

Review Article

Molecular Mechanisms Underlying Hypothermia-Induced Neuroprotection

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Stroke is a dynamic event in the brain involving heterogeneous cells. There is now compelling clinical evidence that prolonged, moderate cerebral hypothermia initiated within a few hours after severe ischemia can reduce subsequent neuronal death and improve behavioral recovery. The neuroprotective role of hypothermia is also well established in experimental animals. However, the mechanism of hypothermic neuroprotection remains unclear, although, presumably involves the ability of hypothermia to suppress a broad range of injurious factors. In this paper, we addressed this issue by utilizing comprehensive gene and protein expression analyses of ischemic rat brains. To predict precise target molecules, we took advantage of the therapeutic time window and duration of hypothermia necessary to exert neuroprotective effects. We proposed that hypothermia contributes to protect neuroinflammation, and identified candidate molecules such as MIP-3 α and Hsp70 that warrant further investigation as targets for therapeutic drugs acting as “hypothermia-like neuroprotectants.”

1. Introduction

Stroke is the second leading cause of death in the world [1], and is a primary cause of long-term disability in adults [2, 3]. One potential approach for treating acute stroke involves neuroprotective agents. Although more than 100 clinical trials of potential agents have been conducted, none of these agents proved to be clinically efficacious except for a free radical scavenger edaravone [4–6]. However, recent progress in understanding the disease have unveiled the cellular and molecular events underlying ischemic cell death, such as loss of metabolic stores, excessive intracellular calcium accumulation, oxidative stress, and neuroinflammatory response. For instance, expression of stress, apoptosis, and inflammation-related genes is known to be upregulated upon reperfusion and reoxygenation [7, 8].

Therapeutic hypothermia is a promising neuroprotective intervention shown to improve outcome from brain ischemia in humans. The neuroprotective role of hypothermia has been well established in experimental animals [9–11]

and in patients with cardiac arrest [12, 13]. Although the key mechanism has not been clarified, hypothermic neuroprotection may provide insight into stroke pathology and suggest novel therapeutic drug targets. To date, reactive oxygen species production [14], NF- κ B activation [15], neutrophil infiltration [16] and cytochrome c release [17] have been observed to be inhibited in hypothermia-treated ischemic brains. However, it is difficult to discern an intrinsic hallmark of hypothermia from sequential outcomes by retrospective approaches. To overcome this drawback, we performed comprehensive gene and protein expression analyses using ischemic brains with or without hypothermia collected at appropriate time points. DNA microarray and proteomic approaches are revolutionary technologies for performance of high-throughput analyses of global gene and protein expression. However, additional devices are needed to elucidate the essential molecules and to identify appropriate targets for therapeutic intervention from massive amount of expression profiles. In this series of study, we focused on the therapeutic time window and duration

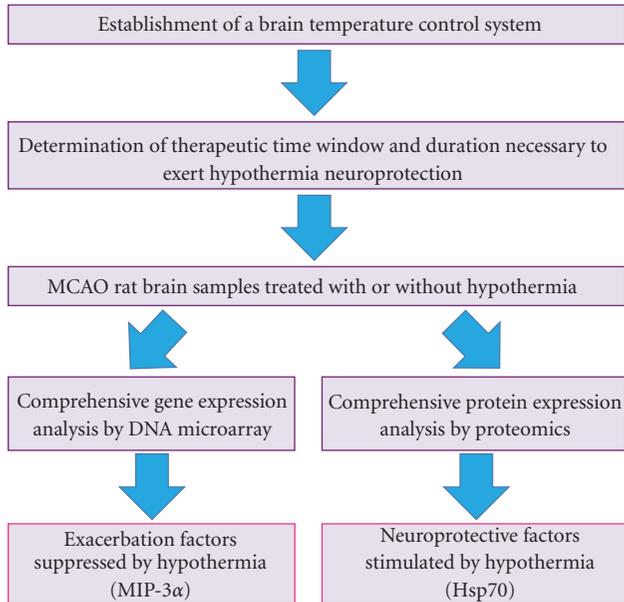


FIGURE 1: An experimental strategy to identify potential drug targets associated with hypothermia.

of hypothermia required for exerting neuroprotective effects (Figure 1).

