Original Article

Influence of selective brain cooling on the expression of ICAM-1 mRNA and infiltration of PMNLs and monocytes/macrophages in rats suffering from global brain ischemia/reperfusion injury

Jianping Cao[1,*], Jianguo Xu[2], Weiyan Li[2], Jian Liu[2]

[1] *Department of Anesthesiology, Hospital No. 455 of the PLA, Shanghai, China;*
[2] *Department of Anesthesiology, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, China.*

Summary **This study sought to evaluate the effects of selective brain cooling on the expression of intercellular adhesion molecule-1 (ICAM-1) mRNA and infiltration of polymorphonuclear leukocytes (PMNLs) and monocytes/macrophages (M*Φ*) during global cerebral ischemia/reperfusion (I/R). Global ischemia of the brain was produced by four-vessel occlusion for 30 min followed by reperfusion for 240 min. Thirty-five SD rats were randomly divided into five groups: group I had no ischemia and reperfusion; groups II, III, IV, and V were subjected to ischemia for 30 min at 37°C and reperfusion for 240 min at 37, 35, 32, and 28°C, respectively. Cerebral tissue samples were taken for pathological examination of the infiltration of PMNLs and M*Φ* and to detect ICAM-1 mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR). The expression of ICAM-1 mRNA and infiltration of PMNLs and M*Φ* increased more markedly in group II than in group I ($p < 0.01$), suggesting that hypothermia evidently inhibited ICAM-1 mRNA expression and PMNL and M*Φ* infiltration in the damaged cerebral tissue. In addition, significant differences were also found between group III and group II ($p < 0.05$) and among groups IV, V, and II ($p < 0.01$). These results suggest that I/R injury induces ICAM-1 mRNA expression and PMNL and M*Φ* infiltration in SD rats and that selective brain cooling, and especially moderate hypothermia (28-32°C), may provide better cerebral protection by markedly inhibiting the expression of ICAM-1 mRNA while decreasing the infiltration of PMNLs and M*Φ* in the brain.**

Keywords: Hypothermia, Ischemia/reperfusion, Intercellular adhesion molecule-1 mRNA, Polymorphonuclear leukocytes, Monocytes/macrophages

1. Introduction

Activation and infiltration of leucocytes, and especially polymorphonuclear leukocytes (PMNLs) and monocytes/macrophages (M*Φ*), is a major factor that results in ischemia/reperfusion (I/R) injury after brain ischemia (*1*). Recent evidence has revealed the crucial role cell-adhesion molecules play in inflammation-induced rolling, adhesion, and accumulation of PMNLs and M*Φ* in tissues (*2*). An important adhesion molecule, intercellular adhesion molecule-1 (ICAM-1) plays a key role in PMNL and M*Φ* adhesion to endothelium and migration into injured tissues (*3*).

Inhibiting the expression of ICAM-1 and blocking the infiltration of PMNLs and M*Φ* have been shown to decrease I/R injury (*4*). Hypothermia provides brain protection by decreasing oxygen consumption, reducing cerebral edema, and inhibiting excitatory amino acid and oxyradical generation (*5*). However, the mechanism of brain protection has not been fully elucidated. The current study investigated whether hypothermia, and especially moderate hypothermia (28-32°C), affects modulation of ICAM-1 expression and PMNL and M*Φ* infiltration in injured cerebral tissue.

**Correspondence to:* Dr. Jianping Cao, Department of Anesthesiology, PLA 455th Hospital, 338 West Huaihai Road, Shanghai 200052, China;
e-mail: cjpcl221019@sina.com.cn

2. Material and Methods

2.1. *Animals*

Sprague-Dawley rats (weight, 180-200 g; n = 35) were used in the experiments. All procedures were approved by the Animal Care and Use Committee of Nanjing University and were in accordance with the NIH guidelines for the ethical use of laboratory animals. Animals were deprived of food for 8 h before surgery and given free access to water. All animals were anesthetized with ketamine (80 mg/kg, i.p.).

