

# Influence of Mild Hypothermia on Inducible Nitric Oxide Synthase Expression and Reactive Nitrogen Production in Experimental Stroke and Inflammation

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Mild hypothermia is neuroprotective, but the reasons are not well known. Inflammation contributes to ischemic damage; therefore, we examined whether the protection by hypothermia may be attributable to alterations in the inflammation. We examined whether hypothermia might alter the inflammatory cell-associated inducible nitric oxide synthase (iNOS) and subsequent nitric oxide (NO) and peroxynitrite generation in experimental stroke and inflammation. Rats underwent 2 hr of middle cerebral artery occlusion (MCAO). Brain inflammation was modeled by intravenous lipopolysaccharide (LPS) (2 mg/kg) injection. Temperature was maintained at 33°C for 2 hr immediately after MCAO and LPS injection, delayed 2 hr after MCAO or maintained at 38°C. Cultured microglia were activated with LPS and then incubated at 33 or 37°C. Both intraischemic and delayed mild hypothermia attenuated infarct size by 40% ( $p < 0.05$ ). Immunohistochemistry was performed to

identify cell type, iNOS, and peroxynitrite. The majority of iNOS- and peroxynitrite-positive cells were activated microglia–macrophages, and mild hypothermia significantly decreased the numbers of immunoreactive cells at 72 hr by  $>50\%$  ( $p < 0.05$ ). After ischemia, mild hypothermia decreased NO production by 40%. Similarly, hypothermia attenuated NO and iNOS in LPS-injected rats, as well as in cultured microglia. Aminoguanidine, an iNOS inhibitor, also attenuated infarct size and NO in ischemic and inflammation models. We conclude that mild hypothermia significantly inhibits the inflammatory response by affecting microglial iNOS–NO generation. Therapies directed against microglia or their activation may be useful in treating stroke.

**Key words:** focal cerebral ischemia; mild hypothermia; inducible nitric oxide synthase; microglia; peroxynitrite; lipopolysaccharide; inflammation

There has been renewed interest in mild hypothermia as a method to protect brain in cerebral ischemia (Ginsberg et al., 1992; Karibe et al., 1994a,b; Maier et al., 1998, 2001). Its neuroprotective effects have often been attributed to a decrease in cerebral blood flow and metabolic requirement for oxygen (Karibe et al., 1994b) and alteration in neurotransmitter release (Ginsberg et al., 1992; Huang et al., 1998). More recently, there have been reports that hypothermia may attenuate the inflammatory response to cerebral ischemia, especially when cooling is delayed. Several studies have provided evidence that postischemic hypothermia protects the brain from cerebral ischemia (Karibe et al., 1994a,b; Maier et al., 1998, 2001; Kawai et al., 2000) when energy stores have already been depleted and glutamate has been released. The observation that delayed cooling still resulted in cerebral protection suggests that mild hypothermia affects some of the injury mechanisms that occur later in the ischemic cascade.

Inflammation plays a central role in the pathogenesis of cerebral ischemia and secondary damage (Barone and Feuerstein, 1999). Inflammation is thought to contribute to the genesis of

secondary damage and develops as a consequence of activation of microglia and resident perivascular and parenchymal macrophages and infiltration of peripheral inflammatory cells (Garcia et al., 1994; Toyoda et al., 1996; del Zoppo et al., 2000). Inflammatory cells generate potentially damaging nitric oxide (NO), oxygen free radicals, and cytokines. Cytokines activate microglia and stimulate expression of adhesion molecules leading to leukocyte infiltration. Activated microglia also potentiate the inflammatory response and generate reactive oxygen species and NO. Inflammatory cells also express more cytokines, leading to more glial cell activation and damage. In the context of brain ischemia, the activity of neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) is broadly deleterious (Eliasson et al., 1999; del Zoppo et al., 2000), and their inhibition is neuroprotective (Iadecola et al., 1995b; Cockroft et al., 1996; Zhang and Iadecola, 1998). NO and superoxide are themselves highly reactive but can also combine to form peroxynitrite, a particularly damaging reactive species. The toxicity of the free radicals and peroxynitrite results from their modification of macromolecules, especially DNA (Love, 1999). We and others have shown that mild hypothermia decreases tissue neutrophils (Toyoda et al., 1996; Maier et al., 1998). Mild hypothermia also attenuates downstream effects of inflammation by consumption of endogenous antioxidants (Kil et al., 1996), blood–brain barrier disruption, and cerebral edema (Karibe et al., 1994b).

Although it is well established that inflammation contributes to cerebral ischemic injury and that mild hypothermia is an effective neuroprotectant, whether and how inflammatory processes are altered to achieve hypothermic protection have not been exten-

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sively studied. In this study, we planned to determine whether mild hypothermia affects expression of iNOS and production of NO–peroxynitrite mediated by activated microglia–macrophages in an experimental model of stroke. Given that any observed differences could be related to the neuroprotective effects of mild hypothermia, we also evaluated the effect of hypothermia in a model of brain inflammation that does not result in cell death.

## MATERIALS AND METHODS

Experiments were performed according to the guidelines for the animal care and use of laboratory animal protocols approved by the Stanford University Administrative Panel on Laboratory Animal Care. Animals were housed with food and water available *ad libitum* under diurnal lighting conditions and a temperature-controlled environment until the day of experiment.