2. Therapeutic Time Window and Duration of Hypothermia

It is not known precisely when the series of events leading to final cell death and dissolution becomes irreversible. However, there is overwhelming evidence that the early recovery phase, before the start of secondary deterioration, represents the effective window of opportunity for initiation of postinsult cooling [18]. A continuous comparison of stroke damage with or without hypothermia would provide massive amount of information that would be difficult to interpret precisely; therefore, it is necessary to compare appropriate samples according to the therapeutic time window and duration of hypothermia required to exert a neuroprotective effect. Precise control of brain temperature is critical for the evaluation of the efficacy of mild postischemic hypothermia and for the acquisition of reproducible gene and protein expression patterns. To predict the effective phase of hypothermia, we established a rat experimental stroke model: a 2-hr transient intraluminal occlusion of the middle cerebral artery (MCAO), which resulted in severe brain infarct with almost complete striatal and extensive cortical damages, was combined with a multichannel computer-controlled brain temperature control system that enabled us to maintain a constant brain temperature for more than 2 days [19]. Rats were implanted with a telemetry brain temperature probe with its temperature sensor inserted approximately 4 mm from the skull surface (lower layer of the cerebral cortex). The rats were individually housed in acrylic cages with wooden chips. To induce hypothermia, each cage was placed in a room maintained at 4°C, and the cortical temperature was

continuously and individually monitored from the freely moving rats. Cooling fans attached to the cage lid were turned on when the brain temperature was higher than 35°C and were stopped when the brain temperature was below 34°C. To maintain normothermia, rats were kept under room temperature. Because the brain temperature increased during MCAO, the term “normothermia” does not refer to a certain controlled temperature. For instance, in our rat model, normothermic rat brain temperature increased over 38°C for more than 18 hr [19]. Similar brain temperature control systems that used water sprays for cooling instead of cold rooms have been reported [20, 21]. The surgical placement of the temperature probes into the brain on the day prior to MCAO should be taken into consideration during further experimentation because the stab wounds might have had a preconditioning effect. The therapeutic time window is considered to depend upon the severity of the insult, depth of damage, and duration of cooling [22–24].

We identified the therapeutic time window of mild hypothermia at 35°C in rats subjected to hypothermia after a 2-hr MCAO [19]. The brain infarct volumes measured 2 days after MCAO were $288.6 \pm 21.5 \text{ mm}^3$ in normothermic rats. In contrast, postischemic hypothermia significantly reduced the volume when introduced at 0 hr ($120.0 \pm 21.7 \text{ mm}^3$, $P < .005$), 2 hr ($147.0 \pm 26.3 \text{ mm}^3$, $P < .005$), and 4 hr ($209.9 \pm 33.5 \text{ mm}^3$, $P < .025$) after normothermic reperfusion. No significant reduction was observed when postischemic hypothermia was introduced 6 hr after reperfusion ($244.3 \pm 35.5 \text{ mm}^3$). The therapeutic time window of hypothermia was therefore concluded as 4 hr after reperfusion in our model. However, even 4 hr of hypothermia when introduced immediately after reperfusion did not significantly reduce brain infarct volumes ($182.3 \pm 29.4 \text{ mm}^3$) as compared to the volumes in normothermic rats ($219.3 \pm 29.9 \text{ mm}^3$). The infarct volume after 24 hr of hypothermia ($63.1 \pm 28.2 \text{ mm}^3$) was significantly smaller than that after normothermia ($219.2 \pm 39.7 \text{ mm}^3$, $P < .01$), suggesting that hypothermia should be maintained for longer than 4 hr in our model to obtain neuroprotective effects. This finding is consistent with reports that mention the importance of prolonged hypothermia for persistent neuroprotection [25, 26]. Hypothermia may exert neuroprotective effects in ischemic brains by regulating particular injurious factors within specific time intervals.

