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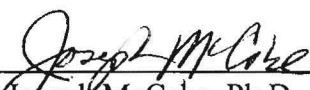
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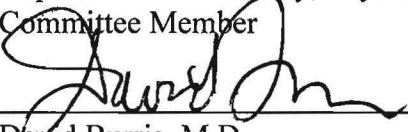
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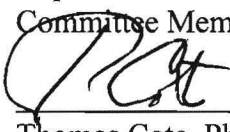
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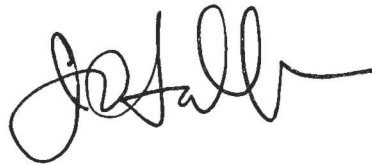
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A handwritten signature in black ink, appearing to read 'J. O'Sullivan', with a long horizontal flourish extending to the right.

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ABSTRACT

THE EFFECT OF DIAZOXIDE UPON HEAT SHOCK PROTEIN EXPRESSION AND PHYSIOLOGICAL RESPONSE TO HEMORRHAGIC SHOCK AND CEREBRAL STROKE

LTC Joseph C. O'Sullivan

Thesis directed by: Joseph T. McCabe, Ph.D., Professor and Vice-Chair of the
Department of Anatomy, Physiology and Genetics; and Professor of Neuroscience and
Molecular and Cell Biology

Our team tested the hypothesis that pharmacological induction of ischemic preconditioning (IPC) can offer cytoprotection and preserve vital tissues after cerebral stroke with hemorrhagic shock. The compound, diazoxide (DZ), is known to mimic IPC through its effects as a mitochondrial K_{ATP} channel opener and succinate dehydrogenase inhibitor. The effect of DZ was examined under two IPC protocols: delayed preconditioning and postconditioning in an anesthetized rat model of hemorrhagic shock, combined in some experimental groups with cerebral vascular occlusion (stroke).

When DZ was administered 24 hours prior to shock (delayed preconditioning), it significantly reduced hyperglycemia, which in vehicle-treated animals persisted after resuscitation. DZ also attenuated hyperlactatemia during the 1 hour shock period. With more severe trauma from combined stroke and shock, DZ also decreased hyperglycemia, but the apparent reduction of hyperlactatemia did not reach statistical significance. DZ

also appeared to increase the survival rate of animals that sustained combined stroke and shock, but not significantly ($p=.058$). The expression levels of heat shock proteins 25 (HSP25) and 70 (HSP70) were used as biomarkers for assessing the response of the kidney, liver, and right cerebral cortex and hippocampus to combined stroke and shock. Compared to vehicle-treated animals, DZ- pretreated rats subjected to stroke and shock exhibited increased HSP25 and HSP70 expression in kidney, liver and brain tissue.

When administered in a postconditioning Stroke + Shock model, where DZ was administered at two time points *after* stroke and shock, DZ again caused a significant increase in HSP25 and HSP70 expression in the ipsilateral cerebral cortex and hippocampus. As a postconditioning trigger, given after stroke and shock, DZ was effective when it was administered 60 minutes after shock immediately prior to reperfusion but not when given 10 minutes after Stroke and Shock (without immediate reperfusion). Taken together, these results suggest DZ attenuates physiological indicators of metabolic stress following shock or combined shock and stroke, that it may increase survivability, and that it enhances the upregulation of cytoprotective heat shock protein expression in key organs.

**THE EFFECT OF DIAZOXIDE UPON HEAT SHOCK PROTEIN AND PHYSIOLOGICAL
RESPONSE TO HEMORRHAGIC SHOCK AND CEREBRAL STROKE**

by

LTC Joseph C. O'Sullivan

US Army Nurse Corps

Doctoral Dissertation submitted to the faculty of the Graduate Program in Neuroscience
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LIST OF ABBREVIATIONS

AIF	apoptotic inducing factor
ABC	ATP binding cassette
ANT	adenosine nucleotide translocator
Apaf-1	apoptosis protease activating factor 1
ARDS	acute respiratory distress syndrome
ATP	adenosine triphosphate
Ca ²⁺	calcium
casp-3	caspase 3, initiator of cell death
CNS	central nervous system
COX-2	cyclooxygenase
cyto-c	cytochrome c
ddwater	double distilled water
DNA	deoxyribonucleic acid
DZ	diazoxide
5-HD	5-hydroxydecanoate
FAD ⁺	flavin adenine dinucleotide
gm	gram
GFAP	glial fibrillary acidic protein

GSK-3	glycogen synthase kinase-3
H ₂ O ₂	hydrogen peroxide
HCT	hemotcrit
HR	heart rate
HSC	heat shock constitutively expressed
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein
ICU	intensive care unit
iNOS	inducible NO synthase
IκB	inhibitor of nuclear factor kappa B
IP	intraperitoneally
IPC	ischemic preconditioning
IV	intravenous
JNK-1	c-jun N-terminal kinase
K ⁺	potassium
K _{ATP}	potassium ATP channel
kg	kilogram
KO	knockout
Kir	potassium inward rectifying
Li ⁺	lithium
MAPK	mitogen-activated protein kinases
Mg ²⁺	magnesium

mg	milligram
ml	milliliter
mmHg	millimeters of mercury
mK _{ATP}	mitochondria potassium ATP channel
mPTP	mitochondrial permeability transition pore
mRNA	messenger ribonucleic acid
MW	molecular weight
Na ⁺	sodium
NaCl	sodium chloride
NFκB	nuclear factor kappa B
NO	nitric oxide
p53	tumor suppressor protein
PIC	phosphate carrier
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
postH	post hemorrhagic shock
postI	post infusion (conclusion of experiment)
preH	pre hemorrhagic shock or exsanguination
preI	pre infusion (before resuscitation)
Rb ⁺	rubidium
RNA	ribonucleic acid
ROS	reactive oxygen species
SC	subcutaneous

SDH	succinate dehydrogenase
Sh	shock (hemorrhagic)
sK _{ATP}	cell surface potassium ATP channel
SOD	superoxide dismutase
Str	stroke
SUR	sulfonylurea receptors
3-NPA	3-nitropropionic acid
USUHS	Uniformed Services University of the Health Sciences
Veh	vehicle

THE EFFECT OF DIAZOXIDE UPON HEAT SHOCK PROTEIN AND PHYSIOLOGICAL RESPONSE TO HEMORRHAGIC SHOCK AND CEREBRAL STROKE

CHAPTER 1

INTRODUCTION

Shock and Trauma

Trauma has an extraordinary impact on our society. More years of life are lost in the US related to trauma and hemorrhagic shock than to heart disease and cancer [1]. Accidental, intentional and physical trauma injuries are the principal cause of death among Americans up to age 38 [1]; they are responsible for more deaths up to age 40 than all other diseases combined [2]. Trauma is also a substantial economic burden. Billions of dollars in direct and indirect lost wages and medical expenses are related to traumatic events. Every year in the U.S. alone, for example, about 500,000 people go into shock eventuating in 50% mortality [3]. Trauma is also the foremost medical care challenge for servicemen and women. In combat, hemorrhagic shock is the most frequent cause of traumatic death and represents almost 50% of the fatalities of military engagement.

There are four basic types of shock. 1) *Cardiogenic shock* involves a severe problem with the pumping of blood to critical organs. Cardiogenic shock can result from myocardial infarction, cardiac tamponade, or cardiac valve failure. 2) *Neurogenic shock* entails critical impairment of the central nervous system or spinal cord related to trauma. Dysfunction of the parasympathetic and sympathetic system with loss of vascular tone [4]

or central respiratory drive quickly leads to physiological demise. 3) Often associated with penetrating trauma, *septic shock* involves cardio-vascular collapse secondary to severe infections. Septic shock is usually seen in the days following trauma. 4) *Hypovolemic shock* occurs with inadequate blood volume related to hemorrhage. All four types of shock share a common pathway—diminished blood supply decreases oxygen supply to vital organs of the body, resulting in the activation of several biochemical processes that lead to cellular apoptosis and necrosis, organ failure, and death of the individual.

Trauma deaths occur in a trimodal temporal distribution. The immediate deaths (one hour or less from time of trauma) are due to significant brain and/or spinal cord injury, or heart and/or great vessel injury. This group comprises approximately 50% of severe traumatic injuries, and is not amenable to medical treatment. The second group of patients (30% of injuries) hemorrhages more slowly from visceral injuries (peripheral arteries) or severe fractures. This group will usually expire within four hours of injuries, but can be saved if effective emergency treatment is applied quickly. Cessation of bleeding and early controlled resuscitation [5] within the ‘golden hour’ will greatly increase survival. The ‘golden hour’ has been defined as the first hour post severe trauma that will lead to increased survival if rapid aggressive medical care is delivered, after the first hour of severe trauma the survival rate greatly decreases. Military and civilian evacuation procedures have greatly reduced the time to treatment and thereby increased survivability. The third and last aggregation of deaths occur secondary to sepsis or multi-organ failure, and peaks days after the injury [6]. Although this group initially survives

resuscitation, the initial ischemic event is too severe and the course to organ failure and death cannot be terminated. One study of trauma ICU patients, examining mitochondria oxidative dysfunction, revealed direct impairment of oxidative function in patients that later developed multiple organ failure [7]. There is an inability to increase oxygen tissue consumption despite increased oxygen delivery [8]. As immediate medical care capabilities improve, the *percentage* of individuals who succumb to later death is increasing. A good deal of current trauma research efforts is focused on the development of procedures that can prevent these delayed lethal complications.

Cerebral Hypoperfusion and Hemorrhagic Shock

An important principle in the management of shock is the preservation of key organs: brain, heart, and lungs. In fact, the body has compensatory mechanisms to shunt blood flow to these organs during hypoperfusion to increase survival since ischemia or injury to a key organ greatly increases lethality. For example, 64% of the hemorrhagic shock patients that died had a coincidental brain injury [2]. Brain ischemia or trauma alters cardiovascular control mechanisms, which affects compensation for hemorrhagic shock. One study demonstrated that a fluid percussion brain injury adversely affected vascular tone during hemorrhagic shock, requiring increased fluid resuscitation to maintain adequate blood pressure [9].

Metabolic Response to Hemorrhagic Shock

Hematological manifestations of severe hemorrhagic shock include a dramatic increase in plasma lactate and glucose levels. Lactate is produced in all tissues but

primarily skeletal muscle, brain, red blood cells and renal medulla. Lactate production is the result of anaerobic glycolysis from the reduction of pyruvate. Pyruvate is the only precursor of lactate, and is produced in the cytoplasm from glucose metabolism [10]. Pyruvate, when administered exogenously in the swine hemorrhagic shock model, improved cerebral metabolism [11] and decreased hepatic apoptosis [12]. If oxygen is available, pyruvate enters the mitochondria, is metabolized to acetyl-CoA and through the citric acid cycle and oxidative phosphorylation, culminates in the generation of water, carbon dioxide, and 32 moles of ATP. Normally, the lactate: pyruvate ratio is 20:1, with the liver and kidney being the primary lactate consuming organs. If hypoxic (shock) conditions exist, pyruvate cannot enter the mitochondria, and is converted to lactate. Under anaerobic conditions, 2 moles of ATP are generated for each mole of glucose but the cost of this reduced energy production is a huge increase in lactate levels. Lactate acidosis occurs when production overwhelms utilization [13]. In fact, in situations of hypoperfusion (shock), the liver no longer utilizes lactate, but becomes a lactate-producing site. Clinically, a lactate level greater than 2.5 mmol/L was an independent predictor of multiple organ failure [14], and higher levels of hyperlactatemia (>5mmol/L) were associated with death [15,16,17]. Effective clinical treatment options are targeted to the cause of the hyperlactemia with the ensuing lactic acidosis, but successful outcomes can be elusive. However, if a medication given early could diminish hyperlactemia in hemorrhagic shock it would have notable clinical implications.

Hyperglycemia also often occurs in response to hemorrhagic shock. Increased glucose production arises primarily from two sources; increased cortisol and glucagon

production, leading to gluconeogenesis, and from increased epinephrine, leading to glycogenolysis. It is hypothesized that increased blood glucose is partially adaptive for two reasons. Higher plasma glucose can give rise to increased plasma (intravascular) refill, via osmotic gradients, and the glucose is needed as a substrate for energy metabolism [15]. Glucose levels rise during the first 5 hrs, then gradually decline over the next 12 hours, but hemodynamic collapse begins before glucose levels are exhausted without resuscitation [16].

Research has shown that increased glucose levels is an independent mortality risk factor in coronary artery bypass and graft surgery [17]. A study of 516 critically ill patients likewise found that early hyperglycemia (blood glucose >200mg/dL), independent of the severity of injury, was associated with an increase in mortality in trauma intensive care patients [18]. Poor outcomes have been noted in patients with hyperglycemia and brain ischemia leading to treatment modalities that encourage tight regulation of blood glucose levels to increase survival [19]. Pharmacological agents that reduce hyperglycemia in hemorrhagic shock should lead to decreased morbidity and mortality.

Ischemic Preconditioning

Ischemic preconditioning may be an effective adjunct treatment that decreases morbidity and increases survival in patients that sustain stroke and hemorrhagic shock. The term *ischemic preconditioning* refers to a mild ischemic episode—followed by reperfusion—that causes no observable pathology. When administered prior to a more

lethal insult, these brief intermittent reductions in blood flow render organs and cells less sensitive to later sustained ischemic insult.

Ischemic preconditioning was originally described in a landmark 1986 study by Murry and associates [20]. Using a cardiac dog model, these investigators found that multiple, brief ischemic episodes protected the heart from a subsequent sustained ischemic insult. An experimental group of dogs experienced circumflex artery occlusion for 5-minute intervals with 5-minute reperfusions. This cycle was repeated a total of four times—clamping for 5 minutes and unclamping for 5 minutes. The circumflex artery was then clamped for 40 minutes. The control group of dogs had their circumflex artery occluded for 40 minutes. Surprisingly, despite the additional 20 minutes of ischemia in the preconditioned animals, cardiac damage, measured by infarct size, was significantly reduced to just 25% of the area of injury observed in the hearts of dogs in the control group. This paper was the first to demonstrate and coin the term, *ischemic preconditioning*. This phenomena has been demonstrated in all species studied to date (rat, mouse, rabbit, dog, human, chicken, sheep, pig and including cell lines), and in all organs studied thus far, including skeletal muscle, brain, kidney, small intestine, heart and liver [21, 22, 23]. Other preconditioning manipulations, such as global hypoxia [24] and thermal injury [25], are also effective in protection from lasting insult. Many studies have investigated the underlying mechanism of this pathway with the aspiration to utilize this powerful protective system clinically. Prior to this 1986 discovery, the best pharmacological treatment (with varying reproducible results) to preserve cardiac muscle from infarction was only 10-20% [26].

Research has determined that ischemic preconditioning can be subdivided into two distinctive types; *classical* and *delayed*. In *classical preconditioning*, the protective effects of ischemia/perfusion cycles are evident within minutes after the insult and persist for 2-3 hours [27]. Classical preconditioning is independent of protein synthesis, and is therefore dependent upon existing cellular pathways. It involves the direct modulation of energy supplies, pH regulation, Na^+ and Ca^{2+} homeostasis and caspase inactivation [28]. Research has shown many *triggers* can activate classical ischemic preconditioning, including agonists of G protein-coupled receptors (bradykinin [29], opioids [30], norepinephrine [31], adenosine [32]), potassium ATP channel (K_{ATP}) openers (diazoxide, pinacidil) [33], succinate dehydrogenase inhibitors (3-nitropropionic acid) [34], and volatile anesthetics, such as sevoflurane or isoflurane [35].

After the appropriate trigger occurs, *intracellular pathways* are activated, which leads to the protected cell phenotype. The actual sequence of these pathways has not been determined, but some components of the cascade have been identified. G-coupled receptors, for example, activate the epsilon isoform of protein kinase C (PKC- ϵ) [36, 37], which has been implicated as the key PKC subtype involved in preconditioning. Also important is the upstream signaling molecule, phosphatidylinositol-3-kinase (**PI3K**) [38], and mitogen-activated protein kinases (**MAPKs**) [39]. PI3K activates the serine/threonine kinase, Akt, which inactivates the pro-apoptotic kinase glycogen synthase kinase-3 (**GSK-3**) via phosphorylation [40]. Phosphorylation of GSK-3, in turn, inhibits the opening of the mitochondrial permeability transition pore (**mPTP**). Cell apoptosis or necrosis often occurs during reperfusion due to opening of the mPTP; a large

nonselective pore traversing both inner and outer mitochondria membranes. Cytochrome c and apoptotic inducing factor (**AIF**) are both released through the mPTP during ischemic reperfusion, leading to the activation of caspase and caspase-independent apoptotic pathways, respectively [41].

Potassium ATP channels (**K_{ATP}**) are key intracellular triggers of early ischemic preconditioning. This channel will be described in more detail below, as there are cell surface K_{ATP} (**sK_{ATP}**) and mitochondrial K_{ATP} (**mK_{ATP}**) forms. Pharmacologically, these are different channels, but opening of this channel (via PKC or pharmacological agents) leads to increased cell survival. One hypothesis is that activation of K_{ATP} channels hyperpolarizes the cell membrane thereby protecting the cell from detrimental depolarization [42]. Studies have shown K_{ATP} channel openers inhibit Ca²⁺ uptake and promote mitochondrial Ca²⁺ release, thereby decreasing the amount of accumulated Ca²⁺ within the mitochondrial matrix. The reduction in calcium influx inhibits Ca²⁺-induced apoptotic cascades [43]. Other investigators have proposed that mK_{ATP} activation increases the mitochondrial matrix volume (K⁺ ion inflow), which would counteract the matrix contraction that would occur during increased rates of ATP synthesis, and stabilize the inner and outer mitochondria membranes [44]. Regardless of the mechanism, blockage of K_{ATP} channels, by the putative K_{ATP} channel inhibitor, 5-hydroxydecanoate (**5-HD**), results in a loss of ischemic preconditioning and cell protection [45].

Although not as effective as the early protection from classical preconditioning, *delayed* or *late preconditioning* becomes apparent approximately 24 hours after initial

preconditioning and it can persist for up to 72 hours [27]. The most significant difference between classical and delayed preconditioning is the requirement of the manufacture of new proteins needed to obtain protection; inhibition of new protein synthesis attenuates the protection derived from delayed preconditioning [46].

The triggers for delayed preconditioning are similar to classical preconditioning. These include cellular stress factors (sub-lethal ischemia, heat stress, cardiac pacing), which release factors such as reactive oxygen species, adenosine, and endogenous nitric oxide (**NO**). NO has been determined to have no effect on classical preconditioning and is a trigger specific only for delayed preconditioning [47]. Endogenous preconditioning agents also initiate delayed preconditioning and include adenosine agonists, bradykinin, opioids, NO donors, acetylcholine and norepinephrine [47]. Exogenous agents that activate the preconditioning pathways include, diazoxide, nicorandil, some hypercholesterolemia agents, and volatile anesthetics [48].

Kinase intracellular pathways play a central role in delayed preconditioning, with activation of the PKC- ϵ isoform being particularly essential. Downstream to PKC- ϵ , tyrosine kinases and other kinases then activate the important transcription factor NF- κ B, which leads to the upregulation of many protective proteins in the nucleus. Some key proteins identified thus far include: inducible NO synthase (**iNOS**) [49], cyclooxygenase (**COX-2**) [50], the antioxidant enzyme superoxide dismutase (**SOD**) [51], and heat shock proteins (**HSP70**, **HSP25** and **HSP 32**) [47]. Delayed preconditioning also reduces apoptosis by upregulating the anti-apoptotic protein Bcl-2, which has been shown to

inhibit opening of the mPTP, leading to cell survival [52]. mK_{ATP} channel opening seems to be the final common pathway for these signaling pathways but it is not yet clear how the opening of these channels affords protection. Delayed preconditioning requires the opening of the mK_{ATP} channels during the ischemic event; its role after 24 hours, when delayed preconditioning occurs, is less clear [53].

Remote Preconditioning

By 1993, an appreciable number of studies had confirmed that the direct application of various stimuli (hypoxia, ischemia, triggering agents) resulted in tissue and organ protection. In that year, however, Przyklenk and colleagues made a startling discovery. Using a canine model in which the circumflex artery was occluded (as in Murry's 1986 study), Przyklenk and colleagues observed that cardiac muscle supplied by the descending left coronary artery was *also* protected from ischemic insult, indicating that the preconditioning stimulus offered protection that was not confined to one area of an organ [54]. Further experiments have shown *remote* classical ischemic preconditioning also is effective in other organs. A preconditioning trigger in one area of an organ offers protection to a different region of that same organ or to a different organ. For example, one group demonstrated that intermittent tourniquet application to a hind limb (ischemic preconditioning) implemented protection in *other* skeletal muscles [55]. Remote classical preconditioning of the heart was also obtained via transient ischemia of the small intestine [56, 57] or the kidney [58]. Other studies have demonstrated remote *delayed* preconditioning. Induction of small intestine ischemia, for example, engenders myocardial protection 24-72 hours later [59, 60].

The exact mechanism of remote ischemic preconditioning is unknown, but putative factors have been identified. Protection from kidney or intestinal preconditioning on cardiac muscle was eliminated with application of the ganglionic blocker, hexamethonium, suggesting involvement of a neuronal pathway [58]. However, stronger evidence exists that a humoral factor may play a more important role. Effluent from a preconditioned heart, transferred by whole blood transfusion, protected a non-conditioned heart from ischemic insult [61, 63]. Remote preconditioning was not activated by adenosine or bradykinin, but was found to be attenuated by the opioid antagonist, naloxone, suggesting opioid receptor involvement [62].

Postconditioning

The term *postconditioning* refers to the time period after ischemic insult but just before reperfusion when a procedure results in cytoprotection. This critical time juncture has significant clinical implications as hemorrhagic shock or stroke can never be planned; negating the practicality of classical or delayed preconditioning. The term postconditioning was first mentioned in a study done by Vinten-Johansen's team [64] that found that very short periods of cardiac artery occlusions in a dog model (three 30-second occlusion-reperfusion events just prior to reperfusion) reduced infarct volume. Further research indicated that the time point for postconditioning intervention is critical to minimize myocardial ischemia [65]. If the postconditioning protocol is not performed within the first minute of reperfusion there is little or no effect on reducing ischemic volume. Experimental findings suggest that the most critical time frame for cell damage after an ischemic event occurs during *reperfusion*. At this time, key cytotoxic elements

are increased, including; tissue edema, reactive oxygen species, invasion and activation of polymorphonuclear neutrophils (inflammation), and lipid peroxidation [64].

Although the exact mechanism of postconditioning remains to be investigated, research has determined that postconditioning does inhibit the opening of the mitochondrial permeability transition pore (**mPTP**); leading to cell survival [66]. The mPTP is nonspecific and spans the inner and outer mitochondrial membrane. MPTP opening results from high mitochondrial Ca^{2+} concentration, low ATP levels and oxidative stress; all present during reperfusion resulting in the collapse of the membrane potential, uncoupled oxidative phosphorylation, and the release of cytochrome c and other apoptotic factors. Another mechanism involved with postconditioning includes a reduction of oxidative generation [65], which is greatly increased during reperfusion, leading to irreversible cellular injury. Interestingly, a canine cardiac study that combined classical preconditioning (ischemic pretreatment before occlusion) and postconditioning (ischemic pretreatment before reperfusion) found there was no further reduction in infarct volume compared to the application of either treatment alone [67].

Summary of Preconditioning

A brief episode of ischemia, as well as other transitory stimuli, causes tissue to be more resistant to injury during subsequent periods of insult. Although far from fully elucidated, five factors are known to be involved: 1) opening of sK_{ATP} channels (see more below), 2) release of reactive oxygen species (and activation of PKC- ϵ), 3) opening of mK_{ATP} channels, 4) fatty acid β -oxidation metabolism changes and 5) the transient

opening and then closing of the mPTP. These factors lead to an overall reduction in reactive oxygen species generation, preservation of Ca^{2+} homeostasis, mitochondrial matrix volume and membrane stabilization, upregulation of protective proteins and inhibition of the mPTP opening—processes that lead to increased cell survival.

Potassium ATP channels

As described earlier, K_{ATP} channels—and in particular the mK_{ATP} —appears to be a common final pathway for the preconditioning effect; inhibition of these channels attenuates the preconditioning effect. Heat stress [68], ischemia [69], adenosine receptor agonists [70], and opioid receptor agonists [71] all offer preconditioning protection by their convergence on K_{ATP} channels. This suggests that the activities of these channels play a common, distal capacity in cytoprotection.

Using patch-clamp recording, Noma and coworkers discovered K_{ATP} channels in mammalian heart cells [72]. These channels are found in other tissues, including the brain, smooth muscle, skeletal muscle, and pancreas. One interesting effect is seen in pancreatic function, where the inhibition of the channel results in increased insulin release. An increase in ATP levels in pancreatic β -cells, due to increased blood glucose levels, closes the K_{ATP} channels, depolarizing the cell membrane. This leads to opening of voltage-dependent calcium channels, which results in Ca^{2+} influx, triggering exocytosis of insulin-containing vesicles [73]. Drugs that inhibit K_{ATP} channels (e.g., glibenclamide) have been used successfully for the treatment of non-insulin dependent diabetes.

Compared to other potassium channels, the structure of the K_{ATP} channel is unique. The K_{ATP} channel is a hetero-octamer comprised of two major subunits that assemble in a 1:1 stoichiometry, with four *outer* sulfonylurea receptors (**SUR**, approximately 160 kDa) and four *inner* potassium inward rectifying (**Kir**, approximately 40 kDa) subunits [74]. The four inner Kir6.x subunits (**Kir6.1** or **Kir6.2**) play critical roles in the formation of the channel's pore as each subunit protein is comprised of two transmembrane domains and a cytoplasmic inhibitory ATP site. Adequate ratio levels of ATP/ADP keep the channel closed. However, during high ATP utilization periods, the ATP/ADP ratio decreases and the K_{ATP} channel opens. Kir channels regulate membrane excitability such as heart rate, vascular tone and in the pancreas, insulin release. The term *inward rectification* is defined as an ion channel that allows greater inflow than outflow of ions. In this case, Mg^{2+} (bound to ADP) and polyamines act to block the outflow of K^+ during depolarization [75].

The outer subunits of the K_{ATP} channel consists of four regulatory subunits, SUR1, SUR2A or SUR2B, which each have three transmembrane domains and two cytoplasmic nucleotide binding domains [76]. The SUR1 subtype predominates in neuronal cells and pancreatic β -cells. SUR2A is found in cardiac and skeletal muscle, whereas SUR2B is found usually in smooth muscle. Pharmacologically, SUR1 can be blocked with glibenclamide, which results in increased insulin release in type 2 diabetes mellitus patients. SUR1 subunits are opened *only* with adequate doses of diazoxide, which can result in hyperglycemia. In contrast, SUR2A and SUR2B can be opened with diazoxide and many other potassium channel openers [76].

The sulfonylurea receptors belong to the ATP binding cassette (**ABC**) transporter superfamily. These proteins are responsible for moving many substrates, including ions, sugars, amino acids, peptides, metabolites, and hydrophobic compounds across organelle and cellular lipid membranes, in an ATP-dependent manner. Mutations in the ABC gene lead to neurological diseases, such as cystic fibrosis, retinal degeneration, and drug resistance. The superfamily consists of seven subfamilies, divided by gene structure and homology, ABCA through ABCG. In this nomenclature, SUR1 and SUR2 are labeled ABCC8 and ABCC9, respectively [77].

As mentioned earlier, the activity of K_{ATP} channels is affected by nucleotide levels; thus these channels couple membrane conductance to metabolism. Opening of K_{ATP} channels shifts the membrane potential toward the equilibrium potential for potassium ions, which is near -80mV in mammalian cells. Closing K^+ channels will depolarize the cell membrane and open voltage-gated Ca^{2+} channels that allow Ca^{2+} to flow into the cell [78]. K_{ATP} channels, therefore, likely regulate the electrical activity of different cells by either controlling the resting membrane potential or modulating action potential. Specifically, cell membrane sK_{ATP} channels are a sensor of the metabolic state. As mentioned earlier, these channels are central to the protective effects of preconditioning. In fact, knockout of Kir6.2 in mice abolished the protective effects of ischemic preconditioning, which links the role of sK_{ATP} modulation to preconditioning [79]. It is logical that a mechanism must be available to link events occurring outside the cell (ischemia, hypoxia, triggering agents, etc.) to inner cellular pathways to up-regulate or modulate cellular protection involving the mitochondria.

An ATP-sensitive K^+ channel in the mitochondria was first identified in 1991 [80] via patch clamp approaches, which used an inside-out configuration on mitochondrial membrane preparations. This channel was inhibited by application of ATP to the matrix side and was activated by the removal of ATP, suggesting that this mitochondrial channel generates a K^+ conductance when the ATP level on the matrix side becomes deficient. However, following this initial finding, much controversy remains regarding the existence and structure of the mK_{ATP} channel. Despite intensive searching, no genome sequence for the mK_{ATP} channel has been found. Research using adenoviral gene-transfer with a dominant negative construct of the Kir gene, in rabbit ventricular myocytes, indicated that neither Kir 6.1 or Kir 6.2 exist in the mitochondria, but do exist in sK_{ATP} channels [81]. Further research has shown that, *in vivo*, ischemic preconditioning was abolished in Kir6.1 or Kir6.2 knockout (KO) mice. The loss of an ischemic preconditioning effect appears to be a result of the incomplete operation of the sK_{ATP} channel. Interestingly, mitochondrial function, as measured by flavo-protein oxidation preservation, was normal, indicating that possibly neither Kir6.1 nor Kir6.2 are components of the mK_{ATP} channel [79].

