Organ dysfunction following regional and global ischemia/reperfusion.

Intervention with Postconditioning and Adenocaine

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INTRODUCTION

Acute myocardial infarction (AMI) is a major cause of death and disability worldwide and accounts for one of every six deaths in the United States.(1) In the years 2007–2009, an average 16.343 persons in Denmark were annually admitted to the hospital with ischemic heart disease as the primary diagnosis and with 6.303 deaths due to ischemic heart disease.(2) About two-thirds of deaths from AMI occur before hospital arrival, mostly from cardiac arrest triggered by ischemia induced lethal arrhythmias.(3) In addition, AMI has been documented in more than 50% of patients suffering from out-of-hospital cardiac arrest.(4) Out-of-hospital cardiac arrest is a major contributor of deaths and each year it affects approximately 275,000 people in Europe.(5) Survival rates are low, with about 24% surviving to hospital admission and with only 7.6% being discharged alive.(6)

While cardiac arrest and AMI are leading causes of death in the middle-aged and elderly, trauma primarily affects the younger segment of the population.(7-9) Hemorrhagic shock, defined as life-threatening blood loss, is responsible for 30-40% of early trauma deaths.7

The aforementioned conditions share several pathophysiological features. AMI, cardiac arrest, and hemorrhagic shock are all characterized by a period of reduced blood flow either regionally in the heart or globally (whole body), and treatment strategies target the restoration of normal blood flow. This is achieved by: 1) percutaneous coronary intervention restoring blood flow to the ischemic myocardium, 2) chest compressions and defibrillations being the treatment for cardiac arrest, and 3) surgery and volume resuscitation being standard treatment for hemorrhagic shock. Together, the treatment strategies restore blood flow to previously closed vascular beds, causing reperfusion. While reperfusion is essential for recovery of organ function, paradoxically it contributes to both reversible and irreversible cellular injury in all three settings.

AMI, cardiac arrest, and hemorrhagic shock are all acute conditions requiring early intervention to prevent progression of tissue injury. However despite early intervention, morbidity and mortality rates remain high, in part due to reperfusion injury. This calls for new early strategies to be implemented at the time of standard care. Ischemic postconditioning (postcon), defined as repetitive periods of ischemia applied during early reperfusion, was in 2003 introduced by the Vinten-Johansen laboratory(10) as a new potential treatment to limit injury following AMI. The concept of postcon demonstrated that reperfusion injury is initiated in the early moments of reperfusion. This early appearance of reperfusion injury implies that what happens first must be treated first. Therefore pharmacological strategies targeting reperfusion injury must be applied immediately at the onset of reflow. Another lesson learned from postcon was that broad-spectrum or combination therapy targeting several aspects of reperfusion injury is more effective than monotherapy. Adenosine, an endogenous signaling molecule formed by the breakdown adenine nucleotides, has shown to be a major mechanism in the cardioprotection of postcon and exerts broad-spectrum effects on reperfusion injury. The combination of adenosine and lidocaine (adenocaine) has been introduced as a new promising treatment with synergistic effects and is currently used as a cardioplegic agent with cardioprotective properties.(11)

This thesis will explore the mechanism of ischemic postcon during regional myocardial ischemia and test the effects of adenocaine as an early pharmacological postcon strategy in models of global ischemia/reperfusion injury.

ISCHEMIA/REPERFUSION INJURY

ISCHEMIA

Ischemia is defined as either partial or total restriction in blood supply to tissues or organs. As a consequence of the reduced blood flow, oxygen delivery is impaired, which subsequently results in ATP depletion, intracellular acidosis, and cellular ionic abnormalities. Cell homeostasis is dependent on the availability of ATP, and a reduction in ATP content reduces the activity of the Na+/K+ -ATPase, resulting in intracellular accumulation of sodium and hydrogen anions.(12) Simultaneously, intracellular calcium levels increase through a release from the sarcoplasmic reticulum and a decreased activity of the normal calcium extrusion mechanism, the Na+/Ca2+ antiporter. Furthermore, osmotic active particles, e.g. sodium, lactate and creatinine, accumulate, leading to cellular edema.(13) As demonstrated in a canine model of

myocardial infarction necrosis progresses over time in a wavefront pattern related to the duration of ischemia.(14) Other determinants of the severity of ischemia in the heart and whole body include the degree of oxygen supply/demand mismatch, the basal tissue metabolic rate, heart rate (for myocardium), and degree of either collateral or supportive blood flow.

REPERFUSION

The concept of timely reperfusion as a strategy to prevent progression of necrosis and to salvage myocardium was established by Maroko et al.(15) This strategy of timely reperfusion is applicable to ischemia in all organs. Restoration of blood supply restores aerobic metabolism and is requisite for salvage of organ function. However; reperfusion also paradoxically triggers additional injury that had not occurred during the preceding ischemic period; this is called ischemia/reperfusion (I/R) injury (Figure 1). Whether the process of reperfusion itself contributes to the pathophysiology of myocardial infarction has been debated for years.(16, 17) Matsumura et al.(18) demonstrated that tissue found to be viable at the end of a period of ischemia lost viability during reperfusion, which supports the concept of reperfusion injury. Furthermore, protective strategies such as postcon and pharmacological interventions administered at the moment of reperfusion can reduce final injury, which provides additional but indirect support for the hypothesis of reperfusion injury and separates it from ischemic injury. Thus, reperfusion injury reduces the therapeutic advantage of timely reperfusion. Reperfusion injury affects all cell types; not only cardiomyocytes or tubular cells in the kidney but also endothelial cells, interstitial cells and circulating cells.(19)

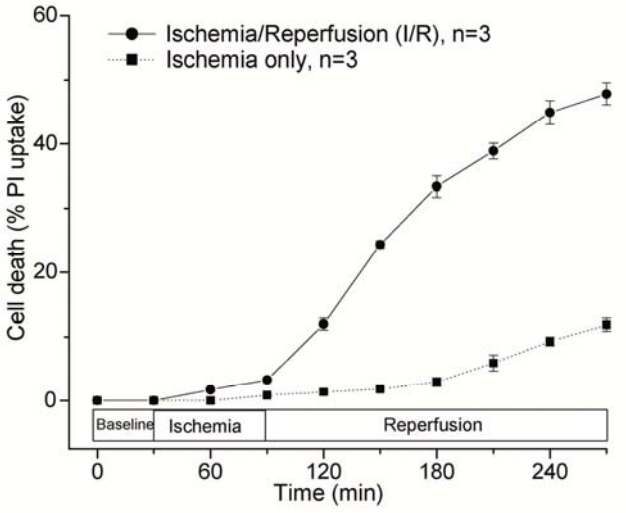

Figure 1 Cell death during either ischemia and subsequent reperfusion or prolonged ischemia. Vanden Hoek T L et al. Am J Physiol Heart Circ Physiol. 2003;284:H141-H150 Reprinted with permission. ©2003 by American Physiological Society

In the myocardium, I/R injury is manifested as either reversible injury, e.g. myocardial stunning and myocardial arrhythmias or irreversible lethal injury (necrosis/apoptosis). Since the mechanisms involved in reversible and irreversible injury differ, I/R injury will be discussed below in light of lethal myocardial injury. However, the mechanisms of tissue/organ injury also apply for I/R injury in other organs following either regional or global ischemia, e.g. cardiac arrest or hemorrhagic shock.

Calcium

Histological analysis of the myocardium after reperfusion reveals that infarcts almost exclusively consist of contraction band necrosis, reflecting excessive activation of the contractile machinery initiated upon reperfusion.(20) Upon reperfusion the mitochondria, if not severely injured, resume ATP production, activating the contractile machinery. However, this occurs before intracellular calcium levels have been normalized. At the end of a prolonged period of ischemia, the cytosol is overloaded with sodium, calcium, and hydrogen anions.(21) Intracellular acidosis is rapidly corrected by the efflux of H+ through the Na+/H+ exchanger and by influx of bicarbonate anion through the Na+/HCO3− symporter, both located in the sarcolemma. This sustains the elevated intracellular levels of sodium, keeping the major calcium extruder, the Na+/Ca2+ exchanger, in reverse mode. The excess calcium is temporarily sequestrated in the sarcoplasmic reticulum. However, if the calcium storage capacity in the sarcoplasmic reticulum is exceeded, it will lead to a cycle of continuous release and reuptake of Ca2+ between the sarcoplasmic reticulum and the cytosol. The activation of the contractile machinery, concomitant with high intracellular calcium levels, promotes excessive force generation and hypercontracture. In addition to hypercontracture, high calcium levels also lead to activation of endogenous proteases.(22)

pH

After a prolonged period of ischemia, anaerobic glycolysis leads to accumulation of H+ and a decrease in intracellular pH. Reperfusion causes a rapid normalization of pH through extrusion of hydrogen anion through the Na+/H+ exchanger and by influx of bicarbonate ions through the Na+/HCO3− symporter.(21) Normally, a low intracellular pH attenuates activation of the contractile machinery; however, the rapid extrusion and normalization of pH removes a potentially protective mechanism. Furthermore, studies have shown that maintenance of a low intracellular pH at reoxygenation protects cardiac cells from reperfusion injury.(23) Therefore, rapid correction of pH favors calcium accumulation and allows for activation of systems that were otherwise inhibited during low pH.

Reactive oxygen species

Although oxygen is essential for tissue survival, the rapid reintroduction of oxygen after a period of ischemia can be detrimental. Oxygen free radicals or reactive oxygen species (ROS) are molecules with unpaired electrons that render the molecule unstable, highly reactive and short-lived. ROS are continuously produced in small amounts as products of normal metabolism where they play important roles in homeostasis and signaling.(24) The formation of ROS is initiated during ischemia, but limited oxygen supply prevents a large-scale production of ROS. However, reperfusion elicits a prominent burst in the ROS production, which can overwhelm endogenous defense mechanisms such as superoxide dismustase (SOD), catalase and glutathione peroxidase that normally scavenges ROS.(25, 26) The excessive production of ROS elicits cellular damage by: 1) causing lipid peroxidation of cell membranes, 2) stimulating chemotaxis of neutrophils (PMNs), 3) causing denaturation of proteins including ion channels, and 4) opening of the mitochondrial permeability transition pore (mPTP). Numerous sources of ROS production have been proposed, with the three most important being 1) xanthine oxidase, primarily within endothelial cells, 2) the mitochondrial electron transport chain, and 3) Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, primarily within leukocytes.(24) Furthermore, evidence that ROS is a mediator of I/R injury has been supported by several studies in which oxidant scavengers

reduced post-ischemic injury.(27) However, in recent years it has emerged that ROS may not always be detrimental but may also serve as important signaling molecules.(28)

Inflammation

Ischemia/reperfusion initiates an acute inflammatory response contributing to myocardial injury. Reperfusion prompts the release of cellular contents from damaged cardiomyocytes to interact with toll-like receptors, and thereby activate the innate immune response.(29) The inflammatory response is complex and composed of both humoral and cellular factors, with the complement system, ROS, and cytokines as humoral factors.(30) In addition to its contribution to myocardial injury, inflammation is also prerequisite for healing and scar formation.(31)

Upon reperfusion, the complement system is activated, and cardiac lymph demonstrates PMN chemotactic activity, which is attenuated by neutralization of the complement factor C5a.(32) In addition to the complement system, cytokines are also up-regulated, leading to an activation of cellular factors.(33, 34) The PMN and its interactions with the endothelium represent a major component of the cellular inflammatory response.

PMNs have been suggested to participate in the pathogenesis of reperfusion injury;(35) this view is, however, controversial.(36) Upon reperfusion, the recruitment of PMNs into the ischemic myocardium is accelerated.(37) The release of pro-inflammatory cytokines and complement activates the coronary vascular endothelium to express P-selectin, which then recruits PMNs to interact with the vascular endothelium. The process of rolling and loose adherence that characterizes the first stage of PMN recruitment primes the PMNs for later full activation of the NADPH oxidase system.(38) Full activation of the PMNs can occur after interactions with the activated endothelium.

Shandelya et al.(39) demonstrated that perfusion of hearts with activated PMNs lead to a marked elevation in the production of ROS when compared to buffer perfused hearts, establishing PMNs as the major contributor of ROS during reperfusion. Activated PMNs can induce myocardial injury in several ways, including lipid peroxidation and damage to DNA secondary to release of ROS, and by direct damage from further release of pro-inflammatory cytokines and proteolytic enzymes. Endothelial dysfunction is, in part, mediated by ROS and PMN adherence.(40) In addition, PMNs can accumulate and embolize in capillaries, thereby contributing to blood flow defects (no-reflow). These events may occur in the early phase of reperfusion before transendothelial PMN migration into the myocardial parenchyma has taken place.(41) Studies have demonstrated that both infarct size and PMN accumulation increase in parallel over time during reperfusion, and that infarct size correlates with PMN accumulation,(37) consistent with but not proving a causative relationship. This correlation has been extended to the clinical setting, with intervention in patients with AMI.(42) Numerous interventions designed to inhibit PMNs and their functions have reduced infarct size. These interventions include 1) depletion of circulating PMNs, (43) 2) inhibition of PMN recruitment by pro-inflammatory mediators, e.g. platelet-activating factor,(44) and 3) inhibitors of PMN-endothelial cell adhesion molecule interactions.(45) However, some in vivo studies fail to show an effect of PMN inhibition strategies.(46) This discrepancy could be due to timing of therapy delivery (i.e. not at onset of reperfusion), dose of the drug, or inefficiency of antibody binding or the redundant pathways of inflammation that are not addressed by mono-therapies. For instance in the study by Tanaka et al.(46), the CD18 antibody was administered at half dose before ischemia and half dose 30 minutes after reperfusion, which could explain the lack of effect with regard to infarct size reduction.