3. DNA Microarray Analysis

DNA microarray analysis is a useful tool for determining gene expression [27–29]. A number of endogenous proteins have been reported to be induced during ischemia, including several involved in inflammation and neuronal death such as the MAP kinase protein and heat shock proteins (HSP) that play roles in both tissue damage and reorganization [30–35]. Lu et al. [36] reported upregulation of immediate early genes, transcription factors, and HSPs as early as 30 min after MCAO in 30 min or 2 hr transient rat MCAO models, and upregulation of genes related to inflammation, apoptosis, the cytoskeleton, and metabolism that peaked within 4–24 hr after MCAO. Figure 2 shows expression patterns of several

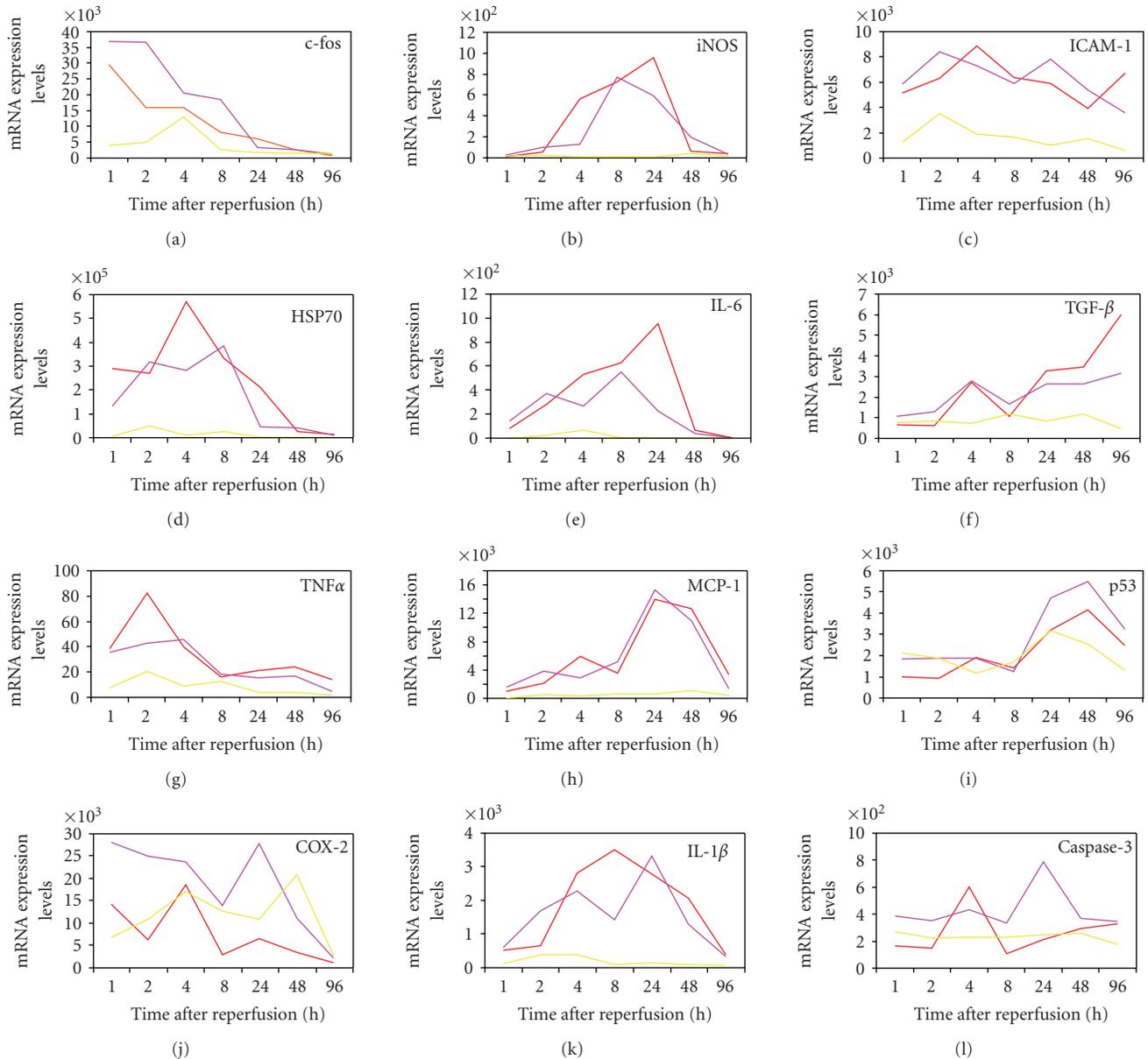


FIGURE 2: Stress-related genes expression in rat brains after reperfusion under normothermia. The expression levels of each mRNA in ischemic core (red), penumbra (purple), and normal (yellow) regions were quantified by real-time PCR, and their mRNA expression levels were shown as a gene copy number per 1 ng of polyA⁺ RNA. The procedure used has been previously described [17].

stress-related genes after reperfusion in ischemic brains of our rat model under normothermia. The expression level of *c-Fos* mRNA reached the peak during MCAO and sustained at least for 1 h after reperfusion. *Hsp70* mRNA expression was also high in the beginning of reperfusion. Interestingly, tumor necrosis factor- α (*TNF- α*), interleukine-1 β (*IL-1 β*), inducible nitric oxide synthase (*iNOS*), and *IL-6* mRNAs were transiently and sequentially