The Karolinska Institutet, Department of Medicine
Cardiology Unit, Karolinska University Hospital
Stockholm, Sweden

ROLE OF ARGINASE IN VASCULAR FUNCTION

Christian Jung

Karolinska Institutet

Stockholm 2015

Published by Karolinska Institutet.
Printed by TMG Sthlm 2015.

ISBN 978-91-7549-909-3

ABSTRACT

Background
Nitric oxide (NO) is central for the integrity of the cardiovascular system, the maintenance of endothelial function and the protection against ischaemic heart disease. The enzyme arginase is up-regulated during ischaemia-reperfusion and by hypoxia in cell culture and animal models which might be of pathophysiological relevance since it competes with NO synthase for their common substrate arginine. The aim of the studies was to clarify the role of arginase in cardiovascular disease related to ischaemia and hypoxia including myocardial ischaemia and reperfusion injury, heart failure and following resuscitation after cardiac arrest by investigating the therapeutic effect of arginase inhibition and its association to increased NO bioavailability.

Studies I-II
To study the relevance of arginase in the context of myocardial ischaemia and reperfusion two different animal models were used. In a rat model, the animals were treated with an arginase inhibitor (N^{ω}-hydroxy-nor-L-arginine, nor-NOHA) alone or together with substances inhibiting NO or its production intravenously before the onset of ischaemia. The infarct size was reduced by 50 % following administration of the arginase inhibitor. The cardioprotective effect was completely dependent on NO synthase activity and NO activity. Ischaemia and reperfusion was associated with increased expression of arginase I in the ischaemic myocardium. Arginase inhibition induced a 10-fold increase in the citrulline/ornithine ratio as an indirect enzyme activity measure, indicating a shift in arginine utilization from arginase towards NO synthase. In a subsequent study this concept was investigated in a large animal (pig) model of myocardial ischaemia and reperfusion with intracoronary drug administration in connection with reperfusion. Administration of nor-NOHA resulted in a profound cardioprotection comparable to that observed in rats. Parallel groups confirmed that the cardioprotective mechanism was dependent on NO production.

Studies III-IV
Circulating levels of arginase I were determined in patients with heart failure and following cardiopulmonary resuscitation as well as in healthy volunteers after global hypoxia in an normobaric hypoxia chamber. These conditions were all associated with increased levels of arginase I. In addition, the effect of topical application of nor-NOHA on the sublingual mucosa on microvascular perfusion was studied using a sidestream darkfield microcirculation camera. The impaired microcirculation in heart failure and in patients following resuscitation was improved by local nor-NOHA incubation via a NO-dependent mechanism.

Conclusions
Inhibition of arginase protects from myocardial ischaemia and reperfusion injury by a mechanism that is dependent on NO production and increased bioavailability of NO by shifting arginine utilization towards NO production. In addition, we showed that heart failure, global hypoxia and cardiopulmonary resuscitation lead to increased plasma levels of arginase I. Impaired microcirculatory perfusion in these patients is improved following topical arginase inhibition by a NO dependent mechanism. Inhibition of arginase is a promising potential treatment target for protection against myocardial ischaemia and reperfusion injury and to ameliorate microcirculatory dysfunction in critically ill patients.

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II. Adrian T. Gonon, Christian Jung, Abram Katz, Håkan Westerblad, Alexey Shemyakin, Per-Ove Sjöquist, Jon O. Lundberg, John Pernow. Local arginase inhibition during early reperfusion mediates cardioprotection via increased nitric oxide production. PLoS One. 2012; 7, e42038.

III. Felix Quitter, Hans-R. Figulla, Markus Ferrari, John Pernow, Christian Jung. Increased arginase levels in heart failure represent a therapeutic target to rescue microvascular perfusion. Clinical Hemorheology and Microcirculation. 2013; 54,75-85.

IV. Christian Jung, Felix Quitter, Michael Lichtenauer, Michael Fritzenwanger, Alexander Pfeil, Alexey Shemyakin, Marcus Franz, Hans-R. Figulla, Rüdiger Pfeifer, John Pernow. Increased arginase levels contribute to impaired perfusion after cardiopulmonary resuscitation. European Journal of Clinical Investigation. 2014; 44, 965-71.

CONTENTS

LIST OF ABBREVIATIONS

°C	Degrees of Celsius
AAR	Area at risk
ADMA	Asymmetric dimethylarginine
apoE	Apolipoprotein E
CA	Cardiac arrest
CHD	Coronary heart disease
CO_2	Carbon dioxide
CPR	Cardiopulmonary resuscitation
CVD	Cardiovascular disease
EF	Ejection fraction
eNOS	Endothelial nitric oxide synthase
HF	Heart failure
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
HR	Heart rate
I/R	Ischaemia and reperfusion
ic	Intracoronary
IL	Interleukin
im	Intramuscular
iNOS	Inducible nitric oxide synthase
IS	Infarct size
IU	International units
iv	Intravenous
LAD	Left anterior descending (artery)
L-NMMA	NG-monomethyl-L-arginine
LV	Left ventricle
MAP	Mean arterial pressure
MI	Myocardial infarction
NaCl	Natriumchloride (Saline)
NADPH	nicotinamide-adenine-dinucleotide phosphate
NO	Nitric oxide
nor-NOHA	N^{ω}-hydroxy-nor-L-arginine
NOS	Nitric oxide synthase
NSE	Neuronal specific enolase
NYHA	New York Heart association (classification)
PCD	Perfused capillary density
PVD	Perfused vascular density
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
ROSC	Return of spontaneous circulation
RPP	Rate pressure product
SDF	Sidestream darkfield imaging
TNF-α	Tumor necrosis factor alpha
TTC	Triphenyltetrazolium chloride

1 INTRODUCTION

1.1 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is common, affecting more than 50% of adults aged 60 years and older. Recently, CVD was estimated to result in 17.3 million deaths worldwide per year (1). For 30-50% of all cases with CVD the underlying entity is coronary heart disease (CHD). The lifetime risk to develop CVD for the population aged 40 years was 49 percent in men and 32 percent in women. Although death rates from CVD decrease, CVD and its related complications remain highly prevalent and expensive to treat (2). For instance, CVD remains the leading cause of death in the majority of developed countries. In addition, prevalence rates of CVD rapidly increase in developing countries (3).

Of central importance for the understanding of CVD is the influence of cardiovascular risk factors. Nine potentially modifiable factors have been described to explain more than 90 percent of the population-attributable risk of a first myocardial infarction (MI): smoking, dyslipidaemia, hypertension, diabetes mellitus, abdominal obesity, psychosocial factors, as well as lack of daily consumption of fruits and vegetables, regular alcohol consumption, and low regular physical activity (4). Especially, diabetes mellitus and its related conditions including insulin resistance, hyperinsulinaemia, and elevated blood glucose levels are associated with atherosclerotic CVD (5). It has been shown that diabetes mellitus accounts for 10 percent of the attributable risk of a first MI and that the all-cause mortality risk associated with diabetes mellitus is equivalent to the all-cause mortality risk associated with a prior MI (6). Furthermore, patients with diabetes mellitus have a greater burden of other atherogenic risk factors, including arterial hypertension and obesity.

Atherosclerosis is accountable for nearly all cases of CHD. This process begins with fatty streaks in the vasculature and these lesions progress into plaques and culminate in thrombotic occlusions and coronary events in later life. In addition to the above mentioned CVD risk factors, several other factors have been described to be associated with an increased risk for atherosclerotic plaques in coronary arteries and other arterial beds (7). For example, male sex is associated with a threefold higher incidence of atherosclerotic CHD (8). An exact estimation of the prevalence of CVD risk factors remains elusive, but the prevalence of identified risk factors has changed over time with increased awareness and changes in diet and lifestyle. Especially the prevalence of obesity has increased dramatically in the developed countries (9-11). In addition, prevalence of diabetes mellitus, arterial hypertension and dyslipidaemia has increased in younger and older subjects (11, 12).

1.1.1 Myocardial infarction and ischaemia and reperfusion injury

Patients with CHD may clinically present with angina pectoris or with acute coronary syndrome that rapidly progresses to a MI. In the majority of the cases the underlying pathophysiological mechanism is a coronary atherosclerotic plaque that in the case of acute coronary syndrome ruptures subsequently resulting in a thrombotic (total) occlusion of the coronary artery. Patients may present with ST-elevation myocardial infarction or non-ST-

elevation myocardial infarction dependening on transmural or subendocardial ischaemia resulting in typical changes in the electrocardiogram. In addition to that, establishing the diagnosis of MI is based upon the typical rise and fall of biochemical markers of myocardial necrosis, such as troponins (13).

The most important treatment in patients presenting with MI is revascularization of the occluded vessel. Nowadays, percutaneous coronary intervention is widely used and allows rapid revascularization (14). Successful reperfusion of the occluded coronary artery combined with pharmacological antiplatelet therapy and anticoagulation has been shown to reduce mortality, infarct size, and to improve left ventricular function following MI. Although a significant progress has been made through reperfusion therapy, it has also been established that the restoration of blood flow to an ischaemic myocardial area is associated with an adverse event. This phenomenon, referred to as "reperfusion injury", leads to cell death and has been estimated to account for up to half of the infarct size (15). During early reperfusion, white blood cells release inflammatory mediators such as interleukins subsequently leading to complement activation. Together with the circumstances in a prothrombotic environment, platelets become activated resulting in platelet aggregation within the microvasculature (16). This occlusive distal event might be further aggravated by microembolic atheromatous debris disrupted during the course of percutaneous coronary intervention (17). In addition, the reintroduction of oxygen potentiates formation of reactive oxygen species (ROS) and accumulation of intracellular calcium which has been proven to be a central hallmark of the reperfusion injury (18)/. This leads to damages in cellular proteins, organelles, and plasma membranes. Also, the activation of proapoptotic signaling cascades aggravates myocyte injury. Due to instability of the inner cell membrane ventricular fibrillation might occur after reperfusion of the infarct-related artery resulting in sudden cardiac death. An important feature contributing to reperfusion injury is endothelial and microvascular dysfunction as a result of reduced production of nitric oxide (NO) and increased inactivation of NO by its reaction with superoxide. The resulting reduction in NO bioavailability will further increase the inflammatory process, the production of ROS and vascular tone (19).

From the clinical perspective, ischaemia and reperfusion (I/R) injury remains an unsolved problem. Different treatment modalities have been proposed. In the experimental setting successful forms of cardioprotection include the application of free radical scavengers, inhibitors of intracellular calcium overload, as well as inhibitors of inflammation (20) but also non-pharmacological approaches such as ischaemic pre-, per- and postconditioning. The latter summarize strategies consisting of brief, repetitive episodes of ischaemia of the affected myocardial area at risk or a remote organ. It has been hypothesized that this leads to the release of endogenous protective factors ameliorating reperfusion injury (21). Furthermore, it has been suggested that remote ischaemic preconditioning contributes to cardioprotection by circulating nitrite confirming supporting the view that treatment strategies aiming at increasing bioavailability of NO seems reasonable (22). This fundamental role of NO in I/R has been investigated in several experimental studies. Mice lacking the gene encoding for the NO producing enzyme endothelial NO synthase ($eNOS^{-/-}$) develop increased infarct size following myocardial I/R (23). Administration of NO, NO donors, nitrite or the NO substrate L-arginine shortly before or at the time of reperfusion have in several studies been demonstrated to reduce infarct size (23-28). However, drawbacks of these approaches

are that L-arginine given systemically may be metabolized by liver arginase or might lead to production of reactive oxygen species in the situation of uncoupling of eNOS. These mechanisms might have been causal for the negative results observed in clinical trials aiming at improving myocardial function following MI by supplementation of L-arginine. There was no benefit of oral L-arginine on left ventricular function and there was a significant increased mortality in the group receiving L-arginine. Although the absolute number of cases was small, the enrollment was terminated by the data safety monitoring committee (29). Also in larger multicenter studies L-arginine supplementation did not lead to improved survival in patients with MI (30). The use of NO donors is limited by a narrow therapeutic window possibly due to pro-inflammatory effects of high concentrations of NO (31). Therefore it is important to develop new therapeutic approaches that increase NO bioavailability via mechanisms that specifically restores normal production of NO and inhibits eNOS uncoupling.

1.1.2 Heart failure

Heart failure (HF) is defined as the condition when the heart is unable to pump sufficiently to maintain blood flow to meet its own and the body's needs. Typical signs and symptoms reported by patients with HF include shortness of breath, excessive tiredness, and leg swelling. Actually, the shortness of breath is typically worse with exercise and when in the supine position. Patients may often be limited in their amount of exercise that they can perform, even under optimal treatment (32).

Common causes of HF include previous MI, high blood pressure, atrial fibrillation, valvular heart disease, and different cardiomyopathies. HF can be classified in different dimensions. One option to distinguish patients is based on reduced ability of the left ventricle to contract or to relax. This creates the entities HF with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF) (33). In the New York Heart Association (NYHA) classification the severity of the disease is graded by how much the patients are limited in their daily life and their ability to exercise. HF is widely occurring, cost intensive, and has a poor prognosis. In Western countries, around 2% of adults have been diagnosed with HF and in those over the age of 65 years, numbers increase up to 6–10%. The risk of death is about 35% in the first year after diagnosis but decreases thereafter to below 10% per year. However, this is still similar to the risks with different types of cancer. HF is the most frequent cause of emergent hospital admissions in most developed countries (34). In addition, HF has a negative impact on quality of life.

Diagnosis of HF is based on the history of the symptoms and a physical examination focusing on clinical hallmarks of HF. In addition, diagnostic workup includes echocardiography, blood tests – especially the measurement of the congestion parameter brain natriuretic peptide, and chest radiography. Subsequently initiated treatment depends on the severity and cause of the disease. In patients with chronic HF, treatment consists of lifestyle modifications including smoking cessation, frequent physical exercise, and dietary changes, as well as pharmacological treatment. Different pharmacological approaches have been confirmed to improve outcome, especially in HFrEF. The key feature in patients with HFpEF is the treatment of the underlying disease such as arterial hypertension. Another treatment modality consists of the implantation of implantable cardiac defibrillator to prevent sudden cardiac

death due to ventricular tachycardia or ventricular fibrillation. In later stages of the disease course, ventricular assist devices may be considered as well as heart transplantation (34).
HF affects the function of a variety of organs, including the gut, the lungs and the brain. However, it also affects the function of the vasculature by decreasing the bioavailability of NO. For example, it was reported that systemic inflammation and endothelial dysfunction were both associated with elevated natriuretic peptide levels, and adverse long-term clinical outcomes in patients with HFrEF (35). Several studies have investigated the underlying mechanisms. In patients with HF increased blood concentrations of the endogenous NO synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA) was observed (36). In patients with acutely decompensated HF, ADMA was markedly increased and negatively correlated with indirect measures of NO (37). In addition to this mechanism a dysfunctional NOS has been documented in HF. Furthermore, eNOS expression and activity have been shown to be decreased in HF (38). Of note, eNOS can become uncoupled in HF leading to the production of superoxide (O_2^-) (39). Therefore it seems reasonable to target NOS or its downstream mechanism to improve endothelial function. It is important to note that available treatment modalities such as phosphodiesterase type 5 inhibitors aiming at increasing cyclic guanosine monophosphate levels as downstream target have failed to show convincing results in HF (40).

1.1.3 Cardiopulmonary resuscitation

Cardiac arrest (CA) remains a major cause of sudden death in developed countries and outcome is often dismal. Despite cardiopulmonary resuscitation (CPR), only the minority of patients return to their former daily life and lifestyle (41, 42). CPR consists of chest compressions as an effort to create artificial circulation by manually pumping blood through the body and an artificial respiration (43, 44).

The primary aim of CPR is to restore partial flow of oxygenated blood to the brain and heart in order to avoid tissue death and to extend the narrow window of opportunity for a successful resuscitation without permanent brain damage. The most important key element of CPR is the delivery of an electric shock (defibrillation) of a shockable rhythm such as ventricular fibrillation or pulseless ventricular tachycardia. In contrast, asystole and pulseless electrical activity are considered not to be shockable. CPR is continued until the patient has a return of spontaneous circulation (ROSC) or is declared dead.

Different efforts have been made to improve outcomes for patients in CA. One key element is the limitation of the time until start of CPR. This includes the education of the population when to start CPR and how to perform it until professional personal arrives. Furthermore, it includes the availability of external automated defibrillators in public places to reduce time until defibrillation takes place (45). Optimization of CPR has been attempted by using mechanical chest compression devices. However, a randomized trial showed no survival benefit in comparison to conventional CPR (46). The use of invasive, extracorporeal CPR with extracorporeal membrane oxygenation in patients without ROSC is an alternative to conventional CPR (47). Extracorporeal membrane oxygenation treatment provides adequate temporary tissue perfusion and oxygenation to organs in CA patients and therefore increases the rate of successful defibrillation, prevents re-arrest due to ischaemia-triggered myocardial

dysfunction, and enables subsequent percutaneous intervention without the limitations of mechanical CPR in patients without ROSC. Even if achieving ROSC, the prognosis remains dismal. In these patients the treatment with therapeutic hypothermia or targeted temperature management has been used to reduce brain damage. Different guidelines support the use of cooling following resuscitation from cardiac arrest (43). However, these recommendations were largely based on two trials showing improved survival and neurological outcome when cooled to 33°C after CA. A large trial from 2013 revealed that a target temperature of 33°C and 36°C results in similar outcomes (48).

