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THE IMPORTANCE OF NITRIC OXIDE AVAILABILITY IN MYOCARDIAL ISCHEMIA-REPERFUSION INJURY: REGULATORY ROLES OF ARGINASE, L-ARGININE AND TETRAHYDROBIOPTERIN

by

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CONTENTS

ABSTRACT

Background. Maintenance of nitric oxide (NO) availability is crucial for cardiovascular homeostasis and protection against myocardial ischemia-reperfusion (IR) injury. The mechanisms underlying reduced NO availability are multifactorial and involve deficiency of the NO synthase (NOS) substrate L-arginine and co-factor tetrahydrobiopterin (BH_4) as well as increased NO inactivation by reactive oxygen species. Increased activity of arginase has emerged as a key factor behind reduced NO availability by competing with NOS for L-arginine as substrate. The aims of the studies were to investigate: 1) the potential of arginase and RhoA/Rho associated kinase (ROCK) inhibition, supplementation of L-arginine with BH_4 and remote ischemic perconditioning (RIPerc) to induce cardioprotection, 2) the mechanisms behind their effects and 3) the influence of diabetes on the cardioprotective effects.

Study I. Arginase inhibition by N-omega-hydroxy-nor-L-arginine (nor-NOHA) prior to reperfusion in rats subjected to myocardial IR reduced infarct size (IS). This effect was abolished by inhibition of NOS, protein kinase C epsilon (PKCε) and blocking of the mitochondrial ATP-dependent potassium channel. The effect of nor-NOHA was associated with enhanced myocardial expression of PKCε.

Study II. Combined administration of L-arginine and BH_4 before and during reperfusion reduced IS in rat and pig models of myocardial IR. At the same time individual administration of L-arginine or BH_4 failed to evoke cardioprotection in both species. NOS inhibition abrogated the cardioprotective effect of L-arginine and BH_4. Myocardial BH_4 levels were higher in pigs given BH_4 with or without L-arginine. The generation of superoxide in the ischemic-reperfused myocardium was reduced only in pigs treated with the combination of L-arginine and BH_4.

Studies III-IV. Pharmacological enhancement of peroxynitrite decomposition and inhibition of ROCK protected from IR injury and attenuated myocardial ROCK and arginase activity in rats subjected to IR. Rats with type 1 diabetes had increased myocardial arginase activity, arginase 2 expression and ROCK activity. In addition, ROCK and arginase inhibition protected against myocardial IR in rats with type 1 diabetes. RIPerc induced by bilateral femoral artery occlusion resulted in reduction of IS, myocardial peroxynitrite formation, ROCK and arginase activity and upregulated myocardial endothelial NOS. The cardioprotective effect of RIPerc and associated changes in arginase and ROCK activity were absent in rats with type 1 diabetes. The cardioprotective effects of RIPerc, ROCK and arginase inhibition were abolished by inhibition of NOS.

Conclusions. Inhibition of arginase before the onset of reperfusion reduced IS via a mechanism dependent on NOS activity, PKCε expression and activation of mitochondrial ATP-dependent potassium channels. Supplementation of L-arginine and BH_4 during late ischemia and reperfusion reduced IS via a NOS-dependent pathway and reduced the generation of superoxide. Peroxynitrite and ROCK signaling pathways are involved in the upregulation of arginase activity during myocardial IR. Inhibition of either ROCK or arginase protected against myocardial IR injury via a NOS–dependent mechanism both in the presence and absence of type 1 diabetes. This suggests that reduction in arginase activity, as a result of reduced formation of peroxynitrite and ROCK activity, is of importance for the NOS-dependent cardioprotective effect of RIPerc. The cardioprotective effect of RIPerc and associated signaling effects on arginase, ROCK and NOS are abolished in type 1 diabetes. Arginase inhibition, supplementation of BH_4 and L-arginine, inhibition of ROCK and RIPerc are potential tools in the treatment of acute myocardial infarction.