The finding that similar durations of I/R in vitro and in vivo result in comparable infarct sizes is used to dispute the role of PMNs in lethal I/R injury.(36, 47) In buffer-perfused hearts, in which PMNs and blood-borne pro-inflammatory mediators, and endogenous anti-oxidants are absent, post-ischemic damage occurs, in part, due to ROS produced by cardiomyocytes and endothelial cells.(39, 48) The early burst of ROS production during reperfusion is associated with a decrease in non-enzymatic endogenous antioxidants in the ischemic heart.(49) This depletion makes the heart dependent on antioxidants delivered by the circulation, and may therefore exacerbate oxidant-induced injury. Glutathione peroxidase and catalase are important antioxidants involved in the removal of hydrogen peroxide, with high levels in erythrocytes.(50, 51) The heart is deprived of these antioxidant defense mechanisms in in vitro studies not using blood as the perfusate. Furthermore, the heart is also deprived of neural (opioids) and humeral mediators (EPO, cytokines) with known cardioprotective effects.(52, 53) In contrast, PMN repletion in in vitro preparations has been associated with a further decrease in post-ischemic endothelial function (40) and contractile function (39, 54) relative to the PMN-free cohort, making buffer perfused hearts less susceptible to damage.

No-reflow

Despite adequate restoration of blood flow at the macrovascular level, reperfusion is associated with a significant impairment of blood flow at the microvascular level, termed the no-reflow phenomenon.(55) This is observed as severely hypoperfused zones within the previously ischemic tissue. This no-reflow phenomenon after ischemia was first observed in the heart by Krug et al.(56) in 1966 and in the brain by Ames et al.(57) in 1968. The impaired post-ischemic blood flow may further compromise the already vulnerable myocardium by imposing secondary ischemia despite restored macrovascular blood flow, and thereby extend post-ischemic injury. The exact mechanism of the no-reflow phenomenon is not fully elucidated, but injury to endothelial cells is a key component. After a period of prolonged ischemia with subsequent reperfusion, endothelial swelling, endothelial gaps, and intravascular accumulation of PMNs and red blood cells are observed.(58) In the normal state, the endothelium expresses a phenotype that appropriately regulates microvascular blood flow and inhibits coagulation and inflammation. Within minutes, I/R causes endothelial dysfunction at both the functional 59 and structural level,(60) changing the phenotype of the endothelium from its normal homeostatic state to one that promotes interactions between endothelial cells, PMNs and platelets, and one that generates oxidant species and pro-inflammatory mediators.(61, 62) This dysfunction is, in part, related to an impaired release or synthesis of NO, which has vasodilatory and anti-PMN properties.(41, 63) PMNs amplify the release of ROS, with inflammatory and vasoconstrictive agents (platelet activating factor) further compromising microvascular flow.

Apoptosis

As referred to above, I/R injury results in lethal cellular injury. Two distinct forms of cell death exist, namely necrosis and apoptosis, both developing over time following I/R.(37, 64) It is not clarified whether apoptosis is triggered during ischemia or reperfusion. However, it is well documented that restoration of oxygen and energy stores at reperfusion accelerates the development of apoptosis.(65) In general, necrosis occurs rapidly with cellular swelling, membrane defects and increased permeability, release of cellular debris and activation of the inflammatory process. In

contrast, apoptosis is an energy-dependent process, with enzyme activation and DNA degradation without inflammation. Apoptosis is generally triggered by a receptor-independent mechanism through factors released from the mitochondria or by receptor-dependent mechanisms through activation of death receptors located at the cellular surface [tumor necrosis factor –alpha (TNF-α) receptor and the FAS receptor].(66) This leads to sequential activation of a series of cytosolic proteases termed caspases.(67) Caspases are a family of pro-enzymes that on activation are involved as both initiators and effectors of apoptosis, which ultimately leads to proteolytic cleavage of proteins involved in cell survival. The receptor-dependent pathway activates caspase-8, while the receptor-independent pathway activates the caspase-9, leading to activation of the final common pathway of apoptosis caspase-3.(68)

Mitochondria

Mitochondria serve as the cell powerhouse by production of ATP through oxidative phosphorylation. The outer mitochondrial membrane is relatively permeable, whereas the inner membrane is impermeable to all, but a few selected molecules. However, at reperfusion, a sudden loss of the mitochondrial membrane potential and opening of an unspecific pore in the inner membrane called the mitochondrial permeability transition pore (mPTP) has been described.(69) The molecular structure of the pore remains unknown, but it has gained much interest in recent years as the final effector of I/R injury.(70) Several factors associated with ischemia reperfusion injury such as calcium overload, oxidative stress, normalization of pH, and adenine nucleotide depletion induce mPTP opening. However, mPTP opening is inhibited by acidosis which prevents opening during ischemia, but upon reperfusion tissue pH is normalized which allows mPTP opening.(69, 71) The opening of the mPTP results in uncoupling of oxidative phosphorylation, swelling, and the release of pro-apoptotic factors such as cytochrome C, which will activate caspase-9 which, in turn, activates caspase-3. The importance of the mPTP during I/R is highlighted by several studies in which modulation of mPTP has attenuated I/R injury.(72)

CARDIAC ARREST

The annual incidence of out-of-hospital cardiopulmonary arrest in Europe is 38 per 100,000 people, 5 with more than 60% of adult deaths from coronary heart disease being due to sudden cardiac arrest.(73) In 25-30% of the victims, the initial heart rhythm is ventricular fibrillation (VF). This percentage has declined over the last 20 years,(74) with more victims found having either asystole or pulseless electrical activity(PEA). Survival rates in victims with VF are around 21%, while overall mortality rates are as low as 8%.(75)

Survival from cardiac arrest is dependent on early recognition, cardiopulmonary resuscitation (CPR), defibrillation, and post-resuscitation care, all linked together in the chain of survival.(76)

Cardiac arrest is the sudden cessation of mechanical activity and the inability of the heart to pump blood to maintain organ perfusion, ultimately leading to global ischemia. Basic CPR, consisting of chest compressions and ventilation, provides blood supply to the heart and brain albeit critically below normal levels.(77, 78) Quality CPR enhances the chance of restoring a normal rhythm when defibrillation is performed in the case of a shockable rhythm or enhances the chance of obtaining a shockable rhythm if asystole or PEA is present.(79) The delivery of defibrillation is a crucial factor following cardiac arrest because of its ability to restore a cardiac rhythm that can support cardiovascular circulation and hemodynamics. In recent years, the importance of post-resuscitation therapy as the final link in the chain of survival has been increasingly recognized, despite its introduction years ago.(80, 81) In a study by Olasvengen et al.,(82) 40% of patients treated with standard care achieved restoration of spontaneous circulation (ROSC), while 30% were admitted to the intensive care unit. However, only 10% survived to hospital discharge, which illustrates the need for effective post-resuscitation care.4 Immediate post-resuscitation treatment has the potential to alter the dismal outcomes after cardiac arrest and resuscitation. Proof of this assertion comes from studies demonstrating that therapeutic hypothermia improves clinical outcome.(83, 84)

One study in this thesis will investigate the effects of treatment in the immediate resuscitation phase with adenocaine on hemodynamic stability, organ function and PMN activation.

Organ dysfunction occurring after ROSC is termed “the post-cardiac arrest syndrome,” and is comprised 1) systemic I/R, 2) cardiovascular dysfunction, 3) brain injury, and 4) the precipitating pathology.(85)

It is important to consider that cardiac arrest post-resuscitation injury has distinct features when compared to focal I/R injury, with a prolonged period of total body ischemia followed by complete macrovascular reperfusion. In the setting of cardiac arrest, the heart is often fibrillating and has an increased oxygen demand, and with the start chest compressions, only insufficient levels of blood flow are provided.(86, 87) Although only low levels of blood flow are provided by chest compressions is also contributes to I/R injury. In addition, cardiac arrest treatment involves repeated periods of ischemia due to interruptions of chest compression and the injection of pharmacological agents. The administration of vasoconstrictive agents during CPR to enhance myocardial and cerebral blood flow may even further compromise visceral organ blood flow.(88) Moreover, epinephrine administered during CPR adds to endogenously released catecholamines, both of which may impair microcirculatory blood flow, (89) while defibrillations my further impair myocardial function.(90) Despite ROSC, hemodynamic instability and the administration of vasopressors may further impair visceral organ blood flow and oxygen consumption in the post-resuscitation phase.(91)

SYSTEMIC ISCHEMIA/REPERFUSION INJURY

Compared to focal tissue ischemia caused by myocardial or cerebral infarction, the ischemic period during cardiac arrest and resuscitation is measured in minutes rather than hours. With the relatively short ischemic period during cardiac arrest, most organs are without any significant degree of necrosis and most extra-cerebral organs tolerate ischemic intervals of extended duration.(92, 93) Despite the lack of obvious tissue necrosis morbidity (organ failure) and mortality remain high. Individually, each organ can tolerate short periods of ischemia; however the ability to tolerate ischemia may be reduced when all organs are affected as during global ischemia. This suggests a synergistic effect of concomitant organ injury reducing individual organ tolerance to ischemia, and that organs affected by I/R, release inflammatory mediators with harmful effects on other organ systems.(81, 94)

Cerchiari et al. (95) were the first to systematically investigate the impact of visceral organ dysfunction after cardiac arrest on cerebral outcome. Using a canine model of VF-induced cardiac arrest, they found that cardiac arrest was associated with systemic bacteremia, transient elevations in liver enzymes, and coagulopathy. In patients resuscitated after cardiac arrest, similar results are found, including a generalized activation of the im-

mune and coagulation system comparable to patients with sepsis.(96-98) This systemic activation of the immune system has been confirmed in other studies in which up to 60% of cardiac arrest patients fulfilled the criteria for the systemic inflammatory response syndrome (SIRS), whereas 36% developed sepsis.99 In another clinical study, endotoxin was detected in blood samples in 46% of the patients.(98, 99) Patients with SIRS had significantly higher levels of soluble P-selectin, which is an adhesion molecule involved in the early events of leukocyte-endothelial interactions, that has been detached from endothelium. Endothelial and leukocyte activation have been demonstrated by other investigators, who have found increased levels of circulating adhesion molecules and an increased release of the enzyme elastase from PMNs.(100-102) Two studies also demonstrated a significant correlation between duration of CPR and either the PMN enzyme elastase or the number of circulating endothelial cells.102, 103 The unspecific activation of the immune system following cardiac arrest also involves production of ROS (104, 105) and the release of cytokines.(98, 106) The increased levels of circulating cytokines are comparable to levels seen in septic patients and with significantly higher levels in non-survivors vs. survivors at hospital admission.(98) However in experimental models of cardiac arrest cytokine levels are in the low range and several of them are below detection limit.(106, 107)

CARDIOVASCULAR DYSFUNCTION

Cardiovascular dysfunction contributes to the high mortality seen after cardiac arrest, especially in the early phase after ROSC.(98, 108) Hemodynamic instability is observed as early as 4 hours after ROSC, with full recovery seen within 72h in surviving patients.(108) The observed hemodynamic instability is primarily attributed to myocardial dysfunction and secondarily attributed to vascular dysfunction.

Several animal studies report a severe abrupt decrease in myocardial function following cardiac arrest that normalizes within 48–72 hours after ROSC.(92, 109, 110) The existence of myocardial dysfunction in patients was first reported by Deantonio et al.(111) and later by others.(108, 112, 113) The decrease in contractile function is characterized by both systolic and diastolic dysfunction.

In a porcine study of cardiac arrest, Gazmuri et al.(110) used left ventricular pressure-volume relationships to evaluate cardiac function after cardiac arrest. After 4 minutes of VF and 8 minutes of chest compressions, systolic function was severely impaired, illustrated by a marked rightward shift of the pressure-volume loops concomitant with a decrease in the slope of the end-systolic pressure-volume relationship (ESPVR). In the same year Kern et al.(92) demonstrated a significant reduction in ejection fraction (EF) and decreased fractional shortening analyzed by Doppler-echocardiographic. Despite a decrease in cardiac function, myocardial blood flow was unchanged, and minimal myocardial necrosis was detected. Diastolic dysfunction was evidenced by increases in left ventricular end-diastolic pressure, the isovolumetric constant tau, and a decrease in compliance.(92, 110)

The phenomenon of reversible post-ischemic myocardial dysfunction in the absence of necrosis is termed myocardial stunning.(114) Myocardial stunning refers to reversible ventricular dysfunction that follows a period of non-lethal ischemia despite restoration of normal blood flow.(114, 115) First described in models of regional ischemia with short durations of coronary occlusion, myocardial stunning was later confirmed in models of cardiac arrest.(92)

In experimental models of cardiac arrest, myocardial recovery was observed within 48–72 hours, while some clinical studies report that recovery is prolonged to weeks.(113) Acute myocardial infarction has been reported in approximately 50% of patients admitted after resuscitation from cardiac arrest.(4, 116) Most experimental studies induce VF in healthy animals by applying an electric current through a pacing catheter placed in the right ventricle. This makes the duration of global myocardial ischemia very short in contrast to patients with cardiac arrest subsequent to myocardial infarction.(4, 116) In patients with cardiac arrest subsequent to myocardial infarction myocardial dysfunction is a result of both regional ischemia due to the AMI and global ischemia due to cardiac arrest. The definition of stunning requires the absence of lethal myocardial injury, which is not be the case for patients with cardiac arrest due to myocardial infarction. Although not significant (p=0.06), Laurent et al. showed a strong trend towards a higher incidence of hemodynamic instability if AMI was the cause of cardiac arrest.(108) In addition, studies comparing pacing and ischemic-induced VF have shown that ischemic-induced VF is characterized by lower survival rates and more profound cardiac dysfunction.(117, 118)

This implies that where myocardial dysfunction following experimental models of VF induced cardiac arrest is simply a result of stunning, it is in cardiac arrest patients with AMI more severe and more than simply just stunning.

