PRECONDITIONING AGAINST ISCHEMIC INJURY OF THE CENTRAL NERVOUS SYSTEM IN AORTIC SURGERY
AN EXPERIMENTAL STUDY IN A PORCINE MODEL WITH REMOTE ISCHEMIC PRECONDITIONING AND DIAZOXIDE
HENRI HAAPANEN

PRECONDITIONING AGAINST ISCHEMIC INJURY OF THE CENTRAL NERVOUS SYSTEM IN AORTIC SURGERY
An experimental study in a porcine model with remote ischemic preconditioning and diazoxide

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Abstract

The repair of thoracoabdominal aortic aneurysms carries a substantial risk of ischaemic perioperative spinal cord injury. Although several protective methods have been developed, the risk of paraplegia has not been eliminated. Moreover, aortic aneurysms, including arch aneurysms, are complex clinical challenges requiring cerebral protection with hypothermic circulatory arrest (HCA). Hypothermia lowers the rate of cerebral metabolism and allows a temporary halt of the systemic circulation. However, there is still a risk for cerebral damage and a need for additional neuroprotective methods.

During the last 15 years, our research group has used a porcine model to investigate a variety of neuroprotective tools. In this thesis, an animal model was utilized to study the efficacy of remote ischaemic preconditioning (RIPC) to ameliorate ischaemic damage to the central nervous system, and to shed light on the potential mechanism. Moreover, diazoxide, the pharmacological mimic of RIPC, was tested in the HCA animal model.

In the first Study (I), RIPC showed beneficial effect on the spinal cord against ischaemic insult as recorded with motor-evoked potentials. Strikingly, the beneficial effect of RIPC was observed even before the ischaemia. In the second Study (II), some beneficial effect of RIPC was seen in the immunohistochemical analysis of the spinal cord ischemia but the result remains inconclusive. Similarly, the diazoxide-treated animals had better hemodynamic status postoperatively and mildly better antioxidant activity of the brain in the third Study (III). The fourth study (IV) was a review of the current knowledge of RIPC from the cardiovascular point of view.

Our studies indicate that RIPC might be a potential adjunct for preventing neuronal ischaemic injury in the setting of thoracoabdominal aortic surgery. Our result indicates that further preclinical studies with diazoxide are required before studies can be conducted in humans.

Keywords: aortic surgery, central nervous system protection, ischemic central nervous damage, ischemic preconditioning, pharmacological preconditioning.
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Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center
Acta Univ. Oul. D 1471, 2018
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä

Meidän tutkimusryhmämme on tutkinut useita keskushermostoa suojaavia tekniikoita ja lääkeaineita viimeisen 15 vuoden aikana. Käytämme sikaa koe-eläimenä mallina, jota on tämän väitetöskirjan osajulkaisuissa käytetty. Tämän väitetöskirjan tarkoituksena on ollut tutkia sekä esialtistavan raajaiskemia (RIPC) että farmakologisen mimeetin, diazoxiden, keskushermostoa suojaavia vaikutuksia sekä niiden mahdollista vaikutuksesta keskushermostoon.


Tutkimuksissamme osoitimme, että esialtistavan raajaiskemiassa on potentiaalia tulla yhdeksi vähenevä keskushermoston iskemiaan vastaan torakoabdominaalisen aortan kirurgiassa. Lisäksi diazoxidin mahdolliset neuroprotektiiviset vaikutukset vaativat lisää koe-eläintutkimuksia ennen ihmiskokeisiin siirtymistä.

Asiakirjat: aortan kirurgia, farmakologinen esialtistus, iskeeminen esialtistus, iskeeminen keskushermoston vaurio, keskushermoston suojaus
To Monika
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I was recruited into this group simultaneously with Oiva Arvola, and we grew up together as a team. Know that without your intelligence and persistence this project would have been impossible. I have always admired your open-minded and ground-breaking approach to research work. The laboratory work with you was more than just a job that needed to be done. During the numerous early mornings and even longer nights, there were good jokes and moments of despair and success. These shared experiences made you my co-worker and friend. Thank you for everything.
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Lastly and most importantly, I thank my family. To my parents, Elina and Heikki, I am privileged to have your unconditional love and support that you have given me every day. You taught me the most important rule of life: hard work is always rewarded. My dear sister and brothers, Tuomas, Jussi, Eveliina, Jaakko, Juho, and Aapo, I know I have not always been the easiest big brother, but after all, you are the world to me and growing up with you has been the greatest pleasure of my life.

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April 2018

Henri Haapanen
Abbreviations

8-OHdG 8-Oxo-2'-deoxyguanosine
acetyl CoA acetyl coenzyme A
ACP antegrade cerebral perfusion
AIF apoptosis-inducing factor
AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AKT protein kinase B
AMP adenosine monophosphate
ADP adenosine diphosphate
ATP adenosine triphosphate
BBB blood–brain barrier
Ca²⁺ calcium (ion)
CAPON carboxyl-terminal PDZ ligand of nitric oxygen synthase
cAMP cyclic adenosine monophosphate
CBF cerebral blood flow
CNS central nervous system
CO₂ carbon dioxide
COX cyclooxygenase
CPP cerebrospinal perfusion pressure
CPB cardiopulmonary bypass
CREB cyclic adenosine monophosphate-responsive element-binding protein
CSF cerebrospinal fluid
DTAA descending thoracic aortic aneurysm
DJ1/PARK7 Parkinson disease protein 7
ERK extracellular-regulated kinase
ETC electron transport chain
EtCO₂ end-tidal carbon dioxide
EtO₂ end-tidal oxygen
FADH₂ flavin adenine dinucleotide
GABA γ-aminobutyric acid
GTP guanosine triphosphate
HIF hypoxia-inducible factor
IL interleukin
IPC ischemic preconditioning
K⁺ potassium (ion)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>K$^+_{\text{ATP}}$</td>
<td>adenosine triphosphate-sensitive potassium ion</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
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<tr>
<td>MEP</td>
<td>motor evoked potential</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MPT</td>
<td>membrane permeability transition</td>
</tr>
<tr>
<td>mPTP</td>
<td>mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>sodium (ion)</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (oxidised)</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>Nfr2</td>
<td>nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>O$_2$</td>
<td>oxygen</td>
</tr>
<tr>
<td>OGG-1</td>
<td>8-Oxoguanine glycosylase</td>
</tr>
<tr>
<td>p53</td>
<td>protein 53</td>
</tr>
<tr>
<td>P$_a$CO$_2$</td>
<td>partial pressure of arterial carbon dioxide</td>
</tr>
<tr>
<td>PLA$_2$</td>
<td>phospholipase A$_2$</td>
</tr>
<tr>
<td>RCP</td>
<td>retrograde cerebral perfusion</td>
</tr>
<tr>
<td>RIPC</td>
<td>remote ischemic preconditioning</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>PEEP</td>
<td>positive end-expiratory pressure</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PPC</td>
<td>pharmacological preconditioning</td>
</tr>
<tr>
<td>Smac</td>
<td>second mitochondria-derived activator of caspase</td>
</tr>
<tr>
<td>SSEP</td>
<td>somatosensory evoked potential</td>
</tr>
<tr>
<td>rt-PA</td>
<td>recombinant tissue plasminogen activator</td>
</tr>
<tr>
<td>Src</td>
<td>proto-oncogene tyrosine-protein kinase</td>
</tr>
<tr>
<td>TAAA</td>
<td>thoracoabdominal aortic aneurysm</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor α</td>
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1 Introduction

Aortic disease, including descending thoracic aortic aneurysms (DTAAs) and thoracoabdominal aortic aneurysms (TAAAs), is the 12th leading cause of overall death in the United States (Svensson et al. 2008). Because of the devastating natural history of DTAAs and TAAAs, the 5-year survival rate remains between 13% and 50% (Elefteriades. 2002, Griepp et al. 1999, Svensson. 1997). Moreover, the mortality is still high in patients with spinal cord ischaemia after open or endovascular surgery. The paraplegia doubles the 5-year mortality (Conrad et al. 2008).

Although a great variety of methods have been developed to protect the spinal cord during and after procedures, the risk for spinal cord ischaemia has not been eliminated. The risk is involved in both endovascular and open repair because of the interruption of circulation to the spinal cord. Ischemic spinal cord injury is a consequence of two mechanisms: either intraoperative lethal interruption of spinal cord blood supply or the postoperative permanent reduction in blood supply secondary to sacrifice of critical vessels (Etz et al. 2015). The risk varies from 2% to 40% after emergency surgery for extensive TAAAs, and the three most important factors are extent of the aortic pathology, the urgency of the surgery, and the presence of acute aortic dissection.

Not uncommonly, TAAAs and DTAAs include the aortic arch. Approximately 15% of thoracoabdominal aneurysms extend proximal to the left subclavian artery (Cronenwett & Johnston. 2010). In these cases, the proximal cross-clamping requires cerebral protection and use of hypothermic circulatory arrest (HCA). This provides a bloodless operating field and eliminates the need for sequential aortic clamping, which, in turn, may reduce the risk for embolization. More importantly, the mortality and morbidity rates compare favourably with those of endovascular and open repair (Kouchoukos et al. 2013).

In 1986, Murry and colleagues (1986) reported on the cardioprotective effect of ischaemic preconditioning. The preoperative intermittent bouts of ischaemia appeared to be beneficial against later ischaemic insult. A decade later, it was discovered that ischaemic bouts focused on a remote part of the body resistant to ischaemia (e.g., hind limb) also protect the heart from ischaemia (Gho et al. 1996). Since then, much evidence has been accumulated on the beneficial effect of remote ischaemic preconditioning (RIPC) of other organs as well. Two decades after the original study of Murry et al., the neuroprotective effect of RIPC was reported (Dave et al. 2006, Vlasov et al. 2005). Additionally, the advantage of RIPC in spinal
cord ischaemia was noted approximately at the same time (Gurcun et al. 2006a).

There is, however, evidence against the neuroprotective effect of RIPC from large clinical trials, although the confounding factors (population heterogeneity, non-standardised anaesthesia protocol, different type of preconditioning stimulus) might be covering the beneficial effect of RIPC (Hausenloy et al. 2015a, Meybohm et al. 2015, Remote Preconditioning Trialists’ Group et al. 2014).

There are reports indicating that the protective effect of RIPC and ischaemic preconditioning targets to mitochondrial $K_{\text{ATP}}$ channels (Fryer et al. 2001, Loukogeorgakis et al. 2007, Pain et al. 2000). Therefore, it is tempting to use a pharmacological mimic that directly targets these channels as a substitute for RIPC. Studies have described the beneficial effect of diazoxide against neuronal ischaemic insult although clinical evidence is lacking (Roseborough et al. 2006).

The effect of RIPC on local spinal cord ischaemia was successfully demonstrated with increased levels of motor evoked potentials (MEPs), as described in the first study (I). In the second article (II), we attempted to shed light on the possible mechanism of RIPC by conducting the immunohistochemical analysis of the spinal cord 24 hours after the local spinal cord ischemia. As we had previously shown the positive effect of RIPC in the HCA-model, we decided to start with the familiar protocol in the third article of this thesis. Therefore, the effects of diazoxide, a potential pharmacological mimetic of RIPC, were tested in the model of 60 minutes HCA (III). In the last study (IV), we summarized the current knowledge of RIPC from the cardiovascular point of view.
2 Review of the literature

2.1 Human central nervous system

The function of the central nervous system (CNS) is to integrate sensory information and form an appropriate response. The CNS has two main components: the spinal cord and the brain. The spinal cord serves as a messenger between the brain and the rest of the body. The brain is responsible for integrating most sensory information and coordinating body function. The brain and spinal cord consist mostly of neuronal cells called neurons and supporting cells, which are commonly referred to as neuroglia cells. Macroscopically, two different types of tissue, white and grey matter, can be identified in the CNS. Grey matter consists of neuron cell bodies, dendrites, and axons, whereas white matter consists mostly of axons, the myelin sheath of which makes it look white. The neuron, which is the key player of grey matter, is the functional unit of the nervous system and a highly specialised, excitable cell. Astrocytes, oligodendrocytes, and microglia, predominantly found in white matter, are considered glial cells, and they constitute the majority of the nerve tissue. Glial cells provide structural support to neurons and maintain local conditions for neuronal function. They have the capacity to proliferate, but do not conduct action potentials.

2.1.1 Characteristics of neuronal tissue

Neurons

The three principal components of the neuron are the cell body, dendrites and axon. The cell body contains the nucleus, where protein synthesis occurs. The dendrites arise as treelike branches from the cell body and form numerous axonal synaptic connections to nearby neurons. Every neuron has only a single axon originating from the cell body. The axons branch extensively at the distal end, and each terminal branch of the axon has an enlarged ending, the synaptic terminal, which forms a connection to the adjacent dendrites. The dendritic tree is specialised for the reception and integration of information, whereas the axon is specialised for the transmission of information. Information passes through the neurons in the form of an action potential. Some axons are sheathed with myelin, which multiplies the velocity of the signal transmission.
Astrocytes

Astrocytes are classically divided into two categories on the basis of morphology: protoplasmic astrocytes are located in grey matter, while fibrous astrocytes are located in white matter. Most brain capillaries and the inner surface of the pia mater are surrounded by the astrocytic end-feet. In addition, neurons and neuronal processes that lack a myelin sheath are surrounded by astrocytes. The main tasks of the astrocytes are to scavenge transmitters released from the synapses, control H⁺ and K⁺ homeostasis, shuttle metabolites and waste products, and participate in the formation of the blood-brain barrier (Chen & Swanson, 2003). Additionally, it has been suggested that astrocytes participate in activity-dependent parenchymal blood flow regulation (Zonta et al. 2003).

Oligodendrocytes

Oligodendrocytes are the myelinating cells of the brain and the spinal cord. They not only insulate axons electrically, but also cluster sodium channels along the axons at the node of Ranvier, which is essential for saltatory conduction (Kaplan et al. 1997). The cytoplasm of the oligodendrocyte contains an extensive Golgi apparatus, many mitochondria, and a large number of microtubules, indicative of extremely high metabolic rates. It has been estimated that oligodendrocytes operate at the highest metabolic rate of any cell in the CNS (Connor & Menzies, 1996). Toxic by-products, such as hydrogen peroxide and reactive oxygen species (ROS), are the result of ATP production and high cellular metabolism (McTigue & Tripathi, 2008). Iron is a necessary co-factor in the production of myelin (Connor & Menzies, 1996), is highly reactive and can induce free radical formation and lipid peroxidation. Thus, the combination of high metabolism and iron make oligodendrocytes particularly vulnerable to damage, such as that caused by ischaemia.

Microglia

Microglia are the resident macrophages of the brain and spinal cord. The microglia have a wide range of responses, from killing invading microorganisms to limiting the effects of trauma and cell necrosis. The response includes the release of superoxide, nitric oxide, proteases, and cytokines and rapid migration and proliferation (Yenari et al. 2010).
2.1.2 **Blood-brain barrier**

The blood-brain barrier (BBB) controls the passage of metabolites, nutrients, and hormones from the systemic circulation to the central nervous system and back. The monitoring is highly precise because of the tight junctions between the BBB-forming cells and special membrane transporters. Moreover, the ion currency through the BBB and the hydrophilicity of the passing compound play an important role.

Three cell types are the most important BBB building blocks: endothelial cells, astrocytes, and pericytes. Endothelial cells have a large number of cytosolic mitochondria, suggesting their capacity for high-energy metabolism. Additionally, the endothelial cells are connected to each other by tight junctions, and their plasma membranes lack fenestrations and thus are selectively permeable to compounds with suitable mass and lipophilicity (Abbott. 2005, Chaudhuri. 2000). The astrocytes have a special role in forming the BBB. The end-feet of the astrocytes attach tightly to neurons on one side and blood vessels on the other, forming a specific station between the neuron and blood. Astrocytes have high concentrations of the water channel aquaporin 4 and K+ channel Kir4.1, implying a role in stabilising the fluid balance (Bernacki et al. 2008). The pericytes are small vessel wall-associated cells that are separated from endothelial cells by basement membrane. Their role in the BBB lies more in the maintenance of brain homeostasis by mediating inflammation and controlling capillary formation and diameter (Bandopadhyay et al. 2001, Cuevas et al. 1984).

2.1.3 **Blood flow**

The regulation of blood flow in the CNS is an integrative process that involves pulmonary gas exchange, cardiovascular function, and mediators of vessel resistance. One of the most effective vessel diameter regulators is the partial pressure of arterial carbon dioxide ($P_a$CO$_2$) (Mandell et al. 2008). Hypoxia also increases the diameter of the vessels, although CO$_2$ has a sensitising effect on vascularity of the CNS, as in the case of respiratory failure (Mardimae et al. 2012). In fact, the response to oxygen appears to be determined by oxygen content rather than the partial pressure of arterial oxygen ($P_a$O$_2$), because reductions in oxygen content resulting from chronic anaemia, hemodilution procedures, and carbon monoxide exposure increase cerebral blood flow (CBF) (Gottesman et al. 2012, Metry et al. 1999, Paulson et al. 1973).
The central nervous system appears to have autoregulation of blood flow, because blood flow seems to be completely stable across a relatively wide range (65–140 mm Hg) of mean arterial pressure (MAP) (Iadecola & Nedergaard. 2007). The vasculature of the CNS is extensively innervated by adrenergic and cholinergic fibers, suggesting the autonomic nervous system is part of blood flow regulation (Cassaglia et al. 2008, Mayhan et al. 1987). According to the current understanding of the brain microvasculature, however, the major factor contributing to the vascular tone is referred to as neurovascular unit. It is a composition of cerebral vascular cells (endothelial cells, pericytes, vascular smooth muscle cells), neuronal terminals, astrocytes and microglia that generates synchronised, cell-to-cell communication processes on demand, causing the changes in vascular diameter. In turn, this linkage between neural activity and cerebral blood flow is called neurovascular coupling, the signalling pathways of which are under intensive research (Filosa et al. 2016). It is a rapidly evolving field of investigation, and understanding of neurovascular coupling in humans is constantly increasing. According to a simplistic and rudimentary explanation of the neurovascular unit’s interactions, the temporal and amplitudinal changes in neuronal activity (e.g., visual cortex excitation) cause changes in cerebral blood flow locally, which is mediated through the astrocytes. Also, neurons modulate vascular tone independently of astrocyte modulation. Depending on the physiological environment, the role between these two cell types can be either antagonistic or synergistic (Phillips et al. 2016). One mechanism is the extracellular glutamate, the interactions of which in neurons are discussed below (2.3.1 Pathogenesis of the ischemic injury). Both neurons and astrocytes are excitable to the glutamate, leading to vasoactive signals in the presence of sufficient intracellular oxygen and glucose (Attwell et al. 2010).

**Cerebral blood flow**

The two internal carotid arteries carry oxygenated blood to cerebral tissue. These two large arteries are responsible for 70% of total CBF; the remaining blood is carried by vertebral arteries arising from the subclavian arteries. Both internal carotid arteries and vertebral arteries anastomose to form the circle of Willis before branching into the main intracerebral arteries (Willie et al. 2014). The vessels at the surface form a dense network of highly vasoactive arterioles within the pia mater, and the pial arterioles are generally considered the site of vascular resistance modulation.
Spinal cord blood flow

During the last two decades, there has been a reassessment of the spinal cord blood supply. In 1993, Svensson et al. concluded that sufficient circulation to the spinal cord is highly dependent on one major artery, the artery of Adamkiewicz (Adamkiewicz. 1882, Svensson et al. 1993b). The artery was named after Albert W. Adamkiewicz, who believed that a single dominant segmental artery in the lower thoracic or upper lumbar region is the most important input into the anterior spinal artery (Adamkiewicz. 1882). Since then, studies have stated that the collaboration of certain vascular networks is responsible for the blood supply (Etz et al. 2011, Griepp & Griepp. 2015). In humans, the subclavian and carotid arteries form the upper portion of the spinal cord vasculature. Multiple segmental arteries (lumbar and intercostal arteries) arising from the aorta form the central cord blood supply, while the hypogastric arteries form the distal spinal cord and cauda equine (Griepp & Griepp. 2007). Additionally, there exists an axial network of small arteries in the spinal canal, including the anterior spinous artery, paraspinous muscles, and perivertebral tissues, that give rise mainly to the segmental arteries (Etz et al. 2011). Thus, the role of the sacrificed segmental arteries has become one of the main topics with respect to the repair of thoracoabdominal aneurysms (Acher et al. 2008, Etz et al. 2006, Etz et al. 2011).

2.1.4 Metabolism

The nervous tissue has a very limited capacity for substrate storage and high metabolic rate; therefore, the precise regulation of blood flow is critical for maintaining a constant supply of nutrients, especially glucose, and oxygen. The O₂ consumption of the human brain averages approximately 20% of the total body resting O₂ consumption, while 90% of the energy needed to maintain neuronal electrical transmission comes from glucose. The muscle and liver have stores of glycogen, which maintains the level of glucose during exercise. Glycogen is found in small amounts in the glia, but it is almost absent in neurons. The other energy store, creatine phosphate, is found in all brain tissue cells, but it is only good up to 5 seconds of cellular activity (Barret et al. 2010).

Energy metabolism in neurons and neuroglia can be divided into three parts. First, in glycolysis, a chain of enzymes produces pyruvate and two molecules of ATP from one molecule of glucose. If oxygen is present, the pyruvate is converted to acetyl coenzyme A (acetyl CoA), which is a substrate for the tricarboxylic acid
(TCA) cycle in mitochondria. Under anoxic conditions, pyruvate is converted by lactate dehydrogenase to lactate while converting nicotinamide adenine dinucleotide (NADH) to NAD⁺. Thus, the lactate/pyruvate ratio is used as an indicator of whether the nervous tissue is under aerobic or anaerobic metabolism.

Second, in the TCA cycle, acetyl CoA is oxidized, producing three molecules of NADH, one molecule of flavin adenine dinucleotide (FADH₂), and one molecule of guanosine triphosphate (GTP). CO₂ is a waste product, from the cell and in the bloodstream to the lungs.

Third, oxygen oxidizes NADH and FADH₂ during oxidative phosphorylation in the electron transport chain (ETC), resulting in the synthesis of ATP. In summary, this three-stage pathway can produce 36 molecules of ATP from one molecule of glucose under aerobic conditions, whereas in the absence of oxygen, only 2 molecules of ATP are produced (Carpenter et al. 2014).

As mentioned earlier, glucose has traditionally been perceived as the primary substrate in most organs. According to recent evidence, neurons may also use lactate for energy production (Carpenter et al. 2014). Lactate is classically branded a waste product, but by 1994, Pellerin and Magistretti had developed a theory positing an astrocyte-neuron lactate shuttle that was responsible for the trafficking of metabolites between astrocytes and neurons (Pellerin & Magistretti 1994). Later, it was found in microdialysis studies that an injured human brain can metabolise lactate via the TCA cycle (Gallagher et al. 2009). The evidence suggests that low extracellular lactate levels are associated with better outcomes (Timofeev et al. 2011). This leads to the conclusion that high extracellular lactate levels may be a sign of severely damaged neurons and additional mitochondria because utilisation via the TCA cycle does not occur.

### 2.2 Essentials of the porcine central nervous system

The use of pigs in experimental surgery has increased during the past few decades. Especially in neuroscience, the potential of pigs has been recognised because of the similarities to humans in anatomy and neurochemistry. Thus, the amount of data on the pig central nervous system continues to increase (Lind et al. 2007).

