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Possibilities for protecting cardiac and vascular function against ischaemia-reperfusion injury

Ph.D. Thesis

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1. LIST OF ABBREVIATIONS

ACh	Acetylcholine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ARNT	Aryl hydrocarbon receptor nuclear translocator protein
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CA	Carbonic anhydrase
cGMP	Cyclic guanosine monophosphate
CO	Carbon monoxide
CO_2	Carbon dioxide
CVD	Cardiovascular disease
DMOG	Dimethyloxalylglycine
DNA	Deoxyribonucleic acid
dP/dt_{max}	Maximal slope of the systolic pressure increment
dP/dt_{min}	Maximal slope of the diastolic pressure decrement
ECG	Electrocardiography
EDTA	Ethylenediaminetetraacetic acid
EDPVR	End-diastolic pressure–volume relationship
EDV	End-diastolic volume
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
FIH	Factor inhibiting HIF
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT	Glucose transporters
H_2O_2	Hydrogen peroxide
HO	Heme oxygenase
HIF	Hypoxia-inducible factor

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HRE	Hypoxia responsible elements
iNOS	Inducible nitric oxide synthase
I/R	Ischaemia–reperfusion
K_{ATP}	ATP-dependent potassium channels
KCl	Potassium chloride
LDL	Low-density lipoprotein
LV	Left ventricular
LVEDP	Left ventricular end-diastolic pressure
LVSP	Left ventricular systolic pressure
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger RNA
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Oxygen
$O_2^{\cdot -}$	Superoxide anion
OCl^-	Hypochlorite ion
$^{\cdot}OH$	Hydroxyl radical
ONOO	Peroxynitrite
PCR	Polymerase chain reaction
PHD	Prolyl hydroxylase domain-containing enzyme
PKC	Protein kinase C
PKG	Protein kinase G
PO_2	Partial oxygen tension

PRSW	Preload recruitable stroke work
qRT-PCR	Qualitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSS	Reactive sulphur species
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
τ (tau)	Time constant of left-ventricular pressure decay
TIMP	Tissue inhibitor of metalloproteinase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau tumour suppressor protein
VSMC	Vascular smooth muscle cells

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2. INTRODUCTION

Cardiovascular disease (CVD), particularly ischaemic heart disease, is responsible for approximately half of all deaths in the developed world and its treatment places a heavy burden on healthcare systems. The increasing prevalence of CVD can partially be attributed to the ageing society.

The difficulty of reducing ischaemia–reperfusion (I/R) injury and hypoxia is a major problem in cardiology and cardiovascular surgery, as well as being related to the problems that occur during organ transplantation. The aim of cardioprotection during and after ischaemia–reperfusion injury in clinical settings is to reduce infarct size, prevent severe cardiac arrhythmias and increase contractile function of the intact myocardium. Cardioprotection during decreased blood and oxygen supply is an extensively discussed and studied field in basic research: pathophysiology and clinical studies. Therapeutic strategies have improved over recent years; at present, thanks to drug research and operative developments, several strategies that result in a better outcome and prognosis even for patients with complicated disorders are in use. However, the development and investigation of new cardioprotective drugs is still required because cardiovascular ischaemia-related disorders remain a leading cause of death in the developed world.

It is impossible to point to one pathway in the ischaemia–reperfusion injury.

This thesis therefore focuses on cardiac and vascular protection against ischaemia–reperfusion injury and attempts to identify potential targets for novel therapeutic options to reduce ischaemia- and reperfusion-related organ damage, loss of function of implanted organs and mortality.

The aim of this study is to describe, using rodent models, the pathophysiological changes to the vascular system and myocardium during and after ischaemia–reperfusion injury. The use of antioxidants and the effect of the hypoxia-inducible factor (Powell et al. 2000) under myocardial or vascular ischaemic conditions were investigated; this may

help improve the understanding of the role of these pathways and form the basis for further developments.

2.1. HISTORY OF OPERATIVE TREATMENT OPTIONS FOR ISCHAEMIC HEART DISEASE

In the early development of surgical treatment for ischaemic heart disease, the clinicians made basic observations and performed extracardiac operations for angina. In 1910, Carrel reported the first form of coronary artery bypass (Carrel 1910). Later, operations were performed directly on the heart with the aim of promoting collateral revascularisation of the myocardium from other tissue. One of the pioneers was Beck, who after several years of experimental design created the first cardiopericardiopexy (or pericardial poudrage) (Gage et al. 1958). The first successful experimental arterial coronary artery bypass autograft was performed by Murray in 1954 (Murray et al. 1954). He used an axillary or free autogenous carotid graft using the subclavian artery. This procedure was the forerunner of the internal mammary artery bypass graft procedure.

Senning reported in 1958 a coronary endarterectomy with direct-vision excision of the plaque followed by patch grafting of the defect in the vessel wall with a split segment of autologous mammary artery (Senning 1958).

From the 1960s, the use of coronary artery bypass grafting was adopted. Favarolo and colleagues used saphenous vein bypass grafts and also used for the first time free interposed saphenous vein autografts (Roncoroni et al. 1973). The first successful heart transplantation was performed in Cape Town by Barnard in 1967 (Barnard et al. 1967).

Along with the development of cardiac surgery, a number of supporting media, solutions and tools were also being developed. One of these was Custodiol® (also called HTK or Histidin-Tryptophan-Ketoglutarat Solution), which was originally developed as a cardioplegia solution but is nowadays used as an organ preservation solution. It is the best alternative to the University of Wisconsin solution (also called UW solution) or other organ transport media. Recently developed solutions such as Custodiol-N® or TiProtec® are more efficient at protecting the myocardium and vessels against ischaemia–reperfusion injury compared with the older generation of preservation

solutions or to ordinary physiological salt solution (Garbe et al. 2011). This is particularly important for the situation of cold ischaemia-warm–reperfusion injury; therefore, it can prolong transport time without severe organ injury (Radovits et al. 2008; Loganathan et al. 2010).

The underlying mechanisms that enable transport media to maintain the physiological function of organs and vessels is the subject of intense investigation. This research has shown that preservation solutions also cause extended tissue injury. This injury is induced by different pathways such as an iron-dependent formation of reactive oxygen species (ROS) or direct cytotoxic effects by histidine (Loganathan et al. 2010). Further research is required to gain further insights into this field.

2.2. THE HISTORY OF INTERVENTIONAL CARDIOLOGY

The first experiments related to interventional cardiology date back several hundred years. One of the first experiments was performed by Harvey, who proved (in 1651) that venous blood flows towards the lungs (Sette et al. 2012). Some years later in 1667, Major became the first to deliver an intravenous injection into a human (Mueller et al. 1995).

The first known cardiac catheterisation was performed by Hales in 1711. During the procedure he inserted a pipe into the vessels of a horse to get into the ventricles, where he measured the pressure with a captured water column. The procedure was named catheterisation by the French physiologist Bernard who also performed other procedures on the heart, such as measurements of left and right ventricular pressure. Chauveau and Marey recorded the pulmonary artery pressure, and they simultaneously registered the aortic and intraventricular pressure (Mueller et al. 1995).

In the last century the field of cardiovascular medicine was characterised by progressive development and refinement of the invasive diagnostic and therapeutic options. New techniques such as cardiac catheterisation, angioplasty and related catheter-based interventions were developed.

One of the greatest innovations was Röntgen´s discovery of X-rays, for which he received the Nobel Prize in 1901. This discovery led to experiments in cadavers with intravenous contrast agents enabling Merkel and Jasmin to publish a book of the coronary arteries in 1907 (Mueller et al. 1995).

Forssmann catheterised his own arm with a urethral catheter via the cubital vein and made an X-ray. Furthermore, he documented for the first time a catheter in the right atrium. This led to the rapid development of catheterisation techniques in humans and was completed by documentation with X-ray and contrast agents (Goerig et al. 2008).

Later, diagnostic cardiac catheterisation was introduced by Cournand and Richards and selective coronary angiography by Sones (Cournand et al. 1945).

Further advancements in the field were achieved through the work of Seldinger, who invented a technique for the percutaneous replacement of an access needle with a catheter over guide wires (Seldinger 1957). This technique allowed the insertion of a catheter in different arteries and veins. At present, coronary angiography and percutaneous coronary intervention are mainly based on the insertion of catheters via the femoral or radial artery.

The importance of these discoveries was recognised when the Nobel Prize in Physiology or Medicine was awarded to Cournard, Richards and Forssmann in 1956 for their pioneering work on cardiac catheterisation (Mueller et al. 1995; Sette et al. 2012).

The cardiac catheter has supported the development of basic scientific understanding of cardiac anatomy and physiology and offered a new perspective which cannot be achieved via noninvasive methods.

Work on a range of catheter techniques continued and in the 1970s Gruentzig developed a balloon catheter. He performed the first coronary angioplasty intraoperatively during bypass surgery. Later, balloon dilatation of renal artery stenosis and coronary and coronary angioplasties were performed (Gruntzig et al. 1977; Gruntzig et al. 1978).

The continuous development of cardiological catheter techniques now permits a broad range of functional measurements of the heart. These techniques are used in critical situations such as acute myocardial ischaemic states, and they enable the use of stents and drug-eluting stents or the implantation of heart valves. Because of the economic benefits and relative low complication rates, the number of cardiological interventions is

rapidly growing. In contrast, the number of cardiac surgeries is decreasing. This shows that in practice only more complicated cases are being surgically treated.

2.3. ISCHAEMIA–REPERFUSION INJURY OF THE MYOCARDIUM AND VESSELS

A compromised blood flow deprives tissues and organs of oxygen and metabolites, such as glucose. This state of deprivation is called 'ischaemia'. Ischaemia may require cardiological interventions and cardiac surgical procedures, which are complex and require myocardial reperfusion after finalisation. However after the onset of myocardial reperfusion, many reactions, such as oxidant generation, activation of neutrophil granulocytes and their adhesion to the coronary vascular endothelium or cell damage due to calcium dyshomeostasis, are induced (Vinten-Johansen et al. 2005).
The injury generated by ischaemia and subsequent reperfusion is called ischaemia–reperfusion injury. The ischaemia–reperfusion-related loss of myocardium due to cell death remains a major issue in cardiovascular protection. In the past decades, various mechanisms for cardioprotection have been discussed and intensively scrutinised. However, it is impossible to single out just one target for cardioprotection because I/R involves different signalling pathways in the mechanisms of tissue injury.

Organs with high O_2-uptake (brain, heart and kidney) need sufficient, continuous blood flow. Blood flow and oxygen supply can be reduced by various pathophysiological conditions, including atherosclerosis, stenosis, reduced blood pressure, shock syndrome and the perioperative period. If the blood flow is insufficient or completely blocked, the organ is subject to hypoxia or anoxia, respectively. The results are tissue injury, cell death and irreversible organ damage when sufficient blood flow is not restored.

The damage caused by reduced oxygen supply can be regional (e.g. because of occluded arteries or myocardial infarction) or global (e.g. via interruption of organ blood flow during organ transplantation or organ transportation). During organ transplantation, two types of ischaemia can be distinguished: warm and cold ischaemia. Warm ischaemia is the period during which a tissue or organ remains at body temperature after its blood supply has been reduced or cut off but before it is cooled or is reconnected to the blood

supply. In clinical terminology this means the following: (1) an ischaemic condition during implantation from the removal of the donor organ from ice until the beginning of reperfusion and (2) ischaemia during organ recovery from the beginning of cross-clamping until perfusion (Halazun et al. 2007). Cold ischaemia is the period between the cooling of a tissue or organ after its blood supply has been reduced or cut off and the time it is warmed by having its blood supply restored. This kind of ischaemia can occur while the organ is still in the body or after it has been removed from the body if it is to be used for transplantation.

If the ischaemic period is short (e.g. a brief spasm of a coronary artery, a rapidly lysed thrombus, the rapid ending of exercise or psychic stress or after coronary bypass surgery) the myocardium will completely survive; however, this depends on the duration and degree of ischaemia. The period of dysfunction is called ‘stunning’, and this results in regional wall motion abnormality, decreased systolic pump function and ECG abnormalities. However, a stunned myocardium is able to react to inotropic stimuli such as dopamine, dobutamine or isoproterenol (Kloner et al. 2001). The role of free radicals in stunning has been investigated in several studies, and it has been shown that pretreatment with enzymes that scavenge O_2-derived free radicals, such as superoxide dismutase and catalase, can inhibit the process of stunning (Bolli et al. 1989).

Ischaemia–reperfusion injury involves both the myocardium and the coronary endothelium–smooth muscle; therefore, protection of the heart should involve these two aspects (He 2005). Myocardial damage after ischaemia–reperfusion injury is characterised by histomorphological changes such as cell swelling, disrupted ultrastructure, contraction bands, deposition of calcium phosphate granules and inflammatory cell responses (Tsao et al. 1990).

To improve the prognosis for patients suffering from ischaemia-related diseases, further research is warranted to answer the multitude of unanswered questions.

2.4. MYOCARDIAL REACTIVE OXYGEN SPECIES GENERATION DURING ISCHAEMIA–REPERFUSION

Atoms, molecules or ions with unpaired electrons are called free radicals. These radicals are most often derived from oxygen (reactive oxygen species, ROS), nitrogen (reactive nitrogen species, RNS) or sulphur (reactive sulphur species, RSS) and are prone to react with other molecules because of their high reactivity.

In cells, energy is generated under physiological conditions in the form of adenosine triphosphate (ATP) by the degradation of glucose during mitochondrial oxidative phosphorylation; this process depends on oxygen. Within the mitochondrial electron transport chain, electrons are transported to molecular oxygen, which is finally reduced to water. As a result, energy is conserved in ATP by mitochondrial synthesis.

If the blood supply of an organ is diminished or completely blocked, this reduces the oxygen supply and subsequently oxidative phosphorylation. When the oxygen supply is insufficient, mitochondria use the available oxygen for the excess generation of mitochondrial ROS. Ongoing hypoxia or ischaemia results in ROS production and causes irreversible oxidative damage to mitochondria followed by cell swelling and cell death (Kiss et al. 2012).

In addition, ROS are generated under normal conditions in cellular metabolism. They not only have detrimental effects but also possess important functions during physiological processes such as cell signalling, apoptosis, gene expression and ion transportation. However, when ROS are generated in excess during pathophysiological conditions (e.g. ischaemia, sepsis or diabetes mellitus), they not only cause mitochondrial damage but also deleterious effects on many molecules including proteins, lipids, RNA and DNA. The process in which ROS damages macromolecules is defined as oxidative stress. Intact cells have the capacity to defend their structures against ROS-induced damage through the use of intracellular enzymes such as superoxide dismutases. The pathophysiological role of oxidative stress is diverse and has frequently been discussed in the literature on inflammatory diseases (e.g. Crohn’s disease), CVD, cancer and ageing (Lu et al. 2010).

2.4.1. ROS IN AN ISCHAEMIC HEART

The generation of ROS in an ischaemic heart is a heterogeneous process that depends on the level of tissue oxygenation, which is generally governed by collateral blood flow (Heyman et al.). Even in the case of severe ischaemia, the heart tissue still retains some oxygen, and this small amount of oxygen is sufficient to generate free oxygen radicals (Murphy et al. 2008). ROS generation also has an important role in the protecting signalling pathway of preconditioning (Chen et al. 1995).

During ischaemia, anaerobic glycolysis and high-energy phosphates (tissue ATP, creatine phosphate) provide energy to cardiomyocytes. However, these reserves of high-energy phosphates rapidly decrease, resulting in the accumulation of adenosine diphosphate (ADP). It is known that late in the reversible phase of ischaemia the adenine nucleotide pool radically decreases (Jennings et al. 1985; Kloner et al. 2001). The ischaemic myocardium switches to anaerobic glycolysis, and the intracellular pH rapidly decreases. To restore intracellular homeostasis, the Na^+/H^+ exchanger pumps out a high amount of H^+, resulting in an excessive Na^+ inflow. When the capacity of ATPases (such as Na^+/K^+ ATPase, ATP-dependent Ca^{2+} reuptake and active Ca^{2+} excretion) are reduced, this leads to intracellular Ca^{2+} overload (Sanada et al. 2004). Ca^{2+} overload or ROS are able to open mPTP (mitochondrial permeability transition pores) on the mitochondrial membrane, which leads to H^+ influx and loss of mitochondrial membrane potential and results in mitochondrial swelling and the induction of apoptotic pathways (Sanada et al. 2004).

The recovery of arterial blood flow to the ischaemic myocardium re-establishes a sufficient oxygen supply, enabling aerobic cell metabolism, followed by the induction of salvage mechanisms. In the first phase, increased blood flow is observed in the coronary arteries (reactive hyperaemia), which leads to equalisation of the metabolic debt developed during ischaemia. Approximately 20 min after reperfusion, the baseline arterial blood flow is re-established. During reperfusion, the blood flow and oxygen supply of the tissue are restored, which results in a large burst of ROS. One reason for this is the ample amount of mitochondria in the myocardium, which not only generate ROS (by the mitochondrial electron transport chain) but also a major target of the ROS-induced damage.

