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Transient changes in blood-brain barrier integrity, thermotolerance, and heat shock protein expression following brief, hyperthermia in an in vitro model

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TRANSIENT CHANGES IN BLOOD-BRAIN BARRIER INTEGRITY, THERMOTOLERANCE, AND HEAT SHOCK PROTEIN EXPRESSION FOLLOWING BRIEF, HYPERTHERMIA IN AN IN VITRO MODEL

BY

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DEDICATION

I dedicate this thesis to my family. Thank you for your continued love, encouragement, and support; without which I would not be where I am today.

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ABSTRACT

TRANSIENT CHANGES IN BLOOD-BRAIN BARRIER INTEGRITY, THERMOTOLERANCE, AND HEAT SHOCK PROTEIN EXPRESSION FOLLOWING BRIEF HYPERTHERMIA IN AN IN VITRO MODEL

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Hyperthermia is being studied as a means of disrupting blood-brain barrier (BBB) integrity. An *in vitro* BBB model was exposed to 10 s of 45, 48, or 51°C, or 5 s of 54°C, and TEER was used to assess integrity. Loss of BBB integrity increased as temperature increased ($r = 0.88$, $P<0.0001$). Thermotolerance was examined by applying a second hyperthermia to models 24 h afterward. Models demonstrated thermotolerance, indicated by less loss ($P<0.001$) of integrity compared to controls. The degree of thermotolerance increased as pre-conditioning temperature increased ($r = 0.42$, $P<0.0001$). Hsp27 and Hsp70 were analyzed by Western blot to examine their contribution to this thermotolerance. Hsp27 was not changed ($P>0.10$) by the pre-conditioning heat treatments. Hsp70 expression increased ($P<0.05$) in response to pre-conditioning for 5 s at 54°C. This thermotolerance cannot be explained by changes in Hsp27 ($r = 0.17$, $P>0.10$) and Hsp70 ($r = -0.17$, $P>0.10$).

INTRODUCTION

The blood-brain barrier (BBB) is a dynamic entity of the central nervous system (CNS) that acts to regulate the permeability of molecules into and out of the brain – an essentiality in maintaining and regulating the neural environment. In this manner, the BBB acts to regulate the composition of cerebral interstitial fluid. As the composition of interstitial fluid is directly related to the function of neurons and glial cells (Brightman and Tao-Cheng, 1993), the maintenance of a BBB is of utmost importance for neural health.

While the BBB is composed of capillary endothelium, pericytes, and astrocytes (Pardridge, 1999) that innervate throughout the brain tissue (Rubin and Staddon, 1999), it is the endothelial cells that are thought to form the basis of this barrier. The endothelial cells of the BBB join together by forming tight junctions. These tight junctions are responsible for severely limiting paracellular transport of molecules (Brightman and Tao-Cheng, 1993; Gloor et al., 2001; Huber et al., 2001). In this anatomical manner, the BBB acts as an important protective structure of the CNS to help ensure that only molecules essential for brain function can enter into the CNS. In a clinical setting, this unique anatomy poses several physiological difficulties: loss of BBB integrity (increase in BBB permeability) can lead to deleterious effects on the CNS, and, contrarily, the presence of a BBB creates extreme difficulty in delivering pharmaceutical drugs to the brain to treat disorders of the CNS. These difficulties prompt the need to

study the BBB to further understand physiological regulation of permeability from two main perspectives: (a) to help enhance the delivery of pharmaceutical drugs to neural tissue, and (b) to understand the role that BBB permeability plays in CNS disease development.

Tight junctions of the BBB are more sensitive to environmental insults than tight junctions of epithelial cells (Wolburg and Lippoldt, 2002). When exposed to environmental stress, the tight junctions of the BBB lose their function and paracellular permeability to blood-borne molecules increases (Sharma and Hoopes, 2003). In order to manipulate the BBB in a clinical setting, it is important to fully understand the mechanistic changes in these tight junctions following stress, such as hyperthermia.

Hyperthermia, or heat stress, is a condition in which the body produces or absorbs more heat than it can dissipate, and is one of the most common forms of environmental stress for humans. Problems of hyperthermia as related to brain health have been known since early times and are responsible for numerous deaths and illnesses. Numerous studies have indicated that the CNS is particularly sensitive to hyperthermia, leading to increased systematic immune responses (Sharma and Hoopes, 2003).

Studies on the effects of heat stress on the BBB are becoming increasingly important in medical science, as new therapies utilizing heat are being developed as an adjuvant therapy for treating brain tumors (Sminia and Hulshof, 1998; Schulze et al., 2004), and as a means to enhance delivery of

pharmaceuticals into the brain (Merritt et al., 1978; Sharma and Hoopes, 2003; Ng et al., 2004). Heat stress can be applied either as whole body hyperthermia or localized hyperthermia. Whole body hyperthermia induces a relatively quick increase in body temperature and is most often used to combat deep seated brain tumors (Sharma and Hoopes, 2003). Enhanced techniques of applying heat stress have allowed for localized heating, allowing for a greater range of temperature and duration used in applied hyperthermia. Localized hyperthermia can utilize much higher temperatures (normally above the physiological range, >44°C), and can be used to locally increase BBB permeability – important in enhancing pharmaceutical drug delivery to the brain. The application of brief, high temperature hyperthermia has recently been shown to increase BBB permeability (Jeliazkova-Mecheva et al., 2006), which may prove beneficial in a clinical setting by providing quicker means of opening the BBB.

A unique phenomenon has been described in cells exposed to repeated bouts of brief, high temperature hyperthermia – acquired thermotolerance (Jeliazkova-Mecheva et al., 2006). Thermotolerance refers to the ability of a cell, that has been "pre-conditioned" with a previous stress, to be resistant to future stresses (Kampinga, 1993). This could play an important role in developing means of closing the BBB under certain disease states in which BBB permeability is undesirably increased, such as in multiple sclerosis. Also, in using heat stress to increase the permeability of the BBB, it is possible that repeated bouts of heat stress could cause thermotolerance. Thus,

thermotolerance is an undesired state in enhancing pharmaceutical drug delivery to the brain.

Thermotolerance has been described in the BBB following both prolonged, mild temperature hyperthermia (Ikeda et al., 1999) and brief, high temperature hyperthermia (Jeliazkova-Mecheva et al., 2006). However, the exact conditions and cellular changes that confer thermotolerance have yet to be defined.

The heat shock proteins, also referred to as the stress proteins or molecular chaperones, are a group of ubiquitous proteins present in all mammalian cell types that show increased cellular expression following stressful stimuli (Kaufmann, 1992; Franklin et al., 2005). Heat shock proteins act as molecular chaperones for various intracellular functions, such as protein-protein interactions, prevention of unwanted protein aggregation, and aid in transporting proteins across cell membranes. Some heat shock proteins are found constitutively expressed in cells, while others are highly induced following metabolic stress (Kaufmann, 1992; Franklin et al., 2005), indicating a role in cellular protection. Some correlation has been noted between thermotolerance and heat shock protein induction (Welch, 1992), suggesting a possible role of heat shock proteins in conferring thermotolerance. However, while the exact mechanisms of heat shock protein induction are still unclear, it is clear that the heat shock response of different cells and organisms are regulated in different ways (Lindquist, 1986), prompting a need to examine the heat shock response in the thermotolerant BBB.

Experiments involving neural cell pre-conditioning demonstrate a strong correlation between the induction of Hsp70 and Hsp27, and protection against subsequent stresses (Franklin et al., 2005), suggesting that these two particular Hsps may play an important role in thermotolerance of the CNS. However, few studies have specifically examined Hsps and thermotolerance of the BBB.

In this study, we investigated the effects of brief, high temperature hyperthermia on BBB permeability and acquired thermotolerance by evaluating changes in BBB transendothelial electrical resistance (TEER) across an in vitro BBB model. We also investigated possible mechanisms for the observed effects by examining changes in Hsp70 and Hsp27 expression in thermotolerant BBB models. Therefore, the following review will summarize BBB anatomy, the current knowledge on BBB permeability and regulation, the effects of heat stress on BBB permeability and thermotolerance, and the role of heat shock proteins following heat stress and their potential role in thermotolerance.

CHAPTER I

LITERATURE REVIEW

Role of Blood-Brain Barrier Permeability as Related to Anatomical and Physiological Function

The unique anatomy of the blood-brain barrier (BBB) provides the brain with a specialized protection system designed to limit the entry of molecules from the blood into the central nervous system (CNS). Allowing only molecules necessary for brain function into the CNS, the BBB inhibits the entry of potentially damaging molecules into sensitive neural tissue, and also maintains ionic homeostasis of the cebral spinal fluid. However, while the BBB provides protection to the brain from foreign molecules, its unique design significantly impairs the delivery of pharmaceutical drugs designed to treat disorders of the CNS, into the brain. Recent research has focused on the manipulation of the BBB to enhance delivery of pharmaceuticals into the brain. In order to better understand the mechanics of BBB manipulation, it is important to understand the unique anatomy and physiological design of this barrier.

Anatomical Properties of the BBB

The BBB is a dynamic entity of the CNS, consisting of specialized blood capillaries that permeate throughout the brain tissue. Paul Ehrlich, a bacteriologist of the late 1800s, was the first to describe the existence of a barrier between the blood and the brain (1885; 1902). He conducted studies on the

CNS using water-soluble Aniline dye injected into the venous system of various animals. His most noted observation from these experiments was that while the aniline dye stained the non-CNS tissues of the body, the brain remained unstained. This observation first described the existence of a barrier of the brain to polar compounds.

In follow-up experiments, water soluble dye injected into the venous system of various animals stained all tissue except the brain (Goldmann, 1909; Goldmann, 1913). Likewise, the same water-soluble dye injected directly into cerebrospinal fluid stained the brain, but the rest of the body remained unstained. These studies helped to further the concept of a BBB.

Observations of cerebral vessels illustrated low permeability to a wide range of organic and inorganic molecules (Krogh, 1946; Reese and Karnovsky, 1967). However, it was unclear whether the capillary endothelium or the surrounding astrocyte foot processes of the cerebral vasculature contributed to the low permeability of the BBB (Kuffler and Nicholls, 1966). Studies of cerebral vasculature permeability were thus conducted.

The development of the electron microscope allowed for the BBB to be further characterized. Horseradish peroxidase (HRP) was used as a tracer molecule to determine the apparent permeability of capillary endothelium (Reese and Karnovsky, 1967). Ten to 60 min following injection of HRP into the tail veins of adult mice, mouse brains were removed and fixed for examination under an electron microscope. Significant amounts of HRP did not pass across the

capillary endothelium into the brain. In previous experiments with heart and skeletal endothelium, HRP significantly permeated the extra-cellular spaces of the heart and skeletal endothelium. These results indicated that capillary endothelium of the brain has distinct characteristics that separate it from endothelium of the heart and skeletal muscle, and that these results were indicative of the presence of zonular tight junctions in capillary endothelium.

Further studies have indicated that the BBB comprises three distinct characteristics when compared to other endothelium of the body: (1) low endocytosis rates of capillary endothelium, (2) the presence of specific transport and carrier molecules on endothelial cells, and (3) the presence of tight junctions between capillary endothelial cells that enhance the low permeability of the barrier (Gloor et al., 2001).

Specific Transport and Endocytosis

The general consensus on BBB permeability is that unless molecules are small and lipophilic, they will not be allowed to cross the barrier (Rubin and Staddon, 1999). Essential molecules to brain function that are small but not lipophilic cross the BBB via means of specialized transport systems. Brain metabolism studies of glucose, ketone bodies, and branched chain amino acids, suggest that these 3 metabolites or their precursors are actively transported across the BBB into the brain (Pardridge, 1981), prompting a closer look at the exact mechanisms of specific transport of various essential molecules.

Specific Membrane Transporter Proteins

In the 1950s, Robert A. Fishman pioneered numerous studies to further the understanding of permeability characteristics of various compounds across the BBB. Most of his work focused on the permeability of proteins. One of his first studies examined plasma albumin exchange rates with cerebrospinal fluid (CSF) in the adult dog (1953). Twenty minutes following intravenous injection of radioiodinated serum albumin (RISA®), RISA® appeared within CSF, with maximum RISA® concentration in the CSF present after 20 hours. This slow exchange rate of RISA® was attributed to the absence of specific albumin transport systems present in the BBB (Kumagai et al., 1987), thus suggesting a relatively low permeability of the BBB to whole proteins.

This finding allowed for the identification of three amino acid carriers: the neutral amino acid carrier, the basic amino acid carrier, and a low-capacity independent carrier (Oldendorf and Szabo, 1976). Specific transport studies found that the neutral amino acid carrier transported phenylalanine, leucine, tyrosine, isoleucine, methionine, tryptophan, valine, dihydroxyl phenylalanine, cysteine, histidine, threonine, glutamine, asparagine, and serine; while the basic amino acid carrier transported arginine, ornithine, and lysine; and finally, the low-capacity independent carrier transported aspartic acid and glutamic acid.

The independent transport systems of the BBB for nucleic acids were then elucidated by injection of ^{14}C-labelled nucleotide bases into the common carotid artery of adult rats (Cornford, 1975). Appreciable uptake of adenine, adenosine, guanosine, inosine, and uridine into the brain were observed, suggesting the

presence of transport systems for these nucleotides. Two independent transport systems were further identified as the mechanisms responsible for uptake into the brain of the aforementioned nucleotides. The first transport system transported adenine, while the second transport system transported adenosine, guanosine, inosine, and uridine. From this study it was learned that essential molecules that do not cross the BBB via diffusion, are transported into the brain via specific transport mechanisms.

Tight Junctions of the BBB

Tight junctions of the BBB were first examined in depth in neuronal tissue of several animal species (Brightman and Reese, 1969). Adult white mice, goldfish, adult domestic chickens, and chicks were injected with either HRP or lanthanum hydroxide as tracer molecules to determine the presence of cellular junctions. Using electron microscopy, only junctions that were present in areas where cell membranes were 30 angstrom or less apart were examined. The junctions present under microscopy were classified into 3 classes, (1) gap junctions, (2) tight junctions, and (3) labile appositions. Tight junctions were only found between endothelial cells of parenchymal blood vessels and between epithelial cells of the choroid plexus. In each location that tight junctions were observed, the junctions were significantly uniform in structure. HRP and lanthanum hydroxide were impermeable through the tight junctions. These data indicate that endothelial tight junctions are an essential component of the structural basis of the BBB.

Following this observation, numerous studies were conducted to further elucidate the structure and function(s) of tight junctions in brain capillary endothelium. Freeze-fractured samples of reptilian brain were examined to determine the presence and structure of tight junctions (Shivers, 1979). Tight junctions of the BBB in the reptile *Anolis carolinensis* were structurally complex and contributed to the impermeability between endothelial cells. The tight junctions also formed a belt-like appearance circumscribing the endothelial cells, indicating a structural contribution to BBB permeability.