**Focal cerebral ischemia model of rat.** Male Sprague Dawley rats weighing between 290 and 320 gm were anesthetized with halothane and maintained during surgical procedures. A femoral artery was cannulated for the continuous monitoring of arterial blood pressure and blood sampling. Physiological parameters were monitored and maintained in the normal range. Blood gases were measured with an automatic pH/blood gas analyzer (model 178; Ciba Corning Diagnostics Corp., Medfield, MA). Ischemia was induced using an occluding intraluminal suture (Maier et al., 1998; Yenari et al., 2000). An uncoated 30-mm-long segment of 3–0 nylon monofilament suture with the tip rounded by flame was inserted into the stump of the common carotid artery and advanced into the internal carotid artery ~19–20 mm from the bifurcation to occlude the ostium of middle cerebral artery (MCAO). At the end of the ischemic period, the suture was removed, and the animal was allowed to recover. Sham-operated animals were treated in the same manner as the ischemic animals, but no ischemia was applied. During surgery, rectal temperature was maintained between 37 and 38°C, corresponding to brain temperature of 38–39°C (Yenari et al., 2000). Mild hypothermia (33°C of rectal temperature, corresponding to brain temperature of 33°C) was achieved as described previously (Maier et al., 1998, 2001; Yenari et al., 2000). Cooling began on ischemia onset (intraischemic hypothermia) or was delayed by 2 hr (delayed hypothermia), cooling begins immediately after the suture is removed. Cooling was maintained for 2 hr, and these conditions were associated with neuroprotection in our hands. At the completion of the experiment, the animals were killed with a halothane overdose and prepared for additional analysis (described subsequently).

**In vivo brain inflammation model.** Animals were anesthetized, and 2 mg/kg lipopolysaccharide (LPS) (*Escherichia coli* serotype 055:B5; Sigma, St. Louis, MO) was administered into the jugular vein. Control animals were given sterile normal saline. LPS-treated animals were kept at 37–38°C of rectal temperature (normothermia) or cooled to 32°C (hypothermia) immediately after injection. Hypothermia was maintained for 2 hr.

**Microglial cultures and activation.** Murine microglial cultures were prepared from astrocyte cultures as described previously (Yenari and Giffard, 2001). Whole brains from postnatal days 1–3 Swiss Webster mice were plated in 75 cm<sup>2</sup> coated flasks at a density of two brains per flask in Eagle's Minimal Essential Medium supplemented with 10% equine serum, 10% fetal bovine serum, epidermal growth factor (1 mg/100 ml), glutamine (2 mM), glucose (21 mM), and bicarbonate (26 mM) (plating media), plus penicillin (100 U/ml) and streptomycin (100 mg/ml). The cultures were maintained in a 37°C humidified incubator with a 5% CO<sub>2</sub> atmosphere. Media was changed every 2–3 d for the first 10 d. Microglia were subcultured using methods described previously (Giulian and Baker, 1986; Sasaki et al., 1989; Smith, 1993) and modified slightly. After 10–14 d *in vitro*, flasks were inspected for microglia growing on top of a confluent cell layer. Microglia containing flasks were shaken at 160 rpm for 30 min at 37°C. The supernatant was collected and spun for 5 min at 800 × g. The resulting pellet was resuspended in plating media and plated at a density of 2–3 × 10<sup>5</sup> cells/ml in uncoated 24-well plates. The plates were returned to the incubator for 1 hr to allow the microglia to attach and then washed and returned to the incubator with fresh media and antibiotics. Cultures were used for experiments 24 hr after plating. Histochemical staining with *Griffonia simplicifolia* B4-isolectin (IB4; Sigma) confirmed that the majority of these cells were indeed microglia. Experiments were repeated four times using cells isolated from two to three different dissections. Microglia were activated by exposure to LPS

(10 µg/ml) and phorbol 12-myristate 13-acetate (PMA) (1 nM in DMSO; a protein kinase C activator; Sigma). Cultures were washed three times in LPS- or PMA- containing media and then returned to the incubator. Control cultures were washed in only plating media. This dose of LPS or PMA was chosen because it was the lowest concentration in pilot experiments that consistently transformed resting microglia into the activated, amoeboid form. To perform hypothermia, cells were kept in an incubator, with the temperature set at 33°C during LPS or PMA treatment.

**iNOS inhibition.** Aminoguanidine, a relatively selective iNOS inhibitor, was administered intraperitoneally to rats after MCAO and LPS treatment. Ischemic rats received aminoguanidine hemisulfate (100 mg/kg in 1 ml of saline; A7009; Sigma) (*n* = 6) immediately after and 8, 24, 32, 48, and 52 hr after MCAO. The dose of aminoguanidine used was found to selectively inhibit iNOS activity (Zhang and Iadecola, 1998). The other set of rats received aminoguanidine hemisulfate (*n* = 6) immediately after LPS injection and 8, 24, 32, 48, and 52 hr later. For primary microglial cultures, aminoguanidine was dissolved in culture media (200 µM) and applied to LPS-stimulated cells.

**Infarct analysis.** Animals were perfused with heparinized saline and were fixed in 3% paraformaldehyde plus 20% sucrose. After fixation, the brains were cut into 3-mm-thick coronal slices: level 1 was from 4 mm anterior to bregma to 1 mm anterior to the bregma; level 2 was to 2 mm posterior to bregma; level 3 was to 5 mm posterior to bregma; and level 4 was to 8 mm posterior to bregma. Cryosections (30 µm) were prepared from each slice, placed on Superfrost Plus slides (Fischer Scientific, Pittsburgh, PA), air dried for 2 hr, frozen, and stored at –80°C until use. To assess ischemic injury, brain sections from four different slices of the brain were stained with cresyl violet. Areas of infarction were measured using an image analysis system described previously (Maier et al., 1998; Yenari et al., 1998). Briefly, infarct was evaluated by light microscopy in a blinded manner. Infarct areas as delineated by areas of nonstaining were measured with an image analysis system (MCID; Imaging Research Inc., Ontario, Canada) and were expressed as a percentage of the total area of ipsilateral hemisphere. Infarct areas from four coronal slices at different levels were summed and expressed as a percentage of the total area of ischemic hemispheres.