The global hypoxia and I/R injury of the entire body after ROSC is a central event that contributes to the so called post-cardiac arrest syndrome (49). This syndrome is characterized by macro- and microcirculatory dysfunction mimicking clinical features of sepsis. However, the unique features of post–cardiac arrest pathophysiology are often superimposed on the disease or injury that caused the CA, as well as underlying comorbidities. Post–cardiac arrest syndrome is characterized by four key features: (1) post–cardiac arrest brain injury, (2) post–cardiac arrest myocardial dysfunction, (3) systemic I/R response manifested by systemic inflammatory response syndrome, impaired vasoregulation, increased coagulation and impaired tissue oxygenation and utilization and (4) persistent precipitating pathology mainly in form of CVD and pulmonary disease (50). The exact pathophysiology remains unclear as several different complex changes seem to occur, including changes in the microcirculation (51-53). Different treatment strategies aim at improving macro- and microcirculation but no specific treatment exits. Of pathophysiological relevance are observations revealing that NO and NOS are influenced following CA. Although the understanding of this field is incomplete especially animal and cell culture studies revealed distinct differences in NO metabolism including the different NOS isoforms following CA. It has been reported that the expression of two different isoforms of NOS, eNOS and inducible NOS (iNOS) in the heart is differentially regulated after CA. Myocardial eNOS was expressed in a pig model prior to cardiac arrest, declined during untreated ventricular fibrillation, increased temporarily during the early postresuscitation period, and finally fell to baseline levels by 6 hours postresuscitation. In contrast, iNOS was not expressed in the myocardium prior to CA, increased after 10 minutes of untreated ventricular fibrillation, decreased slightly during the early postresuscitation period, and then steadily increased up to 6 hours postresuscitation (54-56). It has been assumed that differences in iNOS and eNOS also mediate peripheral vascular alterations following resuscitation in analogy to sepsis (53). Septic shock is characterized by increased expression and activation of iNOS and subsequent production of large quantities of NO (57). It has been hypothesized that excess NO leads to hemodynamic instability and widespread synthesis of reactive nitrogen species subsequently leading to tissue injury. Despite these changes, microcirculatory impairments have been linked to impaired eNOS function, reduced NO production and inflammatory response (58). Due to its prognostic importance, improvement of the microcirculation seems to be an important therapeutic aim. In patients with sepsis, Trzeciak and coworkers tried to improve the microcirculation by inhaled NO in a randomized, sham-controlled clinical trial (59). However, inhaled NO did not augment microcirculatory perfusion in these patients. Although similar studies with patients following CPR are lacking, these observations indicate that alternative treatment strategies targeting NO bioavailability may offer an opportunity to improve vascular function post CA (60).

1.2 THE VASCULATURE

The vasculature is the arrangement of blood vessels in the body consisting of arteries, capillaries and veins. The surface of these vessels has in humans a surface of more than 1000 m^2 and a weight of about 1.5 kg. Three different layers can be distinguished: endothelium, media and adventitia. Throughout the vasculature humans have around 10^{13} endothelial cells (61, 62). Therefore the endothelium is in a sense the largest endocrine organ of the body since it is not only a passive layer between blood and tissue but also actively modulates different processes by the synthesis and release of active mediators modulating vascular function (63). Directly by these mediators and indirectly, shear stress mediated by the flowing blood and the pressure within the blood vessel regulate vascular tone (**Figure 1**).

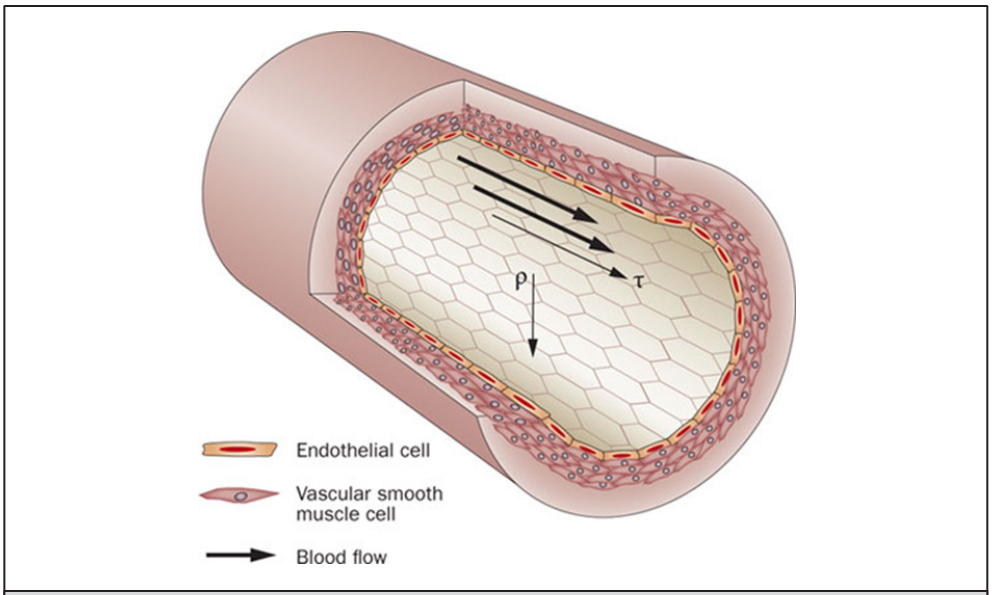

Figure 1: Structure of a blood vessel with special emphasis on hemodynamic forces that act on blood vessels. Shear stress (τ) is a lateral force that activates the endothelium and induces nitric oxide release, which causes vasorelaxation. Pressure (ρ) is a force that acts perpendicular to the arterial wall, stretching myocytes and inducing contraction. Figure and legend reproduced from (64). Permission obtained from Nature Publishing Group.

1.3 THE MICROCIRCULATION

The microcirculation is the perfusion of the smallest vessels. It has been defined as the part of the circulation where exchange of nutrients, water, gas, hormons and waste takes place. Moreover, it can be divided into three main sections: arterioles, capillaries and venules (**Figure 2**). Arterioles are rich in vascular smooth muscle cells and mainly responsible for the regulation of blood flow. The transition from arteries to arterioles is usually defined at a vessel diameter of 100 µm. Both vessels share the same structure including layers with

vascular smooth muscle cells in the media that become thinner with decreasing diameter resulting in a monolayer distally (65). Due to their excellent innervation and local factors mainly released from the endothelium, arteries and arterioles are able to change their diameter rapidly. Capillaries are characterized by their thin walls warranting the exchange between blood and tissues. The capillary network is formed by many branches of vessels that consist of an endothelial tube with a basal membrane and pericytes. The diameter of around 10 µm cannot be actively changed. When several capillaries merge together they are called postcapillary venules. These vessels usually have a diameter of 30-50 µm. Venules have a structure similar to that of capillaries and play a major role in regulating post-capillary resistance and in immunologic processes (66).

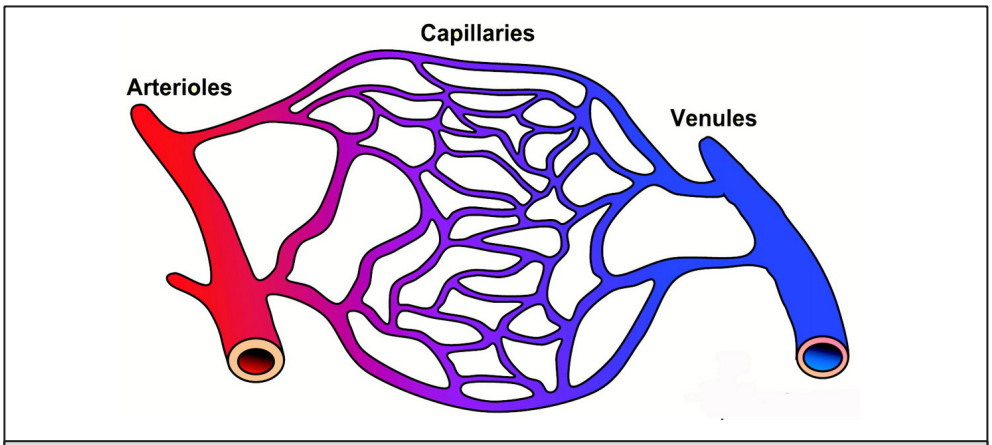

Figure 2: Structure of microcirculatory vessels. Figure reproduced from (67). Permission obtained from the publisher.

One key function of the arterioles is the regulation of blood flow to the different capillary beds and the regulation of the systemic blood pressure. No other segment of the circulation has similar abilities to dilate and constrict. Arterioles are able to dilate to increase the diameter by 50% and can close their lumen completely. This vasomotion is regulated by different stimuli. For example arterioles constrict following augmentation of intravasal pressure and dilate as a consequence of increased flow. In addition, oxygen tension regulates vascular tone (68). Vascular resistance is to a minor extend also influenced by capillaries by changing the properties of the surface and by changing the number of perfused capillaries (69). Venules regulate hydrostatic pressure in the capillaries and modulate capillary perfusion mainly by changed flow properties of erythrocytes dependent on volume status and flow velocity (70).

Other key functions of the microcirculation are to guarantee gas and nutrients exchange as well as to regulate fluid balance. The fluid exchange is mainly influenced by the hydrostatic pressure and the osmotic pressure but also by macromolecules. For instance, inflammatory cytokines may open fluid pores in postcapillary venules leading to extravasation of fluids. In addition, certain cells actively pass these vessels. This transmigration – especially of leukocytes – takes mainly place in the venules and is of high relevance in inflammatory diseases (71).

A large body of knowledge supports the central pathophysiological importance of the microcirculation in the development of organ failure in critically ill patients. This has been made possible due to the development of novel techniques to either directly visualize or indirectly evaluate microvascular perfusion (72). Especially in cardiogenic shock and in sepsis several studies confirmed the existence of microcirculatory impairment and its prognostic relevance (73, 74). This includes reduced vessel density, the proportion of perfused capillaries and microvascular flow (75). Diminished sublingual perfused capillary density (PCD) is associated with development of organ failure and is a predictor of poor outcome in patients with cardiogenic shock (74). Different treatment strategies may lead to microcirculatory improvement in critically ill patients (76) suggesting regulatory factors in the microcirculation as potential therapeutic targets in these patients.

For the clinician, the most frequently used tool to evaluate the microcirculation and tissue hypoxaemia is determination of serum lactate. However, the sublingual microvasculature has frequently been assessed in translational research due to its good accessibility and its close correlation to intestinal perfusion (77). Recent studies were able to confirm that microcirculatory parameters are decreased also in acute decompensated HF. Impaired microcirculation was associated with an increase in factors that augment vascular tone such as endothelin-1 and catecholamines (78). Therapeutic strategies aiming at increasing bioavailability of NO therefore seem reasonable. Nitroglycerine has been shown to improve PCD in patients in severe HF (79), however, systemic administration of this substance is often not possible in these patients due to unfavorable systemic hemodynamic effects. Further research is needed to identify possible novel mechanisms augmenting local bioavailability of NO to improve microvascular and organ perfusion in critically ill patients.

1.4 ENDOTHELIAL FUNCTION

The vascular endothelium plays a central role in the maintenance of normal vascular function by influencing vascular tone, inflammation and platelet function. A healthy endothelium induces vasodilatation, inhibits inflammation, inhibits thrombosis and stimulates fibrinolysis. It takes part in the metabolism of lipoproteins and eicosanoids and is a selective barrier limiting the penetration of high-molecular weight substance to surrounding tissues (80).

Exposure to CVD risk factors eventually leads to endothelial dysfunction. A clear definition of endothelial dysfunction is lacking and therefore the impairment of endothelium-dependent vasodilatation has been widely used (81). Clinically important functional consequences of endothelial dysfunction are vasoconstriction, thrombus formation, arterial hypertension and atherosclerosis. Endothelium-dependent vasodilation can be assessed invasively in several vascular regions including the coronary circulation, non-invasively in the forearm and at the fingertip (82, 83) as well as in the microcirculation of the skin, in the mucosa or in the muscle. Coronary and peripheral endothelial vasodilatations correlate well to each other (84). Of note, impaired endothelial function predicts future cardiovascular events (85). For practical reasons, endothelial function is most often estimated using flow mediated dilatation by provoking vasodilatation caused by increased shear stress induced by reactive hyperaemia (86-88).

In addition, endothelial dysfunction is considered to be of central pathophysiological relevance in several CVD including hypertension, atherosclerosis, vascular complications in diabetes mellitus and organ dysfunction in HF (89). Although the underlying mechanism of endothelial dysfunction is multifactorial, a key circumstance is considered to be the impairment of the bioavailability of NO (90), which is defined as reduced biological activity due to reduced production or increased inactivation of endothelium-derived NO.

NO is produced from the amino acid L-arginine by the three NOS: eNOS, iNOS and neuronal NOS (91). In endothelial cells, NO is primarily produced from by eNOS. The physiologically most important determinants for NO generation are fluid shear stress and pulsatile stretch. Of note, eNOS activity is regulated by a range of transcriptional and posttranscriptional mechanisms including protein phosphorylation and dephosphorylation. Of particular importance in regulating eNOS activity are two amino acids: a serine residue in the reductase domain (Ser(1177)) and a threonine residue (Thr(495)) located within a calmodulin binding domain. Simultaneous changes in the phosphorylation of Ser(1177) and Thr(495) in response to different stimuli are regulated by a number of kinases and phosphatases that continuously associate with and dissociate from the eNOS signaling complex (92, 93). Interestingly, eNOS is further regulated by the presence of cofactors such as tetrahydrobiopterin. Different studies suggested that cellular deficiency of either L-arginine or tetrahydrobiopterin can cause endothelial dysfunction by "uncoupling" eNOS. Uncoupled eNOS is a term used to describe a change in the ratio of NO to O_2^- produced in favour of decreased NO and increased O_2^- production by eNOS. This leads to reduced synthesis and bioavailability of NO and increased levels of superoxide and peroxynitrite. Of note, L-arginine is also a substrate for arginase, which converts L-arginine to L-ornithine and urea (94). This means that the production of NO is dependent on the relative expression and activities of arginase and eNOS. More specifically, increased arginase activity may lead to deficiency of L-arginine available for eNOS and thereby reduce NO production. Increased arginase activity may also cause uncoupling of eNOS due to reduced L-arginine availability. The distinct post-translational regulation mechanisms of eNOS in vascular endothelium have been recently reviewed by Qian and Fulton (95).

1.5 ARGINASE

1.5.1 Localization and regulation of arginase

Arginase is a manganese metalloenzyme hydrolysing L-arginine to urea and L-ornithine. Arginase is present in two isoforms, arginase I and II, that share approximately 60% sequence homology (96). Although both isoforms are expressed throughout the body, arginase I is a cytosolic enzyme mainly localised in the liver. Hepatic arginase I constitutes the majority of the body's total arginase activity and has a central role in the elimination of nitrogen formed during amino acid and nucleotide metabolism in the urea cycle. In addition to that, arginase I expression has been demonstrated in extra-hepatic tissues including endothelial cells, vascular smooth muscle cells and red blood cells (97). In contrast, arginase II is a mitochondrial enzyme with a comparably wide distribution and is expressed in the kidney, prostate, gastrointestinal tract and the vasculature. The role of arginase II is not completely revealed, however, the enzyme is assumed to be involved in the regulation of L-arginine homeostasis and production of L-ornithine for polyamine and proline synthesis for

cell proliferation and development (98). Both isoforms of arginase are expressed in the vasculature but it appears as if the expression is both vessel and species dependent. This has been summarized elsewhere (99).

Increased expression of arginase is stimulated by a variety of pro-inflammatory factors including lipopolysaccharide, tumour necrosis factor (TNF)-α (94, 98, 100-102) as well as interleukin (IL)-4, IL-10 and IL-13 (103). Other stimuli for arginase expression are oxidised low-density lipoprotein (oxLDL) (104), glucose (105), thrombin (106), hypoxia (107, 108) and angiotensin II (109). Reactive oxygen and nitrogen species including H_2O_2 (110) and peroxynitrite (111, 112) derived from eNOS (105) and nicotinamide-adenine-dinucleotide phosphate (NADPH) oxidase (113) increase arginase expression. Furthermore, intracellular signalling pathways activated by these factors include protein kinase C/RhoA/Rho kinase (ROCK) pathway (112, 114), mitogen-activated protein kinase (109), tyrosine kinases and cyclic adenosine monophosphate/protein kinase A (115). This has been illustrated in **Figure 3**. In addition, several transcription factors regulate arginase expression (103).

Arginase activity can be changed independently of alterations in the levels of the arginase protein level. Santhanam and co-workers (116) reported that arginase I is modified by post-translational S-nitrosylation leading to stabilization of the arginase trimer, consequently decreasing the *Km* for L-arginine by a factor of six. Another mechanism resulting in increased arginase II activity was recently proposed by Ryoo et al. (117) who demonstrated that a subcellular redistribution of arginase II from the mitochondria and microtubule cytoskeleton leading to increased enzyme activity occurred after activation of RhoA and ROCK. This might explain the rapid increases in arginase activity induced by oxLDL in endothelial cells well in advance before any changes in protein expression can be expected (104). Further posttranslational modifications (e.g. phosphorylation) are currently not known.

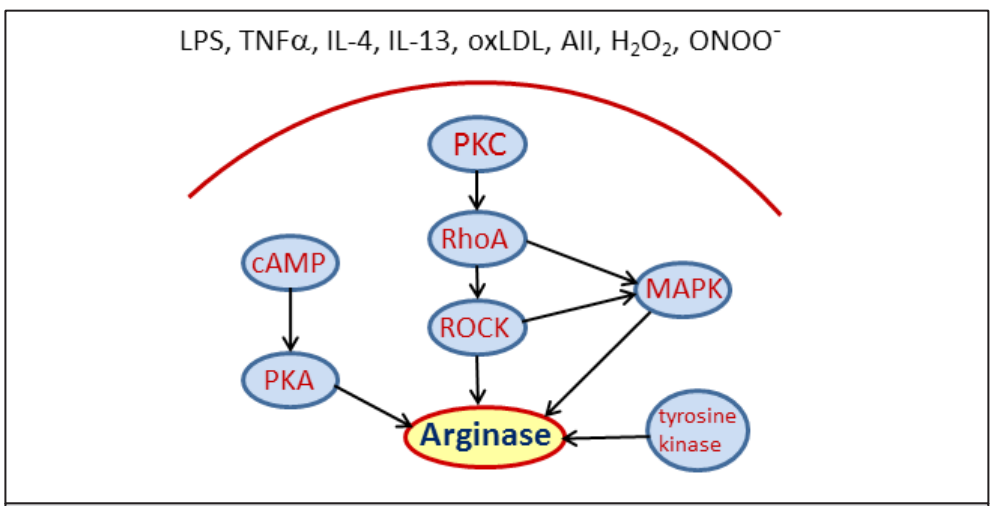

Figure 3. Schematic illustration of factors regulating arginase expression and activity. Various factors including cytokines, oxidised LDL, angiotensin II, reactive oxygen and nitrogen species activate different intracellular signalling pathways. Abbreviations: cAMP: cyclic adenosine monophosphate, MAPK; mitogen-activated protein kinase, PKA; protein kinase A, PKC; protein kinase C, ROCK; Rho kinase. Figure and legend reproduced from (99). Permission obtained from the publisher.

