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Cerebral Protection in Experimental Cardiopulmonary Resuscitation

*With Special Reference to the Effects
of Methylene Blue*

ADRIANA MICLESCU

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2009

ISSN 1651-6206
ISBN 978-91-554-7566-6
urn:nbn:se:uu:diva-106831

Dissertation presented at Uppsala University to be publicly examined in Hedstrandsalen, Akademiska Sjukhuset, entrance no. 70, Uppsala, Friday, September 11, 2009 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Miclescu, A. 2009. Cerebral Protection in Experimental Cardiopulmonary Resuscitation. With Special Reference to the Effects of Methylene Blue. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 466. 79 pp. Uppsala. ISBN 978-91-554-7566-6.

Although survival rates are increasing, brain injury continues to be a leading cause of death after cardiac arrest (CA). Permanent brain damage after CA is determined by limited tolerance to ischemia from CA and cardiopulmonary resuscitation (CPR), as well as the unique cerebral response to reperfusion after return of spontaneous circulation (ROSC). A major pathway leading to neurotoxic cascade and neuronal injury after CA involves the increased presence of reactive oxygen and nitrogen species generated during ischemia and reperfusion. The magnitude of cerebral oxidative injury induced by free radicals increased with the duration of CA (*Paper I*). Nitric oxide (NO), a free radical responsible for the formation of reactive nitrogen species, is increased during global ischemia from CA and reperfusion (*Paper IV*). Hypothetically, the administration of a drug that counteracts the overproduction of NO and also acts as a scavenger of oxygen free radicals might be warranted in order to reduce the damage caused by nitrosative and oxidative stress. For these purposes we used methylene blue (MB), an old dye that has been used in medicine for almost half a century, and an experimental pig model of 20 min of ventricular fibrillation (VF) to reflect a clinical scenario of ischemia/reperfusion injury. Administration of MB added to a hypertonic-hyperoncotic solution (MBHSD) that was started during CPR and continued for 50 min after ROSC increased short-term survival by decreasing myocardial damage, as well as cerebral peroxidation and inflammatory injury (*Paper II*). Immunostaining of cerebral tissue collected at different time points after CA and ROSC (*Paper IV*) provided experimental evidence that cortical blood-brain barrier (BBB) disruption begins as early as during the initial phase of untreated as well as treated CA. The results indicated that MB administration reduced the neurologic injury and BBB disruption considerably, but did not reverse the ongoing detrimental processes. The demonstrated positive effects of MB were related to a decrease of nitrite/nitrate tissue content, and thus to a decrease of excess NO due to the MB inhibitory effects on NOS isoforms. A mixture of MB in hypertonic sodium lactate (MBL) was investigated to facilitate administration of MB in "the field." Based on findings that MBL cardio- and neuroprotective properties were similar to those of MBHSD, there is reason to believe that the use of MBL might be extended during ongoing CPR and after ROSC (*Paper III*). It would therefore make sense to try using MB as a pharmacological neuroprotectant during or after clinical CPR in order to expand the temporal therapeutic window before other measures for neuroprotection such as hypothermia are available.

Keywords: Cardiac arrest, cardiopulmonary resuscitation, reperfusion injury, methylene blue, nitric oxide, nitric oxide synthases, blood-brain barrier

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ISSN 1651-6206

ISBN 978-91-554-7566-6

urn:nbn:se:uu:diva-106831 (<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-106831>)

"NOTHING HAPPENS...BUT FIRST A DREAM"

CARL SANDBURG

To my family

List of papers

This thesis is based on the following papers, which will refer in the text by their Roman numerals:

- I. Basu S, Liu X, Nozari A, Rubertsson S, Miclescu A, Wiklund L: **Evidence for time-dependent maximum increase of free radical damage and eicosanoid formation in the brain as related to duration of cardiac arrest and cardio-pulmonary resuscitation.** *Free Radic Res.* 2003; 37(3):251-6
- II. Miclescu A, Basu S, Wiklund L: **Methylene blue added to a hypertonic-hyperoncotic solution increases short-term survival in experimental cardiac arrest.** *Crit Care Med* 2006; 34(11): 2806-2813
- III. Miclescu A, Basu S, Wiklund L: **Cardio-cerebral and metabolic effects of methylene blue in hypertonic sodium lactate during experimental cardiopulmonary resuscitation.** *Resuscitation* 2007; 75(1):88-97
- IV. Miclescu A, Sharma HS, Cécile Martijn, Wiklund L: **Methylene blue administration during cardiopulmonary resuscitation and early reperfusion protects against blood-brain barrier disruption.** *Submitted*

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Additional publications during my PhD studies, which are not included in this thesis:

- Samar Basu, Adriana Miculescu, Hari S. Sharma, Lars Wiklund: **Propofol lessens “*in situ*” oxidative injury during experimental cardiopulmonary resuscitation.** *Submitted*
- Adriana Miculescu, Torsten Gordh: **Nitric oxide and pain “Something old, something new”.** *Acta Anaesth Scand* 2009; 1-14 (in press)
- Lars Wiklund, Samar Basu , Adriana Miculescu , Per Wiklund, Gunnar Ronquist and Hari S. Sharma: **Neuro- and cardioprotective effects of blockade of nitric oxide action by administration of methylene blue.** *Ann NY Acad Sci* 2007; 1122:231-244
- Miculescu A, Wiklund L: **Finding “solutions“ in CPR: Methylene blue-hypertonic sodium lactate.** *Resuscitation* 2006; 69(1):74
- Miculescu A, Wiklund L: **Methylene blue added to a hypertonic-hyperoncotic solution increases survival in experimental cardiac arrest.** *Acta Anaesth Scand* 2005; 49(117):16
- L.Wiklund, H.S. Sharma, A. Miculescu: **Circulatory arrest as a model for studies of global ischemic injuries and neuroprotection.** *Int J Neuroprotec Neurororegen* 2005; 1(3):187-201

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Abbreviations

- AA
- AMPA
- ANOVA
- BE
- CBF
- CCBF
- CCP
- cGMP
- CePP
- CI
- CK-MB
- CO
- COCR
- COX-2
- CPR
- CVP
- DC
- DO₂
- ECG
- Fr
- FiO₂
- h
- Hb
- HR
- HSD
- kg
- kPa
- LDF
- Log
- LVSWI
- MAP
- MB
- MBHSD
- Arachidonic acid
- α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
- Analysis of variance
- Base excess
- Cerebral blood flow
- Cerebral cortical blood flow
- Coronary perfusion pressure
- Cyclic guanosine monophosphate
- Cerebral perfusion pressure
- Cardiac index
- Creatinin kinase isoenzyme MB
- Cardiac output
- Cerebral oxygen consumption ratio
- Cyclooxygenase 2
- Cardiopulmonary and cerebral resuscitation
- Cardiopulmonary resuscitation
- Central venous pressure
- Defibrillation
- Oxygen delivery
- Electrocardiography
- French
- Inspired fraction of oxygen
- Hour
- Hemoglobin
- Heart rate
- Hypertonic saline dextran
- Kilogram
- Kilopascal
- Laser-Doppler Flow
- Logarithm
- Left ventricular stroke work index
- Mean arterial pressure
- Methylene blue
- Methylene blue hypertonic saline dextran

- MBL
- MBS
- mg
- min
- mL
- mmHg
- mmol
- MPAP
- ng
- NMDA
- NO
- NOS
- O_2^-
- $ONOO^-$
- PaO_2
- PBN
- PCWP
- PEEP
- pg
- PG
- Prot S100 β
- PVRI
- ROS
- ROSC
- RVSWI
- Sal
- SaO_2
- SD
- SE
- S-PBN
- SSP
- SvO_2
- SVRI
- VF
- VO_2
- Temp
- μg
- 8-iso-PGF $_{2\alpha}$
- 15-keto-dihydro-PGF $_{2\alpha}$
- Methylene blue lactate
- Methylene blue saline
- Milligram
- Minute(s)
- Milliliter
- Millimeter mercury
- Millimol
- Mean pulmonary arterial pressure
- Nanogram
- N-methyl-D-aspartate
- Nitric oxide
- Nitric oxide synthase
- Superoxide anions
- Peroxynitrite
- Arterial partial pressure of oxygen
- α -phenyl-tert-butyl nitron
- Pulmonary capillary wedge pressure
- Positive end-expiratory pressure
- Picogram
- Prostaglandin
- Protein S-100 beta
- Pulmonary vascular resistance index
- Reactive oxygen species
- Restoration of spontaneous circulation
- Right ventricular stroke work index
- Saline
- Arterial oxygen saturation
- Standard deviation
- Standard error of the mean
- Sodium 2-sulphophenyl-N-tert-butyl nitron
- Sagittal sinus pressure
- Venous oxygen saturation
- Systemic vascular resistance index
- Ventricular fibrillation
- Oxygen consumption
- Temperature
- Microgram
- 8-iso-prostaglandin $F_{2\alpha}$
- 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$

Introduction

From the depths of sorrow following the death of a loved one arose the desire to reverse that death. But responding to the plea of so many who experience the grief caused by death, voiced in Shakespeare's King Henry VI as 'O, that I could but call these dead to life!' [1, 2] was not easy. Reversing sudden death probably started in Egypt three and a half millennia ago, with methods resembling resuscitative efforts such as when Isis restored the life of her husband Osiris by breathing into his mouth. The first description of resuscitation is probably found in Exodus, when Hebrew midwife Puah "breathed into the baby's mouth to cause the baby to cry". In order to prevent death from taking a person, attempts at resuscitation included warming, whipping, both in early times and later in the 19th century, and freezing the body under a layer of snow and ice, as well as many other methods [3]. Eventually, scientists took interest in the issue of "curing death", and they contributed both human errors and human discoveries to the subject. Modern cardiopulmonary resuscitation (CPR) was based on two major principles, closed chest cardiac massage to restore the blood flow and artificial ventilation to maintain vital gas exchange, both of them pioneered in the 1950s [4, 5]. The enormous potential for chaos related to cardiac arrest (CA) was reduced by introduction of these two major resuscitation attempts to restore the "no-flow" period and start the heart. Unfortunately, CPR did not live up to its early promise. Despite so many efforts and years of research, attempts at resuscitation yielded suboptimal results and regaining a pulse was not enough [6]. Epidemiological data [6] failed to support the expected improvements in hospital discharge following the advances made in critical care during the past half century. Although survival rates are increasing, complete neurologic recovery is often far from certain, and brain injury is the cause of death in 68% of patients after out-of-hospital CA and 23% after in-hospital CA [7]. The scientists who initially thought that return of spontaneous circulation (ROSC) was the "Eureka!"* moment were quickly disappointed when they found out that successful cardiac resuscitation always has "several components that should be tackled and solved" [8], one of which is to mitigate the "reflow" damages. Really convoluted and complicated issues and problems that resist dissection complicate CPR, and these must be dealt with in order to prevent cerebral and myocardial damage after ROSC. Pharmacological strategies have been disappointing, because interventions that were effective for experimental protection-preservation were typically not effective for resuscitation [9]. The final answer always lies one step further

away from experimental breakthroughs. When “God breathed into his nostrils the breath of life” when forming Adam, he was probably aware of “reperfusion injury”. Moreover, he probably knew how to treat it...It is the reperfusion injury after CA, which has been associated with increased oxidant stress, that significantly contributes to tissue injury and subsequent cell death. However, despite the difficulties, an optimistic approach should keep us focusing on the issue. With this same optimism, this series of experimental CA and CPR studies targets preservation of cardiac and cerebral function during cardiac arrest and reduction of reperfusion injury. Using an experimental model of global brain ischemia [10], this research tries to clarify the specific derangements in brain tissue in response to ischemia/reperfusion injury, along with the effects of the pharmacological protection agents.

*'Eureka!' 'Eureka!' (Greek word for 'I have found it!)

1. Background

The following review of the literature concerns classical aspects that are of relevance to this thesis, starting with a brief description of ischemia and reperfusion neuronal injury. The significance of free radicals in reperfusion injury focuses on cerebral oxidative and nitrosative injury. Therefore current concepts regarding eicosanoids and nitric oxide are outlined. The function and integrity of the blood-brain barrier (BBB) in ischemia and reperfusion injury are described. Finally, an overview of methylene blue properties and its effects is summarized. The following review of the literature is not intended to be complete.

1.1 Ischemia and reperfusion neuronal injury

CA and CPR result in a period of global ischemia [10] injury as a consequence of the interruption of blood flow, and a subsequent period of reperfusion injury following the restoration of blood flow [11]. Accordingly, ischemic cerebral injury follows a well documented sequence of events including depolarization, ischemic cascade and reperfusion injury [12]. In spite of their clinical importance, knowledge about the mechanisms of ischemia and reperfusion injury is still incomplete. Although it has been possible to confirm the cerebral pathophysiological events suggesting that both ischemia and reperfusion injury are deleterious to the brain, which of these two phenomena is the more deleterious is still under debate [11]. Both ischemia and reperfusion are responsible for several metabolic and biochemical changes that play major roles in cerebral cell death [12]. Permanent brain damage after CA and resuscitation is determined predominantly by the brain's limited tolerance to ischemia [6] due to CA (no-flow time) and CPR (low-flow time), as well as its unique response to reperfusion after the restoration of blood flow (reflow time) [9].

