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16

Preconditioning and Postconditioning

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1. Introduction

Cerebral ischemic events from trauma, stroke, hemorrhagic shock, or other cerebral perfusion deficit, initiate a cascade of detrimental processes leading to long lasting tissue injury and poor neurological outcome. Correction of the perfusion deficit is vital. However, no interventions have been identified that protect compromised cerebral tissue during the resolution of the ischemic event.

This chapter reviews two emerging concepts: *preconditioning*, which may have therapeutic utility for the protection of patients for *planned* treatments such as surgical intervention, and *postconditioning*, which may have benefits for amelioration of deficits from ischemic events, vascular injury and accidents. Preconditioning (described below) was first described from maneuvers that induced cytoprotection by temporarily occluding vessels serving the tissue or region of interest. There are inherent risks in performing such a maneuver, so that pharmacological agents—particularly for *delayed preconditioning* (described later)—affecting the aforementioned signal transduction and genomic pathways, are a safer, more realistic area of study. Conditioning is still primarily an experimental phenomenon. However, investigators have made considerable strides in uncovering the multiple, albeit complex signal transduction pathways that mediate conditioning effects. This knowledge may help clinicians one day develop schemes for neuroprotection.

2. Ischemic preconditioning: Origins in cardiology

The preconditioning concept has roots in cardiovascular research. Ischemic preconditioning was originally described in a landmark 1986 study by Murry and associates (Murry, et al., 1986). 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 ¼th of the infarct observed in the hearts of dogs in the control group. This paper was the first to demonstrate and coin the term, *ischemic preconditioning*.

2.1 Preconditioning

Preconditioning 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 (Peralta, et al., 1996; Glazier, et al., 1994; Pang, et al., 1995). Other preconditioning manipulations, such as global hypoxia (Emerson, et al., 1999) and thermal injury (Marber, et al., 1993), are also effective in protection from lasting insult. Prior to this 1986 discovery, the best pharmacological treatments (with varying reproducible results) for the protection of cardiac muscle from infarction only preserved 10-20% of tissue compared to the 75% protection afforded by preconditioning (Yellon & Downey, 2003).

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 (Ishida, et al., 1997). 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 (Carini, & Albano, 2003). Investigations have shown many triggers can activate classical ischemic preconditioning, including agonists of G protein-coupled receptors [bradykinin (Goto, et al., 1995), opioids (Schultz, et al., 1998), norepinephrine (Hu & Nattel, 1995), adenosine (Liu, et al., 1991), potassium ATP channel (K_{ATP}) openers, such as diazoxide, pinacidil (Legtenberg, et al., 2002), succinate dehydrogenase inhibitors, such as 3-nitropropionic acid (Ockaili, et al., 2001), and volatile anesthetics, such as sevoflurane and isoflurane (Zaugg, et al., 2002)].

With classic preconditioning, a trigger event, such as brief ischemia, activates a number of intracellular pathways that lead 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 (ε) isoform of protein kinase C (PKC-ε; Mitchell, et al., 1995; Kilts, et al., 2005), which has been implicated as the key PKC subtype involved in preconditioning. Also important is the upstream signaling molecule, phosphatidylinositol-3-kinase (PI3K; Tong, et al., 2000), and mitogen-activated protein kinases (MAPKs; Armstrong, 2004). PI3K activates the serine/threonine kinase, Akt, which inactivates the pro-apoptotic kinase glycogen synthase kinase-3 (GSK-3) via phosphorylation (Tong, et al., 2002). 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 membranes of mitochondria. 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 (Kadenbach, et al., 2011; Penninger, & Kroemer, 2003). K_{ATP} channels are key intracellular triggers of early ischemic preconditioning. This channel will be described in more detail later in this chapter.

Although not as effective as classical preconditioning, *delayed* or *late* preconditioning becomes apparent approximately 24 hours after initial preconditioning and it can persist for up to 72 hours (Ishida, et al., 1997). The most significant difference between classical and delayed preconditioning is the latter's requirement of the manufacture of new proteins needed to obtain protection; inhibition of new protein synthesis attenuates the protection derived from delayed preconditioning (Rizvi, et al., 1999).

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 (Bolli, 2000). Endogenous preconditioning agents also initiate delayed preconditioning and include adenosine agonists, bradykinin, opioids, NO donors, acetylcholine and norepinephrine (Bolli, 2000). Exogenous agents that activate the preconditioning pathways include, diazoxide, nicorandil, some hypercholesterolemic agents, and volatile anesthetics (Gross, 2005).