**Immunohistochemistry.** Cryosections were treated for endogenous peroxidases with 0.03% H<sub>2</sub>O<sub>2</sub>, blocked in 5% normal serum, and then incubated with primary antibody, followed by the secondary antibody (Vector Laboratories, Burlingame, CA). Antibodies were detected using the Elite Vectastain ABC kit (Vector Laboratories) and colorized with diaminobenzidine (Vector Laboratories). For double labeling, the primary antibodies were detected with Cy3- or FITC-conjugated secondary antibody (1:200; Jackson ImmunoResearch, West Grove, PA). The following primary antibodies were used: ED1 (1:200; T3003X; Research Diagnostics, Flanders, NJ) to detect activated microglia; anti-nitrotyrosine antibody (1:50; 06284; Upstate Biotechnology, Lake Placid, NY) to detect peroxynitrite; anti-iNOS antibody (1:500; 482728; Calbiochem, San Diego, CA); anti-microtubule-associated protein-2 (MAP-2) antibody (1:300; M4403; Sigma) to detect neurons; and anti-GFAP antibody (1:100; 556330; PharMingen, San Diego, CA) to detect astrocytes. Counterstain was performed using the nuclear marker 4',6'-diamidino-2-phenylindole (DAPI). The number of immunopositive cells was counted under microscope and normalized to infarct size.

**SDS-PAGE and immunoblotting.** Animals were perfused with saline, and then brains were removed and sectioned into four coronal slices in the same manner described in histology. Slices containing maximal ischemic damage were chosen. Each slice was dissected into four parts: ischemic cortex, ischemic subcortex, contralateral cortex, and contralateral subcortex. Brain tissue was homogenized in Laemmli's lysis buffer plus protease inhibitors. Protein concentrations of each sample solution were determined using the BCA protein assay kit (Pierce, Rockford, IL), and the samples were stored in at –80°C until use. Aliquots containing 50 µg of protein were subjected to 7.5% SDS-PAGE. Protein bands were transferred to polyvinylidene fluoride membrane (IPVH00010; Millipore, Bedford, MA) and probed for the iNOS by incubating in the primary antibody (1:2500; N32020; Transduction Laboratories, Lexington, KY), followed by a horseradish peroxidase-conjugated secondary antibody (sc-2005; Santa Cruz Biotechnology, Santa Cruz, CA). To determine the specificity of the iNOS primary antibody, we used iNOS antibody preabsorbed with anti-iNOS blocking peptide (482729; Calbiochem) instead of primary antibody. Blots were visualized using the ECL system (Amersham Biosciences, Piscataway, NJ) according to the directions of the manufacturer and exposed to x-ray film. Equal protein loading was confirmed by measuring β-actin. Membranes were stripped

**Table 1. Physiological parameters**

	N ( <i>n</i> = 6)	HI ( <i>n</i> = 6)	HD ( <i>n</i> = 6)
Arterial pH	7.38 ± 0.02	7.34 ± 0.02	7.37 ± 0.01
Arterial PCO <sub>2</sub> (mmHg)	37.4 ± 3	38.5 ± 6	43.3 ± 6
Arterial PO <sub>2</sub> (mmHg)	161.7 ± 8	159.4 ± 11	171.6 ± 10
Hematocrit (%)	41 ± 3	42 ± 4	39 ± 3
Blood glucose (mg/dl)	136 ± 7	123 ± 9	145 ± 9
MAP (mmHg)	128 ± 6	133 ± 9	136 ± 5
Heart rate (beat/min)	219 ± 17	178 ± 11	199 ± 14
Temperature (intraischemic)	37.0 ± 0.1	32.9 ± 0.1*	37.0 ± 0.1
Temperature (postischemic)	37.0 ± 0.1	37.0 ± 0.1	33.0 ± 0.1*

Measurements were performed at 1 hr of MCAO and 20 min after reperfusion. Data were analyzed using ANOVA, followed by Tukey's test. Data are mean ± SEM. N, Normothermia; HI, intraischemic hypothermia; HD, delayed hypothermia; PCO<sub>2</sub>, blood CO<sub>2</sub> pressure; PO<sub>2</sub>, blood O<sub>2</sub> pressure; MAP, mean arterial pressure. \**p* < 0.05 versus normothermic ischemia.

and probed for  $\beta$ -actin (1:5000; anti- $\beta$ -actin; A5441; Sigma). Densitometric measurements were made from the film using a GS-700 imaging densitometer (Bio-Rad, Hercules, CA) and then quantified using Multi-Analyst (Bio-Rad). For quantification of relative protein expression, the optical density of the protein band of interest was normalized to the optical density of sham animal brain sample run in an adjacent lane on the same gel. Western blots were repeated two to three times using samples prepared from three different animals or cultures for each experimental condition studied.