2.2. *Four-vessel occlusion model*

A four-vessel occlusion model was used to induce global cerebral ischemia/reperfusion (*6,7*). Under ketamine anesthesia, a midline incision (1 cm in length) was made in the dorsal neck, the paraspinal muscles were separated from the midline, and the right and left alar foramina of the first cervical vertebrae were exposed. As described by Green (*8*), the rat's vertebral arteries travel within the vertebral canal and pass beneath the alar foramen before entering into the posterior fossa. A pin 0.5 mm in diameter was inserted through each alar foramen and both vertebral arteries were cauterized and permanently occluded. The foramina were packed with bone wax, and the muscles and fascia were closed in layers. Through a ventral midcervical incision, each carotid artery was isolated and a 9-0 nylon ligature was looped around it. After 24 h, cerebral ischemia was induced by traction on both carotid ligatures; then, bilateral ligatures were loosened and reperfusion was performed for 4 h. The rats were sacrificed and cerebral tissue was quickly removed. Some cortical samples were immediately frozen in liquid nitrogen and stored at –80°C and other samples were fixed with formaldehyde.

2.3. *Temperature control*

Since some studies have indicated that there was no significant difference between inner ear and brain temperatures (*9*), inner ear temperature was monitored instead of brain temperature in the present experiment. Animals were cooled with ice packs.

2.4. *Experimental grouping*

Animals were randomly divided into the following five groups (Table 1): group I (n = 7) animals had no ischemia or reperfusion, group II animals (n = 7) were subjected to ischemia for 30 min at 37°C and reperfusion for 240 min at 37°C, group III animals (n = 7) were subjected to ischemia for 30 min at 37°C and reperfusion for 240 min at 35°C, group IV animals (n = 7) were subjected to ischemia for 30 min at 37°C and reperfusion for 240 min at 32°C, and group V animals (n = 7) were subjected to ischemia for 30 min at 37°C and reperfusion for 240 min at 28°C.

Table 1. I/R conditions in animal groups

	Animal groups				
	I	II	III	IV	V
Ischemic time (min)	0	30	30	30	30
Reperfusion time (min)	0	240	240	240	240
Temperature of reperfusion (°C)	37	37	35	32	28
Number of animals	7	7	7	7	7

2.5. *Determination of ICAM-1 mRNA level by RT-PCR*

Total cellular RNA was extracted from cortical samples with Trizol reagent (Invitrogen, Carlsbad, CA, USA). The following primers were used in this study: ICAM-1 cDNA (amplification product 590 bp) — sense primer: 5'-AAGGTGTGATATCCGGTAGA-3', antisense primer: 5'-CCTTCTAAGTGGTTGGAACA-3'; β-actin cDNA (amplification product 348 bp) — sense primer: 5'-TA AAGACCTCTATGCCAACAC-3', antisense primer: 5'-TAAAGCCATGCCAAATGTCTC-3'. β-actin was used as an internal control in PCR. ICAM-1 mRNA was reverse-transcripted at 48°C for 45 min. Amplification was performed according to the following conditions: samples were initially denatured at 94°C for 2 min followed by 30 cycles of amplification (30-sec denaturing at 94°C, 1-min annealing at 60°C, and 2-min extension at 68°C) and a final 7-min extension at 68°C. The PCR product was electrophoresed through agarose gel. The gel was dried and autoradiographed. The PCR-amplified DNA bands of ICAM-1 and β-actin were quantitated by phosphorImager analysis. The ratios of ICAM-1 to β-actin were calculated from the co-amplified samples, and relative levels of ICAM-1 mRNA were determined based on the differences of these ratios.

2.6. *Histopathology*

Brain tissues were processed and embedded in paraffin, and 4-μm-thick paraffin sections were stained with hematoxylin-and-eosin for histopathological evaluation. Six random high-double views (magnification, ×100) were taken to count PMNLs and M*Φ*.

2.7. *Statistical analysis*

All values are present as mean ± standard error. Statistical evaluation was performed using ANOVA followed by a Q test. A significant difference was indicated by $p < 0.05$, and a highly significant difference was indicated by $p < 0.01$.