1.1.1 Ischemic neuronal injury

The brain requires a continuous supply of oxygen and glucose to maintain its normal function and viability, but unfortunately the brain has minimal storage capacity. Thus, in sudden normothermic cardiac arrest, brain oxygen stores are lost within 20 seconds, and glucose stores are lost within 5 minutes [9]. Interruption of the continuous oxygen and glucose supplies induc-

es the classical cascade of events: energy source depletion due to the decrease in levels of high energy metabolites such as adenosine triphosphate (ATP) and phosphocreatine, which impairs ATP-dependent processes such as activity of the pumps maintaining ion gradients across the cells membrane [13]. The failure of ATP-dependent Na^+ and K^+ pumps, and K^+ , Na^+ and Ca^{2+} channels, disrupts cellular homeostasis. A sodium/potassium (Na^+/K^+) pump keeps extracellular potassium low and extracellular sodium high compared to intracellular concentrations. Within two minutes without blood flow, neurons lack the energy to power the Na^+/K^+ pump, leading to loss of cellular gradients; thus, potassium ions move out of the cells while sodium and chloride ions rush inward [9]. During ischemia, neurons attempt to produce ATP by anaerobic glycolysis, and ATP breakdown leads to an increase in inorganic phosphate, lactate and H^+ formation, resulting cellular acidification [13]. The loss of ATP and acidosis prevent sequestration of Ca^{2+} from the cell, further aggravating intracellular Ca^{2+} overload [14]. Depolarization of presynaptic membranes results in the release of massive amounts of the excitatory neurotransmitter glutamate (glutamic acid). Postsynaptic membranes contain several types of glutamate receptors, NMDA (N-methyl-D-aspartate) and AMPA (α -amino-3-hydroxy-5-methyl-4 isoxazole propionic acid) receptors, from which NMDA is particularly adept at allowing large amounts of calcium ion to enter the cell. Excessive glutamate release is neurotoxic [15] and causes a sustained and excessive activation of membrane glutamate receptors, further stimulating Ca^{2+} influx [14] resulting in excessive Ca^{2+} entry into cells, which initiates the ischemic brain damage. The cascade initiated by the disruption of Ca^{2+} homeostasis persists hours and days after ischemia and reperfusion [16]. Thus, even after the extracellular levels of glutamate return to normal after reperfusion [17], glutamate receptor activation can persist and act as a mediator of fulminant neuronal death, namely as glutamate neurotoxicity or excitotoxicity [16, 18]. This leads to the activation of all Ca^{2+} -dependent processes that culminate with secondary injury and cell death [13]. These processes include: a) mitochondrial dysfunction; b) activation of proteolytic enzymes (calpains) that break down many cell proteins, particularly those in the cytoskeleton of neurons; c) activation of phospholipase enzymes that attack cell membrane phospholipids causing the release of arachidonic acid. The ischemic brain damage appears mostly in the lipid portion of cell membranes through lipid peroxidation and phospholipase activity [12]. Histologically, the vulnerable neuron subpopulations are located in the hippocampus, cortex, cerebellum, corpus striatum and thalamus [19]. The sensitivity of the neurons is dependent on the duration of ischemia. Thus, a shorter duration of ischemia (3-5 min) is responsible for injury to CA1 pyramidal neurons of the hippocampus, whereas a longer duration (15-20 min) of ischemia induces injury to medium-sized neurons of the striatum. Progression of irreversible neuronal injury after ROSC also has temporal variations, and is observed within 3 hours of estab-

lishing recirculation for striatum neurons and within 48-72 hours for CA1 pyramidal neurons [20].

In contrast to the earlier theory that considered energy failure as the main characteristic of ischemia, the active cellular response to the ischemic insult is now recognized as the major process [21]. Excitotoxicity, oxidative stress and apoptosis propagate through distinctive and mutually exclusive signal transduction pathways and contribute to neuronal loss by activation of programmed cell death [12, 18, 22]. However, the relative contribution of each cell death pathway remains controversial [6].

Figure 1. Cardiac arrest and cardiopulmonary resuscitation trigger a series of biochemical events that cause neuronal death. Excess glutamate is released in the extracellular space, activating glutamate NMDA and AMPA receptors. This causes intracellular calcium overload, leading to activation of production of nitric oxide. These processes are associated with the production of free radicals. Reperfusion leads to free radical production and is followed by additional neuronal injury.

Restarting blood flow after ischemia initiates a second series of injurious events. These are often considered more damaging to the brain than the ischemia itself [12] and they produce additional injury, because the reperfusion leads to secondary brain injury from an influx of neutrophils, an increase of reactive oxygen species (ROS) with damage to intracellular proteins and DNA, and cerebral edema and hemorrhage [20]. The reperfusion injury consists of three molecular phases. **Phase 1** is an immediate response (seconds to minutes) with activation of phospholipase and intracellular cal-

cium. Calcium immediately activates neuronal NOS (nNOS) and later activates inducible NOS (iNOS). In this phase an increase of eicosanoids, prostaglandins, thromboxane, leukotrienes and protein kinases is observed. **Phase 2** (minutes to hours) is marked by active transcription of protein synthesis for those proteins associated with an inflammatory response (tumor necrosis factor- α and IL-1). Immune response and inflammation-pro-inflammatory mediators such as TNF2 α , IL-1 (interleukin-1) are released in large quantities after reperfusion in astrocytes, microglia and endothelial cells. They stimulate accumulation of inflammatory cells in the injured brain as well as the appearance of adhesion molecules on leucocytes and endothelial cells [16]. During **phase 3** activation of kinases (mitogen-activated protein kinase MAPK, extracellular signal regulated protein kinase -ERPK, c-Jun N-terminal kinase JUNK and activation of the transcription factor, nuclear factor-k β) occurs [11, 23].

1.1.2 Biomarkers of reperfusion injury

The generation of free radicals during ischemia and the early reperfusion period (Fig.2) induces cell injury by damage to lipids, DNA and proteins and also produces neuronal death [12, 24]. Brain cells are believed to be more vulnerable to lipid peroxidation and to free radical-mediated injury [18] because they are highly enriched by polyunsaturated fatty acids [25]. The involvement of **oxidative stress** in CPR was demonstrated by our group in previous research [26]. Both free radicals and cyclooxygenase catalyzed oxidation of arachidonic acid during reperfusion injury [26]. F₂-isoprostanes, isomers of PGF_{2 α} , are formed during free-radical catalyzed, non-enzymatic peroxidation of arachidonic acid [27] (Fig.2). 8-Iso-PGF_{2 α} , a major F₂-isoprostane, is a reliable indicator of oxidative stress *in vivo* and has been associated with several risk factors for atherosclerosis including diabetes, obesity, hypercholesterolemia and various types of reperfusion injury, as recently reviewed [28]. Prostaglandin F_{2 α} (PGF_{2 α}) is one of the major prostaglandins formed at sites of inflammation. PGF_{2 α} formation *in vivo* is preferably quantified by measurement of 15-keto-dihydro-PGF_{2 α} , which is a major metabolite of PGF_{2 α} in plasma [29]. 15-Keto-dihydro-PGF_{2 α} has been shown to be a potent indicator of PGF_{2 α} formation and COX-mediated inflammatory processes *in vivo* and is also involved in experimental CPR [29, 31]. In previous research our group demonstrated a significant increase in 8-iso-prostaglandin F2alpha (a metabolite of arachidonic acid and a marker of oxidative injury) and a similar increase in 15-keto dihydro-prostaglandin F2alpha (a marker of inflammatory response) indicating an association between cerebral ischemia during CPR and inflammatory and oxidative response after ROSC [26].

Figure 2. The formation of 8-iso-PGF₂ via non-enzymatic free radicals and 15-keto-dihydro-PGF₂ via cyclooxygenase catalyzed oxidation of arachidonic acid as a result of oxidative injury and inflammation, respectively.

1.1.3 Nitric oxide in ischemia and reperfusion injury

The present chapter summarizes the basic physiology of nitric oxide, a small membrane-permeable gas that serves as a mediator of many physiological events, and the contribution of NO in cerebral ischemia/reperfusion injury.

1.1.3.1 NO and NOS isoforms: basic physiology

In biological systems, NO is generated from L-arginine [32] and molecular oxygen by activity of nitric oxide synthase (NOS). L-citrulline results from this reaction and is shunted into a metabolic pathway that regenerates free L-arginine (Fig.3).

Figure 3. Production of nitric oxide. Nitric oxide synthases (NOS) catalyze an oxidation of one N^{ω} -atom of the guanidine group of L-arginine to form NO (nitric oxide) and L-citrulline, with the intermediate N^{ω} -hydroxy-L-arginine (NOHA). ASS, argininosuccinate synthase; ASL, argininosuccinate lyase; OTC, ornithine carbamoyltransferase.

NO synthases implicated in NO generation were first identified in experiments using rabbit aortas [33]. The different forms of NO synthase have been identified and named based upon the cells in which they were first isolated: neuronal NOS (nNOS, type 1 NOS) [34], inducible NOS in macrophages (iNOS, type 2 NOS), endothelial NOS (eNOS, type 3 NOS) [35], and in some tissues it may exist even in mitochondria (mNOS) [36]. The NOS enzymes differ not only in their localization, but also in their function. The two calcium dependent constitutives of NOS (eNOS and nNOS) generate small amounts of NO, while iNOS production that is independent of intracellular calcium generates NO levels able to damage cells and microorganisms [37]. Important iNOS expression is regulated by cytokines through the transcription factors NF- κ B, AP-1 and STAT-1 α , and transcriptional and post-transcriptional mechanisms play an important role in iNOS regulation. Once produced, NO has a short half-life and undergoes spontaneous oxidation to the metabolites nitrite and nitrate (NO_2^- and NO_3^-). In the absence of substrate and co-factors, NOS no longer produces NO and instead transfers free electrons and produces free oxygen radicals [39]. Thus, during ischemia and early reperfusion NO is increased, probably because it is derived not only from activated NOS isoforms, but also from NOS independent pathways responsible for non-enzymatic production of NO [39, 40]. Tissue acidosis

occurring during ischemia increases NO production independent by NOS [41] and NO is produced from nitrite [42]. Depending on its environment, NO interacts with the superoxide radical (O_2^-) and forms different reactive intermediates such as peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3), which dose-dependently react with the surrounding tissue components resulting in toxic consequences [43]. Although the metabolism of NO is vital for both normal myocardial and cerebral functions, it can be a “double-edged sword” when inappropriate/excessive production of NO occurs [43].

1.1.3.2 Cerebral NO production and NOS expressions during ischemia and reperfusion

The brain expresses all three identified nitric oxide synthase isoforms, and NOS activity in the brain is higher than in any other tissues [44]. Neurons produce NO by activation of nNOS [45], and glial cells (astrocytes, microglia and oligodendrocytes) synthesize NO by iNOS activation [46]. Finally, it should be mentioned that most brain cell types are able to express several NOS isoforms [46]. Although the function of NO in the brain is not entirely clear, most studies suggest that in the brain, NO is involved in synaptic plasticity or that it acts as a neurotoxin when produced in excess [47]. Nitric oxide in excess can lead to an opening of the blood-brain barrier (BBB), eventually leading to vasogenic edema and secondary brain damage [48]. The molecular mechanisms mediating NO-induced neuronal injury and breakdown of the BBB are complex and not yet completely understood [48].

Cerebral ischemia leads to the up-regulation of all three isosforms [49]. Ischemia induces an early increase in the number of nNOS and eNOS immunoreactive neurons in the rat cortex that persists for 24 h [50]. The induction of iNOS following transient ischemia in rats increases progressively with time, with maximal levels in the cortex occurring at 48 h [51]. Several studies on NOS inhibitors in brain ischemia suggest that the NO produced by iNOS and nNOS is detrimental and that NO derived from eNOS is beneficial [46, 49] (Fig.4). Accordingly, selective iNOS and nNOS inhibitors are considered beneficial for brain ischemia. In contrast, non-selective inhibitor may aggravate brain ischemia because they inhibit eNOS.

Figure 4. Neuroprotective versus neurotoxic effects of NO in ischemia/reperfusion. Activation of nNOS in neurons and iNOS in astrocytes leads to neuronal death; activation of eNOS in endothelial cells is neuroprotective.

1.1.3.3 NO-cyclic guanosine monophosphate (cGMP) pathway

NO activates the guanylyl cyclase and increases the synthesis of cyclic guanosine monophosphate [52]. The major target of NO is NO-sensitive guanylyl cyclase or soluble guanylyl cyclase. Activation of the guanylyl cyclase results in conversion of guanosine triphosphate to the second messenger cGMP [52]. The NO/cGMP signaling cascade (Fig.5) is of importance in the cardiovascular and nervous systems, where it controls smooth muscle relaxation and modulation of synaptic transmission [53].

Activation of guanylyl cyclase results in conversion of guanosine triphosphate to the second messenger cGMP [52] that in turn activates two specific cGMP-dependent protein-kinases (PKG I and II) important for vasodilatation and inhibition of platelet aggregation (PKG I) [54]. The second messenger (cGMP) also inhibits the activity of phosphodiesterases (PDE II and III). Inhibition of PDE III elevates concentration of cyclic adenosine monophosphate (cAMP), thereby increasing protein kinase A. A low concentration of cGMP appears to primarily inhibit PDE III, while a higher cGMP concentration activates PKG [39, 54, 55].

Figure 5. NO-cGMP signaling cascade. NO endogenously produced by NO synthases activates NO-sensitive GC and leads to increased synthesis of cGMP. This intracellular messenger in turn modulates the activity of cGMP-dependent kinases, cGMP-gated ion channels, and cGMP-regulated phosphodiesterases.