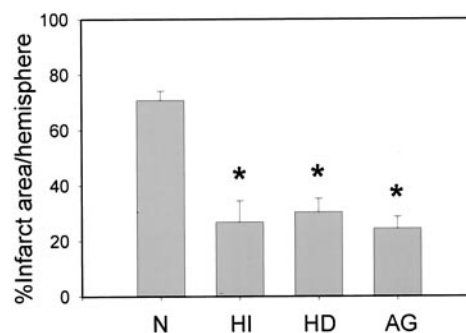
**NO generation measurement.** NO production was evaluated measuring the nitrite, the stable metabolite of NO, content of the tissue homogenates or culture media with the Griess reaction (Salter et al., 1996). Ischemic cortex from the ipsilateral hemispheres or cortex from LPS-treated brains was dissected. Culture media were collected from the microglial cultures. Fresh brain tissue was homogenized in PBS with protease inhibitors at 4°C and then centrifuged at 12,000 × *g* for 1 hr at 4°C. After centrifugation, supernatant was collected and kept at −80°C until use. Duplicates of 100  $\mu$ l of supernatant or culture media were added to 96-well microtiter plates and mixed with 100  $\mu$ l of modified Griess reagent (G4410; 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.

**Statistical analysis.** Data are given as means ± SEM. Comparisons between groups were performed using standard statistical methods using SigmaStat (SPSS, Chicago, IL). The data were analyzed by one-way ANOVA, Kruskal–Wallis one-way ANOVA on ranks, or unpaired *t* test. Statistical significance was determined at the *p* < 0.05 level.

## RESULTS

The physiologic variables of the rats studied are presented in Table 1. Differences in all parameters were not statistically significant between groups, except for temperature.

As we found previously (Maier et al., 1998, 2001; Yenari et al., 2000), mild hypothermia, maintained for 2 hr during the ischemic insult, was significantly neuroprotective. Cooling was still effective when onset was delayed up to 2 hr after ischemia onset. Infarct sizes 72 hr after ischemia onset were 70.8 ± 3.3% of ischemic hemisphere after normothermic ischemia, 26.8 ± 7.7% after intraischemic hypothermia, and 30.4 ± 4.8% after postisch-



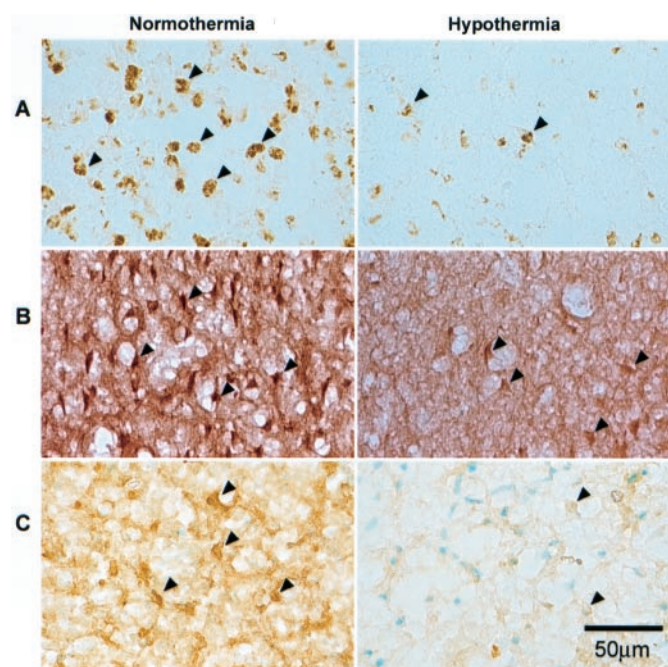
**Figure 1.** Mild hypothermia protects against experimental stroke. The extent of protection is similar to pharmacologic iNOS inhibition by aminoguanidine. Infarct size was measured on cresyl violet-stained coronal sections 72 hr after MCAO. The experimental groups are as follows: normothermia (N), 70.8 ± 3.3% of ipsilateral hemisphere, *n* = 6; intraischemic hypothermia (HI), 26.8 ± 7.7%, *n* = 6; delayed hypothermia (HD), 30.4 ± 4.8%, *n* = 6; aminoguanidine-treated (AG), 24.4 ± 4.2%, *n* = 4. \**p* < 0.05 versus normothermic ischemia. Kruskal–Wallis one-way ANOVA on ranks, followed by a multiple comparisons procedure (Dunn's test).

emic hypothermia (Fig. 1) (*p* < 0.05). To evaluate the damaging role of iNOS in our experimental model, we treated a separate group of ischemic animals with the relatively selective iNOS inhibitor aminoguanidine. Infarct size was similarly decreased by aminoguanidine treatment (Fig. 1).

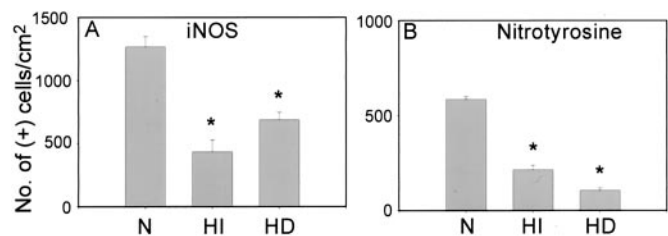
Cells of monocytic lineage (identified by ED1 immunoreactivity) appeared 24 hr after ischemia and increased in number at 72 hr, especially within the peri-infarct area. No ED1-positive cells were observed in sham-operated animals. Mild hypothermia decreased the number of ED1-positive cells (Fig. 2A), whether hypothermia was applied during or 2 hr after ischemia onset. iNOS-positive cells appeared 24 hr after ischemia in the ischemic hemisphere and increased in number by 72 hr. No iNOS-positive cells were observed in sham-operated animals or contralateral hemispheres of ischemic brains. Like ED1-labeled cells, iNOS-positive cells were also observed mainly in the peri-infarct regions. Mild hypothermia decreased the number of iNOS-positive cells (Figs. 2B, 3A). The numbers of ED1-positive cells and iNOS-positive cells in the adjacent sections were counted. Hypothermia decreased the number of ED1-positive cells to 44.8 ± 6.0% (intraischemic hypothermia) and 47.8 ± 4.7% (delayed hypothermia) of normothermia. The ratios of iNOS/ED1-positive cells were 0.79 ± 0.06 (normothermic ischemia), 0.83 ± 0.05 (intraischemic hypothermia), and 0.71 ± 0.11 (delayed hypothermia). Although there were no significant differences between the groups, the intensity of the iNOS stain appeared decreased in hypothermic sections compared with normothermic sections (Fig. 2B). We could not observe any difference in the intensity in aminoguanidine-treated sections.