1.5.2 Role of arginase in vascular function

Since arginase and eNOS utilise L-arginine as their common substrate it is important to note that this leads to reciprocal interactions between these two enzymes. An increase in arginase activity consequently leads to the consumption of L-arginine needed for NO production by eNOS. This may result in reduction in NO production and subsequent endothelial dysfunction. Keeping in mind that a reduction in the bioavailability of NO and the associated endothelial dysfunction are both critically involved in the development of several CVD, upregulation of arginase protein or arginase activity might be an important mechanism (118). In this context it is important to note that L-arginine is also the substrate for the enzymes arginine-glycine amidinotransferase, and arginine decarboxylase being responsible for the production of creatine and agmatine, respectively. However, arginase and NOS are the L-arginine catabolic enzymes with the highest impact on the cardiovascular system (98).

A vast body of evidence has convincingly shown that increased arginase activity is associated with endothelial dysfunction, especially in different experimental models of hypertension (119), atherosclerosis (104), diabetes (105) and ageing (120). It seems clear that the underlying mechanism is due to impaired production of NO secondary to L-arginine deficiency. Another contributing factor is so-called uncoupling of eNOS leading to superoxide production as a result of substrate and/or co-factor deficiency (91). Thus, increased arginase activity leads both to reduced production of NO and to increased superoxide production which further increases NO inactivation. Accordingly, arginase inhibition leads to an increase in the bioavailability of NO and reduces superoxide levels (105, 121) resulting in improved endothelial function (**Figure 4**). Furthermore, increased cytosolic arginase II is co-localised with eNOS during hypoxia (107). This close proximity of the two enzymes that share L-arginine as their substrate hints at an intriguing mechanism for control of NO synthesis. Finally, arginase might also inhibit L-arginine transport in endothelial cells leading to further reduction of substrate availability for eNOS (107). The role of arginase in the development of cardiovascular disease has therefore been recognized to be of importance under different pathological conditions.

1.5.3 Arginase under pathological conditions

Arginase has been implicated in the development of cardiovascular dysfunction in several CVD based on the mechanisms described above. These conditions include myocardial I/R injury, atherosclerosis, hypertension, pulmonary arterial hypertension, HF and vascular complications associated with diabetes (99, 122, 123). In this thesis the primary focus has been to evaluate the pathophysiological role of arginase and the therapeutic potential of arginase inhibition in myocardial I/R, hypoxia and HF.

Myocardial IR injury
It was shown around forty years ago that serum arginase activity was increased in patients with MI and correlated with the extent of myocardial necrosis (124, 125). In addition, Smirnov and co-workers demonstrated increased arginase activity in infarcted human myocardial tissue in comparison with normal myocardium. Interestingly, they found a positive veno-arterial concentration gradient of urea over the coronary vascular bed in patients with ischaemic heart disease, suggesting local production of urea by arginase (126).

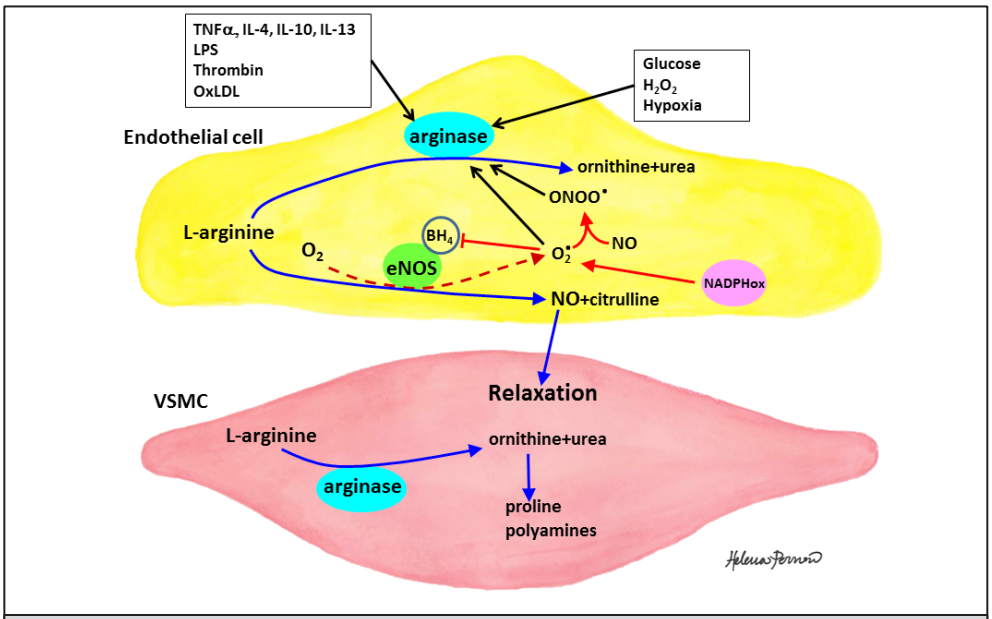

Figure 4: Schematic illustration of the action of arginase in the regulation of NO bioavailability and vascular function. Arginase is expressed in endothelial and vascular smooth muscle cells via regulation of cytokines, thrombin, hypoxia, reactive oxygen species, hyperglycaemia, and oxidized LDL. Increased activity of arginase will via hydrolysis of L-arginine to ornithine and urea reduce the availability of L-arginine for NO synthase (NOS), thereby reducing the production of NO. Lack of L-arginine will also result in 'uncoupling' of NOS whereby the enzyme produces superoxide instead of NO. Generation of superoxide by uncoupled eNOS and NADPH oxidase and peroxynitrite from superoxide and NO will further increase arginase activity and impair NO production via oxidation of tetrahydrobiopterin. Collectively, these changes will reduce the bioavailability of NO and contribute to endothelial dysfunction. In vascular smooth muscle cells, ornithine will increase formation of L-proline and polyamines which stimulate cell proliferation. Abbreviations: Ang II, angiotensin II; BH_4, tetrahydrobiopterin; LDL, low-density lipoprotein; LPS, lipopolysaccharide; NADPHox, nicotinamide adenine dinucleotide phosphate oxidase; NO, nitric oxide; ONOO, peroxynitrite; VSMC, vascular smooth muscle cell. Figure and legend reproduced from: (99). Permission obtained from the publisher.

The relevance of arginase expression following myocardial I/R has been investigated in various experimental models. Hein et al. (127) were able to show that the expression of arginase in coronary arterial endothelial cells and vascular smooth muscle cells was increased following I/R. In a mouse model of I/R, arginase I was not expressed in neutrophils within the myocardium, however, its expression was increased in endothelial cells (128). Myocardial expression of arginase during I/R has been understood to be regulated by inflammatory cytokines (128). In consequence, increased expression and activity of arginase seem to be of central functional importance in myocardial I/R. This is further supported by findings indicating that impairment of *ex vivo* endothelium-dependent vasodilatation in coronary arteries following I/R was prevented following arginase inhibition *in vitro* (127). These observations indicate that inhibition of arginase during myocardial I/R warrant further investigation to determine its effect to reduce infarct size *in vivo*.

Arginase is of importance also in other clinically relevant pathophysiological conditions, including atherosclerosis. Endothelial dysfunction occurs early in the development of atherosclerosis as a result of reduced bioavailability of NO (129, 130). Different studies demonstrated increased expression of arginase protein and arginase activity in experimental models of atherosclerosis. Apolipoprotein E knockout ($apoE^{-/-}$) mice that were fed a cholesterol-rich diet had significantly higher arginase activity in the aorta compared to age matched wild-type mice (106, 131). Of interest, after removal of the endothelium arginase activity was reduced, suggesting an important contribution by endothelial cells (131). Increased arginase activity has also been found in atheromatous lesions of hyperlipidemic rabbits (132). It seems that the predominant isoform of arginase in $apoE^{-/-}$ atherosclerotic mice is arginase II (106, 131, 133). Different mechanisms seem to be involved in arginase activity regulation in atherosclerosis including the RhoA/ROCK pathway (106, 134). The potent proatherogenic oxLDL also increases arginase activity (104, 117, 135) via the endothelial lectin-like oxidized low density lipoprotein scavenger receptor and RhoA/ROCK (117). Further comprehensive studies in atherosclerosis have been summarized elsewhere (99) showing a central relevance of arginase in atherosclerosis indicating a possible treatment target.

Another vascular bed in which I/R is of scientific and clinical interest is the cerebral arterial system. Detailed experimental and clinical information of ischaemic stroke in relation to arginase is limited. However, increased arginase activity following cerebral hypoxia-ischaemia has been documented (136). Upregulation of arginase I has been shown in peripheral white blood cells of patients with ischaemic stroke (137, 138). Yet, there is no information regarding the functional role of arginase in acute ischaemic stroke. In an animal model of subarachnoid haemorrhage, increased arginase resulted in impaired availability of L-arginine and NO production underlining the functional relevance of arginase for central arterial function (139).

Heart failure

Different studies revealed that low bioavailability of NO plays a pivotal role in HF patients due to increased plasma levels of ADMA (140). In addition, there is growing evidence that increased arginase activity might play an additional role in vascular dysfunction in HF patients. It has been speculated that increased plasma arginase activity in HF patients might not arise from enhanced expression but rather from spillover of the enzyme from injured tissues such as a congested liver or damaged myocytes (141). Heusch et al. revealed in a rabbit model of HF induced by left ventricular pacing that the serum arginine concentration was decreased but on the other hand cardiac arginase II expression was increased (142). It has also been shown that cardiomyocytes may regulate contractility via an arginase-mediated reduction of NO bioavailability (143). Thus, increased cardiomyocyte arginase activity may negatively influence HF (144). Of note, combined administration of arginase and ADMA decreased cardiac output and stroke volume in rats (145). Different arginase inhibitors dose-dependently increased contractility in rat myocytes (146). However, very little is known about circulating levels of arginase protein and arginase activity and its influence on vascular function in HF patients.

Hypoxia

Limited conclusive data is available regarding the exact relation between hypoxia and arginase. *In vitro* data suggest that hypoxia induces upregulation of arginase activity as well as

mRNA and protein levels of arginase II in a cell culture model of human lung microvascular endothelial cells (147). Arginase I is not expressed in these cells. In a rat model of chronic intermittent hypoxia arginase activity was measured in lung and heart tissues revealing increased expression of arginase II and higher arginase activity (148). Applying a similar model of chronic intermittent hypoxia, Krause et al. demonstrated increased arginase I protein levels in the carotid artery, whereas eNOS levels were decreased. This was paralleled by impaired endothelial function that was restored by arginase inhibition. This effect was completely blocked by a NOS inhibitor (149). So far, data from studies of global hypoxia on arginase levels in humans are not available.

Collectively, these data suggest a critical role of arginase in the regulation of central pathophysiological processes in several CVD states. Inhibition of arginase activity may thereby provide a novel therapeutic option leading to important beneficial cardiovascular effects.

2 AIMS

Based on these earlier observations the overall aim of this thesis was to investigate the role of arginase for cardiovascular function with special focus on I/R injury and microvascular function.

The specific aims were to:

1) Investigate the protective effect mediated by an arginase inhibitor during myocardial I/R injury and shed light on the mode of action.

2) Test the therapeutic effect of an arginase inhibitor administered by intracoronary infusion during early reperfusion in a large animal model of myocardial infarction.

3) Examine circulating arginase levels in patients with congestive heart failure and to test the therapeutic effect of arginase inhibition on microvascular perfusion.

4) Investigate the influence of global hypoxia on arginase and to determine whether arginase inhibition improves microvascular function following cardiopulmonary resuscitation.

3 METHODS

3.1 ANIMAL MODELS OF MYOCARDIAL ISCHAEMIA AND REPERFUSION

The animal studies were approved by the regional Ethics Committee for laboratory animal experiments in Stockholm and 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).

3.1.1 Rat model of myocardial ischaemia and reperfusion

In **paper I** a rat model of myocardial I/R injury was applied. Male Sprague–Dawley rats (270–400 g) were anaesthetized with sodium pentobarbital intraperitonally (50 mg/kg, followed by an intravenous continuous infusion of 3–5 mg/kg/h), tracheotomized, intubated, and ventilated with air by a rodent ventilator (54 strokes/min, 9 ml/kg tidal volume). The rectal temperature was maintained at 37.5–38.5°C by a heated operation table. The right carotid artery was cannulated and connected to a pressure transducer for measurement of mean arterial pressure (MAP). The heart rate (HR) was determined from the arterial pressure curve. The right jugular vein was cannulated for administration of drugs and Evans Blue at the end of the experiment.

The heart was exposed via a left thoracotomy. Next, a ligature was placed around the left coronary artery (**Figure 5**). After the surgical preparation has been completed, the rats were allowed to stabilize for 15 min and then randomized into five groups. These groups were treated with (i) saline (n=10), (ii) the arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (nor-NOHA, 100 mg/kg, n=8), (iii) the NO scavenger carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO, 1 mg/kg, n =6), (iv) nor-NOHA and cPTIO (n=6), or (v) nor-NOHA and the NOS inhibitor N^{G}-monomethyl-L-arginine (L-NMMA, 10 mg/kg, n=6). All substances were given intravenously as bolus injections 15 min before the onset of ischaemia which was initiated by tightening the coronary artery ligature. An appearance of a cyanotic colour of the myocardial area at risk was noted to confirm ischaemia. The dosages of the substances were based on previous studies (150-153). After 30 min of ischaemia, reperfusion was started by the removal of the snare and was maintained for two hours. The reperfusion was associated with disappearance of the cyanotic colour of the myocardium.

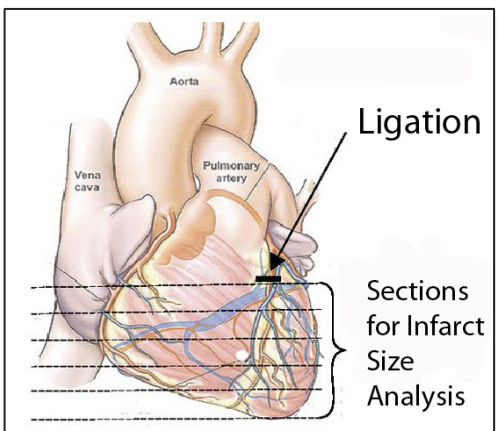

Figure 5: Ligation of the coronary artery. Figure with changes reproduced from (154). Permission obtained from the publisher.

The infarct size was determined after 2 h of reperfusion. Following re-occlusion of the coronary artery, 1.5 ml of 2% Evans Blue was injected in the left jugular vein to mark the ischaemic myocardium (area at risk, AAR). The rats were euthanized with an

overdose of anaesthetics and subsequently the heart excised. The right ventricle and the atria were removed. The left ventricle was cut into 1–1.5 mm thick slices perpendicular to the heart base–apex axis (**Figure 5**). The slices were scanned from both sides for determination of the AAR, weighed, and put in 0.8% triphenyltetrazolium chloride (TTC) for 15 min at 37°C to distinguish the viable myocardium from the necrotic. After 24 h of incubation in 4% formaldehyde, slices were again scanned from both sides, and the extent of myocardial necrosis and the area at risk were determined by planimetry of computer images (Photoshop 6.0; Adobe Systems, San Jose, CA, USA).

3.1.2 Pig model of myocardial ischaemia and reperfusion

In **paper II**, a large animal model of I/R was applied. Twenty-five female farm pigs (27–38 kg) were premedicated with tiletamin (1.5 mg/kg im), zolezepam (1.5 mg/kg im) and medetomidin hydrochloride (0.06 mg/kg im). Anaesthesia was induced by injection of sodium pentobarbital (20 mg/kg iv) and maintained with sodium pentobarbital (2–4 mg kg/h iv) and morphine (0.5 mg/kg/h iv). The animals received heparin 5000 IU/h iv. The animals were intubated and mechanically ventilated with air and oxygen. Arterial blood pH, pO_2 and pCO_2 were used to adjust respiratory rate and tidal volume to keep these values within the physiological range. A heated operating table was used to keep rectal temperature at 39.±0.2°C. A central venous catheter was inserted in the right external jugular vein for drug and fluid administration. Another catheter was positioned in the right femoral artery for blood sampling and for measurement of arterial pressure. Heart rate was determined from the arterial pressure curve. All variables were continuously recorded on a computer equipped with PharmLab V3.0 (AstraZeneca R&D, Mölndal, Sweden). The heart was exposed via a sternotomy and a ligature was positioned around the left anterior descending artery (LAD) at a position from which the distal third of the artery is occluded when tightening the ligature (**Figure 6**). A thin needle connected to a catheter was placed in the LAD distal to the ligature for intracoronary (ic) administration of the experimental drugs during ischaemia and reperfusion into the jeopardized area. An ultrasonic probe (Transonic Systems Inc., New York, USA) was placed around the artery just proximal to the snare for measurement of coronary blood flow to ensure complete coronary artery occlusion and reperfusion during and following coronary artery ligation, respectively. The flow probe was connected to a Transonic 208 blood flow meter.

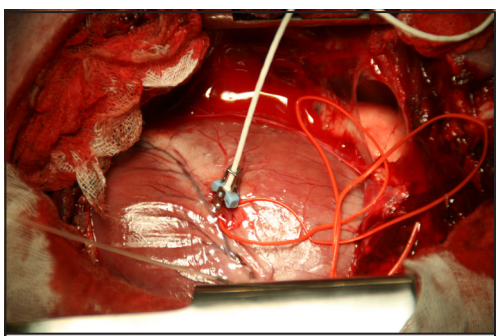

Figure 6: A ligature (orange) was positioned around the left anterior descending artery (LAD). An ultrasonic probe (blue with white cable) was placed around the artery just proximal to the snare for measurement of coronary blood flow.