Observed fracture faces of cerebral endothelium consisted of capillary endothelium that contained multistranded tight junctions extending into the endothelium of the postcapillary venules (Nagy et al., 1984). Capillary endothelium of mouse brain contains an extensive network of tight junctions located between overlaps of the plasma membranes of endothelial cells (Connell and Mercer, 1974). Taken together, these data indicated that tight junctions of the BBB are complex structures.

Tight junctions, likewise, play an important role in BBB development in organisms (Stewart et al., 1994). Tight junctions were examined in the fetal and adult BBB to determine the relationship between development of capillary endothelial tight junctions and the BBB in mouse brain. A low occurrence of tight junctions in fetal brain tissue corresponded to an undeveloped BBB (i.e., in fetal brain tissue, low non-specific permeability had not developed). In the adult brain tissue, a high occurrence of tight junctions was observed, which corresponded to a fully-developed BBB. Contrarily, tissue samples from adult brain tumors

demonstrated a low occurrence of tight junctions (similar to fetal brain tissue) and thus, an undeveloped BBB. From these results, it is apparent that tight junctions are crucial to BBB development and that development of tight junctions and BBB formation is in part dependent on environment.

Tight Junction Function

The presence of tight junctions within capillary endothelium of brain tissue indicates a functional role in regulating the permeability of the BBB (Connell and Mercer, 1974; Nagy et al., 1984; Stewart et al., 1987; Stewart et al., 1994; Petty and Lo, 2002). The significantly low BBB permeability to HRP and lanthanum hydroxide indicates the role of tight junctions in sealing endothelial cells together (Reese and Karnovsky, 1967; Brightman and Reese, 1969). Tight junctions of epithelial cells link cells together by forming a belt around each cell (Van Meer et al., 1986), forming a barrier that limits the paracellular diffusion of molecules and ions (Gumbiner, 1987). However, tight junctions do not form a complete seal. Rather they contain aqueous pores capable of opening and closing depending on various influences, such as cellular signaling for increased phosphorylation of proteins (Claude, 1978). Taken together, these data indicated that tight junctions act as a gate, limiting the molecules that are allowed to diffuse between capillary endothelial cells of the BBB.

Tight junctions, however, are not fusions of cell membranes. Rather, they consist of a plethora of specific tight junctional proteins (Gumbiner, 1987), which under specific regulation, are capable of regulating the paracellular permeability of molecules and ions. While the focus of our research was not on the specific

tight junction proteins, it is still important to understand the various proteins involved in tight junction formation and regulation of permeability.

Tight Junction Proteins

Tight junctional proteins can be classified into two main groups: the transmembrane proteins and the cytoplasmic accessory proteins (Gumbiner, 1987; Petty and Lo, 2002). The identified transmembrane proteins consist of occludin, claudin, and the junctional adhesion molecules (JAMS). Likewise, the identified cytoplasmic accessory proteins of tight junctions consist of the zona occludens, 7H6 antigen, and cingulin.

Transmembrane Proteins: Occludin was first identified using immunofluorescence and immunoelectron microscopy (Furuse et al., 1993). Observed as an isolated 65 kD integral membrane protein, that spanned the cellular membrane four times, occludin was later described as a ubiquitous protein amongst varying mammalian species (Ando-Akatsuka et al., 1996). The cDNA amino acid encoding sequences of human, mouse, and dog occludin were closely related, sharing about 90% identity; while the cDNA amino acid encoding sequences of chicken and rat-kangaroo varied greatly from that of human, mouse, and dog, sharing only about 50% homology. While certain species share close amino acid coding sequences, occludin does not demonstrate homology amongst all mammalian species.

Claudin was the next identified transmembrane protein of tight junctions. Identified as a 22 kD transmembrane protein, claudin spans the membrane four times. Northern blotting techniques were used to elucidate the presence of

different claudin family members in different tissue samples (Morita et al., 1999). Lung and liver tissue demonstrated high presence of claudin-3, which was present in minimal amounts in testis and kidney tissue. Claudin-4, 7, and 8 were expressed solely in lung and kidney tissue, while claudin-5 was expressed in all tissue samples examined. Results from this study indicated a probable role of specific claudin members in tight junction formation of different tissues.

The final group of transmembrane proteins are the junctional adhesion molecules (JAMs). As their name implies, JAMs are involved in cell-to-cell adhesion, which contributes to the control of BBB permeability. The first existence of JAM in tight junctions was described using confocal and immunoelectron microscopy of endothelial and epithelial cells (Martin-Padura et al., 1998). JAMs were concentrated at intercellular junctions of the apical membrane of both endothelial and epithelial cells.

A specific JAM protein was identified by examining expressed sequence tags of a cDNA library from human tonsillar endothelial cells (Palmeri et al., 2000). An immunoglobin-like protein, termed vascular endothelial junction-associated molecule (VE-JAM), was localized at the intercellular boundaries of endothelial cells. VE-JAM was observed as a homologous protein to JAM. This homologous protein contained 2 extra-cellular immunoglobin-like domains, a transmembrane domain, and a short cytoplasmic tail. Due to the localization and structure of JAM, it can be concluded that JAMs are involved in cell to cell adhesion, particularly that of endothelial cell adhesion in the BBB.

Cytoplasmic Accessory Proteins: The first cytoplasmic accessory tight junctional protein isolated was zona occludens 1 (ZO-1) (Stevenson et al., 1986; Hirase et al., 1997; Rubin and Staddon, 1999). Utilizing immunofluorescence, ZO-1 was first identified in the epithelia of colon, kidney, and testis, as well as arterial endothelium (Stevenson et al., 1986). The isolated 225 kD protein was similar in each tissue sample, suggesting that ZO-1 is a ubiquitous protein of mammalian tight junctions. Two other ZO proteins have been isolated and characterized, ZO-2 and ZO-3 (Petty and Lo, 2002).

Since the identification of ZO-2 and ZO-3, the ZO proteins have been grouped into the membrane-associated guanylate kinase proteins family (MAGUK) (Anderson, 1996), because the ZO proteins contain a guanylate kinase region, a *src* homology domain (SH3), and differing numbers of PDZ domains (Haskins et al., 1998). The presence of these particular domains in the ZO proteins, indicate a functional ZO protein role in binding integral membrane proteins (Haskins et al., 1998), helping to seal the tight junction.

The next isolated cytoplasmic accessory protein was 7H6 antigen (Zhong et al., 1993). The 7H6 antigen was isolated in the tight junctions of various tissues, and was found to localize at the periphery of tight junctions, binding with a 155 kD protein. However, 7H6 antigen is found only in tight junctions that are impermeable to macromolecules and ions (Satoh et al., 1996), such as the BBB. Changes in tight junctional proteins during hepatocarcinogenesis display a significant decrease in 7H6 as normal hepatocytes progress towards carcinogenesis (Zhong et al., 1994). These results indicated that under ATP

depletion, 7H6 dissociates from tight junctions, leading to a loss of tight junction integrity (Mitic and Anderson, 1998).

Finally, the next cytoplasmic accessory protein of importance in tight junctional function and structure is cingulin. Cingulin was first identified as a component of tight junctions of avian brush-border cells (Citi et al., 1988). Electron microscopy and immunofluorescence, revealed cingulin as an elongated, heat stable, acidic protein that localized with ZO proteins at the apical region of brush-border cell. Through examination of the tight junctions in *Xenopus laevis*, cingulin was characterized as a 140-160 kD protein localized at the cytoplasmic surface of endothelial and epithelial cells (Cordenonsi et al., 1999). Electron microscopy revealed that cingulin contains globular head and tail domains and a central α-helical rod-like domain. The central rod domain was deemed responsible for coiled-coil dimerization interactions.

The wide array of tight junctional proteins allows for numerous interactions to seal endothelial and epithelial cells together, and to regulate the permeability of the paracellular pathway.

Tight Junctional Protein Interaction: Collectively, the data on endothelial tight junctions indicate the interactions between tight junctions as the primary barrier to regulate the paracellular diffusion of molecules (Gumbiner, 1987; Cereijido et al., 1989; Huber et al., 2001). More specifically, the interaction of tight junction proteins with each other and the apical membrane of endothelial cells is the primary factor in regulating permeability (Huber et al., 2001; Petty and Lo, 2002).

Previous experiments on Madin-Darby canine kidney (MDCK) cells indicated the role of various domains of occludin in regulating paracellular permeability (Balda et al., 2000). The importance of occludin in permeability regulation was examined by observing tight junction formation of various chimeras designed to express different domains of occludin. Extracytoplasmic domains and at least one transmembrane domain were required for the formation of a true tight junction, in which paracellular permeability could be regulated. Chimeric expression of occludin also resulted in accumulation of claudin, indicating a complementary relationship between the two proteins.

The relationship between occludin and ZO proteins was examined by observing occludin and ZO interaction in *E. coli* (Furuse et al., 1994). A specific domain (termed domain E) on occludin was found to interact and bind directly to ZO-1 and ZO-2 proteins in *E. coli*. In vitro studies revealed that when human and bovine epithelial cells were transfected with full-length chick occludin, the chick occludin was delivered and incorporated into pre-existing tight junctions, interacting with ZO. The localization of occludin at tight junctions was related to its association with ZO-1 and ZO-2, thus contributing to linking transmembrane proteins to the cytoskeleton.

Transmission electron microscopy was used to describe the interactions of cingulin with other tight junctional proteins (Cordenonsi et al., 1999). The NH_2-terminal of cingulin was shown to interact with ZO-1, ZO-2, Z0-3, and myosin. Likewise, the COOH-terminal of cingulin interacted with ZO-3 and myosin.

These interactions suggested a role of cingulin in linking the submembrane plaque of tight junctions to the cytoskeleton of endothelial and/or epithelial cells.

Anastomosing strands present in tight junctions were first described in lung epithelium from fetal lamb samples (Schneeberger et al., 1978). By examining lung tissue samples using light and electron microscopy, the appearance of continuous strands present in low permeability tight junctions were noted. The presence of anastomosing strands was attributed to their contribution in regulating paracellular impermeability.

Claudin was found to be an integral protein in forming the anastomosing strands present in tight junctions (Furuse et al., 1999). Cotransfected L cells were observed for interactions of different claudin protein species, specifically claudin-1, -2, and -3. In cells transfected with all three claudin species, claudin-3 formed lateral strands with claudin-1 and claudin-2, forming dimers. However, claudin-1 and claudin-2 did not interact without the presence of claudin-3. From these observations it was apparent that different combinations of claudin interactions could lead to different tight junction anatomy, contributing to varying permeabilities of tight junctions from various locations of the body.

The research on tight junctions, indicate that the specific arrangement of the various transmembrane and cytoplasmic accessory proteins allow for varying degrees of impermeability and regulation of endothelial cell paracellular flux. Research on the tight junctions of the BBB indicates the formation of low permeability junctions that severely limit paracellular flux.

In Vitro Models of the BBB:

Early studies on BBB permeability were normally carried out *in vivo*, as acceptable *in vitro* models had not been developed (Eddy et al., 1997). However, the use of an *in vitro* model for BBB permeability studies provided researchers with a reproducible isolated system that is more easily used to determine isolated events, enhancing the ease of study. Thus, researchers devoted great time to isolating brain capillaries and developing an acceptable *in vitro* model of the BBB.

The first description of isolating brain capillaries involved isolation from tissue collected post-mortem on humans (Siakotos and Rouser, 1969). This method included mechanical dissociation of capillaries from brain tissue, and then further isolation of capillaries by filtering the brain homogenate through a 200 μm pore nylon mesh filter. The resulting isolated capillaries proved to be structurally intact and demonstrated the biochemical functions of brain capillaries *in vivo*. This important discovery would thus allow for *in vitro* permeability studies on the BBB.

Further refinement of capillary isolation and characterization of the properties and physical features of isolated capillaries were performed on mouse brain tissue (DeBault et al., 1979). Capillaries from mouse forebrain were isolated utilizing similar mechanical and filtration techniques as the previous method. Isolated capillaries ranged in size from 4 μm to 25 μm diameter, and isolated vessels ranged from either single segments to large, multibranched segments. Cell viability was approximately 50%, with all isolated capillaries

demonstrating proliferation properties, an important aspect of in vitro models. A significant observation that would later prove useful in primary cell cultures was that the smaller capillaries gave rise to endothelial cells, while the larger capillaries gave rise to both endothelial cells and smooth muscle cells. This important observation led to the derivation of techniques to isolate specific cells (e.g., endothelial cells) from brain capillaries.

Whole brain capillaries, however, cannot be used to measure certain transportation characteristics of the BBB (such as transendothelial transport) (Eddy et al., 1997) as the apical and basolateral membranes of the capillaries are too close together for accurate measurements of transported molecules. Thus the isolation of endothelial cells became a priority in developing an acceptable and accurate *in vitro* model for studying transport of molecules across the BBB.

Both Bowman, et al. (1981) and DeBault, et al. (1981) described techniques of isolating endothelial cells from brain capillary endothelium. Bowman developed a primary culture of capillary endothelial cells derived from rat brain, by using enzymatic dissociation and then employing Percoll™ gradient centrifugation to sort the remaining cell types by size. The resulting product was a homogenous sample of endothelial cells that when cultured displayed properties of the BBB (such as the presence of tight junctions). DeBault developed a method of isolating capillary endothelial cells from Swiss-Webster mice, by utilizing mechanical dissociation, and then subsequent filtration to sort cells by size. The resulting product was a heterogenous mixture of cell types that when cultured, the cells either formed homogenous cell colonies or mixed cell

colonies. Cloning ring techniques were then used to isolate homogenous endothelial cell plaques from the other cell types. Like Bowman's endothelial cell culture, DeBault's resulting endothelial cell line also demonstrated properties of endothelial cells *in vivo* (such as the expression of endothelial cell specific antigens, growth patterns, and morphology). Both of these experiments were hallmarks to cell culture, as now viable endothelial cells could be isolated from brain capillaries and used for *in vitro* permeability studies.

An *in vitro* BBB model was developed by culturing porcine brain capillary endothelial cells on porous Transwell™ membranes (Bobilya et al., 1995). Porcine brain capillary endothelial cells were isolated from weanling Yucatan miniature pigs via mechanical and chemical dissociation followed by filtration through screens of various pore sizes to isolate capillary fragments. Capillary endothelial cells were characterized via labelling with Factor VIII antibody, and by endocytosis of acetylated low-density lipoprotein. A BBB model was established by seeding capillary endothelial cells onto fibronectin coated, porous (4 μm) polycarbonate membranes suspended between two fluid chambers (analogous to the capillary lumen and interstitium of the brain). Barrier integrity (functional integrity of the tight junctions) was assessed by measuring electrical resistance (impedance to current) across the barrier. Following subculture to the polycarbonate membranes, cells grew to confluent monolayers and demonstrated maximum electrical resistance 10-12 days post sub-culture, indicating formation of a tight barrier. These results demonstrated the suitability of this BBB model for *in vitro* studies.