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-κB, which leads to the upregulation of many protective proteins in the nucleus. Some key proteins identified thus far include: inducible NO synthase (iNOS) (Takano, et al., 1998, cyclooxygenase (COX-2) (Shinmura, et al., 2000), the antioxidant enzyme superoxide dismutase (SOD) (Hoshida, et al., 2002), and heat shock proteins, HSP70, HSP25, and HSP32 (Bolli, 2000). 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 (Maulik, et al., 1999). Mitochondrial K_{ATP} (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 (Takano, et al., 2000).

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 (Przyklenk, et al., 1993). 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 (Addison, et al., 2003). Remote classical preconditioning of the heart was also obtained via transient ischemia of the small intestine (Liem, et al., 2005; Patel, et al., 2002) or the kidney (Gho, et al., 1996). Other studies have demonstrated remote delayed preconditioning. Induction of small intestine ischemia, for example, engenders myocardial protection 24-72 hours later (Wang, et al., 2001; Xiao, et al., 2001).

The precise 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 (Gho, et al., 1996). 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 (Dickson, et al., 1999a; 1999b). Remote preconditioning was not activated by adenosine or bradykinin, but was found to be attenuated by the opioid antagonist, naloxone, suggesting opioid receptor involvement (Dickson, et al., 2001).

2.2 Postconditioning

One promising approach for neuroprotective therapies may be derived from *postconditioning*, where supportive measures are employed following an injury. Postconditioning is very similar to preconditioning with the exception of the temporal relationship of the protective maneuver in respect to the prolonged period of ischemia. Preconditioning is an intervention that occurs *prior to* injury while postconditioning interventions occur *after* an injury has occurred and thus may be more clinically relevant. The origin of postconditioning stems from the work of Okamoto and colleagues (Okamoto, et al., 1986). Okamoto's group established that post-ischemic damage could be limited by the use of timely low-pressure reperfusion. Following a period of ischemia, dog hearts were reperfused either with the sudden release of a coronary occlusion, or by low-pressure (40 to 50 mm Hg) coronary reperfusion with normal blood for 20 minutes before completely removing the coronary occlusion. This maneuver focused on the initial stage of reperfusion and established the basis for novel postconditioning approaches to resuscitation. Years later, Mizumura et al. (1995) first demonstrated pharmacological postconditioning. Mizumura's group used the K_{ATP} channel opener, bimakalin. Bimakalin markedly reduced cardiac infarct size in dogs when given 10 minutes before and during the 60-minute coronary reperfusion period following a set time of occlusive hypoxia. Collectively, these initial findings firmly established the concept of postconditioning and emphasized a critical factor that altering the initial moments of reperfusion was beneficial.

In 2003, Zhao and colleagues (2003) first used the term *postconditioning*. They found in a model of occlusive hypoxia in dogs that short, repeated (or stuttered) periods of arterial occlusion and release of previously occluded coronary arteries (three occlusions of 30 seconds each) prior to restoration of perfusion reduced infarct area by 44% as compared to controls. This was an example of a mechanical postconditioning intervention and implied that the first minute of reperfusion is critical in thwarting cellular demise. These findings were further validated by Kin et al. (2004). Kin and colleagues stated that the first minute of reperfusion in the rat was crucial for postconditioning. Three cycles of 10 seconds of coronary occlusion and 10 seconds of coronary release, preceding a full coronary occlusion release, decreased cardiac infarct size by 23%. In the broadest sense, the cellular processes activated by postconditioning are analogous to those activated by preconditioning, and the sole difference between the two interventions is the timing related to the prolonged period of ischemia.

3. Signaling processes linked to preconditioning and postconditioning

Many studies have investigated the mechanism of postconditioning with the aspiration to utilize this powerful protective system in the clinical setting. Recent observations from the

employment of mechanical and pharmacological postconditioning suggest the activation of mitochondrial K_{ATP} (mK_{ATP}) channels initiates a series of events that close the mitochondrial permeability transition pore (mPTP) and converge onto the Reperfusion Injury Survival Kinase (RISK) Pathway.