Many of the pathophysiological features responsible for lethal myocardial I/R injury are also involved in the pathogenesis of myocardial stunning despite the absence of lethal myocardial injury.(114) This includes calcium overload, production of ROS, inflammation, and the mPTP. However, some mechanisms such as apoptosis appear only to play a role in the long-term after focal I/R.(119, 120) During VF, the thickness of the left ventricular wall increases with a concomitant decrease in ventricular compliance.(121) Simultaneously, calcium levels rise, with a decrease in the myofilament responsiveness to calcium.(122) In addition, compelling evidence supports that a burst in ROS production at reperfusion mediates myocardial stunning.(114) Following resuscitation, the levels of ROS increase and in a rat model of VF, the administration of a lipid peroxidation inhibitor improved myocardial function suggesting that ROS plays a causative roll in contractile dysfunction.(104, 105, 123, 124) Also, as with regional I/R, an increase in inflammatory mediators has been associated with myocardial dysfunction.(106, 125) Lately, the importance of the mitochondria in post-resuscitation injury has been highlighted. In a mouse model of cardiac arrest, activity in mitochondrial complex I-III and complex IV was significantly impaired 60 min after ROSC concurrent with an augmented production of ROS and tyrosine nitration from peroxynitrite.(126) The mPTP has been suggested as the final effector of I/R injury. Several models of regional I/R have demonstrated that inhibitors of mPTP opening are protective.(72, 127) The importance of the mPTP in the setting of cardiac arrest was highlighted by Cour et al., who administered two different inhibitors of mPTP opening during resuscitation.(128) Both inhibitors attenuated cellular injury and cardiac function after 15 min of cardiac arrest.

NEUROLOGICAL DYSFUNCTION

In patients resuscitated from cardiac arrest, the cause of death is neurological injury in about 70% of the cases.(82, 129, 130) Compared to the other organs, the brain is very vulnerable to even short periods of ischemia, with ischemic depolarization occurring within minutes of cardiac arrest.(131) Despite chest

compressions, cerebral blood flow is not sufficient to restore membrane polarization. Therefore the duration of global cerebral ischemia is measured from the time from of arrest until ROSC.(132, 133) The chance of achieving ROSC after prolonged cardiac arrest is low therefore total cerebral ischemia time is often below 30 min or less. If the ischemic period is prolonged (>30min) pan-necrosis will develop however since this is not the case after cardiac arrest the pathologic result is varying degrees of selective neuronal death with the most vulnerable neurons placed in hippocampus, cerebellum, striatum and cortex. (134-136)

Ischemia/reperfusion injury in the brain shares many similarities with I/R in other organs, i.e. involving calcium accumulation, ROS generation, protease activation, mitochondrial dysfunction, and inflammation. However, cerebral I/R also has features distinct from other organs. In the brain, excitotoxicity injury is induced by the release of the excitotoxic neurotransmitters, with glutamate as the most abundant. Glutamate increases the expression of cytotoxic cascades and amplifies calcium dyshomeostasis through the opening of calcium channels.(130, 137, 138) Intracellular calcium accumulation increases the proteolytic enzyme phospholipase A2 associated with a rise in free fatty acids which potentiates radical-meditated peroxidation of fatty acids in selective vulnerable regions.(139, 140) Neuronal death progresses over time, a phenomenon called delayed neuronal death. However, the pathogenesis of delayed neuronal death is unresolved but does involve secondary calcium accumulation, ROS, protease activation, and up-regulation of inducible nitric oxide (iNOS) and COX 2.(131, 141)

Following ROSC there is an initial phase of hyperemia in the brain typically lasting 5-40 minutes depending on the insult.(142-144) This is followed by a prolonged phase of cerebral hypoperfusion, in contrast to normal perfusion in other organs. Although delayed secondary hypoperfusion has been proposed as a cause of secondary brain injury after cardiac arrest, human data provide evidence that decreased cerebral blood flow is matched to decreased oxygen consumption,(142) suggesting that cerebral blood flow is under autoregulatory control. Whether the hypoperfusion is a cause of reduced metabolism due to injury or the reduced consumption is due to hypoperfusion remains unanswered.

HEMORRHAGIC SHOCK

Trauma is the leading cause of death in the younger population,(145) and since it primarily affects the younger generation, it represents a heavy burden for society due to the loss of potential years of productive life. Hemorrhage and subsequent exsanguination are the most frequent causes of acute death in the pre-hospital setting. Overall CNS injury is the most common cause of death,(146) while in patients admitted to the ICU multiple organ failure is the leading cause of mortality.(146, 147)

Hemorrhagic shock is a life-threatening condition defined as a period of reduced perfusion of vital organs, leading to inadequate delivery of oxygen and nutrients. During hemorrhagic shock, tissue oxygen levels are inadequate due to hypoperfusion and decreased oxygen delivery resulting in an increase oxygen debt, i.e. oxygen supply-demand mismatch.(148) Oxygen debt is defined by the cumulative difference between the baseline (normal) oxygen consumption and oxygen consumption at any given time point. As with focal ischemia, hypoperfusion causing low-flow ischemia during shock leads to disturbances in cell homeostasis with a reduction in ATP, cell ionic dyshomeostasis and intracellular edema due to accumulation of osmotically active particles.(149, 150) During the early phase of shock, a neuroendocrine response is activated as a compensatory mechanism. However, if the duration of shock is prolonged and resuscitation is inadequate, irreversible shock will develop.149 Hemorrhagic shock is divided into three phases: 1) compensated hemorrhagic shock, 2) decompensated hemorrhagic shock that is reversible, and c) irreversible hemorrhagic shock.(151)

In the first phase compensatory mechanisms are composed of increases in 1) circulating hormones, 2) heart rate, 3) myocardial contractility, and 4) peripheral vascular tone.(149) Peripheral vasoconstriction maintains perfusion of the heart and brain at the expense of blood flow to other organs such as the kidney and intestines. In the later phases of decompensated and irreversible hemorrhagic shock the compensatory mechanisms are insufficient to maintain perfusion of the vital organs.

The primary treatment of hemorrhagic shock is surgical control and volume resuscitation with fluids and blood. Final surgical control is rarely obtained before arrival at the operating theater, and concern has been raised regarding the unrestricted use of fluids before admittance to hospital with regards to the "lethal triad" of hypothermia, acidemia, and coagulopathy.(152, 153) In contrast to unrestricted fluid administration, hypotensive resuscitation is an emerging concept of maintaining a lower systemic arterial pressure that ensures adequate tissue perfusion without prompting re-bleeding in the early out-of-hospital environment.(152, 154) The potential protective mechanisms for hypotensive resuscitation include: 1) providing sufficient perfusion to the organs without causing extravasation and edema, 2) maintaining O2 delivery, and 3) a reduction in the likelihood of re-bleeding by preventing dilution of coagulation factors or by "popping the clot".(155, 156) The optimal composition of fluids is unknown; however, the addition of pharmacologic agents to the resuscitation fluid that target the mechanisms of resuscitation injury represents a novel strategy to improve outcomes after hemorrhagic.(157)

Organ dysfunction following trauma is not only a consequence of hypovolemia, but may also in the later phase develop due to resuscitation injury (I/R injury) (Figure 2).(158)

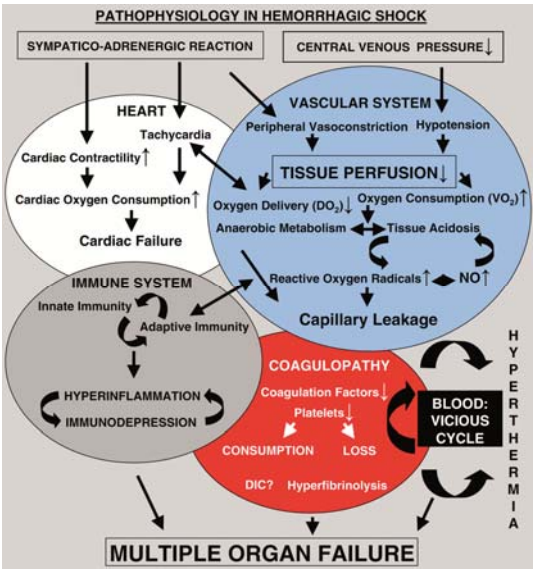

Figure 2 Pathophysiology in hemorrhagic shock; Angele, M. K., Schneider, C. P., & Chaudry, I. H. (2008). Bench-to-bedside review: latest results in hemorrhagic shock. Crit Care, 12(4), 218. Reprinted with permission © 2008, Critical Care

The inflammatory response following hemorrhagic shock can be evoked not only during the shock phase but also during the post-resuscitation phase,(159) and therefore represents a "two-hit" model of injury. Several factors such as iNOS, COX-2, and CD14 are up-regulated during shock, whereas other factors such as nuclear factor-kappa B (NF-κB), IL-6, and G-CSF are up-regulated after resuscitation.(160, 161) This up-regulation of NF-κB, IL-6 and G-CSF is accompanied by an increased accumulation of PMNs in the organs, suggesting that PMNs are an important mediator of organ injury after hemorrhagic shock.(160, 162) That PMNs are mediators of organ injury is further supported by studies demonstrating improved survival when anti-PMN strategies are applied.(163) The first step in PMN extravasation is interaction with endothelial adhesion molecules, enabling rolling, adhesion and extravasation. In both animal models and in patients following resuscitation from hemorrhage shock, endothelial dysfunction has been observed.(164, 165) Subsequent to the early exaggerated inflammatory response, a state of hypo-responsiveness or immune dysfunction eventually follows over time, with an increased risk for sepsis and sepsis-induced organ failure.(166, 167)

CARDIOVASCULAR DYSFUNCTION

During hemorrhagic shock myocardial contractility is initially increased as a compensatory response to hypotension. However, as the shock period is extended myocardial function decreases.(168, 169) Despite adequate resuscitation with fluid and blood myocardial function remains impaired when assessed by echocardiography in a porcine model of shock and resuscitation, and by in vitro perfusion in guinea pigs.(170, 171) In the early post-resuscitation phase TNF-α and IL-1β levels are increased in the myocardium, and when antibodies targeting TNF-α were given before hemorrhagic shock myocardial dysfunction was prevented.(172) Similarly, using an IL-6 knock-out mice model, myocardial function is preserved and activation of NF-κB and expression of ICAM-1 is attenuated.(173) A potential mechanism for the increased expression of cytokines in the myocardium could be by α1 receptor activation since blockage of the receptor improves cardiac function and prevents TNF-α expression.(174) These studies highlight that early anti-inflammatory strategies have the potential to improve myocardial function after hemorrhagic shock.

ACUTE KIDNEY INJURY

Acute kidney injury (AKI) following trauma is a frequent complication and is associated with increased mortality and morbidity.(175) In contrast to the heart, blood flow is red(istributed away from the kidney even after small changes in the circulating blood volume.(176) This decrease in renal blood flow during hemorrhage results in impaired renal function measured as a decrease in glomerular filtration rate (GFR) and despite adequate resuscitation with fluid and blood GFR was in one study not restored to baseline levels until 21 days after the insult.(177, 178) The cause of AKI following shock and resuscitation is multifactorial, as it is in I/R, and involves impaired endothelial function (vascular reactivity), tubular injury, and an accelerated inflammatory response at reperfusion.(179, 180) As with cardiac dysfunction following hemorrhagic shock, the production of inflammatory mediators is proposed as a mechanism of hemorrhage-induced AKI, as evidenced by the amelioration of AKI with inhibitors of either TNF-α or iNOS.(181, 182) Production of ROS and intracellular calcium accumulation are implicated in the pathogenesis of I/R injury, and in rats, strategies targeting both ROS and calcium accumulation attenuate AKI.(177, 183)

In summary, resuscitation from cardiac arrest and hemorrhagic elicits global I/R injury, thus sharing pathophysiological features with regional I/R. However, the unique setting of cardiac arrest and hemorrhagic shock with complex resuscitation and involvement of multiple organs makes it a syndrome of its own; more studies are warranted to explore the mechanisms.

INTERVENTIONAL STRATEGIES

POSTCONDITIONING

The relationship between infarct size and acute mortality, morbidity and heart failure suggests that reducing infarct size may be an important therapeutic goal.(184)

In 2003, Zhao et al.(10) showed that relief of myocardial ischemia in a stuttered manner, a strategy termed postcon, represents a novel and powerful approach to attenuate the deleterious sequelae of I/R injury. In the experimental laboratory setting, postcon was performed by sequentially releasing and reapplying an external ligature around the coronary artery, thereby imposing intermittent interruption of blood flow at reperfusion.(10, 185) More recently, studies have used fluoroscopically guided angioplasty balloon catheters to sequentially occlude and reperfuse the target vessel in closed-chest preparations,(186) a model which more closely simulates the clinical situation of PCI for AMI.The first postcon study demonstrating a protective effect was performed in the canine model using three cycles of 30 seconds of reperfusion and 30 seconds of re-occlusion (ischemia) applied immediately at the onset of reperfusion.(10) Since the original study by Zhao et al. reporting significant reduction in infarct size by postcon, myocardial salvage has been demonstrated in every species tested, e.g. rat,(185) rabbit,(187) canine,(188) swine,(189) and most promising in man.(190) The introduction of postcon highlighted the importance of timing, since the protective effect is abrogated if the start of postcon is delayed.(191, 192) This demonstrates that I/R injury is initiated at the onset of reflow, and what comes first must be treated first.(193) Although infarct reduction is the most investigated endpoint of postcon, the protective phenotype also includes a reduction in the number of arrhythmias(194) and apoptosis.(195) The protective characteristics of postcon can be categorized into 1) molecular triggers, 2) physiological mechanisms, 3) molecular targets, and 4) effectors (Figure 3).