#### 2.2.1 Porcine brain

The cortical surface of the porcine brain resembles human gyrencephalic structure. The porcine brain is 50 times heavier than a rat’s brain, which is much more widely
adopted into stroke research, and its convolutions are also more comparable to those of primates than of rats (Lind et al. 2007). Since the late 1960s, numerous studies have investigated the function of specific cortical regions (Otabe & Horowitz. 1970). For instance, the pig has proven to be a superior experimental subject for evoked potential (both sensory and motor) recordings, because its brain is relatively large and its somatosensory cortical regions are located mainly on the gyral surfaces (Craner & Ray. 1991a, Craner & Ray. 1991b). The pig’s topical, histological, and vascular anatomy is close to that of humans, making it suitable as a model of ischaemic brain injury (Swindle. 2007). In addition to topological structure, the porcine brain has a similar white matter/grey matter ratio compared to the human brain, whereas rodents have roughly five-fold less white matter than humans (Zhang & Sejnowski. 2000).

2.2.2 Porcine spinal cord

The gross anatomy and histology of the spinal cord are similar in humans and pigs. The major difference in humans lies in the vascular anatomy (Figure 1). The blood supply to the porcine spinal cord also involves a collateral network. Subclavian artery branches such as the internal thoracic arteries and subscapular arteries are much larger in a pig, relative to its weight, than in a human. These provide blood supply to the spinal cord through the chest and abdominal walls. In addition, vertebral arteries are relatively large on both sides. The first two branches of the vertebral arteries head toward the cervical spinal cord and are perceived to be of major importance for the upper portion of the spinal cord. The pig also has a large vessel plexus in the cervical area, which is believed to supply both the spinal cord and the base of the brain. Segmental arteries in the thoracic and lumbar areas are relatively small in pigs and generally originate from the aorta as a single branch and divide after 3 to 4 mm. At the aortic bifurcation, the pig has a median sacral artery, the diameter of which is almost comparable to that of the common iliac artery. The blood supply through the median sacral artery to the spinal cord is believed to be remarkable. This vessel is roughly comparable to the hypogastric arteries of humans (Strauch et al. 2007).

Similar to stroke models, rodents have been the golden standard of spinal cord injury research for the last 30 years (Kwon et al. 2015). The clinical testing of promising treatments has relied on small animal studies because many of the tested interventions have failed to show efficiency (Tator. 2006). Therefore, the Stroke Treatment Academic Industry Roundtable (STAIR) has introduced guidelines that
advocate for multiple-species testing before clinical trials (Fisher et al. 2009). Thus, the interest in large animal models of spinal cord injury has increased not only because of the histological similarity, but also because the “translatability” of an experimental therapy to the clinical trials is the greatest concern of preclinical studies (Ramer et al. 2014).

Fig. 1. The most important vessels of the spinal cord blood flow are circled. Modified from Strauch et al. 2003.
2.3 Ischaemic neuronal damage

Ischaemia is the restriction of blood supply to tissues and results in decreased oxygen and glucose. The brain is the organ most sensitive to reductions in blood supply because it has the highest metabolic activity per unit weight of any organ and the lowest levels of protective antioxidants (Adibhatla & Hatcher, 2010, Lee et al. 2000). After only 1–3 minutes without oxygen and glucose there is an ischaemic cascade that involves multiple uncontrolled or controlled events resulting in the death of the neuron.

In cardiac and aortic surgery, decreased CNS perfusion pressure and, especially, reperfusion injury are often present. The possibility of global ischaemic damage is well known in advance of the operation, and thus, in this field, methods for increasing the ischaemic tolerance of the CNS are worth pursuing.

2.3.1 Pathogenesis of the ischaemic injury

The lack of ATP production and the accumulation of lactate cause the ATP-dependent ionic pumps (Na⁺/K⁺-ATPase) in the cell membrane to fail. Hence, Na⁺/K⁺-ATPase is no longer able to maintain resting potential in the cell membrane, and intracellular Na⁺ increases. As a consequence, the ischaemic depolarisation of the cell forces the Na⁺/Ca²⁺ exchanger to work contrariwise, and the intracellular Ca²⁺ concentration increases, resulting in an even more depolarised cell membrane. This anoxic depolarisation is thought to trigger intracellular ischaemic cascades (Taxin et al. 2014).

Acidosis

As discussed earlier, ATP depletion induces the accumulation of lactate. In addition, because the ionic pumps are impaired by the depletion of ATP, the excess CO₂ and H⁺ molecules are not transported out of the cell; thus, acidification strengthens. Acidosis enhances processes that are detrimental for cell survivability, such as ROS production, inactivation of antioxidant defences, and glutamate toxicity (Lewerenz et al. 2010, Siesjo et al. 1996).
Glutamate

Glutamate has been known to have neurotoxic potential since the 1950s (Lucas & Newhouse. 1957). The anoxic depolarisation increases release of the presynaptic glutamate to the extracellular space and inhibits re-uptake. The in vitro study showed that the release of glutamate is mainly due to the reversed operation of neuronal glutamate transporters after anoxic depolarisation (Rossi et al. 2000). Ultimately, excessive glutamate causes an influx of Ca\(^{2+}\), Na\(^+\) and K\(^+\), which further accelerates the signalling pathways for neuronal death (Taxin et al. 2014).

The glutamate that accumulates in the synaptic cleft during ischaemia binds to ionotropic N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which causes persistent activation (excitotoxicity). NMDA receptor activation opens Ca\(^{2+}\) channels, resulting in an even greater intracellular concentration. In contrast, AMPA receptor activation opens Na\(^+\) channels, leading to Na\(^+\) influx. Excessive intracellular Na\(^+\) is thought to induce reversal of the Na\(^+\)/Ca\(^{2+}\) exchanger, leading again to increased Ca\(^{2+}\) influx (White et al. 2000). In fact, excitotoxicity appears to underlie all types of acute injury, including cerebral ischaemia and spinal cord injuries (Fujikawa, 2015). Figure 2 shows the cascade of glutamate-induced excitotoxicity.

Attempts to reduce postischaemic neuronal damage by use of NMDA antagonists have been controversial, but some success has been achieved. Some of the antagonists have failed in many clinical trials not only because of their serious side effects, such as nausea, memory impairment, and psychosis (Villmann & Becker, 2007), but also due to a lack of clinical efficacy (O’Collins et al. 2006). The NMDA receptor antagonists might have been insufficient for blocking the ischaemic cascade because the glutamate also seems to provoke other ischaemic routes. For example, in white matter, oligodendrocyte precursor cells express glutamate receptors, but in mature cells, these receptors are downregulated, and NDMA-independent rise of intracellular Ca\(^{2+}\), which triggers the cell death, has been demonstrated (Hamilton et al. 2016). Although glutamate is the crucial player in the initiation of excitotoxic cell death, it has also been shown that activated NMDA receptors initiate an extended neuronal depolarization, which is extremely injurious for the neuron. However, the receptor activation is not needed for maintaining the depolarized phase of the cell (Limbrick et al. 2003). Therefore, a recent prospective clinical trial showed that xenon inhalation among survivors of out-of-hospital cardiac arrest attenuates white matter injury of the brain (Laitio et
Also, no serious side effects have been reported in patients who were predisposed to xenon inhalation. The ‘NMDA receptor location’ hypothesis is supported by several cell culture studies, which indicate that exciting the extrasynaptic NMDA receptor resulted in Ca\(^{2+}\) entry strongly enough to cause mitochondrial dysfunction and cell death (Hardingham et al. 2002, Stanika et al. 2009, Zhang et al. 2007). The other theory presented to explain the inefficiency of the NMDA antagonists is ‘the subtype hypothesis’. In both in vivo and in vitro studies, activating a certain NMDA subtype will either cause neuronal survival (NR2A) or death (NR2B) (Chen et al. 2008, DeRidder et al. 2006, Liu et al. 2007). When the ischemia occurs, it is suggested that glutamate spill over leads to activation of extrasynaptic NR2B subtype receptors, resulting in excessive Ca\(^{2+}\) influx (Lai et al. 2011).

**Role of white matter**

During the past decade, the evidence of white matter involvement in various CNS pathologies has emerged remarkably. The idea of glutamate-mediated neurotransmission occurring only between neurons was challenged approximately 17 years ago when a functional glutamatergic connection was found between axons and cells that have the capacity to differentiate into oligodendrocytes (Bergles et al. 2000). Moreover, five years later, a study revealed that N-methyl-D-aspartate (NMDA) receptors are expressed in oligodendrocytes, specifically in myelin, and the receptors also are activated during ischemia (Karadottir et al. 2005). The disturbance in glutamate homeostasis is known to be the major contributor to neuronal cell death.

Thereafter, the evolving evidence has pointed out that the glia and axons actually have the necessary components for glutamate signalling. Astrocytes express neurotransmitter receptors and respond to neuronal activity by increasing cytosolic Ca\(^{2+}\). These responses can be evoked by several different transmitters, including glutamate, \(\gamma\)-aminobutyric acid (GABA), and adenosine triphosphate (ATP) (Berridge et al. 2003). When a certain level of activation is reached, the astrocyte releases ATP, which in turn increases Ca\(^{2+}\) in the adjacent astrocytes, resulting in focal activation (Arcuino et al. 2002). More interestingly, neurons do not contain the mitochondrial enzyme glutamine dehydrogenase and therefore cannot produce glutamate, which is the chief excitatory transmitter. Because glutamate does not pass through the blood-brain barrier, excitatory transmission is heavily dependent on the glutamate produced by astrocytes (Nedergaard et al. 2016).
Especially under oxidative stress, cystine/glutamate-antiporter may contribute to excessive glutamate release and eventually will lead to excitotoxicity (Had-Aissouni. 2012). Additionally, the internodal spaces of axons are shown to express glutamate receptor subunits and glutamate transporters, which are crucial for terminating glutamate-mediated signalling (Ouardouz et al. 2009). Interestingly, Sasaki et al. demonstrated that the locally released glutamate activated axonal glutamate receptors and therefore, theoretically, glial-mediated axonal action potential modification might be possible (Sasaki et al. 2011).

The current evidence of white matter injury must be acknowledged, particularly in this thesis, because the histological evaluation of ischemia was based on grey matter injury. Damage of central white matter causes significant functional disability. The majority of ischemic strokes involve both grey and white matter (Goldberg & Ransom. 2003). Evidently, white matter injury consists of the disruption of axon function, in which clinical symptoms might vary between a total loss of motor function and subtle changes in sensory or cognitive performance (Desmond. 2002, Hamner et al. 2011). In addition to histological analysis, we recorded motor-evoked potentials (MEP) of the spinal cord, which gives us indirect information of the signal transmission performance.

**Calcium**

Under physiological conditions, intracellular Ca$^{2+}$ is tightly regulated by the collections of transporters. Neurons use both intracellular and extracellular sources of calcium in a great variety of neuronal processes. Calcium is stored in endoplasmic reticulum, mitochondria, and lysosomes in the cytoplasm, where it is released on request, for example, to trigger the release of neurotransmitters to synaptic junctions (Berridge. 1998, Burgoyne & Haynes. 2014).

More than three decades ago, Schanne et al. (1979) reported that excessive intracellular Ca$^{2+}$ leads to the rupture of cell membranes and cell death triggers multiple pathways that cause either apoptosis or necrosis. The evidence indicates that the route of Ca$^{2+}$ entry into the neuron is more fatal than the concentration of intracellular Ca$^{2+}$ per se. The distinct second messenger pathways appear to have a particular propensity to elicit neurotoxicity (Tymianski et al. 1993).
Fig. 2. Simplified overview of enzyme and protein interactions during neuronal ischaemia. nNOS = neuronal nitric oxide synthase, mPTP = mitochondrial permeability transition pore, ER = endoplasmic reticulum, AIF = apoptosis-inducing factor, Endo G = endonuclease G, Smac = second mitochondria-derived activator of caspase, Cyt C = cytochrome C.

Ca²⁺ enters the cytoplasm after ischaemic insult in three main ways (Szydlowska & Tymianski. 2010). First, there is convincing evidence of the Ca²⁺ influx through the previously described ionotropic glutamate receptors (NMDA and AMPA) (Sattler et al. 1998). Second, non-excitotoxic mechanisms seem to play a small role in neurotoxicity in parallel with or independently of excitotoxicity. These pathways
include the Na⁺/Ca²⁺ exchanger (Pignataro et al. 2004), the family of transient receptor potential channels (Aarts et al. 2003), acid-sensing ion channels (Xiong et al. 2004), and L-type voltage-dependent Ca²⁺ channels (Zhang et al. 2012). These channels increase permeability to extracellular Ca²⁺ under anoxic conditions. Third, the neuron releases intracellular stores of Ca²⁺ under anoxic conditions. Two main locations of stores are the mitochondria and endoplasmic reticulum (Paschen & Doutheil 1999, Schinder et al. 1996).

Excessive free Ca²⁺ ion in the cytoplasm acts as a secondary messenger to multiple pathways. It is not clear which of these pathways plays the most important role. However, all of them seem to result in cellular swelling, acidosis, cell membrane rupture, and cell death. Mitochondrial Ca²⁺ overload is a crucial early event in the excitotoxic cascade leading to mitochondrial swelling, loss of membrane potential, and induction of apoptosis (Liu et al. 1996). Moreover, Ca²⁺ ion activates calcium-dependent proteases, calpains (Higuchi et al. 2005), caspases (Orrenius et al. 2003), phospholipases (Berliocchi et al. 2005), and nitric oxide production (Lai et al. 2014).

**Calpains**

Calpains are calcium-dependent proteolytic enzymes that regulate the proteins involved in calcium homeostasis. In mammals, the calpain system includes three different molecules: μ-calpain, m-calpain, and calpastatin; the function of calpastatin is to inhibit μ-calpain and m-calpain when calcium is present. Under physiological conditions, inactive calpain is localised in the cytosol if calcium is absent. When activated by calcium, the most important function of calpain is to interact in the multiple points of cell cycle and thus regulate the programmed cell death (Janossy et al. 2004).

As discussed earlier, ischaemic insult causes massive Ca²⁺ ion influx into the cytosol of neurons. After ischaemia, the natural inhibitor calpastatin loses its capacity to downregulate calpain activation, resulting in the commencement of the apoptotic pathways. This occurs mainly because members of the apoptotic cascade, such as p53, caspases, and B-cell lymphoma 2 (Bcl-2) family members of cell death regulators, are themselves substrates for calpains (Raghupathi 2004).

At least a dozen different pharmacological reversible or irreversible calpain inhibitors are currently being studied (Yildiz-Unal et al. 2015). The challenge with inhibitors is that calpains are cysteine proteases, which play a crucial role in the cell cycle, apoptosis, differentiation, synaptic plasticity, and CNS development.
Thus, the inhibitors might interrupt multiple physiological functions of calpains, resulting in an adverse outcome (Yuan. 2009).

**Nitric oxide**

Furchgott and Zawadzki made the original discovery of the endothelium-derived relaxing factor in 1980. Thereafter, this factor was identified as nitric oxide. Ten years later, Bredt and Snyder managed to purify the enzyme synthesising NO from rat cerebellum (Bredt & Snyder. 1990). The enzyme was characterised as the neuronal isoform of nitric oxide synthase (nNOS). Two other nonneuronal NOS isoform identified were found two years later from macrophages (inducible NOS [iNOS]) and bovine aortic endothelial cells (endothelial NOS [eNOS]) (Lamas *et al.* 1992, Xie *et al.* 1992). Inducible NOS mostly regulates the immune system, and eNOS has regulatory functions in the cardiovascular system. Neuronal NOS is abundantly expressed in neuronal tissues and has an essential role in the ischaemic cascade but is also expressed in non-neuronal tissues, such as endothelium and smooth muscle cells in humans (Costa *et al.* 2016).

Neuronal NOS couples to NMDA receptor in the plasma membrane through the postsynaptic density protein-95 (PSD-95) scaffold. The coupling exposes nNOS to Ca\(^{2+}\) influx, which is induced by NMDA receptor activation, and initiates immediate NO synthesis from l-arginine (Brenman & Bredt. 1997). This leads to the formation of cyclic guanosine-5'-monophosphate (cGMP) and further activation of cGMP-dependent protein kinases (PKGs) (Hanafy *et al.* 2001). The cGMP/PKG pathway is thought to be the main route for the activation of factors such as protein kinase B (AKT) (Hanada *et al.* 2004), extracellular regulated kinases 1 and 2 (ERKs) (Meini *et al.* 2006), cAMP-responsive element-binding protein (CREB) (Ciani *et al.* 2002), and proto-oncogene tyrosine-protein kinase (Src) (Mishra *et al.* 2009), which are mostly responsible for cell survival.

Nitric oxide itself can be harmful to the cell because it has the ability to form powerful reactive nitrogen species (ONOO\(^{-}\), NO\(_2\), NO\(_2\)O\(_3\)) with the end products of cellular respiration (peroxides, hydroxyl radicals, and hydrogen peroxides), called ROS. Furthermore, reactive nitrogen species have been documented to take part in neuronal apoptosis (Bian *et al.* 2003).

The first studies of nNOS inhibitors were conducted in the 1990s using knockout mice, and the results revealed attenuated glutamate excitotoxicity in cortical neurons (Dawson *et al.* 1991b). However, the physiological roles of NO, such as neuronal differentiation and synaptic plasticity in the brain, limit the use of
nNOS inhibitors (Garthwaite, 2008). More interestingly, it has been stated that the neurotoxicity of NO depends on the source. Endothelial-derived (eNOS) NO seems to be beneficial during ischaemia because it increases CBF and, therefore, the oxygen and substrate supply to the ischaemic area (Stagliano et al. 1997).

**Phospholipase A2**

Phospholipase A2 (PLA2) is a superfamily of enzymes responsible for cleaving the fatty acids of membrane phospholipids; the major free fatty acids cleaved are arachidonic acid and docohexaeonic acid. In the presence of oxygen and sufficient ATP production, the released free fatty acids are converted to acyl-CoA and subsequently returned to membrane phospholipids through lysophospholipid acyltransferases (Sun et al. 2010). The phospholipase A2 superfamily consists of 22 different types that are present in mammalian cells and are classified into three different groups based on molecular structure: calcium-dependent cytosolic (cPLA2), secretory (sPLA2), and calcium-independent (iPLA2) phospholipases A2. The different groups of phospholipases A2 together play a crucial role in mediating the oxidative and inflammatory responses in various CNS pathologies, including stroke and spinal cord injury (Adibhatla & Hatcher. 2008).

The evidence of involvement in the ischaemic cascade is for the calcium-dependent phospholipases A2. In a cell culture study, NMDA-mediated calcium influx activated cPLA2 and subsequently led to the release of arachidonic acid and production of ROS (Windelborn & Lipton. 2008). Arachidonic acid is further used in the production of eicosanoids by cyclooxygenases (COXs) and 5-lipoxygenases. This conversion releases superoxide as a concomitant product (Bazan. 2005). Increased expression and activation of COX-2 have been documented in ischaemic human brains (Sairanen et al. 1998). In rodent studies, the glutamate mediated brain injury attenuated when COX-2 inhibitor was present (Iadecola et al. 2001). There is, however, evidence against the role of COX in ROS generation (Kunz et al. 2007), but it is evident that calcium-dependent PLA2 is activated under ischaemic conditions, and when inhibited, the size of the infarct is smaller (Bonventre et al. 1997).

Many PLA2 inhibitors have been studied so far. Most studies are performed in vitro, and thus, the in vivo effects are not fully clarified. Like all other specific enzymatic blockers/activators, successful treatment requires sufficient duration of activity, ability to bypass the BBB, and absence of harmful side effects (Farooqui et al. 2006).
Mitochondrial failure

The decrease in energy production in mitochondria is the main reason for neuron death. These are the target organelles for most cascades beginning in the cell membrane or the cytoplasm (Budd & Nicholls. 1998). After depletion of ATP, the neuron may undergo either programmed (apoptosis) or disordered (necrosis) cell death. The proposed factors and events are discussed below.

First, the excessive cytosolic calcium ions enter the mitochondria through the electrophoretic uniporter because of the negative membrane potential driving force (White & Reynolds. 1997). Mitochondria become depolarised because of the inward positive ion current, leading to inhibition of oxidative phosphorylation (White & Reynolds. 1996). Thus, ATP generation continues to deteriorate, and as a result, ATP-dependent Ca²⁺ pumps will fail, increasing the accumulation of Ca²⁺ ions. Additionally, Ca²⁺ is known to be the initial factor in opening the inner membrane mitochondrial permeability transition pore (mPTP), increasing MPT. The opening allows more large molecules to enter through the inner membrane (Zoratti & Szabo. 1995). Therefore, the process of excitotoxic neuronal death is enhanced, and ultimately, the process triggers ROS production and death cascades in the mitochondria (Luetjens et al. 2000, Rego et al. 2000).

The mitochondria generate superoxide anions and hydrogen peroxide during oxidative phosphorylation from the electron transport chain under normal physiological conditions. To be exact, oxygen free radicals are generated in complexes I and III. Neurons have developed multiple ROS clearance systems, the most important of which are superoxide dismutases (SOD1-3), glutathione peroxidase, and catalase. These antioxidant enzymes work together to scavenge superoxide to water (Chan. 2001). Therefore, oxygen metabolism is a sensitive process and potential threat to neurons (Boveris & Chance. 1973). The excess Ca²⁺ in the mitochondria collapses membrane potential and leads to the interruption of ETC. Subsequently, free electrons react with oxygen that is supplied after reperfusion, causing the formation of superoxide (Rego et al. 2000). Moreover, the reactive oxygen and nitrogen species themselves inhibit ETC, increasing mitochondrial free radicals (Won et al. 2002). Oxidative stress is known to be another key player in the opening of the mPTP, which is thought to initiate apoptotic or necrotic pathways (Manzanero et al. 2013).

Reactive oxygen species generated from the mitochondria mediate multiple pathways that finally trigger mitochondrial release of pro-apoptotic proteins. Before the apoptotic cascades begin, ROS launch several cytosolic pathways that
determine whether neurons survive or die. The long list of mechanisms accentuates the difficulty of attempts to therapeutically limit the toxic effects of ROS once released. The role of protein 53 (p53) as an inducer of ischaemic apoptosis or necrosis was reported during the last decade (Saito et al. 2005). Protein 53 is a transcription factor that activates or represses the expression of multiple genes related to cell cycle and growth (Riley et al. 2008) and reacts to various cellular stresses, including DNA damage, hypoxia, and oxidative stress (Hu et al. 2012). The Bcl-2 protein family is a group of proteins that are the principal regulators of mitochondrial membrane integrity and function. The family is divided in three subgroups: anti-apoptotic proteins, pro-apoptotic proteins, and BH3-only proteins (Niizuma et al. 2010). Many of these proteins are the products of p53, and inhibition of p53 in rodent studies resulted in a neuroprotective effect (Endo et al. 2006). Additionally, ROS have been documented to trigger p53 binding to a protein called cyclophilin D, which is a regulator of the mPTP. Thus, p53 is another opener of this crucial pore leading to neuron death (Vaseva et al. 2012).