3. Results

3.1. *Infiltration of PMNLs and MΦ*

Table 2 summarizes the absolute values of tissue

PMNL and MΦ counts for the various groups. Group II exhibited significantly more PMNLs and MΦ in cortical tissues than Group I ($p < 0.01$). In comparison to Group II, the counts of PMNLs and MΦ decreased significantly in other groups ($p < 0.05$, $p < 0.01$).

3.2. *Expression of ICAM-1 mRNA in various groups*

Expression of ICAM-1 mRNA in cerebral tissue was detected by RT-PCR. The quantitative data for ICAM-1 mRNA, after normalization to β-actin in each sample, were summarized. As shown in Figure 1, only a low basal level expression of the ICAM-1 mRNA was detected in the non-ischemic rat (group I). The expression of the 590 bp ICAM-1 mRNA was induced by I/R injury (Figure 1, group II). Figure 2 shows the RT-PCR of ICAM-1 mRNA expression at various temperatures. In comparison to group II, significant downregulation of ICAM-1 mRNA was noted in hypothermic groups ($p < 0.05$, $p < 0.01$) (Table 3).

4. Discussion

Recent evidence has indicated that reperfusion itself may be detrimental to the ischemic tissue and that leucocytes, and especially PMNLs and MΦ, play an important role in the development of ischemia-reperfusion injury (*10*). One of the proposed mechanisms is "no-reflow phenomenon"-microvascular occlusion caused by accumulated leucocytes, mechanical obstruction, and vasoconstrictive mediator release (*11*). In addition to acute microvascular occlusion, leukocytes may also facilitate ischemic injury by enhancing the blood-brain barrier, infiltrating ischemic tissue, or initiating thrombosis (*12*). They may also lead to parenchyma injury *via* protein hydrolytic enzyme release, lipid mediator production, or oxygen radical production due to activated inflammatory cell infiltration and the respiratory burst (*13*). Depletion of leucocytes has been found to alleviate brain injury after I/R (*14*). The current results indicate that the number of PMNLs and MΦ closely correlates with the extent of cerebral tissue damage.

The current view is that adhesion of leukocytes to microvascular endothelium is a critical stage in the migration of leukocytes into injured tissues (*15*). The adhesion is regulated in part by ICAM-1 in endothelial cells and a group of CD11/CD18 glycoproteins in leukocytes (*16*). An increase in leukocyte adherence to endothelial cells has been reported in ischemic/reperfused tissue, and interactions between ICAM-1 and CD11/CD18 may be involved in this process (*16*).

A lack of sensitivity to I/R injury has been noted in mice with the ICAM-1 and CD11a/CD18 genes knocked out (*17*). Pretreatment with anti-ICAM-1 antibodies and anti-CD11a/CD18 antibodies significantly reduces cerebral ischemic cell damage by blocking leukocyte endothelial adhesion and migration. A deficiency in ICAM-1 attenuates microcirculatory disturbance and infarction size in focal cerebral ischemia (*18,19*).

In international, hypothermia was plotted out mild hypothermia (33-35°C), moderate hypothermia

Table 2. PMNL and MΦ infiltration in cerebral tissue in various groups

Groups	Infiltration [a,b]	
	PMNLs	MΦ
I	2.1 ± 1.5**	0.2 ± 0.1**
II	10.3 ± 1.1	2.0 ± 0.3
III	8.4 ± 2.4*	1.6 ± 0.6*
IV	6.5 ± 0.7**	1.3 ± 0.4**
V	6.1 ± 0.8**	1.2 ± 0.3**

[a] Data are represented as the mean ± SE; [b] In comparison to group II: * $p < 0.05$, ** $p < 0.01$.

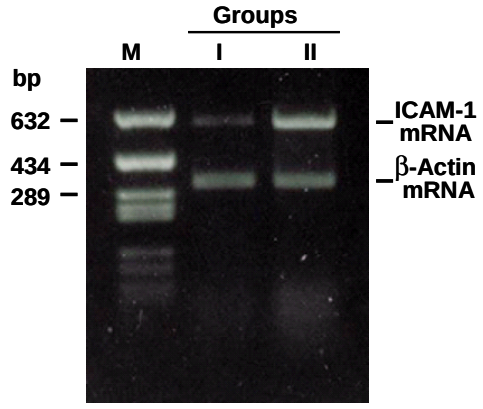

Figure 1. Expression of ICAM-1 mRNA in I/R injury. β-Actin mRNA was used as an internal control in PCR. Lane M, PCR marker.