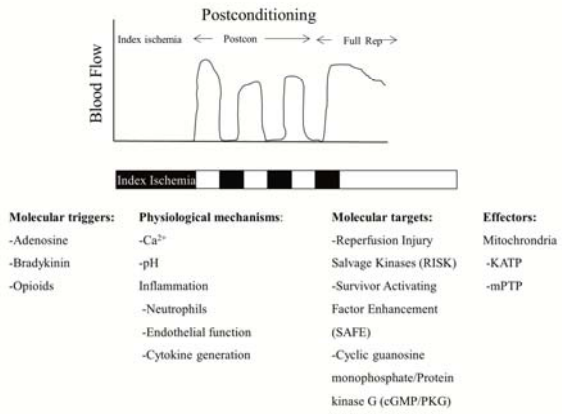

Figure 3 A schematic diagram of the mechanisms involved in the cardioprotective effects of postconditioning

Molecular triggers

The best known local mediators implicated in the protective mechanisms of poston are the autacoids adenosine, bradykinin, and opioids, which trigger cardioprotection by receptor-mediated mechanisms.

The repetitive occlusions and re-occlusions by postcon delay the washout of metabolites accumulated during ischemia, such as adenosine.(196) Kin. et al. demonstrated that postcon delayes the washout of endogenous adenosine, enabling adenosine receptor (AR) activation.(197) As for adenosine, postcon maintains opioid peptide content early in reperfusion, whereas the non-selective opioid-receptor antagonist naloxone-abrogated protection by poston.(198) Finally, studies have demonstrated that postcon activated bradykinin receptor subtype 2 and mediated protection through NO and prostaglandin synthesis.(199) In addition, several other naturally occurring mediators have been demonstrated to exert protective properties when administered early in reperfusion. However, their involvement as a mechanism in postcon has not been confirmed.

Physiological mechanisms

Calcium

It is recognized that calcium accumulation during I/R causes lethal injury. Sun et al. (200) demonstrated in isolated cardiomyocytes that postcon reduced calcium accumulation and lethal injury following hypoxia and re-oxygenation. This finding was recently confirmed by Dong et al., (201) who found a reduction in calcium levels both intracellularly and in the mitochondria.

Recovery of pH

One physiological mechanism by which postcon may protect the heart is by delaying the normalization of tissue pH. Reperfusion results in a rapid normalization of intracellular pH, which in turn triggers hypercontracture and activation of enzyme systems, while delayed pH normalization keeps mPTP in a closed state. When postcon is applied, tissue pH and coronary effluent remain acidotic for a longer period compared with an abruptly reperfused heart.(202, 203) Cohen et al.(204) demonstrated in the isolated rabbit heart that perfusion with an acidotic buffer reduced infarct size to the same extent as conventional postcon and that the infarct sparing effect of postcon was reversed when the heart was simultaneously perfused with alkalotic (vs. neutral pH) perfusate during postcon. Inserte et al. extended these findings by demonstrating that only postcon protocols that delayed recovery of intracellular pH afforded protection against I/R injury.(196) Recovery of pH is abrupt and therefore interventions that address the pH issue must be initiated immediately at reperfusion. The advantage of a prolonged recovery of pH seems to be dependent on an inhibition of gap junction opening, attenuation of calpain activation, and inhibition of mPTP opening.(23, 205)

Inflammation

Numerous studies have suggested that cardioprotection by postcon is, in part, related to a reduction in the inflammatory response. In the original study by Zhao et al.(188) postcon prevented post-ischemic endothelial dysfunction as assessed by the vasodilatory response to acetylcholine, and by a decrease in the surface expression of P-selectin. This suggests a reduced activation of the endothelium that consequently promotes less PMN recruitment and adherence. Accordingly, Zhao et al.(188) demonstrated a reduced adherence of PMNs to the post-ischemic coronary artery endothelium, which is physiologically linked to an impaired basal production of NO.(41, 206, 207) Furthermore, postcon has in both animals and humans attenuated cytokine release following I/R.(208, 209) Together these data suggest that postcon attenuates endothelial cell dysfunction by decreasing oxidant and cytokine generation, and by increasing eNOS activity and NO bioavailability. The improved endothelial function and reduction in P-selectin expression may contribute to a reduced PMN accumulation after both short- and long-term reperfusion. (10, 210) Furthermore postcon has attenuated the cytokine release following I/R in both animals and humans (208, 209, 211).

No-reflow

Two studies have explored the effect of postcon on the area of no-reflow. In one study by Hale et al.,(212) postcon failed to reduce infarct size and area of no-reflow. Whether the lack of effect was due to an insufficient postcon protocol or postcon did not reduce the area of no-reflow remains unknown. However Zhao et al. (207), displayed a 22% reduction in infarct size and a 33% reduction in area of no-reflow. They used a mini-swine model and the fluorescent dye thioflavin S to identify no-reflow zones. The role of postcon on post-ischemic blood flow remains unresolved at this point, and requires studies that focus primarily on the no-reflow phenomenon.

Molecular targets

A number of transduction pathways that convey signals from the cell membrane to the mitochondria have been discovered and implicated in the protective mechanisms of postcon. However, despite recent studies disputing the involvement of certain signaling pathways in larger animal models,(213) compelling evidence in smaller animals supports their involvement.(214, 215) Collectively three major pathways are recognized: the Reperfusion Injury Salvage Kinases (RISK) pathway, the Survivor Activating Factor Enhancement (SAFE) pathway, and the cyclic guanosine monophosphate/Protein kinase G (cGMP/PKG) pathway. The first study implicating the RISK pathway demonstrated that postcon phosphorylated Akt, eNOS, and p70S6K. Inhibition of phosphatidyl inositol 3 kinase (PI3K) and Akt phosphorylation abrogated the protective effects of postcon, suggesting a functional role for the PI3K – Akt pathway.(214) Several other protein kinases such as protein kinase C and extracellular signal-regulated protein kinase 1/2 (ERK1/2) have also been implicated in the RISK pathway.(192, 215) However the mechanisms responsible for recruitment and activation of the RISK pathway remain unclear; evidence supports an interaction between ligands (e.g. adenosine, sphingosine kinase-1) and G-protein-coupled receptors at the cell surface.(216, 217)

An alternative to the RISK pathway is the SAFE pathway. High levels of TNF-α are detrimental and antibodies targeting TNF-α limit infarct size and endothelial dysfunction in animal studies.(33, 218) Furthermore, TNF-α levels following I/R are attenuated by postcon.(195) In contrast, Lacerda et al.(219) demonstrated that TNF-α can mimic postcon and that the TNF-α receptor type 2 is necessary for infarct size reduction by postcon. In addition, both postcon and treatment with TNF-α failed to confer an infarct-sparing effect in signal transducer and activator of transcription-3 (STAT-3) deficient mice.(220) Upon receptor activation, the tyrosine kinase family Janus-activated kinase (JAK) phosphorylates and activates STAT3, which translocate to the nucleus and potentially to the mitochondria.

A possible third signaling pathway to be activated by postcon is the cGMP/PKG-pathway. Following ischemia, cGMP synthesis is attenuated, (221) while preservation of cGMP levels by either NO donors or postcon ameliorates I/R injury.(222, 223) The effects of postcon are abrogated by inhibitors of 1) NO,(192, 224) 2) cGMP

and 3) PKG.(223) The infusion of cGMP, and PKG inhibitors prevents the normal delay in recovery of intracellular pH, suggesting interplay between ion pumps regulating intracellular pH and the cGMP/PKG pathway.

How the aforementioned signaling pathways converge at the mitochondria and stimulate cell survival is debated but may involve altered calcium handling, reduced apoptosis and glycogen synthase kinase 3 beta (GSK3b)(225-227) Inhibition of either of the three pathways leads to complete abrogation of the infarct size reduction by postcon, revealing a potential interplay between signaling pathways, with the mitochondria as the common effector.

Effectors

Mitochondria have gained increased attention as the end-effector of postcon and as the pivotal site for determination of cell survival. The protective effect of postcon is abrogated by inhibition of the mitochondrial KATP channel,(228) while the effect of KATP activation is abrogated by administration of radical scavengers or hypoxic perfusion.(204, 229) While postcon delays normalization of pH and keeps the mPTP in a closed state, reintroduction of oxygen enables a low ROS production by the mitochondria, which in turn activates protective kinase signaling including protein kinase C(204). In addition, studies have shown that postcon reduces the calcium load required to open the mPTP following I/R and that infusion of atractyloside, an opener of the mPTP, abolishes the effect of postcon.(230, 231) However the processes by which receptor activation at the cell surface and intracellular signaling converge and modify the mPTP are still unrecognized.

PHARMACOLOGICAL POSTCONDITIONING

As described previously, studies exploring the mechanisms of postcon demonstrated that a diversity of different triggers, targets, and physiological effects are involved. However, while postcon is clinically applicably in the setting of AMI, it may not be clinically applicable in the setting of cardiac arrest and hemorrhagic shock. Instead pharmacological postcon may be advantageous.

One of the triggers of postcon, adenosine, exerts widespread effects, and in combination with lidocaine, it serves as a broad-spectrum combination therapy.

This combination therapy, adenosine plus lidocaine (adenocaine) will be investigated in models of cardiac arrest (Study II) and hemorrhagic shock (Study III). The following sections provide a background on adenosine and lidocaine as the constituents of adenocaine, and then background on adenocaine itself.

Adenosine

Adenosine is a naturally occurring endogenous nucleoside that is generated both intra- and extracellularly by dephosphorylation of adenine nucleotides in response to cellular stress but also by hydrolysis of S-adenosylhomocysteine (Figure 4). (232)

Following its production, adenosine is transported across cell membranes via bidirectional nucleoside transporters by both facilitated and co-transport mechanisms. Normally adenosine levels range from 10 to 200nM, but under situations with cellular stress, e.g. hemorrhage or ischemia, it can increase many fold.(233-235)

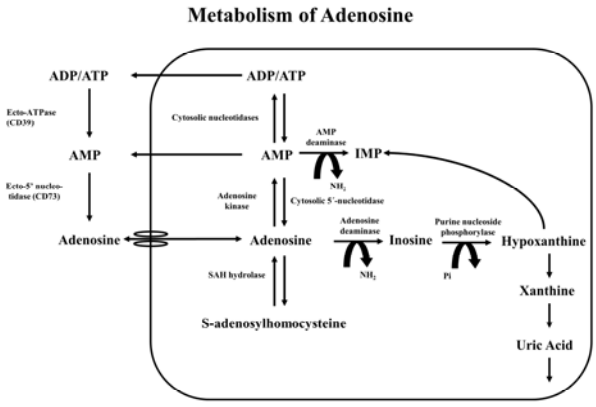

Figure 4 Metabolism of adenosine extra- and intracellular.

Adenosine exerts widespread physiological effects as an autocrine and paracrine signaling molecule through binding to one of its four G-protein coupled AR subtypes (A1, A2A, A2B, and A3).

The receptors subtypes are classified by their different affinities for adenosine and their distinct intracellular signaling pathways, which are specific for each receptor.(236)

Adenosine has many actions and among these are its cardiovascular effects, which include negative chronotropy, dromotropy, and antiadrenergic effects mediated through the A1 receptor. Due to this array of effects, adenosine is used as an anti-arrhythmic agent for the treatment of supraventricular tachycardia in the clinical setting.(237) Furthermore, adenosine acts as a link between energy demand and supply by its endogenous vasodilator effect.(238, 239) In addition, adenosine has a role as an important regulator of inflammation, highlighted by the fact that ARs are abundantly expressed on the endothelium, PMNs and macrophages, and activation of the A2A and A2B receptors are reported to be anti-inflammatory.(240)

Several studies have demonstrated cardioprotective effects of adenosine in the setting of myocardial I/R injury when infused at reperfusion.(241, 242) The protective effects of adenosine early during reperfusion are due to activation of the A2 receptor, with pronounced anti-inflammatory effects: 1) attenuated PMN superoxide generation, 2) decreased PMN adherence to endothelium, and 3) a reduction in the production of cytokines.(243-245)

Besides its cardioprotective and general anti-inflammatory effects, adenosine is also involved in regulation of cerebral blood flow and cerebral metabolic activity.(239, 246) Adenosine inhibits the release

of excitatory neurotransmitters such as glutamate and causes hyperpolarization, which reduces the rate of neuronal firing.(246) This inhibitory effect by adenosine released during hypoxia on excitatory function is to limit cellular activity and help neurons to survive despite a reduction in supply in oxygen.(247) Furthermore, in a rat model, infusion of adenosine for 90 min following cardiac arrest and resuscitation significantly improves outcome and delays hippocampal cell loss.(248)

Finally, relevant to this thesis, adenosine is also involved in the regulation of renal function.

