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**THE EFFECTS OF MODERATE HYPOTHERMIA ON IL-1 β AND
ISCHEMIA-INDUCED CEREBRAL INFLAMMATION IN C57/B16
MICE**

By Ian Sutcliffe

A thesis submitted to the School of Graduate Studies and Research of the University of
Ottawa

In partial fulfillment of the requirements for the degree of M.Sc.

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ABSTRACT

Hypothermia has long been known to be neuroprotective following brain injury, yet the correlates of this neuroprotection are poorly understood. We postulated that moderate hypothermia would attenuate leukocyte rolling and adhesion following IL-1 β or cerebral ischemia induced inflammation. Laser Doppler was used to demonstrate successful induction of global cerebral ischemia in C57/Bl6 mice. ISEL, NeuN, and GFAP staining of brain sections were used to demonstrate temporal and spatial injury in this murine model of 10-minutes global cerebral ischemia. Four hours hypothermia (32°C) induced immediately following normothermic (37°C) cerebral ischemia provided protection against cell death and astrogliosis for up to 7 days post-ischemia. An open cranial window and intravital microscopy were used to visualize leukocyte rolling and adhesion in pial venules at 4-h following injection of IL-1 β , and 0 to 4-h following global cerebral ischemia. Increases in leukocyte rolling and adhesion were observed in IL-1 β injected mice. Four hours of hypothermia begun immediately following injection of IL-1 β reduced both rolling and adhesion. Similarly, increases in leukocyte rolling and adhesion were observed at 0-h and 2-h following cerebral ischemia in normothermic mice, and 2-h of post-ischemic hypothermia reduced both rolling and adhesion. In contrast, MPO and neutrophil immunohistochemistry showed that no significant infiltration of neutrophils into the brain following global cerebral ischemia. Molecular mechanisms of hypothermic effects were investigated *in vitro*. We showed that neither IL-1 β nor hypothermia altered the expression of CD18, or chemotaxis in human neutrophils. Together, these findings suggest that one mechanism by which hypothermia may provide neuroprotection is by reduction of leukocyte-endothelial interactions in the cerebrovasculature.

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List of Abbreviations

2VO	two-vessel occlusion
°C	degree celcius
AP-1	activating protein-1
ATP	adenosine triphosphate
BBB	blood brain barrier
BCCA	bilateral common carotid artery
BNP	bacitracin zinc/neomycin sulfate/polymyxin B
CBF	cerebral blood flow
CEC	cerebral endothelial cell
CD	cluster of differentiation
CNS	central nervous system
DAPI	4', 6-diamidino-2-phenylindole dihydrochloride
ddH₂O	double deionized water
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FITC	fluorescein isothiocyanate
fMLP	N-formylmethionyl-leucyl-phenylalanine
g	gravity
GFAP	glial fibrillary acidic protein
gm	gram
h	hour

H₂O₂	hydrogen peroxide
HBSS	hank's balanced salt solution
HCEC	human cerebral endothelial cell
ICE	IL-1 converting enzyme
IEG	immediate early genes
IFN	interferon
IgG	immunoglobulin
IL-1	interleukin-1
IL-8	interleukin-8
ICAM-1	intercellular adhesion molecule-1
i.p.	intraperitoneal
ISEL	<i>in situ</i> end labeling
kg	kilogram
LPS	lipopolysacchride
M	mole
Mab	monoclonal antibody
MCAO	middle cerebral artery occlusion
mg	milligram
ml	milliliter
mM	millimole
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
NaCl	sodium chloride

NaN₃	sodium azide
NeuN	neuronal nuclear protein
NF-κB	nuclear factor-κB
nm	nanometer
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NSAID	non-steroid anti-inflammatory drugs
pCO₂	carbon dioxide partial pressure
pO₂	oxygen partial pressure
PB	phosphate buffer
PBS	phosphate buffer saline
PECAM-1	platelet-endothelial cell adhesion molecule-1
PFA	paraformaldehyde
PLA₂	phospholipase A₂
PLC	phospholipase C
PMN	polymorphonuclear
rCBF	regional cerebral blood flow
SD	standard deviation
ROS	reactive oxygen species
rpm	revolutions per minute
TBI	traumatic brain injury
TGF	transforming growth factor
TNFα	tumour necrosis factor alpha

tPA	tissue plasminogen activator
U	units
VCAM-1	vascular cell adhesion molecule-1
μl	microliter
μM	micromole

Chapter I: INTRODUCTION

1.1 The Rationale

Cerebral ischemia/reperfusion is an important pathophysiologic mechanism of injury in traumatic brain injury (TBI), cardiac arrest, birth asphyxia and stroke and is a common cause of morbidity and mortality in all age groups. Secondary inflammatory events follow the initial injury and involve breakdown of the blood-brain barrier (BBB) (vasogenic edema) and the transmigration of leukocytes (e.g. neutrophils and monocytes) across the BBB into the brain parenchyma in response to inflammatory mediators. These inflammatory events occur over several hours and days and account largely for the clinical observation that brain edema and brain damage can progress from days to weeks following the initial injury. Of the myriad of therapeutic strategies pursued, hypothermia has been shown to provide potent protection following ischemia/reperfusion injury in heart and brain, although the mechanisms of protection are unresolved. Although considerable evidence suggests that inflammation has a profound impact on cerebral ischemia/reperfusion injury, limited knowledge is available on the effects of hypothermia on leukocyte function and inflammation.

Secondary post-ischemic inflammation begins with neutrophil infiltration into brain parenchyma. These leukocytes then release various injurious molecules including reactive oxygen species, proteases, and pro-inflammatory mediators that contribute to BBB dysfunction and neuronal death. Migration of neutrophils from the microvascular lumen into the brain parenchyma involves a cascade of cellular responses mediated by an adhesion pathway, which has been typified into a rolling→activation→adhesion→

transmigration paradigm. The effects of hypothermia on this recruitment paradigm have not been fully elucidated.

We used *in vivo* and *in vitro* models to study the effects of hypothermia on inflammatory processes including leukocyte rolling→activation→adhesion→transmigration during systemic inflammation induced by IL-1 β or global cerebral ischemia. This knowledge will allow for a more comprehensive understanding of the mechanism of hypothermic neuroprotection, and how hypothermia may be best applied in future therapeutic strategies against ischemic brain injury.

1.2 Cerebral Ischemia – Epidemiology and General Information

Stroke and cardiac arrest (ex: congestive heart failure or cardiovascular disease) are the most important causes of morbidity and mortality in Western society. With an incidence of 2-4/1000 and mortality rate of 30%, stroke (after heart disease and cancer) is the 3rd leading cause of death in industrialized countries (Sarti et al., 2000). Moreover, in the 1980's, the annual incidence of heart failure was 7.2 /1000 in men and 4.7/1000 in women aged \geq 45 yrs, and was shown to increase markedly with age (Garg et al., 1993). Similar to the high prevalence of heart failure in adults, cardiac arrest from cardiorespiratory failure or asphyxia is prevalent in the neonatal to pediatric age groups (Sirbaugh et al., 1999). Furthermore, stroke and cardiac disease imposes a substantial economic burden on individuals and society. In the USA alone in 1993, the economic burden of heart failure and stroke were \$8 billion and \$30 billion respectively (Taylor et al., 1996; Garg et al., 1993).

More than any other organ, the brain critically depends on a continuous supply of oxygen and glucose. Although the brain represents only 2% of total body weight, it receives an overwhelming 15% of total cardiac output. This high oxygen and energy demand is largely due to the necessity for active maintenance of ion gradients (ex: Na^+/K^+ ATPase) in excitable neurons. Furthermore, neuronal mitochondrial oxidative phosphorylation must generate high levels of adenosine triphosphate (ATP) to sustain high cerebral metabolic rate. Thus, the brain is uniquely sensitive to reductions in blood flow.

Ischemia (from the Greek *ischein*, to suppress, and *haima*, blood) is a condition characterized by an insufficient available blood flow to meet tissue metabolic needs. The immediate consequences of ischemia are lack of oxygen and nutrient delivery to the tissue. Reperfusion is the state of reflow following transient reduction of blood flow. Prompt reperfusion of cerebral ischemic brain tissue is critical for restoring normal function. However, in circumstances of prolonged severe ischemia, this return of flow can be detrimental and further damage tissue (Verrier, 1996). Ischemia/reperfusion can occur in a discrete area of the brain, commonly referred to as focal ischemia (e.g. stroke), or in the whole brain, called global ischemia (e.g. cardiac arrest).

Ischemic stroke occurs most commonly by thrombosis or embolism. Global ischemia is incurred differently than focal ischemia in that it is commonly due to hypovolemia, low blood pressure, or cardiac pump failure resulting in systemic hypoperfusion or circulatory failure. The most vulnerable areas following global cerebral ischemia are the vascular border zones (watershed) zones between cerebral arteries. In contrast, thrombosis or embolism blocks blood to one area causing a “core” infarct in the

center of the area supplied by the affected artery, while collateral circulation from other arteries spares brain tissue at the “penumbra” bordering the lesion. This penumbral zone represents a graded cerebral ischemia surrounding the pan-necrotic core where neuronal cells are only at risk of dying, and if not intervened can succumb to delayed death.

1.3 The Mechanisms of injury

1.3.1 The Cerebral Ischemic Insult

Cerebral ischemia is initially a hypoxic, metabolic insult. Reduced blood flow following global and focal cerebral ischemia results in rapid ATP depletion (Lipton, 1999). The combination of ATP breakdown and activation of anaerobic glycolysis leads to increases in inorganic phosphate, lactate, and acidosis. Energy deficiency results in failure of Na^+/K^+ ATPase pump and loss of ionic gradients leading to depolarization. Depolarization leads to somatodendritic and presynaptic activation of voltage-sensitive Ca^{2+} channels, and release of excess neurotransmitters, in particular the excitatory amino acid glutamate. Furthermore, energy-dependent reuptake of glutamate by neurons and astrocytes is impeded further increasing accumulation of glutamate in the extracellular space (Nathaniel and Nathaniel, 1981; Takahashi et al., 1997). Apart from triggering further spread of cellular depolarization, glutamate binds ligand gated ionotropic (i.e. NMDA, AMPA) and G-coupled metabotropic (i.e. mGluR) receptors at the postsynaptic membrane and initiates a cascade of events leading to accumulation of intracellular Ca^{2+} , activation of intracellular messengers, and brain edema. The end results following glutamate cascades include activation of immediate early genes (IEG)(e.g. c-fos, c-jun) (Kogure and Kato, 1993), stress genes (e.g. hsp70, hsp72) (Gonzalez et al., 1989),

kinases (e.g. PKA, PKC, CaMKII, MapK) (Domanska-Janik, 1996), proteases (calpains, caspases) (Suzuki et al., 1995; MacManus and Buchan, 2000), lipolysis (PLC, PLA₂) (Siesjo and Katsura, 1992), and reactive oxygen species (ROS), leading to cell death. There are believed to be two modes of cell death that participate in ischemic brain injury (Majno and Joris, 1995): apoptosis and necrosis. In experimental cerebral ischemia, the severity of damage depends on the density and duration of the reduction of cerebral blood flow (CBF) (Pulsinelli et al, 1982). The temporal and spatial pattern of cerebral ischemic cell death is believed to involve early necrotic death, and substantial delayed apoptotic neuronal death (Lipton, 1999).

Apoptosis is an active cell death that is a part of normal cell homeostasis, whereas necrosis is a passive process that occurs after massive exogenous insult (ex: ATP depletion). Necrotic cell death is characterized by cellular swelling, membrane disruption, and random DNA breakage. Furthermore, an inflammatory response initiated by the release of cellular contents can cause extensive damage to surrounding cells. Contrary to necrosis, apoptotic death is an active gene mediated process (Kerr et al., 1972). Apoptotic death involves nuclear and cytoplasmic condensation, DNA fragmentation, blebbing of the plasma membrane into membrane bound vesicles, and ultimately phagocytosis of these vesicles without induction of an inflammatory response (Kerr et al., 1972). At the biochemical level, apoptosis is a controlled proteolytic death orchestrated by cysteine proteases called caspases (MacManus and Buchan, 2000).

1.3.2 Secondary Ischemic (Reperfusion) Brain Injury

On reperfusion there is quick recovery of energy and ionic gradients. Some cells succumb to the insult itself, while others struggle to survive the toxic environment provoked by ischemia. Recovery of the brain after cerebral ischemia depends greatly on the restoration of nutritive CBF, which may be disturbed and result in secondary brain damage. Secondary brain damage is a cell injury that is not apparent immediately after the insult, but rather develops after a delay of hours to days, and has typically been observed after cerebral insults including ischemia or trauma (Hallenbeck and Dutka, 1990).

Therefore, the final outcome of ischemia is determined not only by the volume of the primary ischemic core, but can be profoundly affected by secondary brain damage to tissues surrounding the ischemic core due to brain swelling, impaired microcirculation, and inflammation (Hossman, 1997; Siesjo and Siesjo, 1996) (Figure 1.1). Secondary ischemic brain injury is mediated by several distinct, but interrelated pathophysiological events: a) the development of brain edema (Siesjo and Siesjo, 1996; Grande et al., 1997); b) post-ischemic microvascular stasis and vasomotor/hemodynamic deficits leading to hypoperfusion (Conger and Weil, 1995) and the "no-reflow" phenomenon (Fischer and Hossmann, 1995; Ames et al., 1968); c) activation of glial cells, commonly known as reactive gliosis (Juurink, 1997), and; d) post-ischemic inflammation involving mobilization of peripheral inflammatory cells (Feuerstein et al., 1997). Overall, hemodynamic disturbances may become a limiting factor for post-ischemic recovery. It is believed that secondary damage can be reduced by therapeutic measures designed to prevent or minimize molecular events leading to cell death.

Figure 1.1. Schematic flowchart of the mechanisms of cerebral ischemic injury.

Primary Ischemic Insult

↓ Cerebral Blood Flow

↓ ATP & O₂ deprivation

↓ Disruption of ion balance:
↑ [Na⁺]_i [Ca²⁺]_i [Cl⁻]_i [K⁺]_e

Reperfusion

Activation/secretion of: ROS,
endonuclease, protease, lipases

Activation of glial and
endothelial cells

Cellular swelling

BBB disruption

Cytokines/chemokines

Adhesion molecules

Cytotoxic

Vasogenic

Leukocyte activation
& infiltration

Brain edema

Inflammation

Microvascular dysfunction

Secondary ischemic brain damage

Cell Death

The mechanisms of delayed hypoperfusion and “no-reflow” following global and focal cerebral ischemia are not fully understood, but current hypotheses include: cytotoxic edema due to cell membrane injury (Tomita and Gotoh, 1992), release of vasoactive substances (prostanoids, ROS, endothelin) (Verrier, 1996), capillary plugging and increased blood viscosity (Fischer and Hossmann, 1995; Obrenovitch and Hallenbeck, 1985), and activation of leukocyte-endothelial interactions leading to mechanical obstruction (Hallenbeck et al., 1986). Although conflicting reports have been made relating to the mechanisms of hypoperfusion, the ultimate consequence is prolonged reduction in CBF contributing to secondary cerebral injury (Hossman, 1997; Dirnagl et al., 1994).

Secondary cerebral ischemic injury due to breakdown of the BBB has been well documented (Greenwood, 1991). Our understanding of the cellular mechanisms that initiate changes in BBB permeability are limited, but evidence suggests mediators such as arachidonic acid and ROS lead to compromised BBB integrity (del Zoppo and Hallenbeck, 2000). If the BBB is sufficiently deteriorated, this can lead to vasogenic edema and hypoperfusion (Siesjo and Siesjo, 1996). Furthermore, the cerebral endothelial cells of the BBB are a target for pro-inflammatory activation, and express adhesion molecules critical for leukocyte infiltration into the brain (discussed below). Once activated, cerebrovascular endothelial cells (CEC) can in turn produce autocooids (ex: prostaglandins, leukotrienes), cytokines, and nitric oxide (NO) (Stanimirovic and Satoh, 2000). These important vasoactive, permeabilizing, and pro-inflammatory mediators contribute to reperfusion and secondary cerebral injury. The inflammatory phenomena which occur in the cerebral circulation, their role(s) in

secondary ischemic brain injury and the ability of hypothermia to modulate these responses are the focus of this thesis.

1.4 Inflammation

1.4.1 General Information

Brain inflammation following cerebral ischemia is believed to develop as a consequence of microglial activation, and the mobilization and infiltration of peripheral inflammatory cells into the brain (Feuerstein et al., 1994). The development of post-ischemic brain inflammation is coordinated by activation, expression and secretion of numerous pro-inflammatory genes/mediators from the brain parenchyma and vascular cells, including cytokines, leukotrienes, and adhesion molecules (Giulian et al., 1993; del Zoppo, 1994).

The recruitment of leukocytes from the circulation into the extravascular space is a central feature of inflammation. In contrast to the lack of human studies, extensive evidence in support of leukocyte invasion following focal cerebral ischemia in animal models has been reported (Hallenbeck et al, 1986; Lee et al., 1999; Barone et al., 1991; Garcia et al., 1994; Zhang et al., 1994). Intra-parenchymal infiltration of neutrophils can be seen as early as 6-24-h after the onset of cerebral ischemia, and monocytes and macrophages invade 2-3 days later (Kochanek and Hallenbeck, 1992). Furthermore, direct evidence for increased leukocyte accumulation in microvessels following global (Villringer et al., 1991) and focal cerebral ischemia (Garcia et al., 1994) as early as 30 minutes following occlusion has been documented. The infiltration of leukocytes into brain tissue occurs earlier following transient as compared to permanent cerebral

ischemia (Zhang et al., 1994). Moreover, a recent finding by Lee and colleagues (1999) suggests that not only infiltrating leukocytes, but also the number of peripheral leukocytes is closely related to delayed neuronal damage in both global and permanent focal cerebral ischemia. Together, these findings support a direct role of leukocytes in the pathogenesis of cerebral ischemic injury.

The hallmark of cerebral ischemic inflammation is neutrophil infiltration (Matsuo et al., 1994; Garcia et al., 1994). Neutrophils are the first exogenous cells to enter ischemic tissue, and are known to release injurious mediators including cytokines, and lipid-derived mediators (Tomita and Fukuuchi, 1996; Siesjo and Katsura, 1992; Hallenbeck, 1996). Neutrophils also generate ROS (O_2^- and H_2O_2) via NADPH oxidase (Ellis et al., 1989). Furthermore, it has been suggested that neutrophils contribute to “no-reflow” and exacerbate post-ischemic hypoperfusion (Ames et al., 1968; del Zoppo et al., 1991). Other neutrophil-mediated effects following cerebral ischemia include: increased BBB permeability, secretion of vasogenic mediators (prostaglandin H_2 , thromboxane A_2), proteases (elastase, collagenase), and production of arachidonic acid (Ellis et al., 1989; Akopov et al., 1996; Hallenbeck and Dutka, 1990).

1.4.2 The Inflammatory Paradigm - Adhesion Molecules

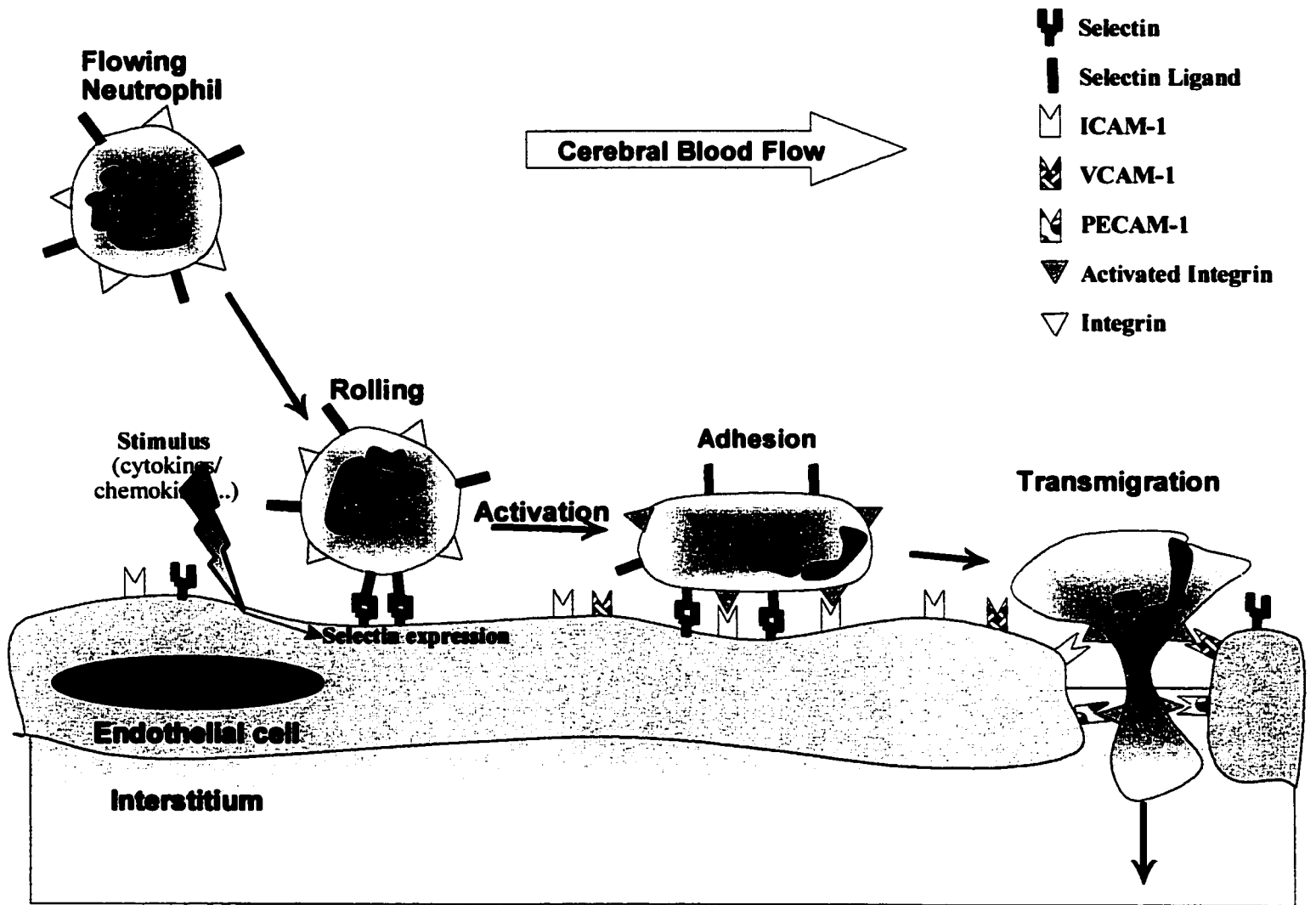
The presence of recruited leukocytes at the site of inflammation is critically dependent upon the coordinated expression of adhesion molecules on inflammatory cells and the activated vascular endothelium. The attachment of circulating blood cells to endothelium involves a variety of complementary receptors and is orchestrated by a host of signaling molecules. Normally quiescent, the endothelium maintains blood fluidity by

inhibiting coagulation and resisting the adhesion of blood leukocytes. In response to tissue injury, local cytokine and endothelial adhesion molecule expression is induced, facilitating the docking of activated immune cells to CEC. Cytokines, chemokines, lipopolysaccharides, and ROS have all been shown to induce expression, or up-regulate transcription and *de novo* synthesis of adhesion molecules (McCarron et al., 1993; Tedder et al., 1995; Lewis et al., 1988).

The prerequisite for leukocyte infiltration into the brain is the complex interaction of leukocytes and CEC mediated by three families of adhesion molecules: selectins, β_2 integrins, and the immunoglobulin (Ig) superfamily (Kochanek and Hallenbeck, 1992). Selectins recognize carbohydrate ligands, and members of the Ig superfamily recognize the β_2 integrins. The classic paradigm of inflammation consists of a rolling-activation-adhesion-transmigration cascade (Figure 1.2). In brief, circulating leukocytes tether to selectins expressed on activated endothelial cells, decelerate, roll, and sense the local environment for pro-inflammatory mediators (i.e. cytokines/chemokines). Following activation by local mediators, the strong adhesion and subsequent transmigration of leukocytes is mediated by activated leukocyte β_2 integrins and endothelial Ig superfamily adhesion molecules (Frenette and Wagner, 1996).

Leukocyte-endothelial cell rolling is mediated by the interaction of selectins with surface glycans (sialyl-Lewis^x structure), or with other selectins (Kriegelstein and Granger, 2001; Bevilacqua et al., 1994). The selectin glycoproteins are designated according to the cell type on which they were identified: E-selectin (endothelium), P-selectin (platelets and endothelial cells), and L-selectin (leukocytes) (Bevilacqua and Nelson, 1993). E-selectin is biosynthesized and expressed in response to ischemic or cytokine stimulation,

Figure 1.2. Inflammatory paradigm of leukocyte rolling-activation-adhesion-transmigration in a venule.



reaches maximal levels by 4-6 hours and returns to basal levels by 24-48 hours (Zhang et al., 1998; Bevilacqua and Nelson, 1993). Preformed P-selectin is a transmembrane protein located in platelets (α -granules) and endothelial cells (Weibel-Palade bodies), and is translocated to the cell surface in as early as 15-minutes following cerebral ischemia (Bevilacqua and Nelson, 1993; Zhang et al., 1998). In contrast to E- and P-selectins, L-selectin is constitutively expressed, and shed shortly following activation. All three selectins are induced following cerebral ischemic insults, and have roles in leukocyte-endothelial interactions (Zhang et al., 1998).

Integrins are transmembrane cell surface proteins that bind to cytoskeletal proteins and communicate extracellular signals. The integrin family of adhesion proteins is divided into three subfamilies defined by their common β subunit (β_1 , β_2 , β_3) (Cronstein and Weissman, 1993). The β_2 integrin subfamily is found on leukocytes and includes heterodimeric proteins composed of noncovalently bound α (α^L , α^M , α^X) and β (β_2) subunits. In the cluster of differentiation (CD) nomenclature, these heterodimers are referred to as CD11a/C18, CD11b/C18, and CD11c/CD18; they are also commonly called LFA-1, Mac-1, and p105 respectively. PMN leukocytes and monocytes express all β_2 integrins, whereas lymphocytes only express LFA-1 (lymphocyte function antigen-1). LFA-1 and Mac-1 are the most abundant CD11/CD18 integrins on neutrophils. Both these molecules have been shown to play roles in the firm adhesion of leukocytes to the endothelium, and are stimulated by inflammatory mediators (Carlos and Harlan, 1994). Surface levels of LFA-1 and Mac-1 on human neutrophils are approximately equal, but regulation of surface expression differs: LFA-1 is controlled by *de novo* synthesis and

expression, while Mac-1 is mediated by rapid mobilization of storage granules (Carlos and Harlan, 1994).

Members of the Ig superfamily adhesion molecules are structurally similar in that they have a variable number of Ig like domains, a transmembrane domain, and a short cytoplasmic sequence (Hogg et al., 1991). Those best characterized include intercellular adhesion molecule-1 (ICAM-1; CD54), ICAM-2 (CD102), platelet-endothelial cell adhesion molecule-1 (PECAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (Feuerstein et al., 1997). ICAM-1 is the crucial adhesion molecule that mediates leukocyte-endothelial adhesion (Sobel et al., 1990). While the mobilization of the leukocyte β_2 integrins is rapid, endothelial cell ICAM-1 is constitutively expressed at low levels and upregulation requires mRNA transcription and protein synthesis. Two transcription factors have been implicated in the regulation of endothelial ICAM-1 expression: NF- κ B and AP-1 (Collines et al., 1995). The time course of ICAM-1 expression and translation are well documented, with ICAM-1 mRNA expression beginning at 1-h, and upregulation of ICAM-1 protein 1 to 2-h following transient cerebral ischemia (Zhang et al., 1995; Clark et al., 1995). Significant increases in ICAM-1 mRNA (significant at 3-h, maximal at 6 to 12-h.) expression are also found following permanent focal cerebral ischemia, and persists for days (Zhang et al., 1995; Wang et al., 1994).

