Neurobiology of Disease 33 (2009) 301–312

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Inflammation and NFκB activation is decreased by hypothermia following global cerebral ischemia

Carla M. Webster [a,b,1], Stephen Kelly [c,1], Maya A. Koike [a,b], Valerie Y. Chock [b], Rona G. Giffard [b], Midori A. Yenari [a,*]

[a] Department of Neurology, University of California, San Francisco and the San Francisco Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121, USA
[b] Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305, USA
[c] Clinical Sciences at South Bristol, University of Bristol, Bristol, BS1 3NY, UK

ARTICLE INFO

Article history:
Received 21 July 2008
Revised 3 October 2008
Accepted 3 November 2008
Available online 19 November 2008

Keywords:
Global cerebral ischemia
Hypothermia
Inflammation
Nuclear factor-kappa B
Microglia

ABSTRACT

We previously showed that hypothermia attenuates inflammation in focal cerebral ischemia (FCI) by suppressing activating kinases of nuclear factor-kappa B (NFκB). Here we characterize the inflammatory response in global cerebral ischemia (GCI), and the influence of mild hypothermia. Rodents were subjected to GCI by bilateral carotid artery occlusion. The inflammatory response was accompanied by microglial activation, but not neutrophil infiltration, or blood brain barrier disruption. Mild hypothermia reduced CA1 damage, decreased microglial activation and decreased nuclear NFκB translocation and activation. Similar anti-inflammatory effects of hypothermia were observed in a model of pure brain inflammation that does not cause brain cell death. Primary microglial cultures subjected to oxygen glucose deprivation (OGD) or stimulated with LPS under hypothermic conditions also experienced less activation and less NFκB translocation. However, NFκB regulatory proteins were not affected by hypothermia. The inflammatory response following GCI and hypothermia's anti-inflammatory mechanism is different from that observed in FCI.

Published by Elsevier Inc.

Introduction

The neuroprotective effects of mild hypothermia have been well documented in experimental models (see reviews Krieger and Yenari, 2004; Liu and Yenari, 2007; Lyden et al., 2006). Furthermore, there is now clinical evidence showing that mild hypothermia significantly protects against neurological damage following cardiac arrest (Bernard et al., 2002; Hypothermia after Cardiac Arrest Study Group, 2002). The precise mechanism(s) by which mild hypothermia protects brain cells remains to be elucidated, but it is likely that hypothermia acts upon multiple pathways to ultimately prevent cell death (Liu and Yenari, 2007; Lyden et al., 2006). Hypothermia has been reported to attenuate cytochrome *c* release, apoptosis inducing factor induction, excitatory amino acid accumulation, free radical generation and increased BBB permeability (Zhao et al., 2007). There is a growing literature on the damaging nature of the inflammatory response to brain ischemia (Tang and Yenari, 2006; Wang et al., 2007).

Earlier findings in studies of various models of brain ischemia, especially focal cerebral ischemia (FCI, a model of stroke) have focused on the notion that hypothermia mitigates damage following ischemia by decreasing metabolic rate (Erecinska et al., 2003; Lanier, 1995; Yenari et al., 2004) and improving ion homeostasis (Sick et al., 1999). However, hypothermia appears to mitigate a variety of toxic factors such as decreasing reactive oxygen species (ROS) (Maier et al., 2002), suppressing the infiltration of neutrophils, and decreasing activation of microglia (Ishikawa et al., 1999; Wang et al., 2002). However, the role in inflammation and especially the effect of hypothermia on inflammation has been studied to a lesser extent in global cerebral ischemia (GCI), an experimental correlate of cardiac arrest.

Inflammation is an orchestrated response involving the rapid upregulation and activation of a variety of genes. Nuclear factor-kappa B (NFκB) is a major transcription factor involved in this response. NFκB is normally sequestered in the cytoplasm where it is bound to a family of inhibitory proteins known as the inhibitor of NFκB (IκB). A major IκB and the one most often studied is IκB-α. Inflammatory stimuli activate a family of upstream kinases (IκB kinase, IKK) which phosphorylates IκB leading to its degradation and the liberation of NFκB to enter the nucleus and induce gene expression (Rothwarf and Karin, 1999).

Previously, we showed that NFκB activation is reduced by mild hypothermia following focal cerebral ischemia (Han et al., 2003), and this reduction may explain some of the anti-inflammatory effects of hypothermia. In the FCI model, we found that hypothermia inhibited NFκB's activating kinases, IKKγ and IKKβ. Here, we characterize the inflammatory response in the brain following GCI, and how it is mitigated by hypothermia.

* Corresponding author. Fax: +1 415 750 2273.
E-mail address: yenari@alum.mit.edu (M.A. Yenari).
[1] Contributed equally to the work presented.
Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.nbd.2008.11.001

Materials and methods

In vivo models of brain ischemia and inflammation

All experimental protocols carried out on animals were approved by the Stanford University Administrative Panel on Laboratory Animal Care and San Francisco VA Medical Center's Animal Care Facility and were in accordance with NIH guidelines.

Rat global cerebral ischemia model

GCI was induced in male Sprague Dawley rats ($n=25$, weight=350 g–450 g; Simonsen Laboratories, Inc., Gilroy, CA, USA) as previously described (Kelly et al. 2002; Zhang et al. 2001). Briefly, rats were anesthetized with isoflurane (5% for induction, 2–3% for maintenance) mixed with medical air and oxygen (700:300 ml/min) via face mask. Depth of anesthesia measured by hindlimb pinch every 15 min and rectal temperature was monitored. The left femoral artery and vein were cannulated for measuring mean arterial blood pressure (MABP) and arterial blood gases. The common carotid arteries were exposed, heparin was infused via the femoral venous catheter (500 IU/kg in normal saline 100 IU/ml), hypotension was induced by withdrawal of venous blood until the MABP was 30 mm Hg. The common carotid arteries were occluded for 8 min with aneurysm clips and MABP maintained at 30 mm Hg. After 8 min, the clips were removed, blood was returned to the animal, and all wound sites were closed. Sham operated animals were exposed to anesthesia and surgery as in experimental groups; however, the carotid arteries remained patent and MABP was not manipulated. We previously established the relationship between core body temperature and brain temp in rats where rectal temperature of 30 °C was found to correspond to brain temperature of 33 °C (Yenari et al. 2000). Hypothermia (rectal temperature=30 °C with a predicted brain temperature of 33 °C) was induced at the onset of ischemia and maintained for 3 h or maintained at normothermia. Posthypothermic rewarming was accomplished by removing the animals from hypothermic conditions, and placing them on a heating blanket. The process of rewarming to a rectal temperature of 37 °C took about 20 min. Following surgery, animals were monitored while being allowed to recover from 24 h to 3 d, then euthanized via halothane overdose and transcardially perfused with 3% paraformaldehyde. Brains were harvested, blocked, embedded in paraffin and stored until use.

To estimate integrity of the blood brain barrier (BBB), some animals ($n=3$/group) were given 1 ml 4% Evan's blue dye I.V., 3 h before sacrifice (Deng et al., 2003; Yenari et al., 2006). Brains were removed 1 and 3 d postischemia and examined for contrast extravasation. An animal that received a focal ischemic insult was used as a positive control.

Mouse global cerebral ischemia model

Male C57BL6 mice (Simonsen Labs, Gilroy, CA), 8–12 weeks old, were anesthetized with isoflurane by face mask and subjected to ischemia using previously published methods (Kelly et al., 2001; Murakami et al., 1998). Monitoring of anesthesia was performed as described above. Common carotid arteries were exposed and aneurysm clips placed to occlude the vessels. After 12 min, the clips were removed, and the wound sites were sutured. Mice were rendered hypothermic (30 °C rectal temperature corresponding to 33 °C in the brain) at the start of reperfusion and maintained for 3 h, or kept at 37 °C (normothermic). Hypothermic mice were rewarmed in a similar fashion as the rats. Mice were euthanized 1 and 3 d later. Since MABP is not reduced in the murine GCI model due to technical considerations, ischemic injury to the hippocampus relies on the absence of posterior communicating arteries (PComms) (Murakami et al., 1998). C57/BL6 mice generally have atretic PComms, and are thus ideal for the study of GCI. Absence of PComms were confirmed by perfusing with 10% carbon black. Hemispheres with patent PComms were excluded from study. After harvest, brains were sunk in 20% sucrose, and frozen at −80 °C in Tissue Tek O.C.T embedding compound (Cat. # 4583, Sakura Finetek, U.S.A, Torrance, CA) for cryosectioning, or immediately frozen at −80 °C for use in Western blots or ELISA.

Brain inflammation model

A model of pure brain inflammation without brain cell injury was studied as a positive control (Deng et al., 2003). Mice were given 5 mg/kg lipopolysaccharide (LPS, *E. Coli* endotoxin, *E. Coli* serotype 055:B5; Sigma, St. Louis, MO) IP to cause transient aseptic meningitis. We elected to use LPS as a model of brain inflammation because it produces a robust inflammatory response without causing cell death. This model has previously been published by us, and leads to peak inflammatory responses 8–24 h post LPS, which resolves without histologic and biochemical evidence of brain injury as far out as 72 h (Deng et al., 2003; Han et al., 2002). Eight to 12 week old male C57BL6 mice were anesthetized with isoflurane by face mask, then given LPS and maintained at either 37 °C or cooled to 33 °C for 3 h immediately after injection. Brains were harvested 24 h later.

In vitro models of ischemia and inflammation

Primary microglial cell cultures were prepared as previously described (Yenari and Giffard, 2001). Whole brains from postnatal day 1–3 Swiss Webster mice were dissected, meninges discarded, and treated with 0.25% trypsin. Tissue was gently triturated, and cell mixtures plated in T75 flasks in Eagle's Minimal Essential Medium supplemented with 10% equine serum, 10% fetal bovine serum, epidermal growth factor (10 μg/ml) (Sigma E4127), glutamine (2 mM), glucose (21 mM), bicarbonate (26 mM)(plating media) with penicillin (100 U/ml) and streptomycin (100 μg/ml) added. Cultures were maintained at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. Two weeks later, microglia were subcultured from mixed glial cultures by shaking at 160 rpm for 30 min at 37 °C, and plated at a density of 2×10^5 cells/ml. Histochemical staining with *Griffonia simplicifolia* B4-isolectin (IB4, Sigma) confirmed the purity of microglia cultures to be approximately 98%. Subcultures were allowed to stabilize for 24 h prior to experimentation.