1.1.4 Blood- brain barrier and ischemic/reperfusion brain injury

The maintenance of an intact BBB is needed to prevent brain damage, but there is still little information about the function and integrity of the BBB during CA and after CPR. The subject is complicated by different results obtained in clinical trials and animal experiments using different methods to assess BBB function and using different animals models. It is also thought that there are temporal changes in BBB integrity [56].

Experimental studies after resuscitation showed an intact BBB using protein tracer techniques [57, 58], whereas BBB disruptions were seen with the use of Evans blue [59]. Dye extravasation without any significant correlation to the magnitude of blood pressure increase occurred not only during CPR, but also several minutes after ROSC. The presence of the dye in the brain reflected plasma protein extravasation into brain tissue [59]. Immature pigs are prone to a delayed increase in permeability, thereby allowing circulating substances greater access to the brain [60, 61]. In piglets exposed to CA followed by CPR vacuolation and luminal discontinuities of endothelial cells were demonstrated with evidence of perivascular astrocytic swelling [62]. Widespread microvascular and neuronal damage is present as early as 4 h after cardiac arrest in infant piglets [62].

Vascular permeability evaluated with horseradish peroxidase (HRP) [63, 64] and [3H] sucrose [65] indicated that BBB disturbances are of biphasic nature [63, 65]. The first phase, appearing immediately after ischemia and persisting during the first postischemic hour, was limited to the cerebral and cerebellar cortex and the central periventricular structures. The second phase

covering the period between 6 and 24 h after resuscitation was characterized by random leakage in all the CNS structures [63]. At 6 h, a delayed intensification of opening was evident in striatum and hippocampus, regions known to undergo selective, delayed neuronal death [65]. There was generalized BBB recovery by 24 h except in experiments that involved prolonged ischemia (25 min) or concomitant brain hyperthermia (41 degrees C, 10 min) protocols that evoked a third opening [65]. The BBB openings did not increase in frequency with longer periods of ischemia and recirculation [64].

During CA, reduced blood flow to the brain alters BBB permeability and regulatory transport functions [66, 67]. Ischemia induces disruptions of BBB, facilitating increased cerebrovascular permeability, an important factor in the development of ischemic brain injury and edema formation [68, 69]. In cerebral ischemia the functions of the BBB may also be compromised because of the hypoxic endothelial damage and the toxic substances released from intravascular blood components such as oxygen free radicals and proteases from leukocytes and platelets [70]. Reperfusion is often hyperemic in the first few minutes after CA because of elevated CPP and impaired cerebrovascular autoregulation [6], which is either absent or right-shifted [71]. The high levels of NO produced during ischemia and early reperfusion may caused cerebral hyperemia [72, 74]. Hyperemia may be detrimental to cells, especially in the brain parenchyma, by causing disruption of the BBB, which may ultimately worsen neurological injury [74]. BBB permeability is increased and allows extravasation of albumin and other high molecular weight compounds [75]. All of these favor formation of cerebral edema and may also increase intracerebral pressure (ICP) [76]. However, there is limited evidence that brain edema and elevated ICP exacerbate post-cardiac arrest brain injury [6]. In contrast to brain edema observed early after ROSC, which is rarely associated with a clinically relevant increase in ICP [77], delayed brain edema occurring days after CA is associated with increased ICP and a poor prognosis [78].

1.2 Methylene blue

Methylene Blue (methylthionine chloride) is a heterocyclic aromatic chemical compound with molecular formula ($C_{16}H_{18}ClN_3S$, $3H_2O$) (Fig.6) with the chemical name [3, 7-bis (Dimethylamino)-phenazathionium chloride Tetramethylthionine chloride] [79, 80]. Methylene blue (MB) is a cationic thiazine dye that is deep blue in the oxidized state while it is colorless in its reduced form (leucomethylene blue) (Table 1). MB and leucomethylene blue exist as a redox couple in equilibrium and together form a reversible oxidation-reduction system or electron donor-acceptor couple [79].

Methylene blue

Figure 6. Chemical structure of methylene blue.

1.2.1 The history of methylene blue

Methylene blue was first prepared by Caro in 1876 as an aniline-derived dye for textiles [80]. However, it is a drug full of surprises that has made history as a histochemical stain, a biochemical reagent and a lead compound in the development of therapeutic agents for diseases ranging from microbial disease to dementia [81]. The staining activity of methylene blue, developed by Paul Ehrlich in 1891, provided the foundation of modern chemotherapy [81]. In the late 19th and early 20th centuries it was used in humans to treat malaria [82], but then ceased to be used as an anti-malarial due to its two inevitable side effects: green urine and blue sclera. Interest in its use has recently been revived, especially because it is very cheap. In the 1920s it proved to be a dramatic antidote for cyanide poisoning, since its reduction potential is similar to that of oxygen and it can be reduced by components of the electron transport chain [81]. Its versatility has allowed MB to be tested against many other poisons; it miraculously reversed toxic methemoglobinemia, it reversed symptoms of cyclophosphamide- induced encephalopathy and, exotically, those of Jamaican ackee fruit poisoning [83].

MB reverses hypotension in septic shock and it may have value in the treatment of protamine reactions. It can help in some cases of anaphylactic shock, and it has helped to treat hypotension related to lithium toxicity, ACE inhibition, and hemodialysis [83]. In cases of profound “vasoplegia” following cardiopulmonary bypass [84] where first-line therapy fails (norepinephrine refractory vasoplegia), the use of MB is a potent approach. Whether MB should be the first drug therapy in patients with vasoplegia is still a matter of debate [84].

1.2.2 Physical and chemical properties and pharmacokinetics of methylene blue

Table 1. Physical and chemical properties and pharmacokinetics of MB

Physical and chemical properties	Values	Pharmacokinetics	Properties
Melting temperature	180°	Ionization at gastric pH	Completely at normal gastric pH [85]
Boiling temperature	No data	Oral absorbtion	53-97% (86)
Solubility in water	35.5g·l ⁻¹	Peak plasma concentration	After 30-60 min [85, 87]
pH value	3 (10g/l H ₂ O)	Volume of distribution	20 ml·kg ⁻¹ [86]
Molecular weight	319 g·mol ⁻¹	Plasma half-life	5-6 hours [85]
Color	Dark blue-green in oxidized form, colorless in reduced form (leukomethylene blue)	Metabolism	Reduced in peripheral tissues to leukomethylene blue (65-85%) [85]
Chemical formula	C ₁₆ H ₁₈ N ₃ ClS	Elimination	Bile, feces and urine as leukomethylene blue [87]

1.2.3 Administration and dose of methylene blue

Table 2. Administration and dose of methylthioninium chloride in humans

Dose	Administration	Duration	Indication
1-2 mg.kg ⁻¹	Bolus i.v.	10-20 min; 1 hour	Vasodilatation with hypotension [88, 89]
	After bolus i.v. continuous infusion	48-72 h	Vasoplegic syndrome [90]
0.25–1 mg.kg ⁻¹ .h ⁻¹	Continuous infusion	6 h, 2 h after bolus	Sepsis [91, 92]
1.5–2 mg.kg ⁻¹	Bolus i.v		Anaphylactic shock [93]
300 mg.day	Oral doses of up to 300 mg.day ⁻¹	Daily	Hereditary methemoglobinemia
150 mg.day	Oral doses or i.v.	50 mg three times daily	Encephalopathy after ifosfamide [94]
2 mg.kg ⁻¹	Bolus i.v.	Before CPB	Surgery for septic endocarditis [95]
0.5 mg.kg ⁻¹ .h ⁻¹	Continuous i.v until 30 min after CPB	3 h	Cardiopulmonary bypass (88, 96), patients on angiotensin-converting agents after start of CPB [93]

1.2.4 Toxicity of methylene blue

Table 3. Dose-related toxicity of MB

Animal studies	Toxic doses	Manifestation
Rat [97]	5-50 mg.kg ⁻¹ 1250 mg.kg ⁻¹ (LD50)	Neuronal apoptosis , reduced MAC isoflurane
Mouse	3500 mg.kg ⁻¹	
Sheep [98]	40 mg.kg ⁻¹	
Dog [99]	10-20 mg.kg ⁻¹	Hypotension, decreased SVR, renal blood flow, pulmonary hypertension
Human studies	Dose (mg/kg)	Toxic manifestations [96, 100]
	2-4	Hemolytic anemia, skin desquamation in infants
	7	Nausea, vomiting, abdominal pain, chest pain, fever, hemolysis
	7.5	Hyperpyrexia, confusion
	20	Hypotension
	80	Bluish discoloration of skin (similar to cyanosis)

1.2.5 Effects of MB on nitric oxide synthase and guanylyl cyclase

MB has direct inhibitory effects on nitric oxide synthases, both constitutive and inducible [101, 102] and blocks accumulation of cyclic guanosine monophosphate (cGMP) by inhibiting the enzyme guanylate cyclase [101]. MB blocks the activity of NO-dependent guanylate cyclase via the oxidation of the active haemo centre [103] or by inactivation of its haemo-deficient apoenzyme [104]. Data suggest that MB is a more specific and potent inhibitor of NOS than guanylyl cyclase, because direct NO-donating compounds in the presence of MB can still partially activate c-GMP signaling pathways [101, 102]. Hence, MB restores vascular reactivity to endogenous catecholamines [105] in the setting of excessive NO production, but has no intrinsic contractile property, as shown in experimental conditions [106]. The effect of MB is due to NO inhibition (effect of NO on K_{Ca} channels), although there are presently no data on the relative inhibition of the NOS isoforms by MB, nor is there any information on regional organ differences in the inhibition of cNOS.

1.2.6 Antioxidant effects of MB

MB possesses unique biochemical properties that are the attributes of a neuroprotective agent, such as being a powerful antioxidant. MB blocks the iron-containing enzymes [107] such as xanthine oxidase an iron containing enzyme that is inhibited by methylene blue due to a competition with molecular oxygen for xanthine derived electrons [108]. MB protects organs from the toxic effects of free oxygen radicals by competing with molecular oxygen for the transfer of electrons by XO and is effective in attenuating ischemia-reperfusion syndrome [109, 110].

Methylene blue is permeable through biomembranes and it readily crosses the blood-brain barrier and selectively stains brain tissue [111]. It has been demonstrated that after administration MB is selectively trapped in the brain and its concentrations are 10-20 times higher in the brain than in circulation one hour after administration [87]. MB has affinity for tissue oxidases, so it concentrates in mitochondria where is able to maintain mitochondrial function by accepting electrons from blocked components of the respiratory chain and by transferring them to cytochrome oxidase or oxygen, improving mitochondrial respiration [112]. The effects of MB are reflected in increased activity in processes coupled with mitochondrial energy production such as Na/K ATPase activity and intermediary metabolism. [113]. For this reason, MB constitutes a metabolic enhancer that accelerates the activity of the electron transport chain. The intracerebroventricular administration of MB has been shown to reduce the minimum alveolar anaesthetic concentration (MAC) of volatile anaesthetics [114] and the intravenous administration reduced the anaesthetic requirement of propofol [115].

1.2.7 MB and platelets aggregation

MB inhibits the arachidonic acid metabolism in human blood platelets [116]. When human platelets are incubated with MB that oxidizes cellular NADPH, a marked inhibition of platelet aggregation and of the metabolism of the arachidonic acid in both the cyclooxygenase and lipoxygenase pathway is found. Thus, MB possesses inhibitory effects on platelet activation, adhesion and aggregation [66, 117] synergistically with an inhibition of platelet thromboxane A₂ and endothelial prostacyclin I₂ (PGI-2) production [118, 119].

2. Aims of the study

The general aim of this thesis was to study the pathophysiology of CA and early post-cardiac arrest brain injury and to clarify the possible neuroprotective effects resulting after administration of pharmacological agents.

The specific aims of this project were to investigate

- The evidence for a time-dependent increase of free radical damage and eicosanoid formation in the brain as related to duration of CA and CPR
- Whether the continuous infusion of MB, an inhibitor of the nitric oxide pathway, added to a hypertonic-hyperoncotic solution, would affect the outcome after CA, in comparison with two other infusions (hypertonic saline/dextran and isotonic saline) without MB
- Whether an alternative mixture of MB in a continuous infusion of hypertonic lactate might be used in CPR, as compared with two other infusions of MB added to hypertonic saline/dextran and MB and isotonic saline, respectively
- The magnitude of nerve cell damage at different time points after CA and after ROSC, investigated through immunostaining of cerebral cortical tissue
- The potential upregulation of nitric oxide synthases (NOS) and consequently quantify the nitric oxide metabolite levels after global brain ischemia from CA
- The possible neuroprotective effects of MB on cortical BBB integrity maintenance after CA and CPR

3. Material & Methods

The Uppsala Institutional Review Board for Animal Experimentation approved the studies. They were prospective, randomized, laboratory animal studies, with the exception of the first study that used some data from previously published articles [26, 120]. The piglets were handled according to the guidelines of the Swedish National Board for Laboratory Animals and the European Convention of Animal Care. Qualified individuals in the experimental laboratory at the University Hospital cared for the animals. Anesthesia was used in all surgical interventions.

3.1 Animals

The following inclusion criteria were applied: no apparent pre-existing disease, PaCO₂ between 5-5.5 kPa, PaO₂ > 10 KPa (75 mmHg) at baseline, which was after stabilization. The animals in all the studies were 12-14 weeks old and were without any evidence of illness (Table 4). They were Swedish country “triple breed” domestic piglets of both sexes obtained from a single provider source. All piglets were kept fasting but had free access to water during the night and were taken to the laboratory on the morning of the experiment.