ICAM-1 binds leukocyte LFA-1 and Mac-1 (Hayflick et al., 1998). More importantly, interaction of β_2 integrins with ICAM-1 requires activation (qualitative and quantitative) of surface integrins by inflammatory mediators including cytokines/chemokines and lipid mediators. Both *in vitro* and *in vivo* models have

demonstrated the relevance of ICAM-1 in regulating leukocyte trafficking into the brain following cerebral ischemia. *In-vitro* experimentation using human cerebromicrovascular endothelial cells (HCEC) demonstrated that ICAM-1 is upregulated by exposure to cytokines (IL-1 β), and simulated oxygen glucose deprivation (Stanimirovic et al., 1997). This study also demonstrated that significant increases in adhesion of neutrophils to HCEC accompanied the up-regulation of ICAM-1. *In-vivo*, increased leukocyte infiltration following both transient and permanent focal ischemia (MCAO models) in rats accompanied increases in the expression of ICAM-1 on CEC (Wang et al., 1994). Similar results have been found in other animals including mice, and baboons (Okada et al., 1994). Furthermore, anti-ICAM-1 antibodies have been shown to limit leukocyte infiltration following cerebral insult in experimental models (Bowes et al., 1993).

Cell-cell adhesion of leukocytes to the endothelium triggers a variety of biochemical processes, including phosphorylation, cytoskeletal modification, and gene regulation (Etienne et al., 1998). Current research has shown that these processes are mediated by intracellular signal transduction within leukocytes (Richardson and Parsons, 1995), and cerebral endothelial cells (Etienne et al., 1998). Evidence suggests that intracellular signal cascades associated with integrin and/or ICAM binding are highly dependent on protein tyrosine kinase activation (ex: PKC, pp125FAK, Lyn) (Richardson and Parsons, 1995; Kansas and Tedder, 1991). Although the complete signal transduction cascade is unknown, various groups have shown that cross-linking of ICAM-1 induces tyrosine phosphorylation of several cellular proteins including Src kinases, FAK, Raf-1, and mitogen-activated protein kinases (ERK, MapKp38, JNK) (Robertson and Parsons, 1995; Holland and Owens, 1997). Although binding to ICAM-1 was found to be

necessary for the migration of leukocytes into the brain, exact signaling cascades that precede this migration are poorly understood. Etienne and colleagues have shown that ICAM-1 intracellular domain interacts with cellular actin and cytoskeleton-associated proteins, and that ICAM binding leads to phosphorylation of Rho, and ultimately cytoskeletal reorganization (Etienne et al., 1998).

Leukocyte-CEC interactions are not limited to the luminal surface; leukocytes undergo transendothelial migration whereby they interact with subendothelial structures. Typically, leukocytes leave the postcapillary venule by extending pseudopodia between apposing CEC and pulling themselves into the adjacent interstitial compartment. Transmigration from microvessels into the tissue (diapedesis) is poorly understood. The transendothelial migration seems to involve interactions between activated integrins and Ig superfamily adhesion molecules (PECAM-1, ICAM-1, VCAM-1) (Kriegelstein and Granger, 2001). However, it appears that migration of leukocytes is not an inevitable consequence of their attachment to CEC, as leukocyte adhesion is not always accompanied by transmigration (Akopov et al., 1996).

1.4.3 Inflammatory Cytokines & Chemokines

Cytokine is a term referring to a broad family of mediators, which include: interleukin-1 (IL-1), IL-6, tumor necrosis factor (TNF α), transforming growth factor (TGF), and interferon (IFN). Cytokines are low molecular weight glycoproteins produced by glia, leukocytes, endothelial cells, platelets, and other cell types (Feuerstein et al., 1998). Cytokines are among the principal mediators of inflammation (Rothwell, 1999). Ischemic brain has been shown to generate cytokines and increased levels of cytokines

have been detected in the CSF of stroke patients (Feuerstein et al., 1998; Tarkowski et al., 1997).

Each cytokine performs distinct but also overlapping functions. IL-1 is a highly inflammatory polypeptide produced following infection, or injury (Dinarello, 1996). Although the macrophage is a primary source of IL-1, leukocytes, neurons, microglia, astrocytes, and CEC are all capable of synthesizing IL-1 (Lechan et al., 1990). There are three members in the IL-1 gene family: IL-1 β , IL-1 α and IL-1 receptor antagonist (IL-1ra). IL-1 β and IL-1 α are synthesized as precursors, and are cleaved by IL-1 converting enzyme (ICE) into their active forms. IL-1 α remains intracellular, whereas IL-1 β is secreted following cleavage by ICE protease. IL-1ra is a naturally occurring IL-1 receptor antagonist. There are two IL-1 receptors: type I (IL-1RI) is a signal transducing receptor and type II (IL-1RII) is a non-signaling receptor (Kuno and Matsushima, 1994). When IL-1 binds IL-1RI, a complex forms with an IL-1 accessory protein (IL-1R-AcP), resulting in signal transduction.

IL-1 β induces several biochemical events within minutes of receptor binding. The overall pattern following IL-1 β activation includes G-protein activation on the IL-1RI, and activation of mitogen activated protein kinases (i.e. p38 MAPK) (Hopp, 1995). One of the principle events following IL-1 β activation is protein phosphorylation of inhibitory κ B (I- κ B). This leads to the dis-inhibition of NF- κ B (p50/65) transcription factor, and translocation of NF- κ B to the nucleus where it binds to promoters of a number of inflammatory genes (ex: ICAM-1, IL-1 β , and IL-8) (Beg et al., 1993; Manning et al., 1995). A downstream effect of NF- κ B activation is stimulation of adhesion molecules expression on cerebral endothelial cells following cerebral ischemia (Collins et al., 1995).

Increased levels of IL-1 β and induced expression of ICE have been demonstrated in the animal brain following injection of bacterial endotoxin, and ischemic injury (Higgins and Olschowka, 1991). A number of laboratories have shown that IL-1 β mRNA is rapidly induced by focal and global cerebral ischemia (Buttini et al., 1994; Liu et al., 1993; Zhang et al., 1998; Yabuuchi et al., 1994). Yabuuchi and colleagues (1994) localized IL-1 β mRNA in glia and perivascular cells 30-minutes following transient forebrain cerebral ischemia, whereas mRNA was detected within 15-minutes and reached a peak after 3-h following focal cerebral ischemia. Furthermore, the presence of the IL-1 β receptors has been confirmed in CEC, and mRNA for the receptor is present at high levels throughout the cerebrovascular system (Dinarello, 1996). In agreement with the time course of IL-1 β mRNA, IL-1 β protein has also been localized in cerebral ischemic brain (Betz et al., 1996). Three genes are highly sensitive to IL-1 β : iNOS, COX-2, and PLA₂. Their products (NO, prostaglandins, PAF, and ROS respectively) are all potent pro-inflammatory mediators.

Chemokines are low molecular weight proteins that entice selective leukocyte recruitment (chemotaxis) to sites of inflammation (Baggiolini, 1998) and are critically involved in leukocyte recruitment across the BBB (Baggiolini, 1998; Ransohoff, 1997). Chemokine selectivity is determined by the distribution of four cysteines in a highly conserved N-terminal domain, such as that α (CXC; prototypic member IL-8) chemokines primarily attract neutrophils, β (CC; prototypic member MCP-1) chemokines attract monocytes and lymphocytes, and δ (CX3C; neurotactin) chemokines attract both neutrophils and monocytes. Recent studies show that chemokines and their receptors are expressed in a variety of cells including HCEC, and glial cells (Zhang et al., 1999).

Chemokines have also been shown to play a role in stimulating leukocyte activation and adhesion to CEC in post-traumatic, and post-ischemic brain inflammation (Ransohoff, 1997). Increased levels of chemokines have been detected in the ischemic rat brain, and systemic administration of anti-IL-8 has been shown to reduce neutrophil infiltration, neutrophil-mediated tissue injury, and the size of cerebral infarction (Ransohoff, 1997; Mukaida et al., 1998; Matsumoto et al., 1997). The presentation of chemokines by CEC and the proximity of chemokines to target immune cells may be the decisive trigger for leukocyte activation and chemotaxis to occur (Baggiolini, 1998).

1.5 Therapeutic Interventions

1.5.1 Acute Interventions

A number of intervening strategies has been shown to inhibit/reduce injury due to mechanisms previously discussed. Genes that are anti-apoptotic (ex: bcl-2) have been overexpressed in transgenic mice and provided moderate resistance to cerebral ischemic damage in both focal and global models (Martinou et al., 1994). COX-2 inhibitors (e.g. NS398), and overexpression of radical scavenging enzymes have reduced cerebral ischemic injury (Nogawa et al., 1997; Sheng et al., 2000; Yamamoto et al., 1997). NMDA receptor antagonists have been studied (ex: MK-801, remacemide) (Small et al., 1999), and although some showed neuroprotection, others did not. Neuroprotection has been shown with Na⁺ and Ca²⁺ channel blockers (ex: tetrodotoxin, SB206284) following both focal and global cerebral ischemia (Small et al, 1999). Nonetheless, despite the large number of therapeutic interventions that decrease brain damage in experimental models, they failed to demonstrate a significant benefit in clinical trials (Grotta, 1997;

The American Nimodipine Study Group, 1992). Currently, tissue plasminogen activator (tPA) is the only approved treatment of thrombo-embolic stroke (Buchan et al., 2000).

1.5.2 Anti-inflammatory Therapies

Manipulation of the expression/release of inflammatory mediators in the brain is being investigated as a therapeutic approach to reduce secondary ischemic brain damage. In principle, anti-inflammatory therapy should be considered an adjuvant therapy to thrombolytics and/or neuroprotectants. Several therapeutic strategies that directly or indirectly target post-ischemic brain inflammation have been developed and tested in animal and human studies. The two most important approaches have been: i) targeting a single inflammatory mediator; ii) reducing the overall immune response with non-selective immunosuppressants.

Non-selective immunomodulation consists of using drugs such as glucocorticoids, cyclosporin A, or non-steroid anti-inflammatory drugs (NSAID). Animal studies have shown that cyclosporin A and NSAID ameliorate brain damage after global and focal ischemia (Li et al., 2000; Zhang et al., 2000; del Zoppo et al., 1997; Jean et al, 1998). Largely, immunosuppression strategies have been considered non-specific and non-effective.

Targeting immune cells and inflammatory mediators (i.e. adhesion molecules, cytokines) has had variable success. Blocking IL-1 β , IL-1ra over-expression, and ICE deficiency have been shown to be neuroprotective following experimental cerebral ischemia (Yamasaki et al., 1994; Yang et al, 1999; Schielke et al., 1998). Neutralizing anti-IL8 antibody, and broad-spectrum inhibitor of chemokines (NR58-3.14.3) reduced

brain edema and infarct size in experimental MCAO models (Matsumoto et al., 1997; Beech et al., 2001). Systemic injection of the TNF α inhibitor CNI-1493, as well as administration of anti-TNF α antibody have been shown to reduce brain infarct volumes after MCAO (Barone et al., 1997). Although anti-TNF α strategies have not been tested in clinical trials of stroke, TNF α neutralization has been documented in trials for heart failure, and rheumatoid arthritis (Camussi and Lupia, 1998). Neutrophil depletion and blocking or deficiency of adhesion receptors on either the neutrophil (i.e. CD18, Mac-1) or the endothelial cell (ICAM-1) has also shown benefit in animal models of cerebral ischemia (Chopp et al, 1994; Chopp et al, 1996; Kogure et al., 1996; Soriano et al, 1996; Zhang et al, 1994). The initial clinical experience with leukocyte adhesion molecule inhibitors has demonstrated safety, and in some cases efficacy as an adjuvant therapy for stroke (The Abciximab in Ischemic Stroke Investigators, 2000). However, in contrast to the protective effect of anti-adhesion molecules in animal models, recent failure of a murine anti-ICAM antibody in human stroke illustrates the difficulties in extrapolating therapeutic effects observed in the laboratory to clinical benefits in patients (DeGraba, 1998). An additional difficulty in these approaches is associated with the redundancy of inflammatory mediators, such as that blocking a single mechanism of inflammatory injury is likely offset by the detrimental affects of an alternate pathway, and is unlikely to be effective in the clinical setting.

1.5.3 Importance of Hypothermia

1.5.3.1 Historical Perspective

Many innovative therapeutic strategies for cerebral ischemia are being pursued, but none except tPA have been shown to have lasting benefit in clinical practice. Temperature modulation has now been proven to have significant benefits following brain injury including positive histological and behavioral outcomes (Corbett and Thornhill, 2000; Colbourne et al., 1997; Ginsberg et al., 1993). Unlike studies using pharmaceutical intervention, the observed histological protection is long lasting (e.g. 60-day to 1-yr) if not permanent. Consequently, hypothermia is being examined as a potential therapy for numerous insults including cerebral ischemic injury (Corbett et al., 2000) and TBI (Clark et al., 1996).

Hypothermia has been defined as mild (34-36°C), moderate (28-32°C) and deep (15-25°C) (Safar et al., 1996). Nonetheless, considerable divergence from this classification occurs so prudence must be utilized in reviewing the literature. Interest in the use of hypothermia to prevent injury during cardiac surgery and global cerebral ischemia dates back more than 50 years (Bigelow et al., 1950). Subsequently hypothermia was used to treat cerebral ischemia following asphyxia and cardiac arrest (Westin et al., 1959). Despite intensive investigations into the use of hypothermia, the temperatures used early on were too hypothermic (4-32°C) and led to complications such as infection (Biggart and Bohn, 1990), and cardiac arrhythmias (Mouritzen and Andersen, 1966).

In the late 1980's, interest in hypothermia was rekindled with reports that lowering brain temperature to a milder degree (32-34°C) provided considerable

neuroprotection following experimental global cerebral ischemia in the laboratory setting (Busto et al., 1987; Buchan and Pulsinelli, 1990; Coimbra and Cavalheiro, 1990). Furthermore, the risks of adverse effects (ex: increased blood viscosity, arrhythmia, sepsis) appear to be minimal following this more mild hypothermia. Known modern-day applications of hypothermia include: cardiothoracic open-heart surgery, neurosurgical procedures, acute head injury, and neonatal resuscitation (Wagner et al., 1999).

1.5.3.2 Intra-Ischemic and Post-Ischemic Hypothermia

Subsequent to the finding in 1990 by Buchan and Pulsinelli, there has been a surge of animal models investigating the benefits of hypothermia following cerebral ischemia. *In vitro* models have provided added support showing that hypothermia protects murine astrocytes and neuronal cells in culture against simulated ischemia (Shuaib et al., 1993). Many animal studies have shown that moderate intra-ischemic hypothermia reduces cerebral ischemic injury (Buchan and Pulsinelli, 1990; Busto et al., 1987; Coimbra and Wieloch, 1992), and provides prolonged behavioral and histological protection (Corbett and Thornhill, 2000). In global cerebral ischemia, intra-ischemic hypothermia provided virtually complete hippocampal CA1 pyramidal cell preservation at 2 months, compared to normothermic ischemic animals (Green et al., 1992). Similar neuroprotection has been reported in other rat models of global cerebral ischemia (Minamisawa et al., 1990; Dietrich et al., 1993), and in focal cerebral ischemic models (Chen et al., 1992; Barone et al., 1997; Connolly et al., 1996), spanning from young to aged animals (Corbett et al., 1997). However, the utility of cooling during cerebral ischemia is a restricted therapeutic tool since most patients who suffer ischemic insults do

not reach treatment until hours after the onset of symptoms. A clinically more fundamental question is whether post-ischemic hypothermia is beneficial.

Numerous global cerebral ischemia studies demonstrate considerable benefits with post-ischemic hypothermia (Busto et al., 1987; Safar et al., 1996; Busto et al., 1994; Coimbra et al., 1990). A large portion of this work has been done in gerbils, but similar results have recently been reported in the mouse (Yang et al., 1997), rat (Colbourne and Buchan, 1999; Coimbra and Wieloch, 1992), pig (Thoresen et al., 1995), and dog (Safar et al., 1996). Moderate post-ischemic hypothermia protects against neuronal loss in the hippocampus (CA1) and improves neurobehavioral outcome following global cerebral ischemia in the gerbil (Colbourne et al., 2000; Corbett et al., 1997). Nonetheless, controversy arises regarding of how long moderate hypothermia must be maintained to provide prolonged neuroprotection. Evidence suggests that short duration (<3h) post-ischemic hypothermia does not provide protection (Welsh et al., 1991), brief duration (3-4-h) simply delays cerebral ischemic damage (Dietrich et al., 1993), and that 12-24 h hypothermia is required to provide long lasting (1 year) protection (Colbourne et al., 1997). Moreover, moderate hypothermia is most beneficial when applied immediately following the cerebral ischemic insult and for longer durations (i.e.48-h), even though hypothermia of 48-h duration is effective even when the onset of hypothermia is delayed 6-h after severe forebrain ischemia (Colbourne and Buchan, 1999).

The investigation of post-ischemic hypothermia in focal cerebral ischemia models has been less extensive. A number of animal models have been used including mouse (Chen et al., 1992; Connolly et al., 1996; Yang et al., 1997), and rat (Kawai et al., 2000; Maier et al., 1998; Huh et al., 2000; Zhang et al., 1993). Brief duration (1-3h) moderate

post-ischemic hypothermia has been shown to provide short-term neuroprotection (Zhang et al., 1993; Maier et al., 1998). Although extended survival times have not been investigated thoroughly, one report does suggest that 24-h post-ischemic hypothermia following MCAO in a rat model does provide prolonged (1 month) neuroprotection (Corbett and Colbourne, 2000).

1.5.3.3 Potential Mechanisms of Hypothermic Neuroprotection

There is a large body of evidence that mild to moderate post-ischemic hypothermia provides both histological and functional neuroprotection. Indeed, no other experimental treatment shows comparable efficacy in different species of all ages, and safety concerns have been thoroughly investigated (Marion et al., 1993). Presently, there are numerous clinical trials comparing moderate hypothermia to normothermia following stroke (Schwab et al., 1998), cardiac arrest (Ziener et al., 2000), neonatal asphyxia (Gunn et al., 1998), and TBI (Jiang et al., 2000; Clifton et al., 2001), yet the mechanisms by which hypothermia evokes protection are still not fully understood. Proposed mechanisms include decreased BBB permeability (Dietrich et al., 1990; Smith and Hall, 1996; Jiang et al., 1992), reduced metabolic rate and O₂ consumption (Thoresen et al., 1995), attenuation of post-ischemic hypoperfusion (Karibe et al., 1994), attenuated glutamate release and enhanced re-uptake (Zhao et al., 1997; Busto et al., 1989), decreased generation of free radicals and lipid peroxidation (Lei et al., 1994; Thoresen and Wyatt, 1997), reduced apoptosis (Leonov et al., 1990; Edwards et al., 1995), and anti-inflammatory effects (Maier et al., 1998; Toyoda et al., 1996; Ishikawa et al., 1999; Whalen et al., 1997).

While cooling inhibits/reduces some deleterious processes, it also increases production of neuroprotective factors such as brain derived neurotrophic factor (BDNF), and contributes to restorative processes (D'Cruz et al., 2001). It is likely that a combination of several mechanisms accounts for the reduced neuronal injury and improved functional outcome produced by hypothermia (Leonov et al., 1990; Clark et al., 1996; Colbourne and Corbett, 1995).

1.5.3.4 Hypothermia and Inflammation

Investigations into the effects of hypothermia on inflammatory mechanisms have been limited. Moderate hypothermia (29°C) has been shown to reduce ovine leukocyte migration toward chemotaxic stimuli *in-vitro* (Akriotis and Biggar, 1985). Several studies have subsequently demonstrated that intra-ischemic (2-3h) moderate hypothermia reduces leukocyte infiltration into the brain following transient focal cerebral ischemia (Toyoda et al., 1996; Maier et al., 1998; Inamasu et al., 2001). Post-traumatic hypothermia provided similar protection (Whalen et al., 1997), and also reduced IL-1 β production (Goss et al., 1995). A recent study has revealed that 16-h post-ischemic hypothermia only delayed neutrophil accumulation from 2 to 3 days following cerebral ischemia, implying that post-ischemic hypothermia may only extend the window of opportunity for further intervention (Inamasu et al., 2000). Nonetheless, these findings support the hypothesis that reducing inflammation in the ischemic brain is a potential mechanism of hypothermic neuroprotection.

The effects of hypothermia on ischemia-induced leukocyte-endothelial interactions in the cerebral microvasculature are unknown. One report by Ishikawa et al.

demonstrates that intra-ischemic hypothermia reduces leukocyte adhesion to CEC following focal cerebral ischemia (Ishikawa et al., 1999). Siemionow and colleagues have shown that hypothermia attenuates leukocyte rolling in postcapillary venules of cremaster muscle flap in the rat after ischemic injury (Siemionow et al., 1993). Furthermore, a recent study has shown that intra-ischemic hypothermia attenuates endothelial ICAM-1 expression following transient focal cerebral ischemia (Inamasu et al., 2001). However, there is a lack of studies on the effects of post-ischemic hypothermia on *in-vivo* leukocyte-endothelial interactions in the brain.

Although there is an impressive amount of data showing that hypothermia is neuroprotective, its mechanisms of action are not fully understood, and require thorough investigation. Properly elucidating the effects of post-ischemic hypothermia on leukocyte-endothelial interactions will strengthen our understanding of the therapeutic influence of hypothermia, and potentially open doors in the future for combination therapeutic modalities.

1.6 Hypothesis and Objectives

The hypothesis tested in this study is that hypothermia will reduce leukocyte-endothelial cell interactions stimulated by inflammatory or ischemic insults in cerebral microvessels

To test this hypothesis, the following objectives were pursued:

1. To determine if systemic IL-1 β administration in mice induces neutrophil rolling-adhesion in pial microvessels at physiological temperature, and whether hypothermia can alter these responses;
2. To develop and characterize a reproducible murine global cerebral ischemia model;
3. To determine if moderate post-ischemic hypothermia provides neuroprotection in the murine global cerebral ischemia model;
4. To determine if the global cerebral ischemia in mice induces leukocyte rolling-adhesion in pial circulation at physiological temperature, and if post-ischemic hypothermia alters these responses;
5. To determine if the global cerebral ischemia in mice induces leukocyte infiltration;
6. To determine possible mechanisms by which hypothermia may affect leukocyte-endothelial interactions *in vivo*, using human neutrophil *in vitro* models

Chapter II: MATERIAL AND METHODS

2.1 Animals

Male C57/Bl6 mice (19-25 g; Charles River, Montreal, PQ) were housed two per cage in a temperature-controlled environment with a 12-h light/dark cycle and access to standard laboratory chow and water *ad libitum*. All animals were treated in strict accordance with the University of Ottawa Animal Care Committee and Canadian Council of Animal Care guidelines.

2.2 In vivo Studies

2.2.1 Anesthesia

Mice were initially anesthetized by intraperitoneal (i.p.) injection of a 10 mg/kg xylazine (Bayer Inc., Toronto, ON) and 135 mg/kg ketamine hydrochloride (BimedamTC, Cambridge, ON) mixture. Two hours following the initial injection, mice were further injected with 4 mg/kg xylazine and 55 mg/kg ketamine hydrochloride to maintain anesthesia.

2.2.2 Temperature Modulation

Temperature was monitored/maintained differently in the two *in vivo* models described in this thesis. For the IL-1 β injection model, both rectal and intra-cerebral temperatures were recorded. Conversely, for the ischemic model there was concern that insertion of a cerebral probe for intra-cerebral recordings may injure the brain, thus a

preliminary series of experiments was performed to determine the optimal mode of indirect measurement of cerebral temperature.

2.2.2.1 IL-1 β Induced Systemic Inflammation Model

Following anesthesia, a burrhole was made in the left parietal bone using a drill (Black & Decker rotary tool) and dissecting microscope. A 40-gauge thermister was then placed intracranially through the burrhole to monitor brain temperature, and a 30-gauge thermister inserted into the rectum to monitor rectal temperature. Anesthesia induced spontaneous hypothermia (32°C within 10 min) in this mouse model. Both rectal and cerebral temperatures were recorded (Dual T thermocouple thermometer, Cole-Parmer Instrument Company, Concord, ON), and rectal temperature was kept within 0.4°C of the target temperature (37 or 32°C) throughout the experimental period (i.e., during 4-h IL-1 β stimulation) using a heated water mat (K-20 Module, Baxter Healthcare Co, Valencia, CA, USA) and overhead heating lamp. Rectal temperature was maintained at 37.3°C \pm 0.3°C during ischemia for all groups. Rectal and intra-cerebral temperatures were continuously monitored and recorded every 10-minutes during IL-1 β treatment.

2.2.2.2. Preliminary Temperature Correlation in Global Cerebral Ischemia Model

Mice were randomized to one of three groups (n=3): sham-operation followed by 1-h normothermic reperfusion, ischemia followed by 1-h normothermic reperfusion (37°C \pm 0.4°C), ischemia followed by 1-h hypothermic reperfusion (32°C \pm 0.4°C).

A burrhole was made in the left parietal bone using a drill (Black & Decker rotary tool) and dissecting microscope, and a 40-gauge thermister was placed intracranially

through the burrhole to monitor intra-cerebral temperature. An incision in the cheek was then made to expose the temporalis muscle and a 40-gauge thermister inserted to measure temporalis temperature. Finally, a 30-gauge thermister was inserted into the rectum to monitor rectal temperature. Temporalis, intra-cerebral, and rectal temperatures were simultaneously monitored and recorded (Dual T thermocouple thermometer, Cole-Parmer Instrument Company, Concord, ON), and rectal temperature was kept at $37 \pm 0.3^{\circ}\text{C}$ throughout pre and intra-ischemic experimental periods, and at $37 \pm 0.3^{\circ}\text{C}$ or $32 \pm 0.3^{\circ}\text{C}$ during post-ischemic periods using a heated water mat and overhead heating lamp. Temporalis, intracranial, and rectal temperature were recorded every minute during ischemia and every 10-minutes during the 1-h reperfusion interval.

2.2.2.3 Global Cerebral Ischemia Model

Temperature modulation in the global cerebral ischemia model was executed as described in the IL-1 β model (2.2.2.1), with modification. Based on the temperature correlation experiments described above, only rectal temperature was kept within 0.4°C of the target temperature throughout the experimental period (i.e. pre-ischemic, intra-ischemic, and during reperfusion). Rectal temperatures were continuously monitored and recorded every minute during ischemia and every 10-minutes during reperfusion.

2.2.3 Experimental Model of IL-1 β Induced Systemic Inflammation

Mice were randomized to one of 4 groups: sham (n=7) operated animals received 0.9% physiological saline (300 μl) i.p. and were maintained at $37^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$ rectally for 4-h; control (n=6) animals were injected with 5 $\mu\text{g}/\text{kg}$ IL-1 β (Upstate Biotechnology,

Lake Placid, NY) i.p. and maintained at $37^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$ rectally for 4-h; hypothermic animals were injected with $5\mu\text{g}/\text{kg}$ IL- 1β i.p. and maintained at $32^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$ rectally for either 1-h (n=7) just prior to video recordings or for 4-h (n=7) commencing immediately after IL- 1β injection. At 4-h post IL- 1β injection, intravital microscopic recordings were made of leukocyte rolling and adhesion in pial venules (Intravital microscopy, see 2.2.5).