3.1 K_{ATP} channels

K_{ATP} channels were first discovered in 1983 by A. Noma in a patch clamp study using cardiac muscle membrane preparations (Noma, 1983). There are cell surface K_{ATP} (sK_{ATP}) and mitochondrial K_{ATP} (mK_{ATP}) forms. Pharmacologically, these are different channels, but their opening (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 (Kirino, 2002). In 1997, Garlid and coworkers presented evidence that mK_{ATP} channels have a cardioprotectve role in ischemia and reperfusion, and were a component in the mechanism for preconditioning (Garlid, et al., 1997). A prototypical mK_{ATP} channel, as reviewed by Aguilar-Bryan and Bryan (1999), is an octameric structure consisting of four sulfonylurea receptor (SUR1 or SUR2) subunits and four K^+ inward-rectifying (Kir6.1 or Kir6.2) subunits. Attached to the SUR subunits are two nucleotide binding domains (NBD). The mK_{ATP} channel is activated in low energy states by ADP, binding to NBDs, allowing the influx of K^+ into the mitochondrial inner matrix. Conversely, the mK_{ATP} channel is inhibited in high energy states when ATP closes the Kir channel. In the brain, it appears the predominant subtypes are SUR2 and Kir6.2, although the SUR1 and Kir6.1 subunits are present in smaller amounts (Lacza, et al., 2003). The mK_{ATP} channel may trigger preconditioning or postconditioning via mechanisms dependent on matrix volume stabilization, respiratory inhibition, controlled production of reactive oxygen species (ROS), and the closure of the mPTP.

The physiological functions of mK_{ATP} channels have been debated. The activities of the mK_{ATP} channel and the K^+/H^+ exchanger are believed to maintain K^+ homeostasis within mitochondria by controlling mitochondrial volume and moderating the outer-to-inner pH gradient needed to drive ATP synthesis. In the presence of hypoxia, whole cell pH decreases and ATP production declines. This causes a switch to anaerobic metabolism. A decrease in pH combined with an increase in the AMP/ADP ratio secondary to ATP metabolism causes the mK_{ATP} channel to open allowing the influx of K^+ into the inner matrix. This, in turn, activates the K^+(out)/H^+(in) exchanger decreasing the hydrogen gradient between the outer membrane and inner matrix (Szewczyk & Marbán, 1999). By doing so, the proton motive forces driving ATP production are attenuated and mitochondria energetics slow. During this time, the mPTP is closed as membrane stability and electrical potential are better maintained by the simultaneous activity of the mK_{ATP} channel and the K^+/H^+ exchanger. In addition, reactive oxygen species (ROS) generation is proportional to the availability of oxygen and activation of mK_{ATP} channel appears to moderate ROS production (Ferranti, et al., 2003; Saitoh, et al., 2006). However, this effect is only protective to a limited extent. For example, a moderate or controlled production of ROS signals promote prosurvival signaling while excessive ROS production promotes apoptotic signaling.

As anaerobic metabolism continues in response to an extended period of severe hypoxia, ATP hydrolysis exceeds ATP generation causing a dramatic rise in H^+ within the cell and mitochondrial inner matrix. At some point H^+ entry becomes lethal as it exceeds the outward pumping capacity of the mitochondrial electron transport chain already hindered

by anaerobic metabolism. This results in a total loss of proton motive force driving ATP production. If prolonged, this loss results in osmotic matrix swelling, mitochondrial degradation, and release of apoptotic proteins such as cytochrome C.

Upon resolution of a perfusion defect, abrupt reperfusion following prolonged ischemia results in a substantial amount of ROS generation. Following restoration of flow to intact but vulnerable cells, ATP levels begin to rise, mK_{ATP} channels close and K^+ transport into the mitochondrial matrix declines. This indirectly decreases the activity of the K^+/H^+ exchanger. As H^+ ions are rapidly removed from the matrix during mitochondrial respiration, the inner matrix quickly alkalinizes, causing the mPTP to open (Vinten-Johansen, et al., 2007). Opening the mPTP rapidly elevates inner matrix osmotic pressure leading to matrix distension and if allowed to remain open, mitochondrial rupture.

In total, mK_{ATP} channel closure associated with abrupt reperfusion cand result in the significant elevation of ROS and increase the mitochondrial inner matrix osmotic pressure, causing the mitochondria to quickly swell or rupture, releasing apoptotic factors such as cytochrome C (Armstrong, 2004). It is reasonable to suggest that maintaining the patency of the mK_{ATP} channel would be beneficial during reperfusion. Allowing the mK_{ATP} channel to remain open during reperfusion could: 1) moderate the generation of ROS, 2) reduce osmotic force within the matrix by promoting ion exchange, and 3) reduce the activity of the mPTP thereby providing a protective effect.