LIST OF ABBREVIATIONS

5-HD	5-hydroxydecanoic acid sodium (mitoK_{ATP} channel blocker)
AAR	area at risk
BH_2	dihydrobiopterin
BH_3	trihydrobiopterin
BH_4	tetrahydrobiopterin
CAD	coronary artery disease
FeTPPS	5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III), chloride ($ONOO^-$ decomposition catalyst)
cGMP	cyclic guanosine monophosphate
eNOS	endothelial nitric oxide synthase
ε-V1-2	specific PKCε inhibitor
HR	heart rate
im	intramuscular
iNOS	inducible nitric oxide synthase
ip	intraperitoneal
IR	ischemia-reperfusion
IS	infarct size
iv	intravenous
LAD	left anterior descending artery
L-NMMA	N^G-monomethyl-L-arginine monoacetate (NOS inhibitor)
MAP	mean arterial pressure
mitoK_{ATP} channel	mitochondrial adenosine triphosphate dependent potassium channel
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
nor-NOHA	N-omega-hydroxy-nor-L-arginine (arginase inhibitor)
NOS	nitric oxide synthase
O_2^-	superoxide
$ONOO^-$	peroxynitrite
PCI	percutaneous coronary intervention
PKCε	protein kinase C epsilon
PKG	protein kinase G
RIC	remote ischemic conditioning
RIPerc	remote ischemic perconditioning
RIPostc	remote ischemic postconditioning
RIPrec	remote ischemic preconditioning
ROCK	RhoA/Rho-associated kinase
ROS	reactive oxygen species
sGC	soluble guanylyl cyclase
TTC	triphenyltetrazolium chloride

List of Original Papers

This thesis is based on the following studies, which will be referred to by their Roman numerals.

I

Yahor Tratsiakovich, Adrian T. Gonon, Anna Krook, Jiangning Yang, Alexey Shemyakin, Per-Ove Sjöquist, John Pernow.

Arginase inhibition reduces infarct size via nitric oxide, proteinkinase C epsilon and mitochondrial ATP-dependent K+ channels.

European Journal of Pharmacology. 2013; 712, 16–21.

II

Yahor Tratsiakovich, Adrian T. Gonon, Attila Kiss, Jiangning Yang, Felix Böhm, Per Tornvall, Magnus Settergren, Keith M. Channon, Per-Ove Sjöquist, John Pernow.

Myocardial protection by co-administration of L-arginine and tetrahydrobiopterin during ischemia and reperfusion.

International Journal of Cardiology. 2013; 169, 83–88.

III

Attila Kiss, Yahor Tratsiakovich, Adrian T. Gonon, Olga Fedotovskaya, Johanna T. Lanner, Daniel C. Andersson, Jiangning Yang, John Pernow.

The role of arginase and Rho kinase in cardioprotection from remote ischemic perconditioning in non-diabetic and diabetic rat in vivo.

PLoS ONE. 2014; 9, e104731.

IV

Yahor Tratsiakovich, Attila Kiss, Adrian T. Gonon, Jiangning Yang, Per-Ove Sjöquist, John Pernow.

Inhibition of Rho kinase protects from ischemia-reperfusion injury via regulation of arginase activity and nitric oxide synthase in type 1 diabetes.

Manuscript.

INTRODUCTION

MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

Myocardial infarction is a cause for millions of new mortality and morbidity cases occurring worldwide each year (White and Chew, 2008; Hausenloy and Yellon, 2013). Diagnosis and treatment of acute myocardial infarction have reached substantial progress over the last decades resulting in improved outcomes and prognosis for the patients. However, despite timely applied primary percutaneous coronary intervention (PCI), a significant number of patients die or develop heart failure (Bulluck et al., 2015).

Occlusion of a coronary artery leads to myocardial ischemic injury which is characterized by a switch to anaerobic respiration in absence of oxygen, adenosine triphosphate depletion, depolarization of mitochondrial membrane, reduction of intracellular pH and ionic imbalance between the cells and extracellular space (Avkiran and Marber, 2002). The current strategy for treating acute myocardial infarction caused by coronary artery occlusion is aimed at rapid and effective reperfusion which minimizes myocardial damage (Hausenloy and Yellon, 2013). Early restoration of blood flow by means of primary PCI and thrombolytic therapy rescues the myocardium at risk and limits infarct size (IS). However, reperfusion may itself induce additional myocardial injury and is therefore characterized as a "double-edged sword". The rescue of ischemic myocardium is inevitably accompanied by hazardous alterations and death of cardiomyocytes (Braunwald and Kloner, 1985; Piper et al., 1998; Hausenloy and Yellon, 2013). Reperfusion injury is characterized by oxidative stress (Zweier et al., 1987), intracellular calcium overload (Miyamae et al., 1996), prompt change of pH (Lemasters et al., 1996), opening of mitochondrial permeability transition pore (Heusch et al., 2010), activation of inflammatory processes, immune response and dysfunction of the microvascular endothelium (Turer and Hill, 2010). Thus, development of novel treatments that limit the extent of the reperfusion injury will have substantial impact on IS and myocardial function following an acute myocardial infarction. Reperfusion injury has therefore gained considerable interest as a target for myocardial salvage and in the design of novel therapeutic strategies. The pathophysiology of ischemia and reperfusion in the heart created by restoration of blood supply to the jeopardized myocardium, are often described together as the phenomenon of myocardial ischemia-reperfusion (IR) injury. The development of appropriate therapeutic strategies protecting against myocardial IR injury by targeting its pathophysiological mechanisms is expected to be of clear clinical benefit.