On a positive note, one study using yeast two-hybrid studies determined that they may have found a clue for further pursuit of the structure of the mK_{ATP} channel [82]. Ardehali and colleagues report that the channel may consist of four mitochondrial inner membrane proteins: 1) ABCB8 (ABC binding cassette) 2) phosphate carrier (PIC), 3) adenosine nucleotide translocator (ANT), and 4) ATP synthase, which all associate with a fifth protein, succinate dehydrogenase (SDH). Although this multiprotein complex was

tested with known inhibitors and activators of mK_{ATP} (including diazoxide), all with correct responses, it differed in molecular mass, ion selectivity, and conductance when compared to findings of other research groups [83]. Therefore, although researchers are confident that the mK_{ATP} channel exists, there presently is no consensus about the nature of the protein components of this important structure.

Diazoxide

Diazoxide (Hyperstat) has been used clinically for many years as a drug of choice for hypertensive crises, such as malignant hypertension or pre-eclampsia. Diazoxide (**DZ**) has been effective in patients with impaired renal function, hypertensive encephalopathy, or hypertension complicated by left ventricle failure and eclampsia. The vasodilation effect is from a direct action on vascular smooth muscle. In these clinical situations, it is given rapidly by the intravenous route to initiate its antihypertensive effect. Due to newer and improved anti-hypertension medications, DZ use has decreased.

Further research has shown that at lower dose levels DZ is a potent opener of mK_{ATP} channels and a weak sK_{ATP} opener. DZ binds 1000 times more potently to mK_{ATP} channels than sK_{ATP} channels [84]. This leads to DZ being touted as a specific mK_{ATP} channel opener, which can assist in further elucidating the properties of this channel. This fact is important, as it is extremely difficult to isolate the components of the mK_{ATP} channel due to its location in the inner matrix membrane of the mitochondria. However, it must be noted that early, overlooked, research showed that DZ directly affected cellular energetics via inhibition of succinate dehydrogenase [85]. This fact alone throws doubt

on the hypothesis that DZ produces ischemic preconditioning via K_{ATP} channel opening *alone*, as had been recently the basis of many papers [44, 84, 86, 87]. The overall hypothesis in these publications seems to be that excessive mitochondrial contraction is deleterious to electron transport, which is extremely sensitive to matrix volume over a very narrow range. Maintenance of matrix volume, secondary to physiological mK_{ATP} opening, may prevent respiratory inhibition due to matrix contraction that would otherwise occur during high rates of ATP synthesis.

Taken together, it may be that cellular protection arises from a specific response to stimulation, via K_{ATP} channel activation, followed by the appearance of a relatively non-specific, end-product effect, via reactive oxygen species (**ROS**) generation. DZ, then, may open K_{ATP} channels, which produces mitochondrial matrix swelling, thereby uncoupling oxidative phosphorylation [88]. Once mitochondria energy production is uncoupled, Complex III is prone to generate ROS. ROS generation may be the common pathway between DZ effects, ischemic preconditioning, and cellular protection [89].

Conflicting evidence of the roles between ROS generation, DZ and ischemic preconditioning merits discussion. One research team indicated that DZ produced a 173% increase in ROS generation in cardiac myocytes leading to cardioprotection [90]. However, other research demonstrated that DZ *reduces* overall ROS generation via inhibition of succinate dehydrogenase and cellular ATPase, therefore preserving myocardial energetics [91]. These conflicting results demonstrate that ROS generation can have both negative and positive effects on cell survival. Historically, it has been

determined that excess ROS generation leads to oxidative destruction of cells overwhelming the natural anti-oxidant buffering mechanisms of the cell. However, the current hypothesis is that the initial mitochondrial uncoupling leads to a small burst of ROS generation, which activates PKC- ϵ and leads to ischemic preconditioning. This results in a later overall reduction of ROS generation that protects the cell. Normally, in cells not protected, cell death occurs during reperfusion due to a dramatic increase in ROS generation [92]. In summary, *early* generation of ROS is one key to creation of the protective mechanism of ischemic preconditioning, which prevents *late* generation of ROS leading to cell death. Further reinforcing this argument, evidence has shown that the protective preconditioning effects of DZ (and related drugs) and ischemic preconditioning are blocked by anti-oxidants [93, 94]. Lastly, studies have shown that DZ when applied shortly before ischemia in isolated heart mitochondria reduced the rate of succinate-supported generation of ROS through inhibition of succinate dehydrogenase via modulation of flavin adenine dinucleotide (FAD⁺) [91]. Regulation of flavoprotein redox reactions are important because succinate dehydrogenase and coenzyme Q are the sites of free radical production, which contributes to oxidative injury [95].

Using a canine hypothermic circulatory arrest model, Shake and colleagues demonstrated that DZ pretreatment (15 minutes before arrest) produced significant neuron preservation [96]. This preconditioning effect was attenuated when the putative mK_{ATP} blocker, 5-hydroxydecanoate (**5HD**), was administered. Another cardiac model demonstrated that when DZ was added to cardioplegia solution in cardiopulmonary

bypass in a pig model, it significantly reduced regional myocardial cell necrosis and apoptosis [87].

Many papers have discussed the effect of 5HD as an agent to directly counteract the effect of DZ on mK_{ATP} channels. However, studies have shown that the use of 5HD as a specific inhibitor of mK_{ATP} , and DZ as a specific activator of mK_{ATP} , could be in error. Recent research has determined that although 5HD application does prevent preconditioning, it does not appear to be via inhibition of the mK_{ATP} channel. Evidence and research has shown that ischemia results in a necessary increase in fatty acid metabolism for cell survival. 5HD (a fatty acid) combines with acyl-CoA and is then transported into the mitochondrial matrix via carnitine palmitoyl transferase I and II (membrane transporter). In other words, 5HD is rapidly converted to 5HD-CoA by mitochondrial fatty acyl CoA synthase and then acts as a weak substrate or inhibitor of respiration [97]. In fact, more detailed studies by Hanley and colleagues suggest that during an ischemic event the beta-oxidation of fatty acids is a key component to cell survival. It has been determined that the beta-oxidation of the 5HD-CoA molecule leads to a bottleneck in enzyme action, preventing beta-oxidation of other fatty acids; leading to cell death [98]. This is in direct contrast to past suggestions that 5HD inhibited or closed the mK_{ATP} channel, therefore eliminating ischemic preconditioning and triggering cell death. For that reason, the assertion that 5HD reverses the preconditioning effect of DZ by closing the mK_{ATP} channels may need re-evaluation. Further research by Hanley *et al.* casts doubt on the assertion that the preconditioning action of DZ is solely related to its ability to open mK_{ATP} channels, and that other independent mechanisms (partial

inhibition of the respiratory chain) may be more important [99]. Also of interest is the hypothesis that the 5HD-CoA molecule may have an effect on the adenine nucleotide translocase (**ANT**) receptor. Fatty acyl-CoA molecules are known to displace adenine molecules (ADP, ATP) from the ANT, which could accelerate cell death by preventing ADP entry in the mitochondria [100].

DZ, as previously mentioned, also inhibits succinate dehydrogenase (**SDH**); a key enzyme in the TCA cycle. This enzyme is required, with FAD, to initiate the reaction, $\text{succinate} + \text{FAD} \leftrightarrow \text{fumarate} + \text{FADH}_2$. Inhibiting this enzyme, leads to an accumulation of succinate and a decrease in the electron transfer to the complexes involved with cellular respiration. SDH is unique as it is embedded in the inner mitochondrial membrane and is the direct link between the TCA cycle and ATP formation. It is believed that the uncoupling of this link with DZ leads to preservation of ATP during an ischemic event. Furthermore, research has shown that the increase of succinate leads to hypoxic inducing factor 1 (HIF-1) activation, which results in increased angiogenesis and glycolysis [101].

Therefore, while the exact mechanism of the pharmacological action of DZ in preconditioning is enigmatic, it remains as a potent drug for classical and delayed preconditioning. The rationale for this study is that no one has examined the potential beneficial effects of DZ for the treatment of hemorrhagic shock or stroke with hemorrhagic shock. Important clinical implications could become evident if DZ

demonstrated significant upregulation of survival proteins (for example, heat shock proteins) in key organs during hemorrhagic shock and stroke.

Heat Shock Proteins

Early research demonstrated that heat shock proteins (**HSPs**) are a highly conserved group of proteins that are expressed when organisms are exposed to elevated temperature. HSPs were originally discovered in *Drosophila busckii*, and were one of the first eukaryotic genes to be cloned [102], for their organization within the genome to be defined [103], and to have their regulatory sequences identified [104]. HSPs are classified according to their size as either high molecular weight (**MW**) HSPs (includes HSP 60, 70, and 90) or small MW HSPs (includes HSP 10, 27, and 40). Some of these HSPs are expressed constitutively and others are upregulated following cellular stress. One key functional difference between the two groups of HSPs is that the high MW HSPs are ATP-dependent chaperones and the small MW HSPs are ATP-independent [105]. Increases in HSP25 and HSP70 expression results in increased resistance to apoptosis [106].

HSP70

The HSP70 family is the most studied of the ATP-dependent chaperones. This protein is highly conserved from mammals to bacteria to plants; in fact, *E. coli* and human homologs have 50% matching amino acids [107]. HSP70 is found mostly in the cytosol of cells and have constitutively (**HSC70**) and inducible components. HSP production can be induced via heat, oxidative stress, and heavy metals [108]. Major

housekeeping functions of the HSP70 family include folding proteins in the cytosol (HSP70), in mitochondria (HSP75) and in endoplasmic reticulum (GRP75). Other functions include dissolution/degradation of unstable proteins, reducing protein aggregation leading to increased neuronal survival [109], importing proteins into cellular compartments including the mitochondria, and control of regulatory proteins [110].

HSP70 appears to block apoptosis at multiple levels. Acting on a caspase-*dependent* pathway, HSP70 inhibits the action of apoptosis protease activating factor-1 (**Apaf-1**) [111], which prevents the recruitment of procaspase-9 to the apoptosome. The apoptosome structure consists of Apaf-1, procaspase-9 and cytochrome c, which together activates caspase-3, the initiator of cell death. HSP70 has also been shown to act further downstream and directly inhibit caspase-3 [112]. Finally, HSP70 prevents cell death by a caspase-*independent* process involving inhibiting apoptosis inducing factor (**AIF**) [113]. AIF is normally found and needed within the mitochondrial membrane for oxidative reactions. During severe cellular stress, AIF is released from the mitochondria and migrates quickly to the nucleus to initiate cell apoptosis via DNA fragmentation [114]. Needless to say, many cancer cells and tumors over-express HSP70, which renders them resistant to treatment [110].

Research has shown that transgenic mice that over-express HSP70 show decreased cytochrome c release into the cytosol. This resulted in decreased apoptosis following cerebral ischemia, as compared to wild type mice [115]. Specifically, the study by Tsuchiya and colleagues demonstrated a significant decrease in infarct area in

transgenic mice (increased HSP70 expression) compared to wild type mice 24 hours after middle cerebral artery occlusion. Furthermore, recent evidence has given merit to the hypothesis that HSP70 proteins that are made from one cell can be passed on to neighboring cells and therefore stress tolerance can be activated via HSPs in extracellular fluid [116]. This could imply that HSP upregulation in affected cells could limit the area of damage from ischemic insult by directly affecting nearby cells.

HSP70 mediates several additional mechanisms known to promote cell survival. In a rat liver model with upregulated HSP70 due to heat shock, for example, it was shown that HSP70 stabilizes I- κ B binding, resulting in the inhibition of NF- κ B. It is hypothesized that the inhibition of NF- κ B by HSP70 prevents the expression of proinflammatory mediators, leading to increased liver cell survival [117, 118]. In a human liver model, HSP70 induction resulted in beneficial effects from reduction of transaminases and fibrinogen synthesis [119]. Increased clinical survival after severe trauma has been correlated to increased serum HSP70 expression [120]. Also, HSP70 has been shown to repair proximal renal tubules in an ischemic kidney model [121]. Photothrombic brain injury elicits the expression of HSP70 and HSP27. HSP70 expression as early as one hour post-trauma delineated the area of necrosis at 24 hours post-thrombic injury in ipsilateral cortex. HSP27 expression also was found to be upregulated and in fact more globally expressed in the entire ipsilateral cerebral cortex, primarily in astrocytes [122].

HSP25 and HSP27

Research demonstrates that HSP 27, the human counterpart of HSP25, also exhibits an anti-apoptotic effect through several proposed mechanisms. HSP 27 (unphosphorylated) has been shown to directly bind to cytochrome c released from the mitochondria and to prevent the interaction between Apaf-1 and procaspase 9 during stress, inhibiting the activation of the mitochondria-caspase (intrinsic) pathway [123]. HSP27 (phosphorylated form) also inhibits the extrinsic CD-95 death pathway via interaction and binding with a key pathway protein- Daxx [124]. HSP 27 also offers protection via activation of serine/threonine kinase Akt, which when activated, inhibits both Bcl-2 pro-apoptotic proteins and caspase-9 [125]. Activated Akt (via HSP27) also is a key component necessary for phosphorylation of I- κ B, which then releases NF- κ B, leading to upregulation of genetic expression of anti-apoptotic proteins [126]. HSP27 has been shown to have other anti-apoptotic effects. For example, HSP27 has been shown to inhibit protein kinase C-delta (PKC- δ), which has been shown to have a negative effect upon cell survival [127]. Using a cellular model, it has been demonstrated that HSP27 binds directly to caspase-3 and thereby inhibits apoptosis and necrosis [128]. Caspase-3 mediated apoptosis was found to cause extensive lymphocyte apoptosis and death in septic patients in the ICU [129].

HSP25 protein expression was found to be decreased prior to the degeneration of motoneurons in amyotrophic lateral sclerosis mice. HSP25 mRNA was found to be unchanged, but post-transcriptionally it was found that motoneuron cell death and muscle weakness was correlated to a prior decrease in HSP25 expression [130]. HSP27 acts as a

molecular chaperone by scavenging misfolded and denatured proteins through formation of large complexes (4000 kDa) in an ATP-independent manner [131]. Those proteins that cannot be refolded into proper configuration are targeted for destruction in the ubiquitin-proteasome pathway, thereby increasing cell survival.

In a study of mice given a lethal dose of acetaminophen, survival at 9, 24 and 48 hours later were 96%, 38% and 36%, respectively. HSP25 expression was noted at 24 hours and was hypothesized to be the key protein expressed that allowed only a 2% decrease in survival [132]. Another study suggests that HSP25 upregulates IL-10, which is a potent down-regulator of monocyte production of pro-inflammatory mediators (TNF- α and IL-8) [133]. This effect can protect cells and organs by decreasing the inflammatory response to injury.

Microfilaments, one of the three components of the cytoskeleton, are essential to many cellular processes, such as endocytosis, exocytosis and signal transduction. HSP25 has been shown to protect actin structure against breakage by actin-severing proteins and it promotes microfilament reorganization. HSP25 has been shown to stabilize the cytoskeleton and promote cell protection by preventing microfilament disruption by stress signals [134].

Project Summary

Hemorrhagic shock is the most frequent cause of traumatic death in combat and represents almost 50% of the fatalities of military engagement. Blood loss decreases

oxygen supply to vital organs of the body, resulting in the activation of several biochemical processes that lead to cellular apoptosis and necrosis, organ failure and death of the individual. Many resuscitative strategies have been developed to improve survival of combat casualties, but too often resuscitation at the aid station is unsuccessful, or initially successful, only to have the patient later succumb to organ failure. The long-term goal of the proposed research is to investigate a treatment that could improve mortality and morbidity from hemorrhagic shock and stroke. The specific aim of this project was to examine the ability of DZ, a mitochondrial K_{ATP} channel opener and succinate dehydrogenase inhibitor, to preserve selected vital tissues (brain, liver, kidney) after exposure to stroke and hemorrhagic shock, and to begin to determine what key molecular mechanisms (HSP70, HSP25) are affected by this drug.

To examine the efficacy of DZ as a treatment for hemorrhagic shock, this research employed two models of trauma: *hemorrhagic shock* that uses anesthetized laboratory rats with 40% blood loss, and hemorrhagic shock combined with *stroke*, precipitated by occlusion of the right common carotid artery. The effectiveness of DZ was tested at three key time points. First, DZ was given ~24 hours before shock to elicit *delayed* preconditioning. Next, DZ was also evaluated in a *postconditional* situation after 40% blood loss combined with stroke. Here, animals received DZ 20 minutes after blood loss; a time when ischemic preconditioning has previously been shown to be effective. DZ was administered to additional rats 60 minutes after blood loss; that is, near the end of what has been described as the ‘golden hour’ after trauma. Separate analyses determined

whether DZ affected the expression of HSP25 and HSP70 in the liver, kidney, and selected regions of the central nervous system.

As outlined in the next chapter, we examined the impact of DZ as a mediator of preconditioning during hemorrhagic shock. In **Experiment 1**, we determined the efficacy of DZ treatment given 24 hours *before* hemorrhagic shock (delayed preconditioning). Tissue examination, with physiological and hematological sampling, were used to evaluate the protective effect of DZ. **Experiment 2** also examined the effect of DZ treatment given 24 hours before surgery, but included right carotid artery occlusion combined with hemorrhagic shock. In **Experiment 3**, we determined an optimal *dose* for DZ administration for postconditional treatment to rats that have sustained hemorrhagic shock, right carotid occlusion and resuscitation. In **Experiment 4**, we assessed the efficacy of DZ by sampling tissues from animals that have sustained hemorrhagic shock and resuscitation, when DZ is administered *before* or *simultaneously* with resuscitation (postconditioning).

Goal of Thesis and Summary of Results

The goal of this project was to determine whether DZ, a pharmacological agent with known preconditioning effects in other models, is effective in a hemorrhagic shock and stroke model in the rat. Two types of preconditioning effects were tested, *postconditional* and *delayed*. The response of rats to shock and combined stroke and shock, was assessed via hematological and physiological parameters as well as HSP25 and HSP70 expression.

It was determined that although DZ had no substantial effect on mean arterial blood pressure during shock or resuscitation, it reduced blood lactate and glucose levels during hemorrhagic shock. These hematological effects, combined with noticeable increases in HSP25 and HSP70 expression 24 hours later, could be part of the explanation for possible increased survival in those rats that received DZ 24 hours prior to insult. Key organs including kidney, liver and brain all expressed increased HSP25 and HSP70 production, which has been shown in many other models to decrease apoptosis of cells and preserve organ function. The postconditional study demonstrated increased HSP25 and HSP70 production in those rats that received DZ just prior to resuscitation (60 minutes post shock), but not in those rats that received DZ 20 minutes after the start of shock.

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CHAPTER 2

OVERVIEW OF EXPERIMENTAL DESIGN AND METHODS

The Effect of Delayed Preconditioning upon the Outcome of Hemorrhagic Shock

Experiment 1 (referred in Chapter 3 as the *Shock Study*) utilized anesthetized rodents to determine the effect of DZ upon the expression of heat shock proteins and upon hematological and physiological parameters following hemorrhagic shock. There have been no previous investigations that have determined the effect of DZ *in vivo* in this animal model of shock. The first experiment consisted of five groups. Group 1 (Sham; **Sham**) received only anesthesia and vascular cannulation. Group 2 rats (Vehicle Infusion; **Sham Inf**) received the same experimental treatment as Group 1, but were given an infusion of normal saline. Group 3 animals (Vehicle Shock; **Veh Sh**) sustained hemorrhagic shock for 1 hour followed by normal saline resuscitation infusion. Group 4 (DZ Sham; **DZ Sham**) received the same experimental treatment as Group 1, but had been given DZ 24 hours prior to surgery. Group 5 (DZ Shock; **DZ Sh**) rats received an injection of DZ 24 hours prior to surgery, and had the same experimental treatment as Group 3.

The brain tissue from these animals was used in preliminary studies of immunocytochemistry, where a number of antisera were used to determine whether shock causes the induction of signals known to be associated with apoptosis. In general, no change in staining intensity for caspase 3, c-Jun, p53, cresyl violet, HSP25 and HSP70 was observed in this study (data not shown). These results from assays of the CNS after

hemorrhagic shock were interpreted as evidence that the brain, under the conditions employed in this study, was not substantially injured by hemorrhagic shock. These findings are in line with general views and recent experimental findings [1] that the CNS is “protected” by a number of compensatory responses following hypotension.

The Effect of Delayed Preconditioning upon the Outcome of Combined Cerebral Stroke and Hemorrhagic Shock

Experiment 2 examined the impact of DZ in animals that sustained combined stroke with hemorrhagic shock (referred in Chapter 3 as the *Stroke + Shock Study*). Group 1 (Control; **Control**) animals did not receive any experimental treatment. Group 2 (Vehicle Stroke; abbreviated **Veh Str**) received anesthesia, right carotid ligation and vascular cannulation only. Group 3 (Vehicle Stroke + Shock; **Veh Str+Sh**) received anesthesia, right carotid ligation, vascular cannulation, hemorrhagic shock (40% blood loss), and resuscitation (normal saline). Group 4 rats (DZ Stroke; **DZ Str**) received DZ 24 hours prior to anesthesia, right carotid ligation, and vascular cannulation. Group 5 (DZ Stroke + Shock; **DZ Str+Sh**) received DZ 24 hours prior to treatments identical to Group 3.

The results of MAP and plasma levels of lactate and glucose are reported in Chapter 3. Liver and kidney protein assays for HSP25 and HSP70 are reported in Chapter 3, while the results from assays of the cerebral cortex and hippocampus of these proteins is summarized in Chapter 4. As described below, Chapter 4 summarizes results from employment of DZ for delayed preconditioning and for postconditioning.

Dose Tolerance Study of Diazoxide in Postconditioning upon the Outcome of Combined Cerebral Stroke and Hemorrhagic Shock

Experiments 1 and 2 assessed the effect of DZ as a cytoprotectant when given 24 hours prior to trauma. A far more useful application of DZ would be if this drug could be given following hemorrhagic shock. As described later, Experiment 4 addresses the utility of DZ when administered after shock, but before or at the time of resuscitation. Before undertaking a full study, however, it was necessary to determine the effects of DZ on blood pressure in animals that experience combined cerebral stroke and hemorrhagic shock. Accordingly, the goal of **Experiment 3** was to determine the effect of DZ when it is administered shortly after anesthetized animals sustain hemorrhagic shock and stroke, and before and during resuscitation.

Only one prior study appears to have extensively investigated the hemodynamic effects of DZ *in vivo*; Evered investigated the impact of DZ in anesthetized *non-hemorrhaged* rats. A dose of 15 mg/kg intravenously (IV) caused a 25% reduction in blood pressure [2]. In Experiment 3, a dose range was chosen that was considerably below this level, since the goal in the present work was to avoid exacerbating hypotension. Previous studies, in fact, have demonstrated far lower doses are sufficient for tissue protection. Sanada and colleagues, for example, showed that a relatively low dose of DZ (0.4mg/kg IV) decreases infarct size in the ischemic canine heart [3].

Experiment 3 was initiated using a 3.2mg/kg intravenous (IV) infusion of DZ. DZ was given *slowly*, over 10 minutes, beginning 10 minutes after the completion of

exsanguination. Vital signs and blood values were recorded and animals were allowed to recover from anesthesia (see below in Hemorrhage Shock and Resuscitation Model).

A criterion was established to assess whether DZ exacerbated hypotension. If any rat experienced a 25% fall of MAP, at any time after DZ infusion commenced until the end of the resuscitation period, it was considered a significant hypotensive event. This dose level of DZ would then be deemed hypotensive, and the next rat would receive a dose of DZ that was 50% of the previous dose.

As Figure 1 demonstrates, the MAP did not decrease significantly in any rats that received DZ. An analysis of variance comparing MAP values, beginning at the time DZ infusion commenced (10' DZ on the abscissa) to the end of the shock period (p60), indicated there was no change in blood pressure ($F=0.945$; $p = 0.527$). Since animals that have sustained combined stroke and shock exhibited no significant alteration in MAP, the dose of 3.2mg/kg of DZ (IV) was utilized for the postconditioning study (Experiment 4).

The Effect of Postconditioning upon the Outcome of Combined Cerebral Stroke and Hemorrhagic Shock

Experiment 4 employed a postconditioning treatment paradigm to assess the efficacy of DZ treatment as a protectant of the brain *following* stroke and hemorrhagic shock. The results of this study are summarized in Chapter 4. Experiment 4 consisted of six separate groups of rats; all animals in the study sustained right common carotid artery ligation. Group 1 (Stroke Vehicle, abbreviated **Str Veh**) served as one of the control groups. This group of rats was anesthetized and cannulated, and received right common

carotid artery occlusion (as described below) to obtain blood values and control measures of physiological parameters, but did not receive DZ or hemorrhagic shock. Group 2 animals (Stroke + Shock Vehicle; **Str+Sh Veh**) received no DZ treatment, but were anesthetized, cannulated, and sustained controlled hemorrhage and reperfusion. Group 3 (Stroke with DZ @ 20 minutes; **Str DZ20**) received an intravenous dose of DZ (dose determined from Experiment 3) 20 minutes after carotid artery ligation—treated identically to Group 1—with no exsanguination. Group 4 animals (Stroke + Shock DZ @ 20 minutes; **Str+Sh DZ20**) were anesthetized, cannulated, exsanguinated and received DZ intravenously, 10 minutes after the end of the 10-minute exsanguination period, and were resuscitated after one hour of shock. Group 5 rats (Stroke + Shock DZ @ 60 minutes; **Str+Sh DZ60**) were anesthetized, cannulated, bled, and received intravenous DZ 60 min after completion of exsanguination; just prior to resuscitation. Group 6 (DZ Stroke + Shock; **DZ Str+Sh**) served as a comparison to the groups of rats that had received DZ as a postconditioning treatment either 20 (**Str+Sh DZ20**) or 60 minutes (**Str+Sh DZ60**) after combined stroke and shock. Group 6 animals (**DZ Str+Sh**), therefore, were a replication of Experiment 2 results (delayed preconditioning); 24 hours prior to sustaining a stroke, shock, and resuscitation, these animals were given DZ. Following experimental treatment, the brain tissue from these animals was used to assess changes in HSP25 and HSP70 levels. The liver and kidneys were harvested and frozen, but not used.

Hemorrhagic Shock and Resuscitation Model

For all animals that experienced hemorrhagic shock, the following procedure was employed. Six days prior to experimental treatment, all rats (male Sprague-Dawley rats, 225-350 gm body weight, Charles River Laboratories) were handled daily and had *ad libitum* access to rat chow and water. Table 1 summarizes the time course for the hemorrhage/resuscitation procedures.

Rats were anesthetized in an induction chamber with 3-5% isoflurane (IsoFlo, Abbott Laboratories) until unconscious (approximately 15-60 seconds). Anesthesia was maintained throughout the experiment with isoflurane via a rodent nose cone with a scavenging system to maintain a constant plane of anesthesia throughout the experiment. A warming blanket was used to maintain euthermia.

A surgical cutdown was performed to the right groin for isolation and cannulation of the right femoral artery and vein. A polyethylene catheter (PE-50, Clay Adams, Piscataway, NJ) was inserted using aseptic technique guided with the aid of a surgical optical scope. The arterial catheter was then connected to a pressure transducer and computerized physiographic system (Ponemah Physiology Platform, Gould Instrument Systems, Valley View, OH) for continuous blood pressure and heart rate recordings. In Experiments 2-4, rats also sustained a unilateral common carotid artery ligation. To induce unilateral cerebral stroke, which only can be precipitated in rats that sustain hypotension from hemorrhagic shock, a midline neck incision was made just before

exsanguination. After dissecting away the vagus nerve, the right carotid artery was ligated by tying its circumference with surgical suture.

After establishing baseline vital signs and hematological indices, rapid hemorrhage (in selected noted groups), was induced over a 10 minute period (27 cc/kg; ~40% of total blood volume based on body weight, after subtracting blood needed for hematological sampling-approximately 6-7ml). Sixty minutes after rapid hemorrhage, resuscitation was commenced by intravenous infusion of normal saline (81cc/kg) through the femoral vein over the next 45 minutes (approximately 22-26ml). The catheters were then removed, the operative site repaired, and the animals were allowed to recover. After regaining consciousness and receiving analgesia (buprenex, 0.03 mg/kg. SC), the rodents were returned to their home cage for 24 hours.

Physiological Monitoring of Plasma Lactate and Glucose, Mean Arterial Pressure and Heart Rate

During the procedure, four heparinized arterial blood samples were obtained from the femoral arterial catheter (0.3cc each); at baseline before hemorrhage, at the completion of exsanguination, at the end of the 60 minute shock period, and at the conclusion of resuscitation. Blood samples are then analyzed by a Stat Profile 2 Blood Gas and Electrolyte Analyzer (Nova Biochemical, Waltham, MA).

