

Mild Hypothermia Inhibits Inflammation After Experimental Stroke and Brain Inflammation

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Background and Purpose—We previously showed that mild hypothermia protects against experimental stroke, even when cooling was delayed by 2 hours. Protection may be due in part to inhibiting inflammation. To clarify, we examined leukocyte infiltration, microglial activation, and adhesion molecule expression in models of stroke and pure brain inflammation.

Methods—Rats underwent 2-hour middle cerebral artery occlusion (MCAO; n=36) or intravenous injection with 5 mg/kg lipopolysaccharide (LPS; n=22). Temperature was lowered to 33°C for 2 hours or kept at 37°C. In MCAO, cooling was applied intraschemically or on reperfusion (delayed). In the LPS model, cooling began after injection. One and 3 days later, brains were assessed for neutrophils, monocytes/microglia, major histocompatibility complex class II antigen, and intercellular adhesion molecule-1 (ICAM-1).

Results—One day after MCAO, both intraschemic and delayed hypothermia decreased ICAM-1 (51% and 60%, respectively, versus normothermia; $P<0.001$), monocytes (63% and 57%; $P<0.01$), and microglia (55% and 53%; $P<0.001$). Similar decreases were seen at 3 days for ICAM-1 (91% and 93%; $P<0.001$), monocytes (62% and 54%; $P<0.01$), and microglia (55% and 53%; $P<0.001$). In the LPS model, ED-1–positive cells were not observed in the brain, but hypothermia decreased ICAM-1 (26%; $P<0.05$), OX6 (56%; $P<0.01$), and microglia (47%; $P<0.01$) at 1 day.

Conclusions—Mild hypothermia decreases inflammatory responses in both brain inflammation and stroke, implicating a direct anti-inflammatory effect of cooling. This suggests that hypothermia can attenuate factors contributing to delayed ischemic injury. (*Stroke*. 2003;34:2495-2501.)

Key Words: hypothermia ■ inflammation ■ ischemia ■ rats

It is well established that mild hypothermia reduces cerebral injury in the laboratory,¹ and this protection has been attributed to preservation of metabolic stores and decreases in excitatory amino acid release.² However, mild hypothermia may protect by other mechanisms because it protects even when delayed for hours after ischemic onset when excitatory amino acids have been released and energy stores are exhausted.^{3,4} Some studies indicate that the acute inflammatory response contributes significantly to injury after ischemia,^{5,6} and protection by mild hypothermia is associated with anti-inflammatory processes.⁷⁻⁹ Infiltrating leukocytes are thought to contribute to secondary ischemic damage by producing toxic substances that kill brain cells and disrupt the blood-brain barrier.^{10,11} Infiltration occurs when leukocytes bind endothelial intercellular adhesion molecule-1 (ICAM-1) and ICAM-1 is upregulated after ischemia.¹² Previously, our group and others have found that intraschemic mild hypothermia decreases ICAM-1 expression,¹³ neutrophil infiltration,^{8,9,14} and blood-brain barrier disruption^{15,16} after experimental stroke.

Although hypothermia has been shown to suppress markers of inflammation in stroke models, observed decreases could be due secondarily to reduced injury from hypothermia. To clarify, we compared 2 separate models of experimental stroke and pure brain inflammation induced by lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, and investigated the influence of mild hypothermia on ICAM-1 expression and inflammatory cell activation/infiltration.

Materials and Methods

Experiments were carried out according to the guidelines for animal care and use. Laboratory animal protocols were approved by the institutional panel on laboratory animal care.