2.2.4 Experimental Model of Cerebral Ischemia

2.2.4.1 General Preparation

Mice were randomized to sham-operated or ischemic groups. BNP (bacitracin-neomycin-polymixin, Vetcom Inc., Upton, PQ) was applied to both eyes to prevent drying/infection. Ischemic animals were subjected to 10-minutes of bilateral common carotid artery (BCCA) occlusion (i.e. 2VO). In short, a midline incision in the neck was made to expose the trachea and the common carotid arteries were carefully isolated. Silk suture (5.0) was wrapped around the carotids, and retracted till blood flow was blocked. All mice were kept normothermic during ischemia (see 2.2.2.3). Immediately following 10-minutes 2VO, bupivacane was applied to the incision to alleviate irritation and the incision was closed using tissue adhesive (VetBond, 3M Animal Care Products, MN, USA). For the sham-operated animals, BCCA were exposed for 10-minutes but no occlusion was performed.

To determine histological brain damage, astrocytosis, and leukocyte infiltration, mice were randomized into the following 4 groups:

- 1) Sham-operation followed by 4-h normothermic reperfusion ($37^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$)
- 2) Ischemia followed by 4-h normothermic reperfusion ($37^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$)

- 3) Ischemia followed by 4-h hypothermic reperfusion ($32^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$)
- 4) Ischemia followed by 4-h hyperthermic reperfusion ($39^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$)

Immediately following the 4-h reperfusion period, animals were returned to housing. Mice were sacrificed and brains removed at 1, 3, or 7 days post-ischemia for histological analysis.

To determine post-ischemic inflammatory events and the effects of post-ischemic hypothermia on inflammation in the pial microcirculation *in vivo*, 7 experimental groups were used:

- 1) Sham (n=4) 5-min normothermic reperfusion ($37^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$)
- 2) Sham (n=4) 2-h normothermic reperfusion ($37^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$)
- 3) Sham (n=4) 4-h normothermic reperfusion ($37^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$)
- 4) 2VO (n=6) 5-min normothermic reperfusion ($37^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$)
- 5) 2VO (n=6) 2-h normothermic reperfusion ($37^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$)
- 6) 2VO (n=6) 4-h normothermic reperfusion ($37^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$)
- 7) 2VO (n=6) 2-h hypothermic reperfusion ($32^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$)

Following the indicated periods of reperfusion, intravital microscopic recordings were made of pial venule leukocyte rolling and adhesion (see 2.2.5).

2.2.4.2 Cerebral Blood Flow Measurement

Mice (n=4) were prepared for BCCA occlusion (see 2.2.4.1). Rectal temperature was monitored (Dual T thermocouple thermometer, Cole-Parmer Instrument Company, Concord, ON), and kept at $37 \pm 0.5^{\circ}\text{C}$ throughout the experimental period using an

overhead heating lamp. The head was fixed in a stereotactic frame and a burrhole was made in the left parietal bone using a drill (Black & Decker rotary tool) and dissecting microscope. Regional cerebral blood flow (rCBF) in the cortical surface vessels was measured with laser-Doppler (Model MP100 with TSD145 micro-needle probe, Biopack system Inc., Santa Barbara, CA), which quantitatively measures changes in microvascular rCBF over a wide range within a tissue volume of approximately 0.5 mm³. CBF measurements were made through the burrhole: 5 and 1 minute before ischemia; at 0, 5, and 10 minutes during occlusion; and 1, 5, 10 and 20 minutes following ischemia. All data were represented as percentages of baseline blood cell perfusion units (BPU; 130 ml/100gm tissue/min).

2.2.4.3 Arterial Blood Gas and pH

Nine mice were sacrificed for arterial blood gas and pH analysis. After anesthesia, all mice were prepared for BCCA occlusion (see 2.2.4.1), and rectal temperature kept at $37 \pm 0.5^{\circ}\text{C}$ using an overhead heating lamp. The left femoral artery was exposed. Arterial blood gases (pO₂, pCO₂) and pH were measured 10 minutes before ischemia (n=3), at 10-minutes during ischemia (n=3), and 5-minutes after reperfusion (n=3). Blood samples (100 μl) were extracted from the left femoral artery for each measurement (stat profile pOHx blood gas analyzer; Nova Biomedical, Mississauga, ON), and mice immediately sacrificed.

2.2.5 Cranial Window and Intravital Microscopy

Three hours post IL-1 β injection, or immediately before BCCA occlusion, 1.5-h and 3.5-h after BCCA occlusion, an open cranial window was made in the right parietal bone as described by Rosenblum et al. (1994). Briefly, the scalp was retracted and craniotomy performed with a small drill (Black & Decker rotary tool) and forceps. The dura was retracted with the bone using very fine tipped forceps (0.2 x 0.12 mm Dumont forceps, Fine Science Tools Inc., North Vancouver, BC). The area of exposed cerebral surface was then superfused with warmed artificial cerebrospinal fluid (150mM Na, 3mM K, 1.4mM Ca, 0.8mM Mg, 1.0mM P, 155mM Cl, 18mM HCO₃) bubbled with a special gas mixture (6%CO₂, 10%O₂, balance N₂) (Praxair, Mississauga, ON).

An intravenous line (27-gauge Winged infusion set, Terumo, Tokyo, Japan) was then inserted into the tail vein. Mice were placed under an intravital microscope (Olympus BHMJ modular focusing mount and BH2-RFCA illuminator on a custom made stand, Hitachi CCD video camera, Sony monitor and Panasonic WJ-810 time code generator). Acridine orange (17 μ g/kg) (Sigma Chemicals Co, St. Louis, MO, USA) was infused intravenously, and the microscope was focused on 20-50 μ m diameter pial venules using a 20X long working distance objective. The microcirculation was illuminated with a mercury vapor lamp through a 495/535 nm excitation/emission filter (BH2-DMV, Olympus) for fluorescence or with a fibreoptic epi-illuminator (Olympus highlight 3000) for bright field illumination.

At 4-h post IL-1 β injection, or 5-min, 2-h and 4-h following ischemia, videocassette recordings were made using a Mitsubishi (model HS-U69) sVHS video recorder for 30 seconds each at 2-4 venules per animal. Mocha (Jandel Scientific Inc.,

San Rafael, CA) image analysis software was used for measuring microvessel diameter and distances in the microvessels following capture of an image to a personal computer using a Targa+ (Truevision Inc., Indianapolis, USA) framegrabber.

The number of leukocytes rolling and adhering to pial venules was determined in a blinded fashion during playback of the videotapes (Dirnagl et al., 1994). Rolling leukocytes were quantified as the number of rolling cells passing a reference line in the pial venules over 30 seconds. Adhering leukocytes were quantified as those adhering along a 100 μm length of the pial venule wall for longer than 2 seconds over the 30 second recording.

2.2.6 Immunohistochemical Detection of DNA Fragmentation Using *In Situ* End Labeling

Ischemia-induced DNA fragmentation was assessed using *in situ* end labeling (ISEL) as described by Xu et al. (1997). Mice whose brains were used for ISEL (n=4/group) were sacrificed by i.p. injection of 250 mg/kg sodium pentobarbital (MTC Pharmaceuticals, Cambridge, On) at 3 days post-ischemia or sham-operation. Brains from each group were removed and frozen in 2-methylbutane (Sigma-Aldrich, Mississauga, ON) on dry ice, and stored at -80°C in an upright freezer. Coronal sections (10 μm) were made at bregma 1.1, -0.22, -1.94, and -3.16 mm using a microtome cryostat (Microm HM 5000 M).

Fresh frozen cryostat sections were thawed and fixed with 1% glutaraldehyde in 0.1 M PBS (pH 7.3) for 15-minutes at room temperature. Sections were washed three times for 5-minutes in 0.01 M PBS followed by permeabilization in methanol/acetone

(1:1) for 10-minutes, and another three washes in PBS. Sections were then incubated with 20 µg/ml proteinase K in 25 mM Tris-HCl (Boehringer Mannheim, Laval, PQ) (pH 6.6) for 15-minutes at room temperature. The tissue was then washed three times for 5 minutes each with PBS, and incubated in ISEL reaction mixture containing 2 mmol/L CoCl₂ (Boehringer Mannheim, Laval, PQ), 10 µmol/L biotin-16-dUTP (Boehringer Mannheim), terminal transferase buffer (Boehringer Mannheim), ddH₂O and 25 U terminal transferase (Boehringer Mannheim, Laval, PQ) for 1-h at 37°C in a dark humid chamber.

The reaction was terminated by washing the sections three times (1 minute per wash) with PBS and incubating them with 250 µl/slide of staining solution containing 2.5 µg/ml avidin-FITC (Sigma Chemicals Co, St. Louis, MO, USA), sodium citrate buffer, 0.1% triton-X and 0.5% powdered milk for 30-minutes at room temperature in the dark. The sections then underwent a final three washes in PBS (1 minute each) and were coverslipped with antifade solution containing 1 mg/ml *p*-phenylenediamine in 90% glycerol in PBS. FITC positive nuclei were visualized using an upright epifluorescence microscope (Zeiss Axiophot microscope and Zeiss Plan-Neofluoar objectives, Germany). Images were captured using Northern Eclipse software and a SONY 3CCD color video camera.

2.2.7 NeuN Immunohistochemistry

NeuN immunoreactivity was detected using the monoclonal antibody A60, generously donated by Dr. M. McBurney. A60 (or NeuN) was generated against brain cell nuclei and recognizes the neuron-specific protein NeuN (neuronal nuclei). The

antibody labels nuclei and perikarya of most neuronal cell types found in the nervous system of mice, and crossreacts immunohistochemically with neural tissue from rats, chicks, humans, and salamanders (Mullen et al., 1992, Wolf et al, 1996).

Mice brains used for NeuN immunohistochemistry (n=4/group) were sacrificed at 7 days post-ischemia or sham-operation with i.p. injection of 250 mg/kg sodium pentobarbital (MTC Pharmaceuticals, Cambridge, ON). Mice were transcardially perfused with 10 ml 0.9% saline, followed by 10 ml 4% paraformaldehyde (PFA) (pH 7.6) in 0.1 M phosphate buffer. Perfused tissues were postfixed in 4% PFA overnight, then cryopreserved in a 10% sucrose phosphate buffered solution for 48-h at 4°C. Brains were frozen using gaseous carbon dioxide, and coronally sectioned (10 µm) using a microtome cryostat (Microm HM 5000 M) at bregma 1.1, -0.22, -1.94, and -3.16 mm for analysis. Sections were stored floating in 0.01 M phosphate buffered saline (PBS) at 4°C in 24-well plates.

Immunohistochemistry was performed using a standard procedure described by Xu et al. (1997). Free-floating sections were washed 10-minutes with 0.01 PBS and then incubated for 10-minutes in PBS containing 0.3% hydrogen peroxide to block endogenous peroxidase activity. Sections were then washed three times in 0.01 M PBS, and incubated for 48-h at 4°C in NeuN primary antisera (1:200, containing PBS with 0.3% Triton-X). Sections were then washed three times in PBS and incubated in biotin-labeled donkey anti-mouse secondary antibody (1:200, Jackson laboratories, Mississauga, ON) for 12-h at 4°C. Once again, sections were washed three times in PBS, followed by incubation for 3-h at room temperature in PBS containing 0.3% Triton X-100 and streptavidin-horseradish peroxidase (1:200, Amersham Life Science Inc., Oakville, ON).

After three more washes, the sections were rinsed in 0.1M acetate buffer (pH 6.0) and the reaction was visualized using a glucose oxidase-diaminobenzidine (DAB) nickel method (Shu et al., 1988). The reaction was terminated by washing in 0.1 M acetate buffer (pH 6.0) and sections were mounted on gelatin-coated slides, dehydrated through a graded series of alcohols and xylene, and coverslipped for bright field microscopic observation. Two negative controls were always included: one processed as described above but lacking the addition of the NeuN antibody, the other processed as described above but using a non-specific mouse IgG2a primary antibody (1:200, Sigma-Aldrich, Mississauga, ON) in the place of the NeuN antibody.

2.2.8 Quantification of NeuN Immunoreactivity: Damage Score

NeuN immunoreactivity was quantified to evaluate extent of damage 7 days post-ischemia. Damage was evaluated using light microscopy (Wild Leitz Inc., Rockleigh, NJ, USA) by a blinded neuropathologist (Dr. J. Michaud, Children's Hospital of Eastern Ontario), and scored on a 5-point scale (0= no damage, 1= one small focus, 2= two small foci, 3= more than two small foci, 4= one or more large foci with widespread damage).

2.2.9 Glial Fibrillary Acidic Protein Immunohistochemistry

Activation of astrocytes or reactive gliosis is characterized by cellular hyperplasia of astrocytes as well as hypertrophy of the cell bodies and processes (Nathaniel and Nathaniel, 1981). The gliotic response at the molecular level is characterized by an increase in the metabolism of cytoskeletal intermediate filaments (i.e. glial fibrillary acid

protein; GFAP). Due to the specificity of GFAP in astrocytes, it is a good marker for activation of astrocytes in response to CNS injury.

Mice whose brains were used for GFAP immunohistochemistry were sacrificed at 3 days (n=4/group) and 7 days (n=4/group) reperfusion. Mice were transcardially perfused with 10 ml 0.9% saline, followed by 10 ml 4% PFA (pH 7.6) in 0.1 M PB. Perfused tissues were post-fixed in 4% PFA overnight, then cryopreserved in a 10% sucrose phosphate buffered solution for 48-h at 4°C. Brains were frozen using gaseous carbon dioxide, and coronally sectioned (10 µm) using a microtome cryostat (Microm HM 5000 M) at bregma 1.1, -0.22, -1.94, and -3.16 mm onto slides for analysis.

Immunohistochemical detection of GFAP was performed using a rabbit anti-mouse polyclonal antibody that strongly reacts with human GFAP protein. Slide mounted sections were washed three times with 0.01 M PBS and then incubated for 48-h at 4°C with the primary GFAP antibody (1:3000, Dako Diagnostics Canada Inc., Mississauga, ON) in 0.01 M PBS containing 0.3% Triton X-100. Sections were washed another three times in 0.01 M PBS and incubated in the dark for 3-h at room temperature in PBS containing 0.3% Triton X-100 and FITC labeled donkey anti rabbit IgG (1:200, Amersham Life Science Inc., Oakville, ON). Sections were further washed three times in 0.01 M PBS in the dark, and coverslipped using antifade (1 mg/ml *p*-phenylenediamine in 90% glycerol in PBS).

FITC positive immunoreactivity was visualized using an upright epifluorescence microscope (Zeiss Axiophot microscope and Zeiss Plan-Neofluoar objectives, Germany). Images were captured using Northern Eclipse software and a SONY 3CCD color video camera. Two negative controls were included: one processed as described above but

lacking the addition of the GFAP antibody, the other processed as described above but using a non-specific mouse IgG2a primary antibody (1:200, Sigma-Aldrich, Mississauga, ON) instead of the GFAP antibody.

2.2.10 Myeloperoxidase Assay

Myeloperoxidase (MPO) is a heme lysosomal enzyme present in the azurophilic granules of neutrophils. MPO activity was used as an indicator of tissue polymorphonuclear leukocyte (PMNL) accumulation in ischemic mouse brains (Bradley et al., 1982). The assay is based on the ability of MPO to oxidize o-dianisidine (O-D) in the presence of H₂O₂ to generate a colored complex, which can be monitored spectrophotometrically. We used the procedure described by Barone et al. (1991) with minor modifications, for the quantification of MPO activity.

To eliminate intravascular blood, 1-d post-ischemic or sham-operated mice were intracardially perfused with 10 ml cold 0.9% NaCl (n=4/group). Brains were removed and frozen in 2-methylbutane (Sigma-Aldrich, Mississauga, ON) on dry ice, and stored at -80°C in an upright freezer. Brains were thawed, weighed in milligrams wet weight, and put on ice. Each brain was homogenized at 10,000 rpm (Model PT/1035 Polytron, Brinkman Instruments Inc., Westburg, NY, USA) in 1:20 (wt/vol.) 5mM PB (pH 6.0) for three on/off cycles at 10-second intervals. Brain homogenate was centrifuged (Beckman Avanti J-25, Beckman Instruments, Palo Alto, California, USA) at 30,000 g for 30 minutes at 4°C, and the supernatant discarded. The pellet was washed again as described above, and the pellet extracted by suspension in 1:2 (wt/vol.) 0.5% hexadecyltrimethylammonium bromide (HTAB; Sigma Chemicals Co, St. Louis, MO,

USA) in 50 mM potassium phosphate buffer (pH 6.0, 25°C) for 2-minutes. The samples were immediately frozen on dry ice. Freeze and thaw cycles were then performed three times. Samples were incubated at 4°C for 20-minutes, and centrifuged at 12,500 g for 15 minutes at 4°C. The supernatants (100 µl aliquots) were mixed with 580 µl reaction solution of 50 mM PB (pH 6.0), containing *o*-dianisident dihydrochloride (0.167 mg/ml; Sigma Chemicals Co, St. Louis, MO, USA), and hydrogen peroxide (0.005%). The reaction was measured with a spectrophotometer (Beckman DU-7) for 10-minutes at 460 nm, in duplicate. Aliquots of neutrophil rich peritoneal exudate were used as controls (see 2.2.12). Results were reported in U/gm wet weight, where 1 unit of MPO activity is defined as that degrading 1µmol peroxide/minute at 25°C.

2.2.11 Neutrophil/DAPI Double Labeling

Neutrophil immunoreactivity was determined by labeling with a rat anti-mouse monoclonal neutrophil antibody (CL899AP, Cedarlane[®], Hornby, ON), and counterstaining with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes, Oregon, USA). This neutrophil antibody recognizes a polymorphic myelomonocytic antigen 7/4 found at high levels on polymorphonuclear leukocytes (PMNL), but absent from macrophages, and expressed at very low levels on monocytes (Gordon et al., 1992). Blue fluorescent DAPI is a nuclei acid stain that associates with AT clusters in the minor groove of dsDNA, producing a 20-fold nuclear fluorescence enhancement.

Mice whose brains were used for mouse neutrophil immunohistochemistry were sacrificed at 1 day (n=4/group) reperfusion. Mice were transcardially perfused with 10

ml. 0.9% saline, followed by 10 ml 4% PFA (pH 7.6) in 0.1 M PB to wash out neutrophils in the microcirculation and thus quantify only infiltrating neutrophils. Perfused tissues were postfixed in 4% PFA overnight, then cryopreserved in a 10% sucrose phosphate buffered solution for 48-h at 4°C. Brains were frozen using gaseous carbon dioxide, and coronally sectioned (10 µm) onto slides using a microtome cryostat (Microm HM 5000 M) at bregma 1.1, -0.22, -1.94, and -3.16 mm for immunohistochemical analysis.

Immunohistochemical detection of CNS infiltrated neutrophils was performed on slide mounted sections. Sections were fixed in 4% PFA for 5-minutes, and washed three times with 0.01 M PBS (10-minutes each). Sections were then incubated 30-minutes at room temperature in prehybridization blocking solution containing 5% goat serum and 0.3% Triton X-100 in 0.01 PBS. Slides were shaken dry, and incubated 1-h at room temperature with the neutrophil antibody (1:50) in PBS containing 0.1% goat serum and 0.3% Triton X-100. Sections were washed three times in 0.01 M PBS and incubated in the dark for 3-h at room temperature in PBS containing 0.3% Triton X-100 and FITC labeled goat anti-rat IgG (1:200, Amersham Life Science Inc., Oakville, ON). Sections were further washed three times in 0.01 M PBS in the dark, and slides were counterstained with DAPI (1:1000) in PBS containing 0.3% Triton X-100 for 5-minutes at room temperature. Slides were washed a final three times in 0.01 M PBS, and coverslipped using antifade (1 mg/ml *p*-phenylenediamine in 90% glycerol in PBS).

Neutrophil peritoneal exudate slides were used as positive controls (see 2.2.12). Two negative controls were also included: one processed as described above but lacking the addition of the neutrophil antibody, the other processed as described above but using

a non-specific mouse IgG primary antibody (1:200, Sigma-Aldrich, Mississauga, ON) instead of the neutrophil antibody.

Neutrophil and DAPI positive immunoreactivity was visualized using an upright epifluorescence microscope (Zeiss Axiophot microscope and Zeiss Plan-Neofluoar objectives, Germany). Images were captured using Northern Eclipse software and a SONY 3CCD color video camera.

2.2.12 Isolation of Neutrophil Exudate: Positive Controls

Samples of neutrophil peritoneal exudate were collected from animals (n=4) 12-h following injection of 2% glycogen (Sigma Chemicals Co, St. Louis, MO, USA). Briefly, mice were injected i.p. with 2ml 2% glycogen. Approximately 12-h post injection, mice were sacrificed, and injected i.p. with 2 ml ice-cold Hank's (Calcium Magnesium free) balanced salt solution (HBSS; Sigma Diagnostics, St. Louis, MO, USA) containing 5 U/ml heparin. The abdomen was massaged two minutes with thumb and forefingers to bath the intestines, mesenteries, and crevices in order to suspend the inflammatory cells. Following this, the peritoneal cavity was opened, and the suspension collected. The peritoneal cavity was flushed again with 2 ml ice-cold heparinized HBSS, and total suspension centrifuged at 1000 rpm, 10-minutes at 4°C. The pellet was resuspended in 0.01 M PBS (final dilution 1:48). Aliquots of neutrophil rich peritoneal exudate were used as controls for MPO, while others were cytopun (Cytospin 2, Shandon Southam Products Ltd., Cheshire, UK) 10-minutes at 60 rpm onto charged slides and stored at -20°C for use as positive controls in neutrophil immunohistochemistry.

2.3 In-vitro Studies

2.3.1 Neutrophil Isolation

The protocol was approved by the Children's Hospital of Eastern Ontario Human Ethics Committee. Human neutrophils were isolated from fresh, EDTA-treated venous blood obtained from healthy adult volunteers. The neutrophil-containing band was separated by centrifugation at 1580 rpm for 30-minutes at room temperature through discontinuous polysucrose-sodium diatrizoate (Histopaque1077/1119; Sigma Diagnostics, St. Louis, MO, USA). The neutrophil band was collected and contaminating erythrocytes removed by two cycles of brief hypotonic lysis in ice-cold 0.15% NaCl, and centrifugation at 1400 rpm for 10-minutes at room temperature. Neutrophils were counted using a hemocytometer, then labeled with 10 μ m calcein-AM (Molecular Probe, OR, USA) in PBS for 20-minutes at 37°C. The cells were centrifuged at room temperature for 5-minutes at 1400 rpm, washed twice in Hank's Balanced salt solution (HBSS; Sigma Diagnostics, St. Louis, MO, USA), and re-suspended in HBSS to achieve 60,000 cells/well.

2.3.2 Neutrophil Chemotaxis

Neutrophils were submitted to the following conditions: (1) 2-h HBSS at 32°C or 37°C, (2) 2-h IL-1 β (100 u/ml) stimulation at 32°C or 37°C, (3) 1-h hypoxia (<2% O₂ in hypoxic chamber; Anaerobic System model 1024, Forma Scientific) followed by 1-h recovery (exposure to ambient air) at 32°C or 37°C. Following exposure to the appropriate condition, chemotaxis of labeled neutrophils were assessed by a quantitative *in vitro* method using a 96-well chemotaxis assembly membrane (ChemoTx[®], Neuro Pore

Inc., MD, USA) consisting of a polycarbonate filter, as described by Junger et al. (1993). Briefly, the wells of the plates were loaded with Buffer or IL-8 (0.2 µg/ml, Biosource International, Camarillo California). Labeled neutrophils (60,000) from each condition were also added to one well for each condition in order to provide a maximum fluorescence reading per treatment. A framed filter was positioned on top of the plate, 60,000 labeled neutrophils were applied on the top of each membrane/well (except for the wells for maximum readings), and the assembly incubated 80-minutes at 37°C. The number of neutrophils which transmigrated into the wells of the 96-well plate were quantified by measuring intensity of fluorescence (excitation/emission: 485/530 nm) in a CytoFluor 2350 fluorescence microplate reader (Millipore, Bedford, MA, USA), and reported as relative ratios compared to maximum readings.

2.3.3 Analysis of Neutrophil CD18 Expression by Flow Cytometry

Samples of whole blood were collected from healthy volunteers (n=3) into heparinized tubes, and were analyzed within 2-h. Whole blood was diluted 1:10 in PBS, and aliquoted into 24-well culture plates (Falcon, Lincoln Park, NJ). Control cells and cells exposed to IL-1β (100 u/ml; Upstate Biotechnology, Lake Placid, NY), peptide neutrophil chemoattractant fMLP (5mM; Sigma Chemicals Co, MO, USA), or bacterial lipopolysaccharide (LPS; 1 µg/ml; Sigma Chemicals Co, MO, USA) were incubated: 15 minutes at 32°C, 15-minutes at 37°C, 1-h at 32°C, or 1-h at 37°C. Cells were harvested and washed with PBS/0.1% NaN₃. Contaminating erythrocytes were removed by two cycles of brief hypotonic lysis in ice-cold 0.15% NaCl. Cells were then resuspended in PBS/NaN₃, and IgG (2 mg/ml) added for 10-minutes to block non-specific binding.

Surface expression of CD18 was examined using one-color flow cytometric analysis. Stimulated and control cells were incubated with 10 μ l (determined by titration) mouse anti-human FITC-CD18 mAb (BD Biosciences, Mississauga, ON) for 15-minutes at room temperature in the dark, washed and resuspended in PBS/0.1% NaN₃. Autofluorescence and isotype control (FITC-IgG1/2; Sigma Chemicals Co, MO, USA) was determined in all groups. Neutrophils were gated based on forward scatter/side scatter characteristics. CD18 expression was then measured in these gated populations as mean channel fluorescence intensity (Becton-Dickinson FACScan Flow Cytometer). A total of 50 000 events were recorded for each sample. This data was analyzed using the WinMDI software package (J. Trotter, Scripps Institute, San Diego, CA).

2.4 Statistical Analysis

All data are expressed as mean \pm SD. Differences between experimental groups were compared using one-way ANOVA followed by Bonferroni's multiple comparison. Pearson correlation ($r > 0.9$) was used for determination of temperature correlation. Statistical significance was considered at $p < 0.05$ for all experiments.