In the past few decades, several studies have confirmed the positive effects of antioxidant treatments during ischaemia–reperfusion injury of the heart. However, the literature also includes controversial reports on the effectiveness of antioxidant strategies after ischaemia–reperfusion injury of the heart (Bolli et al. 1989; Chen et al. 1995; Powell et al. 1997; Tang et al. 1997; Carden et al. 2000; Korichneva 2006; Lu et al. 2010).

2.4.2. ROS IN VESSELS

Arteries play a key role in the regulation of organ blood flow. They have a thin inner lining, a single-layer endothelium, which is known not only to be a physical barrier but also acts as an important regulator of vascular tone. Outside this, the tunica media is composed of smooth muscle cells, which are the effectors in the regulation of blood flow. Fibroblasts, pericytes, dendritic cells, endothelium (from the vasa vasorum), adipocytes and filaments of innervating adrenergic nerves form the outer layer of the vessel, the adventitia. Some authors, such as Stenmark et al., have proposed that the adventitia not only has a barrier function but also plays a complex role in the regulation of vascular physiology (Stenmark et al. 2013).

ROS such as hydrogen peroxide, hypochlorite and superoxide anions are involved in the pathogenesis and progression of various cardiovascular diseases. Under pathophysiological conditions, activated neutrophil granulocytes secrete myeloperoxidase (MPO), an enzyme which in the presence of chloride, converts hydrogen peroxide (H_2O_2) into hypochlorite. Hypochlorite and H_2O_2 injure the endothelium and myocardium. This leads to endothelial and myocardial dysfunction, and subsequently contributes to processes such as atherosclerosis and reperfusion injury (Sand et al. 2003).

During pathophysiological conditions, excessive ROS release leads to a reduction in the production of nitric oxide (NO) in the endothelium. As a result, its ability for vasorelaxation is diminished.

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2.5. NITRIC OXIDE IN CARDIOVASCULAR PHYSIOLOGY AND PATHOPHYSIOLOGY

NO is a polyvalent molecule with a wide range of biological effects. It is a gaseous molecule without charge; it has an important signalling role, and it can easily diffuse between compartments without the need for a transport system. In cardiovascular biology and pathology, the important functions of this molecule are vasodilatation, the regulation of platelet adhesion, involvement in vascular remodelling and mediating cell growth and apoptosis (Furchgott 1983; Postovit et al. 2005).

NO is synthesised by nitric oxide synthase (NOS) (Figure 1) and has three isoforms: endothelial NOS or eNOS is constitutively expressed by the endothelium; neuronal NOS or nNOS is mainly produced by neuronal tissues and inducible NOS or iNOS, which is produced as a response to inflammation in vascular smooth muscle cells and inflammatory cells and results in a large, uncontrolled burst of NO (Zebger-Gong et al. 2010). Both eNOS and nNOS are calcium dependent but iNOS is not.

In the cardiovascular system NO serves as a substrate for protein S-nitrosylation or nitration. In addition, further effects of NO are mediated by the activation of soluble guanylate cyclase (sGC) and the subsequent generation of cyclic guanosine monophosphate (cGMP). Cellular functions are regulated by cGMP binding to cyclic nucleotide phosphodiesterases and by activation of protein kinase G (PKG). PKG is involved in the regulation of the myocardium via regulation of contraction. In the vessel wall, PKG modulates the vascular tone, endothelial permeability and vascular proliferation (Kass et al. 2007).

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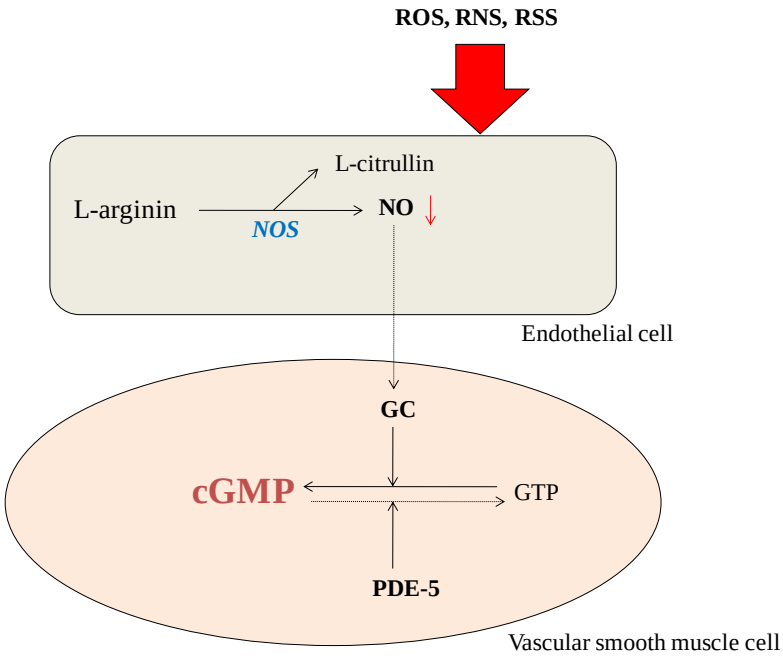

Figure 1. Synthesis of NO. NOS induces synthesis of NO from L-arginine. This leads to an elevated guanylate cyclase level in the vascular smooth muscle cell, which causes endothelium-dependent vasorelaxation through increased intracellular cGMP levels. During oxidative or nitrosative stess by ROS, RNS or RSS, NO production is reduced followed by a decrease in the cGMP level, which results in reduced vasorelaxation and endothelial dysfunction.

NO reacts with the superoxide anion ($O_2^{\cdot -}$) under pathophysiologycal conditions; this leads to the formation of peroxynitrite ($ONOO^-$), which causes nitration of proteins, lipid peroxidation and cytotoxicity (Motterlini et al. 2002). Overproduction of NO has been identified as a potent cytotoxic agent against infection, inflammation and cancer (Nathan 1997; Motterlini et al. 2002). The Ca^{2+}-independent isoform of NOS, iNOS, is activated under stress conditions caused by oxygen free radicals, endotoxins or cytokine release (Ca^{2+}-independent isoform), causing excessive NO production (Motterlini et al. 2002).

2.5.1. DECREASED NO PRODUCTION

Cardiovascular diseases and pathophysiological conditions associated with ROS excess, such as during hypertension, atherosclerosis, diabetes mellitus, cardiac hypertrophy, heart failure, ischaemia–reperfusion injury or stroke lead to a reduced NO production. Increased $^{\cdot}O_2^-$ levels inactivate NO leading to endothelial dysfunction (Paravicini et al. 2008). Consequently, the effects of decreased NO production are smooth muscle contraction, smooth muscle proliferation, platelet aggregation, increased oxidation of LDL, increased endothelin production, monocyte and platelet adhesion and increased expression of adhesion molecules.

2.5.2. NO AND VASCULAR INTEGRITY IN ORGAN TRANSPLANTATION

NO is important for vascular homeostasis. It helps to maintain the critical balance between endothelium-derived relaxation and contraction factors. When this balance is disrupted, the vasculature is predisposed to vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, pro-oxidation, thrombosis, impaired coagulation, vascular inflammation and in heart transplant patients cardiac allograft vasculopathy (Colvin-Adams et al. 2013). In the 1970s and 80s, there was intensive investigation of NO and its central role in vasorelaxation (Furchgott 1983).

Vascular integrity is a major problem, e.g. during bypass surgery or percutaneous coronary intervention and in general in organ transplantation. The short- and long-term outcomes of organ transplantations (e.g. kidney, liver and heart) are mainly determined by an efficient, continuous blood flow (Woestenburg et al. 2008; Colvin-Adams et al. 2013).

In the initial period after reperfusion, severe endothelial cell dysfunction rapidly occurs and can develop without morphological alteration of cell injury. Those morphological alterations that accompany reperfusion after prolonged ischaemia generally cause cell swelling, a loss of pinocytic vesicles, lifting of the endothelial cells from the underlying basement membranes and the attachment of activated neutrophil granulocytes to the endothelial cell surface. Profound changes in the production of ROS and NO are observed early after reperfusion.

After global or regional tissue ischaemia–reperfusion injury, long-term tissue survival is mainly dependent on the extent of the early vascular injury. In general, after organ transplantation, the most important factors responsible for reduced graft survival are occlusive vascular changes. These changes occur because of progressive proliferation of intima, also referred to as allograft arteriosclerosis or transplant vasculopathy. The function of the graft mainly depends on NO provided by intact endothelial cells (Zebger-Gong et al. 2010).

Key factors which determine vascular integrity in the early phase are partly dependent on the organ donor. For live organ donation, the age of the organ donor and pre-existing diseases (diabetes mellitus, hypertension, atherosclerosis) play an important role. Brain death of the organ donor is also problematic because it leads to an excessive cytokine release, which can initiate a diffuse, generalised inflammatory response and severe endothelial injury. In addition, the method of transport and subsequent storage of the transplant need to be considered; transport media cause cold ischaemia. After clamping the blood supply, the subsequent perfusion with a cold preservation solution leads to the iron-dependent formation of ROS or directly to cytotoxic effects, e.g. by histidine (Loganathan et al. 2010). These days, transportation methods and duration have been extended because of the year-on-year decrease in the number of organ donors, which makes it necessary to include matching donors from an extended region.

2.6. INTRACELLULAR ANTIOXIDANTS

Biological intracellular antioxidants are naturally occurring molecules that are able to protect cells and biological structures from uncontrolled oxidant injury. This injury can be provoked by activated oxygen species or free radicals. The reduction of free radicals and ROS is mainly based on the oxidation of endogenous antioxidants by scavenging and reducing molecules (Chaudiere et al. 1999; Squier 2001).

2.6.1. NON-ENZYMATIC ROS SCAVENGERS

Non-enzymatic scavengers are mainly compounds of oxidising free radicals, activated oxygen species, or physical quenchers of excited species such as singlet oxygen, hypochlorous acid or hypervalent iron species (Fe^{2+}, Fe^{3+}). The kinetics of scavenging reactions are very fast. The free radical products of scavengers decay through dismutation, recombination or reduction by secondary scavengers (Chaudiere et al. 1999).

2.6.2. ENZYMATIC ROS SCAVENGERS

The elimination of extremely oxidising species, such as $O_2^{\cdot -}$ and hydroxyl ($OH^{\cdot}$) radicals, is mainly performed by superoxide dismutases (SODs) (Chaudiere et al. 1999). The hydroxyl radical is formed from superoxide by the Haber-Weiss and Fenton reactions and reacts with phenylalanine to form hydroxylated derivates (Kloner et al. 2001).

The most common isoform is Zn^{2+}/Cu^{2+} (zinc and copper)-SOD (SOD-1) accounting for approximately 50–80% of all SODs in the vascular smooth muscle cells. This is mainly located in the nucleus and cytosol but is absent from mitochondria. In contrast, Mn^{2+} (manganese)-SOD (also called SOD-2) is abundant in mitochondria. The third form, SOD-3, is anchored to the extracellular matrix (Mendez et al. 2005). SOD-3 has a role in primarily protecting the brain and lungs against oxidative stress.

2.6.3. OXIDATIVE/NITROSATIVE STRESS AND HEME OXYGENASES

Nitrosative stress is similar to oxidative stress. Cells are able to respond to increased ROS and RNS through adaptation or resistance to toxicity, depending on the severity of the nitrosative and/or oxidative stress.

NO plays an important role in cytoprotection against nitrosative and oxidative injury. NO and NO-related species induce heme oxygenase (HO) expression and activity, particularly in vascular cells. Cells pretreated with different NO-releasing agents increase their resistance to H_2O_2-mediated cytotoxicity while HO is highly activated. NO-mediated activation of HO-1 during a stress response has still not been completely explored and needs further investigation.

During reperfusion injury, activated neutrophil granulocytes can generate ROS. The production of hypochlorite can cause endothelial dysfunction because it can react with amino acids or proteins. This dysfunction manifests as a reduced response to vasodilative agents such as acethylcoline (Sand et al. 2003; Zhang et al. 2004).

HO has three isoforms. The first, HO-1, is a 32-kDa protein that can be induced by numerous stimuli such as ROS, heavy metals, oxidants, hypoxia. HO-1 plays a pivotal role in vascular function under conditions of increased ROS generation. It is inducible and is involved in the oxidative stress response, and it mediates protective effects. HO catalyses the breakdown of heme to equimolar amounts of biliverdin, iron and carbon monoxide (CO). Biliverdin is reduced to bilirubin by bilirubin reductase, and the free iron is either used in intracellular mechanisms or sequestered in ferritin. Because of its antioxidant property, bilirubin is able to protect cells against peroxynitrite-mediated apoptosis. In addition, it is able to suppress oxidant-induced microvascular leukocyte adhesion and ameliorate post-ischaemic myocardial function. High to normal serum bilirubin levels are inversely related to atherogenic risk, possibly because of inhibitory effects against low-density lipoprotein oxidation and the scavenging of oxygen radicals (Idriss et al. 2008). CO acts as a cellular messenger and has been implicated in vascular tone regulation and neurotransmission. CO acts on vasculature in a manner similar to that of nitric oxide by increasing intracellular cGMP levels (Morita et al. 1995).

Iron released during heme catabolism is reported to have a number of effects. It has been suggested that increased levels of ferritin reduce the cellular oxidant potential by further decreasing the cellular concentration of free iron. On the other hand, ferrous iron has a cytotoxic potential through the generation of ROS. However, these negative effects are negligible in comparison with the cytoprotective potential of bilirubin and CO.

In addition to cellular processes, nutritional deficiencies can also weaken antioxidant protection. However, it should be emphasised that there are many situations of oxidative stress in which an increased intake of elements such as selenium, copper, zinc or manganese will not improve the antioxidant status; this is because these processes are

regulated by other factors and cannot be influenced by the nutritional intake of these elements (Chaudiere et al. 1999).

2.7. MYOCARDIAL PREVENTION

2.7.1. MYOCARDIAL PRECONDITIONING

Cardiologists reported the so-called ‘warm up phenomenon’ in the 1980s. They observed that patients who had experienced at least one episode of prodromal angina pectoris before unstable angina or acute myocardial infarction clinically presented with less variation in ST-segments, less cardiac dysfunction and a smaller infarcted area (Jaffe et al. 1980). Following the experiments of Murry, this phenomenon became known as ischaemic preconditioning. In 1986 Murry et al. published results on an experimental model showing the beneficial effect of short ischaemic periods before the onset of a 40-min myocardial coronary occlusion (Murry et al. 1986). Numerous studies confirmed that the cardioprotective effects of ischaemic preconditioning are not present only during myocardial stunning and global or regional acute ischaemia; beneficial effects also appear in chronic cardiac disorders such as hibernating myocardium, contractile dysfunction or myocardial remodelling (Sanada et al. 2004).

Ischaemic preconditioning is a biphasic phenomenon consisting of an early and a late phase. The early phase marks the period starting within minutes following exposure to the stimuli and lasts only 1–2 h. The second window of preconditioning, the late phase, develops more slowly (after 12–24 h) but lasts 3–4 days. The protective effects of acute preconditioning are protein-synthesis independent, while the effects of delayed preconditioning requires protein synthesis (Carden et al. 2000).

2.7.2. CARDIOPROTECTION THROUGH EARLY/CLASSICAL PRECONDITIONING

The potent but short-term protective effect from classic preconditioning is not entirely understood. It has been reported that the early phase developed through rapid posttranslational modification of pre-existing proteins through a series of signalling cascades. In addition, it has been suggested that multiple signal transduction pathways

converge on the mitochondria, either preserving ATP synthesis or preventing the onset of mPTP formation after reperfusion or both (Ferdinandy et al. 2007).

2.7.3. CARDIOPROTECTION VIA DELAYED/LATE PRECONDITIONING

The late preconditioning phase is mediated by gene expression and the subsequent synthesis of cardioprotective proteins. This mechanism involves the redox-sensitive activation of transcription factors through the protein kinase C (PKC) and tyrosine kinase signalling pathways that are also involved in the early phase of preconditioning.
Furthermore, the expression of protective mediators such as HO, heat shock proteins, vascular endothelial growth factor (VEGF) and erythropoietin (EPO) is increased after hypoxic preconditioning and plays an important role in protection against tissue injury. During the late phase of ischaemic preconditioning, these protective mediators are mainly regulated by hypoxia-sensing mechanisms through the stabilisation of HIF (Powell et al. ; Heyman et al. 2011).

2.7.4. MYOCARDIAL POSTCONDITIONING

It has been demonstrated in several studies that brief periods of reperfusion alternating with re-occlusion applied during the very early minutes of reperfusion can protect the myocardium against extended ischaemia reperfusion injury (Zhao et al. 2003). Generally, postconditioning has a protective effect not only on the cardiomyocytes, but it also reduces infarct size and apoptotic changes and protects the endothelium (Vinten-Johansen et al. 2005; Sanada et al. 2011). In contrast to several smaller trials reported previously, some human studies found no significant effects of ischaemic postconditioning on infarct size or secondary study outcomes (Hahn et al. 2013; Limalanathan et al. 2014).