Measuring BBB Permeability In Vitro

Transwell™ plates offer a reliable and efficient method of evaluating BBB permeability *in vitro* (Eddy et al., 1997), thus selection of a useful method of measuring permeability is of utmost importance. Most studies observe extravasation of large (M.W. greater than 350 (Eddy et al., 1997)) tracer molecules to measure permeability, such as fluorescein (Merritt et al., 1978), Evans blue dye (Ikeda et al., 1994; Oztas and Kucuk, 1998), and HRP (Reese and Karnovsky, 1967). Recently, transendothelial electrical resistance has been employed as a more sensitive means of measuring changes in BBB permeability (Butt et al., 1990; Hurst and Clark, 1998; Jeliazkova-Mecheva et al., 2006).

Electrical resistance of bovine brain microvessels was found to be significantly greater than that of bovine aortic endothelial cells (Rutten et al., 1987). Bovine brain microvessel (BMEC) and aortic endothelial cells were isolated from individual clones, and grown as monolayers on a permeable support glutaraldehyde-treated collagen gel. Transendothelial electrical resistance of BMEC ranged from 157-783 Ω^*cm^2, while the TEER of the aortic endothelial cells was much lower, with a mean TEER of 13.5 Ω^*cm^2, indicating that BBB impedence to ions is much greater than aortic ion impedence. Sensitivity of TEER was also measured on BMEC exposed to calcium free medium for 10 min. Following exposure to calcium free medium, electrical resistance of BMEC decreased to 75% of pre-exposure values. Following addition of calcium back to the medium, TEER returned to the original pre-

exposure values within 1 h. These results indicated that TEER can be a sensitive method of measuring changes in BBB permeability.

Transendothelial electrical resistance across the BBB in anaesthetized rats aged between 17 days gestation and 33 days after birth increased as the rats aged (Butt et al., 1990). The 17 day-old fetuses displayed a BBB TEER of 310 Ω*cm^2, while at 21 days gestation the resistance increased significantly to 1128 Ω*cm^2. From birth to 33 days after birth the TEER of the BBB leveled to 1462 Ω*cm^2. These results indicated that the increase in electrical resistance indicates a decrease in paracellular BBB ion permeability, and that TEER does not significantly change in the rat BBB following birth.

Effects of Heat Stress on BBB Permeability

Heat stress, or hyperthermia, can be described as a condition in which the body temperature of an organism is raised above normal homeostatic temperatures, and is normally due to a failure in an organism's heat dissipating mechanisms and/or overproduction of heat. Heat stress can be caused by numerous circumstances, such as exposure to a hot environment, exercise, or fever caused from bacterial and viral infections (Sharma and Hoopes, 2003). Excessive heat stress (caused by long-term exposure or extreme temperatures) in an organism can elicit severe stress responses, which could lead to coma or death of the organism. Studies on the effects of heat stress on the BBB have become increasingly important in medical science, as new therapies utilizing heat are being developed to treat brain tumors, and enhance the delivery of pharmaceuticals into the brain.

Deleterious Effects of Heat Stress on the CNS

The use of hyperthermia in medicine has prompted several concerns regarding severe effects heat stress can elicit on the CNS. Since heat therapy is often used to treat tumors of the CNS, and new methods of heat therapy are being employed to enhance therapeutic drug delivery into the brain, it is important to understand the effects that heat therapy will have on brain tissue. Various studies have been performed to determine what pathologies manifest in the brain after heat stress in several animal models.

Pathological changes induced by acute heat stress were observed in young rat brains (Sharma and Cervos-Navarro, 1990). Young rats exposed to 38°C for 4 hours demonstrated a 4.4% increase in brain water content (edema). Visualization under electron microscopy showed collapsed microvessels. These results indicate that in the young rat, 38°C exposure for 4 hours causes significant damage to the BBB and brain.

Age-related pathophysiological changes following heat stress were then observed in the rat model (Sharma et al., 1992). Young rats (8-9 weeks old) and adult rats (24-32 weeks old) were exposed to 38°C temperatures for 4 hours, using a biological oxygen demand incubator. The young rats demonstrated significant increases in brain water content, plasma and brain serotonin levels, and a significant reduction in cerebral blood flow. Morphological examination revealed significant increases in glial fibrillary acidic protein and vimentin immunoreactivities indicating neuronal damage. Adult rats, however, demonstrated either mild or no changes in brain edema, plasma and brain

serotonin levels, changes in cerebral blood flow, or significant morphological changes. These results indicated a greater tolerance to hyperthermia in adult rats compared to young rats, indicating an age dependent development of heat stress related pathophysiologies.

Changes in BBB disturbance due to higher temperature hyperthermia was observed in male Wistar rats following whole body hyperthermia (Urakawa et al., 1995). Rats were exposed to 44°C for 30 minutes, with histological examinations performed either 2 h, 6 h, 3 d, or 7 d following heat stress. Two animals died before histological examination could be completed. One died of respiratory trouble and the other of epidural hematoma (both presumably due to heat stress). Of the remaining animals, mean arterial blood pressure and partial pressure of oxygen were significantly lower post-hyperthermia treatment than pre-hyperthermia treatment. In the 6 h to 3 d groups, brain tissue consisted of distinct necrotic, reactive, and permeable zones, indicating a distinct change in cellular morphology of the brain. The necrotic zones contained cells that were "spongy" and had lost their cellular structure. In the reactive and permeable zones, astrocyte feet appeared swollen. In the 7 d group, the necrotic zone consisted of "liquified" tissue, and the reactive zone demonstrated distinct hypertrophic astrocytes. From these data it was apparent that severe heat stress (defined as 30 min at 44°C) in the rat model causes extreme brain pathologies (necrosis and loss of cellular integrity) that are not reversible, as the pathologies worsened over time.

These data on the deleterious effects of severe heat stress demonstrate the need for further research examining the heating limits of the mammalian brain, so that appropriate heat therapy can be applied without causing pathologic changes to the brain.

Heating Limits of the Brain From Localized Hyperthermia

If we are to use hyperthermia as a therapy in clinical medicine, it is important to define the heating limits of brain tissue. The current research indicate that different heating limits of brain tissue are dependent on species and heating method. However, as more research is conducted, our knowledge of the heating limits of brain tissue expands and we can correlate safe heating limits from observed maximum tolerated doses.

The threshold heating limits of dog normal brain were defined following 8 MHz interstitial hyperthermia (Ikeda et al., 1994). Histological changes in brain tissue were examined following *in vivo* exposure to various temperatures and duration. Forty-eight healthy adult dogs were exposed to set temperatures of 42°C, 43°C, or 44°C for either 15, 30, 45, or 60 min. Histological changes were observed after staining with haematoxylin and eosin, thionin, and luxol fast blue. Significant, irreversible brain damage was defined as, in grey matter, pyknosis of neurons or neuronal death, and in the white matter as mild to severe separation of myelinated nerve fibers and necrosis. The results of this study demonstrated that the threshold heating limits of dog normal brain are 42°C for 45 minutes or 43°C for 15 minutes – as irreversible brain damage occurred in temperature and exposure lengths exceeding these values. This finding is important, as it

indicated the severe sensitivity of brain tissue to heat – as the temperature was increased by 1°C, the tolerable heating limit of dog brain decreased by 30 min.

Heat Stress and Increased BBB Permeability Studies

Numerous studies abound describing the changes in BBB permeability to various molecules in several animal models. The majority of the early work on hyperthermia studies was conducted *in vivo* with heat stress applied at physiological temperatures for long duration (from 10-15 min to several hours). Several methods of employing heat stress are used for hyperthermia studies; the most common methods included microwave radiation, localized radio-frequency, and infusion of hyperthermic substances (such as saline). These early studies on BBB permeability following hyperthermic stress were vital in enhancing our understanding of physiological changes to the BBB, which would later allow for the enhancement of pharmaceutical drug delivery into the brain.

Changes in BBB permeability to fluorescein were examined in adult rats following hyperthermic exposure (Merritt et al., 1978). Adult rats were exposed to 30 min of 1.2 GHz radiation at power densities ranging from 2-75 mW/cm^2. No increase in BBB permeability to fluorescein was observed following microwave-radiation. However, when rats were made hyperthermic in a warm-air environment, fluorescein uptake across the BBB was observed. The results of this study indicate that hyperthermia, not non-hyperthermic microwave-radiation, was the cause of BBB breakdown, and increased permeability to fluorescein.

Changes in BBB permeability to Evans blue dye was examined in normal dog brain made hyperthermic by exposure to 8 MHz radiofrequency interstitial

hyperthermia (Ikeda et al., 1994). Adult dogs were exposed to various hyperthermic temperatures and durations. Significant BBB breakdown, and observance of Evans blue extravasation did not occur until dog brain was heated to either 43° C for 60 min, or 44° C for 30 min. However, the heating limits of normal dog brain are 42° C for 45 min or 43° C for 15 min – after which irreversible brain damage occured. These results thus demonstrated a clear problem, in that in order to achieve adequate BBB breakdown in dog normal brain, heating limits must be exceeded.

An increase in BBB permeability (to Evans blue bound to albumin) was observed following induction of hyperthermia in rats (Oztas and Kucuk, 1998). Reversible BBB opening following intracarotid hyperthermic saline infusion was examined in adult Wistar rats. Evans blue extravasation was observed in rats that were injected with 43° C saline solution for 30 seconds at a rate of 0.12 ml/s. However, researchers observed that the increased BBB permeability to Evans blue was reversible; an important finding. Increased Evans blue extravasation remained for 3 hours after infusion, however, at 6 hours after infusion, normal levels (pre-hyperthermia) of Evans blue were noted in the brain.

Brief heat shock significantly reduced BBB function in an *in vitro* model (Jeliazkova-Mecheva et al., 2006). The BBB model was exposed to temperatures between 44 and 58°C for 3 to 60 s. To determine BBB function, immediately before and following treatment, transendothelial electrical resistance (TEER) was measured. Significant reduction of barrier function occurred following 30 s exposure to 41-45°C, 10 s exposure to 50-53°C, and 3 s exposure

to 54°C and greater. Blood-brain barrier models were all able to regain barrier function following heat treatment, however, the rate of recovery was dependent on temperature and duration of heat treatment. For example, BBB models exposed to 51°C for 10 s regained full barrier function within 5 min following heat treatment, but BBB models treated at 54°C for 10 s required 10 h for barrier recovery, and BBB models treated to 58°C for 3 s or longer did not regain full barrier function within 24 h following treatment, suggesting this temperature significantly damaged the BBB. The results from this study indicated that brief-heat shock can significantly reduce BBB function *in vitro*, however this reduction is highly dependent on the temperature and duration of heat treatment.

Acquired Tolerance

Acquired tolerance describes a state in which stressed cells are made resistant to subsequent episodes of stress. Theoretically, stresses of any kind, as long as they are non-lethal, can induce a state of tolerance in cells. These tolerant cells would then be resistant to future stresses, possibly even lethal stresses.

Thermotolerance, acquired tolerance due to hyperthermia, presents several issues in clinical hyperthermia. As hyperthermia is being extensively studied to enhance the delivery of pharmaceuticals, and in combination with radiation therapy for cancers, the acquisition of thermotolerance is undesired. It is thus important to understand the mechanisms and conditions that induce a thermotolerant state.

In human glioma cells, the acquisition of thermotolerance was dependent on the method of hyperthermic preconditioning (Raaphorst et al., 1995). Human glioma cells, from the U87MG cell line, were tested for the development of either chronic or acute thermotolerance. To examine the development of chronic thermotolerance, glioma cells were heated at 39 - 42°C for up to 48 h. However, little to no thermotolerance developed, and as the temperature increased, thermal toxicity increased, as indicated by a decrease in cell survival. These results indicate that chronic hyperthermia at 39 - 42°C for up to 48 h does not induce acquired thermotolerance in glioma cells. To examine the development of acute thermotolerance, glioma cells were heated at 40 - 42°C for either 4, 8, 24, or 48 h, immediately following which cells were exposed to 45°C. At all pre-conditioning temperatures, maximum acute thermotolerance was observed after 8 h of heating, with a gradual loss of thermotolerance noted at 24 h and 48 h, respectively. These results indicated that acute hyperthermia can induce thermotolerance.

Thermotolerance of the BBB

In Vivo Thermotolerance Studies: Hyperthermic pre-conditioning conferred BBB tolerance to hypoxia-ischemia in the newborn rat (Ikeda et al., 1999). Seven-day old rats were preconditioned with whole body hyperthermia of 41.5°C - 42°C for 15 minutes. Twenty-four h following preconditioning treatment, rats were exposed to hypoxic-ischemic insult for 2 h. Brains were perfused and fixed 24 h following hypoxia-ischemia, and brain samples observed for extravasation of IgG via immunoreactivity staining. Compared to controls,

newborn rats that were preconditioned before hypoxic-ischemic insult demonstrated significantly less IgG staining, suggesting hyperthermia preconditioning prevented BBB disruption following hypoxia-ischemia.

In Vitro Thermotolerance Studies: In response to brief heat shock, brain microvascular endothelial cells developed thermotolerance (Jeliazkova-Mecheva et al., 2006). Porcine brain microvascular endothelial cells were cultured onto Transwell™ membrane inserts to develop a model of the BBB. Thermotolerance was observed in BBB models exposed to heat treatments that significantly reduced BBB function - 52°C for 5 or 10 s, 53°C for 5 s, and 54°C for 3 s. The BBB models were treated with temperatures between 44°C and 58°C for 3 to 60 s. Immediately before and following heat treatment, TEER was measured to determine the degree of reduced BBB function. Twenty-four hours following the initial heat treatment, BBB models were again heat treated at the same temperature and duration and TEER was measured before and after heat treatment. Thermotolerance was defined as a reduced loss of barrier function following the subsequent heat treatments. The observations of thermotolerance in the BBB model at the higher heat treatments are of critical importance for clinical applications of heat treatment, and suggest the need for further studies on thermotolerance of the BBB both *in vitro* and *in vivo*.

The Heat Shock Proteins

Following heat stress, all cell types respond by synthesizing a specific set of proteins, termed the heat shock proteins (Hsp). This increased Hsp synthesis following heat stress is termed the "heat shock response," and has been found to

occur in cells after a variety of other stressors as well – such as exposure to heavy metals, hypoxia, and ischemia. Many think that the heat shock response guards the cell against further damage, helping to initiate repair of damage sustained during the initial stress (Lindquist, 1986; Mizzen and Welch, 1988; Franklin et al., 2005). Several studies have shown the Hsps are induced in response to denatured proteins produced during heat stress (Sharp et al., 1999), and act by binding and stabilizing unstable conformers of proteins (Rokutan et al., 1998), thereby indicating a protective role. However, the exact mechanisms of the heat shock response on the BBB still remain to be fully clarified – as little direct evidence exists linking Hsps to a specific cellular event.