After a post-surgery stabilization period of 30 min the pigs were subjected to myocardial ischaemia induced by tightening the ligature around the LAD. The animals were randomized to receive ic infusion of either (i) saline (0.9% NaCl, vehicle, n= 8), (ii) the arginase inhibitor nor-NOHA (2.0 mg/min, n = 8) or (iii) the NOS inhibitor L-NMMA (0.35 mg/min) together with nor-NOHA (n=6). A fourth group received nor-NOHA 2 mg/min as a systemic iv

infusion (n= 5). In addition, a fifth group of sham operated pigs (n =5) was added to be used as a control group for the analysis of arginase I and II and arginase activity. Two pigs (one pig randomized to the vehicle group and one in the nor-NOHA group) were excluded from the study due to irreversible ventricular fibrillation occurring during ischaemia. As shown in **Figure 7**, the infusions of vehicle or nor-NOHA were started at 30 min of ischaemia and continued up to 5 min after start of reperfusion. The infusion of L-NMMA was started at 25 min of ischaemia and continued until 5 min after initiation of reperfusion. All infusions were given at a rate of 2 ml/min. After 40 min of ischaemia LAD was reperfused for 4 h by removal of the ligature. At the end of reperfusion the LAD was reoccluded and 1 mg/kg of 2% Evans Blue was injected iv to outline the area at risk, after which the animals were sacrificed by an iv injection of potassium chloride. The heart was rapidly extirpated. The atria and the right ventricle were removed. The left ventricle was cut into 1 cm thick slices perpendicular to the heart base-apex axis. Myocardial pieces of the third slice from the apex were used for expression analyses of arginase I and II using immunohistochemistry and Western blotting. The remaining myocardial slices were placed in 0.8% TTC at 37°C for 10 min which stained viable myocardium red. The extent of area risk and myocardial necrosis were determined by planimetry using Adobe PhotoshopC5. Five ml of blood was sampled from the abdominal aorta before ischaemia and at 5, 20, and 60 min of reperfusion for determination of nitrite.

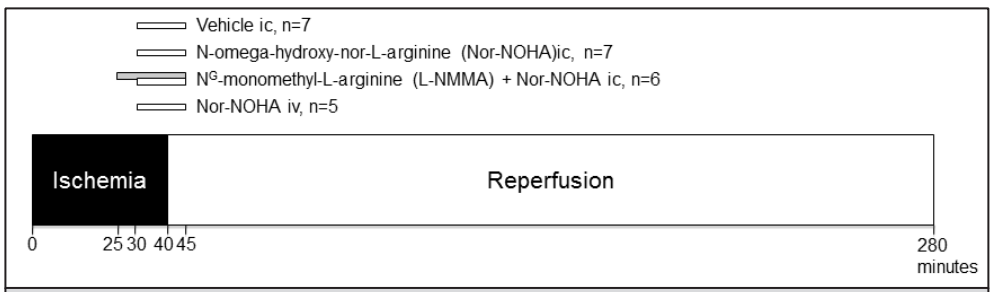

Figure 7: Experimental protocols. The animals were subjected to 40 min of ischaemia and 240 min reperfusion. The groups were randomized to vehicle ic, nor-NOHA ic, nor-NOHA combined with L-NMMA ic or nor-NOHA iv. All infusions were started at 30 min of ischaemia except L-NMMA which was started at 25 min of ischaemia. The infusions were maintained until 5 min of reperfusion. Figure and legend reproduced from (155). Permission obtained from the publisher.

3.2 CLINICAL STUDIES

The clinical studies were approved by the ethical committee of the Friedrich-Schiller-University (Jena, Germany). Four different populations were studied:

3.2.1 Stable heart failure patients

Eighty patients were included in **study III** following consecutive recruitment in a referral outpatient clinic for patients with chronic HF or from a cardiology ward at the University of Jena. Inclusion criteria were a) symptomatic HF irrespectively of its genesis and b) willingness to participate. Exclusion criteria were: a) acute coronary syndrome within the

last three months, b) systemic inflammatory disease, c) renal insufficiency with a serum creatinine >250 mmol/l, d) known malignant diseases, or e) significant anaemia defined as a haematocrit <25.0%. The past medical history including cardiovascular risk factors, cardiovascular events, current drug treatment and vital signs were obtained from a personal interview. Patients were divided into two groups based on their NYHA class (Group 1: NYHA classes I/II, group 2: NYHA classes III/IV). Diabetes mellitus was assessed by determination of fasting blood glucose (>6.0 mmol/l) and/or by medical history and an elevation of HbA1c above 5.3%. Healthy control subjects were recruited from the personnel of the Cardiology department, University of Jena (n=6). Blood was taken after their signed informed consent. Echocardiogram evaluations (Philips iE33, Philips, Germany) were performed by cardiologists blinded to the study. Left ventricular ejection fraction (LVEF%) was derived using Simpson's modified biplane method. Presence of diastolic dysfunction was assessed using current echocardiographic standards. Especially, the mitral inflow Doppler echocardiogram was used with a reversal of the normal E/A ratio defining diastolic dysfunction grade I. In addition, left atrial size and estimates of left ventricular filling pressures were used (156).

3.2.2 Decompensated heart failure patients

In order to perform a mechanistic part of the study in **paper III** investigating the influence of the topical application of pathway-specific blockers, eight patients with severe HF (NYHA IV) were included, applying the same inclusion and exclusion criteria. The experimental protocol is described below.

3.2.3 Subjects undergoing global hypoxia

To study the effect of global hypoxia on circulating arginase levels in **paper IV**, fourteen healthy subjects were recruited from our personnel. The study was performed in an air-conditioned, normobaric hypoxia chamber, which has a carbon dioxide (CO_2) scrubber to eliminate CO_2. The oxygen concentration can be adjusted to achieve a certain degree of hypoxia or a simulation of an altitude. The chamber is situated in a gym and regularly used by athletes to train under hypoxic conditions to simulate high altitude training. Any kind of disease associated with hypoxia or any acute disease within the two weeks before the experiment were exclusion criteria. A first blood sample was obtained before hypoxia. Subsequently, the oxygen concentration was adjusted to an altitude equivalent to a height of 5500 m (oxygen concentration = 9.9%) to achieve hypoxic conditions with a peripheral oxygen saturation of around 75%, and a second blood sample was taken after at least 6 h of hypoxia and 2 h at 5500 m. For safety reasons, any subject whose oxygen saturation would fall below 70% or who experience discomfort would be excluded from the experiment.

3.2.4 Patients following cardiopulmonary resuscitation

A total of 31 patients undergoing CPR were included as an additional cohort in **paper IV** in order to confirm the relevance of hypoxia for arginase also in the clinical setting. Inclusion criteria were: (i) ROSC within 60 min of CPR, (ii) nontraumatic CA independent of primary rhythm and (iii) estimated time of hypoxia including nonwitnessed CA ≤ 15 min. In addition,

only unconscious patients who survived at least 72 h were included to study the effect of time and possible influence of therapeutic hypothermia due to a hypothermia period of 24 h and the subsequent rewarming. It is important to note that 19 patients were treated with mild hypothermia while 12 patients received standard therapy. The responsible intensive care specialist on duty decided whether to apply hypothermia or standard therapy. Therapeutic hypothermia was applied by either surface or intravascular cooling (Coolgard 3000 Thermal Regulation System, Alsius Corporation, Irvine, CA, USA). A rapid infusion of two liters of 4°C normal saline was administered to accelerate the induction of hypothermia. The body core temperature was measured using an urine bladder temperature probe to keep the temperature between 32.5 and 33.5°C for 24 h. Rewarming was achieved passively in patients treated with surface cooling and actively (0.3°C/h) in patients treated with intravascular cooling. Cerebral Performance Category Scale was obtained to assess neurological outcome after four weeks. Blood sampling was performed directly after arrival at the intensive care unit and 72 hours thereafter. In **paper IV**, another control population was recruited consisting of 21 healthy subjects from the personal.

3.3 INVESTIGATION OF ARGINASE IN BLOOD AND TISSUE

3.3.1 Western blotting

A standard Western Blot protocol was applied in **paper I**. Myocardium obtained from the ischaemic and non-ischaemic myocardium was pre-treated with saline and frozen at -80°C for later evaluation of the expression of arginase I and II by immunoblotting. Then, frozen samples were homogenized in ice cold lysis buffer containing 20 mM Tris (pH 7.8), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1% Triton X-100, 10% (w/v) glycerol, 10 mM NaF, 1 mM ethylenediaminetetraacetic acid, 5 mM Na-pyrophosphate, 0.5 mM Na_3VO_4, 1 µg/ml leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride, 1 µg/ml aprotinin, and 1 mM benzamidine. The homogenates were centrifuged at 5000 g for 20 min at 4°C and the concentration of protein in the supernatant in each aliquot was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Protein extracts (50 µg/lane) were loaded onto a 10% SDS gel and separated by electrophoresis. Extracts from two separate groups were loaded on one gel and the amount of protein was accordingly compared pairwise. Proteins were transferred to nitrocellulose membranes (Hybond-C pure, Amersham Biosciences UK Ltd, Little Chalfont, UK), and Ponceau staining was used to confirm efficiency of transfer and to visualize protein loading. Membranes were incubated overnight at 4°C with antibodies against arginase I (BD Biosciences Pharmingen, CA, USA) and arginase II (Santa Cruz Biotechnology, CA, USA) followed by anti-mouse (BD Biosciences Pharmingen) and anti-goat secondary antibody (Santa Cruz Biotechnology), respectively. Proteins were visualized by enhanced chemiluminescence with ECL advance Western blotting detection kit (Amersham Biosciences UK Ltd) and quantified using densitometry.

To determine possible differences in subcellular compartments regarding their arginase expression during ischaemia and reperfusion subcellular fractions were obtained in **paper II**. In close analogy to the handling of the rat hearts, frozen pig heart pieces were transferred into ice-cold buffer consisting of (in mM): sucrose, 250; KCl, 20; EDTA, 1; and Hepes, 5

(pH 7.4). Following thawing, the tissue was minced while in the buffer with cold scissors and then homogenized with a loose fitting motor-driven Teflon pestle in a glass homogenizor. Homogenization was performed at low speed on ice for 2 min (15 s on, 5 s off). Samples were then centrifuged at 2°C for 10 min at 750 g. The pellet was discarded and the supernatant was centrifuged at 2°C for 10 min at 12000 g. The supernatant was used as the cytosolic fraction and the pellet was resuspended (2 µl/mg wet weight) in a buffer consisting of (in mM): Hepes, 20; NaCl, 150; EDTA, 5; KF, 25; Na_3VO_4, 1; 20% glycerol; 0.5% Triton X, and protease inhibitor cocktail (Roche, Basel, Switzerland) 1 tablet per 50 ml, and this represented the mitochondrial fraction. Fractions were frozen at -80°C until processed. Correctness of this method was confirmed by Western blots of the fractions for cytochrome oxidase where strong bands are seen in the mitochondrial fractions and weak or no bands are seen in the cytosolic fractions. To further determine arginase expression, cytosolic and mitochondrial fractions were diluted with reduced Laemmli buffer and heated at 70°C for 10 min. Fifteen (arginase I) or 5 µg (arginase II) of protein were loaded onto each well and separated by electrophoresis using NuPAGE Novex 4–12% Bis-Tris Gels (Invitrogen) and transferred onto polyvinylidine fluoride membranes (Immobilon FL, Millipore). Membranes were blocked for 1 h at room temperature in Li-Cor Blocking buffer (LI-COR Biosciences), followed by incubation overnight at 4°C with the following primary antibodies diluted in blocking buffer: mouse anti-dihydropyridine receptor (DHPR; 1:500, #ab2864, Abcam), mouse anti-oxphos complex IV subunit I (cytochrome oxidase, 1:1000, #459600, Invitrogen), rabbit anti-arginase I (1:2000, #HPA003595, Sigma), and rabbit anti-arginase II (1:2000, #HPA000663, Sigma). Membranes were then washed in TBS-T and incubated for 1 h at room temperature with IRDye 680-conjugated goat anti-mouse IgG and IRDye 800-conjugated goat anti-rabbit IgG (1:15,000, LI-COR) blocking buffer and 0.01% SDS. Membranes were then washed in TBS-T and imunoreactive bands visualized using infrared fluorescence (IR-Odyssey scanner, LI-COR Biosciences). Band densities were analysed with Image J (NIH, USA; http://rsb.info.nih.gov/ij/).

3.3.2 Arginase activity assay

Arginase activity was determined in **paper II** by using a colorometric assay that has been previously described by Berkowitz and coworkers (157). The assay measures the urea content using α-isonitrosopropiophenone. To detect arginase activity only the inhibitable fraction of urea was used in the analysis. A lysis buffer consisting of PBS, 1 mM EDTA, Triton X (0.1%) and protease inhibitors (Roche) was freshly prepared. Lysates of homogenized myocardial tissue were centrifuged for 15 min at 14000 g at 4°C. 50 µl of the supernatant were added to 75 µl of Tris-HCl (50 mM, pH 7.5) containing 10 mM $MnCl_2$. The mixture was activated by heating for 10 min at 56°C. Each sample was then incubated at 37°C for 1 h under three conditions: (1) with L-arginine (50 µl, 0.05 M, in Tris-HCl pH 9.7), (2) with only Tris-HCl (50 µl, pH 9.7) and (3) with L-arginine (50 µl, 0.05 M, in Tris-HCl pH 9.7) and 30 min pre-incubation with the arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH; 100 µM; Enzo Clinical Labs, Farmingdale, NY, USA). The reaction was stopped by adding 400 µl of an acid solution (H_2SO_4–H_3PO_4–H_2O= 1:3:7). 25 µl of a-isonitrosopropiophenone (9% in ethanol) was added to each sample, and the mixture was then heated at 100°C for 60 min. The samples were placed in the dark for 10 min and the urea concentration was determined at 550 nm using spectrophotometry. The urea inhibitable fraction was then calculated and used in statistical analyses.

3.3.3 Enzyme linked immunosorbent assay

In **papers III / IV** arginase I protein amount was determined in plasma. Therefore, plasma was separated (centrifuged within 60 min at 2000 g for 20 min) and stored at –80°C for further analysis. Arginase I was determined using a commercially available ELISA (BioVendor, Heidelberg, Germany) according to the manufacturer's instructions.

3.4 DETERMINATION OF NITRITE AND AMINO ACIDS

To determine the effect of the intravenous bolus of nor-NOHA on the levels of nitrite, arginine, citrulline, and ornithine a separate series of rat experiments was performed in **paper I**. Blood was collected in anaesthetized rats before and 15 min after iv bolus injection of nor-NOHA (100 mg/kg, n=6) or nor-NOHA (100 mg/kg) together with L-NMMA (10 mg/kg, n=5) or saline (n=6). The blood was centrifuged immediately at 1200 g for 10 min at 4°C and stored at -70°C until analysis. Nitrite was measured using a highly sensitive chemiluminescence method while arginine, citrulline, and ornithine were measured with an HPLC method representing an application of the method of Lindroth and Mopper (158). The samples were mixed with an equal amount (10 µl of an orthophthaldialdehyde/mercaptoethanol reagent by a CMA/200 autoinjector (CMA Microdialysis AB, Sweden). After 60 s reaction at 8°C, 15 µl was injected on the HPLC column (60 x 4 mm Nucleosil 100 C18, 5 µm, Knauer GmbH, Germany). The elution was achieved with a Na acetate buffer (0.03 M), methanol 2.5% v/v, and tetrahydrofuran 2% v/v. A gradient of methanol 0–60% was established between 4 and 28 min. The column was then regenerated with the initial buffer for 3 min. The detection was performed with a fluorescence detector (CMA/280), with excitation and emission band around 350 and 495 nm, respectively. The quantification was done with an integration program (EZ Chrom Chromatography Data system, Scientific software Inc., CA, USA) comparing peak heights. In **paper II**, plasma nitrite was measured with the same method.

3.5 DETERMINATION OF MICROVASCULAR FUNCTION

There are a variety of different methods to evaluate the microcirculation in the clinical settings as well as in research. In general, it is important to distinguish between methods directly visualizing the smallest vessels with methods without visualization. In the clinical setting the most frequently used method is to evaluate indirect signs of malperfusion of the microcirculation. This includes among others malperfused skin, the capillary refill, impaired renal output but most importantly also increased serum lactate.

Measurements of the microcirculation without visualization include Laser-Doppler flow measurements that allow quantification of flow after the signal has been reflected by erythrocytes without distinguishing between arterioles, capillaries and venules. Currently, only measurements at the skin are possible (159). To evaluate the balance between oxygen transport and oxygen consumption it is furthermore possible to measure oxygen concentration within a tissue of interest by special electrodes. A central limitation of this method is that oxygen concentration is also dependent on other parameters independently of the microcirculation (159). Carbon dioxide can also be measured with the same information and limitation. Microdialysis allows the analysis of substances present in the extracellular space including lactate. This method is not very reproducible and also easily to influence by comorbid factors and circumstances.

Direct visualization is possible by the use of microvideoscopic techniques. They emit light on the surface of different organs or tissues and clear the signal of reflecting light by the most surface tissue layers and use signals of the microcirculation beneath. Established is nailfold video capillaroscopy illuminating the border between the nail and the skin using a light microscopy and transparent oil (160). These techniques have further been developed resulting in Orthogonal Polarization Spectral Imaging and Sidestream Darkfield Imaging (SDF) (161). Polarization filters allow only reflecting light by deeper layers to pass and eliminate surface reflection. In SDF light emission is pulsed and the wave length of the emitted light has been optimized to allow light depolarization by red blood cells to focus on blood vessels (**Figure 8**) (77, 159). Using online or offline computer-aided analysis a number of different parameters characterizing the microcirculation is possible. First, the perfused vessels density (PVD, 10-100 µm) and the perfused capillary density (PCD, 10-20 µm) can be determined (77). Flow in these vessels is considered to be present if at least sluggish or continuous and absent if there is no perfusion or intermittent perfusion. This has been proven to determine microvascular flow in the smallest vessels with high prognostic relevance (74, 162, 163).