Stroke Model

Male Sprague-Dawley rats (Charles River) weighing between 290 and 350 g were anesthetized with isoflurane with a face mask and maintained with 1% to 2% isoflurane in 200 mL/min oxygen and 800 mL/min air. Every 15 minutes, rectal temperature was assessed during the period the animals were under anesthesia (4 to 5 hours). Physiological parameters were also monitored and maintained in normal ranges. Middle carotid artery occlusion (MCAO) was per-

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formed as previously described by our group.^{7,9} In brief, a midline incision in the neck exposed the common carotid, external carotid, internal carotid, and pterygopalatine arteries. MCAO was produced by inserting an uncoated 3-0 monofilament suture 18 to 20 mm from the bifurcation of the internal and external carotid arteries. The suture was kept in place for 2 hours. After MCAO induction, rats were maintained at normothermic conditions (37°C) throughout MCAO (n=12), subjected to intras ischemic hypothermia (33°C) immediately after ischemia onset (n=12), or subjected to delayed hypothermia with cooling beginning on reperfusion (n=12). Cooling was maintained for 2 hours. Animals were euthanized 1 or 3 days after ischemia by a CO₂ overdose and perfused with normal saline, and brains were prepared as described below.

Brain Inflammation Model

Male Sprague-Dawley rats weighing between 350 and 450 g were anesthetized as described above. Physiological parameters were also monitored and recorded as described above. Inflammation was induced by intrajugular administration of 5 mg/kg bacterial LPS in sterile normal saline (*Escherichia coli* serotype 055; B5; Sigma). After injection, the experimental animals were maintained at 37°C (n=11) or 33°C (n=11) for 2 hours. In the hypothermic group, cooling began immediately after injection, and temperature decreased within 15 minutes. Sham animals were injected with normal saline. Animals were euthanized after 1 or 3 days with a CO₂ overdose and then perfused intracardially. Brains were quickly removed and prepared as for Western blots or histochemistry. Because previous meningitis models involving injections of endotoxins directly into the central nervous system reported direct injury and blood-brain barrier disruption,^{17,18} some animals were injected with 1 mL 4% Evans blue dye in 0.9% NaCl into the jugular vein 1 hour before sacrifice at 24 hours. Similarly, transferase dUTP nick-end labeling (TUNEL) staining was performed to determine whether LPS resulted in brain cell death.

Histochemistry

Microglia, Neutrophil, and Monocyte Histochemistry

Brains were fixed in 2% paraformaldehyde for 7 days, embedded in paraffin, cut into 10- μ m coronal sections, deparaffinized, microwaved in 0.1 mol/L citric acid, and treated for endogenous peroxidases with 0.03% hydrogen peroxide. To identify microglia, sections were incubated for 3 hours with 10 μ g/mL *Griffonia simplicifolia* isolectin B4 (IB4; L5391; Sigma), and visualized with diaminobenzidine. For other markers, sections were blocked with 5% normal serum, followed by a 1-hour incubation in ED1 antibody (MCA341R; 1:400; Serotec), a marker for macrophages and phagocytic microglia, or anti-myeloperoxidase antibody (MPO; A0398; 1:500; Dako), a marker for neutrophils. Sections were incubated with a biotinylated secondary antibody that had been preabsorbed to rat serum (Elite Vectastain ABC Kit, Vector Labs), followed by a tertiary binding complex (ABC), and visualized with diaminobenzidine (Sigma Fast Diaminobenzidine, Sigma).

ICAM-1 and Major Histocompatibility Complex Class II Immunohistochemistry

Fresh-frozen tissue sections were prepared from brains sunk in 20% sucrose/phosphate-buffered saline (PBS) solution for 24 hours at 4°C. Brains were frozen and cut into 25- μ m-thick sections. After fixing in 75% acetone:25% ethanol, sections were placed in 0.05% H₂O₂ and blocked in 3% horse serum. Sections were then exposed to antibodies against major histocompatibility complex (MHC) class II antigen (OX-6; 554926; 1:100; PharMingen) or ICAM-1 (MCA1333; 1:50; Serotec), followed by biotinylated secondary antibody, and visualized as before. Some sections were double labeled with OX-6 and IB4. After blocking in normal serum, sections were labeled with OX6 using a cy3-conjugated secondary antibody (1:200; JacksonImmunoResearch), followed by incubation with fluorescein isothiocyanate-conjugated IB4 (Sigma).