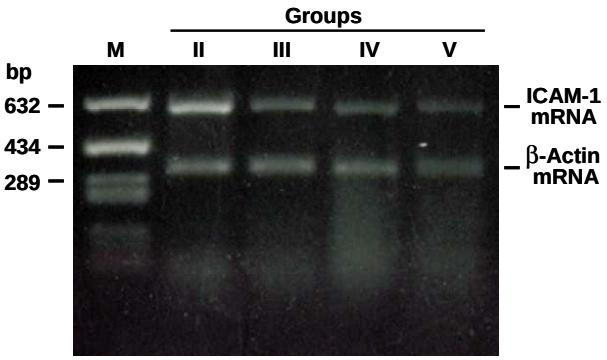

Figure 2. Expression of ICAM-1mRNA at various temperatures. β-Actin mRNA was used as an internal control in PCR. Lane M, PCR marker.

Table 3. ICAM-1 mRNA levels after normalization to β-actin in each sample

Groups	ICAM-1/β-actin mRNA [a,b]
I	0.264 ± 0.073**
II	1.825 ± 0.265
III	0.872 ± 0.151*
IV	0.447 ± 0.065**
V	0.433 ± 0.059**

[a] Data are represented as the mean ± SE; [b] In comparison to group II: * $p < 0.05$, ** $p < 0.01$.

(28-32°C), deep hypothermia (17-27°C) and super deep hypothermia (2-16°C) (*3*). Mild and moderate hypothermia offer acceptable brain protection. Possible contributing factors include i) decrease in oxygen consumption, a reduction in cerebral edema, inhibited excitatory amino acids, acetylcholine, dopamine, norepinephrine, 5-hydroxytryptamine, NO, and oxyradicals, ii) blockage of Ca^{2+} overload, iii) a decrease in the destruction or a replenishment of structural proteins of cerebral cells, and iv) alleviation of diffuse axial injury (*20*). Research has yet to be done on the effect of hypothermia on the expression of adhesion molecules or infiltration of PMNLs and M*Φ*. The current study found up-regulation of ICAM-1 mRNA expression and significant infiltration of PMNLs and M*Φ* after I/R injury. Hypothermia, and especially moderate hypothermia (28-32°C), could significantly inhibit the expression of ICAM-1 and infiltration of PMNLs and M*Φ*. These data suggest an optimal temperature (28-32°C) for the treatment of cerebral ischemia/reperfusion injury.

The mechanism by which ICAM-1 upregulation acts on cerebral endothelium during I/R injury is unclear. ICAM-1 is upregulated in the human brain and in microvascular endothelial cells by proinflammatory cytokines, tumor necrosis factor-α, and interleukin-1 (*21*). The mechanism of oxidation-reduction plays a role in modulating ICAM-1 expression (*22*). Another possibility is that the endothelium itself upregulates ICAM-1 in response to the stimulus of ischemia-reperfusion. The current study discovered a new mechanism of hypothermic brain protection *via* inhibition of ICAM-1 expression and blocking of PMNL and M*Φ* infiltration in I/R injured tissue. The issue of whether this hypothermic mechanism inhibits ICAM-1 expression directly or indirectly is unclear.