Statistical Analysis

Results for the physiological and hematological data were summarized using a Treatment x Trials design analysis of variance (SigmaStat, Version 2.03) with repeated measures on the second factor and *post hoc* comparisons (all pairwise multiple comparisons using the Holm-Sidak method). In Experiment 1, preliminary analyses indicated there was no difference in responses for Sham animals (no exsanguination) that had received either vehicle (normal saline) or diazoxide 24 hours before physiological monitoring. To reduce animal number, fewer rats in each treatment condition were used, and the data from these sham-treated animals were pooled. There then were four levels of the treatment factor. The Trial factor refers to repeated measures on the same animal for physiologic and hematological measures during shock and resuscitation. Blood pressure measurements were taken every 1 min immediately after exsanguination for 5 min and then every 5 min during the remaining periods of shock and resuscitation.

Western blots were performed to measure changes in HSP25 and HSP70 (relative to β -actin levels) in samples of the kidney (Experiment 2), liver (Experiment 2) and brain (Experiments 2 and 4) of rats from the Stroke + Shock Study. The Fuji image analysis program (Fuji Film LAS-1000plus) was used to determine western blot band density for HSP25, HSP70 and for β -actin levels in each sample. All western blot membranes were prepared with five samples each from two groups; Group 1 (the Control Group in Experiment 2, or the Str Veh Group in Experiment 4), and samples from an experimental group (Groups 2, 3, 4, 5 or 6). In cases where a protein sample from the same rat was used on more than one blot, the mean band density for this animal was computed.

Comparisons between membranes were performed by standardizing all membranes to the mean band intensity of the Group 1 samples, before a single-factor analysis of variance and *post hoc* comparisons were performed. Preliminary inspection indicated there occasionally were samples with excessively high density scores. To determine whether these values were outliers, the mean and standard deviation were computed, and the data for each data set was converted to z-scores. Values that exceeded 2.5 times the standardized values ($z\text{-score} > 2.5$) were considered candidate outliers. This test was reiterated on each sample, after removal of candidate outliers, until no outliers were detected [4].

Immunocytochemistry

A small pilot study was conducted to assess the utility of employment of immunocytochemistry as a means of evaluating changes in HSPs as a function of trauma and DZ treatment. The result of this work, which was only preliminary, is summarized in Chapter 5. This approach was abandoned due to time constraints and the fact that quantitative western blotting was a more efficient means of determining protein changes.

Twenty-four hours after surgery, the rats were anesthetized and transcardially perfused with 100mM buffered saline (PBS) and then with 4% paraformaldehyde in PBS using methods familiar to this lab [5, 6, 7]. Specimens were immediately removed and post fixed in 4% paraformaldehyde in 0.1M PBS at 4°C overnight and then transferred into 30% sucrose. Frozen coronal sections of the cerebrum (40µm thickness) were prepared and collected for storage in cryoprotectant at -20°C. Selected specimens were

then incubated in phosphate-buffered saline-Triton X-100 (PBS-T) containing 0.1% H₂O₂ for 30 min. Subsequently, sections were washed three times for 5 min in 0.1M PBS-T and then incubated for 1 hour in 0.1M PBS-0.3% Triton containing normal goat serum diluted 1:10. After further rinses in 0.1 M PBS-T, separate sections were incubated overnight at 4°C with HSP70 (mouse monoclonal SPA-810, Stressgen) and HSP25 (rabbit polyclonal, Stressgen). After overnight incubation, the sections were washed extensively in buffer and then incubated in biotinylated secondary antibody appropriate for conjugation to the primary antiserum. Subsequently, the sections were washed extensively in buffers and processed for visualization of product with the chromogen 3, 3'-diaminobenzidine tetrahydrochloride and a Vectastain Elite ABC kit. Controls to evaluate the specificity of immunostaining include: 1. Substitution of the primary antiserum with pre-immune serum, 2. Pre-absorption of the antisera with their authentic peptide or peptide residues, and 3. Pre-absorption of antisera with the authentic peptide or peptide residues of other antisera to determine cross-reaction.

Table 1 Hemorrhagic Shock and Resuscitation Protocol				
Cumulative Time	0'	10'	70'	115'
Duration	0'	10'	60'	45'
Exsanguination	→			
Hemorrhagic Shock	→			
Resuscitation	→			
Hematological Sampling *	*		*	*
MAP, HR q 5 minutes *	*	→		

* Refers to instances when hematological or vital signs will be obtained.

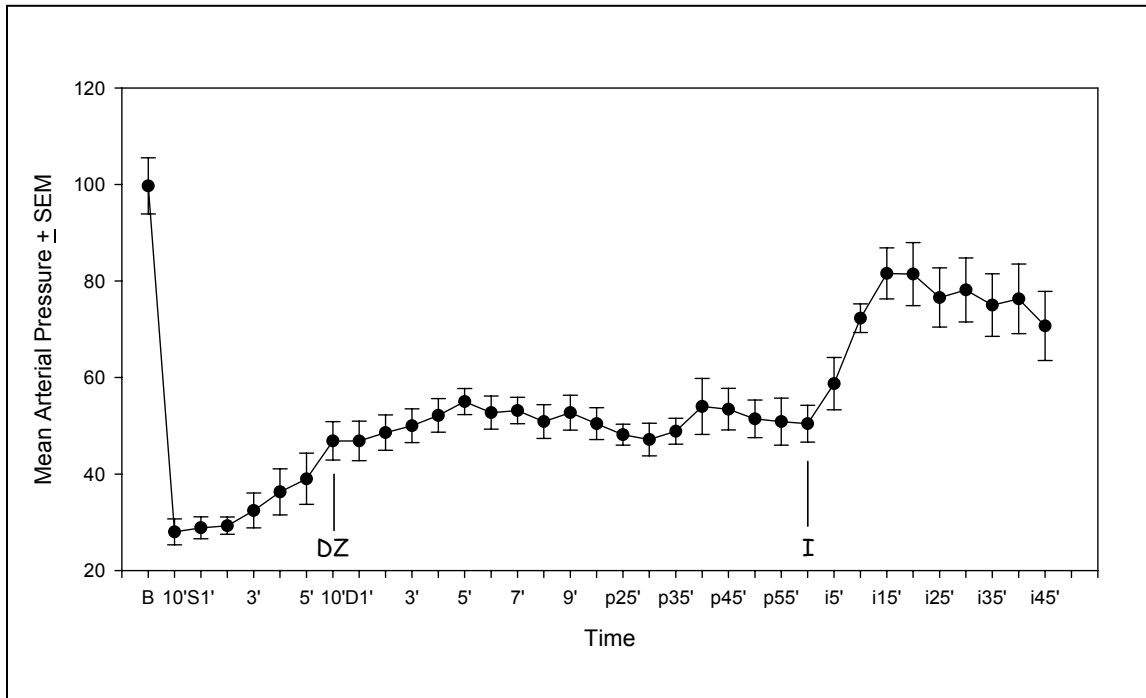


Figure 1. Experiment 3 results of mean arterial blood pressure (MAP) after combined Stroke and Shock, and intravenous administration of 0.32mg/kg/min DZ (dose 3.2mg/kg infused over 10 minutes). Infusion of DZ began 10 minutes after the end of the 10-minute exsanguination period. Rats (n=7) that sustained combined Stroke + Shock, exhibited no significant fall in MAP during DZ infusion or over the entire shock period, which was terminated by saline infusion, 60 minutes after shock. **DZ** on the graph indicates when the animals began infusion of diazoxide, over the next 10 minutes, and **I** indicates the time when resuscitation (saline infusion) was initiated. (Abcissa scale: B, baseline; 10', 10 minute exsanguination period; S1'-S10', shock period of 10 minutes; D1'-D10', diazoxide infusion over 10 minutes; p25'-p60', post shock period; i5'-i45', infusion of normal saline over 45 minutes)

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CHAPTER 3

DIAZOXIDE PRECONDITIONING INCREASES HSP25 AND HSP70 IN LIVER AND KIDNEY AFTER COMBINED HEMORRHAGIC SHOCK AND CEREBRAL STROKE

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ABSTRACT

Our team tested the hypothesis that pharmacological induction of ischemic preconditioning can offer cytoprotection and preserve vital tissues after hemorrhagic shock and stroke. The compound, diazoxide (DZ), is known to induce preconditioning through its effect as a mitochondrial K_{ATP} channel opener and succinate dehydrogenase inhibitor. When DZ was administered 24 hours prior to shock, it significantly reduced hyperglycemia, which in vehicle-treated animals persisted after resuscitation. DZ also attenuated hyperlactatemia during the 1 hour shock period. With more severe trauma from combined shock and stroke, DZ also decreased hyperlactatemia and hyperglycemia levels but the reduction was only significant for hyperglycemia. DZ also seems to increase the survival rate of animals that sustained combined stroke and shock, but not significantly ($p=.058$). The expression levels of heat shock proteins 25 (HSP25) and 70 (HSP70) were used as biomarkers for response of the kidney and liver to DZ and combined stroke and shock. Compared to vehicle-treated animals, DZ-treated rats subjected to shock and stroke exhibited increased HSP25 and HSP70 in kidney and liver tissue. Taken together, these results suggest DZ attenuates physiological indicators of metabolic stress following shock or combined shock and stroke, that it may increase

survivability, and that it enhances the upregulation of cytoprotective heat shock protein expression.

KEY WORDS

Mitochondria, Ischemic Preconditioning, Ischemia, Lactate, Glucose, Survival

INTRODUCTION

Ischemic preconditioning was first described in a 1986 landmark study by Murry and colleagues (1). In this canine cardiac model, the circumflex artery of the control group was clamped for 40 minutes. The experimental group experienced 5 minutes of circumflex artery clamping, followed by 5 minutes of reperfusion. This was repeated three times followed by circumflex artery occlusion for 40 minutes. Compared to the control group, the hearts of dogs from the experimental group exhibited a stunning 75% reduction of infarct area. Except for coronary artery bypass and graft surgery, this was the first proven method to limit myocardial infarction and cellular damage. Other investigations quickly followed and reported that this phenomenon occurs in brain, liver, skeletal muscle and across species (2). It has been hypothesized that the mild chest pain felt before a major cardiac infarction is an ischemic preconditioning phenomenon that could help increase survival or limit muscle damage. In fact, those patients that experienced angina before a myocardial infarction had an increased chance of survival compared to those patients that did not experience angina prior to their heart attack (3).

Studies on the proposed mechanism(s) of ischemic preconditioning remain controversial, but research indicates that adenosine, bradykinin, opioids, catecholamines and oxygen radicals activate phospholipase C or D via G-protein coupled receptors. Phospholipase C/D activates protein kinase C (PKC), which leads to a complex kinase cascade that ultimately ends in the opening of mitochondrial potassium ATP channels (mK_{ATP}) (2). The opening of mK_{ATP} channels appears to be involved near the end of the pathway, although further research is required to determine how the influx of K^+ ions into the mitochondrial matrix through the K_{ATP} channel achieves preconditioning.

Research has demonstrated that there are two phases of preconditioning; classical and delayed. Classical preconditioning occurs within minutes after a brief triggering stimulus, and lasts for several hours. It involves the direct modulation of energy supplies by profound uncoupling of the mitochondria, Na^+ and Ca^{2+} homeostasis that reduces Ca^{2+} uptake into the mitochondria, and pH regulation. Classical preconditioning, the phase originally described by Murry *et al.*, has been used successfully in clinical trials including cardiac (4) and liver tissue (5) surgeries. In these situations, the target organ is cycled between clamped and unclamped arterial flow, which allows better survival of the target organ when cessation of blood flow is necessary for surgery or transplantation.

Although less protective than classical preconditioning, delayed preconditioning begins 12-24 hours after the triggering stimulus and persists for 2-3 days. It is assumed that PKC activates NF- κ B and other transcription factors, which leads to an upregulation of protective gene products. Delayed preconditioning leads to heat shock protein

induction, which can reduce the nuclear binding of proinflammatory transcription factors and increase the antioxidant capacity of cells (6). Both of these effects attenuate the inflammatory response in preconditioned livers and kidneys. In liver, some of the signals necessary for preconditioning may include changes in the expression of adenosine, nitric acid, and reactive oxygen species, which in turn activate PKC and p38 mitogen-activated protein kinase (7). Regardless of what pathway(s) are induced, the essential consequences are that preconditioning decreases energy demand of ischemic tissues (8) and increases cell survival after insult.

Diazoxide (DZ; also known as Hyperstat) has previously been used to treat severe hypertension. This mechanism of action involves activation of membrane K_{ATP} channels and hyperpolarization of vascular smooth muscle, resulting in arteriolar vasodilation and decreased peripheral resistance. Through the activation of mK_{ATP} channels in pancreatic β -cells, DZ also decreases insulin secretion leading to increased blood glucose levels. However, further research has shown that DZ, at lower dose levels, activates the preconditioning effect and has been identified as a specific mK_{ATP} channel opener, binding to mK_{ATP} channels 2,000 times more than sarcolemmal K_{ATP} channels (9).

DZ induction of preconditioning may involve several processes. Some work, for example, suggests the ability of DZ to open mK_{ATP} channels is a key event in preconditioning (10). This results in Ca^{2+} stabilization and homeostasis in isolated cardiac mitochondria (11). Thus, the opening of mK_{ATP} channels, and K^+ influx, could prevent mitochondria demise by avoiding Ca^{2+} overload and cell death. Alternatively,

DZ may exert preconditioning effects *independent* of mK_{ATP} channel activation (12).

Three decades ago, for example, DZ was noted to block succinate dehydrogenase activity in liver mitochondria (13), resulting in the inhibition of complex II action in the electron transport respiratory chain. Furthermore, research has shown that it is the inhibition of the respiratory chain and the uncoupling of respiration that tempers Ca²⁺ uptake (14); not the opening of the mK_{ATP} channels located on the inner mitochondria membrane. During ischemia and reperfusion, for example, limited energy availability and elevated Ca²⁺ levels activates a number of enzymes including proteases, phospholipases and calpain. Calpain activation leads to cytochrome c release from the mitochondria, which plays a key role in initiation of the apoptotic pathway (15). Further study may find that the opening of mK_{ATP} channels and inhibition of the mitochondrial respiratory cycle/electron transport chain are complementary effects of preconditioning that facilitate the ultimate survival of compromised cells and organ systems.

Hemorrhagic shock is one of the most serious conditions in trauma medicine. It is the most frequent cause of traumatic death in combat, representing almost 50% of the fatalities of military engagement, and it represents the presenting condition in 30% of the admissions to civilian trauma centers (16). No studies have previously assessed the efficacy of DZ in a delayed preconditioning model of hemorrhagic shock, hereafter referred to as *Shock*. Since shock is often combined with cerebral injury in vehicle accidents, and results in increased fatality, we also examined DZ's effect in animals that sustained both shock and unilateral common carotid occlusion (*Stroke*). We found that DZ pretreatment of animals that sustained shock alone, or in combination with cerebral

stroke, resulted in significant changes in physiological and hematological parameters. In animals that sustained stroke and shock, DZ caused significant increases in heat shock protein 25 and 70 expression in the kidney and liver. These findings suggest that the pharmacological induction of delayed preconditioning offers cytoprotective and energy protective effects that may be relevant to therapeutic care in trauma medicine.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (275-350 grams, n=68) were purchased from Charles River Laboratories (Wilmington, MA). Rats were housed with a 12 hour light/dark cycle, 24°C constant temperature, and had unlimited access to water and rat chow. All procedures were approved by the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences.

Drugs

DZ (Sigma, St. Louis) was dissolved in 50µl of 0.5M NaOH, and then diluted with normal saline to a final concentration of 2.5mg/ml. In all cases DZ (5mg/kg) was given intraperitoneally (IP) 24 hours before hemorrhagic shock. Isoflurane (Abbott Labs, Chicago) with air was utilized as the anesthetic agent during surgery, titrated to respiration rate. Lidocaine 2% (Phoenix, St. Louis) was infiltrated into incision areas to reduce anesthetic requirements. Buprenex (Reckitt Benckiser Pharm, Richmond, VA) was given subcutaneously for post-surgical discomfort. For euthanization, the rats

received a combination of 80mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) with xylazine (Phoenix, St. Louis) 10mg/kg IP prior to harvesting tissue.

Experimental Groups

Two experimental treatment regimes were employed; hemorrhagic shock alone (*Shock Study*) and hemorrhagic shock combined with stroke (unilateral common carotid artery occlusion; *Stroke + Shock Study*). In both studies there were five treatment conditions (see Table 1). In the *Shock Study*, Group 1 received only anesthesia and hematological and physiological monitoring. Group 2 rats received anesthesia, vascular cannulation, and resuscitation by an infusion of normal saline. No differences were observed between Groups 1 and 2, which hereafter are combined and described as the Vehicle Sham (Veh Sham) Group. Group 3 (Veh Shock) received anesthesia, shock, monitoring, and resuscitation. Groups 1-3 received a saline injection (Veh) 24 hours prior to further experimentation. Group 4 rats received an injection of DZ (5mg/kg IP), and the next day these rats received anesthesia and monitoring, but no shock or resuscitation by infusion of saline (DZ Sham Group). Group 5 rats (DZ Shock Group) received the same treatments as Group 3, except this group received DZ (5mg/kg IP) 24 hours prior to surgery instead of vehicle.

The *Stroke + Shock Study* was comprised of five treatment groups (see Table 1). Group 1 (Control) rats did not receive any surgical procedure and were euthanized immediately. Group 2 (Veh Str) rats received an injection of normal saline vehicle the

day before they were anesthetized, sustained a permanent stroke (Str) by right carotid ligation, vascular cannulation for monitoring, but no shock. Group 3 (Veh Str+Sh) rats also received a saline vehicle (Veh) injection 24 hours before anesthesia, right carotid artery ligation (Str), and then sustained shock (Sh) followed by resuscitation. Group 4 (DZ Str) rats received DZ 24 hours before carotid ligation stroke (Str) but no hemorrhagic shock. Group 5 (DZ Str+Sh) rats received DZ 24 hours before anesthesia, right carotid ligation (Str), and hemorrhagic shock (Sh).

Protocol

All rats (except Group 1 in the Stroke + Shock Study) were anesthetized in an induction chamber with 3-5% isoflurane (IsoFlo, Abbott Laboratories) until unconscious (15-60 seconds). To ensure a constant plane of anesthesia throughout the experiments, isoflurane anesthesia was maintained via a rodent nose cone with a scavenging system. Euthermia was maintained with a heat therapy isothermal pad (Gaymar T/Pump model TP-500). A surgical cutdown was performed to the right groin for isolation and cannulation of the femoral vein and artery. Using aseptic technique, a polyethylene catheter (PE-50, Clay Adams, Piscataway, NJ) was inserted; guided with the aid of a surgical optical scope. The arterial catheter was then connected to a pressure transducer and computerized physiographic system (Ponemah Physiology Platform, Gould Instrument Systems, Valley View, OH) for continuous blood pressure and heart rate recordings. For the Stroke + Shock Study, a small midline neck incision was made to expose the right common carotid artery. The vagus nerve was carefully separated from

the artery, and a sterile suture was tied around the artery, proximal to its bifurcation into external and internal branches.

Table 2 summarizes the experimental protocol. After establishing baseline vital signs, a rapid hemorrhage was induced over 10 minutes. The exsanguination was defined as a loss of 27cc/kg of blood; ~40% of total blood volume based on body weight, less 1.2 cc needed for the four blood draws that were sampled over the duration of the experiment. Beginning 60 minutes after the conclusion of rapid hemorrhage, resuscitation commenced and continued over the next 45 minutes, by intravenous infusion of normal saline (81cc/kg) through the right femoral vein catheter. The catheters were then removed, the femoral artery and vein ligated, the operative site repaired, and the animals were allowed to recover. After regaining consciousness and receiving analgesia (buprenex, 0.03 mg/kg SC), the rodents are returned to their home cage for 24 hours with free access to food and water. During the procedure four arterial blood samples were obtained; at baseline before hemorrhage, at the completion of exsanguination, at the end of the 60-minute shock period and at the conclusion of resuscitation. Blood samples were then analyzed on a Stat Profile 2 Blood Gas and Electrolyte Analyzer (Nova Biochemical, Waltham, MA).

Western Blot Analysis

Samples from the liver and kidney from each animal of the Stroke + Shock Study were homogenized by sonication in lysis buffer (10X PBS-50cc, 10% SDS-5cc, NP-40 5cc, sodium deoxycholic acid-2.5grams and ddwater to 500cc) with protease inhibitor

(Complete Mini, Roche Diagnostics, Mannheim, Germany). Lysates were centrifuged at 12000 for 20 minutes at 4°C. Total protein concentration was determined in each sample by measuring the ultraviolet absorbance at a wavelength of 595nm by comparison to a standard curve using known protein concentration samples (Bovine Serum Albumin). Thirty or forty micrograms of total protein from each sample in loading buffer was boiled for 5 minutes, then loaded on Nu-Page gels (Invitrogen, Carlsbad, CA) and separated electrophoretically. Proteins were transferred to nitrocellulose membranes at room temperature overnight. Membranes were blocked with 5% dry milk, and incubated with 1:2000 concentration of monoclonal HSP70 (Stressgen, Ann Arbor, MI) or 1:10000 concentration of polyclonal HSP25 (Stressgen, Ann Arbor, MI) overnight at 4°C on a shaker table. Membranes were washed in washing buffer, and then incubated with the appropriate alkaline phosphatase-conjugated secondary antibody (1:5000 for HSP70, and 1:10000 for HSP25) for 1.5 hours at room temperature. The membranes were then washed and incubated with chemiluminescent detection reagents (Pierce, SuperSignal West Femto Maximum Sensitivity Substrate) for 5 minutes, visualized with a Fuji Film LAS-1000 camera, and quantified with Fuji Film LAS-1000plus software. Relative protein levels were determined from the optical densities of the corresponding protein bands after subtraction of background values obtained from the same lane. All protein samples were standardized with β -actin for each membrane.

Statistical Analysis

Results for the physiological and hematological data were summarized using a Treatment x Trials design analysis of variance with repeated measures on the second

factor and *post hoc* comparisons (SigmaStat, Version 2.03). Preliminary analyses indicated there was no difference in responses for Sham animals (no exsanguination) that had received either vehicle (normal saline) or diazoxide 24 hours before physiological monitoring. To reduce animal number, fewer rats in each treatment condition were used, and the data from these sham-treated animals were pooled. There then were four levels of the treatment factor. The trial factor refers to repeated measures on the same animal for physiologic and hematological measures. For the hematological measurements during shock and resuscitation, there were repeated measures taken at baseline, at the end of exsanguination, at the beginning of resuscitation, and at the end of reperfusion. Blood pressure measurements were taken every 1 min immediately after exsanguination for 5 min and then every 5 min during the remaining periods of shock and resuscitation.

Western blots were performed to measure changes in HSP25 and HSP70 (relative to β -actin levels) in samples of the kidney and liver of rats from the Stroke + Shock Study. The Fuji image analysis program (Fuji Film LAS-1000plus) was used to determine western blot band density for HSP25, HSP70 and for β -actin levels in each sample. All western blot membranes were prepared with five samples each from two groups; the Control Group (Group 1), and then samples from an experimental group (Groups 2, 3, 4 or 5). In cases where a protein sample from the same rat was used on both blots, the mean band density for this animal was computed from the two measures. Comparisons between membranes were performed by standardizing all membranes to the mean band intensity of the Control samples, where a single factor analysis of variance and *post hoc* comparisons were performed. Preliminary inspection indicated there were

samples that resulted in apparently excessively high density scores. To determine whether these values were outliers, the mean and standard deviation were computed, and the data for each data set was converted to z-scores. Values that exceeded 2.5 times the standardized values ($z\text{-score} > 2.5$) were considered candidate outliers. This test was reiterated on each sample, after removal of candidate outliers, until no outliers were detected (17).

RESULTS

Comparison of Physiological Responses to Shock Alone and Combined Stroke and Shock

Exsanguination resulted in a precipitative fall in mean arterial pressure (MAP) in the Shock (Figure 1, left histogram, Veh Shock and DZ Shock groups) and the Stroke + Shock (right histogram, Veh Str+Sh and DZ Str+Sh) animals. In both experimental models, MAP was approximately 20mm/Hg at the end of the exsanguination period (10' time points in Figure 1), but levels in bled animals slowly increased as shock continued. A difference between the Shock and Stroke + Shock experiments was seen, where cerebral stroke impaired compensatory mechanisms. Over time, the animals that had sustained shock (left graph) slowly compensated to a blood pressure of approximately 60mm/Hg. However, in the animals that sustained both shock and stroke (right graph), MAP increased to only ~40mm/Hg and achieved 60mm/Hg only after resuscitation by saline infusion. Figure 2 directly compares changes in MAP during the 60 minute shock period. By 40 minutes after exsanguination, animals that sustained Stroke + Shock

exhibited statistically lower MAPs compared to animals that received shock alone. In the Shock Study and the Stroke + Shock Study, no significant differences were noted in MAP between the sham groups that received saline infusions (Veh) and those that received DZ.

Blood Lactate Levels

Plasma lactate levels were measured at four time points during the study; at Baseline (before exsanguination, 0' preH in Figure 3), 10 min after exsanguination (10' postH), just before resuscitation (70' preI), and at the conclusion of resuscitation (115' postI). As shown in Figure 3 (left), in the Shock Study, plasma lactate levels in the shocked animals (Veh Shock and DZ Shock Groups) were elevated to levels that were higher than the Veh Sham and DZ Sham Groups. However, lactate levels were significantly less in the DZ Shock Group than in the Veh Shock Group. At the conclusion of the hemorrhagic shock period, lactate levels only remained elevated in the shocked animals that had not received DZ (Veh Shock Group). Following resuscitation, (115' postI time point), lactate levels in all of the four treatment groups were no longer different.

In the Stroke + Shock Study (right histogram), plasma lactate levels at the end of exsanguination (10' postH) and at the end of the shock period before resuscitation (70' preI), in both groups of rats that had sustained stroke (Str) and shock (Sh), were significantly greater than the animals that had sustained carotid artery occlusion, but no

exsanguination (Veh Str and DZ Str Groups), and were no different whether the animals had received Veh or DZ injections.

Blood Glucose Levels

In the Shock Study, plasma glucose levels in the shocked animals (Veh Shock and DZ Shock Groups in Figure 4, left histogram) were elevated to levels that were higher than the Veh Sham Group (the 10' postH time); although at this time the shocked groups were not significantly greater than the DZ Sham Group. At the conclusion of the hemorrhagic shock period, however, both groups that received hemorrhagic shock had levels of plasma glucose that were significantly greater than the Sham Groups (70' preI time). Following resuscitation (115' postI time), levels of glucose remained significantly greater for the Veh Shock animals compared to all other groups (at time 115' postI), while glucose levels in the animals that had received DZ were not different from levels in the Sham animals.

In the Stroke + Shock Study (Figure 4, right histogram), plasma glucose levels at the conclusion of the shock period (70' preI time) were significantly greater in both the Veh Str+Sh and DZ Str+Sh Groups, compared to the animals that sustained right carotid occlusion (Veh Str and DZ Str Groups), but no exsanguination. However, at the conclusion of resuscitation glucose levels were still elevated only in the Veh Str+Sh Group, compared to the Veh Str Group (time 115' postI), while the DZ Str+Sh animals at

this time no longer differed from the Veh Str and DZ Str groups. The plasma glucose levels at this time for the Veh Str+Sh Group also were greater than levels for this group at baseline (0' preH).

Heat Shock Protein 25 and Heat Shock Protein 70 in the Kidney

Quantitative western blotting was used to measure HSP25 and HSP70 protein levels following sham treatment, stroke (by carotid artery occlusion) and combined stroke and hemorrhagic shock. In the kidney, there was a dramatic increase in levels of HSP25 in rats that had received DZ treatment prior to sustaining stroke and shock (Figure 5, left histogram and Figure 6, left side). HSP70 levels (Figure 5, right histogram and Figure 6, left side), however, exhibited a different pattern, where DZ administration to animals that sustained the combined treatment (DZ Str+Sh) had significantly higher levels of HSP70, compared to sham animals and rats that had received vehicle or DZ and carotid occlusion alone (Veh Str and DZ Str bars). Animals that had received vehicle and the combined treatment (Veh Str+Sh) exhibited an intermediate increase in HSP70. That is, the animals that received vehicle before combined stroke and shock had elevated levels of HSP70, which were not significantly less than levels in the animals that sustained the same trauma, but that were pretreated with DZ, although the elevated levels in the kidneys of vehicle-treated animals was only significantly elevated when compared to the vehicle-pretreated animals that sustained stroke alone (Veh Str+Sh vs. Veh Str).

Heat Shock Protein 25 and Heat Shock Protein 70 in the Liver

In the liver, pretreatment with DZ elevated levels of HSP25 in animals that received stroke alone (Figure 7, left histogram, DZ Str Group) or combined Stroke + Shock (DZ Str+Sh Group, Figure 6, right side). Levels of HSP25 were the highest in animals that received DZ and sustained the combined trauma, while DZ and stroke alone treatment resulted in an intermediate level of HSP25 levels (levels of HSP25 were significantly greater in the DZ Str Group compared to the Veh Str Group). Measurement of changes in HSP70 in the liver (Figure 7, right histogram) shows a similar pattern of expression except the levels of HSP70 in the liver samples from the DZ Str group did not significantly change, although an increase in HSP70 expression is discernable. However, HSP70 expression in the DZ Str+Sh group is significantly larger than all other groups (Figure 6, right side).

