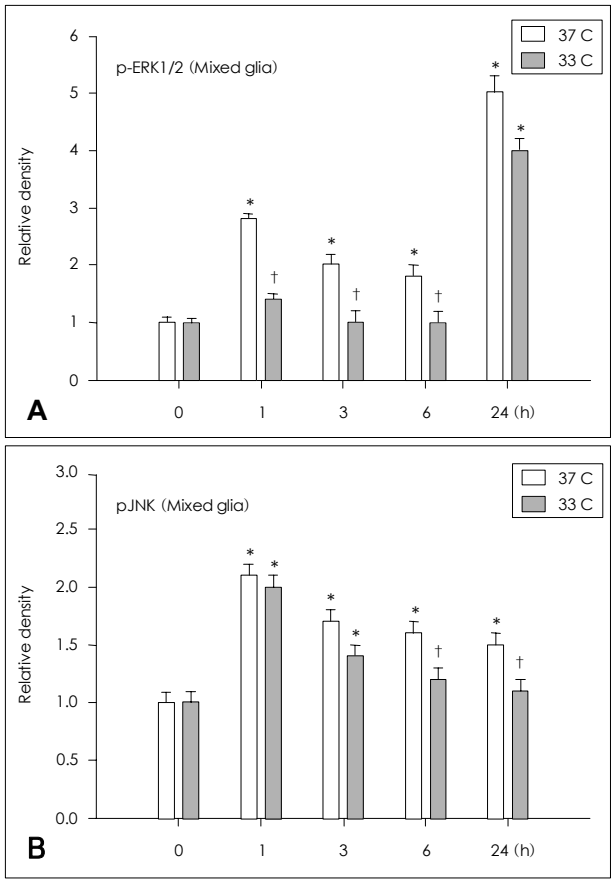

Figure 5. Phosphorylation of ERK1/2 and JNK was observed using Western blot analysis in the mixed glial cells. ERK1/2 phosphorylation showed 2 peaks at 1 and 24 hour. Hypothermia reduced the activation of ERK1/2 (Fig. 5A). Phosphorylation of JNK was peaked at 1 hour after LPS treatment. Hypothermia attenuated JNK activation (Fig. 5B). *p<0.05 versus control (0 hour) and †p<0.05 versus normothermia (37℃). ERK: extracellular signal-regulated kinase, JNK: c-jun N-terminal kinase, LPS: lipopolysaccharide.

Discussion

In this study, we showed that hypothermia inhibited iNOS expression and NO production in BV2 and mixed glial cells in common. The amount of NO produced was higher in mixed glia than BV2 cells. In mixed glia, TNF-alpha gene expression also was reduced by hypothermia. Hypothermic attenuation was not shown in IL-1beta and COX-2 while IL-6 was increased in hypothermic condition. In mixed glial cells, LPS activated both ERK1/2 and JNK but only ERK1/2 was enhanced in BV2 cells. Hypothermic attenuation of phosphorylation of ERK1/2 and JNK was shown both in mixed glia and BV2 cells. Our data demonstrate that hypothermia is not an omnipotent suppressor of gene expression and mixed glia and BV2 cell react to LPS in a slightly different manner.

NO, TNF-alpha, IL-1beta, IL-6 and COX-2 are known as the representative inflammation related markers in the brain diseases and it is suggested that suppression of inflammation may underlie the neuroprotective effects of mild hypothermia In our previous report we demonstrated that MAPK pathway was modulated by hypothermia in stroke model.[11] Therefore we focused on MAPK signal pathway as well. Among the factors we investigated in this study, iNOS, IL-1beta and COX-2 are known as the typical proinflammatory factors while the role of TNF-alpha or IL-6 is ambiguous or dependent on the experimental conditions. Since it is believed that hypothermia attenuates all the proinflammatory factors, we expect that most of these factors will be down regulated by hypothermia But gene expression pattern was not consistent among the genes and cell type also made the result more complicated. This data made us to realize again that the real situation in the brain is not easy to predict and development of microenvironment monitoring technology is strongly needed.