Table 4. Number of animals and mean weight and SD

<i>Study</i>	<i>Number of pigs</i>	<i>Weight (mean±SD)</i>
<i>Paper I</i>	53	24 ± 2 kg
<i>Paper II</i>	63	25.1 ± 2.1 kg
<i>Paper III</i>	30	24.8 ± 1.7 kg
<i>Paper IV</i>	60	25 ± 1.05 kg

3.2 Anesthesia

Immediately after arrival to the laboratory, the animals received an intramuscular injection of 6 mg·kg⁻¹ Zoletil® (tiletamine and zolazepam, Reading, France) combined with 2.2 mg·kg⁻¹ Rompum® (xylazine, Bayer, Germany) and atropine 0.04 mg·kg⁻¹ (Atropin®, NM Pharma, Stockholm, Sweden). A peripheral venous catheter (18 Gauge) inserted in an ear vein was used for

induction and maintenance of anesthesia, as well as for fluid administration. All the piglets received an intravenous injection of 20 mg morphine (Morfin Bioglan®). If needed, 100 mg ketamine (Ketaminol®, Veterinaria AG, Switzerland) was added as an intravenous bolus to achieve surgical anesthesia. Absence of motor response to painful stimuli was considered indicative of an adequate level of anesthesia. An intravenous infusion of 8 mg·kg⁻¹·h⁻¹ sodium pentobarbital (Apoteket, Sweden), morphine (Morphine®, Pharmacia, Uppsala, Sweden) 0.5 mg·kg⁻¹·h⁻¹ and 0.25 mg·kg⁻¹·h⁻¹ pancuronium bromide (Pavulon®, Organon, Netherlands) dissolved in 2.5% glucose solution was started and used for maintenance of anesthesia. The piglets were secured in a supine position and were tracheostomized, which was done to secure a free airway during the experiment. The animals were mechanically ventilated (Servo 900C, Siemens-Elema, Solna, Sweden, in the first three studies and Servo-i V3.1, Siemens Medical, Solna, Sweden, in the fourth study with a 30/70 mixture of oxygen/nitrous oxide during the preparation and 30% oxygen in air after the preparation. In all studies mechanical ventilation was volume-controlled with I:E ratio of 1:2, a fixed frequency of 25/min and a minute volume set to maintain arterial PaCO₂ at an average of 5-5.5 kPa. Positive end-expiratory pressure (PEEP) at 5 cm H₂O was applied to prevent atelectasis. The capnogram and saturation were displayed continuously until extubation (CO₂SMO Plus-8100, Novametrix, Wallingford, CO, USA), as were leads II and V5 of the electrocardiogram.

3.3 Fluid administration

Fluid replacement with acetated Ringer's solution (Ringer-acetat®, Freze-nius Kabi, Stockholm, Sweden) was provided to all the animals with an infusion pump (Life Care®, Abbot Shaw, USA), as follows: 30 mL·kg⁻¹ during the first hour of preparation in order to obtain a pulmonary artery wedge pressure of 7-10 mmHg, and a continuous infusion of 10 mL·kg⁻¹·h⁻¹ thereafter.

3.4 Preparation and procedures

A 14-G saline-filled double lumen catheter was placed into the right atrium via a cutdown of the right external jugular vein to measure right atrial pressure and for drug administration. Along with that, a 7 Fr fluid filled pulmonary artery catheter (Criticath Ohmeda®), equipped with a thermistor, was floated into wedge position in the pulmonary artery to measure cardiac output and capillary wedge pressure and to sample mixed venous blood. These catheters were connected to pressure transducers, which were calibrated to atmospheric pressure at the level of the right atrium. In the first two studies the left internal jugular vein was cannulated in the cephalad direction with a

catheter (18G) advanced to the jugular bulb. In order to limit contamination from extra-cerebral blood, the position of the catheter tip was confirmed by lateral neck fluoroscopy and a contrast injection. A catheter tip placed as close to the base of the skull as possible and above disc C1/C2 on lateral radiographic view was considered to have the correct position. Jugular venous bulb saturation ranging between 55% and 71% was considered reliable for the positioning of the catheter close to the jugular bulb.

In studies II and III a 10-mm burr hole was made between the middle and the coronal suture of the right hemisphere and the dura was opened. Through this hole, a Laser-Doppler flow probe (MT B500-43, Periflux PF 2B Laser-Doppler flowmeter, Perimed, Stockholm, Sweden) was placed directly on the surface of the right frontal cortex. Care was taken to avoid placing the probe over visible blood vessels. The probe was fixed by suturing dura and skin. In the third study, the bone lamella corresponding to the frontal sagittal sinus was resected over an area measuring 2 x 3 cm directly above the sagittal sinus. Any bleeding from the bone was coagulated and sealed with bone wax. A 22-G cannula was inserted into the sagittal sinus for measurement of sagittal sinus pressure (SSP) and blood sampling. The scalp was then closed and the animals were left undisturbed for an equilibration period. No surgical procedures were performed on the skull in the histological studies except at the end of the experiment. A minor vesicotomy was performed and a urinary catheter was introduced into the bladder for urine collection.

3.5 Measurements

3.5.1 Hemodynamic variables

During CPR and after ROSC, hemodynamic parameters including leads II and V5 of the ECG, heart rate (HR), systemic arterial blood pressure, right atrial pressure and pulmonary artery pressure were continuously displayed (Solar 8000 monitor, Marquette Medical Systems, Milwaukee, WI, USA) and recorded (Workbench 3.0, Strawberry Tree Inc., Sunnyvale, CA, USA). Cardiac output (CO) was measured as a mean of three measurements, using the thermodilution technique at baseline and repeatedly during the postresuscitation period. Pulmonary capillary arterial wedge pressure (PCWP) was recorded at the same time points as CO. Coronary perfusion pressure (CPP) was calculated as the difference between the diastolic aortic pressure and simultaneously measured right atrial pressure. Left and right ventricular stroke indexes (LVSWI, RVSWI), systemic and pulmonary resistance vascular indexes (SVRI, PVRI), cardiac index (CI), delivery of oxygen (DO_2), and oxygen consumption (VO_2), were derived from their conventional formulae [121, 122].

3.5.2 Cerebral variables

Cerebral cortical blood flow was recorded every 5 seconds by a computer and presented as a fraction of the steady state baseline flow level before the induction of ventricular fibrillation. The cerebral oxygen extraction ratio was calculated as the ratio between arterial-jugular bulb oxygen content difference and arterial oxygen content $[(CaO_2 - CjO_2)/CaO_2]$. The sagittal sinus pressure (SSP) was recorded in study III and cerebral perfusion pressure (CePP) was measured as the difference between the mean arterial pressure and sagittal sinus pressure.

3.6 Samples

Samples of jugular vein blood in studies I and II and sagittal sinus blood in study III, and samples of arterial blood in all four studies were taken for blood gas analysis and acid-base balance (ABL 300, Radiometer, Copenhagen, Denmark) at baseline, and at 14, 16, and 19 min during CRP and after ROSC at 5, 15, 30, 60, 120, 180 min, and, in the first three studies, at 240 min. The same analyses were performed from blood samples taken from the pulmonary artery at baseline, and at 60, 120, 180, and 240 min. Oxygen saturation (SaO₂) and hemoglobin (Hb) were determined simultaneously on an OSM3 Hemoximeter (Radiometer, Copenhagen, Denmark) at the same time points. In addition, jugular vein samples in the first two studies and sagittal sinus vein blood in study III were collected before cardiac arrest (baseline) and after ROSC at 5, 15, 30, 60, 120, 180, and 240 min. After centrifugation (3500x g for 12 min), plasma was stored at -70 °C until analyzed for 8-iso-PGF_{2α}, 15-keto-dihydro-PGF_{2α} and protein S-100β. Plasma obtained after centrifugation (4000x g for 5 min) of arterial blood drawn at baseline and at 120 and 240 min was used to determine troponin I and CK-MB. Glucose, lactate, and electrolyte concentrations were determined (ABL 700, Radiometer, Copenhagen, Denmark) at baseline and 120 min after ROSC.

3.7 Analytical methods

Plasma concentrations of isoprostane 8-iso-PGF_{2α} (an indicator of oxidative injury) and 15-keto-dihydro-PGF_{2α} (an indicator of inflammation) were measured according to a highly specific and validated radioimmunoassay (RIA) method at our laboratory, as previously described [29, 123]. The detection limit for 8-iso-prostaglandin F_{2α} was 23 pmol/L and the detection limit of the assay for 15-keto-dihydro-PGF_{2α} was 45 pmol/L. Myocardial and cerebral damage were assessed by serum concentrations of cardiac troponin I and astroglial protein S-100. Serum S-100β levels were measured by an immunoluminometric assay (LIA-mat, Sangtec® 100) with a detection limit of

0.02 ng/mL and a cutoff level of <0.12 ng/mL. Troponin I and CK-MB were measured with monoclonal/policlonal mass immunoassays (ADVIA Centaur, Bayer) with normal values under 0.12 µg/L for troponin I and under 6µg/L for CK-MB.

3.8 Experimental protocol (Fig.7)

After preparation, the piglets were ventilated with 30% oxygen in air, stabilized for one hour, and then baseline measurements were obtained before cardiac arrest. Thereafter, to induce ventricular fibrillation (VF), a 50-Hz, 40-60-V alternating transthoracic current was applied via two subcutaneous needle electrodes. Cardiopulmonary arrest was defined as the decrease of aortic blood pressure below 25 mm Hg and the presence of ventricular fibrillation on the electrocardiogram. Mechanical ventilation was stopped at this point. Closed-chest CPR (The Rentsch Cardiac Press Device) [124] with a compression rate of 80 /min and mechanical ventilation with 100% O₂ were initiated in the first study. In all the other studies closed-chest CPR was performed after 12 min of untreated cardiac arrest with a pneumatically driven automatic piston device for CPR (Lucas[®], Lund, Sweden), and mechanical ventilation with 100% oxygen was resumed with the same ventilatory settings as before induction of cardiac arrest. After commencement of CPR all animals received, at one min, 0.4 U·kg⁻¹ of vasopressin (Arg⁸-vasopressin, Sigma Chemicals Co, St. Louis, MO, USA) as a bolus administered via the right atrial catheter. After 8 min of external chest compressions, a monophasic countershock was delivered through defibrillation electrode pads (Medtronic Physio-Control Corp., Seattle, WA, USA) at an energy level of 200 J. If restoration of spontaneous circulation (ROSC) was not accomplished, another two defibrillatory shocks (200 J, 360 J) and a bolus injection of epinephrine 20 µg ·kg⁻¹ were administered. DC shocks were then applied at the same energy level of 360 J during a maximum period of 5 min. CPR was discontinued if ROSC was not achieved during this time. ROSC was defined as return of co-ordinated electrical activity resulting in a systolic blood pressure greater than 60 mm Hg for at least 10 consecutive minutes. If arterial pH was less than 7.20 or the base deficit more than 10 mmol/L at 5 min after ROSC, acidosis was corrected with a tris buffer mixture (Tribonat[®], Kabi Fresenius, Stockholm, Sweden), 1 mmol·kg⁻¹, and by increasing minute ventilation, aiming a PaCO₂ within the range of 5.0 to 5.5 kPa. No additional intervention was performed during the resuscitation phase in the second study. After the resuscitation phase the only intervention in studies II, III and IV was the administration of dobutamine (Dobutrex[®], Eli Lilly) in a solution of 12.5 mg/ml starting at 5µg·kg⁻¹· min⁻¹ when the systolic blood pressure decreased to less than 70 mm Hg. Hemodynamic parameters and blood gases were observed for 4 h after ROSC. After completion of the

study, all animals received an injection of 10 mL of potassium chloride (KCL) 20 mmol/mL and were sacrificed. Only five animals (*Paper I*) served as controls and received no further interventions except anesthesia and placement of catheters. In *paper IV*, before KCL induced circulatory arrest, Evans blue dye was administered, the skull was opened, and the brain was taken out for immunohistochemical analysis.

3.8.1 Protocol I

Seven groups of animals were studied. The first group (n=6, VF2) was subjected to 2 min of untreated VF followed by 5 min of CPR. The second (n=6) group, denoted VF5, was subjected to 5 min of untreated VF followed by 5 min of CPR. The third (n=14) and fourth (n=18) groups, denoted VF8, were subjected to 8 min of untreated VF followed by 5 or 8 min of closed-chest CPR, respectively. The fifth group (n=8), denoted VF10, was subjected to 10 min of VF followed by 8 min of CPR. The sixth group (n=3), denoted VF12, was subjected to 12 min of VF followed by 8 min of CPR. Five animals served as controls (group 7) with no further interventions except anesthesia and placement of catheters.

Figure 7. Experimental protocol for studies II, III, IV. After stabilization for 1 h, animals were subjected to 20 min of ventricular fibrillation (VF) including 12 min of cardiac arrest and 8 min of cardiopulmonary resuscitation (CPR). HSD (hypertonic saline dextran); MB (methylene blue); MBL (methylene blue lactate); SAL (saline); ROSC (restoration of spontaneous circulation); DC (defibrillatory shocks); KCL (potassium chloride).