Survival Following Hemorrhagic Shock or Combined Stroke and Shock

No animals in the Shock Study died as a result of sham surgery or hemorrhage (data not shown). In the Stroke + Shock Study, however, comparisons between groups of animals that had or had not sustained hemorrhagic shock and carotid ligation exhibited significant differences in fatality ($X^2(3) = 11.82, p < 0.01$). While there were no deaths of rats that were treated with either Vehicle or DZ, followed by Stroke without Shock (Table 3; the Veh Str and DZ Str Groups), vehicle (normal saline administration) pretreatment before combined Stroke + Shock resulted in ~33% deaths within 24 hours (Veh Str+Sh Group). However, when rats were pretreated with DZ, there was over a four-fold reduction in percent of deaths to ~7% (DZ Str+Sh Group). A one-tailed Fisher

Exact Test indicated the difference in number of deaths between the animals that received vehicle or DZ, and combined stroke and shock, almost reaches statistical significance ($p=0.058$).

DISCUSSION

Stroke + Hemorrhagic Shock Model

Delayed preconditioning was pharmacologically induced by the administration of the mK_{ATP} channel opener, diazoxide. During the 1 hour shock period, DZ pretreatment was found to attenuate hyperlactatemia that is ordinarily sustained by exsanguinated rats. In animals that sustained hemorrhagic shock, DZ also significantly reduced hyperglycemia, even after resuscitation, which in vehicle-treated animals remained elevated. With more severe trauma from combined stroke and shock, DZ also decreased hyperlactatemia and hyperglycemia levels, but the reduction was only significant for hyperglycemia.

Anatomically, the rat circle of Willis in the cranium is as patent as in humans, and this property provides an effective compensatory circuit for ipsilateral or bilateral cerebral occlusions. A study utilizing a bilateral carotid ligation model did not exhibit ischemia unless the mean arterial blood pressure was decreased below 50mm/Hg (18). Similarly, unilateral carotid occlusion often has no ischemic effect given patency of the remaining cerebral vascular supply. When carotid artery occlusion was present during

hemorrhagic shock, the apparent combined effects of these injuries overwhelms the compensatory vascular supply in the circle of Willis and increases lethality through ipsilateral central nervous system ischemia. The increased severity is perhaps not surprising. Analysis of multi-center clinical databases in the United Kingdom, for example, has shown a doubling of the risk of death when moderate brain injury is combined with hemorrhagic shock and/or tissue injury (19). Nevertheless, the chosen dose and timing for preconditioning with diazoxide may not have been optimal for effecting stark differences in lactic acid and glycemic response to combined injury.

Mean Arterial Blood Pressure

While mean arterial pressure levels were observed to be essentially equivalent between the DZ and Vehicle Shock groups with either model during shock or resuscitation—and arterial pressures in non-shocked animals that had been given saline or DZ—a difference between compensatory mechanisms based on the two injury models was evident. It is probable that the normal sympathetic response to hemorrhagic shock (increased sympathetic vascular tone) and the ensuing increase in blood pressure as a compensatory mechanism is attenuated by the ipsilateral carotid ligation combined with hypovolemia, anemia and hypoxia. Other research has confirmed a decrease in vascular tone during compensation from hemorrhagic shock in brain injured rats, which require much larger volumes of intravenous fluids to maintain adequate blood pressures (20). This was aptly demonstrated in our experiments by the MAP slowly rising to approximately 60mm/Hg in the Shock Study before resuscitation, while animals that had sustained stroke and shock exhibited a statistically significant lower level of pressure

(~40 mm/Hg) just prior to resuscitation (Figure 2). In this case, fluid resuscitation was an apparent necessary condition to bring the Stroke + Shock rats to 60mm/Hg.

Hyperlactatemia

Other studies have likewise found that more severe injuries, such as trauma in combination with cerebral injury or ischemia, results in poorer outcome, often preceded by elevated plasma lactic acid and glucose levels (21). In the first study, we administered DZ 24 hours prior to shock (no stroke) and observed a significant difference in lactate levels, compared to vehicle-treated rats, during the 1 hour shock period. These results appear to be in line with previous reports. One hemorrhagic shock study, which employed delayed preconditioning (24 hours) but utilized arterial clamps instead of a preconditioning pharmacological agent, showed that both intestinal lactate levels and lung edema were reduced with delayed preconditioning (22). Furthermore, a recent study demonstrated that DZ classical preconditioning reduced lactate levels in a perfused rat heart preparation (23).

Hyperlactatemia can occur from either an increase in production or an underutilization of lactate. In a study of critically ill ICU patients, it was determined that lactic levels at or above 5mmol/L resulted in only 17% of the patients being discharged from the hospital, with only 21% leaving the ICU (24). Lactate production and utilization is a normal process that occurs in almost all tissues and is regulated by the cytosolic

enzyme lactate dehydrogenase. Increased lactate production is the result of anaerobic metabolism of pyruvate. This process requires NADH, which produces a hydrogen ion and NAD^+ . Under hypoxic conditions, there is a shift in the equation: $\text{H}^+ + \text{NADH} + \text{pyruvate} \leftrightarrow \text{NAD}^+ + \text{lactate}$, to the right. The increased NAD^+ manufactured from this process is used in glycolysis to produce ATP. Once produced, lactate can only be re-oxidized to pyruvate or used by tissues for gluconeogenesis. Hypoxic states normally lead to anaerobic metabolism with an increase in lactate glyconeogenesis. Therefore, the cell can maintain (diminished) energy production during anaerobic environment, but at the expense of producing a lactic acidosis.

DZ has a dramatic effect on the mitochondria via succinate dehydrogenase inhibition and mK_{ATP} channel opening with the result that the citric acid cycle and respiratory cycle are partially inhibited (12). It is assumed that through these mechanisms that ATP production is “stunned,” which saves ADP-ATP molecules for ischemic or post-ischemic insult; resulting in better cell and tissue survival. Succinate dehydrogenase inhibition may prevent a shift to the right in the equation, resulting in a decrease in NAD^+ production with the ensuing hyperlactatemia. During hemorrhagic shock, cerebral and skeletal muscle increase the production of lactate and lactate excretion is diminished as blood is shunted away from the kidney and liver, which diminishes lactate excretion. Increased production and retention leads to acute hyperlactatemia. DZ is assumed to decrease the production side to this formula, leading to reduced levels of hyperlactatemia.

In the Stroke + Shock Study, exsanguination dramatically increased lactate levels, regardless of whether the rats had received vehicle or DZ. Importantly, however, we observed a trend that was consistent with the results of the Shock Study. There was a noticeable, consistent difference between the Veh Stroke + Shock and the DZ Stroke + Shock Groups in lactate levels during shock and resuscitation, even though the difference was not statistically significant. This lack of statistical significance could be due to three reasons. First, the severity of lactate accumulation from combined stroke and shock may be so overwhelming that it is unaffected by DZ administration, such that one cannot demonstrate a difference. Second, given the nature of the model, the dose of DZ (5mg/kg IP) may be inadequate and require a higher dose or more frequent dosing to affect lactate levels. Lastly, a significant difference in lactate levels between the DZ Stroke and the DZ Stroke + Shock Groups may have been evident if the sample sizes had been larger.

Hyperglycemia

As an osmotic agent and substrate for energy metabolism, an alteration in plasma glucose levels can serve as an adaptive physiological response to hemorrhagic shock. One of the immediate roles of hyperglycemia in hypovolemic shock, for example, is to act as an inert solute that increases plasma osmolality and draws fluid into the vascular compartment (25). However, despite this positive effect, early hyperglycemia (over 200mg/dl) is an independent indicator of infection and mortality independent of injury characteristics (26), and there is a positive correlation between plasma glucose levels and 30 day mortality in patients receiving coronary artery bypass grafting (27).

Compared to coupled oxidative phosphorylated states, research has shown a significantly lower glucose concentration in hemorrhagic shocked animals with uncoupled mitochondria (28). DZ, with its effect on mK_{ATP} channels and/or succinate dehydrogenase inhibition, and possibly some other sulfonylurea-related receptor pathways, uncouples mitochondria phosphorylation and decreases hyperglycemia in shock. Our study did not determine if the decrease in hyperglycemia is related to decreased production of glucose or more utilization of glucose due to the uncoupling of oxidative phosphorylation. However, a hint may be offered from an experiment with perfused rat hearts, which utilized DZ in a classical preconditioning model. This study demonstrated decreased myocardial glycogen depletion, compared to untreated hearts, which suggests decreased production of glucose may occur as a result of DZ preconditioning (23).

Heat Shock Proteins

Heat shock proteins (HSP), are divided into two major categories based upon molecular weight (HSP10, 25, 60, 70, and 90) and the requirement for ATP (larger HSPs require ATP, smaller HSPs do not). In general, HSPs are a set of highly conserved inducible proteins associated with a positive survival response to cellular stress and injury. Heat shock proteins prevent protein denaturation and incorrect polypeptide aggregation during exposure to cellular insults, they act as molecular chaperones that fold nascent peptides, eliminate damaged or misfolded proteins from the cytoplasm, stabilize

cytoskeleton, and inhibit apoptotic (programmed cell death) pathways (29). In our study we examined the effect of DZ on liver and kidney expression of heat shock proteins 25 and 70 in a delayed preconditioning model.

In the clinical setting, acute traumatic insults from hemorrhagic shock are often successfully treated by emergency first responders. Days later, however, patients can expire due to sepsis, end-organ failure, or both, such that secondary sequelae remain a leading cause of post-injury death (16). The pathogenesis of organ failure remains elusive despite decades of investigation. Liver and/or kidney failure is often the cause of death in these patients as these organs are often affected by the development of refractory tissue hypoxia. In the present study, we measured changes in liver and kidney protein levels of heat shock protein 25 and 70. The selection of these as biomarkers was based in part on previous studies, which demonstrated that delayed preconditioning elevates levels of these key anti-apoptotic proteins, and that elevated levels are associated with increased cell survival (30).

Heat Shock Protein 70

Located in the cytoplasm or mitochondria, HSP70 is expressed constitutively. It is inducibly expressed by cells during cellular hyperthermia and ischemic episodes. HSP70 is an ATP-dependent key protein required for translocation of mitochondrial proteins synthesized in the cytosol into the mitochondria, where it can act to interfere with oxidative stress and block apoptosis (31). Increased HSP70 production leads to increased survival of cells due to inhibition of the construction of the apoptosome (a

caspase-dependent apoptotic activator. which includes the proteins apaf-1, procaspase 9 and cytochrome c) by blocking apaf-1 (32).

HSP70 also blocks a caspase-*independent* pathway. Apoptosis inducing factor (AIF), which is released from the mitochondria during severe cell stress, is regulated by the Bcl-2 family of proteins and migrates to the nucleus to initiate DNA cleavage (33). AIF is directly bound to HSP70 preventing its migration to the nuclear compartment. Other studies have shown that HSP70 can inhibit cell death by inhibiting the opening of mitochondrial permeability transition pores (34), and has been shown to bind and inhibit the pro-apoptotic protein p53 (35). In a study of sixty-seven patients with severe trauma, all patients with high serum levels of HSP72 survived, whereas a significant number of patients with low HSP72 died from their injuries (36).

Heat Shock Protein 25

HSP25, a non-ATP dependent stress protein, also functions as a key anti-apoptotic protein. This protein has been shown to enhance the degradation of ubiquitinated proteins by the 26S proteasome (35), directly inhibit caspase 3 (the executioner of apoptosis), and directly inhibit cytochrome c (which forms part of the apoptosome and activates caspase 3) (35). HSP25 has also been noted to inhibit the PKC- δ isozyme, which has been associated with apoptosis and been shown to inhibit cell cycle progression (37). Finally, the overexpression of HSP25 results in stronger stability of the actin cytoskeleton and leads to faster recovery after disruption; all further contributions to cell survival (38).

Liver and kidney were chosen as the primary organs to examine for HSP25 and 70 expression due to the common cellular and end-organ failure which occurs after severe hemorrhagic insult. Blood flow during hemorrhagic shock is shunted away from these organs to ensure survival of the heart, lungs and brain, but this leads later to kidney and liver failure. Analysis of the changes in HSP25 and HSP70 in animals that sustained combined stroke and shock would permit us to assess the impact of DZ administration in these two organs that undergo profound stress during trauma.

Kidney

As expected, there was minimal constitutive expression of HSP25 and HSP70 in the kidney samples from animals that underwent sham procedures. Analysis of the effect of DZ on HSP25 regulation in this model in the rat kidney (Figure 5) shows a slightly different profile from the HSP25 expression in the liver. Similar to the HSP25 expression in the liver in the DZ Str+Sh Group, there is a substantial increase in HSP25 expression in the kidney in the same group. However, the kidney in this model did not show a concurrent increase in HSP25 expression in the DZ Str Group compared to the Veh Str Group. It is assumed that certain cells in the kidney are not as sensitive as compared to the liver to the effects of stroke without shock in HSP25 expression. The possible effect of centrally mediated sympathetic vascular constriction due to stroke on such a vascular organ as the liver leads to significant upregulation of HSP25 with DZ, which was not seen in the kidney.

HSP70 upregulation in the kidney was the greatest in the DZ Str+Sh group, further verifying the dramatic effect that DZ has on upregulating these anti-apoptotic proteins. However, the temporal expression of HSP25 and HSP70 differ as was shown by a gene chip assay analysis performed by Raghavendra *et al.* following traumatic brain injury (43). HSP70 expression seems to peak at approximately 9 hours post-trauma whereas HSP25 is still increasing at 24 hours post-trauma. In our model it appears that HSP70 has been intermediately expressed in the Veh Str+Sh Group due to the hypoxic effect of shock. If we had chosen an earlier time point we may have observed greater expression of HSP70 in this group. DZ appears to ramp up and maintain a higher level of HSP70 expression in our stroke and shock model at 24 hours post-trauma as compared to the Veh Str+Sh Group.

Liver

There was minimal constitutive expression of HSP25 and HSP70 in the liver samples from animals that did not sustain exsanguination. However, under the condition of sustained stroke and shock DZ had a significant effect upon levels of HSP25 and HSP70 in the liver (Figure 7). In fact, it appears that DZ further significantly upregulates HSP25 in animals that received DZ 24 hours before common carotid occlusion. Literature shows that the upregulation of HSP25 in the liver can be a crucial determinant of survival (39) and HSP70 upregulation offers beneficial effects to human liver after sustaining ischemia (40).

The mechanisms for increased HSP expression in the injured liver, as a function of DZ treatment, are not known. However, delayed preconditioning occurs 12-24 hours after the presentation of a preconditioning trigger, and leads to the upregulation of gene protective products, including heat shock proteins (41). Among the important candidates are the heat shock transcription factors (HSFs). HSFs are normally bound to HSPs inhibiting their respective functions. Misfolded proteins from stress, heat or ischemia, dislodge HSFs from HSPs. Cellular stress, or in this case some direct or indirect mechanism of DZ, can cause the trimerization of HSF (primarily HSF1), which then binds to the heat shock element (HSE) on the promoter region of the HSP gene. HSF1 is negatively regulated by binding to HSP's via phosphorylation in a kind of negative feedback loop, which then inhibits further production of HSPs (42).

Increased levels of HSP70 after trauma correlate with increased survival (36). Taken together, it is evident that there is a statistically significant increase in HSP25 and HSP70 expression in those rats that received DZ 24 hours prior to combined stroke and shock. This is the first study performed that shows a direct relationship between early DZ administration, an ischemic event followed by increased stress proteins expression.

Mortality Following Shock and Combined Stroke and Shock

Our model of shock resulted in no mortality; its utility is therefore limited in terms of evaluating the effects of DZ. Combined stroke and shock, however, is evidently a more lethal condition, and suggests that DZ (given in a delayed preconditioning model) improves survivability (Table 3). Although there appears to be a dramatic difference in

survivability (16 survive: 8 dead following Stroke+Shock in Vehicle-treated rats, compared to 14 survive: 1 dead when rats were pretreated with DZ), the Fisher exact test did not indicate a difference in survival ($p=.058$). Examining possible relationships between death and body weight revealed no correlation. Rats that are heavier due to increased fat content, for example, would have sustained proportionally greater blood loss since fat tissue is relatively avascular. We found no relationship between death and higher body weight. Although the association did not reach statistical significance, it would appear the lower death rate can be attributable, at least in part, to DZ.

This work does not address the hypothesis that survivability was a direct result of the higher levels of HSP25 and HSP70 seen in the kidney and liver samples from rats that received DZ, although the upregulation of HSP25 and HSP70 both block cell destructive proteins released from the mitochondria, apoptotic inducing factor (AIF) and cytochrome c, respectively (35). As such, mitochondria function within the cell has been implicated to having a central involvement in apoptotic and cell survival pathways. Therefore, DZ's direct (mK_{ATP} channel opening and succinate dehydrogenase inhibition) as well as indirect effects (upregulation of stress HSP70 proteins and HSP25) leads to the ischemic preconditioning effect of increased cell survival (35).

Diazoxide and HSPs

The two known pathways of the effect of DZ (mK_{ATP} channel opening and succinate dehydrogenase inhibition) center upon the mitochondria, a link has not been established as how these effects can upregulate HSPs. Although the structure of the cell

membrane K_{ATP} channel is well known (SUR regulatory subunit and Kir pore-forming subunit), the structure of the mK_{ATP} channel has been controversial (44, 45). The mK_{ATP} channel for example, has not been cloned, many reports differ concerning whether the Kir subunit is a constituent of the mK_{ATP} channel. Evidence thus far supports the argument that the pore forming subunits of the K_{ATP} channel may differ between the mitochondria and cell membrane (46).

It has been suggested that succinate dehydrogenase is a component of the mK_{ATP} channel. Succinate dehydrogenase, associates with four other proteins including, mitochondrial ATP binding cassette protein (mABC1-same family as SUR), adenine nucleotide translocator (ANT- an ADP and ATP membrane transporter), an inorganic phosphate carrier (PIC-phosphate membrane transporter) and ATP synthase (complex V in cellular respiration) (47). It is unknown if a relationship exists between DZ activation of the mABC1 receptor with its associated proteins including ANT (45), PIC and ATPase in regulating HSP70, but evidence does exist that the function of HSP70 requires an intrinsic ATPase activity that is modulated by nucleotide exchange factors (48). If ATPase activity or some nucleotide exchange factor displaces HSF1 from these heat shock proteins, this would lead to an upregulation of these anti-apoptotic proteins.

Likewise, recent research has demonstrated that the SUR component of the K_{ATP} channel may not exclusively combine with Kir. One report has shown that a SUR1-regulated nonselective cation channel, called the NC Ca_{ATP} channel, is activated by Ca^{2+} and by the depletion of intracellular ATP. It is equally permeable to monovalent cations

such as K^+ , Na^+ , Rb^+ and Li^+ (49). This channel is hypothesized to have a SUR1 regulatory subunit and distinct (yet to be identified) pore forming subunits. DZ has been shown to activate this channel through its SUR1 subunit, but the normal physiological role of the NC Ca_{ATP} channel and its downstream pathway remains to be determined.

Summary

The present work is the first *in vivo* demonstration that DZ holds potential as a clinically efficacious pretreatment to protect organs that sustain shock or combined stroke and shock. Our work indicates that DZ, given 24 hours before shock, decreases hyperglycemia after shock and that hyperlactatemia was decreased in the DZ Str+Sh Group during the 1 hour shock period. DZ was also found to increase HSP25 and HSP70 expression in the liver and kidney. HSP25 and HSP70 are key anti-apoptotic stress proteins that increase cell survival. A possible implication for survivability in the Stroke + Shock model with DZ was not proven, but initial results here suggest DZ was beneficial. Identification of the exact mechanisms responsible for these significant findings could lead to new pharmacological agents that could mimic or enhance DZ's action.

Table 1. Experimental Groups for Shock (HS) and Stroke + Shock Studies	
Shock Study*	
Group	Experimental Treatment
Group 1	Sham (received anesthesia only)
Group 2	Sham + Inf (anesthesia and intravenous infusion of normal saline)
Group 3	Vehicle Shock (Veh Sh) (anesthesia, hemorrhagic shock and intravenous infusion of normal saline as resuscitation)
Group 4	DZ Sham (diazoxide 24 h before anesthesia and intravenous infusion of normal saline)
Group 5	DZ Shock (diazoxide 24 h before anesthesia, shock, and intravenous infusion of normal saline as resuscitation)
Stroke + Shock Study	
Group 1	Control (no anesthesia, surgery, monitoring or infusion of saline)
Group 2	Vehicle Stroke (Veh Str) (anesthesia, monitoring, carotid ligation with no hemorrhagic shock or infusion)
Group 3	Vehicle Stroke and Shock (Veh Str+Sh) (anesthesia, monitoring, hemorrhagic shock, carotid ligation, with saline infusion for resuscitation)
Group 4	DZ Stroke (DZ Str) (diazoxide 24h before anesthesia, carotid ligation with no hemorrhagic shock or infusion)
Group 5	DZ Stroke and Shock (DZ Str+Sh) (diazoxide 24h before anesthesia, carotid ligation, hemorrhagic shock and saline infusion for resuscitation)

*MAP data for Groups 1 and 2 in the Shock Study are pooled, and labeled Veh Sham in Figs. 3 and 4 (left graphs)

Table 2. Hemorrhagic Shock and Resuscitation Protocol				
Time (minutes)	0'	10'	60'	45'
Cumulative Time	0'	10'	70'	115'
Exsanguination	————→			
Hemorrhagic Shock	————→			
Resuscitation			————→	
Hematological Sampling	*	*	*	*
MAP, HR q 5 minutes	*	————→		

*Refers to times when hematological blood samples were obtained.

Table 3. Mortality from Stroke and Shock after Vehicle or Diazoxide (DZ) Pretreatment

Group	Survived	Died	% Deaths
Vehicle-Stroke	12	0	0
Vehicle-Stroke + Shock	16	8	33
DZ- Stroke	12	0	0
DZ-Stroke+Shock	14	1	7

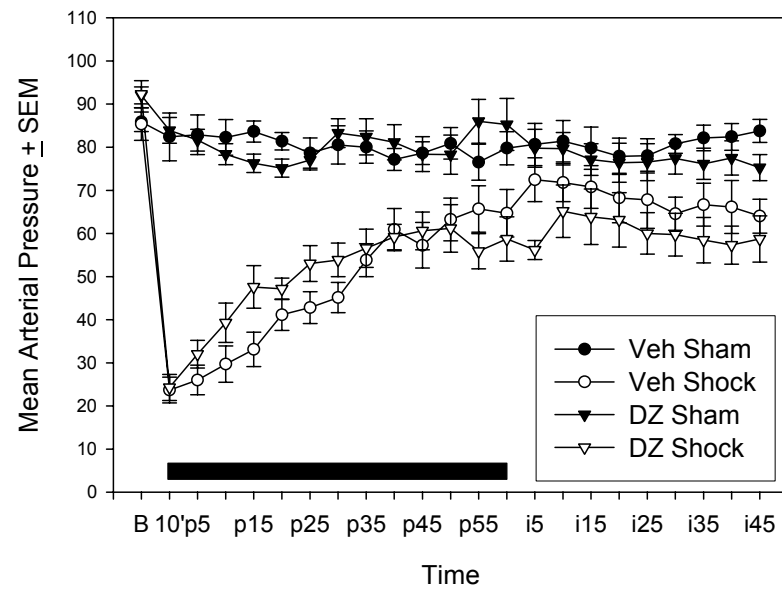
Fig. 1. Mean arterial pressure of Shocked vs. Stroke and Shocked animals.

MAP of animals in the Shock (left) and in the Stroke + Shock Study (right) that had received normal saline (Veh) or diazoxide (DZ) 24 hr prior to surgery.

In the Shock Study, preliminary tests indicated there were no significant differences in MAP at any time point for the sham-treated animals (that were anesthetized and monitored, but were not exsanguinated), whether or not they had received a saline infusion. The data for these groups were therefore collapsed and treated as a single sham group (Veh Sham). At Baseline (B on abscissa), there were no differences in MAP across the groups, and MAP did not change over time for the Veh Sham or DZ Sham groups. As shown in the graph on the left, MAP for rats that had been pre-treated with vehicle (Veh Shock) or with diazoxide (DZ Shock), and were exsanguinated, exhibited significantly lower levels of MAP 10 min after exsanguination and for the next 60 min post-shock (time point p60), compared to the non-exsanguinated groups (illustrated by the black horizontal bar for times 10' to p60). Compared to their MAP at Baseline, levels remained significantly less at all time points after bleeding for the DZ Shock animals, including MAP levels during infusion (i5-i45). For the Veh Shock group, MAP was significantly less at all time points (10', p5-p60, and i25-i45), except during the initial phase of resuscitation (i5-i20). With the exception of MAP measures at i5, at no time was there a difference in MAP between the two exsanguinated groups (Veh Shock and DZ Shock), suggesting that pretreatment with diazoxide had no impact upon MAP at baseline, during recovery (p1-p60), or during resuscitation (i10-i45). In the Stroke + Shock Study

(right graph), there was no difference in MAPs between the four groups at Baseline, and MAP did not change over time for the DZ Stroke (DZ Str) group. For the Vehicle Stroke (Veh Str) group, MAP at times p10 and p15 were the only periods where levels were less than Baseline. MAP for groups of rats that had been pre-treated with vehicle (Veh Str+Sh) or with diazoxide (DZ Str+Sh) and were exsanguinated, however, exhibited significantly lower levels of MAP 10 min after exsanguination and for the next 60 min (time point p60), compared to the non-exsanguinated groups. Compared to their MAP at Baseline, levels remained significantly less at all time points after bleeding for the Veh Str+Sh animals, including MAP levels during infusion (i5-i45). For the DZ Str+Sh group, MAP was significantly less at all time points during shock (10', p5-p60), but not during resuscitation (i5-i45).

Shock Model



Stroke + Shock Model

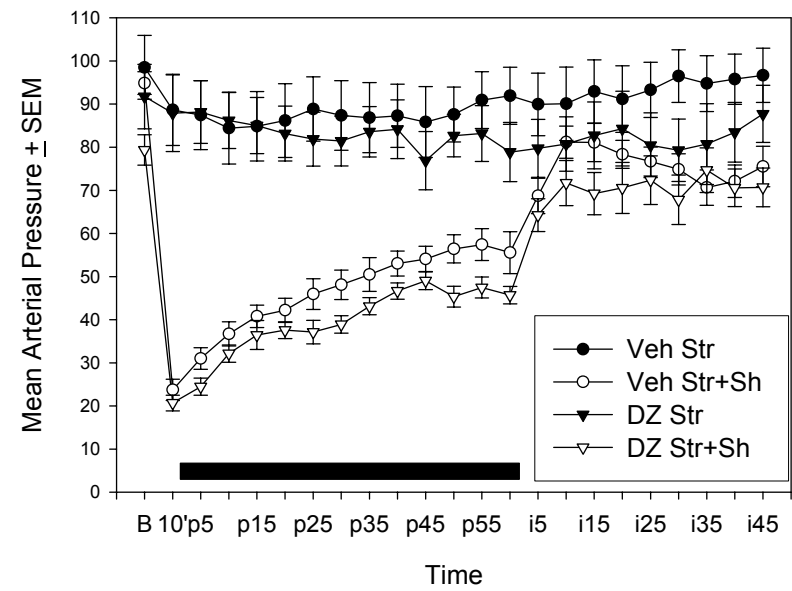


Fig. 2. MAPs for the exsanguinated animals in the Shock and the Stroke + Shock Studies. This figure re-plots the data shown in Figure 1, but only the MAPs during the shock period for the groups of exsanguinated animals are illustrated (data from animals of groups that received either Veh or DZ are pooled). Beginning ~20 minutes (p20) into the shock period, the animals that sustained both stroke and shock (Stroke+Shock line with open circles) begin to exhibit a blunted recovery. Levels of MAP for the Shock Groups (line with filled circles) were significantly greater than the Stroke + Shock Group during the last 25 min of shock (i.e., p40-p60 time points, $*p<0.05$).