Body Temperature

Group	MCAO	LPS	
Normothermic	37.0 \pm 0.1	36.8 \pm 0.1	
	Intraischemic	Delayed	
Hypothermic	32.9 \pm 0.1	33.0 \pm 0.1	33.0 \pm 0.2

Temperature was monitored only during the time the animals were under anesthesia and represent the average of the mean value for each animal. Values are mean \pm SEM.

TUNEL Stain

TUNEL staining was performed on brains of LPS-treated animals that survived to 3 days to determine whether cell damage occurred. This method has previously been published by our laboratory.¹⁹ In brief, brains were fixed in 3% paraformaldehyde plus 20% sucrose and then cryosectioned into 25- μ m-thick sections. TUNEL staining was performed with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Intergen) according to kit instructions with some modifications. Cryo sections were treated with 10 μ g/mL proteinase K (Sigma) at room temperature for 15 minutes and then incubated in 0.3% hydrogen peroxide, followed by steps outlined in the kit. Sections were counterstained with hematoxylin and eosin.

Western Blot

Brains were perfused with normal saline; then, predefined brain regions of the cortex and striata²⁰ were quickly dissected on dry ice. Tissue was homogenized in Laemmli's lysis buffer plus protease inhibitors. Protein concentrations of each sample solutions were determined with a BCA protein assay kit (Pierce), and samples were stored at -80°C until use. Then, 25 μ g protein from cell lysates was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (IPVH00010, Millipore). Membranes were blocked with 5% milk for 1 hour and then probed for 1 hour with ICAM-1 antibody (MCA773; 1:500; Serotec), washed in PBS containing 0.1% Tween 20, and incubated with horseradish peroxidase-conjugated anti-goat IgG for 1 hour. Bound antibody was visualized with the ECL system (Amersham). Membranes were then stripped and probed for β -actin to confirm equal protein loading. Optical densities were measured with a BioRad Multianalyst GS 700.

Data and Statistical Analyses

In the stained sections, positive cells were counted from 6 adjacent fields in the peri-infarct area in the stroke model and 6 adjacent fields in both lateral cortexes for each animal in the LPS model. Brain regions selected for counts have previously been detailed.¹³ MPO-positive cells with morphology consistent with neutrophils were counted per \times 400 field; ED-1-, OX-6-, and IB4-positive cells were also counted per \times 400 field. ICAM-1-positive vessels were counted per \times 100 field.

Statistical analyses were performed with 1-way analysis of variance, followed by Tukey's multiple comparisons procedure with SigmaStat (SPSS). Data are shown mean \pm SE. Values of $P < 0.05$ were considered significant.

Results

Temperature is shown for each group in the Table. For rats undergoing MCAO, mean temperature for the intras ischemic hypothermia group was 32.9 \pm 0.1°C, and mean temperature for the delayed hypothermia group was 33.0 \pm 0.1°C. For rats undergoing LPS injection, the mean temperature for the hypothermic group was 33.0 \pm 0.2°C. Physiological parameters were otherwise similar.

Mild Hypothermia and ICAM-1 Expression After MCAO

Cell lysates from the striatum and the peri-infarct area of the ipsilateral hemisphere were assayed for ICAM-1 expression with Western blot. Previous studies by our group indicated that intras ischemic mild hypothermia significantly lowers densities of ICAM-1-immunopositive vessels.¹³ Western blots show similar significant decreases in ICAM-1 expression for both intras ischemic and delayed hypothermia at 1 and 3 days compared with normothermic controls ($P < 0.001$ for all comparisons; Figure 1A and 1B). At 24 hours, intras ischemic hypothermia decreased the expression of ICAM-1 by 51%, and delayed hypothermia decreased the expression by 60%. ICAM-1 expression was decreased even more at 3 days, with 91% reduction by intras ischemic hypothermia and 93% reduction by delayed hypothermia.