Chapter III: RESULTS

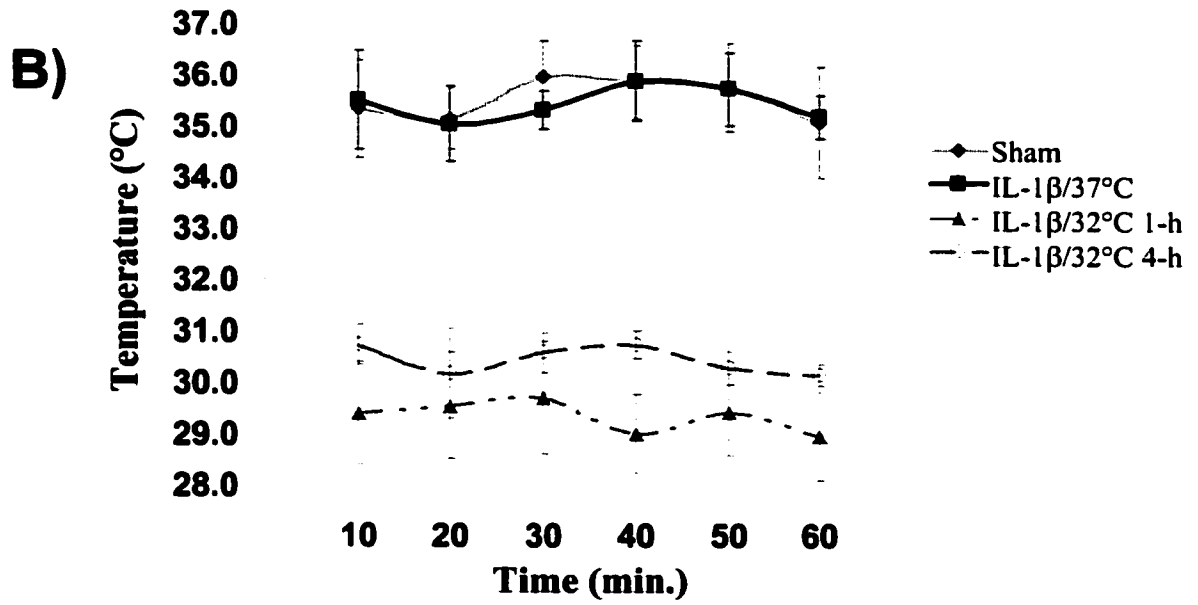
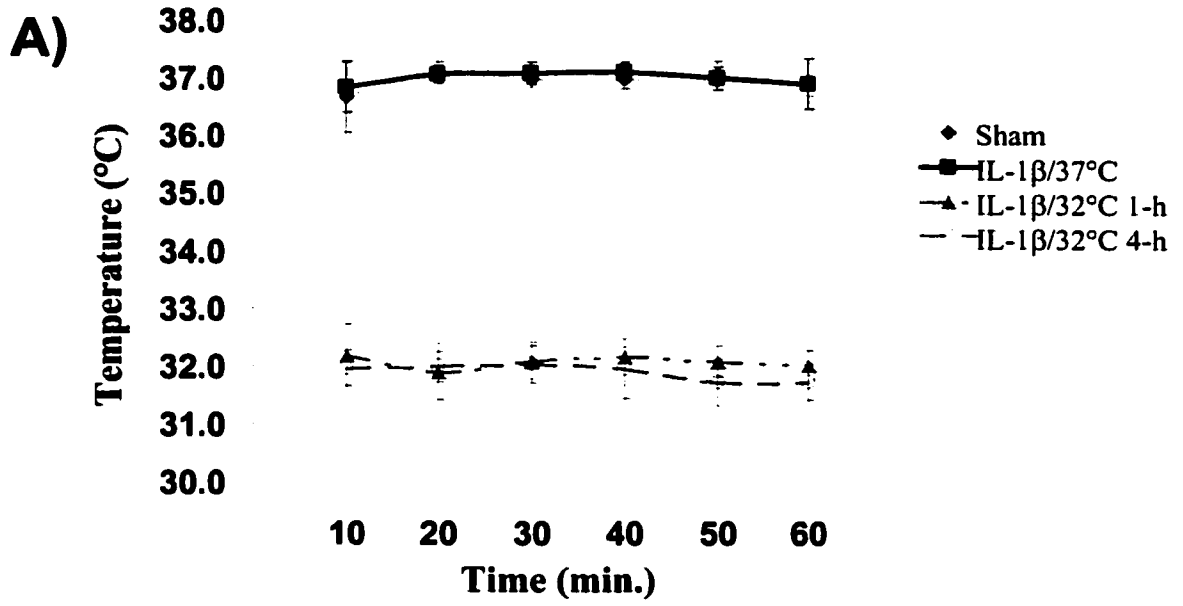
Two models were employed to induce cerebral inflammation: IL-1 β injection and cerebral ischemia. The first model (IL-1 β injection) was used to investigate the effects of hypothermia on cytokine-induced inflammation. These experiments were followed by the development and characterization of a murine cerebral ischemia model required to subsequently evaluate the effects of hypothermia on cerebral ischemia-induced inflammation.

3.1 Effects of Moderate Hypothermia on IL-1 β Mediated Cerebral Inflammation

3.1.1 Temperature Recordings Following IL-1 β Stimulation

Temperature was monitored and recorded throughout the experimental period in order to determine effective maintenance of normothermic (37°C) and moderate hypothermic (32°C) core and intra-cerebral temperatures. Both rectal (Figure 3.1A) and intra-cerebral (Figure 3.1B) temperatures were measured at 10-min intervals, while rectal temperature was tightly maintained normothermic or hypothermic. Both rectal and intra-cerebral temperatures in the hypothermic groups were significantly reduced compared to the normothermic groups by 4.5-5°C (Figure 3.1A & 1B), indicating effective maintenance of moderate hypothermia in this mouse model. No significant variations in single animals or between animals within any group were found. Cerebral temperature was lower than rectal temperature in all experimental groups. Similar observations have been reported by other research groups (Maier et al., 1998).

Figure 3.1. Temperature recordings in four experimental groups of mice (Sham, 37°C - closed diamond; IL-1 β , 37°C- closed square; IL-1 β , 32°C for 1 h-closed triangle; IL-1 β , 32°C for 4 h-open circle) used in the study. Rectal (A) and intra-cerebral (B) temperatures were recorded every 10-minutes over the 4-h period after i.p. administration of IL-1 β (5 μ g/kg). Rectal temperature was maintained using a heated water mat and heating lamp. The data shown are means \pm SD of temperature recordings taken from 6-7 animals per group at 10-min intervals over the 1-h period just prior to videomicroscopy recordings.



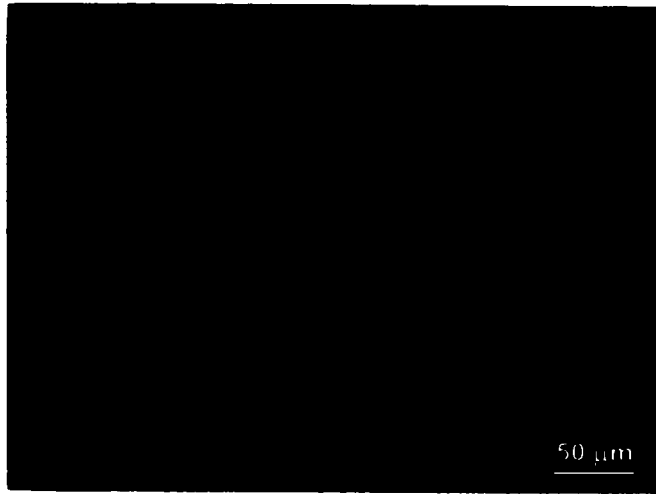
3.1.2 Effects of Hypothermia on Leukocyte Rolling-Adhesion Induced by Systemic Injection of IL-1 β

Intravital microscopy and an open cranial window were used to visualize mouse pial venules 4-h following IL-1 β or saline injection. Leukocyte rolling and adhesion were assessed by staining leukocytes *in vivo* with the fluorescent dye acridine orange, which has been previously shown to have no effect on leukocyte trafficking (Janssen et al., 1994). In the saline injected (sham) group there was virtually no rolling or adhering leukocytes observed (Figure 3.2A). An increase in numbers of rolling and adhering leukocytes was seen in animals injected with IL-1 β as compared to sham controls (Figure 3.2A & B).

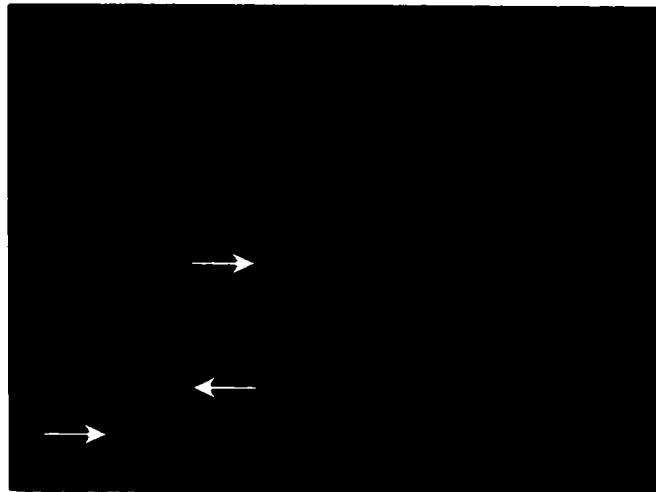
At 4-h post IL-1 β injection videocassette recordings were made of 2-4 venules per animal. Quantitative analysis showed that IL-1 β stimulation at 37°C induced significant increases in both rolling (Figure 3.3) and adhering (Figure 3.4) leukocytes after 4-h. In animals subjected to 1-h 32°C hypothermia 3-h after IL-1 β injection, the numbers of rolling and adhering leukocytes were not different from those recorded in the normothermic IL-1 β injected group (Figure 3.3 & 3.4). However, in animals subjected to a 32°C hypothermia for 4-h after IL-1 β injection, a complete inhibition of leukocyte rolling (Figure 3.2C; Figure 3.3) and attenuation of leukocyte adhesion (Figure 3.4) was detected. For all pial vessels in which rolling and adhesion were assessed, vessel diameter was measured (Figure 3.5). This measurement was taken since vessel size could influence shear flow rates and the area of vessel wall available for rolling and adhesion. All venules chosen for measurements of leukocyte rolling and adhesion were 20-50 μ m

Figure 3.2. Representative images of pial venules obtained during open cranial window recording of leukocyte rolling and adhesion with a CCD camera. A) Sham-operated control at 37°C; B) IL-1 β (5 μ g/kg i.p.) injected animals maintained at 37°C for 4-h; C) IL-1 β (5 μ g/kg i.p.) injected animals maintained at 32°C for 4-h. Intravital microscopy (20X long objective) recordings were done after systemic injection of acridine orange as described in Materials and Methods. Arrows indicate leukocytes adhering to endothelial cells lining the walls of the microvessels. Bar=50 μ m.

A)



B)



C)



Figure 3.3. The effects of hypothermia on IL-1 β -induced leukocyte rolling in the pial microcirculation of mice. Average numbers of rolling leukocytes in mouse pial venules were determined over a 30-second period 4-h after injection of saline (sham control) or IL-1 β (5 μ g/kg i.p.) as described in Materials and Methods. Hypothermia (32°C) was applied to IL-1 β -injected groups for either 1-h or 4-h. Values shown are means \pm SD, n = 7 mice/group. * Indicates a significant difference (p<0.01; one way ANOVA followed by Bonferroni's multiple comparisons of means) compared to sham. # Indicates a significant difference (p<0.01) compared to IL-1 β -treated cells for 4-h at 37°C.

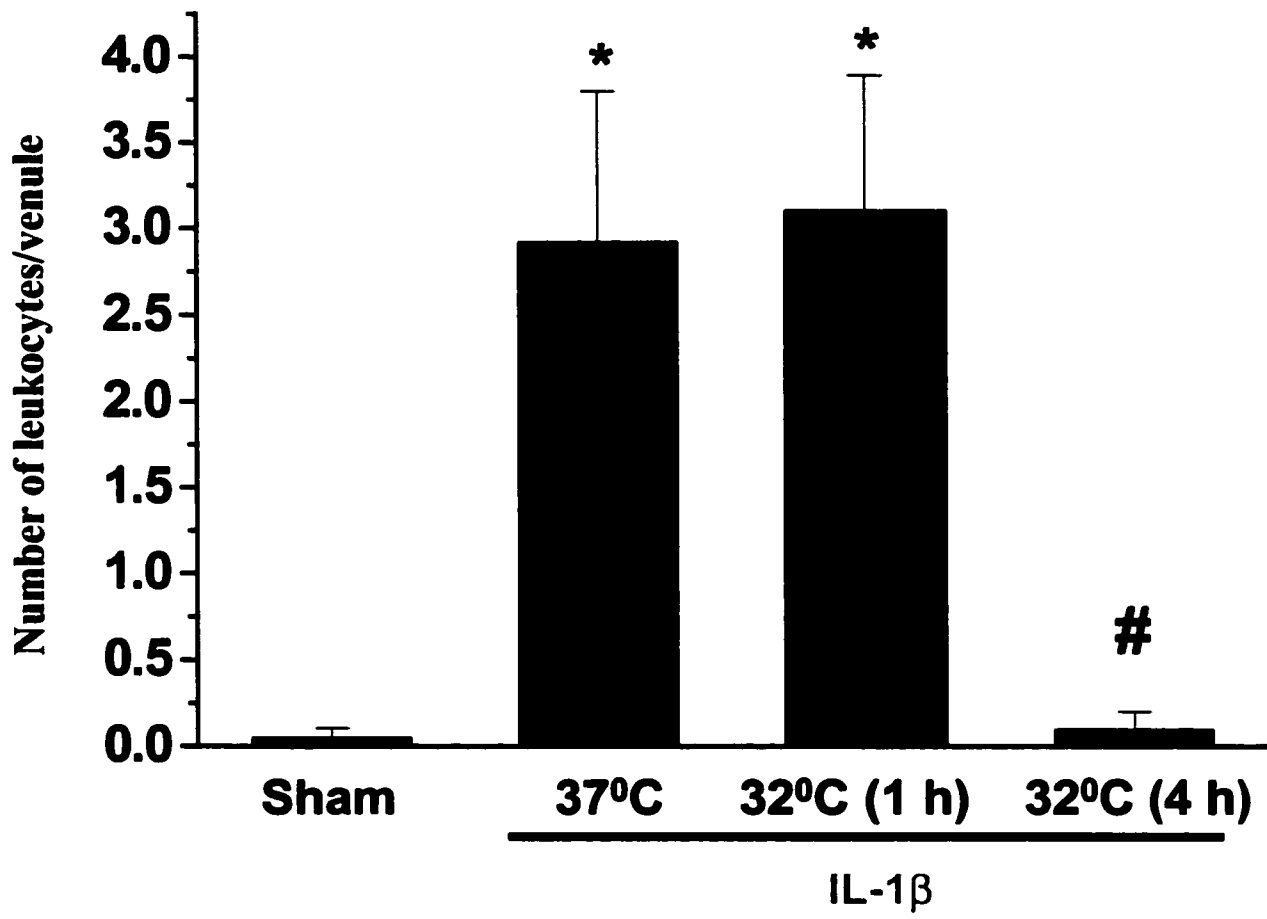


Figure 3.4. The effects of hypothermia on IL-1 β -induced leukocyte adhesion in the pial microcirculation of mice. Adhering leukocytes were quantified as those adhering to the walls of pial venules for longer than 2 seconds over the 30 second recording. Average numbers of adhering leukocytes was determined 4-h after injection of saline (sham control) or IL-1 β (5 μ g/kg i.p.) as described in Materials and Methods. Hypothermia (32°C) was applied to IL-1 β -injected groups for either 1-h or 4-h. Values shown are means \pm SD, n = 7 mice/group. * Indicates a significant difference (p<0.01; one way ANOVA followed by Bonferroni's multiple comparisons of means) compared to sham. # Indicates a significant difference (p<0.01) compared to IL-1 β -injected mice treated for 4-h at 37°C.

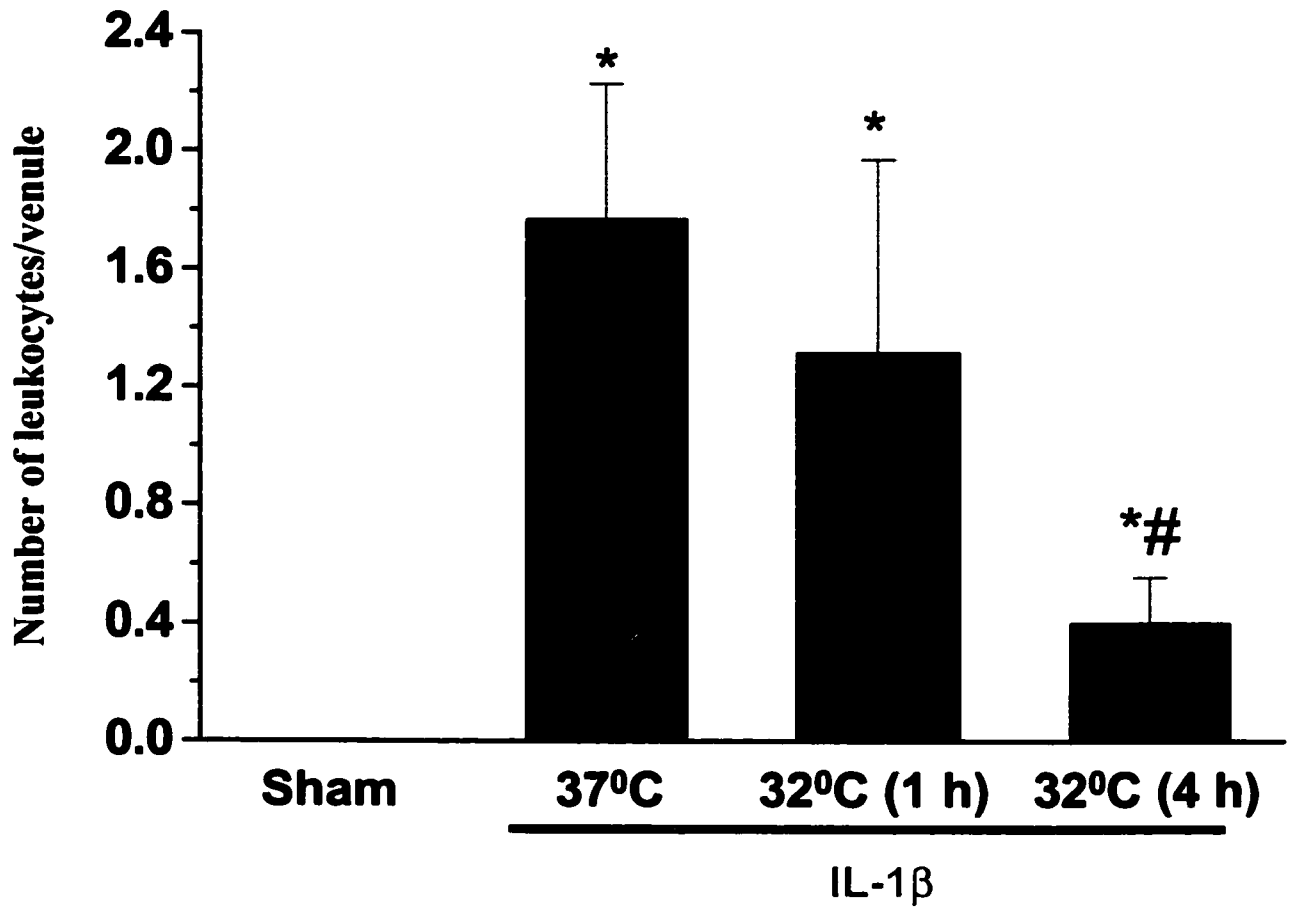
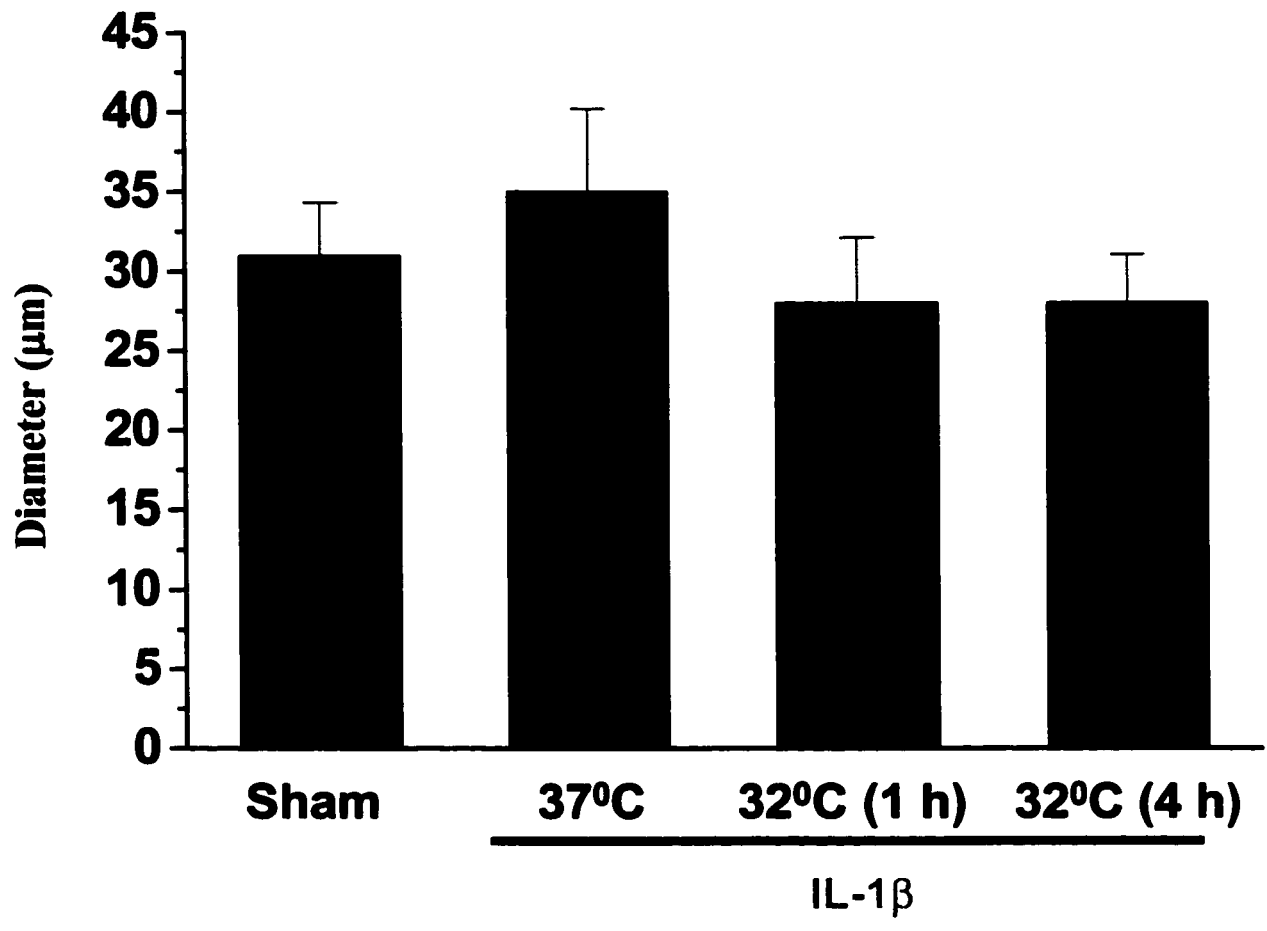


Figure 3.5. Average diameter of pial venules used for measuring leukocyte rolling and adhesion in different experimental groups was measured. Venule diameter was measured using Mocha (Jandel Scientific Inc.) image analysis software. Values shown are means \pm SD, n = 7 mice/group.



and no significant differences in mean venule diameter between groups were found, suggesting that observed differences in leukocyte rolling/adhesion were not due to variations in vessel diameter.

3.1.3 Effects of Hypothermia on Neutrophil CD18 Expression

The β_2 integrins (CD11/CD18) are known to mediate leukocyte adherence to the cerebral endothelium via binding ICAM-1 following activation (Carlos and Harlan, 1994). To further investigate the effects of hypothermia on IL-1 β mediated neutrophil activation and trafficking, CD18 expression was determined in isolated human neutrophils exposed to IL-1 β *in vitro* using flow cytometry. Two known stimulants of neutrophil β_2 integrin activity, LPS and fMLP were used as positive controls (Gahmberg, 1997). Whereas both LPS and fMLP significantly increased neutrophil CD18 expression (Figure 3.6; Table 3.1), IL-1 β had no significant effect after either 15 min or 1-h of exposure (Figure 3.6; Table 3.1). Hypothermia applied for either 15-minutes or 1-h did not significantly affect neutrophil CD18 expression under either control or stimulated (i.e., IL-1 β , fMLP or LPS) conditions (Table 3.1). These findings suggest that the expression of CD18 by human neutrophils is not altered by IL-1 β or hypothermia.

3.1.4 *In-vitro* IL-1 β and/or IL-8 Stimulated Neutrophil Chemotaxis

The reduction in leukocyte rolling and adhesion by 4-h post-inflammatory hypothermia suggests a possible modulation of neutrophil activation by hypothermia. In an attempt to elaborate possible effects of hypothermia on neutrophil mobility,

Figure 3.6. Representative histogram of the surface expression (fluorescence intensity) of CD18 antigen from whole blood from humans subjected to IL-1 β , fMLP and LPS determined by flow cytometry. Control media, 100 u/ml of IL-1 β , 5 mM of fMLP, or 1 μ g/ml LPS were added to whole blood for 1-h at 37°C. Neutrophils were then isolated and labeled with anti-CD18-FITC antibody and subjected to flow cytometry as described in Materials and Methods. The histogram is representative of experiments performed in blood samples from three volunteers. Fluorescence intensity is displayed on a log-scale versus cell count.

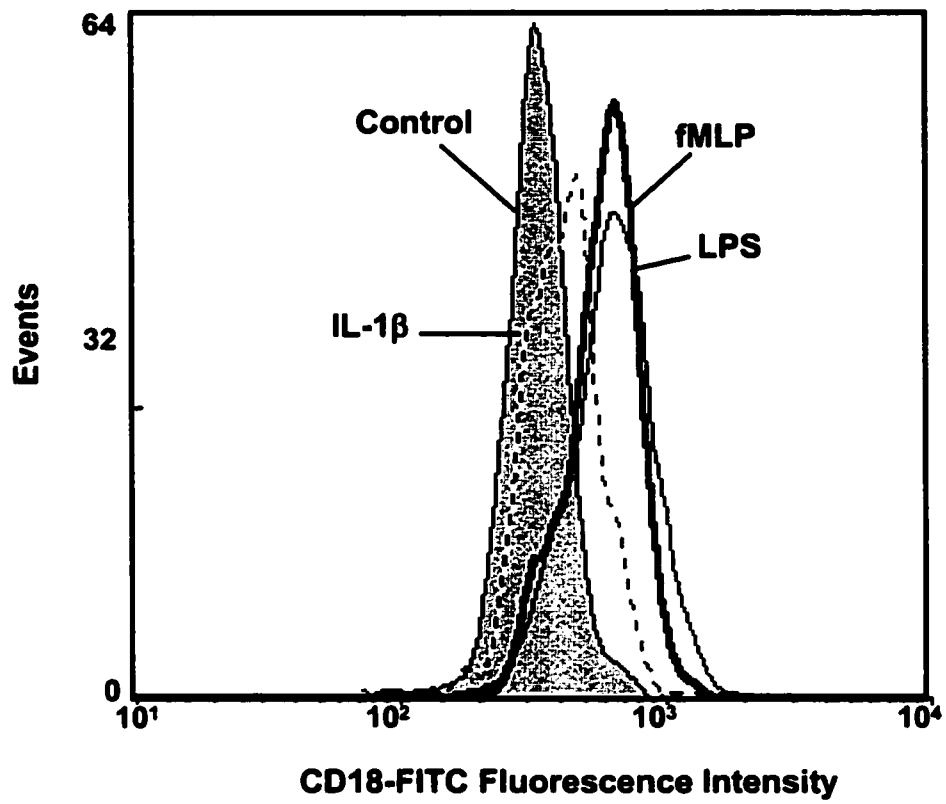


Table 3.1. CD18 expression in neutrophils subjected to IL-1 β , LPS or fMLP at 37° C or 32°C for 15-min or 1-h determined by flow cytometry.