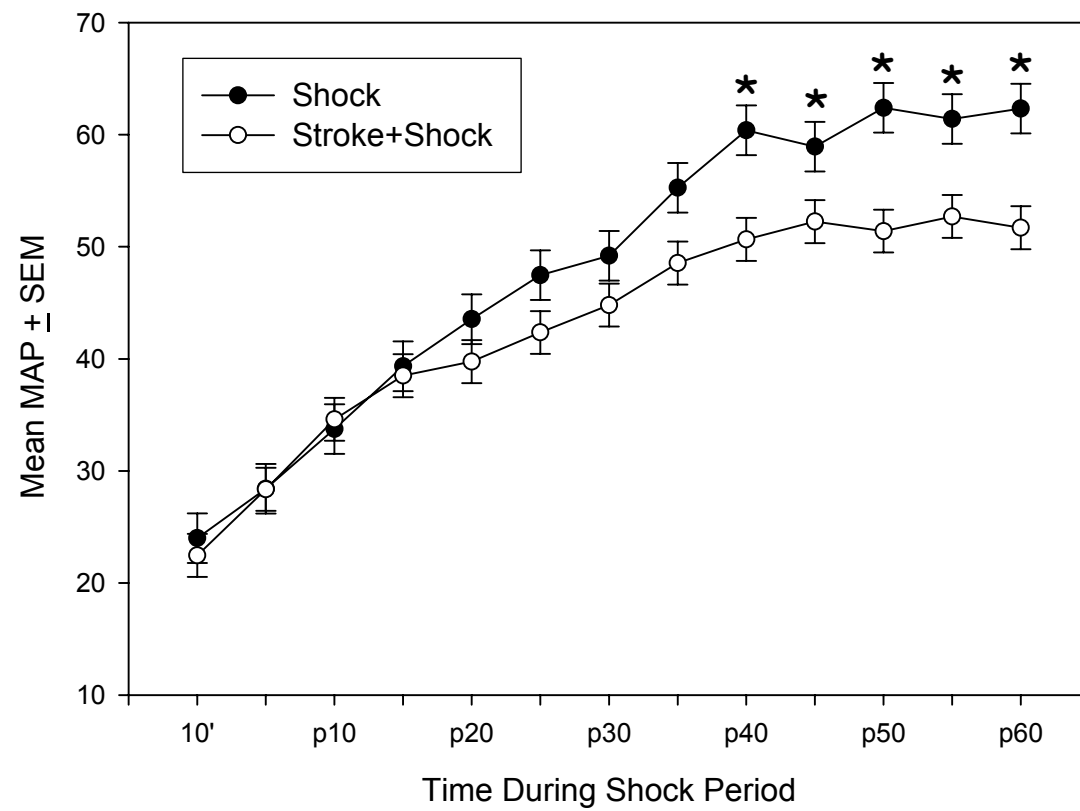
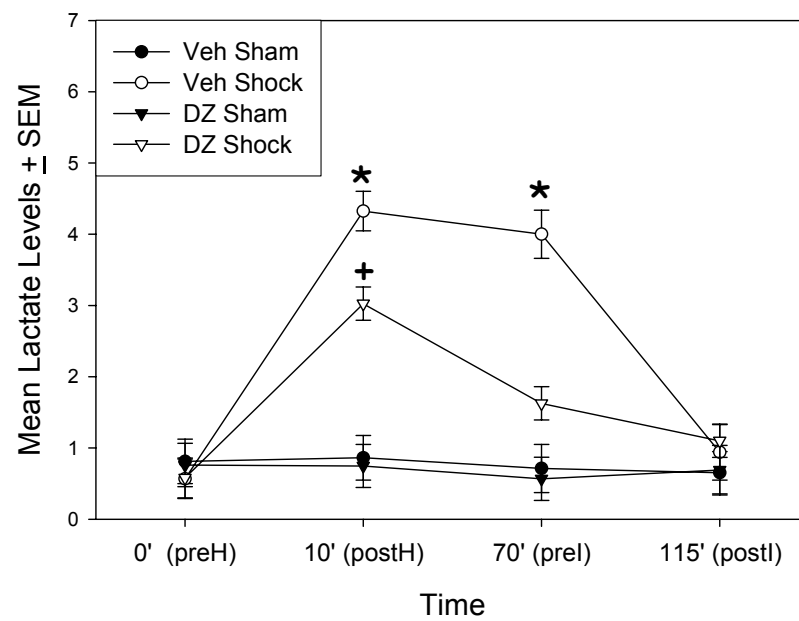


Fig. 3. Plasma lactate levels of Shocked vs. Stroke and Shocked rats. Plasma lactate levels were measured at four time points during the study; at Baseline (before exsanguination, 0' preH, on the abscissa), 10 min after exsanguination (10' postH), just before resuscitation by saline infusion (70' preI), and at the conclusion of resuscitation (115' postI). In the Shock study (left graph), plasma lactate levels in the shocked animals that had received vehicle injection (Veh Shock Group) was elevated to levels that were higher than the Veh Sham Group (the postH time; indicated by * symbol) and was higher than the DZ shock group. Lactate levels in animals of the DZ shock group (+ symbol) were significantly higher than levels in sham (non-exsanguinated) rats, but were also significantly *less* than in the rats of the Veh Shock Group (* symbol). At the conclusion of the hemorrhagic shock period (70' preI), lactate levels only remained elevated in the shocked animals that had not received DZ (Veh Shock Group). Following resuscitation, (115' postI time point), lactate levels in all of the four treatment Groups were no longer different.

In the Stroke+Shock study (right graph), plasma lactate levels at the end of exsanguination (10' postH) and at the end of the shock period before resuscitation (70' preI), for both groups of rats that had sustained Stroke+Shock, were significantly greater than the Sham Groups, and were not different whether the animals had received Veh or DZ injections (^ symbol).

Shock Study



Stroke + Shock Study

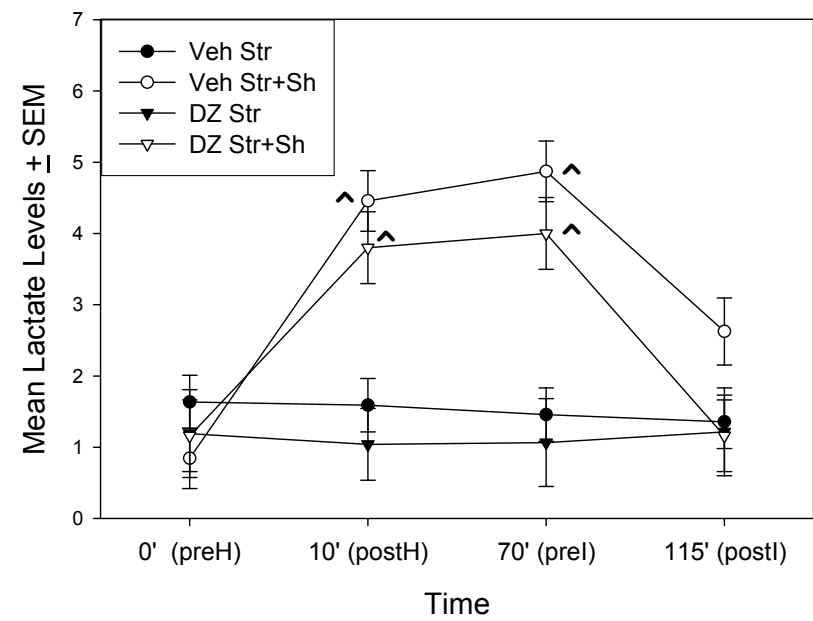


Fig. 4. Plasma glucose levels of Shocked vs. Stroke and Shocked rats. Plasma glucose levels at four time points during hemorrhagic shock (left figure) and during stroke and shock (right figure). In the Shock Study (left), plasma glucose levels in the shocked animals (Veh Shock and DZ Shock Groups) were elevated to levels that were higher than the Veh Sham Group (the 10' postH time; indicated by # symbol), although at this time the shocked groups were not significantly greater than the DZ Sham Group. At the conclusion of the hemorrhagic shock period, both groups that received hemorrhagic shock had levels of plasma glucose that were significantly greater than the Sham Groups (70' preI time; ^ symbol). Following resuscitation (115', postI time) levels of glucose remained significantly greater for the Veh Shock animals compared to all other groups (* symbol at time 115'), while glucose levels in the animals that had received DZ were not different than levels in the Sham animals.

In the Stroke + Shock Study, plasma glucose levels at the conclusion of the shock period (70' preI time, ^ symbol) were significantly greater in both the Veh Str+Sh and DZ Str+Sh Groups, compared to the Sham Groups, while levels at the conclusion of resuscitation were still elevated only in the Veh Str+Sh Group, compared to the Veh Str Group (# symbol at time 115' postI), and compared to the plasma glucose levels for this group at baseline (0' preI time). The DZ Str+Sh animals at this time (115' postH) no longer differed from the Sham Groups.

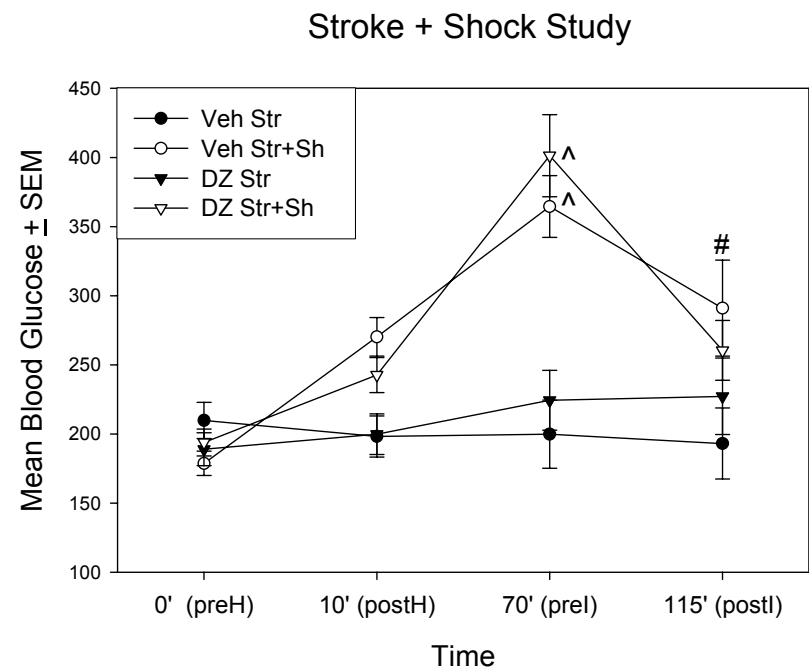
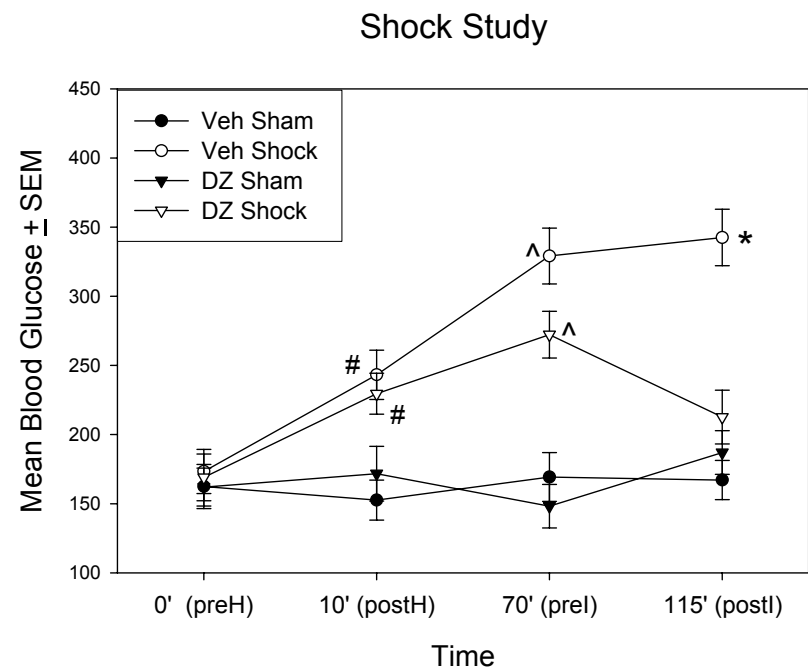
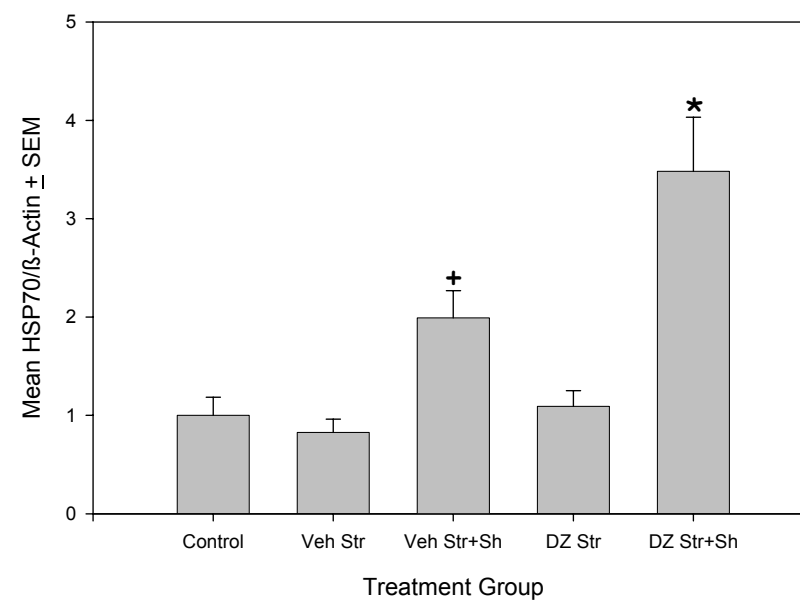
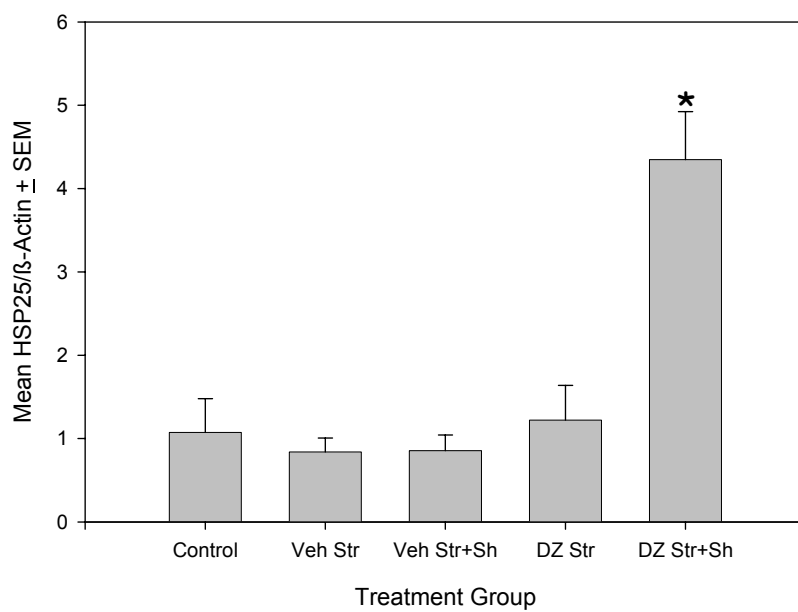


Fig 5. Protein levels of HSP25 and HSP70 in the kidney following treatment with DZ and stroke and hemorrhagic shock. Levels of HSP25 were significantly greater in samples of the kidneys from animals that had received DZ 24 hours before sustaining hemorrhagic shock and unilateral common carotid ligation (left histogram, labeled DZ Str+Sh, $*p < 0.02$), than were observed in all other treatment groups. HSP70 levels in the kidney (right histogram) were significantly elevated in animals that had received DZ 24 hours before sustaining Stroke + Shock ($*p < 0.02$, DZ Str+Sh Group $>$ Sham, Veh Str and DZ Str Groups), but did not differ significantly from levels observed in the animals that had also received combined trauma, but no DZ ($+p < 0.02$, where the Veh Str+Sh Group $>$ Veh Str Groups, but not different from all other groups).



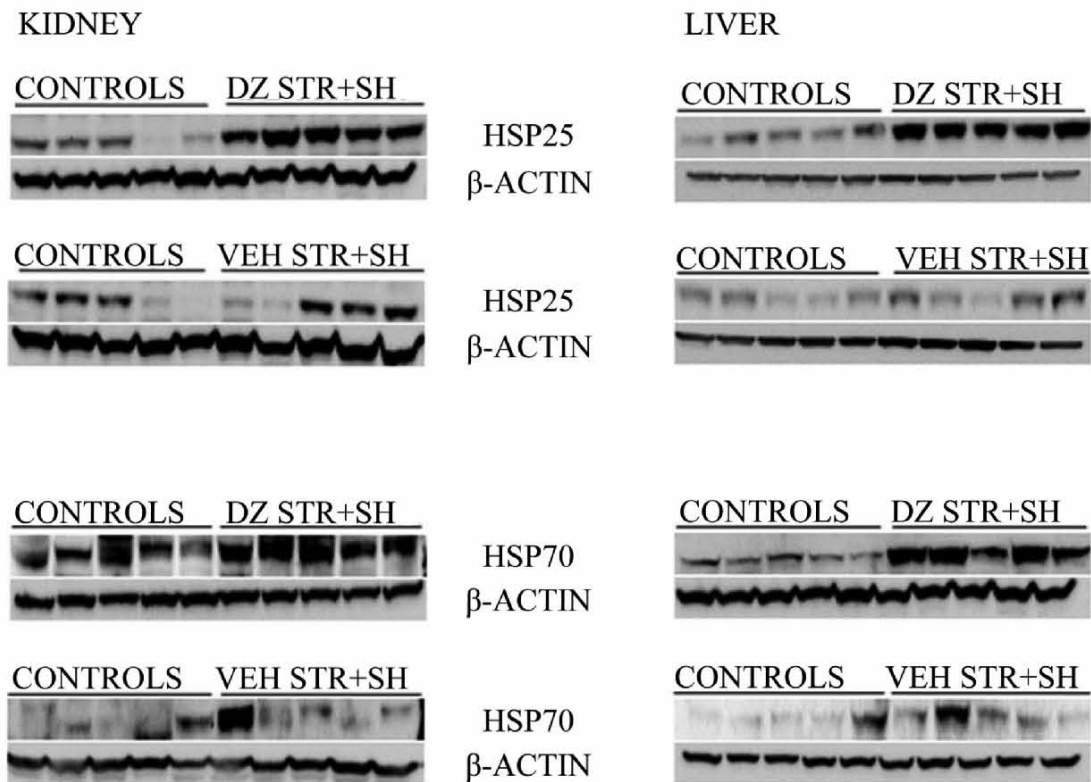
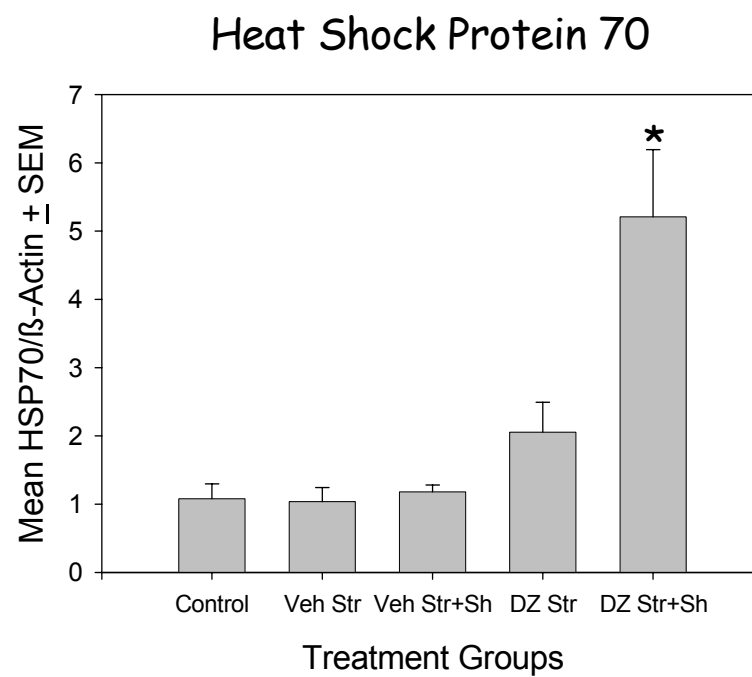
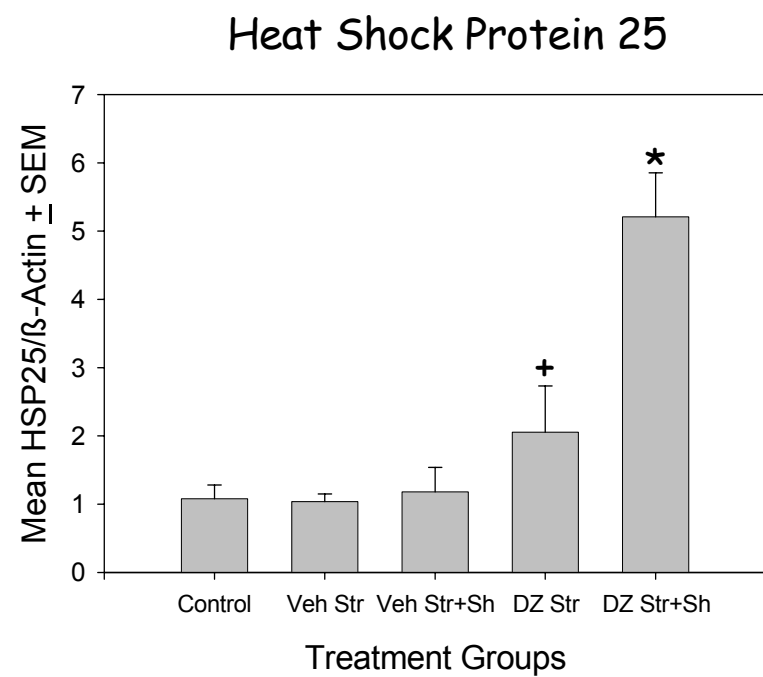


FIGURE 6

Immunoblots of HSP25 and HSP70 in selected groups of kidney and liver tissue. As indicated by the representative immunoblots, there was a noticeable increase in HSP25 (top two rows of blots) and HSP70 (bottom rows) expression in kidney (left) and liver (right) tissue in the DZ Str+Sh groups as compared to the Veh Str+Sh groups.

Fig. 7. Protein levels of HSP25 and HSP70 in the liver following treatment with DZ and stroke and hemorrhagic shock. A similar increase in HSP25 levels was seen in the liver of animals (left histogram) that received DZ 24 hours before sustaining Stroke + Shock treatment (DZ Str+Sh Group, $*p<0.02$, where levels were greater than all other groups except the DZ Str Group). Levels of HSP25 in the DZ Str Group were intermediate between the DZ Stroke + Shock Group and all other groups, and differed significantly only from the animals that had also received carotid ligation, but no pretreatment with DZ ($+p<0.02$, DZ Str vs. Veh Str Groups). HSP70 levels in the liver (right histogram) were significantly elevated in the DZ Str+Sh group compared to all other groups ($*p<0.001$), and a noticeable slight upregulation of HSP70 is noted in the DZ sham group (similar to the upregulation of HSP25 in the DZ Str group).



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CHAPTER 4

DIAZOXIDE, AS A POSTCONDITIONING AND DELAYED PRECONDITIONING TRIGGER, INCREASES HSP25 AND HSP70 IN THE CENTRAL NERVOUS SYSTEM FOLLOWING COMBINED CEREBRAL STROKE AND HEMORRHAGIC SHOCK

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ABSTRACT

Combined hemorrhagic shock (Shock) and unilateral common carotid artery occlusion (Stroke) results in a decrease of oxygen availability to peripheral tissues and organs and the central nervous system (CNS). A variety of biochemical processes ensue, including organ failure, cellular apoptosis and necrosis. Using heat shock protein 25 and 70 (HSP25, HSP70) as biomarkers, we tested the hypothesis that pharmacological induction of ischemic preconditioning can offer cytoprotection from combined Stroke and Shock. The compound, diazoxide (DZ), is known to induce preconditioning through its effect as a mitochondrial K_{ATP} channel opener and succinate dehydrogenase inhibitor. When administered 24 hours prior to Stroke and Shock (delayed preconditioning), DZ increased cerebral cortical and hippocampal levels of HSP25 and HSP70. A more clinically relevant treatment paradigm was tested, where DZ was administered after the induction of Stroke and Shock (postconditioning). When administered 60 minutes (but not 10 minutes) after the induction of Stroke and Shock, DZ significantly increased HSP25 and HSP70 expression in the ipsilateral cerebral cortex and hippocampus. Taken together, these results suggest that DZ treatment may be efficacious for CNS injury resulting from blood loss and anoxia from combined cerebral ischemia and hemorrhagic shock. “Postconditioning” treatment with DZ, immediately before resuscitation, is a potentially effective treatment for ischemia-reperfusion injury from combined Stroke and Shock.

KEY WORDS-Ischemic Preconditioning, Postconditioning, Ischemia, Shock, HSP

INTRODUCTION

First described by Murry and colleagues in 1986, *ischemic preconditioning* was found to result in a 75% reduction in cardiac infarction area from 40 minutes of circumflex artery occlusion (Murry, Jennings et al. 1986). Preconditioning was induced *in vivo* by short, 5-minute intervals of circumflex artery occlusion, with 5 minute periods of reperfusion, immediately before extended occlusion. Later work of other investigators determined that ischemic preconditioning could be induced by a variety of stimuli, across a wide range of species, and in many organs; liver (Peralta, Closa et al. 1996), skeletal muscle (Pang, Yang et al. 1995), and brain (Glazier, O'Rourke et al. 1994). Further research initiated by Marber, *et al.* in 1993 demonstrated that there are two phases of preconditioning; classical and delayed (Marber, Latchman et al. 1993). While Murry's, *classical preconditioning* (Murry, Jennings et al. 1986), occurs rapidly after a brief triggering stimulus, and persists for 1-3 hours, it is followed by a subsequent 24-72 hour *delayed preconditioning* phase. Delayed preconditioning is associated with the synthesis of new proteins, but may offer approximately 50% less protection than classical preconditioning (Yellon and Downey 2003).

With continued study, Zhao and colleagues identified a third phase of ischemia-induced cytoprotection, which was named *postconditioning* (Zhao, Corvera et al. 2003). Although the term can be confusing, postconditioning refers to the time frame immediately prior to reperfusion, but well after the ischemic event, when short alternate bouts of arterial reperfusion

and ischemia was found to be as effective as preconditioning. Zhao and colleagues employed a dog ischemic cardiac model (Zhao, Corvera et al. 2003) and found that postconditioning led to a significant reduction in myocardial infarct area. Since trauma or hypoxic events are most commonly unplanned occurrences, delayed or classical preconditioning are of limited utility. However, “postconditioning” intervention *after* the ischemic event has important clinical implications (Diaz and Wilson 2004).

Following cell and tissue ischemia, the involvement of the mK_{ATP} channel appears to be central for the protection of cells against necrosis and apoptosis (Carroll, Gant et al. 2001; Zaugg, Lucchinetti et al. 2002). Blockage of this channel, for example, eliminates the preconditioning effect (Fryer, Eells et al. 2000). DZ has been proven to be an effective delayed and classical preconditioning agent in many models, but has not previously been assessed in either a stroke/hemorrhagic shock model or in the postconditioning paradigm.

An increase in heat shock protein (HSP) expression has been shown in delayed and classical preconditioning models. However, to date an effect of DZ on HSP levels has not been performed. HSPs are a family of highly conserved, inducible proteins associated with a positive survival response to cellular stress and injury (Pittet, Lee et al. 2002; Boeri, Dondero et al. 2003). Mammalian stress proteins consist of two major groups; high molecular weight proteins (HSPs 90, 70, 60) that require ATP, and low molecular weight proteins (HSPs 10 and 25), which do not require ATP for their cytoprotective effects. As important molecular chaperones, HSPs prevent protein denaturation and incorrect polypeptide aggregation during exposure to cellular insults (Yenari 2002), they fold nascent and denatured peptides, stabilize the cytoskeleton, and inhibit

apoptotic (programmed cell death) pathways (De Maio 1999). HSPs eliminate damaged or misfolded proteins from the cytoplasm via the proteasome (Parcellier, Gurbuxani et al. 2003). HSP25 and HSP70 inhibit cytochrome c and apaf-1, respectively, which are key components of the apoptosome; an apoptotic signaling complex which activates caspase-3 (Bruey, Ducasse et al. 2000). Finally, HSP25 stabilizes the actin cytoskeleton (Mounier and Arrigo 2002) and HSP70 inhibits the apoptotic inducing factor (Ravagnan, Gurbuxani et al. 2001). Both of these stress proteins can directly inhibit caspase-3; the ‘initiator’ of cell death (Jaattela, Wissing et al. 1998; Concannon, Orrenius et al. 2001). In the context of CNS injury, the stress proteins examined in the present study are noteworthy because HSP25 and HSP70 are preferentially expressed in astrocytes and neurons, respectively (Plumier, Armstrong et al. 1997; Acarin, Paris et al. 2002)(Yenari 2002; Tidwell, Houenou et al. 2004).

To maintain survival, mammal physiological systems under shock will shunt blood from secondary organs (kidney, liver, skin, intestine and skeletal muscle) to primary organs (heart, lungs, and brain). Since shock alone will not injure neurons and glia significantly, we combined hemorrhagic shock (Shock) with cerebral hypoperfusion arising from unilateral occlusion of the right common carotid artery (Stroke). Using combined Stroke + Shock, we examined the effect of DZ on HSP25 and HSP70 expression in the rat ipsilateral cerebral cortex and hippocampus in postconditioning and delayed preconditioning experiments. Research was initiated to determine if DZ is effective in the upregulation of stress proteins in delayed preconditioning (given 24 hours prior to Stroke + Shock) as well as in postconditioning (given *after* Stroke + Shock).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (275-350 grams, n=127) from Charles River Laboratories (Wilmington, MA) were housed with a 12-hour light/dark cycle, 24°C constant temperature, and unlimited access to water and rat chow. All procedures were approved by the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences, Bethesda, Maryland.

Experimental Groups

In Experiment 1 (Table 1, top), a delayed preconditioning model was employed. DZ was given 24 hours prior to combined Stroke + Shock, and HSP25 and HSP70 levels were assessed in the right and left cerebral cortex and right hippocampus. HSP25 and HSP70 changes in liver and kidney were reported in another publication (O'Sullivan, *et al.*, submitted). In Experiment 2 (Table 1, bottom), DZ was administered at one of two time points *after* Stroke + Shock, prior to resuscitation, to assess postconditioning effects on cortical and hippocampal changes in HSP25 and HSP70.