induced after reperfusion. In contrast, monocyte chemotactic protein-1 (*MCP-1*), transforming growth factor- β (*TGF- β*), and *p53* mRNAs gradually increased along with progression of the disease. Gene expression analysis provides a large amount of information regarding expression changes of

thousands of genes, and time-dependent changes should therefore be analyzed to grasp the entire picture. Hence, the information of therapeutic time window and time duration of hypothermia was exploited. The therapeutic time window of hypothermia in our model was 4 hr, so we hypothesized that gene expression changes occurring prior to this time point are important for hypothermic neuroprotection. Of the 8000 transcripts represented on the Affymetrix Rat U34A GeneChip, $45 \pm 8\%$ showed a present call (a specific signal with statistical significance) in both hypothermia and normothermia brains during the 4 hr reperfusion [19]. Approximately 400 of the genes were upregulated more than 2-fold, but no genes were reproducibly suppressed.

Of the upregulated genes, 22 were upregulated more than 20-fold, including *Hsp70* mRNA. Interestingly, most of the genes whose expression was significantly suppressed by hypothermia were inflammatory-related molecules including IL-1 β and osteopontin (OPN), suggesting that the suppression of neuroinflammation primarily contributes to neuroprotection. OPN has a proinflammatory role in some diseases [37–40], although the neuroprotective role of OPN is also reported [41]. Further studies are needed to elucidate the roles of OPN in ischemic pathophysiology.