In vitro injury paradigms

Microglia were first subjected to glucose deprivation (GD). Cultures were deprived of glucose by washing in glucose free balanced salt solution containing (in mM) NaCl 116, $CaCl_2$ 1.8, $MgSO_4$ 0.8, KCl 5.4, NaH_2PO_4 1, $NaHCO_3$ 14.7, N-(2-hydroxyethyl)piperazine-N′-ethane-sulfonic acid (HEPES) 10; and phenol red 10 mg/l at pH 7.4 (BSS_0). Control cultures were incubated in a similar balanced salt solution containing 5.5 mM glucose ($BSS_{5.5}$). Cultures were maintained in these solutions for 30 h based on prior work showing that GD led to >60% cell death by this time point (Yenari and Giffard, 2001). Cell death was assessed by trypan blue staining. Cell counts were performed from at least 5 high power fields by an investigator blinded to the experimental conditions.

To simulate ischemic conditions, microglial cultures were subjected to oxygen glucose deprivation (OGD) for 2 h (to simulate conditions that result in significant neuronal death, and activation but little death of microglia) or 24 h (to cause significant microglial cell death in order to assess cytoprotection) by placing cultures into an anaerobic chamber ($O_2<200$ ppm) in BSS_0. After this period of OGD, cultures were “reperfused” by adding glucose to a final concentration of 5.5 mM and returned to normoxia for 4 or 24 h. Control cultures were maintained in balanced salt solutions containing 5.5 mM glucose ($BSS_{5.5}$) at normoxia. These OGD conditions do not lead to significant amounts of microglial death, but do

reproducibly cause activation and generation of inflammatory mediators.

In vitro inflammation

To model activation, cultured microglia were activated with LPS (10 μg/ml, same type used in the *in vivo* model) then maintained at incubator temperatures of 37 °C (normothermia) or 33 °C (hypothermia) in a 5% CO_2 incubator for 24 h.

Assays

Enzyme linked immunosorbent assay (ELISA)

Inflammatory cytokine levels of TNF-α (OptEIA #2698KI, BD Biosciences, San Jose, CA) and IL-1β (Cat. # 559111, BD Biosciences) were measured in culture supernatants using ELISA per the manufacturer's protocol. NFκB's p65 activity was assessed in nuclear extracts using a DNA binding ELISA (Cat. # 40096, TransAM NFκB kit, Active Motif, Carlsbad, CA) following the kit instructions (Han et al., 2003).

Western blot

Brains were perfused with normal saline, then each hemisphere was isolated on dry ice and lysed with Laemmli's lysis buffer. After homogenizing, protein concentration was determined via a BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL); samples were stored at −80 °C until use. One hemisphere per mouse was used for Western blot, while the other hemisphere was used for immunohistochemistry or ELISA. 25 μg protein per well was separated on 10% SDS-polyacrylamide gel electrophoresis (BioRad, Hercules, CA) and transferred onto polyvinylidene difluoride membranes (IPVH0010, Millipore, Billerica, MA). Transfer was assessed with Ponceau S Solution (Sigma), then membranes were probed with antibodies against IKKγ (sc-8330, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), IκBα (sc-1643, Santa Cruz) and p-IκB-α which recognizes only the

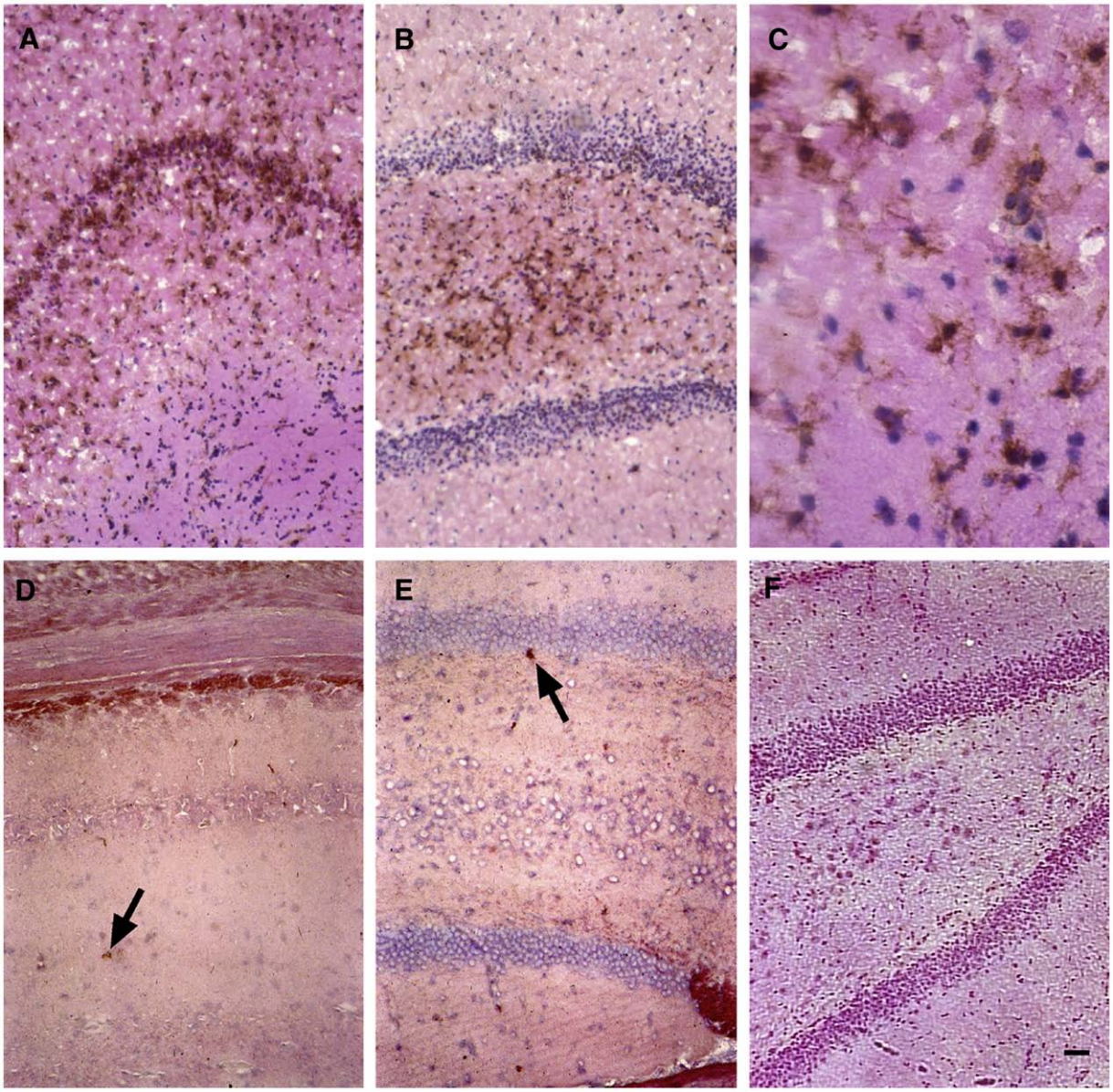

Fig. 1. Inflammatory reaction after global cerebral ischemia: increased OX42 staining in microglia is observed in the region between the blades of the dentate (A) and within hippocampal CA1 (B) 72 h post GCI in the rat, compared to a sham animal where little staining is seen (F). OX42 cells usually possessed processes (C), consistent with a microglial morphology. In contrast, rare cells near the dentate (D), and CA1 (E) stained for a neutrophil marker (myeloperoxidase, MPO), but the morphology of these cells was not consistent with them being neutrophils (arrows).

phosphorylated form of IκBα (sc-8404, Santa Cruz), all dilutions 1:200. Membranes were stripped then probed again for β-actin (clone AC-315, Sigma) as a loading control.

Immunohisto/immunocytochemistry

Paraffin embedded, fixed rat brains were cut into 9 μm coronal sections, deparaffinized, microwaved in 0.1 M citric acid, and treated for endogenous peroxidases with 0.03% hydrogen peroxide. Sections were then incubated for 3 h with 10 μg/ml isolectin B4 from *Griffonia simplicifolia* (IB4, L5391, Sigma) to identify microglia, and visualized with DAB. For other markers, after hydrogen peroxide treatment, sections were blocked with 5% normal serum, and reacted with the OX42 antibody (1:500, anti-CD11b, Serotec, Raleigh, NC) to identify monocytes/macrophages and activated microglia, anti-myeloperoxidase antibody (MPO, 1:500, A0398, Dako, Carpinteria, CA) to identify neutrophils, or antibodies against the p65 subunit of NFκB (1:200, sc-109, Santa Cruz) and IκB-α (1:200, sc-317, Santa Cruz) was applied for 1 h. After washing with PBS, sections were incubated with a biotinylated secondary antibody which had been preabsorbed with rat serum (Elite Vectastain ABC Kit, Vector Labs, Burlingame, CA) followed by a tertiary binding complex (ABC), and visualized with diaminobenzidine and H_2O_2 (Sigma Fast DAB, Sigma). Sections were counterstained with hematoxylin and eosin (H&E) or methyl green.

Primary microglia cultures were first washed with ice cold PBS, then fixed with ice cold 1:1 methanol to acetone for 15 min. The cultures were then washed again with ice cold PBS three times and blocked with 5% BSA (Sigma, Cat.# A788) in PBS plus 0.1%Tween20 (PBST) overnight, then incubated with anti-p65 antibody (rabbit polyclonal, 1:200 dilution, Santa Cruz) for 4 h, washed, then incubated in Alexa fluor 488 conjugated secondary antibody (1:500, cat. A21206 Invitrogen, Carlsbad, CA) for 2 h. Cells were then washed and mounted and counter stained with DAPI (Vector Labs). Cell were viewed with a Zeiss Axiovert fluorescent microscope with Axiovision v4.4 acquisition software (Carl Zeiss Microimaging, Inc., Thornwood, NY).