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2.8. INHIBITION OF PROLYL-HYDROXYLASES AND THE MECHANISM OF HYPOXIC ADAPTATION

Hypoxia means an inadequate supply of oxygen in cells (Carden et al. 2000). Mammals have an oxygen sensing mechanism that helps to adapt cells to hypoxia by increasing cell respiration or blood flow (Eltzschig et al. 2011). Organs and areas within an organ have a variable partial pressure of oxygen (PO_2). This pressure is mostly in the range 20–45 mmHg. The kidney medulla, bone marrow and the intrauterine foetal compartment normally have a lower PO_2 of 10–25 mmHg. In some solid tumours, PO_2 can be lower than 1 mmHg. During evolution, certain cell groups differentiated to sense O_2 tension; these cells can be found in the carotid body, pulmonary artery, and adrenal chromaffin cells (Aragones et al. 2009).

During hypoxia the transcription factor, hypoxia-inducible factor (HIF-1), becomes activated. HIF-1 is a heterodimeric transcription factor consisting of the consecutively expressed HIF-β (aryl hydrocarbon receptor nuclear translocator protein or ARNT) and a regulatory HIF-α subunit (mainly regulated post-translationally). After dimerisation, the α and β subunits of HIF regulate an overlapping but a distinct set of genes with HIF-2α and HIF-3α. The HIF complex regulates a variety of genes with biological functions including vessel growth (VEGF), vasodilatation (NOS), HO-1, oxygen transport, and metal and energy metabolism to cell fate decisions (e.g. EPO, glucose transporters (GLUT) and carbonic anhydrase (CA)). The target genes mediate adaptive responses to hypoxia/ischaemia at organism, organ and cellular levels (Czibik 2010).

The HIF-α subunit does not sense O_2 directly; this activity is performed by the prolyl-hydroxylase domain (PHD) proteins and a single asparaginyl-hydroxyalse, known as factor inhibiting HIF (FIH) (Aragones et al. 2009). These enzymes differ in their affinity for O_2. FIH remains active at reduced O_2 tensions, when PHDs have already lost their activity. PHD and FIH are 2-oxoglutarate-dependent iron (II)-dioxygenases that use one of the atoms in an O_2 molecule to hydroxylate prolyl- or asparagyl-residues, respectively. The second atom of the O_2 molecule is used to convert 2-oxoglutarate to carbon dioxide (CO_2) and succinate. Iron, maintained in a reduced state by ascorbate, is a necessary cofactor. PHDs hydroxylate N-and C-terminal prolyl residues of the

α-subunit. When the prolyl residues are hydroxylated under normal oxygen tension, HIF-α subunits are recognised by the von Hippel-Lindau (VHL) protein in the multiprotein E3 ubiquitin ligase complex and will be ubiquitinated and degraded by the proteasome. When the tissue O_2 supply drops, PHD and FIH become progressively inactive, resulting in stabilised (elevated) levels of transcriptionally active HIF complexes (Figure 2).

Three isoenzymes of prolyl 4-hydroxylases specific to HIF-1α have been described in mammals: PHD1, PHD2 and PHD3; these have homology in the C-terminal catalytic domain (Siddiq et al. 2007).

The PHD1 protein is localised in the nucleus and is constitutively expressed and stimulates cell proliferation. Hypoxia does not affect PHD1 gene expression, but it is induced by oestrogen. It is highly expressed in the testis and can be found at low levels in the kidney, liver and heart.

The PHD2 protein is located mainly in the cytoplasm, with lower levels in the nucleus. Its expression can be regulated by hypoxia and hypoxia mimetics. Basal expression levels are high in the heart and moderate in the brain. A hypoxia-responsible element has been found in the PHD2 gene in humans. In rats, analysis of PHD2 mRNA expression levels in different organs following hypoxia showed reduced levels in the brain but no changes in the heart and kidney (Metzen et al. 2005; Willam et al. 2006).

PHD3 is localised in the nucleus and cytoplasm and its expression can be regulated by hypoxia or hypoxia mimetics.

In the cardiovascular system, PHD2 is the predominant isoform and its expression is induced by hypoxia or hypoxia mimetics such as desferroxamine and cobalt (II) chlorite and by pharmacological PHD inhibitors (such as dimethyloxalylglycine, DMOG) (Czibik 2010).

If oxygen tension is reduced, PHD becomes less active and HIF-α accumulates in the cytosol, and HIF-αβ-heterodimers are formed and translocate into the nucleus. There

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they bind to the promoter region of genes known as hypoxia-responsive elements (Humphrey et al.).

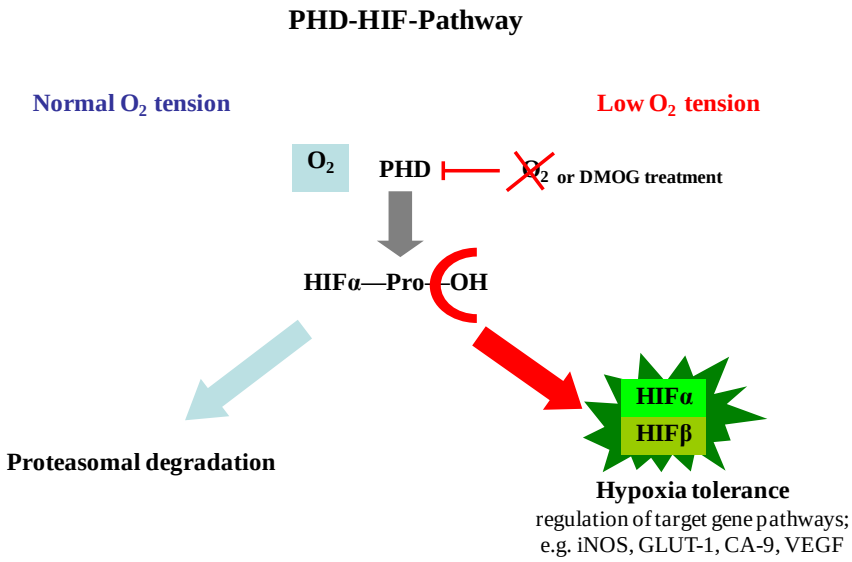

Figure 2. Regulation of HIF via the O_2-dependent prolyl-hydoxylase domain containing enzymes under normal oxygen tension and under hypoxic conditions. In normoxia (left side), PHD hydroxylates a specific proline residue that directs the degradation of constitutively synthetised HIF-1α. During hypoxic conditions (right side), inhibition of hydroxylation leads to an increase in HIF-1α protein levels; HIF heterodimers are then formed and this results in hypoxia tolerance via binding to DNA hypoxia-responsive elements.

The importance of HIF in cardiac ischaemic conditions was suggested when it was discovered that HIF-1α in animal infarct models improves myocardial perfusion and left ventricular function. Furthermore, the overexpression of cardiac HIF-1α reduces myocardial infarct size and promotes post-ischaemic function and capillarisation. This suggests that HIF-1α activation/stabilisation is beneficial in cardiac ischaemic syndromes (Czibik 2010). Additionally, it was reported that PHD inhibition (with FG-2216) did not reduce infarct size, but improved left ventricular function and prevented remodelling (Philipp et al. 2006).

The mechanisms that explain how HIF activation can protect cells against ischaemia–reperfusion injury have not completely been explored. However, it is known that several pathways are involved, such as antioxidant pathways, angiogenesis, cell death and anti-apoptotic pathways.

Drugs that can induce HIF stabilisation under normoxic conditions could provide a new treatment option for myocardial infarction, stroke, renal or liver injury, peripheral vascular disease, or severe anaemia.

In addition, it should be noted that HIF stimulation not only has protective effects for cells and organs, but also can enhance tumour growth and promote fibrosis and may induce pre-eclampsia in pregnant women (Heyman et al. 2011).

In tumours with a high proliferation rate, abnormal vessels and vessel arborisation have been reported. These signs are particularly prevalent in the central regions which became hypoxic or anoxic. This is a consequence of intratumoral hypoxia, which induces overexpression of HIF. This enables tumour cells to adapt to extreme conditions present in solid tumours. Tumour patients with overexpressed HIF have a bad prognosis and higher mortality.

Further research to target HIF pharmacologically and ameliorate the negative effects is therefore warranted.

2.9. THE ROLE OF ZINC IN CYTOPROTECTION DURING ISCHAEMIA–REPERFUSION INJURY

Zinc plays a vital role in physiological cellular functions. Zinc deprivation results in severe disorders related to growth and maturation and also in stress responses. In the heart, zinc affects the differentiation and regeneration of cardiac muscle, cardiac conductance, acute stress responses and recovery after heart transplantation (Korichneva 2006).

Disruption of zinc homeostasis is associated with severe pathophysiological conditions such as decreased erythrocyte copper-zinc superoxide dismutase, increased low-density lipoprotein cholesterol, decreased high-density lipoprotein cholesterol, decreased

glucose clearance, decreased methionine and leucine encephalins and abnormal cardiac function (Sandstead 1995). The central position of zinc in the redox signalling network is based on its chemical nature. Being itself redox inert, zinc creates a redox active environment when it binds to sulphur as a ligand. The most important property of zinc-sulphur ligand interaction is the release of zinc under oxidative circumstances. PKC is one of the redox-sensitive signalling molecules. Under oxidative conditions zinc is released from PKC. Oxidative stress during ischaemia–reperfusion in the myocardium probably triggers changes in the redox status and zinc content of PKC, as well as that of other cellular redox-sensitive proteins, thereby affecting myocardial zinc homeostasis.

The acute protective role of zinc ions for myocardial tissue is mainly due to changes in redox homeostasis: a decrease in generation of $^{\cdot}OH$ from H_2O_2 due to antagonism of redox-active metals such as iron and copper. The other mechanism is the stabilisation of sulfhydryl, where zinc protects several enzymes (e.g. delta-aminolevulinate dehydratase, dihydroorotase and tubulin) (Powell 2000).

Chevion (1988) discussed the site-specific formation of free radicals. Copper- or iron-binding sites are prevalent in macromolecules such as DNA, peptides or proteins but also exist in nucleotides or glucose. These molecules are the source of the production of hydroxyl radicals via the Fenton reaction. Prevention of site-specific free radical damage can be achieved using selective iron or copper chelating substances. Furthermore, it is possible displace these metals with other redox-inactive metals such as zinc by introducing high concentrations of hydroxyl-radical scavengers and spin trapping agents and by applying protective enzymes that remove superoxide or hydrogen peroxide (Chevion 1988).

In addition, Powell (2000) suggested that the push-versus-pull reaction can reduce $^{\cdot}OH$ formation. The metal is removed from its binding site by the pull mechanism via a high affinity chelator. This contrasts with the push mechanism where the metal is forced off its binding site via a chemically similar, but redox inactive, agent. As a result, the metal is displaced into the cytosol and undergoes hydrolytic polymerisation, precipitation or possibly redistribution to other less critical sites, thereby shifting the site of $^{\cdot}OH^{-}$ formation. It has been suggested that zinc is able to compete with copper and iron on

specific binding sites. This was confirmed in different heme proteins, where zinc is able to compete with Cu^{2+} for site-specific binding (Powell 2000).

The family of matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases capable of degrading extracellular matrix proteins. Their activity is controlled by limited gene transcription, the synthesis of pro-enzymes, endocytosis and endogenous inhibitors (tissue inhibitors MMP and TIMP). MMP-2 and MMP-9 digest denatured collagens (gelatins). The role of MMPs in chronic heart failure has been confirmed. They contribute to myocardial remodelling through reorganisation of the connective tissues. Activation of MMPs is observed after elevated levels of ROS or vasoconstrictors such as norepinephrine. Increased MMP activity results in apoptosis. During chronic heart failure, MMPs disrupt the myocardial connective tissue and cause a loss of myocardial integrity, which causes reduced left ventricular function and left ventricular dilatation (Wohlschlaeger et al. 2005). However, after ischaemia–reperfusion injury, an acute release of MMP-2 contributes to cardiac mechanical dysfunction. Pharmacological inhibition of MMP-2 in rats resulted in cardioprotection similar to the effect of ischaemic preconditioning. MMPs are generally inhibited by compounds that contain reactive zinc chelating groups, which opens up potentially new therapeutic options against ischaemia-related cardiac remodelling (Talbot et al. 1996; Giricz et al. 2006; Cheung et al. 2008; Dorman et al. 2010).

Numerous scientific papers on the protective effects of zinc have been published over the last 10–20 years and several authors have even published data on different zinc complexes, such as chloride salt or zinc complexed with, for example, carnosine, histidinate or aspartate. However, the major facilitator of the effects seen was zinc in all these studies.

In the present project we investigated the potential beneficial effects of Q50, an iron-chelating and zinc-complexing agent belonging to the 8-hydroxyquinoline family. Therefore, Q50 may be a good candidate as therapeutic agent because of its iron-chelating potential; in addition, it acts on the intracellular source of zinc forming a protective complex.

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3. OBJECTIVES

The **first aim** of this work focuses on the role of the hypoxia-inducible factor in vascular cold ischaemic storage and warm reperfusion injury. We therefore treated isolated rat aortic rings with DMOG and simulated reperfusion injury in an organ bath experiment by adding hypochlorite. In addition to the vascular functional measurements, we performed experiments on the cellular and molecular changes.

The **second aim** of this work was to investigate the activity and the characteristics of the newly developed iron-chelating and zinc-complexing agent, Q50. This work was carried out in rodent models of regional and global myocardial ischaemia–reperfusion. Regional myocardial ischaemia was induced in the rodent model by ligation of the left anterior descendent coronary artery. Global ischaemia was induced by orthotopic heart transplantation. Cellular and molecular changes of the heart were investigated after the cardiac functional measurements were performed.

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4. METHODS

4.1. EFFECTS OF PROLYL HYDROXYLASE INHIBITION ON VASCULAR FUNCTION

4.1.1. ANIMALS

Sprague–Dawley rats (male, 250–350 g; Charles River, Sulzfeld, Germany) were used in the experiments. The animals were housed in a room at a constant temperature of 22 ± 2 °C with 12 h light/dark cycles and were fed a standard laboratory rat diet and water ad libitum. The rats were randomly assigned to different groups. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures and handling of animals during the investigations were reviewed and approved by the Ethical Committee for Animal Experimentation.

4.1.2. PREPARATION OF AORTIC RINGS

Rats were anaesthetised with an injection (60 mg/kg) of intraperitoneal pentobarbital. After bilateral thoracotomy, the thoracic aorta was removed and immediately placed in cold (4 °C) Krebs-Henseleit solution (118 mM sodium chloride (NaCl), 4.7 mM potassium chloride (KCl), 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.77 mM $CaCl_2$, 25 mM $NaHCO_3$ and 11.4 mM glucose; pH = 7.4). After dissection of adhering fat and connective tissue, 4 mm length segments were placed in a testing tube in different solutions (NaCl or DMOG supplemented NaCl solution, as described in the following section).

4.1.3. EXPERIMENTAL GROUPS

The aortic segments for the organ bath experiments were randomised into 3 groups:
1) in the *control group*, the aortic rings were immediately mounted in the organ bath;
2) in the *NaOCl group*, the aortic rings were preserved in saline at 4 C for 24 h after explantation;

3) in the *DMOG group*, the aortic rings were stored at 4 °C for 24 h in saline or in 10^{-4} M DMOG-supplemented saline.

Vascular smooth muscle cells (VSMC) were divided into 3 groups:
1) the *control group*: without cold ischaemia and warm reperfusion,
2) the *NaCl group*: cells were stored at 4 °C in saline for 24 h followed by 6 h warm reperfusion in normal medium at 37 °C,
3) the *DMOG (10^{-3}M) group*: cells were stored at 4 °C in DMOG-supplemented saline for 24 h followed by 6 h warm reperfusion in a normal medium at 37 °C.
The DMOG concentrations used were based on previous literature data and our pilot studies on aortic rings and cell culture (VSMC).

4.1.4. MODEL OF IN VITRO COLD ISCHAEMIC STORAGE – WARM REPERFUSION-INDUCED VASCULAR INJURY

After 24 h of cold storage in different solutions (NaCl or DMOG-supplemented NaCl), we investigated *in vitro* vascular function in an organ bath experiment. As the major source of free radicals and oxidants produced during ischaemia–reperfusion are activated leukocytes *in vivo*, which are absent from the present *in vitro* model, it was necessary to add an external oxidant source to the aortic rings for improved simulation of the clinical situation. The aortic rings were therefore investigated in a similar manner, with additional exposure to hypochlorite (200 μM) for 30 min and rinsing before phenylephrine pre-contraction. Special attention was paid during the preparation to avoid damaging the endothelium. The different preservation solutions were aerated with nitrous oxide to reduce oxygen concentration, simulating hypoxic conditions.