Quantitative measurement of iNOS protein in whole brain lysates was performed using Western blot analysis. We detected iNOS protein bands in samples prepared from peri-infarct areas of the ischemic brains at 72 hr after MCAO. iNOS was not detectable in sham-operated animal brains. Both intraischemic and delayed hypothermia decreased iNOS levels in ischemic brain compared with normothermic ischemic brain (Fig. 4, MCAO).

In the ischemic hemisphere, nitrotyrosine-positive cells were detected 72 hr after insult. Rare cells stained with anti-nitrotyrosine antibody were detected in sham-operated animals. Within ischemic brain, nitrotyrosine was seen within cells, as well



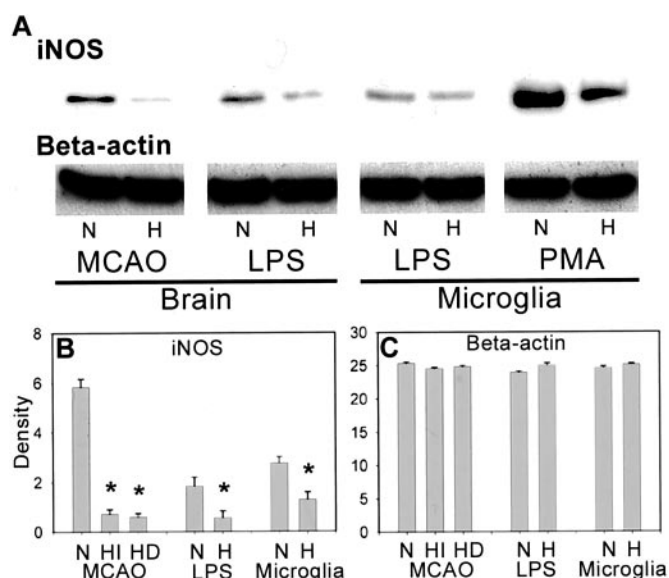
**Figure 2.** Mild hypothermia attenuates microglia–monocytes, iNOS, and peroxynitrite after MCAO. Immunohistochemical stains of ED1 (to identify activated microglia–monocytes), iNOS, and nitrotyrosine (to identify peroxynitrite) 72 hr after MCAO. Within the infarct, ED1 immunoreactivity is seen mainly in the cortex (A). The majority iNOS-positive cells reside in peri-infarct area of the ischemic hemisphere (B). The presence of nitrotyrosine immunoreactivity, a nitration product of tyrosine in proteins by peroxynitrite, suggests active NO production in the ischemic area (C). The densities of ED1-, iNOS-, and nitrotyrosine-positive cells are decreased by mild hypothermia (A–C, *Hypothermia*, respectively). Arrowheads show immunoreactive cells. Images are taken from the normothermic and delayed hypothermic brain sections. Scale bar, 50 μm.



**Figure 3.** Mild hypothermia decreases iNOS and nitrotyrosine immunoreactivity in ischemic brain. Mild hypothermia reduced the number of iNOS-positive cells within ischemic brain 72 hr after ischemia. A, The experimental groups are as follows: normothermic ischemia (N),  $1265 \pm 83$  cells/cm<sup>2</sup>,  $n = 6$ ; intraischemic hypothermia (HI),  $435 \pm 93$  cells/cm<sup>2</sup>,  $n = 6$ ; delayed hypothermia (HD),  $688 \pm 59$  cells/cm<sup>2</sup>,  $n = 6$ . Mild hypothermia reduced densities of peroxynitrite-positive cells 72 hr after ischemia. B, The experimental groups are as follows: normothermic ischemia (N),  $585 \pm 14$  cells/cm<sup>2</sup>,  $n = 6$ ; intraischemic hypothermia (HI),  $214 \pm 23$  cells/cm<sup>2</sup>,  $n = 6$ ; delayed hypothermia (HD),  $106 \pm 12$  cells/cm<sup>2</sup>,  $n = 6$ . \* $p < 0.05$  versus normothermia. ANOVA, followed by Tukey's test.

as extracellularly, consistent with previous reports (Gursoy-Ozdemir et al., 2000). Intraischemic and delayed mild hypothermia decreased densities of nitrotyrosine-positive cells compared with normothermia (Figs. 2C, 3B).