Meanwhile, we also speculated that the interruption of hypothermia is implicated in ischemic damage because the duration necessary to exert hypothermic neuroprotection was longer than 4 hr. To identify genes involved in the recovery phase before the secondary deterioration, we compared gene expression in ischemic brains kept in hypothermia for 8 hr during reperfusion with those kept in hypothermia for the first 4 hr followed by 4 hr normothermia. We identified 12 genes functionally related to neuroinflammation, including *c-Fos*, early growth response 1 and 4 (*Egr-1*, and *Egr-4*), MAPK phosphatase-1 (*MKP-1*), macrophage inflammatory protein-3 α (*MIP-3 α*), *MCP-1*, and *IL-1 β* [19]. *Egr-1* is a master switch activated by ischemia to trigger the expression of pivotal regulators of inflammation, such as IL-1 β , MCP-1, and macrophage inflammatory protein-2 (MIP-2), in addition to coagulation and vascular hyperpermeability [43]. Deng et al. [44] demonstrated that postischemic mild hypothermia decreases inflammatory responses by suppressing intercellular adhesion molecule-1 (ICAM-1), an essential molecule for peripheral leucocyte recruitment into brain parenchyma. Stroke-mediated release of proinflammatory cytokines and adhesion molecules is a relevant component of the complex pathophysiological response [45]. Those inflammatory mediators enable leukocytes to adhere to the vascular endothelial cells and to infiltrate inflammatory cells from the circulation into the ischemic brain within hours after ischemic damage. Studies addressing efficacy of mild hypothermia also indicate the importance of microglia activation [16, 44], nuclear-factor kappaB (NF- κ B) activation [46], or nitric oxide production [47].

4. Proteomic Analysis

Proteomic analysis is another powerful tool for examining the physiopathologies of diseases. Hence, proteomic studies using brain tissues, blood, or cerebrospinal fluids of stroke patients [48] or experimental models [42, 49] have been performed to elucidate the pathogenesis of stroke and to identify novel diagnostic biomarkers. Proteomic profiling generally consists of 2 technologies: two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for the comparison of protein expression and LCQ-advantage micro-LC/MS/MS system for protein identification. In our experiments, the criteria for altered protein expression between hypothermia and normothermia were set as a ratio of greater than 1.5-fold or less than 1/1.5-fold. Out of approximately 1200 spots on 2D-PAGE, 44 were identified with a statistically significant difference in mean values. Thirteen of the 44 spots were upregulated by hypothermia more than 1.5-fold

(1.50–1.85) compared with normothermia. Among them, a spot identified as Hsp70 was studied further because it was upregulated by MCAO and was further induced by hypothermia, although hypothermia itself did not induce Hsp70 in nonischemic brains [50]. This observation was confirmed by Hsp70-specific enzyme-linked immunosorbent assay (ELISA), western blotting, and immunohistochemistry of ischemic brain tissues. Hsp70 is a chaperone protein that can fold or refold proteins, coordinate protein trafficking, inhibit protein aggregation or degradation, and exhibit antiapoptotic and anti-inflammatory activities under physiological conditions [51–53]. Several reports have suggested that Hsp70 inhibits NF- κ B transcription by stabilizing NF- κ B and preventing its phosphorylation [54]. Zheng et al. [55] recently demonstrated that the interaction between Hsp70 and the NF- κ B:I κ B complex significantly inhibits NF- κ B activation and leads to a decrease in NF- κ B-regulated genes in MCAO-treated Hsp70 transgenic mice. The expression of Hsp70 and Hsp27 can be induced in glial cells and neurons by a wide range of noxious stimuli including ischemia [56, 57], epileptic seizure [58], and hyperthermia [59]. Neuroprotective effects of Hsp70 against ischemic injury have been reported for drugs that induce Hsp70 expression [60] and in Hsp70 transgenic mice [61]. However, the induction of Hsp70 by hypothermia has not been reported to date. Hsp27 was also induced by ischemia, but was slightly suppressed by hypothermia (Figure 3). GAP43, a constitutive protein in the brain, and other Hsps, including Hsp40, Hsp90, Grp78, Grp94, PDI, and ORP150, were detectable under normal conditions and were not affected by either hypothermia or ischemia (Figure 3 and unpublished data). Hence, Hsp70 accumulation by hypothermia was unique. Recently Hagiwara et al. [62] reported that hypothermia at 34°C increased the Hsp70 expression level in lipopolysaccharide (LPS)-stimulated RAW264.7 cells, although IL-1 β , IL-6 and TNF- α expression levels were reduced under the same conditions. In contrast to the protein level of Hsp70, *Hsp70* mRNA expression in our model was suppressed by hypothermia. Rubtsova et al. [63] reported that the 5'-untranslated region of human *Hsp70* mRNA represents an internal ribosome entry site (IRES) with relative activity similar to that of the classical picornaviral IRESs, implying posttranscriptional regulation of Hsp70. While it is unknown whether there is a similar IRES element in rat *Hsp70* mRNA and if the IRES is activated under hypothermia, this finding merits further investigation because it hints at the identity of neuroprotectants that increase Hsp70 by a novel mechanism. The neuroprotective roles of Hsp70 in hypothermia are also worthy of further investigation.