4.1.5. IN VITRO ASSESSMENT OF VASCULAR FUNCTION ON AORTIC RINGS

Isolated aortic rings were mounted on stainless steel hooks in individual organ baths (Radnoti Glass Technology, Monrovia, CA, USA) containing 25 ml of Krebs–Henseleit solution at 37°C and aerated with 95% O_2 and 5% CO_2. Isometric contractions were recorded using the isometric force transducers of a myograph (159901A, Radnoti Glass

Technology, Monrovia, CA, USA) and digitised, stored and displayed with the IOX Software System (EMKA Technologies, Paris, France). The aortic rings were placed under a resting tension of 2 g and equilibrated for 60 min. During this period, tension was periodically adjusted to the desired level and the Krebs–Henseleit solution was changed every 30 min. At the beginning of each experiment, maximal contraction forces in response to KCl (80 mM) were determined and the aortic rings were washed until the resting tension was again obtained. Aortic preparations were preconstricted with phenylephrine (10^{-6} M), the α-adrenergic receptor agonist, until a stable plateau was reached, and relaxation responses were examined by adding cumulative concentrations of endothelium-dependent dilator acetylcholine (10^{-9}–10^{-4} M). For testing the relaxation responses of smooth muscle cells, a direct nitric oxide-donor, sodium nitroprusside (SNP, 10^{-10}–10^{-5} M), was used. Half-maximal effective concentration (EC_{50}) values were obtained from individual concentration–responses by fitting experimental data with a sigmoidal equation using Origin 7.0 (Microcal Software, Northampton, USA). Contractile responses to phenylephrine are expressed as a percentage of the maximal contraction induced by KCl. The sensitivity to vasorelaxants was assessed using pD2=−log EC50 (M); vasorelaxation (and its maximum, R_{max}) are expressed as a percentage of the contraction induced by phenylephrine (10^{-6} M).

4.1.6. INVESTIGATION OF COLD ISCHAEMIC STORAGE – WARM REPERFUSION INJURY ON AORTIC SMOOTH MUSCLE CELLS IN CELL CULTURE

Vascular smooth muscle cells were isolated from rat aorta with Liberase® following manufacturer instructions, resuspended in base medium and plated and incubated on 6-well plates. The cells were grown over 70% of the plate. To verify the quality of the cells, the α-smooth muscle was immunostained. We performed the experiments with 3–5 passages of the cells. The medium was changed for saline or DMOG-supplemented saline solution, incubated for 24 h and stored for hypothermic ischaemia at 4 C. After the cold storage, the complete cell culture medium was added and reperfusion was simulated by further incubation at 37 C for 6 h. Samples were harvested in a RLT lysis

buffer and stored at −80 C for later quantitative real-time polymerase chain reaction (qRT-PCR) measurement of mRNA expression.

4.1.7. AORTIC AND VASCULAR SMOOTH MUSCLE CELL mRNA EXPRESSION BY QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Aortic rings and smooth muscle cells used for qRT-PCR were snap-frozen in liquid nitrogen after harvesting and were homogenised. Total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Quiagen, Hilden, Germany**)** ß-mercaptoethanol completed Buffer RLT. RNA concentration and purity were determined photometrically (at 260, 280 and 230 nm). RNA (1 μg from each group) was reverse transcribed with QuantiTect Reverse Transcription Kit (Quiagen, Hilden, Germany). Real-time PCR reactions were performed on the Light Cycler 480 Real-time PCR detection system using the LightCycler 480 Probes Master and Universal Probe Library probes (Roche, Mannheim, Germany). The expression of HO-1 in aortic rings and VSMCs was determined. mRNA was isolated from aortic rings after 0, 2, 4, 6 h of warm reperfusion**.** The conditions for PCR were: 95 °C for 10 min (1 cycle); 95 °C for 10 s; 60 °C for 30 s (single; 45-cycle quantification) and 40 °C for 10 s (1 cycle). The reaction volume was 20 μl. Efficiency of the PCR reaction was confirmed with standard curve analysis. Sample quantifications were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression using a pool of all the cDNAs from the control group (positive calibrator). Primers were obtained from TIB Molbiol (Berlin, Germany) and their sequences were as follows:

HO-1:

forward: 5‘-GTCAAGCACAGGGTGACAGA-3‘

reverse: 5'-CTGCAGCTCCTCAAACAGC-3'

GAPDH:

forward: 5'-AGCTGGTCATCAATGGGAAA-3'

reverse: 5'-ATTTGATGTTAGCGGGATCG-3'.

Evaluation used Light Cycler 480 SW 1.5 software (Roche, Mannheim, Germany).

4.1.8. TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE-MEDIATED dUTP NICK END LABELLING REACTION

Aortic segments were fixed in 4% buffered formalin, dehydrated and embedded in paraffin and 3-μm-thick sections were placed on adhesive slides. A terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay was performed for the detection of DNA strand breaks. This was carried out using a commercial kit following the protocol provided by the manufacturer (Chemicon International, Temecula, CA, USA). Rehydrated sections were treated with 20 μg/ml of DNase-free Proteinase K (Sigma-Aldrich, Germany) to retrieve antigenic epitopes, followed by 3% hydrogen peroxide to quench endogenous peroxidase activity. Free 3′-OH termini were labelled with digoxigenin-dUTP for 1 h at 37 °C utilising a terminal deoxynucleotidyl transferase reaction mixture (Chemicon International, Temecula, CA, USA). Incorporated digoxigenin-conjugated nucleotides were detected using a horse-radish peroxidase-conjugated anti-digoxigenin antibody and 3,3′-diaminobenzidine. Sections were counterstained with methyl green. Dehydrated sections were cleared in xylene and mounted with Permount (Fischer Scientific, Germany) and coverslips were applied. Four representative pictures were made from each aortic ring with 200x magnification. TUNEL positive and negative cell nuclei were counted and the TUNEL positive cell nuclei were calculated as a percentage of the total cell number.

4.1.9. STATISTICAL ANALYSIS

Statistical analysis was performed using the Origin 7.0 program. Data distribution was tested for normality with the Shapiro-Wilk test. Normally distributed data are expressed as mean ± standard error of mean (SEM). Two groups were compared with Student's *t*-test; more than two groups (e.g. PCR, immunohistochemical scores in the media, VSMC assay) were compared using one-way analysis of variance (ANOVA) and Bonferroni-corrected post hoc test. Values of $P < 0.05$ were considered as statistically significant.

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4.1.10. REAGENTS

DMOG was provided by Cayman Chemical (Ann Arbor, Michigan, USA) and diluted in saline to concentration of 10^{-3} and 10^{-4} M. sodium phenobarbital (MerialGmbH, Hallbergmoos, Germany) was used for the anaesthetic. Phenylephrin, acetylcholine and sodium nitroprusside were obtained from Sigma-Aldrich (Taufkirchen, Germany). Sodium hypochlorite solution was produced by Grüssing (Filsum, Germany).

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4.2. Q50 IN THE RAT MODELS OF ISCHAEMIA / REPERFUSION

Lewis and Sprague-Dawley rats (male, 250–350 g; Charles River, Sulzfeld, Germany) were housed in a room at 22 ± 2 °C under 12 h light/dark cycles and were fed a standard laboratory rat diet and water ad libitum. The rats were acclimatised for at least 1 week before the experiments. All animals received humane care in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources and published by the US National Institutes of Health (NIH Publication No. 86-23, revised 1996). This investigation was reviewed and approved by the appropriate institutional review committees.

4.2.1. RAT MODEL OF MYOCARDIAL I/R INJURY: SURGICAL PREPARATION OF REGIONAL I/R

Rats were anaesthetised with sodium pentobarbital (60 mg/kg, intraperitoneally, ip.). An intratracheal tube was inserted, and the animals were artificially ventilated using a rodent ventilator (Föhr Medical Instruments, Seeheim Ober Beerbach, Germany). Body temperature was maintained at 37 °C with a controlled heating pad. The chest was opened via a left thoracotomy, followed by a pericardiotomy. A 6-0 single silk suture was passed around the left anterior descending (LAD) coronary artery (Loor et al. 2008; Zhang et al. 2008; Zhong et al. 2008; Nagel et al. 2011; Kiss et al. 2012) and the ends of the tie were pulled through a small pledget to form a snare and then tightened. After 45 min of ischaemia, reperfusion was achieved by releasing the snare. After surgery, the thorax was closed, the skin was sutured and the rats were allowed to recover on a heating pad. Sham-operated animals were subjected to the same surgical procedures, except that the suture around the LAD coronary artery was not tied.

4.2.1.1. EXPERIMENTAL GROUPS

Sprague-Dawley rats were randomised into four groups each of 6–8 rats:
1) *Sham* animals received vehicle but no tightening of the coronary suture,
2) *Sham + Q50* rats received Q50 and the ligature placed around the LAD but without occlusion,
3) *I/R* rats were treated with vehicle and subjected to I/R and
4) *I/R + Q50* animals were given Q50 and subjected to I/R, undergoing 45 min of myocardial ischaemia followed by 24 h of reperfusion.
Vehicle (10% Solutol® HS15) or Q50 (10 mg/kg) were given as an intravenous bolus 5 min before the onset of reperfusion. The dose of Q50 was chosen on the basis of our pilot studies.

4.2.1.2. IN VIVO HEMODYNAMIC PARAMETERS

After 24 h of reperfusion, the rats were anaesthetised with sodium pentobarbital (60 mg/kg ip.), tracheotomised, intubated and artificially ventilated. To assess cardiac function, left ventricular (LV) pressure – volume analysis was performed with a 2F microtip pressure – volume catheter (SPR-838, Millar Instruments, Houston, TX, USA).

4.2.1.3. DETERMINATION OF AREA AT RISK AND INFARCT SIZE

After haemodynamic measurements, the hearts were excised and quickly attached to a Langendorff apparatus. Next, 1.5 ml of Evans blue dye (1% w/v) was injected into the aorta and coronary arteries to demarcate the ischaemic risk (non-stained) and nonrisk (stained) areas of the heart. Heart tissue was excised and transverse slices were incubated with 1% TTC (2,3,5-triphenyltetrazolium chloride) for 30 min at 37 C.

4.2.1.4. BIOCHEMICAL ESTIMATION

Blood collected from the rats into EDTA tubes was immediately centrifuged and the plasma separated. Cardiac troponin-T concentrations were determined by automatic biochemistry analyser.

4.2.2. RAT MODEL OF HETEROTOPIC HEART TRANSPLANTATION

Transplantations were performed in an isogenic Lewis to Lewis rat strain, so organ rejection was not expected. The experimental model was established according to the reported method (Loganathan et al. 2010). Briefly, the donor rats were anesthetised intraperitoneally with a mixture of ketamine (100 mg/kg) and xylazine (3 mg/kg) and heparinised (400 IU/kg). Cardiac arrest was induced by Custodiol® solution. After 1h of ischaemia, the hearts were implanted intra-abdominally, anastomosing end-to-side the aorta and pulmonary artery of the donor heart with the abdominal aorta and inferior caval vein of the recipient, respectively. To minimise variability between experiments, the duration of the heart implantation was standardised at 60 min. After completion of the anastomoses, the vessels were released and the heart was perfused in situ.

4.2.2.1. EXPERIMENTAL GROUPS

The rats were randomly divided into four groups:
1) *control*: heart explanted without any treatment,
2) *control + Q50*: Q50 administered 1 h prior to explantation,
3) *I/R*: donor rats received vehicle 1 h prior to explantation, then hearts were subjected to 1 h ischaemia and transplanted and
4) *Q50 + I/R*: Q50 treatment of the donor animals 1 h prior to explantation, then hearts were subjected to 1 h ischaemia and transplanted.
Vehicle (10% Solutol® HS15) or Q50 (30 mg/kg) were given intravenously. There were 6 male Lewis donor and 6 recipient rats in each group and for each measurement.

4.2.2.2. HEMODYNAMIC MEASUREMENTS

After 1 h of reperfusion, rats were anesthetised intraperitoneally with a mixture of ketamine (100 mg/kg) and xylazine (3 mg/kg) and a 3F latex balloon catheter (Edwards Lifesciences Corporation, Irvine, CA, USA) was introduced into the left ventricle via the apex to determine LV systolic pressure, LV end-diastolic pressure, maximal slope of the systolic pressure increment (dP/dt_{max}) and the maximal slope of the diastolic pressure decrement ($dP/dt_{min,}$) using a Millar micromanometer (Millar Instruments, Houston, TX, USA) at different LV volumes. From these data, LV pressure – volume relationships were constructed using PVAN 3.6 software (Millar Instruments, Houston, TX, USA).

4.2.2.3. DETERMINATION OF HIGH-ENERGY PHOSPHATE LEVELS

For this analysis, 1 g of heart tissue was homogenised and centrifuged. Next, 5 ml of supernatant was neutralised with 1 ml of triethanolamine-HCl/K_2CO_3 solution. ATP degradation was assessed with standard photometry. Using an enzyme kinetic assay, the content of each of ATP, ADP and AMP was expressed as micromoles per gram of dry weight. The energy charge potential was calculated as (ATP + 0.5ADP)/(ATP + ADP + AMP).

4.2.2.4. QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Reverse transcription was performed with the QuantiTect Reverse Transcription Kit from Qiagen using 1000 µg RNA in a volume of 20 µl. Quantitative real-time PCR reactions were performed on the LightCycler480 system using the LightCycler480 Probes Master and Universal ProbeLibrary probes (Roche, Mannheim, Germany). The conditions for qRT-PCR were as follows: 95 °C for 10 min (1-cycle); 95 °C for 10 s; 60 °C for 30 s (single; 45-cycle quantification) and 40 °C for 10 s (1-cycle). The reaction volume was 20 µl. The efficiency of the PCR reaction was confirmed with standard curve analysis. Sample quantifications were normalised to GAPDH expression by using a pool of all the cDNAs from the control group (positive calibrator). Primers

were obtained from TIB Molbiol (Berlin, Germany) and their sequences were as follows:

Cytochrome-c oxidase:

forward: 5'-AGCCAAATCTCCCACTTCC-3'

reverse: 5'-ATAGCTCTCCAAGTGGGATAAGAC-3'

SOD-1:

forward: 5'-GGTCCAGCGGATGAAGAG-3'

reverse: 5'-GGACACATTGGCCACACC-3'

GAPDH:

forward: 5'-AGCTGGTCATCAATGGGAAA-3'

reverse: 5'-ATTTGATGTTAGCGGGATCG-3'.

Evaluation was performed with the Light Cycler 480 SW 1.5 software (Roche, Mannheim, Germany).

4.2.2.5. WESTERN BLOTTING

Myocardial proteins were extracted into a solution containing 8 M urea, 5 mM EDTA, 0.002% trasylol, 0.05 mM PMSF and 0.003% tritonX-100 containing protease inhibitors (Roche, Mannheim, Germany). Protein concentration was determined using a commercial kit according to the manufacturer's protocol (BCA protein assay kit; Thermo Scientific, Rockford, USA). Total protein homogenates (30 μg) were denatured, separated on SDS-PAGE gradient gels (Invitrogen, Darmstadt, Germany) and transferred to a PVDF membrane (Invitrogen, Darmstadt, Germany). The membranes were blocked with 5% milk in Tris-Buffered Saline Tween 20 before incubation overnight at 4 °C with primary antibodies specific to SOD-1 (1:10000, Abcam, Cambridge, UK), cytochrome-c oxidase (1:1000, New England Biolabs GmbH, Frankfurt am Main, Germany) and MMP-2 (1:100, Dianova GmbH, Hamburg, Germany). After washing the blots to remove excessive primary antibody binding, they were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:5000, Santa Cruz Biothechnology, Heidelberg, Germany) for SOD-1 and cytochrome-c oxidase and with peroxidase-conjugated secondary antibodies (1:10000,

dianova GmbH, Hamburg, Germany) for MMP-2. The immunoreactive protein bands were developed using the Enhanced Chemiluminescence system (PerkinElmer, Rodgau-Juegesheim, Germany or GE Healthcare Europa GmbH, Freiburg, Germany). The intensity of immunoblot bands was detected with a Fujifilm LAS-3000 Imager or Hyperfilm™ ECL (GE Healthcare Europa GmbH, Freiburg, Germany).

4.2.3. CARDIAC MYOCYTE PROTECTION STUDIES IN VITRO

H9c2 rat embryonic cardiac muscle cells (ATCC, Rockville, MD, USA) were cultured in Dulbecco's Modified Eagle's Medium and treated with and without Q50 (5 μM) 30 min after exposure to hydrogen peroxide (H_2O_2; 100 μM). Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and cDNA synthesis was performed with the cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). A real-time cell electronic sensing cardioprotection assay measured changes in the impedance of individual microelectronic wells. This correlates linearly with cell index (reflecting cell number, adherence and cell growth), thereby allowing the continuous determination of cell viability during oxidative stress. This assay was performed as described previously with slight modifications (Ozsvari et al. 2010). On the following day, H9c2 rat embryonic cardiac muscle cells were post-treated (30 min after H_2O_2 treatment) with Q50 or a solvent (dimethyl sulfoxide; negative control cells) of the compound. The absolute control group did not receive H_2O_2 treatment. The H_2O_2 concentration used here to elicit cell injury (100 μM) was previously optimised for H9c2 cells according to their sensitivity to oxidative stress. Cells were dynamically monitored over 24 h by measuring the electrical impedance every 5 min. The raw plate reads for each titration point were normalised relative to the cell index status immediately before the addition of H_2O_2.