Effect of Mild Hypothermia on Leukocyte Infiltration and Microglial Activation After MCAO

Mild hypothermia decreased the density of ED-1-positive cells, which detects phagocytic cells, including macrophages and phagocytic microglia. Under normothermic conditions, ED-1-positive cells appear at 1 day and increase at 3 days, especially within the peri-infarct area.¹³ As shown in Figure 1C, both models of mild hypothermia significantly reduced the numbers of macrophages detected at both time points. At 1 and 3 days, both intras ischemic and delayed mild hypothermia reduced the numbers of ED-1-positive cells to a similar extent.

IB4-positive cells were also decreased by mild hypothermia. IB4 detects resident microglia (resting and activated), peripheral monocytes, and macrophages. Although it is not possible to distinguish between peripheral macrophages and fully activated microglia, the pattern of IB4-positive cell appearance was similar to that of ED-1, which detects macrophages and microglia once they have become phagocytic. Under normothermic conditions, IB4-positive cells were present at 1 day and increased in number and intensity at 3 days. Although many of the IB4-positive cells were round and amoeboid in shape, many were also ramified with processes. Compared with normothermic controls, treatment with either hypothermia paradigm significantly reduced the number of IB4-positive cells at both time points (Figure 1D). IB4 marked both rounded and process-bearing cells. The proportions of round and ramified cells were not obviously different between the hypothermic and normothermic groups, but the overall intensity of staining was higher in the normothermic group.

LPS-Induced Brain Inflammation Model

LPS administration caused animals to develop piloerection and decreased motor activity, but no mortality was observed. LPS also resulted in increased meningeal cell infiltrate compared with sham-injected brains (Figure 2). This infiltrate was observed as early as 8 hours, peaked at 1 day, and subsided by 3 days. Mild hypothermia markedly decreased densities of meningeal cell infiltrates (Figure 2B). This dose of LPS did not reveal any evidence of brain cell death or