Treatment	MFI (15 min)		MFI (60 min)	
	37°C	32°C	37°C	32°C
Control	1158 \pm 54	1156 \pm 197	416 \pm 24	372 \pm 40
100 u/ml IL-1β	1174 \pm 28	1405 \pm 145	509 \pm 37	442 \pm 30
5mM fMLP	1730 \pm 171*	1692 \pm 84*	632 \pm 31*	618 \pm 40*
1μg/ml LPS	1686 \pm 239*	1875 \pm 65*	672 \pm 48*	541 \pm 45*

Mean fluorescence intensity (MFI) is presented as x-intercept of population mean and represents semiquantitative data on the number of fluorescent-binding molecules expressed by the cells. Data are means \pm S.D. of measurements performed in blood samples of 3 volunteers. * Indicate significant difference ($p < 0.01$; one-way ANOVA followed by Bonferroni's multiple comparison) from unstimulated control at given temperature.

neutrophils were isolated from human blood (n=3), exposed to IL-1 β or phosphate buffer control, and chemotaxis of neutrophils towards IL-8 or phosphate buffer control was measured. No significant induction of chemotaxis was found following stimulation of isolated human neutrophils with IL-1 β , as compared to control (Figure 3.7A). Similarly, IL-8 elicited no significant increase in chemotaxis of neutrophils, either in the presence or absence of IL-1 β , (Figure 3.7B). Furthermore, hypothermia had no effect on basal or stimulated chemotaxis of neutrophils (Figure 3.7A & B). These findings support flow cytometry data, and argue that neutrophil chemotaxis is not altered by IL-1 β or hypothermia. Moreover, the finding that isolated neutrophils are unresponsive to exogenous IL-1 β or IL-8 activation suggests that IL-1 β and IL-8 may primarily target other cell types, most notably CEC.

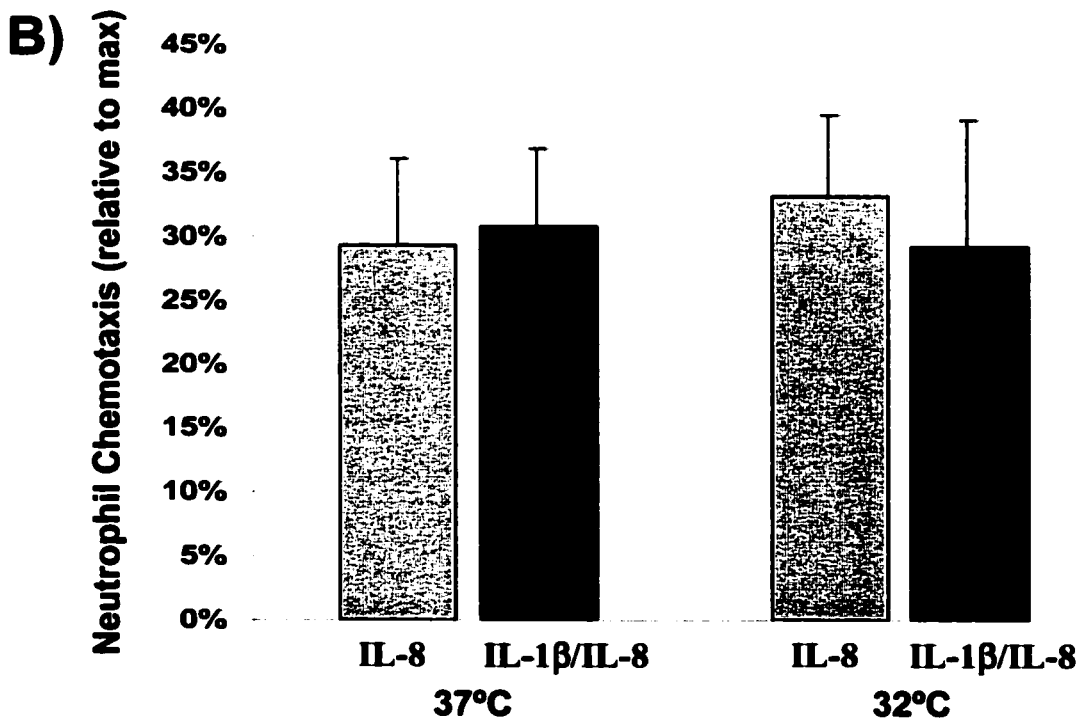
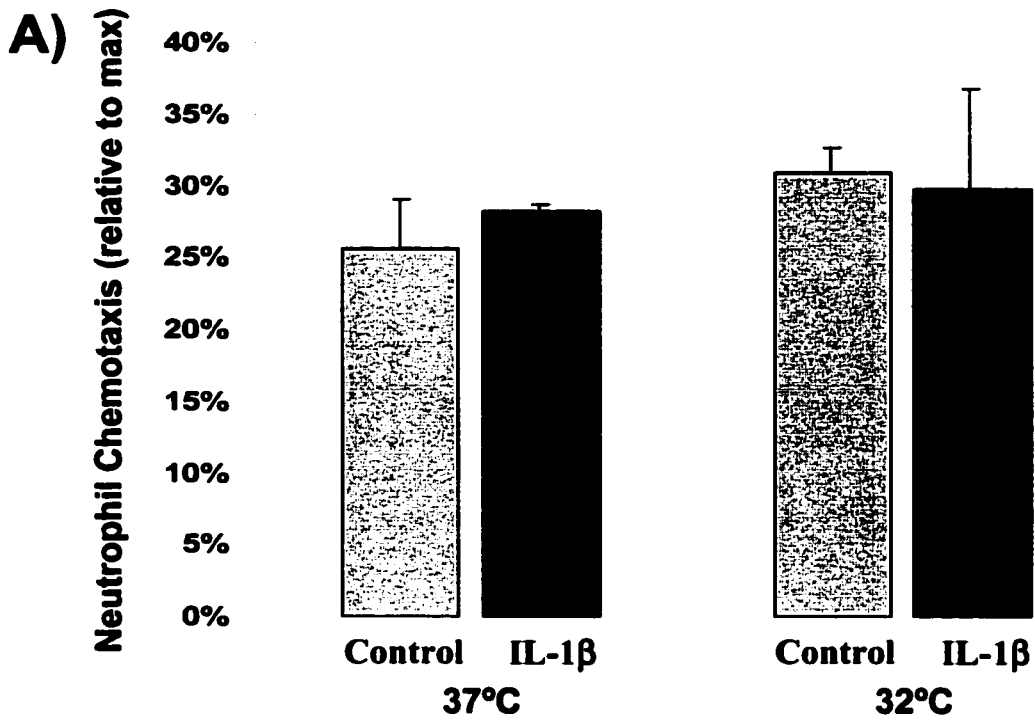
3.2 Characterization of the Murine Global Cerebral Ischemia Model

In order to determine the effects of moderate post-ischemic hypothermia on inflammation, a murine global cerebral ischemia model was developed. To provide evidence that this is an effective and appropriate model, a thorough characterization of the model was essential.

3.2.1 Temperature Correlation for the Global Cerebral Ischemia Model

Studies have clearly established that brain temperature may vary independently of core body temperature during a cerebral ischemic insult, and that small variations in brain temperature markedly influence the extent of neuronal pathology (Ginsberg and Busto, 1989). Accurate measurement/manipulation of core and cerebral temperatures must

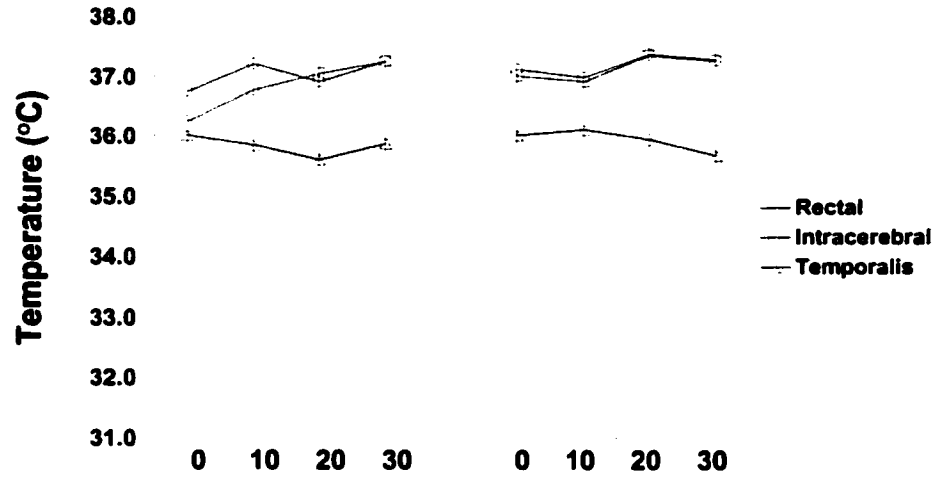
Figure 3.7. The effects of hypothermia on IL-1 β (A) and IL-8 (B) induced chemotaxis of isolated human neutrophils. Neutrophils were tagged with calcein-AM. A) Neutrophils were incubated in the presence or absence of 100 u/ml IL-1 β at 37°C or 32°C for 2-h, and chemotaxis quantified by measuring intensity of following transmigration through a micropore filter. Data are reported as ratios compared to relative maximum readings. B) Chemotaxis of neutrophils incubated in the presence or absence of IL-1 β at 37°C or 32°C for 2-h was also quantified towards IL-8 (0.2 μ g/ml), and reported as ratios compared to relative maximum readings. Each bar represents the mean \pm SD of four replicates in a representative experiment. No significant difference ($p < 0.05$; one way ANOVA) was found between any groups. Similar results were obtained in 2 separate neutrophil isolations.



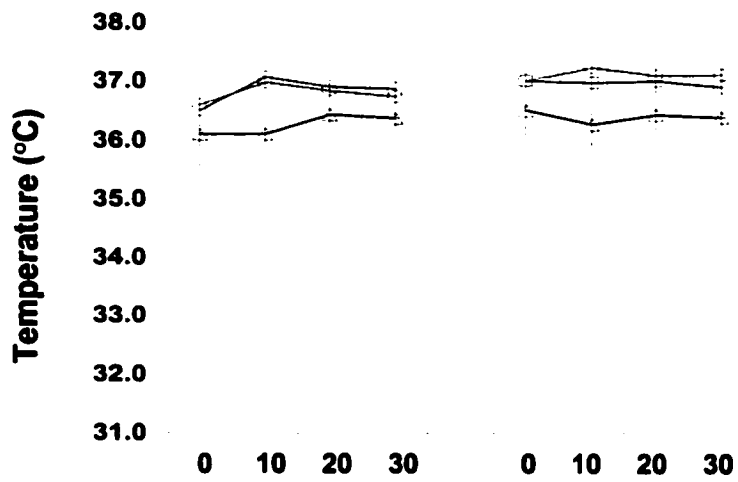
therefore be achieved to produce an effective temperature sensitive murine cerebral ischemia model. To avoid placement of an intra-cerebral probe, as it may induce inflammation, we conducted preliminary experiments monitoring temperature at several sites in order to determine an appropriate means of monitoring brain temperature indirectly. Rectal, intra-cerebral, and temporalis temperatures were monitored simultaneously every 10-minutes pre-ischemia and post-ischemia for 1-h (Figure 3.8), and every minute during sham-operation or 10-min ischemia in mice (Figure 3.9). Post-ischemic temperature recordings were reported beginning 10-minutes following recirculation to allow sufficient time for post-ischemic hypothermia to be induced (Figure 3.8). Rectal temperature was maintained normothermic throughout the 10-minutes cerebral ischemic period. All three regions had highly linear temperature correlation throughout the entire experimental period, for all groups (Pearson Correlation, $r > 0.9$, $p < 0.01$). For the sham, pre-ischemic, and post-ischemic periods (mice in prone position), brain and rectal temperatures were not significantly different in any group, while temporalis muscle temperature was significantly lower than both brain and rectal temperatures (Figure 3.8A-C). Contrarily, during sham-ischemia (mice supine), no difference was found between temperature readings (Figure 3.9A). Moreover, during 10-minutes global ischemia, brain temperature was found to be 0.3°C lower than rectal and temporalis temperatures (Figure 3.9B & C). In light of these results, rectal temperature was monitored exclusively in all subsequent global cerebral ischemia/reperfusion experiments in mice. Furthermore, rectal temperature was kept at 37.3°C during ischemia (to maintain normothermic intra-cerebral temperature), and at 37°C pre-ischemic and

Figure 3.8. Rectal (open square), intra-cerebral (open circle) and temporalis (open triangle) temperature recordings *pre-ischemia and during reperfusion* following 10-minutes experimental global cerebral ischemia for mice receiving sham-operation (A), 37°C reperfusion (B), and 32°C reperfusion (C). Rectal temperature was maintained at the appropriate temperature using a heated water mat and over-head heat lamp. Temperature recordings were made every 10-minutes for 30-minutes pre-ischemic, and 30-minutes post-ischemic periods (begun 10-minutes following recirculation). The data shown are means \pm SD of temperature recordings taken from 3 animals per group at the indicated time-points. Rectal and intra-cerebral temperatures were not significantly different in any group ($p < 0.05$; one-way ANOVA), while temporalis temperatures was lower than both rectal and intra-cerebral temperatures ($p < 0.05$) in all groups at all time-points.

A)



B)



C)

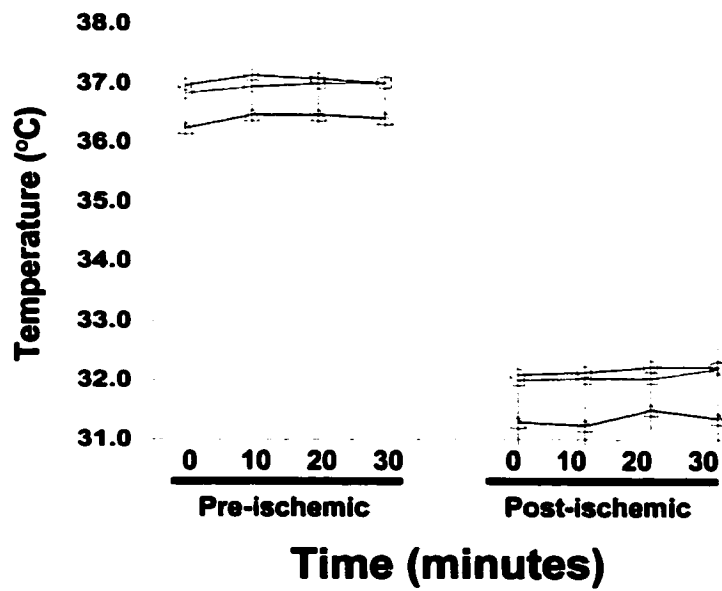
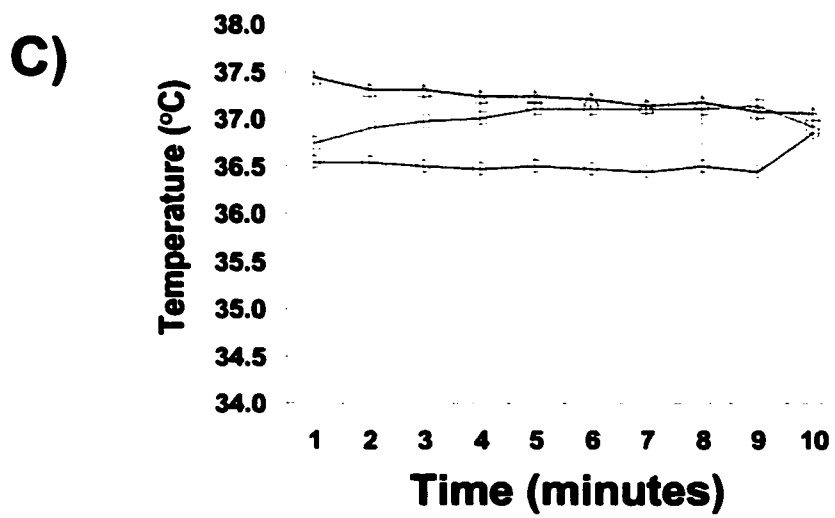
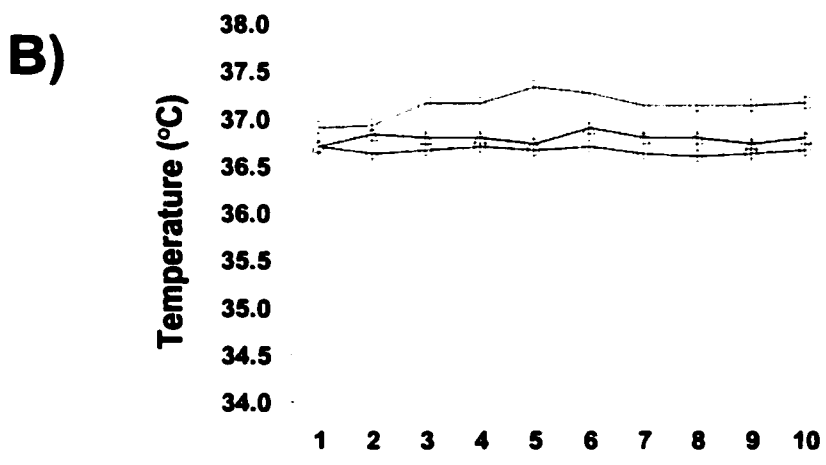
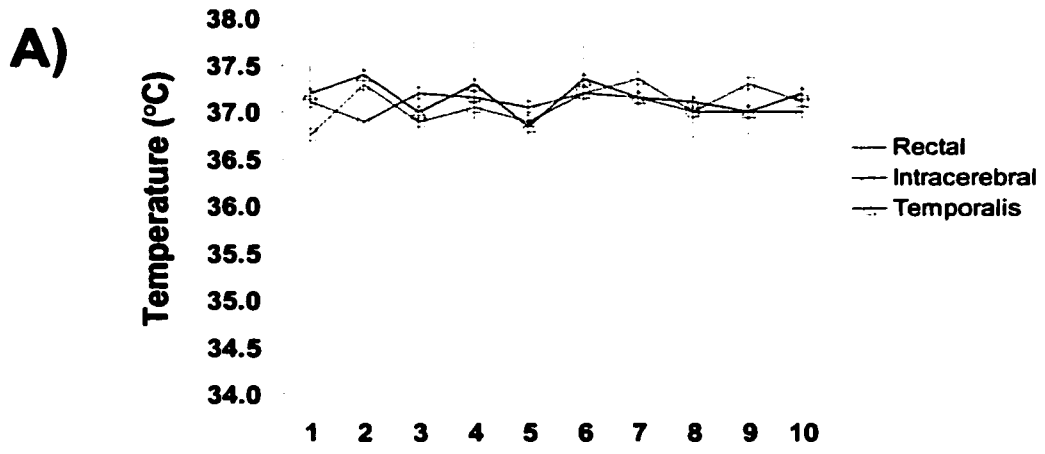


Figure 3.9. Rectal (open square), intra-cerebral (open circle) and temporalis (open triangle) temperature recordings *during* 10-minutes experimental global cerebral for mice receiving sham-operation (A), 37°C reperfusion (B), and 32°C reperfusion (C). Rectal temperature was maintained at 37°C ± 0.3°C, using a heated water mat and over-head heat lamp. Temperature recordings were made every 1-minute over the 10-minute ischemic period. The data shown are means ± SD of temperature recordings taken from 3 animals per group at the indicated time-points. Rectal and intra-cerebral temperatures were significantly different between ischemic groups ($p < 0.05$; one-way ANOVA).



during reperfusion. An important point to emphasize is the ability to induce hypothermia within minutes (10-minutes) following global cerebral ischemia in this murine model.

3.2.2 Determination of Cerebral Blood Flow, Arterial Blood Gases, and pH

To characterize the physiological effects of this murine cerebral ischemia model, Laser Doppler was used to measure rCBF throughout the experimental period, and arterial blood was analyzed for physiological parameters. Laser Doppler flowmetry are presented as percentages of baseline blood cell perfusion units (BPU) (Figure 3.10). Measurements were made pre, intra, and post-ischemia in mice (n=4). During the pre-ischemic interval, rCBF was unchanged. Immediately upon BCCA occlusion for global cerebral ischemia, rCBF decreased to $20.5 \pm 4.6\%$ of baseline (Figure 3.10). Moreover, rCBF continued to decrease over the 10-minute ischemic period to $12 \pm 4.8\%$ of baseline. This rapid microvascular low flow state was also observed during separate *in-vivo* experiments during which intra-ischemic intravital microscopic recordings of pial vessels were made for determination of leukocyte rolling and adhesion (not shown). Immediately upon reperfusion, a brief attempt for recovery of rCBF occurred, reaching $38 \pm 1\%$ of baselines at 1-minute reperfusion. Although rCBF during this brief recovery period was significantly lower than baseline, it was significantly increased relative to intra-ischemic readings (Figure 3.10). Within minutes following this brief rise in rCBF, blood flow dropped to $24 \pm 5.5\%$ of baseline. Hypoperfusion lasted till the end of the observation period (20-minutes), with rCBF progressively improving to $43 \pm 9.6\%$ of baseline. All rCBF readings subsequent to BCCA occlusion were significantly lower than baseline (pre-ischemia) (Figure 3.10).

Figure 3.10. The effects of transient global cerebral ischemia on cortical microvascular perfusion in the C57/Bl6 murine model. Regional cerebral blood flow (rCBF) in the cortical surface vessels was measured with laser-Doppler flowmetry. Rectal temperature was monitored and kept at $37 \pm 0.5^{\circ}\text{C}$ throughout the experimental period using a warming mat and overhead heating lamp. CBF measurements were made through a burrhole: 5 and 1 minute before ischemia; at 0, 5, and 10 minutes during occlusion; and 1, 5, 10 and 20 minutes following ischemia. Laser Doppler flowmetry are presented as percentages of baseline blood cell perfusion units (BPU). Values shown are means \pm SD of four mice. * Indicates a significant reduction in rCBF ($p < 0.05$; one way ANOVA followed by Bonferroni's multiple comparisons of means) compared to baseline. # Indicates a significant difference ($p < 0.05$) compared to intra-ischemic (0-10 min.) rCBF.

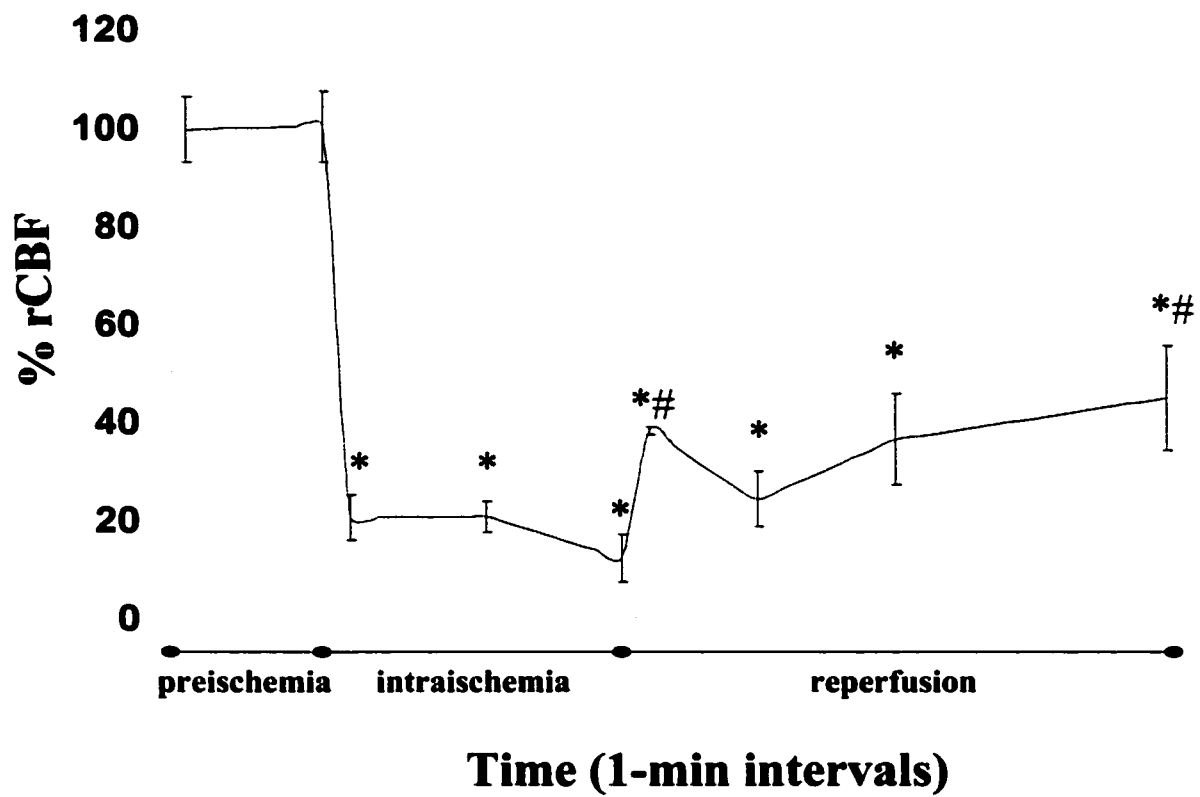


Table 3.2. Arterial blood gas and pH in transient global ischemia

	Pre-ischemic (n=3)	Intra-ischemic (n=3)	Post-ischemic (n=3)
pO ₂	72.067 ± 3.75	68.30 ± 4.84	69.9 ± 8.10
pCO ₂	48.167 ± 6.15	53.80 ± 4.33	52.7 ± 6.81
pH	7.324 ± 0.017	7.297 ± 0.012	7.295 ± 0.023

Data are means ± S.D. No significant difference in blood gas or pH was found throughout the experimental period (p<0.05; one-way ANOVA).

Physiological parameters are shown in Table 3.2. Analysis of arterial blood for pH, pCO₂, and pO₂ revealed no significant differences between groups (n=3). There were insignificant trends towards increased pCO₂ and decreased pO₂ during and after ischemia, raising the possibility that anesthetic may alter ventilation or perfusion in these animals.