Adenosine infusion in man lowers GFR by constricting the afferent arterioles and at the same time causing dilatation of the

deep cortical arterioles in the medulla.(249, 250) In the kidney, adenosine is involved in regulation of the tubuloglomerular feedback through the A1 AR.(251) If production of ultrafiltrate increases, energy requirements in the tubules are increased, leading to increased formation of adenosine and hence a reduction in GFR. In this way, adenosine works as an important feedback circuit whereby excess transport work in the renal tubules is attenuated by adenosine formation.(252) Ischemia/reperfusion of the kidney elicits an inflammatory response,(179, 180) and, in accordance with its cardioprotective effects, infusion of adenosine early during reperfusion protects the kidney from I/R injury.(253, 254) The potential protective effects are through an activation of the A2A AR attenuating medullar hypoxia by vasodilatation but also by an attenuation of the inflammatory response.(255)

The ability of adenosine to improve long-term outcome is always questioned because of its very short half-life. In in vitro studies using whole blood, addition of low doses of adenosine have shown that the half-life of adenosine is below 10 sec.(256) Moser et al. (257) also using in vitro whole blood confirmed the very short half-life of adenosine, but showed that the half-life of adenosine was prolonged when using a higher dose of adenosine. Adenosine is metabolized by either deamination to inosine or phosphorylation to adenosine monophosphate; however which pathway dominates is dependent on adenosine concentrations and thereby its half-life.(258) In healthy volunteers, infusion of adenosine, in increasing doses from of 2.5 mg to 10 mg, increased plasma concentrations of adenosine and its plasma half-life from 0.92 minutes to 1.86 minutes.(259) The studies included in this thesis used even higher concentrations of adenosine, which could lead to higher plasma concentrations and longer half-lives. Adenosine may be cleared from the circulation within minutes; however the effects mediated by the binding of adenosine to its receptors my last for hours due to activation of intracellular signaling pathways.(248, 260)

Lidocaine

Lidocaine is a local anesthetic agent normally used in the clinic for its nerve-blocking effects through inhibition of voltage sensitive fast Na+-channels. Lidocaine is approved as an anti-arrhythmic agent,(261) and, before amiodarone, it was used as the anti-arrhythmic drug of choice during resuscitation.(262) It also possesses negative chronotropic and inotropic effects and serves as a vasodilator.(263) However, data show that lidocaine exerts (possible) anti-inflammatory effects independent of the fast Na+ blockade mechanism, including inhibition of leukocyte-endothelial interactions, the release of inflammatory mediators, and a decrease in superoxide anion production.(264-266) Lidocaine has also been shown to protect from myocardial I/R injury by reducing final infarct size(267, 268) and the degree of apoptosis after cerebral I/R.(269)

In contrast, the effects of lidocaine on renal function are controversial, with some studies showing both protective effects after murine septic peritonitis and exacerbation of renal dysfunction after renal I/R.(270, 271)

Magnesium

Magnesium is a naturally occurring ion in the human body and is involved in the regulation of several important functions. Normally only 1% of total body magnesium is located in the blood, whereas approximately 35% of the total magnesium content is in metabolic tissues bound to ATP. In situations of ATP depletion, e.g. hemorrhagic shock or ischemia, the levels of free magnesium are increased due to hydrolysis of ATP.(272, 273) Magnesium is involved in several critical cellular functions such as 1) maintenance of cardiac conduction, 2) vascular tone, 3) membrane ion channel activity, including Na+/K+ pump activity and intracellular Ca2+ handling, and 4) as a cofactor for ATP utilization or synthesis.(274, 275) Magnesium has been used as an adjunctive in surgical cardioplegia since the early 1970s 276 due to its anti-inflammatory properties and its role as a natural calcium blocker, protecting cells against I/R-induced Ca2+ overload.(277, 278) In addition, treatment with magnesium reduces infarct size following myocardial I/R, potentially through an adenosine-mediated effect.(279)

An apparent limitation to the experimental model of I/R using the snare technique to induce ischemia is the absence of micro-embolization, which is often seen in patients.(298) The rat model was chosen for the PMN inhibition contributes to cardioprotection by postconditioning".

depletion study since the PMN serum was commercially available, and a large number of studies have demonstrated the effects of PMN depletion in the rat.(299, 300) PMN depletion has only been performed in few large animal studies, and this was by PMN depletion filters, injections of mechlorethamine and home-made anti-serums.(301, 302)

However, the small size of the rat heart precludes direct sampling of coronary venous blood from the myocardial area at risk (AAR). To determine whether •O2 production by PMNs originating from the AAR was attenuated by postcon, we therefore used a canine model of I/R in which coronary venous blood was sampled from the anterior ventricular vein draining the left anterior descending artery perfused myocardium.(303)

In both the rat and the canine model, the protective effect of postcon has been associated with a reduction in PMN accumulation, inflammation and oxidative stress, making it relevant to compare the models. Therefore, the results may pertain to both species.

The pig as an experimental model of cardiac arrest and hemorrhagic shock

The pig is very comparable with humans with regard to size, physiology and anatomy. The porcine kidney is as in humans multirenculate, multipapillate and renal electrolyte regulation is very similar to what is seen in man.(304) Furthermore, both the porcine heart and cardiovascular physiology share many similarities with humans.(3059 In addition, the gyrated pig brain is more similar to the gyrated primate brain than the rodent lissencephalic brain, making the pig a highly relevant model of cardiac arrest-induced neurological injury.(306) The use of large animals also allows for accurate measurements of urine production, uniform consecutive blood sampling, and accurate hemodynamic monitoring of cardiac function with pressure catheters.

The cardiac arrest model

Several different animal models of cardiac arrest exist. It can be induced by either asphyxia, infusion of potassium, electrical stimulation of the heart, or myocardial ischemia.(117, 126, 307) Cardiac arrest due to asphyxia is the most common etiology in children, while cardiogenic cardiac arrest (i.e. AMI) is most common in adults.(308) In our model, cardiac arrest was induced by the placement of a pacing lead and delivery of a 9-volt electrical signal to the right atrial septum or ventricular wall, resulting in immediate VF.

The duration of cardiac arrest is highly predictive of outcome, and with increasing duration of cardiac arrest, post-resuscitation organ function decreases.(121, 132, 309, 310) In the first pilot studies conducted to determine the appropriate duration of VF, resuscitation started after 5 min of VF. However, due to high

rates of ROSC the duration of arrest was then increased to 10 minutes. This resulted in unsuccessful resuscitation attempts, so the duration of cardiac arrest was set at 7 minutes, which resulted in acceptable ROSC rates(Figure 6).

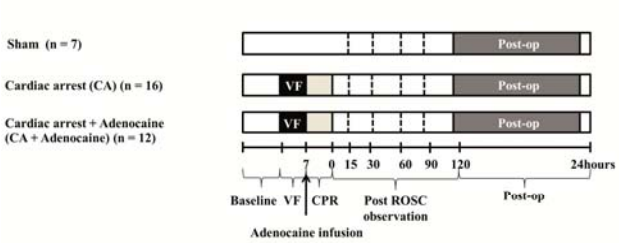

Figure 5 Schematic diagram of the study protocol used in the cardiac arrest study

The pigs were resuscitated according to the 2005 advanced life support guidelines, however with the exception of calcium and bicarbonate administration.(311)

The administration of calcium in the case of PEA was based on guidelines stating that intravenous calcium chloride is indicated in the presence of hypocalcemia, which frequently occurs following resuscitation.(312) Ten ml of 8.4% bicarbonate were injected after 1 minute of chest compressions despite that routine of administration of bicarbonate is not recommended. Administration of bicarbonate was used due to improvements in the rate of ROSC in a porcine pediatric cardiac arrest study performed simultaneously at the cardiothoracic research laboratory, Emory University.

The model of electrically induced VF was chosen to explore the effects of adenocaine following global I/R injury from cardiac arrest, since the majority of cardiac arrests are of cardiac origin. Animal models have demonstrated that organ dysfunction after cardiac arrest is dependent on the method used to induce cardiac arrest, with differences between cardiac arrest induced by either electricity, asphyxia, or ischemia.(117, 118, 313) As previously stated, adenocaine reduces infarct size following regional I/R injury in the heart.(284, 288) If a model of ischemia-induced VF had been used, it would not be possible to differentiate whether the systemic effects were due to a reduction in infarct size and hence improved cardiac function or due to systemic effects of adenocaine. Therefore, a model of electrically induced cardiac induced was chosen instead of a model of ischemia-induced cardiac arrest.

The hemorrhagic shock model

Hemorrhagic shock can be divided into models of fixed-pressure, fixed-volume, or uncontrolled hemorrhage with or without concomitant tissue injury.

In the hemorrhagic shock study we used a pressure controlled hemorrhagic shock model without concomitant tissue injury. Tissue injury was not induced so that the study could solely explore the effects of adenocaine treatment on global I/R, without influencing factors from damaged tissue.

The pressure-controlled hemorrhagic shock model was first described by Wiggers et al.,(314) who bled animals to a predefined MAP for a specific time period. The pressure-controlled hemorrhagic shock model is very reproducible and standardized making it an excellent model for the comparison of treatment effects. Furthermore, the first studies demonstrating a protective effect of treatment with adenocaine during hemorrhagic shock was performed in pressure-controlled models.(291, 315) However, the model has certain drawbacks. In contrast to the pressure-controlled model, bleeding in the clinical scenario is often uncontrolled and with concomitant tissue injury.(316, 317) With ongoing bleeding, the coagulation system is activated, and, in addition, in the presence of tissue injury, inflammatory mediators are released into the circulation.(161) Furthermore, in pigs the volume of blood needed to reach a specific level of shock is reduced in the presence of concomitant tissue injury or if bleeding is uncontrolled.(318, 319) A clear limitation of our model was the use of heparin, which is clinically irrelevant. Heparin was used primarily for 2 reasons: 1) to maintain the patency of the sheaths during withdrawal of blood, and to avoid the infusion of thrombus material 2) due to the extensive catheterization with two catheters placed in the left ventricle, a swan ganz catheter placed in the pulmonary circulation and a sizing ballon placed in the vena cava. During the study microspheres was injected through the pigtail catheter to measure regional blood flow, blood samples was withdrawn from the swan ganz to calculate oxygen consumption and hypertonic saline was injected through the sizing ballon to calculate parallel conductance. If heparin was not to be used the catheters would clot and we would not have been able to measure blood flow, calculate oxygen consumption and cardiac function by pressure-volume analysis. Studies have demonstrated protective effects of pre-heparinization on microvascular function following hemorrhagic shock, (320, 321) however at doses 5 to 10 times higher than used in the current study. Furthermore, all groups were treated with the same dose of heparin, making adenocaine the only true difference between groups.

In the current hemorrhagic shock study, a model of two-staged bleeding was used, with a fast bleeding phase (2.15 ml/kg/min) lasting 7 min followed by a slower bleeding phase (1.15 ml/kg/min) until the target MAP of 35 mmHg was reached (Figure 7).

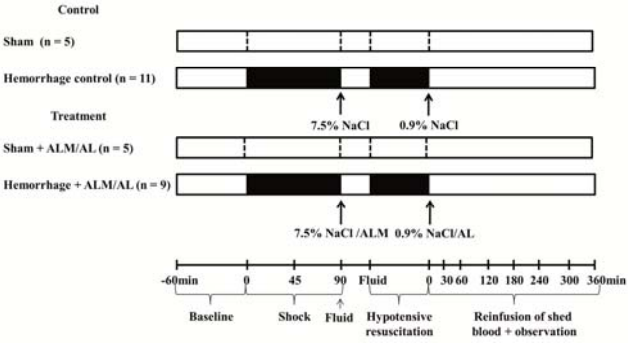

Figure 6 Schematic diagram of the study protocol used in the hemorrhagic shock study

This two-phase approach results in a more relevant physiological response that resembles the clinical scenario, with fast bleeding during the early stage of hemorrhage and a slower rate as arterial pressure decreases.(322) The MAP of 30–35mmHg was maintained for 90 min, resulting in eight pigs dying due to VF, and a total blood loss of approximately 74%, demonstrating the severity of the model. The choice of a 90-min shock period was based on pilot experiments demonstrating a 40% reduction in GFR at the end of the experiment.

ENDPOINTS

Infarct size

Infarct size (study 1) was assessed using triphenyltetrazolium chloride (TTC). TTC is reduced by cellular dehydrogenase enzymes and cofactors to a red compound (formazan). In the infarcted myocardium, cellular dehydrogenase activity is lost, which is incompatible with cellular survival. Cells that are viable reduce TTC and are stained red. At the end of the experiment the AAR was determined by injecting 20% unisperse blue dye via the external jugular vein after the left coronary artery was ligated. This way normal tissue is stained blue while the AAR is left unstained. Extra-left ventricular tissue was removed and the left ventricle was sliced transversely into five to six slices. The non-stained AAR was separated from the blue-stained non-ischemic zone myocardium, and the AAR was incubated in a 37°C 1% solution of buffered (pH 7.4) TTC for 15 minutes to identify the area of necrosis (AN) within the AAR (Figure 8).

Figure 7 One representative slice from a canine heart subjected to regional I/R. The blue area represents the normal tissue, while the non-blue area represents area at risk. Within the area at risk, viable tissue stains red and necrotic tissue is pale

The heart was then stored in 4% formaldehyde buffer to enhance color contrast. The TTC-negative area and TTC-positive areas were meticulously separated and weighed. The gravimetric technique of infarct size quantitation has been used for years,(198, 323) and several studies(324, 325) have demonstrated a very high correlation between planimetric and gravimetric analysis.(328, 329) From the study by Toombs et al.,(325) it was shown that the correlation between planimetry and gravimetric methods is 0.826 for the AAR/LV and 0.874 for An/AAR.

Coronary Perfusion Pressure

Normally the myocardium is supplied by blood primarily during diastole due to the development of high left ventricular tissue pressures and vascular resistance that impede flow through the intramural vessels during systole. During CPR (study II), there is no contraction of the myocardium, and the resistance vessels are fully dilated, making the pressure gradient the main determinant of myocardial blood flow, which is often below 30% of normal levels.(326, 327) Niemann et al.(328) and Ditchey et al.(329) proposed that the coronary perfusion pressure (CPP) is the difference in pressure between the aorta and the right atrium and that myocardial blood flow was dependent on the pressure difference. During the compression phase the coronary flow is retrograde, whereas it is antegrade during the decompression phase.(330, 331)

The importance of the CPP is illustrated by the close relationship between CPP and myocardial blood flow and successful resuscitation.(332, 333) Studies demonstrated that higher CPP results in higher rates of ROSC and both short-term and long-term survival.(332, 334-336)

The correlation between CPP and survival following cardiac arrest highlights CPP as an important research tool to monitor resuscitation efforts.

The Utstein-style guidelines for uniform reporting of laboratory CPR research specify that the point just before compression (end-diastole) should be used for calculating the CPP by subtracting the aortic and right atrium pressures (Figure 9).(337)

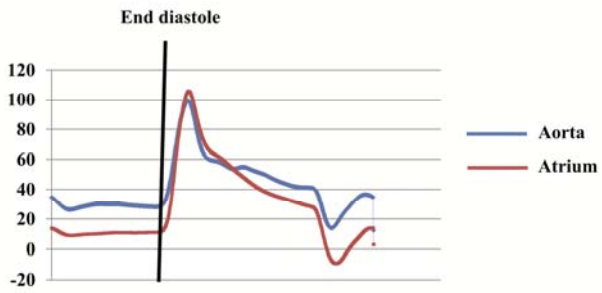

Figure 8 Example of calculation of the CPP at the point just before compression

However, controversies exist regarding the optimal method for calculating the CPP with regard to the time point of subtraction or whether to use an integrated approach.(338) Kern et al.(331) demonstrated that during the compression phase of CPR, coronary flow was retrograde (blood moving from the coronary arteries into the aorta) independent of the CPP.