The Hsps are highly conserved amongst species, and are normally constitutively expressed in most cell types (Franklin et al., 2005). Five families of Hsp have been identified based on amino acid sequence and molecular weight: the low molecular weight Hsp's (27-32 kDa), the 60 kDa family, the 70 kDa family, the 90 kDa family, and the 110 kDa family (Welch, 1992; Franklin et al., 2005).

Heat shock proteins have gained increased interest in medicine, as several pathologies of the CNS are marked by increased expression of the Hsps within the brain. Several studies have shown increased expression of Hsp70 in patients with Alzheimer's disease (Fonte et al., 2002; Clarimon et al., 2003), and recent research has indicated an increased expression of Hsp27 in patients with epilepsy (Bidmon et al., 2004). However, the functional aspects of Hsp70 and Hsp27 upregulation in CNS diseases are still unclear.

Experiments involving cell pre-conditioning demonstrated a strong correlation between the induction of Hsps and protection against subsequent stresses, suggesting that the Hsps may play an important role in thermotolerance (Franklin et al., 2005). Therefore, studies regarding heat shock and thermotolerance in the brain can help to determine a relationship between Hsp expression and protection of the brain during stress. Since Hsp70 and Hsp27 appear to play important roles in the CNS, this review will focus on the current knowledge of Hsp70 and Hsp27 in terms of expression following heat shock and in thermotolerance.

Heat Shock Protein 70

The heat shock protein 70 family is regarded as the most highly conserved of the Hsps (Lindquist, 1986) and consist of both constitutively expressed and inducible proteins (Welch, 1992). Of the Hsp70 family, the two main Hsp's are Hsp72 and Hsp73. Heat shock protein 73 is regarded as the constitutive form of Hsp70, as it is constitutively synthesized in mammalian cells, while Hsp72 is referred to as the inducible form of Hsp70, as it is upregulated during stress (Welch, 1992).

Since Hsp70 is regarded as the most inducible Hsp following cell stress, much of the research that exists on Hsp expression focuses on Hsp70. The following highlights the current research on Hsp70 following heat stress of the CNS in both *in vitro* and *in vivo* studies.

Hsp70 Expression Following Heat Stress

Synthesis and accumulation of a 71 kDa protein (termed "P71") was observed following a 15 min hyperthermic shock at 42-42.5°C in various tissues of 6-week-old rats (Currie and White, 1983). Synthesis of P71 was determined via fluorography, while accumulation of P71 was observed using Coomassie brilliant blue staining. In brain tissue, P71 synthesis was greatest 120 min following hyperthermic shock, and then declined to undetectable levels by 1 day following shock. Accumulation of P71 was barely detectable at 120 min following stress, but increased to "easily detectable" levels 1 day following stress, and then declined to no detectable accumulation by 4 days following hyperthermia. The authors concluded that brain tissue was capable of synthesizing large amounts of P71 following hyperthermic stress, and both synthesis and accumulation of P71 were time dependent.

Concomitantly, a delayed and maximum synthesis of Hsp70 in heat stressed mammalian cells was observed (Mizzen and Welch, 1988). The heat shock response was examined in several mammalian cell lines exposed to heat stress at 43°C or 45°C for 30 min to 90 min. An interesting observation of this experiment was that the inhibition of protein synthesis and then subsequent level of Hsp70 induction was dependent on the temperature and incubation time of applied heat stress. Cellular protein synthesis was inhibited for 2 h following 1.5 h of 43°C hyperthermia, and for 5 h following 30 min of 45°C hyperthermia. Likewise, maximum expression of Hsp70 occurred 4 h following 1.5 h of 43°C hyperthermia, and 13 h following 30 min of 45°C hyperthermia. Moreover,

relative Hsp70 expression was significantly greater in cells exposed to 30 min of 45°C hyperthermia than in cells exposed to 1.5 h of 43°C hyperthermia. These results suggest that Hsp70 induction and expression is dependent on the duration and temperature of hyperthermia.

In endothelial cells, the expression of Hsp70 in human umbilical vein endothelial cells (HUVECs) was strongly correlated to heat stress (Wagner et al., 1999). Northern blot analysis was used to determine the relative expression of Hsp70 mRNA and Hsp70 in heat stressed HUVECs. Heat stress was applied for 3 h at 42.5°C, after which cells were allowed to recover for 4 h at 37°C. Levels of Hsp70 mRNA reached a maximum 2 h post-heat stress, while Hsp70 levels began increasing 60-90 min post-heat stress and continued to increase until 6 h post-heat stress. These results indicated a strong correlation between heat stress and increased Hsp70 expression in an endothelial cell type. Interestingly, however these data also demonstrated a delayed heat shock response, suggesting a delay in any protection Hsp70 might confer in endothelial cells post heat stress. While this presents an essential view of the Hsp70 response in endothelial cell types, this study only addressed the heat shock response under one defined circumstance. As such, different applied methods of heat stress could induce a different pattern of Hsp70 response.

Expression of Hsp70 following heat stress is also dependent on the tissue type (Manzerra et al., 1997). Differences in Hsp70 expression were examined in neural and non-neural brain tissues of rabbits made hyperthermic. Rabbits were injected with d-lysergic acid diethylamide to initiate hyperthermia at 42.5°C for 1

h. Compared to basal levels of Hsp70, non-neural tissues displayed a significant increase in Hsp70 expression following hyperthermia, while neural tissues displayed only a modest increase in Hsp70 expression. This difference in Hsp70 expression was attributed to a possible dampened Hsp response in neural tissues and also suggests that the level of Hsp70 induction during the stress response may be dependent on the amount of constitutive Hsp70 already present in the tissue.

BBB Expression of Hsp70

Relatively little data exists outlining the mechanisms of the heat shock response in the BBB. Few studies have been done in this area and in some aspects the results are not in agreement in terms of Hsp70 induction in the BBB following hyperthermia.

In the BBB, Hsp70 synthesis was correlated to heat shock (Shivers et al., 1988). Bovine brain capillary endothelial cells (BBMEC), a model of the BBB, were heat stressed at 43°C for 1 – 3 h and allowed to recover at 37°C for 2 – 6 h. Cell lysate collected from BBMEC during heat stress and following recovery from stress was used to compare the relative synthesis of Hsp70 during the heat shock response. Maximum Hsp70 synthesis was observed during heat stress and then declined by approximately 5% following 2 h recovery, and continued to decline 6 h post recovery to "normal" Hsp70 expression levels in non-stressed cells. These results contradict Wagner (1999), who observed maximum Hsp70 expression 6 h post heat stress. Thus, it is apparent that Hsp70 expression in

heat stressed cells of the BBB could be dependent on several factors: cell type, and the method of applying heat stress (temperature, time, recovery).

The expression of Hsp70 in BBMEC following heat stress differed between BBMEC that were exposed to mild or moderate heat stress (Ng et al., 2004). Mild heat stress was delivered via ultrasound, and maintained at 41°C for 20 min. Cells were allowed to recover from the heat stress for 20 min, 1 h, 4 h, or 24 h, at which point cells were lysed and the lysate analyzed for Hsp70 expression. There was no significant difference between Hsp70 expression in heat stressed cells vs. control cells (non-stressed) at any of the recovery time points. Moderate heat stress was applied to BBMEC by submerging cells in a 43°C water bath for 3 h. Cells that received moderate heat treatment demonstrated a significant increase in Hsp70 levels compared to control cells. These data indicate that Hsp70 expression is sensitive to the method of applying heat stress. However, it is unclear as to whether the difference in Hsp70 expression was due to the temperature difference (41°C for 20 min vs. 43°C for 3 h) or the method at which heat was applied (ultrasound vs. water bath).

From these studies it is apparent that hyperthermia induces the heat shock response in the BBB by induction of Hsp70. Also, the degree of Hsp70 induction and time at which Hsp70 synthesis begins following heat stress is dependent on the method of applying heat stress, and the temperature and duration at which heat stress is applied. Due to the induction of Hsp70 during heat stress, it is possible that Hsp70 might play a role in acquired thermotolerance.

Short-Duration High Temperature Stress and Hsp70 Induction: The majority of the studies on Hsp induction have been conducted utilizing exposure to mild to moderate (38-44°C) temperatures for long duration, as would be found in a fever state or heat exhaustion. However, the potential application of high temperature for short duration in enhancing the delivery of pharmaceuticals to the brain prompts the need for studying the relationship between short duration high temperature heat stress and Hsp induction.

Heat shock protein 70 was induced in keratinocytes briefly exposed to high temperatures (Bowman et al., 1997). Normal human epidermal keratinocytes (NHEK) were exposed to high temperatures, ranging from 53 - 60°C for 1 s. Synthesis of Hsp70 following high temperature exposure was observed in a dose-dependent manner peaking at 56°C, with the relative quantity of Hsp70 about 80% greater in the 56°C cells compared to controls. In NHEK exposed to 57°C and above, Hsp70 quantity abruptly declined. These results demonstrate that short duration high temperature stress to 56°C can induce Hsp70 synthesis in NHEK.

Acquired Tolerance and Hsp70 Induction

Very little data exists outlining the correlation between Hsp70 induction and thermotolerance of the BBB. However, several studies have been conducted in regards to Hsp70 and acquired tolerance of other mammalian tissues and cell types. This review will focus on those studies to examine a possible correlation between thermotolerance and Hsp70 induction of the BBB.

Thermotolerance and Hsp70 Induction: Mizzen and Welch (1988) were one of the first teams to observe a connection between the thermotolerant cell and Hsp70 expression. Mammalian cell lines were made tolerant by a 43°C, 1.5 h heat shock with a subsequent 8 h recovery at 37°C before a 45°C challenge for 20 – 40 min (dependent on the cell type). Thermotolerant cells recovered translational activity more quickly than non-thermotolerant cells, but the duration of Hsp70 synthesis following the 45°C challenge was significantly reduced in thermotolerant cells compared to non-thermotolerant cells. These data suggested that (1) due to the quicker translational recovery, thermotolerant cells are better able to cope with cellular stresses, and (2) Hsp70 synthesis is under strict regulation. However, it is still difficult to correlate Hsp70 translational activity with thermotolerance because thermotolerance can be dependent on a number of different parameters; and although thermotolerant cells recovered translational activity more quickly, relative expression of Hsp70 was not compared in thermotolerant vs. non-thermotolerant cells.

Thermotolerant cells of the hippocampal region of rat brains do not have increased expression of Hsp70 compared to non-thermotolerant cells (Lu et al., 2004). Rats were made thermotolerant with whole-body hyperthermia (41°C - 42°C for 15 min) 24 h prior to induction of BBB opening via 1.6 M D-mannitol infusion. Compared to sham treated rats, there was a significant induction of Hsp70 following whole-body hyperthermia. However, there was no significant difference in Hsp70 levels in rats solely treated with whole-body hyperthermia vs. rats treated with both whole-body hyperthermia followed by D-mannitol infusion.

These results suggest that D-mannitol treatment did not alter expression of Hsp70 following hyperthermia, suggesting that once made thermotolerant, cells do not further increase expression of Hsp70 following a second stress.

Ischemic Tolerance and Hsp70 Induction: Increased levels of Hsp70 were observed in hippocampal tissue of rats made tolerant by ischemic stress (Liu et al., 1993). Rats were exposed to either a pre-conditioning 3 min ischemic stress or a sham treatment 72 h prior to a 6 min ischemic stress treatment. Neurons from the sham treated and pre-conditioned rats were examined for cell damage following the 6 min ischemic stress. Immunohistochemical staining was performed on rat hippocampal sections and Hsp70 immunoreactivity was observed 2 h, 1 d, 3 d, and 7 d following the 6 min ischemic stress. Neuron damage was not present in either treatment group until 3 d following the 6 min ischemia; at which point, the sham treated group demonstrated neuronal damage and death. However, the pre-conditioned neurons were preserved through the 7 days. Compared to sham treated rats, pre-conditioned rats demonstrated Hsp70 immunoreactivity in the hippocampus before and 2 h post ischemic stress. The relative amount of Hsp70 at 2 h, however, was significantly less than the amount present before ischemia, indicated by intense and mild immunoreactivity respectively. However, at 1 and 3 days following ischemic stress both the sham treated rats and pre-conditioned rats displayed a significant amount of Hsp70 indicated by intense immunoreactivity. Seven days following ischemic stress, the sham treated rats had minimal Hsp70 remaining, while the preconditioned rats still had a significant amount of Hsp70, indicated by moderate immunoreactivity.

The initial presence of Hsp70 after the 6 min ischemic stress was due to pre-existing Hsp70 synthesized following the preconditioning treatment (as new Hsp70 can take 4-6 h for synthesis to occur). This accumulation of Hsp70 possibly aided in protecting the cells from damage due to the 6 min ischemic stress. This indicated a correlation between Hsp70 induction and conferment of ischemic tolerance.

To determine whether Hsp70 was a major mediator of ischemic tolerance, the abundance of Hsp70 in ischemic tolerant rat brains was analyzed (Dhodda et al., 2004). Rats were pre-conditioned with a 10 min ischemic stress, and 3 days following were exposed to a 1 h ischemic stress. Heat shock protein 70 content was analyzed by immunocytochemistry and western blot analysis. Control rats (sham operated) displayed no Hsp70 immunoreactivity, while pre-conditioned rats had Hsp70 immunopositive cells observed 4 h following pre-conditioning, with peak Hsp70 immunoreactivity observed at 8 h. Twelve h following pre-conditioning, immunopositive cells were not observed. Rats that were subjected to pre-conditioning had a significantly smaller infarct volume after the 1 h ischemic stress compared to control rats, indicating that the greater levels of Hsp70 in pre-conditioned rats provide a protective role in ischemic stress.

Heat Shock Protein 27

Heat shock protein 27 is a member of a family of small stress proteins that provide cellular protection against injury and apoptosis (Valentim et al., 2003). In the brain Hsp27 is constitutively expressed, and its levels can increase by as

much as 10-20 fold (Welch, 1992) following stressful stimuli, such as heat stress. Increased induction of Hsp27 has also been observed in thermotolerant cells.

Induction of Hsp27 in the Brain Following Stress

Heat shock protein 27 induction was increased 1 day following excitotoxic lesion in immature rat brain (Acarin et al., 2002). Nine day old rats received an intracortical injection of N-methyl-D-aspartate to induce excitotoxic lesions (neural damage) in the brain. Brains were removed at various time points following injection, and probed for Hsp27 using immunohistochemistry. Astrocytes displayed Hsp27 as early as 1 day following excitotoxic lesion, with maximal expression occurring 3-5 days post-lesion. The increased induction of Hsp27 in rat brain indicates its possible role in protecting brain tissue against further damage.