Activation of mK_{ATP} channels have been shown to be protective during reperfusion in cardiac and brain tissue (Obal, et al., 2005; O'Sullivan, et al., 2007; Penna, et al., 2007; Wu, et al., 2006). Obal and colleagues (2005), for example, demonstrated the utility of inhaling volatile anesthetics as a postconditioning trigger through mK_{ATP} channel activation. In rats subjected to cardiac ischemia, postconditioning was invoked by administering 1 minimum alveolar concentration (MAC) of sevoflurane for 2 minutes with the onset of reperfusion. This resulted in a significant decrease in cardiac infarct size. Penna et al. (2007, isolated rat hearts and exposed them to an ischemic period followed by reperfusion. Their results suggested that postconditioning mechanisms are activated by a bradykinin or a diazoxide mechanism resulting in the upregulation of protein kinase G (PKG). This upregulation was dependent on early ROS generation triggered by mK_{ATP} channel activation. They emphasized that their results were different from mechanical manipulations by showing that pharmacological agents, such as bradykinin or diazoxide, administered during the reperfusion period could induce protection. ROS also regulate the activity of heat shock proteins (HSPs). Using an *in vitro* vascular smooth muscle preparation, Madamanchi and colleagues (2001) discovered that the application of H_2O_2 significantly upregulated HSP70.

3.2 mPTPs

As previously mentioned, the mPTP is inhibited with the activation of mK_{ATP}. The existence of the mPTP was confirmed in 1992 in rat liver mitoblast membranes (Szabó & Zoratti, 1992). The primary components of the mPTP are the voltage-dependent anion channel in the outer membrane, the adenine nucleotide translocator, and the cyclophilin D protein within the matrix (Lin & Lechleiter, 2002). In general, it is thought that the opening of the mPTP occurs with a decrease in the inner matrix potential, decreased AMP and ADP levels, increased matrix Ca^{2+}, with alkalinization, or during oxidative stress (Gateau-Roesch, et al., 2006). mPTP opening blocks ATP formation and allows for the equilibration of small molecules (Gateau-Roesch, et al., 2006; Halestrap, 2004). mPTP opening increases osmotic

forces within the mitochondria inner matrix and leads to degradation of the matrix membrane, causing the release of apoptotic factors, especially cytochrome C (Honda, et al., 2005). Also, as the mitochondrial membrane potential is perturbed, ATP synthase reverses its primary function and serves as an ATPase; further depleting cellular ATP concentrations and increasing H^+ levels.

Feng and colleagues (2005) determined that volatile anesthesia-induced postconditioning prevented the opening of the mPTP by inhibiting glycogen synthase kinase 3β (GSKβ). This inactivation was a result of PI3K-AKT signaling pathway inactivation with the resulting phosphorylation and inactivation of GSKβ, which protected against reperfusion damage. Argaud et al. (2005) found that mechanical postconditioning decreased cellular Ca^{2+} and protected *in vivo* rabbit hearts, suggesting that the mPTP could be inhibited by the PI3K-AKT-eNOS cascade. Bopassa et al. (2006), using a rat heart preparation undergoing postconditioning, concluded that PI3K signaling regulates the closure of mPTP. In addition, Cohen, Yan, and Downey (2007) observed that postconditioning prevented mPTP opening as a result of inhaled CO_2-induced acidosis during the first minutes of reperfusion. They suggested that low cellular pH inhibits the opening of mPTP in heart tissue, but as the cellular pH normalizes, the inhibition of mPTP is lost. They hypothesized that by maintaining the cellular pH at a lower level while introducing oxygen during reperfusion, it was possible to keep the mPTP closed allowing the redox signaling necessary to trigger preconditioning-like protection. Cohen, Yan, and Downey further suggest that moderate acidosis during reperfusion might be protective. This hypothesis was addressed through the use of sodium bicarbonate ($NaHCO_3$) during postconditioning. In isolated rabbit hearts, acidic CO_2 perfusate at the time of reperfusion mimicked postconditioning while an alkaline $NaHCO_3$ perfusate blocked that effect. They hypothesized that an acidic environment inhibited mPTP opening while an alkaline environment favored mPTP opening. Fujita and colleagues (2007) also hypothesized that $NaHCO_3$ would blunt the protective properties of postconditioning. Using *in vivo* dog hearts that underwent ischemia, the administration of $NaHCO_3$ during four intermittent cycles of one-minute reperfusion with one-minute reocclusion of a coronary vessel completely abolished the postconditioning effects. Their results suggested that postconditioning leads to the opening of mK_{ATP} channels as a result of decreased pH, leading to the attenuation of cardiac infarct size.