The first Stroke + Shock experiment (Experiment 1) was comprised of five treatment conditions (Table 1, top). Group 1 rats served as a negative control; they did not receive any surgical stress or DZ and were euthanized immediately. Group 2, the vehicle stroke group (Veh Str), were

anesthetized, sustained a right common carotid artery ligation, and vascular cannulation for physiological and hematological monitoring. This group did not sustain shock. Group 3 animals, the vehicle stroke + shock (Veh Str+Sh) group, received anesthesia, right carotid artery ligation, monitoring, and hemorrhagic shock with normal saline infusion for resuscitation (see below). The rats in a fourth group, DZ with stroke (DZ Str Group), tested the function of DZ without shock. These rats were treated the same as Group 2, but received DZ. Group 5 (DZ Str+Sh) tested the effectiveness of DZ in reducing organ stress. This group experienced the same experimental variables as Group 3, except they received DZ.

The second experiment (Stroke + Shock with a postconditional treatment with DZ) was performed to determine if HSP protein levels in the cerebral cortex and hippocampus were altered when DZ was given at two different time points *after* hemorrhagic shock. This postconditioning experiment consisted of six treatment groups (Table 1, bottom). Group 1 (Str Veh) received anesthesia, physiological monitoring and right carotid ligation with vehicle administration (~0.6cc sterile normal saline) 20 minutes after the beginning of the experiment. Group 2, vehicle Stroke + Shock (Str+Sh Veh) rats, received anesthesia, physiological monitoring, right carotid ligation, shock, vehicle administration 20 minutes after the onset of Stroke + Shock, and normal saline resuscitation 1 hour later. Group 3 rats (the Str DZ20 Group) were treated the same as Group 1. These animals received carotid ligation but no shock, but DZ was given 20 minutes after the start of the protocol. Group 4 (Str+Sh DZ20) rats sustained Stroke + Shock, and were given DZ 20 minutes after shock initiation. At the end of the 1-hour period of shock these animals then received sterile saline for resuscitation. Group 5 (Str+Sh DZ60) animals sustained Stroke + Shock, and at the end of the 60-minute shock period they received

DZ, immediately prior to normal saline infusion. Group 6 (DZ Str+Sh) rats received DZ 24 hours prior to Stroke + Shock (delayed preconditioning).

Before the postconditioning experiment was initiated, a study was conducted to determine whether DZ could be given intravenously (IV) without detrimentally affecting blood pressure in animals that had sustained Stroke + Shock. Animals received Stroke + Shock, and 10 minutes after the end of the exsanguination period they received DZ (0.32mg/kg/minute IV) over the following 10 minute period. Mean arterial pressure (MAP) was recorded before the onset of shock, during and after DZ infusion, and during resuscitation. DZ was considered to have a significant effect if MAP decreased by 25% during IV administration or during the subsequent stages of shock or resuscitation. All seven rats that were tested exhibited no significant reduction of blood pressure when 3.2mg/kg DZ was infused over a 10-minute period.

Drugs

DZ (Sigma, St. Louis) was dissolved in 50µl of 0.5M NaOH and then diluted with normal saline (2.5mg/cc). As shown in Table 1, for delayed preconditioning DZ (5mg/kg, intraperitoneally) was given 24 hours before hemorrhagic shock, while in the postconditioning study, DZ (3.2 mg/kg/10 minutes, IV) was administered via a femoral venous catheter at one of two different time points (see below). Isoflurane (Abbott Labs, Chicago) with air was utilized as the anesthetic agent during surgery, titrated to respiration rate. Lidocaine 2% (Phoenix, St. Louis) was infiltrated into incisional areas to reduce anesthetic requirements. Buprenex (Reckitt Benckiser Pharm, Richmond, VA) was given subcutaneously (0.03 mg/kg) for post-surgical discomfort. The last medication utilized was a combination of ketamine (80mg/kg; Fort Dodge

Animal Health, Fort Dodge, IA) and xylazine (10mg/kg IP; Phoenix, St. Louis) that was administered prior to euthanization, which occurred 24 hours after the Stroke and Shock protocol.

Protocol

Table 2 provides a temporal overview of the protocol. All rats (except Group 1 in the delayed preconditioning experiment) were anesthetized in an induction chamber with 3-5% isoflurane (IsoFlo, Abbott Laboratories) until unconscious (15-60 seconds). Anesthesia was maintained with isoflurane, via a rodent nose cone with a scavenging system, to maintain a constant plane of anesthesia throughout the experiment. Euthermia was maintained with a heat therapy isothermal pad (Gaymar T/Pump model TP-500). A surgical cutdown, after shaving and lidocaine injection (SQ), was performed to the right groin for isolation and cannulation of the right femoral vein and artery. A polyethylene catheter (PE-50, Clay Adams, Piscataway, NJ) was inserted using aseptic technique guided with the aid of a surgical optical scope. The arterial catheter was then connected to a pressure transducer and computerized physiographic system (Ponemah Physiology Platform, Gould Instrument Systems, Valley View, OH) for continuous blood pressure and heart rate recordings. After establishing baseline vital signs, a midline incision was made to expose and ligate the right common carotid artery, after the vagus nerve was carefully separated from the artery. A rapid hemorrhage was then induced over 10 minutes. Exsanguination (27cc/kg) resulted in ~ 40% loss of total blood volume based on body weight (less 1.2 cc needed for four blood draws that occurred either during or at the end of resuscitation). Beginning 60 minutes after the conclusion of rapid hemorrhage, resuscitation was initiated by intravenous infusion of normal saline (81cc/kg), over the next 45 minutes, through

the right femoral vein catheter. The catheters were then removed, the femoral artery and vein were ligated, the operative site repaired, and the animals received analgesia (buprenex, 0.03 mg/kg SC) and were allowed to recover. After regaining consciousness, the rodents were returned to their home cage for 24 hours before they were anesthetized by ketamine and xylazine administration. Cortical and hippocampal specimens were dissected and stored in liquid nitrogen.

During the procedure four arterial blood samples are obtained; at baseline before hemorrhage, at the completion of exsanguination, at the end of the 60 minute shock period and at the conclusion of resuscitation. Blood samples were then analyzed by a Stat Profile 2 Blood Gas and Electrolyte Analyzer (Nova Biochemical, Waltham, MA).

Western Blot Analysis

For isolation of total protein, 30-50 mg of the right and left cerebral cortex and right hippocampus from each rat were crushed with mortar and pestle in liquid nitrogen and dispersed with a sonicator (Kontes micro-ultrasonic cell disrupter) in lysis buffer (10X PBS-50ml, 10% SDS-5ml, NP-40 5ml, sodium deoxycholic acid 2.5grams, and double-distilled water to 500ml) containing 1x TBS, 0.1% Triton-X-100, and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Boehringer-Mannheim). Lysates were centrifuged at 12000rpm for 20 minutes at 4°C. Total protein concentration was determined according to the manufacturer's instructions (Protein Assay Kit, BioRad). Thirty micrograms of total protein from each sample, in loading buffer, were boiled for 5 minutes, then loaded on Nu-Page gels (Invitrogen, Carlsbad, CA) and separated electrophoretically. Proteins were transferred to nitrocellulose membranes at

room temperature overnight. Nitrocellulose membranes were first incubated in blocking buffer (5% dry nonfat milk in TTBS: 20mM Tris-HCL, pH 7.4, 150 mM NaCl and 0.1% Tween-20) for 1-2 hours, followed by incubation in antisera to HSP70 at 1:2000 concentration (Stressgen, SPA-810) or HSP25 at 1:10000 concentration (Stressgen, SPA-801) overnight at 4°C on a shaker table. Membranes were washed in washing buffer, and then incubated with the appropriate HRP-conjugated secondary antibody (1:5000 for HSP70 and 1:10000 for HSP25) for 90 minutes at room temperature. The membranes were then washed and incubated with chemiluminescent detection reagents (Pierce, SuperSignal West Femto Maximum Sensitivity Substrate) for 5 minutes, and quantified with a Fuji Film LAS-1000 camera and LAS-1000plus software. Relative protein levels were determined from the optical densities of the corresponding protein bands after subtraction of background values obtained from the same lane. All protein samples were then standardized with an internal control; β -actin.

Statistical Analysis

Results for the physiological and hematological data were summarized using a Treatment x Trials design analysis of variance with repeated measures on the second factor and *post hoc* comparisons (SigmaStat, Version 3.1). *Trials* refers to repeated measures on the same animal for physiologic and hematological measures. For the hematological measurements during shock and resuscitation, there were repeated measures taken at baseline, at the end of exsanguination, at the beginning of resuscitation, and at the end of reperfusion. For physiological data (heart rate, blood pressure) measurements were taken every 5 minute immediately after exsanguination and during resuscitation.

As noted, western blots were performed to measure changes in HSP25 and HSP70 (relative to β -actin levels) in samples of the cerebral cortex and hippocampus of rats from the delayed preconditioning and postconditioning Stroke + Shock studies. Each western blot consisted of five individual samples from the Control Group (Group 1), and five samples from one of the experimental groups (Groups 2, 3, 4, 5 or 6). In cases where a protein sample from the same rat was used on more than one blot, the mean band density for this animal was computed. Comparison between blots was performed by standardizing all blots to the mean band intensity of the Control samples, and data were analyzed by a single factor analysis of variance and *post hoc* comparisons.

RESULTS

Physiological Response to DZ with Stroke and Shock

In the delayed preconditioning Stroke + Shock study (Experiment 1), mean arterial blood pressure (MAP) exhibited a profound and sustained episode of hypotension after exsanguination. MAP was approximately 20mm/Hg immediately following exsanguination, and slowly rose over time to a maximum of ~40mm/Hg until the rats received resuscitation. MAP increased to ~60mm/Hg only with fluids, but never achieved baseline blood pressures (data not shown; results reported in O'Sullivan, *et al.*, submitted). Similar changes in MAP were seen in the postconditioning Stroke + Shock study (Experiment 2; Fig. 1). In both the delayed preconditioning (data not shown) and the postconditioning Stroke + Shock studies (Fig. 1), DZ appeared to have no effect on MAP during shock or resuscitation. Heart rate (HR; data not shown) demonstrated a similar pattern as MAP. HR decreased after the 10 minutes of

exsanguination (profound bradycardia) but rapidly increased to near baseline and was not affected by DZ or resuscitation.

Hematological Response to DZ with Stroke and Shock

As expected, there was a statistical difference in lactate and glucose levels between animals that sustained shock and animals that received no exsanguination (results for the delayed preconditioning study are reported in O'Sullivan, *et al.*, submitted). In the postconditioning study (Fig. 2), there was a significant increase in blood glucose and lactate levels during the 1 hour shock period (the 10' postH and the 70' preI time points) in the Stroke + Shock animals (Str+Sh Veh, Str+Sh DZ20 and Str+Sh DZ60 Groups). Lactate and glucose levels, however, both returned to near baseline levels after normal saline infusion, although glucose levels from the Stroke + Shock Groups remained significantly elevated after resuscitation (115' postI time point) in comparison to levels for the Control Group. Ninety-five minutes after drug infusion (115' postI time point), the Str DZ20 Group, which received DZ 20 minutes after initiation of the experiment, exhibited an increase in blood glucose levels; perhaps related to the known hyperglycemic effect of DZ (Alemzadeh and Holshouser 1999).

Delayed Preconditioning Effects on HSPs Levels in the Cerebral Cortex and Hippocampus

When DZ was administered 24 hours before Stroke + Shock, it caused a significant upregulation of HSP25 in the ipsilateral cerebral cortex (Fig. 3, top right histogram, and Fig. 4; DZ Str+Sh Groups). However, there was no difference between any of the groups for HSP25 in the left cerebral cortex (Fig. 3, top left). HSP70 expression in the right cerebral cortex from the DZ Str+Sh and the Veh Str+Sh rats was significantly higher than all other groups (Fig. 3, lower

right graph, and Fig. 4). There was no difference in HSP70 expression in the uninjured left cerebral cortex across any of the treatment groups (Fig. 3, left bottom histogram).

The expression of HSP25 and HSP70 in the right hippocampus (Fig. 5, left and right histograms, respectively) of the DZ Str+Sh Groups exhibited a significant increase compared to all other treatment groups—including the Veh Str+Sh Groups.

Postconditioning Effects on HSP Levels in the Cerebral Cortex and Hippocampus

As indicated earlier, DZ administration 24 hours before Stroke + Shock (DZ Str+Sh Group) resulted in elevated protein levels of HSP25 and HSP70 in the ipsilateral cerebral cortex (Fig. 6, top and bottom histograms, respectively and Fig. 7, left side) in comparison to levels observed in the Veh Str, Str+Sh Veh, and Str DZ20 Groups. A similar pattern of elevation was seen in the “postconditioned” animals. The Str+Sh DZ60 Group exhibited significantly elevated levels of HSP25 as compared to the Veh Str and Str DZ20 groups. HSP70 levels were significantly greater in the Str+Sh DZ60 animals compared to all other treatment groups, with the exception of the DZ Str+Sh Group (Fig. 6, bottom histogram, and Fig. 7, left side).

A similar effect was seen in the hippocampus. Administration of DZ at the end of the shock period (Str+Sh DZ60) resulted in a significant increase in HSP25 (Fig. 7, right side; Fig. 8, top) and HSP70 (Fig. 7, right side; Fig. 8, top), compared to all other groups, and levels were similar to those observed in the delayed preconditioned animals (DZ Str+Sh Group in Fig. 7 and Fig. 8).

DISCUSSION

Stroke and Hemorrhagic Shock

The present study demonstrated that DZ—as a delayed preconditioning and a postconditioning trigger—significantly increased HSP25 and HSP70 expression in the ipsilateral cerebral cortex and hippocampus of animals that had sustained combined Stroke and Shock. To our knowledge, this is the first report of DZ's effect on heat shock protein expression and the first assessment of DZ as a postconditional treatment for hemorrhagic shock.

Neither shock nor carotid artery occlusion independently result in apoptosis and necrosis in the central nervous system. As previously demonstrated, however, the combination of transient, unilateral common carotid arterial occlusion with hypotension leads to cerebral ischemia (Glazier, O'Rourke et al. 1994). In addition to causing cerebral injury, the Stroke + Shock model results in a higher incidence of lethality than either of these traumas singly. A multi-center study, for example, found a higher rate of fatalities when trauma occurs with brain injury (McMahon, Yates et al. 1999). It should be noted that the trauma induced in the present study differs slightly from other models. First, hypotension was induced by a permanent 40% blood loss. Other models include maintaining animals at a constant hypotensive MAP for a specified time, followed by reinfusion with blood. In the present case, severe hemorrhagic Shock + Stroke was initiated without reinfusion of blood. This precipitated a state of anemia. Secondly, the ipsilateral common carotid artery occlusion was permanent. Shock, combined with stroke injury, elicited secondary inflammation, anemia, and edema leading to greatly diminished ipsilateral perfusion pressures and continued autonomic dysregulation. These effects presumably resulted in more deleterious ipsilateral cerebral injury.

Notwithstanding differences in animal models of combined stroke and shock, the present results demonstrate that delayed preconditioning and postconditioning induced neuroprotective biomarkers, HSP25 and HSP70, in brain regions that sustained ischemia. Correspondingly, DZ was found to also elicit HSP25 and HSP70 expression in the liver and kidney of rats that sustained combined Stroke and Shock, and it may also reduce fatality (O'Sullivan, *et al.* submitted).

Mean Arterial Pressure Changes from Combined Stroke + Shock, and Response to Diazoxide

Analysis of MAP shows that significant central nervous system autonomic dysfunction occurs after Stroke + Shock. In our previous study (O'Sullivan, *et al.*, submitted for publication), which compared MAP responses to Shock alone vs. Stroke + Shock, we found that MAP levels in both models fell precipitously to ~20mm/Hg. In the animals that sustained shock alone, however, MAP gradually increased to about 60mm/Hg during the hour-long shock period, and MAP did not change as a result of resuscitation (normal saline infusion). However, in animals that sustained Stroke + Shock, MAP levels reached a plateau of ~40mm/Hg by the end of the shock period, and only reached ~60mm/Hg following resuscitation (O'Sullivan, *et al.*, submitted for publication). A similar profile was observed in the postconditioning study (Fig. 1). MAP in the Stroke + Shock animals slowly increased to ~40mm/Hg by the end of the shock period, and increased to a maximum of 60mm/Hg only following resuscitation. Finally, MAP results suggest that DZ, at the dose and infusion rate used here, had no detrimental, hypotensive effect; even in animals that sustained Stroke + Shock.

Postconditioning by Diazoxide

In light of its clinical relevance, it is important to identify the critical parameters and mechanisms that underlie postconditioning benefits. One critical element may be the temporal relationship between the postconditioning trigger and the onset of reperfusion or resuscitation. Kin and colleagues, found that very short arterial occlusions of cardiac tissue (3x 10 seconds each) just prior to reperfusion reduced the infarct area (Kin, Zhao et al. 2004). These results implied that the first *minute* of reperfusion is critical to cellular protection by postconditioning (Kin, Zhao et al. 2004). In a dog cardiac model, Mizumura *et al.* demonstrated that the K_{ATP} channel opener, bimakalin, markedly reduced infarct size when given 10 minutes before and during the 60-minute reperfusion period (Mizumura, Nithipatikom et al. 1995). Finally, Zhao and colleagues demonstrated the importance of the temporal variable in deriving benefits from preconditioning. A reduction in canine cardiac infarct area was seen when very short arterial occlusion/reperfusion triggers were performed one hour *after* the ischemic event but just *before* resuscitation (Zhao, Corvera et al. 2003).

The present findings also support the importance of temporal contiguity between trigger presentation and reperfusion (resuscitation). DZ treatment 40 minutes before reperfusion failed to increase heat shock protein expression in animals that had sustained combined stroke and shock, while administration beginning at the onset of reperfusion (resuscitation) resulted in a robust increase in heat shock protein levels in the injured cerebral cortex and hippocampus. Taken together, these studies suggest that the “therapeutic window” for cytoprotection is narrow,

or that it is critical to administer the postconditioning agent just before and/or during the initiation of reperfusion to trigger the protective effects.

Mechanisms of Diazoxide Action in Postconditioning

While previous reports have demonstrated that DZ is effective for eliciting both delayed (Shake, Peck et al. 2001) and classical (Dzeja, Bast et al. 2003) preconditioning, the present work is the first to demonstrate its utility as a postconditioning agent. The specific mechanism(s) underlying its efficacy as a postconditioning agent, presumably by way of its ability to forestall reperfusion injury, requires further study. Normally, ischemic areas of tissues unleash large amounts of reactive oxygen species (ROS; H_2O_2 , $\text{OH}^\cdot$, NO , and superoxide radicals) during reperfusion. ROS leads to cell death by lipid peroxidation of the mitochondria cell membrane and the release of apoptotic proteins (Yellon and Downey 2003). Ischemic preconditioning offers cell protection by inhibiting oxidant generation; especially at reperfusion (Kin, Zhao et al. 2004). Other effects of reperfusion injury include opening of the mitochondrial permeability transition pore (mPTP); related to a substantial depletion of ATP and Ca^{2+} overload (Argaud, Gateau-Roesch et al. 2005). Opening of the mPTP also results in the collapse of the mitochondrial membrane potential and release of apoptotic proteins. It is for these reasons that current thought is that the benefits of preconditioning are not during the ischemic event, but more likely during reperfusion (Downey and Cohen 2005). The present results demonstrate that DZ administration just before reperfusion (postconditioning) also upregulates neuroprotective stress proteins.

Diazoxide and HSPs

While ischemic preconditioning has been shown to induce HSP production (Raghavendra Rao, Dhodda et al. 2003; Dhodda, Sailor et al. 2004), there is little information regarding how DZ alters HSP levels in the stressed cell. Some key facts, however, may shed light on this relationship. First, although the traditional working hypothesis is that DZ acts via the activation (opening) of the mK_{ATP} channel, its channel structure has not been firmly elucidated (Seharaseyon, Ohler et al. 2000; Brustovetsky, Shalbuyeva et al. 2005). The mK_{ATP} channel has not been cloned and reports differ concerning whether the Kir subunit is a constituent. Evidence thus far supports the argument that the pore-forming subunits of the K_{ATP} channel in mitochondrial and cell membranes may be different (Hanley and Daut 2005). Germane to the present work, some research has suggested that the mK_{ATP} channel may consist of a succinate dehydrogenase component, which associates with four other proteins, including a mitochondrial ATP binding cassette protein (mABC1, a member of the family of SUR components found in the cell membrane K_{ATP} channel), an adenine nucleotide translocator (ADP/ATP membrane transporter), a phosphate inorganic carrier (phosphate membrane transporter) and an ATP-synthase (complex V in cellular respiration) (Ardehali and O'Rourke 2005). Secondly, it was always assumed that the SUR1 component of the K_{ATP} channel—the DZ-targeted protein—only associates with Kir components. However, one team has shown that SUR1 is part of a nonselective cation channel, which they labeled the NC Ca_{ATP} channel. It is activated by Ca²⁺, depletion of ATP, and DZ. The pore-forming unit and the physiological role of the NC Ca_{ATP} channel has yet to be identified (Chen and Simard 2001; Ardehali and O'Rourke 2005). Thirdly, although it was initially believed that the opening of the mK_{ATP} channel was the key event for

protection by DZ, further research suggests succinate dehydrogenase inhibition may be equally important (Hanley and Daut 2005). Since this inhibition occurs at the mitochondrion, it may be that succinate dehydrogenase inhibition and the mK_{ATP} channel activation by DZ are essentially one and the same actions.

Finally, DZ may regulate HSP levels via heat shock factors (HSF), and heat shock factor 1 in particular. HSF1 is normally associated with the major HSPs in a dormant state. However, in response to cellular stress, damaged proteins bind to HSPs, dislodging HSF1 from its site, leading to trimerization. Trimerized HSF1 then migrates to the nucleus, binds with the heat shock promoter element (HSE) on selected genes and executes transcriptional activation. Increased HSP production acts as a negative feedback loop to decrease unbound HSF1-initiated transcription (Morimoto 1998). Evidence exists that the function of HSP70 requires an intrinsic ATPase activity that is modulated by nucleotide exchange factors (Moro and Muga 2006). If ATPase activity, or some nucleotide exchange factor is modified by DZ through the mK_{ATP} channel, the NC Ca_{ATP} channel or some other pathway, and displaces HSF1 from these heat shock proteins, this would lead to an upregulation of these anti-apoptotic proteins. Therefore, DZ's direct (mK_{ATP} channel activation) and indirect preconditioning effects (upregulation of HSP25 and HSP70) could lead to increased cell survival.

Summary

The present studies demonstrate the utility of DZ as a regulator of two key stress proteins—HSP25 and HSP70—in the CNS when used as a delayed preconditioning and postconditional trigger for cytoprotection from combined stroke and hemorrhagic shock. HSP upregulation is

one of the key factors in providing cellular neuroprotection. The exact mechanism(s) of DZ's action as a cytoprotectant for combined stroke and shock, however, remains to be elucidated. DZ may elicit its preconditioning and postconditioning effects through its ability to enact succinate dehydrogenase inhibition, cellular membrane K_{ATP} channel opening, mK_{ATP} channel opening, SUR activation of the NC Ca^{2+}_{ATP} channel, or another presently unknown pathway. Of worthy note, DZ did not significantly increase HSP production in the DZ Str groups, suggesting that DZ does not increase HSP levels unless central nervous system cells are subjected to insult.

These findings suggest two different implications. Surgical classical preconditioning with DZ was shown to be cardioprotective (Wang, Wei et al. 2003; Wang, Wei et al. 2004). Based on the delayed preconditioning effects seen here, DZ may have some applications for neuroprotection. Patients that are about to undergo central nervous system surgery, for example, could receive DZ 24 hours prior to surgery to increase stress protein production, which may provide supplemental neuroprotection. Stress protein production has been shown to increase cell survival (Chen, Graham et al. 1996; De Maio 1999; Beere, Wolf et al. 2000). Secondly, DZ may have utility as a postconditional agent, given before the onset of reperfusion or resuscitation. Our results, at the present dose and duration, indicated DZ precipitated no detrimental effect upon mean arterial pressure at any time point when given over 10 minutes in our rat Stroke and Shock model. Further research to understand DZ's cytoprotective effects, via histological analysis, is warranted, and may further validate this drug as a therapeutic pharmacological preconditioning agent.

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Table 1. Summary of Treatment Groups

<i>Group</i>	<i>n</i>	<i>Experimental Treatment</i>
<i>Experiment 1-Delayed Preconditioning Study</i>		
Group 1	6	Control: No Anesthesia, carotid ligation, or hemorrhagic shock
Group 2	12	Vehicle Stroke (Veh Str): Anesthesia and carotid ligation only
Group 3	16	Vehicle Stroke + Shock (Veh Str+Sh): Anesthesia/carotid ligation/hemorrhagic shock/resuscitation
Group 4	12	DZ stroke (DZ Str): DZ treatment 24 hours prior to anesthesia and carotid ligation only
Group 5	14	DZ stroke + shock (DZ Str+Sh): DZ 24 hours prior to anesthesia/carotid ligation/hemorrhagic shock/resuscitation
<i>Experiment 2-Postconditioning Study</i>		
Group 1	10	Vehicle Stroke (Veh Str): Anesthesia and carotid ligation only
Group 2	10	Stroke + Shock Vehicle (Str+Sh Veh): Anesthesia/carotid ligation/hemorrhagic shock/resuscitation
Group 3	10	Stroke with DZ @ 20min (Str DZ20): Anesthesia/carotid ligation/diazoxide @ 20 minutes
Group 4	10	Stroke + Shock DZ @ 20min (Str+Sh DZ20): Anesthesia/carotid ligation/hemorrhagic shock/diazoxide @ 20 minutes/resuscitation at 60 minutes
Group 5	10	Stroke + Shock DZ @ 60min (Str+Sh DZ60): Anesthesia/carotid ligation/hemorrhagic shock/diazoxide @ 60 minutes/resuscitation at 60 minutes
Group 6	10	DZ Stroke + shock (DZ Str+Sh): Anesthesia/carotid ligation, hemorrhagic shock, resuscitation with DZ 24 hours prior to Stroke + Shock

Table 2. Hemorrhagic Shock and Resuscitation Protocol

Cumulative Time	0'	10'	70'	115'
Duration	0'	10'	60'	45'
Exsanguination	————→			
Hemorrhagic Shock		————→		
Resuscitation			————→	
Hematological Sampling *	*	*	*	*
MAP, HR q 5 minutes	*	————→		

* Refers to instances when hematological or vital signs will be obtained.

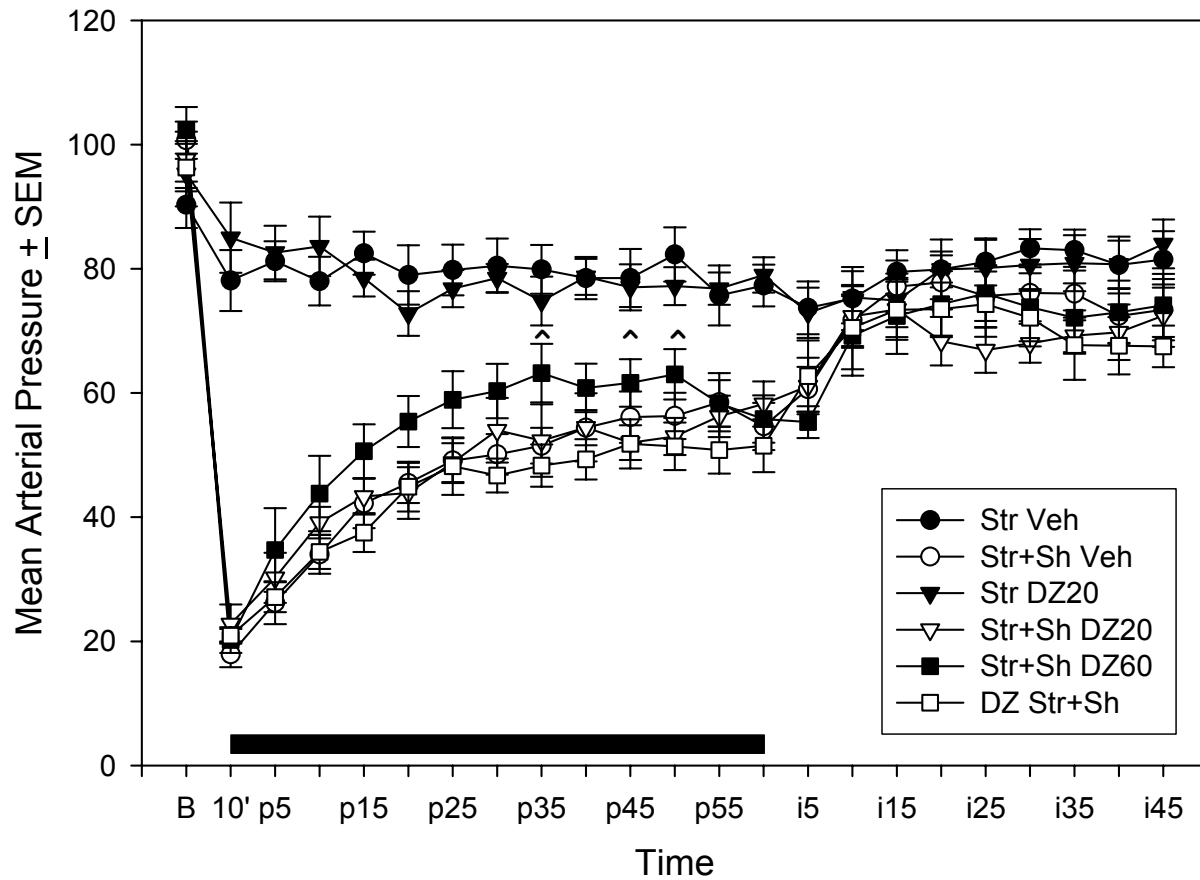


FIG. 1. Mean arterial pressure (MAP) following stroke treatment or combined stroke and shock. The horizontal bar above the abscissa indicates that all Shock Groups (Str+Sh Veh, Str+Sh DZ20, Str+Sh DZ60, and DZ Str+Sh) exhibited significant decreases in MAP compared to the non-shocked groups (Str Veh and Str DZ20 Groups), except at the three time points indicated by the ^ symbol, when the Str DZ20 and Str+Sh DZ60 Groups did not differ. Following resuscitation (Str+Sh Veh, Str+Sh DZ20, Str+Sh DZ60, and DZ Str+Sh Groups) by venous infusion of saline, there was no significant difference in MAP between the treatment groups.