Glutamate levels

Glutamate levels generated by microglia were measured using high performance liquid chromatography (HPLC) (courtesy of Robert Newcomb, PhD; Newcomb et al., 1997). Separate cultures of microglia were prepared and stimulated as described above in serum and glutamine free media. Media was then collected and frozen at −80 °C prior to assaying. After precolumn derivatization with o-phthaldialdehyde, amino acids were separated on a 4.5×250-mm Phenomonex

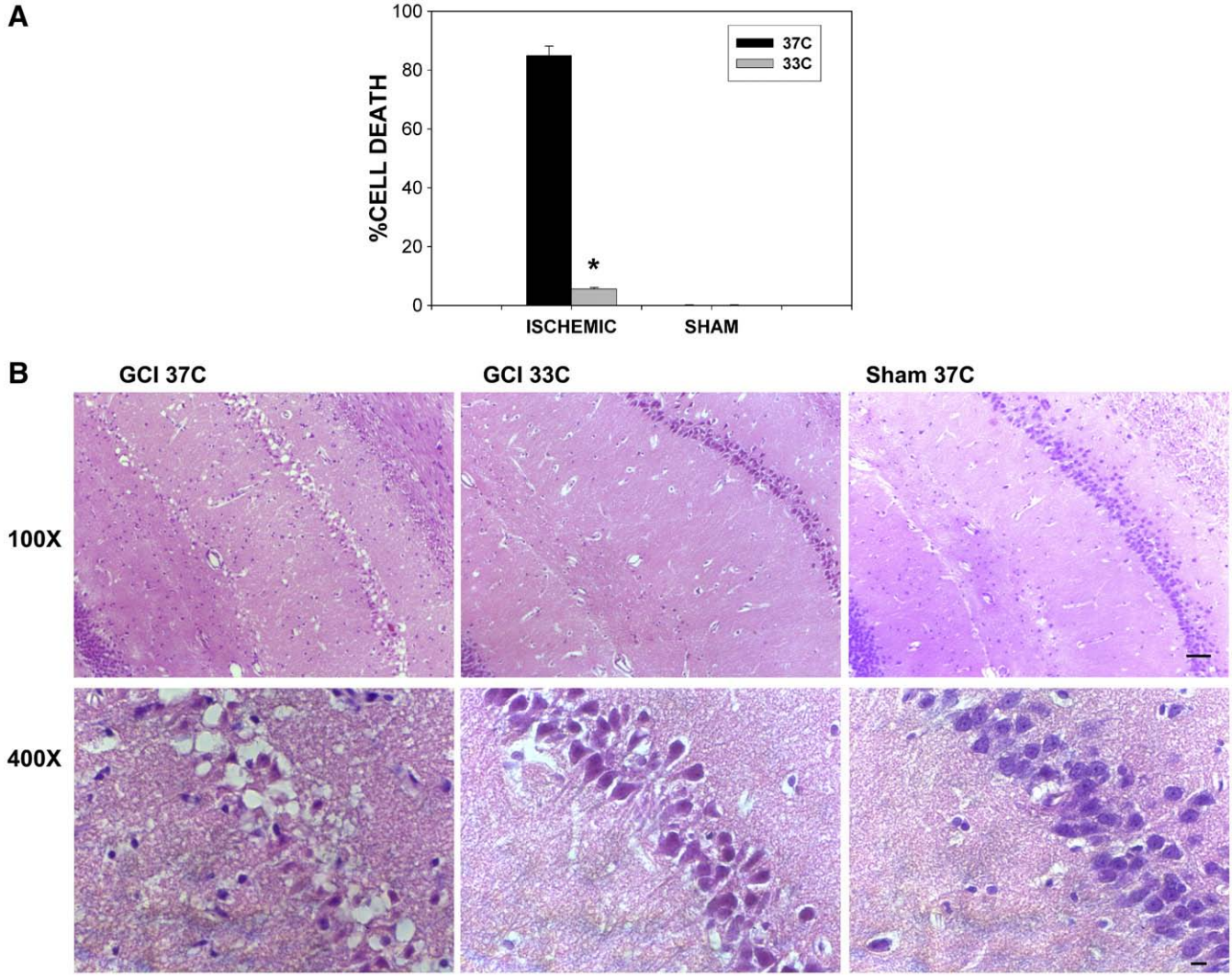

Fig. 2. Mild hypothermia reduced ischemic damage in the CA1 hippocampus region 72 h following GCI treatment. Hematoxylin and eosin staining revealed less cell death in the 33 °C and sham operated rats (n=4–6 per group) quantified as the percent of ischemic damage after 72 h (A) (*P<0.05). Images (B) are representative of H&E stained CA1 sections 72 h post GCI shown at both 100× and 400× magnification. Scale bars=100 μm 100×, 20 μm.

Primesphere "HC" octadecylsilica column (dp5 μm) using a gradient of 0–35% methanol in 15 mM sodium phosphate, pH 6.2 (0.13% tetrahydrofuran), over 65 min at 1 ml/min. (0.13% tetrahydrofuran), over 65 min at 1 ml/min. A Hewlett-Packard model 1046A fluorimetric detector (excitation 340 nm, emission 420 nm) was used.

Histological assessments

All histological assessments were made by researchers blinded to the conditions of the experiments. For assessment of cell counts from H&E stained sections, 10 consecutive high power fields were sampled from the dorsal hippocampal CA1 subfield. Counts of intact neurons were performed from the ischemic and sham brains. The extent of cell death was estimated by numbers of intact cells from the sham minus numbers from the ischemic brain and divided by counts from the shams. For NFκB stains, all immunoreactive cells in hippocampal CA1 on two coronal sections were counted, then numbers of cells with nuclear staining were counted separately. For IκB-α and ED1, a semi-quantitative score was assigned. Cells in the pyramidal layer of the dorsal hippocampus CA1 subfield (IκB-α) or cells within the blades of the dentate (ED1) on two coronal sections were assessed and assigned a score of 0–3 (0=no immunoreactive cells; 1=a few immunoreactive cells; 2=many immunoreactive cells; 3=almost all immunoreactive cells).

Statistical analysis

All statistical analyses were performed using Sigma Stat 2.03 (Systat Software, Inc., San Jose, CA). Quantitative data were presented as mean ± S.E. Two-group comparisons were performed by two-tailed Student's *t*-test. Multiple-group comparisons were performed using one-way ANOVA followed by Tukey HSD *post hoc* test. Non parametric tests were used for noncontinuous data. $P<0.05$ was considered statistically significant.

Results

The inflammatory response following GCI

GCI increased microglial reactivity for OX42 in the rat. 72 h post GCI, OX42 staining was increased in intensity with increased size and numbers of microglia. Microglia were especially noticeable between the blades of the dentate as well as around the CA1 region of the hippocampus (Figs. 1A–C). Myeloperoxidase staining (Figs. 1D, E) did not convincingly demonstrate the presence of neutrophils, although rare positive cells were observed. There was also no evidence of Evan's blue dye extravasation, indicating that BBB disruption did not occur, at least to the extent that serum molecules the size of albumin did not

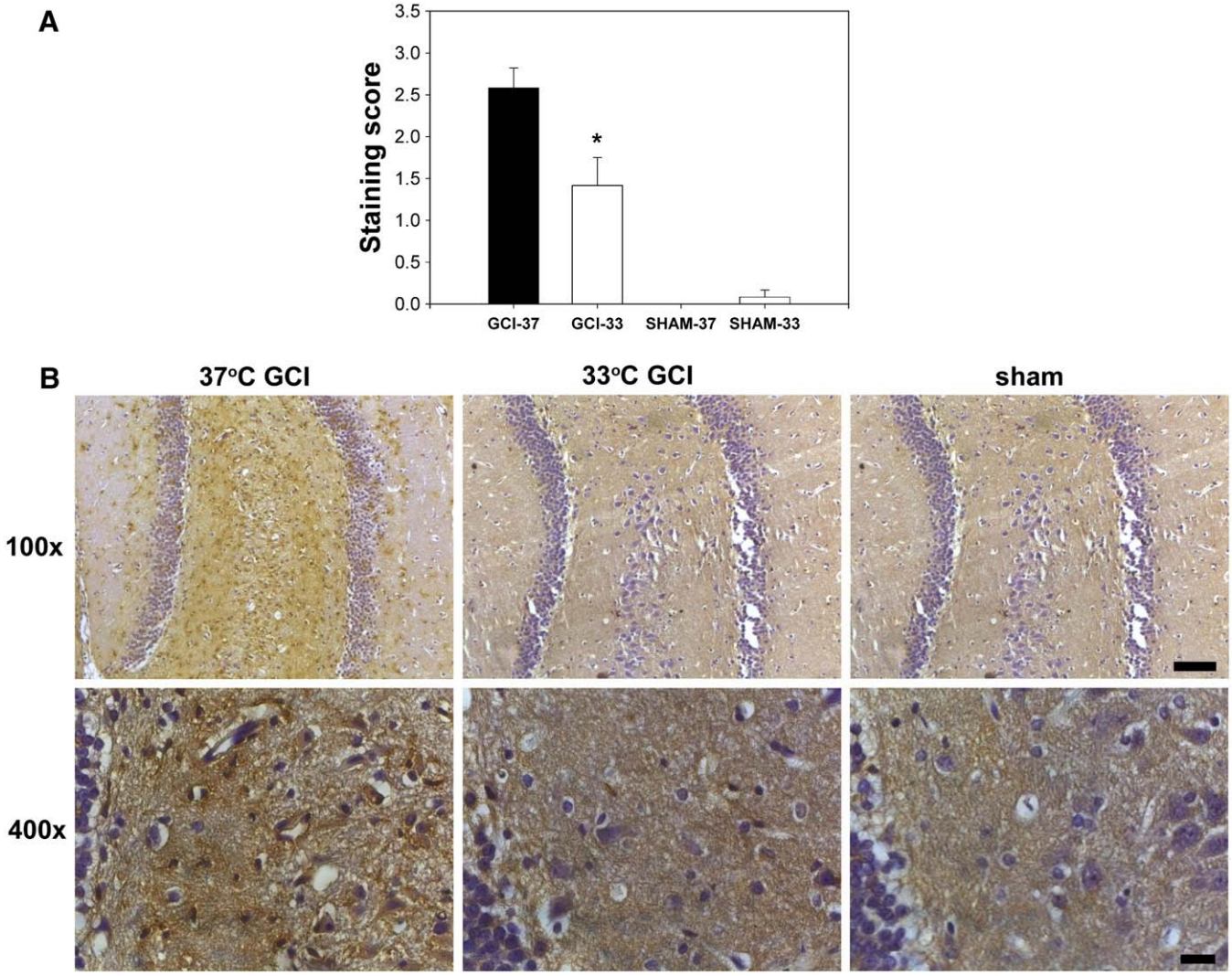

Fig. 3. Mild hypothermia decreases microglial staining following GCI. Histological scores (see text) from the isolectin B4 (IB4) stained sections were assessed for each section and quantified at 72 h. Staining scores were significantly lower in hypothermic ischemic rats (GCI-33) than normothermic (GCI-37, *$P<0.05$ vs. GCI37, $N=6$/group). IB4 staining was nearly undetectable among sham animals, and scores were no different between hypothermic (SHAM-33) and normothermic (SHAM-37) groups. Representative histochemical stains (B) of the regions between and around the blades of the dentate gyrus 72 h following GCI are shown. Scale bars=100 μm 100×, 20 μm.