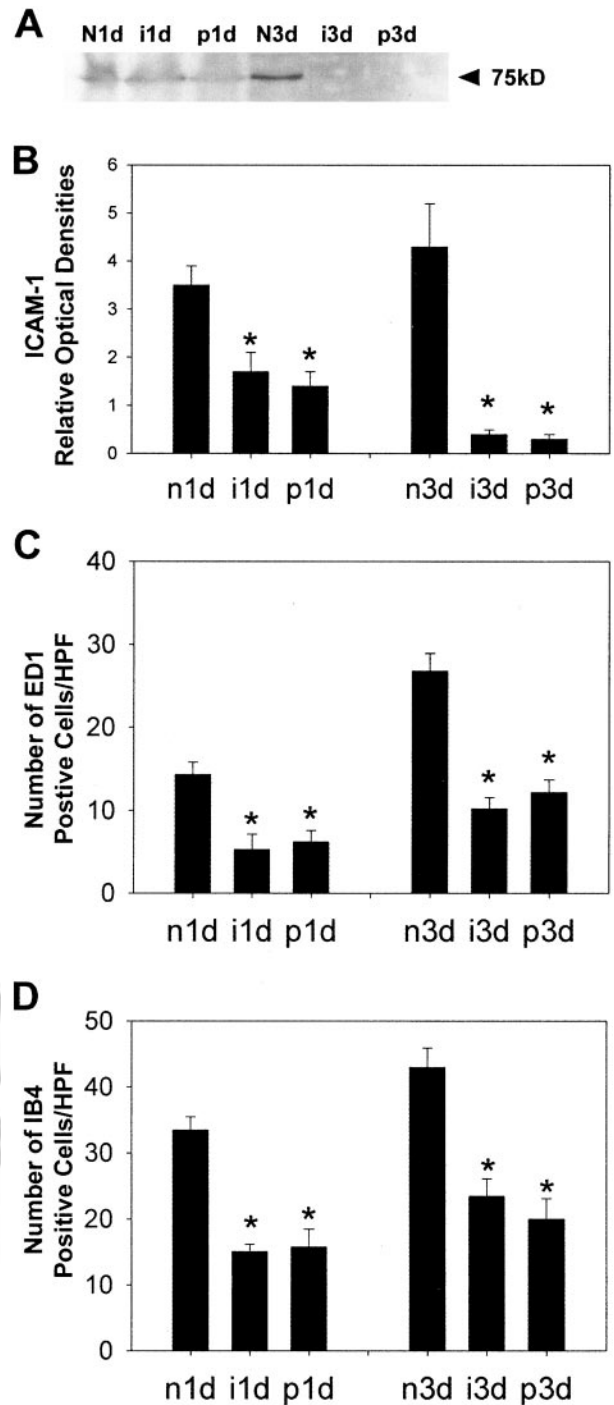


Figure 1. Mild hypothermia reduces ICAM-1 expression and monocyte infiltration/microglia activation after experimental stroke. Western blots of ischemic brain regions show increased ICAM-1 expression in normothermic brains at 1 and 3 days after insult (N1d, N3d) vs hypothermic animals at the same time point whether cooling began at the onset of ischemia (i1d, i3d) or on reperfusion (p1d, p3d) (A; $*P < 0.05$). Quantification of relative optical densities (B) show that ICAM-1 expression is significantly reduced by both hypothermia paradigms. Similarly, densities of macrophages/activated microglia were decreased by mild hypothermia with 2 different markers (C: ED1, $*P < 0.01$; D: IB4, $*P < 0.001$). HPF indicates high-power field.

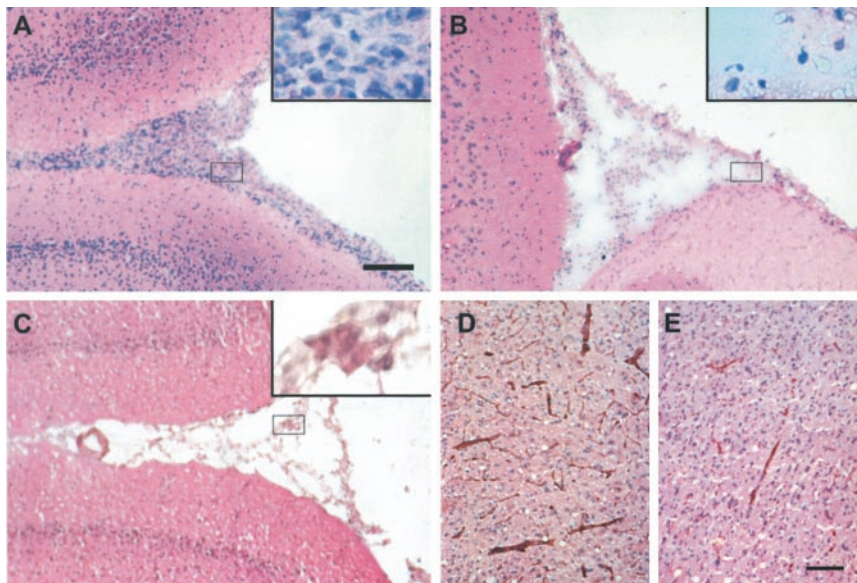


Figure 2. Mild hypothermia inhibits inflammatory cell infiltration in a model of pure brain inflammation. A, Meningeal leukocyte influx after normothermic conditions after LPS administration. Note that no obvious cell death in the brain was observed on routine light microscopy. B, Leukocytes in the meninges after hypothermic conditions after LPS injection. This infiltrate is clearly reduced by hypothermia. C, Leukocyte influx after sterile PBS injection for comparison. Inserts show higher detail of the meningeal infiltrates. Immunostains of ICAM-1 show increased expression after LPS injection under normothermic conditions (D) but is decreased under hypothermic conditions (E). Scale bar=100 μm (A) and 20 μm (E).

DNA damage by conventional histology or TUNEL staining. Blood-brain barrier disruption also did not occur in this model, as evidenced by a lack of Evans blue dye extravasation into brain parenchyma from the vessels (data not shown).