Otlewski et al.(338) demonstrated nicely that the CPP is very dependent on the choice of methods, yielding differences in the CPP up to 27 mmHg. The integrated approach is favored by some, since it also incorporates pauses in CPR and subtracts retrograde flow during the compressions phase; however during advanced life support where the pig is intubated, chest compressions are uninterrupted. Furthermore, when using the integrated approach, the negative CPP during the compression phase is subtracted from the positive CPP during the relaxation phase, which could potentially cause an underestimation of the CPP. We chose to measure the CPP according to the Utstein guidelines as the difference between aortic pressure and right atrial pressure at the point just before compression calculated as the average of the last 10 beats of the 1st cycle of compressions.

Cardiac function

Evaluation of cardiac function (study II and III) is complex, and many indices are used as measures of cardiac function. A correct assessment of ventricular systolic and diastolic functions is fundamental for understanding cardiovascular pathophysiology and to evaluate the efficacy of interventions. However, indices of cardiac function are influenced by many factors such as preload, afterload, and heart rate. In the present thesis, clinical indices, pressure-derived indices, and pressure-volume relationships were used to evaluate cardiac function. The clinical indices used were cardiac index (CI) and ejection fraction EF. Cardiac index is defined by cardiac output divided by body surface area or, as here, divided by body mass, while cardiac output is the volume of blood being pumped by the heart in a minute. The other clinical index used, EF, is the percent of the end-diastolic volume that is ejected during systole per heart beat and is calculated accordingly: (end-diastolic volume – end-systolic volume) / end-diastolic volume. The pressure derived factors used to assess cardiac function were

the maximum positive development of ventricular pressure over time (dP/dtmax) and the maximum negative development of pressure over time (dP/dtmin) (Figure 10).

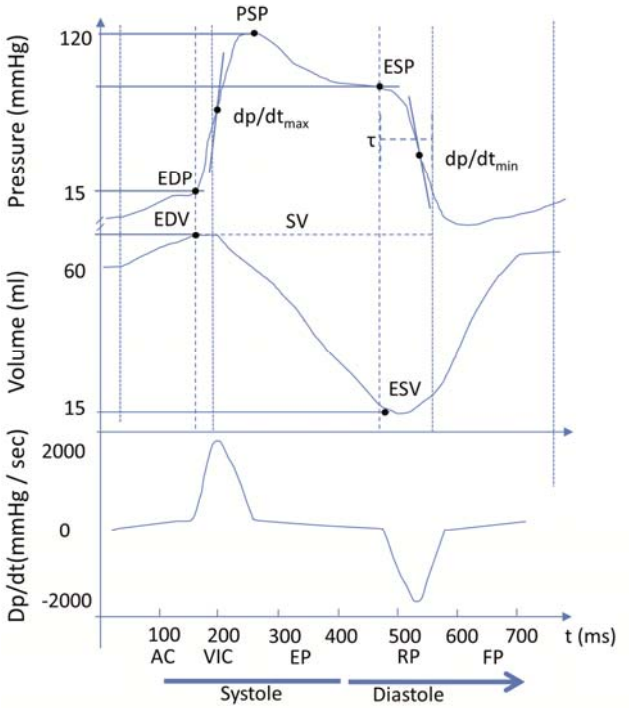

Figure 9 A representative example of a cardiac cycle. Pressure (upper graph) and volume (lower graph) curves illustrate the various phases of one single cardiac cycle. Peak systolic pressure (PSP), end-diastolic and end-systolic pressure (EDP, ESP), end-diastolic and end-systolic volume (EDV, ESV), relaxation time (τ), stroke volume (SV), maximum and minimum rates of pressure rise and decline during ejection (dP/dtmax) and relaxation phase (dp/dtmin).; VIC, ventricular isovolumetric contraction; EP, ejection phase; diastole: RP, relaxation phase; FP, filling phase; AC, atrial contraction. Reprinted with permission: Yerebakan C. et al.; Interact CardioVasc Thorac Surg 2009;9:163-168 Copyright ©2009 The European Association for Cardio-thoracic Surgery

The clinical indices and pressure-derived factors are all easily measured, but they are all sensitive to changes in preload, afterload and heart rate. However, some discrepancy exists with regard to the degree of sensitivity to changes in afterload for dP/dtmax, In some papers (339, 340) an increase in afterload is associated with an increase in dP/dtmax while in other papers no effect of afterload on dP/dtmax is found.(341, 342) It is argued that if the afterload increases, the rate of pressure development must be increased due to a fixed duration of the systolic phase. However, some studies show that at low pressures, dP/dtmax is obtained right at aortic valve opening due to premature shortening, while at higher pressure, dP/dtmax is obtained before aortic valve opening. These results indicate that aortic valve opening pressure does not usually affect dP/dtmax unless arterial pressure is low enough to limit its full development.(342, 343) However it is generally believed that pressure-derived measurements should be interpreted with caution in cases where there are changes in loading conditions, which will be encountered in the cardiac arrest and hemorrhagic shock studies.

To circumvent the influence of loading conditions, we also assessed cardiac function using pressure-volume indices, which allows determination of ventricular performance independent of loading conditions. The following parameters were calculated using instantaneous pressure-volume data: the end-systolic pressure–volume relationship (ESPVR), preload recruitable stroke work (PRSW), and the end-diastolic pressure–volume relationship (EDPVR).

ESPVR and PRSW characterize the systolic function of the ventricle, while EDPVR describes the passive properties of the ventricle (compliance), with the muscles in a relaxed state, hence changes in slope of the EDPVR are due to changes in myocardial material properties.(344) During the cardiac cycle, the pressure-volume "loop" progresses in a counter clockwise fashion, with the lower right pressure-volume point coinciding with the end of diastole, while the upper left corner coincides with the end of systole (Figure 11a). During changes in loading conditions by either preload reduction or afterload elevation, a series of evenly declining or increasing pressure-volume loops are produced (Figure 11b).

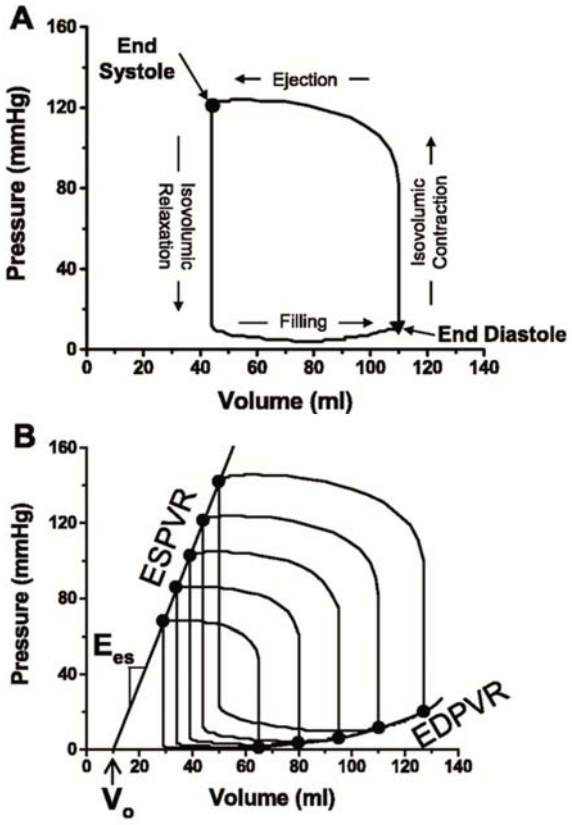

Figure 10 A) Schematic diagram the cardiac cycle B) Schematic diagram of the cardiac cycle during a preload occlusion resulting in a reduction in ventricular filling. The lines represent connected end-systolic and end diastolic pressure. Reprinted with permission. Burkhoff D et al. Am J Physiol Heart Circ Physiol 2005;289:H501-H512 ©2003 by American Physiological Society

The ESPVR relationship is constructed by connecting end-systolic pressure-volume points (upper left hand corner of each loop), while the EDPVR relationship is constructed by connecting end-diastolic pressure-volume points (lower right hand corner of each loop). The third parameter derived from the pressure-volume loops is PRSW, that is, the relationship between internal stroke work and end-diastolic volume.(345) The stroke work is derived from the area inscribed by each pressure-volume loop.

The ESPVR and PRSW are both characterized by a slope and volume axis intercept (V0) portraying a linear relationship. The slope of PRSW has the unit (mmHg · ml · ml-1) and is regarded independent of heart size, making comparisons of contractility between species possible.(344, 345) In contrast, the slope of the EDPVR is nonlinear, and a variety of curve fits have been applied to describe the EDPVR.

ESPVR, EDPVR, and PRSW are generally accepted to be load-independent measures of ventricular function with sensitivity to contractile state and inotropic agents.(344, 346, 347)

The technique requires simultaneous recordings of instantaneous left ventricular volumes and intraventricular pressures in vivo. The ventricular volume is measured using the conductance technique.(348) The pressure-volume catheter is equipped with equally spaced electrodes with a current applied between the first and last electrode, while the inner electrodes measure the voltage changes that correspond to the conductivity of the blood. These electrode pairs divide the LV cavity into segments that are stacked and summated to give the total conductance, which is the sum of blood conductance and conductance of contiguous tissues including myocardium, pericardium and lungs: Gmeasured = Gblood + Gtissue. Gtissue is referred to as parallel conductance. During injections of hypertonic saline, the conductivity of blood changes while tissue conductivity is unchanged, which allows the separation of blood conductance from tissue conductance (parallel conductance) so that the tissue conductance component can be subtracted. Blood conductivity (using hypertonic saline) was measured at baseline and after infusion of shed blood while the electric resistance of blood (rho) was measured repeatedly throughout the hemorrhagic shock study because of the large fluid fluctuations.

The pressure-volume-derived measurements have the clear advantage of being insensitive to changes in preload and afterload. However, they are still sensitive to large changes in heart rate, and since they require simultaneous recordings of instantaneous left ventricular volumes and intraventricular pressures, it is technically more difficult than the indices derived from pressures only.

The use of pressure-volume loops is also associated with limitations that have to be accounted for:

In general the ESPVR are not always linear (349); however if the slope of the relationship is calculated from data points within a narrow pressure and volume range, the assumption of a linear relationship is acceptable.

During preload occlusions achieved by transiently occluding the inferior vena cava to generate declining loops, arterial pressure decreases, with potential sympathetic reflex-mediated increases in heart rate and contractility, which can affect measurements. To avoid this, preload occlusions were performed in triplicate over a relative short period of time (10 seconds), with sufficient pauses in between to avoid sympathetic reflex-mediated increases in heart rate and contractility and with a sufficient number of beats (minimum of 10)

Changes in calcium levels during arrhythmias can potentially affect the myocardial contractility; this was circumvented by excluding pressure-volume loops in pressure-volume data sets containing arrhythmias.

Blood flow

The injection radioactive microspheres and the collection of a reference blood sample have been used for decades to estimate organ perfusion in different experimental settings.(350, 351) Measurements using microspheres serve as the gold standard for evaluation of tissue perfusion and were used in study II and III. The use of polystyrene neutron-activated microspheres circumvents the disadvantages of radioactive microspheres and the handling of radioactive waste.(351, 352) However, important considerations must be taken into account to ensure precision of the measurements. A uniform distribution of microspheres to all organs is required, and is ensured by injection into the left atrium/ventricle for adequate mixing of microsperes. Signal strength for optimal counting characteristics is ensured injecting a sufficient number of microspheres.(353) It is recommended that a minimum of 400–600 microspheres is needed per tissue sample.(354) The following equation provided by the manufacturer was used to calculate the number of microspheres to be injected: Y = 1.2·106 + 1.9·105·weight. One gram tissue samples are generally sufficient to ensure entrapment of enough microspheres for optimal counting. Furthermore, it is important that the microspheres are completely entrapped in the vascular beds downstream from the site of injection during the first circulation and that the microspheres must remain entrapped until counted. The 15-µm microspheres are in general accepted due to a distribution similar to red cells, and at the same time the degree of non-entrapment is minimal.(354)

A last consideration is that injection and entrapment of the microspheres must have no effect on either the general circulation or on local organ hemodynamics despite the entrapment in the microcirculation. Studies have demonstrated that injection of even large numbers of microspheres does not cause any significant physiological effects.(355, 356) Less than 1% of the capillaries are plugged by entrapped microspheres per injection, which is not sufficient to induce tissue ischemia. In the cardiac arrest and hemorrhagic shock studies, the following technique was used:

Neutron-activated microspheres (15 µm diameter, BioPhysics Assay Laboratory, Inc, Worcester, MA, USA) were delivered through a pig-tail catheter placed in the left ventricle to measure local organ blood flow. Just before microsphere injection, a reference sampling of blood from the femoral artery was started, and a reference sample was withdrawn for 90 seconds at a rate of 7 ml/min in study II and 6.6 ml/min in study III. The reference blood sample was centrifuged and the supernatant was removed. At the end of the experiment, 1 to 1.5 gram of tissue samples was harvested from the organs of interest. The reference blood sample and harvested tissue were dried overnight and shipped to the manufacturer. The microspheres are activated by neutron bombardment, enabling measurement of radioactive decay.

The regional blood flow (expressed as ml/min/g tissue) is then calculated as ((flow in the reference sample · counts per min in tissue samples)/ counts per min in the reference sample)/tissue weight (g).