5. Novel Targets for Therapeutic Drugs Acting as “Hypothermia-Like Neuroprotectants”

Neuroinflammation is involved in the pathogenesis of many CNS diseases. In stroke, excess inflammatory activation results in brain injury and ultimately causes severe neuronal apoptosis [64]. Anti-inflammatory therapies using immunosuppressants [65] or biogenetics such as an anti-ICAM-1-neutralizing antibody [66] have been applied in

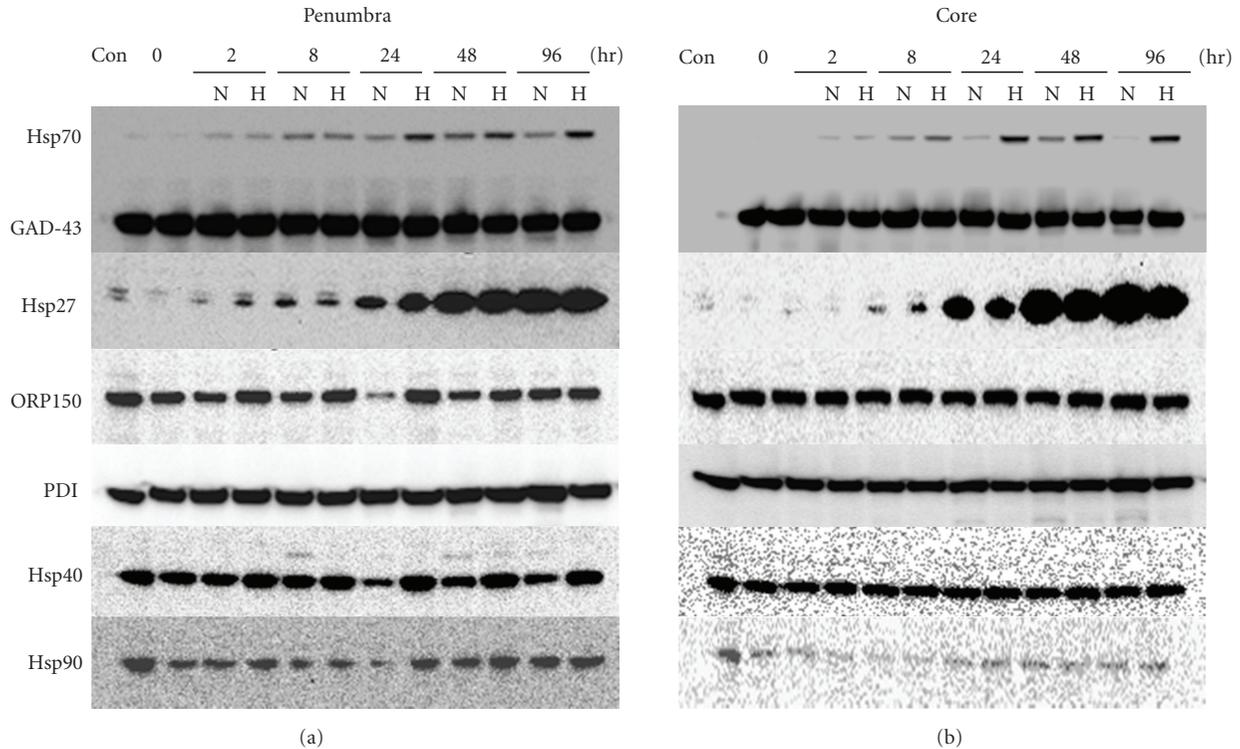


FIGURE 3: Effect of occlusion of the middle cerebral artery (MCAO) and hypothermia on chaperones expression in rat brains. Western blot analysis for Hsp70, Hsp27, Hsp40, Hsp90, ORP150, PDI, and GAD-43 was performed using protein samples from the penumbra and core regions of rat ischemic brains at the indicated time points after reperfusion. GAD43 protein served as an internal control. N: normothermia, H: hypothermia. "Con" denotes protein samples from normal rat brains. The procedure used has been previously described [42].

both preclinical and clinical contexts. MCP-1 is also considered a promising drug target due to its possible role in exacerbating ischemic injury, controlling blood-brain barrier permeability, and driving leukocyte infiltration into the brain parenchyma in stroke [67, 68]. Our DNA microarray analysis revealed that both cerebral *MIP-3 α* and *CC-chemokine receptor 6 (CCR6)* genes were significantly induced in the core and penumbra regions of MCAO rat brains, and hypothermia suppressed expression of both genes [69]. The *MIP-3 α* chemokine is expressed in macrophages, dendritic cells, and lymphocytes. Depending on the conditions, *MIP-3 α* acts both constitutively and inducibly and serves as a chemoattractant, especially in epithelial immunological systems such as those of the skin and mucosa [70, 71]. In CNS, *MIP-3 α* expression has been reported in autoimmune encephalomyelitis [72] and stroke [36, 73], but its full role has not yet been determined. *CCR6*, the only receptor for *MIP-3 α* , is expressed in multiple leukocyte subsets, and is implicated in