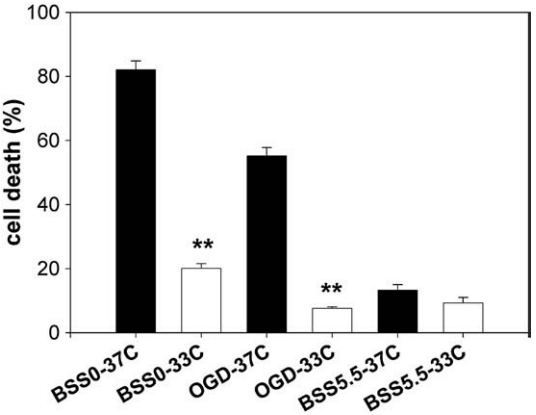

Fig. 4. Hypothermia reduces microglial cell death due to ischemia-like insults. Less cell death is observed when microglia are subjected to glucose deprivation (BSS0) or combined oxygen glucose deprivation (OGD) under hypothermic (33 °C) conditions compared to normothermic (37 °C). Control cultures (BSS5.5) suffered low levels of cell death, and this was not affected by temperature. (**$P<0.001$ vs. cultures studied at 37 °C).

pass into the brain parenchyma. Responses in murine brains subjected to GCI were similar in character (not shown).

Mild hypothermia protects against ischemia and ischemia-like injury, and reduces microglial activation

Consistent with previous findings in our lab (Zhang et al., 2001), mild hypothermia significantly reduced damage to CA1 neurons following GCI shown here in the rat. Quantification of remaining, viable neurons showed 75% reduction in cell death in the 33 °C brains relative to the 37 °C brains at 72 h (Fig. 2). Rapid rewarming is known to cause detrimental effects in humans, but this was not observed in our model as no animal died prematurely, and inspection of all brains did not reveal any signs swelling or herniation, regardless of whether the animal was cooled or not. Mild hypothermia significantly decreased the microglial response 72 h after ischemia (Fig. 3). Brains of animals subjected to GCI with 3 h hypothermia exhibited significantly less microglial (IB4) staining than ischemic brains at normothermia. Microglial activation was not observed in sham brains, and was not affected by cooling. Similar reductions in cell death and microglial activation were observed in the mouse.

Hypothermia also protected microglia from ischemia-like insults *in vitro*. Cultured microglia were exposed to 30 h glucose deprivation (GD) or 24 h OGD at either 33 °C or 37 °C. A marked reduction in cell death was observed in both hypothermic groups (Fig. 4). Furthermore, hypothermia tended to prevent transformation of cultured microglia to an amoeboid morphology (Fig. 5).

Less nuclear NFκB staining, translocation, and activation observed under hypothermic conditions

We previously showed that in the focal cerebral ischemia model, NFκB activation was markedly suppressed by mild hypothermia (Han et al., 2003); thus, we explored the effect of hypothermia on NFκB in the GCI model. At the *in vivo* level in rats, immunostaining for NFκB's p65 subunit showed that NFκB is primarily cytoplasmic with faint nuclear staining of cells in the hippocampal CA1 region after GCI at 33 °C. In contrast, after GCI at 37 °C, p65 staining was increased in intensity both in the nucleus and the cytoplasm (Fig. 6A). In shams at either temperature, staining was faint, and there was virtually no NFκB immunoreactivity in the nucleus. Counts of all cells with p65 immunoreactivity were increased by GCI, and decreased by hypothermia (Fig. 6B). Counts of cells with nuclear p65 staining revealed significantly fewer cells in the 33 °C group versus the 37 °C group (Fig. 6C).

To quantitatively evaluate NFκB activation, we performed a NFκB DNA binding assay (Han et al., 2003; Zheng et al., 2008) using nuclear extracts from brain hemispheres of mice exposed to GCI, LPS or controls at either 33 °C or 37 °C and assayed 24 h later. NFκB binding activity was increased by both insults, and was significantly reduced in hypothermic animals (Fig. 7).

At the *in vitro* level, similar patterns emerged. Cultured microglia were exposed to OGD for 2 h followed by 4 h of reperfusion at either 33 °C or 37 °C for the duration of the experiment. OGD at 37 °C led to increased NFκB staining within, and adjacent to, the nucleus. In contrast, microglia exposed to OGD at 33 °C and sham cells showed very faint NFκB staining, which was more diffuse and cytoplasmic with virtually no staining within the nucleus (Fig. 8). Phase contrast images show that under sham conditions, microglia possessed a resting morphology with elongated shape and processes. This morphology was retained in microglia exposed to OGD at 33 °C.

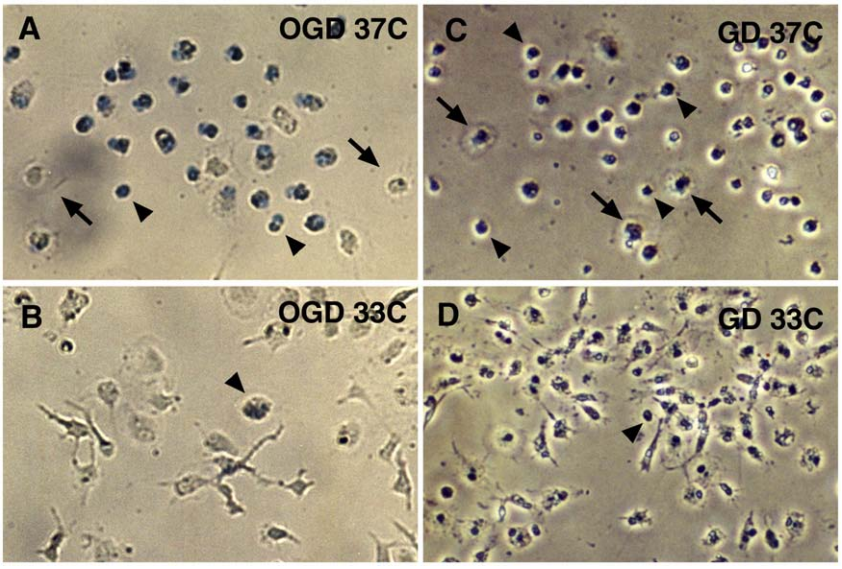

Fig. 5. Microglia suffer less cell death and are less activated following ischemia-like insults under hypothermic conditions. Less cell death is observed when microglia are subjected to oxygen glucose (OGD) or glucose deprivation (GD) at 33 °C (B, D) compared to 37 °C (A, C). Arrows indicate representative dead cells. Fewer amoeboid (and thus, activated) microglia were observed at 33 °C compared to 37 °C as well (arrowheads), indicating that hypothermia might also prevent microglial activation.