4.2.3.1. MEASUREMENT OF HUMAN MATRIX METALLOPROTEINASE ENZYME ACTIVITY

The SensoLyte® MMP Assay Kit was used for the continuous spectrophotometric assay of MMP-2 and MMP-9 activities according to the manufacturer's protocol (Anaspec Inc., San Jose, CA, USA). Briefly, the MMP proenzyme was activated by trypsin

treatment, then the chromogenic substrate, a thiopeptolide, was cleaved by the MMPs, releasing a sulfhydryl group with or without Q50. The sulfhydryl group reacts with Ellman's Reagent (5,5'-dithiobis(2-nitrobenzoic acid)). The final product of this reaction, 2-nitro-5-thiobenzoic acid (TNB), can be detected at 412 nm (Victor 2, Perkin Elmer). Each reaction was done in four technical replicas and the mean value was calculated.

4.2.3.2. STATISTICAL ANALYSIS

All data are expressed as mean ± SEM. For the heart transplantation haemodynamic parameters, Student's t-test was used to analyse the differences between groups. In all other cases, the means were compared between groups by 1-way ANOVA with Bonferroni correction for multiple post hoc comparisons. $P < 0.05$ was considered statistically significant.

4.2.3.3. REAGENTS

Q50 was synthesised at Avidin Ltd (Szeged, Hungary), dissolved in 10% Solutol® HS15, a polyethylene glycol 660 hydroxystearate as non-ionic solubiliser for injection solutions. Custodiol® was purchased from Dr Franz Köhler Chemie GmbH (Alsbach-Hähnlein, Germany).

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5. RESULTS

5.1. EFFECTS OF PROLYL HYDROXYLASE INHIBITION ON VASCULAR FUNCTION

5.1.1. ENDOTHELIUM-DEPENDENT AND ENDOTHELIUM INDEPENDENT VASORELAXATION OF AORTIC RINGS

Endothelial dysfunction induced by cold ischaemic storage followed by warm reperfusion and with additional hypochlorite (the NaOCl group) was indicated by reduced R_{max} and right shift of the concentration-response curves of aortic segments to acethylcholine when compared with the control group (Figure 3). Treatment of aortic rings with DMOG 10^{-4} M significantly improved the acethylcholine-induced, endothelium-dependent, NO-mediated vasorelaxation after cold ischaemic storage and warm reperfusion (Figure 3, Table 1).

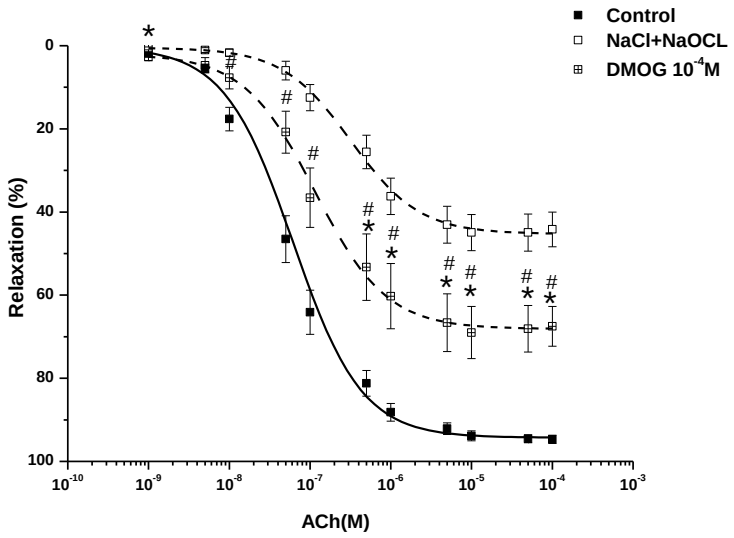

Figure 3. Relaxation of rat aortic rings to acethylcholine (ACh). Vascular function after 24 h cold storage, concentration-response curves of acethylcholine. Treatment with DMOG after reperfusion injury resulted in improved endothelium-dependent vasorelaxation. Each point of the curves and column represents the mean ± SEM. Significance ($P < 0.05$): #, vs. control; *, vs. NaOCl. Case numbers: control, n = 32; NaOCl, n = 12; DMOG, n = 17.

As indicated by the vasorelaxation of aortic rings to SNP, the endothelium-independent vascular smooth muscle function was not be significantly altered by cold storage followed by warm reperfusion injury compared with the control group (Table 1).

5.1.2. CONTRACTILE RESPONSES OF THE AORTIC RINGS

The effects of hypochlorite on the contraction forces induced by KCl (80 mM) and phenylephrine (10^{-6} M) are shown in Table 1. The contractile response to high K^+-induced depolarisation was significantly reduced compared with the control group at a high concentration of DMOG (10^{-4} M). The contraction induced by the α_1-adrenergic receptor agonist phenylephrine did not differ among the groups.

Table 1. Contractile responses and vasorelaxation ability in the three groups. Values of maximal relaxation (R_{max}) and pD_2 to acethylcholine and sodium nitroprusside in the control, NaOCl-exposed and DMOG-treated aortic rings. Values represent mean ± SEM. Significance ($P < 0.05$): #, vs. control; *, vs. NaOCl. Case numbers: control, n = 32; NaOCl, n = 12; DMOG, n = 17.

	Control	**NaOCl**	**DMOG 10^{-4} M**
R_{max} to ACh (%)	95 ± 1	44 ± 4 $^{\#}$	68 ± 5 $^{\#,*}$
pD2 to ACh	7.23 ± 0.1	6.46 ± 0.11	6.20 ± 0.44 $^{\#}$
R_{max} to SNP (%)	101 ± 1	101 ± 1	100 ± 1
pD2 to SNP	8.26 ± 0.06	8.11 ± 0.06	8.33 ± 0.10
KCl (g)	3.83 ± 0.15	2.52 ± 0.20$^{\#}$	2.74 ± 0.20$^{\#}$
Phenylephrine (g)	3.25 ± 0.15	3.45 ± 0.15	3.10 ± 0.16

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5.1.3. RESULTS OF HISTOPATHOLOGICAL STAINING

TUNEL-staining was used to determine the effect of hypochlorite and DMOG on apoptosis. The measurements showed pronounced DNA damage in the wall of aortic segments in the hypochlorite-treated group compared with the control group, reflected by quantitative assessment of the TUNEL-staining. Compared with NaOCl, DMOG treatment significantly reduced the DNA strand breaks induced by cold ischaemic storage and warm reperfusion, which were measured as an indicator of apoptosis (Figure 4).

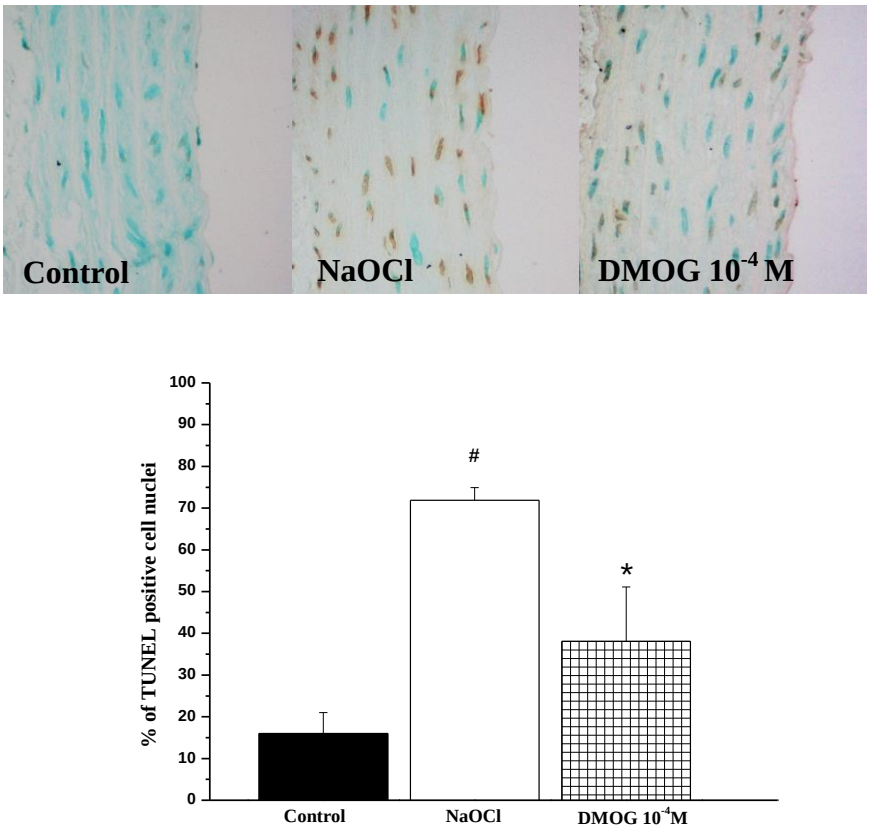

Figure 4. TUNEL-staining after 24 h of cold ischaemic storage and 6 h of warm reperfusion. The stained nuclei (brown coloured, top of the figure) were counted for the three groups. In the NaOCl group the number of cell nuclei with damaged DNA (bottom) was significantly increased compared with the control group. This was reduced in the group with DMOG pretreatment. Values represent mean ± SEM. Significance ($P < 0.05$): #, vs. control; *, vs. NaOCl. Case numbers: control, n = 40; NaOCl, n = 32; DMOG, n = 16.

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5.1.4. EFFECTS OF PROLYL HYDROXYLASE INHIBITION ON GENE EXPRESSION

5.1.4.1. EFFECTS OF DMOG ON HO-1 GENE EXPRESSION IN AORTIC RINGS

As described earlier, isolated rat aortic rings were stored for 24 h at 4 °C in different solutions and warm reperfusion was simulated by the addition of hypochlorite, after we isolated mRNA and determined gene expression.

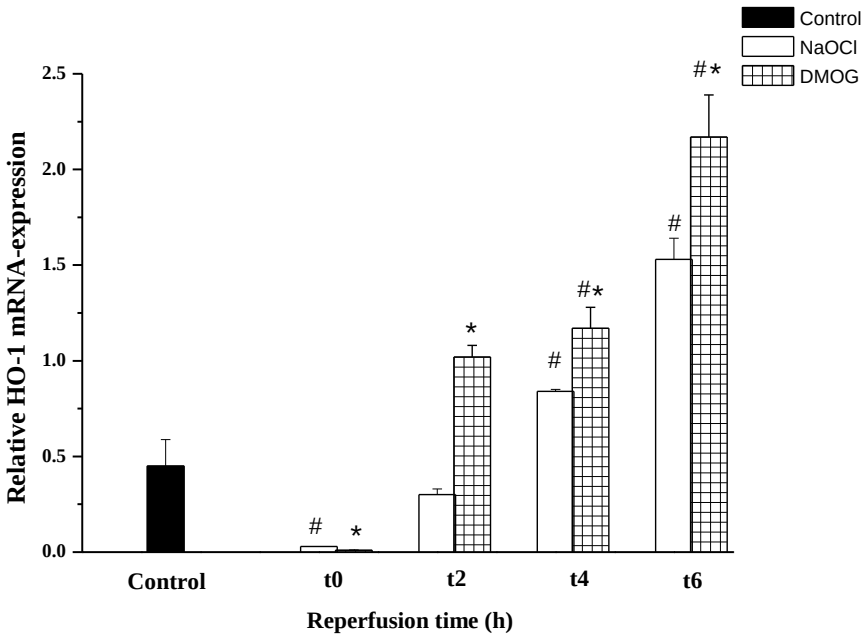

Figure 5. HO-1 mRNA-expression. Relative expression of HO-1 in aortic segments compared with expression of GAPDH after 24 h of cold ischaemic storage followed by 0, 2, 4 and 6 h of warm reperfusion in the control, NaOCl and DMOG groups. After the second hour the DMOG treated group had significantly higher HO-1 mRNA levels. Values represent mean ± SEM. Significance ($P < 0.05$): #, vs. control; *, vs. NaOCl. Case numbers in the different groups: control, n = 18; NaOCl, n = 16; DMOG t0, n = 8; DMOG t2, n = 12; DMOG t4, n = 12; DMOG t6, n = 16.

HO-1 is an inducible enzyme and is involved in the oxidative stress response that protects cellular structures. Isolated aortic rings were treated with DMOG (10^{-4} M) or NaOCl and the expression of *HO-1* was determined at different time points during the

24 h of cold ischaemic storage followed by up to 6 h of warm reperfusion. We observed that immediately after cold ischaemia storage and at the beginning of the warm reperfusion (time t0) the expression of *HO-1* in the NaOCl group was significantly reduced compared with the control group. Starting from the second hour of 'reperfusion' of the aortic segments, those treated with the prolyl hydroxylase inhibitor DMOG showed significantly increased levels of *HO-1* expression compared with the aortic rings stored in NaOCl (Figure 5). This change in expression was maintained throughout the treatment time and reached its maximum at 6 h of reperfusion (Figure 5).

5.1.4.2. THE IMPACT OF DMOG ON HO-1 GENE EXPRESSION IN AORTIC SMOOTH MUSCLE CELL CULTURE

After 24 h of cold storage followed by 6 h of warm reperfusion, relative mRNA-expression of HO-1 was significantly higher in the DMOG group when compared with that in the NaCl group (Figure 6).

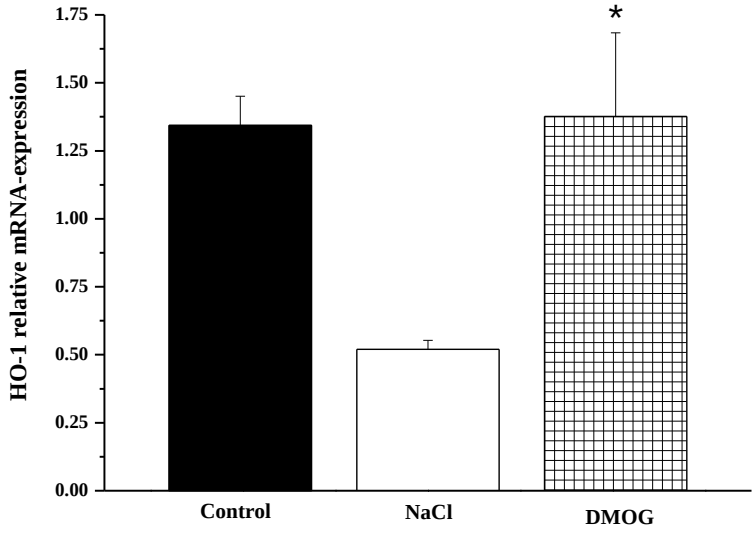

Figure 6. HO-1 mRNA expression. Relative expression of HO-1 in vascular smooth muscle cells compared with expression of GAPDH after 24 h of cold ischaemic storage followed by 6 h of warm reperfusion in the control, NaCl and DMOG groups. Relative mRNA-expression of HO-1 was significantly higher in the DMOG group when compared with that in the NaCl group. Values represent mean ± SEM. Significance ($P < 0.05$): *, vs. NaCl. Case numbers: control, n = 7; NaCl, n = 7; DMOG, n = 4.

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5.2. THE IMPACT OF TREATMENT WITH Q50 ON THE RODENT MODEL OF REGIONAL AND GLOBAL MYOCARDIAL ISCHAEMIA

5.2.1. EFFECTS OF Q50 POST-TREATMENT ON REGIONAL MYOCARDIAL ISCHAEMIA / REPERFUSION INJURY

5.2.1.1. MYOCARDIAL INFARCT SIZE

In the experiment of regional myocardial ischaemia and reperfusion we tested the effects of Q50 on the myocardium, beginning by testing the size of myocardial infarction compared with the control group. In rats subject to coronary artery occlusion and reperfusion, no difference was observed in the area at risk between the vehicle- and Q50-treated rats. This is a strong indication that a comparable degree of ischaemia was induced in both groups. Post-ischaemic treatment with Q50 did not reduce myocardial infarct size compared with the non-treated group suffering ischaemia–reperfusion injury (the I/R group) (I/R + Q50: 43 ± 12% vs. I/R: 41 ± 6%).