The other important molecular pathway triggered by ROS generation is activation of protein kinase B (Akt). This is the key molecule between neuronal death and survival after an ischaemic insult (Noshita et al. 2003). This is a major downstream target of the phosphoinositide 3-kinase (PI3K) pathway. Akt inactivates one member of the Bcl-2 family (Bad), which is then unable to inhibit other pro-survival proteins, leading to inactivation of cell death pathways. Additionally, some caspases are inhibited by the PI3K/Akt pathway (Cardone et al. 1998). Thus, this pathway is one major factor pushing the cellular balance toward cell survival.

After interacting with other Bcl-2 family members, the pro-apoptotic protein Bax is activated and translocated to the mitochondria, leading to neuronal apoptosis (Okuno et al. 2004). The combined effect of the opening of mPTP, loss of membrane potential, and cytosolic signalling of the Bcl-2 proteins leads to a release of the following proteins in the mitochondrial intermembrane space: cytochrome c, apoptosis inducing-factor (AIF), second mitochondria-derived activator of caspase (Smac), and endonuclease G. There is no turning back after the release of these proteins, and thus, the neuron faces inevitable death (Niizuma et al. 2010).

The most common downstream pathway from mitochondrial protein release is caspase activation. Cytochrome c induces a caspase cascade resulting in caspase-3 activation, which liberates proteins that damage DNA (Li et al. 1997). Caspase-3 also activates other caspases, which in turn have their own signalling system, leading to apoptosis (Krupinski et al. 2000). Smac protein is also related to the
caspase cascade (Saito et al. 2003), whereas AIF and Endo G are known to translocate straight to the nucleus and cause DNA fragmentation after ischaemic insults (Culmsee et al. 2005, Lee et al. 2005).

**Reactive oxygen species**

In addition to the metabolism of arachidonic acid and mitochondrial production, several cellular oxidative metabolic processes such as xanthine oxidase and NADPH oxidase are important for excessive ROS generation during and after ischaemia. For example, NADPH oxidase has a special role in ROS production during reperfusion, and it has been considered a potential target of stroke therapy because it seems that this enzyme has no essential function other than the production of ROS (Radermacher et al. 2012). ROS themselves cause macromolecular damage, such as lipid peroxidation, protein oxidation, and DNA oxidation, all of which can lead to cell injury and death (Chan. 1994, Chan. 2001).

Ischaemic insult-mediated oxidative stress greatly outweighs the antioxidant defence systems in neurons, but this has provided an opportunity for pharmacological enhancement of antioxidants. The pharmacological agents either enhance upregulation of endogenous antioxidants or enhance the effect through exogenous delivery. A key antioxidant is SOD2, an enzyme responsible for the dismutation of superoxide produced during oxidative phosphorylation (Keller et al. 1998). Studies in rodents have reported that SOD2-deficient mice have higher levels of oxidative stress (Kim et al. 2002), and also, the deactivating genes responsible for SOD2 expression led to increases in the size of brain infarcts (Jung et al. 2009). Unfortunately, it seems that the genetic pathway underlying SOD2 activation might be too complex to target pharmacologically.

Another antioxidant pathway under intensive research examines the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and its corresponding controller, Kelch-like ECH-associated protein 1 (Keap1). ROS or reactive nitrogen species dissociate Keap1 and Nrf2, and free Nrf2 is able to translocate to the nucleus and bind to antioxidant-responsive elements, inducing expression of antioxidant proteins (Itoh et al. 1999). In a rodent model, Nrf2 was found to reduce infarct size and improve neurological scores after three days of focal cerebral ischaemia (Son et al. 2010). Interestingly, the preliminary results of the phase II clinical trial indicated that blocking ROS and/or reactive nitrogen species at the source, rather than scavenging already produced oxidant agents, is
more beneficial (Hill et al. 2012). Hill et al. used an inhibitor of PSD-95 protein, which is known to be crucial in coupling NMDA receptor to nNOS signalling.

**Apoptosis and necrosis**

Although the result differs, all ischaemic events start with a similar set of cellular events. Apoptosis and necrosis are conceptually and morphologically distinct forms of cell death. Apoptosis leads to nuclear condensation and DNA degradation (ATP generation is required for the final phase (Leist et al. 1997)) and is characterised by cell shrinkage and the preservation of cellular contents. Necrosis leads to membrane lysis, swelling, release of cellular components, and thus activates an inflammatory response. Moreover, necrosis is more fatal to tissue because the leakage of cell contents predisposes the surrounding cells to proteolytic enzymes and excitatory amino acids. However, these two types of extinction can occur simultaneously in cell culture studies and seem to react to the same stimulus and share, at least partially, the same pathways heading to mitochondrial failure (Shimizu et al. 1996).

In fact, studies during the last decade have pointed out that morphologically apoptotic cells are not seen after acute brain injury in adults. However, naturally occurring neuronal apoptosis peaks are seen in the first postnatal week in rats. The central effector of the apoptotic cascade is the previously discussed caspase-3, and by postnatal day 60, there is no evidence of this enzyme in cerebral ischaemia (Hu et al. 2000, Ikonomidou et al. 1999, Zhu et al. 2005).

In conclusion, the research implies that neuronal death after acute ischemic insult is caspase independent. The best option is calpain activation, which results in neuronal death (Vosler et al. 2009). More interestingly, calpain cleaves the same death-promoting Bcl-2 family proteins (Takano et al. 2005). As a result, the same proteins are released from mitochondria, and similar injury occurs, but morphologically, it is necrosis. It has been suggested that this phenomenon be called programmed necrosis or necroptosis (Fujikawa. 2015).

**2.3.2 Ischaemia-reperfusion injury**

The fatality of ischaemia is dependent on both the magnitude and the duration of the insult. Therefore, restoration of blood flow to the injured area as soon as possible is still the main therapeutic approach. In cardiac and aortic surgery, this usually means decreasing aortic clamping time as much as possible. However, it
has been known since the 1960s that the restoration of blood supply accelerates necrosis after ischaemia (Jennings et al. 1960), and that it involves ROS generation has been known since the early 1980s (Bulkley. 1987, Granger et al. 1981). Paradoxically, the oxygen depletion caused by ischaemia and the restoration of the oxygen supply during reperfusion both contribute to the total injury in nervous tissue (Figure 3).

The increase in ROS has been suggested to contribute to the pathogenetic mechanisms underlying ischaemia-reperfusion injury. Reperfusion restores oxygen levels to the neurons, but due to the mitochondrial ETC dysfunction and the electron leak, oxygen is used to generate massive amounts of ROS (Seet et al. 2011). Mitochondrial Ca\textsuperscript{2+} overload and ROS production burst open the mPTP, leading to loss of ionic balance and the release of death-signalling proteins. Also induced by ischaemia-reperfusion injury is an inflammatory response characterised by marked recruitment of neutrophils, production of cytokines and chemokines, and, ultimately, exacerbation of ischaemia-reperfusion injury (Kvietys & Granger. 2012). Subsequently, methods for increasing tolerance to reperfusion injury, especially in cardiac surgery, are needed.
Fig. 3. Schematic of the relationship between ischaemia and reperfusion. The graph illustrates the importance of reperfusion injury. The earlier the blood supply is restored, the less severe the tissue injury. On the other hand, if reperfusion is delayed, the injury is more severe. The injurious effect of reperfusion decreases over time, and if there is no reperfusion at all, the ischemic injury will eventually destroy the tissue (Pundik et al. 2012).

**Inflammatory response**

Inflammation includes a great variety of cells and molecular signalling cascades that are vital to defence against invading pathogens. The inflammatory response is roughly divided into two parts. Innate immune cells such as macrophages (microglia in nervous tissue) and neutrophils are the primary defenders and migrate to the injured area and produce additional cytokines and chemokines that lead to the activation of lymphocytes and adaptive immune responses. The sterile innate
inflammatory response induced by acute ischaemia-reperfusion injury is similar to the response to invading pathogens (Kvietys & Granger, 2012).

The central nervous system has been previously described as an immunologically inactive organ, but currently, it is widely accepted that the nervous tissue and immune system are engaged in bidirectional crosstalk. Microglia and astrocytes seem to play a pivotal role in inflammatory response after ischaemia-reperfusion injury (Lucas et al. 2006, Swanson et al. 2004). Both cell types are able to produce inflammatory factors such as cytokines, chemokines, and reactive oxygen species.

Cytokines are a group of small glycoproteins that are upregulated in the nervous tissue in various diseases, including ischaemic insult. The most studied cytokines are tumour necrosis factor α (TNF-α), transforming growth factor β (TGF-β), and interleukins (ILs) such as IL-1β, IL-6, IL-20, and IL-10 (Chen & Chang, 2009, Ferrarese et al. 1999, Spera et al. 1998, Yamasaki et al. 1995, Zhu et al. 2002). Higher expression of interleukins seems to correlate with infarct severity in rodent studies, but some cytokines, especially IL-10 and TGF-β, may have a neuroprotective effect because infarct size is smaller when these cytokines are expressed. Some cytokines have the ability to guide the migration of blood-borne inflammatory cells toward the cytokine source. These are called chemokines, and they have a deleterious effect because they increase leucocyte infiltration to the injured area (Kim et al. 1995).

Cellular adhesion molecules are the proteins responsible for leukocyte adherence to the endothelium and migration. There are three main groups of proteins: selectins, the immunoglobulin gene superfamily, and integrins. The leucocyte is activated by these proteins. First, leucocyte rolls over the endothelial surface and then adheres to the endothelial cell before migrating into the nervous tissue. During the early stages of ischaemia, a rise in circulating cellular adhesion molecules has been documented in both rodent and clinical studies, implying that higher leucocyte recruitment results from ischaemia (Rallidis et al. 2009, Zhang et al. 1998).

Matrix metalloproteinases are proteolytic enzymes that remodel the extracellular matrix. Their expression in the adult brain is very low, but they have been found to be expressed after ischaemic insults. Neurons, microglia, and astrocytes all have the ability to express these proteins, and studies indicate that BBB breakdown results from the activation of matrix metalloproteinases (Asahi et al. 2001).
Postischemic inflammatory changes lead to nervous tissue dysfunction. BBB rupture, cerebral oedema, and neuronal cell death are consequences of complex pathways initiated by ischaemia-reperfusion injury.

2.4 Thoracoabdominal aortic aneurysm

The first successful end-to-end anastomosis of the thoracic aorta after resection of an aortic co-arctation was performed in 1944 in Sweden (Crafoord & Nylin, 1945). During the 1950s, there were multiple reports concerning the repair of descending thoracic aortic aneurysms (DTAAs) and thoracoabdominal aortic aneurysms (TAAAs). The first DTAA repair with homograft was reported by Lam and Aram in 1951. Thereafter, English surgeon Charles Rob (1955) was credited with the first description of a TAAA repair with direct manipulation (Rob. 1955), but at the same time, Etheredge et al. (1955) documented one of the first successful repairs of a TAAA in the United States. Ten years later, DeBakey et al. (1965) developed the polyester tube graft (Dacron), which was sutured to the aorta proximal and distal to the aneurysm, and the visceral arteries were attached to the conduit one after another. In 1974, Crawford improved the technique for TAAA repair, with only one death in 23 consecutive cases. He reconstructed the aortic branches from within the aneurysm via excised patches in the main aortic graft (Crawford. 1974). Crawford’s approach most resembles the techniques currently used, along with the utilization of cardiopulmonary bypass, hypothermic circulatory arrest, and cerebrospinal fluid drainage.

2.4.1 Definition, pathogenesis, and risk factors

Dilatations of the thoracic and abdominal aorta secondary to weakening of the aortic wall are considered TAAAs. TAAAs can occur from the left subclavian artery to the aortic bifurcation.

Formation of a TAAA is a complicated and dynamic process that involves gene factors, cellular imbalance, and altered hemodynamic factors. The medial layer of the aortic wall contributes to aortic capacitance and elasticity. This layer consists mostly of structural proteins such as elastin and collagen, and the degradation of these proteins weakens the aortic wall. Overactivity of the extracellular matrix proteinases, specifically MMPs, has been found to be increased in the wall of TAAAs, correlating with inflammatory degenerative process of the media layer (Ikonomidis et al. 2005). The diameter of the aortic wall increases because of
weakened wall structure, and as defined by the law of Laplace, when diameter increases, wall tension increases, resulting in a vicious cycle.

Genetic connective tissue disorders, such as Marfan syndrome, are known etiologies of aortic aneurysms. Patients with Marfan syndrome produce abnormal fibrillin, resulting in weakened connective tissue formation. The vast majority of TAAAs are deemed degenerative and are the result of atherosclerotic disease. As a consequence, risk factors are the same as for atherosclerosis itself, including age, smoking, hypertension, obesity, hyperlipidaemia, chronic obstructive pulmonary disease, and family history. Eighty percent of TAAAs are secondary to medial degeneration, and approximately 15% to 20% are caused by aortic dissection; in addition, patients with TAAAs secondary to aortic dissection are younger, and the aneurysms more extensive (Cronenwett & Johnston 2010). Moreover, it seems that specifically, a diastolic blood pressure greater than 100 mm Hg is a consistent risk factor associated with progression of dissection to aneurysm formation (Dapunt et al. 1994, Juvonen et al. 1999).

2.4.2 Classification

In 1986, Crawford et al. proposed a four-type classification for thoracoabdominal aortic aneurysms (Figure 4). The proximal start of type I TAAAs is just distal to the left subclavian artery and reaches the level of renal arteries. Type II TAAAs cover the complete thoracoabdominal aorta distally from the left subclavian artery to the aortic bifurcation. Type III TAAAs include more distal thoracic aorta and end at the aortic bifurcation or lower. Type IV TAAAs start from the level of the diaphragm and end at the aortic bifurcation or lower (Crawford et al. 1986). Safi and Miller (1999) added type V TAAAs, which include variable lengths of the distal thoracic aorta and end distally at the origins of the celiac and superior mesenteric arteries, but not the renal arteries.

The DTAAs are separated into three types based on the risk of developing spinal cord ischaemia during open repair (Figure 5). Type A DTAAs start just distal to the left subclavian artery and end at the level of the sixth rib (T6). Type B DTAAs start from there and end at the level of the diaphragm. Type C DTAA covers the total descending thoracic aorta (Estrera et al. 2005).
2.4.3 Incidence

The large Swedish register study of 14,229 patients documented the incidence of thoracic aortic disease (including aneurysms and dissections) in women as 16.3 per 100,000 per year and 9.1 per 100,000 per year in men, respectively. Additionally, there was an obvious increase in the incidence in men (52%) and women (28%) during the 15-year follow-up (Olsson et al. 2006). Although ascending (40% of all thoracic aortic aneurysms) aortic aneurysms are more common than DTAAs (35%) or TAAAs (10%), these two are not rare. Researchers have placed the incidence of TAAA at between 5.9 and 10.4 per 100,000 per year and constantly increasing. The factors in this rise may include improved imaging techniques, an aging population, and increased patient and physician awareness (Cronenwett & Johnston. 2010).
2.4.4 Mortality and morbidity in the open repair

Open repair of thoracic aortic aneurysms is associated with high morbidity and mortality, which are highly dependent on the type of aneurysm. Estrera et al. published extensive results of the open repair of DTAAs (Estrera et al. 2005). According to their experience, the 30-day mortality is 8%. Additionally, they found that cerebrospinal fluid (CSF) drainage was beneficial with respect to the incidence of neurologic deficits compared with the lack of adjunctive measures (1.3% versus 6.5%).

The greater extent of the TAAA requires both thoracotomy and laparotomy, and thus, the operative treatment is more complex. The worldwide 30-day mortality rate varies between 3% and 20% (Cambria et al. 2002, Coselli et al. 2007, Svensson et al. 1993b). According to Rigberg et al.’s (2006) population study, the 30-day mortality was 19% and the one-year mortality was as high as 31%. Additionally, the correlation between age and one-year mortality was obvious, as the mortality increased up to 40% among octogenarians (Rigberg et al. 2006). The findings on mortality were even more sobering for emergent TAAA repairs, for which 30-day mortality rose by as much as 50%.

Paraplegia or paraparesis is one of the most devastating complications of TAAA repair, and in patients who do not recover, it is the major cause of morbidity (Crawford et al. 1991). The risk for spinal cord ischaemia lies around 3–7% of all
TAAA patients and in the presence of extended aortic pathology (type II TAAAs), the risk increases up to 22% (Greenberg et al. 2008).

### 2.4.5 Surgical management

The objective in the surgical treatment of DTAA and TAAA is to prevent death from rupture. The original clamp and sew technique with revascularization of major aortic arteries has been modified during recent decades and now may involve cardiopulmonary bypass, hypothermic circulatory arrest, single-lung ventilation, cerebrospinal fluid drainage, and epidural cooling (Riga & Jenkins. 2012a). Although open surgical repair remains the best option for many patients, in the late 1980s, Volodos et al. introduced an alternative technique for isolated TAAAs (Volodos’ et al. 1988). Endovascular treatment of thoracic aneurysms has since evolved and is now considered primary therapy for many patients with TAAAs. The third and most recent technique, called hybrid repair, was used for the first time in 1998 in a patient with a type IV TAAA (Quinones-Baldrich et al. 1999).

**Extracorporeal circulation**

John Gibbon was the first surgeon to operate on an intracardiac defect in 1953 using cardiopulmonary bypass (CPB) (Gibbon et al. 1954). He was not the inventor of extracorporeal circulation, but he is credited with the development of the first “modern” CPB because the machine he used was equipped with a roller pump and an oxygenator, which are essential for full cardiopulmonary bypass and enable hypothermic circulatory arrest.

The CPB maintains extracorporeal circulation. Venous blood is first drained from the patient’s body into the CPB machine’s reservoir through venous cannulae. The blood passes through tubing into a roller pump that leads into an oxygenator. CO₂ and O₂ diffusion restores the oxygenation level of the blood, which is then returned to the patient via arterial cannulas. Additional roller pumps and reservoirs can be used depending on the type of operation, and a heat exchanger is usually used to control the patient’s core temperature.

**Left heart bypass.** The goal of extracorporeal circulation in thoracic aortic aneurysm repair is to provide sufficient blood supply to the mesenteric arteries, renal arteries, and spinal cord during cross-clamping of the thoracic aorta. Most frequently used for DTAA and TAAA repair is the method called left heart bypass. This method does not require an oxygenator because venous cannulation is
performed through the left atrial appendage or the left inferior pulmonary vein. The oxygenated blood is returned into the left femoral artery cannula using a centrifugal pump. This method is superior to a full CPB in operations on aneurysms that do not involve the aortic arch because it requires only a modest dose of heparin; therefore, there are fewer complications from bleeding, and the technique is simpler than a full CPB (Schepens. 2016).

**Full cardiopulmonary bypass.** The full CPB requires cannulation of the femoral vein and artery to support sufficient blood supply to the systemic circulation. Generally, this method is used in larger TAAAs (types I–III), but the choice between left heart bypass and full CPB is dependent on the repair techniques. There are, however, multiple variations of the cannulation technique with TAAA or DTAA repair. For example, cannulations of the ascending aorta and left axillary artery and bidirectional perfusion through the left common carotid and femoral arteries have been used. Additionally, selective perfusion of the celiac axis, superior mesenteric artery, and renal arteries may be required depending on the extent of the aneurysm (Cronenwett & Johnston. 2010).

**Aortic arch involvement.** It is not uncommon for DTAAs and TAAAs to involve the distal or even proximal aortic arch. In these cases, cross-clamping proximal to the left subclavian artery is often necessary. For example, Girardi *et al.* (2005) reported cross-clamping between the left carotid and left subclavian arteries in 42% of type I and II TAAAs (Girardi *et al.* 2005). If the extent of arch involvement is extreme, proximal clamp control may be impossible, and a complete CPB with hypothermic circulatory arrest (HCA) may be required. These cases are unique surgical challenges and may require the maintenance of CNS blood flow during aortic clamping (selective perfusion) to minimize the ischaemic injury (Cronenwett & Johnston. 2010).

**Open thoracoabdominal aortic aneurysm repair**

Because the extent of an aneurysm may vary tremendously, the approach to each case is individualised. The main goal for the approach is to provide adequate visualization of the diseased aorta. This might require collapse of the left lung with a double-lumen endotracheal tube. Left posterolateral thoracotomy provides sufficient exposure of the descending thoracic aorta, but clear visualization of the aorta may require resection of the sixth and fifth ribs. If the abdominal aorta is also diseased, the incision is extended over the costal margin in an oblique line toward the umbilicus. The final extent of the incision is dictated by the size of the aneurysm.
Selection of the technique for extracorporeal circulation varies depending on the type of TAAA, the familiarity of the surgeon with the technique, and availability. Most frequently used is distal aortic perfusion by left heart bypass. Other techniques include full CPB with or without HCA and/or antegrade cerebral perfusion (ACP), especially when the aortic arch is involved. Ischaemic injury to a visceral organ must be avoided, and thus, in some cases, selective perfusion of visceral and renal arteries is required. Almost every procedure includes hypothermia, or at least a spontaneous decrease in temperature to 32°–33°C.

The proximal clamp position on the aorta is carefully planned. Usually, it is positioned distal to the left subclavian artery, but if necessary, the clamp can be placed more proximally, and then sufficient blood supply to the brain must be secured by selection of the extracorporeal circulation method. The distal clamp position varies, but during surgery on a Crawford extent II TAAA, surgeons usually require control from the proximal descending aorta, and thus, the clamp is applied at the junction of the upper and middle thirds of the descending aorta. The proximal anastomosis is then tailored into the aorta. After the proximal anastomosis is completed, the focus turns to the visceral and renal arteries. The distal clamp and aortic cannula are removed, and a longitudinal incision is made down to the aortic bifurcation (TAAA type II). Furthermore, some surgeons favour the reattachment of intercostal arteries to the graft to ensure spinal cord blood supply. Thereafter, the proximal clamp can be positioned lower to the graft, and spinal cord circulation is ensured (sequential clamping). At the distal part of the aorta, the visceral and renal arteries are selectively perfused, and the distal anastomosis of the graft is sutured. There are large-scale methods for anastomosis of the visceral and renal arteries, and some of the grafts include separate branches for the arteries (Cronenwett & Johnston. 2010, de la Cruz et al. 2012).

**Endovascular approach**

Given the high mortality in surgical practice, there must be innovations to reduce the risk. Since the first report of the stent-graft exclusion for the thoracic aorta in 1994, endovascular treatment of the thoracic aorta has become more and more popular (Dake et al. 1994). Many concepts of endovascular treatment of the thoracic aorta have been explored. One of the main challenges was preservation of all four visceral arteries. Chuter et al. (2001) performed the first total endovascular repair of a TAAA with branched grafts. The advantages of endovascular repair include a smaller incision, less postoperative pain, limited pulmonary
complications, and more rapid recovery. The endovascular treatment is also more suitable for high-risk (heart failure, age >80 years, chronic obstructive lung disease, renal failure) patients (Guillou et al. 2012).

A retrospective study of the endovascular treatment of 420 patients documented a 4.8% 30-day mortality. The spinal cord ischaemia was permanent in 1.7% of patients and temporary in 7.9%, respectively. Significantly, the study also included aortic arch (218 patients) and descending aorta (193) pathologies; only 35 of the patients had thoracoabdominal aortic pathology (Patel et al. 2014). A larger study of 724 patients found no differences in mortality between endovascular and open repair of TAAAs after 12 months (15.6% versus 15.9%, respectively) (Greenberg et al. 2008). The incidence of spinal cord ischaemia was higher in the open repair group, but it was also highly dependent on the type of aneurysm (type I, II, III, or IV).