NITRIC OXIDE IN REGULATION OF CARDIOVASCULAR FUNCTION

Nitric oxide (NO) is a key regulator of vascular tone, inhibitor of platelet adhesion and aggregation, modulator of smooth muscle cell proliferation and its adequate basal synthesis is crucial for proper cardiovascular function (Moncada and Higgs, 2006). The synthesis of NO is performed by the enzyme nitric oxide synthase (NOS), which exists in three isoforms: neuronal (nNOS), expressed constitutively in neurons; inducible (iNOS), expressed in macrophages in response to immunological stimulation by cytokines; endothelial (eNOS), expressed constitutively in endothelial cells (Moncada and Higgs, 2006). Expression and

activation of iNOS are observed in neoplasms, during infections and inflammation (Kröncke et al., 1998) and are of minor importance under physiological conditions. The most important physiological effects of nNOS include the regulation of neuronal communication and the release of neurotransmitters in the nervous system (Zhao et al., 2015). It should be noted that nNOS is also expressed in cardiomyocytes (Xu et al., 1999), smooth muscle cells (Boulanger et al., 1998) and endothelial cells (Bachetti et al., 2004). It has been shown that nNOS-derived NO participates in regulation of vascular tone independently from eNOS (Ichihara et al., 1998). However, eNOS is the most crucial isoform involved into the regulation of cardiovascular function via synthesis of NO in endothelial cells (Förstermann and Sessa, 2012; Zhao et al., 2015).

NO is synthesized in endothelial cells by eNOS from the substrate L-arginine (Palmer et al., 1988) (**Figure 1**). The production and release of endothelium-derived NO is stimulated by several factors, including shear stress (Andrews et al., 2010), acetylcholine (Kellogg et al., 2005), bradykinin (Bae et al., 2003), histamine (Kishi et al., 1998) and other organic chemicals. NO produced by endothelial cells exerts constitutive vasodilating (**Figure 1**) effect (Furchgott, 1988) via stimulation of soluble guanylate cyclase (sGC) in the vascular smooth muscle cells,