FIG. 2. Plasma levels of lactate (left) and glucose (right) following stroke treatment or combined stroke and shock. Lactate levels were altered by combined stroke and shock, where all Shock Groups (Str+Sh Veh, Str+Sh DZ20, Str+Sh DZ60, and DZ Str+Sh) exhibited significant increases in lactate levels (denoted by the horizontal bar above the abscissa), compared to the non-shocked groups (Str and Str DZ20' Groups), at the end of the exsanguination period (10' postH) and after 1 hour of combined stroke and shock (70' preI). Following resuscitation, levels did not differ across groups. By the end of the combined stroke and shock period, glucose levels were significantly elevated in all groups that had sustained combined stroke and shock (70 preI time point). At the end of the resuscitation (115' postI), all groups that sustained combined stroke and shock continued to have elevated glucose plasma levels (horizontal bar). In addition, animals that had sustained stroke alone and had received DZ (Str DZ 20) 20 minutes later exhibited an elevation in plasma glucose levels, compared to the Control animals, and at this time their glucose levels were significantly greater than at the 0' preH and 10' postH time points (denoted by ^).

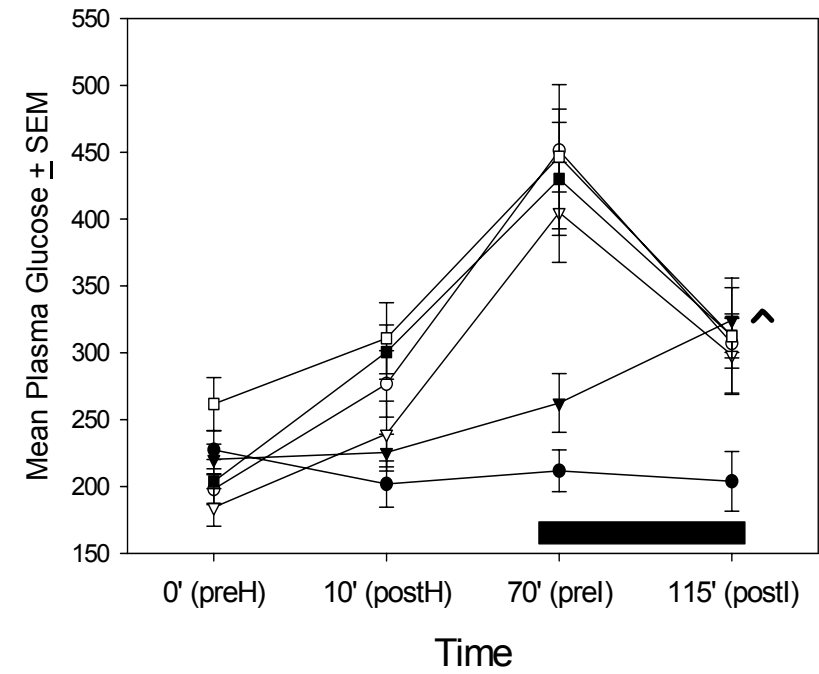
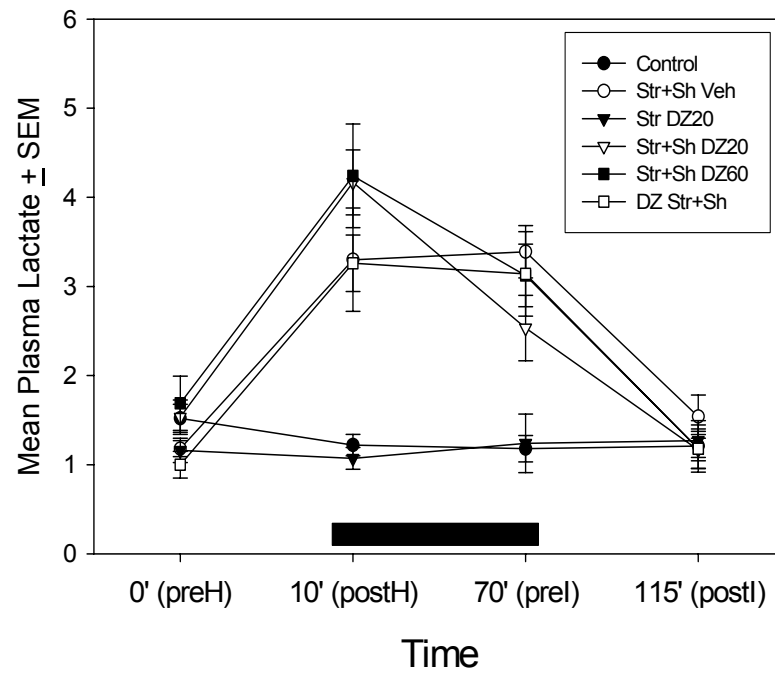
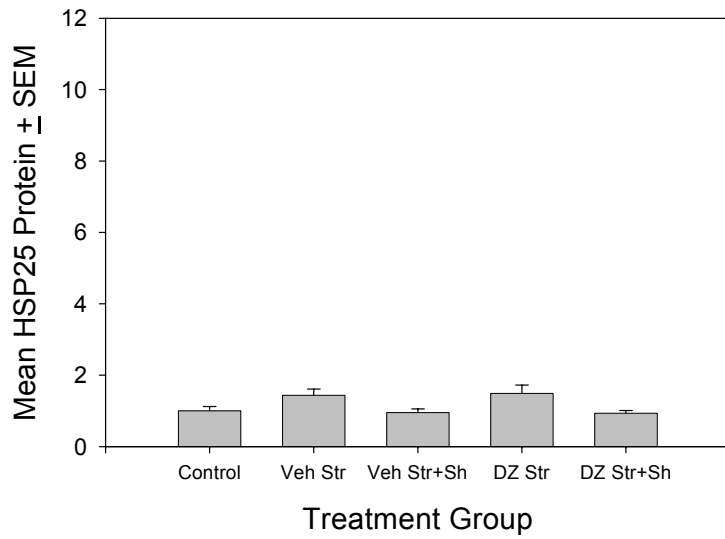
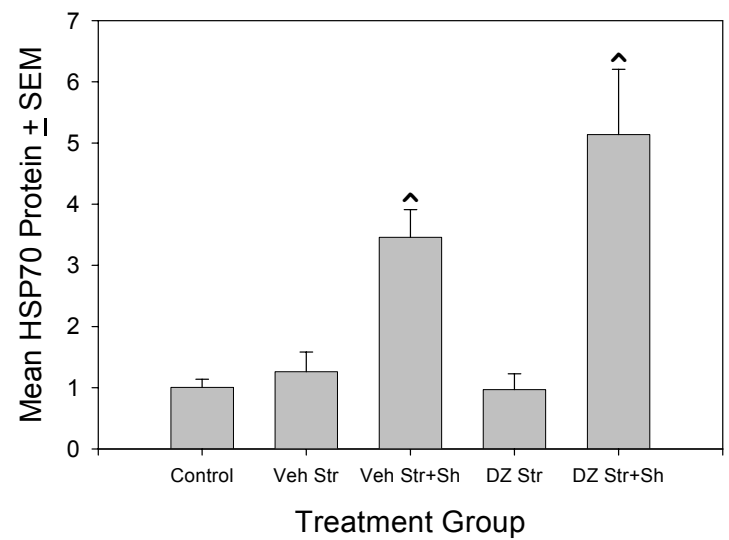
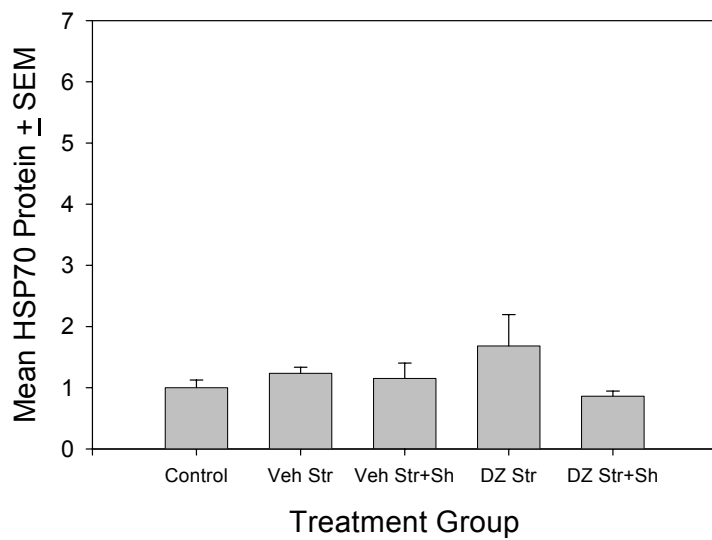
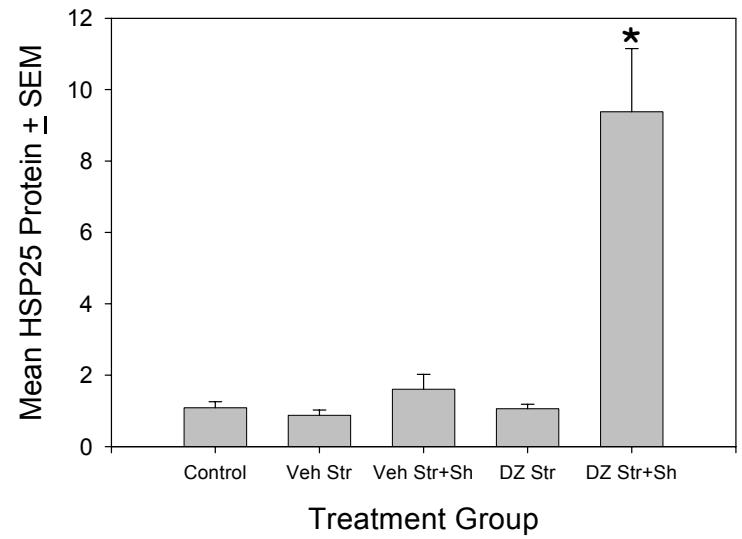


FIG. 3. HSP25 and HSP70 expression in the left and right cerebral cortex following delayed preconditioning. There was no significant difference in HSP25 and HSP70 expression between any of the groups in the left cerebral cortex (left side, top and bottom) after various experimental treatments. In the right cerebral cortex (top right graph), the asterisk (*) indicates that HSP25 levels were significantly higher, 24 hours later, in rats that received DZ 24 hours before combined Stroke + Shock. HSP70 levels in the right cerebral cortex (bottom right) were significantly increased in the Veh Str+Sh group and DZ Str+Sh Group (labeled ^) compared to the Control, Veh Str and DZ Str Groups.

Left Cerebral Cortex



Right Cerebral Cortex



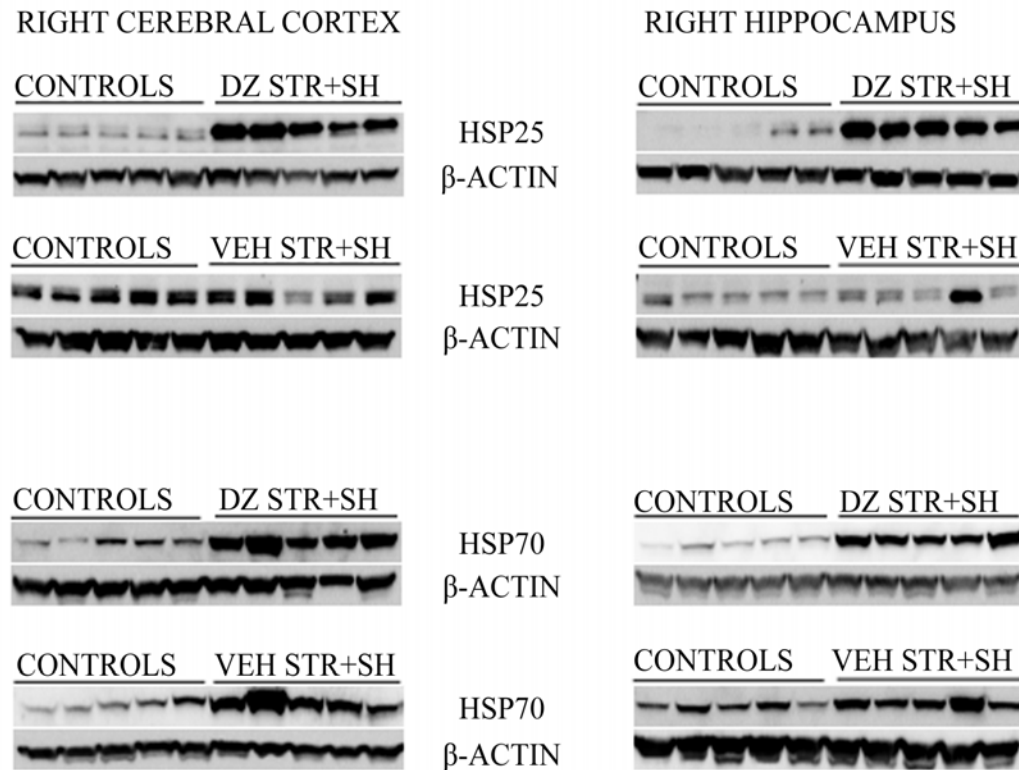
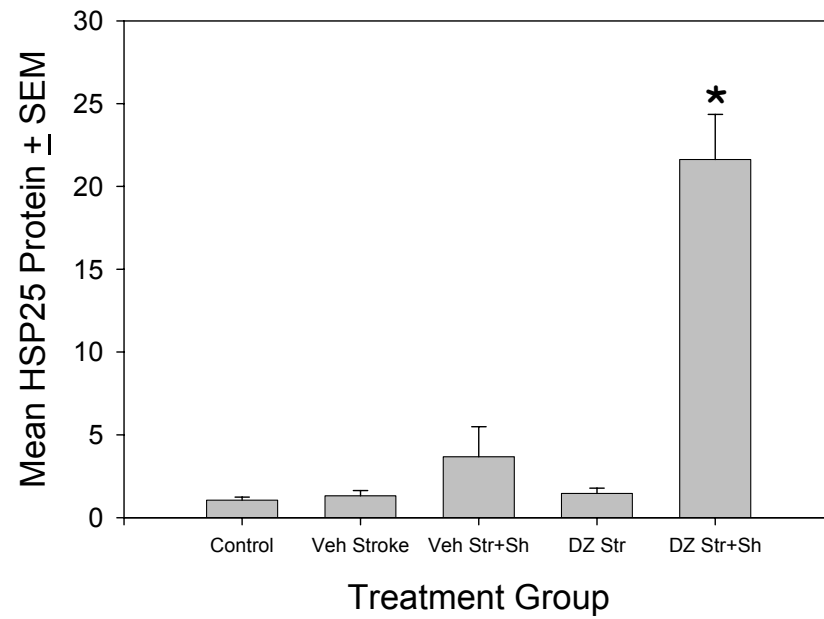


FIGURE 4

Immunoblots of HSP25 and HSP70 in selected groups of the right cerebral cortex and hippocampus in the delayed preconditioning study. As indicated by the representative immunoblots, there was a noticeable increase in HSP25 (top two rows of blots) in the right cerebral cortex and hippocampus in the DZ Str+Sh Groups as compared to the Veh Str+Sh Groups. HSP70 expression (bottom two rows of blots) shows an increase in the DZ Str+Sh Groups, but an increase was also evident in Veh Str+Sh Group in the right cerebral cortex.

FIG. 5. HSP25 and HSP70 expression in the right hippocampus following delayed preconditioning. The asterisk (*) indicates that compared to all other treatment groups, HSP25 (left graph) and HSP70 levels (right graph) in the right hippocampus of rats that received DZ 24 hours before combined Stroke and Shock were significantly higher 24 hours later.

Heat Shock Protein 25



Heat Shock Protein 70

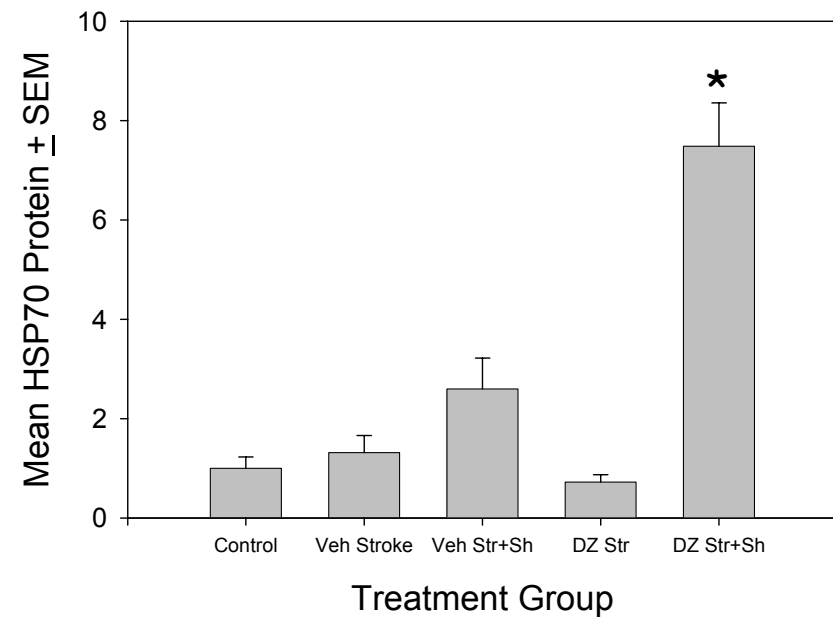


FIG. 6. HSP25 and HSP70 levels in the right cerebral cortex following postconditional treatment with DZ. HSP25 levels (top) in the DZ Str+Sh Group were significantly greater than levels observed in the Str Veh, Str+Sh Veh, and Str DZ20 Groups (but not different from the Str+Sh DZ60 Group; denoted by the # symbol). The symbol (^) indicates that HSP25 levels in the Str+Sh DZ60 Group were significantly greater than levels observed for the Str Veh and Str DZ20 Groups (but not the Str+Sh Veh or Str+Sh DZ20 Groups). Compared to levels in the Str Veh, Str+Sh Veh, Str DZ20, and Str+Sh DZ20 Groups, HSP70 levels (bottom, denoted by *) were significantly higher in the animals that received DZ as a postconditional stimulus (the Str+Sh DZ60 Group) and when given as a delayed preconditioning trigger (the DZ Str+Sh Group).

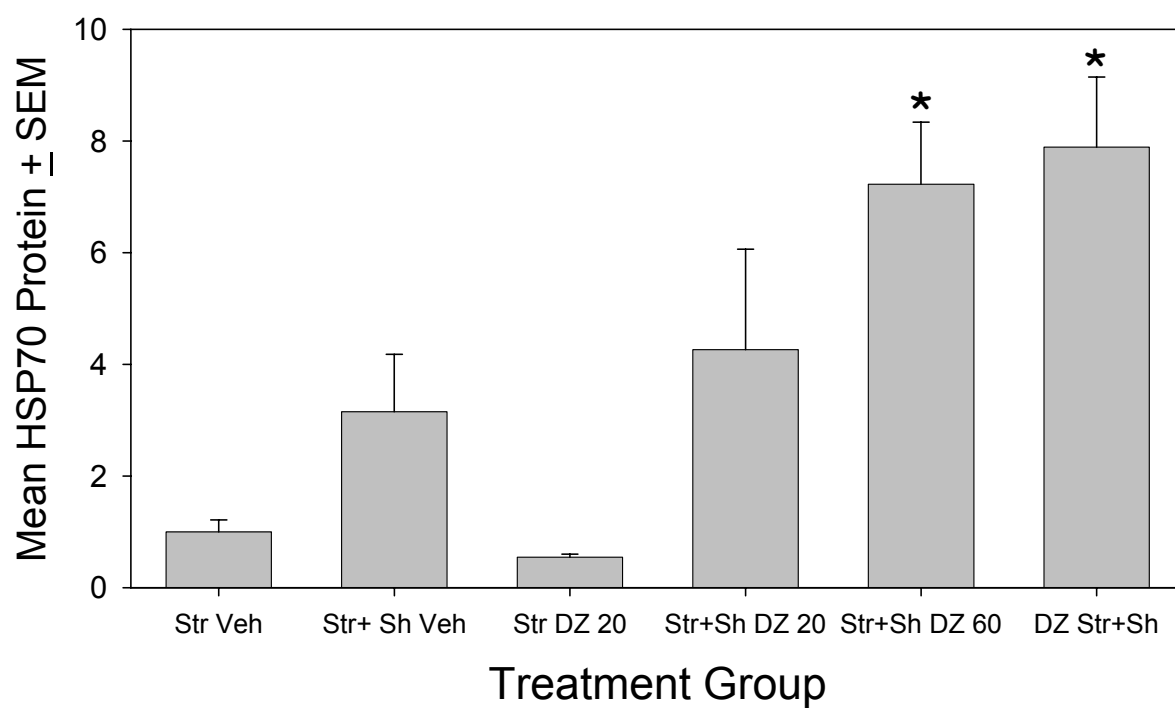
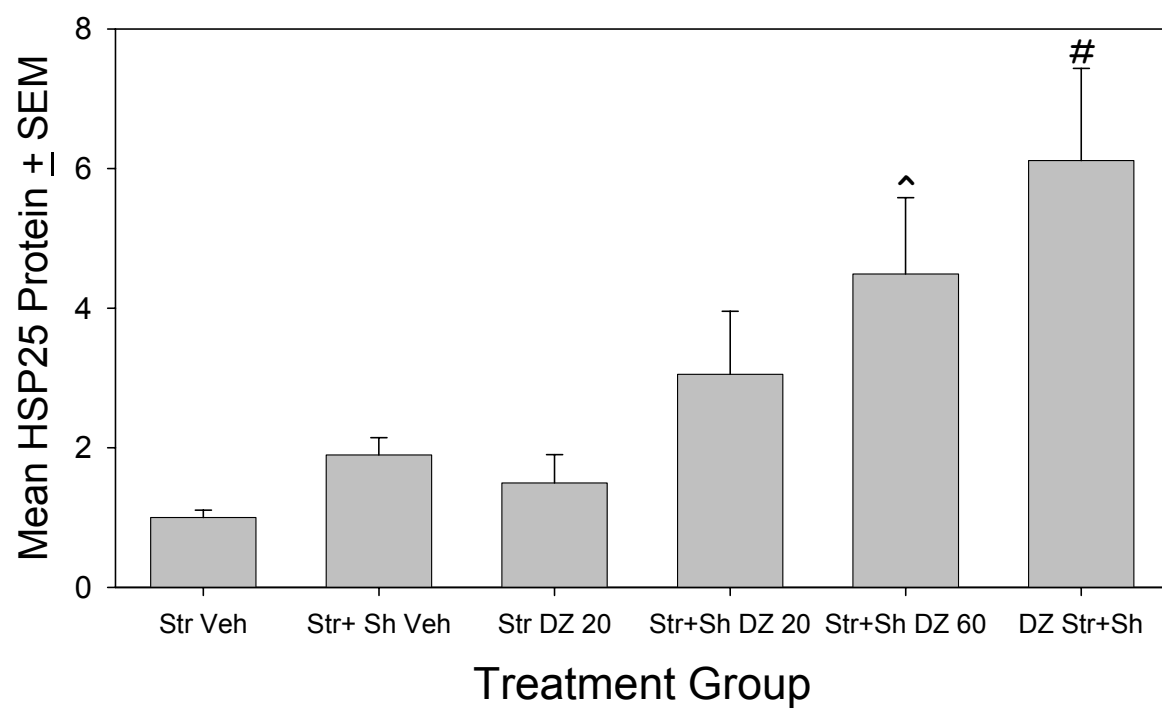


FIG. 7. Immunoblots of HSP25 and HSP70 in the right cerebral cortex and hippocampus of selected groups in the postconditional study. As indicated by the representative immunoblots, there was a noticeable increase in HSP25 (top three rows of blots) in the right cerebral cortex and hippocampus in the DZ Str+Sh and Str+Sh DZ60 Groups compared to the control and Str+Sh DZ20 Groups. HSP70 expression (bottom three rows of blots) demonstrates there was an increase in HSP70 levels in the DZ Str+Sh and Str+Sh DZ60 Groups, compared to the Str+Sh DZ20 Group.