The results for endovascular treatment of DTAAAs also seem encouraging. A recent systematic Cochrane review concluded that stent grafting of the thoracic aorta seems to be a preferable method in terms of the early outcome measures, such as paraplegia, mortality, and hospital stay (Abraha et al. 2016). However, no randomized clinical trials have been performed to date, or none is on-going (www.clinicaltrials.gov, January 2018). A recent review also emphasized the favourable long-term outcome of the surgical repair, although endovascular repair has become the first-line treatment of descending thoracic aorta aneurysms since the technique seems to carry a lower operative risk (Clare et al. 2016).

Hybrid repair

Sometimes, the patient’s comorbidities limit the use of open repair, and as such, this has led to the development of hybrid repairs. Hybrid operations are usually performed in two stages and combine the advantages of the open and endovascular strategies. Instead of thoracotomy, a transperitoneal abdominal approach is more common because of better visualization of the origins of the renal arteries, celiac axis, and superior mesenteric artery. The inflow site for retrograde renal and visceral artery bypass is usually the distal aorta or iliac vessels, depending on the extent of the aneurysm. The procedure is completed with endovascular aortic aneurysm grafting and subsequent aneurysm exclusion. The advantages of the hybrid procedure are the lack of aortic cross-clamping and the absence of a thoracotomy in patients with significant pulmonary comorbidity (Riga & Jenkins. 2012b).
In 2007, Donas et al. performed a meta-analysis of patients treated with hybrid open-endovascular repair. Their study, which included 58 patients, reported a 30-day mortality in elective and emergency surgeries of 10.7%, and none of the patients had spinal cord injury (Donas et al. 2007). A meta-analysis of 19 publications comprising 660 patients compared the results of single-stage and staged operations (Canaud et al. 2013). Thirty-day mortality varied between 0% and 44%, and spinal cord ischaemic injury, between 0% and 15.3%. They did not find a significant difference between operative methods. However, hybrid procedures are targeted to patients with severe comorbidities, and therefore, the mortality and morbidity rates tend to be higher compared with those for open procedures.

2.5 Neuroprotection during thoracoabdominal aortic aneurysm surgery

As discussed earlier, spinal cord protection is an important entity during thoracoabdominal aortic procedures, including both endovascular and open repair approaches. During recent decades, various methods to improve surgical outcomes have been introduced. Deep hypothermia (either systemic or regional) minimises the metabolic rate of nervous tissue during an aortic operation when oxygen delivery is jeopardised. Intraoperative detection of spinal cord ischaemia is necessary to avoid permanent ischaemic injury. The technique most often used to detect spinal cord ischaemia involves somatosensory and motor-evoked potentials. The intraoperative spinal cord circulation is maintained with both sufficient supply (e.g., systemic hypertension) and cerebrospinal fluid drainage.

2.5.1 Spinal cord protection

Hypothermia

Therapeutic hypothermia is used in various forms in aortic surgery. In clinical practice, core temperatures (nasopharyngeal) between 28° and 34°C are usually referred to as mild hypothermia, and those between 20° and 28°C as moderate hypothermia. Core temperatures below 20°C are considered deep hypothermia. Temperatures between 15° and 23°C have been used for deep HCA and in
Hypothermia is known to increase the risk for coagulopathy, arrhythmia, and lung dysfunction. Mild hypothermia also seems to reduce the risk of complications, but also increases the ischaemic tolerance of the spinal cord (Luehr & Etz. 2014). Griep and Di Luozzo (2013) studied the safety of hypothermia in clinical and experimental settings and found that the ischaemic tolerance of the spinal cord was significantly longer than that of cerebral tissue under normothermia. The spinal cord seems to tolerate 20 minutes of ischaemia, whereas the brain tolerates only 5 minutes. (Griep & Di Luozzo. 2013). However, some criticism of the benefits of mild hypothermia has been shown, although not in the context of thoracoabdominal aorta repair. Nielsen et al. demonstrated that six months after cardiac arrest, the neurological outcome or mortality was not different, whether the patient had targeted temperature management at 33 °C or 36°C (Nielsen et al. 2013).

According to experimental and clinical studies, hypothermia decreases the demand for oxygen of CNS cells. It has been calculated that every 1°C drop in core temperature decreases cellular metabolism by an average of 5%–7% (Erecinska et al. 2003). The most efficient proven neuroprotective mechanism for hypothermia is the reduction of extracellular levels of excitatory neurotransmitters, including glutamate (Okuda et al. 1986). Thus, the initiation of excessive Ca²⁺ influx and concomitant activation of cell death pathways are both inhibited. Moreover, hypothermia has been documented to block the release of almost all factors responsible for caspase-independent apoptosis or necroptosis (Zhao et al. 2007). Furthermore, ROS production and the subsequent inflammatory response are significantly reduced in experimental studies (Horiguchi et al. 2003, Xiong et al. 2009).

Hypothermic circulatory arrest

Hypothermic circulatory arrest was conceived of almost 60 years ago (Niazi & Lewis. 1957). Even before the time of CPBs, Bigelow et al. (1950) conducted several human and animal studies using hypothermia as a protective factor during cardiotomy. Twenty-five years later, Griep et al. (1975) introduced a technique for total arch replacement using deep HCA at 12° to 18°C, which significantly reduced the metabolic rate of cerebral tissue and provided a bloodless operative field for the surgeon (Griep et al. 1975). The HCA is an operative procedure that lowers the patient’s target core temperature and shuts down all blood flow in a patient’s
cardiovascular system using extracorporeal circulation. The method provides a bloodless operating field with a limited time window. McCullough et al. (1999) documented an obvious decrease in cerebral metabolic rate associated with lower core temperature. They predicted that the safe duration of HCA at 15°C is 29 minutes without neurological deficits (McCullough et al. 1999).

Deep hypothermia is one option for spinal cord preservation (Kouchoukos et al. 2001, Kouchoukos et al. 2013), although mild hypothermia is also used (Safi et al. 1998, Strauch et al. 2004). To date, several studies have been conducted on the depth of hypothermia in aortic surgery. Weiss et al. (2012) performed a retrospective study comparing deep HCA with mild hypothermia methods without circulatory arrest. The study involved 240 patients. Patients who underwent deep HCA had better postoperative outcome with respect to renal and liver function, but the two groups had a similar incidence of paraplegia. A large meta-analysis compared the outcome of deep HCA and moderate HCA with antegrade selective cerebral perfusion in aortic arch surgery (Tian et al. 2013a). The neurological outcome of patients who underwent moderate HCA with selective antegrade cerebral perfusion was significantly better. Tian et al. also reported another meta-analysis of comparing deep HCA with or without selective antegrade perfusion in aortic arch surgery, and mortality was significantly lower with the patients who underwent the procedure with selective antegrade perfusion (Tian et al. 2013b).

**Epidural cooling**

Epidural cooling is a method of local therapeutic hypothermia. The first clinical report is that of Davison et al. (Davison et al. 1994), who found an incidence of postoperative paraplegia as low as 3% with this method. The ideal use would be a combination of relatively aggressive epidural cooling and mild systemic hypothermia (Shimizu & Yozu. 2011).

Epidural cooling is usually introduced through a catheter into the intrathecal space. Cooling is achieved with cold saline injection (4°C). However, this application might cause a deleterious increase in CSF pressure and, thus, exacerbate neurological injury (Motoyoshi et al. 2004). This adverse effect has led to innovation of the epidural catheter by addition of an isolated countercurrent lumen that does not elevate CSF pressure. Shimizu et al. (2010) introduced the catheter in a clinical setup of six patients with DTAAs or TAAAs.
Cerebrospinal perfusion pressure

Cerebrospinal perfusion pressure (CPP) is the difference between systemic mean arterial pressure and CSF pressure or right atrial pressure. If right atrial pressure increases above CSF pressure, it decreases the CPP, and conversely, if systemic MAP increases, CPP increases. Right atrial pressure might rise as a result of increased preload or increased intrathoracic pressure caused by positive end-expiratory pressure during the aortic operation (Subramaniam et al. 2011).

Modern spinal cord neuroprotection includes maintenance of the perioperative systemic MAP in the high physiologic range (80–100 mmHg) in both endovascular and open strategies (Augoustides et al. 2014, Grabenwoger et al. 2012). Additionally, postoperative maintenance of high pressure for at least 24–48 hours has been found beneficial. The explanation might include the adaptation of the collateral network of the spinal cord after loss of arterial input resulting from the aortic repair. The increase in diameter of the anterior spinal artery and epidural arterial network has been documented in an experimental setup during the five days after thoracic aortic repair (Etz et al. 2011). Thus, the systemic hypertension gives the critical time to the network for adaptation.

The more common and extensively used method for CPP control is CSF drainage. This method has a strong evidence-based benefit during DTAA and TAAA operations (Hiratzka et al. 2010). Although CSF drainage is indicated in most extensive open TAAA repairs, its role in endovascular operations is evolving. A large systematic review (n = 4,936) of CSF drainage in endovascular operations demonstrated the advantages of the method. The incidence of spinal cord ischaemia was 3.89% (Wong et al. 2012). On the other hand, a smaller Cochrane meta-analysis (n = 287) evaluated CSF drainage in open type I and II TAAA operations and reported an 80% decrease in the relative risk of spinal cord ischaemia when CSF drainage was used (Khan & Stansby. 2012). CSF drainage is important in the open repair of TAAAs, because after aortic cross-clamping, CSF pressure might rise sharply despite the left heart bypass (Drenger et al. 1997).

The complication associated with CSF drainage is bleeding with subsequent subdural or epidural hematoma. These complications occur significantly more frequently in patients with excessive drainage (Dardik et al. 2002). Dardik et al. reported an incidence of 3.5% for subdural hematomas, and a recent large study of 504 patients reported an incidence of 2.8% (Youngblood et al. 2013).
Evoked potential monitoring

The monitoring of motor (MEPs) and somatosensory (SSEPs) evoked potentials is very useful in the intraoperative determination of spinal cord function. SSEP monitoring is used to monitor sensory pathways during surgery. The method was first used in scoliosis surgery (Brown & Nash. 1979). In clinical practice, for SSEP monitoring, the peripheral nerves are stimulated, and the response is recorded through the area of surgery. Typical stimulation sites are the posterior tibial nerve in the leg and median nerve in the arm. Tibial nerve stimulation is recorded from the popliteal area, cervical spine, and scalp, whereas median nerve stimulation can be detected from the brachial plexus, supraclavicular fossa, cervical spine, and scalp. The tibial nerve stimulation is used when the surgical site is below the cervical level, and the median nerve is used if the site is above this level.

Somatosensory evoked potentials have been documented to reduce the incidence of spinal cord injury in scoliosis surgery (Dawson et al. 1991a); however, several well-documented studies indicate that SSEP monitoring misses significant spinal cord injury (Jones et al. 2003, Wiedemayer et al. 2004) that are related to vascular injury. In theory, the majority of sensory pathways conduct in the dorsal columns of the spinal cord (Yamada. 2000), and the blood supply differs from the anterior of the spinal cord. Insufficient blood flow through the anterior spinal artery places the anterior part at risk, but the dorsal side remains intact (Shimizu & Yozu. 2011). Thus, the tools of anterior horn nerve pathways monitoring were devised. MEP monitoring is considered superior to SSEP in the detection of paraplegia because of the motor pathway monitoring.

After the development of MEP monitoring in the 1950s in studies on monkeys, MEPs have proven a useful tool in various operations, including aortic surgery (Patton & Amassian. 1954). To date, several MEP recording techniques have been described, but the most commonly used technique is transcranial electrical stimulation (Tabaraud et al. 1993). The stimulation electrodes are placed on top of the motor cortical areas in the scalp, and recordings are made with intramuscular or subcutaneous needles placed in muscles of the arms and legs.

In thoracic aortic surgery, the role of SSEP monitoring has been under discussion, mainly because of the inability to detect infarction in the anterior horn. However, better results have been achieved when these two methods are combined (MacDonald & Janusz. 2002). SSEP recording requires at least a 5-minute interval before a new SSEP stimulus can be generated. MEP recording is much faster, and information can be updated multiple times during the critical phase of surgery.
Studies indicate that MEP signal changes occur ≥3 minutes after aortic clamping, whereas SSEPs take 15–30 minutes (Pajewski et al. 2007). This is important in reducing the risk of paraplegia because through monitoring MEPs, ischaemia can be detected before irreversible damage occurs, and adjustments (e.g., elevation of distal perfusion pressure, increased CSF drainage) for protection can be made in time.

The major disadvantage of MEP monitoring is that neuromuscular blockade has to be minimised and preferably avoided. Subsequently, every MEP stimulus causes movement of a limb and facial muscles. Surgeons have to stop working for 15–30 seconds during the stimulus to avoid an unfavourable outcome. Masseter muscle contraction might result in tongue lacerations, tooth fractures, or even mandible fractures. MEP monitoring should be avoided in patients with epilepsy, increased intracranial pressure, or skull defects. Nevertheless, the incidence of complications has been very low (MacDonald & Janusz. 2002).

Concern has arisen over the reliability of evoked potential recordings because multiple confounding factors affect evoked potentials during vascular surgery. First, anaesthesia must be stabilised. In particular, propofol has been documented to reduce the amplitude of MEPs (Nathan et al. 2003). The patient's core temperature has an effect on evoked potentials. This must be taken into consideration when using hypothermia because both MEPs and SSEPs seem to disappear below 28°C. In contrast, hyperthermia increases the conduction velocity. The safe range for reliable evoked potential measurements appears to vary 2°–2.5°C above or below baseline temperature (Oro & Haghighi. 1992). Additionally, hemodynamic changes and further CPP changes may result in a decrease in amplitude, which is reversible if pressure is increased (Pajewski et al. 2007).

**Segmental arteries**

In the presence of an insufficient blood supply, the risk of spinal cord injury rises. One source is the segmental arteries (i.e., intercostal and lumbar arteries), which branch from the thoracic and abdominal aorta and provide blood flow to the spinal cord. These vessels form an integral part of the vascular network for the spinal cord (discussed in Section 2.1.3). The mean number of these arteries that can be sacrificed without spinal cord ischaemia has been found to be 9 ± 3 in experimental series (Strauch et al. 2003). In addition, the importance of the segmental arteries was illustrated in an experimental setting, in which the CPP dropped approximately 60-70% after all segmental arteries were sacrificed (Etz et al. 2007). The CPP
returned to baseline after five days postoperatively, which led to the conclusion that TAAA repair should be performed in two stages. A clinical study of 90 patients revealed that patients operated on in two stages had significantly better outcomes (Etz et al. 2010). Similar results were obtained in an experimental setting (Geisbusch et al. 2014). This knowledge has been utilised in hybrid operations, as discussed in Section 2.4.5.

Should the segmental arteries be reattached, there are no clear answers. Some studies have shown the benefit of re-implantation (Ueda et al. 2000), while another study did not report significant benefits from re-implantation of segmental arteries (David et al. 2012). A relatively small number of patients were included in that study (n = 40), with two cases of extensive type II TAAA. Also, a larger retrospective analysis did not show a statistically significant decrease in spinal cord injury with segmental artery reimplantation (Wynn et al. 2016).

One method for utilising segmental arteries is the selective perfusion of the arteries during thoracoabdominal aortic surgery. In theory, this method provides sufficient blood supply to the spinal cord throughout the critical span. Work with the experimental pig model revealed a clear advantage of selective segmental artery perfusion during 60 minutes of aortic clamping (Meylaerts et al. 2000). On the other hand, a small clinical study found no significant benefit for selective perfusion (Ueda et al. 2000).

2.5.2 Brain protection

Just as with the protection of the spinal cord, the cerebral neuroprotection is a major challenge in aortic procedures. The principles are similar to those with spinal cord protection and include the extensive use of hypothermia, minimisation of ischemic time, and maintenance of a sufficient blood supply. The aortic arch is involved in about 15% of patients with DTAAs and TAAAs. Traditional repair of combined aneurysmal disease usually requires two stages. The aortic arch is repaired in the first stage, and the descending thoracic aorta in the second. This method is challenging because some patients do not return for the second operation for various reasons. The endovascular strategy has partially replaced two-stage repair. A single-stage repair using a hybrid prosthesis is another possibility for combined aortic arch and descending aortic aneurysms. Regardless, both these methods carry a risk for spinal cord injury (3%–17%) and stroke (5%–7%) (Dias et al. 2015, Mommertz et al. 2009, Svensson et al. 1993a). Current strategies for cerebral and spinal cord protection during aortic arch surgery include three perfusion methods:
deep HCA, antegrade cerebral perfusion, and retrograde cerebral perfusion (RCP). Hypothermic circulatory arrest provides some technical benefits, although it is not proven to be any safer a method than the other two. The stroke rate in elective aortic arch repairs with deep HCA is at best below 2%, but obviously, the rate will increase up to 13% when the safe time of HCA is exceeded (Dumfarth et al. 2013, Gega et al. 2007, Ziganshin & Elefteriades. 2013).

2.6 Remote ischemic preconditioning: A tool for neuroprotection

Since the mid-1980s, preconditioning has been a topic of extensive research. Murry et al. (1986) introduced a concept called ischemic preconditioning, four 5-minute cycles of coronary occlusion, resulting in significant reduction in the area of infarction, after 40 min of coronary occlusion (Murry et al. 1986). The same concept was tested in cerebral tissue four years later by Kitagawa et al., who performed brief (2-minute) bilateral carotid occlusions in a gerbil model to protect against subsequent ischaemic insult resulting from longer bilateral carotid occlusion (Kitagawa et al. 1990). A significant decrease in the area of infarction has led to different applications of ischaemic preconditioning. Pryzklenk et al. tested one of the first concepts of remote ischemic preconditioning in 1993, when transient ischemic conditioning to circumflex coronary artery reduced myocardial infarct size following left coronary artery occlusion (Przyklenk et al. 1993). The study demonstrated that the protective effect of ischemic conditioning could be transferred from one region of the heart to another.

2.6.1 Definition of remote ischaemic preconditioning

One of the most interesting concepts is remote ischaemic preconditioning (RIPC). Sub-lethal ischaemic occlusions are targeted to a remote region resistant to ischemic injury (e.g., limb) before a more vulnerable organ (e.g., brain, spinal cord) is subjected to major ischaemia (circulatory arrest, thoracic aortic clamping). Brief consecutive ischemic periods can be targeted, for example, with a blood pressure cuff (up to 200 mm Hg, or 30 mm Hg above the patient’s systolic pressure) to an arm or leg for 5–10 minutes in 2–4 cycles prior to injurious ischaemia.
2.6.2 Safety of remote ischemic preconditioning

Remote ischaemic preconditioning is beneficial in many ways and is smoothly applicable to aortic arch and descending aortic aneurysm operations, using both endovascular and open procedures. It is not surprising that in clinical studies of RIPC, arm or leg ischaemia is most often used because of availability. Compared with pharmacological protective agents, RIPC is substantially more cost effective. Furthermore, RIPC is generally well tolerated and safe. Bilgin-Freiert et al. (2012) conducted a microdialysis study measuring possible injurious effects of RIPC to the limb and did not find any indications of permanent cell damage. The clinical trial in phase I tested the safety and feasibility of RIPC and did not find indications of any neurovascular injury. The only disadvantage was temporary pain caused by inflation-deflation cycles (Gonzalez et al. 2014, Koch et al. 2011).

2.6.3 Mechanism: Pathway from remote organ to target protection

The fascinating part of RIPC is its ability to produce protective factors from distance to the actual target. The studies on RIPC have proposed three possible pathways: the neuronal pathway, the humoral pathway, and systemic response. The pathways are more widely studied in the heart; however, nervous tissue protection is a somewhat newer entity. The underlying pathways of the potential protective effect of RIPC are not clearly identified, and large numbers of different mediators have been suggested (Table 1). The plasma analyses of the experimental settings have suggested a protective factor, which is thermolabile, hydrophopic and 3.0–8.5 kDa in size (Breivik et al. 2011b, Dickson et al. 2001, Lang et al. 2006, Serejo et al. 2007, Shimizu et al. 2009). The effectiveness of RIPC seems not to be related to ischemic injury repair, but more to the attenuation of reperfusion injury (Figure 6). Thus, ischaemic tolerance might be the key mechanism of RIPC. The most important molecular pathways are discussed below and are summarised in Table 2.
Fig. 6. Graph illustrates the effect of ischaemic preconditioning on reperfusion injury (thick black line). Modified from Garcia-Dorado et al. The reperfusion injury is attenuated by the effect of RIPC (grey line). Cell death is more severe if ischaemic preconditioning is not performed before reperfusion (black line). If no blood flow is restored at all, cell death is complete (dotted black line) (Garcia-Dorado & Piper. 2006).

**Neuronal pathway**

The hypothesis that signals from remote areas to the target organ are transmitted through neuronal pathways is based on studies of nerve blockers and electrical nerve stimulations. The neuronal pathway includes the somatosensory and autonomous nervous systems and the spinal cord. It has been proposed that endogenous substances (adenosine, bradykinin, calcitonin gene-related peptide) are
released in a remote preconditioned organ and further stimulate afferent nerve fibres, which transmit the effect to the target organ, promoting protective cellular processes (Hausenloy & Yellon. 2008).

Local nerve stimulation seems to have an effect similar to that of RIPC (Merlocco et al. 2014), whereas a sensory nerve blocker and transection of the peripheral nerve abolished the protection by RIPC (Donato et al. 2013, Redington et al. 2012). Additionally, Donato et al. assessed the role of the afferent nervous system and found that the vagus nerve has a role as an RIPC transmitter to the heart. They also found that sectioning the spinal cord abolished the RIPC effect. In contrast, the evidence for CNS protection and neuronal pathway involvement is not as strong because the effect of RIPC on the spinal cord was not abolished with the ganglionic blocker, but with antioxidants (Dong et al. 2010).

**Humoral pathway**

It has been suggested that humoral factors or substances generated in remote preconditioning organs need to be washed out into the bloodstream toward the more susceptible organ (heart, brain). Konstantinov et al. (2005) constructed an experimental setup in which a pig with a denervated donor heart was treated with limb RIPC. The myocardial infarct size was reduced in the donor hearts, although the heart was denervated. However, the afferent sensory nerve pathway from the limb cannot be excluded (Konstantinov et al. 2005a).

Several studies have attempted to identify a specific circulating factor that might be the transmitter of RIPC (Table 1). The proposed factors are rather similar to those with neuronal pathway activators (e.g., adenosine, bradykinin, calcitonin gene-related peptide, endocannabinoids, and opioids). The nonselective adenosine receptor antagonist abolished the cardioprotective effect of RIPC, and moreover, remote preconditioning elevated adenosine plasma levels, whereas local preconditioning did not (Takaoka et al. 1999). Bradykinin was similarly shown to be involved with a specific bradykinin receptor antagonist, and increased calcitonin gene-related peptide levels were detected after RIPC. Calcitonin gene-related peptide is one of the PKC epsilon activators, at least in the heart (Schoemaker & van Heijningen. 2000, Wolfrum et al. 2005).

Our knowledge of mediating factors grows continuously, and it is increasingly evident that the protective cascade is not a simple, but rather an extremely complex process, including both humoral and neuronal pathways. More information is
needed about the effect of different organs because it has not been fully confirmed that the same factors that affect the CNS affect, for example, the heart.