Microglia and astrocytes play an important role in regulating inflammatory pathways by acting independently and interacting with each other. One of the major mechanisms by which glial cells are thought to exhibit cytotoxic activity is the production of soluble factors. Among these, NO has been implicated in the pathogenesis of various diseases.[12] In glial cells, NO is synthesized from iNOS.[13] iNOS mRNA as well as immunoreactivity has been found in glial cells in brains from patients with multiple sclerosis, but not in brains of control persons,[14] and increased expression of iNOS mRNA was also found in experimental autoimmune encephalomyelitis.[15] NO is known to damage neurons and oligodendrocytes.[16,17] In the context of brain ischemia, the activity of neuronal nitric oxide synthase (nNOS) and iNOS is broadly deleterious, and their inhibition is neuroprotective.[10]

NO and superoxide are highly reactive but can also combine to form peroxynitrite, a particularly damaging reactive species. The toxicity of peroxynitrite results from their modification of macromolecules, especially DNA.[18]

Regarding the regulation of NO synthesis, it seems that MAPK plays an important role. ERK1/2 and p38 have been shown to regulate iNOS and TNF-alpha gene expression in endotoxin-stimulated primary glial cultures and the key roles for ERK1/2 and p38 cascades was demonstrated in the transcriptional and post-transcriptional regulation of iNOS and TNF-alpha gene expression in endotoxin-activated glial cells.[19] In other study, it is reported that endotoxin/cytokine-stimulated phosphorylation of ERK1/2 is critical to iNOS expression.[20]

However there are contrasting reports on MAPK role in NO regulation. In C6 glioma cells, iNOS expression was mediated by p38 but not ERK1/2.[21] Watters and colleagues reported that ERK activation is not a required function of LPS-mediated signaling events and illustrated that alternative or additional pathways for LPS action exist.[22] TNF-alpha is induced at the site of injury, or at pathologically-damaged sites of the brain such as those of multiple sclerosis, acquired immunodeficiency syndrome dementia, and ischemia. The TNF-alpha has been generally considered to be cytotoxic for oligodendrocytes and neurons, and to be involved in the progression of inflammation.[23]

MAPKs consist of three subgroups: p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase/stress -activated protein kinase (JNK/SAPK). These kinases are activated by phosphorylation of both tyrosine and threonine residues that is catalyzed by specific upstream MAPK. Activated MAPK phosphorylate their specific substrates on serine and/or threonine residues ultimately leading to activation of specific subsets of transcription factors. The standard model suggests that ERK sustains cell viability, whereas the 2 major stress-activated protein kinases, p38 and JNK, may promote cell death.[24] However, many exceptions to this general rule exist. For example, although JNK is known to mediate Fas-induced apoptosis in neuronal cells,[25] it can also be beneficial by interrupting p38 kinase and preventing cell death after tumor necrosis factor-alpha exposure in cardiomyocytes.[26] Similarly, both deleterious[27,28] and beneficial[29] actions of p38 signaling have been documented. Contradictory effects have also been described for ERK. Whereas ERK activation prevented apoptosis after growth factor withdrawal in cerebellar neurons and PC12 cells,[24] a couple of different laboratories[30-33] have shown that ERK inhibition protected against cortical neuronal injury. MAPK pathways have been implicated in inflammatory activation of astrocytes.[34-36] Overall, the literatures suggest that contributions of individual MAPK depend on the cell types and the nature and severity of the insult involved.

Regarding the role of hypothermia on MAPK pathway, there are very limited numbers of studies were performed.[37-39] One report demonstrates that both ERK and JNK pathways are activated during the first 24 hour of reperfusion after global cerebral ischemia, and that hypothermia increases the activation of ERK relative to JNK. Thus, an increase in ERK activation may be associated with improved neuronal survival after ischemic injury.[37] However two reports showed downregulation of MAPK by hypothermia.[38,39]

Acknowledgments

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