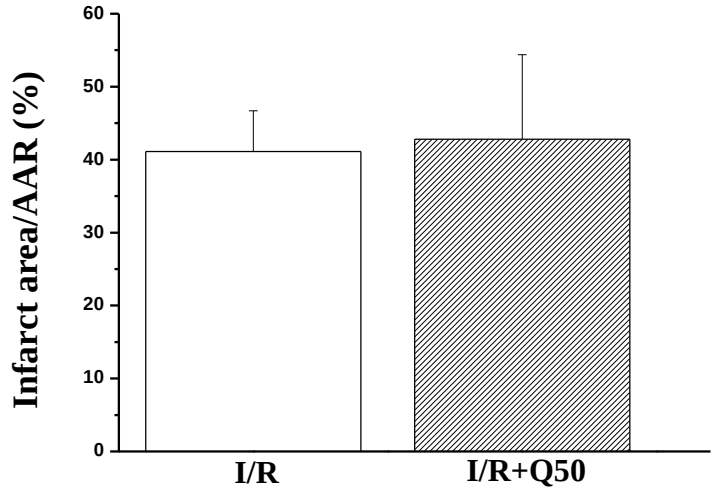

Figure 7. Infarct area compared with the area at risk (AAR). In rats subjected to coronary artery occlusion and reperfusion (n = 7) no difference was observed in the area at risk between the vehicle- and the Q50-treated rats (each with n = 6). (Statistical test: Student *t*-test). Values represent mean ± SEM.

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5.2.1.2. PLASMA CARDIAC TROPONIN-T AFTER MYOCARDIAL INFARCTION

After 24 h of reperfusion, the levels of plasma cardiac troponin-T in the I/R-group (n = 9) were significantly increased compared with the sham (n = 10) and sham + Q50 (n = 6) groups (I/R: 2820 ± 584 pg/ml vs. sham: 487 ± 118 pg/ml vs. sham + Q50: 399 ± 114 pg/ml, $P < 0.05$). Post-ischaemic treatment with Q50 (n = 4) did not significantly decrease plasma levels for this enzyme (I/R + Q50: 2210 ± 784 pg/ml).

5.2.1.3. CARDIAC FUNCTION AFTER MYOCARDIAL INFARCTION

After heart catheterisation, the cardiac parameters derived from pressure–volume analysis that compared myocardial infarcted rats with the controls are shown in Table 2. There was no significant difference between the groups in heart rate, LV end-diastolic pressure, stroke volume, cardiac output, stroke work, or slope of the EDPVR values. However, increased end-systolic and end-diastolic volumes in myocardial infarcted rats were significantly reduced after post-ischaemic treatment with Q50. In the I/R-group, decreased LV load-dependent (dP/dt_{max}) and decreased load-independent (slope of dP/dt_{max}/end-diastolic volume relationship and maximum time-varying elastance) contractility parameters were significantly increased after post-ischaemic treatment with Q50 (Table 2 and Figure 8). Moreover, the ejection fraction was significantly increased in the I/R + Q50 group when compared with the I/R group.

Systolic and diastolic blood pressures and mean arterial pressure were significantly reduced in the I/R, I/R + Q50 and sham + Q50 groups compared with the sham-operated rats. When compared with the sham group, rats with myocardial infarction showed significantly decreased LV end-systolic pressure, PRSW, dP/dt_{min} and impaired cardiac relaxation as reflected by a prolonged τ (a preload-independent measure of isovolumic relaxation). Post-ischaemic treatment with Q50 did not significantly restore these parameters.

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Table 2. Cardiac haemodynamic parameters in the rat model of myocardial infarction. (LV: left-ventricular; PRSW: preload recruitable stroke work; dP/dt_{min}: maximal slope of the diastolic pressure decrement; τ: time constant of left-ventricular pressure decay; EDPVR: end-diastolic pressure–volume relationship). Values represent mean ± SEM. Significance, $P < 0.05$: * vs. sham, # vs. I/R. The case numbers in the four groups for haemodynamic measurements: sham group, n = 7; I/R group, n = 8; I/R + Q50, n = 6; sham + Q50, n = 7.

Parameters	**Sham**	**Sham+ Q50**	**I/R**	**I/R + Q50**
	Basic haemodynamic data			
Heart rate [beats/min]	404 ± 8	431 ± 19	383 ± 17	410 ± 21
Systolic blood pressure [mmHg]	157 ± 3	139 ± 4* #	116 ± 3*	129 ± 7*
Diastolic blood pressure [mmHg]	126 ± 3	109 ± 3* #	93 ± 4*	102 ± 5*
Mean arterial pressure [mmHg]	136 ± 3	119 ± 3* #	101 ± 3*	111 ± 6*
	LV pressures and volumes			
LV end-systolic pressure [mmHg]	136 ± 8	127 ± 5#	110 ± 3*	118 ± 8
LV end-diastolic pressure [mmHg]	15 ± 2	14 ± 3	16 ± 2	15 ± 4
End-systolic volume [µl]	52 ± 10	64 ± 20	93 ± 8*	43 ± 8#
End-diastolic volume [µl]	113 ± 17	120 ± 19#	177 ± 15*	97 ± 13#
Stroke volume [µl]	61 ± 24	85 ± 7	84 ± 10	86 ± 9
Ejection phase and pressure–volume relationship indexes				
Ejection fraction [%]	46 ± 15	51 ± 8	47 ± 3	52 ± 10#
Cardiac output [ml/min]	46 ± 6	38 ± 4	50 ± 9	35 ± 3
Stroke work [mmHg.µl]	10129±1895	8373±1838	11790±2031	6762±1623
PRSW [mmHg]	93 ± 14	114 ± 12#	75 ± 4*	93 ± 11
Indexes of the active phase of relaxation				
$-dP/dt_{min}$ [mmHg/s]	12625±1678	11910±1119#	7217±275*	8653±967
τ [ms]	10.4 ± 0.9	10.4 ± 1.1#	14.6 ± 0.7*	13.9 ± 1.1*
Index of the passive phase of relaxation				
Slope of EDPVR [mmHg/µl]	0.043±0.011	0.070±0.007	0.062±0.011	0.050 ± 0.008

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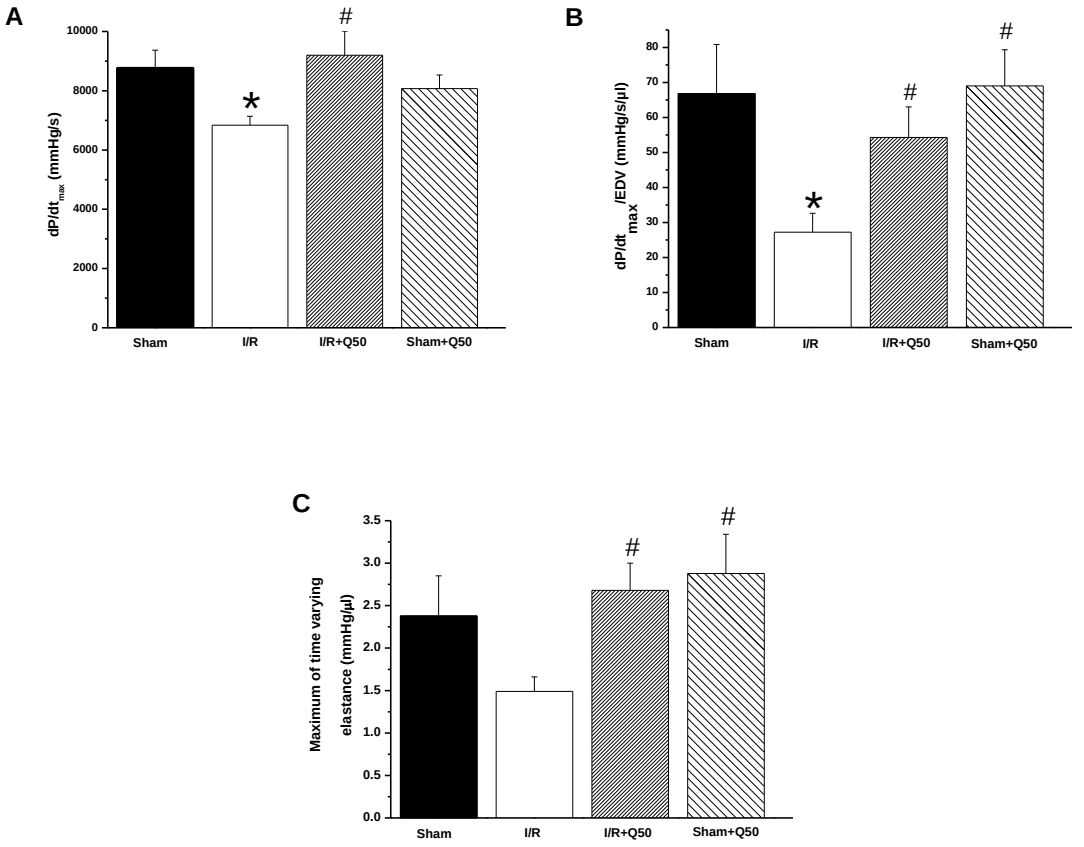

Figure 8. Cardiac functions in the four groups after myocardial infarction. In rats subjected to a 45 min occlusion of the left anterior descending coronary artery followed by 24 h reperfusion (I/R): **(**A) maximal slope of the systolic pressure increment dP/dt_{max} ; (B) dP/dt_{max}/end-diastolic volume (EDV) and (C) time-varying elastance. Q50 treatment of the I/R group resulted in an ameliorated LV function. Contractility parameters were significantly increased after post-ischaemic treatment with Q50 Values represent mean ± SEM. Significance, $P < 0.05$: * vs. sham, $^{\#}$ vs. I/R.

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5.2.2. EFFECT OF Q50 PRE-TREATMENT ON GLOBAL ISCHAEMIA / REPERFUSION INJURY

5.2.2.1. EFFECT OF Q50 ON GRAFT FUNCTION AFTER HEART TRANSPLANTATION

After heterotopic heart transplantation and 1 h after the onset of myocardial reperfusion, LV systolic pressure and dP/dt_{max} were significantly increased in the Q50-treated group compared with the I/R-group, indicating improved myocardial contractility (Table 3, Figure 9A and B). Moreover, Q50 treatment resulted in a significant increase in dP/dt_{min} values compared with the I/R-group, reflecting improved myocardial relaxation (Table 3). LV end-diastolic pressure, as a marker of the standardised balloon-catheter measurements, did not show any major differences (Table 3, Figure 9C).

Table 3. Effects of Q50 on graft function after heart transplantation. Left-ventricular peak systolic pressure (LVSP), maximal slope of the systolic pressure increment (dP/dt_{max}), left-ventricular end-diastolic pressure (LVEDP) and maximal slope of the diastolic pressure decrement (dP/dt_{min}) at an intraventricular volume of 80 µl, 1 h after reperfusion. Q50 treatment resulted in a significant increase in LVSP, dP/dt_{min} and dP/dt_{max} values compared with the I/R-group. Case numbers: I/R = 6, Q50 + I/R = 6. Values represent mean ± SEM. $P < 0.05$: * vs. I/R.

Parameters	**I/R**	**Q50 + I/R**
LVSP [mmHg]	80 ± 2	105 ± 5*
dP/dt_{max} [mmHg/s]	1781 ± 94	3219 ± 190*
LVEDP [mmHg]	5.5 ± 2.0	7.1 ± 4.1
dP/dt_{min} [mmHg/s]	989 ± 115	2477 ± 424*

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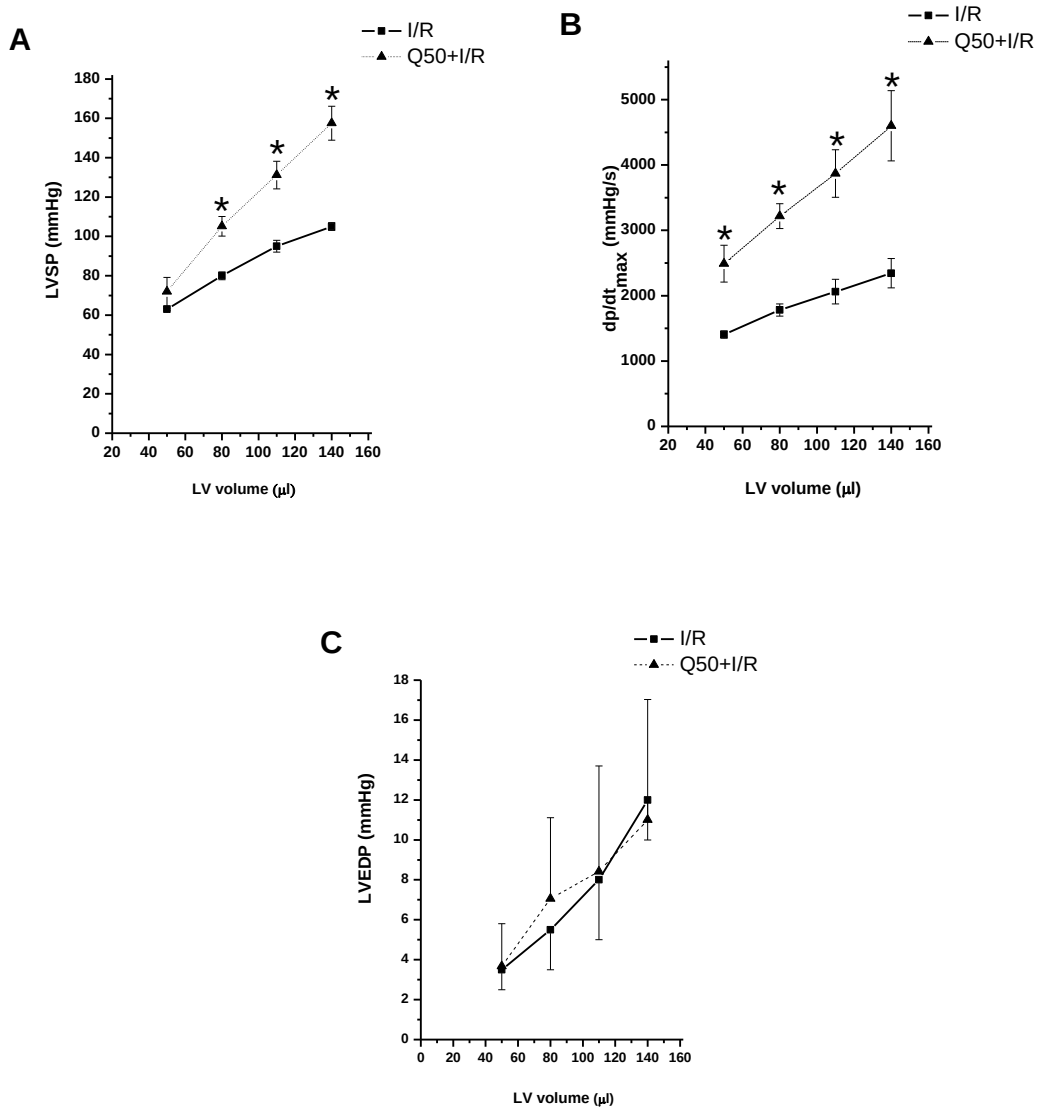

Figure 9. Diagrams of left ventricular function by global myocardial ischaemia. (A) Left-ventricular peak systolic pressure (LVSP), (B) maximal slope of the systolic pressure increment (dP/dt_{max}) and (C) left-ventricular end-diastolic pressure (LVEDP) after 1 h of reperfusion. LVSP and dP/dt_{max} were significantly higher in the Q50 treated group. (Statistical test: Student t-test). Values represent mean ± SEM. $P < 0.05$: * vs. I/R. Case numbers: I/R, n = 6; Q50 + I/R, n = 6.

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5.2.2.2. EFFECT OF Q50 ON GRAFT MYOCARDIAL HIGH-ENERGY PHOSPHATE CONTENTS AFTER HEART TRANSPLANTATION

After heart transplantation, myocardial high-energy phosphate contents, ATP and ADP levels were preserved by Q50 preconditioning as compared with the I/R-group (Table 4). AMP level did not show any relevant changes between the groups. Energy charge potential, as an indicator of the myocardial energy level showed a significant improvement in Q50-pretreated rats when compared with the I/R-group.

Table 4. Effects of Q50 on myocardial ATP, ADP, and AMP contents in the rat model of heart transplantation. Myocardial high-energy phosphate contents, ATP and ADP levels and energy charge potential were preserved by Q50. I/R: ischaemia/reperfusion; ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate. Values represent mean ± SEM. $P < 0.05$ * vs. other groups. Case numbers: control, n = 6; I/R, n = 6; Q50 + I/R, n = 6.

Parameters	**Control**	**I/R**	**Q50 + I/R**
ATP [μmol/g]	6.58 ± 1.12	1.86 ± 0.41*	6.66 ± 0.63
ADP [μmol/g]	3.48 ± 0.16	2.05 ± 0.42*	5.01 ± 0.43
AMP [μmol/g]	1.91 ± 0.22	2.07 ± 0.22	2.59 ± 0.61
Energy charge potential	0.69 ± 0.07	0.49 ± 0.04*	0.85 ± 0.08

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5.2.2.3. EFFECT OF Q50 ON GRAFT GENE EXPRESSION AFTER HEART TRANSPLANTATION

Quantitative real-time PCR from myocardium RNA extracts revealed that relative mRNA expression for SOD-1 and cytochrome-c oxidase remained unchanged in the control, control + Q50 and I/R-groups. However, their expression was significantly up-regulated in the Q50 pretreated I/R group when compared with the control group (Figure 10A and B).