Systemic response

Evidence of suppression of the inflammatory response is detected after RIPC. It is obvious that specific adhesion molecules (ICAM-1, P-selectin) are decreased in rat liver cells after RIPC. Thus, leucocyte-endothelium interactions are regulated by RIPC, and a reduced number of inflammatory cells is recruited to the ischaemic area (Peralta et al. 2001). Moreover, RIPC seems to reduce pro-inflammatory gene expression in circulating leucocytes. These genes are associated with leucocyte activation, cell adhesion, and intracellular signalling (Konstantinov et al. 2004). Moreover, these studies suggest that RIPC has a direct effect on the inflammatory system and thus prevents the exacerbation of ischemic injury. Additionally, the anti-inflammatory responses have been shown to occur in the target tissues also, pointing to the versatility of the mechanism (Table 1).

Other mechanisms: Hypoxia-inducible factor

The hypoxia-inducible factor (HIF) has gained major support as a delayed protective mechanism of RIPC. In mammals, the HIF, especially subtype HIF1α, is hydroxylated under normoxia by an oxygen- and iron-dependent prolyl hydroxylase, whereas under hypoxic conditions, this hydroxylase is non-functional. Thus, HIF1α enters the nucleus, dimerises with HIF1β, and promotes the transcription of at least 100 genes that enhance hypoxic resistance (Dirnagl et al. 2009). Cai et al. (2013) and Kalakech et al. (2013) provide controversial results. A decrease in HIF expression after RIPC was noted in patients undergoing CPB (Albrecht et al. 2013). Additionally, HIF1α knockout mice maintained their ability to develop ischaemic tolerance after ischaemic conditioning. This suggests that HIF1α is not essential for conferring robust neuroprotection, but may be one of the routes activated after RIPC (Baranova et al. 2007).
<table>
<thead>
<tr>
<th>Potential mediator</th>
<th>Species</th>
<th>RIPC model</th>
<th>Finding</th>
<th>Pathway</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>Rat</td>
<td>Mesenteric artery occlusion</td>
<td>Cardioprotection</td>
<td>Effect was abolished with ganglion blocker and adenosine receptor antagonist.</td>
<td>(Liem et al. 2002)</td>
</tr>
<tr>
<td>Hypoxia inducible factor-1</td>
<td>Mice</td>
<td>Hindlimb</td>
<td>Cardioprotection</td>
<td>HIF-1α knock-out mice did not have protective effect. HIF-1α activates IL-10 gene transcription.</td>
<td>(Cai et al. 2013)</td>
</tr>
<tr>
<td>Multiple circulating cytokines and immune cell populations</td>
<td>Rat</td>
<td>Hindlimb</td>
<td>Cerebral protection</td>
<td>Increase of TNF-α, noninflammatory monocytes, B-cell population and reduction in T cell population.</td>
<td>(Liu et al. 2016b)</td>
</tr>
<tr>
<td>Endogenous opioids</td>
<td>Rat</td>
<td>Mesenteric artery occlusion</td>
<td>Cardioprotection</td>
<td>Naloxone attenuated the cardioprotective effects.</td>
<td>(Patel et al. 2002)</td>
</tr>
<tr>
<td>Neurally mediated bradykinin release</td>
<td>Rat</td>
<td>Mesenteric artery occlusion</td>
<td>Cardioprotection</td>
<td>Sensory nerve bradykinin receptor blocking abolished the effect.</td>
<td>(Schoemaker &amp; van Heijningen. 2000)</td>
</tr>
<tr>
<td>Glukacon-like peptide-1</td>
<td>Rat</td>
<td>Hindlimb</td>
<td>Cardioprotection</td>
<td>GLP-1 blockade abolished pro-survival kinase AKT phosphorylation.</td>
<td>(Basalay et al. 2016)</td>
</tr>
<tr>
<td>Nitrite</td>
<td>Mice</td>
<td>Hindlimb</td>
<td>Cardioprotection</td>
<td>Pharmacological and genetic inhibition of nitrite abolished the effect</td>
<td>(Rassaf et al. 2014)</td>
</tr>
<tr>
<td>microRNA-144</td>
<td>Mice</td>
<td>Hindlimb</td>
<td>Cardioprotection</td>
<td>MicroRNA-144 blockade abolished RIPC effect.</td>
<td>(Li et al. 2014)</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>Rat</td>
<td>Hindlimb</td>
<td>Cardioprotection</td>
<td>Inhibition of specific cardiac receptor blocked the protective effect.</td>
<td>(Davidson et al. 2013)</td>
</tr>
<tr>
<td>Neurally mediated erythropoietin release</td>
<td>Mice</td>
<td>Hindlimb ischemia</td>
<td>Cardioprotection</td>
<td>Renal EPO release. Renal denervation abolished the effect.</td>
<td>(Oba et al. 2015)</td>
</tr>
<tr>
<td>CGRP</td>
<td>Rat</td>
<td>Mesenteric artery occlusion</td>
<td>Cardioprotection</td>
<td>PKC blockade abolished the effect.</td>
<td>(Wolfrum et al. 2005)</td>
</tr>
</tbody>
</table>
Vagus nerve Rat Hindlimb Cardioprotection Vagotomised rats did not have the protective effect of dialysate collected from RIPC treated rats. (Pickard et al. 2016)

<table>
<thead>
<tr>
<th>Potential mediator</th>
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<th>Finding</th>
<th>Pathway</th>
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<tr>
<td>Vagus nerve</td>
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<td>Hindlimb</td>
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<td>Vagotomised rats did not have the protective effect of dialysate collected from RIPC treated rats.</td>
<td>(Pickard et al. 2016)</td>
</tr>
</tbody>
</table>

EPO, erythropoietin; CGRP, calcitonin-gene related peptide; PKC, protein kinase C; IL, interleukin; TNF-a, tumour necrosis factor alpha; SDF-1a, stromal cell-derived factor-1alpha

2.6.4 Mechanism: What happens inside the cell

The pathways of RIPC may include the release of blood-borne factors from preconditioned limb (Breivik et al. 2011a) neuronal pathway activation (Lim et al. 2010) or direct systemic inflammatory response participation (Shimizu et al. 2010), as discussed earlier. Because ischaemia is an unspecific injury that causes disturbances in multiple cellular processes, one likely explanation is that RIPC activates the release of several humoral factors and involves multiple endogenous protective mechanisms. The preconditioning stimulus has to be recognised by the cellular sensor, and the cell must become more prepared for the upcoming stress. Preventing the upcoming reperfusion injury, the ischemic conditioning has been shown to recruit prosurvival signalling pathways, which are shown in Table 2 and discussed below. Also, the final phase of ischemic conditioning has been hypothesized to preserve the mitochondrial function. The possible mechanisms are summarised in Table 2 and discussed further below. The elements of the RIPC might include the sensor of the stress signal, transducers of the stimulus, and effectors of the tolerance (Gidday. 2006).

Table 2. The suggested prosurviving signalling pathways after RIPC insult.

<table>
<thead>
<tr>
<th>Suggested pathway</th>
<th>Mechanism</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>RISK pathway</td>
<td>Pro-survival kinases: Akt, Erk 1 &amp; 2, PKC, PI3K</td>
<td>(Hausenloy et al. 2005, Hausenloy &amp; Yellon. 2007)</td>
</tr>
<tr>
<td>SAFE pathway</td>
<td>TNF and JAK/STAT3 components</td>
<td>(Hausenloy et al. 2011)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Preventing calcium overload.</td>
<td>(Yellon &amp; Downey, 2003)</td>
</tr>
<tr>
<td>ROS</td>
<td>Attenuated ROS production.</td>
<td>(Hausenloy, 2013)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>MPTP inhibition.</td>
<td>(Ong et al. 2015)</td>
</tr>
</tbody>
</table>

RISK, Reperfusion injury salvage kinases, SAFE, Survivor activating factor enhancement; ROS, reactive oxygen species; MPTP, mitochondrial permeability transition pore
Timing of remote ischaemic preconditioning

There are two temporally distinct types of RIPC: early and delayed protection. Both types of ischaemic tolerance have been detected in the brain and heart. It is accepted that acute protection is independent of protein synthesis, and the protective effect is brief (minutes), whereas the delayed pattern involves new protein synthesis and lasts hours to days (Bhuiyan & Kim. 2010).

Sensors of the stress signal

The RIPC is like a double-edged sword because a frequent ischaemic stimulus must activate the same ischemic pathways. If RIPC is too robust, it might cause permanent injuries; on the other hand, if RIPC is too weak, it will not elicit a response. Therefore, the same molecules that cause ischaemic brain injury may respond as a particular preconditioning stimulus at lower levels. In general, the stress stimulus sensors of the cell are neurotransmitter receptors, ion channels, redox-sensitive enzymes, or endogenous substances (adenosine, bradykinin). Subsequently, these sensors or factors may enter the bloodstream and remotely activate multiple enzymes and pathways that transduce the signal and initiate adaptive responses (Gidday. 2006).

Transducers of the stimulus

Mitochondrial dysfunction is thought to be the major contributor to ischaemia-reperfusion-mediated injury. Thus, it seems logical that the protective effect of RIPC is targeted to preservation of mitochondria during ischaemia and reperfusion. In fact, many reviews of the mechanisms underlying RIPC and IPC point to evidence that several protein kinases (ERK, Akt, PKC epsilon) are activated during ischaemic preconditioning and targeted toward maintenance of mitochondrial function, especially inhibition of the opening of the mPTP (Bhuiyan & Kim. 2010, Gidday. 2006, Miura et al. 2010, Thompson et al. 2015).

Initially, sub-lethal ischaemic insults lead to transient decreases in cellular ATP levels. Two decades ago, a study showed that the ATP/ADP ratio decreases minutes after of mitochondrial ATP synthase inhibition (Budd & Nicholls. 1996). The consequent increase in ATP metabolites, such as ADP, AMP, and adenosine, may act to induce ischaemia tolerance pathways. For example, adenosine pre-treatment has been observed to attenuate ischaemic insults in in vivo and in vitro studies.
(Blondeau et al. 2000, Hiraide et al. 2001). The decreased ATP/ADP ratio may also directly or indirectly activate mitochondrial K⁺ATP channels. Thus, these channels seem to play a vital role in ischaemic tolerance (Blondeau et al. 2000).

**Mitochondrial K⁺ATP channels**

These channels are on the inner mitochondrial membrane, but the completed molecular structure has not yet been elucidated. Nevertheless, this inner membrane ion channel is believed to be one of the gatekeepers of life and death (O'Rourke et al. 2005). The function of this channel has been more extensively studied in the heart, but the mitochondria itself behaves similarly in any cell. In fact, brain mitochondria contain seven times more mitochondrial K⁺ATP channels compared with the liver or heart mitochondria, which reflects the importance of these channels in neuroprotection (Bajgar et al. 2001). The physiological role of mitochondrial K⁺ATP channels is linked to energy production through ETC. For instance, during increased ATP production, the increased current flow through ETC causes the inner membrane potential to decrease. Thus, abundant ATP closes the mitochondrial K⁺ATP channels, osmotically maintaining the matrix (Garlid et al. 2003). On the other hand, when ATP production decreases, the channels open, decreasing the membrane potential and thus promoting the ETC rate and further ATP production.

Upstream from mitochondrial K⁺ATP channel activation, multiple pathways target channel opening. One key player is protein kinase C, especially subtype epsilon, which is proposed directly to be activated after stress sensation during RIPC. PKC epsilon can also be activated by ATP metabolites and ROS, mostly via the PI3K/Akt pathway (RISK pathway). This kinase can activate mitochondrial K⁺ATP channels (Thompson et al. 2015).

The evidence suggests that mitochondrial K⁺ATP channels are crucial to the RIPC mechanism. At least in the heart, the protective effect of ischaemic conditioning is abolished by selective mitochondrial K⁺ATP channel blockers (Fryer et al. 2001, Pain et al. 2000). The rodent model also supports the theory of mitochondrial K⁺ATP channel involvement in ischaemic conditioning (Yoshida et al. 2004). These studies do not directly support the mechanism of RIPC, but K⁺ATP channels, either sarcolemmal or mitochondrial, also seem to be involved in the remote concept. In human studies, the RIPC seems to have a protective effect on endothelial cells of the contralateral arm and is dependent on K⁺ATP channel activation (Loukogeorgakis et al. 2007).
The opening of mitochondrial K\textsuperscript{+}\textsubscript{ATP} channels leads to a mild increase in ROS generation, which is thought to induce ischaemia tolerance pathways. This is supported by the study of antioxidants and ischaemic preconditioning. The neuroprotective effect of preconditioning was abolished by pre-treatment with antioxidants (Ravati et al. 2001). Another discovery was a significant decrease in pro-apoptotic protein release. Most importantly, mitochondrial K\textsuperscript{+}\textsubscript{ATP} channel activation is known to inhibit the influx of Ca\textsuperscript{2+} into the mitochondria during ischaemia. Additionally, K\textsuperscript{+} ion influx is believed to inhibit the opening of the mPTP, which is the most crucial event between cell survival and death (O'Rourke et al. 2005).

**Mitochondrial K\textsuperscript{+}\textsubscript{Ca} channels**

There is another mitochondrial inner membrane channel with a protective effect similar to that of the mitochondrial K\textsuperscript{+} channel. These Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels have an analogous neuroprotective effect when activated with a specific opener, but the activation route appears to be different (Sato et al. 2005). Because these two channels work in parallel, it is not impossible that this channel also plays a partial role in RIPC mechanism.

**Effectors of the tolerance**

The final manifestations of the protective effect of RIPC are anti-apoptosis, anti-inflammation, and anti-oxidant mechanisms. These outcomes develop in minutes to days. All these effectors and mediators are a complex combination of receptors, cytokines, peptides, cell interaction factors, and transcription factors, and the role of a single factor is extremely difficult to distinguish. However, mitochondrial K\textsuperscript{+}\textsubscript{ATP} channel activation is considered a major factor in rapid phase protection, whereas delayed protection requires new protein synthesis (Dimagl et al. 2009). Moreover, because RIPC as an intermittent limb ischaemia is safe to produce, its linkage to major cellular cascades is relatively easy to study, even in clinical trials.

**2.6.5 Summary of mechanisms**

RIPC induces the same factors induced by ischaemic insult, but it seems that when generated in mild amounts, these factors promote protective pathways. After RIPC induction, rapid phase protection is initiated, and subsequently, the delayed new
protein synthesis of cell survival enzymes will follow. The more the route of the protective factors or mediators approaching the target tissue is investigated, the more multidimensional it appears to be. A recent study demonstrated that in response to RIPC, 51 different proteins were expressed (Hepponstall et al. 2012). A study of children undergoing congenital cardiac surgery found that RIPC up-regulated 48 peptides, whereas the placebo-group expressed six peptides (Hepponstall et al. 2015). Some of the factors involved in the neuronal and humoral pathways are the same, and thus, it is expected that both pathways have a role in mediating the protective effect.

The described pathway to mitochondrial $K^+_\text{ATP}$ channel activation might be the endpoint of the collaboration of multiple different enzymes (Figure 7). The activated enzymes have other protective effects and trigger various pathways than only direct mitochondrial preservation. The brief ischaemic insults conducted by RIPC thus trigger a rather complex combination of different neuroprotective pathways, and pharmacologically mimicking one route might not be sufficient for improving clinical outcomes. Nevertheless, the mitochondria are the energy factories of the cell, and if it is preserved, cell function is clearly better. The obvious connection between the mitochondrial $K^+_\text{ATP}$ channel and RIPC provides an attractive opportunity for pharmacological approaches that are discussed below.

### 2.6.6 Diazoxide-mimicking remote ischaemic preconditioning

Diazoxide was discovered more than five decades ago in a study designed to examine possible nondiuretic mechanisms by which benzothiadiazines lower blood pressure. Diazoxide was found to cause direct vasodilatation of blood vessels (RUBIN et al. 1962). Only two years later, diazoxide was reported to elevate plasma glucose levels (Wolff. 1964). Furthermore, in 1976, the U.S. Food and Drug Administration (FDA) approved diazoxide (in tablet form) for the treatment of symptomatic hypoglycaemia. Officially, intravenous diazoxide was indicated for the emergency reduction of blood pressure in severe hypertension in hospitalised adults and children. Additionally, hypoglycaemia in children with congenital hyperinsulinism is usually treated with diazoxide. The mechanism underlying diazoxide’s clinical action is related predominantly to the opening of pancreatic and smooth muscle $K^+_\text{ATP}$ channels.
Protective mechanisms of diazoxide

The cardioprotective effect of diazoxide has been documented in experimental studies (Garlid et al. 1997, Nakai & Ichihara. 1994). Its cardiac protection is, once again, a subject of clinical studies (Menander et al. 2012). Diazoxide is being studied both in vivo and in vitro in a variety of animal species at concentrations between 1 and 10 mg/kg, administered intravenously (Coetzee. 2013).

Although diazoxide also opens other $K^+_ATP$ channels expressed in cells (pancreatic, smooth muscle, cardiac sarcolemma), the most interesting target as a means of preconditioning is the mitochondrial $K^+_ATP$ channels. The opening of these channels requires 2.3 to 27 µM of diazoxide, as proven in isolated cell culture studies, but the range varies between 10 and 100 µM, and in studies in vivo, the lowest effective dose was 1 mg/kg. Because diazoxide is not a specific mitochondrial $K^+_ATP$ channel opener, some researchers have concluded that doses greater than 50 µM have effects that are independent of mitochondrial $K^+_ATP$ channels (Coetzee. 2013, Kowaltowski et al. 2001). The suggested protective effect of mitochondrial $K^+_ATP$ channel opening is the decrease in mitochondrial membrane potential. Results, however, are mixed regarding the effect of diazoxide on membrane potential. Some have reported depolarization of the mitochondrial membrane (Busija et al. 2005), while others have not reported any effect (Carroll et al. 2001). More interestingly, diazoxide prevents membrane depolarisation caused by anoxia, and thus, it seems that diazoxide plays a critical role in the maintenance of mitochondrial membrane potential, which is essential for energy production (Mykytenko et al. 2008).

Those studies, which did not recognise any effect on mitochondrial membrane potential, noticed a slight increase in ROS production (Carroll et al. 2001). In fact, diazoxide was recognised as an inhibitor of complex II protein of the mitochondrial cellular respiratory chain as early as 1969 (Schafer et al. 1969). Additionally, to link mild ROS generation to neuroprotective effects, the inhibition may relate to the partial and reversible uncoupling of oxidative phosphorylation (Kopustinskiene et al. 2003), which indeed is beneficial during ischaemia-reperfusion because excessive ROS are not generated.

Diazoxide also has other effects at the molecular level. For example, some authors have concluded that the primary effect of diazoxide is PKC epsilon activation, which is the major effector in the cell survival cascade, as previously described (Kim et al. 2006). Whatever the mechanism(s), the clear improvement in mitochondrial function has been detected in many experimental studies. Most
studies are carried out in the manner of cardioprotection, but they are mainly cell culture studies, and thus, the results are at least partially applicable to CNS injury. In fact, an experimental study showed protective effect of diazoxide in the brain (Wang et al. 2011).

Fig. 7. Suggested common molecular pathways of remote ischaemic preconditioning and diazoxide in the simplified scheme. The elements of the RIPC might include the sensor of the stress signal (1), transducers of the stimulus (2), and effectors of the tolerance (3). Diazoxide effects straightforwardly on the mitochondrion leading to better preservation of the function. CGRP = calcitonin gene-related peptide, ROS = reactive oxygen species, mPTP = mitochondrial permeability transition pore, K_{ATP} channel = mitochondrial K_{ATP} channel.
2.7 Remote ischaemic preconditioning and diazoxide: From laboratory to clinical trials?

As discussed, mitochondrial preservation may be the key in neuroprotection (Figure 7), and the methods used to provide it may be smoothly applicable to spinal cord protection during thoracic aortic aneurysm repairs. The major challenges in thoracic aortic aneurysm repair are spinal cord injury and ischaemia, and the risk is not likely to disappear. However, even one patient with paraplegia is too many. Remote ischemic preconditioning is an attractive technique since it is inexpensive and available for everyone. The translation of RIPC to a clinical setting has been challenging, which is further discussed below. The Stroke Treatment Academic Roundtable (STAIR) has announced the outcome measures criteria for preclinical neuronal damage studies (Fisher et al. 2009). The outcome should be based on functional response and infarct volume and should be tested at least in two different species, favourably in larger species. The preclinical studies have shown both histological and functional benefit of RIPC, as well as diazoxide in spinal cord protection (Table 3). Therefore, in light of current knowledge of RIPC and diazoxide, spinal cord protection needs to be carefully evaluated before moving toward clinical trials. This thesis is mainly focused on spinal cord protection in the experimental large mammal model. Therefore, this data will bring new information on the possible beneficial effects of RIPC and diazoxide and also, additional preclinical results into the decision-making, such as whether to go for clinical trials. Also, it must be kept in mind that the effect or RIPC and diazoxide might be different various parts of the central nervous system i.e., the detected cerebral protection might not be translatable to spinal cord protection and vice versa.

2.7.1 Diazoxide and organ protection

To date, several clinical studies have shown the cardioprotective effect of diazoxide after coronary artery bypass surgery (Deja et al. 2009, Mennander et al. 2012, Shalaby et al. 2011, Wang et al. 2003, Wang et al. 2004), but to our best knowledge, no clinical trials have been conducted regarding the possible neuroprotective effect of diazoxide. Diazoxide has shown to be beneficial in spinal cord protection (Table 3) and in cerebral protection (Hou et al. 2016, Nakagawa et al. 2005, Shake et al. 2001, Simerabet et al. 2008) in preclinical studies. Because pharmacological adjuncts would work much faster than bouts of ischaemia, diazoxide has a clinical potentiality although the research has not been as intensive as it has been with RIPC.
One tempting idea is to evaluate if RIPC and diazoxide potentiate the effect of each other’s effect, especially in the context of spinal cord ischaemia.

2.7.2 RIPC and neuroprotection

Experimental and clinical studies on the neuroprotective effect of RIPC on the CNS have been rather limited so far, but provocative. To date, no randomized clinical trials of RIPC or diazoxide for spinal cord protection have been conducted in aortic or cardiac surgery. Adults undergoing carotid endarterectomy benefitted from RIPC, as documented by the significant reductions in saccadic latency (Walsh et al. 2010). Also, a placebo-controlled trial with smallish study population (N = 26) concluded that remote ischemic conditioning may improve neurological outcome when performed with patients suffering from acute ischemic stroke (England et al. 2017). Spinal cord protection was found in a small study of 40 patients who underwent cervical decompression surgery. A significantly better recovery rate was obtained for RIPC-treated patients three months from surgery (Hu et al. 2010). More interestingly, RIPC has been associated with significantly better motor learning in healthy adults (Cherry-Allen et al. 2015). In children, however, RIPC did not have any effect on white matter injury after CPB surgery (Gaynor et al. 2018). In an experimental model of one hour HCA, a significantly better EEG recovery and behavioural scores was obtained after seven days postoperatively (Jensen et al. 2011). At the moment, there are on-going clinical trials regarding the potential neuroprotective effect of RIPC (Gasparovic et al. 2014, Tulu et al. 2015).

