OULU 2013
D 1203

ACTA UNIVERSITATIS OULUENSIS

Fredrik Yannopoulos

REMOTE ISCHEMIC PRECONDITIONING AS A MEANS TO PROTECT THE BRAIN AGAINST HYPOTHERMIC CIRCULATORY ARREST

AN EXPERIMENTAL STUDY ON PIGLETS

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU, FACULTY OF MEDICINE,
INSTITUTE OF CLINICAL MEDICINE,
DEPARTMENT OF SURGERY,
DEPARTMENT OF ANAESTHESIOLOGY;
CLINICAL RESEARCH CENTER;
OULU UNIVERSITY HOSPITAL

ACTA UNIVERSITATIS OULUENSIS
D Medica 1203

FREDRIK YANNOPOULOS

REMOTE ISCHEMIC PRECONDITIONING AS A MEANS TO PROTECT THE BRAIN AGAINST HYPOTHERMIC CIRCULATORY ARREST

An experimental study on piglets

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 1 of Oulu University Hospital, on 7 June 2013, at 12 noon

UNIVERSITY OF OULU, OULU 2013

Acta Univ. Oul. D 1203, 2013

Supervised by
Professor Tatu Juvonen

Reviewed by
Doctor Antti Vento
Doctor Ari Mennander

Opponent
Professor Tómas Guðbjartsson

ISBN 978-952-62-0130-6 (Paperback)
ISBN 978-952-62-0131-3 (PDF)

ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)

Cover Design
Raimo Ahonen

JUVENES PRINT
TAMPERE 2013

Yannopoulos, Fredrik, Remote ischemic preconditioning as a means to protect the brain against hypothermic circulatory arrest. An experimental study on piglets

University of Oulu Graduate School; University of Oulu, Faculty of Medicine, Institute of Clinical Medicine, Department of Surgery, Department of Anaesthesiology; Clinical Research Center, P.O. Box 5000, FI-90014 University of Oulu, Finland; Oulu University Hospital, Kajaanintie 50, FI-90220 OYS, Finland

Acta Univ. Oul. D 1203, 2013
Oulu, Finland

Abstract

Open aortic arch surgery almost always requires a bloodless operating field which necessitates the use of hypothermic circulatory arrest. Hypothermic circulatory arrest is a technique where the core temperature of a patient is lowered so that the systemic blood circulation can be stopped momentarily. This can cause unwanted damage to the brain. The risk for neurological impairment is at its highest when corrective surgery has to be performed in emergency situations. This highlights the need for additional neuroprotective methods.

Our research group has used a porcine model described in this thesis for about 12 years in various setups to study many neuroprotective hypotheses. We have tested and researched surgical and CPB strategies that could be useful in a HCA and aortic arch reconstruction setting. In this thesis we have combined both chronic surviving animal data with acute experiments and aim to shed light on the mechanisms and efficacy of RIPC as neuroprotective method.

In our experimental model, RIPC provided a mitigation of inflammatory response and cerebral injury after prolonged HCA. In general, the collected data showed homogeneity as similar biochemical results were seen in study I and II. Also interestingly, study III and IV possibly shed some light as to the mechanisms of the neuroprotective effect seen in Study II. These results seem to corroborate each other in a logical way.

In study I which was acute experiment we saw faster EEG recovery rates in the intervention group. Additionally we recorded beneficial biochemical changes from samples that were collected from the brain. In our chronic study, were the animals were followed for a 7 day period after hypothermic circulatory arrest, we saw a statistically significant neuroprotective effect of remote ischemic preconditioning. In studies III and IV we attempted to shed light on the mechanisms. Study III revealed that an altered oxygen usage profile during hypothermic circulatory arrest and recovery phase might have a role in the neuroprotection. In study IV we saw a reduced microcirculatory leukocyte accumulation in cerebrocortical vessels was noted using an intravital microscope. The intravital microscope also provided results that indicated a difference in the redox state of the mitochondria via NAD+/NADH autofluorescence measurements.

Keywords: brain protection, cardiac surgery, ischemic brain damage, ischemic preconditioning

Yannopoulos, Fredrik, Esialtistavan raajaiskemian aivoja suojaavat vaikutukset sydän- ja aorttakirurgiassa. Kokeellinen tutkimus porsailla

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Kliinisen lääketieteen laitos, Kirurgia, Anestesiologia; Kliinisen tutkimuksen keskus, PL 5000, 90014 Oulun yliopisto; Oulun yliopistollinen sairaala, Kajaanintie 50, 90220 OYS

Acta Univ. Oul. D 1203, 2013
Oulu

Tiivistelmä

Sydän- ja aorttakirurgiassa tarvitaan jossain tilanteissa täysin veretöntä leikkausaluetta. Verettömän leikkausalueen saavuttamiseksi joudutaan joskus turvautumaan potilaan elimistön jäähdytyksen jälkeiseen verenkierron pysäytykseen. Tämän menetelmän haittana on kuitenki aivokudokselle aiheutuva hapenpuute ja tästä mahdollisesti seuraava vaurioituminen. Vaurioitumisen riski on korkeimillaan erityisesti päivystyksellisissä tilanteissa.

Tämän tutkimuksen tavoitteena on ollut selvittää, onko esialtistavalla raajaiskemialla kykyä suojata aivokudosta hapenpuutostilanteissa. Tutkimusryhmämme on viimeisen 12 vuoden aikana tutkinut sianporsailla eri keinoja, joilla voitaisiin parantaa aivojen suojausta sydän- ja aorttakirurgian aikana. Esialtistava raajaiskemia toteutetaan kiristämällä mansetti eläimen oikean takajalan ympärille. Tämän jälkeen mansetti täytetään viiden minuutin välein neljästi. Täyttökertojen välissä pidettään viiden minuutin tauko, jolloin mansetti on avatuna ja jalan verenkierto palautuu normaaliksi.

Ensimmäisessä tutkimuksessamme totesimme, että esialtistava raajiskemia vaikuttaa aivojen sähkökäyrän toipumista nopeuttavasti. Toisessa tutkimuksessamme seurasimme koe-eläimiä seitsemän päivän ajan kokeen jälkeen. Tämän tutkimuksen yhteydessä toteutessa aivokudoksen mikroskooppiananalyysissä havaitsimme, että raajaiskemia vaikutti suojaavan aivokudosta hapenpuutteen aiheuttamilta aivovaurioilta. Kolmanessa tutkimuksessa selvitimme, että raajaiskemia vaikuttaa aivojen happipitoisuuteen sekä verenkierron pysäytyksen aikana että toipumisvaiheessa. Viimeisessä tutkimuksessa kuvasimme aivojen pintaverisuonia mikroskoopilla. Seurasimme kokeessa valkosolujen käyttäytymistä aivokudoksessa käyttäen fluoresoivia lääkeaineita. Havaitsimme, että raajaiskemiaryhmässä valkosoluja oli aivokudoksen pintaverisuonissa merkittävästi vähemmän. Lisäksi samalla menetelmällä tutkimme sitruunahappokiertoon osallistuvan NAD+/NADH parin suhteita autofluoresenssi ilmiöllä. Autofluorensenssi tutkimuksen tulokset viittaavat siihen, että mitokondrioiden hapetus/pelkistys kyky oli parempi raajaiskemia ryhmässä.

Kokeissamme esialtistava raajaiskemia vähensi tulehdussolujen määrää aivokudoksessa sekä vähensi aivovauriota hapenpuutteen jälkeen.

Asiasanat: aivojen suojaus, aivovaurio, esialtistava iskemia, sydänkirurgia

Medicine is a science of uncertainty and an art of probability

– Sir William Osler (1849–1919)

Acknowledgements

The present thesis and the studies contained in it were completed at the Cardiothoracic Research Laboratory of the Department of Surgery at Oulu University Hospital during the years 2006–2012.

First of all I owe my deepest and sincerest gratitude for this thesis to Professor Tatu Juvonen. I had the good fortune to be recruited into his group of well-established researchers during the early years of my studies at the Faculty of Medicine at Oulu University. Without your perseverance, guidance and energy this thesis would not have been possible. I thank you warm-heartedly for recruiting me into your research group and giving me the possibility to learn, innovate and do science.

I extend my heartfelt gratitude to Docent Kai Kiviluoma. I was privileged to have the opportunity to learn from his extensive knowledge in the fields of anesthesia and experimental surgery. Kai has always had the time to help me with any scientific or medical challenge that I have confronted. I thank you, Kai, for everything.

Docent Vesa Anttila, I thank you warmly for all the support and help you have given me. You are a calm and warm-hearted professional whom I could always ask for help. Your help, advice and encouragement have been instrumental in completing this work.

My warmest gratitude also goes out to Professor Petri Lehenkari who helped me when I took my first steps into the amazing world of experimental science. You taught me that one should always strive to find success not only in science and medicine but also in one's civilian life. You taught me to keep things in balance.

Jussi Mäkelä was the first person with whom I worked in the laboratory. I owe so much to Jussi's guidance that I cannot even begin to describe it. You showed me my first surgical knots, and you took me under your tutelage and taught me the essentials of doing research. Admittedly, performing experimental surgery while trying to study full time and still maintain healthy relationships with loved ones was sometimes difficult. In those difficult times you were the one I looked up to and the one I asked for help and guidance. It is rare to meet a person of such honesty, integrity and intelligence. It is even rarer to befriend one.

I also express my deepest and sincerest gratitude to Hanna Jensen who was likewise my mentor in the research laboratory and taught me the other half of what I know about experimental thoracic surgery. Thank you for your guidance.

I was recruited into this group simultaneously with Tuomas Mäkelä, and during the years Tuomas became my co-worker and friend. With Tuomas I could discuss plans for future experiments with the same ease I could talk about new camping gear and games. Your sense of humor is also amazing; there were numerous times when your cheerful spirit and good (bad) jokes helped to keep us energized and happy in the research laboratory. Thank you for everything.

I am grateful to my other co-workers Eija Rimpiläinen, Kirsi Alestalo, Oiva Arvola, Henri Haapanen, and Johanna Herajärvi with whom I have had the opportunity to share joys and sometimes hardships of life in the research laboratory. I will never forget the moments of joy, trial and sorrow that we have been through together.

I sincerely thank you Seija Seljanperä for all your efforts and work in facilitating our experiments. You have tirelessly worked in the laboratory to make sure we have the instruments and medicines. You have shared the pains, hardships and joys of experimental surgery and life with the junior team. Thank you for everything.

I am also grateful to the other professionals at Oulu University's Laboratory Animal Center for caring for the animals and us. In addition I sincerely thank the Finnish Cardiovascular Foundation and the Sigrid Juselius Foundation for their financial support.

Lastly I would like to thank my family. All my life I have been surrounded by a loving and caring family: my sister Marina, my brother Sebastian, and my mother and father. My dear sister Marina, you loved me and cared for me from the day I was born and continued to do so even after I managed to break your tooth at the playground.

To my brother I am indebted for my present ability to remain calm and collected in many situations. You are still my longest-lasting roommate. Now you are a grown man, and I am deeply proud to be able to call you my brother.

Thank you mother and father for the support and care you have given me. You have always encouraged me in my endeavors, no matter how odd and foolish they might have been. You nurtured me and taught me how important it is to trust my own thinking and always ask questions. The love you have given me was never a constricting kind but one of encouraging honesty. You showed me the door to the world but never closed it, so I could always come back home if the world got too dark. I like to believe that my inquisitive mind is a gift from my parents. Do not doubt yourselves, you have done an exquisite job at parenting.

Finally I would like to thank the one person who has sustained me throughout the six years we have known each other. We have grown together through hardships and the good times. On the first day I saw you I somehow knew I would like you. This proved true on the very same day as you managed to kick me hard enough to leave a mark. You have given me so much. You have supported me and you have guided me. You know me. You have the keys to me. I love you so much Kaisa.

Oulu, Finland, September 2012 Fredrik Yannopoulos

Abbreviations

α-Stat	Alpha-stat acid-base management strategy
AIF	Apoptosis inducing factor
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
B1R	Bradykinin receptor 1
B2R	Bradykinin receptor 2
BDNF	Brain-derived neurotropic factor
Ca2+	Calcium (ion)
CBF	Cerebral blood flow
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CO_2	Carbon dioxide
COX-2	Cyclooxygenase 2
CPB	Cardiopulmonary bypass
CVP	Central venous pressure
DNA	Deoxyribonucleic acid
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay
$EtCO_2$	End-tidal carbon dioxide
eNOS	Endothelial nitric oxide synthase
ETC	Electron transport chain
F	Abbreviation of the French catheter unit scale
HCA	Hypothermic circulatory arrest
HE	Hematoxylin eosin
IGF-I	Insulinlike growth factor 1
K_{ATP}	ATP-sensitive potassium
iNOS	Inducible nitric oxide synthase
NfH	Neurofilament heavy chain
MAO	Mesentery artery occlusion
MnSOD	Manganese superoxide dismutase
mitoK_{ATP}	mitochondrial ATP-sensitive potassium channel
MPT	Mitochonrial permeability transition
MPTP	Mitochondrial permeability transition pore
NAD+	Oxidized form of nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide

NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
O_2	Oxygen (molecule)
ONOO-	Peroxynitrite
$PbrO_2$	Partial cerebral oxygen tension
pH	Hydrogen ion concentration i.e. acidity
pH-stat	pH-stat acid-base management
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
pCO_2	Partial pressure of carbon dioxide
pO_2	Partial pressure of oxygen
RER	Rough endoplasmic reticulum
RIPC	Remote ischemic preconditioning
ROS	Reactive oxygen species
S100-B	Glial-specific S100 calcium binding protein B
SD	Standard deviation
TEM	Transmission electron microscopy
TnI	Troponin I
TNF	Tumour necrosis factor
TNF-R1	Tumour necrosis factor receptor 1
TRI	Transient remote ischemia

List of original publications

This thesis is based on the following articles.

I Yannopoulos FS, Mäkelä T, Niemelä E, Tuominen H, Lepola P, Alestalo K, Kaakinen H, Kiviluoma K, Anttila V & Juvonen T (2010) Improved cerebral recovery from hypothermic circulatory arrest after remote ischemic preconditioning. Ann Thorac Surg 90(1): 182–188.

II Jensen HA*, Loukogeorgakis S*, Yannopoulos F, Rimpiläinen E, Petzold A, Tuominen H, Lepola P, Macallister RJ, Deanfield JE, Mäkelä T, Alestalo K, Kiviluoma K, Anttila V, Tsang V & Juvonen T (2011) Remote ischemic preconditioning protects the brain against injury after hypothermic circulatory arrest. Circulation 123(7): 714–721.

III Yannopoulos F, Mäkelä T, Arvola O, Haapanen H, Anttila V, Kiviluoma K & Juvonen T (2012) Remote ischemic precondition preserves cerebral oxygen tension during hypothermic circulatory arrest. Scand Cardiovasc J 46(4): 243–248.

IV Yannopoulos F, Arvola A*, Haapanen H*, Jensen H, Herajärvi J, Miinalainen I, Kiviluoma K & Juvonen T (2013) Leg ischemia before circulatory arrest alters brain leukocyte count and respiratory chain redox state. Manuscript.

* = equal contribution in article

Contents

Abstract
Tiivistelmä
Acknowledgements **9**
Abbreviations **13**
List of original publications **15**
Contents **17**
1 Introduction **21**
2 Review of literature **23**
2.1 The human brain ... 23
2.1.1 Cells of the brain ... 23
2.1.2 Cerebral blood flow ... 24
2.1.3 Metabolism ... 25
2.2 Cardiopulmonary bypass ... 25
2.2.1 Hypothermic circulatory arrest ... 26
2.3 Ischemic brain damage ... 26
2.3.1 Ischemic cascade ... 27
2.3.2 Inflammatory response ... 32
2.4 Remote ischemic preconditioning ... 33
2.4.1 Mechanism ... 33
2.5 Limitations of this study ... 37
3 Aims of the present study **39**
4 Materials and methods **41**
4.1 The porcine model ... 41
4.2 Test animals and preoperative management ... 41
4.3 Remote ischemic preconditioning ... 42
4.4 Anesthesia ... 43
4.5 Hemodynamic monitoring ... 43
4.6 Blood transfusion ... 43
4.7 Intracranial monitoring ... 44
4.7.1 Electroencephalographic analysis ... 44
4.8 Microdialysis ... 44
4.9 Intravital microscopy ... 45
4.10 Transmission electron microscopy ... 47
4.10.1 Assessment ... 48
4.11 Additional measurements ... 48

4.12 Behavioural score ... 48
4.13 Cardiopulmonary bypass ... 49
4.14 Histopathology ... 49
4.15 Biochemical assay ... 50
4.16 Statistical analysis ... 50
5 Results **51**
5.1 Study I ... 51
5.1.1 Comparability of study groups ... 51
5.1.2 Electroencephalographic analysis ... 51
5.1.3 Cerebral microdialysis ... 51
5.2 Histopathology ... 52
5.3 Additional measurements ... 53
5.4 Study II ... 54
5.4.1 Comparability of study groups ... 54
5.4.2 Histopathology ... 54
5.4.3 Cerebral measurements ... 55
5.4.4 Behavioural score ... 56
5.5 Study III ... 56
5.5.1 Comparability of study groups ... 57
5.5.2 Cardiopulmonary bypass results ... 57
5.5.3 Cerebral oxygen tension ... 58
5.6 Study IV ... 58
5.6.1 Comparability of study groups ... 59
5.6.2 Leukocyte count ... 59
5.6.3 Intravital microscopy ... 59
5.6.4 Transmission electron microscopy ... 61
5.7 Summary of results ... 61
6 Discussion **63**
6.1 Study design ... 63
6.2 Hemodynamic data ... 63
6.3 Neurological data ... 63
6.4 Intracerebral measurements ... 64
6.4.1 EEG ... 64
6.4.2 Microdialysis ... 65
6.5 Intravital microscopy ... 65
6.5.1 Leukocytes ... 66
6.5.2 NADH ... 66

6.6 Cerebral oxygen tension 67
6.7 Transmission Electron Microscopy 67
6.8 Summary 68
7 Conclusions **69**
References **71**
Original publications **81**

1 Introduction

The human brain and central nervous system is very susceptible to all kinds of insults. At a macroscopic level our brains are very soft and gelatinous organs that albeit vital seem to be very vulnerable to damage. Evolution has done its part in developing encasements to protect our central nervous systems from the many rigors of the physical world. However, the brain is most susceptible to damage at a microscopic level. The cells of our brain, called neurons, are in constant need of energy and oxygen. Even the slightest interruption to either one will quickly cause symptoms and eventually lead to unrepairable cellular dysfunction.