3.8.2 Protocol II

The animals were randomly assigned to receive an infusion of either 55 mL·kg⁻¹·h⁻¹ of saline (SAL group, n=21), or 10 mL·kg⁻¹·h⁻¹ of the plasma volume expander RescueFlow®, (Biophausia, Uppsala, Sweden) (HSD group, n=21), or 7.5 mL·kg⁻¹·h⁻¹ MB (Metyltioninklorid 10 mg/mL, Apoteket, Umeå, Sweden) with 10 mL·kg⁻¹·h⁻¹ RescueFlow® (MBHSD group, n=21), through the right atrial catheter, started after 1 min of CRP. After ROSC the infusions were reduced as follows: saline 16.5 mL·kg⁻¹·h⁻¹, RescueFlow® 3 mL·kg⁻¹·h⁻¹, MB 2.25 mL·kg⁻¹·h⁻¹, and were continued at these rates for 50 min after ROSC, at which point the infusions were stopped.

3.8.3 Protocol III

One minute after the start of CPR the animals were randomly assigned to receive an infusion of 55 mL·kg⁻¹·h⁻¹ of normotonic saline (MBS group, n=10), or 10 mL·kg⁻¹·h⁻¹ of the hypertonic-saline (7.5%) dextran (6%) solution RescueFlow®, (Biophausia, Uppsala, Sweden) (MBHSD group, n=10). To both groups, 7.5 mg·kg⁻¹·h⁻¹ of MB (Metyltioninklorid 10 mg·mL⁻¹ equivalent to 8.56 mg·mL⁻¹ water free MB, Apoteket, Umeå, Sweden) was administered continuously. The third group received 10 mL·kg⁻¹·h⁻¹ of a solution prepared in our pharmacy from a mixture of MB and hypertonic sodium lactate (sodium lactate 0.63 M =7%, containing 630 mmol/L Na⁺, and methylene blue 0.86 g/L, pH=6.7) (MBL group, n=10). The relative proportions between rates of administration of normal saline vs. hypertonic solutions were calculated based on the relative distribution of crystalloid and colloid solutions in the intra- and extravascular fluid compartments at equilibrium 30 min to 60 min after administration [123]. After ROSC the infusions were reduced as follows: normotonic saline 16.5 mL·kg⁻¹·h⁻¹ with MB 2.25 mg·kg⁻¹·h⁻¹, hypertonic saline dextran 3 mL·kg⁻¹·h⁻¹ with MB 2.25 mg·kg⁻¹·h⁻¹, and MBL 3 mL·kg⁻¹·h⁻¹.

3.8.4 Protocol IV

To test the disruptions of the blood–brain barrier (BBB) and the water and electrolyte content of the brain, 38 pigs were divided into three groups. The first group (CA) with 16 animals underwent CA without CPR and the brain of the animals was isolated immediately after CA (n=5), and at 5 min (n=3), 15 min (n=2), 20 min (n=3), and 30 min (n=3) after CA. The other two groups with CA and CPR received either an infusion of saline (CA-MB group, n=10) or an infusion of saline with MB (CA+MB, n= 12) in the same doses as in the previous protocols (II and III). In the CA-MB and CA+MB groups the brains were removed for histological analysis in three animals (CA-MB) and in four animals (CA+MB), respectively, at each of the following time points: 30, 60, 180 min after ROSC.

3.9 Neurological injury, immunohistochemistry, western blot, brain water and electrolyte content (Paper IV)

3.9.1 Fixation

One of the brain hemispheres was immersed in 4% buffered formalin and stored at 4° C for 1 week. Small tissue pieces (<3x5 mm) were cut from the cerebral cortex and processed for histology or immunohistochemistry. The tissue pieces were dehydrated in a graded series of alcohol, rinsed in xylene, and embedded in low-temperature paraffin (56-58° C) according to a standard protocol [126]. Multiple 3-5- μ m-thick sections (6 to 8) were cut from each tissue block and collected on glass slides. After deparaffinization, duplicate sections were stained with either Nissl (Cresyl violet) or hematoxylin and eosin using a commercial protocol [10].

3.9.2 Cerebral tissue injury (evidenced histologically)

Three-micrometer paraffin sections from identical tissue blocks from the cerebral cortex were cut and stained with Haematoxylin and Eosin or Nissl for analysis of cellular changes. The number of distorted neurons in one whole section was counted in each animal at least three times in a blinded fashion and the median values were recorded for data analysis.

3.9.3 Microphotography and processing of digital images

Microphotographs were taken on Kodak Supra 100 ASA Colour negative film under a Leica bright field microscope. The digital images (size 52 cm $\times$ 32 cm, 80 pixels/inch) were processed on a CD by Kodak Colour laboratories (Stockholm, Sweden). These digital images were modified using the Adobe Photoshop 3.5 program on a G-4 Macintosh computer. The images obtained were processed (final image size 8 $\times$ 13 cm, 300 pixels/inch) using identical color filter and color balance [127].

3.9.4 Albumin immunostaining

Endogenous albumin immunostaining to detect leakage across the BBB is a well-standardized method in neuropathological laboratories and has been used by several workers in the past [126, 128]. Immunohistochemistry analysis for albumin was performed on paraffin embedded (3- μ m thick) sections using a sheep polyclonal anti-rat albumin antibody (Sigma, USA) and the streptavidin-HRP-biotin technique as described previously [129, 130]. Brief-

ly, the endogenous peroxidase activity was blocked with 3% H₂O₂ and 5% normal goat serum, followed by incubation with the primary antibodies (1:500 for albumin). This was followed by incubation with biotinylated linking antibody and HRP (Dako), with brief rinses in PBS between incubations. The reaction was visualized using 3-amino-9-ethycarbazole (Vector Laboratories, Burlingame, USA) and counterstained with Haematoxylin. Reagent controls (omitting the primary antibody or substituting nonimmune serum for the primary antibody in the staining protocol) on tissue sections were used to confirm the specificity of the primary antibodies used [131, 132]. The numbers of albumin-positive cells were counted in one identical area of the cortex from each animal three times, in a blinded fashion. It is often difficult to differentiate glia from neurons, particularly when edema and neural damage alter the shape of cells. Although most albumin present inside cells appears to be in neurons, a few glial cells can also be positive. However, albumin leakage into the extracellular environment is also seen in these cases. Therefore, albumin-positive cells appear to represent both damaged neurons and glial cells [131].

3.9.5 Immunohistochemistry of NOS

Immunostaining was performed on 3- μ m thick paraffin sections of cerebral cortex (Fig.8) using a monoclonal NOS antiserum as described earlier [133, 134, 135]. In brief, the antibodies of NOS were diluted 1:5000 and applied for 48 h with continuous shaking at room temperature. The immune reaction was developed using a peroxidase-antiperoxidase technique and visualized in a light microscope. No immunoreactivity was detected in controls where the primary antibody step was omitted. The number of NOS positive cells in each group was counted in a blind fashion.

3.9.6 Western blot

Protein homogenates of brain samples (Fig.8) were prepared by rapid homogenization in Tissue Extraction Reagent II (Invitrogen Corporation) according to the manufacturer's instructions. The protein concentration was determined using the Bio-Rad RC DC kit. Protein extracts (50 μ g) for each group were pooled from 3 animals, resolved by electrophoresis on 12% SDS-acrylamid gels and transferred to Hybond-P PVDF membrane (GE Healthcare). The membranes were blocked with Phosphate-buffered saline (PBS) containing 0.2% Tween-20 and 5% non-fat dry milk, prior to incubation, overnight at 4°C, with either anti NOS1 (1:10,000, Euro-Diagnostica AB), anti-NOS2 (1:10,000, abcam), anti-NOS3 (1:200, Santa Cruz Biotechnology) or anti-actin (1:200, Sigma) in PBS-Tween-20. After washing, membranes were incubated (1 h at RT) with anti-rabbit IgG-conjugated to horseradish peroxidase (1:10,000). After washing, immunoreactive bands were

visualized by enhanced chemiluminescence using Lumi-Light^{plus} (Roche Diagnostics) and protein band densities were digitally quantified and normalized to the loading control (β actin) using Quantity One software (Biorad).

Figure 8. The areas from the brain where tissues samples prelevations for immuno-histochemical analysis (1,5) and for western blot (1,2,5) analysis were made for NOS identifications.1-cortex parietal (1) and temporal (2, 5).

3.9.7 Brain water and electrolyte content

Small tissue pieces of pig cerebral cortex were used to measure water and electrolyte content according to standard protocol [136]. The water content was calculated from the differences between dry and wet weights of the samples, and ion content (Na^+ , K^+ and Cl^-) was measured from the dry weight of the samples as described earlier [137]. These measurements were made in slices from the same hemisphere of the brain at the level of the parietal cerebral cortex. To measure water content, after obtaining the wet weight, the samples were placed in an oven, maintained at 90 °C, for 72 h or until three dry weight measurements were constant. Water content was calculated according to the formula: $\text{wet weight-dry weight/wet weight} \times 100$ [138, 139]. To measure ions, after obtaining the dry weight, the samples were heated at 550 °C for 24 h to produce ash. The ash was dissolved in 5 mL of 3 mmol nitric acid and diluted with deionized water (1: 10). Na^+ , K^+ and Cl^- were determined at 330, 404 and 512 nm, respectively, with an Atomic Absorption Spectrophotometer (Packard Instruments, Downers, IL, USA) using an air-acetylene flame [140, 141].

3.9.8 Measurement of nitrite and nitrate in the cerebral cortex of pigs

3.9.8.1 Sample preparation

Immediately after the experiments, the brain tissue was rapidly taken out and a small volume of cerebral cortex, 250-300 mg, was dissected out at 4° C and immediately frozen on dry ice and stored at -90 °C until assay.

3.9.8.2 Tissue

For sample preparation, the tissue was thawed and homogenized in 20 mM Tris buffer, 10 mM EDTA (pH 7.4), and centrifuged at 4000 x g for 15 min and the protein content in cytosol was determined [142].

3.9.8.3 Fluorometric assay of nitrite and nitrate

Nitrite or nitrate levels were measured using fluorescence of 2, 3-diaminonaphthotriazole as described earlier [143, 144]. The cytosol was filtered to decontaminate hemoglobin through a 10 kDa cutoff filter at 15 000 g for 90 min and then processed for nitrite or nitrate measurement [145]. The NO_x levels were measured following reduction of nitrate to nitrite with nitrate reductase and NADPH regenerating system (G-6-P/G-6-PDH). The complete reaction was developed using nitrate reductase 30mU; NADPS 3 μM; G-6-P 750 μM; G-6-PDH 48 mU in a total reaction volume of 100 μl. The samples were incubated at room temperature for 90 min. At the end of incubation 30 μl DAN reagent (50 μg/ml, 0.62 N HCl) was added after 10 min, and 30 μl of 1.4 N NaOH was added to the incubation mixture. The fluorescence was measured in an Aminco-Bowmann Spectrophotofluorometer (USA) at an excitation wave length of 360 nm and 450 nm.

4. Statistical Analysis

In all the experiments a statistically significant difference was considered to be present when $p < 0.05$.

4.1 Paper I

The maximum increase in the eicosanoids was calculated as a multiple of the control values of each animal. These maximum values were collected for each group of different duration of VF and CPR. The results obtained for all groups were compared using Kruskal Wallis non-parametrical analysis of variance, and if a statistical difference was detected, post-hoc analysis of each group compared to the control group was performed using Dunn's multiple comparisons test. Maximum eicosanoid values were correlated to the duration of VF and CPR using Pearson correlation.

4.2 Papers II and III

Resuscitation variables were calculated with the chi-square test and the t-test. Data are presented as means $\pm$ SD (Table 5). Repeated measures two-way analysis of variance was used for comparisons of the groups over time after the data were shown to be normally distributed. In order to secure normally distributed data, analyses of 8-iso-PGF_{2 α} and 15-keto-dihydro-PGF_{2 α} were done after logarithmic transformation. Where ANOVA showed significant differences, a Bonferroni multiple comparison test was used. Survival analysis was carried out using the method of Kaplan and Meier (GraphPad PRISM® version 4, GraphPad Software, Inc. San Diego, CA, USA), and comparisons between groups were made using the log-rank test as well as the log-rank test for trend, as survival was considered to be a variable of the ordinal type.

4.3 Paper IV

Water content, ion analysis, and hemodynamic data were analyzed using ANOVA followed by Bonferroni's test for multiple group comparison. The histological and immunohistochemical results obtained for all groups were compared using Kruskal Wallis non-parametrical analysis of variance, and if a statistical difference was detected, post-hoc analysis of each group compared to the control group was performed using Dunn's multiple comparisons test.

5. Results

5.1 Survival

5.1.1 Paper II

A significant difference in survival to the end of the 4-hr experimental protocol between the MB and SAL groups ($p = 0.02$) was demonstrated using the Kaplan-Meier survival curve and the log-rank test, and when testing the three groups together using the log-rank test for trend ($p = 0.03$), while there were no differences between the MB and HSD groups ($p = 0.107$). Survival time was longer in the MB group in comparison with both other groups ($p < 0.0001$) (Table 5). The greatest post-arrest mortality occurred in the first hour after ROSC in the SAL group, in which it was associated with arrhythmias, low cardiac output, and decreasing blood pressure (eight animals).

5.1.2 Papers III and IV

Survival did not differ between the groups (*paper III*) and no differences were seen in resuscitation variables (Table 6). In paper IV, 15 of a total of 60 piglets did not achieve ROSC. During CPR, end-tidal CO₂ levels in the piglets that did not achieve ROSC were lower than the end-tidal CO₂ values of the animals that were successfully resuscitated ($P < 0.0001$) (Fig. 9).

Figure 9. End-tidal CO₂. During resuscitation in the pigs that achieved return of spontaneous circulation (ROSC), end-tidal CO₂ (with white circles) was higher ($p < 0.0001$) than end-tidal CO₂ in the pigs that did not achieve ROSC (with black circles) (Paper IV).