Arsenite induced the expression of Hsp27 in cultured rat astrocytes (Fauconneau et al., 2002). One-hour exposure to 50 μM arsenite induced a moderate increase in Hsp27 expression 24 h following treatment. However, 6 h following treatment Hsp27 expression was not increased compared to controls. These results indicated a delayed and modest induction of Hsp27 in astrocytes following heavy metal stress.

Whole body hyperthermia induced increased expression of Hsp27 in several tissue types of the adult rat brain (Krueger-Naug et al., 2000). Male Sprague-Dawley rats were exposed to a brief, hyperthermic stress of 42°C for 15 min. Following hyperthermic stress, rats were allowed to recover for 1.5 h to 6 d at which point the brain was removed and fixed for immunohistochemistry and

Western blotting for Hsp27. Increased levels of Hsp27 were evident in the cerebral cortex, hippocampal formation, cerebellum, and brain stem following hyperthermia. In astrocytes throughout the CNS, increased levels of Hsp27 were time dependent, peaking at 24 h following hyperthermic stress, at which point levels gradually declined. However, the increase in astrocytic Hsp27 was not uniform across brain tissue, with astrocytes of the hippocampal formation and lateral septal area displaying stronger induction of Hsp27 compared to astrocytes in the remainder of the brain. These results indicated both a time-dependent and brain tissue specific activation of Hsp27 following brief hyperthermia.

Hsp27 Induction in the BBB: Heat shock protein 27 levels increased in the human endothelial cell line EA.hy926 following heat stress and microwave radiation (Leszczynski et al., 2002). To determine the effect of hyperthermia and microwave radiation on the cellular stress response, expression status of Hsp27 was examined in semiconfluent EA.hy926 cultures exposed to either 1 h of 900 MHz radio-frequency-modulated electromagnetic field (RF-EMF) or 3 h of 43°C hyperthermia. Both indirect immunofluorescence staining and Western blotting was used to detect Hsp27. Immunofluorescence studies revealed that control and sham treated cells expressed high constitutive levels of Hsp27. Likewise there was an increase in Hsp27 levels in cells exposed to RF-EMF, and an even greater increase in Hsp27 in cells exposed to heat stress. These increased levels were evident immediately following RF-EMF and heat stress, respectively. Compared to sham treated cells, in cells exposed to RF-EMF, Hsp27 levels peaked 1 h following treatment, and then steadily declined to sham treated levels

at 2 h, and by 6-8 h post stress Hsp27 levels were about 50% of sham treated levels. This observation contradicts previous findings in the brain that Hsp27 induction peaks 24 h following stress, suggesting that either the type of stress or cell type influences Hsp27 induction. Unfortunately this is difficult to determine, as the authors did not report time-dependent induction of Hsp27 following heat stress in EA.hy926.

Hsp27 and Acquired Tolerance

Several studies have indicated a relationship between Hsp27 expression and resistance to a lethal stress. Very little data exist on the relationship of thermotolerance and Hsp27 expression in the BBB, however much can be learned from studies on other cells of the body and the brain.

Cells with increased expression of Hsp27 had an increased survival rate following lethal heat stress compared to control cells (Landry et al., 1989). A variant of Chinese hamster lung fibroblasts, 023 cells, were transfected with a human Hsp27 gene prior to exposure to 3.5 h, 43-44°C heat stress treatment. Immediately following heat stress, cells were trypsinized and plated at 37°C for survival rate determination. Survival was determined from the number of cell colonies formed 7-10 d following heat stress. Compared to control cells, the survival rate of transfected cells following heat stress was significantly greater. Transfected cells also displayed a greater relative expression of Hsp27 following heat stress compared to controls. These results indicate a role of Hsp27 in conferring thermotolerance in cell lines, in that elevated levels of Hsp27 are sufficient in protecting cells from lethal heat stress.

Increased immunocontent of Hsp27 was associated with acquired tolerance of rat organotypic hippocampal cultures to oxygen and glucose deprivation (Valentim et al., 2003). Hippocampal cultures were exposed to 5 or 10 min of oxygen and glucose deprivation (OGD) 24 h prior to exposure to a 40 min OGD. Cultures were allowed to recover for 24 h following the 40 min OGD before assessment of cellular damage and Hsp27 content. Compared to controls, cultures preconditioned to 5 or 10 min of OGD displayed a greater cellular quantity of Hsp27. Likewise, control cultures experienced a higher degree of cellular damage following the 40 min OGD compared to pre-conditioned cultures. These results also suggest a role of Hsp27 in providing cellular protection against OGD.

Summary

The specialized design of the BBB provides the brain with a protection system that limits the paracellular entry of potentially damaging blood-borne molecules into the CNS and regulates the ionic composition of the CSF. Initial BBB permeability studies indicated a barrier impermeable to large macromolecules such as HRP (Reese and Karnovsky, 1967) and serum albumin (Fishman, 1953; Kumagai et al., 1987). The presence of endothelial tight junctions were attributed to this low paracellular permeability (Brightman and Reese, 1969), acting as gates capable of opening and closing depending on various influences (Claude, 1978). The ability of tight junctions to open and close is attributed to the plethora of specific proteins that aggregate at, and form, the structural basis of tight junctions (Gumbiner, 1987).

The unique anatomy of the BBB poses several difficulties: loss of BBB integrity can lead to deleterious effects of the CNS, and contrarily, the presence of a BBB creates extreme difficulty in delivering pharmaceutical drugs to treat disorders of the CNS. The development and use of in vitro BBB models (Bowman et al., 1981; DeBault et al., 1981; Bobilya et al., 1995) have allowed for more BBB permeability studies to be conducted, enhancing our knowledge of BBB permeability regulation.

Heat stress has been shown to significantly disrupt the BBB (Sharma and Cervos-Navarro, 1990; Sharma et al., 1992), allowing for increased permeability of various molecules that would normally be excluded by the BBB, such as fluorescein (Merritt et al., 1978) and Evans blue dye (Ikeda et al., 1994; Oztas and Kucuk, 1998). Studies on the effects of heat stress on the BBB are becoming increasingly important in medical science, as new therapies utilizing heat are being developed to treat brain tumors (Schulze et al., 2004), and enhance the delivery of pharmaceuticals into the brain (Ng et al., 2004). As enhanced techniques of applying localized heat stress are developed, a greater range of temperature and duration can be used in applied hyperthermia. For example brief, high temperature hyperthermia significantly increased BBB permeability in an *in vitro* model (Jeliazkova-Mecheva et al., 2006).

Acquired thermotolerance has been observed in the BBB exposed to repeated bouts of heat stress (Ikeda et al., 1999; Jeliazkova-Mecheva et al., 2006). Thermotolerance describes a state in which cells (such as endothelial cells of the BBB) upon pre-exposure to hyperthermia become resistant against

future heat stresses (Kampinga, 1993). Thermotolerance has been described in cells exposed to mild hyperthermia (Raaphorst et al., 1995; Ikeda et al., 1999), and in cells exposed to brief, high temperature hyperthermia (Jeliazkova-Mecheva et al., 2006).

Following heat stress, all cell types respond by synthesizing the Hsps. The Hsps are a group of highly conserved proteins grouped into five families by amino acid sequence and molecular weight. Experiments involving cell pre-conditioning in the CNS demonstrate a strong correlation between the induction of Hsp70 and Hsp27 and protection against subsequent stresses (Franklin et al., 2005), suggesting that Hsps may play an important role in thermotolerance.

In this study, we investigated the effects of brief, high temperature hyperthermia on BBB integrity and acquisition of BBB thermotolerance by evaluating changes in BBB TEER across an in vitro BBB model. We also investigated possible mechanisms for the observed effects by examining changes in relative Hsp70 and Hsp27 expression in thermotolerant BBB models.

CHAPTER II

MATERIALS AND METHODS

Isolation of Brain Capillary Endothelial Cells

The University of New Hampshire Animal Care and Use Committee (ACUC) have previously approved all procedures in which animals were used experimentally. All reagents, unless otherwise specified, were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO.

Porcine brain capillary endothelial cells (BCEC) were isolated following the procedure outlined by Bobilya et al. (1995). Briefly, grey matter from the cerebral cortices of 3-4 month old Yucatan miniature swine was aseptically removed and mechanically minced in collection medium (CM) composed of HEPES modified minimum essential medium (MEM with Earle's Salts, L-glutamine and 20 mM HEPES) supplemented with sodium bicarbonate (2.2 mg/mL), amphotericin B (5 μg/mL), gentamicin (50 μg/mL) and 2% fetal bovine serum (FBS). Then the tissue was enzymatically digested in an equal volume of Type IA collagenase solution (1 mg/mL, 270 IU/mg in CM) at 37°C for 60 min. Following enzymatic digestion, differential centrifugation utilizing an albumin gradient (25% bovine serum albumin in CM) was employed to separate out capillaries from brain tissue. The resulting pellet consisting of capillaries and single cells was resuspended in CM, and then the suspension was filtered through decreasing

pore size Nitex™ nylon screens (Sefar America, Inc., Briarcliff Manor, NY) to separate out capillary fragments and astrocytes. The resulting suspension wasthen passed through a glass bead column (12 mm diameter column, 0.5 mm diameter beads supported by a 20 μm Nitex™ nylon screen), in which capillary fragments attached to the glass beads and Nitex™ screen. Glass beads and the screen were then rinsed with CM to detach the capillary fragments. The resulting suspension was centrifuged and the pellet re-suspended in a primary growth medium composed of MEM (with Earle's salts and L-glutamine) supplemented with 15% platelet-poor horse serum, bicarbonate, endothelial cell growth supplement (50 μg/mL), and heparin (100 μg/mL). Endothelial cell growth supplement was prepared from freeze-dried hypothalamic extract previously isolated in our lab by the procedure of Maciag, et al. (1979). Isolated capillary fragments were seeded into 75 cm^2 fibronectin-coated (2 $\mu g/cm^2$) cell culture flasks (Corning, Inc., Corning, NY). Cells were incubated in a water-jacketed incubator at 37°C with 5% CO_2 and 95% humidity.

Migration of single endothelial cells from capillary fragments was evident on day 2. Starting on day 4, cells were grown in a secondary growth medium (primary growth medium plus 2% FBS). Confirmation of BCEC cultures was done by visual examination (using phase contrast microscopy), in which characteristic BCEC morphology was observed (elongated, cigar-shaped cells with poorly defined borders). Previous studies have determined confirmation of BCEC by presence of Factor VIII antigen and accumulated ac-LDL (Bobilya et al., 1995).

To establish an in vitro BBB model, once cells reached 60-70% confluency (on day 5-6) they were subcultured, via trypsinization, and seeded at 75,000 cells/cm^2 onto fibronectin-coated (2 $\mu g/cm^2$) porous Transwell™ membrane inserts (12 mm diameter, 0.4 μm pore size, 1 cm^2 surface area, Corning Inc., Corning, NY). Figure 2.1 demonstrates a BCEC monolayer grown on a Transwell™ membrane.

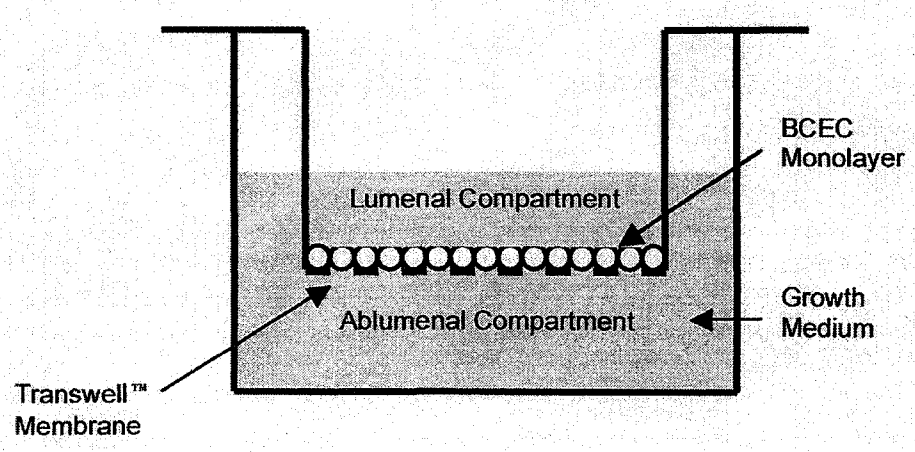
Assessment of BCEC Monolayer Integrity

BCEC monolayer integrity was assessed via electrical resistance measurements across the barrier. Transendothelial electrical resistance (TEER) was measured using an Endohm chamber connected to an EVOM voltohmmeter (World Precision Inst., Inc., Sarasota, FL). TEER measures the impedence of ions across the BCEC monolayer, and is recognized as one of the most sensitive methods of measuring changes in BBB permeability (Butt et al., 1990). TEER was calculated from the displayed reading by subtraction of the electrical resistance of a Transwell™ membrane without cells. Once a tight BCEC monolayer was established (usually on days 5-6 post-subculture), as determined by steady TEER readings (usually between 40-50 $\Omega \times cm^2$) our BBB model was used in heat stress experiments.

Heat Stress Treatments

To examine the longitudinal changes in BBB integrity and acquired thermotolerance in our BBB model exposed to different temperatures, a heat stress assay was performed (usually on day 6 post-subculture). The BBB models were randomly assigned to 1 of 5 treatment groups: 37°C, 45°C, 48°C,

Figure 2.1: *In vitro* BBB model.



Porcine BCEC were grown to confluency on porous Transwell™ membranes to create our BBB model.

51°C, and 54°C. On the first day of the assay, TEER of an insert was measured immediately before submersion into a beaker containing sterile, pre-warmed (to the treatment temperature) MEM for 10 s. The 54°C treatment group was submerged for only 5 s – as previous studies have observed that longer exposure to 54°C caused BBB models to not fully recover from heat stress within 24 h (Jeliazkova-Mecheva et al., 2006). Immediately following heat treatment, TEER was again measured. BBB models were then returned to the incubator at 37°C for recovery. TEER was measured 30 min following treatment to assess recovery of the BBB from heat treatment.

To examine acquired thermotolerance in the BBB, 24 h following the initial heat treatment (pre-conditioning treatment), all BBB models were submerged for 10 s into a beaker containing sterile MEM warmed to 51°C. As with the pre-conditioning treatment, TEER was measured immediately before and after heat treatment. Immediately following the after treatment TEER measurement, cells were lysed in a lysis buffer (7.9 g/L Tris, 8.7 g/L NaCl, 2.9 g/L EDTA, 1% triton X-100, 0.02% Na azide) containing 1% proteolytic enzyme inhibitor (100 μL lysis buffer/insert). Lysates collected from each treatment group (37°C - 54°C) were pooled to obtain an adequate amount of lysate for Western blot analysis. Lysates were stored at -80°C until Western blotting. Table 2.1 summarizes our heat stress experimental design.