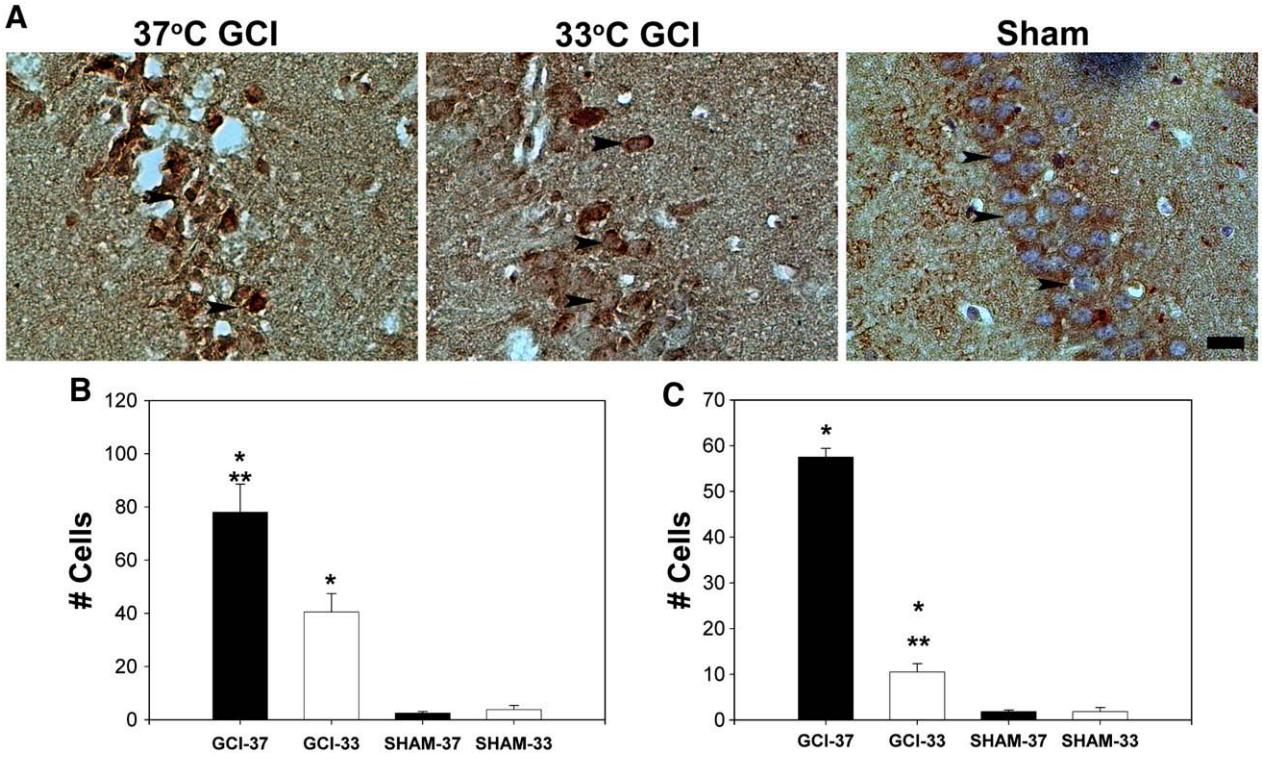

Fig. 6. Mild hypothermia reduces NFκB translocation following GCI. Normothermic, hypothermic, and sham operated rat brain sections were stained for the p65 subunit of NFκB and counterstained with hematoxylin to identify nuclei. Once NFκB is activated, it translocates into the nucleus. Images (A) are representative of the hippocampal CA1 region 72 h after GCI. In the normothermic GCI group (37 °C GCI), NFκB staining was seen in the nucleus of most cells (arrowheads) and cytosol. In hypothermic brains (33 °C GCI), staining was mostly confined to the cytosol (arrowheads) with very faint staining in the nucleus. Among sham animals (Sham), cytosolic staining was observed, but no nuclear staining was seen as evidenced by the bluish appearance of nuclei (arrowheads) from the hematoxylin stain (Scale bar = 20 μm). The extent of NFκB expression and translocation was quantified in figures B and C. All positive cells in the CA1 region were counted and quantified per high power field. Ischemia led to higher numbers of positive cells, and fewer positive cells were observed in hypothermic brains (GCI-33) compared to normothermic ischemic brains (GCI-33, *P<0.001 vs. shams, **P<0.01 vs. GCI-33). Furthermore, numbers of cells that exhibited nuclear NFκB staining per field were much fewer among GCI-33 compared to GCI-37 (C) (*P<.001 vs. shams, **P<0.001 vs. GCI-37).

Microglia exposed to OGD at 37 °C transformed into a phagocytic morphology with a typical rounded, "fried egg" appearance (Kauppinen and Swanson, 2005).

Hypothermia does not alter expression of NFκB regulatory proteins, IκB-α, or IKK-γ

Our previous work showed that mild hypothermia decreased IκB-α phosphorylation and activity of IκB's inhibitory kinase, IKK-γ in the focal ischemia model (Han et al., 2003). In mice subjected to GCI or LPS exposure, Western blots showed increased expression of IKK-γ and phosphorylated IκB (p-IκB) (Figs. 9A, D–F) relative to shams, but no changes in total IκB (Figs. 9C, G). However, we did not observe significant differences in expression of any of these proteins under conditions of hypothermia (Fig. 9). Similar observations were made in the rat GCI model.

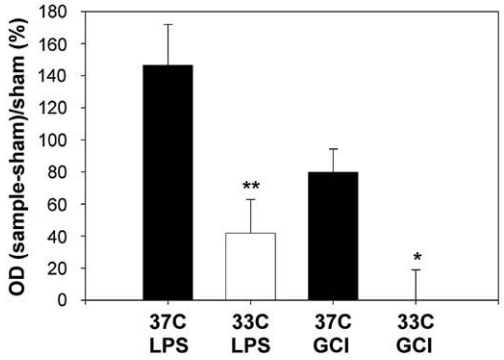

Fig. 7. Mild hypothermia decreases NFκB activation. A NFκB p65 DNA binding assay was used to measure NFκB activity in nuclear extracts of brain samples taken from mice exposed to LPS injection or GCI. Data represent the % increase in optical density (OD) relative to the activity detected in sham samples. Following both GCI and LPS, lower temperature led to decreased DNA binding (*P<0.05, **P<0.01 vs. 37C, n=4–8 samples/group).

Hypothermia decreases elaboration of inflammatory mediators by microglia

Inflammatory cytokines TNF-α and IL1-β were measured in microglial culture supernatants after stimulation with LPS or 2 h OGD exposure followed by 24 h reperfusion. Microglia increased generation of inflammatory cytokines following LPS stimulation and OGD, and this was attenuated by hypothermia (Figs. 10B–D). Microglia also secrete glutamate when activated, and glutamate is known to be neurotoxic in the setting of ischemia. Glutamate levels were increased in microglia stimulated with LPS at 37 °C, but less glutamate was detected when stimulated at 33 °C (Fig. 10A).

Discussion

At both the laboratory and clinical levels, mild hypothermia has been shown by several labs to improve neurological outcome from the devastating effects of global cerebral ischemic brain injury at both the histological and behavioral levels (Colbourne and Corbett, 1994, 1995; Colbourne et al., 1999; Dietrich et al., 1993; Lyden et al., 2006). Several mechanisms by which mild hypothermia exerts its protective effects have been characterized, including salutary effects on blood flow, reduction in excitotoxin accumulation, preservation of brain

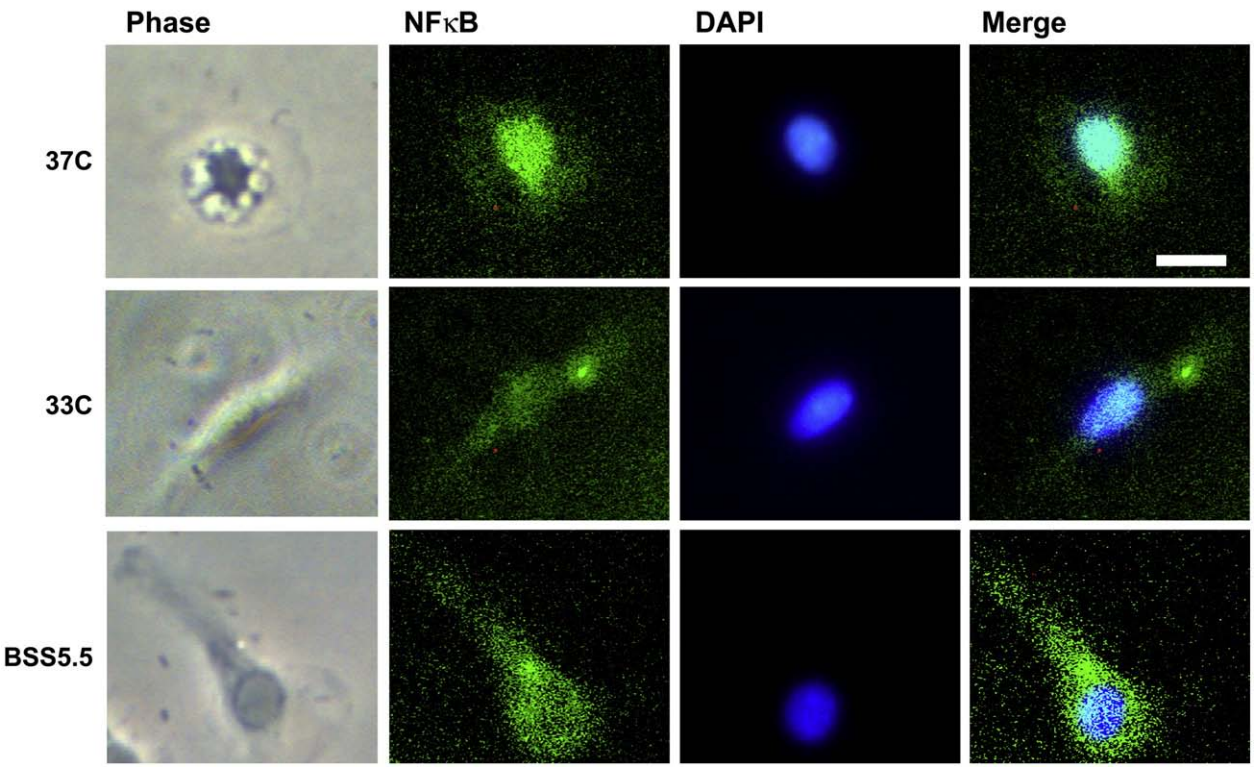

Fig. 8. Hypothermia inhibits nuclear NFκB translocation in primary microglia following 2 h OGD and 4 h reperfusion. Primary microglia subjected to OGD and reperfusion were stained for NFκB's p65 subunit. In the absence of injury, microglia appeared elongated with processes (BSS5.5, phase), but OGD at normothermic conditions caused microglia to assume a phagocytic phenotype (37 °C) which was not observed under hypothermic conditions (33 °C). Under normothermic conditions, OGD led to increased nuclear NFκB staining (37 °C, nuclei delineated by DAPI staining), compared to hypothermic and control conditions (33 °C and BSS5.5, respectively). Under hypothermic conditions, NFκB was primarily cytosolic, and less intense than microglia exposed to OGD at normothermia. Both morphology and NFκB staining patterns were similar between control and hypothermic cultures. (scale bar = 10 μm).

metabolites, and inhibition of molecular pathways such as apoptosis and inflammation (see reviews Liu and Yenari, 2007; Lyden et al., 2006; Zhao et al., 2007).

The role of inflammation in GCI models has been less studied compared to FCI. In contrast to FCI where abundant infiltrating leukocytes have been documented by several investigators (Stevens et al., 2002; Tang and Yenari, 2006; Wang et al., 2007), we found very little neutrophil influx and no blood brain barrier disruption in GCI. However, this is not to say that inflammation does not accompany ischemic brain injury following GCI, as we found robust increases in microglial immunoreactivity, size and cell number consistent with activation. We found similar responses to GCI in rat and mouse brain. While not especially surprising, it is important to document since the study of genetic mouse models is becoming increasingly popular, but most reports of GCI are in the rat.