3.3 Reperfusion Survival Kinase Pathway

The Reperfusion Injury Survival Kinase (RISK) pathway begins with the activation of PI3K and ERK to promote cell survival. RISK can be activated by insulin, urocortin, atorvastatin, adenosine, bradykinin, opioid agonists, volatile anesthetics, or diazoxide (Bell & Yellon, 2003a,b; Chiari, et al., 2005; Gross, et al., 2004; Jonassen, et al., 2001; Schulman, et al., 2002; Wang, et al., 2004; Yang, et al., 2004). The RISK pathway promotes pro-survival signaling while inhibiting pathways associated with apoptosis. In 2004, Tsang and colleagues (2004) reported that in isolated rat hearts, which had undergone mechanical postconditioning following a period of ischemia, postconditioning is mediated by the PI3K-AKT-eNOS/p70s6K pathway. They also suggested MEK 1/2-ERK 1/2 pathways were indirectly involved. Zhu and coworkers (2006) followed by finding that cardioprotection from postconditioning in the remodeled rat myocardium is regulated through PI3K-AKT signaling. The role of ERK 1/2 was addressed by Darling et al. (2005) and Krolikowski et al. (2006). Darling and colleagues utilized mechanical postconditioning in isolated rabbit hearts

and found ERK1/2 but not PI3K activity provided cardiac protection. Krolikowski and colleagues exposed rabbits to isoflurane before and during early reperfusion and suggested a central role of ERK1/2, p70s6k, and eNOS in anesthetic-induced postconditioning.
Downstream in the RISK pathway, phosphorylation of AKT occurs with the subsequent phosphorylation of protein kinase C (PKC) and GSKβ. When PKC is phosphorylated it is stimulated while the phosphorylation of GSKβ inhibits its activity. In a rabbit model, Philipp and colleagues (2006) demonstrated through inhibitor studies that adenosine, PKC, and PI3K mediated the effects of mechanical postconditioning. In their investigation, they concluded that protection was conferred through the activation of adenosine receptors by endogenous adenosine, a cellular metabolite. This, in turn, activated the PI3K component of the RISK pathway resulting in activation of PKC. In regards to GSKβ, Feng and colleagues (2005), using isoflurane as a postconditioning trigger along with an AKT inhibitor, showed that when inhaled early in reperfusion, isoflurane phosphorylated AKT and GSKβ. Phosphorylated GSKβ was inhibited and could not promote the opening of mPTP. They also determined that while the PI3K-AKT signal was strong, the ERK1/2-p38 MAPK was not altered. This suggests a primary role of PI3K-AKT in the RISK pathway and in mPTP closure. Recently, in human tissue, it has been found that the cytoprotective proteins, HSP25 and HSP70, are upregulated by the PI3K-AKT pathway (Dickson, et al., 2001).

3.4 The heat shock response

Both preconditioning and postconditioning upregulate proteins identified as Heat Shock Proteins (HSPs), specifically HSP25 and HSP70. The heat shock response was discovered in 1962. *Drosophilia* larvae, when heated, developed puffing patterns in certain chromosomal regions. This suggested a change in the synthetic activity of the chromosomal bands concerned (Ritossa, 1962). Sixteen years later, the RNA for *Drosophilia* exposed to a thermal stimulus was coded using hybrid-arrested translation and indicated that proteins of 83, 72, 70, 68, 28, 26, 23 and 22 kilodaltons were upregulated (Livak, et al., 1978). Over the following decades, the investigation of the heat shock response has confirmed that a family of highly conserved HSPs is upregulated following a variety of sublethal stressors, possibly as a result of non-native proteins accumulating in a stressed cell (Voellmy & Boellmann, 2007). These proteins are subcategorized by their molecular weight and are either inherently present or can be induced following sublethal stress (O'Sullivan, et al., 2008). In particular, HSP25 and HSP70 have been thoroughly investigated with the consensus being they are protective when upregulated following stress (Beere, et al., 2000; Garrido, et al., 2006; Takayama, et al., 2003).