Table 2.1: Heat stress experimental design.

	0 h		24 h	
Treatment Group	Pre-Condition Temperature	Pre-Condition Duration	Second Heat Stress Temperature	Second Heat Stress Duration
37°C	37°C	10 s	51°C	10 s
45°C	45°C	10 s	51°C	10 s
48°C	48°C	10 s	51°C	10 s
51°C	51°C	10 s	51°C	10 s
54°C	54°C	5 s	51°C	10 s

Following an initial (pre-condition) brief, high temperature heat treatment, BBB models were returned to the 37°C incubator for recovery. On day 1 (0 h) TEER was measured before treatment (BT), after treatment (AT), and 30 min AT. On day 2 (24 h), TEER was measured BT and AT. Following the final TEER measurement, BCEC were lysed for Western blot analysis.

Analysis of Hsp Quantity in Heat Treated BBB

Protein Determination

Protein concentration was determined using the bicinchoninic acid (BCA) method (Smith et al., 1985). Briefly, 50 μL of lysate was added to 1 mL of BCA. Standards were prepared with 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL bovine serum albumin and 1 mL of BCA was added to each standard. Standards and samples were incubated in a circulating 37°C water bath for 30 min. Absorbance was measured at 562 nm. Measurements were performed in triplicate. A standard curve was constructed to determine protein concentration of the lysates.

Western Blot Analysis for Hsp70 and Hsp27

Lysate (containing 6-8 μg protein) was combined with an equal volume of loading buffer (Laemmli sample buffer with 5% 2-mercaptoethanol (Biorad Labs, Richmond, CA) and heated for 4 min at 95°C. Samples were then loaded into a well of a SDS-PAGE gel consisting of a 4% acrylamide stacking gel and a 12% acrylamide separation gel. Hela cell lysate was also loaded into one well of the gel to serve as a positive control. Gels were placed in a discontinuous buffer system (1% tris-glycine with 0.2% SDS) and electrophoresis (200 V) was employed to separate proteins by size. Following separation, proteins were electrophoretically transferred (100 V for 1 h) onto a polyvinylidene fluoride (PVDF) membrane. The buffer used during electrophoretic transfer contained 20% methanol, 1% tris-glycine, and 0.05% SDS.

Following transfer, the PVDF membrane was blocked in 20 mL of a 5% milk solution (1.0 g non-fat powdered milk in 20 mL tris buffered saline with

0.05% Tween 20 – TBST) overnight on a rotary shaker set at 65 rpm. Membranes were then probed with the respective primary antibody for 1 h. For Hsp70, membranes were incubated in a 1:3000 dilution of monoclonal mouse anti-Hsp70 (Sigma-Aldrich, St. Louis, MO) in 10 mL 5% milk solution. For Hsp27, membranes were incubated in a 1:3000 dilution of polyclonal rabbit anti-Hsp27 (Stressgen Bioreagents, Assay Designs, Ann Arbor, MI) in 10 mL 5% milk solution. Following incubation, membranes were washed for 10 min in TBST, and then exposed to a secondary antibody for 30 min. For Hsp70, the secondary antibody was HRP conjugated anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) prepared in a 1:10,000 dilution in 10 mL 5% milk solution. For Hsp27, the secondary antibody was HRP conjugated anti-rabbit IgG (Stressgen Bioreagents, Assay Designs, Ann Arbor, MI) prepared in a 1:10,000 dilution in 10 mL 5% milk solution. Following secondary antibody incubation, membranes were washed 3 times in 10 mL of TBST for 10 min.

PVDF membranes probed for Hsp70 were then incubated in 2.5 mL 3,3',5,5'-Tetramethylbenzidine (TMB) colorimetric substrate until a signal developed (usually by 5 minutes of incubation). Excess TMB was blotted from the membrane, and the membrane scanned for densitometry.

PVDF membranes probed for Hsp27 were incubated in 5 mL of Super Signal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) for 5 min. Following incubation, excess Super Signal West was carefully blotted from the membranes, and x-ray film was exposed to the membranes for 15 s. Following development, the exposed film was scanned for densitometry.

Following probing of Hsp70 or Hsp27, PVDF membranes were stripped using a buffer consisting of 9.85 g/L Tris, 2% SDS, and 10% 2-mercaptoehtanol (Biorad Labs, Richmond, CA). PVDF membranes were incubated in 15 mL of 55°C stripping buffer for 15 min. They were then washed 3 times for 30 min each, in 10 mL of TBST. Following washing, membranes were then incubated for 1 h in a 1:1500 dilution of monoclonal mouse anti β-actin (Sigma-Aldrich, St. Louis, MO) in 10 mL 5% milk solution. Following incubation, membranes were washed for 10 min in TBST, and then incubated in a 1:10,000 dilution of HRP conjugated anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) in 10 mL 5% milk solution. Following secondary antibody incubation, membranes were washed 3 times in 10 mL of TBST for 10 min. PVDF membranes were then incubated in 5 mL of Super Signal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) for 5 min. Following incubation, excess Super Signal West was carefully blotted from the membranes, and x-ray film was exposed to the membranes for 15 s. Following development, the exposed film was scanned for densitometry.

Densitometry was used to determine the relative expression of Hsp70 or Hsp27 relative to the 37°C treatment group. Briefly, an image of each PVDF membrane was scanned and loaded into Un-Scan-It Gel™ 6.1 digitizing software (Silk Scientific Inc, Orem, UT).

Statistics

One-way ANOVA and Tukey's test were used for statistical analysis. Pearson correlation was used to determine the degree of linear relationship between temperature and BBB integrity loss or thermotolerance. Statistical tests

were performed using Systat 10 software (Systat Software Inc, San Jose, CA). Significance was defined as $P \leq 0.05$. Unless otherwise noted, values expressed are means ± standard deviations.

CHAPTER III

RESULTS

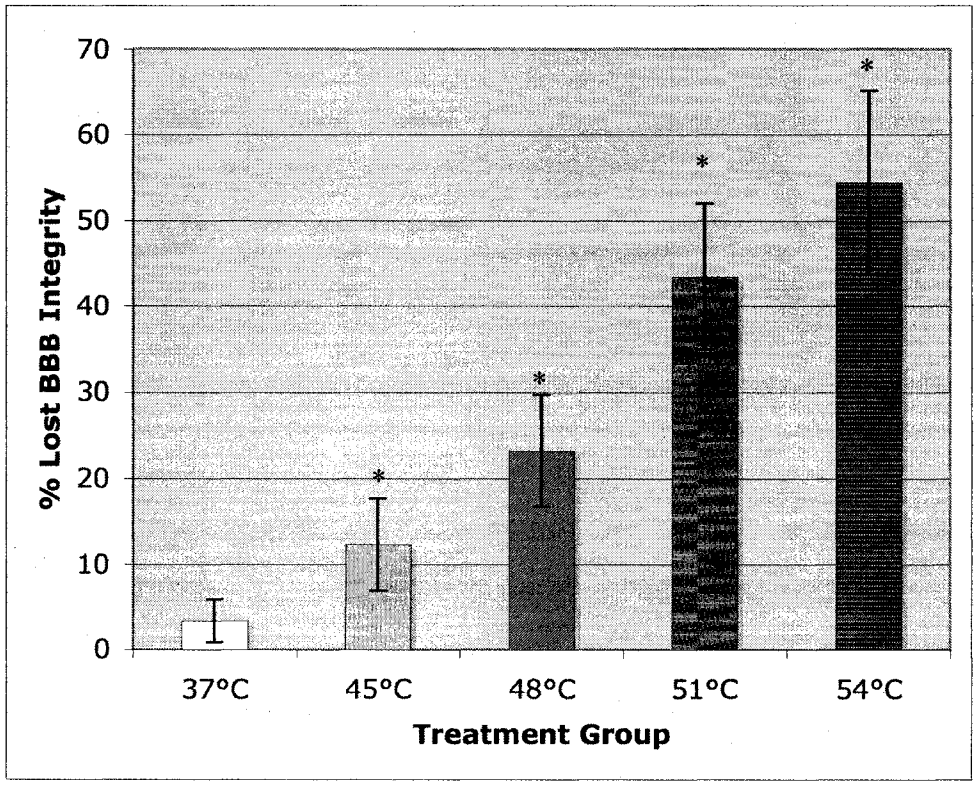
The effects of pre-conditioning temperature on BBB integrity and acquired thermotolerance were examined in an in vitro BBB model. Relative expression of Hsp70 and Hsp27 were examined in thermotolerant cells to assess if the thermotolerance could be explained by relative changes in Hsp expression.

Effect of Heat Stress Temperature on BBB Integrity

Following brief, high temperature heat stress there was a significant ($P<0.0001$) loss of BBB integrity (increase in BBB permeability) (Figure 3.1). Loss of BBB integrity in all treatment groups was different ($P<0.0001$) from sham treated BBB models (controls, treated for 10 s at 37°C), and different ($P<0.0001$) from each other. Sham treated BBB models did not display a significant ($P>0.10$) decrease in BBB function following sham treatment. However, exposure of BBB models to 45, 48, and 51°C for 10 s, and 54°C for 5 s resulted in an increase in BBB integrity loss as treatment temperature increased ($r = 0.8811$, $P<0.0001$).

Following treatment of 45°C for 10 s, BBB models lost 12.3 ± 5.4% BT integrity. Following treatment of 48°C for 10 s, BBB models lost 23.2 ± 6.5% BT integrity. Models treated at 51°C for 10 s lost 43.5 ± 8.5% BT BBB integrity following treatment, and models treated at 54°C for 5 s lost 54.5 ± 10.7% BT BBB integrity following treatment.

Figure 3.1: BBB integrity loss following brief, high temperature heat treatment.



TEER was measured immediately following a brief, high temperature heat treatment to determine the degree of BBB integrity compared to before treatment (BT). Y-axis represents % decrease in TEER compared to 0 h BT. Values expressed are means ± standard deviation. Data from 3 experiments were pooled (37°C n=23, 45°C n=23, 48°C n=24, 51°C n=25, 54°C n=24). All treatment groups are different ($P<0.0001$) from controls and from each other.

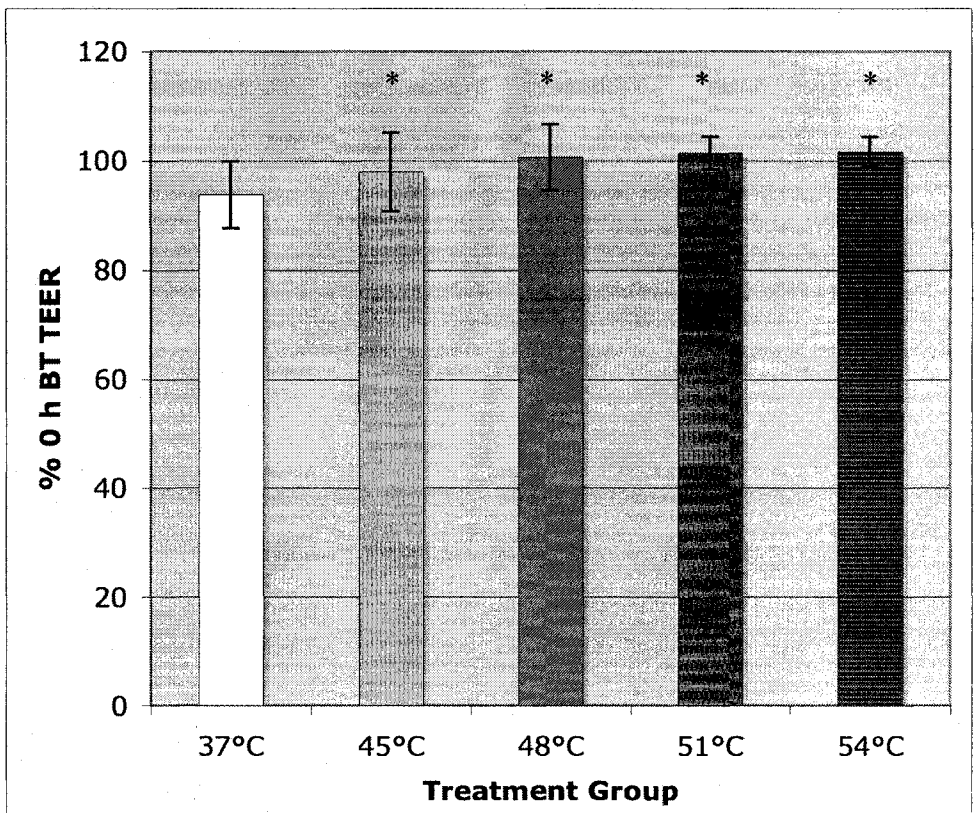
Transendothelial electrical resistance was assessed 30 min following heat stress treatment to determine the degree of BBB recovery (Figure 3.2). At 30 min post-treatment, sham-treated BBB models displayed TEER that was 92.7 ± 6.1% BT integrity, and were the only models that did not recover 100%. Models treated at 45, 48, and 51°C for 10 s, and 54°C for 5 s displayed TEER measurements that were greater than controls ($P<0.05$); 98.7 ± 7.2%, 100.2 ± 6.2%, 101.1 ± 3.1%, and 101.2 ± 2.8% respectively, indicating recovery from heat stress.

Twenty-four h following pre-condition treatment, TEER was measured on all BBB models immediately before a second heat treatment to assess BBB integrity difference relative to BT (0 h) integrity. There was no difference ($P > 0.10$) in BBB integrity compared to BT in all treatment groups (Figure 3.3). Control models displayed TEER measurements that were 92.5 ± 14.2% original TEER. Models treated at 45, 48, and 51°C for 10 s, and 54°C for 5 s displayed TEER measurements that were 97.2 ± 12.7%, 92.6 ± 10.3%, 96.8 ± 7.7%, and 93.6 ± 15.5%, respectively, of original BT TEER measurements.