After the advent of the cardiopulmonary bypass (CPB) method and its subsequent development surgeons were able to repair congenital and acquired defects in the cardiovascular system that previously were untreatable. During the 1950's hypothermia was discovered as a way to increase the ischemia tolerance of the brain (Niazi & Lewis 1957) and quickly after that the concept of hypothermic circulatory arrest (HCA) was first introduced. During the 1970s HCA was first applied successfully to aortic arch repairs in humans. Although it was known by that time that profound and deep hypothermia has substantial protective properties it was unclear what the limits were. In the early 1980s it was first shown that there were no clear-cut boundaries that could be drawn to define a completely safe duration of HCA. It was noted that the risk of damaged and necrotic neurons grew almost exponentially after 36 minutes of circulatory arrest (Treasure 1984). The noteworthy fact was that HCA was not completely safe even in the time window of 0 to 36 minutes.

Approximately at the same time in other laboratories experimental surgeons were investigating another interesting technique called ischemic preconditioning. It was discovered that the effects of cessation of blood flow (i.e. ischemia) in the heart could be alleviated by exposing the myocardium to small intermittent periods of ischemia beforehand (Murry *et al.* 1986). This phenomenon was later shown to have similar effects in several other organs such as the kidney, liver and brain. After local ischemic preconditioning was discovered it took several years before the first evidence of the so called remote ischemic preconditioning (RIPC) effect was discovered. As the name implies the method involved causing intermittent non-lethal ischemia to remote organs to protect the intended organ, which in the first reported experiments was the heart (Gho *et al.* 1996). Since then studies have shown that other organs besides the heart could possibly be protected by this method. It took almost another decade before the first studies were

published in which researchers tried to mitigate ischemic damage of the brain (Dave *et al.* 2006, Vlasov *et al.* 2005). Additionally some studies have shown promising results in ameliorating ischemic sequelae in kidney and liver tissue (Ali *et al.* 2007, Theodoraki *et al.* 2011).

Remote ischemic protection of the brain was first tested in 2006 in a rat model of global cerebral ischemia and the results were promising (Dave *et al.* 2006). Subsequently it was shown to be neuroprotective also in a focal ischemic animal model (Ren *et al.* 2008). Although some negative results have been published (Saxena *et al.* 2009), it seems that most of the experimental studies published have shown RIPC to have some neuroprotective effects (Malhotra *et al.* 2011, Tropak *et al.* 2011).

The first paper of this thesis was completed to assess whether RIPC had any measurable effects in our established HCA porcine model. In this setup we use a CPB pump to lower the core temperature of the animals to 18°C after which the whole circulation is stopped for 60 minutes. We were able to show significant differences in the acute recovery phase from HCA (I). For the second paper the efficacy of RIPC was tested in a chronic model in collaboration with our colleagues from Great Ormond Street Hospital, London (II). The third study tested the hypothesis that RIPC might affect the oxygen usage profile of the cerebrum during HCA (III). Lastly this thesis presents the yet unpublished manuscript of our study where we use intravital microscopy to directly assess in-vivo cerebral leukocyte adhesion after HCA (IV).

2 Review of literature

2.1 The human brain

The brain consists almost purely of neuronal cells called neurons and the supporting cells which are commonly referred to as neuroglia cells. Macroscopically the brain or cerebrum contains as its most prominent structures two large hemispheres. The outermost layer, cerebral cortex, is densely convoluted. This maximises the surface area of the cortex without an increase in volume. Grey matter and white matter are visible in anatomical sections of the brain. The white matter consists mostly of neuronal axons and the grey matter mostly of neuronal cell bodies.

2.1.1 Cells of the brain

The neuron

The neuron has several distinct parts. The body, called soma, is where protein synthesis occurs and where the nucleus is. The soma has outward reaching extensions like branches of a tree called dendrites, which function as the main input channels for the neuron. An axon is the part responsible for the transmission of efferent (i.e. away from the soma) signals. A multitude of variation occurs in soma size, dendrite amount and axon length. The axon itself can be either myelinated or non-myelinated. The myelinisation is done by another group of cells called Schwann cells (Figure 1). The myelinisation of the axon multiplies the transmission velocity of the axon.

Neuroglial cells

Neuroglial cells can roughly be divided into four different cell types.

Astrocytes are a major type of neuroglial cells; this cell type covers practically all vascular surfaces of the brain with its specialised dendritic endfeet. They comprise a major fraction of the brain's biomass and have a vital role in maintaining ion and neurotransmitter homeostasis and keeping the brains blood-brain barrier effective (Parpura *et al.* 2012). It has even been suggested that the

immense complexity and diverse functionality of the astrocytes is the distinguishing factor between human and primate brains (Oberheim *et al.* 2006).

The main function of oligodendrocytes is to produce myelin with which to cover the axons of the brain and spinal cord. Oligodendrocytes such as Schwann cells produce large amounts of myelin daily. The production of myelin requires a high metabolic rate and consumes large quantities of adenosine triphosphate (ATP). Unfortunately, oligodendrocytes are very susceptible to injury and dysfunction due to their high metabolic rate and a low concentration of anti-oxidants (McTigue & Tripathi 2008).

Microglial cells are the "third" major glial subtype and are the macrophage-monocyte cells of the central nervous system (CNS). They respond to tissue damage and infection and play a major role in the immunological response of the CNS. The final subtype of neuroglial cells is the ependymal cell. Ependymal cells coat the inside of cerebral ventricles and the spinal canal.

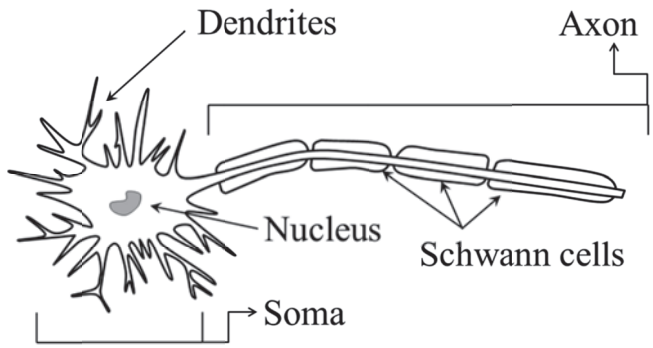

Fig. 1. The basic structure of a neuron

2.1.2 Cerebral blood flow

The brain is in constant need of energy substrates such as oxygen (O_2) and glucose that are provided by the blood flow. The cerebral blood vessels and capillaries have the ability to sustain normal levels of cerebral blood flow (CBF) even when there are large changes in systemic arterial pressure (Harper 1966). This is generally referred to as the autoregulatory capability of the brain.

2.1.3 Metabolism

Although the brain as an organ weighs approximately 2% of total body mass it receives a major share of the cardiac output volume. If cerebral perfusion pressure is adequate to sustain normal CBF, and oxygen and glucose are present in the blood flow, then neurons and neuroglial cells operate in aerobic conditions. In this state one mole of glucose is metabolised into 38 moles of ATP.

Although ATP can be produced by several other mechanisms the most efficient one is the electron transport chain (ETC). The ETC uses a chain of enzymes that are in sequence increasingly electronegative to generate ATP. The process consumes the reduced form of nicotinamide adenine dinucleotide (NADH) that is in turn generated most efficiently in the citric acid cycle. In anoxic conditions the same amount of glucose generates only two moles of ATP. The prevalent dogma for the past three decades has been that only glucose can be utilised for this process. Some emerging studies have shown, however, that lactate could be a major energy substrate in aerobic conditions, although its significance during anoxia or hypoxia is yet to be determined (Overgaard *et al.* 2012, Wyss *et al.* 2011).

2.2 Cardiopulmonary bypass

The honour of the first successful open heart surgery with a cardiopulmonary bypass pump goes to Dr. John Gibbon and his surgical team who repaired an intracardiac defect in 1953. Although the idea of extracorporeal circulation was not his own, Dr. Gibbons and his team developed the first "modern" type CPB machine with a roller pump and an oxygenator that was used first in animal studies and later with human patients (Gibbon *et al.* 1954)

Extracorporeal circulation

The modern CPB maintains extracorporeal circulation. Venous blood is first drained by gravity from the patient's body into the CPB machines reservoir using venous cannulae. Then the oxygenated blood passes via tubing into a roller pump that leads it to an oxygenator that facilitates carbon dioxide (CO_2) and O_2 diffusion. From there blood is then directed back into the patient via arterial cannulae. Supplementary roller pumps and reservoirs can be used depending on

the type of the operation performed. A heat exchanger is usually used in conjunction to maintain adequate core temperature or to cool the patient.

2.2.1 Hypothermic circulatory arrest

Hypothermia means literally “low-temperature”. Even before the advent of the CPB machine some scientist were experimenting on using hypothermia to lower the oxygen usage of the body to facilitate longer cardiac surgery times. The general use of hypothermia in cardiac surgery is accredited to Dr. Bigelow who conducted several human and animal studies in the 1940s and 1950s even before the invention of the CPB machine (Bigelow *et al.* 1950). Almost at the same time several studies were started that used severe hypothermia to enable surgeons to operate in a total circulatory arrest, which led to the concept of hypothermic circulatory arrest (HCA) (Niazi & Lewis 1957). The HCA is an operative procedure in which the patient is cooled via extracorporeal perfusion to a target temperature, the heart is stopped and the extracorporeal circulation is stopped effectively shutting down all blood flow in a patient’s cardiovascular system. The method has a strict “safe” operating time that is dependent on the depth of hypothermia. At 15°C the safe operating time is considered to be only 30 minutes (McCullough *et al.* 1999). An HCA prolonged beyond this timeframe increases the risk of neurological injury significantly (Treasure 1984).

Today HCA enables surgeons to operate on certain types of aortic diseases and defects that require a bloodless operating field. The mortality rate in elective aortic arch surgeries requiring HCA is approximately 2%, whereas in emergency aortic arch repairs it is almost 10%. A similar significant difference is seen in the risk of neurological injury (8% vs. 17%, respectively) (Czerny *et al.* 2011). Additional factors such as patient age further increase the mortality rate significantly (Biancari *et al.* 2011). Although the risk of neurological injury and mortality is quite high, it is noteworthy that an untreated acute dissection of the ascending aortic arch carries a mortality rate of almost 100%.

2.3 Ischemic brain damage

Ischemic brain damage can roughly be defined as either a global ischemic insult or a focal ischemic insult. Hypothermic circulatory arrest to some degree causes the former and plaque-induced thrombi the latter. A prolonged HCA and manipulation of the large arteries of the heart increase the risk of ischemia.

However, the hypothermic component of the HCA method mitigates some of the most harmful effects as it lowers the energy metabolism rate of the brain significantly (Erecinska *et al.* 2003). The porcine model is very suitable for studying the effects of cerebral ischemia during hypothermia, because the metabolic suppression rate of the brain of the animal is very similar to that of humans (Ehrlich *et al.* 2002).

Selective vulnerability

Although a depletion of oxygen or glucose is detrimental to all cells in the brain the dose response ratio varies greatly with different cell types. The most vulnerable cells of the brain are located in the hippocampus and are called CA1 type pyramidal neurons (Johansen *et al.* 1984, Kirino & Sano 1984). In a focal ischemic insult the totally ischemic core is surrounded by an area that has lowered CBF values. This area, termed penumbra, can functionally recover if adequate CBF values are restored (Olsen *et al.* 1983).

Pathogenesis

Ischemic brain damage is always a result of an imbalance between the metabolic needs of the brain cells and the capability of the circulatory system to provide the required metabolic substrates. This can be a consequence of a decreased cerebral perfusion or a decreased oxygen and glucose content in an otherwise normal CBF. The neurons and the glial cells of the brain rely on a constant supply of O_2 and glucose to function properly. The determinant of ensuing cellular dysfunction and brain damage is directly related to the severity and duration of the ischemia. It has been established that a CBF of 20 mL/min/100g is the critical threshold after which the levels of intracellular ATP drop rapidly (Obrenovitch *et al.* 1988). Once the critical threshold of CBF has been reached neurons no longer can maintain cellular function and homeostasis. This causes breakdown of ionic membrane potentials, and depolarization occurs.

2.3.1 Ischemic cascade

A metabolic imbalance launches a series of events that in general can be called ischemic cascade. The first step of the ischemic cascade is a loss of the electrochemical membrane potential. This depolarization of the neuronal cell

membrane leads to glutamate spillage into synaptic clefts, and simultaneously the reuptake of glutamate and other excitatory amino acids is hindered. This in effect leads to even greater concentration of excitotoxic glutamate in the synaptic cleft (Figure 2) (Wroge *et al.* 2012).

Glutamate

Glutamate-specific receptors, such as *N*-methyl-D-aspartate (NMDA), are then constantly activated and contribute to a rise in intracellular Ca^{2+}, which quickly translates into a marked increase in O_2 consumption (Gleichmann *et al.* 2009). In vitro experiments have implicated a dose-dependent death of cultured neuronal cells when exposed to glutamate (Voulgari-Kokota *et al.* 2012). The dose dependency in vivo is likely to be more complicated however. The activated NMDA receptors then cause Ca^{2+} influx through them and simultaneously Ca^{2+} flows into the cytosol via other routes such as the Na^{+}/Ca^{2+} exchanger due to the depolarization of the cell membrane (Brittain *et al.* 2012). This is a possible reason why the NMDA receptor antagonist proved ineffective in attenuating ischemic damage in humans (Davis *et al.* 1997, Davis *et al.* 2000). The route through which Ca^{2+} is deposited into the cytosol is unconnected to its subsequent harmful effects (Stanika *et al.* 2012), and a moderate rise in cytoplasmic Ca^{2+} paradoxically increases the metabolic needs of neurons when there is already a depletion of O_2 and glucose, which further deteriorates homeostasis (Wang *et al.* 1994).

Calpains

Calpains is a proteinase family that is calcium activated. The function of proteases is to regulate cell functions and cleave produced proteins into their mediatory or final structure. They also have a role in disintegrating unwanted proteins. Although calpains were discovered over 30 years ago their exact role is yet unknown. What makes calpains interesting in this case is that the required Ca^{2+} concentrations to activate some of them are not achievable in any known normal physiological conditions (Zadran *et al.* 2010). The calcium ion has a major role in the ischemic cascade. A simplified depiction of the interplay between effectors is shown in Figure 2. Solid in vitro evidence has emerged that some calpains are the final effectors in programmed cell death resulting from glutamate excitoxicity (D'Orsi *et al.* 2012).

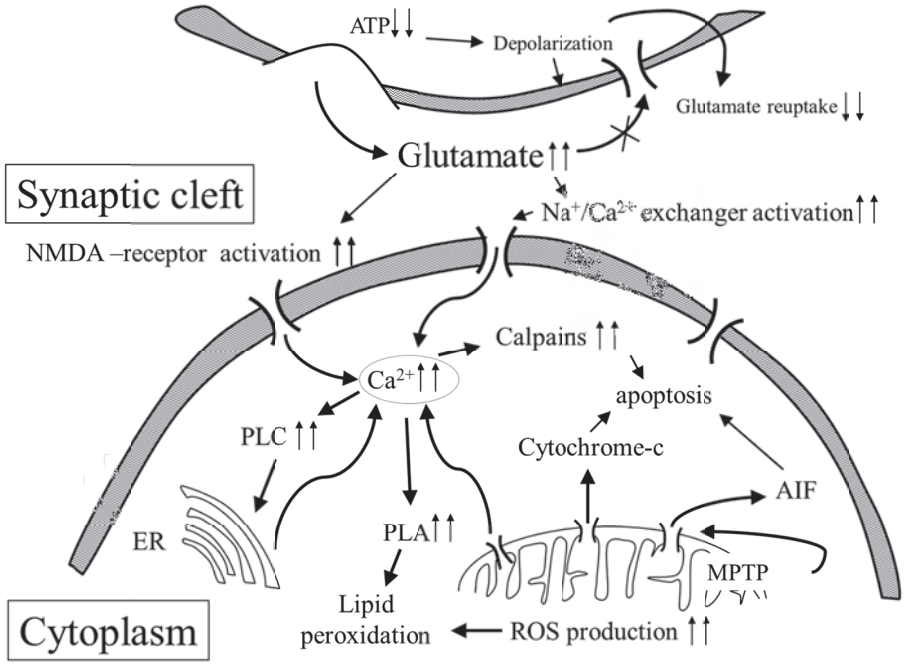

Fig. 2. A simplified overview of ischemic cascade; PLC = phospholipase C, PLA = phospholipase A, ROS = reactive oxygen species, AIF = apoptosis inducing factor, MPTP = mitochondrial permeability transition pore.