Table 5. Resuscitation variables (paper II)

Resuscitation Variables	MB (n=20)	HSD (n=19)	Sal (n=20)
Number of deaths before ROSC	1	4	3
Number of deaths after ROSC	3	4	8
Survival (number of survivors /total)	16/20	12/19	9/20
Counter shocks (number of shocks and mean± SD)	34 <hr/> (1.7±0.6)	38 <hr/> (2±1.5)	33 <hr/> (1.65±0.7)
Epinephrine (number of administrations and mean ± SD)	11 <hr/> (0.29±0.3)	12 <hr/> (0.28±0.3)	11 <hr/> (0.22±0.3)
Time to ROSC (s) (mean±SD)	115±38	123±25	124±27
Tribonate (mmol) (mean±SD)	25.35±8	18.76±11.6 (p=0.04) Vs MB	23.62±14.5
Survival time (min) (mean±SD)	199±84	149±110 (p< 0.0001) Vs MB	133±105 (p<0.0001) Vs MB

Table 6. Resuscitation variables (Paper III)

Variables	MBL	MBHSD	MBSAL
DC shocks (number of counter shocks /number of pigs that survived)	14/8	12/9	14/8
Epinephrine (number of administrations /number of pigs that survived)	3/10	2/10	2/9
Tribonate (number of administrations /number of pigs that survived)	10/10	10/10	9/9

5.2 Cerebral oxidative injury

As the indicator of oxidative injury, 8-iso-PGF_{2α} levels were determined in jugular bulb plasma in the first two papers and in sagittal sinus plasma in the third paper. The levels of 8-iso-PGF_{2α} increased significantly in all intervention groups compared to baseline levels with a maximum increase at 15-30 min in jugular bulb plasma and at 30 -60 min in sagittal vein plasma. The levels gradually decreased during the next 150 min and returned to baseline levels 240 minutes after ROSC. The concentration of 8-iso-PGF_{2α} sampled in the sagittal sinus in *paper III* was approximately double that in jugular bulb plasma as determined in the paper II (Fig.10).

Figure 10. The levels of 8-iso-PGF_{2α} at baseline and after ROSC (Papers II, III). The levels of 8-iso-PGF_{2α} measured in sagittal sinus plasma (S) (Paper III) and jugular bulb plasma (J) (Paper II) in the groups that received methylene blue added to a hypertonic hyperoncotic solution. P***<0.001; P**<0.01 between groups at 30 min and 60 min after ROSC.

5.2.1 Paper I

Jugular bulb plasma 8-iso-PGF_{2α} levels increased significantly in all the groups of animals that underwent CA and CPR compared to the control group in which no interventions were done. The levels of 8-iso-PGF_{2α} in the group subjected to 12 min of VF and 8 min of CPR (VF12) increased up to 33-fold from baseline during the period 30 min after ROSC, considerably more than in any of the other groups. The total duration of VF and CPR was correlated to the maximum increase in the 8-iso-PGF_{2α} level ($r^2=0.20$; $P<0.05$). Inter-individual differences in the increase of 8-iso-PGF_{2α} were small in the lowest VF groups (VF2 and VF5 min) but increased gradually in

the groups with longer durations of VF. Out of the animals subjected to a total of 20 min of ventricular fibrillation and CPR of low flow, those that exhibited signs of severe cerebral blood flow derangements or brain death as detected by no-flow in the cerebral cortex at 30 min after ROSC showed a 5-fold increase in 8-iso-PGF_{2α} levels at 15 min after ROSC.

5.2.2 Paper II

After ROSC, the jugular bulb concentration of 8-iso-PGF_{2α} increased to a maximum at 15 min after ROSC, and thereafter gradually decreased towards baseline values during the remaining part of the investigation. The jugular bulb 8-iso-PGF_{2α} concentration was significantly ($P < 0.001$) greater in the SAL group than in the MB group, as well as in comparison with the R group ($P < 0.001$). No differences between MB and HSD groups were seen.

5.2.3 Paper III

No differences were recorded between groups in the levels of 8-iso-PGF_{2α}. The sagittal sinus plasma concentration increased by 16-fold, with a maximum 30-60 min after ROSC. The highest levels of isoprostanes were determined in the jugular bulb in the group that received saline and lowest levels in the group that received hypertonic saline-dextran with or without methylene blue.

5.3 Cerebral inflammatory response

5.3.1 Paper I

Jugular bulb plasma levels of 15-keto-dihydro-PGF_{2α} increased in all intervention groups within 5 min, with a maximum increase at 15-30 min after ROSC, and then gradually decreased within 120 min. A slight increase in 15-keto-dihydro-PGF_{2α} was observed at 30 min in the control group but not to the same extent as in the intervention groups. Maximum plasma 15-keto-dihydro-PGF_{2α} levels increased in a non-duration dependent manner with the greatest increase in the intervention group subjected to 8 min of VF and 5 min of CPR. The total duration of VF plus CPR was not correlated to the maximum levels of 15-keto-dihydro-PGF_{2α}. The levels of 15-keto-dihydro-PGF_{2α} increased 8-fold from baseline during a period of 30 min after ROSC. Inter-individual differences were small between VF2 and VF5, but increased to a maximum in the VF8 group. Out of the animals subjected to 20 min of low-flow, the animals that exhibited signs of severe cerebral blood flow derangements or brain death at 30 min after ROSC showed a 2-fold increase in 15-keto-dihydro-PGF_{2α} from the basal levels at 15 min after ROSC, which then rapidly decreased to the basal level at 30 min after ROSC.

5.3.2 Papers II and III

The baseline jugular bulb concentration of 15-keto-dihydro-PGF₂ increased in both experiments during the early reperfusion phase immediately after ROSC. Thereafter, this level decreased gradually, returning to baseline level at the end of the study. There was a significantly lower level in the MB group ($P < 0.001$) compared to the other two groups in paper II and there were no differences in paper III.

5.4 Cerebral neurological injury (Protein S-100 β)

5.4.1 Paper II

In all the groups, protein S-100 β increased during the first hour after ROSC and returned to baseline levels towards the end of the study. There were statistically significant differences between the three groups, with lower levels in the MB group in comparison with the SAL group ($P < 0.0001$) and the HSD group ($P < 0.001$). No statistically significant difference was found between the SAL group and the HSD group.

5.4.2 Paper III

No differences were recorded between groups in the levels of 8-iso-PGF_{2 α} , 15-keto-dihydro-PGF_{2 α} , and protein S-100 β . The sagittal sinus plasma concentration increased by 16-fold and 3-fold with a maximum 30-60 min after ROSC for the oxidative and inflammatory markers, respectively, and a maximum at 5-15 min after ROSC for the protein S-100 β .

5.5 Cerebral cortical blood flow

5.5.1 Paper II

During the early reperfusion phase (5 minutes after ROSC), the mean CCBF increased in all groups in comparison with baseline values ($P < 0.001$). Thereafter this blood flow started to decrease below control values, but there were no significant differences compared with baseline. Towards the end of the study, the CCBF gradually returned to baseline in all the groups. During the first 60 min after ROSC, the CCBF differed between the groups ($P < 0.001$); it was greatest in the MB group and lowest in the SAL group, with an intermediate value in the HSD group.

5.5.2 Paper III

There were no differences between groups in cerebral perfusion pressure. During the early reperfusion phase (5-10 min after ROSC), the mean CCBF increased in all groups in comparison with baseline values ($P < 0.001$). Thereafter, this blood flow started to decrease and gradually returned to baseline in all the groups. Differences were observed at 5 min after ROSC, with an increase in CCBF in the MBS group in comparison with the MBHSD group ($P = 0.023$) and the MBL group ($P = 0.021$). At the same time point, the increased CCBF was associated with a numerical difference in the oxygen extraction ratio in the MBS group in comparison with both of the other groups, but it failed to reach statistical significance.

5.6 Myocardial enzymes

5.6.1 Paper II

Both biochemical markers of myocardial injury increased during the experiment with maximums towards the end of the experiment. Statistically significant differences appeared between troponin I levels in the MB and SAL groups at 240 min ($P < 0.01$) as well as between the MB and SAL groups regarding CK-MB levels at 120 and 240 min ($P = 0.03$ and $P < 0.01$, respectively). In contrast, there were no statistically significant differences between the HSD and SAL groups.

5.6.2 Paper III

The MBS group exhibited significantly increased levels of myocardial tissue injury enzymes as follows: at 240 min troponin I was increased in comparison with MBHSD ($P = 0.019$) and MBL ($P = 0.037$). CK-MB was also increased in this group in comparison with both other groups at 120 min and 240 min ($P < 0.001$).

5.7 Hemodynamic parameters

In all the experiments, heart rate increased significantly after ROSC and then gradually returned to baseline values by the end of the first hour after ROSC in all the groups. Arrhythmias associated with a negative ST-segment appeared within the first hour after ROSC in all the groups. The ST-segment returned to normal levels in the first hour after ROSC in surviving pigs. After ROSC, there was a period of 5-10 min of spontaneous overshoot in arterial and pulmonary arterial blood pressures, and cardiac output in all groups ($P < 0.0001$). After that a hypotensive period gradually followed in all

groups, with the lowest levels at 30 minutes compared with baseline levels ($P < 0.001$). The decreased blood pressure lasted approximately 1 h after ROSC, followed by a gradual increase to baseline levels.

5.7.1 Paper II

There was a statistically significant increase in arterial blood pressure at 15 min in the MB group in comparison with the SAL group ($P < 0.01$), and increased diastolic blood pressures at 15, 30 min in the same group ($P < 0.01$). CO was increased in both the HSD and the MB groups in comparison with the SAL group ($P < 0.05$) 5 minutes after ROSC. Thereafter significant differences were seen only between MB and SAL at 15 minutes ($P < 0.0001$), 30 minutes ($P = 0.037$) and 60 minutes ($P = 0.04$) after ROSC, with better cardiac output in the group that received MB. A greater coronary perfusion pressure at 15 minutes after ROSC ($P < 0.05$) was observed in the MB group in comparison with both other groups. The pulmonary arterial pressure was increased in the MB group in comparison with the SAL group ($P < 0.05$) at 5 and 15 min after ROSC. (*Addendum I*)

5.7.2 Paper III

No differences between groups were seen in the number of pigs that received dobutamine. PCWP was increased at 180 min after ROSC in the MBS group ($P = 0.023$) and remained increased until the end of the experiment. Tendencies toward higher pressures in the pulmonary artery and right atrium were observed at 120 min after ROSC in the same group but they failed to reach statistical significance.

5.8 Systemic and jugular bulb acid-base status

In all of the experiments the arterial blood pH gradually decreased during the 8 min of CPR, but acidemia was not profound in any of the three groups. In contrast, acidemia was profound (< 7.2) 5 min after ROSC and needed correction with tris buffer mixture and increased ventilation. In contrast to the arterial pH, the jugular bulb blood pH and sagittal sinus blood pH decreased immediately after commencement of CPR and started to increase at 16 minutes after arrest.

5.8.1 Paper III

The total dose of buffer administered to the three groups did not differ. The pH returned to normal values at 120 min after ROSC in the MBL group, with a mean value that was slightly higher in comparison with the MBS ($P = 0.01$)

and the MBHSD groups ($P=0.03$). In parallel with the pH effects, the base excess was higher in the MBL group from 120 min until the end of the study.

5.9 Electrolytes, lactate, glucose

5.9.1 Paper II

Plasma lactate levels increased during the experiment in all three groups (significant differences in all the groups at 120 min in comparison with baseline) ($P < 0.0001$). There was a significant difference between the SAL group and the MB group at 120 min ($P < 0.001$) with lower levels of lactate in the MB group. Significant differences were found between the MB and the SAL groups at 120 min ($P < 0.0001$) and between the HSD and the SAL groups ($P < 0.001$) at the same time point, while there were no differences between the HSD and MB groups. Significant differences appeared at 120 min in all the groups in comparison with baseline ($P = 0.04$ in HSD group, $P = 0.03$ in MB group and $P < 0.001$ in SAL group). The sodium level was lower than the control value in the SAL group, while the other two groups exhibited increased sodium concentrations in comparison with the control value. Serum potassium concentration increased in all the groups from 3.72 ± 0.1 mmol/L to 4.2 ± 0.2 mmol/L in the SAL and HSD groups and to 4.1 ± 0.4 mmol/L in the MB group, while calcium levels decreased from 1.33 mmol/L to 1.21 mmol/L in all the groups.

5.9.2 Paper III

No differences in the baseline blood levels of lactate (Fig. 11) in the three study groups were seen. Lactate concentrations increase after ROSC with higher levels in MBHSD group.

The sodium load administered to the MBHSD group increased the plasma sodium concentration after ROSC compared to prearrest conditions ($P < 0.001$), and compared to the other treatment groups ($P < 0.001$). Hypertonic sodium lactate administration had no effect on serum sodium concentration, while administration of normal saline decreased serum sodium 120 min after ROSC ($p < 0.001$). Hypertonic saline dextran (MBHSD) increased the serum chloride concentration ($P < 0.001$) in comparison with baseline, whereas lactate administration (MBS) substantially decreased it ($P < 0.001$). There was a significant difference between these two groups ($P < 0.001$). Calcium decreased in the MBL group in comparison with both other groups ($P < 0.05$) and baseline ($P < 0.05$). There was no effect on serum potassium or on blood glucose in any of the groups.