To determine whether hypothermia attenuates NO production in ischemia, we measured nitrite content in the ischemic brain using the Griess reaction. Like iNOS, NO production was significantly increased at 72 hr after MCAO. Compared with normo-

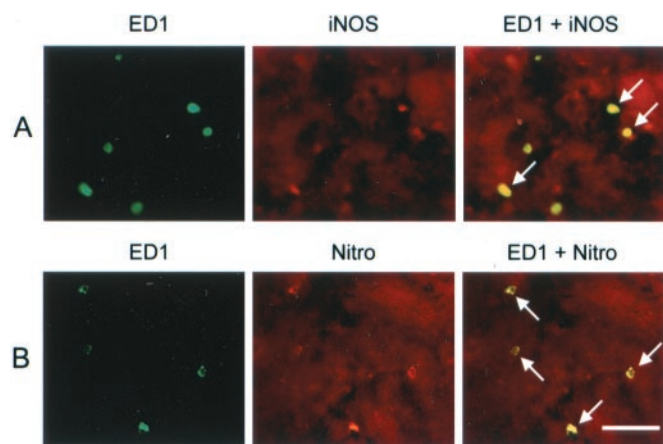


**Figure 4.** Mild hypothermia reduces iNOS in the brain and within microglia. Protein samples taken from ischemic brains 72 hr after MCAO or LPS treatment and cultured microglia 72 hr after LPS or PMA treatment were subjected to SDS-PAGE and probed for iNOS. iNOS was detected in ischemic brain (MCAO), LPS-treated brain (LPS), cultured microglia treated with LPS (Microglia), or PMA (PMA) under normothermic conditions (N) but was not detectable in sham animals or inactive cultured microglia. Intraischemic (HI) and delayed hypothermia (HD) in MCAO brains, hypothermia (H) in LPS brains, and cultured microglia show decreased iNOS expression. Representative Western blots of iNOS and  $\beta$ -actin (A). Optical densities of iNOS and  $\beta$ -actin bands in B and C, respectively. \* $p < 0.05$  versus normothermia. ANOVA, followed by Tukey's test or unpaired  $t$  test.

thermia, both intraischemic and delayed hypothermia attenuated NO production at 72 hr (see Fig. 6, MCAO). NO production was decreased by aminoguanidine treatment to a similar extent as hypothermia (see Fig. 6, MCAO).

To identify the cells that expressed iNOS and nitrotyrosine, double immunofluorescent labeling was performed. Cell type markers to identify neurons (MAP-2), astrocytes (GFAP), and activated microglia–macrophages (ED1) were used. MAP-2 and GFAP failed to colocalize with iNOS, but the majority of ED1-positive cells were iNOS positive (Fig. 5A). To evaluate the effect of hypothermia on the proportion of ED1-positive, iNOS-expressing cells, the numbers of iNOS/ED1-colabeled cells and ED1-positive cells in the same sections were counted. The proportion of colabeled iNOS/ED1-positive cells among all ED1-positive cells was  $0.73 \pm 0.04$  (normothermic ischemia),  $0.74 \pm 0.09$  (intraischemic hypothermia),  $0.63 \pm 0.11$  (delayed hypothermia), and  $0.81 \pm 0.05$  (aminoguanidine-treated group). There were no significant differences between the groups.

Nitrotyrosine-labeled cells were also colocalized using the same cell type markers. Similarly, the majority of the ED1-positive cells were nitrotyrosine positive, although extracellular staining was also observed, and is consistent with the diffusible nature of NO or peroxynitrite (Fig. 5B). Mild hypothermia decreased the overall numbers of colabeled cells, but the proportion of colabeled cells was unchanged from normothermia. We used DAPI staining to identify the nuclei of the stained cells. Several cells that were not labeled with DAPI or appeared pyknotic were also nitrotyrosine positive. These cells were also MAP-2 positive. Most of the cells with MAP-2 and nitrotyrosine label appeared



**Figure 5.** Colocalization of iNOS- and nitrotyrosine (Nitro)-producing cells in brains 72 hr after MCAO. Double immunofluorescent staining was performed for a microglial–monocyte marker (ED1, green), iNOS (A, red), and nitrotyrosine (B, red). The majority of ED1-positive cells colocalized with iNOS (A, ED1 + iNOS, arrows). Similarly, the majority of ED1-positive cells also colocalized with nitrotyrosine (B, ED1 + Nitro, arrows). Scale bar, 40  $\mu$ m.

dysmorphic with pyknotic DAPI-stained nuclei, suggesting that these cells were damaged.

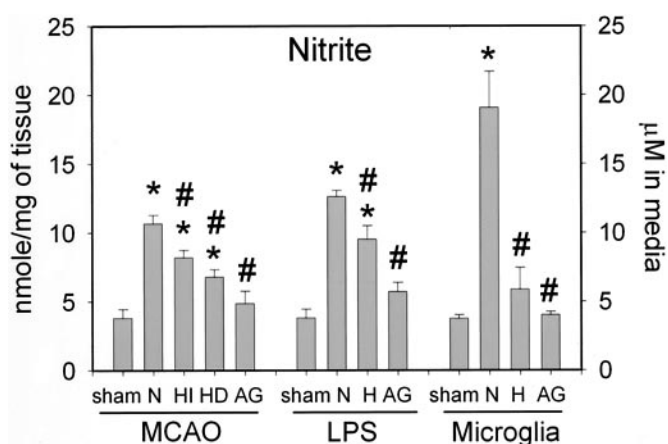
To further confirm the significance of hypothermia on the inflammatory response, we also studied a separate set of animals using a model of pure brain inflammation to determine whether any of the observed changes were not influenced by the smaller infarcts seen with mild hypothermia. To test the hypothesis that brain temperature independently altered the inflammatory response, we administered LPS to rats. LPS elicits an inflammatory response, characterized by infiltration of leukocytes, activation of microglia, expression of adhesion molecules, and iNOS. Notable in this model was that these histological changes are not associated with neuronal death up to 72 hr after LPS injection. NO production significantly increased from basal to peak levels 72 hr after LPS treatment in both whole brain and cultured microglia. Compared with normothermia, hypothermia decreased iNOS and NO production in LPS-treated brains (Figs. 4, 6, LPS).