Calcium

The rise in cytosolic Ca^{2+} caused by synaptic glutamate instigates additional harmful effects. Phospholipase-C (PLC), which is an integral part of G-protein-coupled-receptor-mediated calcium signalling in cells, is calcium activated. The activated PLC then causes the Ca^{2+} stores of cellular endoplasmic reticulum (ER) to be released into the cytosol further deteriorating the homeostasis of the cell (Kim *et al.* 2011). Additionally the rise in intracellular Ca^{2+} is rapidly mirrored in the mitochondria of neurons; (Rizzuto *et al.* 1992) inhibiting ATP-production of neurons completely by disrupting the electrochemical gradient of the mitochondria that is vital for the process. Furthermore a rise of intramitochondrial Ca^{2+} causes a marked increase in reactive oxygen species (ROS) production which in turn can lead to neuronal programmed cell death called apoptosis or direct cell death termed necrosis (Maciel *et al.* 2001). This multi-layered ischemic

process is simultaneously exacerbated by pro-apoptotic factors leaked from the mitochondria due to mitochondrial permeability transition (MPT) (Zoratti & Szabo 1995).

Mitochondrial permeability transition

The MPT is a process where the permeability of the mitochondrial membrane through a mitochondrial permeability transition pore (MPTP) increases. Pro-apoptotic factors, such as cytochrome-c and apoptosis inducing factor (AIF), and Ca^{2+} are leaked into the cytosol en masse (Kroemer & Reed 2000). This leakage and the loss of intramitochondrial antioxidants such as glutathione (Schild & Reiser 2005, Starkov *et al.* 2004) further aggravate the harm that the already heightened ROS production of mitochondria causes intracellularly. When a single mitochondrion has had its electrochemical gradient sufficiently disrupted it can undergo MPT. At some point in the ischemic process the cell will start a programmed cell death via mitochondria going through MPT, or it will spontaneously die due to massive cellular structure damage caused by lipid peroxidation from ROS and phospholipase A (PLA) (Kroemer & Reed 2000).

Phospholipase A

Phospholipase A's are a group of enzymes that hydrolyse glycerophospholipids and seem to have a role in the production of eicosanoids in cell signalling. Their function is closely related to another enzyme called cyclooxygenase-2 (COX-2) (Kishimoto *et al.* 2010). An overexpression of COX-2 enzyme has been shown to worsen cerebral infarction (Dore *et al.* 2003). Moreover, the activation of PLA is Ca^{2+} dependent which causes it to translocate from the cell cytosol to the nuclear envelope where it causes localized lipid hydrolysis and thereby additional cell damage (Peters-Golden *et al.* 1996).

Reactive oxygen species

Oxygen is a key ingredient in sustaining normal neuronal function. It is used by the mitochondria in the electron transfer chain to produce ATP via oxidative phosphorylation. Unfortunately, an impartial reduction of the O_2 can lead to the production of some highly oxidizing molecules, such as hydrogen peroxide, superoxide anion, and nitric oxide (NO) (Boveris & Chance 1973), known as

ROS. Because these highly oxidising molecules also occur under physiological, i.e. non-ischemic, conditions, cells have adapted to various ROS-scavenging methods with which to mitigate ROS induced peroxidation of lipids and proteins (Niizuma *et al.* 2009).

Although ischemia causes several simultaneous deleterious effects, the sensitivity of various cells to these effects varies. Hippocampal cells and glial cells have shown to be specifically more vulnerable to ROS than neurons (Feeney *et al.* 2008).

Nitric Oxide

Nitric oxide (NO) is produced mainly by three routes: the endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and neuronal nitric oxide synthase (nNOS) (Bredt 1999). It functions as a physiological neurotransmitter as well as a regulator of perfusion in the brain (Iadecola 1997). Specifically the nNOS is capable of producing either neuroprotective or neurodestructive NO species (Iadecola 1997). Moreover, the synthase in question is Ca^{2+} activated and, as previously discussed, represents a hallmark event in ischemic damage. Following the rise in cytosolic Ca^{2+} nNOS produces excessive amounts of NO which then reacts with superoxide anions to form a highly toxic compound called peroxynitrite ($ONOO^-$) (Lipton *et al.* 1993). Although the rate response between NO production and ONOO- formation is not linear, it has been shown in an in vivo animal study that $ONOO^-$ concentrations increase with NO concentrations (Yang *et al.* 2008).

Apoptosis and Necrosis

Cell death can be divided into two different and distinct pathways: apoptosis and necrosis. Apoptosis is a vital part of normal tissue development where unnecessary cells are removed in a controlled manner. Apoptosis is an extremely complex cascade of events, which culminates in strong protein and lipid oxidation, DNA fragmentation, and the formation of apoptotic bodies that are subsequently removed via phagocytosis (Kerr *et al.* 1972). Although apoptosis and necrosis have been considered to be two separate and distinct pathways, there is some emerging evidence to suggest that they share some common elements. The actual severity of the ischemic insult, the degree of Ca^{2+} elevation, and the subsequent mitochondrial dysfunction with ATP loss determine whether the cell

undergoes apoptosis or necrosis. Some researchers are referring to it as an apoptosis-necrosis continuum as cell death can occur in multiple variants with similar pathways coexisting (Formigli *et al.* 2000, Yakovlev & Faden 2004, Yamashima & Oikawa 2009). However, apoptosis elicits only a slight immunologic response because the cellular structure is broken down in a controlled fashion (Savill 1997), whereas necrosis elicits a stronger inflammatory response (Stoll *et al.* 1998) leading to neutrophil activation and pro-inflammatory cytokine release. This exacerbates ischemic damage by causing accumulation of leukocytes in the capillary bed of the cerebrum and aggravates tissue oedema, which further deteriorates perfusion (Waldner *et al.* 2012).

2.3.2 Inflammatory response

During ischemia a complex and intricate ischemic cascade is activated, and it has several detrimental effects. Unfortunately the restoration of blood flow triggers additional harmful processes.

Postischemic inflammation is triggered via activated neuroglial cells called microglial cells. These function as leukocyte-macrophages of the brain. In response to noxious stimuli they can secrete chemokines and transform into phagocytic cells, and as such, play a major part in the brains immunological response. The exact mechanisms of microglial activation are not yet known, but evidence suggests that microglial cells have the capability to be both detrimental and neuroprotective in the injured brain (Ekdahl *et al.* 2009). The protective effects are suspected to be mediated via anti-inflammatory mediators such as brain-derived neurotropic factor (BDNF) and insulin-like growth factor I (IGF-I) (Arroba *et al.* 2011, Mizoguchi *et al.* 2009). More recently it was also discovered that microglial cells had a marked neuroprotective role in alleviating NMDA receptor-mediated exitotoxicity (Vinet *et al.* 2012). However, at the same time the microglial cells are able to secrete various proinflammatory chemokines and generate harmful ROS (Hur *et al.* 2010). Chemokines cause neutrophil activation and expression of endothelial and intercellular adhesion molecules. They also activate the immunological system, which in some situations, such as bacterial infection, may be desired but in postischemic tissue is unwanted (Kielian *et al.* 2001).

In addition to microglial cells, astrocytic cells also have a role in the inflammatory process. In physiological homeostasis they have several functions such as glutamate uptake, working as NO sink, and maintaining the blood-brain

barrier intact. They do, however, also contribute to delayed cell death after ischemia by producing themselves ROS, releasing pro-inflammatory chemokines, and causing oedema through overexpression of water-specific channels called aquaporins (Ribeiro Mde *et al.* 2006, Ronaldson & Davis 2012). The chemokines attract several types of leukocytes to the afflicted site, including T lymphocytes. T lymphocytes have been shown to have a major role in postischemic recovery and ischemia/reperfusion injury; the removal of a CD4+ T cells was found to improve functional recovery, whereas the removal of another subtype, CD25 positive T lymphocytes, degraded recovery (Saino *et al.* 2010).

2.4 Remote ischemic preconditioning

Local ischemic preconditioning was discovered during the 1980s in the myocardium (Murry *et al.* 1986). Later it was discovered that the brain was affected similarly (Kitagawa *et al.* 1990). It took some time, however, before it was discovered that ischemic preconditioning was also a systemic phenomenon. First, RIPC was induced by invasive methods such as arterial occlusion using an open surgical approach. Remarkably it was then discovered that RIPC was inducible by simply inflating a blood pressure cuff on the upper or lower limb of the patient (Kharbanda *et al.* 2002). This finding greatly simplified the inducing procedure.

Promising results have been published in the cardio- and renoprotective field in humans but so far clinical trials have been limited and small (Alreja *et al.* 2012, Candilio *et al.* 2011, Hong *et al.* 2012). To date, no clinical trials have been published concerning the possible neuroprotective effects of RIPC in humans. A simplified summary of the possible mediators and mechanisms of RIPC is shown in Figure 3.

2.4.1 Mechanism

The exact mechanism of RIPC is not entirely clear at the moment, but an increasing amount of research is being conducted on the matter. Short non-lethal ischemia seems to activate several kinases that act in conjunction to mitigate subsequent more severe ischemia (Burley *et al.* 2007, Hausenloy & Yellon 2004).

The cardioprotective effects, the most researched at the moment, have shown to be partly induced by a potassium-dependent ATP (K_{ATP}) channel (Loukogeorgakis *et al.* 2007). The activated K_{ATP} channels, especially the

mitochondrial ones, have the ability to limit the opening of MPTPs, thus causing a marked improvement in cell survival (Ma *et al.* 2011, Robin *et al.* 2011). This neuroprotective effect also applies to cerebral tissue (Wang *et al.* 2011).

Although not yet verified the current dogma is that the effects of RIPC are transferred systemically in three separate modalities.

Neuronal pathway

The hypothesis for the neuronal pathway is based on the fact that using autonomic ganglion blockades abolishes the cardioprotective effects of RIPC when the preconditioning ischemic insult is performed via mesenteric artery occlusion (MAO) (Gho *et al.* 1996). A more recent study confirmed that this is also an important transit mechanism for the neuroprotective effect (Malhotra *et al.* 2011) Interestingly enough the same ganglion blockade did not abolish RIPC when the preconditioning was performed with coronary artery occlusion instead of MAO, which only goes to show that the exact mechanisms can vary depending as much on the "target" organ to be protected as on the preconditioned tissue.

Additionally, adenosine receptors, particularly the subtype A1, have been implicated as the mediators of neuroprotection in RIPC (Nayak *et al.* 2011). It has been shown to increase specific antioxidants and nitric oxide production (Hu *et al.* 2012). Nitric oxide has a dual role as it can function as a neuroprotective agent in homeostatic conditions (Calabrese *et al.* 2007) and to a certain extent in ischemic events but can also induce highly oxidising molecules such as peroxynitrite in severe anoxic conditions (Iadecola 1997).

Humoral pathway

Before the turn of the millennium it was discovered that cardioprotective effects of RIPC might be transferrable to a recipient via whole blood transfusion (Dickson *et al.* 1999a, Dickson *et al.* 1999b). Newer studies done with transplanted hearts corroborate this finding, although to date there has not been certainty of what exactly the humoral agents are or what their exact role is in the process (Konstantinov *et al.* 2005, Redington *et al.* 2012).

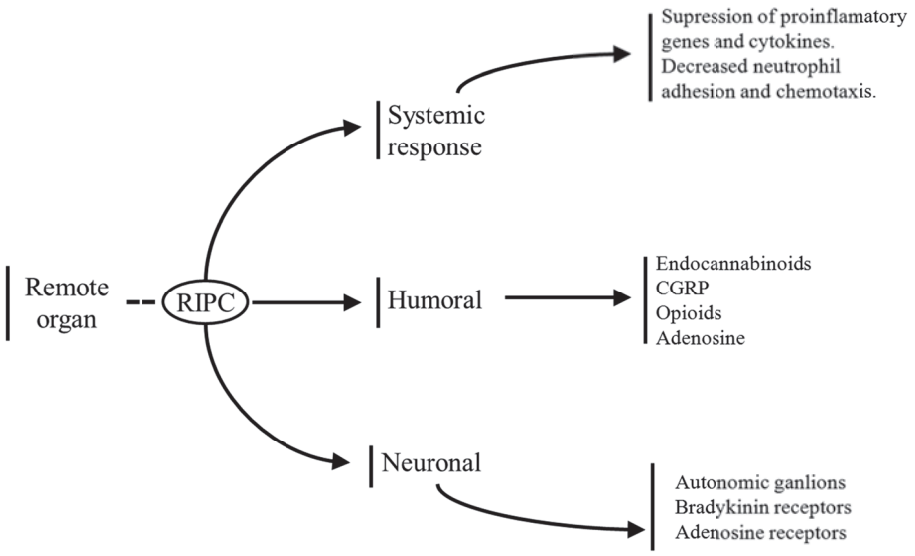

Fig. 3. Suggested mediators of remote ischemic preconditioning; CGRP = calcitonin gene-related peptide.

Adenosine is released from tissues in response to infection and ischemia (Barletta *et al.* 2012), and it affects cellular function through its receptors. Adenosine receptors are responsible for relaying opioid-induced remote cardioprotective effects in animals (Yao *et al.* 2011) and some results indicate that adenosine and adenosine receptors also have a role in relaying some parts of the ischemic preconditioning stimuli to the brain (Hu *et al.* 2012). Endogenous cannabinoids have likewise been implicated as neuroprotective agents. Endocannabinoid receptor blockers abolish RIPC and it has been suggested that they also play an integral part in the process (Su *et al.* 2009). In addition to the factors mentioned above, calcitonin gene-related peptide (CGRP) is also involved in mediating RIPC (Rehni *et al.* 2007). Other possible humoral mediators include even erythropoietin (Ruscher *et al.* 2002).

Bradykinins are a group of proinflammatory peptides that are released into the blood stream in response to various noxious stimuli. Their receptors B1R and B2R are numerous in the CNS. Animal studies have shown that supressing B1R is neuroprotective (Austinat *et al.* 2009), but the role of B2R is more contradictory. Neuroprotective effects have been induced with both B2R receptor activation (Su *et al.* 2012) and B2R receptor suppression (Lumenta *et al.* 2006). The immense complexity of the interplay between peptides and their receptors in the brain is

further evident in a study were bradykinin inhibitor did not abolish the effects of limb RIPC in healthy human volunteers (Pedersen *et al.* 2011).

Although knowledge about neuroprotective mediators and mechanics of RIPC is slowly accumulating, it is apparent that the process is extremely complex.

Systemic response

There is somewhat strong evidence that RIPC modifies several different modalities of the immunological system. It has been shown to alter neutrophil adhesion and phagocytosis and to modify their cytokine expression R1 (Shimizu *et al.* 2010). Interestingly the changes include the suppression of the proinflammatory tumour necrosis factor (TNF) production but not the suppression of its receptor, TNF-R. This would directly translate to less circulating free TNF. The activated TNF-R1 promotes the production of manganese superoxide dismutase (MnSOD), which is a strong antioxidant and provides protection against ROS. The suppression of anti-inflammatory genes extend to proapoptotic, chemotactic, and cell adhesion molecules that promote cellular extravasation (Konstantinov *et al.* 2004). Other immunological changes include the suppression of proinflammatory cytokines that promote chemotaxis and leukocyte activation (Liang *et al.* 2011).

Effectors and summary

RIPC is a highly researched method. However, most of the studies do date have been performed on the cardioprotective effects. This is evident because the use of RIPC in cardioprotection has reached clinical human trials. Conversely, as the neuroprotective possibilities of RIPC are a somewhat newer discovery, research on this area has not reached the same phase yet. The neuroprotective mediators and effectors are continuously but slowly emerging through in vitro and animal studies. Local in vitro neuron cultures have shown that protein kinase C (PKC) (Zhang *et al.* 2011) and lymphocyte kinase (Bae *et al.* 2012) are some of the possible end-effectors in ischemic preconditioning of the brain. In addition to the ones already mentioned there are a few other pathways and molecules (Lee *et al.* 2008) that are suspected to have a role in causing ischemic preconditioning of the brain. The PI3K/Akt pathway has been shown to cause ischemic tolerance (Gao *et al.* 2010) apparently through reducing release of proapoptotic factors from mitochondria (Miyawaki *et al.* 2008). Not surprisingly, a key transcriptional

factor, nuclear factor kappa B, is activated in neuronal cultures of preconditioning and seems to facilitate some parts of the ischemic preconditioning process via COX-2 expression (Kim *et al.* 2010).

All these effectors and mediators form a complex mesh of peptides, receptors, cytokines, cell interactions and transcription factors. It is therefore extremely difficult to distinguish the exact role and the interactions of a single factor until all of the mediators and effectors have been identified. However, as the method of inducing RIPC by intermittent limb ischemia is known to be quite safe, we can study its efficacy and safety at macroscopic level even before we understand the full complexity of its mechanisms.

2.5 Limitations of this study

Animal models are used routinely in almost all aspects of scientific research. Although they provide a significant amount of data, it is important to acknowledge the limitations of these models.