3.4.1 Heat Shock Protein 25/27

HSP25 is the rodent equivalent of the primate HSP27 and often the terms are used interchangeably. HSP27 confers protection at different levels as it can interact with several proteins implicated in cell death based upon its phosphorylation and oligomerization condition and not upon ATP. The main mechanisms for how HSP27 confers cytoprotection appear to be: molecular chaperoning, interference with cell death pathways, signaling of antiapoptotic pathways, stabilization of the cytoskeleton, and antioxidant activities. Serving as a chaperone, HSP27 can bind folded intermediate non-native proteins, inhibiting their aggregation, and in the presence of HSP70 these HSP27-bound proteins can be reactivated (Ehrnsperger, et al., 1997). Within the cytosol, HSP27 can sequester cytochrome C;

interfering with the formation of the apoptotic protease activating factor-1 (APAF-1)-cytochrome c multimeric apoptosome and the activation of procaspase 9 (Bruey, et al., 2000; Concannon, et al., 2001; Garrido, et al., 1999). HSP27 also directly interacts with procaspase-3, decreasing the activity of activated caspase-3 (Concannon, et al., 2001). HSP27 serves as a signaling messenger by causing the activation of serine/threonine kinase Akt thereby inhibiting Bcl-2 and caspase-9 (Cardone, et al., 1998). HSP25/27 has other actions. Phosphorylated HSP27 can stabilize F-actin and increase the number of cells retaining microfilament organization thus stabilizing membrane structure (Lavoie, et al., 1995). Additionally, HSP27 is able to increase glutathione levels, thereby reducing levels of ROS (Kretz-Remy, et al., 1996).

3.4.2 Heat Shock Protein 70

Over the last three decades, HSP70 has become the most thoroughly investigated protein of the HSP family of proteins. Like HSP25, HSP70 can inhibit cell death at various sites within the cell. However, unlike HSP25, HSP70 function is "ATP-dependent." HSP70 is typically found *in vivo* bound by ATP and HSP70 function is typically based upon the hydrolysis of the attached ATP molecule. HSP70 serves as a chaperone protein, inhibits stress signaling, prevents mitochondrial membrane permeabilization, and inhibits apoptotic pathways. HSP70 may chaperone kinases by binding to an unfolded carboxyl terminus, preventing aggregation, and allowing re-autophosphorylation of the kinase enzyme; thus stabilizing the enzyme and restoring function (Gao & Newton, 2002). HSP70 also binds the death receptors, DR4 and DR5, inhibiting Apo-2L/TRAIL-induced cell death (Guo, et al., (2005), and HSP70 blocks Bax translocation into the mitochondrial outer membrane. The latter effect prevents the permeabilization of the mitochondrial membrane and subsequent release of apoptosis-inducing factor (AIF) and cytochrome C (Stankiewicz , et al., 2005). HSP70 binds AIF within the cytosol; inhibiting its nuclear translocation and limiting nuclear condensation (Ruchalski, et al., 2006). Similar to HSP25, HSP70 prevents cell death by binding to Apaf-1 and interfering in the formation of the apoptosome complex and subsequent recruitment of procaspase-9 (Beere, et al., 2000). Lastly, HSP70 suppresses apoptotic signaling by binding precursor forms of caspase-3 and caspase-7; preventing their cleavage and activation (Komarova, et al., 2004).

3.5 Cleaved caspase 3

Both HSP25 and HSP70 inhibit the cleavage of caspase-3 (Concannon, et al., 2001; Komarova, et al., 2004). Cleaved caspase-3 (CC3) is a primary executioner of apoptosis as it is responsible for the total or partial proteolytic cleavage of numerous key cellular survival proteins (Fernandes-Alnemri , et al., 1994). One of those proteins being the abundant nuclear enzyme polymerase, which functions in DNA repair and protein modification during oxidative stress (Smith, 2001). Thus, induction of HSP25 and HSP70 may alleviate cerebral ischemic injury and resuscitation injury that results from the mitochondrial release of cytochrome C with subsequent cleavage of caspase-3.