Mild Hypothermia and ICAM-1 Expression in LPS-Induced Brain Inflammation

Similar to its effects in the stroke model, mild hypothermia also attenuated ICAM-1 expression in the LPS model of pure brain inflammation. Figure 2D and 2E shows representative immunohistochemical sections that illustrate the decrease in vessel density by hypothermia. Overall ICAM-1 expression was measured with Western blot, with 26% reductions in total protein in the cortex and striatum by hypothermia (Figure 3A and 3B). However, immunostains showed that although ICAM-1 was mainly in small vessels and occasionally in medium-sized vessels, it also stained some microglia and monocytes. Counts of only immunopositive vessels showed that 2 hours of mild hypothermia decreased the number of ICAM-1 positive vessels by 69% (Figure 3C).

Mild Hypothermia and Leukocyte Infiltration/Microglial Activation in LPS-Induced Brain Inflammation

The effect of hypothermia on microglial activation was determined with IB4 and OX-6. At 1 day, immunostaining indicated that mild hypothermia decreased the number of OX-6- and IB4-positive cells (Figure 3D). Compared with normothermic controls, OX-6-positive cells were decreased by 56% and IB4-positive cells by 47%. Figure 4A through 4D shows representative sections that demonstrate the decrease in IB4- and OX6-positive cells by hypothermia.

With double immunofluorescent labeling, OX6 colocalized with IB4, indicating that these stellate cells are indeed microglia (Figure 4E through 4G). In this model, OX6 (to identify MHC II antigen and thus activated microglia) labeled many microglia that had not undergone complete transformation and were still stellate (Figure 4C and 4D), although some cells possessed rounded morphology.

Cells positive for ED-1 were found mainly in the meninges, consistent with brain macrophages that normally reside in the meninges. Only a few neutrophils were identified with MPO. Accurate cell counting in the meninges for all brains was not possible because parts of the meninges were lost in the tissue preparation process. Although rare cells stained positive for ED-1 and MPO in the brain under both conditions, no obvious differences were noted.

Discussion

We show here that mild hypothermia inhibits adhesion molecule expression and microglia-monocyte activation and infiltration in experimental stroke and pure brain inflammation. Although there are no good markers to distinguish peripheral macrophages from activated microglia, our results indicate that suppression of inflammation, whether central or peripheral, may underlie the neuroprotective effects of mild hypothermia. Furthermore, our observations in the LPS model suggest the anti-inflammatory effects of hypothermia even when cell death does not occur.

Postischemic inflammation is characterized partly by leukocyte influx. ICAM-1 is essential for peripheral leukocyte recruitment into brain parenchyma and was decreased in both models by hypothermia. ICAM-1 has been shown to be upregulated in the brain microvasculature during ischemia and reperfusion.²¹ Consistent with prior reports that pharmacological¹² or genetic²² ICAM-1 inhibition prevents peripheral leukocyte infiltration into the brain, hypothermia may also act in a similar manner. Recently, Ishikawa and colleagues²³ showed that, after MCAO, moderate hypothermia suppresses leukocyte adhesion in vessels. Others have shown significant reductions in ICAM-1 mRNA by prolonged hypothermia after MCAO²⁴ and trends toward lowered ICAM-1 expression in vessels.²⁵ Hypothermia also reduced leukocyte rolling and adhesion in vessels after systemic injection of interleukin-1 β in mice, although it did not alter ICAM-1