Because we noted reduced iNOS staining in the cells of monocyte lineage (Fig. 2B) but no alteration in the proportion of iNOS-positive cells, we assessed the contribution of temperature to microglial activation by studying pure culture. In these experiments, hypothermia reduced iNOS generation (Fig. 4, Microglia) and subsequent NO production (Fig. 6, Microglia) after stimulation by LPS. Cultured microglia were also activated using another stimulator, PMA. PMA-stimulated microglia markedly induced iNOS expression. iNOS induction was suppressed by hypothermia (Fig. 4, Microglia).

NO production was completely blocked by aminoguanidine treatment, suggesting that NO production is mainly attributable to LPS-induced iNOS expression (Fig. 6, Microglia).

## DISCUSSION

In this study, we show that mild hypothermia inhibits microglia–monocytes activation and infiltration. Furthermore, these inflammatory cells appear to be the primary source of iNOS, NO, and peroxynitrite, which are inhibited by mild hypothermia. Such effects were observed when cooling was applied during or 2 hr



**Figure 6.** Mild hypothermia decreases NO generation in brain and microglia. Seventy-two hours after exposure, NO production was measured by determining nitrite content in ischemic brain (MCAO) and after systemic LPS administration (LPS) and in cultured microglia exposed to LPS (Microglia). In ischemic brains, intras ischemic (HI;  $n = 4$ ) and delayed (HD;  $n = 4$ ) hypothermia decreased brain nitrite levels compared with normothermia (N;  $n = 4$ ). Aminoguanidine (AG;  $n = 4$ ) also decreased nitrite levels. In a model of pure brain inflammation, LPS-induced NO generation in the brain was decreased by mild hypothermia (H;  $n = 5$ ) and aminoguanidine treatment (AG;  $n = 6$ ) compared with the normothermic group (N;  $n = 5$ ). Cultured microglia exposed to 10  $\mu$ g/ml LPS generated NO at 37°C (N;  $n = 6$ ), but NO generation was significantly decreased at 33°C (H;  $n = 4$ ). Aminoguanidine (AG;  $n = 4$ ) also decreased NO production in cultured microglia. \* $p < 0.05$  versus sham; # $p < 0.05$  versus normothermia. ANOVA, followed by Tukey's test.

after ischemia. Thus, inhibition of iNOS induction by microglia–macrophages presumably contributed to the robust protective effect of mild hypothermia against stroke injury. We further showed in *in vivo* and *in vitro* models of LPS-induced inflammation that mild hypothermia inhibited the inflammatory response in a similar manner. Ischemic damage and increased NO production was inhibited by iNOS inhibitor. All of these results suggest that mild hypothermia directly inhibits the inflammatory response and iNOS induction.

After ischemia, endothelial cells upregulate adhesion molecules (Okada et al., 1994; Zhang et al., 1995), allowing entry of peripheral leukocytes, which then release reactive oxygen species (Traystman et al., 1991), cyclooxygenase products, NO, and cytokines (del Zoppo et al., 2000), which contribute to secondary injury. Several studies have shown that inhibition of leukocyte infiltration by blocking various adhesion molecules reduced the infarction (Bowes et al., 1995; Goussev et al., 1998; Yenari et al., 1998). Furthermore, mild hypothermia has been shown to decrease adhesion molecule expression and inflammatory cell infiltration (Maier et al., 1998; Inamasu et al., 2000, 2001; Kawai et al., 2000).

Microglial activation has been observed as early as 6 hr after insult (Lyons et al., 2000), and macrophages–microglia increased in number for several days before reaching a plateau (Garcia et al., 1994; Schroeter et al., 1994; Stoll et al., 1998; Barone and Feuerstein, 1999). Inhibition of microglial activation can protect against stroke (Yrjanheikki et al., 1999). After ischemia, microglial activation results in a series of functional and morphological modifications that involve proliferation (Kato and Wood, 1998). Although microglia play an important role in ischemia, there are few studies that investigate the effect of hypothermia on microglial action, especially in the transient MCAO model. The hypo-

thermic inhibition of microglial activation in global ischemia was shown previously (Kumar and Evans, 1997; Abraham and Lazar, 2000). Inamasu et al. (2000) suggested the possibility that post-ischemic hypothermia might delay microglial activation.

This study demonstrates the importance of the inflammation in hypothermic neuroprotection. Not only did mild hypothermia decrease densities of microglia–monocytes, but these cells also generated less damaging substances. This was the case even when cooling was delayed by 2 hr, a time when energy stores are already depleted (Hoehn-Berlage et al., 1995) and glutamate is already released (Graham et al., 1990; Huang et al., 1998). Therefore, the protective effect of mild hypothermia may be attributable to other downstream factors, including inflammation. Furthermore, we show that mild hypothermia also decreased microglial–monocyte generation of iNOS, NO, and peroxynitrite after LPS treatment. Therefore, the neuroprotective effect of mild hypothermia may be primarily attributable to suppression of inflammatory cell activation and infiltration. Decreased ischemic damage and NO production by aminoguanidine suggest that NO plays important roles in inflammation after ischemia.