4. Evidence of preconditioning and postconditioning in the brain

Wu and colleagues (Wu, et al., 2006) directly examined the roles of mPTP and the mK_{ATP} channel in preconditioning and postconditioning in a rat model of cerebral stroke. These

investigators activated the mK_{ATP} channel with diazoxide 20 minutes before middle cerebral artery occlusion followed by reperfusion, or inhibited the mPTP by infusion of cyclosporin A 15 minutes before reperfusion. It was discovered that both measures significantly increased functional performance scores and reduced infarction volumes. Importantly, both of these effects were abolished by blocking the adenine nucleotide port located on the mPTP. Their results strongly suggested that the mK_{ATP} channel and mPTP activity during reperfusion share a common protective pathway; the Reperfusion Survival Kinase Pathway (RISK). More recently, Feng, Rhodes, and Bhatt (2010) discovered that hypoxic preconditioning could invoke neuroprotection through the activation of AKT, a kinase that is part of the aforementioned RISK pathway. These investigators subjected newborn rats to 3 hours of 8% oxygen followed by 24 hours of reoxygenation. Following reoxygenation, the right carotid artery was permanently ligated and again the rats were subjected to 8% oxygen but for 140 minutes instead of 3 hours. Compared to rats subjected to normoxia prior to carotid ligation, preconditioned rats had a significant reduction in cerebral injury. It was found that preconditioning preserved RISK pathway signaling and attenuated caspase-3 activity.

Acute models of postconditioning have emphasized the benefit of cerebral reperfusion under controlled conditions. For example, several groups have shown that carefully controlled periods of reperfusion, before the full return of cerebral circulation, results in reduced injury. Zhao and colleagues (2006) employed permanent middle cerebral artery occlusion in combination with transient common carotid artery occlusions. Shorter periods of repeated common carotid occlusion resulted in a reduction in infarct size. Pignataro, et al. (2008) also employed middle cerebral artery occlusion for 100 minutes. Reperfusion of the artery that included a 10-minute period of occlusion was found to be the most effective, although intermittent occlusions were also beneficial. Gao, Ren and Zhao (2008) found that three cycles of reperfusion of the common carotid artery, in conjunction with permanent middle artery occlusion, reduced infarct size, while ten cycles was not effective. These publications, as well as numerous reports with cardiac models, emphasis the criticality of the duration of cerebral ischemia (longer periods of ischemia result in more cerebral damage, including irreversibility), as well as the essential specifics of the timing, duration, number of cycles, and inter-reperfusion intervals for effective postconditioning.

As well, recent work has shown the benefit of pharmacological postconditioning in cerebral ischemia. O'Sullivan and colleagues (2007) employed a rat model of combined hemorrhagic shock and permanent unilateral common carotid artery occlusion. The administration of diazoxide at the time of hemorrhagic resuscitation significantly increased the expression of heat shock proteins in the cerebral cortex and hippocampus. Robin and colleagues (2011), using a middle cerebral artery occlusion model, found that in Wistar strain rats ischemic postconditioning decreased infarct size by 40% and improved neurological outcomes. Specifically, pharmacological postconditioning by diazoxide administration decreased cerebral infarct by 60%. In addition, these beneficial effects in both ischemic postconditioning and diazoxide postconditioning were blocked through the use of the K_{ATP} blocker, 5-hydroxydecanoate (5-HD), which blocked the inhibition of the mPTP opening caused by ischemic postconditioning and diazoxide.

In 2011, Wang and colleagues discovered that selective delta opioid peptide [D-Ala2, D-Leu5] enkephalin (DADLE) provided a postconditioning effect by protecting hippocampal CA1 neurons in a model of forebrain ischemia. In this investigation, DADLE triggered

postconditioning neuroprotection for hippocampal CA1 neurons and improved spatial learning and memory in rats. This protection was dependent upon DADLE-induced activation of the PI3K/Akt signaling.

5. Conclusion

As reviewed, the majority of research related to pre- and post-conditioning has not been performed in studies related to cerebral ischemia. As recently stated by Keep and colleagues (2010), the question remains—"Is there a place for cerebral preconditioning in the clinic?" The clinical utility of cerebral conditioning is potentially limited by issues of safety, the relatively narrow therapeutic window, and the need to present the stimulus before the injury.