Prior reports studying inflammation in related GCI models have described neutrophil adhesion to cerebral vasculature and neutrophil invasion into brain tissue, and this was correlated to worsened outcome (Lenzser et al., 2007; Ritter et al., 2008). However, a few reports suggest that leukocyte adhesion may actually protect the brain from ischemia. Beck et al. (2007) found that adhesion of leukocytes to brain endothelium was correlated to decreased brain injury following 15 min GCI in gerbils. The same investigative group also found that blocking leukocyte rolling using an antibody against P-selectin actually decreased survival rates (Lehmberg et al., 2006). Some investigators have described disruption of the BBB following 20 min GCI in rats (Dietrich et al., 1990; Lenzser et al., 2007), and increased BBB disruption correlated to increased temperature. Thus, potential discrepancies between the work presented here and those in the literature could be due to the duration of ischemia. Here, we occluded vessels no longer than 8 min in the rat, and 12 min in the mouse. In other studies, vessels were occluded for 15 min or longer, and this may be sufficient to cause disruption of the BBB and entry of circulating leukocytes. Furthermore, the study by Ritter et al. (2008) which described a correlation between leukocyte adhesion and worsened outcome, studied aged animals, whereas we studied younger animals. However, the study by Lenzser et al., (2007) appeared to use animals of similar size, and presumably age as we, but found detectable levels of myeloperoxidase (a neutrophil marker) in the brain. The main difference between our study and theirs, is that the duration of ischemia was longer (20 min). At the clinical level, it is known that autopsy specimens of brains from comatose survivors of cardiac arrest ("respirator brains") differ from brains of stroke patients. Whereas infiltrating leukocytes have been well described in brains of stroke victims (Garcia, 1985), in brains of comatose patients who survived cardiac arrest, there is a relative absence of inflammatory infiltrates, whereas gliosis and activated microglia are prominent (Garcia, 1985; Norenberg and Gregory, 1985), much like what was observed in our model. Thus, it is possible that inflammatory responses due to global cerebral ischemia and its clinical counterparts may be mediated primarily by microglia.

We found that mild hypothermia significantly reduced microglial activation and generation of a variety of cytotoxic immune mediators. This would be consistent with the numerous reports that largely suggest that ischemia-induced inflammation is detrimental (Tang and Yenari, 2006; Wang et al., 2007). While mild hypothermia inhibited transformation of microglia to a phagocytic phenotype *in vitro*, it also reduced overall microglial cell death. This latter observation might suggest a potentially added benefit of hypothermia, in that by preserving microglial viability, microglia may be available to perform restorative functions such as phagocytosing necrotic debris and elaborating pro-survival factors.

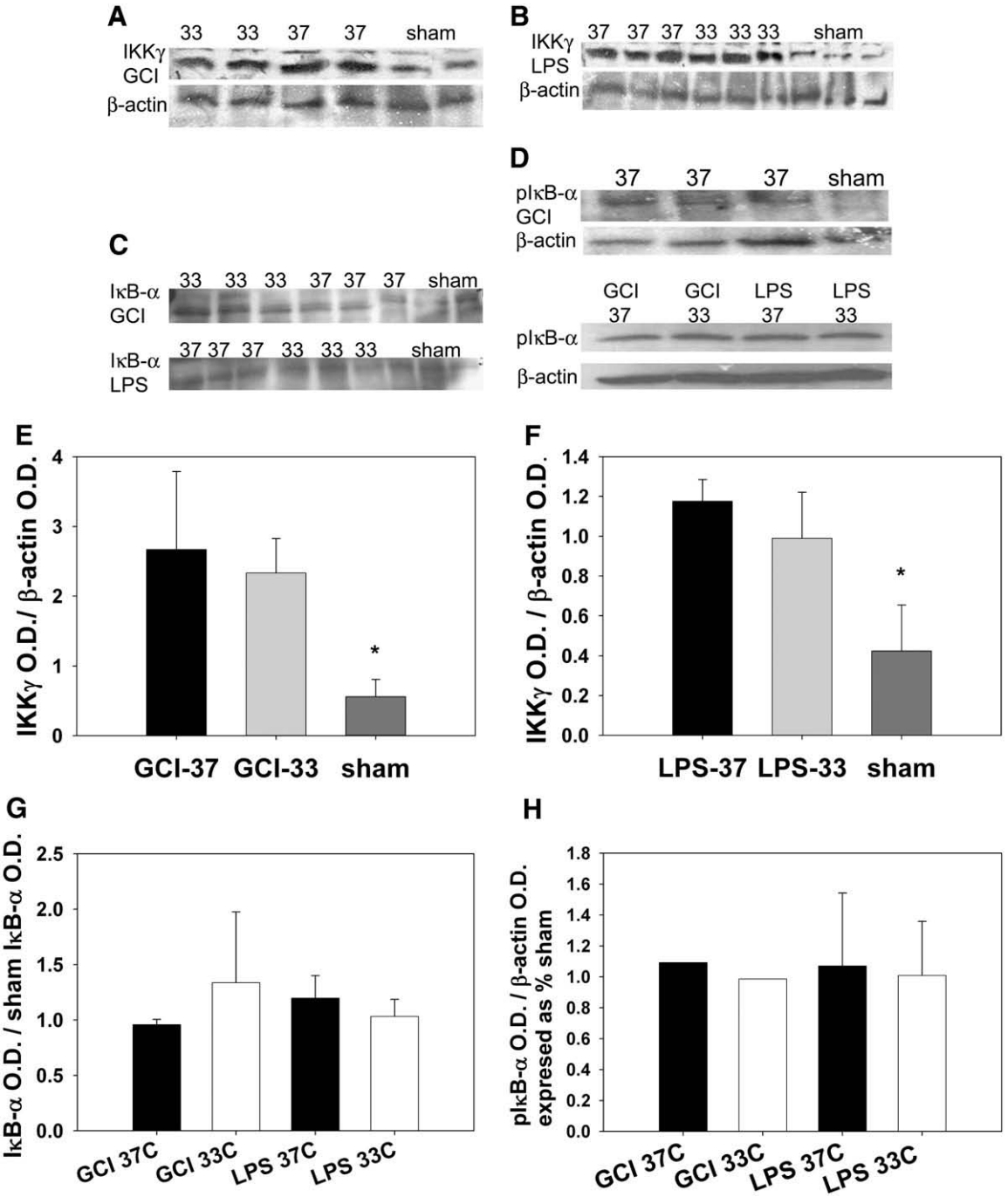

Fig. 9. The inhibition of NFκB translocation by hypothermia is not due to differences in the expression of NFκB modulatory proteins. Western blots of IKKγ following GCI (A, E) and LPS injection (B, F) showed increased expression compared to sham, but expression was no different between normothermic and hypothermic brains. Total levels of NFκB's inhibitory protein, IκB-α was unchanged by GCI or LPS, and levels were no different with hypothermia (C, G). GCI led to increases in IκB-α phosphorylation (D, upper gel), but hypothermia failed to alter phosphorylated IκB-α levels (D, lower gel, H). (*$P<0.05$, β-actin shown as a housekeeping control, $n=6$/group).

Consistent with our prior observation in the FCI model (Han et al., 2003), we also observed decreased nuclear NFκB staining in brains following GCI, and decreased NFκB activity with hypothermia. We found similar decreases in NFκB activation in brains of mice given LPS. These latter observations are an important control, since the extent of inflammation in ischemia models is likely directly correlated to the amount of damage. Since hypothermia decreases overall cell death, the amount of inflammation should also be proportionately decreased, and any anti-inflammatory effects may be secondary to an upstream mechanism. However, in the LPS model, a robust inflammatory response occurs, but in the absence of cell death (Deng et al., 2003). Thus, hypothermia appears to have direct effects on NFκB activation. Further evidence supporting a direct anti-inflammatory effect of hypothermia is that we observed less overall and nuclear NFκB staining in primary cultured microglia after OGD with hypothermia.

Prior reports by other investigators on the effect of temperature on NFκB are varied. Irazuzta et al. (2002) studied mild hypothermia in a rodent model of group B streptococcal meningitis and found similar attenuation of NFκB and improved neurobehavioral scores. However, other reports have presented results in contrast to ours. Fairchild et al. (2005) studied a macrophage cell line and found increased and prolonged NFκB activation and increased expression of inflammatory cytokines. Gibbons et al. (2003) studied BV2 cells stimulated with LPS