Renal Function

Renal function during hemorrhagic shock (study III) was estimated using GFR, plasma and urinary markers of renal function. GFR is the gold standard in evaluating renal function, and, in the pig, chromium-51-ethylenediaminetetraacetic acid (Cr-51-EDTA) clearance has been shown to accurately estimate GFR (357). In the hemorrhagic shock study, we determined Cr-51-EDTA clearance using a continuous infusion clearance technique with a bolus injection at baseline followed by at steady infusion during the remainder of the experiment. The disadvantage of this method is some imprecision when urine volumes are small; however, it allows for measurement of renal function sequentially over time, and to quantify changes from hour to hour. In pilot experiments

baseline was extended by 1 hour to ensure that chromium levels were stable before bleeding was started (Figure 12). The low urine production during bleeding caused Cr-51-EDTA concentrations to increase dramatically in the pilot experiments. Due to this, Cr-51-EDTA infusion was turned off during the bleeding phase and was turned on again at fluid resuscitation in order to ensure a steady-state concentration.. Blood and urine samples were counted twice using a gamma counter (Cobra II, Packard, Meriden, CT, USA). GFR was calculated using the equation: GFR = (Vurine · CPMurine, 1ml) / CPMplasma, 1ml: Vurine: Volumen of urine; CPM: Counts per minute. Counts were corrected for background activity and physical decay using the equation: CPMdecay = (CPM-background) / (e(-ln2*(elapse time/t½))), t½ (51CrEDTA) = 27.8 days.

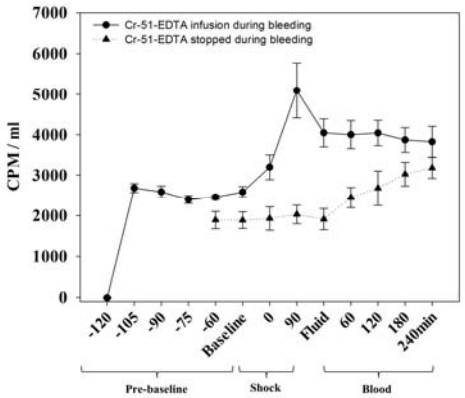

Figure 11 Representaive example of Cr-51-EDTA levels during experiments where infusion was continued during bleeding, and experiments where infusion was paused during bleeding.

RESULTS

The majority of results obtained for this dissertation are reported in the individual papers, while only a summary of the results from each paper will be presented below.

STUDY I:

To aim of study I was to investigate whether the infarct sparing effect of postcon involves inhibition of PMN functions. The main finding in study I was that postcon initiated immediately at reperfusion reduced infarct size and PMN accumulation in a rat model of myocardial I/R (Figure 13).

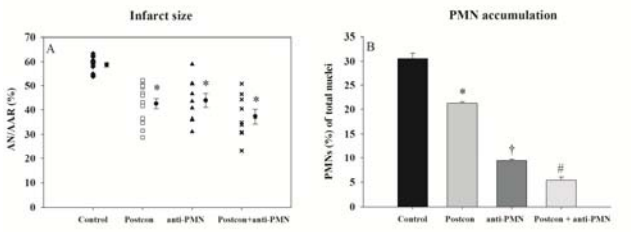

Figure 12 Left figure (A): Infarct size expressed as a percentage of the area at risk in each rats for all four groups. Values represent mean ± SEM. *P < 0.05 vs. control. (AN/AAR, area of necrosis/area at risk). Right Figure (B): Treatment with postcon, PMN antiserum, and postcon + PMN antiserum significantly decreased PMN accumulation. Values are mean ± SEM; *P < 0.05 vs. values in control group; †P < 0.05 vs. values in postcon group; #P < 0.05 vs. values in PMN antiserum group

However when postcon was applied in already PMN-depleted rats, no further reduction in infarct size was found. Furthermore, in the canine model, postcon reduced infarct size and attenuated superoxide anion production by PMNs in coronary venous blood draining the AAR (Figure 14).

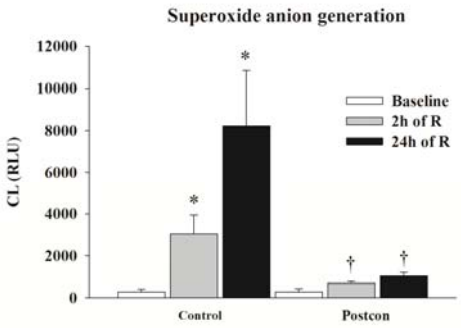

Figure 13 Superoxide anion generation in local coronary venous blood at baseline, 2 h and 24 h of reperfusion in control and postcon animals. Values represent mean SEM. *P < 0.05 vs. baseline, †P < 0.05 vs. control.

STUDY II:

Study II tested the hypothesis that adenocaine attenuates 1) myocardial dysfunction, 2) systemic inflammation and 3) brain injury in a porcine model of cardiac arrest. Median time to ROSC was 360 seconds in both groups, with 11 out of 16 (69%) pigs achieving ROSC in the cardiac arrest group and 7 out of 12 (58%) pigs in the cardiac arrest + Adenocaine group (p=0.57), summarized in table 1.

Table 1

	Group (Median; Interquartile range (IQR))	
	Cardiac arrest	Cardiac arrest + Adenocaine
ROSC	5/16	5/12
Time to ROSC (seconds)	360(240-420)	360(240-260)
Total dose of EPI	1.44(0.74-1.7)	0.91(0.69-1.84)
Number of shocks per surviving pig	2(1-3)	2(1-2)
CPP	17.8(13.7-22.1)	32.1(22.4-37.5)
Myocardial blood flow during CPR (ml/min/g tissue)	0.07-(0.04-0.21)	0.11(0.03-0.28)

Treatment with adenocaine during resuscitation significantly improved early myocardial function, evidenced by significant improvements in dp/dtmax and dp/dtmin and by a lack of a rightward shift in the V0-intercept of the ESPVR. (Figure 15)

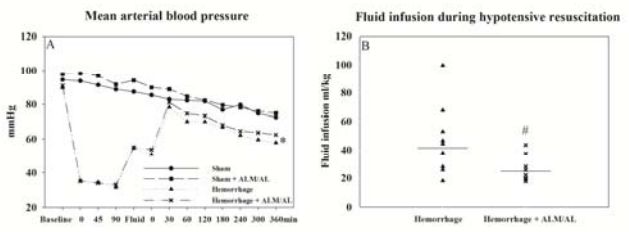

Figure 17 Left figure (A): MAP, clearly showing the different phases of the study (Median: within-animal SD, 0.08; total SD, 0.12). *Significant time/group interaction compared with both sham groups (ANOVA). Right figure (B): Volume of fluid required to maintain a MAP af minimum 50mmHg for 30min; # p=0.02

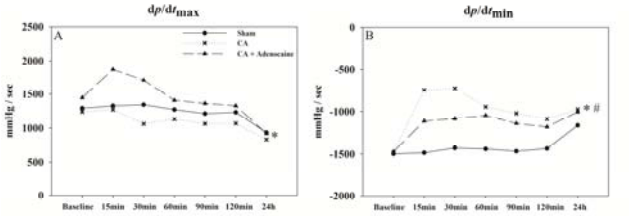

Figure 14 Left figure (A): Maximum positive development of ventricular pressure over time (dP/dtmax) (Mean: within-animal SD, 265.6; total SD, 337.4). Right figure (B): Maximum negative development of pressure over time (dP/dtmin) (Mean: within-animal SD, 173.9; total SD, 283.5): * Significant time/group interaction between cardiac arrest (CA) and CA +Adenocaine; #Significant time/group interaction between sham and cardiac arrest (ANOVA)

Neurological function was assessed by neurologic deficit scores (0= normal; 500= brain death) and histological evaluation (score of 0-4). The median neurologic deficit score was 17.5 (IQR 0:75) in the cardiac arrest group and 35 (IQR 15:150) in the cardiac arrest + Adenocaine group, both being significantly higher than shams 0(IQR 0:0). The histological score in hippocampus and cortex tended to be lower in the cardiac arrest + Adenocaine group vs. the cardiac arrest group; however, the histological score was not different in either of the CA groups when compared to shams (Figure 16).

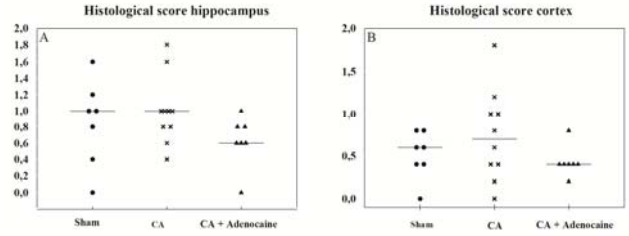

Figure 15 **Left figure (A): Histology scoring in the hippocampus showed no difference between groups; Sham 1(IQR 0.4:1.2) cardiac arrest 1(IQR 0.8:1) cardiac arrest + Adenocaine 0.6(IQR 0.6:0.8) (p=0.19) Right figure (B): Similar to hippocampus no difference in histology scoring was found in cortex; Sham 0.6(IQR 0.4:0.8) cardiac arrest 0.7(IQR 0.4:1) cardiac arrest + Adenocaine 0.4(IQR 0.4:0.4) (p=0. 32)**

Systemic inflammation was evaluated using superoxide anion production after stimulation by opsonized zymosan (OPZ) and by plasma cytokine levels. In the cardiac arrest group (n=5) OPZ-induced superoxide anion production increased significantly during the course of the experiment, whereas this was significantly attenuated 120 min after ROSC in the cardiac arrest + Adenocaine group (Figure 17). Overall, cardiac arrest and resuscitation were not associated with an increased production of the measured cytokines (IL-1β, IL-6, IL-8, IL-10, and TNF-α).

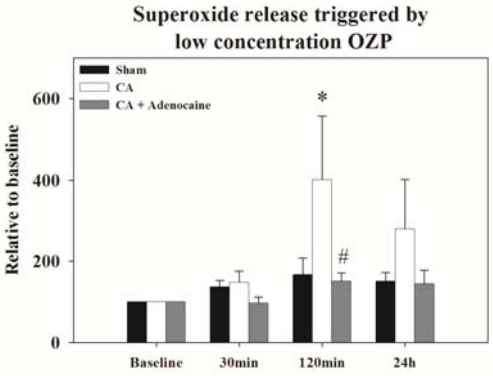

Figure 16 Total superoxide anion generation induced by OPZ (0.2mg/ml) at different time points and normalized to the basal production. Superoxide anion production was significantly attenuated by AL 120min after ROSC (Control: 402±155.6 vs. Adenocaine: 151.1±20.1; p<0.05). Means ± SEM *Significant vs. baseline and 30-min CA; # Significant vs. 120min CA + AL

STUDY III

In the third study, the aims were to investigate the effect of ALM/AL on the initial fluid requirement during the fluid hypotensive resuscitation phase, and the effects of adenocaine on cardiac and renal function in a porcine model of hemorrhagic shock. In accordance with the protocol, MAP during hypotensive resuscitation was similar in the hemorrhage groups, but the fluid volume needed to maintain a target MAP of 50 mmHg was 41.4 (CI: 27.7 – 61.8) ml/kg in the hemorrhage control group and 24.7 (CI: 19.4–31.5) ml/kg in the hemorrhage + ALM/AL group (p=0.02).

Infusion of ALM during fluid resuscitation was associated with a significant increase in systemic vascular resistance index and cardiac contractility (dP/dtmax) (Figure 19).

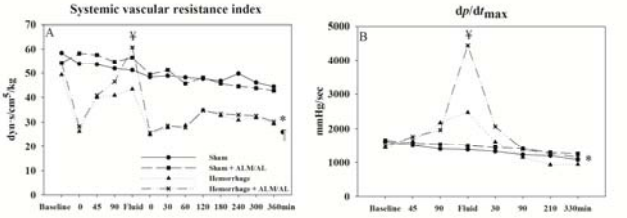

Figure 18 Left figure (A): Systemic vascular resistance index (Median: within-animal SD, 0.18; total SD, 0.23). Right figure (B): Maximum rate of pressure development over time (dP/dtmax). *Significant time/group interaction compared with both sham groups (ANOVA); ¶ Average mean level significantly different during reperfusion when compared to sham groups; ¥ Significant difference between hemorrhage groups during hypotensive resuscitation.

Infusion of high dose AL in 0.9% NaCl with return of shed blood transiently reduced whole body oxygen consumption (VO2). The decrease in VO2 was due to an increase in mixed venous oxygen content in the ALM/AL group, without difference in cardiac index between hemorrhage groups. (Figure 20).

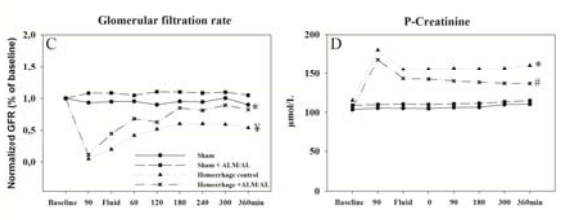

Figure 19 Left panel (A) Cardiac index (Median: within-animal SD, 0.18; total SD, 0.23). Right panel (B) Whole body oxygen consumption decreased by 27% after infusion of high dose AL consumption (Median: within-animal SD, 0.19; total SD, 0.22). *Significant time/group interaction compared with both sham groups (ANOVA); ¥ t-test on difference from start of blood infusion to 30 min after blood infusion between hemorrhage groups. Cardiac function evaluated by ESPVR and PRSW, and renal function evaluated by GFR and plasma creatinine, were significantly improved with ALM/AL (Figure 21).