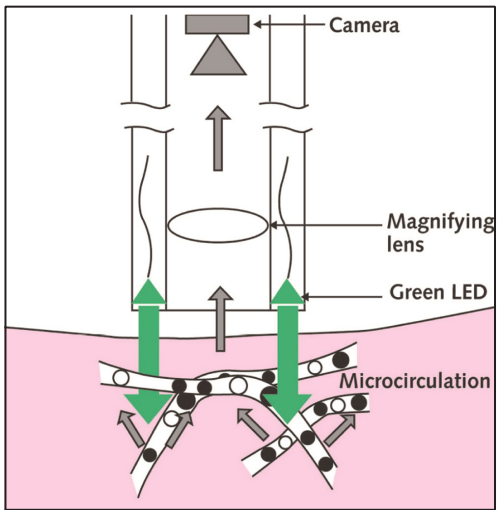

Figure 8: Sidestream dark-field (SDF) imaging. This imaging technique is an improved method of observing the human microcirculation at the bedside. SDF imaging consists of a light guide surrounded by green light-emitting diodes (wavelength 530 nm) whose light penetrates the tissue and illuminates the microcirculation from within. The light is absorbed by hemoglobin of the red blood cells and scattered by leukocytes. A magnifying lens projects the image onto a video camera. Placed on organ surfaces, SDF imaging provides crisp images of the red blood cells and leukocytes flowing through the microcirculation. Figure and legend reproduced from (164). Permission obtained from the publisher.

In **paper III and IV**, the sublingual microvascular network was studied as previously described using the SDF microcirculation camera (72, 73, 75, 78, 165-167). In summary, the camera emits green light that is absorbed by red blood cells within microvessels. In this way, red blood cells are used as the contrast agent to visualize sublingual blood flow in the microvessels. A handheld device (Microscan, Microvision Medical, Amsterdam, The Netherlands) was gently applied on the sublingual mucosa in an area approximately 2 to 4 cm from the tip of the tongue to obtain two-dimensional video images of sublingual microcirculatory blood flow. Saliva was gently removed. At least three records in each step were taken with a length of at least 30 seconds without any movement artefacts and saliva and free from repetitive structures with a mobile computer connected to the microscopic camera. The sequences were later analysed by a blinded investigator using special software (Automated Vascular Analysis, AVA 3.0, Microvision Medical). Capillaries were defined as microvessels with a diameter ≤20 mm. PCD was calculated by measuring total length of perfused capillaries divided by the image area. Capillaries were regarded as perfused if they had either of the following flow classifications obtained by visual inspection:

sluggish, continuous, or hyperdynamic. Unperfused capillaries (i.e. capillaries with absent or intermittent perfusion) were judged not to take part of the circulation. All used electronic devices were tested by the Department for Medical Instruments (Friedrich-Schiller-University) for bedside use.

To investigate the pathophysiological relevance of arginase and the effect of arginase inhibition on microvascular function eight patients with HF NYHA class III or IV were recruited in **study III**. Baseline microvascular measurements were recorded on the right and left side of the sublingual frenulum at the base of the tongue, the commonly used region of interest in studies of the microcirculation. Then, two previously prepared cotton tissue balls were placed in this area. One was prepared with 10 mg of nor-NOHA dissolved in 3 ml of saline. The other one was prepared with 10 mg nor-NOHA together with 10 mg of L-NMMA in 3ml saline. Measurements of microvascular flow in the two areas were performed after five minutes of incubation of the substances (nor-NOHA alone right side of the frenulum, nor-NOHA together with L-NMMA on the left side). The same protocol was used in **study IV** with eight patients who were successfully resuscitated and were not treated with therapeutic hypothermia within 48 h following resuscitation.

3.6 STATISTICAL ANALYSES

In **paper I**, comparisons within the groups over time were done with one-way ANOVA followed by Bonferroni's post hoc test. For comparisons between groups over time and at single time points, one-way ANOVA followed by Dunnett's test was used. Groups were compared using one-way ANOVA together with Bonferroni's post hoc test. Nitrite, amino acid, and protein levels were compared with paired and unpaired t-test. A value of $p<0.05$ was considered statistically significant. In **paper II**, Mann-Whitney U test and Kruskal Wallis followed by Dunns test were used.

In **paper III and IV**, continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test. Non-normally distributed continuous variables groups were compared by the Mann-Whitney U test, while normally distributed were analysed by t test (two sided, including Levene's test for equality of variances). Groups were furthermore compared using Fisher's exact test. Correlation analysis was performed using Spearman's test. Statistical significance was assumed if a null hypothesis could be rejected at $p\leq0.05$. In **paper III**, a multiple linear regression analysis was performed. Age, body-mass index, left ventricular ejection fraction, NYHA classification, creatinine and pulmonary artery pressure were included in the model. Furthermore the model was controlled for the presence of coronary artery disease, arterial hypertension, diabetes mellitus and hypercholesterolaemia. Stepwise multiple regression analysis was performed to further analyse the most significant interrelation to the variation of arginase levels. First, all variables were entered into the model simultaneously. In each following step, the variable having the least p value was excluded from the model. Finally, all variables with $p<0.05$ remained in the model.

In all papers, statistical analyses were performed with IBM SPSS statistics, version 19.0 (IBM Inc.) or GraphPad Prism (Version 4.0/5.0; GraphPad Software Inc., La Jolla, CA, USA).

4 RESULTS

4.1 ARGINASE INHIBITION PROTECTS FROM MYOCARDIAL ISCHAEMIA AND REPERFUSION INJURY (PAPERS I AND II)

In **paper I**, MAP, HR, and rate pressure product (RPP) before and after drug administration, during ischaemia, and during reperfusion in all experimental groups are presented in appendix 1. No significant difference was evident in MAP, HR, or RPP between groups before treatment. In comparison with the control group, MAP during the entire I/R protocol was significantly higher in the cPTIO group ($p<0.05$). A significant transient increase in MAP ($p<0.01$) was observed following administration of nor-NOHA together with L-NMMA. HR was lower in animals belonging to the groups given nor-NOHA and cPTIO, cPTIO alone, and nor-NOHA with L-NMMA than in those of the control group (**Appendix 1**).

Of all pigs included in **paper II**, a total number of eight pigs developed ventricular fibrillation during 15–30 min of ischaemia. Six of them randomized to vehicle (n= 3), nor-NOHA (n =2) and the combination of nor-NOHA and L-NMMA (n =1) were successfully converted to sinus rhythm by defibrillation with 20–30 Joule. The remaining two pigs (one pig randomized to vehicle and one to nor-NOHA group) developed irreversible ventricular fibrillation before drug administration and were therefore excluded from the study. The hemodynamic changes during the experiments are presented in **Appendix 2**. There were no statistically significant differences in HR, MAP, or RPP or coronary blood flow between the groups.

Analysing area at risk and infarct size of the studies, it is important to note that there were no significant differences between study groups regarding area at risk within the rat or the pig studies. In **paper I**, the infarct size of rats in the control group was 79±4% of the area at risk (**Figure 9**). Iv administration of the arginase inhibitor nor-NOHA before the onset of ischaemia was accompanied by a significant reduction of infarct size to 39±7% ($p<0.001$). Administration of cPTIO or L-NMMA abolished the reduction in infarct size induced by arginase inhibition (**Figure 9**). Administration of cPTIO alone had no influence on infarct size.

In the pig experiments (**paper II**), the cardioprotective treatment was given as ic infusions starting before the onset of reperfusion. Infarct size was significantly reduced in the group given ic infusion of nor-NOHA in comparison with the vehicle group (**Figure 10**, 46±5% vs. 80±4% of the area at risk, $p<0.01$). Co-administration with the NOS inhibitor L-NMMA abolished the cardioprotection mediated by ic nor-NOHA. For control purposes, nor-NOHA was also administered as a systemic iv infusion at a dose similar to that given ic. Nor-NOHA did not influence infarct size when given iv, demonstrating that the cardioprotective effect of ic nor-NOHA was mediated by a local effect within the jeopardized myocardium.

Taken together, using two different animal models of I/R these data demonstrate that iv or ic administration of an arginase inhibitor effectively reduces infarct size. The cardioprotective effect of nor-NOHA is mediated via an effect dependent of NOS activity and NO formation.

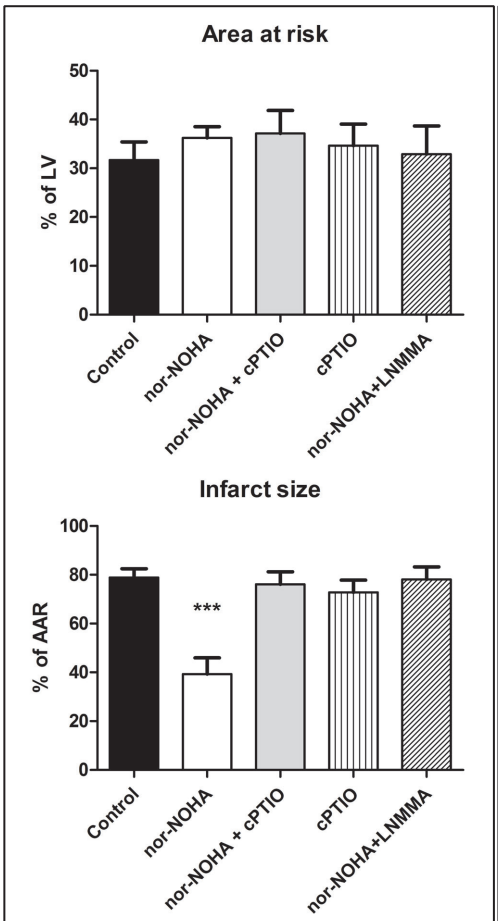

Figure 9: Upper panel: Area at risk (AAR) expressed as percentage of left ventricle (LV). Lower panel: Infarct size (IS) presented as percentage of AAR. The rats were given either saline, the arginase inhibitor nor-NOHA, nor-NOHA together with the NO scavenger cPTIO, nor-NOHA together with the NOS inhibitor L-NMMA, or cPTIO only. Infarct size was significantly lower in the nor-NOHA group in comparison with all other groups (***p<0.001). Data are presented as mean and SEM, n = 6–7.

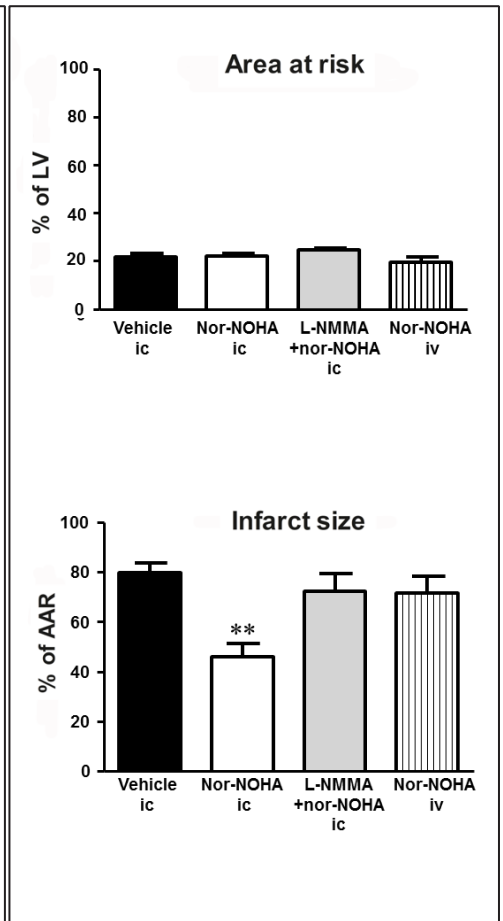

Figure 10: Area at risk (upper panel), infarct size (lower panel) of the groups given intracoronary (ic) vehicle, the arginase inhibitor nor-NOHA, the combination of the NOS-inhibitor L-NMMA and nor-NOHA, or intravenous (iv) administration of nor-NOHA. Data are presented as mean and SEM; n = 5–7. Significant difference from the vehicle group is indicated; **p<0.01. Figure and legend reproduced with modifications reproduced from (155). Permission obtained from the publisher.

4.2 MOLECULAR MECHANISM OF CARDIOPROTECTION

4.2.1 Myocardial arginase expression (papers I and II)

To understand the molecular mechanism of cardioprotection different analyses were performed. To reveal whether arginase protein levels change during I/R myocardial arginase expression was determined.

In **paper I**, the expression of arginase I was significantly higher in rat myocardium subjected to I/R compared to the non-ischaemic myocardium (**Figure 11**). Expression of arginase II was not detectable in ischaemic or non-ischaemic myocardium.

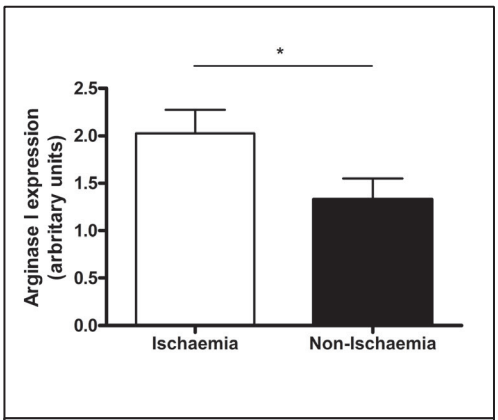

Figure 11: Quantification of arginase I expression in ischaemic-reperfused and non-ischaemic myocardium. Data are presented as mean and SEM, n=5. Significant difference between the groups is shown; *p<0.05. Figure and legend reproduced with modifications from (168). Permission obtained from the publisher.

In **paper II**, the successful separation of mitochondrial membrane fractions was confirmed by expression of cytochrome oxidase in the mitochondrial fraction and only weak or no expression in the cytosolic fraction (**Figure 12**). Cytosolic and mitochondrial fractions of non-ischaemic, ischaemic-reperfused and sham operated myocardium were analysed for arginase I and II expression. Expression of arginase I in the cytosol was similar in all groups. In contrast, arginase II was not expressed in the cytosolic fraction. Arginase II was clearly expressed in the mitochondrial fraction, but there were no significant differences between the ischaemic-reperfused, non-ischaemic or sham myocardium.

The expression analysis revealed higher myocardial arginase I levels in the rat following I/R but no change in arginase expression in the pig.

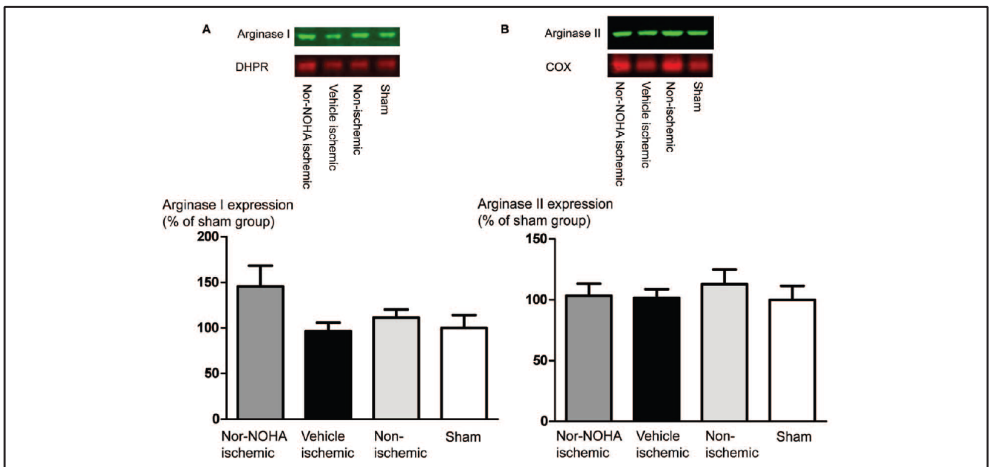

Figure 12: Representative blots (upper panel) and quantitative analysis of the expression (lower panel) of arginase I in the cytosolic fraction (A) and arginase II in the mitochondrial fraction (B) of myocardial samples. Expression of arginase I is normalized to that of the surface membrane L-type Ca^{2+} channel, dihydropyridine receptor (DHPR) and arginase II to that of cytochrome oxidase (COX) and presented in % of the mean expression in the sham group. Data are presented as mean and SEM, n=6. There were no significant differences between the groups. Figure and legend reproduced from (169). Permission obtained from the publisher.

4.2.2 Myocardial arginase activity (paper II)

Since total activity of the enzyme arginase is dependent on total arginase protein level and enzyme activity, myocardial arginase activity was determined. Since this method had to be established the methodology was not available in **paper I** yet.

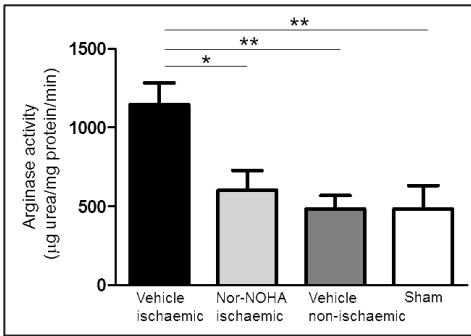

Figure 13: Arginase activity in myocardial samples from ischaemic and non-ischaemic myocardium of pigs subjected to ischaemia and reperfusion and myocardium from sham operated pigs. Mean and SEM, n = 6–7. Significant differences between the groups are shown; *p<0.05, **p<0.01. Figure and legend reproduced with modifications from (169). Permission obtained from the publisher.

In **paper II**, the activity of arginase was similar in the myocardium of sham operated pigs and in the non-ischaemic myocardium from pigs subjected to ischaemia-reperfusion (**Figure 13**). Arginase activity was 2-fold higher in ischaemic-reperfused myocardium than in non-ischaemic myocardium of pigs given vehicle and in comparison with the sham group ($p<0.01$). Nor-NOHA administration during early reperfusion significantly reduced arginase activity in the ischaemic-reperfused myocardium in comparison with the vehicle group ($p<0.05$).