Ultimately, the animal models in use involve relatively small study groups; even in the largest of experiments the animals usually number in the tens rather than in the hundreds. This might cause false positives because even a few animals that display altered behaviour or results in a specific group can bias the difference between the control and intervention animals. Alternatively, small study groups are also prone to false negatives because small or minute differences are difficult to distinguish.

Additionally one must consider that animal models are almost always homogenous experiments as the animals involved are usually healthy individuals with almost no differences. This can cause difficulties in trying to extend the results to human patients who are an extremely heterogeneous population even within one specific disease group.

Finally there is the fact that animal experiments cannot provide us with all the answers because they are just that, experiments done with animals. Nevertheless, they can provide us with vital information about the preliminary efficacy and safety of a proposed intervention. One can also study the intervention and try to understand it at cellular and biochemical levels. Once all means of ascertaining the efficacy and safety of a method or hypothesis have been exhausted in animal models, only clinical human trials can provide further information on these topics.

3 Aims of the present study

This thesis was completed to ascertain whether RIPC is a viable method for producing neuroprotection in an HCA setting. The present thesis comprises the following studies:

Study I	To examine if RIPC is a viable method for inducing enhanced ischemia tolerance in the central nervous system of a porcine.
Study II	To ascertain whether RIPC has neuroprotective effects after HCA in a surviving experimental set-up.
Study III	To examine whether RIPC alters the oxygen usage profile or consumption during HCA.
Study IV	To assess whether RIPC has effects on leukocyte adherence and rolling, redox state of the brain, or vessel diameter on the cortex of the brain; and also, to evaluate possible neuronal and neuroglial ultrastructural changes by using transmission electron microscopy.

4 Materials and methods

4.1 The porcine model

The studies were completed at the Department of Experimental Surgery at Oulu University using a porcine model. The porcine model was developed by Professor Randall Griepp and his group at the Mount Sinai School of Medicine. Later on Professor Tatu Juvonen established a similar experimental porcine model at the Department of Experimental Surgery at Oulu University. The model has been further developed and altered to study different hypotheses. The basic layout of all our studies is shown in Figure 4. The actual weaning from respirator and anesthesia was performed only in chronic surviving experiments. In acute experiments the animals were terminated by pentobarbital injection after the follow-up period. Study I and Study IV were acute studies where the experiment was terminated at the end of the eighth hour of the postoperative follow-up period. Study III was a hyperacute study that was terminated at the end of the rewarming period. Study II had a seven-day follow-up period after weaning. Study IV was also an acute study in which we performed a large decompressive hemicraniectomy to directly visualize the cerebral cortex using an intravital microscope.

4.2 Test animals and preoperative management

Pigs from a native stock and provided by a piggery in Oulu, Finland, were used as test animals in all the four studies presented in this thesis. No cloned or genetically manipulated animals were used. All animals received humane care in accordance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources, National Research Council (published by National Academy Press, revised 1996). The study was approved by the Research Animal Care and Use Committee of the University of Oulu. The animals were cared for by a professional staff of trained nurses and their wellbeing was monitored daily before and after the operations.

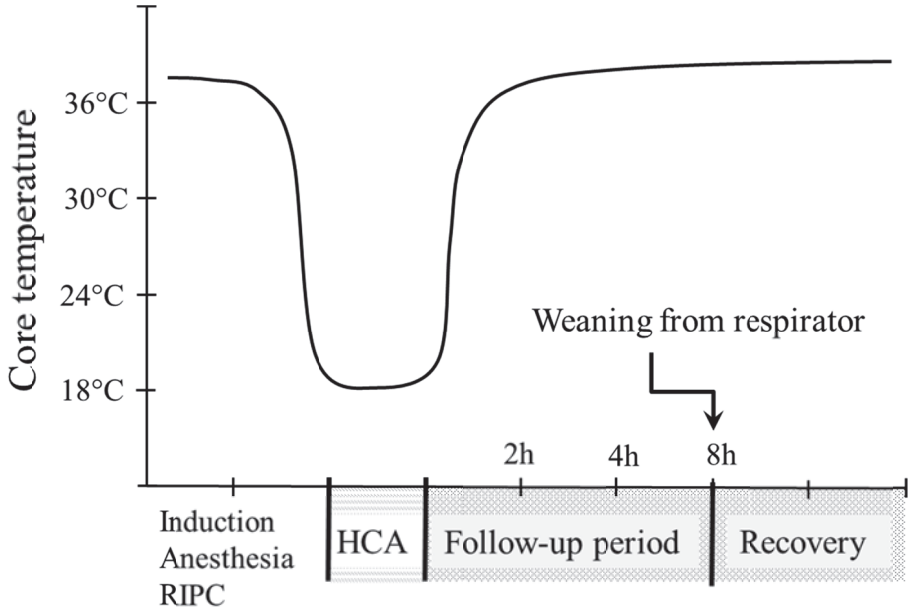

Fig. 4. Simplified depiction of the experimental protocol; HCA = hypothermic circulatory arrest, RIPC = remote ischemic preconditioning.

4.3 Remote ischemic preconditioning

The actual ischemic preconditioning was performed by an experienced nurse. Preconditioning was induced with a children's blood pressure cuff by applying a static pressure of 230 mm Hg to the right hind leg. Systemic arterial pressure readings were recorded simultaneously to confirm the reactive hypertension and hypotension after the occlusion and reperfusion of the femoral artery. Four cycles of 5-minute ischemia intermittent with three 5-minute reperfusion periods were completed. Cardiopulmonary bypass was initiated 60 minutes after the RIPC, and HCA was achieved in 90 minutes from the RIPC. The ischemic preconditioning protocol was identical in all of the four studies. The term transient remote ischemia (TRI) was used in Study I as an interchangeable term with RIPC.

4.4 Anesthesia

Although this thesis contains four separate publications, the preoperative anesthesia and preparation were similar in all four studies.

The animals were sedated with ketamine (350 mg intramuscularly) and midazolam (40–50 mg intramuscularly). After endotracheal intubation, a balanced anesthesia was maintained by inhaled isoflurane (0.5%) and continuous infusion of fentanyl (25 µg/kg/h) midazolam (0.25 mg/kg/h), and pancuronium (0.2 mg/kg/h) in both study groups throughout the experiment. The animals were maintained on positive-pressure ventilation with 50% oxygen. Studies I, II, and III were conducted using isoflurane inhalation anesthetic. In study IV, sevoflurane was used instead. End-tidal carbon dioxide ($EtCO_2$) content was kept at 5%. A catheter was inserted into the bladder of the animals. Also a rectal thermometer was used to monitor the body temperature of the animals.

4.5 Hemodynamic monitoring

A 7F Schwann-Ganz catheter (CritiCath; Ohmeda GmbH, Erlangen, Germany) was inserted through the left femoral vein into the pulmonary artery, allowing blood sampling and invasive hemodynamic monitoring of central venous and pulmonary artery pressure in addition to various other parameters. Invasive arterial pressure measurements were recorded through an arterial needle inserted into the left femoral artery.

4.6 Blood transfusion

We used donor pigs to compensate for the effects of hemodilution during extracorporeal bypass. Preoperatively, a membrane oxygenator (D905 Eos; Dideco SpA, Mirandola, Italy) was primed using 500 mL of Ringer's acetate solution, 3 to 4 units of donor blood, and heparin (5000 IU).

The donor pigs were sedated, intubated, and kept under anesthesia as previously described and 3 to 4 units (1200 mL) of blood was collected. Equal amounts of donor and receiver blood were mixed in a separate test tube and tested for any cross reactions. If hemolysis or coagulation was detected, the priming was done again. After the collection, donor animals were euthanized using pentobarbital (60 mg/kg).

4.7 Intracranial monitoring

The cranial procedures varied slightly in the performed studies. In studies I and II we performed a 7-cm midline longitudinal incision on top of the scalp, and a total of four electroencephalography (EEG) leads were placed directly on the periostium over the frontal cortex. In the first study (Study I) we used a separate temperature probe (Thermocouple Temperature Catheter-Micro-Probe; Experimental Setting GMS GmbH, Mielkendorf, Germany), whereas in Study III we used a combined temperature/oxygen tension probe that was inserted through a burr hole into the right parietal cerebral cortex (Licox CC1.P1; Integra LifeSciences, Plainsboro, NJ). In study IV we performed a decompressive hemicraniectomy on the right side of the skull.

4.7.1 Electroencephalographic analysis

In studies I and II we registered EEG data. These data were recorded using 5-minute intervals at baseline and every full hour after HCA. Recovery was measured using dedicated software that evaluated the EEG data and calculated the proportion of bursts to burst suppression periods. The anesthetic protocol was kept constant during and between the time points to maintain good comparability between experiments. The EEG hardware and software were operated by an experienced biophysicist who was blinded to the experimental setup.

4.8 Microdialysis

Microdialysis refers to a sample collecting technology wherein a sample is collected through a semipermeable membrane from the interstitial fluid of an organ. With this method one can collect extracellular fluid and analyze its content for various markers. In studies I, II, and IV we inserted a cerebral microdialysis catheter (CMA Brain MD 70 Catheter;CMA/Microdialysis AB, Solna, Sweden) into the right parietal parenchyma of the animals. The catheter was connected to a microdialysis pump (CMA 107 Microdialysis Pump;CMA/Microdialysis AB), and the microdialysis samples were collected using a flow setting of 2.0 μL/min and analyzed with a CMA 600 microdialysis analyzer (CMA/Microdialysis AB). The analyzer was used to measure extracellular glutamate, glucose, pyruvate, glycerol and lactate concentrations. The first samples were collected for 30 to 60

minutes and always discarded to mitigate the influence of unavoidable insertion trauma.

4.9 Intravital microscopy

In study IV we used an intravital microscope (Leica Model MZFL III; Leica, Heerbrugg, Switzerland) placed over the cranial window to analyze vascular diameter changes, leukocyte amount and adhesion and also to obtain real-time measurements about the redox state of NADH in the cortex. The cranial window was created using a neurosurgical drill. We used the three sets of filters: a violet filter (450–490 nm excitation, > 515 nm emission wavelength) to visualize microvascular perfusion, a green filter (536–556 nm excitation, > 590 nm emission wavelength) for visualization of rhodamine-labeled leukocytes, and ultraviolet filter for NADH analysis. A sample image that was used to calculate leukocytes is shown in Figure 5.

The image was captured using a charge-coupled device video camera (CCD 300-ETRCX; Dage-MTI, Michigan City, Ind), transferred to a monitor (LCD SyncMaster 710 mp; Samsung Elec- tronics Co, Ltd, Seoul, South Korea), and videotaped. A frame grabber (Kudo Interactive Frame Grabber; Kudo Interactive, Westminster, Colo) and a computer-assisted image analysis system (Scion Corporation, Frederick, Md) were used for offline analysis. The final magnification on the monitor was ×400. At baseline the piglet received a 2-mL (4 mg/mL) loading dose of rhodamine 6G chloride MW 479 (Sigma Chemical Co, St Louis, Mo) 5 minutes before the initial recording.

Video recordings were taken at 11 time points: at baseline; at 5 minutes before CPB cannulation; at 10 and 20 minutes of cooling; at 50 minutes after institution of HCA; at 5, 15, and 45 minutes of rewarming; and finally at 90, 120, and 180 minutes after HCA. The epi-illumination was restricted to 1 minute to avoid thermal injury.

Fig. 5. A sample image used for leukocyte counting.

Violet filter

Once proper magnification and resolution were acquired, a 1-mL (50 mg/mL) intravenous bolus of fluorescein isothiocyanate-labeled dextran (150 kd; Fluka Chemicals, St Louis, Mo) was administered to label the plasma of the cerebral vessels. Subsequently we recorded video image using the violet filter of the microscope. The diameters of 20- to 50-µm arterial and venous cerebrocortical micro vessels were measured from still images by using an image analysis program. Three arterioles and three venules were measured from each animal. Each measurement was referred to baseline, so that the baseline diameter of the vessel was 100%.

Green filter

A 1-mL (4 mg/mL) intravenous bolus of rhodamine was used to stain the activated leukocytes in circulation. A postcapillary venule was used to calculate the adherent and rolling leukocytes. The exact number of adherent leukocytes was calculated from an easily determined portion of the vessel, and the number of cells was then related to the surface area rendered. The actual counting was done manually by an analyst who was blinded to the setup. To assess and count rolling leukocytes, a specific point of the vessel was chosen, and the number of leukocytes rolling past that point was observed during approximately 10 to 15 seconds in each recording.

Ultraviolet filter

The ultraviolet filter was used to capture video image for the analysis of NADH. The monitoring of NADH is based on the absorption difference of NADH and the oxidized form of nicotinamide adenine dinucleotide (NAD+). The NAD+ does not absorb light at 320 to 380 nm, but NADH does, whereas the fluorescence emission of NADH is peaked at 450 nm (Chance *et al.* 1973).

4.10 Transmission electron microscopy

In Study IV we used both intravital and transmission electron microscopy (TEM) to analyze intracellular morphological changes. Biopsies were taken bilaterally from cortex of temporal lobes. We took biopsies from the same area of the cortex that we had captured on video using the intravital microscope. After collecting the specimens, the animals were euthanized using pentobarbital (60 mg/kg) while anesthetized. To establish a baseline for the effects of rhodamine, fluorescein, and lamination of the cortex we also took samples from an animal that did not undergo HCA (zero-control). The biopsies were fixated using 1% glutaraldehyde 4% formaldehyde mixture in 0.1 M phosphate buffer. They were postfixed in 1% osmiumtetroxide, dehydrated in acetone, and embedded in Epon LX 112 (Ladd Research Industries, Williston, Vermont, USA). Thin sections were cut with Leica Ultracut UCT ultramicrotome, stained in uranyl acetate and lead citrate, and examined in Tecnai G2 Spirit transmission electron microscope (FEI Europe, Eindhoven, The Netherlands). Images were captured by Veleta CCD camera (Olympus Soft Imaging Solutions GMBH, Munster, Germany).

4.10.1 Assessment

The TEM picture was analyzed by a blinded experienced analyst. Changes in mitochondria and rough endoplasmic reticulum (RER) of neuroglial cells and neurons, and structural changes in astrocytic perivascular endfeet were assessed and scored. The observed mitochondrial changes included swelling, disrupted cristal integrity, and loss of matrix density; the observed changes in RER consisted of dilation and presence of vacuoles; and the observed changes in the ultrastructural features of astrocytes included swelling of astrocytic soma and perivascular endfeet, ruptured cytoplasmic and mitochondrial membranes, and electron-lucent cytoplasm. Structural alterations were scored as follows: normal = 0, mild = 1, moderate = 2, severe = 3. If a sample contained both cells with normal features and cells with mildly affected features, a mitochondria score of 0,5 was given. The total score was summed of these ultrastructural scores, maximum being 18.

4.11 Additional measurements

In all of our experiments we measured blood gas values, pH, electrolytes, plasma ionized calcium, plasma lactate levels, glucose, hemoglobin, and hematocrit levels at baseline, at end of cooling, and at 30 minutes from the end of the HCA period using a portable blood gas analyzer (i-STAT Analyzer; i-STAT Corporation, East Windsor, NJ, USA). We also recorded several other important data such as central temperature readings (pulmonary artery, intracerebral, rectal), cardiac output, urine amount, amount of fluids infused, and amount of donor blood.

4.12 Behavioural score

In the chronic surviving animal study (Study II) the mental status of the animals was assessed daily by a nurse that was blinded to the experimental setup. The assessment was done using a quantifying scale in which three basic categories are scored as follws: behavior (coma = 0, lethargy = 1, depression = 2, normal = 3); appetite (does not drink or eat = 0, does not eat = 1, decreased appetite = 2, normal appetite = 3); and motor function (unable to stand = 0, unable to walk = 1, unsteady gait = 2, normal = 3). The category scores were summed with a

maximum score of 9. A high score indicates normal neurological function and lower values signify neurological injury.

4.13 Cardiopulmonary bypass

The CBP protocol was kept similar in all of our studies to maintain a good degree of comparability. The CPB was performed through right lateral thoracotomy. The right atrial appendage was cannulated using a 24F venous cannula. A 16F arterial straight tip arterial cannula was used for the aortic cannulation. Flow was adjusted to maintain a mean arterial pressure of 55 to 60 mm Hg.

Cooling was initiated using pH stat acid-base management (pH-stat) strategy, and the core temperature was lowered to 18°C using a heat exchanger. After a 30-minute cooling phase, perfusion flow was stopped and HCA was initiated. The intracerebral and rectal temperatures were monitored continuously and maintained at 18°C with ice packs. At the beginning of rewarming, furosemide (40 mg), mannitol (15 g), lidocaine (40 mg), methylprednisolone (40 mg), and calcium glubionate (90 mg) were administered into the CPB circulation.

The pH-stat strategy was used for the 45 minutes of rewarming, and the piglets were warmed to 37°C while attempting to maintain a mean arterial pressure of 60 mm Hg. With pH-stat strategy we attempted to maintain the pH value of blood constant despite the decrease in temperature. This is achieved by increasing the CO_2 content in the blood.

The heart was defibrillated if necessary at 25°C to 30°C. Ventilation was started 10 minutes before weaning from CPB, which was performed 45 minutes after the start of rewarming. During cooling and rewarming perfusion line temperature, rectal temperature, brain temperature, venous blood saturation, pump flow, and central venous pressure (CVP) were measured and recorded. Furthermore, arterial pH and CO_2 values were measured at regular intervals.