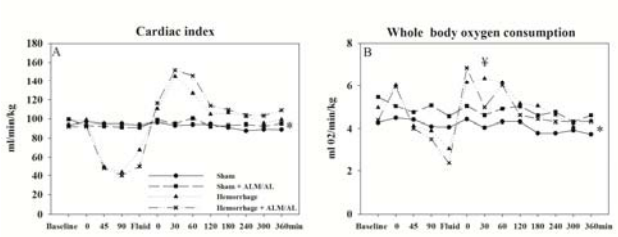

Figure 20 Upper left figure (A): Preload recruitable stroke work (Mean: within-animal SD, 0.36; total SD, 0.52). Upper right figure (B): End-systolic pressure-volume relationship (Mean: within-animal SD, 0.36; total SD, 0.62). Lower left figure (C): Glomerular filtration rate (Mean: within-animal SD, 11.3; total SD, 16.6) Lower right figure (D): Plasma creatinine levels (Mean: within-animal SD, 6.9; total SD, 16.75). *Significant time/group interaction compared with both sham groups (ANOVA) # Significant time/group interaction between hemorrhage groups during reperfusion (ANOVA). ¥ t-test on difference from baseline to end of experiment between hemorrhage groups

DISCUSSION

The role of PMNs in the cardioprotective effects of postconditioning

In the first study, using a rat model of regional ischemia and reperfusion, it was demonstrated that postcon reduced infarct size. However, when postcon was applied in PMN-depleted rats, no further reduction in infarct size was observed. Furthermore, in a canine model of regional I/R, postcon attenuated PMN superoxide production, implying that cardioprotection by postcon involves direct inhibition of PMNs.

The first study by Zhao et al. (10) that introduced postcon, demonstrated that postcon was associated with a reduction in expression of P-selectin, PMN accumulation, and PMN adherence to the endothelium, suggestive of a potential effect of postcon on PMN function. However postcon is also cardioprotective in PMN-free environments, disputing a significant role of PMNs as a part of postcon.(47) When PMNs are added to in vitro preparations, a further decrease in post-ischemic endothelial function (40) and contractile function (39, 54) relative to the PMN-free cohort have been reported. This suggests that PMNs do participate in I/R injury and that the dependence of postcon on circulating factors should be investigated in the presence of these factors using in vivo models.

If the application of postcon is delayed or too many repetitive occlusions are used, the protective effect is abolished.(358) This demonstrates that the early minutes of reperfusion are essential, and, that despite the short time window of postcon, it may affect long-term effects of I/R. Several studies have shown that the duration of postcon is sufficient to activate G-protein coupled receptors (i.e. adenosine receptors),(197) phosphorylate NO,(214) reduce surface expression of P-selectin, and reduce cytokine production. Ultimately, this leads to a reduction in PMN adherence to the coronary vascular endothelium. Extravascular accumulation of PMNs occurs several hours after reperfusion, whereas intravascular activation, adhesion to endothelium, and accumulation occurs within minutes of reperfusion, manifested as PMNs rolling along the endothelium. Entman et al.(359) demonstrated that immediately upon adhesion, the neuptrophils release superoxide anions, suggesting a rapid adhesion-dependent activation of the NADPH oxidase. This demonstrates that PMN-mediated injury occurs in the early phase of I/R and coincides with the existence of endothelial dysfunction within 2.5 min after reperfusion.(59) Thus, postcon despite its short duration seems to prevent endothelial dysfunction during the early minutes of reperfusion via inhibition of PMN adherence, superoxide release, subsequent accumulation and damage.(10) However it is difficult to completely separate cause and effect in this experiment. The changes in PMN function and oxygen radical generation following postcon could all be due to the smaller infarct size produced by postcon.

Effect of adenocaine on global ischemia-reperfusion injury

In the case of cardiac arrest and hemorrhagic shock, ischemic postcon may not be applicable, whereas pharmacological postcon could be a suitable alternative. Studies exploring the effects postcon demonstrate that pharmacological strategies targeting reperfusion injury must be initiated at the onset of reperfusion and they must target several aspects of reperfusion injury, e.g. PMNs. Adenocaine is a promising agent to trigger pharmacological postconditioning because it can be administered at reperfusion, and, due to the combination of the two drugs adenosine and lidocaine, it exerts broad-spectrum effects.

Despite that select pharmacotherapy during resuscitation increases the rate of ROSC, hospital survival to discharge has not been correspondingly increased.(82) In study II in this thesis, early infusion of adenocaine during resuscitation was used to target the post-cardiac arrest syndrome. Infusion of adenocaine improved early myocardial function, augmented myocardial and pulmonary blood flow and reduced systemic inflammation, but without effects on neurological dysfunction or cerebral blood flow.

Both adenosine and lidocaine are known to induce vasodilation and a drop in blood pressure.(238, 263) The infusion of vasodilators during cardiopulmonary resuscitation may seem counterintuitive when the primary treatment is epinephrine, which has a vasoconstrictive effect due to its alpha-adrenergic stimulation. This vasoconstriction causes an increase in systemic pressure, which in turn increases the CPP. Even though administration of epinephrine increases the rate of ROSC, it is also associated with post-resuscitation myocardial dysfunction and a decrease in cerebral microcirculatory function.(89, 360) Therefore therapies such as adenocaine that target the post-resuscitation syndrome without abolishing the effects of epinephrine on the rate of ROSC are warranted. Actually, in the current study, there was a strong trend toward a higher CPP during chest compressions in the adenocaine group compared to the untreated cardiac

arrest group. This difference in CPP was due to a significantly higher aortic pressure in the adenocaine group, while right atrial pressures were comparable between groups.

The dose of adenocaine was based on in vitro experiments demonstrating inhibitory effects on PMN function,(287) and the inhibitory effect on PMN function was confirmed in study II. In a previous study in rats, prolonged infusion of adenosine for 60 or 90 minutes improved survival and delayed cell loss in the hippocampus.(248) In our study, adenocaine was infused over a period of only 6 min during resuscitation. This infusion period was chosen since a short infusion duration is more clinically applicable in contrast to a prolonged continuous infusion. We can only speculate whether a prolonged infusion or repeated boluses of adenocaine could have further improved myocardial function and neurological outcome.

In study III, treatment with ALM/AL during the early phase of resuscitation was tested in a porcine model of hemorrhagic shock. Resuscitation with 7.5% NaCl and ALM reduced fluid requirements during fluid resuscitation in the pig following severe hemorrhagic shock, which was also associated with improved hemodynamic stability and cardiac function. Furthermore, treatment with 0.9% NaCl and AL administered with the return of shed blood transiently reduced whole body O2 consumption and improved cardiac and renal function over a 6-hour period. Resuscitation with fluid or blood is essential to the effective treatment of hemorrhagic shock; however, resuscitation with fluid or blood also initiates reperfusion injury within seconds.(193, 361) The strategy to introduce treatment at both fluid resuscitation and re-infusion of shed blood was applied to target reperfusion injury occurring at both fluid resuscitation and re-infusion of blood, the latter related to the reintroduction of inflammatory cells and soluble pro-inflammatory factors. The rationale for combining adenocaine and Mg2+(ALM) diluted in 7.5% saline was derived from the study by Letson et al.,(291) demonstrating a protective effect using a rodent hemorrhagic shock model with ultra-small volume resuscitation. The high dose of adenocaine infused at re-infusion of shed blood was based on study II in this thesis where it demonstrated a protective effect on myocardial function after cardiac arrest.(292)

As in the model of cardiac arrest, it seems counterintuitive to infuse vasodilating drugs with negative chronotropic effects during hemorrhagic shock, where vasoconstriction and tachycardia are major compensatory mechanisms. In a study by Tisherman and colleagues (362) adenosine was administered intraperitoneally to circumvent these effects of intravenous adenosine administration. However, as demonstrated here, if administered over a few minutes, the vasodilatory and hypotensive effects of adenodcaine effects are only temporary and treatment with 7.5% NaCl ALM actually decreased fluid requirements during hypotensive resuscitation and increased systemic vascular resistance, demonstrating prolonged protective effects.

It is unknown how the combination of two vasodilators can cause an increase in arterial pressures during cardiopulmonary resuscitation and decrease fluid requirements and increase systemic vascular resistance during hemorrhagic shock. Cardiac arrest and hemorrhagic shock are known to induce substantial endothelial and microcirculatory dysfunction due to changes in membrane potential, electrolyte imbalance, the release of inflammatory mediators and adherence of PMNs to the endothelium.(102, 164) Both adenosine and lidocaine have been shown to attenuate PMN adherence to the endothelium, and are associated with micro-vascular protection. However the exact mechanisms responsible for the increase in arterial pressure remain speculative and further studies are warranted to explore the effects of adenocaine in the setting of cardiac arrest and hemorrhagic shock.

An interesting finding in the hemorrhagic shock study was that infusion of high-dose adenocaine at re-infusion of shed blood resulted in a 27% decrease in whole body oxygen consumption. Much adenosine research has revolved around the role of adenosine as a metabolic regulator of organ function, matching blood flow to energy consumption (demand).(363) As described, adenosine increases in response to hypoxia, causing vasodilatation and thereby an increase in blood flow and supply which relieves the cause of hypoxia.(238) However adenosine not only increases supply but also decreases demand in the heart, brain and kidney. Stimulation of the A1 AR lowers body temperature and mediates metabolic suppression during torpor. (247, 364, 365) In the brain, metabolic rate decreases in response to hypoxia, an effect mediated through the A1 AR. In addition infusion of adenosine decreases renal oxygen consumption.(250, 364) Adenosine infusion has also been reported to decrease whole body VO2, (366, 367) while lidocaine at high doses has been reported to decrease cerebral oxygen consumption. (368) Therefore, there is scientific precedent for an oxygen consumption lowering effect of adenocaine. It is, however, noteworthy that, despite the decrease in VO2 30 minutes into reperfusion, neither cardiac nor renal function was decreased. In our study it is possible that adenocaine reduced whole body VO2 in part by blunting the metabolic effects of catecholamines via anti-adrenergic receptor modulation.(369) Plasma catecholamines are well known to increase in response to shock, and may increase oxygen consumption through a beta-adrenergic mechanism or so called “oxygen wasting”.(370) Adenosine alone is also known to decrease heart rate and cardiac contractility as determinants of cardiac VO2 in the presence of a beta-adrenergic stimulus. A reduction in adrenergically increased VO2 is supported by the observation that the same dose of adenocaine did not cause a decrease in VO2 in the sham group. This illustrates that a potential mechanism for organ protection induced by treatment with adenocaine is a decrease in oxygen demand, and a reduction in potential oxygen supply/demand mismatch.

LIMITATIONS

Some of the limitations of this thesis have been addressed in the material and methods section.

Limitations in the present studies are primarily related to the experimental animal models that were used. In general animal models should approximate the clinical scenario as close as possible. In all three studies, the animals were anesthetized due to practical and ethical reasons, which is not the case in a clinical setting. The administration of anesthetics affects the cardiovascular system, the inflammatory response, and metabolism, and the effects are dependent of the type of anesthesia. In the cardiac arrest study isoflurane was used as the anesthetic agent which may have preconditioned the heart and brain and activated protective mechanisms at reperfusion.(371) This may have influenced the results; however, the anesthetic protocols were identical in all groups, making the treatment the only difference between groups.

In study I, two different animals models of I/R injury were used, which is an apparent limitation. In study III, a pressure-controlled hemorrhagic shock model was used that does not

completely reflect the clinical scenario of combined uncontrolled hemorrhage and soft tissue injury.

In study II and III the pigs were observed for relatively short observation periods being only 6 hours in the hemorrhagic shock study. In study II, the cardiac arrest study, pigs were observed for 24 hours; however neurological injury is an ongoing process and continues to develop over time, why 24 hours may have been to short an observation period. Furthermore neurological scoring may have been affected by opioid anesthesia (fentanyl patch 25 µg/h) used to control post-procedural pain as required by the Institutional Animal Care and Use Committee of Emory University

CONCLUSION

This thesis demonstrated that early intervention with either postconditioning or adenocaine attenuates I/R injury and organ dysfunction in animal models of acute myocardial infarction, cardiac arrest or hemorrhagic shock. Study I showed that postconditioning inhibits PMN function, and implies that future therapies for the treatment of I/R injury should encompass strategies targeting PMNs and inflammation. In line with this, it was demonstrated in study II that early infusion (pharmacological postconditioning) of adenocaine attenuated PMN production of superoxide anions following cardiac arrest.

The protective effects of adenocaine in models of cardiac arrest and hemorrhagic shock are encouraging, but further studies in more clinically relevant models exploring the underlying effects are warranted. Postconditioning and adenocaine may be promising new therapies for protection against I/R after acute myocardial infarction, cardiac arrest and hemorrhagic shock.

SUMMARY

Cardiac arrest and acute myocardial infarction are leading causes of death in the middle-aged and elderly, whereas trauma primarily affects the younger segment of the population. The three conditions are all characterized by a period of reduced blood flow either regionally in the heart or globally, and treatment strategies target the restoration of normal blood flow. Paradoxically, reperfusion of ischemic tissue contributes to cellular injury in all three settings. Ischemic postconditioning initiated immediately at reperfusion was in 2003 introduced as a new potential treatment to limit injury following acute myocardial infarction.

The aim of this dissertation was explore the mechanism of ischemic postconditioning during regional ischemia and test the effects of early pharmacological postconditioning using adenocaine in models of global I/R injury.

In the first study, the mechanisms of postconditioning were explored. In a rat model of regional ischemia, it was demonstrated that postconditioning reduced infarct size. However when postconditioning was applied in already PMN-depleted rats, no further reduction in infarct size was observed. Furthermore, in a canine model of regional ischemia, postconditioning attenuated PMN superoxide production, implying that cardioprotection by postconditioning involves inhibition of PMNs.

In the second study, treatment with adenocaine as pharmacological postconditioning during the immediate phase of cardiopulmonary resuscitation, attenuated early post-resuscitation myocardial dysfunction, augmented pulmonary and cardiac blood flow and reduced PMN superoxide production in a porcine model of cardiac arrest.

In the third study, treatment with ALM/AL during the early phase of resuscitation was tested in a porcine model of hemorrhagic shock. Resuscitation with ALM/AL reduced fluid requirements during fluid resuscitation, transiently reduced whole body O2 consumption and improved cardiac and renal function.

In conclusion, early intervention with either postconditioning or adenocaine attenuates I/R injury and organ dysfunction in animal models of acute myocardial infarction, cardiac arrest or hemorrhagic shock. Postconditioning and adenocaine may be promising new therapies for protection against I/R after acute myocardial infarction, cardiac arrest and hemorrhagic shock.

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