Myocardial arginase activity was increased following I/R and treatment reduced arginase activity, confirming the achievement of the designated pharmacological effect.

4.2.3 Effect of arginase inhibition on amino acid levels (paper I)

In **paper I**, amino acid products of arginase and NOS were quantified as a measurement of arginase and NOS activities. To this end, the effect plasma and myocardial amino acid levels were determined in separate groups of rats that were not subjected to myocardial I/R given the arginase inhibitor nor-NOHA and saline, respectively. Administration of nor-NOHA significantly altered plasma amino acid levels indicating changed metabolic activities. Plasma citrulline (product of NOS) increased from 95.2 ± 9.7 to 126.6 ± 7.4 µM and ornithine (product of arginase) decreased from 63.8 ± 8.2 to 37.8 ± 1.8 µM ($p< 0.05$, **Figure 14**) 15 min after administration of nor-NOHA, whereas they remained unchanged in the saline group. In contrast, arginine levels remained unchanged in both groups. As a quantitative indicator of the consumption of arginine by NOS versus arginase, citrulline/ornithine ratio was used. This ratio increased markedly in animals receiving nor-NOHA in comparison with control animals. The citrulline/arginine ratio, reflecting NOS activity, was increased by nor-NOHA in comparison with saline-treated animals (**Figure 15**). To further reveal treatment effects within the heart myocardial amino acids levels were also determined. Ornithine levels were undetectable in all except three myocardial samples. In consequence, only the citrulline/arginine ratio was determined in myocardial tissue. This showed a higher ratio in animals treated with nor-NOHA ($p<0.05$) than in saline-treated animals.

Collectively, these observations suggest that arginase inhibition causes a change in arginine utilization from arginase to NOS resulting in increased citrulline and NO formation.

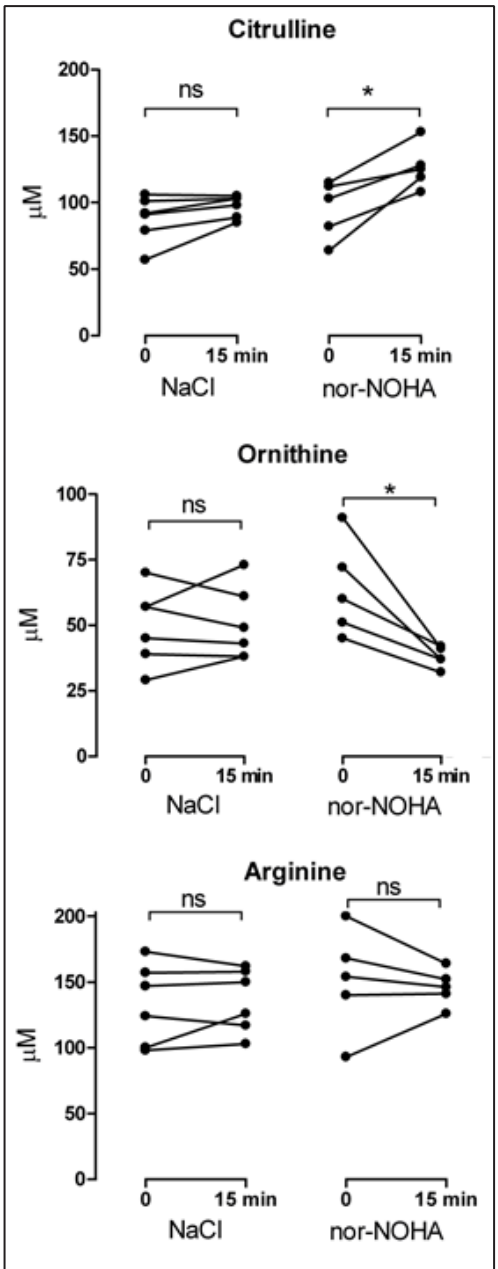

Figure 14: Plasma amino acid levels before and 15 min following administration of saline and nor-NOHA. Significant changes from baseline (time 0) are shown; *p<0.05; NS, not significant, (n=6). Figure and legend with modifications reproduced from (168). Permission obtained from the publisher.

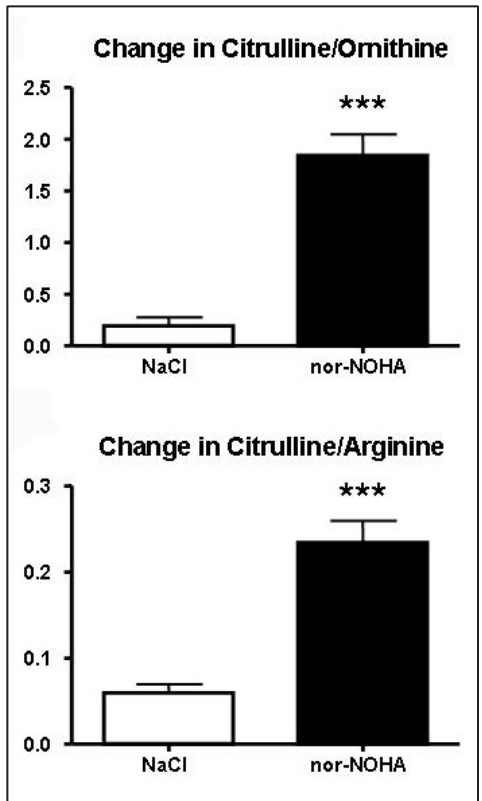

Figure 15: Change in plasma citrulline/ornithine and citrulline/arginine ratio from baseline to 15 min following administration of saline and nor-NOHA. Data are presented as mean and SEM, n=6. Significant difference from the control group are shown; ***p<0.001. Figure and legend with modifications reproduced from (168). Permission obtained from the publisher.

4.2.4 Plasma nitrite levels (papers I and II)

To further determine the effect of arginase inhibition on NO formation, the oxidation product nitrite was determined. In **paper I** plasma nitrite levels increased from an initial value of 304 ± 45 nM by 102 ± 30 nM ($p<0.05$) 15 min following administration of nor-NOHA but remained unchanged in the saline-treated group. In **paper II**, arterial plasma nitrite concentration did not change significantly following administration of nor-NOHA. Plasma nitrite was 680 ± 160 nM before the onset of ischaemia, 692 ± 116 nM 5 min after the onset of reperfusion (during nor-NOHA infusion) and 681 ± 119 nM at 60 min of reperfusion. Furthermore, arterial plasma nitrite was unchanged in the vehicle group.

The increased nitrite levels in the rat model support the notion of increased NO production following arginase inhibition. Unchanged systemic arterial nitrite levels in the pig suggest that the effect of ic arginase inhibition is attributed to an increased bioavailability of NO within the heart.

4.3 CHARACTERISTICS OF THE STUDY POPULATIONS

4.3.1 Heart failure populations (paper III)

The baseline characteristics of the patients included in **paper III** are summarized in **Table 1**. Of the 80 patients included, 55 were in NYHA class I/II and 25 in NYHA class III/IV. All patients were male. Patients with NYHA class III/IV were significantly older, had lower LVEF and higher creatinine and brain-natriuretic peptide levels. The majority of the patients had systolic impairment while only a minority had isolated diastolic dysfunction. There were no differences in comorbidities and medications between NYHA classes I/II and NYHA classes III/IV.

To evaluate the influence topical administration on the sublingual mucosa in patients with HF another patient cohort with decompensated HF was recruited in **paper III**. The characteristics of the patients undergoing the experimental protocol are shown in **Table 2**. All patients suffered from severe HF with NYHA class III/IV.

4.3.2 Subjects undergoing global hypoxia (paper IV)

Clinical characteristics of the 14 volunteers (12 male, 2 female) undergoing global hypoxia in **paper IV** are presented in **Table 3**.
All subjects were free of symptoms of acute mountain sickness during the experimental period. A 100% compliance rate was obtained for the study.

4.3.3 Patients following cardiopulmonary resuscitation (paper IV)

Characteristics of the further study group of **paper IV** are summarized in **Table 4**. The majority of the patients were male, resuscitated within a medical institution, treated with therapeutic hypothermia and did not survive day 28.

Table 1: Baseline characteristics of the study population			
	NYHA I / II	NYHA III / IV	Comparison
N	55	25	
Demographic parameters, blood pressure and heart rate			
Age (years)	58 ± 12	67 ± 11	p=0.002
Height (cm)	177 ± 0.7	174 ± 0.9	n.s.
Weight (kg)	89 ± 15	96 ± 15	n.s.
Body mass index (kg/m²)	28.8 ± 4.4	31.0 ± 4.3	n.s.
Heart rate	70 ± 14	68 ± 9	n.s.
Systolic blood pressure (mmHg)	138	132	n.s.
Diastolic blood pressure (mmHg)	87	84	n.s.
Comorbidities (%)			
Arterial hypertension	75	83	n.s.
Diabetes	25	41	n.s.
Coronary artery disease	38	60	n.s.
Medication (%)			
Betablocker	96	100	n.s.
ACE-Inhibitor	73	76	n.s.
Echocardiographic parameters			
Ejection fraction (%)	41 ± 10	31 ± 12	p=0.001
Diastolic dysfunction (%)	62	64	n.s.
Isolated diastolic dysfunction (%)	5	8	n.s.
Laboratory values			
Haematocrit (%)	43.8 ± 3.8	44.9 ± 6.2	n.s.
Creatinine (mmol/l)	102 ± 43	137 ± 45	p=0.001
Brain natriuretic peptide (pg/ml)	361 ± 690	990 ± 680	p=0.022
Aspartate transaminase (µmol/l*s)	0.54 ± 0.21	0.60 ± 0.31	n.s.
Alanine transaminase (µmol/l*s)	0.76 ± 0.26	0.62 ± 0.31	n.s.

Table 2: Characteristics of the patient population included in the arginase inhibitor study protocol. All patients were in NYHA class III or IV.	
N	8
Demographic parameters, blood pressure and heart rate	
Age (years)	73 ± 13
Sex (male / female)	6 / 2
Heart rate	77 ± 14
Systolic blood pressure (mmHg)	122 ± 22
Diastolic blood pressure (mmHg)	74 ± 19
Comorbidities (%)	
Arterial hypertension	87
Diabetes	25
Coronary artery disease	63
Medication (%)	
Betablocker	100
ACE-Inhibitor	100
Echocardiographic parameters	
Ejection fraction (%)	36 ± 16
Diastolic dysfunction (%)	63
Laboratory values	
Haematocrit (%)	37.1 ± 5.3
Creatinine (mmol/l)	126 ± 54
Brain natriuretic peptide (pg/ml)	2007 ± 1174

Table 3: Clinical characteristics of individuals included in the global hypoxia study. Values are mean ± standard deviation, n=14, *p<0.05 baseline vs. hypoxia. These values have been published previously (170).

	Baseline	Hypoxia
Age (y)	30±6	
Height (cm)	178±8	
Weight (kg)	75±13	
Heart rate (beats/min)	73±9	87±11*
Systolic blood pressure (mmHg)	129±10	119±16*
Diastolic blood pressure (mmHg)	82±6	73±10*
O_2 saturation (%)	97±2	78±3*
Breathing frequence (1/min)	13±2	21±6*
Smoking status (n/%)	2/14	

Table 4: Characteristics of the patient population following successful cardiopulmonary resuscitation.

N	31
Demographic parameters	
Age (years)	64 ± 15
Sex (male/female)	22/9
Underlying disorder n (%)	
Acute myocardial infarction (AMI)	9 (29)
Coronary artery disease other than AMI	5 (16)
Pulmonary disease	5 (16)
Gastroenterologic disorder	5 (16)
Other	7 (23)
Conditions related to resuscitation	
Out of hospital resuscitation (%)	45.2
Duration of resuscitation until ROSC (min)	24.3 ± 31.6
Delay until start of resuscitation (min)	6.2 ± 8.5
Witnessed cardiac arrest (%)	66
Primary rhythm:	
Ventricular fibrillation, n (%)	10 (32)
Asystolia, n (%)	12 (39)
Other, n (%)	9 (29)
Treatment	
Therapeutic hypothermia n (%)	19 (61)
Coma duration (days)	9 ± 9
Duration of controlled ventilation (days)	10 ± 10
Glasgow Outcome Scale after 28 days	
Dead, n (%)	17 (55)
Vegatative state, n (%)	3 (10)
Severe disability, n (%)	1 (3)
Moderate disability, n (%)	5 (16)
Good recovery, n (%)	5 (16)

A cohort that underwent investigation of microvascular function by topical application with arginase and NOS inhibitors on the sublingual mucosa following resuscitation was recruited in **paper IV**. The characteristics of these patients are shown in **Table 5**.

Table 5: Characteristics of resuscitated patient in the interventional protocol.	
N	8
Demographic parameters	
Age (years)	68 ± 16
Sex (male/female)	6/2
Underlying disorder n (%)	
Acute myocardial infarction (AMI)	3 (38)
Coronary artery disease other than AMI	1 (13)
Pulmonary disease	2 (25)
Gastroenterologic disorder	0 (0)
Other	2 (25)
Conditions related to resuscitation	
Out of hospital resuscitation (%)	63
Duration of resuscitation (min)	12.6 ± 12.0
Delay until start of resuscitation (min)	3.5 ± 4.2
Witnessed cardiac arrest (%)	50
Primary rhythm:	
Ventricular fibrillation, n (%)	1 (13)
Asystolia, n (%)	5 (62)
Other, n (%)	2 (25)

4.3.4 Control subjects

To compare arginase I levels with healthy controls, six healthy people from the personnel of the Cardiology department, University of Jena served as healthy controls in **paper III**. All subjects were male, healthy, did not take any drugs and they were 32 ± 8 years old. In parallel, 21 healthy subjects from the personnel of the Cardiology department, University of Jena served as controls in **paper IV** (male: 20; female: 4, mean age 29 ± 5 years).

4.4 INCREASED ARGINASE I IN HEART FAILURE PATIENTS (PAPER III)

Different enzymes and compounds that actively influence NO production have been described in the past. The aim of the study was to evaluate whether arginase is increased in HF patients. Circulating arginase I levels were significantly higher in patients with HF compared to controls ($p<0.001$, **Figure 16, paper III**). Subclassification according to NYHA class

revealed that NYHA III/IV patients had significantly higher arginase I levels than patients with NYHA class I/II ($p<0.01$). There were no significant correlations between circulating arginase I levels and blood pressure, age or BMI. Furthermore, the presence of CVD had no influence on arginase I levels. In multiple linear regression analysis, which was controlled for the presence of CVD, arterial hypertension, diabetes mellitus and hypercholesterolaemia, age, BMI, left ventricular ejection fraction, NYHA class, creatinine and pulmonary artery pressure, only NYHA status remained in the final model ($p<0.001$, standardized beta: 0.498). Arginase I levels did not differ between patients with normal hepatic transaminases (30% of the patients) and those with elevated transaminases (70%).

These findings reveal for the first time increased levels of arginase I in patients with HF.

4.5 INCREASED ARGINASE I FOLLOWING GLOBAL HYPOXIA (PAPER IV)

Until now, no data was available regarding the effect of global hypoxia on arginase in humans. Therefore, circulating arginase I was determined in experimental human global hypoxia.

Exposure of healthy subjects to hypoxia corresponding to an altitude of 5500 m for 6 h induced a significant increase in plasma arginase I levels (**Figure 17**). During exposure to hypoxia heart rate, systolic blood pressure and breathing frequency increased whereas diastolic blood pressure and oxygen saturation decreased (**Table 3**).

To the best of our knowledge this study reports for the first time increased levels of arginase I following global hypoxia in humans.

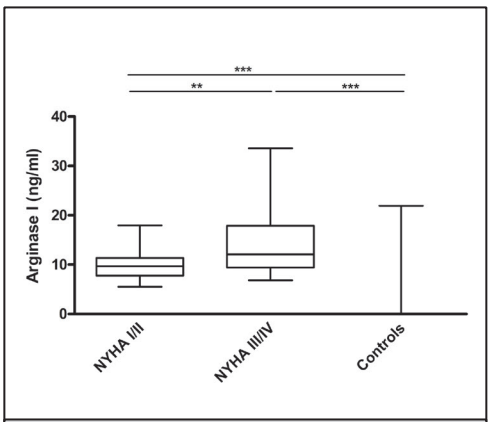

Figure 16: Circulating levels of arginase I in patients with heart failure in New York Heart Association (NYHA) functional class I/II and III/IV and in healthy controls. Significant differences between groups are shown; **p<0.01; ***p<0.001, n=55 for NYHA I/II, n=25 for NYHA III/IV, n=6 for controls.

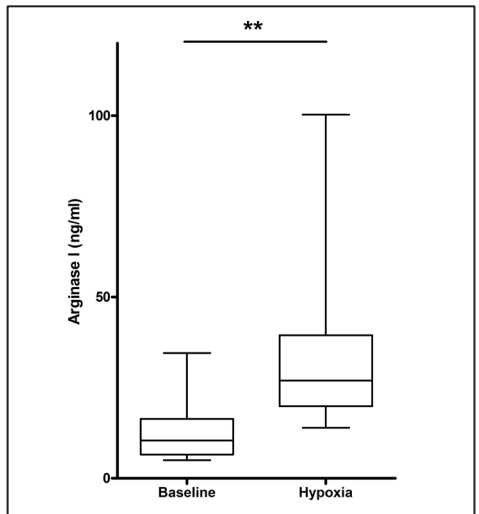

Figure 17: Circulating levels of arginase I subjects before and after completion of the global hypoxia protocol. Significant differences between groups are shown; **p<0.01, n=14.

4.6 INCREASED ARGINASE I IN PATIENTS FOLLOWING CARDIO-PULMONARY RESUSCITATION (PAPER IV)

Based on the data described above the aim was to determine arginase levels in a patient cohort that had undergone global hypoxia in the clinical setting of resuscitation.