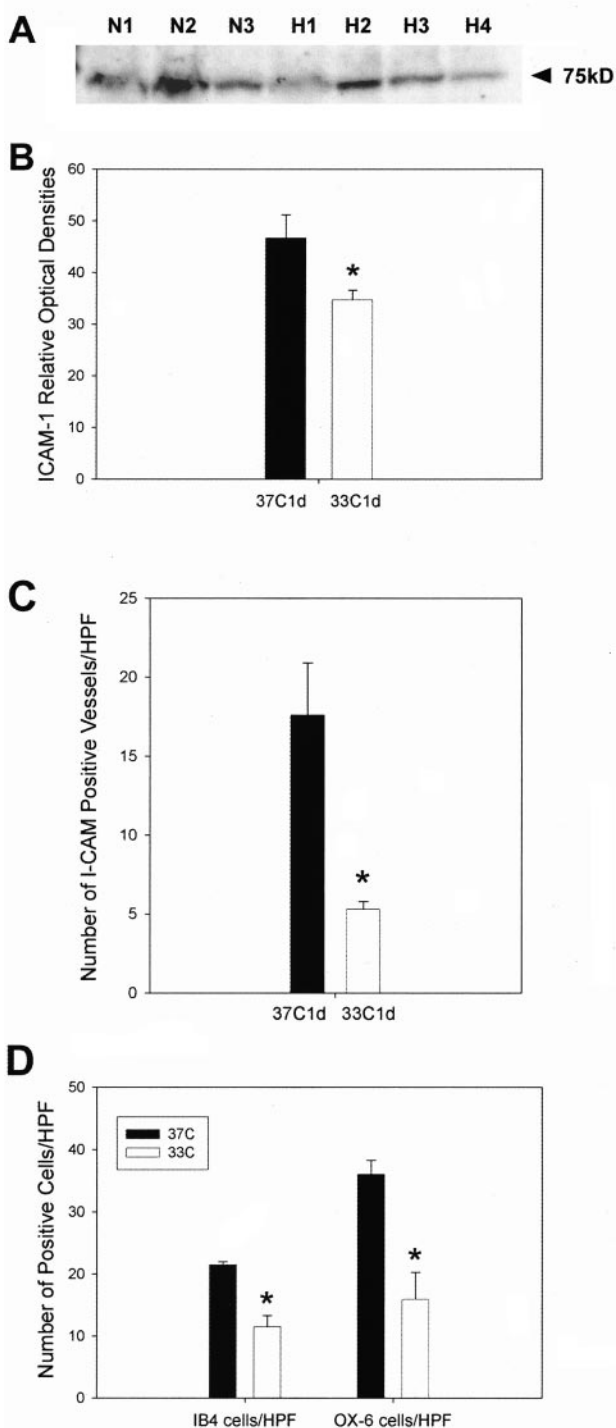


Figure 3. Mild hypothermia suppresses ICAM-1 expression and monocyte infiltration/microglial activation in a model of brain inflammation. Western blots of ICAM-1 and β -actin in LPS-treated brain under normothermic (N) and hypothermic (H) conditions (A). Relative optical densities of ICAM-1 protein in tissues collected 1 day after LPS injection indicate that hypothermia reduces its expression (B, $*P < 0.05$). Numbers of ICAM-1-positive vessels 1 day after LPS administration were significantly decreased by hypothermia (C). Microglial densities as identified by IB4 labeling are significantly decreased by hypothermia vs normothermia (D). Microglial activation as identified by OX6 is also significantly decreased by hypothermia (C, D, $*P < 0.01$). HPF indicates high-power field.