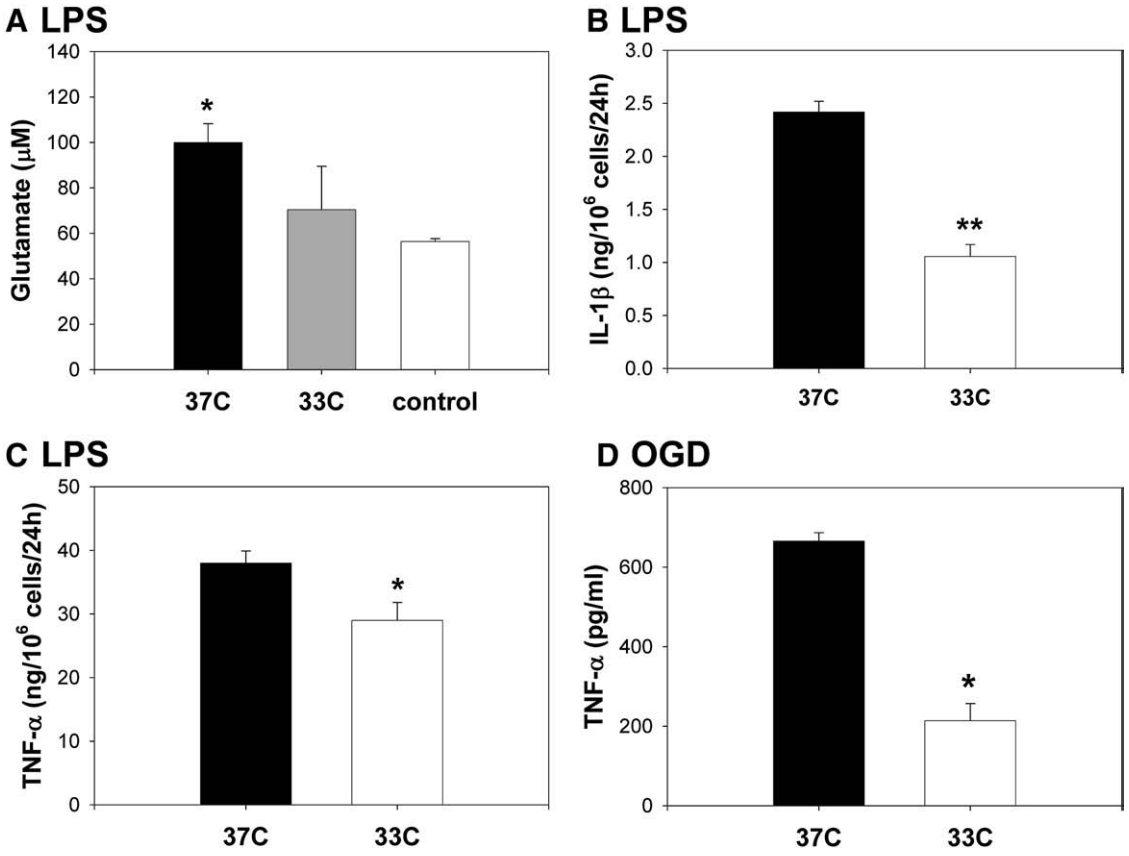

Fig. 10. Mild hypothermia decreases inflammatory mediator secretion by cultured microglia. Primary microglial cultures were stimulated with LPS (A–C) at either 37 °C or 33 °C for 24 h. Measurement of glutamate (A), IL-1β (B) and TNF-α (C) showed decreased levels in culture supernatants at lower temperatures. Similarly, microglia exposed to 2 h OGD followed by 24 h reperfusion at 33 °C led to less TNF-α secretion compared to 37 °C (D). (*$P<0.05$, **$P<0.01$).

under 37 °C vs. 33 °C. While finding decreased expression of inducible nitric oxide synthase (iNOS), a NFκB regulated gene, they found increased expression of cyclooxygenase-2 (COX-2), another NFκB regulated gene. Yet, they found no differences in NFκB translocation or TNF-α production. The reasons for these discrepancies are not clear, but may in part be explained by the study of cell lines compared to primary cell culture and *in vivo* models studied here.

In contrast to our previous study in FCI (Han et al., 2003) where we found that hypothermia inhibited NFκB activation by inhibiting IKKβ and IKKγ expression and function leading to less IκB phosphorylation, here, we did not find significant differences in IκB phosphorylation or IKK expression. Reasons for this discrepancy are unclear, but clearly indicate that mechanism for protection and anti-inflammatory effects in GCI are different from FCI. Our observations might be explained by temperature dependent changes in membrane fluidity. The nuclear membrane is dynamic and fluid at physiological temperatures. It is possible that changes in membrane properties associated with mild hypothermia could inhibit the nuclear translocation of NFκB, and downstream inflammatory events. It is clear that lowered temperatures decrease membrane fluidity and alter the biophysical properties of membrane lipids (Aloia and Raison, 1989; Yatvin et al., 1980). Evidence suggests that membrane properties, particularly in regions of higher cholesterol and sphingomyelin have significantly decreased water permeability at 30 °C compared to 35 °C (Rawicz et al., 2008). Thus, it is conceivable that lower temperatures render NFκB less capable of penetrating the nuclear membrane. This mechanism would explain decreases in nuclear NFκB staining and DNA binding, and would be independent of expression and function of its regulatory proteins which were not changed by temperature. We also observed less overall NFκB staining. This is not surprising since NFκB can drive its own expression (http://www.superarray.com/chipqpcr.php).

Numerous studies have sought to better understand the protective role of hypothermia following ischemia-like insults, particularly in neurons. One hypothesis is that hypothermia acts by reducing the accumulation of toxic metabolic substrates following ischemia insults, such as glutamate (Busto et al., 1989; Yenari et al., 2004). Consistent with this hypothesis is our observation that microglia generate less glutamate at lower temperatures. However, delayed induction of hypothermia at times well after depletion of metabolic stores and release of at least neuronal glutamate is still protective (Baker et al., 1992; Huang et al., 1998; Xue et al., 1992; Yenari et al., 2004), and the Q10 for glucose metabolism suggest additional actions of hypothermia (Xue et al., 1992; Yenari et al., 2004).

Much of the work attempting to elucidate the mechanisms of hypothermic protection in GCI models has focused on pathways largely involving neurons. However, other cell types are likely to participate in determining the ultimate fate of the brain following GCI, but this has not yet been studied extensively. Prior studies have shown that activated microglia and circulating macrophages increase neuronal injury following hypoxia treatment when co-cultured with neurons (Flavin et al., 1997; Flavin and Ho, 1999; Flavin et al., 2000; Giulian et al., 1993; Giulian and Vaca, 1993; Lehnardt et al., 2003), and inhibiting microglia seems to lead to reduced cell death in GCI (Yrjänheikki et al., 1998). Hypothermia leads to decreased inflammation including less microglial activation and activation of NFκB following FCI (Deng et al., 2003; Han et al., 2003; Inamasu et al., 2000; Maier et al., 1998; Wang et al., 2002). However, the salutary effects of NFκB deserve some comment, as it is capable of upregulating several survival factors such as several trophic factors and inducible superoxide dismutase (Carroll et al., 2000). Whether NFκB plays detrimental or protective role(s) may depend on the nature of the insult or the cell type in which it is activated. Our findings here

suggest, but do not prove, that NFκB may play an overall damaging role in brain ischemia which hypothermia, by decreasing its activation, ameliorates. The data that we present here in the GCI model show similar findings to the FCI model, although the mechanism appears to differ somewhat. In conclusion, our data demonstrate that hypothermia following global cerebral ischemia is associated with decreased microglial activation, and decreased nuclear translocation of NFκB, thereby reducing activation of the downstream inflammatory pathway.

Acknowledgments

This work was made possible by the following institutions and grants: NIH RO1 NS40516 (MAY), AHA EIA0540066N (MAY), NIH P50 NS14543 and P01 NS37520 (RGG, MAY), NIH R01 NS53898 (RGG).

References

Aloia, R.C., Raison, J.K., 1989. Membrane function in mammalian hibernation. Biochim. Biophys. Acta 988, 123–146.

Baker, C.J., Onesti, S.T., Solomon, R.A., 1992. Reduction by delayed hypothermia of cerebral infarction following middle cerebral artery occlusion in the rat: a time-course study. J. Neurosurg. 77, 438–444.

Beck, J., Stummer, W., Lehmberg, J., Baethmann, A., Uhl, E., 2007. Activation of leukocyte–endothelial interactions and reduction of selective neuronal death after global cerebral ischemia. Neurosci. Lett. 414, 159–164.

Bernard, S.A., Gray, T.W., Buist, M.D., Jones, B.M., Silvester, W., Gutteridge, G., Smith, K., 2002. Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. N. Engl. J. Med. 346, 557–563.

Busto, R., Globus, M.Y., Dietrich, W.D., Martinez, E., Valdes, I., Ginsberg, M.D., 1989. Effect of mild hypothermia on ischemia-induced release of neurotransmitters and free fatty acids in rat brain. Stroke 20, 904–910.

Carroll, J.E., Hess, D.C., Howard, E.F., Hill, W.D., 2000. Is nuclear factor-kappaB a good treatment target in brain ischemia/reperfusion injury? NeuroReport 11, R1–4.

Colbourne, F., Corbett, D., 1994. Delayed and prolonged post-ischemic hypothermia is neuroprotective in the gerbil. Brain Res. 654, 265–272.

Colbourne, F., Corbett, D., 1995. Delayed postischemic hypothermia: a six month survival study using behavioral and histological assessments of neuroprotection. J. Neurosci. 15, 7250–7260.

Colbourne, F., Li, H., Buchan, A.M., 1999. Indefatigable CA1 sector neuroprotection with mild hypothermia induced 6 hours after severe forebrain ischemia in rats. J. Cereb. Blood Flow Metab. 19, 742–749.

Deng, H., Han, H.S., Cheng, D., Sun, G.H., Yenari, M.A., 2003. Mild hypothermia inhibits inflammation after experimental stroke and brain inflammation. Stroke 34, 2495–2501.

Dietrich, W.D., Busto, R., Halley, M., Valdes, I., 1990. The importance of brain temperature in alterations of the blood–brain barrier following cerebral ischemia. J. Neuropathol. Exp. Neurol. 49, 486–497.

Dietrich, W.D., Busto, R., Alonso, O., Globus, M.Y., Ginsberg, M.D., 1993. Intraischemic but not postischemic brain hypothermia protects chronically following global forebrain ischemia in rats. J. Cereb. Blood Flow Metab. 13, 541–549.

Erecinska, M., Thoresen, M., Silver, I.A., 2003. Effects of hypothermia on energy metabolism in Mammalian central nervous system. J. Cereb. Blood Flow Metab. 23, 513–530.

Fairchild, K.D., Singh, I.S., Carter, H.C., Hester, L., Hasday, J.D., 2005. Hypothermia enhances phosphorylation of I{kappa}B kinase and prolongs nuclear localization of NF-{kappa}B in lipopolysaccharide-activated macrophages. Am. J. Physiol. Cell Physiol. 289, C1114–1121.

Flavin, M.P., Ho, L.T., 1999. Propentofylline protects neurons in culture from death triggered by macrophage or microglial secretory products. J. Neurosci. Res. 56, 54–59.

Flavin, M.P., Coughlin, K., Ho, L.T., 1997. Soluble macrophage factors trigger apoptosis in cultured hippocampal neurons. Neuroscience 80, 437–448.

Flavin, M.P., Zhao, G., Ho, L.T., 2000. Microglial tissue plasminogen activator (tPA) triggers neuronal apoptosis in vitro. Glia 29, 347–354.

Garcia, J.H., 1985. Circulatory disorders and their effects on the brain. In: Davis, R.K., Robertson, D.M. (Eds.), Textbook of Neuropathology. Williams & Wilkins, Baltimore, pp. 548–631.

Gibbons, H., Sato, T.A., Dragunow, M., 2003. Hypothermia suppresses inducible nitric oxide synthase and stimulates cyclooxygenase-2 in lipopolysaccharide stimulated BV-2 cells. Brain Res. Mol. Brain Res. 110, 63–75.