4.14 Histopathology

Studies I and II include a histopathological analysis. Studies III and IV were hyperacute and thus histopathological analysis was not used. We collected the brains from these studies and kept them in formalin for two weeks after which an experienced neuropathologist scored five regions: the cerebral cortex and thalamus, cerebellum, pons, and medulla. The scoring was based on the presence of oedema (0–3), hemorrhages (0, 2–3), neuron degeneration (0, 2–3) and the

presence of infarcted tissue (0, 3). The sum of these five regions comprises the total histopathological score.

4.15 Biochemical assay

In study II we collected serum samples that were analyzed by our colleagues at the Great Ormond Street Hospital, London. The samples were assessed for serum concentrations of neuronal damage-specific markers at each time point. Serum neurofilament heavy chain (NfH) levels were measured using an enzyme-linked immunosorbent assay (ELISA) method. The soluble fraction of NfH measured is indicated with the capture of antibody in the superscript (NfHSMI35). The analysis focused on NfHSMI35 instead of the neurofilament light chain due to the fact that NfHSMI35 is relatively resistant to proteolysis (Petzold *et al.* 2003). Serum glial-specific S100 calcium binding protein (S100-B) levels were measured using a modified ELISA method perfected in Great Ormond Street Hospital (Green *et al.* 1997).

4.16 Statistical analysis

Statistical analysis was performed by an experienced biostatistician using SPSS (version 18.0; SPSS, Chicago, IL, USA) and SAS (version 9.2; SAS Institute, Cary, NC, USA) statistical software packages. Continuous and ordinal variables are expressed as mean values with standard deviation (SD) in parenthesis or median values with 25th–75th percentile. Simple between group comparisons were performed using Mann-Whitney *U* test for continuous variables. The repeatedly measured data were analyzed using a linear mixed model with patients fitted as random, and the best covariance pattern was chosen according to Akaike's information criteria. Two-tailed significance levels are reported. In Study II, analysis of variance (ANOVA) was used instead. Reported p values are as follows: p between groups (p*g) indicates a level of difference between the groups; p for time (p*t) indicating difference between time; p for time by group (pt*g) that indicates behaviour difference between the groups with time.

5 Results

5.1 Study I

5.1.1 Comparability of study groups

Study I included 12 animals that were randomized to a control group or a RIPC group. The weight of the animals between the groups was comparable. Additionally there were no differences in baseline hematocrit. Likewise, blood, rectal, and intracerebral temperatures had no statistically significant differences at baseline, during HCA, or at any time point postoperatively.

5.1.2 Electroencephalographic analysis

The EEG burst recovery can be used as an easily achievable mathematically derived variable to measure neurological recovery. Furthermore, we have shown in our previous study that it is predictive of neurological infarction in an experimental setting that uses HCA (Pokela *et al.* 2003). The EEG burst recovery ratio was recorded at 5-minute intervals by dedicated software as described previously. A simplified depiction of the EEG data shows that the RIPC group had a better EEG burst recovery ratio than the control group (Figure 6).

5.1.3 Cerebral microdialysis

Microdialysis collected directly from the brain tissue showed higher concentrations of measured metabolites in the control group implying an altered cerebral metabolism. Glucose concentration had a tendency to be higher in the control group with statistically significant differences right after HCA. Glycerol content rose sharply at the end of HCA ($p < 0.05$) and remained at a higher level during follow-up at time points 90 minutes through 4 hours reaching a statistically significant level of difference at 4 hours. Pyruvate and lactate quantities rose slowly and steadily during the follow-up period after HCA reaching statistically significant levels at several time points.

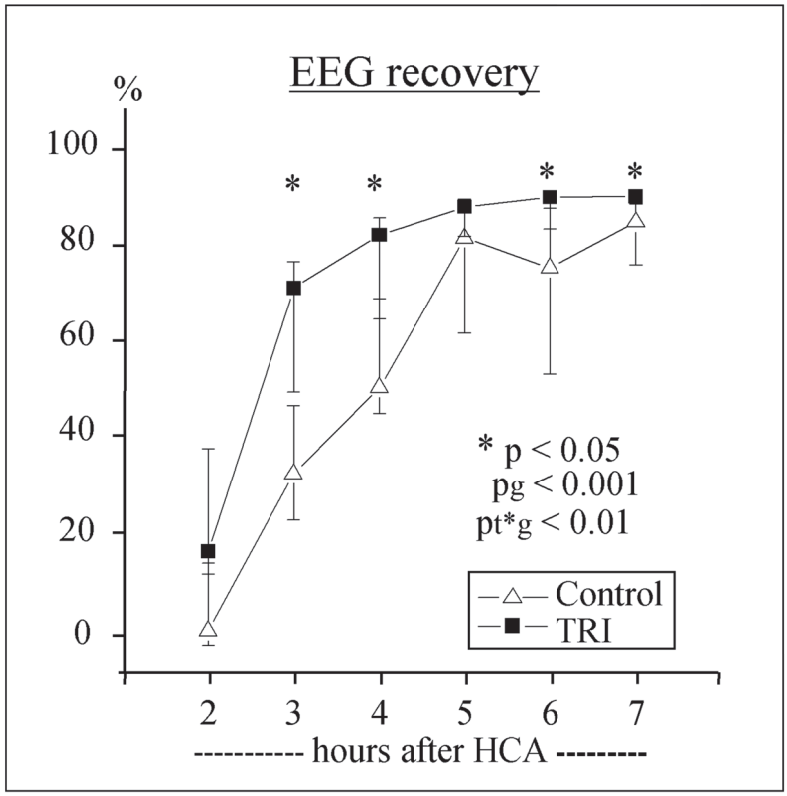

Fig. 6. EEG burst recovery ratio; TRI = transient remote ischemia (Yannopoulos et al 2010, published with permission from Elsevier).

5.2 Histopathology

The histopathological samples were left to fixate in formalin for two weeks, after which they were processed and dyed by experienced laboratory technicians. After the hematoxylin-eosin (HE) staining was complete a qualified neuropathologist assessed the samples. Samples were taken from the cerebral cortex, thalamus, cerebellum, pons, and medulla. The median total histopathological score of the control group was 8 (3–11), and that of the transient remote ischemia (TRI) group was 5 (3–9); the corresponding total oedema scores were 3 (2–5) and 4 (1–5), with no statistically significant differences.

5.3 Additional measurements

In addition to the above-mentioned data we collected copious amounts of hemodynamic data. There were no major differences between groups. In other CPB and hemodynamic parameters we measured statistically significant differences between the groups at few isolated time points. During the follow-up period the control group was more acidic at 2 hours. Additionally, CVP and calculated O_2 consumption seemed to be higher at one time point. Furthermore, similarly to several previous animal studies, we also saw a cardioprotective effect of RIPC, as a statistically significant release of cardiac-specific enzymes occurred in the control group (Figure 7). For more detailed information we kindly ask the reader to consult the reprint of the article.

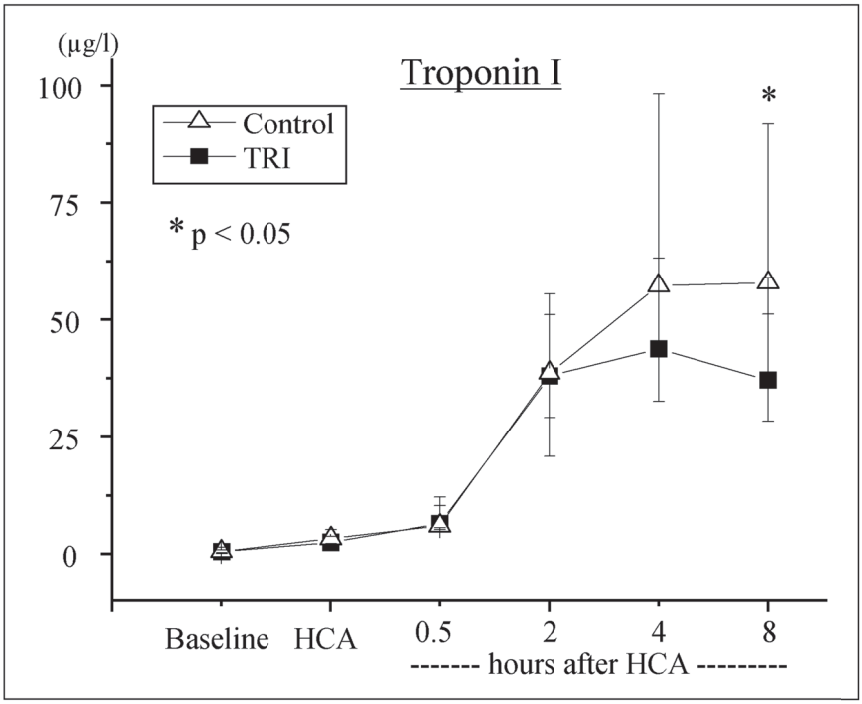

Fig. 7. Cardiac troponin concentrations (Yannopoulos et al 2010, published with permission from Elsevier)

5.4 Study II

In Study II, we examined whether RIPC had neuroprotective effects in a chronic surviving animal model. This study was a joint study with our colleagues at Great Ormond Street Hospital and had a follow-up period of seven days. It showed that RIPC group had a better although not statistically significant recovery of EEG burst suppression ratio compared with the control group. Neuronal damage-specific S-100B and NfH concentrations were measured and showed that the animals in the RIPC group had significantly smaller quantities of these agents in their serum samples. Histopathology strongly corroborated these biochemical findings, as the RIPC group has a statistically significant better histopathological score (Table 1). In addition, this study further corroborated the cardioprotective effect of RIPC, apparent in lowered troponin I (TnI) concentrations in the RIPC group.

5.4.1 Comparability of study groups

The study groups included twelve piglets from a native stock. The piglets were preoperatively randomized to a RIPC group and a control group using sealed envelopes. The study groups did not have any statistically significant baseline differences. Baseline temperatures, weight and venous glucose were similar in both groups at baseline.

5.4.2 Histopathology

Histological samples were fixated in formalin and embedded in formalin as described earlier. After the samples were stained with HE, the neuropathologist assessed the median total histopathological scores, which were 1.5 (0.5–2.5) in the RIPC group and 5.8 (3.8–7.5) in the control group ($p = 0.001$). Signs of ischemic injury, such as hemorrhages and neuronal damage, were seen only in samples collected from the control group. Additionally the samples of the control group showed higher oedema score compared with the RIPC group ($p = 0.002$). Histopathological results are shown in Table 1.

Table 1. Histopathological scores in Study II.

Protocol	Oedema score	Total score
RIPC		
Mean	1.0 (0.0–2.0)	1.5 (0.5–2.5)
Control		
Mean	3.5 (2.8–5.0)	5.8 (3.8–7.5)
P Value	0.002	0.001

Modified from Jensen et al. Circulation. 2011;123:714–721

5.4.3 Cerebral measurements

In Study II we used microdialysis, EEG monitoring, intracerebral pressure recording, and biochemical marker analysis to evaluate the efficacy of RIPC. Additionally a behavioral score was assigned to each animal at each postoperative day.

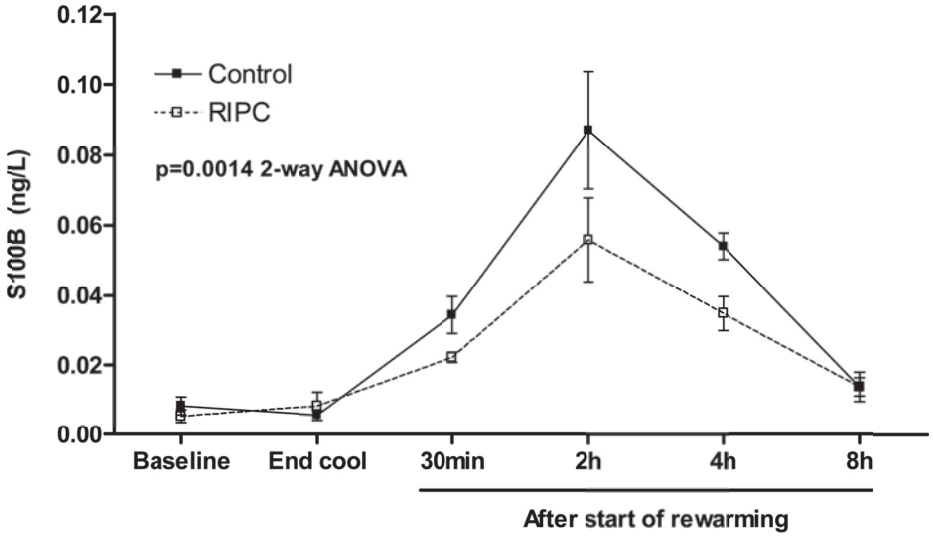

Fig. 8. Serum S-100B concentrations (Jensen et al 2011, published with permission from Lippincott Williams & Wilkins).

Microdialysis

The microdialysis samples were collected after a stabilization period to avoid the effect of placement trauma. There were no observable differences in

concentrations of cerebral glucose, glutamate, pyruvate or glycerol between the groups. Cerebral lactate concentration was higher in the RIPC group right after HCA and towards the end of the follow-up period (2-way ANOVA $p < 0.001$).

EEG

The EEG recovery ratio returned to preoperative values during the follow-up period in both groups; however, the rate of recovery was faster in the RIPC group (2-way ANOVA $p < 0.01$). For a more detailed view of the EEG results the reader is kindly asked to consult the article.

Biochemical markers

There were no differences in serum NfH and S-100B baseline values between the groups. Both biochemical markers showed similar behavior during the experiments. Highest concentrations were measured at approximately 2 hours after HCA. This was followed by a slow decline in measured concentrations that reached nearly baseline levels at the end of the 8-hour follow-up. NfH levels were statistically significantly higher in the control group. Serum S-100B concentrations are shown in Figure 8.

5.4.4 Behavioural score

Study II used a surviving animal model in which all 12 animals survived the experiments and were assessed daily for the entire duration of the follow-up. The animals of the RIPC group recovered faster ($p = 0.0001$, 2-way ANOVA), a difference most notable during the first 2 postoperative days. The daily scores were added together to obtain a cumulative behavioral recovery score that was 54 (52–56) in the RIPC group and 50 (44–52) in the control group ($p = 0.078$).

5.5 Study III

Study III was performed to assess whether there were any discernible differences between the groups in their cerebral oxygen profile before, during, and right after HCA. In Study I and Study II we saw a faster EEG recovery and better biochemical profile, including lower lactate concentrations, in RIPC animals.

These findings lead to our hypothesis that RIPC might affect the oxygen usage profile of the cerebrum during HCA.

5.5.1 Comparability of study groups

The mean weight of the piglets was 21.4 kg in the control group and 22.0 kg in RIPC group. The median baseline hematocrit and amount of transfusion blood received were similar. Additionally haematocrit, cardiac index, and CVP levels remained comparable throughout the experiments and did not have any statistically significant differences at any time points. Measured core temperatures were similar preoperatively and during cooling and rewarming.

Although nearly all measured variables between the groups were comparable, two statistically significant differences were seen. Partial pressure of carbon dioxide (pCO_2) in venous blood was higher in the control group (6.69 kPa [6.36–7.66 kPa]) than in the RIPC group (6.08 kPa [5.66–6.28 kPa]) ($p = 0.041$). Another statistically significant difference appeared in the blood potassium levels; in the control group the potassium level was 3.8 mmol/L (3.8–4.0 mmol/L) and in the RIPC group it was 3.6 mmol/L (3.4–3.6 mmol/L) ($p = 0.026$). Arterial blood O_2 content was similar in both groups with no statistically significant differences at any time point.

5.5.2 Cardiopulmonary bypass results

Because testing our hypothesis in this study was based on direct measurement of cerebral tissue oxygen tension ($PbrO_2$), it was imperative that CPB parameters and hemodynamic parameters be kept stable and similar in both groups.

All of the measured CPB parameters, including hematocrit, venous/arterial CO_2, and arterial/venous pH, were similar throughout the cooling phase of CPB as well as the rewarming period, with no statistically significant differences between the groups. Line temperature, rectal temperature, brain temperature, venous blood saturation, pump flow, and CVP values showed no statistically significant differences at any time point. There were no statistically significant differences in arterial O_2, pH, and CO_2, and mean arterial pressure during CPB.

5.5.3 Cerebral oxygen tension

Absolute pressure values (mmHg) were recorded using a dedicated probe that measures both temperature and oxygen tension (Licox CC1.P1; Integra LifeSciences, Plainsboro, NJ, USA). The measured $PbrO_2$ during HCA is shown in Figure 9. Oxygen tension values rapidly began to drop in the control group after the initiation of HCA. No statistically significant differences were measured in the $PbrO_2$ during cooling and rewarming perfusion.