Acquired Thermotolerance

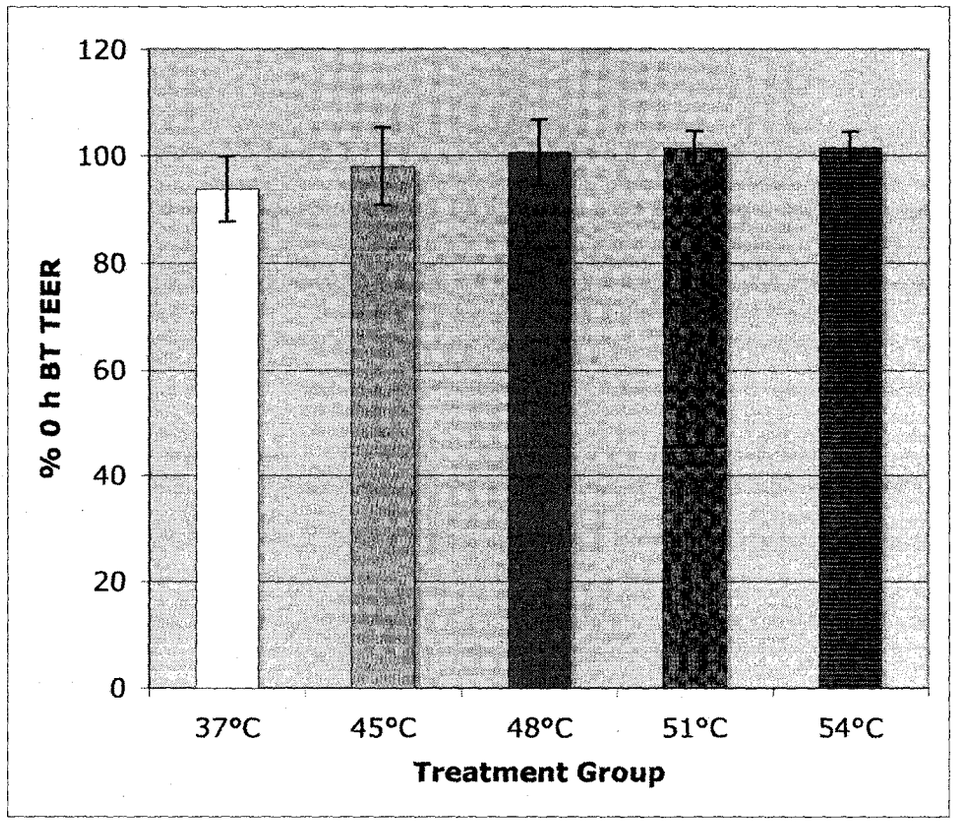
The phenomenon of thermotolerance was examined in our models by applying a second hyperthermia (10 s at 51°C) to all BBB models, 24 h following pre-conditioning treatments (Figure 3.4). Control BBB models lost more integrity than the pre-conditioned models. The degree of acquired thermotolerance was different ($P < 0.001$) amongst treatment groups depending on the temperature of

Figure 3.2: TEER 30 min following initial brief, high temperature heat treatment.



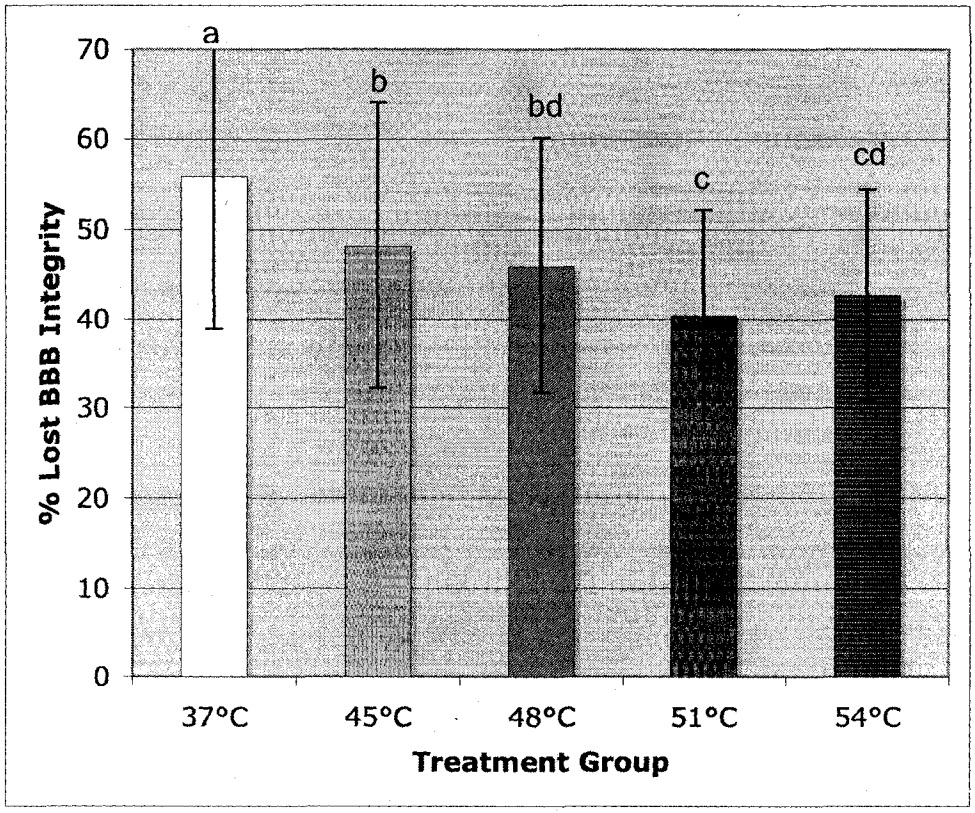
TEER was measured 30 min following an initial brief, high temperature heat treatment to determine degree of recovery of BBB monolayers. Y-axis represents % TEER compared to 0 h BT TEER. Values expressed are means ± standard deviation. Data from 3 experiments were pooled (37°C n=23, 45°C n=23, 48°C n=24, 51°C n=25, 54°C n=24). All treatment groups are different from controls ($P<0.05$).

Figure 3.3: BBB integrity before second brief, high temperature heat treatment.



TEER was measured prior to a second brief, high temperature heat treatment (10 s at 51°C) to determine degree of BBB integrity compared to 0 h BT. Y-axis represents % TEER compared to 0 h BT TEER. Values expressed are means ± standard deviation. Data from 3 experiments were pooled (37°C n=23, 45°C n=23, 48°C n=24, 51°C n=25, 54°C n=24). None of the treatment groups are different (P>0.10) from controls nor each other.

Figure 3.4: BBB integrity loss following a second brief, high temperature heat treatment.



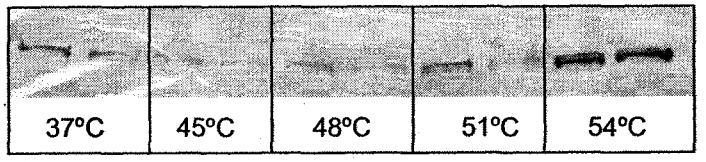
TEER was measured immediately following a second brief, high temperature heat treatment (10 s at 51°C) to determine degree of acquired thermotolerance. Y-axis represents % decrease in TEER compared to 0 h BT. Values expressed are means ± standard deviation. Data from 3 experiments were pooled (37°C n=23, 45°C n=23, 48°C n=24, 51°C n=25, 54°C n=24). All treatment groups are different from controls ($P<0.0001$).

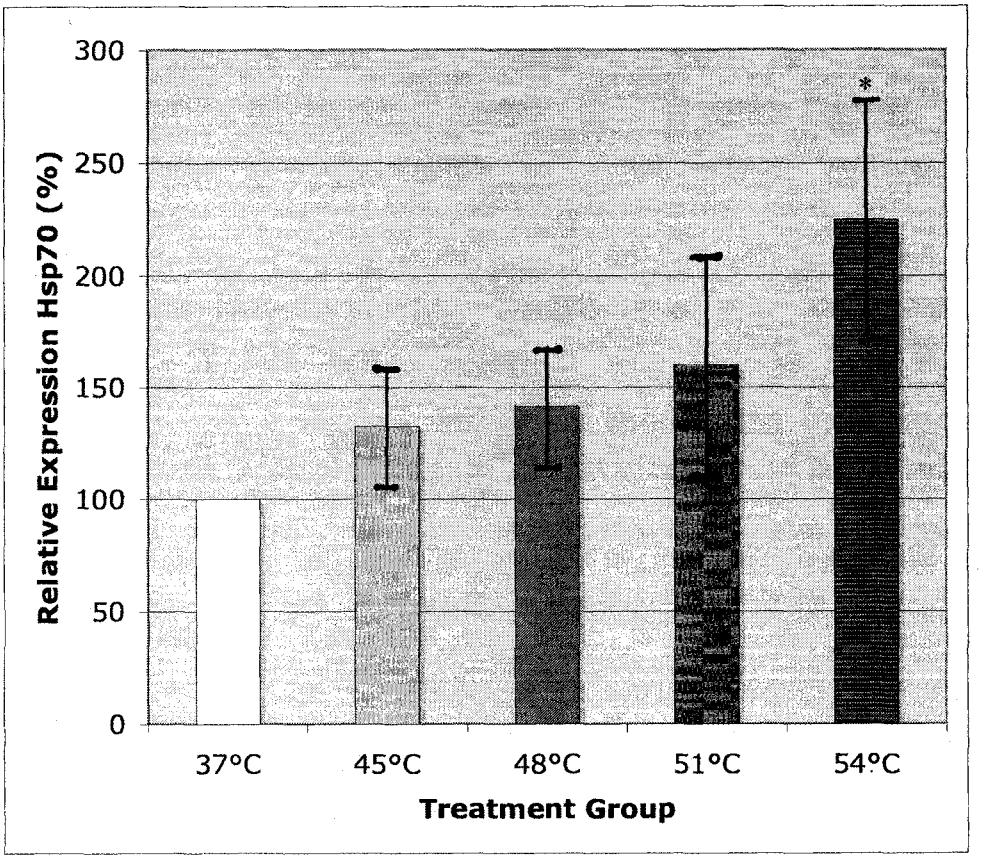
heat stress applied 24 h previously (pre-conditioning treatment) ($r = -0.4156$, $P < 0.0001$). Following a 10 s, 51°C heat treatment, control models (sham-treated) maintained 38.3 ± 17.0% BT integrity; the 45°C pre-conditioning treatment group maintained 47.4 ± 15.9% BT integrity; the 48°C pre-conditioning treatment group maintained 49.9 ± 14.3% BT integrity; the 51°C pre-conditioning treatment groupmaintained 55.9 ± 11.8% BT integrity; and the 54°C pre-conditioning treatment group maintained 54.1 ± 11.8% BT integrity. BBB models pre-conditioned at 51°C for 10 s and 54°C for 5 s displayed the greatest degree of thermotolerance, by maintaining BBB integrity that was approximately 35.3% and 30.1% (respectively) greater than controls ($P<0.0001$). Models preconditioned at 48°C for 10 s maintained 22.7% greater BBB integrity than controls ($P<0.0001$), while models preconditioned at 45°C for 10 s maintained 17.6% greater BBB integrity than controls ($P<0.0001$).

Hsp70 Expression 24 h Following Pre-Condition Heat Treatment

Relative expression of Hsp70 was dependent on treatment group (Figure 3.5). Compared to controls (pre-conditioned at 37°C), relative expression of Hsp70 was not different ($P>0.10$) in treatment groups pre-conditioned at 45, 48, and 51°C for 10 s. In response to the pre-conditioning for 10 s at 45°C, 10 s at 48°C, and 10 s at 51°C relative expression of Hsp70 was 132.6 ± 26.8%, 141.5 ± 26.8%, and 160.0 ± 49.4%, respectively, of the expression of Hsp70 in control treated models. Values expressed are means ± standard deviations. Hsp70 increased significantly ($P<0.05$) in response only to the pre-conditioning for 5 s at 54°C. Relative expression of Hsp70 in the 54°C, 5 s treatment group was

Figure 3.5: Relative expression of Hsp70 following pre-conditioning treatment.





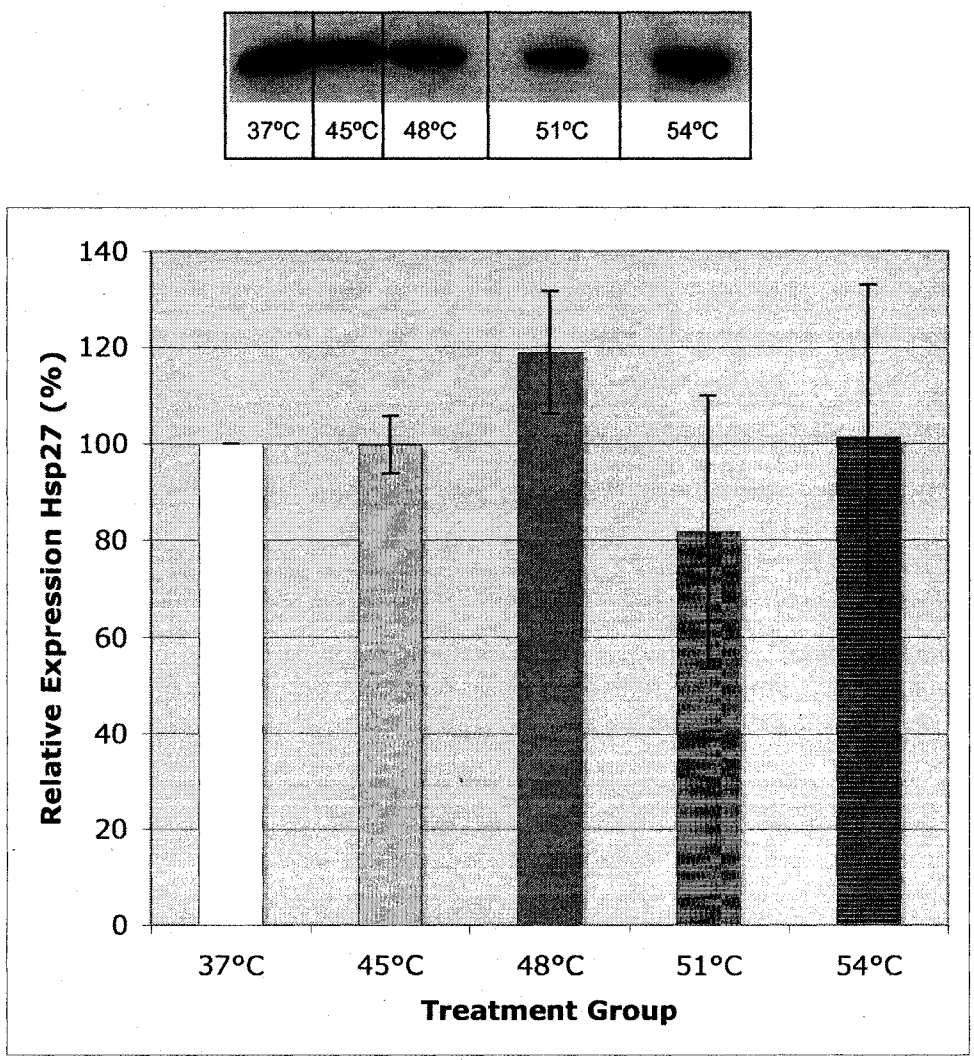
Immediately following a second brief, high temperature heat treatment (10 s at 51°C), BCEC were lysed, and using Western blot analysis, relative expression of Hsp70 was determined. Data was pooled from 3 experiments (n=3). Values expressed are means ± standard deviations. The 45, 48, and 51°C treatment groups were not different ($P>0.10$) relative to controls (37°C). The 54°C treatment group was different ($P<0.05$) relative to controls.

approximately 225.2 ± 52.8% of the expression of Hsp70 in control treated models. The degree of acquired thermotolerance was not correlated with relative Hsp70 expression ($r = -0.17$, $P>0.10$).