Lactate

Figure 11. The lactate levels (mmol/L) in Figure A. MBS group (methylene blue saline), Figure B. MBHSD group (methylene blue hypertonic saline dextran) and Figure C. MBL group (methylene blue lactate) at baseline and 120 minutes after return of spontaneous circulation. The lactate concentration was increased 2 hours after ROSC in all groups ($P < 0.05$). Differences in lactate were recorded between groups, with higher levels in the MBL group only in comparison with the MBS group ($P = 0.034$), with an intermediate level in the MBHSD group.

5.10 Immunohistochemistry (albumin, NOS), neurological injury, water content, electrolytes, nitrite/nitrate content, western blot (Paper IV)

Albumin immunoreactivity (Fig.12) and neuronal injury (Fig.13) were lowest after 5 min of cardiac arrest (CA) and at 30 min after ROSC in the other two groups. In all the groups increased albumin, water, electrolytes and neuronal injury were demonstrated after cardiac arrest. Immunohistochemistry analysis indicated decreased blood brain barrier disruption in the animals that received MB as evidenced by decreased albumin leakage ($P = 0.004$). The water content ($P = 0.02$), potassium ($P = 0.04$), and neuronal injury ($P = 0.008$)

decreased in this group in comparison with the group that was not treated with MB (Fig.13). Albumin, water and neuronal damage were decreased at every point of observation (30, 60, 180 min) in CA+MB compared to CA-MB ($P<0.001$), as were sodium and potassium, most at 30 and 180 min ($P<0.001$). Differences between the CA+MB and CA-MB groups in chloride content were seen only at 30 min ($P<0.01$).

Figure 12. Representative examples of albumin immunoreactivity in the cerebral cortex (parietal a, c, e and temporal b, d, f) following cardiac arrest without CPR in the CA group (e, f), with CPR without treatment with methylene blue (arrows; a, b), cardiac arrest with MB (arrowheads; c, d). Marked albumin immunoreactivity is seen in the cortical neurons (arrows) after cardiac arrest and CPR without MB.

Figure 13. Nissl-stained, paraffin section (3 μm thick) from pig brain obtained from the parietal cerebral cortex region in the vicinity of Evans blue extravasation. Many dark and distorted nerve cells (arrows) are seen in deeper layers of the cortex. Some normal nerve cells (arrowheads) are also seen in the periphery. Sponginess and edema (*) are clearly evident. Bar: 50 μm .

Pigs subjected to a 5-min survival after cardiac arrest showed a mild to moderate degree of nerve cell damage in the cortical regions (Fig. 13). Moderate to severe nerve cell damage was also present in the subcortical regions, for example the mid-thalamic nuclei and the brain stem reticular formation. The magnitude of nerve cell damage was most pronounced in the brain stem region [10].

Similarly, MB treatment reduced the nitrite/nitrate content ($P=0.02$), iNOS expression ($P<0.01$), and nNOS expression ($P<0.01$). In the control condition there was very little expression of any of the NOS isoforms. Experimental global ischemia from cardiac arrest rapidly led to upregulation of the nNOS and iNOS isoforms. After only 5 min of untreated CA, significant nNOS expression was obvious. Immunohistochemical analysis from temporo-parietal cortex indicated no inhibition of eNOS. Western blot analysis of pooled protein extracts of superficial cerebral tissue of all animals at respective time points indicated results which varied somewhat from the immunohistochemical analysis for nNOS and iNOS but reasonably consistent with microscopical evaluation of immunostained sections of cerebral

cortex in the temporo-parietal region. No increase in eNOS protein expression could be shown using western blot technique.

Figure 14. Cerebral cortical immunoreactivity for albumin and signs of brain edema (water and electrolyte content: sodium, potassium, chloride), as well as neuronal injury. Untreated animals (CA-MB group) exhibited a profound increase in BBB permeability for albumin, and an increase in the cerebral content of water, sodium, and potassium. In the CA-MB group, a significant increase in the number of distorted neurons as a measure of neuronal injury was observed. The groups described were: the CA group (cardiac arrest without CPR) at 5, 15, 30 min after CA; the CA-MB group and the CA+MB group (cardiac arrest with MB administration) at 30, 60, 180 min after ROSC.

6. Discussion

To answer to the eternal question “to be or not to be?” or translated in CPR terms “to survive or not to survive?” after cardiac arrest, efforts were made to find solutions for restarting the heart and protecting all the organs, especially the brain and the heart, from ischemia and reperfusion injury. There are still no answers, partly because the events of human cardiac arrest and CPR cannot be repeated satisfactorily in vitro, and thus a large portion of this type of research is conducted in animals. Therefore the experimental CPR model that was selected warrants some consideration.

6.1 The experimental model of CPR

The time selected for CA and CPR in our experimental pig model was 20 min of VF in order to reflect a typical clinical scenario. For example, in a retrospective cohort study in Sweden, the interval between cardiac arrest and paramedics’ arrival was approximately 7-14 min (median time from witnessed out-of-hospital cardiac arrest to alarm is 3-4 min and there is a median time of 4-10 min from arrest to ambulance arrival), and the initial drug therapy was administered after 20 min of cardiac arrest [146]. The pathophysiology of CA and reperfusion injury seems to be related to the duration of CA, and Weisfeld and Becker therefore proposed that CA involves three distinct phases, or the “three-phase model of cardiac arrest”[147]. During the initial phase, known under the name “electrical” (from 0-4 min after CA), VF responds well to countershock. The second phase (from 4–10 min after CA) is the “circulatory phase.” During this phase, promotion of cardiac work and oxygen delivery after initiation of CPR and supplemental administration of vasopressors before attempting defibrillation seems to yield better survival outcome. The third phase of CA (metabolic phase) occurs beyond 10 minutes. Despite the fact that this last phase corresponds to the time of administration of resuscitative drugs and interventions, few studies have been performed beyond 10 minutes after CA, obviously because the longer duration of circulatory arrest has a major influence on outcome. The 20-min VF time used in the present research might be considered as a **prolonged-time VF** and also reflects the metabolic phase of CA. During the metabolic phase, the consequences of prolonged periods of tissue ischemia may resemble a state of sepsis, resulting in release into the circulation of tumor necrosis factor, endotoxins and cytokines [148]. This was illustrated in *Paper I* where

a time-dependent free radical damage related to the duration of CA was demonstrated. The magnitude of post-resuscitation collapse is related to VF duration, and thus when VF is prolonged as in our study, post-resuscitation cardio-vascular collapse may occur [149], and this requires exogenous support. When comparing post-resuscitation hemodynamics in *Paper II* with that in *Papers III and IV* in which the animals received dobutamine when systolic blood pressure decreased below 70 mmHg after VF, we can conclude that after prolonged VF, post-resuscitation hemodynamics can neither be stabilized nor undergo spontaneous recovery without exogenous support.

Whether **fluids** should be infused routinely during CPR is still controversial and the guidelines for CPR neither support nor refute [150] the routine use of intravenous fluids. There is no protocol for fluid administration during resuscitation, except for rapid infusion of 2 L of ice-cold saline for induction of mild hypothermia [151]. However evidence from human [152] and animal studies indicates that infusion of hypertonic saline during CPR increases the resuscitation success rate and improves myocardial and cerebral reperfusion during CPR. Evidence from animal studies supports the use of intravenous fluid administration during CPR to improve cerebral perfusion during open-chest CPR [153], significantly increasing myocardial blood flow and myocardial perfusion pressure [154, 155, 156]. In porcine experimental studies infusion of hypertonic solutions increased resuscitation success and survival rates [155, 156] as was demonstrated in *Paper II*. The results of our studies support these findings.

The protocol used in all the studies for performing CPR before defibrillation during the circulatory phase is in accordance with the recommendations of the 2005 guidelines for CPR [150], although our research started before implementation of these guidelines. The choice to start CPR before defibrillation came after randomized clinical trials [157, 158] in which patients with out-of-hospital CA received either standard care with immediate defibrillation or 3 min of basic CPR before defibrillation. In the latter study, the group that received CPR first achieved 58% ROSC and 22% survival in comparison with 38% ROSC and only 4% survival in the group with defibrillation first. In contrast, no such effect was observed in an Australian study [159] with only a 4-5% survival rate to hospital discharge. However, in a recent animal study that used a porcine model of 5-8 min of VF, it was demonstrated that increasing the duration of CPR prior to defibrillation had no effect on ROSC and survival [160]. The strategy chosen will inevitably influence the results of the study. In searching the literature it was surprising to note the relative differences in percentages of survival and ROSC in porcine models. In our opinion, the better outcome results depend on the coronary perfusion pressure during CPR, which depends not only on the chest compressions, but also on the type of vasopressor. Interestingly, the hemodynamic effects of epinephrine are influenced by the quality of chest compressions [161]. An important observation was that the coronary perfusion

pressure increased after vasopressin administration in the first two minutes of CPR, but decreased at the end of the 8 minutes of CPR. Prior studies with even shorter periods of ventricular fibrillation and CPR prior to defibrillation were associated with worse outcome without vasopressor administration [160]. Ewy et al [162] demonstrated 50% ROSC and survival with CPR during a duration of VF up to 12 min before defibrillation using epinephrine. Aufderheide et al [163] demonstrated 86% survival in a porcine model after 6 min of VF and follow-up CPR. Hayes et al [164] evaluated ventilation scenarios in a swine model and reported 25–41% ROSC and survival after 8 min of uninterrupted VF followed by 3 cycles of 2 min of continuous chest compressions prior to defibrillation. The relatively higher survival rate in studies II and III in comparison with the above studies might also be related to the improved quality of chest compressions that were performed with the LUCAS device. It was demonstrated in our group that chest compressions with the LUCAS device during experimental resuscitation resulted in higher cerebral blood flow and cardiac output than standard manual external chest compressions [165]. In animal studies, superior survival rates with drugs have been demonstrated in comparison with humans, probably due to the fact that drug administration occurs some 10 min earlier in humans than in animal studies [166]. Shorter drug delivery time in animal models of CA may thus be one reason for the failure of animal studies to translate successfully into the clinical arena [16, 166]. Using a swine model, it was demonstrated that the duration of resuscitation may be extended throughout the use of a vasoactive drug prior to rescue countershock [167]. There are many similarities between a porcine animal model and humans regarding metabolic and cardiovascular function [168,169,170]. The model has human hemodynamic characteristics [171] and similar coronary anatomy compared with human cardiac anatomy, with the exception of the left azygous vein, which enters the coronary sinus rather than the precava [168, 169, 170]. However, in addition to the limitation that all animals were anesthetized as required by our ethics committee, the present studies have other limitations. An important one is that we used young, healthy pigs that were free of atherosclerotic disease. Second, the swine myocardium is poorly collateralized compared to the myocardium of patients with chronic obstructive coronary artery disease, who are likely to demonstrate collateralization [172,173]. Poor collateralization influenced the CPR-generated regional myocardial blood flow, and possibly the resuscitation rate and degree of postresuscitation myocardial dysfunction [173, 174]. A model of ischemically induced VF followed by standard resuscitative interventions would have replicated more closely the conditions of patients with out-of-hospital cardiac arrest resuscitation [174] than the method of electrically induced VF. The hemodynamic response to exogenously administered arginine vasopressin may be different in humans because the vasopressin receptors in pigs (lysine vasopressin) and humans (arginine vasopressin) are different [175]. In addition, however, the pig

brain, which is gyrencephalic, resembles the human brain more in anatomy, growth and development than do the lissencephalic brains of commonly used small laboratory animals [16, 176, 177].

6.2 Cerebral oxidative, nitrosative, inflammatory and neurological response after resuscitation from cardiac arrest

Early ischemia is associated with accumulation of free fatty acids, especially arachidonic acid that is rapidly metabolized during reperfusion and generates oxygen radicals. Free radicals oxidize phospholipids of the cell membrane, resulting in structural damage. Ischemia leads to inflammation, the earliest stage of which appears to be invasion of microglia within 2-4 hours after the event, followed by production and release of proinflammatory cytokines.

The sagittal sinus plasma concentration increased 16-fold and 3-fold in *Paper III* with a maximum 30-60 min after ROSC for the oxidative (8-iso-PGF_{2α}) and inflammatory markers (15-keto-dihydro-PGF_{2α}), respectively, and at 5-15 min after ROSC for the protein S-100β. Thereafter they gradually decreased towards baseline values during the remaining part of the investigation. In *Paper III* the cerebral venous blood was sampled from the sagittal sinus vein, while it was taken from the internal jugular sinus in the *Paper II*. When comparing the results of the analyses from the two sites it was found that 8-iso-PGF_{2α} was more than doubled in plasma sampled in the sagittal sinus but peaked later than in plasma samples from the internal jugular vein. 8-Iso-PGF_{2α} levels increased about 16-fold from the basal levels in sagittal sinus plasma while a 9-fold increase was observed in the internal jugular vein after ROSC. The magnitude of the difference was similar for 15-keto-dihydro-PGF_{2α}, while the difference was less for the astroglial protein S-100β in the sagittal sinus plasma compared to the plasma collected from the jugular bulb. Interpretation of this comparison implies that the first two indicators are more specific for cerebral injury than the astroglial protein S-100β in connection with experimental porcine cardiac arrest and CPR. The blood obtained from the sagittal sinus drains the frontal parts of the the brain, compared to the relatively mixed blood from the internal jugular vein that also drains extracranial tissues. Thus, evidence of free radical formation following ROSC is greater in the blood collected from the sagittal sinus than the jugular vein, a significant manifestation of oxygen deprivation in a cerebral hypoxic state. This corroborates our earlier statement that cardiac arrest and ROSC may lead to a severe state of oxidative stress in the brain compared to the rest of the body, which may cause neurodegeneration in the future [26]. The neurological outcome 24 h after experimental CPR

was shown to correlate with cerebral plasma 8-iso-PGF2 concentrations after ROSC [120].