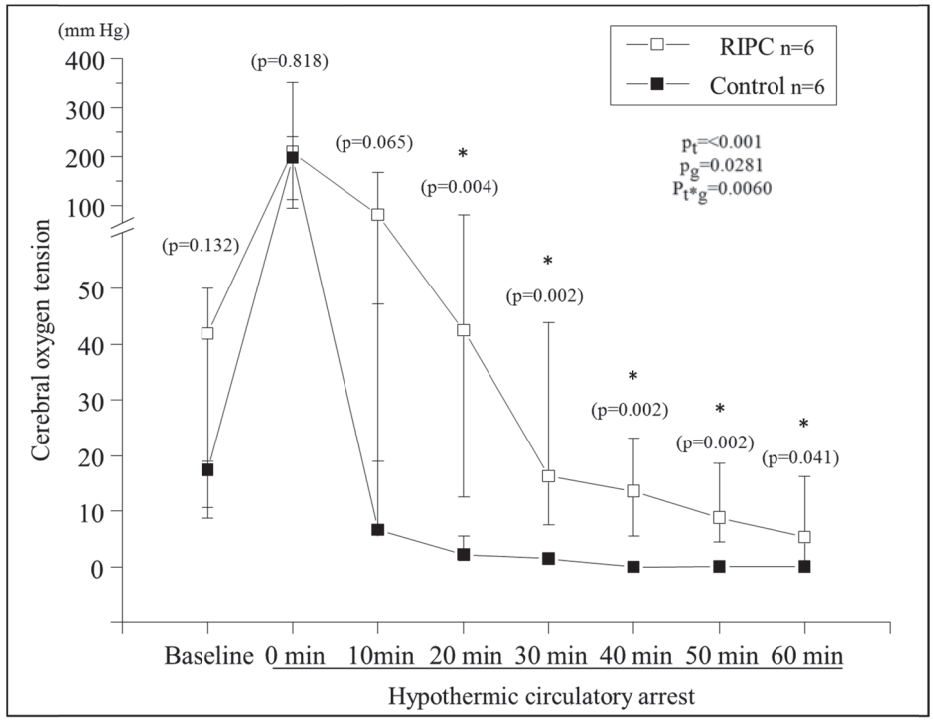

Fig. 9. Cerebral oxygen tension during HCA (Yannopoulos et al 2012, published with permission from Informa Healthcare)

5.6 Study IV

Study IV was a hyperacute study that was conducted using an intravital microscope. The basic protocol was similar to the three other studies; however, we performed a large decompressive hemicraniectomy to observe the cerebral

cortex directly. The method is explained in more detail in the "Materials and methods" section of this thesis.

5.6.1 Comparability of study groups

The median weight was comparable between study groups with no statistically significant differences between groups. The baseline hematocrit slightly differed between groups, as the median hematocrit was 25% (23–27) in the RIPC group and 22% (21–23) in the control group. After baseline measurements the pigs were given 61.9 mL/kg (54.9–70.1 mL/kg) of donor blood with no statistically significant difference between groups. Blood gas analysis and electrolyte concentrations were comparable at baseline, with a significant difference only in pCO_2, which was slightly higher in the control group than in the RIPC group: 5.53 kPa (5.46–5.60 kPa) and 5.33 kPa (4.86–5.36 kPa), respectively ($p < 0.05$). There were no statistically significant differences in mean arterial pressure, CVP, and pulmonary capillary wedge pressure between the groups at baseline.

5.6.2 Leukocyte count

Whole leukocyte count was 24.4×10^9/L (16.1×10^9/L to 25.7×10^9/L) in the RIPC group and 18.0×10^9/L (16.9×10^9/L to 21.4×10^9/L) in the control group at baseline, with no statistically significant difference between groups. Correspondingly, the lymphocyte and neutrophil counts were similar in the study groups. After HCA, the whole white blood cell count, and the neutrophil count tended to be higher in the RIPC group, but the difference did not reach statistical significance.

5.6.3 Intravital microscopy

Median baseline venous vessel diameter in the RIPC group was 57 μm (46–66 μm), and in the control group 77 μm (72–78 μm). The difference between the groups was not significant ($p = 0.117$). The diameters of selected venous vessels did not differ significantly at any time point during the experiment.

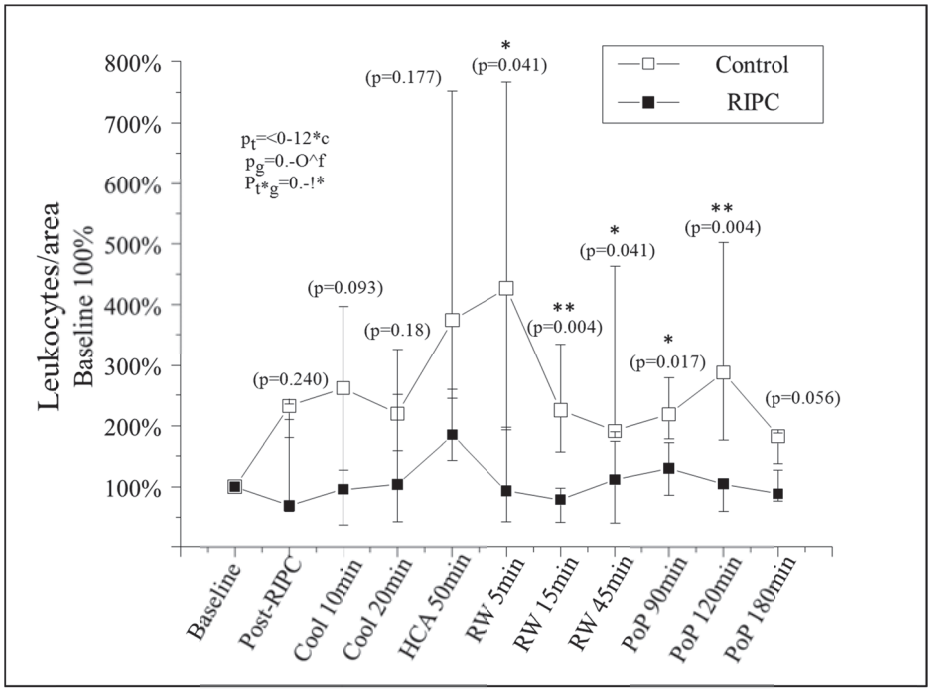

Fig. 10. Leukocyte count.

Leukocytes

The number of leukocytes per area unit in cerebral microvasculature was higher in the control group, and several statistically significant differences were seen (Figure 10). The total systemic leukocyte counts did not differ between groups. During the rewarming period, cerebral leukocyte count did not fluctuate in the RIPC group, whereas in the control group a clear peak in leukocyte count was seen.

The total and rolling leukocytes were counted from still images obtained by the intravital microscope. The rolling leukocytes were calculated using the same vein that was used for calculating adherent leukocytes. There were no statistically significant differences between groups. In both groups, the largest numbers of rolling leukocytes were observed during perfusion phase.

NADH

The NADH autofluorescense showed statistically significant differences between the study groups at 2 and 3 hours postoperatively. At these time points the control group exhibited higher autofluorescense values indicating a higher concentration of the NADH molecule. During the cooling phase, values increased in both groups with no statistically significant differences.

5.6.4 Transmission electron microscopy

The score for ultrastructural changes of cerebrum in the control group was 4.66 (SD 1.44), and in the RIPC group the score was 4.58 (SD 1.36) ($p = 0.97$). The score for cerebellum in the control group was 4.41 (SD 0.89), and in the RIPC group 3.08 (SD 0.27) ($p = 0.31$). The total combined score of both cerebral and cerebellar changes was 9.66 in the control group (SD 1.71) and 7.66 in the RIPC group (SD 1.40) ($p = 0.054$). Statistically significant differences were seen only in the RER analysis of the cerebellar samples. Interestingly, all animals in the RIPC group had ultrastructurally normal RERs in their cerebellar tissue, whereas the control group animals had a mean score of 1.06 (SD 0.41) ($p = 0.026$). For comparative purposes a porcine was operated that underwent similar anesthesia with similar surgical procedures without HCA. The cerebral score of the sham-control animal was 3, and the cerebellar score was 0.5, summing up the total score to 3.5.

5.7 Summary of results

In our experimental model RIPC mitigated inflammatory response and cerebral injury after prolonged HCA. In general the collected data showed homogeneity; for example, similar biochemical results were seen in studies I and II. Interestingly, studies III and IV may also have shed some light on the mechanisms of the neuroprotective effect seen in Study II. These results seem to corroborate each other in a logical way, although this is not the same as scientifically proving a correlation between these results. A summary of the results is presented in Table 2.

Table 2. Summary of results.

Study	Main result of preoperative RIPC
I	Faster EEG recovery, beneficial intracerebral biochemical changes
II	Cerebral protection against injury after HCA
III	Better preservation of intracerebral oxygen tension during HCA
IV	Reduced microcirculatory leukocyte accumulation in cerebrocortical vessels

6 Discussion

6.1 Study design

Cardiac surgery and particularly aortic arch surgery always includes a certain risk for neurological sequelae. Open aortic arch surgery almost always requires a bloodless operating field, which necessitates the use of HCA in operations. The risk of neurological impairment is in some cases quite high, especially when corrective aortic surgery has to be performed in emergency and when the patients are very elderly (Biancari *et al.* 2011). This highlights the need for additional neuroprotective methods.

Our research group has used a porcine model described in this thesis for about 12 years in various setups to study many neuroprotective hypotheses. We have tested and researched surgical and CPB strategies that could be useful in a HCA and aortic arch reconstruction setting. In this thesis we have combined chronic surviving animal data with acute experiments aiming to shed light on the mechanisms and efficacy of RIPC as a neuroprotective method.

6.2 Hemodynamic data

Remote ischemic preconditioning was first noted to have cardioprotective effects (Przyklenk *et al.* 1993), and data about such effects are abundant. Our results substantiate this concept. Although not our primary end point, we found larger TnI quantities in control animals both in Study I and Study II supporting the earlier studies that indicated cardioprotective effects of RIPC.

6.3 Neurological data

In Study II we saw a marked improvement in the neuropathological score of the RIPC group compared to controls. In Study I we did not find a similar improvement in histopathological scores. We hypothesized that this could have been a consequence of the acute nature of the study. We did however find in Study I that the RIPC group had a better EEG recovery rate when compared with control animals, and this finding is substantiated in Study II. This could partially be explained by a better respiratory chain function we found in Study IV, although this correlation has to be studied further to be confirmed. Mitochondrial

respiratory function is critical in maintaining neuronal aerobic metabolism. Several studies have shown that mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}) is affected by ischemic preconditioning (Konstantinov *et al.* 2005, Watanabe *et al.* 2008). The effects of ischemic preconditioning can be nullified by blocking this mitoK_{ATP} channel, which suggests that mitoK_{ATP} activation is crucial in propagating neuroprotection (Sack 2006). A better EEG recovery and reduced NADH autofluorescence after RIPC could to some extent both be consequences of the mitochondrial inner membrane depolarization.

In our chronic surviving study (Study II) we gave a daily neurological recovery score for each animal. We found that the RIPC group had a faster recovery of behavioural score postoperatively. Although no similar results have yet been discovered in large animals, some studies carried out with smaller animals, such as rodents, have produced comparable results (Ren *et al.* 2009). The behavioural score is a good secondary end point in assessing neurological recovery, even though its actual clinical correlation to human patients is minimal.

6.4 Intracerebral measurements

6.4.1 EEG

Electroencephalographic monitoring was used in studies I and II. The EEG is an uninvasive method and can be used with comparative ease to assess neurological impairment after trauma and ischemia in humans (Abend *et al.* 2009, Diedler *et al.* 2009). It can also be used with ease to measure burst suppression ratio. Higher burst suppression (low EEG recovery) is indicative of neurological impairment after ischemic events (Myles *et al.* 2009, Pokela *et al.* 2003).

In Study I we found a statistically significant difference in EEG burst suppression between the study groups (Figure 6). The collected data showed higher burst suppression in the control group during the follow-up period. To mitigate disparities caused by the inhalation anesthetic to the EEG burst suppression the isoflurane quantity was kept steady in both groups. In study II we saw similar results in the EEG burst recovery ratio; RIPC produced a faster EEG recovery (i.e. low burst suppression) in both Study I and Study II. Study II also corroborates some of our earlier findings that linked low EEG recovery to brain infarction.

6.4.2 Microdialysis

We collected microdialysis data in studies I and II. Cerebral microdialysis is a method that enables obtaining direct information about the metabolic state of tissue. The data we collected imply that cerebral metabolism is altered by RIPC after HCA.

We found higher glucose levels in the control group after HCA in Study I, but this coincided with a reduced EEG recovery. In our earlier studies we found that low cerebral glucose content was a predictive factor of postoperative death (Pokela 2001). Judging from the EEG data from the same time points in studies I and II, the function and recovery of the cortical neurons were suppressed. We detected a simultaneous glycerol release in the control groups, which could also indicate cellular membrane breakdown (Merenda *et al.* 2008), although it is possible that higher glucose, lactate, and glycerol contents are just an indication of an altered, more anaerobic glucose metabolism (Clausen *et al.* 2011). This thought is supported by Study IV where we found weaker mitochondrial respiratory chain function in the control group indicating some level of anaerobic metabolism.

These findings are further corroborated in studies I and II where we saw that lactate concentrations increased after HCA and towards the end of the follow-up period. In study I, the heightened lactate content coincided with a heightened pyruvate concentration, possibly indicating some level of lactate-based metabolism. Recent research has highlighted lactate as a possible alternative source of energy during high-energy-demand situations in the brain (Overgaard *et al.* 2012).

The microdialysis data of Study I and Study II show that RIPC alters cerebral metabolism with a yet undetermined mechanism. Microdialysis is a valuable additional tool for assessing neurological impairment when combined with other end points. Combined with our EEG data and histopathological data, the microdialysis data support the hypothesis of RIPC as a neuroprotective method.

6.5 Intravital microscopy

In Study IV we used an intravital microscope to directly measure capillary diameter changes and to directly assess leukocyte concentrations and adherence in vivo. There were no statistically significant vascular diameter changes at any time. The observed arterioles were located in the cortical capillary bed on the

surface of the brain and as such they do not play a major role in the autoregulatory vessel diameter changes that occur in deeper cerebral vessels (Yoshino *et al.* 2009).

6.5.1 Leukocytes

Leukocytes are known to become activated via several mechanisms; contact with the extracorporeal circulation tubing, mechanical shear stress, ischemia and inflammatory signals cause activation of leukocytes. We examined leukocyte behaviour directly in vivo using an intravital microscope.

From the start of rewarming to 3 hours postoperatively, RIPC group demonstrated significantly lower number of leukocytes in cerebral microvasculature compared to baseline (Figure 10). Due to the in-vivo intravital microscopy method it was difficult to find identical cerebrocortical vessels between different experiments. There were considerable variations in vessel form, length and width. Therefore we felt it was more prudent to compare the measured leukocytes to the baseline values. The same vessels were used for measurements in one experiment at all the subsequent time points. Difference between groups in cerebral leukocytes equalized at 3 hours. Our findings are limited to the number of leukocytes in the superficial cerebral venules; their function and reactivity were not assessed.

In fact, total white blood cell counts were even higher in RIPC group 1 hour postoperatively to 3 hours postoperatively. This observation might imply that systemic leukocyte count does not reflect the leukocyte amount in the cerebrocortical vessels.

6.5.2 NADH

Regulation of NAD^+/NADH redox state is of utmost importance in maintaining vital cell processes and integrity. NADH crucially partakes in the production of ATP, another vital molecule (Wilhelm & Hirrlinger 2012). Interestingly, cytosolic NADH concentrations affect also Ca^{2+} signalling (Requardt *et al.* 2012) which is crucial mediator in cellular signalling. A higher NADH concentration causes increased Ca^{2+} signalling which in turn can instigate several detrimental effects if the energy metabolism of the cell is disrupted.

The increase in NADH implicates worse tissue oxygenation; thus, the highest NADH autofluorescence levels are measured in anoxic conditions (Chance *et al.*

1973). Our finding in Study IV implies that after weaning from CPB, RIPC contributes to improved preservation of cerebral tissue oxygenation. This coexists with our previous study, in which we showed that RIPC preserves oxygen tension during HCA and may produce better outcome after CPB.

We succeeded in demonstrating the correlation between RIPC and lesser NADH autofluorescence, implicating better tissue oxygenation in the cerebrum. Further studies are needed to explain the linkage between TRI preconditioning and adhesion of the leukocytes and tissue oxygenation.

6.6 Cerebral oxygen tension

In Study III we saw a statistically significant difference in the $PbrO_2$ during HCA between the two study groups. We measured a rapid decrease in $PbrO_2$ after the start of HCA in the control group. The RIPC group did not show a similar pattern; the group maintained higher oxygen tension throughout the circulatory arrest. It is notable that there was no difference in arterial pO_2. This was probably due to the fact that arterial oxygen content was measured from femoral artery samples. Any correlation between pO_2 of the femoral artery and $PbrO_2$ would have been difficult to rationalize.

6.7 Transmission Electron Microscopy

Several cellular organelles were identified and their ultrastructural change quantified. These included the analysis of neuronal mitochondria and RER in both cerebrum and cerebellum. Changes in perivascular endfeet of astrocytes were also assessed and scored. There was only one statistically significant difference in TEM score for ischemia-related ultrastructural changes between the RIPC and control groups. The control group had a statistically significantly higher cerebellar RER score than RIPC group. The clinical relevance of this finding remains unclear, however, as it is an isolated result in our TEM data. It is noteworthy, though, that all animals in the RIPC group in Study IV had ultrastructurally normal RERs. We feel that it would be worthwhile to examine TEM samples from chronic surviving animals.