Arginase I levels were 69.1 ± 83.3 ng/ml on day 1 and 44.2 ± 65.6 ng/ml on day 3 following CPR. These levels were significantly higher compared to the control group (**Figure 18**). However, arginase I levels did not differ between patients with good versus bad neurological outcome or between patients treated with versus without therapeutic hypothermia. Serum levels of neuronal specific enolase (NSE) on day 1 were 26.9 ± 15.2 µg/l and 68.4 ± 63.1 µg/l on day 3, respectively. NSE and arginase I correlated significantly with each other on day 1 ($p=0.026$, $r=0.40$) but not on day 3. In addition, circulating arginase I levels on day 3 correlated significantly with the duration until ROSC ($p=0.002$; $r=0.58$).

These data demonstrate that plasma arginase I is increased in patients with global hypoxia following CPR.

4.7 ARGINASE AND MICROVASCULAR FLOW IN HEART FAILURE (PAPER III)

To further characterize the functional relevance of increased arginase levels an arginase inhibitor was administered by topical application to patients with HF in **paper III** as described above. The procedures were well tolerated and no adverse effects were detected. Furthermore, topical administration did not lead to significant changes in blood pressure or heart rate. Following application of nor-NOHA, PCD increased significantly (**Figure 19**). This effect was abolished when nor-NOHA was given together with L-NMMA. On the other hand, nor-NOHA did not induce any change in the proportion of perfused vessels.

These findings indicate that topical arginase inhibition improves microvascular perfusion by a NOS dependent mechanism in patients with HF.

4.8 ARGINASE AND MICROVASCULAR FLOW FOLLOWING CARDIO-PULMONARY RESUSCITATION (PAPER IV)

To study the functional relevance of increased arginase levels, the effect of arginase inhibition on microvascular flow was determined in patients following CPR. Topical application of nor-NOHA increased PCD from 8.7 ± 4.2 mm/mm² to 14.1 ± 1.0 mm/mm² and PVD from 9.5 ± 4.2 mm/mm² to 15.9 ± 1.6 mm/mm² (both $p<0.001$). Coincubation with the NOS inhibitor L-NMMA abolished the effect of arginase inhibition (**Figure 20**).

In parallel with the findings in patients with HF, topical arginase inhibition also improves microvascular perfusion by a NOS dependent mechanism in patients following CPR.

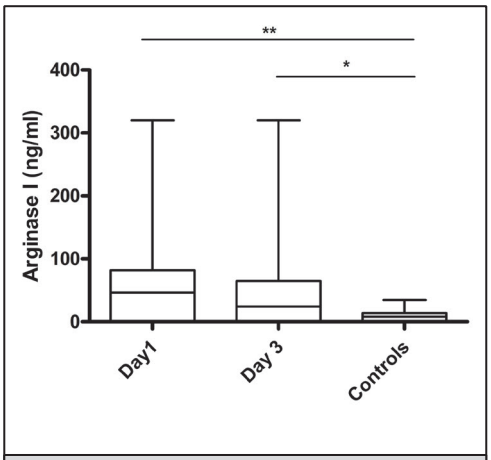

Figure 18: Plasma levels of arginase I on days 1 and 3 following successful cardiopulmonary resuscitation (n = 31) and in a group of healthy controls (n = 21). Significant differences between groups are shown; *p<0.05; **p<0.01.

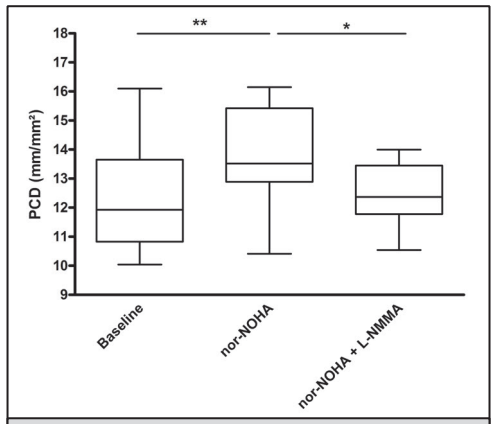

Figure 19: Perfused capillary density (PCD) in the sublingual mucosa in patients with severe heart failure at baseline and after administration of the arginase inhibitor nor-NOHA alone or together with the nitric oxide synthase inhibitor L-NMMA. Significant differences between groups are shown; **p<0.01, *p<0.05, n=8.

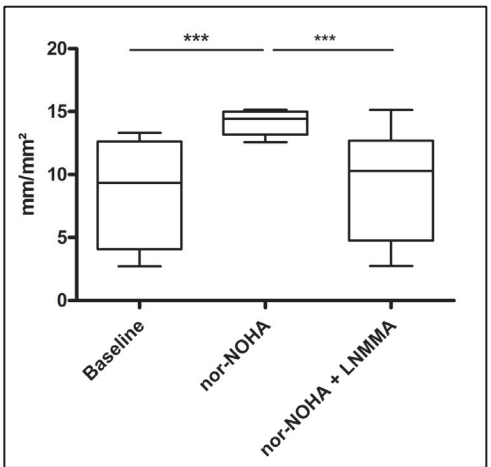

Figure 20: Quantification of the microcirculation by measuring perfused capillary density at baseline and after application of the arginase inhibitor nor-NOHA alone and together with the NOS inhibitor L-NMMA in patients following CPR. Significant differences between groups are shown; ***p<0.001, n=8. Figure reproduced from (171). Permission obtained from the publisher.

5 GENERAL DISCUSSION

In summary, **papers I-IV** indicate that arginase represents a promising therapeutic target due to its important regulation of cardiovascular function in different clinically relevant scenarios such as MI, HF and CPR. In two different animal models of myocardial I/R injury (**paper I and II**) arginase inhibition mediates cardioprotection with substantial reduction in infarct size. Arginase levels are increased in patients with HF and following CPR and arginase inhibition improves microvascular perfusion in these patient groups (**paper III and IV**).

5.1 ARGINASE INHIBITION AND MYOCARDIAL ISCHAEMIA AND REPERFUSION INJURY

It is well established that NO plays a central role in regulation of vascular function and in particular during I/R. Furthermore, it has been documented that reduced bioavailability of NO is associated with undesired effects such as leukocyte recruitment, increased oxidative stress and tissue injury (172). In consequence, restoration of NO bioavailability by supplying of the substrate L-arginine or NO donors has been shown to protect from I/R injury in various experimental models, possibly suggesting that the limited pool of L-arginine might be one of the limitations for the function of eNOS. This led to the idea that arginase activity is of importance for the development of I/R injury. Before we applied this treatment mechanism in myocardial I/R, Jeyabalan et al. demonstrated a protective effect of arginase inhibition in hepatic I/R injury (150). **Paper I** clearly shows that blockade of arginase profoundly protects from I/R injury as demonstrated by the 50% reduction in infarct size following administration of nor-NOHA. The additional groups studied convincingly revealed the NO dependence of the treatment effect. Of note, nor-NOHA did not significantly affect blood pressure, HR or RPP in comparison with the control group indicating that the protective effect was unrelated to changes in myocardial oxygen demand. These data present a novel strategy to increase bioavailability of NO which results in protection against I/R injury. These results have subsequently been confirmed in additional studies using nor-NOHA (173, 174) or structurally different arginase inhibitors (175).

To further underline the treatment effect of arginase inhibition on the relative activity of the two enzymes (NOS and arginase) the ratio between amino acids (citrulline and ornithine) that are produced by the two enzymes were studied. Most strikingly, it could be shown that the citrulline/ornithine ratio increased following arginase inhibition without any change in arginine concentrations. This indicates an increase in NOS activity and a shift of arginine utilization from the arginase to the NOS pathway. This is further supported by the increases in absolute citrulline levels and plasma levels of the NO metabolite nitrite. Although these experiments were performed in a group of animals not subjected to myocardial I/R, the findings support increased NOS activity following arginase inhibition.

Considering all possibilities regarding the mode of action that contributes to the protection following arginase inhibition in I/R, the increase in extra-cardiac NO generation may contribute to cardioprotection via endocrine-like effects. Accumulating evidence suggests

that the supposedly inert anion nitrite is a stable circulating reservoir of NO (176-178). Nitrite is reduced to NO in blood and tissues, especially accelerated under hypoxic conditions. In this context it is important to note that a cardioprotective effect of low dose nitrite administration in I/R injury models seems comparable to that of arginase inhibition (179). Therefore, the increase of plasma nitrite by 35% following arginase inhibition might contribute to the cardioprotective effects of nor-NOHA in addition to its effects on NO bioavailability in the heart.

Therefore, **paper II** sheds additional light on the cardioprotection mediated by arginase inhibition. This study not only demonstrates a significant cardioprotective effect of an arginase inhibitor administered shortly before reperfusion in a clinically relevant large animal model, it also localizes the treatment effect to the jeopardized myocardium. The different groups in the pig study confirmed the treatment mechanism of arginase inhibition in myocardial I/R and the involvement of NO as the underlying mechanism of cardioprotection. In addition, the arginase inhibitor was only effective when given by local ic infusion while the same dose of nor-NOHA given iv did not confer cardioprotection. This observation excludes an endocrine-like systemic effect of arginase inhibition. The protection seems to be related to a change of the activity of enzymatic pathways within the jeopardized myocardium. This is supported by the unchanged arterial nitrite levels following arginase inhibition in the pig model.

Further key aspects of **paper I and II** were the detailed evaluation of arginase expression and protein activity. In the rat study, analysis of protein expression revealed that arginase I was upregulated in the ischaemic myocardium, whereas arginase II was undetectable. In contrast, there were no changes in arginase expression in the pig study. However, a clear increase in arginase activity was demonstrated in the ischemic myocardium of the pig, suggesting upregulation of enzyme activity. A previous study demonstrated that arginase I is upregulated in coronary artery vascular smooth muscle cells and endothelial cells following I/R (127). In contrast, myocardial arginase I expression was not changed after seven weeks of chronic ischaemia (180). However, increased arginase activity and/or increased expression has been documented in several subsequent studies of myocardial I/R (173, 174, 181, 182). Although increased expression of arginase II induced by hypoxia has been documented in human pulmonary artery smooth muscle cells (183), its role in myocardial I/R remains to be established. However, arginase I expression was identified as the strongest and fastest transcriptional adaptation during myocardial I/R (184). The mechanisms leading to this effect have been recently reviewed by Schlüter et al (185): First, hypoxia and reoxygenation leads to cardiomyocytes damages by creating initially excessive calcium load during ischaemia. Reoxygenation is associated with energy generation that allows strong contraction subsequently disrupting the sarcolemmal membrane. This leads to a release of intracellular material into the extracellular compartment. In addition to this, other proteins released into the extracellular space might lead to subsequent activation of arginase expression via TNF-α (186). This is further supported by the lack of induction of arginase I in TNF-$\alpha^{-/-}$ mice following I/R (128). TNF-α has been understood to trigger arginase expression via activation of the transcription factor AP-1. It has been documented that hypoxia directly recruits c-jun to AP-1 binding sites of the arginase I promoter (187). Subsequently, c-jun binds together with activating transcription factor-2 at the AP-1 site, which leads to the initiation of the transactivation (188). In this context it is important to note that (myocardial) hypoxia induces assembly and activation of AP-1 within several minutes. This increase in

arginase activity might theoretically be an advantage during ischaemia by reducing oxygen-demanding NO production, but mediates deleterious effects as soon as reperfusion starts. This opens an attractive therapeutic window of arginase inhibition during early reperfusion as shown in the present pig study. This has recently been translated into the clinical setting. In a first study using arginase inhibition in patients with CAD Kövamees and coworkers (189) investigated if arginase inhibition protects from endothelial dysfunction induced by I/R. Endothelium-dependent vasodilatation was assessed before and after 20 min of I/R in the arm during intra-arterial infusion of nor-NOHA or saline. The authors were able to show that I/R decreased endothelial function during saline administration. However, nor-NOHA prevented the decrease in endothelial function. In conclusion, a single brief administration of intra-arterial arginase inhibition was well tolerated in patients suggesting a clinical trial with myocardial I/R is warranted.

In the light of our studies and the papers published in the field so far, it is not possible to determine which of the two arginase isoforms is of functional importance during myocardial I/R. Currently, it seems more likely that arginase I is a key player in this context. A major limitation of research in this field is that no isoform specific arginase inhibitors are available. Another limitation is that knockdown of arginase I in genetic animal models results in a lethal model due to its central relevance of arginase I in ammonium detoxification.

Another aspect is the cellular source of arginase during I/R. It is attractive to speculate that endothelial arginase is of importance by regulating endothelial NO production. Inhibition of arginase results in increased endothelium-derived NO that mediates the cardioprotective effects (174). It was recently suggested that arginase in red blood cells plays an important role by regulating NO export from these cells. This function seems to be of special relevance during I/R (97). Thus, arginase inhibition mediated cardioprotective effects in the isolated rat and mouse heart only in the presence of red blood cells, and this effect was completely dependent on the presence of eNOS in red blood cells and export of NO. This observation demonstrates a novel interesting cellular source of the arginase-NO pathway in myocardial I/R. It remains to be clarified, however, to which degree red blood cells arginase modulates I/R injury in the in vivo situation.

In conclusion, inhibition of arginase reduces myocardial infarct size in two different animal models of myocardial I/R. These findings represent an important and novel mechanism in cardiac I/R injury in which inhibition of arginase activity increases the bioavailability of NO by shifting utilization of the substrate arginine from arginase to NOS.

5.2 ARGINASE INHIBITION AND MICROVASCULAR PERFUSION

In **paper III and IV** three different scenarios with clinical relevance were evaluated regarding the circulating level of arginase I. These included patients with HF, often associated with tissue hypoxia, a model of global hypoxia in healthy volunteers and a patient cohort after successful CPR. In all three settings increased levels of arginase I were demonstrated. Of further importance, in patients with HF and in resuscitated patients mucosal application of nor-NOHA improved microvascular function via a NO dependent mechanism demonstrating a functional role of the elevated arginase.

In **paper III**, we were able to show for the first time that plasma arginase I levels are elevated in patients with HF, especially among those with severe HF. As outlined above, increased arginase levels or increased arginase activity has been documented previously in different diseases like hypertension, atherosclerosis and diabetes (99). Recently Toya and coworkers (190) published an excellent study about the pathophysiological role of arginase in heart failure. In a model using doxorubicin-induced cardiomyopathy in mice, protein expression and activity of arginase in the lungs, the aorta and liver were increased. Further analysis revealed that administration of an arginase inhibitor completely reversed doxorubicin-induced decrease in the ejection fraction. In addition, arginase inhibition reversibly lowered systolic blood pressure and recovered doxorubicin-induced decline in NO concentration in serum, lungs, and aorta (190). These findings are in line with our findings confirming increased arginase translating into functional impairment in HF. The exact mechanism leading to this increase remains to be determined. One option is general tissue hypoxia which is present at a low level in HF. Another option is that increased arginase I levels are related to liver congestion due to its high expression in this organ. However, in the current study, arginase I levels were not associated with elevated liver enzymes. However, no final conclusions can be made from the current study regarding this issue.

Clear evidence is available that endothelial dysfunction is present in patients with HF (36, 191). As described by others (37) this has been linked to increased ADMA levels, limiting the bioavailability of NO subsequently leading to macro- and microvascular dysfunction. Therefore, increasing the bioavailability of NO seems reasonable to improve microvascular perfusion in HF patients. Of central importance and proof-of-principle are the studies performed by den Uil and coworkers. They found improved microcirculation by NO donors in a dose-dependent manner in patients with cardiogenic shock and acute decompensated heart failure (74, 79, 192). The authors demonstrated that nitroglycerin dose-dependently decreased mean arterial pressure and cardiac filling pressures and increased cardiac index. In addition, nitroglycerin increased sublingual PCD at low doses before changes in systemic hemodynamics were evident. In the present study it was demonstrated that administration of an arginase inhibitor improved microvascular function via a mechanism related to NO production from NOS. This illustrates that increased arginase is of pathophysiological importance for the impaired microvascular function by attenuating NO bioavailability in these patients. The mechanism of improved microcirculation seems to be related to a recruitment of vessels taking part in perfusion since the density of perfused vessel increases. Which of the microcirculatory parameters that has the highest potential to predict improved survival needs to be determined since conflicting data exits (74, 193, 194).

In **paper IV**, we were able to demonstrate that global hypoxia leads to an increase in systemic arginase I levels in healthy volunteers and in patients following CPR. It is well documented that patients after CPR are characterized by impaired microcirculatory perfusion. Although the understanding of the role of microcirculation in critically ill patients has grown significantly in the past two decades, the role of the microcirculation after CA is not extensively studied. Key publications by Donadello et al. and Omar et al. describing that microcirculatory dysfunction appears early in these patients and that a better microcirculatory function may be associated with improved neurological outcome (195, 196). Of additional value is the study by van Genderen et al. (197) showing a decreased microvascular flow in patients subjected to therapeutic hypothermia, possibly due to vasoconstriction and decreased metabolism. It

is well documented that an impaired macrocirculation with low mean arterial pressure in the critically ill patients subsequently leads to impaired microcirculation (198). Still, the complex interaction between the macrocirculation and the microcirculation is not fully understood, but adequate perfusion pressure seems to be a key requirement to achieve sufficient microvascular perfusion. In contrast, optimal systemic hemodynamics does not necessarily result in adequate microcirculation. Although no control groups of healthy controls were included in **paper III/IV**, microcirculatory parameters were abnormal in the patient cohorts despite the hemodynamic stability of these patients. This confirms several earlier published studies that investigated patients following resuscitation, septic shock, cardiogenic shock and HF with optimized systemic hemodynamics (73, 75, 196, 199, 200). It is important to note that the pathophysiology of impaired microvascular perfusion in post-cardiac arrest patients remains to be determined in detail. Different possibilities include tissue I/R injury and associated inflammatory activation and decreased bioavailability of NO (172), leukocyte and platelet activation and activated coagulation cascade (201). We were able to show that circulating arginase I is increased in human global hypoxia and that this seems to contribute to microvascular dysfunction. As described above, it is well documented that hypoxia activates arginase activity (185, 202). The functional relevance of elevated arginase for the regulation of microvascular flow was confirmed in a subgroup of resuscitated patients. Sublingual incubation of nor-NOHA significantly increased microvascular function as revealed by the increase in perfused capillary and vessel density. Of note, this was prevented by the NOS inhibitor L-NMMA revealing the involvement of NO formation. Although the prognosis after successful CPR is dependent on the management of different organ complications, one of the most important determinants is neurologic outcome. The influence on cerebral microcirculation remains to be determined but an interesting hypothesis built on the present observation is the demonstration in **paper IV** that neuronal specific enolase (as measure of neurologic damage) and arginase I concentrations correlated significantly to each other on day 1.