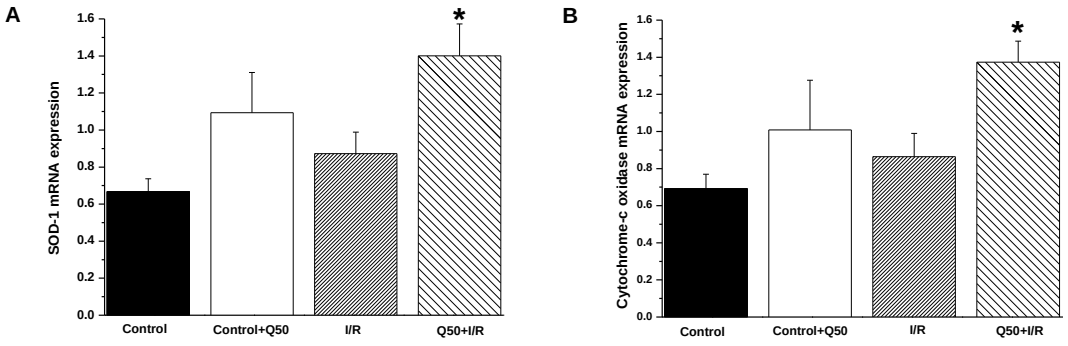

Figure 10. Effects of Q50 on gene expression after heterotopic heart transplantation. (A) SOD-1, (B) cytochrome-c oxidase mRNA expression in the myocardium. SOD-1 and cytochrome-c oxidase were significantly up-regulated in the Q50 pretreated I/R group when compared with the control group Values represent mean ± SEM. $P < 0.05$: * vs. control. Case numbers: control, n = 6; control + Q50, n = 6; I/R-group, n = 5; Q50 + I/R, n = 7.

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5.2.2.4. Effect of Q50 on graft protein levels after heart transplantation

Densitometric analysis of bands for SOD-1 and cytochrome-c oxidase did not show any differences between the control, control + Q50 and I/R-groups. However, after heart transplantation, Q50 treatment significantly up-regulated the protein expression of SOD-1 when compared with the controls and I/R group and increased cytochrome-c oxidase protein level compared with controls (Figure 11).

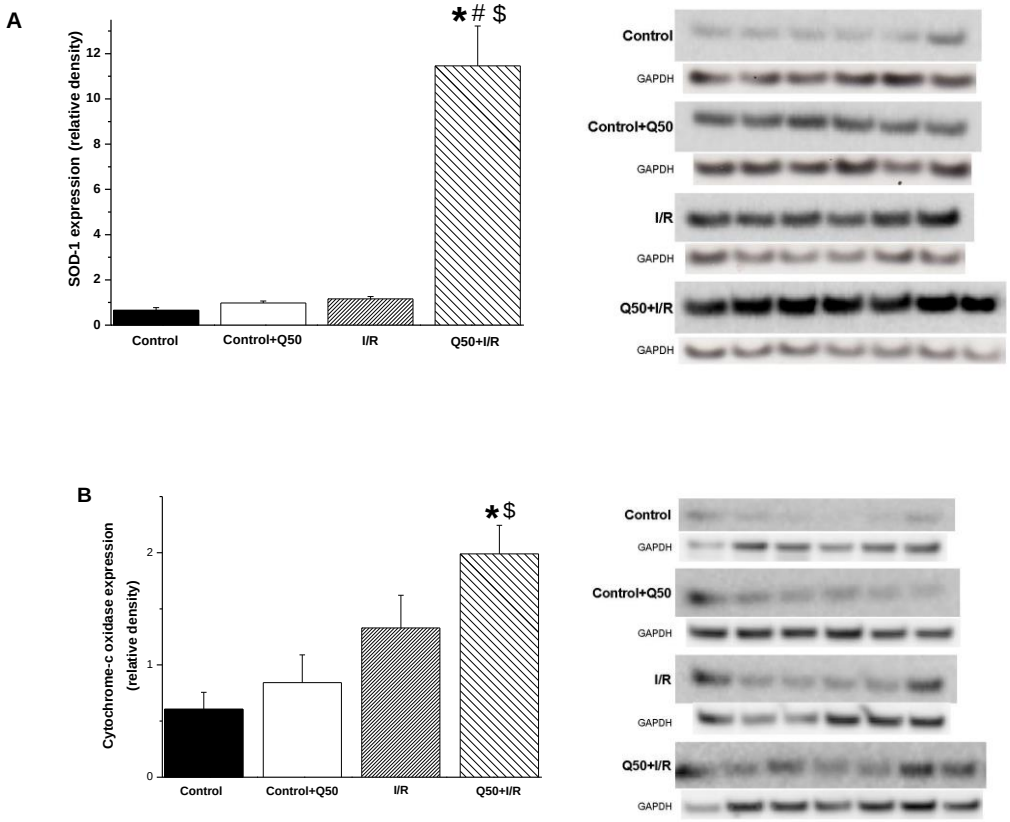

Figure 11. Protein levels of SOD-1 and cytochrome-c oxidase from the myocardium after heterotopic heart transplantation in the four groups. Immunoblot analysis for (A) SOD-1 and (B) cytochrome-c oxidase protein band densities in the myocardium. Q50 treatment significantly up-regulated the protein expressions. Values represent mean ± SEM. $P < 0.05$: * vs. control, $^{\$}$ vs. control + Q50, $^{\#}$ vs. I/R. Case numbers: control, n = 6; control + Q50, n = 6; I/R-group, n = 5; Q50 + I/R, n = 7.

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5.2.3. H9C2 RAT MYOCARDIAL CELLS AND THE POST-TREATMENT EFFECT OF Q50 AFTER OXIDATIVE STRESS

5.2.3.1. CYTOPROTECTIVE EFFECT OF Q50 MEASURED USING A REAL-TIME CELL-MICROELECTRONIC SENSING TECHNIQUE

Cells were attached and grown overnight and subjected to H_2O_2-induced oxidative stress (arrow 1, Figure 12). After 30 min, Q50 was added to the wells (arrow 2) at specified concentrations. Normalisation of cell index was calculated at the time point of the start of H_2O_2 application. Exposure of H9c2 cells to 100 μM H_2O_2 resulted in a rapid decrease of the cell index, while the cell index of the absolute control continued to slightly increase. Post-treatment with Q50 exerted a dramatic dose-dependent cytoprotective effect after H_2O_2 stress: concentrations as low as 0.5 μM maintained the cell index near absolute control levels after the initial 3 h of the experiments, and cell index remained markedly elevated during the course of the entire experiment.

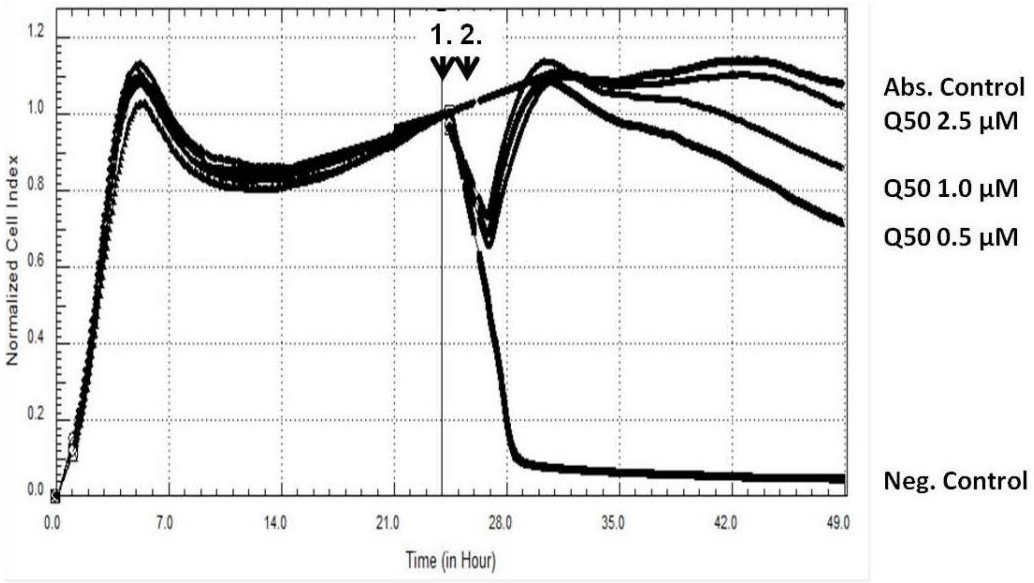

Figure 12. Cytoprotective effect of the Q50 post-treatment of H9c2 cells after oxidative stress. Determination of the effects of Q50 on the cell index, measured using a real-time cell electronic sensing method in cultured rat cardiomyocytes. H9c2 rat embryonal cardiac muscle cells were subjected to oxidative stress (100 μM H_2O_2; arrow 1). After 30 min, Q50 was added to the wells (arrow 2). Curves in the figure are measurements of single wells. The normalised cell index shows the relative viability of

cells per well. The black vertical line in the middle of the graph indicates the time of H_2O_2 application.

5.2.3.2. Effect of Q50 on relative HO-1 gene expression in H9c2 rat myocardial cells after H_2O_2-induced oxidative stress

HO-1 gene expression ratios were determined after 1 h, 3 h and 24 h post-treatment (Figure 13). We found pronounced induction (on average by a factor of 7) after 3h. This decreased to a 2-fold increase but remained significantly higher in stressed cells in comparison with untreated cells. Q50 alone mimicked the effects of H_2O_2 on *HO-1* expression; however, when relative mRNA levels were determined in treated and stressed groups no significant differences could be recorded compared with Q50 treatment without applying H_2O_2.

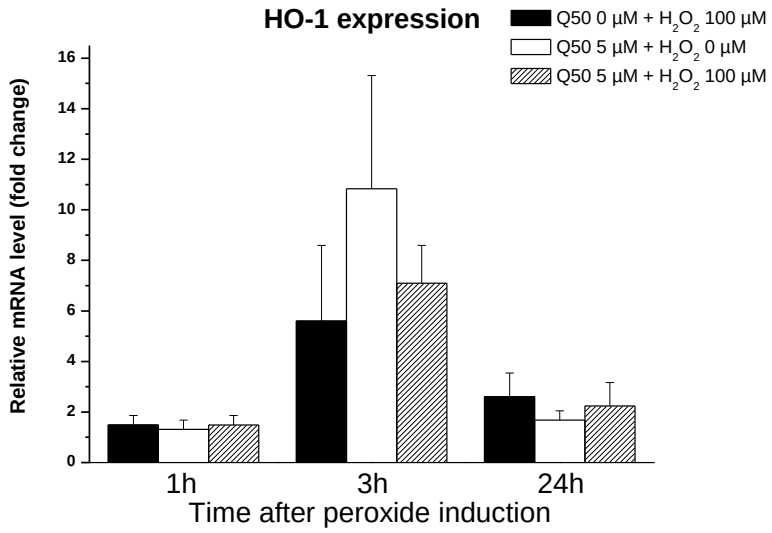

Figure 13. HO-1 mRNA expression in H9c2 myocardial cells. Effect of Q50 on relative HO-1 gene expression in H9c2 rat myocardial cells after H_2O_2-induced oxidative stress. Expression ratios were determined after 1 h, 3 h and 24 h post-treatment. Values represent mean ± SEM.

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5.2.3.3. EFFECT OF Q50 ON MATRIX METALLOPROTEINASES

Q50 concentrations ranging from 0.3 to 10 μM had no significant effect on the inhibition of either human MMP-2 or MMP-9 enzymes activities (not illustrated). After heart transplantation, heart graft protein expression of MMP-2 was significantly increased compared with the controls. Q50-treatment of the donor animals 1 h prior to explantation resulted in significantly down-regulated graft MMP-2 expression (Figure 14).

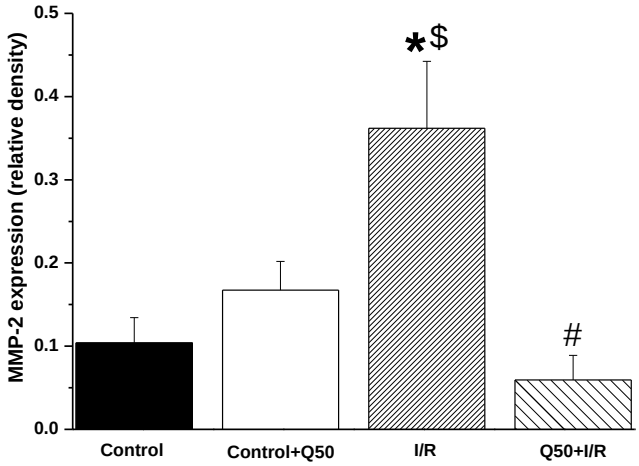

Figure 14. Protein expression of MMP-2 in the four groups in the model of heterotopic heart transplantation. Effects of Q50 on myocardial MMP-2 protein expression after heart transplantation. After heart transplantation, heart graft protein expression of MMP-2 was significantly increased compared with the controls. Values represent mean ± SEM. $P < 0.05$: * vs. control, $ vs. control + Q50, $^{\#}P < 0.05$ vs. I/R. Case numbers: control, n = 6; control + Q50, n = 6; I/R group, n = 5; Q50 + I/R, n=7.

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6. DISCUSSION

6.1. MECHANISM FOR PHD INHIBITION BY DMOG IN THE MODEL OF COLD ISCHAEMIA – WARM REPERFUSION INJURY

The main problem caused by cold ischaemia is that during warm reperfusion a number of damaging mechanisms lead to excessive endothelial injury. This injury is independent of the type of vessel and occurs during the first phase of vessel or organ transplantation. After implantation, the degree of endothelial injury is one of the factors that determine the functional integrity of the organ and the success of the transplantation.

The literature on organ preservation describes a number of new substances which could help to conserve endothelial function. These substances may contribute further to improving existing conservation protocols. The development process targets different pathways, including for example antioxidants, poly (ADP ribose) polymerase and NO-cGMP-PKG. The need for further research in the field of organ and vessel preservation is mandated by the declining number of organ donors and consequently the increasing distance on average between organ donor and recipient.

In our experimental model we isolated aortic rings from male rats and performed functional tests with the aim of establishing how vascular function is affected by the inhibition of PHDs by DMOG. DMOG was applied in the preservation solution during the cold ischaemic period. The role of the PHD-HIF system in I/R injury and other hypoxia-related disorders has already been shown in different animal models, such as myocardial, cerebral ischaemia, liver ischaemia–reperfusion and cancer models (Zebger-Gong et al. 2010).

The data presented in this thesis focus on the role of oxygen-sensing systems under pathophysiological conditions in a model of cold ischaemia–warm reperfusion. The prolyl hydoxylase inhibitor DMOG was used to modulate the oxygen-sensing system. DMOG stabilises HIF under normoxic conditions. HIF-1 is a transcription factor that

plays a central role in the response to hypoxia and ischaemia through the regulation of gene expression (inducing and suppressing, e.g. HO, NOS, CA, VEGF, EPO or GLUT).

Cold ischaemic storage is a common way to preserve tissues and organs against the loss of functional integrity during the ischaemic period. In vascular grafts and in transplanted organs the acute cold ischaemia and warm reperfusion injury leads to loss of the functional integrity of the vessels, which manifests as an acute endothelial dysfunction and later as transplant vasculopathy. Furthermore, vascular integrity of the graft is critically dependent on nitric oxide production by intact endothelial cells (Garbe et al. 2011). Endothelial integrity is crucial in the protection of vascular grafts because the vascular endothelium contributes to the prevention of platelet aggregation, to smooth muscle proliferation and to maintaining an adequate vascular tone. Therefore, the protection of endothelial integrity is essential. Experiments performed by He and colleagues have indicated that short-term storage of vessels in saline causes loss of endothelial function (He 2005). Intact endothelial and vascular smooth muscle function is particularly important for the prevention of postoperative graft thrombosis and stenosis (Garbe et al. 2011). Work by other groups has highlighted that the saline solution often used for cold storage was unable to prevent the loss of functional integrity of the vasculature. This is reflected in the reduced ability for endothelium-dependent relaxation and also the decreased development of smooth muscle tone to a high potassium concentration (Radovits et al. 2008). Previous published data from our research group showed that short-term storage was not able to induce a marked deficit in functional integrity (Radovits et al. 2009). Therefore, an *in vitro* or *ex vivo* model of cold ischaemic storage is not suitable for reliable pharmacological trials (Sand et al. 2003; Stocker et al. 2004; Zhang et al. 2004; Radovits et al. 2007).

It has been shown in various models of vascular diseases (e.g. for diabetes, atherosclerosis and ischaemia–reperfusion injury) that leukocyte-derived myelo-peroxidase plays an important role (because of the formation of ROS) in vascular injury (Zhang et al. 2004). Hydrogen peroxide is a substrate of MPO, which results in the generation of hypochlorous acid (Radovits et al. 2007). Hypochlorite was used to simulate reperfusion injury; acetylcholine-induced vasorelaxation was reduced by

hypochlorite treatment by approximately 50% compared with the control animals, and was normalised in the group with DMOG-supplemented preservation. This type of experimental endothelium injury had previously been established in our laboratory (Hunter et al. 2005; Radovits et al. 2007).