6.8 Summary

In this thesis we present primary end points such as histopathological and neurological recovery data that support the hypothesis that RIPC has neuroprotective effects. We also have several secondary end points that individually would be too weak to be convincing indicators of any neuroprotective effects of RIPC but in conjunction with other secondary and primary end points present a convincing case that RIPC could have substantial neuroprotective effects.

There are of course some limitations in this thesis that have to be kept in mind. Firstly, the results of this thesis are only based on a small number of animals, 60 animals in total. Secondly, the data are purely from animal experiments. Although results from animal experiments cannot be directly extended to humans they still in some cases give indications about the safety, efficacy and applicability of a proposed intervention. Our large animal model uses the same techniques and instruments that are used in human patients undergoing HCA or aortic arch surgery. Therefore, our results could be used in planning human studies with RIPC.

7 Conclusions

I RIPC provides enhanced cerebral recovery and quicker EEG recovery and seems to alter cerebral metabolism after prolonged HCA.

II RIPC confers significant neuroprotective effects after HCA in an experimental animal model.

III RIPC seems to alter the oxygen usage profile of the brain during HCA maintaining a better cerebral oxygen tension during HCA.

IV RIPC modifies the leukocyte response in the cerebral vessels and leads to a decreased amount of adherent leukocytes in cerebral circulation after HCA. Additionally it seems to provide a better mitochondrial respiratory chain function during late recovery phase.

References

Abend NS, Topjian A, Ichord R, Herman ST, Helfaer M, Donnelly M, Nadkarni V, Dlugos DJ & Clancy RR (2009) Electroencephalographic monitoring during hypothermia after pediatric cardiac arrest. Neurology 72(22): 1931–1940.

Ali ZA, Callaghan CJ, Lim E, Ali AA, Nouraei SA, Akthar AM, Boyle JR, Varty K, Kharbanda RK, Dutka DP & Gaunt ME (2007) Remote ischemic preconditioning reduces myocardial and renal injury after elective abdominal aortic aneurysm repair: a randomized controlled trial. Circulation 116(11 Suppl): I98–105.

Alreja G, Bugano D & Lotfi A (2012) Effect of remote ischemic preconditioning on myocardial and renal injury: meta-analysis of randomized controlled trials. J Invasive Cardiol 24(2): 42–48.

Arroba AI, Alvarez-Lindo N, van Rooijen N & de la Rosa EJ (2011) Microglia-mediated IGF-I neuroprotection in the rd10 mouse model of retinitis pigmentosa. Invest Ophthalmol Vis Sci 52(12): 9124–9130.

Austinat M, Braeuninger S, Pesquero JB, Brede M, Bader M, Stoll G, Renne T & Kleinschnitz C (2009) Blockade of bradykinin receptor B1 but not bradykinin receptor B2 provides protection from cerebral infarction and brain edema. Stroke 40(1): 285–293.

Bae ON, Rajanikant K, Min J, Smith J, Baek SH, Serfozo K, Hejabian S, Lee KY, Kassab M & Majid A (2012) Lymphocyte cell kinase activation mediates neuroprotection during ischemic preconditioning. J Neurosci 32(21): 7278–7286.

Barletta KE, Ley K & Mehrad B (2012) Regulation of neutrophil function by adenosine. Arterioscler Thromb Vasc Biol 32(4): 856–864.

Biancari F, Vasques F, Benenati V & Juvonen T (2011) Contemporary results after surgical repair of type A aortic dissection in patients aged 80 years and older: a systematic review and meta-analysis. Eur J Cardiothorac Surg 40(5): 1058–1063.

Bigelow WG, Callaghan JC & Hopps JA (1950) General hypothermia for experimental intracardiac surgery; the use of electrophrenic respirations, an artificial pacemaker for cardiac standstill and radio-frequency rewarming in general hypothermia. Ann Surg 132(3): 531–539.

Boveris A & Chance B (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 134(3): 707–716.

Bredt DS (1999) Endogenous nitric oxide synthesis: biological functions and pathophysiology. Free Radic Res 31(6): 577–596.

Brittain MK, Brustovetsky T, Sheets PL, Brittain JM, Khanna R, Cummins TR & Brustovetsky N (2012) Delayed calcium dysregulation in neurons requires both the NMDA receptor and the reverse Na+/Ca2+ exchanger. Neurobiol Dis 46(1): 109–117.

Burley DS, Ferdinandy P & Baxter GF (2007) Cyclic GMP and protein kinase-G in myocardial ischaemia-reperfusion: opportunities and obstacles for survival signaling. Br J Pharmacol 152(6): 855–869.

Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA & Stella AM (2007) Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. Nat Rev Neurosci 8(10): 766–775.

Candilio L, Hausenloy DJ & Yellon DM (2011) Remote ischemic conditioning: a clinical trial's update. J Cardiovasc Pharmacol Ther 16(3–4): 304–312.

Chance B, Oshino N, Sugano T & Mayevsky A (1973) Basic principles of tissue oxygen determination from mitochondrial signals. Adv Exp Med Biol 37A: 277–292.

Clausen F, Hillered L & Gustafsson J (2011) Cerebral glucose metabolism after traumatic brain injury in the rat studied by 13C-glucose and microdialysis. Acta Neurochir (Wien) 153(3): 653–658.

Czerny M, Krahenbuhl E, Reineke D, Sodeck G, Englberger L, Weber A, Schmidli J, Kadner A, Erdoes G, Schoenhoff F, Jenni H, Stalder M & Carrel T (2011) Mortality and neurologic injury after surgical repair with hypothermic circulatory arrest in acute and chronic proximal thoracic aortic pathology: effect of age on outcome. Circulation 124(13): 1407–1413.

Dave KR, Saul I, Prado R, Busto R & Perez-Pinzon MA (2006) Remote organ ischemic preconditioning protect brain from ischemic damage following asphyxial cardiac arrest. Neurosci Lett 404(1–2): 170–175.

Davis SM, Albers GW, Diener HC, Lees KR & Norris J (1997) Termination of Acute Stroke Studies Involving Selfotel Treatment. ASSIST Steering Committed. Lancet 349(9044): 32.

Davis SM, Lees KR, Albers GW, Diener HC, Markabi S, Karlsson G & Norris J (2000) Selfotel in acute ischemic stroke : possible neurotoxic effects of an NMDA antagonist. Stroke 31(2): 347–354.

Dickson EW, Lorbar M, Porcaro WA, Fenton RA, Reinhardt CP, Gysembergh A & Przyklenk K (1999a) Rabbit heart can be "preconditioned" via transfer of coronary effluent. Am J Physiol 277(6 Pt 2): H2451–7.

Dickson EW, Reinhardt CP, Renzi FP, Becker RC, Porcaro WA & Heard SO (1999b) Ischemic preconditioning may be transferable via whole blood transfusion: preliminary evidence. J Thromb Thrombolysis 8(2): 123–129.

Diedler J, Sykora M, Bast T, Poli S, Veltkamp R, Mellado P, Steiner T & Rupp A (2009) Quantitative EEG correlates of low cerebral perfusion in severe stroke. Neurocrit Care 2009;11(2):210–6.

Dore S, Otsuka T, Mito T, Sugo N, Hand T, Wu L, Hurn PD, Traystman RJ & Andreasson K (2003) Neuronal overexpression of cyclooxygenase-2 increases cerebral infarction. Ann Neurol 54(2): 155–162.

D'Orsi B, Bonner H, Tuffy LP, Dussmann H, Woods I, Courtney MJ, Ward MW & Prehn JH (2012) Calpains are downstream effectors of bax-dependent excitotoxic apoptosis. J Neurosci 32(5): 1847–1858.

Ehrlich MP, McCullough JN, Zhang N, Weisz DJ, Juvonen T, Bodian CA & Griepp RB (2002) Effect of hypothermia on cerebral blood flow and metabolism in the pig. Ann Thorac Surg 73(1): 191–197.

Ekdahl CT, Kokaia Z & Lindvall O (2009) Brain inflammation and adult neurogenesis: the dual role of microglia. Neuroscience 158(3): 1021–1029.

Erecinska M, Thoresen M & Silver IA (2003) Effects of hypothermia on energy metabolism in Mammalian central nervous system. J Cereb Blood Flow Metab 23(5): 513–530.

Feeney CJ, Frantseva MV, Carlen PL, Pennefather PS, Shulyakova N, Shniffer C & Mills LR (2008) Vulnerability of glial cells to hydrogen peroxide in cultured hippocampal slices. Brain Res 2008 Mar 10; 1198: 1–15.

Formigli L, Papucci L, Tani A, Schiavone N, Tempestini A, Orlandini GE, Capaccioli S & Orlandini SZ (2000) Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. J Cell Physiol 182(1): 41–49.

Gao X, Zhang H, Steinberg G & Zhao H (2010) The Akt pathway is involved in rapid ischemic tolerance in focal ischemia in rats. Transl Stroke Res 1(3): 202–209.

Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ & Verdouw PD (1996a) Myocardial protection by brief ischemia in noncardiac tissue. Circulation 94(9): 2193–2200.

Gibbon JH Jr, Miller BJ, Dobbel AR, Engell HC & Voigt GB (1954) The closure of interventricular septal defects in dogs during open cardiotomy with the maintenance of the cardiorespiratory functions by a pump-oxygenator. J Thorac Surg 28(3): 235–240.

Gleichmann M, Collis LP, Smith PJ & Mattson MP (2009) Simultaneous single neuron recording of O2 consumption, [Ca2+]i and mitochondrial membrane potential in glutamate toxicity. J Neurochem 109(2): 644–655.

Green AJ, Keir G & Thompson EJ (1997) A specific and sensitive ELISA for measuring S-100b in cerebrospinal fluid. J Immunol Methods 205(1): 35–41.

Harper AM (1966) Autoregulation of cerebral blood flow: influence of the arterial blood pressure on the blood flow through the cerebral cortex. J Neurol Neurosurg Psychiatry 29(5): 398–403.

Hausenloy DJ & Yellon DM (2004) New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. Cardiovasc Res 61(3): 448–460.

Hong DM, Jeon Y, Lee CS, Kim HJ, Lee JM, Bahk JH, Kim KB & Hwang HY (2012) Effects of remote ischemic preconditioning with postconditioning in patients undergoing off-pump coronary artery bypass surgery--randomized controlled trial. Circ J 76(4): 884–890.

Hu S, Dong H, Zhang H, Wang S, Hou L, Chen S, Zhang J & Xiong L (2012) Noninvasive limb remote ischemic preconditioning contributes neuroprotective effects via activation of adenosine A1 receptor and redox status after transient focal cerebral ischemia in rats. Brain Res 1459: 81–90.

Hur J, Lee P, Kim MJ, Kim Y & Cho YW (2010) Ischemia-activated microglia induces neuronal injury via activation of gp91phox NADPH oxidase. Biochem Biophys Res Commun 391(3): 1526–1530.

Iadecola C (1997) Bright and dark sides of nitric oxide in ischemic brain injury. Trends Neurosci 20(3): 132–139.

Jensen HA, Loukogeorgakis S, Yannopoulos F, Rimpilainen E, Petzold A, Tuominen H, Lepola P, Macallister RJ, Deanfield JE, Makela T, Alestalo K, Kiviluoma K, Anttila V, Tsang V & Juvonen T (2011) Remote ischemic preconditioning protects the brain against injury after hypothermic circulatory arrest. Circulation 123(7): 714–721.

Johansen FF, Jorgensen MB, Ekstrom von Lubitz DK & Diemer NH (1984) Selective dendrite damage in hippocampal CA1 stratum radiatum with unchanged axon ultrastructure and glutamate uptake after transient cerebral ischaemia in the rat. Brain Res 291(2): 373–377.

Kerr JF, Wyllie AH & Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26(4): 239–257.

Kharbanda RK, Mortensen UM, White PA, Kristiansen SB, Schmidt MR, Hoschtitzky JA, Vogel M, Sorensen K, Redington AN & MacAllister R (2002) Transient limb ischemia induces remote ischemic preconditioning in vivo. Circulation 106(23): 2881–2883.

Kielian T, Barry B & Hickey WF (2001) CXC chemokine receptor-2 ligands are required for neutrophil-mediated host defense in experimental brain abscesses. J Immunol 166(7): 4634–4643.

Kim EJ, Raval AP, Hirsch N & Perez-Pinzon MA (2010) Ischemic preconditioning mediates cyclooxygenase-2 expression via nuclear factor-kappa B activation in mixed cortical neuronal cultures. Transl Stroke Res 1(1): 40–47.

Kim JK, Choi JW, Lim S, Kwon O, Seo JK, Ryu SH & Suh PG (2011) Phospholipase C-eta1 is activated by intracellular Ca(2+) mobilization and enhances GPCRs/PLC/Ca(2+) signaling. Cell Signal 23(6): 1022–1029.

Kirino T & Sano K (1984) Selective vulnerability in the gerbil hippocampus following transient ischemia. Acta Neuropathol 62(3): 201–208.

Kishimoto K, Li RC, Zhang J, Klaus JA, Kibler KK, Dore S, Koehler RC & Sapirstein A (2010) Cytosolic phospholipase A2 alpha amplifies early cyclooxygenase-2 expression, oxidative stress and MAP kinase phosphorylation after cerebral ischemia in mice. J Neuroinflammation 7: 42.

Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K & Mikoshiba K (1990) 'Ischemic tolerance' phenomenon found in the brain. Brain Res 528(1): 21–24.

Konstantinov IE, Arab S, Kharbanda RK, Li J, Cheung MM, Cherepanov V, Downey GP, Liu PP, Cukerman E, Coles JG & Redington AN (2004) The remote ischemic preconditioning stimulus modifies inflammatory gene expression in humans. Physiol Genomics 19(1): 143–150.

Konstantinov IE, Li J, Cheung MM, Shimizu M, Stokoe J, Kharbanda RK & Redington AN (2005) Remote ischemic preconditioning of the recipient reduces myocardial ischemia-reperfusion injury of the denervated donor heart via a Katp channel-dependent mechanism. Transplantation 79(12): 1691–1695.

Kroemer G & Reed JC (2000) Mitochondrial control of cell death. Nat Med 6(5): 513–519.

Lee JY, Kim YJ, Kim TY, Koh JY & Kim YH (2008) Essential role for zinc-triggered p75NTR activation in preconditioning neuroprotection. J Neurosci 28(43): 10919–10927.

Liang J, Wang J, Saad Y, Warble L, Becerra E & Kolattukudy PE (2011) Participation of MCP-induced protein 1 in lipopolysaccharide preconditioning-induced ischemic stroke tolerance by regulating the expression of proinflammatory cytokines. J Neuroinflammation 8: 182.

Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, Loscalzo J, Singel DJ & Stamler JS (1993) A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature 364(6438): 626–632.

Loukogeorgakis SP, Williams R, Panagiotidou AT, Kolvekar SK, Donald A, Cole TJ, Yellon DM, Deanfield JE & MacAllister RJ (2007) Transient limb ischemia induces remote preconditioning and remote postconditioning in humans by a K(ATP)-channel dependent mechanism. Circulation 116(12): 1386–1395.

Lumenta DB, Plesnila N, Klasner B, Baethmann A, Pruneau D, Schmid-Elsaesser R & Zausinger S (2006) Neuroprotective effects of a postischemic treatment with a bradykinin B2 receptor antagonist in a rat model of temporary focal cerebral ischemia. Brain Res 1069(1): 227–234.

Ma H, Huang X, Li Q, Guan Y, Yuan F & Zhang Y (2011) ATP-dependent potassium channels and mitochondrial permeability transition pores play roles in the cardioprotection of theaflavin in young rat. J Physiol Sci 61(4): 337–342.

Maciel EN, Vercesi AE & Castilho RF (2001) Oxidative stress in Ca(2+)-induced membrane permeability transition in brain mitochondria. J Neurochem 79(6): 1237–1245.

Malhotra S, Naggar I, Stewart M & Rosenbaum DM (2011a) Neurogenic pathway mediated remote preconditioning protects the brain from transient focal ischemic injury. Brain Res 1386: 184–190.

McCullough JN, Zhang N, Reich DL, Juvonen TS, Klein JJ, Spielvogel D, Ergin MA & Griepp RB (1999) Cerebral metabolic suppression during hypothermic circulatory arrest in humans. Ann Thorac Surg 67(6): 1895–9; discussion 1919–21.

McTigue DM & Tripathi RB (2008) The life, death, and replacement of oligodendrocytes in the adult CNS. J Neurochem 107(1): 1–19.

Merenda A, Gugliotta M, Holloway R, Levasseur JE, Alessandri B, Sun D & Bullock MR (2008) Validation of brain extracellular glycerol as an indicator of cellular membrane damage due to free radical activity after traumatic brain injury. J Neurotrauma 25(5): 527–537.

Miyawaki T, Mashiko T, Ofengeim D, Flannery RJ, Noh KM, Fujisawa S, Bonanni L, Bennett MV, Zukin RS & Jonas EA (2008) Ischemic preconditioning blocks BAD translocation, Bcl-xL cleavage, and large channel activity in mitochondria of postischemic hippocampal neurons. Proc Natl Acad Sci U S A 105(12): 4892–4897.

Mizoguchi Y, Monji A, Kato T, Seki Y, Gotoh L, Horikawa H, Suzuki SO, Iwaki T, Yonaha M, Hashioka S & Kanba S (2009) Brain-derived neurotrophic factor induces sustained elevation of intracellular Ca2+ in rodent microglia. J Immunol 183(12): 7778–7786.

Murry CE, Jennings RB & Reimer KA (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74(5): 1124–1136.