In conclusion, we showed that clinically relevant HF and global hypoxia leads to increased plasma levels of arginase I. Similar observations were made in patients that underwent CPR. Impaired microcirculatory perfusion in patients with decompensated HF and following CPR is improved following topical arginase inhibition by a NO dependent mechanism. Inhibition of arginase is a promising potential treatment target to ameliorate microcirculatory disorders in critically ill patients. Further (clinical) studies are needed to determine treatment protocols, mode of application and timing of treatment in different clinical scenarios.

5.3 LIMITATIONS

There are certain limitations associated with the studies. In all functional studies using arginase inhibition it is not possible to determine which isoform of arginase is of relevance. This is due to the fact that selective arginase inhibitors are not available. Arginase II knockout mice may be used to evaluate this isoform but arginase I knockout is a lethal phenotype. Future studies may be performed using conditional knockout models with targeted deletion of arginase I in endothelial and haematopoetic cells. Furthermore, we cannot from the present studies determine which isoform of NOS that was regulated by arginase since non-selective NOS inhibitors were used. It was recently demonstrated that arginase regulated NO formation from eNOS in red blood cells in mice using red blood cells from eNOS knockout animals (97). The

myocardial I/R studies were performed in open chest models. Open chest will increase the inflammatory response that may further stimulate arginase activity. The advantage of open chest models is that coronary flow can be continuously monitored in the pig model allowing proper determination of complete coronary artery occlusion and reperfusion. Appropriate control populations are lacking in the functional experiments in **papers III** and **IV**. The effect of arginase inhibition on microvascular function can therefore not be related to the response in healthy subjects. A previous study has demonstrated that arginase inhibition does not affect endothelial function in healthy subjects (203) suggesting that arginase is not of functional importance in vascular regulation under healthy conditions. Although baseline measurements in the analysis of the microcirculation did not differ between the two sides of the tongue, we cannot exclude that changes occur during the interventional protocol, possibly leading to scattering of results. In **paper III**, differences in the baseline characteristics were evident with differences for age and impaired renal function between patients with HF NYHA I/II and NYHA III/IV that might have influenced arginase I levels independent of HF severity.

5.4 CONCLUSIONS

1) Pharmacologic inhibition of arginase mediates cardioprotection in a rat model of myocardial I/R by a NO dependent mechanism shifting the utilization of arginine from arginase to NOS and increased NO production.

2) Intracoronary infusion of an arginase inhibitor during early reperfusion in a pig model of myocardial infarction also mediates cardioprotection by a local NO dependent mechanism.

3) Circulating arginase I levels are increased in patients with congestive heart failure and topical sublingual administration of an arginase inhibitor improves microvascular perfusion.

4) Global hypoxia increases circulating arginase I levels in healthy volunteers. Furthermore, circulating arginase I levels are increased in patients following CPR. Topical arginase inhibition improves microvascular function following CPR.

Collectively these data demonstrate an important regulatory function of arginase in CVD. Inhibition of arginase is a promising potential treatment target for protection against myocardial I/R injury and to ameliorate microcirculatory dysfunction in critically ill patients.

6 FUTURE PERSPECTIVES

Accumulating evidence clearly suggest that increased activity of arginase is of importance in different cardiovascular diseases. It seems clear that arginase is critically involved in the development of endothelial dysfunction in (among others) I/R and diabetes and possibly heart failure and in patients post-resuscitation. The central effects seem to be exerted mainly via interference with NO bioavailability by limiting L-arginine sources and contributing to oxidative stress. In consequence, arginase thereby represents an attractive and promising pharmacological target. Although great achievements have been made in modern medicine some issues remain to be solved. Two of such issues are: 1) the I/R injury which has not been fully understood and in which no convincingly effective treatment options exist and 2) microcirculatory disorders of critically ill patients. For these two conditions novel pharmacological approaches that target specific mechanisms are warranted. Considering the findings presented in this thesis inhibitors of arginase may represents such a specific therapeutic option. In these scenarios short-term administration of an arginase inhibitor may provide an effective treatment. This makes potential negative effects of arginase inhibition less likely. However, these have to be kept in mind since arginase plays an important role in ammonia detoxification in the urea cycle and little is known about inhibition of hepatic arginase and possible side effects. A next step in the future will be to dissect the exact role of different arginase isoforms in different species including humans in the vasculature and in the myocardium to improve treatment strategies and clarify the underlying mechanisms. In summary, these promising data warrant further development of potent isoform-selective arginase inhibitors to be tested in well-designed studies in disease animal models and larger clinical trials.

7 ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to all who helped me making this thesis possible. In particular, I would like to acknowledge the following:

John Pernow for being the perfect supervisor, trainer, discussant and much more. It was (and is) really fun to be part of your research group. You stand for an ideal combination of scientific excellence, warm atmosphere, believing in people, being integrative and sharp understanding. I will always remember a lot of moments that made our collaboration more than a normal business. For example sitting in the sauna drinking ice cold beer while discussing science, barbecuing in your garden, ordering some excellent fish in Barcelona or writing SMS during important soccer games. Did you know that we exchanged more than 1000 emails in the last years which ranks you on number 1 in my email box (my wife is second place)? I deeply hope that our collaboration will continue for a long time!

Per-Ove Sjöquist, better known as "P-O", co-supervisor, for being always enthusiastic about new results and always knowing the next helpful step, even when the original plan was lost somewhere. Your thoughts during group meetings were very much appreciated independently of teaching me that Sweden has a West coast which is "of course" much more beautiful. Still, I didn't get a receipt of *Västkustsallad*…

Adrian Gonon, co-supervisor, for a lot of interesting discussions, including research, politics, medicine, cardiology, Stockholm, Germany, Switzerland,… . You were one of the most important persons during my stay, not only for teaching me the pig animal model, showing me infrastructure and people. Most importantly, thank you for all the nice evenings with you and **Gunilla** in your summerhouse, the uncountable beers we had and a lot of moments that I will not forget such as my first pike that I fished under your supervision, the *svampsoppa* that we had in the archipelago, teaching me to cook *renskarv*, …It is good to know that you are there.

Marita Wallin, the queen of the lab. Without you this thesis would have been impossible. First, it was you who showed me everything in the former lab (now the parking space) and the current lab and helped me to get things running. You supported me with a lot of experiments but also taught me how to behave in a Swedish lab (don't forget the dishwasher! Well, I wasn't good in that discipline...). I can easily remember when you told me the first time that it is *fika time* and you know why. Thank you not only for your big support in the lab but also most appreciated company during evening activities, for knitting a *mössa* for Linus (he is still wearing it), sharing the newest gossip, creating a welcoming atmosphere and never giving up to teach me Swedish. *Tack så mycket!*

Alexey Shemyakin, my neighbor in the office. Thank you for all the interesting discussions about science and arginase, but also politics, whisky, economics and everything else not to forget the nice evenings and your hospitality. Indeed, one of my favorite stories is when we corrected an abstract of a colleague from another group and started 45 minutes before the abstract deadline was. I am sure you remember. Привет.

Julia Grönros, for always being nice and friendly and giving input into the arginase field. Thank you for teaching me doing echocardiography in mice and the nice collaboration.

Norbert Gerdes, for all the fun we had in the lab but also for all the science we discussed. Thank you for teaching me flow cytometry even if you think microparticles are uninteresting rubbish. I hope I contributed well enough in spending half of your poster prize money in the nightlife of Boston that you will continue collaborating with me… Thanks also to **Christin** for some nice evenings and afternoons with the Gerdes family. Nobbi, hope to see you soon ;-)

Thanks also to "the other Germans" **Daniela Strodthoff** and **Roland Klingenberg,** for nice chats during coffee breaks and more…, eller hur?

Of central importance to find my way to patient samples and in the clinics were **Kerstin Höglund** and colleagues. Kerstin, thank you for being always helpful!

Always good for a nice chat and laugh was **Arnar Rafnsson**. Thank you for understanding my irony and being a strong Icelandic Viking. In addition, thanks for the nice cooperation regarding the EPC study. I am sure Sweden misses your humor, communication skills and ability to say and know something about everything. At least I do.

Special thanks to **Peder Sörensson** regarding the CMR study and your patience when new data was needed for another revision of the manuscript…

I have to acknowledge the help of **Annika Van Vollenhoven** who helped me establishing different FACS protocols and who helped me understanding this machine.

Special thanks to **Aliaksandr Bulhak** who showed me the rat model of ischemia and reperfusion with a deep systematic approach and your support even during extra hours.

I wish to express my admiration to **Jon Lundberg** and **Eddie Weitzberg,** always ready to estimate the next steps in scientific projects. Jon, you had several very good ideas to push my projects forward and I like your open door and quick email replies.

Special thanks to everybody who made CMM 3rd floor a creative and friendly working environment, especially to **Maggie Folkerson, Ulf Hedin, Björn Gustafsson, Johan Ekstrand, Magnus Bäck, Daniel Kethelhuth, Göran Hansson, "Jack" Tseng, Maria Johansson, Anna Lundberg, Francisco Rios, Zhong-Qun Yan, Gabrielle Paulsson-Berne, Olga Ovchinnikova, Hannah Agardh, Peder Olofsson, Anders Gabrielsen, Anton Razuvaev, Lotta Tamik.**

A special thank goes to **Lars Rydén**. We had a rehearsal for the ESC meeting in 2008 and your feedback for my presentation was tough but so constructive that I still hear your voice while preparing talks. You never found out that the two back-up slides you suggested answered all questions from the auditorium.

Very appreciated was / is the help of **Raquel Binisi** solving issues with any document and giving quick smiling advice when I was lost in some KI administrative stuff.

Eva Wallgren: thank you for putting the thesis together and that printing of all that stuff really happens.

Thanks to **Pellina Janson** for your help in the animal facility.

I would like to acknowledge the former and present heads of clinical cardiology, **Cecilia Linde** and **Fredrik Gadler** for contributing to an academic atmosphere.

It is important to acknowledge my “research family” in Jena. Regarding the projects within the thesis I thank especially **Felix Quitter** for his engagement in microcirculatory research. Of central importance is my German scientific, clinical and personal mentor **Hans-Reiner Figulla**. I learned a lot the last ten years! I cannot mention all collaborators from Jena, but special thanks deserve **Björn Göbel, Michael Fritzenwanger, Marcus Franz, Alexander Pfeil, Friedhelm Küthe, Attila Yilmaz, Angelika Jahn, Romy Scholze, Ulrike Rabia, Michael Lichtenauer, Markus Ferrari, Alexander Lauten, Stefan Betge, Tudor Pörner, Annett Schmidt, Yvonne Schlenker, Josi Ratka, Katja Grün** and all those I cannot mention. My time in Jena is over now and it was an excellent time I will always remember.

Clinical research is impossible without the understanding and patience of patients. Thanks to all patients who gave me the opportunity to do clinical research.

In this acknowledgements section it is important to mention **Isabell Steger** and **Carl Jorns**. We got to know each other by incidence in Florida but you were one major reason why we came to the idea to come to Stockholm. The both of you were of central importance why our stay in Stockholm was so much fun and I cannot count all the activities. Thank you for letting me build your boat house ☺. It is good to have you as friends!

I wish to thank my parents **Wolfgang and Sabine Jung** for their never ending loving support and encouragement to do the right thing, as well as my sisters **Stefanie and Nina Jung** being the core of the Jung-clan.

Last but not least I deeply thank my beloved wife **Eva Jung** for your love, flexibility and warmth as well as for being my everything and in addition everything between my nutritionist and my prime minister. **Linus** and **Hannes**, thank you for all the fun and all the new words you find! High five!

This work supported by the Swedish Research Council Medicine (10857), the Swedish Heart and Lung Foundation, the Stockholm County Council (ALF), Karolinska Institutet/ Stockholm County Council Strategic Cardiovascular Programme, Gustav V and Queen Victoria Foundation, European Foundation for the Study of Diabetes Research, Wellness Foundation Diabetes, Interdisziplinäres Zentrum für Klinische Forschung (IZKF) Jena and Novo Nordisk Foundation

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9 APPENDIX

Appendix 1: Haemodynamic variables in **paper I**.

Group	Parameter	Before drug	7,5 min after drug	Before ischaemia	Before reperfusion	Reperfusion 15min	Reperfusion 30min	Reperfusion 60min	Reperfusion 120min	*P* vs. NaCl (AUC)
NaCl	MAP	92 ± 6	87 ± 6	80 ± 4	75 ± 5	75 ± 6	86 ± 6	77 ± 7	66 ± 5	-
	HR	451 ± 12	458 ± 21	476 ± 16	441 ± 8	435 ± 9	421 ± 13	408 ± 12	390 ± 12	-
	RPP	41439 ± 2367	40150 ± 3011	38205 ± 2104	33139 ± 1914	32708 ± 2868	36128 ± 2630	31363 ± 3079	25640 ± 2039	-
nor-NOHA	MAP	83 ± 5	83 ± 4	84 ± 5	79 ± 5	79 ± 6	87 ± 6	81 ± 8	73 ± 5	ns
	HR	439 ± 13	431 ± 10	450 ± 15	429 ± 8	419 ± 6	428 ± 32	385 ± 9	377 ± 17	ns
	RPP	36488 ± 3216	35934 ± 2230	37777 ± 2086	33747 ± 2072	32980 ± 2452	37858 ± 5023	31331 ± 3642	27520 ± 1573	ns
nor-NOHA + cPTIO	MAP	75 ± 6	83 ± 8	79 ± 6	85 ± 14	70 ± 12	91 ± 14	87 ± 6	79 ± 5	ns
	HR	416 ± 21	432 ± 25	427 ± 18	403 ± 17	396 ± 12 *	396 ± 9	365 ± 7	374 ± 7	<0.05
	RPP	31469 ± 3429	36377 ± 4951	34101 ± 4056	34696 ± 6102	28535 ± 5261	36326 ± 5935	31926 ± 2707	29606 ± 1945	ns
cPTIO	MAP	87 ± 8	93 ± 11	88 ± 11	88 ± 14	95 ± 14	107 ± 15	91 ± 9	88 ± 12	<0.05
	HR	432 ± 9	433 ± 7	432 ± 7	390 ± 12 *	380 ± 8 **	388 ± 14	383 ± 14	385 ± 15	<0.05
	RPP	37608 ± 3734	40242 ± 5080	37989 ± 4686	35033 ± 6137	36550 ± 6113	42019 ± 6803	35289 ± 4525	34038 ± 4801	ns
nor-NOHA + L-NMMA	MAP	77 ± 5	124 ± 11 **	73 ± 3	85 ± 5	95 ± 9	92 ± 7	95 ± 9	79 ± 8	ns
	HR	396 ± 14	417 ± 9	396 ± 15 **	397 ± 14 *	387 ± 12 **	392 ± 14	387 ± 5	390 ± 16	<0.01
	RPP	30571 ± 2504	51694 ± 4459	28631 ± 1425	33925 ± 2477	36544 ± 3430	35964 ± 3024	36607 ± 3380	30926 ± 3618	ns

Abbreviations: MAP, mean arterial pressure; HR, heart rate; RPP, rate pressure product. Significant differences from the control group (NaCl) at the individual time points and during the entire experimental protocol (area under curve; AUC) are indicated. *P< 0.05; **P<0.01. Data are presented as mean+SE.

Appendix 2 Haemodynamic variables in **paper II**.

	Pre-isch	**60 min rep**	**120 min rep**	**180 min rep**	**240 min rep**
Vehicle ic					
Heart rate (bpm)	93±9	109±5	116±9	133±10	135±19
MAP (mmHg)	95±6	70±6	62±6	62±5	52±7
LAD flow (ml/min)	20.1±1.8	24.6±6.8	18.0±4.1	16.9±3.7	8.8±2.1
RPP (bpm x mmHg)	9014±1466	7800±822	7106±862	8197±894	7482±1321
Nor-NOHA ic					
Heart rate (bpm)	97±7	121±5	117±3	120±6	125±10
MAP (mmHg)	95±4	71±4	67±5	62±4	56±5
LAD flow (ml/min)	17.0±1.9	25.6±7.4	19.9±2.8	14.1±2.6	9.9±1.7
RPP (bpm x mmHg)	9175±750	8555±589	7897±567	7301±329	6768±621
L-NMMA + nor-NOHA ic					
Heart rate (bpm)	79±6	106±7	109±9	116±11	117±10
MAP (mmHg)	97±5	72±4	72±5	65±6	60±4
LAD flow (ml/min)	15.7±1.3	21.3±6.2	22.4±3.2	14.8±1.5	10.1±0.7
RPP (bpm x mmHg)	7737±794	7747±848	7922±835	7507±924	7001±780
Nor-NOHA iv					
Heart rate (bpm)	105±8	105±8	110±7	117±9	115±5
MAP (mmHg)	96±5	65±5	67±7	65±7	55±8
LAD flow (ml/min)	17.4±2.1	17.0±4.8	16.7±5.6	11.1±2.0	5.6±0.8
RPP (bpm x mmHg)	9985±505	6704±388	7473±1123	7567±908	6313±884

Abbreviations: MAP, mean arterial pressure; LAD, left anterior descending coronary artery; RPP rate pressure product (RPP); ic, intracoronary; iv, intravenous. Data are presented as mean±SEM. There were no significant differences between the groups.