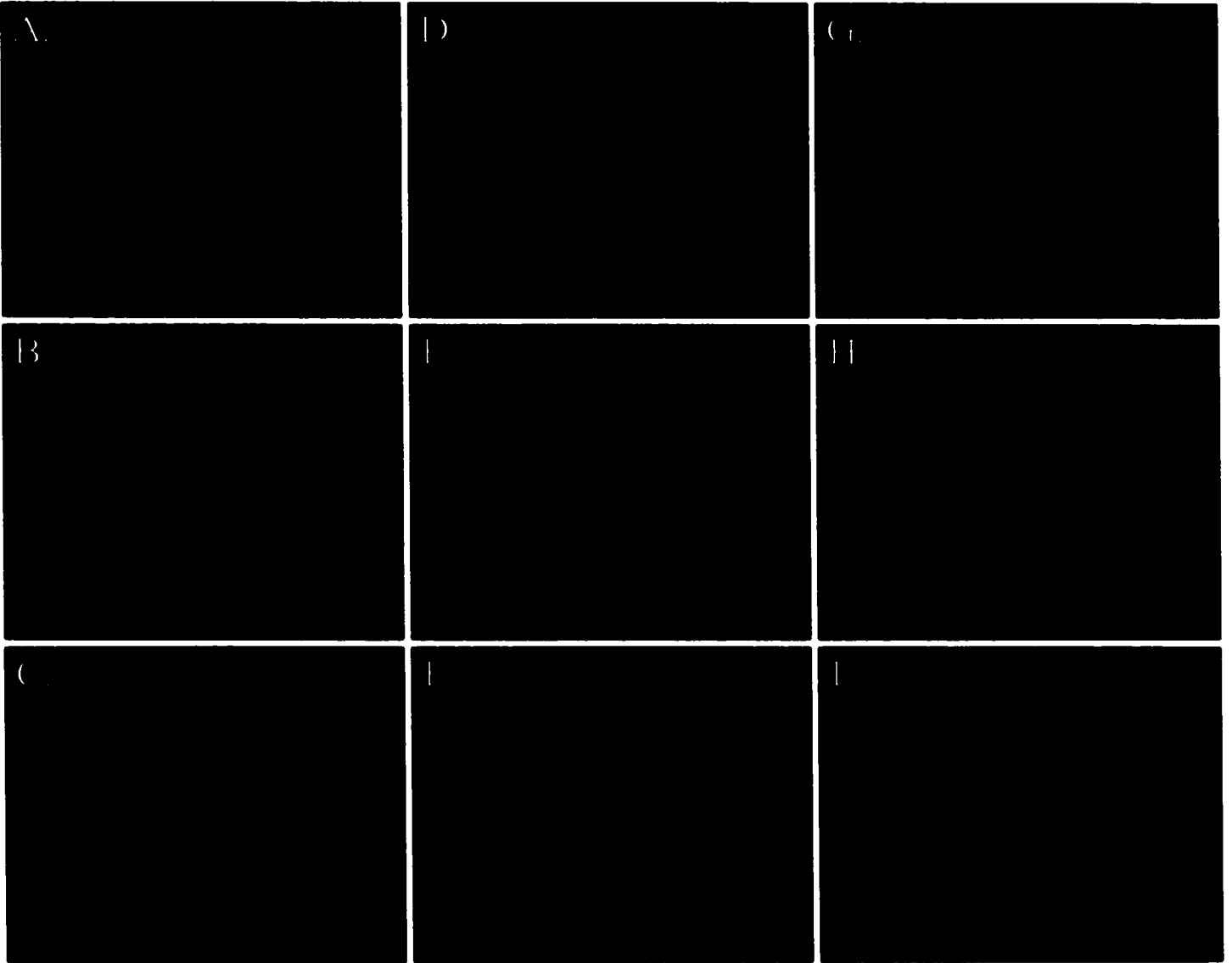
3.2.3 Histological Characterization of the Global Cerebral Ischemia Model

In order to determine if a 4-h duration of moderate post-ischemic hypothermia leads to protection in this murine model of 10-minutes global cerebral ischemia, we quantified cell death and astrocytosis 3 and 7 days following injury. These experiments included 4 groups: sham-operation, ischemia followed by 4-h normothermia, ischemia followed by 4-h hypothermia, and ischemia followed by 4-h hyperthermia. Due to excessively high mortality rates in the group reperfused 4-h at hyperthermic temperatures (81.8%), this group was excluded from analysis.

3.2.3.1 Apoptotic-like Neuronal Death

DNA fragmentation was assessed using *in situ* end labeling (ISEL) on mice survived 3 days. No ISEL positive cells were found anywhere in sham-operated brain sections (Figure 3.11A, D & G). Extensive ISEL positive cells were observed in the cortex (Figure 3.11B), striatum (Figure 3.11E), and hippocampus (Figure 3.11H) of ischemic mice reperfused at 37°C. Cortical ISEL positive labeling was apparent in neocortical layers 2 through 5. Consistent hippocampal CA1 positive labeling was observed, though frequent dentate and CA3 positive labeling of DNA fragmentation also

Figure 3.11. Representative *in situ end* labeling (ISEL) of apoptotic-like neuronal death 3-days following 10-minutes global cerebral ischemia in mice. Fluorescence identification of ISEL labeling (green) was performed on coronally sectioned mouse brains, and visualized at 10X power. Cortical (A-C), striatal (D-F), and hippocampal (G-I) areas were evaluated in sham-operated (A, D, G) or ischemic mice that received 4-h reperfusion at 37°C (B, E, H) or 32°C (C, F, I). Similar results were found in four animals per group. ISEL positive cells are seen in all areas of the ischemic brain maintained normothermic, while considerable reductions in ISEL positive cells were found in mice maintained hypothermic 4-h following global cerebral ischemia.



**A-C=cortex; D-F=Striatum; G-I=Hippocampus
A,D,G=Sham; B,E,H=Normothermia; C,F,I=Hypothermia**

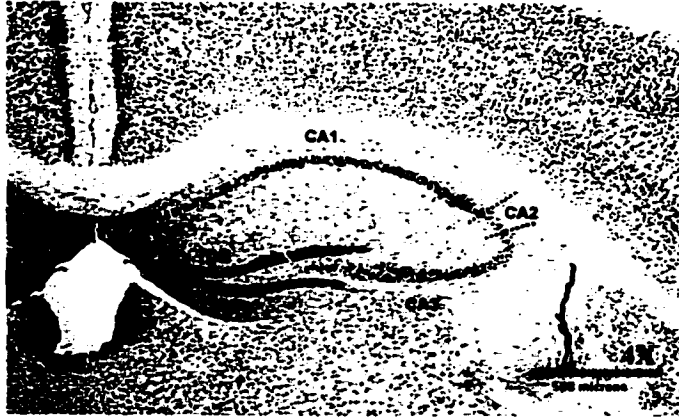
occurred. Striatal and neocortical ISEL positive cells appeared in distinct foci, and often unilaterally. Profound reductions in ISEL positive cells were observed in the 4-h post-ischemic hypothermic group in all areas of the brain, suggesting significant neuroprotection by moderate transient post-ischemic hypothermia (Figure 3.11 C, F & I).

3.2.3.2 Neuronal Loss

To determine the extent of neuronal loss in the global cerebral ischemia model, we attempted to identify viable neurons 7 days following ischemia. Mice were either maintained normothermic or moderately hypothermic 4-h immediately following ischemia or sham-operation. Viable neurons were identified using immunohistochemistry with a primary antibody (A60) to a neuron-specific nuclear protein marker (NeuN) (Figure 3.12 & 3.13). Lack of NeuN staining was regarded as indicative of neuronal loss. Dark background staining was seen around areas of severe damage in ischemic tissue. Immunohistochemical negative controls also showed these consistent darkened areas around severe damage (Figure 3.13D) indicating possible infiltration of blood components and presence of cellular debris. No neuronal loss was observed in the sham-operated group (Figure 3.12A & 3.13A). We observed considerable neuronal loss in the ischemic group reperfused 4-h at 37°C. Profound and consistent CA1 neuronal loss was observed (Figure 3.12B; black arrows), as well as substantial striatal and neocortical (layer 2 through 5) death (Figure 3.13B; area indicated by arrow). Sporadic neuronal loss was also observed in other areas of the hippocampus including CA3, and dentate gyrus. These histological findings are similar to those previously reported by Pulsinelli et al. (1982), who demonstrated a similar pattern of neuron death following 4VO in rats.

Figure 3.12. Immunohistochemical detection of viable neurons in the mouse hippocampus using a monoclonal antibody (A60) against the neuron-specific marker NeuN. Mice received sham-operation (A), or 10-minutes global cerebral ischemia and 4-h reperfusion at 37°C (B) or 32°C (C). Immunohistochemical analysis of coronal sections was performed following 7 days survival. Consistent neuronal loss in the CA1 was found in mice maintained normothermic following ischemia (black arrows), while no damage was found in ischemic mice maintain hypothermic 4-h during reperfusion. DG=dentate gyrus.

A)



B)



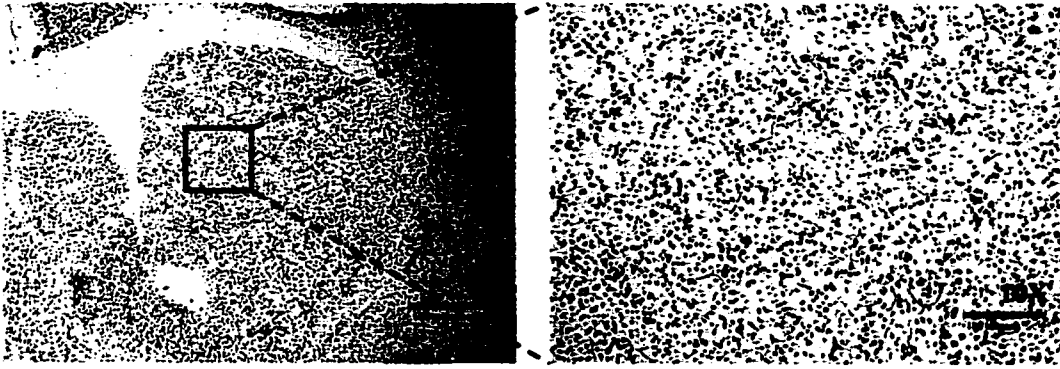
C)



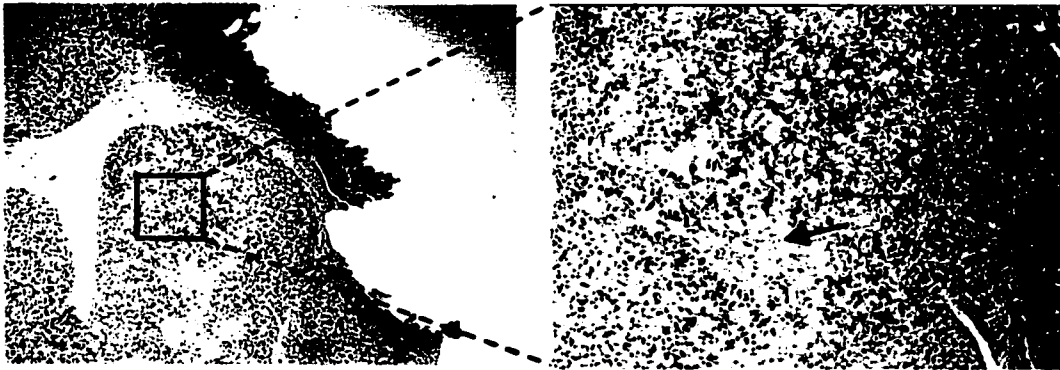
A=Sham; B= Normothermia; C= Hypothermia

Figure 3.13. A-C) Immunohistochemical detection of viable neurons in the mouse striatum using a monoclonal antibody (A60) against the neuron-specific marker NeuN. Immunohistochemical analysis of brain sections was performed following 7-days of survival on each of 3 groups. Mice received sham-operation (A), or 10-minutes global cerebral ischemia and 4-h reperfusion at 37°C (B) or 32°C (C). Immunohistochemical analysis of coronal sections was performed following 7 days survival. Histological representative figures at both 4X and 10X demonstrate considerable neuronal loss in the striatum in mice maintained normothermic following ischemia, while little to no damage was found in ischemic mice maintained for 4-h at 32°C during reperfusion. D) Representative negative control for NeuN immunohistochemistry. Negative controls were generated by processing brain sections without addition of NeuN antibody. Negative control histological representative image of mouse section which received ischemia and was reperfused 4-h at 37°C illustrates the dark background staining in areas of severe damage.

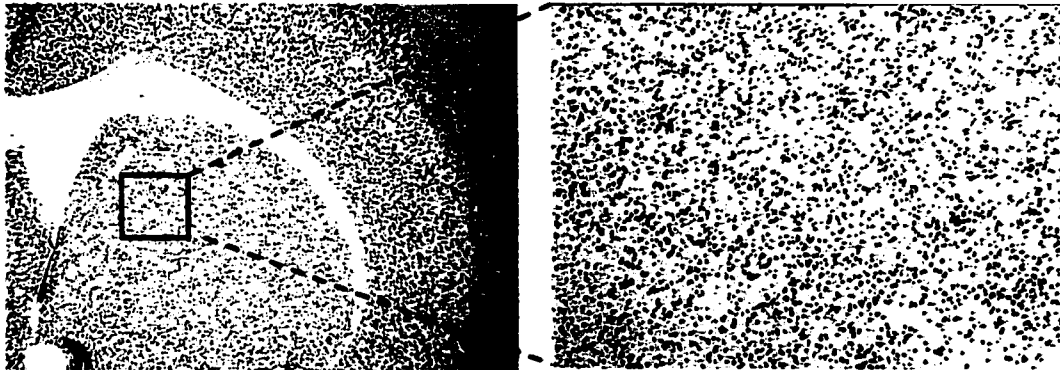
A)



B)

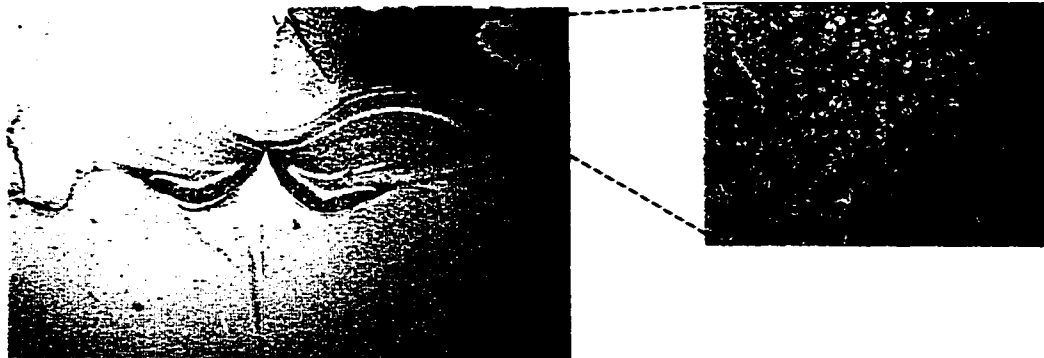


C)



A=Sham; B= Normothermia; C= Hypothermia

D)



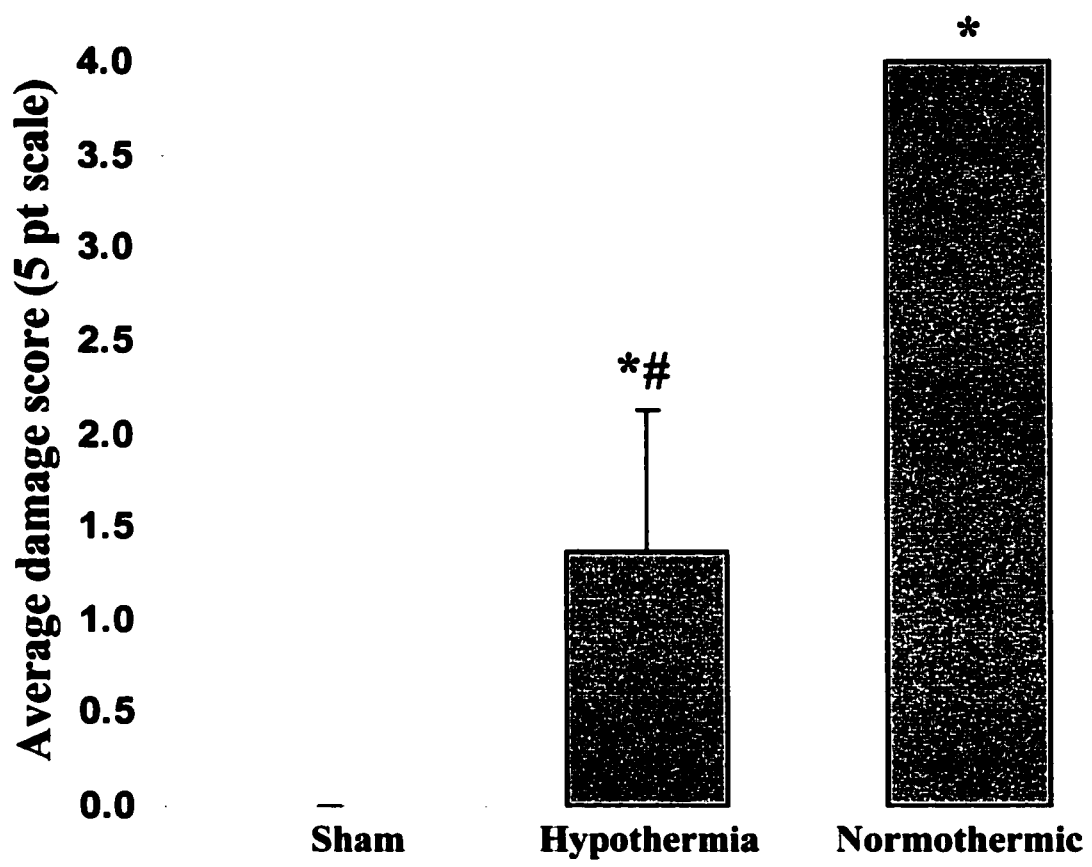
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In the ischemic group maintained hypothermic for 4-h reperfusion, either no damage or minimal neuronal loss was found, with sparse “patchy” neuronal loss in the striatum and neocortex (Figure 3.13C). Complete protection of the hippocampus was attained (Figure 3.12C). Quantification of neuronal damage performed on coronal sections stained with NeuN was assessed using light microscopy by a pathologist blinded to the treatments. Overall damage was scored for each animal on a 5 point scale: 0-4 (no damage to severe damage). No damage was reported in any of the sham-operated mice (Figure 3.14) and severe damage was recorded in all mice maintained normothermic during reperfusion. A significant reduction in neuronal damage was found in ischemic mice reperfused 4-h at 32°C, as compared to ischemic mice reperfused at 37°C (Figure 3.14). Neuronal loss in the post-ischemic hypothermic group ranged from no damage, to identification of 1-2 small foci. This reduction in damage 7 days following 10-minutes global cerebral ischemia suggests that short duration post-ischemic hypothermia provides considerable neuroprotection in this murine model.

3.2.3.3 Determination of Post-ischemic Astrocytosis

Gliosis has been characterized by cellular hyperplasia and hypertrophy of the astrocytic cell bodies and processes (Kumar et al., 1996). Previous reports have demonstrated an increase in the number of activated astrocytes following cerebral ischemia (Kindy et al., 1992). In an effort to elucidate the effects of this global cerebral ischemia model on reactive astrocytosis, immunohistochemical analysis was performed using a glial fibrillary acid protein (GFAP) antibody. Mice received sham-operation or

Figure 3.14. Quantification of neuronal damage 7-days following global cerebral ischemia. A pathologist who was blinded to the treatments evaluated damage on coronal sections stained with NeuN using light microscopy. Overall damage was scored for each animal on a 5-point scale: no damage (0) to severe widespread damage (4). Bars shown are means \pm SD for each group, n = 4 mice/group. * Indicates a significant difference ($p < 0.01$; one way ANOVA) compared to sham. # Indicates a significant difference ($p < 0.01$) compared to ischemic mice reperfused 4-h at 37°C.



ischemia, followed by either 4-h normothermic or hypothermic reperfusion. Mice were survived 3 (n=4/group) and 7 days (n=4/group), and coronal brain sections stained for GFAP. Basal GFAP immunoreactivity was identified in sham-operated mice at both 3 days (Figure 3.15A) and 7 days (Figure 3.15D). Increased numbers and thickened processes of GFAP-positive cells was noted in the hippocampal CA1 region of ischemic mice maintained normothermic during 4-h reperfusion, as compared to sham-operated mice, at both 3 days and 7 days (Figure 3.15B & E). Hypothermia significantly reduced both hypertrophy and quantity of GFAP staining seen at 3 and 7 days post-ischemia (Figure 3.15C & F). These data are in agreement with previous findings reported by Kumar et al. (1996) using intra-ischemic hypothermia during forebrain ischemia in gerbils.

3.2.4 Histological Evaluation of Neutrophil Infiltration Following Global Cerebral Ischemia

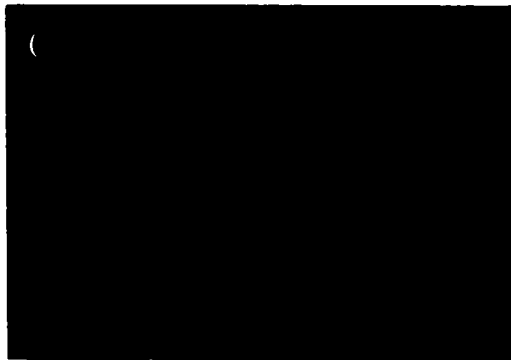
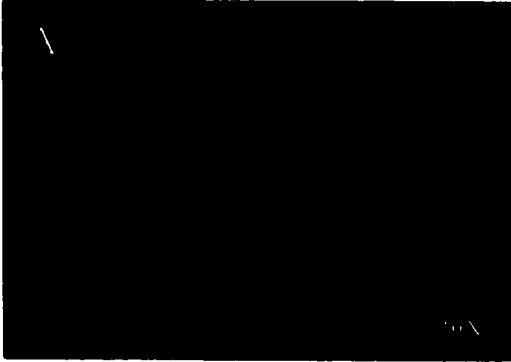
In the effort to evaluate the effects of moderate post-ischemic hypothermia on inflammation, infiltration of neutrophils into ischemic brain was determined.

3.2.4.1 Myeloperoxidase assay (MPO)

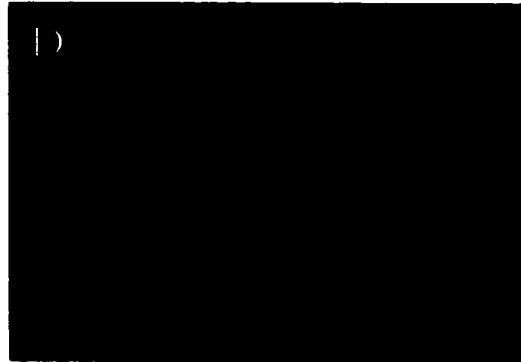
Following 24-h reperfusion, MPO activity was measured in brain tissue homogenates from sham-operated, and ischemic mice maintained normothermic or hypothermic for 4-h following global ischemia. Although neutrophil positive controls showed substantial MPO activity (1.03 ± 0.19 U), no significant change in MPO activity was found in any group, compared to sham-operated mice (Figure 3.16). These findings

Figure 3.15. The effects of 10-minutes transient global cerebral ischemia on reactive astrocytosis in mice. Immunofluorescent identification of glial fibrillary acid protein (GFAP) positive immunoreactivity (green) was performed on coronally sectioned mouse brains, and visualized at 10X power. Mice received sham-operation (A&D) or ischemia, followed by either 4-h normothermic (B&E) or hypothermic (C&F) reperfusion. Mice were survived 3 (A-C) and 7 (D-F) days. Increased intensity and number of GFAP immunoreactivity is seen in ischemic mice reperfused at 37°C, relative to sham-operation and mice reperfused 4-h at 32°C.

3d

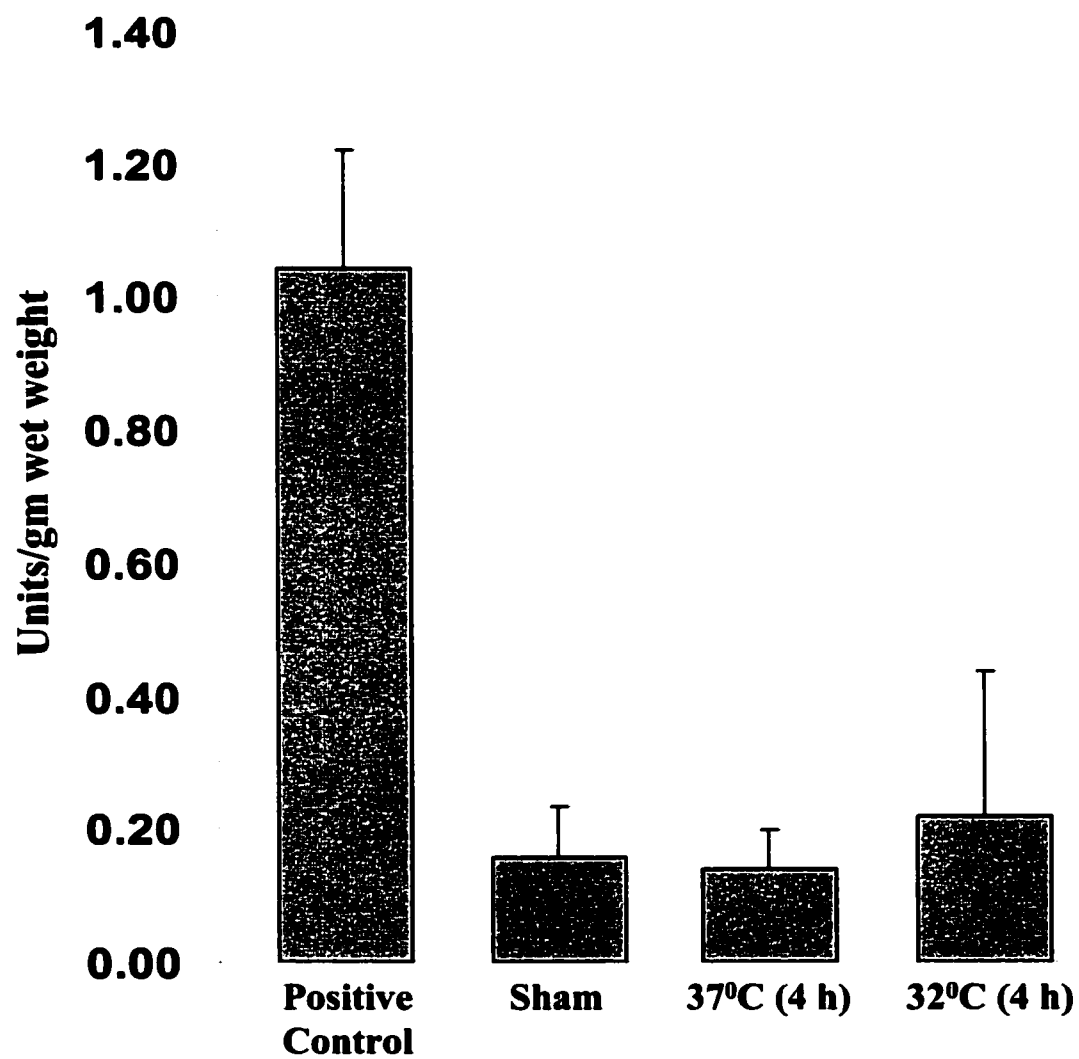


7d



A,D= Sham, B,E= Normothermia, C,F= Hypothermia

Figure 3.16. Myeloperoxidase (MPO) assay determination of neutrophil infiltration following 10-minutes global cerebral ischemia in mice. Following 24-h reperfusion, MPO activity was measured in brain tissue homogenates from sham-operated, and ischemic mice maintained normothermic or hypothermic 4-h following global ischemia. MPO activity was determined in isolated murine PMN exudate from normal mice as a positive control. Results are reported in U/gm wet weight, where 1 unit of MPO activity is defined as that degrading 1 μ mol peroxide/minute at 25°C. Each bar represents mean \pm SD, n = 4 mice/group. No significant difference (p<0.05; one way ANOVA) was found between any groups.



suggest that no significant infiltration of neutrophils into cerebral parenchyma occur 24-h following this global ischemia/reperfusion model.