Brief periods of ischemia can enact classical and delayed conditioning. These momentary periods of ischemia have been shown to protect neuronal cells *in vitro* and to reduce injury *in vivo* in several experiment models and species (Koch, 2010). Since safety issues prevent deliberately inducing conditioning by cerebrovascular occlusion, research has focused on pharmacological agents, including volatile anesthetics, inhibitors of cellular metabolism, K_{ATP} channel activators, and inflammatory mediators (Keep, et al., 2010). Other agents that have been effective in producing conditioning are hyperbaric oxygen, cooling and hyperthermia, and acupuncture (Keep, et al., 2010). Recent research has also given credence to remote conditioning where ischemia to a hindlimb (e.g., by application of a tourniquet) protects the brain from later middle cerebral artery occlusion. Remote preconditioning or ischemia probably has the most practical use for clinical utilization. However, currently there are no clinical data to strongly support the use of any type of conditioning for brain protection.

From a clinical standpoint, a major problem with the application of conditioning is timing. With the exception of a planned neurosurgical intervention, classically employed technique such as vascular clamping is impractible as a pretreatment. Pharmacological agents, then. Given at the time of reperfusion may hold promise. Agents such as $MgSO_4$, erythropoietin, anti-hypertension drugs, anticoagulants, and statins all given to patients at risk for stroke have shown limited damage from a stroke should it occur (Keep, et al., 2010).

In addition, there is still little *clinical* evidence from basic research regarding the use of preconditioning for neuroprotection. Research models currently in use have at least four important limitations. First, experiments are routinely conducted on young, disease-free animals (Koch, 2010). The majority of patients who suffer cerebral ischemic events are older, and may have artherosclerosis, cardiac or kidney disease, or other co-morbidities, such as obesity, hypertension, diabetes, as well as additional risk factors such as sedentary lifestyle, and tobacco, alcohol, or illicit drug abuse. The 'chronic' ischemic state of these patients, with a chronic conditioning compensatory state, may not allow further conditioning protection with interventions. Secondly, both Keep, et al. (2010) and Koch (2010) noted that the effect of medications used by patients has a potential to interfere with preconditioning effects. Do certain prescribed medications or self-administered substances such as herbal products interfere with the conditioning signaling pathways? Third, the neuroprotective cascade might be very specific to gender, diet, genetic background, and age (Dirnagl, et al., 2009). Lastly, major issues to be resolved include determination of doses of preconditioning drugs that are safe and whether premorbid conditions, for example intermittent transient ischemic

events, act as a conditioning stimulus event (Dirnagl, et al., 2009; Keep, et al., 2010; Koch, 2010).

Finally, optimal neuroprotection may be a combination of physiological manipulations (e.g., body temperature regulation) and pharmacological treatment(s). Gidday (2010) provides an excellent overview of the current state of pharmacological approaches for neuroprotection. Related to the present review, the translational possibilities require continued bench science to characterize the signal transduction pathways mediating neuroprotection, and whether or not they have potential clinical applicability. There are many "gaps" in understanding the mechanisms of action of the >20 drugs presently known to be beneficial (Gidday, 2010), and we must determine how best to employ these agents.

The landmark study by Murry and colleagues on cardiac tissue heralded new and exciting research regarding classic and delayed and remote preconditioning as well as the more clinically important postconditioning effect. Research continues with pharmacological or physical manipulations that can mimic pre- or post- conditioning and this could eventually have significant clinical ramifications. Further work is needed that considers the aforementioned limitations. Reducing the long-term effect of stroke or traumatic brain injury by preserving ischemic tissue can vastly improve the quality of life for patients. Likewise, billions of dollars saved from long-term care requirements, lost wages, family care-giver issues, and the reduced burden on our health care system will all stand to benefit from progress in this critical field of study.

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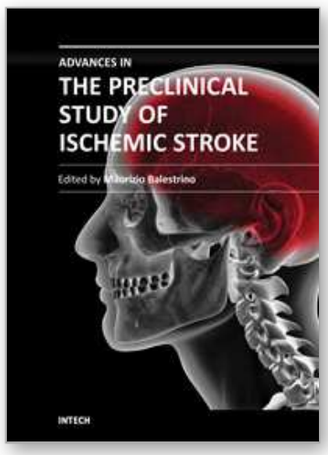

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This book reports innovations in the preclinical study of stroke, including - novel tools and findings in animal models of stroke, - novel biochemical mechanisms through which ischemic damage may be both generated and limited, - novel pathways to neuroprotection. Although hypothermia has been so far the sole "neuroprotection" treatment that has survived the translation from preclinical to clinical studies, progress in both preclinical studies and in the design of clinical trials will hopefully provide more and better treatments for ischemic stroke. This book aims at providing the preclinical scientist with innovative knowledge and tools to investigate novel mechanisms of, and treatments for, ischemic brain damage.

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