These studies support the concept that isoprostanes not only increase in models of ischemia–reperfusion but may also play a role in the pathophysiological sequelae of this injury [178].

In blood collected from the sagittal sinus, contamination from extracerebral tissue is avoided, but these measurements necessitated craniotomy. It cannot be ruled out that this preparation might have influenced the inflammatory response and the subsequent magnitude of oxidative injury.

The astroglial protein S-100 β is a sensitive marker of hypoxic brain damage after cardiac arrest, and it correlates well with the extent and prognosis of ischemic brain injury [179,180]. Protein S-100 β also reflects the extent of experimental brain damage after cerebral ischemia and serves as a useful biomarker for the assessment of effects of neuroprotective drugs [179, 180]. In addition, the association between the observed histopathological cerebral injury in *Paper IV* and the plasma indicators of neurological injury recorded in *Papers II and III* might be speculated upon. The levels of protein S-100 β remained the same in the groups that received MB (*Paper III*) with other fluids and it was significantly lower during the course of the experiments in the MB group (*Paper II*) in comparison with the groups without MB. This finding indicates a neuroprotective effect of MB administration that was later confirmed histopathologically in *study IV* by counting the injured cortical neurons. MB acts as a neuroprotective agent by inhibiting brain NOS activity *in vivo* and interfering with the NO-cGMP system [181].

The short half-life of NO *in vivo* makes it difficult to measure NO levels directly, and cerebral tissue contents of nitrite and nitrate were therefore determined in *Paper IV*. Nitrite and nitrate are stable metabolites of NO, accessible for quantitative analysis, and their measurement is considered to be the most suitable and practical method for assessing NO synthesis [182]. Nitrite has been reported to represent both circulating and tissue storage forms of NO which are activated during ischemia by reduction of nitrate [183]. In contrast, tissue nitrate content decreases only after long-term NOS inhibition [184], while nitrite levels respond rapidly to NOS inhibition, as also demonstrated in *Paper IV*.

6.3 Blood-brain barrier disruption after CA and CPR

It has been long been accepted that global cerebral anoxia induced by circulatory arrest can result in neurological damage and neurological dysfunction after only 5 min of cardiac arrest and subsequent restoration of spontaneous circulation [79]. *Paper IV* contains evidence for early neurological injury after ROSC and indicates that the magnitude of neurologic injury increases with time from the initial ischemic insult. *Paper IV* provides experimental

evidence for leakage of albumin, water and electrolytes to the brain parenchyma after cardiac arrest and ROSC. BBB disruption already began during the initial phase of untreated as well as treated cardiac arrest and progressed throughout the time these animals were observed. These results are contradictory to previous studies on piglets indicating that young animals might be more prone to a delayed (4 h) increase in BBB permeability after cardiac arrest and CPR [59, 60] and to other animal studies indicating a biphasic nature of BBB disruptions [63, 65].

6.4 Methylene blue in CA and CPR

In *Paper IV* MB administration during CPR and the initial phase after ROSC reduced the BBB disruption and neurologic injury considerably, but did not, on the other hand, reverse the ongoing detrimental process. Other NOS inhibitors such as the non-selective NOS inhibitor N^G-monomethyl-L-arginine (L-NAME)[71], N^G-nitro-L-arginine (L-NNA) [185] and selective inhibitors such as aminoguanidine (iNOS inhibitor), 1-2 (trifluoromethylphenyl) imidazol (TRIM), a nNOS inhibitor, were also tested in experimental models of cardiac arrest [186, 187]. Schleien et al [71] demonstrated that non-specific NOS inhibition with L-NAME attenuated the post-ischemic cerebral and myocardial hyperemia. In *Paper IV*, counting the distorted neurons in parietal-temporal cortex was used to compare the severity of post cardiac arrest brain damage, and cerebral albumin and water content expressed the magnitude of BBB disruptions. The results unequivocally demonstrate that MB markedly reduced BBB disruption and subsequent neurologic injury after ischemia/reperfusion from cardiac arrest.

6.4.1 Methylene blue and NOS inhibition

Zhang et al [188] studied non-selective inhibition of NOS with L-NNA in a swine resuscitation model, demonstrating that pre-arrest NOS inhibition did not improve survival, but reduced requirements for epinephrine and closed-chest compression. Adams et al [186] suggested that an intact basal nNOS activity is vital for survival after ischemia/reperfusion injury and that iNOS inhibition prior to ischemia reperfusion protects myocardial function after ROSC. Using a method of endothelial stimulation to produce endothelial derived NO (periodic acceleration pGz), superior myocardial function post-resuscitation was demonstrated [187], possibly indicating that complete eNOS inhibition during CA is deleterious to cardiac function in resuscitation from CA. Hence, it was also considered that the beneficial effects of non-selective inhibitors were limited, because they inhibit eNOS to a similar degree and may therefore aggravate effects of cardiac and brain ischemia. In cerebral tissue the large NO tissue content produced a few hours after ROSC

by activation of iNOS is considered detrimental, but the much smaller tissue content of NO derived from eNOS is beneficial due to its vasodilating effects after cerebral ischemia [187]. In comparison with these NOS inhibitors it must be mentioned that MB interferes with several pathways and it may be that the protective effects of MB in ischemia/reperfusion injury are not only explained by the inhibition of nitric oxide production. Along with the inhibition of NOS, MB reacts directly with NO [101], with superoxide molecules (which react rapidly with NO)[102], and inhibits guanylate cyclase (the vessel-relaxing factor for NO)[103].

All three NOS isoforms were expressed in the brain after global ischemia from cardiac arrest. However, the iNOS induction occurs transcriptionally long after activation of posttranscriptionally activated eNOS and nNOS, suggesting that iNOS does not contribute to early cerebral injury [49]. *Paper IV* demonstrates reasonable agreement between immunohistochemical analysis and Western blot regarding the inhibitory effects of the MB on the expressions of iNOS and nNOS, but the opposite effects on eNOS. Changes in NOS expression were investigated by immunohistochemistry in temporoparietal cortex and in cerebral tissue by Western blotting, thus differences may occur between the two methods knowing that NOS have cerebral regional distribution. MB administration attenuated morphologic changes suggesting that NO and consequent peroxynitrite formation during ischemia-reperfusion injury contributes to cerebral injury.

6.4.2 The dose of methylene blue

The total dose of MB that was administered was similar (2.7-3.4 mg/kg) in all the papers. Dose-related toxicity of methylene blue is described [86] for doses over 2 mg/kg, but considerably higher doses have also been used without untoward effects. Dose-related neurotoxicity of MB affecting the central nervous system with higher doses (between 5 mg/kg and 50 mg/kg) than those used in the present study, as well as possible interspecies differences [97, 100], have been described. The clinical efficacy and safety of MB are well documented with both intermittent [189, 190] and continuous administration [91]. Further, the cost-effectiveness ratio of MB could be more advantageous than for the newer compounds with inhibitory effect on NOS.

6.4.3 Methylene blue and hemodynamics

Paper IV confirmed the prognostic value of end-tidal CO₂ during cardiopulmonary resuscitation and demonstrated it to be associated with survival. In *Paper II* it was demonstrated that MB not only increased short-term survival after CA and CPR as compared to a control group given normal saline, but also induced less hypotension, better coronary pressure and cardiac performance early after ROSC, thus improving post-resuscitation hemodynamics.

In the same paper, MB decreased myocardial injury as assessed by troponin I. The following mechanisms could be responsible for the observed beneficial effects of this combination of events:

1) An improvement was observed in coronary perfusion pressure initially after ROSC. It has previously been demonstrated that nonselective inhibition of NO by NG monomethyl-L-arginine increases coronary blood flow after ischemia, thus improving contractility [191]. NO affects myocardial contraction in a dose-dependent fashion, with low doses of NO resulting in positive inotropic effects and higher concentrations exerting negative inotropic effects [153]. Resuscitation studies using hypertonic–hyperoncotic solutions have usually reported an improvement in myocardial blood flow during CPR [154] as a result of improved microcirculation during ischemia [152], whereas only minor effects have been found during recirculation [152, 192]. In our investigation, significant statistical differences, although rather small and of relatively short duration, occurred in mean arterial blood pressure, coronary perfusion pressure, and left ventricular stroke work, not only between the MB and SAL groups at 5–15 mins after ROSC, but also between the MB and HSD groups. Thus, these results could not be related solely to the administration of HSD solution.

2) MB blocks the formation of several reactive oxygen species, particularly superoxides, by competing with molecular oxygen for the transfer of electrons by xanthine oxidase [108]. Peroxynitrite ($\cdot\text{ONOO}^-$), a product of NO and superoxide anion ($\cdot\text{O}_2^-$) interaction, is known as a powerful oxidant that exerts vasorelaxant effects on peripheral blood vessels [193]. Peroxynitrite depresses myocardial contractility by decreasing the ability of Ca^{2+} to trigger contraction, an effect that is partly mediated by the cGMP/cGMP-dependent protein kinase pathway [194]. The presence of MB significantly inhibits the relaxation induced by peroxynitrite [195], and it promotes myocardial contraction [89].

7. Conclusions

Prolonged duration of CA leads to a worsening of oxidative injury. The jugular bulb plasma concentration of 8-iso-PGF2 α (indicating free radical-mediated lipid peroxidation) is associated with the duration of ischemia. The cyclooxygenase derived PG does not increase to the same magnitude as free radical mediated isoprostane formation (*Paper I*).

- MB co-administered with small volumes of hypertonic-hyperoncotic solutions improves early hemodynamics and decreases inflammatory, oxidative and neuronal and myocardial injury. Consequently, it increases early survival (vs. saline) and survival time (vs. both other groups) after CA and CPR (*Paper II*).
- The mixture of MB with hypertonic-sodium lactate may be an alternative for administering MB during ongoing CPR, with similar myocardial and cerebral effects as MB co-administered with hypertonic-saline dextran (*Paper III*).
- BBB disruption occurs during the initial phase of untreated as well as treated CA. MB administration during CPR and the initial phase after ROSC reduced BBB disruptions considerably but, on the other hand, did not reverse the ongoing detrimental process. MB administration attenuated neurologic injury, suggesting that NO and consequent peroxynitrite formation during ischemia-reperfusion injury contributes to cerebral injury (*Paper IV*).
- All three NOS isoforms were expressed after global ischemia from cardiac arrest, but iNOS induction occurs transcriptionally long after posttranscriptionally activated eNOS and nNOS, suggesting that iNOS does not contribute to early cerebral injury. There are nonselective inhibitory effects of MB on NOS (*Paper IV*).

8. Acknowledgements

I would like to express my sincere gratitude to all those who helped me on this journey:

*My tutor and admirable friend **Lars Wiklund**, for being a distinguished source of inspiration. "Entirely original ideas are rare, rediscoveries are frequent and credit should go to those individuals who convinced the world " (W.O. Safar), and you, **Lars**, convinced the world.*

***Monica Hall, Anders Nordgren, Elisabeth Pettersson** for skillful technical guidance and invaluable help in the research laboratory:" Genius is one per cent inspiration, ninety-nine per cent perspiration"(Thomas A. Edison). Thank you.*

*My second tutor **Samar Basu** and co-authors **Hari Shanker Sharma and Cécile Martijn** for expert analytical and histopathological preparations: "All truths are easy to understand once they are discovered; the point is to discover them" (Galileo Galilei).*

***Torsten Gordh** for encouragement and support and for keeping me updated so that "I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale"(Marie Curie).*

***Stephen Butler** for your profound open mind and for all the "coffees" together. "Genius is of no country"(Charles Churchill).*

***My dear friends from the pain clinic**, intensive therapy, anesthesia and those from everywhere else for offering me "fantastical moments"and making me able to paint again, because"Without friends no one would choose to live, though he had all other goods"(Aristotle).*

***Björn Zarèn and in the memory of Doina Gladkvist**, those who started my Swedish journey "Everything you can imagine is real"(Pablo Picasso).*

***Torbjörn Karlsson**, for creating such an extraordinary environment for advanced clinical research in our department. "I'm an idealist. I don't know where I'm going, but I'm on my way" (Carl Sandburg)*

***Sten Rubertsson**, for being an enthusiastic researcher and always an inspiration "Nothing great was ever achieved without enthusiasm"(Ralph Waldo Emerson)*

Ala Nozari, Miklos Lipcsey, Rolf Gedeberg, for your help and suggestions “If we knew what it was we were doing, it would not be called research, would it?”(Albert Einstein)

For all the members of my family who are still alive and for all those who passed away to whom this thesis is dedicated because “To love and be loved is to feel the sun from both sides” (David Viscott).

For Lucia, Vasile, Sara si Vlad “Who, being loved, is poor?”(Oscar Wilde). Thank you for your love, support, patience and for the long discussions (including those about the revival therapy after a stress-related attack in women).

For syster Augusta (tusa Reghina) “Fără credință, fara Dumnezeu, omul devine un animal rațional care vine de nicăieri și merge spre nicăieri”(Petre Tutea).

Vincent van Gogh, Salvator Dali, Georgia O`Keeffe and all the painters who provided me with “cerebral protective colors” and inspired me. “When this war is over I shall confine myself entirely to writing and painting”.(Winston Churchill)

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