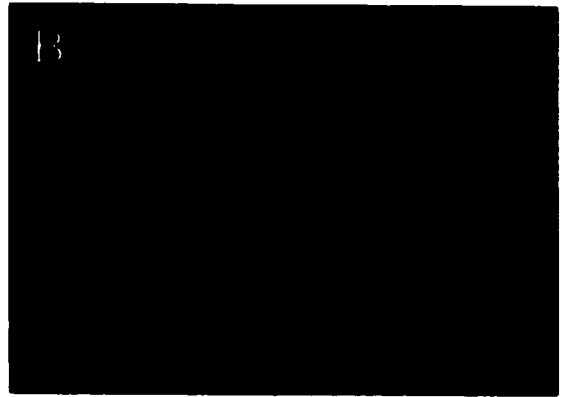
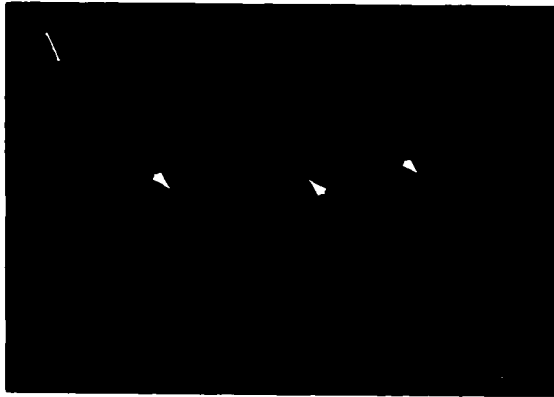
3.2.4.2 Neutrophil/DAPI Double Labeling

To further elucidate neutrophil infiltration in this global cerebral ischemia model, sham-operated and ischemic mice were maintained normothermic or hypothermic 4-h following global cerebral ischemia. After 1-d reperfusion, dual immunohistochemical labeling with DAPI and a monoclonal neutrophil antibody was performed on coronally sectioned brain. Although numerous neutrophil/DAPI immunoreactive cells were found in the murine neutrophil positive control (Figure 3.17A), no neutrophil positive immunoreactivity was found in any sham or hypothermic animal (Figure 3.17B & D). In the four ischemic mice maintained normothermic 4-h post-ischemic, a total of two neutrophil/DAPI positive cells were found, both in the cerebral neocortex (Figure 3.17C). These findings suggest negligible infiltration of neutrophils into cerebral parenchyma occurs 24-h following this global ischemia model, and supports our aforementioned MPO findings.

3.3 Effects of Hypothermia on *In-vivo* Leukocyte Rolling-Adhesion Induced by Global Cerebral Ischemia

Subsequent to the thorough characterization of this murine global cerebral ischemia model, direct *in-vivo* assessment of the effects of moderate post-ischemic hypothermia on inflammation was achieved.

Figure 3.17. Immunohistochemical identification of infiltrated neutrophils into mouse neocortex using double labeling with a monoclonal mouse FITC-neutrophil antibody, and DAPI. Isolated murine neutrophil exudate was used as a positive control (A). Mice received sham-operation (B) or 10-minutes global cerebral ischemia followed by 4-h normothermic (C) or hypothermic (D) reperfusion. After 1-d survival, dual immunohistochemical labeling was performed on coronally sectioned brain, and visualized using a 40X objective and fluorescence microscopy. Arrows indicate positive immunoreactivity for both DAPI and mouse neutrophils. (n = 4 mice/group)



A= Positive Control, B= Sham, C= Normothermia, D= Hypothermia

Bright field recordings were taken pre-ischemia, intra-ischemia, and post-ischemia for mice in the 5-min reperfusion group (not shown). Playback analysis allowed for the visualization of considerable reductions in blood flow during ischemia (maximal after 5 minutes), and occurrence of incomplete recovery upon reperfusion (no-reflow). These microscopic observations are in support of findings reported using laser Doppler measurement of rCBF.

To evaluate post-ischemic rolling-adhesion, and the effects of post-ischemic hypothermia on these processes in the pial microcirculation, mice were randomized to one of 7 groups: sham-operation followed by 5-min, 2-h and 4-h recovery at 37°C; global cerebral ischemia followed by 5-min, 2-h and 4-h reperfusion at 37°C; and global cerebral ischemia followed by 2-h reperfusion at 32°C. Following the indicated periods of reperfusion, intravital microscopic recordings (3-4 per animal) were made of pial venule leukocyte rolling and adhesion. Fluorescence recordings using an open cranial window and intravital microscopy showed an increase in the number of rolling leukocytes in ischemic mice reperfused 5-min and 2-h at 37°C, as compared to sham-operated animals (Figure 3.18). Similarly, an increase in total neutrophil adhesion was found in ischemic mice following 5-min, 2-h and 4-h reperfused at 37°C (Figure 3.19). However, in ischemic mice reperfused 2-h at 32°C hypothermia, a complete inhibition of leukocyte rolling (Figure 3.18) and reduced total leukocyte adhesion (Figure 3.19) were detected, as compared to ischemic mice reperfused either 5-min or 2-h at 37°C. All pial venules chosen for measurements of leukocyte rolling and adhesion were 19-42 μm and no significant differences in average venule diameter between groups were found

Figure 3.18. The effects of post-ischemic hypothermia on leukocyte rolling in the pial microcirculation of mice. Average numbers of rolling leukocytes in mouse pial venules were determined over a 30-second period. Sham-operated mice or mice subjected to 10-minutes global cerebral ischemia were reperfused 5-min, 2-h or 4-h at either hypothermic or normothermic temperatures. Hypothermia (32°C) or normothermia (37°C) was applied to mice immediately following ischemia as described in Materials and Methods. Values shown are means \pm SD. * Indicates a significant difference ($p < 0.05$; one way ANOVA followed by Bonferroni's multiple comparison) compared to sham. # Indicates a significant difference ($p < 0.05$) compared to ischemic mice reperfused 2-h at 32°C. (sham $n = 4$; ischemia $n=6$)

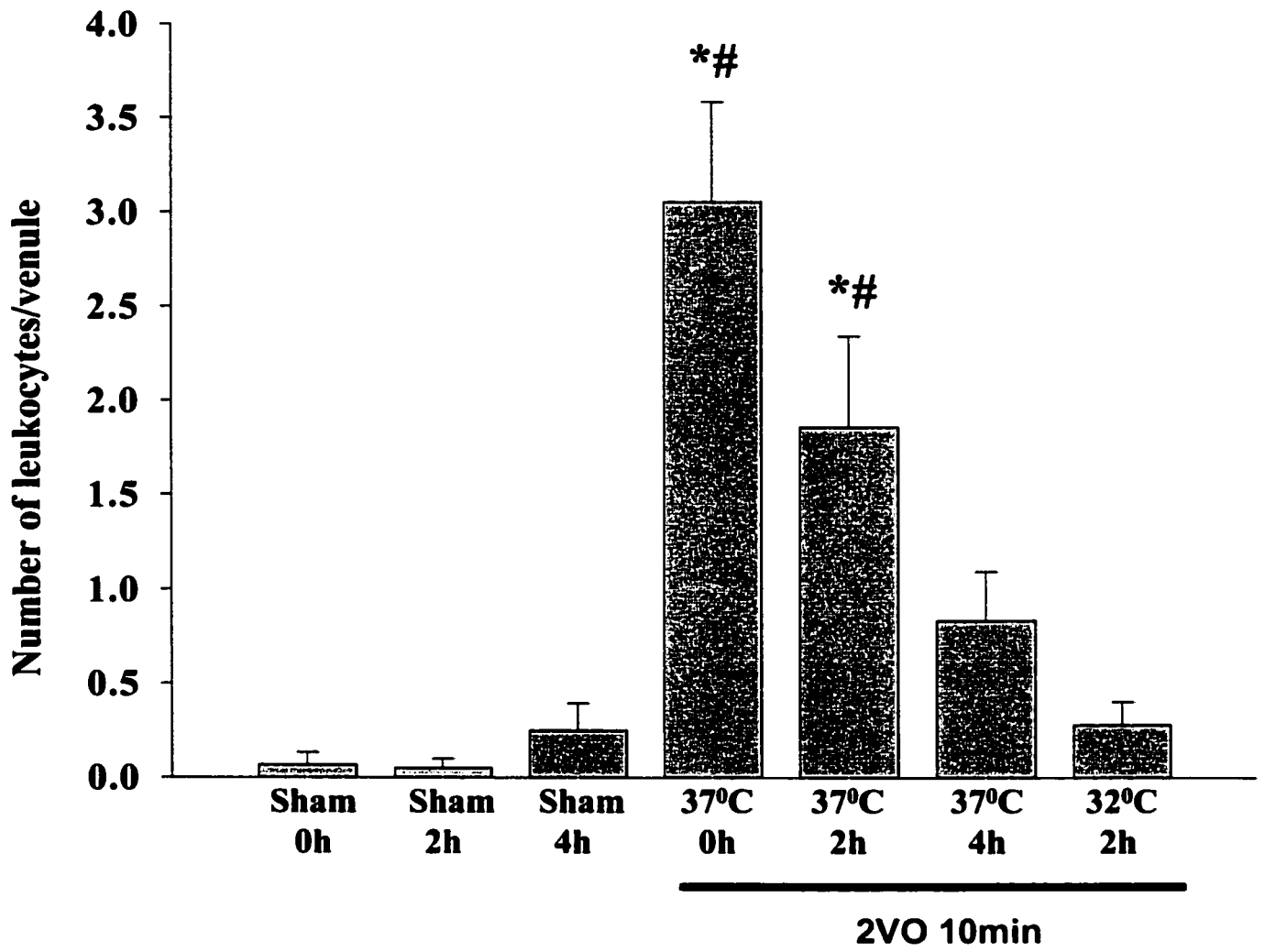


Figure 3.19. Effects of post-ischemic hypothermia on leukocyte adhesion in the pial microcirculation of mice. Sham-operated mice or mice subjected to 10-minutes global cerebral ischemia were reperfused 5-min, 2-h or 4-h at either hypothermic or normothermic temperatures. Following the appropriate durations of reperfusion, average numbers of adherent leukocytes in mouse pial venules were determined as those adhering to the walls of pial venules for longer than 2 seconds over the 30 second recording. Hypothermia (32°C) or normothermia (37°C) was applied to mice immediately following ischemia as described in Materials and Methods. Values shown are means \pm SD. * Indicates a significant difference ($p < 0.05$; one way ANOVA followed by Bonferroni's multiple comparison) compared to sham. # Indicates a significant difference ($p < 0.05$) compared to ischemic mice reperfused 2-h at 32°C. (sham n = 4; ischemia n=6)

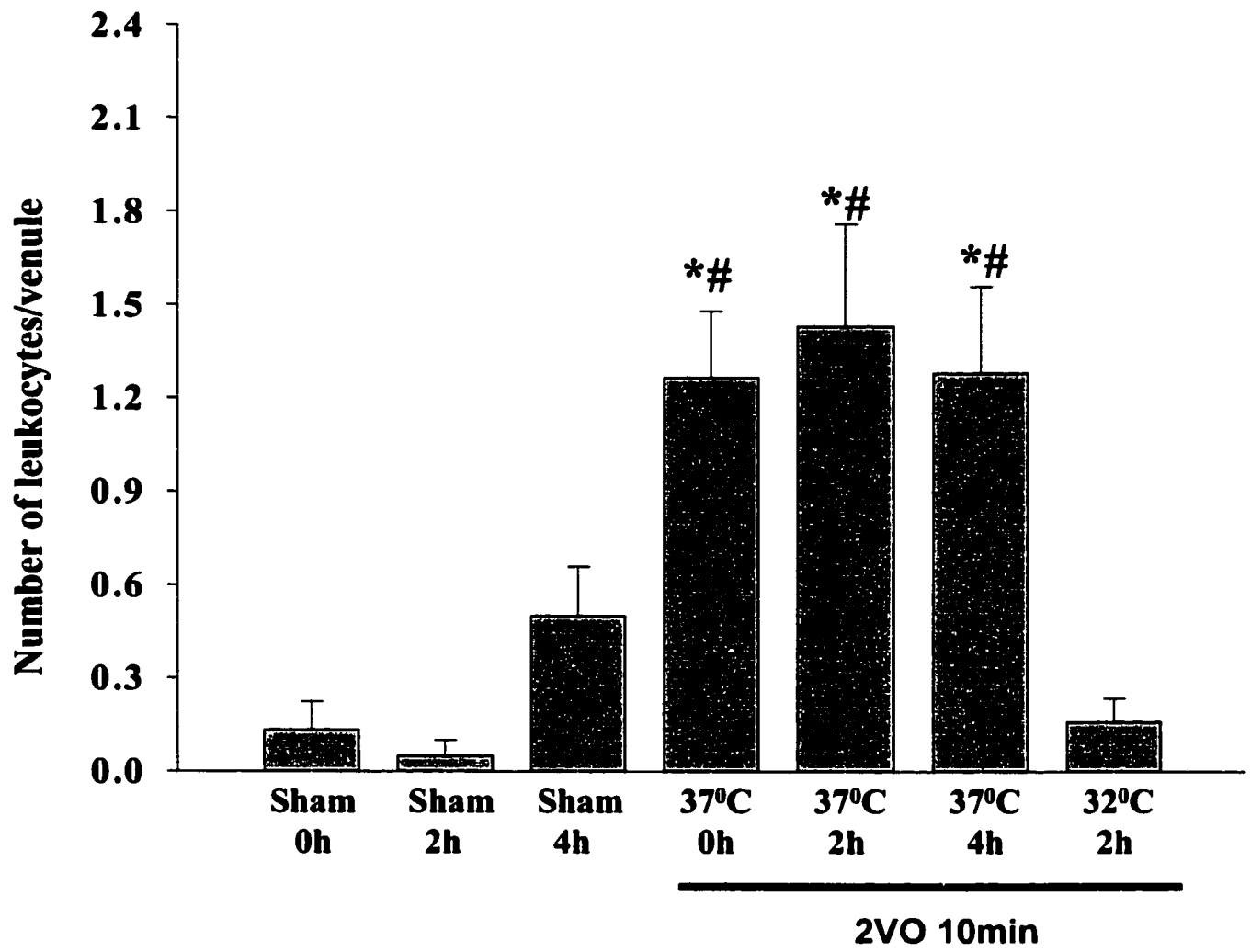
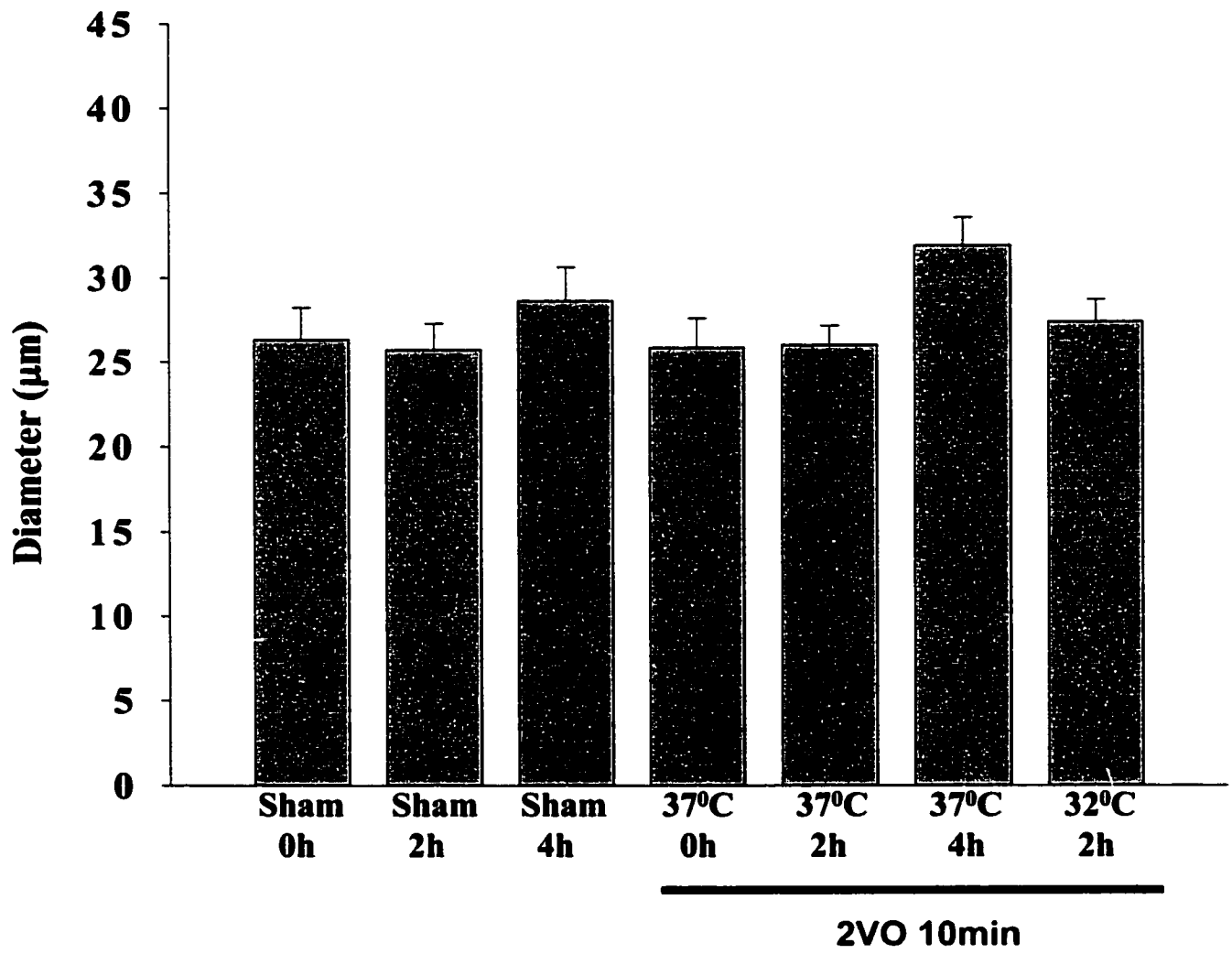


Figure 3.20. Average diameter of pial venules used for monitoring leukocyte rolling and adhesion in the different experimental groups for global cerebral ischemia was measured. Venule diameter was measured using Mocha (Jandel Scientific Inc.) image analysis software. Values shown are means \pm SD. No significant difference ($p < 0.05$) between any group was found.

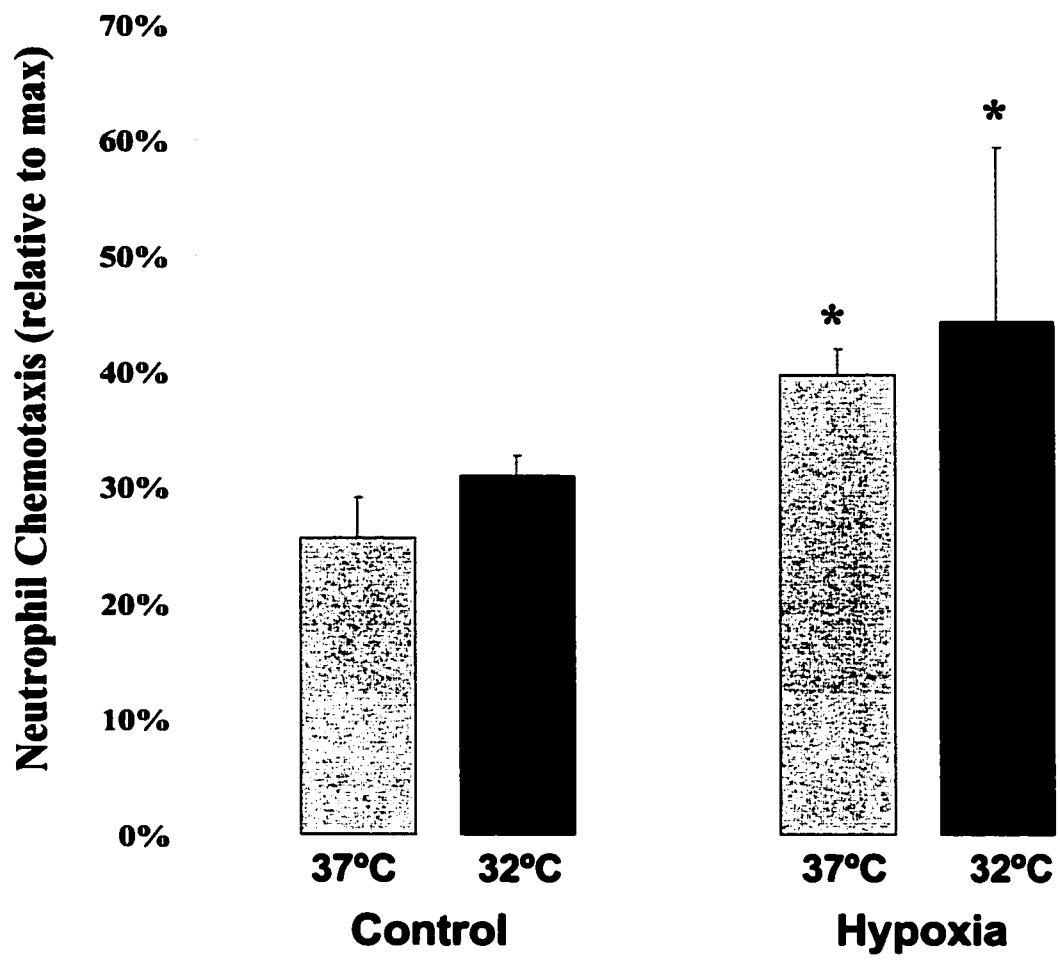


suggesting that observed differences in leukocyte rolling and adhesion were not due to differences in vessel diameter (Figure 3.20).

3.4 In-vitro Effects of Hypothermia on Hypoxia Stimulated Neutrophil Chemotaxis

In order to evaluate the potential effects of hypothermia on neutrophil functioning, we exposed isolated human neutrophils to 1-h hypoxia followed by 1-h recovery at 32°C or 37°C. 1-h hypoxia recovered at both 32°C and 37°C induced a significant increase in neutrophil chemotaxis relative to control unstimulated cells (Figure 3.21). Furthermore, recovery at 32°C had no significant effect on hypoxia stimulated chemotaxis, as compared to recovery at 37°C. Previous experiments in our laboratory have found that hypothermia failed to affect inflammatory activation of human cerebral endothelial cells stimulated with hypoxia *in vitro* (unpublished).

Figure 3.21. Effects of moderate hypothermia on hypoxia/reoxygenation-induced chemotaxis of human neutrophils. Neutrophils were labeled with calcein-AM, and incubated 1-h in a hypoxic chamber (<2% O₂) followed by 1-h recovery in ambient air at 32°C or 37°C. Chemotaxis was quantified by measuring intensity of fluorescence in a fluorescence microplate reader, and reported as ratios compared to relative maximum readings. Each bar represents the mean ± SD of four replicates in a representative experiment. Similar results were obtained in 2 separate neutrophil isolations. * Indicates a significant difference (p<0.05; one way ANOVA) from unstimulated control at given temperature.



Chapter IV: DISCUSSION

4.1 Major Findings

This study provides evidence that moderate hypothermia reduces rolling and adhesion of peripheral leukocytes to pial venules induced by the systemic application of the pro-inflammatory cytokine IL-1 β and global cerebral ischemia in C57/Bl6 mice. Global cerebral ischemia does not stimulate significant transmigration of neutrophils into the brain parenchyma in this model. We further demonstrate that 4-h hypothermia protects against cell death and astrocyte activation following global cerebral ischemia. Finally, we show that neither IL-1 β nor hypothermia alters neutrophil CD18 expression or chemotaxis *in vitro* suggesting that other neutrophil adhesion receptors or cerebral endothelial cells may be the principle target of hypothermic anti-inflammatory effects. These data suggest that one mechanism by which moderate hypothermia provides neuroprotection following inflammatory brain injury or cerebral ischemia may be by reducing leukocyte-endothelial interaction at the blood brain barrier.

4.2 IL-1 β Stimulated Inflammation

Considerable evidence demonstrates that endogenous IL-1 β is a key mediator of endothelial activation, inflammation, and secondary brain damage in cerebral ischemia and trauma (Feuerstein et al., 1997; Betz et al., 1996). Studies have shown that IL-1 β mRNA and protein are synthesized early in ischemia, and that the injection of IL-1 β into ischemic brain enhances edema formation (Betz et al., 1996). Exogenous IL-1 β has also been shown to significantly up-regulate inflammatory mediators (Beg et al., 1993),

leukocyte rolling (Thorlacium et al., 1997), and transmigration (Nourshargh et al., 1995) in peripheral tissues. Given the ability of IL-1 β to stimulate inflammatory processes, and its critical involvement in cerebral ischemic injury, we investigated the effects of hypothermia on IL-1 β stimulated leukocyte-endothelial interactions in mouse pial vessels. Leukocyte-endothelial interactions are generally seen in venules, and rarely in arterioles prepared for intravital microscopy (Thorlacium et al., 1997; Uhl et al., 2000). In this study intravital microscopy was used to visualize *in vivo* leukocyte rolling and adhesion along mouse pial venules 4-h following systemic administration of IL-1 β . IL-1 β injection increased leukocyte rolling and adhering at physiological temperature. As IL-1 β is known to be produced/secreted following brain injury (Buttini et al., 1994; Zhang et al., 1998; Yabuuchi et al., 1994; Betz et al., 1996), our findings suggest that endogenous IL-1 β may be inducing leukocyte rolling-adhesion in the cerebrovascular bed.

We provide novel evidence that a moderate (32°C) whole body hypothermia strongly reduces IL-1 β induced leukocyte rolling and adhesion in murine cerebral microvasculature. This reveals a novel role for hypothermia in suppressing inflammatory brain responses. It is worth emphasizing that anti-inflammatory effects of hypothermia in pial venules were seen only when hypothermia was protracted over a 4-h period during IL-1 β stimulation, whereas transient 1-h hypothermia was ineffective. These findings are in agreement with previous reports that hypothermia is neuroprotective only when maintained for extended length of time (Colbourne et al., 1997; Welsh et al., 1991).

Molecular mechanisms involved in hypothermic suppression of leukocyte rolling and adhesion *in vivo* are not well understood. Studies have emphasized the significance of β_2 integrins (CD11/CD18) in leukocyte-endothelial adhesion (Carlos and Harlan,

1994). We show that neither IL-1 β stimulation nor hypothermia have an appreciable effect on CD18 expression in isolated neutrophils, as compared to potent CD18 stimulation by LPS and fMLP (Lynn et al., 1991). In this study we also provide novel evidence that neither exogenous IL-1 β (stimulant of activation) nor IL-8 (stimulant of motility) induces chemotaxis of isolated neutrophils. Furthermore, hypothermia had no effect on neutrophil chemotaxis. Akriotis and Biggar (1985) previously showed that *in vitro* hypothermia of 29°C reduced the ability of neutrophil to migrate under agarose, however hypothermia was applied during neutrophil chemotaxis. Several possible explanations for our findings are proposed: 1) IL-1 β receptors have been demonstrated in neutrophils, although predominantly the non-signaling type II IL-1R (Parker et al., 1989). It has also been shown that neutrophils bind IL-1, and can function to concentrate IL-1 at sites of inflammation (van der Laken et al., 1997). Therefore, although we show that neutrophils are unresponsive to direct IL-1 β stimulation, perhaps IL-1 β bound to the neutrophil type II receptor function as an IL-1 β receptacle, transporting it to sites of inflammation. Consequently, this may elevate the local concentration of IL-1 β , which in turn stimulates other cells (e.g. cerebral endothelial cells; CEC) capable of activating neutrophils. Furthermore, activated neutrophils have been shown to release IL-1 β , potentially exacerbating this cycle exponentially (Lord et al., 1991). 2) The β_2 integrins are known to be regulated both by expression, and binding affinity (Cronstein and Weissman, 1993). Accordingly, IL-1 β may stimulate neutrophil β_2 integrin binding affinity rather than surface expression. Furthermore, as we show that hypothermia suppresses *in vivo* rolling-adhesion but does not affect neutrophil CD18 expression, we cannot exclude the possibility that hypothermia affects affinity of neutrophil integrins or

neutrophil selectin expression such that it prevents effective neutrophil interactions with endothelial cells. 3) Hypothermia may exert its effects via cells other than neutrophils, most notably CEC. Cerebral endothelial cells are believed to assume divergent physiological postures, from a quiescent to an activated state, and this ability is thought to critically regulate homeostasis of the vascular milieu. While we used isolated neutrophils, studies generally examine IL-1 β stimulation in co-incubation (neutrophil-endothelium) models, or add neutrophils to stimulated endothelial cells (Liu et al., 1998; Bittleman and Casale, 1995). These studies imply that endothelial cells are decisive participants in the regulation of inflammatory stimulation, and that they may secrete factors required for the subsequent mobilization of neutrophils. In support of this idea, *in vitro* hypothermia (25°C) has been shown to transiently inhibit transcriptional activation and surface expression of E-selectin and neutrophil adherence to human umbilical vein endothelial cells stimulated with IL-1 β or TNF α (Haddix et al., 1996). Neutrophil migration across endothelial mono-layers stimulated with IL-1 β also appears to be due to endogenous IL-8 produced by the stimulated endothelium (Smith et al., 1993). In addition, we recently showed that hypothermia inhibits IL-1 β -induced NF- κ B activation, expression of IL-1 β and expression/secretion of IL-8 in human cerebral endothelial cells (HCEC) (Sutcliffe et al., 2001). Therefore, the ability of hypothermia to reduce IL-1 β -induced inflammatory activation of HCEC provides a tentative explanation for the observed reduction in leukocyte rolling/adhesion in the pial circulation *in vivo*. These studies, together with our findings, promote the likelihood that IL-1 β , IL-8 and hypothermia exert their effects predominantly via CEC to elicit their respective responses observed *in vivo* (Sutcliffe et al., 2001).