2.7.3 RIPC and other organs

Several randomized clinical trials regarding the cerebral, myocardial or renal protective benefit of RIPC have been published, and the results remain controversial. High-quality randomized clinical trials have shown cardiac (Thielmann et al. 2013) and renal protection (Zarbock et al. 2015) and also a reduced rate of adverse cardiac and cerebral events six months postoperatively (Davies et al. 2013). Two large meta-analysis have shown beneficial effects of RIPC in morbidity measures but not in mortality (Pierce et al. 2017, Sardar et al. 2016). Also, a systematic review of 11619 patients evaluated ischemic conditioning in various settings and showed possible effects on acute kidney injury and stroke (Sukkar et al. 2016).
However, three recent, large multicentre randomized, clinical trials showed no benefit of RIPC on patients undergoing coronary artery bypass grafting (Meybohm et al. 2015), heart surgery utilising CPB (Hausenloy et al. 2015a), and cardiac surgery (Hong et al. 2014). The subsequent report of one-year follow-up results from RIPHeart was not able to demonstrate any benefit of RIPC (Meybohm et al. 2018). Also, single centre trials showed that RIPC did not have a beneficial effect on children undergoing congenital cardiac surgery and one may speculate that in children, the preoperative hypoxic state might also confer the effect of RIPC (Jones et al. 2013, McCrindle et al. 2014).

The studies by Meybohm et al. (RIPHeart study) and Hausenloy et al. (ERICCA study) have been criticized by using propofol anaesthesia, whereas the studies reporting positive findings have used halogenated agents, which are known to have an additive effect on survival compared to propofol (Kottenberg et al. 2012, Landoni et al. 2013, Zangrillo et al. 2015). Similar conclusions about propofol being a potentially confounding factor were drawn from a sub-analysis of the RIPHeart study (Ney et al. 2018). Additionally, it must be kept in mind that other factors may have affected the renal, cardiac and neuroprotective effect of RIPC. The acute myocardial injury in cardiac surgery is not purely caused by ischemia-reperfusion injury but is multifactorial, including coronary microembolization, handling of the heart, inflammation, etc. Also, medications, especially beta-blockers (Zhou et al. 2013), and coexisting comorbidities, such as older age, diabetes, and hypertension might reduce the beneficial effect of RIPC. The RIPC seemed to have myocardial protective effect in the settings, in which the injury is more specifically caused by ischemia-reperfusion injury, such as acute myocardial infarct (Botker et al. 2010, Crimi et al. 2013, Eitel et al. 2015, Liu et al. 2016a, Prunier et al. 2014, Rentoukas et al. 2010, White et al. 2015, Yellon et al. 2015). Therefore, a multicentre trial is recruiting to investigate whether RIPC reduces cardiac death after acute myocardial infarction at one year (Hausenloy et al. 2015b) and one trial is recruiting in the field of vascular surgery (Healy et al. 2015).
Table 3. Preclinical studies indicating the effect of RIPC and diazoxide on the spinal cord.

<table>
<thead>
<tr>
<th>Study</th>
<th>Model</th>
<th>Stimulus</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Dong et al. 2010)</td>
<td>Aortic occlusion of 20 minutes distal to the renal arteries in New Zealand White rabbits.</td>
<td>Two cycles of 10 minutes bilateral femoral artery occlusion 30 minutes before ischemia – 10 minutes reperfusion.</td>
<td>RIPC reduced histologic damage in the anterior spinal cord via antioxidant pathways correlating with higher Tarlov score after 48 hours.</td>
</tr>
<tr>
<td>(Sapmaz et al. 2015)</td>
<td>Aortic occlusion of 30 minutes distal to the renal arteries in New Zealand White rabbits.</td>
<td>RIPC: Three cycles of 5 minutes axillary artery clamping just before ischemia – 5 minutes reperfusion. IPost: Three cycles of 5 minutes abdominal aortic clamping after ischemia.</td>
<td>RIPC and IPost reduced ultrastructural (EM) injury of spinal cord, which correlated with improved Tarlov score after 24 hours.</td>
</tr>
<tr>
<td>(Fukui et al. 2018)</td>
<td>Aortic occlusion of 15 minutes distal to the renal arteries in New Zealand White rabbits.</td>
<td>RIPC: Two cycles of 10 minutes bilateral femoral artery clamping – 10 minutes reperfusion. IPC: Two cycles of 5 minutes abdominal aorta – 15 minutes reperfusion.</td>
<td>RIPC did not have spinal cord protective effect seven days after reperfusion, whereas IPC improved neurological outcome and preserved higher number of normal neurons.</td>
</tr>
<tr>
<td>(Gurcun et al. 2006b)</td>
<td>Aortic occlusion of 40 minutes distal to the renal arteries in New Zealand White rabbits</td>
<td>RIPC: Two cycles of 5 minutes left renal artery clamping just before ischemia – 15 minutes reperfusion. IPC: Two cycles of 5 minutes abdominal aorta – 15 minutes reperfusion.</td>
<td>RIPC and IPC improved neurological outcome 24 and 48 hours after reperfusion and reduced neuronal damage in spinal cord.</td>
</tr>
<tr>
<td>Study</td>
<td>Model</td>
<td>Stimulus</td>
<td>Inference</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>(Jing et al. 2017)</td>
<td>Fourteen minutes cross-clamping of aorta between LCA and LSA in Sprague-Dawley rats</td>
<td>Three cycles of 3 minutes right femoral artery occlusion 30 minutes before ischemia – 3 minutes reperfusion.</td>
<td>RIPC preserved the integrity of blood-spinal cord barrier and improved functional neurological outcome.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RIPC, remote ischemic preconditioning; IPost, Ischemic post-conditioning; IPC, ischemic preconditioning; EM, electron microscopy; LCA, left carotid artery; LSA, left subclavian artery</td>
</tr>
<tr>
<td>(Roseborough et al. 2006)</td>
<td>Aortic occlusion of 30 minutes distal to the renal arteries in New Zealand White rabbits</td>
<td>Five mg/kg intravenous dose of diazoxide 10 minutes before ischemia</td>
<td>Diazoxide reduced mitochondrial and ROS-related injury and improved neurological functional score after 48 hours.</td>
</tr>
<tr>
<td>(Kim et al. 2008)</td>
<td>Aortic occlusion of 20 minutes distal to the renal arteries in New Zealand White rabbits</td>
<td>Two groups: 5mg/kg intravenous dose of diazoxide 48 hours and 15 minutes before ischemia</td>
<td>Significant improvement in neurological scores and preserved motor neuron numbers in the spinal cord were observed in both diazoxide groups.</td>
</tr>
<tr>
<td>(Caparrelli et al. 2002)</td>
<td>Aortic occlusion of 20 minutes distal to the renal arteries in New Zealand White rabbits</td>
<td>5mg/kg intravenous dose of diazoxide 15 minutes before ischemia.</td>
<td>Tarlov score was significantly better in diazoxide-treated animals 24 and 48 hours after ischemia. Histopathological analysis showed no differences.</td>
</tr>
<tr>
<td>(Yamanaka et al. 2018)</td>
<td>Four minutes cross-clamping of aorta between LCA and LSA in Sprague-Dawley rats</td>
<td>Twenty mg/kg oral dose of Diazoxide</td>
<td>Diazoxide-treated animals performed improved motor function 48 hours after ischemia.</td>
</tr>
</tbody>
</table>
3 Aims of the study

The purpose of the studies described in this thesis was to assess whether RIPC or diazoxide produces neuroprotection in an experimental setting that is mimicking open thoracic aortic aneurysm repair. The present thesis comprises following studies:

I To ascertain whether RIPC has neuroprotective effects against spinal cord ischaemia in terms of neurological assessment and MEP recordings
II To assess the effect of RIPC on oxidative stress of the spinal cord after local ischemic insult.
III To examine if diazoxide, a possible pharmacological RIPC mimetic, has neuroprotective effects after HCA.
IV To summarize the current knowledge of the RIPC and shed light on the potential mechanism of the RIPC
4 Author’s contribution

The projects (referred as Study I, II, III and IV in the future chapters) included in this thesis were supervised by Professor Tatu Juvonen and Docent Vesa Anttila in collaboration with the research team. The author’s contribution in the projects was as follows:

He participated in the planning of projects I, II and III, performed all surgical procedures in collaboration with the research team, did the statistical analyses together with biostatistician, and reported the results. Additionally, the author of this thesis was responsible for the design of these projects, interpretation of the results as well as for the drafting the works for publication. The Study IV is mainly based on the literature review of this thesis and therefore, the author has a crucial role in the article. The manuscript layout and writing were conducted by the first author.
5 Materials and methods

This thesis is constructed on the basis of four different studies, and the materials and methods are summarised in this section. The studies I, II and III were subacute models. The first and second (I and II) studies assessed the impact of RIPC on the spinal cord without cardiopulmonary bypass, whereas the Study III was a HCA-model with diazoxide. The Study IV was a review of the current knowledge of the RIPC from the cardiovascular point of view.

5.1 The porcine model

Professor Randall Griepp and his team were the first to employ the surviving experimental animal model at the Mount Sinai School of Medicine in New York. The research group of Professor Tatu Juvonen brought the model to Finland, and the first experiments were conducted at the Cardiothoracic Research Laboratory in Oulu in the autumn of 1997. The model has been under continuous critical improvement ever since. All three studies are randomised and controlled setups consisting of two comparative groups.

5.2 Test animals and preoperative management

Only native stock pigs were used. They were raised in a local piggery near Oulu, Finland, and the animals were taken into experimental use at the age of 6–10 weeks. It must be noted that all pigs were allowed to adjust to new surroundings in the same cage at least 7 days before experimentation and the environment was kept non-stressful pre- and postoperatively by the staff of the Laboratory Animal Centre and researchers. All animals received humane care in accordance with the Principles of Laboratory Animal Care formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals (http://www.nap.edu/catalog/5140.html). The Research Animal Care and Use Committee of the University of Oulu approved all the studies used in this thesis.

5.3 Anaesthesia

Every animal was sedated with an intramuscular injection of ketamine (350 mg), midazolam (45 mg), and medetomidine (1.5 mg). If required, an intravenous bolus of thiopental was administered through a peripheral catheter placed in the right ear.
vein during intubation. The 6.5-mm cuffed endotracheal tube was connected to the ventilator (GE Aisys Carestation, GE Healthcare, Madison, WI, USA). The anaesthesia was inducted with an intravenous bolus of fentanyl (50 μg/kg) and inhalation of 1.0% sevoflurane. Ventilation was maintained at 20 times per minute with positive-end-expiratory-pressure (PEEP) of 5 cm H₂O. With the help of end-tidal-control of the ventilator, end-tidal carbon dioxide (EtCO₂) and oxygen (EtO₂) in expired air were strictly maintained at 5.0 kPa and 50%, respectively.

The continuous infusion anaesthesia was as described below in Study III (HCA model), but was modified in studies I and II. In parallel with inhalation anaesthesia, a combination of fentanyl 25 μg/kg·h, midazolam 25 mg/kg·h, and pancuronium 0.2 mg/kg·h was administered throughout the experiment in the HCA study (III). Obviously, both inhalation and intravenous anaesthesia were discontinued during HCA.

Motor evoked potential recordings are very sensitive to certain anaesthetic agents; thus, midazolam and pancuronium were replaced with ketamine 15mg/kg·h in the Study I. Small boluses of rocuronium (0.1 mg/kg) were used for relaxation during surgery, but were not given after MEP baseline recordings. Additionally, sevoflurane was also discontinued before baseline measurements and was not given afterward.

5.4 Haemodynamic and temperature monitoring

An arterial line was introduced into the left femoral artery and a pulmonary artery thermodilution catheter (CritiCath 7 French, Ohmeda, Erlangen, Germany) was placed in the pulmonary artery via the left femoral vein. In addition to blood sampling, these lines were used for monitoring pulse, systemic and pulmonary pressures, cardiac output, pulmonary capillary wedge pressure, and central venous pressure and for recording blood temperature. Rectal core temperature was also recorded. Mean arterial pressure was held over 60 mm Hg throughout the experiment, and sodium chloride (0.9%) and/or noradrenalin were infused if required. Thus, fluid balance was carefully recorded and urine output was monitored through a 10 French catheter placed in the urinary bladder. Electrocardiographic monitoring was carried out throughout the experiment.
5.5 Biochemical measurements

In Study III, samples of venous and arterial blood were collected at baseline, at the end of cooling (10 min before institution of HCA), at 30 min, 1, 2, 4, and 8 hours after the start of rewarming and analysed. The Study I setup did not include HCA, and thus, the sampling points were at baseline, 15 min after RIPC, immediately and 1, 2, and 4 hours after the ninth segmental artery occlusion. The following parameters were analysed for all blood samples (i-STAT Analyzer, i-STAT, East Windsor, NJ): pH, oxygen and carbon dioxide partial pressures, oxygen saturation, haematocrit, haemoglobin, sodium, potassium, lactate, ionised calcium, glucose. Additionally, leucocyte differential count (Cell-Dyn analyser, Abbot, Santa Clara, CA, USA], troponin I, creatine kinase (CK), and its isoenzyme (CK-MB) were analysed in studies I and III (Hydrasys LC-electrophoresis, Hyrysis-densitometry, Serbia, France).

5.6 Cardiopulmonary bypass (Study III)

The right atrial appendage and aortic arch were revealed through the right fourth intercostal space. The pericardium was opened to provide access to the aortic arch and right atrium. The pig was first systemically heparinised (500 IU/kg), and then the venous cannula (24 French) was placed through right appendage; a 14 French arterial straight-tip cannula was used for aortic arch cannulation (Figure 8).

Preoperatively, a membrane oxygenator (D905 Eos, Dideco, Mirandola, Italy) was primed with 800 mL of Ringer acetate solution and heparin (15,000 IU). The mean arterial pressure was maintained between 50 and 70 mm Hg by adjusting the flow rate of nonpulsatile CPB. The heat exchanger was used for core cooling and warming. Cardiopulmonary bypass was conducted using a pH-stat strategy by adding CO₂ to the inflowing gas and correcting the PₐCO₂ and pH levels for the actual core temperature. Sampling every 5 min during the cooling and rewarming periods ensured the pH strategy.

The cooling was started immediately after cannulation, and after the target core temperature was reached, the aortic arch was cross-clamped just distal to the aortic cannula. Potassium chloride (40 mmol) administered through the arterial cannula was used as a cardioplegic to stop the heart. The core temperature was maintained at 18°C with ice packs over the head and body during the period of HCA. Rectal, blood, and intracerebral temperatures were recorded at 10-min intervals, ensuring similar handling of the study animals during HCA.
5.6.1 Donor blood transfusion

Animals tended to be anemic after weaning from CPB, and therefore donor pig blood was used to prime the membrane oxygenator. The donor pigs were sedated, intubated, and kept anaesthetised, as previously described. The pigs were heparinised (1,000 IU/kg), and blood (25th and 75th percentiles: 54.9–79.8 mL/kg) was suctioned from the right femoral artery to the oxygenator. Thereafter, donor pigs were sacrificed using pentobarbital (60 mg/kg) while anaesthetised.
5.7 Remote ischaemic preconditioning (studies I and II)

Remote ischaemic preconditioning was performed with a 9-cm-wide blood pressure cuff. The cuff was placed around the left hind limb and inflated to 250 mm Hg for 5 min and then deflated for 5 min. The total protocol included four inflation–deflation cycles, and thus the total duration of RIPC was 40 min. The control group without RIPC was observed for only 40 min.

5.8 Diazoxide infusion (Study III)

Diazoxide (D9035 Sigma, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ~10 mL of sodium hydroxide (1 mol/L). This solution was diluted to 40 mL sodium chloride and infused into the systemic blood circulation via a pulmonary artery catheter. The infusion (5 mg/kg diazoxide) was carried out during the cooling phase, and mean arterial pressure was held at greater than 60 mm Hg with the assistance of the heart–lung machine. Additionally, blood glucose and arterial and venous pH levels were systemically controlled throughout the experiment. At the end of infusion, the pulmonary artery catheter was rinsed with an additional 10 mL of saline. The control group was infused with only 60 mL of saline during the cooling phase of CPB.

Fig. 9. Protocol for Study I and II. The upward arrows represent the time points for blood sampling in Study I. The protocol was similar in Study II, but no MEP recordings were assessed (Haapanen et al. 2015, published with permission from Elsevier).
5.9 Experimental protocols

5.9.1 Model using motor evoked potentials (Study I)

The surgical protocol is illustrated in Figure 9. The setup for motor evoked potential monitoring was started with a U-shaped incision in the scalp; the periosteum was removed from the top of the skull. Four wire leads were attached with sterile stainless screws into the skull and placed 10 mm lateral to the sagittal suture, two on the left side (8 mm anterior and 8 mm posterior to the coronal suture) and two on the right side, respectively. The leads were connected to an electrical stimulator (Cadwell, TCS-1). This constant-voltage stimulator (train length 4, interstimulus 2 ms, stimulus pulse width 75 μs) was used for eliciting transcranial electrical MEPs. To measure the response, stainless-steel needle electrodes were placed through the skin in both hind limbs (musculus tibialis anterior). The intraoperative neuromonitoring system (Cadwell Cascade Elite Version .2.6, Cadwell, Kennewick, WA, USA) was used for recordings.

Afterward, MEP data were analysed with a separate computer program (Cascade software Version 2.6, Microsoft Windows 7 platform). Peak-to-peak amplitude, onset latency, and difference between peak and onset latencies were determined from the data (Figure 10).

In Study I, a left anterolateral thoracotomy was performed through the 4th and 7th intercostal spaces to expose the left subclavian artery and upper thoracic segmental (intercostal) arteries arising from the descending aorta. The rest of the segmental arteries to the level of the diaphragm were dissected through the left 11th intercostal space incision. First, all segmental arteries to the level of diaphragm and left subclavian artery were revealed and rounded by threads. Then the baseline MEP amplitude was recorded, and RIPC performed (or sham). Thereafter, meticulous left subclavian artery and all segmental arteries beyond the diaphragm were ligated and cut at 5-min intervals. The MEP recordings were made in parallel to arterial occlusion at the following time points: baseline, after RIPC or sham procedure, during the period of segmental artery occlusion at 1-min intervals, for a period of 30 min after ischaemia at 5-min intervals, and every 30 min throughout the 4-hour follow-up (Figure 11).

After follow-up, animals were extubated and transferred to a recovery room. After 24 hours from the onset of arterial occlusion, the animals were sacrificed using intravenous pentobarbital (60 mg/kg).
Fig. 10. Motor evoked potential response. The figure is modelled from one experiment. Onset latency, peak latency, and peak-to-peak amplitude are represented by arrows (Haapanen et al. 2015, published permission from Elsevier).
5.10 Behavioural assessment (Study I)

Neurological assessment of spinal cord function performed only in Study I. The evaluation was conducted using the Tarlov score 24 hours after occlusion of the last segmental artery (Tarlov. 1957). The scale is outlined in Table 4. The purpose of the evaluation was more likely to provide a rough assessment of hind limb function as the general anaesthesia was a confounding factor at the time of the evaluation. Nevertheless, obvious paraplegia could be documented with this method.
Table 4. Tarlov score.

<table>
<thead>
<tr>
<th>Score</th>
<th>Neurological function of the hind limbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Spastic paraplegia, no movements</td>
</tr>
<tr>
<td>1</td>
<td>Paraparesis, slight movements</td>
</tr>
<tr>
<td>2</td>
<td>Paraparesis, powerful movements but not standing</td>
</tr>
<tr>
<td>3</td>
<td>Able to stand but not walk</td>
</tr>
<tr>
<td>4</td>
<td>Full recovery, normal walking function</td>
</tr>
</tbody>
</table>

5.11 HCA model (Study III)

After the cannulation of the aortic arch and right appendage, CPB was initiated and cooling was started immediately using the pH strategy. Core temperature (rectal and intracerebral temperatures) was lowered to the target temperature of 18°C in 30 minutes using the heat exchanger. After the cooling phase, perfusion was stopped for 60 min to initiate HCA.

Reperfusion was started after HCA with 5 min of cold perfusion at 18°C. During this period, furosemide (40 mg), lidocaine (40 mg), methylprednisolone (40 mg), and calcium gluconate (90 mg) were administered to the CPB circulation. Ten minutes later, mannitol 100 mL (15mg/100 mL) was also infused after the first blood samples were taken. The reperfusion period lasted 45 minutes until the core temperature reached 35°C-37°C. If necessary, the heart was defibrillated at 30°C and ventilation was started cautiously after 10 min of reperfusion with the assistance of the ventilator.

After weaning from CPB, the systemic heparinisation was revoked by administration of protamine sulphate. The heat-exchanger mattresses and heating lamps prevented the drop in core temperature. The follow-up lasted 24 hours under systemic anaesthesia until the animal was euthanised. The protocol is illustrated in Figure 12.
Fig. 12. A simplified protocol for Study III. The follow-up lasted 24 hours. HCA = hypothermic circulatory arrest, CPB = cardiopulmonary bypass.

5.12 Histopathological analysis

The hematoxylin–eosin staining method was similar for brain and spinal cord. The assessment was conducted using the same scale (Table 5), and the same experienced neuropathologist who was blinded to the grouping analysed the samples in studies I, II and III. Four signs of injury were visually evaluated: haemorrhage, oedema, neuronal damage, and infarcts. Immunohistochemical staining was used in studies II and III to reveal oxidative stress and ischemic injury in a more detailed manner.

5.12.1 Hematoxylin–eosin staining

Immediately after intravenous injection of pentobarbital (60 mg/kg), the brain/spinal cord was harvested and immersed in 10% neutral formalin for
histopathological analysis. The brain/spinal cord was allowed to fix in formalin for 1 week before sectioning. Thereafter, 3-mm coronal samples were sliced from the frontal lobe (both left and right), thalamus, and hippocampus and sagittal samples from the brainstem (medulla oblongata and pons) and cerebellum in Study III. The spinal cord was sectioned horizontally similarly from five predetermined regions: thoracic vertebra nerve roots 1–3, 4–6, 7–9, and 10–13(14) and lumbar vertebra nerve roots 1–4(5). The lengths of the thoracic and lumbar spinal column varied by one vertebra among the pigs.

Subsequently, nervous tissue was fixed in fresh formalin for another week. Thereafter, the sample processing was as follows: rinsing in water for 20 min and immersion in 70% ethanol for 2 hours, 94% ethanol for 4 hours, and absolute ethanol for 9 hours. Then the specimens were kept in an absolute ethanol–xylene mixture for 1 hour and in xylene for 4 hours and embedded in warm paraffin for 6 hours. The final thickness of the sample was 6 μm; samples were ultimately stained with hematoxylin and eosin.

Table 5. Hematoxylin-eosin staining score.