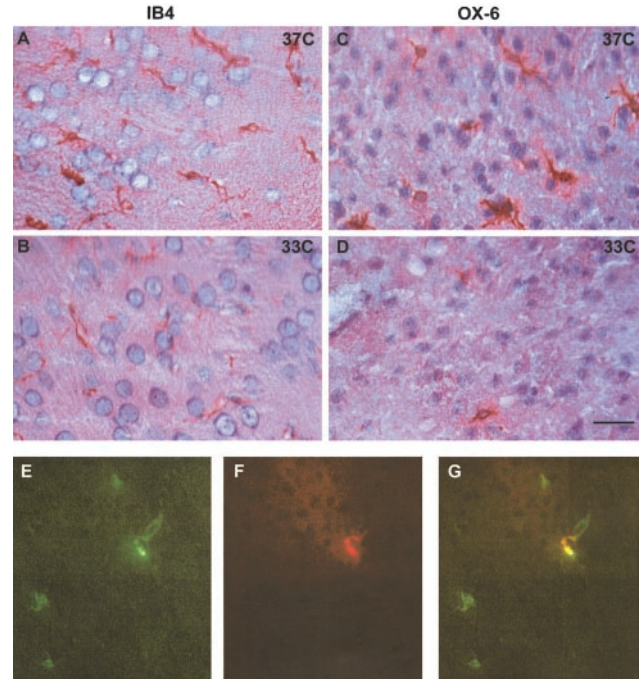


Figure 4. Mild hypothermia inhibits microglial activation and MHC class II molecule expression. After LPS injection, brains were stained for IB4 to identify cells of monocyte origin and OX6 to identify MHC class II molecules. Under normothermic conditions (37°C), numerous IB4-positive cells with processes consistent with microglia are apparent (A). Rare cells possessed rounded or amoeboid morphology consistent with a phagocytic state. Many of these cells were also positive for OX6 (C). Under hypothermic conditions (33°C), markedly fewer cells stained positive for IB4, and those that did also possessed processes (B). Rare cells were OX6 positive (D). Double immunofluorescent labeling on a representative normothermic brain of an LPS-injected rat is shown in E through G. IB4-positive cells are observed (E), some of which are also positive for OX6 (F). Merged image indicates colocalization of the 2 markers (G) (scale bar = 25 μ m).

expression in interleukin- 1β -stimulated cultured human cerebral endothelial cells.²⁶

In line with the above observations, we previously showed that, after MCAO, densities of ICAM-1-positive cells and leukocytes were reduced by intras ischemic hypothermia.¹³ However, such a reduction could be explained by the application of hypothermia during the ischemic period when it might be expected to protect by preserving metabolic stores and preventing glutamate accumulation.^{27,28} In the present study, hypothermia similarly suppressed leukocyte infiltration and ICAM-1 expression even when cooling was initiated 2 hours later, a time when adenosine triphosphate is depleted and glutamate has already been released. Mild hypothermia also suppressed ICAM-1 expression in the LPS model, suggesting a direct effect of hypothermia on inflammation. Unlike the stroke model, ICAM-1 expression in the LPS model does not appear to be associated with leukocyte influx. Although leukocytes were present in the stroke model, there was little peripheral leukocyte infiltration into the parenchyma in the LPS model, regardless of temperature. ED1-positive cells were found in the meninges, and there was no

disruption of the blood-brain barrier, suggesting that leukocyte infiltration depends on the presence of necrotic cells or other factors not stimulated by LPS. Nevertheless, our data imply a direct effect of the temperature dependence of inflammation and hypothermic neuroprotection.

Microglia also participate in the progression of ischemic injury, are activated in the brain as early as 6 hours after MCAO,²⁹ and generate a variety of damaging substances.³⁰ A few studies have shown that hypothermia can prevent cultured microglia from generating less superoxide, nitric oxide, and inducible nitric oxide synthase, indicating a direct effect of hypothermia on these cells.^{7,31} Here, double immunofluorescent labeling showed that LPS administration could activate microglia to express MHC class II antigen without full transformation into an amoeboid morphology. Although the reasons for this are not clear, hypothermia could suppress microglial activation as it reduced the numbers of OX6-positive cells.

It should be noted that whether all inflammatory responses are necessarily damaging to the ischemic brain is far from clear. Although early inflammatory responses appear to be involved in potentiating ischemic injury, chronic inflammation may be necessary for repair.^{1,32} Although several studies have now shown that preventing early neutrophil influx into ischemic brain reduces damage, it is not clear whether preventing influx and activation of other leukocytes at later time points is also protective. Our data here suggest but do not prove that, because parenchymal leukocytes are decreased by hypothermia, they may be involved in potentiating damage. Further studies addressing both early and late components of this response are clearly needed.

Our data support the notion that brain inflammation can be modulated by mild hypothermia, and this may be a mechanism by which hypothermia works to protect against stroke. To date, the only effective neuroprotectant at the clinical level is hypothermia in the setting of cardiac arrest.^{33,34} Therefore, strategies to limit ischemic injury might include hypothermia plus a pharmacological neuroprotectant or anti-inflammatory therapies.

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