Myles PS, Daly D, Silvers A & Cairo S (2009) Prediction of neurological outcome using bispectral index monitoring in patients with severe ischemic-hypoxic brain injury undergoing emergency surgery. Anesthesiology 110(5): 1106–1115.

Nayak GH, Prentice HM & Milton SL (2011) Neuroprotective signaling pathways are modulated by adenosine in the anoxia tolerant turtle. J Cereb Blood Flow Metab 31(2): 467–475.

Niazi SA & Lewis FJ (1957) Profound hypothermia in the monkey with recovery after long periods of cardiac standstill. J Appl Physiol 10(1): 137–138.

Niizuma K, Endo H & Chan PH (2009) Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival. J Neurochem 109 Suppl 1: 133–138.

Oberheim NA, Wang X, Goldman S & Nedergaard M (2006) Astrocytic complexity distinguishes the human brain. Trends Neurosci 29(10): 547–553.

Obrenovitch TP, Garofalo O, Harris RJ, Bordi L, Ono M, Momma F, Bachelard HS & Symon L (1988) Brain tissue concentrations of ATP, phosphocreatine, lactate, and tissue pH in relation to reduced cerebral blood flow following experimental acute middle cerebral artery occlusion. J Cereb Blood Flow Metab 8(6): 866–874.

Olsen TS, Larsen B, Herning M, Skriver EB & Lassen NA (1983) Blood flow and vascular reactivity in collaterally perfused brain tissue. Evidence of an ischemic penumbra in patients with acute stroke. Stroke 14(3): 332–341.

Overgaard M, Rasmussen P, Bohm AM, Seifert T, Brassard P, Zaar M, Homann P, Evans KA, Nielsen HB & Secher NH (2012) Hypoxia and exercise provoke both lactate release and lactate oxidation by the human brain. FASEB J 26(7): 3012–3020.

Parpura V, Heneka MT, Montana V, Oliet SH, Schousboe A, Haydon PG, Stout RF,Jr, Spray DC, Reichenbach A, Pannicke T, Pekny M, Pekna M, Zorec R & Verkhratsky A (2012) Glial cells in (patho)physiology. J Neurochem 121(1): 4–27.

Pedersen CM, Schmidt MR, Barnes G, Botker HE, Kharbanda RK, Newby DE & Cruden NL (2011) Bradykinin does not mediate remote ischaemic preconditioning or ischaemia-reperfusion injury in vivo in man. Heart 97(22): 1857–1861.

Peters-Golden M, Song K, Marshall T & Brock T (1996) Translocation of cytosolic phospholipase A2 to the nuclear envelope elicits topographically localized phospholipid hydrolysis. Biochem J 318(Pt 3): 797–803.

Petzold A, Keir G, Green AJ, Giovannoni G & Thompson EJ (2003) A specific ELISA for measuring neurofilament heavy chain phosphoforms. J Immunol Methods 278(1–2): 179–190.

Pokela M, Jantti V, Lepola P, Romsi P, Rimpilainen J, Kiviluoma K, Salomaki T, Vainionpaa V, Biancari F, Hirvonen J, Kaakinen T & Juvonen T (2003) EEG burst recovery is predictive of brain injury after experimental hypothermic circulatory arrest. Scand Cardiovasc J 37(3): 154–157.

Przyklenk K, Bauer B, Ovize M, Kloner RA & Whittaker P (1993) Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. Circulation 87(3): 893–899.

Redington KL, Disenhouse T, Strantzas SC, Gladstone R, Wei C, Tropak MB, Dai X, Manlhiot C, Li J & Redington AN (2012) Remote cardioprotection by direct peripheral nerve stimulation and topical capsaicin is mediated by circulating humoral factors. Basic Res Cardiol 107(2): 241-011-0241-5. Epub 2012 Jan 10.

Rehni AK, Singh N & Jaggi AS (2007) Possible involvement of insulin, endogenous opioids and calcitonin gene-related peptide in remote ischaemic preconditioning of the brain. Yakugaku Zasshi 127(6): 1013–1020.

Ren C, Gao X, Steinberg GK & Zhao H (2008) Limb remote-preconditioning protects against focal ischemia in rats and contradicts the dogma of therapeutic time windows for preconditioning. Neuroscience 151(4): 1099–1103.

Ren C, Yan Z, Wei D, Gao X, Chen X & Zhao H (2009) Limb remote ischemic postconditioning protects against focal ischemia in rats. Brain Res 1288: 88–94.

Requardt RP, Hirrlinger PG, Wilhelm F, Winkler U, Besser S & Hirrlinger J (2012) Ca(2)(+) signals of astrocytes are modulated by the NAD(+)/NADH redox state. J Neurochem 120(6): 1014–1025.

Ribeiro Mde C, Hirt L, Bogousslavsky J, Regli L & Badaut J (2006) Time course of aquaporin expression after transient focal cerebral ischemia in mice. J Neurosci Res 83(7): 1231–1240.

Rizzuto R, Simpson AW, Brini M & Pozzan T (1992) Rapid changes of mitochondrial Ca2+ revealed by specifically targeted recombinant aequorin. Nature 358(6384): 325–327.

Robin E, Simerabet M, Hassoun SM, Adamczyk S, Tavernier B, Vallet B, Bordet R & Lebuffe G (2011) Postconditioning in focal cerebral ischemia: role of the mitochondrial ATP-dependent potassium channel. Brain Res 1375: 137–146.

Ronaldson PT & Davis TP (2012) Blood-brain barrier integrity and glial support: mechanisms that can be targeted for novel therapeutic approaches in stroke. Curr Pharm Des 18(25): 3624–3644.

Ruscher K, Freyer D, Karsch M, Isaev N, Megow D, Sawitzki B, Priller J, Dirnagl U & Meisel A (2002) Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model. J Neurosci 22(23): 10291–10301.

Sack MN (2006) Mitochondrial depolarization and the role of uncoupling proteins in ischemia tolerance. Cardiovasc Res 72(2): 210–219.

Saino O, Taguchi A, Nakagomi T, Nakano-Doi A, Kashiwamura S, Doe N, Nakagomi N, Soma T, Yoshikawa H, Stern DM, Okamura H & Matsuyama T (2010) Immunodeficiency reduces neural stem/progenitor cell apoptosis and enhances neurogenesis in the cerebral cortex after stroke. J Neurosci Res 88(11): 2385–2397.

Savill J (1997) Recognition and phagocytosis of cells undergoing apoptosis. Br Med Bull 53(3): 491–508.

Saxena P, Bala A, Campbell K, Meloni B, d'Udekem Y & Konstantinov IE (2009) Does remote ischemic preconditioning prevent delayed hippocampal neuronal death following transient global cerebral ischemia in rats? Perfusion 24(3): 207–211.

Schild L & Reiser G (2005) Oxidative stress is involved in the permeabilization of the inner membrane of brain mitochondria exposed to hypoxia/reoxygenation and low micromolar Ca2+. FEBS J 272(14): 3593–3601.

Shimizu M, Saxena P, Konstantinov IE, Cherepanov V, Cheung MM, Wearden P, Zhangdong H, Schmidt M, Downey GP & Redington AN (2010) Remote ischemic preconditioning decreases adhesion and selectively modifies functional responses of human neutrophils. J Surg Res 158(1): 155–161.

Stanika RI, Villanueva I, Kazanina G, Andrews SB & Pivovarova NB (2012) Comparative Impact of Voltage-Gated Calcium Channels and NMDA Receptors on Mitochondria-Mediated Neuronal Injury. J Neurosci 32(19): 6642–6650.

Starkov AA, Chinopoulos C & Fiskum G (2004) Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury. Cell Calcium 36(3–4): 257–264.

Stoll G, Jander S & Schroeter M (1998) Inflammation and glial responses in ischemic brain lesions. Prog Neurobiol 56(2): 149–171.

Su B, Dong H, Ma R, Zhang X, Ding Q & Xiong L (2009) Cannabinoid 1 receptor mediation of spinal cord ischemic tolerance induced by limb remote ischemia preconditioning in rats. J Thorac Cardiovasc Surg 138(6): 1409–1416.

Su J, Tang Y, Zhou H, Liu L & Dong Q (2012) Tissue kallikrein protects neurons from hypoxia/reoxygenation-induced cell injury through Homer1b/c. Cell Signal 24(11): 2205–2215.

Theodoraki K, Tympa A, Karmaniolou I, Tsaroucha A, Arkadopoulos N & Smyrniotis V (2011) Ischemia/reperfusion injury in liver resection: a review of preconditioning methods. Surg Today 41(5): 620–629.

Treasure T (1984) The safe duration of total circulatory arrest with profound hypothermia. Ann R Coll Surg Engl 66(4): 235–240.

Tropak MB, Shi H, Li J, Dai X, Redington AN & Askalan R (2011) Potent neuroprotection induced by remote preconditioning in a rat model of neonatal cerebral hypoxic-ischemic injury. J Thorac Cardiovasc Surg 142(1): 233–235.

Vinet J, Weering HR, Heinrich A, Kalin RE, Wegner A, Brouwer N, Heppner FL, Rooijen N, Boddeke HW & Biber K (2012) Neuroprotective function for ramified microglia in hippocampal excitotoxicity. J Neuroinflammation 9: 27.

Vlasov TD, Korzhevskii DE & Polyakova EA (2005) Ischemic preconditioning of the rat brain as a method of endothelial protection from ischemic/repercussion injury. Neurosci Behav Physiol 35(6): 567–572.

Voulgari-Kokota A, Fairless R, Karamita M, Kyrargyri V, Tseveleki V, Evangelidou M, Delorme B, Charbord P, Diem R & Probert L (2012) Mesenchymal stem cells protect CNS neurons against glutamate excitotoxicity by inhibiting glutamate receptor expression and function. Exp Neurol 236(1): 161–170.

Waldner MJ, Baethmann A, Uhl E & Lehmberg J (2012) Bradykinin-induced leukocyte- and platelet-endothelium interactions in the cerebral microcirculation. Brain Res 1448: 163–169.

Wang GJ, Randall RD & Thayer SA (1994) Glutamate-induced intracellular acidification of cultured hippocampal neurons demonstrates altered energy metabolism resulting from Ca2+ loads. J Neurophysiol 72(6): 2563–2569.

Wang L, Zhu QL, Wang GZ, Deng TZ, Chen R, Liu MH & Wang SW (2011) The protective roles of mitochondrial ATP-sensitive potassium channels during hypoxia-ischemia-reperfusion in brain. Neurosci Lett 491(1): 63–67.

Watanabe M, Katsura K, Ohsawa I, Mizukoshi G, Takahashi K, Asoh S, Ohta S & Katayama Y (2008) Involvement of mitoKATP channel in protective mechanisms of cerebral ischemic tolerance. Brain Res 1238: 199–207.

Wilhelm F & Hirrlinger J (2012) Multifunctional Roles of NAD(+) and NADH in Astrocytes. Neurochem Res 37(11): 2317–2325.

Wroge CM, Hogins J, Eisenman L & Mennerick S (2012) Synaptic NMDA Receptors Mediate Hypoxic Excitotoxic Death. J Neurosci 32(19): 6732–6742.

Wyss MT, Jolivet R, Buck A, Magistretti PJ & Weber B (2011) In vivo evidence for lactate as a neuronal energy source. J Neurosci 31(20): 7477–7485.

Yakovlev AG & Faden AI (2004) Mechanisms of neural cell death: implications for development of neuroprotective treatment strategies. NeuroRx 1(1): 5–16.

Yamashima T & Oikawa S (2009) The role of lysosomal rupture in neuronal death. Prog Neurobiol 89(4): 343–358.

Yang Y, Ke-Zhou L, Ning GM, Wang ML & Zheng XX (2008) Dynamics of nitric oxide and peroxynitrite during global brain ischemia/reperfusion in rat hippocampus: NO-sensor measurement and modeling study. Neurochem Res 33(1): 73–80.

Yannopoulos FS, Makela T, Niemela E, Tuominen H, Lepola P, Alestalo K, Kaakinen H, Kiviluoma K, Anttila V & Juvonen T (2010) Improved cerebral recovery from hypothermic circulatory arrest after remote ischemic preconditioning. Ann Thorac Surg 90(1): 182–188.

Yao L, Wong GT, Xia Z & Irwin MG (2011) Interaction between spinal opioid and adenosine receptors in remote cardiac preconditioning: effect of intrathecal morphine. J Cardiothorac Vasc Anesth 25(3): 444–448.

Yoshino H, Sakurai T, Oizumi XS, Akisaki T, Wang X, Yokono K, Kondoh T, Kohmura E & Umentani K (2009) Dilation of perforating arteries in rat brain in response to systemic hypotension is more sensitive and pronounced than that of pial arterioles: simultaneous visualization of perforating and cortical vessels by in-vivo microangiography. Microvasc Res 77(2): 230–233.

Zadran S, Bi X & Baudry M (2010) Regulation of calpain-2 in neurons: implications for synaptic plasticity. Mol Neurobiol 42(2): 143–150.

Zhang N, Yin Y, Han S, Jiang J, Yang W, Bu X & Li J (2011) Hypoxic preconditioning induced neuroprotection against cerebral ischemic injuries and its cPKCgamma-mediated molecular mechanism. Neurochem Int 58(6): 684–692.

Zoratti M & Szabo I (1995) The mitochondrial permeability transition. Biochim Biophys Acta 1241(2): 139–176.

Original publications

I Yannopoulos FS, Mäkelä T, Niemelä E, Tuominen H, Lepola P, Alestalo K, Kaakinen H, Kiviluoma K, Anttila V & Juvonen T (2010) Improved cerebral recovery from hypothermic circulatory arrest after remote ischemic preconditioning. Ann Thorac Surg 90(1): 182–188.

II Jensen HA, Loukogeorgakis S, Yannopoulos F, Rimpiläinen E, Petzold A, Tuominen H, Lepola P, Macallister RJ, Deanfield JE, Mäkelä T, Alestalo K, Kiviluoma K, Anttila V, Tsang V & Juvonen T (2011) Remote ischemic preconditioning protects the brain against injury after hypothermic circulatory arrest. Circulation 123(7): 714–721.

III Yannopoulos F, Mäkelä T, Arvola O, Haapanen H, Anttila V, Kiviluoma K & Juvonen T (2012) Remote ischemic precondition preserves cerebral oxygen tension during hypothermic circulatory arrest. Scand Cardiovasc J 46(4): 243–248.

IV Yannopoulos F, Arvola A, Haapanen H, Jensen H, Herajärvi J, Miinalainen I, Kiviluoma K & Juvonen T (2013) Leg ischemia before circulatory arrest alters brain leukocyte count and respiratory chain redox state. Manuscript.

Original publications are not included in the electronic version of the dissertation.

1186. Härkönen, Pirjo (2012) Elämäntyytyväisyys ja terveys : Voimavarasuuntautunut ikääntyvien henkilöiden seurantatutkimus

1187. Laukkanen, Päivi (2012) Occurrence of high risk human papillomaviruses and cervical cancer among fertile-aged women in Finland

1188. Junttila, Eija (2012) Cardiovascular abnormalities after non-traumatic intracranial hemorrhage

1189. Hookana, Eeva (2012) Characteristics of victims of non-ischemic sudden cardiac death

1190. Murugan, Subramanian (2012) Control of nephrogenesis by Wnt4 signaling : mechanisms of gene regulation and targeting of specific lineage cells by tissue engineering tools

1191. Nikkilä, Jenni (2013) *PALB2* and *RAP80* genes in hereditary breast cancer predisposition

1192. Määttä, Mikko (2012) Assessment of osteoporosis and fracture risk : axial transmission ultrasound and lifestyle-related risk factors

1193. Kaakinen, Marika (2012) Genetic and life course determinants of cardiovascular risk factors : structural equation modelling of complex relations

1194. Nevalainen, Mika (2012) Gene product targeting into and membrane trafficking from the endoplasmic/sarcoplasmic reticulum in skeletal myofibers

1195. Meriläinen, Sanna (2013) Experimental study of acute pancreatitis in a porcine model, especially tight junction structure and portal vein cytokines

1196. Koivikko, Minna (2013) Cardiac autonomic regulation and repolarisation during hypoglycaemia in type 1 diabetes

1197. Aro, Ellinoora (2013) Prolyl 4-hydroxylases, key enzymes regulating hypoxia response and collagen synthesis : the roles of specific isoenzymes in the control of erythropoiesis and skeletogenesis

1198. Koivumäki, Janne (2013) Biomechanical modeling of proximal femur : development of finite element models to simulate fractures

1199. Paavola, Liisa (2013) Salla disease – rare but diverse : a clinical follow-up study of a Finnish patient sample

1200. Vesterinen, Soili (2013) Osastonhoitajien johtamistyylit osana johtamiskulttuuria

1201. Huusko, Tuija (2013) Genetic and molecular background of ascending aortic aneurysms

Book orders:
Granum: Virtual book store
http://granum.uta.fi/granum/

UNIVERSITY OF OULU P.O.B. 7500 FI-90014 UNIVERSITY OF OULU FINLAND

ACTA UNIVERSITATIS OULUENSIS

ISBN 978-952-62-0130-6 (Paperback)
ISBN 978-952-62-0131-3 (PDF)
ISSN 0355-3221 (Print)
ISSN 1796-2234 (Online)

UNIVERSITY of OULU
OULUN YLIOPISTO