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemorrhage</td>
<td>No</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
</tr>
<tr>
<td>Oedema</td>
<td>No</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
</tr>
<tr>
<td>Neuronal damage</td>
<td>No</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Infarcts</td>
<td>No</td>
<td>—</td>
<td>—</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Other organ samples in the Study III

Samples of heart apex and cortex of the right kidney were harvested in the Study III for assessment of the systemic effect of diazoxide. These specimens were stained in hematoxylin and eosin and handled as previously described. Additionally, the left parietal cortex of the brain was biopsied 8 hours after HCA. A disposable perforator was used to reveal the dura mater. A small window was made in the dura, and a biopsy was taken with a specific brain needle. Haemostasis was ensured with cautious use of diameter. No complications were documented in any animal used in this study. The cortex, heart, and kidney samples were also immunohistochemically analysed.
5.12.2 Immunohistochemical staining

Immunohistochemical staining was used in studies II and III to gather detailed information on oxidative stress and cell death. Six different factors of the cell death cascade were determined: nuclear erythroid related factor 2 (Nrf-2), 8-oxoguanine glycosylase (Ogg-1), 8-hydroxy-2’-deoxyguanosine (8-OhdG), protein deglycase DJ-1/Parkinson disease protein 7 (DJ-1/PARK 7), cytochrome C, and caspase-3. Nrf-2, cytochrome C, and caspase-3 were discussed earlier (Section 2.3.1). 8-Hydroxy-2’-deoxyguanosine is one of the major products of DNA oxidation and thus it can be considered a measure of oxidative stress within a cell. Furthermore, 8-oxoguanine glycosylase is a main enzyme in repair of injuries caused by 8-OhdG in DNA (de Souza-Pinto et al. 2001). Protein deglycase DJ-1, also known as Parkinson disease protein 7 (PARK 7), is expressed mainly in neurodegenerative diseases that are tightly related to oxidative stress. However, experimental models of ischemic brain injury have also revealed elevated DJ-1 levels (Yanagida et al. 2009). Both neurons and glial cells express DJ-1, and the function is related to neuroprotection (Ariga et al. 2013).

The staining method was as follows: Sections (4,5 μm) cut from paraffin-embedded specimens were deparaffinised in xylene and rehydrated through graded alcohols. For antigen retrieval, the sections were pretreated with either Tris-EDTA, pH 9, or citrate buffer, pH 6, in a microwave oven. After neutralisation of endogenous peroxidase activity, the sections were incubated at room temperature with diluted antibodies. Bound antibodies were detected using the Envision Flex system (Dako, Agilent Technologies, Santa Clara, CA, USA) or Invitrogen system (Thermo Fischer Scientific, Waltham, MA, USA). Deaminobenzidine was used as the chromogen, and hematoxylin as the counterstain (Table 6).

The scoring system was a four-point scale: 0 = negative, 1 = mild, 2 = moderate, 3 = strong stain intensity. The same specialised neuropathologist who was blinded to the grouping evaluated both hematoxylin–eosin- and immunohistochemically stained specimens.
Table 6. Immunohistochemical staining method for oxidative stress markers.

<table>
<thead>
<tr>
<th>Antibody (clone)</th>
<th>Dilution</th>
<th>Retrieval</th>
<th>Detection kit</th>
<th>Incubation time</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf-2 (ab 62352)</td>
<td>1:300</td>
<td>Tris-EDTA</td>
<td>Envision</td>
<td>60 min</td>
<td>ABCam</td>
</tr>
<tr>
<td>Ogg-1 (NB 100-106)</td>
<td>1:1000</td>
<td>Citrate buffer</td>
<td>Envision</td>
<td>Over night</td>
<td>Novus Biologicals</td>
</tr>
<tr>
<td>8-OhdG (N45.1)</td>
<td>1:75</td>
<td>Citrate buffer</td>
<td>Invitrogen, Hististain-Plus</td>
<td>45 min at 42°C</td>
<td>Japan institute For the Control of Aging</td>
</tr>
<tr>
<td>DJ-1 (ab 18257)</td>
<td>1:2000</td>
<td>Citrate buffer</td>
<td>Envision</td>
<td>60 min</td>
<td>ABCam</td>
</tr>
<tr>
<td>Cyt C (ab90529)</td>
<td>1:1500</td>
<td>Citrate buffer</td>
<td>Envision</td>
<td>30 min</td>
<td>ABCam</td>
</tr>
<tr>
<td>Casp-3 (AF835)</td>
<td>1:400</td>
<td>Citrate buffer</td>
<td>Envision</td>
<td>30 min</td>
<td>R&amp;D Systems</td>
</tr>
</tbody>
</table>

Cyt C = cytochrome; Casp-3 = active caspase 3.

5.13 Statistical analysis

Statistical analysis was performed with SPSS (IBM, Armonk, NY, USA) Versions 20.0 (Study I) and 22.0 (Studies II and III) and SAS Version 9.2 (SAS Institute, Cary, NC, USA) statistical software packages. In all studies, Continuous variables in tables and figures are expressed as medians with 25th–75th percentiles and categorical data is presented as proportions. As the sample size was relatively small in all studies, the percentiles are more representational. The repeated measurements were analysed using a linear mixed model with animals fitted as random, and the best covariance pattern was chosen according to Akaike’s information criteria. Complete independence was assumed across animals (by random statement). Reported \( P \) values are as follows: \( P \) between groups (\( P_g \)) indicates a level of difference between groups, \( P \) time \( \times \) group (\( P_{t*g} \)) indicates behaviour between groups over time. The Student \( t \) test or Mann–Whitney \( U \) test was used as appropriate to assess the distribution of variables between study groups. Normally distributed data were evaluated with Student’s \( t \) test, but when the normality assumption failed (Shapiro-Wilk test of normality), the Mann–Whitney \( U \) test was run. Two-tailed significance levels are reported. \( P < 0.05 \) was considered statistically significant.

Because the individual MEP conduction velocities in the spinal cord vary greatly between study subjects i.e. absolute values are not comparable between individuals, the data are presented as relative changes from initial values in the Study I. Hence, the \( P \) values of multiple testing (\( P_g \) and \( P_{t*g} \)) are not reported on MEP values. Therefore, only cross-sectional analysis was performed.
6 Results

The total number of the animals used in the work described here was 44. The median weight of all animals was 20.45 kg (18.9–21.9 kg), and the age varied between 6 and 8 weeks. However, a band of animals was excluded from the studies for a variety of reasons (Table 7). Overall, 18 animals were included in studies I, II and III.

Table 7. Numbers of animals excluded from the studies.

<table>
<thead>
<tr>
<th>Indication for exclusion</th>
<th>Study I and II</th>
<th>Study III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>C</td>
</tr>
<tr>
<td>Pilot experiment</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Massive intraoperative bleeding</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Unsuccessful weaning from CPB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Failure to protect myocardium</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

I = Intervention group (RIPC or diazoxide), C = control group, CPB = cardiopulmonary bypass. Operation-related survival (excluding pilot studies) was 90 % (18/20) in studies I, II and III, respectively.

6.1 Study I

Study I had a 24-hour follow-up. Spinal cord function was evaluated using MEP recordings. We examined whether RIPC had a neuroprotective effect on the spinal cord in the model where the left subclavian artery and all thoracic segmental arteries were sacrificed. The study indicated that MEP recordings were multiplied after RIPC was performed on the left hind limb, and the effect was constant up to 4 hours of surveillance, whereas a decreasing trend in amplitude was recorded in the control group. Additionally, a significantly shorter onset of MEP response was detected in the RIPC group after the intervention.

6.1.1 Comparability of study groups

There were no clinically relevant differences in weight, blood, or rectal temperatures, mean arterial pressure, or haemoglobin between the study groups at baseline. All animals had stable hemodynamics throughout the experiment. The reader is advised to consult the original article for more detailed experimental and metabolic data.
The study included 20 animals, 2 of which had to be terminated because of intraoperative bleeding and thus excluded from the analysis. A total of 18 animals survived 4 hours of follow-up (10 in the RIPC group, 8 in the control group) and 14 animals survived 24 hours of follow-up (7 in each group). Thus, 4 animals were lost during observation, mainly because of respiratory problems.

6.1.2 Motor evoked potentials

Peak-to-peak amplitude

Fig. 13. Peak-to-peak amplitude of the right hind limb. Recordings were referred to baseline, and the percentage changes are illustrated. A 50% decrease in amplitude is represented by the dotted line. SA = segmental artery (Haapanen et al. 2015, published with permission from Elsevier).

Peak-to-peak amplitude was significantly increased in the RIPC group in the right hind limb. Strikingly, the RIPC itself caused the increase in amplitude before the onset of spinal cord ischaemia. There were several statistically significantly
different time points in the right hind limb, whereas left hind limb recordings showed a similar trend (Figures 13 and 14).

The control group reached a 50%-decrease in amplitude sooner than the RIPC group in the right hind limb ($P = 0.044$). All animals in the RIPC group lasted throughout the 4-hour follow-up (345 min), whereas the median time to a 50% amplitude decrease was 195 min (143–345 min) in the control group. No significant difference was recorded from the left hind limb ($P = 0.271$).

![Graph showing percentage change in amplitude over time for different groups.]

**Fig. 14.** Peak-to-peak amplitude of the left hind limb. Recordings were referred to baseline, and percentage changes are illustrated. A 50% decrease in amplitude is represented as a dotted line. SA = segmental artery (Haapanen et al. 2015, published with permission from Elsevier).

**Onset and peak latency**

Onset latency was significantly shortened in the right hind limb in the RIPC group after intervention, and the more sensitive reactivity lasted until the last segmental artery was occluded (Figure 15). The left hind limb did not differ between groups. Furthermore, neither peak latency nor the difference between peak and onset latencies differed significantly between groups.
Fig. 15. Onset latency of the right hind limb. SA = segmental artery (Haapanen et al. 2015, published with permission from Elsevier).

6.1.3 Neurologic evaluation

Fourteen animals were assessed after 24 hours using the Tarlov score (Figure 16). The median score of the RIPC group was 3.0, whereas the control group scored 1.5 ($P = 0.169$). The difference was not statistically significant, but the RIPC group tended to have a better score, and additionally, one animal from the control group had a Tarlov score of 0, whereas the minimum Tarlov score in the RIPC group was 2.

6.1.4 Histological analysis

There were no statistically significant differences between groups with respect to hematoxylin–eosin staining of the spinal cord specimens. Median sums of the score were similar: in the RIPC group, 9 (7.5–10.5), and in the control group, 9 (6–11),
respectively \((P = 0.713)\). The main findings were oedema and haemorrhage. The damage was not specifically pronounced in either of the sides of the spinal cord.

Fig. 16. Tarlov Score evaluation of the study groups 24 hours postoperatively. Median score for the RIPC group was 3.0 (2.0–3.0) and for the control group 1.5 (1.0–3.0), respectively. The white boxes represent the 25th to 75th interquartile variation. The thick horizontal black line is the median value. The range of scores is demonstrated with the vertical black line.

6.2 Study II

In the Study II, immunohistochemical analysis of the ischemic spinal cord was conducted 24 hours after the insult. We examined whether RIPC had a neuroprotective effect regarding the specific oxidative stress analysis. The experiment was performed in a similar manner as in the Study II. The study indicated that RIPC had some antioxidative effect on the ischemic area of the spinal cord. No difference was seen in the serum 8-OHdG levels.
6.2.1 Immunohistochemical analysis of the spinal cord

The antioxidant markers OGG1 and DJ1 were more activated in RIPC group in the single sections \( (P = 0.038 \) and \( P = 0.047 \), respectively), although the overall scores in these factors were not statistically significant. The oxidative stress marker, 8-OHdG, did not differ significantly between groups although the response was strong in both groups (Table 8). The reader is advised to consult the original article for more detailed immunohistochemical analysis.

Table 8. Results of immunohistochemical analysis of the spinal cord.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Th 1 – 2</th>
<th>Th 3 – 4</th>
<th>Th 5 – 6</th>
<th>Th 7 – 8</th>
<th>Th 9 – 10</th>
<th>Sum score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ogg-1 RIPC</td>
<td>2.0 (1.0–2.0)</td>
<td>3.0 (1.0–3.0)</td>
<td>2.0 (1.0–2.0)</td>
<td>2.0 (1.0–3.0)</td>
<td>2.0 (1.5–2.5)</td>
<td>9.0 (6.5–13.0)</td>
</tr>
<tr>
<td>Control</td>
<td>1.0 (1.0–1.5)</td>
<td>1.0 (1.0–1.0)</td>
<td>1.0 (1.0–2.0)</td>
<td>1.0 (1.0–1.5)</td>
<td>1.0 (1.0–2.0)</td>
<td>6.0 (5.0–8.5)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.232</td>
<td>0.038</td>
<td>0.430</td>
<td>0.258</td>
<td>0.337</td>
<td>0.096</td>
</tr>
<tr>
<td>8-OHdG RIPC</td>
<td>3.0 (2.5–3.0)</td>
<td>3.0 (3.0–3.0)</td>
<td>3.0 (3.0–3.0)</td>
<td>3.0 (3.0–3.0)</td>
<td>3.0 (3.0–3.0)</td>
<td>14.0 (14.0–15.0)</td>
</tr>
<tr>
<td>Control</td>
<td>3.0 (3.0–3.0)</td>
<td>3.0 (3.0–3.0)</td>
<td>3.0 (3.0–3.0)</td>
<td>3.0 (3.0–3.0)</td>
<td>3.0 (3.0–3.0)</td>
<td>15.0 (15.0–15.0)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.147</td>
<td>1.0</td>
<td>0.337</td>
<td>0.356</td>
<td>0.337</td>
<td>0.096</td>
</tr>
<tr>
<td>DJ1/PARK7 RIPC</td>
<td>1.0 (1.0–2.0)</td>
<td>1.0 (1.0–2.5)</td>
<td>2.0 (2.0–2.5)</td>
<td>2.0 (1.0–2.0)</td>
<td>1.0 (1.0–1.0)</td>
<td>8.0 (6.5–9.5)</td>
</tr>
<tr>
<td>Control</td>
<td>2.0 (1.5–2.5)</td>
<td>1.0 (1.0–1.5)</td>
<td>1.0 (1.0–1.0)</td>
<td>1.0 (1.0–2.0)</td>
<td>1.0 (1.0–2.0)</td>
<td>7.0 (6.0–9.5)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.337</td>
<td>0.310</td>
<td>0.047</td>
<td>0.902</td>
<td>0.218</td>
<td>0.739</td>
</tr>
</tbody>
</table>

RIPC n = 7, Control n = 7. Values are shown as medians and 25th and 75th percentiles.

6.2.2 8-OHdG Concentration

The difference in the level of 8-OHdG (Figure 17) was not statistically significant at any time point \( (P_g = 0.325) \).
6.3 Study III

The experimental protocol was conducted using HCA with 24 hours of surveillance. We preconditioned the intervention group with pharmacological agent mimicking the effect of RIPC (diazoxide) to see whether the metabolic or histological result would improve significantly.
6.3.1 Comparability of study groups

Weight did not differ between groups; the median weight of the animals in the diazoxide group was 20.3 kg, whereas that in the control group was 20.7 (P = 0.412). Amount of donor blood received was 76.4 mL/kg (73.2–79.8) in the diazoxide group and 74.5 mL/kg (69.1–78.4) in the control group, respectively (P = 0.242).

Rectal and pulmonary temperatures were similar at baseline and throughout the entire experiment (P = 0.40 and P = 0.58, respectively), but rectal temperature differed between groups over time (P = 0.0058). Most importantly, the clinical difference was not significant; furthermore, the temperatures were comparable and similar during HCA (P = 0.18).

Haematocrit differed significantly between groups at baseline (P < 0.05). Thus, haemoglobin behaved similarly (P < 0.05). The differences levelled out until the next time point, and there were no differences between groups (P = 0.84 and P = 0.88, respectively).

A total of 18 animals survived 8 hours of follow-up. Two animals from the control group died before the end of the experiment. The cause of death seemed be heart failure.

6.3.2 Intraoperative measurements

Venous lactate

The venous lactate level was significantly lower in the diazoxide group (P = 0.02). After the initiation of CPB, the lactate level did not reach as high as in the control group, and more importantly, the decreasing trend was significantly steeper in the diazoxide group after weaning from CPB (Figure 18).
Fig. 18. The venous lactate during the experiment. PoP = postoperatively. (Haapanen et al. 2017, published with permission from Forum Multimedia Publishing, LCC).

**Oxygen metabolism**

The oxygen consumption ratio was significantly higher in the control group (Figure 19). The ratio increased significantly in the control group after HCA and exceeded the baseline level until the end of 8 hours of follow-up. The most obvious difference is seen 1 hour after HCA, when a steep increase in consumption was measured in the control group, whereas the baseline consumption level was not achieved until 4 hours after HCA in the diazoxide group. The ratio was determined as a relation between oxygen consumption and delivery. The definitions of the two variables are presented in the study of McLellan and Walsh (McLellan & Walsh. 2004).
Cardiac function

The cardiac index trended to be better in the diazoxide group throughout the experiment but did not reach statistical significance ($P_{tg} = 0.087$). When the cardiac index is referred to baseline, significant improvement is seen in the diazoxide group after weaning from CPB, whereas cardiac function was worse than at baseline in the control group (Figure 20). During cardiopulmonary bypass, cardiac output is the same as the pump flow of the heart–lung machine. Because diazoxide is an effective vasodilatory agent, the higher cardiac index values in the intervention group indicate lower mean arterial pressure during CPB.

We also recorded hemodynamically unstable arrhythmias postoperatively. The arrhythmia was considered as unstable if the decrease in MAP was greater than 20% from baseline value. Three animals in the diazoxide group and seven animals in the control group had severe arrhythmias during the postoperative follow-up ($P = \ldots$).
Additionally, there were no statistically significant differences in either troponin I or creatine kinase isoenzyme MB release.

Fig. 20. Cardiac index referred to baseline and frequency of arrhythmias postoperatively. PoP = postoperatively. (Haapanen et al. 2017, published with permission from Forum Multimedia Publishing, LCC).

6.3.3 Histological analysis

Hematoxylin–eosin staining

There were no significant differences between the groups with respect to brain biopsy 8 hours after HCA or brain, cardiac, and kidney histology 24 hours after HCA. The main findings on brain histology were oedema ($P = 0.261$) and haemorrhage ($P = 0.483$); there were no infarctions in either group.

Immunohistochemistry

Interestingly, cytoplasmic Nrf-2 tended to be more activated in the group of diazoxide-treated animals (Table 9). On the other hand, the response to oxidative stress measured by 8-OHdG was rather mild in both groups, without differences between groups. Mitochondrial injury was more significant in the cerebellum in the control group as measured with cytochrome c and caspase 3 ($P = 0.021$ and $P = 0.016$, respectively). Moreover, 8-ogoguanine DNA glycosylase (OGG-1) and DJ-1 did not differ between groups in brain immunohistological analysis.
Analysis of brain biopsy harvested 8 hours postoperatively did not reveal statistically significant differences between the groups. Similarly, no differences were found in heart and kidney analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Frontal cortex</th>
<th>Hippocampus</th>
<th>Pons</th>
<th>Cerebellum</th>
<th>Thalamus</th>
<th>Sum score</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-OhdG</td>
<td>1.0 (0.0-1.0)</td>
<td>0.0 (0.0-2.0)</td>
<td>1.0 (0.0-1.0)</td>
<td>0.0 (0.0-1.0)</td>
<td>0.0 (0.0-1.0)</td>
<td>3.0 (2.0-4.0)</td>
</tr>
<tr>
<td>Control</td>
<td>1.0 (0.0-1.0)</td>
<td>0.0 (0.0-1.0)</td>
<td>1.0 (0.5-2.0)</td>
<td>0.0 (0.0-1.0)</td>
<td>0.0 (0.0-1.0)</td>
<td>4.0 (1.0-5.0)</td>
</tr>
<tr>
<td>P value</td>
<td>0.900</td>
<td>0.243</td>
<td>0.619</td>
<td>0.414</td>
<td>0.243</td>
<td>0.939</td>
</tr>
<tr>
<td>NRF-2</td>
<td>1.0 (0.0-2.0)</td>
<td>1.0 (0.0-2.0)</td>
<td>1.0 (0.0-1.0)</td>
<td>1.0 (1.0-3.0)</td>
<td>2.0 (1.0-2.0)</td>
<td>5.0 (3.0-13.0)</td>
</tr>
<tr>
<td>Control</td>
<td>0.0 (0.0-1.0)</td>
<td>0.0 (0.0-1.0)</td>
<td>0.0 (0.0-1.0)</td>
<td>1.0 (0.5-1.0)</td>
<td>0.0 (0.0-1.0)</td>
<td>2.0 (2.0-4.5)</td>
</tr>
<tr>
<td>P value</td>
<td>0.342</td>
<td>0.237</td>
<td>0.312</td>
<td>0.069</td>
<td>0.06</td>
<td>0.099</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>3.0 (3.0-3.0)</td>
<td>2.0 (2.0-2.0)</td>
<td>2.0 (2.0-3.0)</td>
<td>1.0 (1.0-1.0)</td>
<td>2.0 (2.0-3.0)</td>
<td>10.5 (10.0-11.0)</td>
</tr>
<tr>
<td>Control</td>
<td>3.0 (2.0-3.0)</td>
<td>2.0 (1.0-2.0)</td>
<td>2.0 (2.0-2.0)</td>
<td>2.0 (1.5-2.5)</td>
<td>3.0 (2.0-3.0)</td>
<td>11.0 (10.0-11.0)</td>
</tr>
<tr>
<td>P value</td>
<td>0.411</td>
<td>0.449</td>
<td>0.375</td>
<td>0.016</td>
<td>0.435</td>
<td>0.710</td>
</tr>
<tr>
<td>Cyt C</td>
<td>2.0 (1.0-2.0)</td>
<td>2.0 (2.0-2.0)</td>
<td>3.0 (2.0-3.0)</td>
<td>1.0 (1.0-2.0)</td>
<td>2.0 (2.0-2.0)</td>
<td>10.0 (7.0-11.0)</td>
</tr>
<tr>
<td>Control</td>
<td>2.0 (1.5-2.0)</td>
<td>2.0 (2.0-2.0)</td>
<td>2.0 (2.0-3.0)</td>
<td>2.0 (2.0-3.0)</td>
<td>2.0 (2.0-2.0)</td>
<td>10.0 (8.5-11.5)</td>
</tr>
<tr>
<td>P value</td>
<td>0.861</td>
<td>1.0</td>
<td>0.898</td>
<td>0.021</td>
<td>0.531</td>
<td>0.442</td>
</tr>
<tr>
<td>DJ-1/PARK7</td>
<td>1.0 (0.0-1.0)</td>
<td>0.0 (0.0-0.5)</td>
<td>1.5 (0.5-2.0)</td>
<td>0.0 (0.0-0.5)</td>
<td>1.0 (0.0-1.0)</td>
<td>3.5 (1.0-6.5)</td>
</tr>
<tr>
<td>Control</td>
<td>1.0 (1.0-1.0)</td>
<td>0.0 (0.0-1.0)</td>
<td>1.0 (0.0-1.0)</td>
<td>0.0 (0.0-0.5)</td>
<td>0.5 (0.0-1.0)</td>
<td>3.0 (2.0-5.0)</td>
</tr>
<tr>
<td>P value</td>
<td>0.942</td>
<td>0.732</td>
<td>0.373</td>
<td>0.887</td>
<td>0.250</td>
<td>0.579</td>
</tr>
</tbody>
</table>

6.4 Study IV

In the Study IV, we reviewed the current knowledge of the potential mechanism of RIPC. We gathered the data from other ischemia sensitive organs as well. The numerous experimental and clinical studies have reported positive results of RIPC protective effect against ischemia-reperfusion injury. However, the protective signal from remote limb to the ischemia sensitive organ is not clear yet. The studies indicate the signalling pathways may include both neuronal and humoral pathways and the pathway may not be the same in all organs (Figure 21). Although the results have been promising in the experimental models, the translation of RIPC from experimental settings to clinical trials have been somewhat disappointing. The more detailed review of the RIPC is reported in the original article.
6.5 Summary of results

The experimental model of the spinal cord ischemia demonstrated that RIPC has beneficial effects against ischaemic insult after in studies I and II. On the basis of these results, however, I cannot draw watertight conclusions on the molecular mechanism underlying RIPC or diazoxide, but some light is shed on it in studies II and III. Most importantly, Study I provides valuable preclinical data supporting the possible clinical studies of RIPC effect in the elective surgery of TAAAs. The overall review of the RIPC conducted in the Study IV revealed that the experimental results have been promising, although the translation to clinical trials has been controversial. The current knowledge of RIPC mechanism is still inadequate. The results are summarised in Table 10.
<table>
<thead>
<tr>
<th>Study</th>
<th>Main result</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>RIPC preserves spinal cord function after ischaemic insult</td>
</tr>
<tr>
<td>II</td>
<td>RIPC has some antioxidant effect on spinal cord after ischaemic insult</td>
</tr>
<tr>
<td>III</td>
<td>Diazoxide has beneficial anti-ischaemic effects in cerebral tissue</td>
</tr>
<tr>
<td>IV</td>
<td>RIPC mechanism pathways are not clear yet</td>
</tr>
</tbody>
</table>
7 Discussion

7.1 Study design

Ischaemic spinal cord injury is still the major and most devastating complication of repair of descending thoracic and thoracoabdominal aortic aneurysms. However, several approaches have been developed to minimise the risk of open and endovascular surgical repair, including partial (left heart) or total CPB and epidural cooling or HCA, cerebrospinal fluid drainage, segmental artery perfusion, and monitoring of motor and somatosensory evoked potentials.