There has been interest in the exact mechanism and regulation of NO and NOS in ischemic damage (Zhang and Iadecola, 1998; De Alba et al., 1999; Forster et al., 1999; Loihl et al., 1999; Fassbender et al., 2000; Hirabayashi et al., 2000). Recently, it is known that ischemia causes a surge in nNOS activity, followed later by increases in iNOS in a range of cells, including infiltrating neutrophils and macrophages, activated microglia, and astrocytes (Love, 1999). The effects of ischemia on the activity of nNOS are thought to be secondary to the activation of NMDA receptors. However, iNOS upregulation and activity is mediated by transcriptional inducers (Love, 1999). In contrast to nNOS, which generates NO early after ischemia onset (Iadecola, 1997; Eliasson et al., 1999), iNOS appears somewhat later in inflammatory cells and contributes to the evolution of the brain injury (Love, 1999). In fact, NO produced by iNOS is a major mechanism of cytotoxicity in models of inflammation (MacMicking et al., 1997). Others have shown that iNOS null mice had smaller infarcts and better neurological outcome than wild-type littermates (Iadecola et al., 1997; Zhao et al., 2000). Treatment with antisense oligodeoxynucleotide to iNOS protected against ischemia-induced brain injury (Parmentier-Batteur et al., 2001). Administration of iNOS inhibitors reduced infarct volume (Iadecola et al., 1995b; Nagayama et al., 1998; Zhang and Iadecola, 1998).

Although the protective effects of mild hypothermia against ischemic brain injury have been studied in the past, there have been very few reports on the interaction between mild hypothermia and induction of NOS in brain injury. Mild hypothermia inhibited total NO synthesis in cerebral ischemia models (Kader et al., 1994; Kumura et al., 1996; Fabian Loidl et al., 1997). However, all of these studies focused on the nNOS activity, and there have been no reports to our knowledge on the effect of mild hypothermia on iNOS expression by microglia in focal cerebral ischemia. One study showed that mild hypothermia attenuated astroglial iNOS activity in global ischemia (Nomura, 1998). Chatzipanteli et al. (1999) reported that hypothermia decreased early constitutive NOS activation and prevented the delayed induction of iNOS in the traumatic brain injury model. One study using microglial cultures suggested that hypothermia inhibited proliferation, superoxide, and nitric oxide production (Si et al., 1997). We show here that mild hypothermia decreases microglial expression of iNOS and NO–peroxynitrite production by cultured microglia. Our results are consistent with those of Si and col-

leagues in that hypothermia inhibited microglial generation of reactive nitrogen species.

From the brain tissue sections, we found that the majority of microglia–monocytes and iNOS expression occurred in the peri-infarct area. Hypothermic protection was observed mainly in the cortical regions but not in the subcortical regions, as we described previously (Maier et al., 1998, 2001). This suggests that inhibition of microglial iNOS expression may be an important mechanism of hypothermic protection. To our knowledge, this is the first report to directly show that mild hypothermia inhibits expression of iNOS and reactive nitrogen species by microglia in cerebral ischemia.

Hypothermia inhibited microglial expression of iNOS and reactive nitrogen species at 3 d after ischemia, although cooling occurred at earlier time points. It is possible that hypothermia interferes with iNOS regulation during or shortly after ischemia. Nuclear factor  $\kappa$ B is known to regulate the expression of iNOS and other inflammatory mediators; therefore, mild hypothermia may be exerting its anti-inflammatory effects by interfering with this mechanism. This deserves additional investigation.

Whether mild hypothermia inhibited microglia or peripheral blood monocytes cannot be inferred from the results presented here. To our knowledge, there are no specific antibodies or other markers to reliably differentiate between activated microglia and peripheral monocytes–macrophages. Given that the functions of both cell populations are very similar, we do not believe that this will affect our interpretation of the results.

The protection by hypothermia, especially delayed hypothermia, is especially important because microglial activation is a delayed and long-lasting phenomenon after ischemia, which may be an attractive therapeutic target for human stroke. We show that intact microglia–monocytes generate iNOS and NO–peroxynitrite 3 d after ischemia and are inhibited by both intraischemic and postischemic hypothermia. An active response by microglia is believed to contribute to cerebral damage (Gonzalez-Scarano and Baltuch, 1999); therefore, it is possible that the potential neuroprotective mechanisms of hypothermia are mediated in part through the suppression of microglia–monocyte activation. A minority of other iNOS-positive cells were not labeled with ED1, GFAP, or MAP-2 and could represent other leukocyte populations. Neutrophil infiltration is also present in the ischemic brain at 3 d (Iadecola et al., 1995a; Maier et al., 1998). Therefore, these other iNOS-producing cells could be neutrophils. However, the predominant inflammatory cell population at 3 d after ischemia was microglia–monocytes rather than neutrophils. NO produced by the other inflammatory cells or neurons themselves (via nNOS) at earlier time points probably caused the tissue damage, leaving nitrotyrosine remnants in injured cells. However, at 3 d after ischemia, microglia plays major role in NO production.

Hypothermia may provide an approach to potentially reduce ongoing damage during reperfusion in stroke patients. Our studies showed that hypothermia that was initiated after 2 hr of ischemia and persisted during reperfusion significantly reduced the cortical infarct volume. These findings led us and others (Garcia et al., 1993; Iadecola et al., 1995a; Du et al., 1996) to the notion that focal ischemic injury is an ongoing process that persists into the post-ischemic period, and postischemic hypothermia can suppress the deleterious processes such as iNOS induction and reactive nitrogen species generation, even days after treatment.

In summary, we show that (1) mild hypothermia protects against experimental stroke when applied during ischemia and

after 2 hr of delay, (2) mild hypothermia attenuates iNOS expression and NO–peroxynitrite production in experimental stroke, (3) mild hypothermia also attenuates NO production in a model of pure brain inflammation and in cultured microglia, and (4) microglia may be an important source of reactive nitrogen species production, and mild hypothermia appears to inhibit this. Hypothermic suppression of iNOS expression by activated microglia is a novel finding and provides insight into the mechanisms of such neuroprotection.

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