Giulian, D., Vaca, K., 1993. Inflammatory glia mediate delayed neuronal damage after ischemia in the central nervous system. Stroke 24, I84–90.

Giulian, D., Corpuz, M., Chapman, S., Mansouri, M., Robertson, C., 1993. Reactive mononuclear phagocytes release neurotoxins after ischemic and traumatic injury to the central nervous system. J. Neurosci. Res. 36, 681–693.

Han, H.S., Qiao, Y., Karabiyikoglu, M., Giffard, R.G., Yenari, M.A., 2002. Influence of mild hypothermia on inducible nitric oxide synthase expression and reactive nitrogen production in experimental stroke and inflammation. J. Neurosci. 22, 3921–3928.

Han, H.S., Karabiyikoglu, M., Kelly, S., Sobel, R.A., Yenari, M.A., 2003. Mild hypothermia inhibits nuclear factor-kappaB translocation in experimental stroke. J. Cereb. Blood Flow Metab. 23, 589–598.

Huang, F.P., Zhou, L.F., Yang, G.Y., 1998. Effects of mild hypothermia on the release of regional glutamate and glycine during extended transient focal cerebral ischemia in rats. Neurochem. Res. 23, 991–996.

Hypothermia after Cardiac Arrest Study Group, 2002. Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. N. Engl. J. Med. 346, 549–556.

Inamasu, J., Suga, S., Sato, S., Horiguchi, T., Akaji, K., Mayanagi, K., Kawase, T., 2000. Post-ischemic hypothermia delayed neutrophil accumulation and microglial activation following transient focal ischemia in rats. J. Neuroimmunol. 109, 66–74.

Irazuzta, J.E., Pretzlaff, R.K., Zingarelli, B., Xue, V., Zemlan, F., 2002. Modulation of nuclear factor-kappaB activation and decreased markers of neurological injury associated with hypothermic therapy in experimental bacterial meningitis. Crit. Care Med. 30, 2553–2559.

Ishikawa, M., Sekizuka, E., Sato, S., Yamaguchi, N., Inamasu, J., Bertalanffy, H., Kawase, T., Iadecola, C., 1999. Effects of moderate hypothermia on leukocyte–endothelium interaction in the rat pial microvasculature after transient middle cerebral artery occlusion. Stroke 30, 1679–1686.

Kauppinen, T.M., Swanson, R.A., 2005. Poly(ADP-ribose) polymerase-1 promotes microglial activation, proliferation, and matrix metalloproteinase-9-mediated neuron death. J. Immunol. 174, 2288–2296.

Kelly, S., McCulloch, J., Horsburgh, K., 2001. Minimal ischaemic neuronal damage and HSP70 expression in MF1 strain mice following bilateral common carotid artery occlusion. Brain Res. 914, 185–195.

Kelly, S., Zhang, Z.J., Zhao, H., Xu, L., Giffard, R.G., Sapolsky, R.M., Yenari, M.A., Steinberg, G.K., 2002. Gene transfer of HSP72 protects cornu ammonis 1 region of the hippocampus neurons from global ischemia: influence of Bcl-2. Ann. Neurol. 52, 160–167.

Krieger, D.W., Yenari, M.A., 2004. Therapeutic hypothermia for acute ischemic stroke: what do laboratory studies teach us? Stroke 35, 1482–1489.

Lanier, W.L., 1995. Cerebral metabolic rate and hypothermia: their relationship with ischemic neurologic injury. J. Neurosurg. Anesthesiol. 7, 216–221.

Lehmberg, J., Beck, J., Baethmann, A., Uhl, E., 2006. Effect of P-selectin inhibition on leukocyte–endothelium interaction and survival after global cerebral ischemia. J. Neurol. 253, 357–363.

Lehnardt, S., Massillon, L., Follett, P., Jensen, F.E., Ratan, R., Rosenberg, P.A., Volpe, J.J., Vartanian, T., 2003. Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. Proc. Natl. Acad. Sci. U. S. A. 100, 8514–8519.

Lenzser, G., Kis, B., Snipes, J.A., Gaspar, T., Sandor, P., Komjati, K., Szabo, C., Busija, D.W., 2007. Contribution of poly(ADP-ribose) polymerase to postischemic blood–brain barrier damage in rats. J. Cereb. Blood Flow Metab. 27, 1318–1326.

Liu, L., Yenari, M.A., 2007. Therapeutic hypothermia: neuroprotective mechanisms. Front. Biosci. 12, 816–825.

Lyden, P., Krieger, D.W., Yenari, M.A., Dietrich, W.D., 2006. Therapeutic hypothermia for acute stroke. Int. J. Stroke. 1, 9–19.

Maier, C.M., Ahern, K.v., Cheng, M.L., Lee, J.E., Yenari, M.A., Steinberg, G.K., 1998. Optimal depth and duration of mild hypothermia in a focal model of transient cerebral ischemia: effects on neurologic outcome, infarct size, apoptosis, and inflammation. Stroke 29, 2171–2180.

Maier, C.M., Sun, G.H., Cheng, D., Yenari, M.A., Chan, P.H., Steinberg, G.K., 2002. Effects of mild hypothermia on superoxide anion production, superoxide dismutase expression, and activity following transient focal cerebral ischemia. Neurobiol. Dis. 11, 28–42.

Murakami, K., Kondo, T., Kawase, M., Chan, P.H., 1998. The development of a new mouse model of global ischemia: focus on the relationships between ischemia duration, anesthesia, cerebral vasculature, and neuronal injury following global ischemia in mice. Brain Res. 780, 304–310.

Newcomb, R., Sun, X., Taylor, L., Curthoys, N., Giffard, R.G., 1997. Increased production of extracellular glutamate by the mitochondrial glutaminase following neuronal death. J. Biol. Chem. 272, 11276–11282.

Norenberg, M.D., Gregory, G.A., 1985. Central nervous system manifestations of systemic disease. In: Davis, R.K., Robertson, D.M. (Eds.), Textbook of Neuropathology. Williams & Wilkins, Baltimore, pp. 403–467.

Rawicz, W., Smith, B.A., McIntosh, T.J., Simon, S.A., Evans, E.A., 2008. Elasticity, strength, and water permeability of bilayers that contain raft microdomain-forming lipids. Biophys. J. 94, 4725–4736.

Ritter, L., Funk, J., Schenkel, L., Tipton, A., Downey, K., Wilson, J., Coull, B., McDonagh, P., 2008. Inflammatory and hemodynamic changes in the cerebral microcirculation of aged rats after global cerebral ischemia and reperfusion. Microcirculation 15, 297–310.

Rothwarf, D.M., Karin, M., 1999. The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. Sci. STKE 1999 (5), re1.

Sick, T.J., Tang, R., Perez-Pinzon, M.A., 1999. Cerebral blood flow does not mediate the effect of brain temperature on recovery of extracellular potassium ion activity after transient focal ischemia in the rat. Brain Res. 821, 400–406.

Stevens, S.L., Bao, J., Hollis, J., Lessov, N.S., Clark, W.M., Stenzel-Poore, M.P., 2002. The use of flow cytometry to evaluate temporal changes in inflammatory cells following focal cerebral ischemia in mice. Brain Res. 932, 110–119.

Tang, X.N., Yenari, M.A., 2006. Inflammation in stroke. In: Yenari, M.A., Giffard, R.G. (Eds.), Glia and Inflammation in Neurodegenerative Disease. Nova Science Publishers, Inc., Hauppauge, pp. 85–115.

Wang, G.J., Deng, H.Y., Maier, C.M., Sun, G.H., Yenari, M.A., 2002. Mild

hypothermia reduces ICAM-1 expression, neutrophil infiltration and microglia/monocyte accumulation following experimental stroke. Neuroscience 114, 1081–1090.
Wang, Q., Tang, X.N., Yenari, M.A., 2007. The inflammatory response in stroke. J. Neuroimmunol. 184, 53–68.
Xue, D., Huang, Z.G., Smith, K.E., Buchan, A.M., 1992. Immediate or delayed mild hypothermia prevents focal cerebral infarction. Brain Res. 587, 66–72.
Yatvin, M.B., Schmitz, B.J., Dennis, W.H., 1980. Radiation killing of *E. coli* K1060: role of membrane fluidity, hypothermia and local anaesthetics. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 37, 513–519.
Yenari, M.A., Giffard, R.G., 2001. Ischemic vulnerability of primary murine microglial cultures. Neurosci. Lett. 298, 5–8.
Yenari, M.A., Onley, D., Hedehus, M., deCrespigny, A., Sun, G.H., Moseley, M.E., Steinberg, G.K., 2000. Diffusion- and perfusion-weighted magnetic resonance imaging of focal cerebral ischemia and cortical spreading depression under conditions of mild hypothermia. Brain Res. 885, 208–219.
Yenari, M.A., Wijman, C., Steinberg, G.K., 2004. Effects of hypothermia on cerebral metabolism, blood flow, and autoregulation. In: Mayer, S., Sessler, D. (Eds.), Therapeutic Hypothermia. Marcel Dekker, Inc, New York, pp. 141–178.
Yenari, M.A., Xu, L., Tang, X.N., Qiao, Y., Giffard, R.G., 2006. Microglia potentiate damage to blood–brain barrier constituents: improvement by minocycline in vivo and in vitro. Stroke 37, 1087–1093.
Yrjänheikki, J., Keinänen, R., Pellikka, M., Hökfelt, T., Koistinaho, J., 1998. Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. Proc. Natl. Acad. Sci. U. S. A. 95, 15769–15774.
Zhang, Z., Sobel, R.A., Cheng, D., Steinberg, G.K., Yenari, M.A., 2001. Mild hypothermia increases Bcl-2 protein expression following global cerebral ischemia. Brain Res. Mol. Brain Res. 95, 75–85.
Zhao, H., Steinberg, G.K., Sapolsky, R.M., 2007. General versus specific actions of mild-moderate hypothermia in attenuating cerebral ischemic damage. J. Cereb. Blood Flow Metab. 27, 1879–1894.
Zheng, Z., Kim, J.Y., Ma, H., Lee, J.E., Yenari, M.A., 2008. Anti-inflammatory effects of the 70 kDa heat shock protein in experimental stroke. J. Cereb. Blood Flow Metab. 28, 53–63.