References

1. Shin DH, Bae YC, Lee JH. Polyphenolamentoflavone affords neuroprotection against neonatalhypoxic–ischemic brain damage *via* multiple mechanisms. J Neurochem 2006; 96:561-572.
2. Cao JP, Xu JG. Effect and significance of ICAM-1 in ischemia/reperfusion injury. J Med PG 2001; 1:65-68.
3. Cao JP, Xu JG. The expression of ICAM-1m RNA and the infiltration of PMNL, M*Φ* in rat being suffered with ischemia/reperfusion injury of global brain. J Med PG 2000; 5:291-294.
4. Storini C, Rossi E, Marrella V. C1-inhibitor protects against brain ischemia–reperfusion injury *via* inhibition of cell recruitment and inflammation. Neurobiol Dis 2005; 19:10-17.
5. Mitsui Y, Schmelzer JD, Zollman PJ, Mitsui M, Tritschler HJ, Low PA. Alpha-lipoic acid provides neuroprotection from ischemia-reperfusion injury of peripheral nerve. J Neurol Sci 1999; 163:11-16.
6. Pulsinelli WA, Brierley JB. A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke 1979; 10:267-271.
7. Furlow TW. Cerebral ischemia produced by four-vessel occlusion in the rat: a quantitative evaluation of cerebral blood flow. Stroke 1982; 13:852-855.
8. Greene E. Anatomy of the rat. Transactions Am Philosophical Soc, 1935.
9. Krieger DW, Yenari MA. Therapeutic hypothermia for acute ischemic stroke: what do laboratory studies teach us? Stroke 2004; 35:1482-1489.
10. Won MH, Kang T, Park S, Jeon G, Kim Y, Seo JH, Choi E, Chung M, Cho SS. The alterations of *N*-methyl-D-aspartate receptor expressions and oxidative DNA damage in the CA1 area at the early time after ischemia-reperfusion insult. Neurosci Lett 2001; 301:139-142.
11. Imai H, Graham DI, Masayasu H, Macrae IM. Antioxidant ebselen reduces oxidative damage in focal cerebral ischemia. Free Radic Biol Med 2003; 34:56-63.
12. Liu SJ, Zhou SW, Xue CS. Effect of tetrandrine on neutrophilic recruitment response to brain ischemia/reperfusion. Acta Pharmacol Sin 2001; 22:971-975.
13. Caimi G, Canino B, Ferrara F, Montana M, Musso M, Porretto F, Carollo C, Catania A, Lo Presti R. Granulocyte integrins before and after activation in acute ischaemic stroke. J Neurol Sci 2001; 186:23-26.
14. Chen H. Neutropenia reduces the volume of cerebral infarct after transient middle cerebral artery occlusion in the rat. Neurosci Res Commun 1992; 11:93-99.
15. Kapadia R, Tureyen K, Bowen KK, Kalluri H, Johnson PF, Vemuganti R. Decreased brain damage and curtailed inflammation in transcription factor CCAAT/enhancer binding protein beta knockout mice following transient focal cerebral ischemia. J Neurochem 2006; 98:1718-1731.
16. Clark WM, Madden KP, Rothlein R, Zivin JA. Reduction of central nervous system ischemic injury in rabbits using leukocyte adhesion antibody treatment. Stroke 1991; 22:877-83.
17. Matsuo Y, Onodera H, Shiga Y, Shozuhara H, Ninomiya M, Kihara T, Tamatani T, Miyasaka M, Kogure K. Role of cell adhesion molecules in brain injury after transient middle cerebral artery occlusion in the rat. Brain Res 1994; 2:344-352.
18. Clark WM, Madden KP, Rothlein R, Zivin JA. Reduction of central nervous system ischemia injury by monoclonal antibody to intercellular adhesion molecule. J Neurosurg 1991; 75:623-627.
19. Chen H, Chopp M, Zhang RL, Bodzin G, Chen Q, Rusche JR, Todd RF 3rd. Anti-CD11b monoclonal antibody reduces ischemic cell damage after transient focal cerebral ischemia in rat. Ann Neurol 1994; 35:458-463.
20. Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci 2003; 4:399-415.
21. Stanimirovic D, Shapiro A, Wong J. The induction of ICAM-1 in human cerebromicrovascular endothelial cells (HCEC) by ischemia-like conditions promtoes enhanced neutrophil/HCEC adhesion. J Neuroimmunol 1997; 76:193-205.
22. Abate C, Patel L, Rauscher FJ 3rd, Curran T. Redox regulation of fos and jun DNA-binding activity *in vitro*. Science 1990; 249:1157-1161.

(*Received December 12, 2008; Revised December 20, 2008; Accepted December 20, 2008*)