Hypothermic circulatory arrest is a technique used to protect the central nervous system during complex aortic arch and cardiac procedures. Although the method has several advantages, HCA has been more frequently used in the repair of thoracoabdominal aortic aneurysms when the aortic arch is involved. The method has several benefits since it provides a bloodless operating field and eliminates the need for sequential aortic clamping, which, in turn, might increase the risk for embolization. Moreover, mortality and morbidity are favourably comparable to those for endovascular and hybrid surgical techniques (Canaud et al. 2013, Greenberg et al. 2008, Kouchoukos et al. 2013). Despite the remarkable improvements in central nervous protection, the incidence of permanent paraplegia can be as high as 20% depending on the extent of the aortic pathology.

We have developed a model of spinal cord ischaemia in which to test the neuroprotective effect of RIPC. In Study I, we showed that RIPC enhanced motor-evoked potentials after spinal cord ischemia. Encouraged by the results, we wanted further to investigate the ischemic insult immunohistochemically. Our research laboratory team has been using the described HCA porcine model for about 17 years in various setups to study many neuroprotective hypotheses. Most recently, the hypothesis of a pharmacological mimic of RIPC was tested in the setup of HCA in Study III.

7.2 Remote ischemic preconditioning against spinal cord ischemia

7.2.1 Motor-evoked potentials

Protection of the central nervous system from ischaemia and reperfusion injury is still one of the most important considerations during surgical thoracoabdominal
aortic aneurysm repair, including aortic repair. We made a finding with our spinal cord ischaemia model, suggesting that RIPC might be beneficial against ischaemic insults encountered during elective surgery. In this study, we wanted to examine a clinically relevant process in a clinically relevant animal model.

Motor-evoked potentials are widely used to measure spinal cord function during various surgical procedures, including TAAA repair. Intraoperative neurophysiological monitoring allows early intervention before ischaemia evolves to infarction. In a clinical sense, the objective of MEP monitoring is to ensure sufficient spinal cord perfusion pressure. Motor-evoked potentials will decrease when critical vessels for spinal cord perfusion are occluded, and thus, neuroprotection can be ensured, for example, by establishing adequate MAP or re-implanting vessels.

In our experimental model, we clamped subsequently the left subclavian artery and nine segmental arteries to the level of the diaphragm to cause local ischaemia of the thoracic spinal cord. Surprisingly, the MEP amplitude was significantly higher immediately after RIPC was performed, that is, before the initiation of ischaemia. The response lasted throughout the experiment. The decrease in latency to onset supported our finding as well. We cannot, however, explain this excitation, but it is possible that RIPC sensitises motor neurons, and thus, spinal cord “conductivity” improves significantly and becomes more resistant to ischaemia. This result is consistent with previous studies, which have also reported dissociation between histological and functional protection of preconditioning-advocating functional tests (Dooley & Corbett. 1998, Dowden & Corbett. 1999). In this respect, motor-evoked potentials provided clinically relevant evidence of the efficacy of RIPC.

There was a difference in MEP recordings between the hind limbs. Left hind limb MEP amplitudes were lower compared with those for the right hind limb in both the RIPC and control groups. There is a possibility that the weakening of the left-side vascular network might explain the differences between the hind limbs. In addition to segmental artery occlusion, the left subclavian artery, which is a crucial supplier of blood to the spinal cord, was ligated, which led to an interruption of the left-side circulation (Strauch et al. 2007). This finding has some indications of the theory that the vascular network is responsible for spinal cord blood flow rather than one dominant artery arising from the lower lumbar region, often referred to as the artery of Adamkiewicz (Griep & Griep. 2015). However, our result is controversial because the ischaemic damage was not pronounced in either of the sides in the histological analysis of spinal cord grey matter. Also, we did not analyse
white matter injury where the myelinated axons run. The other explanation might be that RIPC causes reversible peripheral neuron injury that attenuates the MEP amplitude. On the other hand, every RIPC-treated animal was able to move its hind limbs 24 hours after ischaemia, and in addition, the RIPC tended to have better neurological scores.

Motor-evoked potential monitoring includes a great variety of confounding factors that need to be taken into consideration. First, labile hemodynamics has deleterious effects on the spinal cord because the stable perfusion pressure guarantees sufficient oxygen supply (Etz et al. 2009). Anaesthetic agents, especially propofol and halogenic inhalational agents such as sevoflurane, as well as the neuromuscular blocking agents, significantly reduce the MEP response. On the other hand, opioids have little or no effect, and ketamine has been found to be favourable when considering MEP monitoring (Pajewski et al. 2007). Thus, we designed our anaesthetic technique carefully, and only infusion anaesthesia with ketamine and fentanyl was used during MEP recordings. Moreover, changes in core temperature affect spinal cord conductivity (Pajewski et al. 2007). The explanation for the temporary rise in MEP amplitude of control animals two hours postoperatively might be higher core temperature.

There were significant decreases in blood pressure and cardiac index in RIPC-treated animals after baseline to the next time point. We, however, believe that this can be explained by the series of transient hind limb ischaemic insults performed in the intervention group. The arterial pressure rose during hind limb vascular occlusion and subsequently dropped approximately 10 to 20 mm Hg after the release. In our opinion, this variation would not cause marked changes in spinal cord perfusion pressure because MAP and cardiac index remained within safety limits throughout the experiment. We observed continuous MAP levels during the experiment, and there were no significant differences between groups; more importantly, MAP remained within the safety zone.

7.2.2 Neurological assessment

Neurological function of the spinal cord was assessed using the Tarlov score (Tarlov. 1957). The scale is graded from 0 (unable to walk) to 4 (normal walking), being a rather rough evaluation of neurological status. Nevertheless, the Tarlov score tended to be higher in the RIPC group; also, one animal from the control group had a Tarlov score of 0 points, whereas the minimum Tarlov score in the RIPC group was 2. This finding is supportive for the MEP results, albeit no statistically
significant difference was seen. We feel that the evaluation was suitable for our purposes because the scores were calculated as early as 24 hours postoperatively, when the confounding effect of systemic anaesthesia is still present. Thus, a more detailed analysis might have resulted in false-positive findings.

7.2.3 Oxidative stress in the spinal cord

The hematoxylin-eosin staining findings on the spinal cord were minimal in both groups without a statistically significant difference between groups. On the contrary, the ischemia-reperfusion injury generates oxidative stress to the ischemia-sensitive area, which was seen in the further immunohistochemical analysis of the spinal cord. However, the immunohistochemical results were somewhat modest although the functional and histological results of rodent and rabbit models have been more impressive (Table 3). It might suggest that RIPC has lower efficiency in the large mammal models regarding the neuroprotection after ischemic insult. On the other hand, these results are from the acute model where we performed the immunohistochemical analysis 24 hours after the ischemia. In the future studies, longer follow-up might bring more information. Also, the p-values of the immunohistochemical analysis should be treated with caution due to a large number of dependent comparisons performed.

According to current knowledge, the generation of the reactive oxidative species triggers the several pathological cascades that at worst lead to cell death. In the rodent models, the application of transient ischemia episodes to a remote organ has been shown to increase several ischemia-mediated factors, which in turn indirectly or directly opens mitochondrial $K^+_{\text{ATP}}$ channels and provokes free radical production and as a counter-measure, the antioxidant activity increases (Hu et al. 2014, Mehrjerdi et al. 2015, Shahid et al. 2008). Most interestingly, the protective effect of RIPC in the heart and brain was abolished when the $K^+_{\text{ATP}}$ channel blocker was introduced preoperatively. Thus, there is a possibility the lack of difference in serum 8-OHdG in Study II might reflect the initial release of free radicals caused by RIPC but based on these results, we are unable to say whether the speculated mild ROS release after RIPC is beneficial or not.

The OGG-1 and DJ-1/PARK7 were significantly more activated in a single section of the spinal cord in Study II. DJ-1 is a multifunctional protein that is activated by oxidative stress and is thought to act independently against ischemia-reperfusion injury (Aleyasin et al. 2007). On the contrary, OGG-1 is a member of base excision repair enzymes that primarily cleaves the damaged DNA by 8-OHdG
and has been shown to be activated by RIPC (Li et al. 2006). Although the overall findings of immunohistochemical were rather mild, the significant findings were located in the mid-spine, which was the most affected area of the spinal cord model. This might indicate RIPC has beneficial antioxidant effects on the spinal cord. Based on this study, we cannot conclude whether RIPC acted through the mitochondrial K<sup>+</sup>ATP channel; however, the result is in congruence with the theory.

7.3 Diazoxide against global neuronal ischemia

7.3.1 Hemodynamic and metabolic data

In Study III, the hemodynamic and metabolic status of the study animals was similar in both groups in a clinical sense. Although heart function was not our primary endpoint in this study, it seemed to be significantly better in the diazoxide group after HCA. Additionally, there was a tendency toward higher TnI and CK-MB quantities in the control group after HCA, although the difference did not reach statistical significance. This result supports earlier experimental and clinical studies (Garlid et al. 1997, Mennander et al. 2012). Also, we have shown in our recent study that diazoxide had cardioprotective effect in the isolate cardiac ischemia model (Sarja et al. 2017). Thus, there is a possibility the global ischemia caused by HCA suppressed the cardioprotective effect of diazoxide in Study III.

7.3.2 Lactate

In Study III, a trend of higher lactate levels was recorded in the bloodstream of control animals. Lactate is produced from pyruvate under anoxic conditions and traditionally branded a marker of tissue injury. Furthermore, injured human brain microdialysis analysis showed that the brain has the capability to use lactate as a fuel in the TCA cycle (Gallagher et al. 2009). This means that a high lactate level may indicate severely damaged mitochondria because utilisation via the TCA cycle does not occur. In any case, lower lactate levels are associated with better neurological outcomes (Timofeev et al. 2011). Diazoxide showed a similar beneficial effect on the preservation of cellular metabolic capacity after HCA. This result does not directly point to the same protective molecular pathway but indicates that the end result might be in congruence. On the other hand, the better
hemodynamic condition within the diazoxide treated animals might also be the reason for favourable lactate levels postoperatively.

7.3.3 Oxygen consumption

Oxygen consumption was significantly lower in animals treated with diazoxide. This finding implies that diazoxide might be able to preserve mitochondrial function after ischaemic insult. Diazoxide is known to improve mitochondrial function, and this result indirectly supports the theory. The molecular mechanism underlying diazoxide is not clear, nor is it known whether multiple pathways are affected. Opening the mitochondrial $K_{ATP}$ channel has proven beneficial in a cardioprotective context (Garlid et al. 1997), but diazoxide has also been recognised as an inhibitor of mitochondrial complex II (succinate dehydrogenase) (Schafer et al. 1969). Alternatively, it has been suggested that diazoxide causes mild swelling of the mitochondria through opening of the $K_{ATP}$ channel. The swelling prevents the accelerated ATP hydrolysis caused by ischaemia, and therefore, there will be more energy on reperfusion (Kay et al. 1997). This might explain the better oxygen metabolism status after ischemic insult in diazoxide-treated animals. Overall, this outcome is additional indirect evidence that mitochondria are crucial in the development of ischaemic injury.

7.3.4 Immunohistochemical analysis of the brain

The histopathological findings after diazoxide treatment were minimal and statistically non-significant for hematoxylin-eosin staining, but some findings were found in immunohistochemical analysis with respect to early ischemic injury, such as in the RIPC Study II. Similarly, the results are more encouraging in the rodent and rabbit models (Table 3). Also, the p-values should be treated with caution. However, no infarcts were seen in any of the animals despite the prolonged HCA. Although prolonged HCA is generally considered a global ischemic insult, it was initially innovated to protect the central nervous system. Therefore, there is a possibility that protective elements of HCA have covered some of the diazoxide benefits.

There were no differences between groups in 8-OhdG, which is a sensitive marker for DNA injury (Valavanidis et al. 2009). This might indicate that ROS function as second messengers of pharmacological preconditioning. Mitochondria are the major source of ROS, and therefore, it is suggested that opening
mitochondrial K\textsuperscript{+}_{ATP} channels induces a mild increase in ROS, which in turn may trigger ischaemia tolerance pathways (Thompson et al. 2015). The cellular ROS level is also observed to increase after ischaemic preconditioning (Ravati et al. 2001). Moreover, the Nrf-2 activation seemed to be higher in diazoxide group, although it did not reach statistical significance. This is interesting because ischaemic preconditioning seemed to cause Nrf-2 activation as well (Bell et al. 2011, Herajarvi et al. 2017). It can be speculated that the mild ROS release through the mitochondrial K\textsuperscript{+}_{ATP} channel opening triggers the antioxidant pathway, just like RIPC. However, our result is controversial because we found Nrf-2 only in cytoplasm without nuclear activity. Additionally, cytochrome C release and caspase-3 activation were significantly higher in the cerebellum of the control group. Although a significant finding was only recorded from one brain area, this indicates mitochondrial preservation after treatment of ischaemia with diazoxide. Immunohistochemical analysis revealed the beneficial effects of diazoxide locally, and the results might be congruent with the proposed mechanisms of ischaemic preconditioning. It seems that mitochondrial K\textsuperscript{+}_{ATP} channels may be the key in neuroprotection during ischaemia, and therefore, both pharmacological and remote ischaemic preconditioning are worthy methods to investigate further in challenging aortic surgery.

7.4 Limitations

This thesis describes studies of central nervous system protection during aortic surgery performed in pigs. Studies in animals are widely used in cardiothoracic surgery research because the data collected from these setups are usually homogenous with highly controlled environmental factors. The effects of new applications can be more easily detected than in human trials, in which the study subjects usually have many comorbidities and are taking a variety of medications that influence the results. Animal studies, however, have certain limitations.

The animals used in experiments are usually young and healthy. Difficulties may be encountered when trying to extend the study results to an extremely heterogeneous population. For example, HCA in the pig model usually reveals only the effect of cardiac arrest and possible protective effect of the new intervention, whereas the heterogeneous nature of human studies may mask the effect of the intervention.

In terms of the results, mitochondrial “stabilisation” might be one of the key mechanisms in both remote ischaemic preconditioning and diazoxide. We could
not, however, directly determine the molecular and cellular bases for the beneficial effects of these two protective entities, but there are indirect indications of cellular oxygen consumption. Also, we focused solely on grey matter injury, and therefore, we cannot establish arguments of the possible protective effect of these two adjuncts to white matter. Intraoperative changes in MEP amplitudes might not always be due to spinal cord ischaemia. The functioning of the peripheral nerve is also required to generate MEPs. Vascular malperfusion or mechanical nerve injury can cause the loss of MEP without real spinal cord ischaemia (Etz et al. 2015). In the animal model we used, there is no risk for lower extremities malperfusion, although we cannot rule out possible peripheral nerve injury caused by RIPC.

The number of study groups in animal studies is usually in the tens rather than hundreds. The results were gathered from a relatively small number of study animals and therefore, the research of this thesis is explorative. These studies lack the power to generate significant differences in outcome. False-positive results may be collected because small study groups might bias the difference between the intervention and control groups. On the other hand, because the results may include false negatives, the small differences between groups might be impossible to distinguish.

Finally, and most importantly, animals are not humans. This is a particularly important issue in neuronal ischemia research because the brain histology, i.e., the distribution of white and grey matter, varies between the species. We did not evaluate white matter injury in this thesis, and that must be acknowledged as a limitation. Thus, the effects of drugs can differ in animal models. Additionally, the processes of injury and repair are similar but not identical to those of humans. Nevertheless, animal studies provide excellent preliminary efficacy and safety information on new drugs and methods. Furthermore, the biochemical processes of the cells and possible mechanisms of the interventions are essential topics of the experimental setups. Although animal studies provide us the best possible forum for preclinical results, they will never be capable of replacing human trials.

7.5 Summary and future prospects

Since the first successful repair of a thoracoabdominal aortic aneurysm, ischaemic spinal cord injury has remained the most devastating complication. The risk is relevant and associated with both endovascular and open approaches (Coselli et al. 2007, Greenberg et al. 2008, Coselli et al. 2016). In addition to spinal cord protection, the viability of cerebral tissue must be taken to consideration, especially
when the aortic arch is involved in TAAA. In these clinically challenging cases, cross-clamping proximal to the left subclavian artery is often necessary, and therefore, it is necessary to ensure preservation of cerebral tissue with complete CPB and HCA.

In this thesis, we showed that RIPC improves functional outcome (MEP) of the spinal cord in the experimental model. Our previous study, the functional and histological outcomes of RIPC in experimental HCA setting were significantly better (Jensen et al. 2011). One substantial difference was the length of follow-up. We can speculate the spinal cord perfusion returns near to the baseline 120 hours after the SA sacrifice (Etz et al. 2007) and therefore, the hemodynamic changes in the acute phase may have confounded the early neurological evaluation. Furthermore, the ischemic insult we caused in the spinal cord model might be too mild compared to the possible injurious effect of prolonged HCA. Therefore, we must be cautious when interpreting the results of the spinal cord injury of this study.

We wanted to take one step further and present the beneficial effect of a RIPC-mimicking agent. Pharmacological adjuncts such as diazoxide are employed much faster than bouts of ischaemia, but the complexity of the ischemic cascade might cover the beneficial effect of the drug. Regarding our results and the existing data (Table 3) on diazoxide, the findings about it are not as comprehensive as what was seen with RIPC. Also, diazoxide is less investigated in the field of spinal cord and cerebral injury after major cardiac surgery. Our results showed some functional and positive biomarkers, but the histological findings were modest. Also, our model was acute, and we do not have neurological functional data. Further preclinical studies are warranted for diazoxide before entering into the clinical trials and employing two or more beneficial adjuncts at the same time, such as RIPC and diazoxide, which might lead to a better outcome.

Moreover, in light of the present and existing data, there might be room for clinical trials if in future studies, the positive long-term neurological functional outcome in preclinical studies could be well established. In cardiac surgery, the cardiac and cerebral protective effects of RIPC in high-quality, randomized phase II and III clinical trials have been neutral, but RIPC has not been used in the setting of TAAA in clinical trials to date. In consideration of the time required to perform RIPC, the method may be a useful tool in elective rather than emergency surgery.

In the last study of this thesis (Study IV), we gathered the current knowledge of RIPC from the cardiovascular point of view. We especially wanted to investigate whether the literature has shed light on the possible mechanism of RIPC. Several studies have implicated the role of K+ATP channels in mediating the cardio-
neuroprotective effect of RIPC (Hu et al. 2014, Konstantinov et al. 2005b, Kristiansen et al. 2005, Mehrjerdi et al. 2015, Shahid et al. 2008, Wu et al. 2011). The conclusions of this thesis were that the RIPC may have similar effect on the spinal cord. The cascades producing the protective effect may include reactive oxygen species and other factors, such as adenosine and NO. Also, based on this study, the translation of RIPC from laboratories to clinical setting has been slow.
8 Conclusions

I RIPC improved the conductivity and ischaemic tolerance of the spinal cord as recorded by MEPs.
II RIPC increased mildly antioxidant factors in the spinal cord 24 hours after the local ischemia but the result remains debatable.
III Diazoxide showed a tendency of beneficial effect after HCA in terms of neuroprotection but urges further investigations.
IV The experimental studies have shown beneficial effect of RIPC but the translation to cardiac surgery has been controversial.
References


Botker HE, Kharbanda R, Schmidt MR, Bottcher M, Kaltoft AK, Terkelsen CJ, Munk K, 
Andersen NH, Hansen TM, Trautner S, Lassen JF, Christiansen EH, Krusell LR, 
Kristensen SD, Thuesen L, Nielsen SS, Rehling M, Sorensen HT, Redington AN & 
Nielsen TT (2010) Remote ischaemic conditioning before hospital admission, as a 
complement to angioplasty, and effect on myocardial salvage in patients with acute 


Bredt DS & Snyder SH (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring 

Breivik L, Helgeland E, Aarnes EK, Mrdalj J & Jonassen AK (2011a) Remote 
postconditioning by humoral factors in effluent from ischemic preconditioned rat hearts 
is mediated via PI3K/Akt-dependent cell-survival signaling at reperfusion. Basic Res 
Cardiol 106(1): 135-145.

Breivik L, Helgeland E, Aarnes EK, Mrdalj J & Jonassen AK (2011b) Remote 
postconditioning by humoral factors in effluent from ischemic preconditioned rat hearts 
is mediated via PI3K/Akt-dependent cell-survival signaling at reperfusion. Basic Res 
Cardiol 106(1): 135-145.

7(3): 374-378.


Budd SL & Nicholls DG (1996) A reevaluation of the role of mitochondria in neuronal Ca2+ 

Biochem 33: 43-52.

Suppl: 66-73.

Biochim Biophys Acta.

of ATP-sensitive potassium channel activators diazoxide and BMS-191095 on 
membrane potential and reactive oxygen species production in isolated piglet 

Cai Z, Luo W, Zhan H & Semenza GL (2013) Hypoxia-inducible factor 1 is required for 
remote ischemic preconditioning of the heart. Proc Natl Acad Sci U S A 110(43): 
17462-17467.

Cambria RP, Clouse WD, Davison JK, Dunn PF, Corey M & Dorer D (2002) 
Thoracoabdominal aneurysm repair: results with 337 operations performed over a 15-

Canaud L, Karthikesalingam A, Jackson D, Cresswell L, Clif f M, Markar SS, Maytham G, 
Black S & Thompson M (2013) Clinical outcomes of single versus staged hybrid repair 


127


Fryer RM, Hsu AK & Gross GJ (2001) Mitochondrial K(ATP) channel opening is important during index ischemia and following myocardial reperfusion in ischemic preconditioned rat hearts. J Mol Cell Cardiol 33(4): 831-834.


Schinder AF, Olson EC, Spitzer NC & Montal M (1996) Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. J Neurosci 16(19): 6125-6133.


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PRECONDITIONING AGAINST ISCHEMIC INJURY OF THE CENTRAL NERVOUS SYSTEM IN AORTIC SURGERY

AN EXPERIMENTAL STUDY IN A PORCINE MODEL WITH REMOTE ISCHEMIC PRECONDITIONING AND DIAZOXIDE

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