RIGHT HIPPOCAMPUS

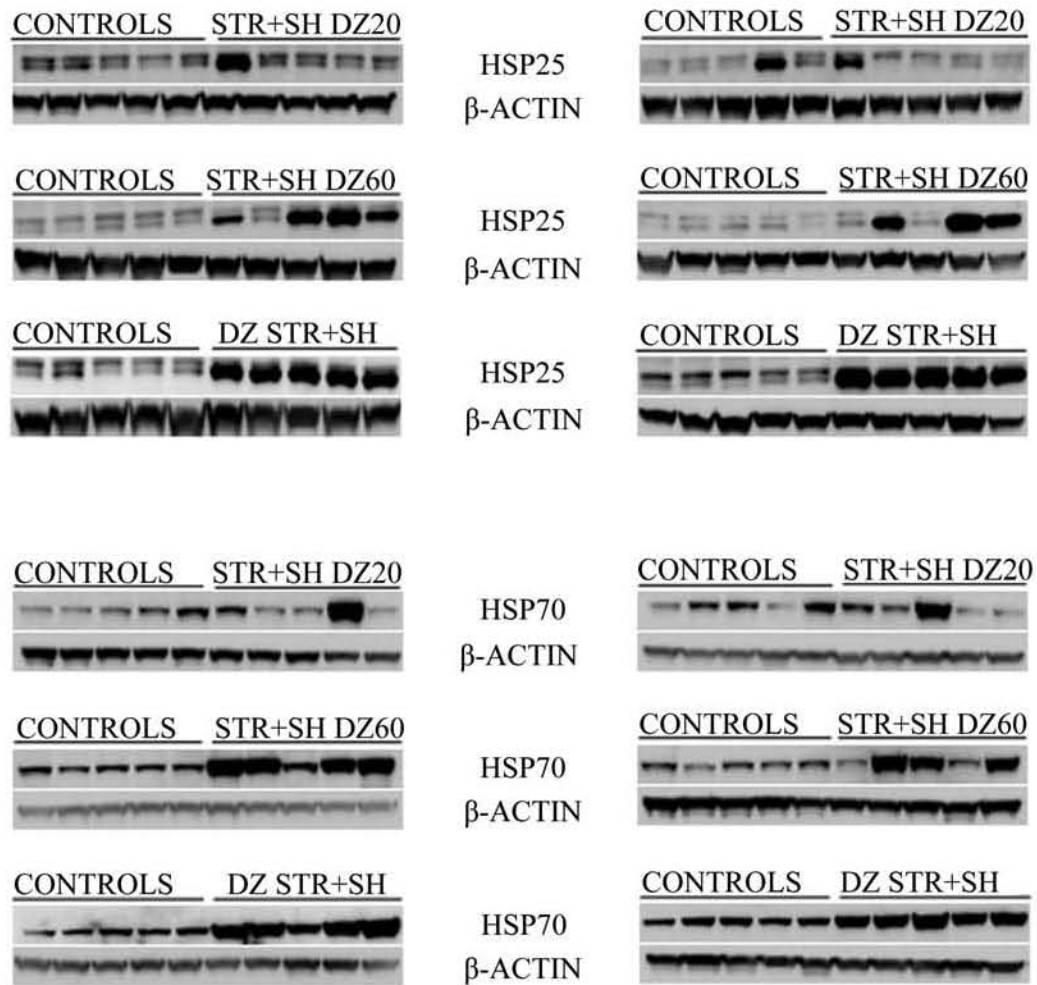
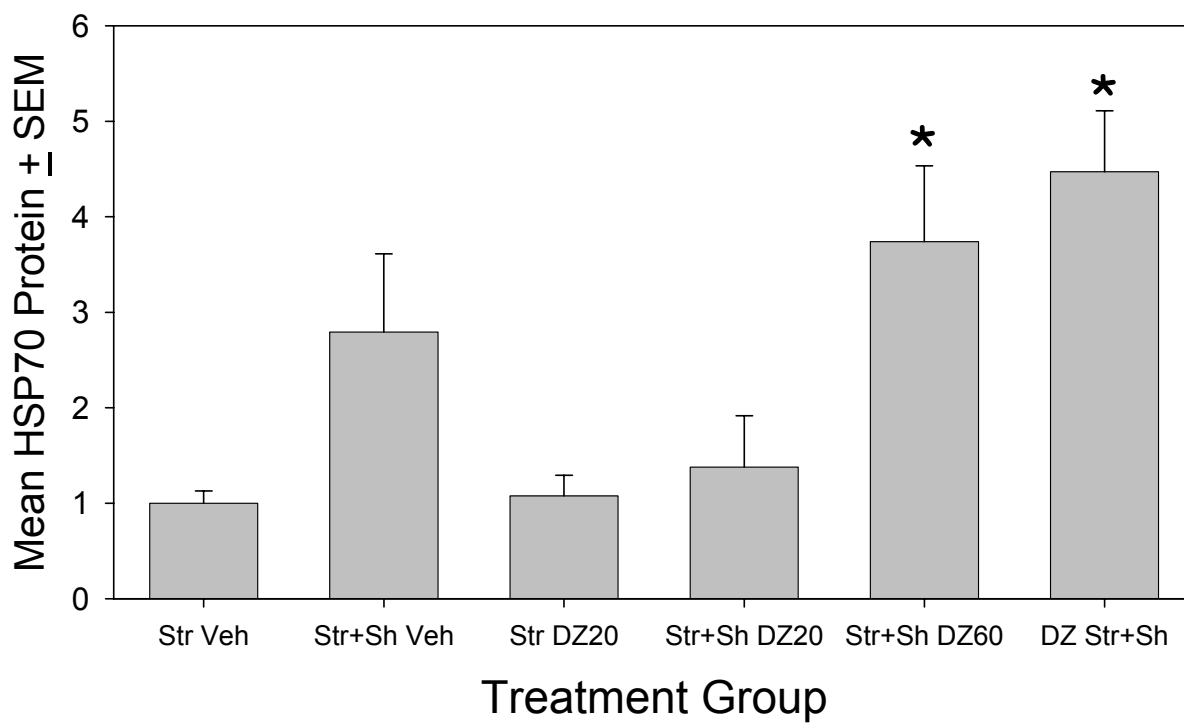
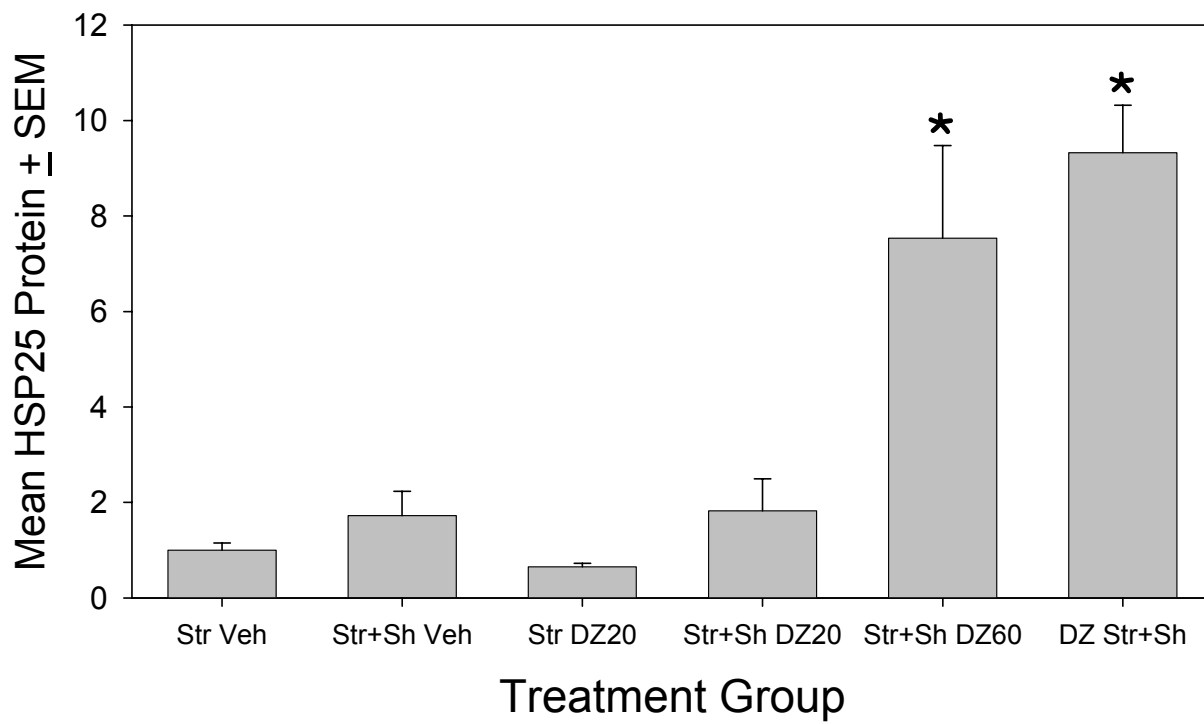


FIGURE 7

FIG. 8. HSP 25 and HSP70 levels in the right hippocampus when DZ was administered as a postconditional trigger. HSP25 expression (top; represented by the *) was significantly increased in Str+Sh DZ60 and DZ Str+Sh Groups of animals, compared to all other groups, while they were not different from each other. HSP70 levels (bottom; * symbol) were significantly greater in the Str+Sh DZ60 and DZ Str+Sh Groups than in all other treatment groups (except Str+Sh Veh), and were not different from each other.



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CHAPTER 5

SUMMARY AND CONCLUSIONS

Objective

Our goal when designing this study was to investigate, using a known pharmacological preconditioning agent, the possible positive effects of DZ in a hemorrhagic shock vs. shock and cerebral stroke model. Literature review indicated that this had never been investigated previously, yet basic research on the efficacy and further development of the application of pharmacologically-induced cytoprotection would have significant clinical implications. Epidemiological findings substantiate the magnitude of trauma incidence in the U.S. For example, three times as many American citizens die each year than were killed in the Vietnam conflict, trauma is the leading cause of death in the population between the ages of 1 and 44 [1], and—notably—the death rate from trauma continues to rise at the rate of about 1 percent per year, while deaths from heart disease and stroke has steadily declined over the past decades [2].

Trauma

Increasingly, military operations are occurring in remote locations where medics and nurses are the first-line responders for medical care of wounded personnel. As in many previous conflicts, methods of immediate care in the field are limited to portable items that ensure mobility. Classically, massive fluid resuscitation had been the standard for resuscitation in order to maintain adequate organ perfusion of vital organs—the brain, lung, kidneys and liver [3, 4]. However, postoperative complications related to this therapy, such as acute respiratory distress syndrome (ARDS), sepsis and acute renal

failure, increases morbidity and mortality [3]. Another option is hypotensive resuscitation which theoretically would limit the hemodilution of important clot forming components of the blood. Unfortunately, this therapy can result in poor oxygen delivery and acidosis and appears to result in no increased survival benefit [5]. Therefore, if a lightweight portable drug is found that could reduce morbidity or mortality from trauma it may also have a significant impact with civilian vehicle accidents or homicides as well, since they far outstrip battle casualties further adding relevance to this research.

Studies have shown that the mortality from trauma doubles when injury to the central nervous system occurs concurrently [6], and that vascular tone is adversely affected when brain injury is combined with hemorrhagic shock [7]. Our studies have shown that our hemorrhagic shock (Shock) model without stroke resulted in no deaths, but there was a dramatic increase in fatalities when shock was combined with stroke. Although the differences were not statistically different, a trend exists of decreased fatalities in animals that had been pretreated with DZ before sustaining combined Stroke + Shock.

Trauma and Lactate/Glucose Levels

Many studies have shown the beneficial effect of reduced lactate and glucose levels in post-trauma situations. Stacpoole *et al.* studied lactic acidosis levels in critically ill ICU patients and determined that lactic levels at or above 5mmol/L resulted in only 17% of the patients being discharged from the hospital, with only 21% leaving the ICU [8]. Hypoxic states normally lead to anaerobic metabolism with an increase in lactate

glycogenesis [9] and other studies have found that more severe injuries, such as trauma in combination with cerebral injury or ischemia, results in poorer outcome, which are often preceded by elevated plasma lactic acid and glucose levels [10, 11]. Our studies have demonstrated a significant decrease in blood plasma lactate levels during the shocked period in those rats that received DZ 24 hours prior. The reduced lactate generation during this pre-resuscitation period from DZ administration could have important clinical ramifications, although the practicality of this paradigm remains limited.

Perhaps more importantly, DZ has been shown to significantly decrease plasma glucose levels in shocked rats. Unlike plasma lactate levels, glucose levels remained elevated after resuscitation in those groups that did not receive DZ. Furthermore, DZ allowed a more rapid normalization of glucose levels after resuscitation than those groups that did not receive DZ. As discussed in Chapter 1, increased glucose levels are an independent predictor of mortality following trauma and results in poor outcome with brain injury [12].

Given the known hyperglycemic effect of DZ, finding a *decrease* in blood glucose levels after shock was surprising. There may have been a transient increase in blood glucose levels after injection but we did not test plasma glucose levels over the ensuing 24 hours. However, at the beginning of surgery, baseline glucose levels were measured and did not differ across groups, indicating that our dose of DZ probably did not suppress insulin secretion 24 hours after administration. In the postconditioning study (Experiment 2, Chapter 4), animals that were given DZ and sustained Stroke but

not Shock (the Str DZ 20 Group) exhibited an elevation in plasma glucose levels, compared to the Str Veh animals, and in comparison to their plasma glucose levels before DZ administration (the 0' preH and 10' postH time points, Figure 2 in Chapter 3). In animals that sustained Shock, DZ's known effect on the energetics of the mitochondria was probably a key factor in significantly reducing blood glucose levels. It has been proven that DZ protects the mitochondria from anoxia [13], therefore it is assumed that blood glucose levels are decreased due to the mitochondria being less damaged from hypoxia than their counterparts that did not receive DZ.

Heat Shock Proteins

Heat shock proteins (HSPs), perhaps better termed *stress proteins*, are a family of highly conserved inducible proteins associated with adaptation to cellular stress and injury [14]. A multiplicity of functions have been ascribed to stress proteins, including the prevention of protein denaturation and incorrect polypeptide aggregation during exposure to cellular insults [15], molecular chaperone functions, where they assist in the folding, assembly and transport of proteins across membranes or to the proteasome for destruction. They function as direct caspase and caspase-independent pathway inhibitors [16], and as cytoskeleton (f-actin)-stabilizers. As the summaries of the experiments below indicate, DZ administration in a Shock or Stroke + Shock model had a significant effect on the upregulation of key stress proteins, implying it may have a significant effect in promoting cell survival or reducing the stress of ischemia.

Experimental Summaries Review

Experiment 1-Shock only

Experiment 1 involved hemorrhagic shock without stroke and the brain tissue from these rats was examined for histological analyses. As mentioned earlier, none of the rats in this study died and—perhaps due to the mammalian compensatory mechanisms which protect the primary organs (brain, heart and lungs) in hypovolemic shock—we found there was no substantial alteration in staining for a range of markers (immunocytochemistry for HSP25, HSP70, caspase 3, c-Jun and p53; and gross assessment of neuropathology by cresyl violet staining). However, some key facts were determined. First, DZ administration 24 hours before shock reduced lactate plasma levels during the 1-hour period of hemorrhagic shock. Second, in animals that were exsanguinated, DZ treatment, compared with untreated animals, significantly reduced plasma glucose levels. Third, we observed that as shock progressed, exsanguinated animals exhibited partial mean arterial pressure (**MAP**) compensation, although they never re-attained their baseline MAP levels. Lastly, DZ preconditioning had no apparent effect on MAP during shock and during resuscitation.

Experiment 2- Stroke + Shock (Delayed Preconditioning)

Experiment 2 examined the effects of delayed preconditioning with DZ in animals that sustained combined right cerebral stroke and hemorrhagic shock. Compared to results when animals experienced shock alone, DZ administration to animals that sustained Stroke + Shock was not able to significantly affect blood lactate levels. This

has been interpreted as a demonstration of the increased metabolic severity arising from this multi-trauma, and it may indicate that under these circumstances delayed preconditioning is less able to modify organism adaptation. On the other hand, it is possible that a higher dose of DZ may have been effective in reducing increased lactate levels. It should be kept in mind, however, that in addition to added deficits in compensatory autonomic responses to exsanguination, these animals actually sustained additional tissue damage. The ischemic stroke would have also generated an additional lactate load. In contrast, DZ preconditioning had a significant impact upon plasma glucose levels. Glucose levels remained elevated in the Stroke + Shock group of animals that did not receive DZ.

Three other important points can be gleaned from this experiment. First, although there was no significant difference in MAP between groups that received DZ and those that did not, there was a significant difference noted between this model (Stroke + Shock) vs. Shock alone. The addition of the stroke component to this model blunted the compensatory mechanism after hypovolemia, such that MAP compensation only attained 40mm/Hg prior to resuscitation. Second, in those groups that received DZ 24 hours prior to stroke and shock, there was a significant increase in HSP25 and HSP70 expression in the liver, kidney, right cerebral cortex and right hippocampus. As expected, there was no upregulation of HSP25 or HSP70 in the left cerebral cortex. Lastly, there seems to be a trend of increased survival in those rats that received DZ 24 hours before stroke and shock and those that did not (Vehicle Stroke + Shock) although it did not reach significance ($p=0.058$).

Experiment 3- Dose Tolerance Study

This study was undertaken to determine whether DZ administration to animals that had sustained Stroke + Shock would exacerbate hypotension. We believed that if we administered DZ slowly (over 10 minutes) starting approximately 10 minutes after exsanguination, that the rats could tolerate DZ. Clinically, DZ is given quickly (over 30 seconds) to reduce MAP. Our criteria was that if the rats experienced a 25% reduction in MAP from the initiating time frame (10 minutes post exsanguination) or any later time point, then the experiment would be repeated with a 50% reduction in the dose of DZ. Our results indicated there was no change in MAP when animals were given a dose of DZ of 3.2mg/kg IV given over a 10-minute period.

Experiment 4- Stroke + Shock (“Postconditional” Preconditioning)

Experiment 4 examined the utility of DZ when given at two different time points *after* Stroke + Shock to examine the effect of DZ in a postconditioning study. To confirm our earlier results, we also included animals that received DZ 24 hours prior to Stroke + Shock. As in Experiment 2, DZ did not have a significant effect on MAP, nor lactate plasma levels, but glucose plasma levels remained elevated in the Stroke + Shocked groups. In addition, there was a steady increase in glucose levels in animals that were not exsanguinated and received DZ 20 minutes after the start of the experiment. As mentioned earlier, it was expected that DZ, administered 20 minutes after the onset of shock, would increase heat shock protein expression. However, DZ administration was only effective when it was given 1-hour later, just when resuscitation commenced. Our

results, therefore, are similar to other research, which has shown that postconditioning is only effective when it occurs immediately prior to reperfusion [17,18,19].

It should be mentioned that in all of the experiments reviewed here, we observed variability in the expression levels of the HSPs. We believe this was due to the fact that variability arises from the intrinsic complexity of the animal models and that there would be some degree of genetic variability and consequential response to trauma. In no treatment group did we observe a consistent response (level of HSP protein) in all animals. For example, in the Str+Sh DZ60 Group only seven out of ten rats were observed to exhibit an upregulation of HSP25 and HSP70; in the DZ Str+Sh Groups, it was approximately 8-9 out of 10; and in the Veh Str+Sh Groups, approximately 3-4 out of 10 expressed upregulation of the stress proteins. As previously described in this thesis, outliers, which were defined as a z value greater than 2.5, were removed from the study.

Cerebral Edema

A criterion was established that a rat survive for 24-hours post-procedure in order to be included in these studies. In the Stroke + Shock studies, rats that did not survive the required 24 hours were examined for cause of death. At autopsy, right cerebral edema was observed. Figure 1 is a photo from an animal that expired soon after surgery. Note the increased cerebral edema and paler coloration of the right cerebral hemisphere, which had sustained combined Stroke + Shock. Severe right cerebral edema leads to increased intracranial pressure, respiratory depression, and death. Often death was preceded by changes in respiratory pattern and seizures and direct observation of these rats prior to

death (when observed) revealed a very rapid respiratory rate (150-200/min) followed later by profound respiratory depression (20-30/min; the mean respiratory rate for a healthy rat is 70-115/min). Research shows that endothelial gaps related to an inflammatory stimulus such as hemorrhagic shock, leads to plasma leakage (failure of $\text{Na}^+ - \text{K}^+$ ATPase pumps) and result in expanded intracranial pressure, pulmonary, intestinal or cerebral edema and can often result in death [20, 21, 22]. No gross changes were noted in the organs of the abdominal and chest cavity although post-mortem evaluation of pulmonary edema, other lethal tissue edema or infections cannot be ruled out.

Importantly, other research has shown that DZ preconditioning, with reperfusion after just 30 minutes of ischemia, decreases cerebral edema formation and likely decreases blood-brain barrier disruption [23]. The present model of severe shock combined with ligation of the ipsilateral carotid artery, provides a robust cerebral ischemic model. There was no noticeable right-sided cerebral edema of the harvested rats at the 24 hour time point, although specific tests for cerebral edema were not performed. It is assumed, however, that despite the lack of gross cerebral edema in survivors, some cellular leakage, disruption of the blood brain barrier, or increased intracranial pressure was sustained.

Behavior Tests

Ligation of the right femoral artery and vein resulted in right leg ischemia, with a bluish hypoxic color, requiring analgesia (Buprenex) doses until harvest. Due to the

ischemic limb, any test for examining locomotion or balance such as the *rotor rod test* or *balance beam test* could not be used to assess stroke damage. Another test considered for use that judges the motor function (front limbs) is called the *forelimb placing test*. The *forelimb placing test* consists of brushing the vibrissae on the corner of a countertop edge while holding the rat by their torso. Intact animals place the forelimb ipsilateral to the stimulated vibrissae quickly on the countertop [24]. Our results were inconclusive, possibly due to the effect of the analgesia, the anemia from shock, weakness from lack of appetite post-surgery (weight loss was noted in all rats from day of surgery to harvest), possible painful tension on the midline neck incision, which would occur if the rat reached up to the table, or a true sensory/motor component to the cerebral ischemia from the stroke. Therefore, behavior tests that attempted to assess ipsilateral motor or sensory stroke damage were inconclusive.

Immunocytochemistry¹

Immunocytochemistry results of HSP25 of cerebral cortex tissue (Figure 2) and hippocampus (Figure 3) demonstrates some key facts about the location and morphology of HSP25 expression. No cells in the left cerebral cortex or left hippocampus in any group were noted to express HSP25. In the brains from animals that sustained Stroke + Shock, numerous cells, occurring in focal areas in the right cerebral cortex and right hippocampus, were observed to stain for HSP25. Although no double-staining procedures were undertaken for verification, it appeared that the HSP25-positive cells were astrocytic in morphology. This observation would be consistent with earlier

¹ Immunocytochemistry studies were supported in part by a USUHS Graduate Student Dissertation Award, T270PG.

research, which demonstrated HSP25 is expressed primarily in GFAP-staining astrocytes when the brain was exposed to stress [25, 26].

Examination of the HSP70 immunocytochemistry staining in the cerebral cortex (Figure 4) and hippocampus (Figure 5) confirms that the Stroke + Shock model elicited staining almost exclusively in the cerebral hemisphere that sustained ipsilateral hypoxia and ischemia. These figures confirm the location and morphology of primarily neuronal upregulation of HSP70. Generally, the neurons that express HSP70 appear in focal isolated areas, although dense global areas of HSP70-positive cells in the right cerebral cortex and right hippocampus have also been noted. However, the left cerebral cortex and hippocampus in all rats (n=16) examined had no staining for HSP70, whereas the ipsilateral cerebral cortex and hippocampus (right hemisphere) demonstrated significant upregulation of HSP70 in neuronal-appearing cells. Due to time constraints, and the fact that quantitative western blotting was a more efficient means of determining protein changes, no attempt was made to quantify these results through immunocytochemistry studies.

Clinical Implications

Although the current research offers some very interesting findings, it would be premature to expect direct clinical ramifications. Research using animal models has often yielded very promising results only to lead to failures in human trials. However, on the positive side, DZ has been used safely for years in humans (albeit for different reasons) with minimum sequelae. Clinically, DZ could easily be utilized, with proper

screening of patients, in a controlled clinical situation. For example, surgical candidates that are going to receive major kidney, liver or brain surgery could be screened for contraindications to DZ. If acceptable, it would be very easy to administer one dose of DZ 24 hours prior to surgery to upregulate key stress proteins, which could help mitigate cellular insults in these key organs. As mentioned earlier, physicians have already used the pre-clamping method of classical preconditioning in liver and cardiac surgeries resulting in positive effects [27].

A second clinical application that could be life-saving would be to employ DZ for hemorrhagic shock. DZ use in this instance, however, is riskier. Although it was shown in rats that slow administration of DZ was beneficial, especially when presented slowly after hemorrhagic shock, the risk may be too high to attempt in humans. Emergency medical personnel, acting in the fast paced ‘life saving mode’ for trauma, would inevitably give DZ rapidly, causing irreparable harm. Diluting DZ in the resuscitation fluids may solve the problem, but this was not tested in this model, although DZ IV infusion has been tested with positive results in a cardiopulmonary bypass model [28]. However, there is a new mK_{ATP} channel opener called BMS-191095, which seems to also possess ischemic preconditioning qualities without hypotensive effects [29]. It may be fruitful to repeat these experiments using this new drug, or DZ diluted in a resuscitation fluid in rats (and other species) before considering human applications in trauma.

Future Studies

Much work remains to be done and can be divided into 3 major areas; *drugs*, *models* and *tests*. As was just mentioned regarding *drugs*, it may be fruitful to examine a different mK_{ATP} channel opener that also crosses the blood brain barrier but does not possess the possible hypotensive or hyperglycemic properties of DZ. The current K_{ATP} channel openers include cromakalim, pinacidil, minoxidil (Rogaine), aprikalim, nicorandil, and DZ, as well another compound, BMS-191094, which is still in development. These drugs vary in their effect on the mK_{ATP} channel vs. cell membrane K_{ATP} channel, but all confer the preconditioning effect [30, 31, 32]. Concurrent application of mK_{ATP} channel inhibitors (e.g. tests with 5-HD) or succinate dehydrogenase inhibitors (3-NPA) alone in some groups could better define the channel function in protecting cells in the face of trauma and differentiating the protective action of DZ. Also, this particular study utilized normal saline as the primary resuscitation fluid but other fluids are commercially available, are utilized, have an impact on protein expression during hemorrhagic shock [33], and should be investigated with DZ.

There are changes to the *model* which could yield valuable insight into the drug effects. For example, valuable information could be determined if the model was extended out from 1 hour post-shock to 2 or 4 hours to find the therapeutic window of DZ with resuscitation. Also, although it was an unexpected finding, this model could be repeated with more rats; allowing investigators to more fully assess if DZ improves survival. Another problem to consider investigating through a model change is the species. Although DZ seems to have an effect with the rat model, is the same true for

rabbits or dogs? Species variability must be taken into account and tested before human applications.

This study *tested* the upregulation of two key stress proteins (HSP25, HSP70), but other anti-apoptotic proteins should be examined. Behavior tests were not done in our study primarily due to ischemic right leg secondary to arterial ligation. Behavior tests would be valuable to incorporate into the study to determine if DZ has an impact on motor skills/brain function, but this would entail a change in the model to achieve arterial access while sparing the femoral artery. Another test that should be considered is mitochondrial DNA/RNA function. DZ (and mK_{ATP} channel openers) function primarily on the mitochondria. What changes occur in trauma in the mRNA expression with and without DZ in the mitochondria?

In conclusion, this study has been only the first small step in studying the use of a pharmacological ischemic preconditioning agent in a hemorrhagic shock or combined stroke and shock model. Although the results are very encouraging and important, there is much work to be done before the transition can be made from bench to bedside. As research continues into the pathway and major players of ischemic preconditioning and postconditioning, we will move closer to formulating an intervention or better therapeutic agent that can save and improve people's lives—which makes all this worthwhile.

Fig 1. **Photo of cerebral cortex of non-survivor (rat).** This is a typical photo of a rat that did not survive the 24 hours necessary to be included in the study. Note the significant right-sided edema secondary to stroke and shock. It is assumed that the non-survivability was caused by increased ICP, leading to respiratory depression and death.



Fig 2. Immunocytochemistry HSP25 of right and left cerebral cortex of a rat subjected to stroke and shock. No expression of HSP25 is noted in the left cerebral cortex. HSP25 expression in the right cerebral cortex appears to be primarily located in astrocytic looking cells.

Cerebral Cortex- Shock + Stroke- HSP25

LEFT CEREBRAL CORTEX



RIGHT CEREBRAL CORTEX



Fig. 3. **Immunocytochemistry HSP25 of right and left hippocampus of a rat subjected to stroke and shock.** No expression of HSP25 is noted in the left hippocampus. HSP25 expression in the right hippocampus appears diffuse and located primarily in astrocytic looking cells.

Hippocampus- Stroke and Shock- HSP25

LEFT HIPPOCAMPUS



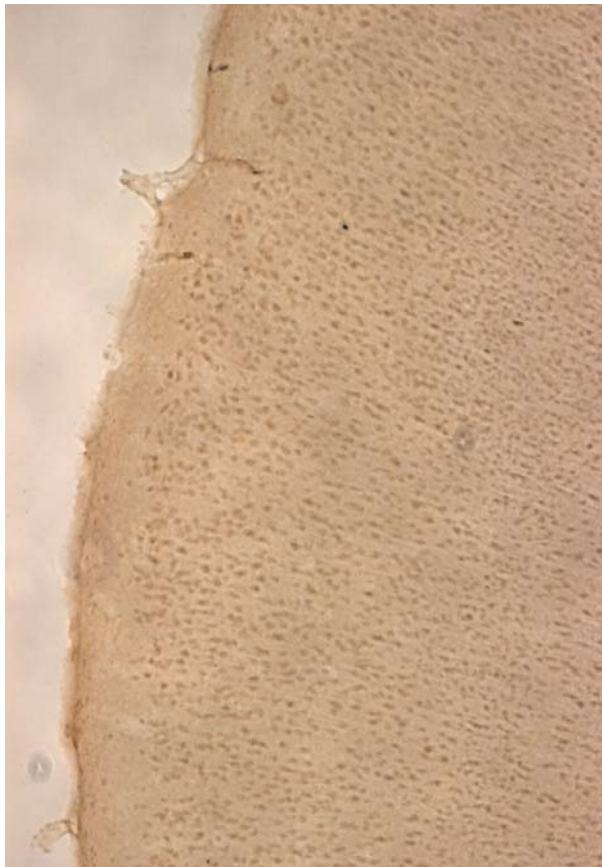
RIGHT HIPPOCAMPUS



Fig 4. Immunocytochemistry HSP70 of right and left cerebral cortex of a rat subjected to stroke and shock. No expression of HSP70 is noted in the left cerebral cortex. HSP70 expression in the right cerebral cortex appears in focally centralized areas in neuronal looking cells.

Cerebral Cortex- Stroke + Shock- HSP70

LEFT CEREBRAL CORTEX



RIGHT CEREBRAL CORTEX

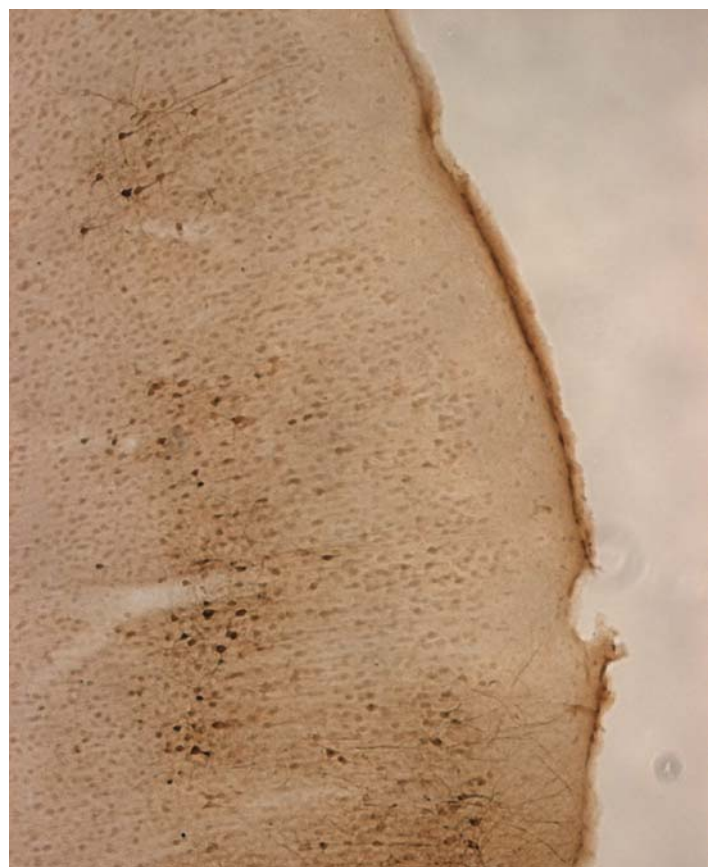


Figure 5. **Immunocytochemistry HSP70 of right and left hippocampus of a rat subjected to stroke and shock.** No expression of HSP70 is noted in the left hippocampus. HSP70 expression in the right hippocampus appears to be in focally centralized areas in neuronal looking cells.

Hippocampus- Stroke + Shock- HSP70

LEFT HIPPOCAMPUS



RIGHT HIPPOCAMPUS



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