The detection of DNA fragmentation using TUNEL staining is a widely used assay that acts as an indirect method for assessing apoptosis (Philip et al.). This study demonstrated that the exposure of aortic vascular segments to cold ischaemic storage followed by warm reperfusion resulted in the formation of DNA strand breaks in the vessel walls as evidenced by TUNEL staining; this was significantly reduced in the DMOG group.

To the best of our knowledge, this work shows for the first time the vascular effects of DMOG. The results presented clearly demonstrate that pharmacological inhibition of PHDs by DMOG results in significantly improved vasorelaxation after 24 h of cold ischaemia and hypochlorite-induced warm reperfusion injury. In the NaOCl group (injured by hypochlorite), we showed an endothelial function that was severely impaired.

The kinetics of expression of HO-1 of aortic rings in the NaOCl group was significantly lower compared with the DMOG group. The same trend was observed for vascular smooth muscle cells. It could be suggested that the phenomenon is caused by the early protective effects of HIF-stabilisation due to prolyl hydroxylase inhibition. This effect may protect the endothelium against I/R injury. The aortic rings without preconditioning probably suffer a stronger but delayed ischaemia–reperfusion injury. In the DMOG group, the observed ameliorated endothelial function was probably caused by *HO-1*-mediated CO release.

Bateman et al. found that during hypoxic conditions, mRNA level as well as in the protein level of HIF 1α was significantly rapidly elevated in the first 2 h followed by increased levels of the target genes (Bateman et al. 2007). This has also been reported by other authors (Czibik et al. 2009). Czibik et al. also reported that after gene therapy with HIF-1α in a murine model, the cardioprotective effect was associated with elevated serum bilirubin levels. This effect was mimicked by remote HO-1 treatment.

CO has a physiological role in the regulation of vascular tone similar to that of nitric oxide. One mechanism for this may be through increased intracellular cGMP (Morita et al. 1995). CO produced by HO-1 has only local effects; therefore, only the same cell (autocrine) or a neighbouring cell (paracrine) can be affected. Lim et al. found HIF-1 activation after treatment with DMOG on a human microvascular endothelial cell line and also a highly activated HO-1 protein level particularly after 24 h in the cell culture media (Lim et al. 2011).

The *in vitro* measurements identified the role of the oxygen-sensing system in endothelium-dependent vasorelaxation but not in smooth muscle dependent relaxation. The hypothesis for this was that either the smooth muscle layer is not as sensitive to changes of oxygen tension as the endothelial layer or the hypochlorite-induced *in vitro* injury could induce damage of endothelial cells but could not penetrate to the deeper tissue layers. The role of modulation of the oxygen-sensing system on isolated rat smooth muscle cell culture has also been investigated. In the NaCl group of vascular smooth muscle cells we were able to detect a significantly decreased level of *HO-1*, in contrast to the DMOG group.

Morita et al. found and suggested that endogenous accumulation of CO derived from smooth muscle cells suppresses the induction of HO under hypoxic conditions (Morita et al. 1995). This is a plausible explanation because HO-*1* expression shows a biphasic characteristic with decreasing mRNA levels after long periods (48 h) of hypoxia (Ockaili et al. 2005).

This work indicated that the pharmacological modulation of the PHD-HIF pathway improved endothelium-dependent vasorelaxation through HIF stabilisation-induced *HO-1* up-regulation after short-term storage. Based on our results, we concluded that the usage of prolyl hydroxylase inhibitors will be useful in targeting the prevention of vascular dysfunction of grafts. Research on the transcription factor HIF-1 and the identification of hypoxia-induced genes could lead to development of new treatments and pretreatments for hypoxia-related pathophysiological conditions such as myocardial ischaemic conditions, transplant vasculopathy or graft failure.

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6.2. THE EFFECTS OF Q50 IN A RODENT MODELS OF REGIONAL AND GLOBAL MYOCARDIAL ISCHAEMIA

6.2.1. EFFECTS OF Q50 POST-TREATMENT ON CARDIAC DYSFUNCTION AFTER MYOCARDIAL INFARCTION

An *in vivo* experimental model was used to study the cardioprotective effect of Q50. The agent was administrated to the animal after occlusion of the artery and before reperfusion to simulate a clinical situation. Myocardial infarction is characterised by significantly decreased systolic performance and impaired ventricular relaxation; it causes an increase in end-diastolic volume, indicative of chamber dilation. End-systolic volume is a marker of ventricular contractility; this also increased in the myocardial infarction group. The present study revealed that administration of Q50 before the onset of reperfusion can improve left ventricular systolic function. The major indicator of the transition from reversible to irreversible I/R injury is the release of intracellular cardiac enzymes or markers such as troponin-T, lactate dehydrogenase, or creatine kinase into the circulation (Ravkilde et al. 1995). These enzymes are evidence for major cellular membrane damage and/or the death of cardiomyocytes (Letienne et al. 2006). In the present study, the increased plasma levels of cardiac troponin-T were not reduced by Q50 post-administration. This is in accordance with the observation by Letienne et al. who showed that there is a linear relationship between myocardial infarct size and plasma levels of biochemical markers (Loganathan et al. 2008). Moreover, the administration of Q50 failed to induce a reduction in myocardial infarct size after temporary occlusion followed by reperfusion compared with controls. However, although the study demonstrated improved cardiac function after myocardial ischaemia, application of Q50 did not decrease the elevated concentration of cardiac troponin-T or myocardial infarct size, indicating no protective effect on damaged cardiomyocytes. It should be noted that this enzyme-biomarker reflects mainly the amount of irreversible injured myocytes and necrosis but not the amount of dysfunctioning cardiomyocytes without irreversible injury. Taken together, these observations support the view that the ability of Q50 to improve cardiac performance may partially be because this iron-chelating and zinc-complexing agent rescues cardiomyocytes from border zones and

remote regions of infarcted hearts, improving their function, which in turn leads to improved global cardiac performance.

6.2.2. EFFECTS OF Q50 PRE-TREATMENT ON GRAFT DYSFUNCTION AFTER HEART TRANSPLANTATION

Fast recovery of myocardial function is essential for the success of cardiac transplantation. Therefore, the effects of Q50 therapy on the early phase (1 h) after heart transplantation were investigated. We attempted to simulate clinical conditions encountered during heart transplantation in investigating the potential use of Q50 to enhance current protective strategy. We previously described that crystalloid cardioplegia associated with cardiac arrest and reperfusion results in a decline of cardiac function (Loganathan et al. 2010). In contrast to our infarcted animals where only myocardial contractility was improved by Q50 post-ischaemic treatment, our data show that the treatment of donor rats with Q50 restores both altered systolic and diastolic LV functions after heart transplantation. The different results of these models may be explained by the type of I/R (irreversible versus reversible) and the timing of application (pre- versus post-ischaemic treatment).

6.2.3. MECHANISM FOR CARDIOPROTECTIVE EFFECTS OF Q50 AGAINST I/R INJURY

ROS generation in the ischaemic heart depends on the tissue oxygenation, although during the reperfusion phase a massive ROS excess is observed. ROS generated during reperfusion initiates before injury before it can be scavenged by SODs or catalase. Controversial data have been published about the efficiency of antioxidant treatments during local or global myocardial ischaemia.

One of the most cited mechanisms of reperfusion injury is the generation of free oxygen radicals at the time of reperfusion, namely superoxide anion, hydroxyl radicals and hydrogen peroxide. Therefore, we studied the effects of Q50 on oxidative stress induced by hydrogen peroxide on cultured cardiomyocytes. Using a cell-microelectronic sensing technique for the screening of cytoprotective compounds (Hooper 1994; Ozsvari et al. 2010), we demonstrated pronounced and concentration-dependent cardioprotective

effects of Q50 on H_2O_2-treated rat embryonic heart cells in a 30-min post-treatment *in vitro* model. We found that Q50 induced HO-1 gene expression with similar kinetics to H_2O_2 stress *in vitro*. MMPs are a family of zinc-dependent endopeptidases (Cheung et al. 2008) capable of degrading extracellular matrix proteins, and zinc is essential for their proteolytic capacity in this process. Matrix metalloproteinases have an important role as proteolytic enzymes through the degradation of extracellular proteins and remodelling of the extracellular matrix morphogenesis, cartilage and bone repair, wound healing, cell migration and angiogenesis. MMPs belong to a family of more than 25 enzymes; however, in cardiovascular pathophysiology (atherosclerosis, restenosis, ischaemic heart disease and heart failure) the main enzyme is matrix metalloproteinase-2 (or gelatinase A) or matrix metalloproteinase-9 (or gelatinase B). MMP-2, a constitutive enzyme, is found in almost all cell types and degrades denatured collagen (gelatin) and collagen type IV as well as other extracellular matrix proteins. MMP-9 is a cytokine-inducible MMP, which is most commonly located in leukocytes.

However, inappropriate, prolonged or excessive expression of these enzymes has deleterious consequences. It has been shown that an acute release of MMP-2 during reperfusion after ischaemia contributes to cardiac mechanical dysfunction (Giricz et al. 2006) and pharmacological inhibition of MMP-2 in rats produced cardioprotection similar to the effect of ischaemic preconditioning (Dorman et al. 2010). As a result, MMPs are considered to be promising drug development targets (Talbot et al. 1996) and pharmacological inhibition of MMPs may be a strategy for the treatment of I/R injury. MMPs are generally inhibited by compounds containing reactive zinc-chelating groups (Ferdinandy et al. 2007). In the present study, regardless of the zinc-binding capacity of Q50, this agent did not show enzymatic inhibition of human MMP-2 and MMP-9 in a biochemical assay. However, Q50-treatment of the donor animals 1 h prior to explantation significantly down-regulated increased graft MMP-2 expression after heart transplantation. Taken together, we can speculate that an indirect *in vivo* inhibitory mechanism (binding zinc, which is essential for the catalytic activity of MMPs) may be possible. During I/R injury, the return of oxygen to ischaemic tissues is accompanied by an increased production of ROS (Menasche et al. 1990). Iron plays a role in the

formation of free radicals that contribute to oxidative stress, and its chelation makes it unavailable for this kind of reaction. Chelation of ferric iron with deferroxamin, an iron-chelator, has been shown to reduce the production of the hydroxyl radical, thereby reducing myocardial I/R injury (Humphrey et al. 1987). In our model of reversible global myocardial I/R, Q50 treatment resulted in a significant increase in SOD-1 protein expression, one of the first line of defence antioxidant enzymes, indicating both its cytoprotective function and its free radical scavenging effect.

In the present study, I/R injury after heart transplantation leads to a significant decrease in high energy phosphate contents compared with the control group. The present data clearly demonstrate that Q50 pre-treatment results in a better preservation of the high energy phosphate pool, primarily by increased myocardial ATP content, resulting in an improved energy status, as expressed by the significant higher energy charge potential. Under aerobic metabolism the heart prefers fatty acids to supply myocardial ATP; however, during ischaemia, the metabolism of myocardial tissue switches to anaerobic glycolysis and becomes an important source of ATP for the preservation of ion gradients. However, during ischaemia the mitochondria are unable to preserve adequate support for oxidative phosphorylation. With reperfusion, fatty acid oxidation recovers and again predominates. The loss of cellular energetic pools in turn has an important effect on myocardial function.

Based on the results of the present study, we propose that Q50 may contribute to a better recovery of cellular ATP, and thereby improve myocardial contractility. In pathologic conditions this iron-chelating and zinc-complexing agent may provide promising antioxidant defence mechanisms.

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7. CONCLUSIONS

Exploring research areas of experimental cardiology and heart surgery for better understanding of myocardial and vessel protection in the period of ischaemia and hypoxia is necessary to improve our knowledge regarding the circumstances of these conditions.

The first aim of this study focused on the role of the hypoxia-inducible factor in vascular cold ischaemic storage and warm reperfusion injury. Therefore, we treated isolated rat aortic rings with DMOG and simulated reperfusion injury in an organ bath experiment by adding hypochlorite. We found that HIF stabilisation leads to an improvement in vasorelaxation, which was mediated mainly via HO-1.

Our second aim was to investigate the activity and the characteristics of the newly developed iron-chelating and zinc-complexing agent, Q50, in rodent models of regional and global myocardial ischaemia–reperfusion. In rats with regional myocardial ischaemia induced by ligation of the left anterior descendent coronary artery, we found that treatment with Q50 showed improved contractility, although the size of myocardial infarct was not influenced. Rats with global myocardial ischaemia from an orthotopic heart transplantation that were treated with Q50 showed a better left ventricular function and increased ATP levels compared with the control groups.

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8. SUMMARY

Storage protocols for vascular grafts need further improvement for protection against ischaemia–reperfusion injury. Hypoxia elicits a variety of complex cellular responses by altering the activity of many signalling pathways; my thesis investigated the role of the oxygen-dependent prolyl hyroxylase domain-containing (PHD) enzyme. Reduction of PHD activity during hypoxia leads to stabilisation and accumulation of hypoxia inducible factor (HIF-α). Cold ischaemic preservation- and hypochlorite-induced severe endothelial dysfunction was significantly improved by dimethyloxallilglycin (DMOG) supplementation, as it was demonstrated by maximal relaxation of aortic segments to acetylcholine. DMOG treatment significantly decreased apoptosis as well. Furthermore, it was shown that in aortic rings and on VSMCs, *HO-1* mRNA levels were significantly higher in the DMOG group than in the control group. Through inhibition of PHD with DMOG in an *in vitro* model of vascular I/R, the pharmacological modulation of the oxygen-sensing system may effectively preserve the endothelial function.

Iron-chelators, and zinc or zinc-complexes have already been shown to protect the heart from reperfusion injury. Therefore, in the second part of this thesis the possible beneficial effects of an iron-chelating and zinc-complexing agent, Q50, was investigated in rat models of I/R-induced myocardial infarction and on global reversible myocardial I/R injury following heart transplantation. In one part of the experiment rats underwent myocardial (regional) ischaemia by left anterior descending coronary artery ligation followed by reperfusion. In myocardial infarcted rats, end-systolic and end-diastolic volumes were significantly decreased by Q50 post-treatment compared with a sham group. Moreover, in I/R rat hearts dP/dt_{max} decreased and load-independent contractility parameters were significantly increased after Q50. However, Q50 treatment did not reduce infarct size. In a heart transplantation model of global myocardial ischaemia, transplanted animals treated with Q50 showed decreased left-ventricular systolic pressure, while dP/dt_{max}, dP/dt_{min}, myocardial ATP-content, and myocardial protein expression of superoxide dismutase-1 was significantly up-regulated 1 h after reperfusion. In the two experimental models of regional and global myocardial ischaemia reperfusion injury, administration of Q50 improved myocardial function.

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9. ÖSSZEFOGLALÁS

Az erek iszkémia-reperfúziós károsodásának megelőzése, csökkentése ma is kiterjedt kutatómunkát és fejlesztést igényel. Hipoxia során számos jelátviteli út aktiválódik, tézisem során az oxigénfüggő prolil-hidroxiláz (PHD) enzim jelentőségét vizsgáltam. Hipoxia alatt a PHD aktivitása csökken, mely a HIF-α stabilizációjához vezet. Hideg iszkémiát követően hipoklorittal idéztünk elő reperfúziós endotélkárosodást, mely DMOG kezeléssel kivédhető. Ugyancsak csökkent az apoptózis mértéke a DMOG-kezelt csoportban. Ezen túlmenően az aorta gyűrűk és az izolált simaizomsejtek esetében a HO-1 mRNS szintje szignifikánsan magasabb volt a DMOG-kezelt csoportban a kontrollhoz képest. Az oxigén érzékelő rendszer PHD gátláson keresztül történő befolyásolása DMOG-gal az endotélfunkciót javítja iszkémia-reperfúzió során.

A vaskelátorok, cink és cink-komplexek védő szerepe a szív iszkémia-reperfúziós károsodásának megelőzésében jól ismert. Tézisem második felében a vaskelátor és cink-komplexáló Q50 hatásait vizsgáltuk lokális (szívinfarktus) és globális (szívtranszplantáció) iszkémia-reperfúziós károsodás kisállat modelljében. Az infarktust elszendvedett, és Q50-nel kezelt állatok végszisztolés és végdiasztolés bal kamrai térfogata jelentősen csökken az ál-operált-csoporttal szemben, illetve a Q50-kezelés hatására a dP/dt_{max} és egyéb kontraktilitást jellemző funkciós paraméterek is jelentősen csökkentek az iszkémia-reperfúziós csoporthoz képest. Az infarktus területe nem változott Q50 kezelés hatására. A szívtranszplantált modellben Q50-nel történő előkezelés hatására 1 óra reperfúziót követően a csökkent bal kamrai szisztolés nyomás, dP/dt_{max}, dP/dt_{min}, illetve az ATP-tartalom és a szuperoxid-diszmutáz koncentrációja szignifikánsan javul a beteg csoporthoz képest. A regionális és globális iszkémia modelljében a Q50 kezelés jelentősen javította a bal kamra pumpafunkcióját.

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