diverse inflammatory responses in animal models, such as allergic airway disorders, inflammatory bowel disease, and autoimmune encephalitis [74, 75]. Strikingly, intracerebral administration of an anti-*MIP-3 α* -neutralizing antibody significantly reduced infarct volumes in MCAO rats ($187.5 \pm 37.0 \text{ mm}^3$) compared with those of vehicle ($304.0 \pm 32.8 \text{ mm}^3$) and of control mouse IgG ($291.3 \pm 33.5 \text{ mm}^3$), suggesting that *MIP-3 α* -*CCR6* signaling

is dominant in neuroinflammatory cascades involved in brain ischemia. Interestingly, *MIP-3 α* administration into the striatum dose-dependently induced *CCR6*, but not *CCR1* or *CCR2*, gene expression. Intrastriatal injection of *IL-1 β* and *TNF- α* upregulated *MIP-3 α* and *CCR6* mRNA expression levels in a sequential fashion. Robust induction of *IL-1 β* and *TNF- α* was observed at an acute phase of MCAO prior to *MIP-3 α* expression, suggesting that these cytokines may be directly involved in *MIP-3 α* production.

MIP-3 α mRNA expression was markedly induced by *IL-1 β* and *TNF- α* in rat astrocytes but not in microglia or neurons. Astrocytes are reported to emerge around the damaged area after ischemic injury [76] and to produce *MIP-3 α* in a mouse experimental autoimmune encephalomyelitis model [77]. In contrast, rat primary microglia constitutively expresses the *CCR6* gene in normal culture conditions while astrocytes or neurons do not. Strikingly, the expression of both *iNOS* and *IL-1 β* were induced in *MIP-3 α* -treated microglia. Microglia are activated and accumulate around the injured area following ischemia [78]. Because hypothermia reportedly inhibits *NF- κ B* directly by decreasing *I κ B* kinase activity in MCAO rat brains [15], one of the suppressive effects of hypothermia on *MIP-3 α* induction may be a direct inhibition of *NF- κ B* activity in astrocytes. Another possibility is that the upregulation of Hsp70 expression by hypothermia inhibits *MIP-3 α* expression by the interaction

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