Hsp27 Expression 24 h Following Pre-Condition Heat Treatment

Relative expression of Hsp27 was not different ($P>0.10$) in any of the pre-condition treatment groups compared to controls (Figure 3.6). In response to the pre-conditioning for 10 s at 45°C, 10 s at 48°C, 10 s at 51°C, and 5 s at 54°C, relative expression of Hsp27 was 99.8 ± 6.0%, 118.9 ± 12.8%, 81.7 ± 28.3%, and 101.6 ± 31.5%, respectively, of the expression of Hsp27 in control treated models. The degree of acquired thermotolerance was not correlated with relative expression of Hsp27 ($r = 0.17$, $P>0.10$).

Figure 3.6: Relative expression of Hsp27 following pre-conditioning treatment.



Immediately following a second brief, high temperature heat treatment (10 s at 51°C), BCEC were lysed, and using Western blot analysis, relative expression of Hsp27 was determined. Data was pooled from 3 experiments (n=3). Values expressed are means ± standard deviations. Treatment groups were not different (P>0.10) relative to controls.

CHAPTER IV

DISCUSSION

The present study investigated the effects of brief, high temperature hyperthermia on BBB permeability and acquired thermotolerance utilizing an in vitro BBB model. Changes in relative expression of Hsp70 and Hsp27 were evaluated to examine a possible correlation for the observed effects. The majority of heat stress studies on the BBB have examined the effect of prolonged, mild heat treatment utilizing in vivo experiments (Merritt et al., 1978; Sharma and Cervos-Navarro, 1990; Sharma et al., 1992; Ikeda et al., 1994; Urakawa et al., 1995). All of these studies examined both pathological changes and permeability changes to the BBB by employing heat stress at 38 - 44°C applied for 0.5 - 4 h. However, there has only been one other *in vitro* report of brief, high temperature hyperthermia on the BBB (Jeliazkova-Mecheva et al., 2006), and to our knowledge no reports of BBB changes in Hsp expression following brief, high temperature hyperthermia.

Use of *In Vitro* BBB Models and TEER to Assess Permeability

In vitro models allow for greater manipulation and control of applied heat stress compared to *in vivo* models. The *in vitro* BBB model provides a reproducible isolated system that is more easily used to determine isolated events. We utilized an *in vitro* BBB model previously developed in our laboratory (Bobilya et al., 1995). Cultured BCEC from weanling Yucatan miniature swine were subcultured onto porous Transwell™ membranes suspended between two

fluid filled chambers: analogous to the capillary lumen and interstitium of the brain *in vivo*. This model allowed us to assess changes in BBB integrity by measuring TEER across the BCEC monolayers, which offers a reliable and efficient method of evaluating BBB permeability *in vitro* (Eddy et al., 1997).

Transendothelial electrical resistance refers to the impedance of ions across the BCEC monolayers, and has been used as a sensitive tool in measuring BBB integrity both *in vivo* (Butt et al., 1990) and *in vitro* (Hurst and Clark, 1998; Jeliazkova-Mecheva et al., 2006). The use of TEER in measuring changes in permeability is beneficial because the sensitivity of TEER can detect small changes in permeability, compared to other methods that measure extravasation of macromolecules, such as horseradish peroxidase (molecular weight 44 kDa) (Reese and Karnovsky, 1967), fluorescein (M.W. 332 g) (Merritt et al., 1978), and Evans blue dye (M.W. 960 g) (Ikeda et al., 1994; Oztas and Kucuk, 1998). These methods require large changes in BBB permeability to produce a detectable change in permeability of molecules into the brain.

Effect of Heat Stress Temperature on BBB Integrity

Our results confirmed that the BBB could be opened by brief, high temperature hyperthermia. We observed a significant, transient loss of BBB integrity (loss of barrier function) when our BBB models were exposed to 45, 48, and 51°C for 10 s, and 54°C for 5 s. The degree of integrity loss increased as the applied treatment temperature increased. This agrees with the observation by Jeliazkova-Mecheva et al. (2006) who observed significant loss of BBB integrity in BBB models exposed to 41°C or greater for 30 s, 50°C or greater for

10 s, and 54°C or greater for 3 s. However, in our study we observed a significant difference in barrier integrity following 10 s of 45°C or greater temperatures. *In vivo* studies in dogs demonstrated significant BBB breakdown following a 60 min, 43°C hyperthermia and a 30 min, 44°C hyperthermia (Ikeda et al., 1994), and studies in rats demonstrated significant BBB breakdown following infusion of 43°C saline solution (Oztas and Kucuk, 1998). Moreover, in our study as evidenced by the higher TEER measurements 30 min following heat stress (compared to controls), it is likely that the heat stress activated cell repair mechanisms, such as the re-folding of denatured tight junction proteins.

Acquired Thermotolerance

We observed significant acquisition of thermotolerance in our BBB models pre-conditioned at 45, 48, and 51°C for 10 s, and 54°C for 5 s. Thermotolerance was expressed as a less significant reduction in barrier function compared to controls. This agrees with the observations of Jeliazkova-Mecheva et al (2006), who observed thermotolerance in BBB models 24 h following pre-conditioning at 52°C for 5-10 s, 53°C for 5 s, and 54°C for 3 s. This also agrees with the *in vivo* report by Ikeda et al (1999), who observed BBB tolerance to hypoxia-ischemia 24 h following a hyperthermic pre-condition treatment in the newborn rat. However, unlike the report by Jeliazkova-Mecheva et al (2006), who examined thermotolerance in BBB models pre-conditioned at the same temperature and duration as the second heat treatment, we observed thermotolerance in BBB models pre-conditioned at different temperatures and durations than the second heat treatment.

The observation of BBB thermotolerance can potentially be both beneficial and detrimental in the clinical application of hyperthermia. Loss of BBB integrity can lead to deleterious effects of the CNS, allowing for the paracellular transport of potentially damaging molecules into the brain. For example, in multiple sclerosis, the tight junctions of the BBB open allowing for leukocytes to cross the BBB, leading to neuroinflammation (Couraud, 1998). In our study, the observed thermotolerance demonstrates a means of reducing BBB tight junction permeability. Conversely, hyperthermia is used as a means to enhance delivery of pharmaceuticals to the brain by opening of the tight junctions (Merritt et al., 1978; Sharma and Hoopes, 2003; Ng et al., 2004). In this application, thermotolerance would be an undesired state.

Heat Shock Protein Expression 24 h Following Pre-Condition Heat Treatment

The heat shock proteins are a group of multigene families that are induced in all cell types following exposure to stress, such as hyperthermia (Lindquist, 1986; Welch, 1992). Induction of the Hsps following a non-lethal heat stress is believed to provide a protective role against subsequent stresses (Lindquist, 1986) by acting as molecular chaperones in assisting with refolding and folding of denatured proteins (Lu et al., 2002). Although the Hsps appear to provide an important role in cellular protection, there are few reports on Hsp induction in the BBB. Our study investigated changes in relative expression of Hsp70 and Hsp27 in thermotolerant BBB models to examine a possible association between thermotolerance and the Hsps.

Hsp70

A significant increase in Hsp70 expression was observed in thermotolerant BBB models pre-conditioned at 54°C for 5 s. In this treatment group, relative Hsp70 expression was approximately twice that of Hsp70 expression in controls. In thermotolerant BBB models pre-conditioned at 45-51°C for 10 s, we observed no significant relative change in Hsp70 expression, although we did observe a trend of increased Hsp70 expression as the pre-conditioning temperature increased. Due to our small sample size (n=3) we were not able to confidently demonstrate that Hsp70 increased. However, Hsp70 has demonstrated physiological relevance in other studies, in that the related Hsp70 proteins are intimately involved in facilitating protein folding and assembly (Welch, 1992).

Relatively few reports exist outlining the heat shock response of the BBB, and of those, to our knowledge none exist identifying the association between Hsp70 and thermotolerance of the BBB. In vitro studies on bovine brain microvessel endothelial cells (BBMEC), an alternative model of the BBB, revealed that the Hsp70 response following heat stress is dependent on the method of applying heat stress (Shivers et al., 1988; Ng et al., 2004). Relative expression of Hsp70 following a 1-3 h 43°C heat stress reached maximum by 2 h post-stress, and declined to "normal" (control) levels by 6 h post-heat stress (Shivers et al., 1988). Ng et al (2004) observed an association between duration and temperature of heat stress and Hsp70 expression. No significant difference was observed in BBMEC Hsp70 expression following 20 min of a 41°C heat

stress, but following a 3 h, 43°C heat stress, a significant increase in Hsp70 expression was noted 20 min, 1 h, 4 h, and 24 h following heat treatment. Likewise, following a 1 s heat stress of 53°C - 60°C in keratinocytes, Hsp70 quantity increased in a dose dependent manner, peaking at 56°C (Bowman et al., 1997), indicating that brief, high temperature hyperthermia can induce Hsp70 synthesis. This report is in agreement with our observation of a two-fold increase in relative Hsp70 quantity in response to a 5 s, 54°C heat stress.

Thermotolerance was observed in all of our treatment groups. However, a significant increase in relative expression of Hsp70 was observed only in the 54°C treatment group, suggesting that our observed thermotolerance was not correlated with an increased Hsp70 expression. Our observed thermotolerance is probably dependent on a number of parameters. Lu et al (2004) report similar observations in thermotolerant cells of the rat hippocampal BBB. Following a second heat stress, thermotolerant cells of the rat hippocampus BBB did not have increased synthesis of Hsp70 compared to non-thermotolerant cells. These results suggest that once made thermotolerant, cells do not increase expression of Hsp70 following a second stress, indicating a threshold for Hsp70 expression. Several reports have demonstrated increased levels of Hsp70 in neural tissue made tolerant by ischemia (Liu et al., 1993; Dhodda et al., 2004), where it was concluded that the increase of Hsp70 conferred tolerance to ischemia in the rat brains. Due to this discrepancy in observations it is possible that the different stressors induce a different cellular Hsp response, or that the Hsp response is dependent on the degree of stress. This is particularly evident in that our BBB

models pre-conditioned at 54°C for 5 s displayed a two-fold increase in Hsp70 expression relative to controls, but none of our other models displayed an increased expression of Hsp70 relative to controls

Hsp27

In thermotolerant BBB models, we observed no relative change in Hsp27 expression following any of the pre-condition treatment groups, suggesting that our observed thermotolerance is not associated with increased expression of Hsp27. While, to our knowledge, there are no other reports on Hsp27 and thermotolerance of the BBB, Landry et al (1989) observed thermotolerance to a 3.5 h, 43-44°C heat stress in Chinese hamster lung fibroblasts induced to increase expression of Hsp27. Likewise, rat hippocampus made tolerant to oxygen and glucose deprivation displayed increased cellular expression of Hsp27 (Valentim et al., 2003), suggesting an association between Hsp27 and acquired tolerance in rat hippocampus. However, unlike our study that examined Hsp27 expression immediately following a second heat stress, in the report of Landry et al (1989), thermotolerance was not determined until 7-10 days following a second lethal stress, and in the report of Valentim et al (2003) neuronal damage and Hsp27 content was not analyzed until 24 h following the second stress. Based on these two reports, it is possible that we might have observed changes in Hsp27 expression if we had examined our BBB models at a later time point following the second heat stress. However, as we still observed thermotolerance in our BBB models 24 h following an initial heat treatment, the role of Hsp27 in association with *in vitro* BBB thermotolerance is still unclear.

Our observations did not confirm that thermotolerance in our BBB model is correlated with increased relative expression of Hsp70 and Hsp27. Thermotolerance is likely dependent on several cellular mechanisms. In the rat hippocampal BBB, Hsp70 translocated to the tight junctions following mild hyperthermia, and subsequent acquisition of thermotolerance (Lu et al., 2004). Following hyperthermic stress in the rat brain, Hsp70 localized to the synapses of neurons (Bechtold et al., 2000). The observations from these *in vivo* studies suggest the possibility that in our study, instead of increasing expression, Hsp70 and/or Hsp27 translocated to the tight junctions, aiding in acquisition of thermotolerance.

It is also possible that other families of Hsps may increase expression following brief, high temperature hyperthermia, conferring thermotolerance in the BBB. Shivers et al (1988) observed an increased synthesis of Hsp100 and Hsp90 in bovine brain capillaries following a 1-3 h, 41°C heat stress. Mizzen and Welch (1988) observed induction of Hsp90 and Hsp32 following mild hyperthermia, and subsequent acquired thermotolerance in rat embryo fibroblasts. Although Mizzen and Welch did not observe cells of the BBB, the results suggest that these two Hsps might have a mechanistic role in conferring thermotolerance. However, thermotolerance is probably dependent upon a number of different parameters, prompting the need for further investigations on thermotolerance of the BBB.

CHAPTER V

SUMMARY

The findings of our research can be summarized by the following in vitro BBB responses to brief, high temperature hyperthermia:

1. Significant loss of barrier integrity was observed following exposure to 10 s of 45, 48, or 51°C, or 5 s of 54°C hyperthermia in our BBB models.
2. The degree of lost BBB integrity increased as the temperature of hyperthermia increased.
3. Thermotolerance was observed in BBB models exposed to a 10 s, 51°C heat stress 24 h following an initial (pre-conditioning) heat stress.
4. The degree of acquired thermotolerance increased in BBB models that were exposed to higher pre-conditioning temperatures.
5. There was a two-fold increase in Hsp70 expression 24 h following a 5 s, 54°C heat treatment compared to controls. Relative expression of Hsp70 was not different 24 h following a 10 s heat treatment of 45, 48, and 51°C in BBB models.
6. Relative expression of Hsp27 was not different 24 h following a 10 s heat treatment of 45, 48, or 51°C, or a 5 s heat treatment of 54°C.

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March 2, 2005

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IACUC #: 050103
Approval Date: 02/25/2005
Review Level: B

Project: An invitro model of the blood-brain barrier

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category B on Page 4 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the study involves either no pain or potentially involves momentary, slight pain, discomfort or stress.* The IACUC made the following comment on this protocol:

1. The investigator needs to contact other users of miniature swine (e.g., Carey, Wells) in order to schedule use of animals at the end of their experiments.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:

1. All cage, pen, or other animal identification records must include your IACUC # listed above.
1. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Van Gould at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Robert G. Mair, Ph.D.
Chair

cc: File