IL-1 β stimulation is involved in various diseases/insults including arthritis, stroke and cardiac arrest (Dinarello, 1996). Although the ability of hypothermia to reduce *in vivo* leukocyte-endothelial cell interactions does not provide direct evidence that hypothermia is protective, it is enticing to speculate that the suppression of IL-1 β -stimulated leukocyte rolling and adhesion is an important mechanism of hypothermic protection following insults characterized by inflammatory processes. An important consideration in these experiments is that IL-1 β causes systemic inflammation rather than a localized brain insult. While in a systemic inflammatory model all vasculature including the cerebromicrovasculature and leukocytes are stimulated, cerebral injury such as ischemia produces a local inflammatory response. Hence, the effects of hypothermia on inflammatory processes in the brain microvasculature must be evaluated following an experimental model of cerebral ischemia to make more compelling inferences.

4.3 Effects of Moderate Hypothermia in Cerebral Ischemia

4.3.1 The Global Cerebral Ischemia Model

Much of the current understanding of cerebral ischemia has come from the ability to model cerebral ischemia in animals, dating back to pioneer work in the 1970's (Ehklöf and Siesjö, 1972; Hossman and Kleihues, 1973). The growing availability of genetically altered mice has led to an increasing use of murine models of cerebral ischemia. A murine global cerebral ischemia model was used here to evaluate the effects of post-ischemic hypothermia on inflammatory processes.

Only recently has information been reported about experimental models of global cerebral ischemia in mice (Wellons et al., 2000; Murakami et al., 1998; Yang et al., 1997; Fujii et al., 1997). Although experimental parameters differ between studies, the C57/B16 strain was consistently shown to be most susceptible to global cerebral ischemia by bilateral carotid artery (BCCA) occlusion. This increased susceptibility in C57/B16 mice to global cerebral ischemia is due to the vascular anatomy of their circle of Willis, which have underdeveloped anastomosis between carotid and basilar arteries (i.e. posterior communicating arteries) (Wellons et al., 2000; Murakami et al., 1998; Yang et al., 1997; Fujii et al., 1997; Barone et al., 1993). Furthermore, Wellons et al. (2000) recently reported that BCCA occlusion in C57/B16 mice produced similar magnitudes of injury regardless of whether hypotension was used. In this study, as BCCA without hypotension is a less invasive global cerebral ischemia model, we used BCCA occlusion without hypotension in male C57/B16 mice.

In order to investigate the effects of moderate post-ischemic hypothermia in this C57/B16 cerebral ischemia model, appropriate management of temperature had to be ascertained. Although most animal experiments have been performed with systemic hypothermia, there is some experimental evidence to suggest that selective head cooling may also be effective (Gunn et al., 1998). The main controversy between models is whether selective hypothermia can effectively cool the deeper brain structures to render the same level of protection that has been demonstrated in animal models with systemic hypothermia. Gradients of temperature and oxygen consumption within the brain may exist with deeper structures and those closest to major vessels being the warmest (Gunn et al., 1998). Another consideration is that cytokine cascades and neutrophil activation

have an impact on vascular endothelium, and brain damage that will not be addressed by selective head cooling because blood and blood components perfusing the brain will be normothermic. Although any hypothermia may be beneficial, moderate systemic hypothermia addresses these problems more thoroughly, and was the method of choice in this study.

4.3.2 Characterization of the Global Cerebral Ischemic Model

Elucidation of physiological parameters is imperative for assessment of an experimental cerebral ischemic model. Regional cerebral blood flow (rCBF), arterial blood gases, and pH have recently been documented in C57/Bl6 mouse models of cerebral ischemia (Wellons et al., 2000; Sheng et al., 1999; Connolly et al., 1996). Arterial blood gases and pH in this model were comparable among groups throughout the experimental period, and similar to reported values. Laser Doppler flowmetry showed that rCBF was severely reduced immediately following BCCA occlusion, and continued to fall until the end of the 10-minute cerebral ischemia. Although a brief attempt at recovery of rCBF occurred upon re-circulation, the rCBF increased to only 40% of pre-ischemic levels. After this initial increase, deeper hypoperfusion persisted for at least 20-minutes. Hypoperfusion has been described to occur following severe ischemic insults, and arises predominantly due to persistent vasomotor disequilibrium (Verrier, 1996). Post-ischemic hypoperfusion following both focal and global cerebral ischemia is of considerable importance in reperfusion injury, and contributes to secondary ischemic damage (Seisjo and Seisjo, 1996). The finding that profound hypoperfusion occurs during reperfusion after global cerebral ischemia in this model confirms that the cerebral

ischemic insult was severe, and has characteristics of secondary ischemia in the reperfusion phase (Seisjo and Seisjo, 1996).

Characterization of neuronal death following global cerebral ischemia in mice indicated substantial neuronal loss in the hippocampal CA1/CA3 areas, striatum, and neocortical layers 2-5. The density and distribution of neuronal loss were comparable to work describing neuronal damage following 4VO global ischemia in the rat (Pulsinelli et al., 1982; Smith et al., 1984), 2VO in gerbils (Kirino, 1982), and more recently in mice (Yang et al., 1997; Murakami et al., 1998; Sheng et al., 1999). The areas determined to have profound neuronal loss in this model correspond to the selectively vulnerable areas previously described following global cerebral ischemia (Pulsinelli et al., 1982; Smith et al., 1984; Kirino, 1982). Most notably, 4-h post-ischemic hypothermia reduced neuronal loss at 3 and 7 days of reperfusion following global cerebral ischemia. The finding that 4-h post-ischemic hypothermia prevents short-term neuronal loss throughout the ischemic brain is in support of work previously done in the gerbil and rat (Coimbra et al., 1990; Colbourne and Buchan, 1999).

Reactive astrogliosis in the hippocampus following global cerebral ischemia was detected by GFAP staining. It has been previously shown that reactive astrogliosis that accompanies structural brain damage persists for prolonged periods and is characterized by increased number and hypertrophy of astrocytes (Petito et al., 1990). In this study, GFAP immunoreactive cells increased in number and intensity in the CA1 region of coronal brain sections at both 3 and 7 days of reperfusion. 4-h post-ischemic hypothermia reduced the occurrence of increased GFAP immunoreactivity. Considerable evidence indicates that inflammatory mechanisms modulate both the proliferation and

biosynthetic activities of reactive astrocytes, which may exert both harmful and beneficial actions during recovery (Stoll et al., 1998). The development of reactive astrocytosis in this model suggests a cerebral response to ischemic injury, and supports previous work done in the gerbil (Kindy et al., 1992). Furthermore, intra-ischemic hypothermia has previously been shown to be neuroprotective in the gerbil, and following focal cerebral ischemia in the rat (Inamasu et al., 2000). Since glial cell activation as evidenced by increased GFAP immunoreactivity accompanies neuronal damage, and since hypothermia inhibits glial cell activation in the 3 and 7-day post-ischemic brain, it appears that post-ischemic hypothermia inhibits those processes that activate glial cells including neuronal damage in this model.

Through in-depth exploration of this global cerebral ischemia model, we provide strong evidence that supports the utility of this model for investigating effects of post-ischemic hypothermia on cerebral ischemia-induced inflammatory processes. Both post-ischemic hypoperfusion and astrocytosis were detected, suggesting development of secondary ischemic injury. 4-h of moderate post-ischemic hypothermia was shown to reduce neuronal loss and astrocytosis, supporting previous claims of hypothermic neuroprotection.

4.3.3 Leukocyte Rolling-Adhesion-Transmigration Following Global Cerebral Ischemia

Following resuscitation from cardiac arrest or thrombo-embolic stroke, interactions between leukocytes and post-ischemic tissue may lead to secondary reperfusion injury (Hallenbeck and Dutka, 1990). Despite evidence that activated

leukocytes contribute to cerebral ischemic injury (Hallenbeck et al., 1986; Akopov et al., 1996), limited information exists concerning *in vivo* leukocyte-endothelial interactions following cerebral ischemia (Abels et al., 2000; Dirnagl et al., 1994; Beck et al., 1997; Uhl et al., 2000). This study reveals the novel finding that increased leukocyte rolling occurs during the early stages (0 to 2-h) of reperfusion, and increased adhesion occurs throughout a 4-h reperfusion period following 10-minute global cerebral ischemia in mice, while no infiltration of neutrophils was found in ischemic brain following 24-h reperfusion.

Conflicting *in vivo* studies have been published citing the incidence of rolling and adhesion following global cerebral ischemia (Abels et al., 2000; Uhl et al., 2000). Beck et al. (1997) show increases in rolling and adhesion at 1-h and 2-h, respectively, following 15-minute global cerebral ischemia in gerbil. Similarly, Uhl et al. (2000) show persistently elevated rolling 40-180 minutes following 15-minute global cerebral ischemia in gerbil, and increased adhesion at 2-h reperfusion. Although, in this study a very strict exclusion criteria was employed and large vessels examined, that may have influenced their results. Furthermore, Caceres et al. (1995) show increased leukocyte adhesion to cerebral venules and capillaries following 8-min cardiac arrest in the piglet. Contrarily, Abels et al. (2000) reported no significant induction of rolling or adhesion following 4-VO in rat. Interestingly, a rapid but brief increase in rolling was seen immediately upon re-circulation in this 4-VO model, but did not reach significance. A considerable amount of rolling/adhesion was present before occlusion of the carotid arteries, possibly due to the permanent occlusion of vertebral arteries the previous day.

There are essentially two mechanisms by which an accumulation of neutrophils in vasculature occurs: adhesion to the venular wall with or without infiltration, and leukocyte plugging. Here we show that rolling and adhesion are induced immediately upon reperfusion and last for several hours following global cerebral ischemia. This demonstration of rapid leukocyte-endothelial interaction indicates early activation of adhesion molecule cascades indicative of cerebral inflammation (Feuerstein et al., 1997). Furthermore, as hypoperfusion was shown to occur during reperfusion in this model, rapid activation of leukocytes during early reperfusion might contribute to mechanical obstruction of microvessels, development and exacerbation of hypoperfusion, and secondary ischemic injury. The postulated involvement of neutrophils in post-ischemic hypoperfusion and vascular obstruction is supported by studies showing improvement of hypoperfusion after depletion of neutrophils before global forebrain cerebral ischemia in the rat (Grogaard et al., 1989; Schurer et al., 1990), and direct quantification of leukocyte microvascular plugging following focal cerebral ischemia (del Zoppo et al., 1991; Zhang et al., 1993). In addition, depletion of circulating neutrophils during focal cerebral ischemia suppressed post-ischemic edema formation and reduced size of ischemic lesion at 24-h (Matsuo et al., 1994). Nonetheless, other reports have shed doubt on the contribution of neutrophils to microvascular plugging: Aspey et al. (1989) showed no reduction in plugging using leukocyte-depletion following 30-minutes global cerebral ischemia in the gerbil, and Dirnagl et al. (1994) showed no significant leukocyte rolling, adhesion or plugging using confocal laser microscopy following 10-minutes global cerebral ischemia in the rat.

A divergence exists between findings cited by Dirnagl et al. (1994) and those reported in this thesis. The experimental set-up and analysis were significantly different between these studies. Factors that contributed to the deviation in outcome could include animal (rat versus mouse), or model (2VO with hypotension versus 2VO) variations. Also, the lowest level of post-ischemic hypoperfusion attained in the study was 54% baseline, possibly indicating development of a less severe reperfusion injury, as compared to our values. Furthermore, a mild increase in leukocyte rolling, adhesion, and plugging during the 4-h reperfusion was observed, although inter-animal variance resulted in lack of statistical significance.

Neutrophil accumulation in brain tissue following focal cerebral ischemia (Barone et al., 1991; Kochanek and Hallenbeck, 1992; Garcia et al., 1994; Zhang et al., 1994) and cerebral air embolism (Hallenbeck et al., 1986) have been reported. In our model of transient global cerebral ischemia no significant neutrophil infiltration was detected. It is possible that activated neutrophils had entered the parenchyma after the time-point used in this study, or entered at an earlier time-point (<24-h) and subsequently died before neuropathological analysis. Nonetheless, it is critical to point-out that infiltration of neutrophils into intra-parenchymal brain tissue is not a prerequisite for neutrophil-mediated injury. Considerable evidence indicates that, apart from potential obstruction of blood flow, intra-vascular interactions between activated leukocytes and the endothelium produces substantial injury (del Zoppo and Hallenbeck, 2000; Hallenbeck and Dutka, 1990). Activated neutrophil can release cytotoxic proteases (e.g. matrix metalloproteinase), ROS, lipid-derived mediators (e.g. prostaglandins), and cytokines (e.g. IL-1 β and TNF α) (Tomita and Fukuuchi, 1996; Hallenbeck, 1996; Akopov et al.,

1996). These products can lead to endothelial injury, development of vasogenic brain edema and potentially secondary ischemic injury. Therefore, the early appearance of activated luminal leukocytes in cerebral ischemic microvasculature indicates that these cells may contribute to the genesis of ischemic reperfusion injury in this model, even without migration into parenchymal tissue.

4.3.4 Effects of Moderate Hypothermia on Ischemia-Induced Inflammation

Thus far, only one *in vivo* investigation of the effects of hypothermia on leukocyte-endothelial interactions has been reported (Ishikawa et al., 1999). In this report, intra-ischemic hypothermia (30-32°C) was shown to attenuate leukocyte adhesion in rat pial venules following focal cerebral ischemia (Ishikawa et al., 1999). Here, we provide evidence that moderate hypothermia applied in reperfusion attenuates both rolling and adhesion following 10-minutes global cerebral ischemia in mice.

In order to investigate how hypothermia affects neutrophil dynamics *in vivo*, we used hypoxia/reoxygenation to simulate ischemia/reperfusion *in-vitro*, as described in other studies (Zhang et al., 2000; Strasser et al., 1997; Arnould et al., 1993). As leukocyte chemotaxis involves activation and transcellular migration of inflammatory cells, the hypothesis that hypothermia reduces ischemia-induced chemotaxis of neutrophils would provide a potential explanation for the reduction in leukocyte rolling→adhesion observed in pial circulation *in vivo*. We provide novel evidence that 1-h hypoxia/1-h reoxygenation stimulates *in vitro* chemotaxis of isolated neutrophils. Conversely, 1-h hypothermia during reoxygenation had no significant impact on hypoxia-induced neutrophil chemotaxis. Literature suggests that leukocytes respond to

hypoxic/ischemic conditions in various ways, including priming and activation of neutrophils (Scannell, 1996). Furthermore, it has been suggested these altered local microenvironments might contribute to leukocyte chemotaxis. We support the premise that an altered microenvironment effects neutrophil motility by showing that 1-h hypoxia (2% O₂) with reoxygenation increases neutrophil chemotaxis. Interestingly, while no significant neutrophil infiltration was found in our *in-vivo* model, the finding that hypothermia does not alter hypoxic-induced neutrophil chemotaxis support our histological demonstrations that neutrophil infiltration were not affected by hypothermia. Overall, these *in vitro* findings imply that reduced neutrophil chemotaxis is not implicated in the *in-vivo* anti-inflammatory actions of hypothermia observed following our murine cerebral ischemia model.

In vivo inflammatory phenomena such as leukocyte rolling→adhesion→transmigration are influenced by shear flow rates, vascular tone, and other adjacent cell types that are all absent in our *in vitro* neutrophil model. The importance of a cellular network is exemplified by a recent report by Zhang et al. (2000) showing that human astrocytes and HCEC co-operate in regulating endothelial expression of inflammatory cytokines and chemokines. Hypothermia likely influences multiple components of the cerebrovascular bed to effect the changes in physiological responses observed *in vivo*. For example, we previously published that hypothermia reduced pro-inflammatory activation of cerebral endothelial cells (Sutcliffe et al., 2001). Furthermore, studies have provided evidence that other cells are affected by hypothermia including macrophages (Abraham and Lazar, 2000) and glial cells (Inamasu et al., 2000; Kumar and Evans, 1997). Regardless, the combined *in vitro/in vivo* approach used in this study has

generated valuable clues into potential molecular mechanisms of anti-inflammatory actions of hypothermia.

Reduced rolling and adhesion by post-ischemic hypothermia may represent a unique and valuable mechanism of hypothermic neuroprotection. Colbourne and Buchan (1999) have previously indicated that moderate hypothermia is most beneficial when applied immediately following the cerebral ischemic insult. The demonstration that protection provided by hypothermia is most favorable when applied during early reperfusion suggests that events, such as leukocyte-endothelial interactions, that ensue in the early phase of post-ischemic re-circulation contribute to disruption of microvascular homeostasis and cerebral injury. Thus, it is reasonable to propose that reduced rolling and adhesion is one of the mechanisms of short-term neuroprotection provided by moderate hypothermia following cerebral ischemia.

It is generally accepted that one, but not the sole mechanism by which hypothermia protects the brain from ischemic challenge is by reducing the metabolic rate (Thoresen et al., 1995; Baldwin et al., 1991). As brain ischemia (energy depletion) and reperfusion (hypoperfusion) leads to a state of inappropriate metabolism with ensuing cytotoxic edema and cellular death, hypothermia conveys protection by virtue of improving the ratio between metabolic demand and substrate supply. Although a general metabolic slow-down could provide a predictable explanation for reduced leukocyte function, previously published observations that hypothermia induces vasoconstriction (Thorlacius et al., 1998), increases blood viscosity and leukocyte adhesions due to reduced shear blood flow (Chen and Chien, 1978), as well as our *in vitro* finding that hypothermia does not affect neutrophil chemotaxis, support the notion that hypothermia

does not confer a ubiquitous depression in cell function. Consequently, other mechanisms of neuroprotection by hypothermia have been proposed including reduced glutamate accumulation (Zhao et al., 1997) and reduced BBB disruption (Dietrich et al., 1990). Based on the results of these studies, we propose that hypothermia reduces injury by two cooperating mechanisms: 1) Hypothermia reduces mechanical obstruction of microvessels by preventing leukocyte activation and leukocyte-endothelial interactions, consequently improving post-ischemic rCBF and assisting early recovery processes. 2) By reducing both IL-1 β and ischemia-induced leukocyte-endothelial interactions, hypothermia provides protection from ramifications of neutrophil-mediated inflammatory processes such as BBB deterioration, induction of pro-inflammatory mediators, and secretion of proteases. Consequently, these proposed neuroprotective mechanisms of hypothermia could assist in the restoration of balance between metabolic demand and supply. These conclusions are in part supported by further reports indicating that hypothermia reduces post-ischemic hypoperfusion, delayed energy failure, and neutrophil infiltration following focal cerebral ischemia (Karibe et al., 1994; Thoresen et al., 1995; Toyoda et al., 1996).

Although not experimentally proven, we believe that the link between early (5min-2h) actions of hypothermia on leukocyte rolling and adhesion following brain ischemia impacts on reduction of brain injury measured after 3-7 days. This is based on: 1) strong literature evidence that blocking adhesion molecules, including leukocyte Mac-1 (Chopp et al., 1994), endothelial ICAM-1 ((Bowes et al., 1993) reduces post-ischemic brain injury; 2) ICAM-1 null mice are less susceptible to ischemic brain injury; 3) establishing prompt reperfusion (within 3 hours of stroke) is essential for good stroke

outcome; 4) hypothermia prevents early circulatory events that can lead to cascades responsible for prolonged hypoperfusion (i.e., secondary ischemia), including microcirculatory obstruction by leukocytes and platelets. Therefore, the consequences of improved perfusion in early post-ischemic period may translate into improved neuronal survival measured at later time points after ischemia.

As a number of reports suggest that transient post-ischemic hypothermia simply delays ischemic injury, it remains possible that transient moderate hypothermia in this model only delays leukocyte-endothelial interactions (Dietrich et al., 1993; Inamasu et al., 2000). If indeed hypothermia only delays leukocyte-endothelial interactions in the cerebromicrovascular bed, the prospective benefits are still substantial by opening the therapeutic window with anti-inflammatory therapy. For example, the only currently approved treatment of thrombo-embolic stroke is tPA (Buchan et al., 2000). Evaluation of the benefits of tPA has indicated that it must be administered within several hours (3-h) following the ischemic insult, or therapeutic benefit is lost. If hypothermia simply delays ischemic injury (primary or secondary), then the therapeutic benefit of post-ischemic hypothermia may lie in its ability to prolong the therapeutic window for intervention by tPA.

4.4 Final Remarks & Conclusion

Previous studies have reported valuable experimental global cerebral ischemia models in the rat and gerbil (Pulsinelli et al., 1982; Kirino, 1982). Here we characterize a new murine 2VO model of global cerebral ischemia that provides a simplified means of future exploration into cerebral ischemia in wild type and genetically modified animals.

Although some controversy in relation to the occurrence of inflammation following global cerebral ischemia has been reported, our studies indicate that inflammation and potentially secondary ischemia are part of the pathophysiology in 2VO global cerebral ischemia in mice.

This study was the first to provide evidence that moderate post-ischemic and post-inflammatory hypothermia exerts anti-inflammatory actions by reducing leukocyte rolling and adhesion in brain microvessels. Inflammatory processes have been associated with secondary ischemic brain injury. Considerable prior research suggests that hypothermia is neuroprotective. Therefore, the suppression of inflammatory events by hypothermia observed in these studies may be a part of the beneficial effect of hypothermia on brain damage. Although the mechanisms of neuroprotection by hypothermia are still not fully understood, clinical trials are underway to determine the therapeutic benefits of hypothermic intervention following traumatic and ischemic brain injuries (Ziener et al., 2000; Schwab et al., 1998; Clifton et al., 2001).

Many mechanisms of hypothermic neuroprotection have been proposed which likely work in concert to elicit long-term benefit. Since short duration hypothermia was not protective, and the suppression of leukocyte rolling-adhesion by hypothermia was observed during early reperfusion in our model, one can argue that anti-inflammatory effects of hypothermia are not the crucial determinants of protection following cerebral ischemia. Nonetheless, the clinical relevance of these findings is compelling, as the demonstration of anti-inflammatory actions of hypothermia will allow for better clinical application of appropriate combination therapies in patients with cerebral ischemia. In addition to cerebral ischemia, neuroinflammation is an integral component of TBI and

encephalitis (Whalen et al., 1997; Sobel et al., 1990). Thus, these reported reductions in leukocyte-endothelial interactions indicate a potential therapeutic benefit of hypothermia in other states of neuroinflammation.

For imminent clinical use of hypothermia, vigilant awareness of some limitations must be stressed. Foremost is the finding that both anti-inflammatory and neuroprotective effects of hypothermia are temperature-dependent (32°C) and duration-dependent (≥ 1 -h) in our model. Furthermore, possible side effects of prolonged hypothermia (ex: hypotension, hypovolemia, lowered immune response) may not be acceptable particularly for the elderly. In light of these restrictions, combination treatment with mild hypothermia may be found to bestow optimal clinical outcome in the future.

It is commonly believed that early therapeutic intervention with hypothermia will provide long-term benefit. Although considerable progress has been made, abundant room still remains for further characterization of hypothermic neuroprotection. Our novel findings of suppression of brain inflammation by hypothermia may provide important clues how to best apply hypothermia to achieve neuroprotection in various neuroinflammatory injuries, including cerebral ischemia.

4.5 Future Experiments & Initiatives

1. Inflammation is believed to be an important component of secondary ischemic injury following focal cerebral ischemia. Although findings in this study could be projected to a focal cerebral ischemic milieu, benefit of hypothermia would be better ascertained by evaluating leukocyte-endothelial interactions in an experimental focal

cerebral ischemia model. A limiting factor is that the cranial window permits visualization of surface vessels, while focal arterial occlusion induces severe ischemia predominantly to deep brain structures. To effectively visualize microvascular dynamics following focal cerebral ischemia, use of advanced equipment would be required.

2. Hypothermia is presently shown to have anti-inflammatory effects. A major repercussion of this anti-inflammatory ability is believed to be subsequent reduction of neutrophil-mediated injury via proteases, ROS, and/or cytokines. Providing experimental evidence that hypothermia reduces BBB dysfunction or secretion of proteases by neutrophils following ischemia in our model would notably strengthen this argument.
3. Previous reports indicate that long duration hypothermia is required for long-term neuroprotection (Colbourne et al., 1997). Our findings only show short-term effects of hypothermia. Therefore, it would be valuable to investigate long-term protection by hypothermia in this model.
4. Some groups suggest that brief transient post-ischemic hypothermia only delays ischemic injury (Dietrich et al., 1993; Inamasu et al., 2000). A valuable extension to our *in-vivo* findings would be to evaluate leukocyte-endothelial interactions beyond 2-h reperfusion in a group maintained hypothermic. These findings would indicate if hypothermia permanently decreases, or only delays leukocyte-endothelial interactions.
5. Previous studies have shown neuroprotection following cerebral ischemia using delayed post-ischemic hypothermia (Colbourne et al., 1995). Although it is

recognized that hypothermia provides neuroprotection via numerous mechanisms, the contribution of anti-inflammatory effects of hypothermic to neuroprotection is unresolved. The future experiments to resolve this issue should include delayed post-ischemic hypothermia initiated after rolling-adhesion had already taken place. This would assist in characterizing the contribution of early inflammation to secondary ischemic injury, and verify the prospective benefit of anti-inflammatory actions provided by post-ischemic hypothermia found in this study.

6. Our study has proposed a potential effect of hypothermia on post-ischemic hypoperfusion, although no direct evidence has been presented. Further evaluation of the effects of hypothermia on hypoperfusion would be helpful, and could include prolonged measurements of rCBF using laser Doppler flowmetry during both normothermic and hypothermic reperfusion
7. It is believed that hypothermia confers neuroprotection via several mechanisms. We provide evidence that hypothermia elicits anti-inflammatory actions by reducing ischemia-induced leukocyte rolling and adhesion. We further suggest that these anti-inflammatory effects contribute to hypothermic neuroprotection. A potential means of demonstrating a causal association between neuroprotection and anti-inflammatory effects of hypothermia following ischemia-reperfusion would be to reproduce our *in-vivo* experiments in adhesion molecule (e.g., E-selectin/L-selectin to eliminate rolling behaviors and ICAM-1 to eliminate adhesion of leukocytes) knockout mice. If hypothermic reduction in leukocyte rolling and adhesion represents a key mechanism by which hypothermia confers neuroprotection, then hypothermia should not provide additional neuroprotection to that provided by adhesion molecule knockout itself.

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