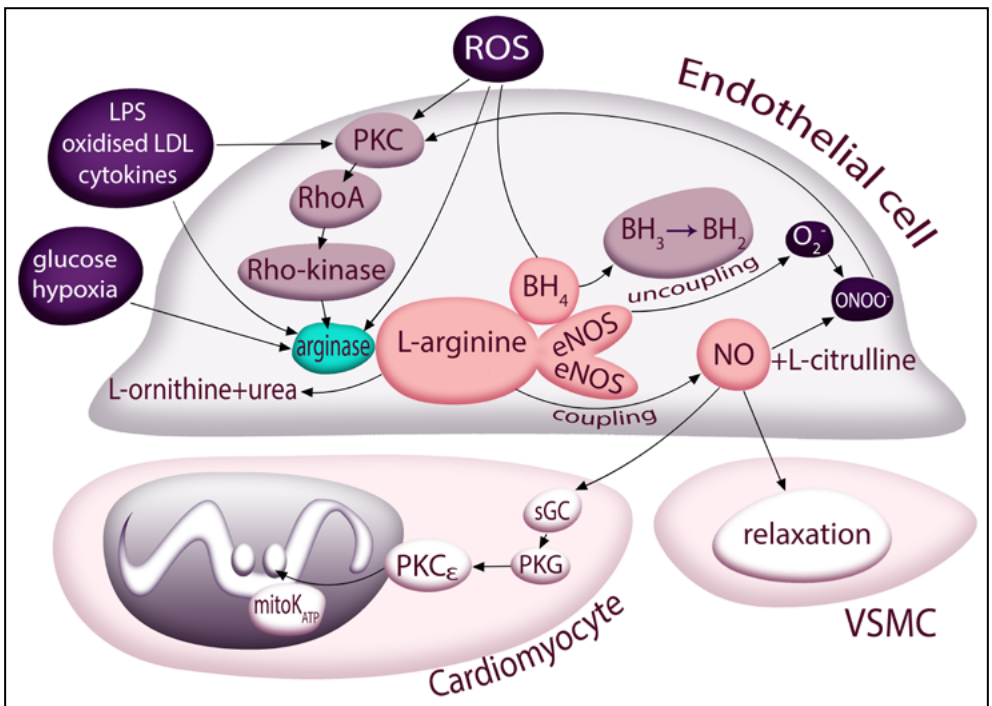

Figure 1. Roles of arginase, RhoA/Rho-associated kinase, L-arginine, tetrahydrobiopterin and principal pathophysiological stimuli for endothelial NO availability and cardiovascular function. BH_2 – dihydrobiopterin; BH_3 – trihydrobiopterin; BH_4 – tetrahydrobiopterin; eNOS – endothelial nitric oxide synthase; LDL – low-density lipoprotein; LPS – lipopolysaccharide; $mitoK_{ATP}$ – mitochondrial adenosine triphosphate dependent potassium channel; NO – nitric oxide; O_2^- – superoxide; $ONOO^-$ – peroxynitrite; PKC – protein kinase C; PKCε – protein kinase C epsilon; PKG – protein kinase G; ROS – reactive oxygen species; sGC – soluble guanylyl cyclase; VSMC – vascular smooth muscle cell.

leading to the formation of cyclic guanosine monophosphate (cGMP), which in turn stimulates protein kinase G (PKG)-mediated intracellular calcium depletion, resulting in the relaxation of smooth muscle (Zhao et al., 2015). In addition to vessel relaxation, NO inhibits apoptosis (Li et al., 1999), reduces neutrophil adherence to the vascular endothelium (Ma et al., 1993), prevents platelet aggregation (Walter and Gambaryan, 2009), scavenges superoxide (O_2^-) (McCall et al., 1989), suppresses the proliferation of vascular smooth muscle cells (D'Souza et al., 2003). In cardiomyocytes NO induces the opening of mitochondrial adenosine triphosphate-dependent potassium channel (mitoK_{ATP} channel) via the activation of sGC, PKG and protein kinase C epsilon (PKCε) (Costa and Garlid, 2008) (**Figure 1**). The activation of mitoK_{ATP} channel leads to reduced anoxic cell injury due to restoration of a membrane potential (Xu et al., 2001). However, not all the effects of NO depend on sGC and cGMP/PKG pathway. Another mechanism of action involves reaction of NO or its derivatives with cysteine residues of target proteins, referred to as S-nitrosylation (Stamler et al., 2001). Unlike sGC signaling, S-nitrosylation requires higher concentrations of NO, and is among other processes implicated in apoptosis, proliferation and secretion of protein and in the regulation of NOS activity (Qian and Fulton, 2013).

The regulation of eNOS expression and activity and therefore control of NO production are performed at the transcriptional, posttranscriptional and posttranslational levels (Qian and Fulton, 2013). Posttranslational mechanisms cause urgent changes in eNOS activity and are crucial for the normal function of NO pathways. The regulation of eNOS activity at the posttranslational level includes availability of enzyme substrate and co-factors, phosphorylation, S-nitrosylation, acylation of fatty acids and interactions between proteins (Qian and Fulton, 2013). Similarly to the other NOS isoforms, eNOS is a monomer and needs to organize as a dimer in order to catalyze the production of NO (Rafikov et al., 2011). Stability of the dimeric form (coupling) is maintained by optimal cellular concentrations of the substrate L-arginine and the co-factor tetrahydrobiopterin (BH_4) which plays a fundamental role in the production of NO in endothelial cells (Rafikov et al., 2011; Förstermann and Sessa, 2012) (**Figure 1**). In contrast to that, the uncoupled (monomeric) form of eNOS is unable to produce NO and generates O_2^- instead (Förstermann and Sessa, 2012) (**Figure 1**). Phosphorylation of various eNOS sites via protein kinases is involved in regulation of its activity. Thus, phosphorylation of human isoforms of serine residue Ser1177 or threonine residue Thr495 results in increase or decrease of eNOS activity, respectively (Qian and Fulton, 2013). However, dephosphorylation is mediated by phosphatases and causes an opposite effect (activation of eNOS after Thr495 dephosphorylation). S-nitrosylation is a feedback instrument to downregulate eNOS activity by targeting enzymes cysteine residues with NO when its concentrations are high (Lima et al., 2010). Fatty acids acylation mediates subcellular distribution of eNOS (Qian and Fulton, 2013). The activity of eNOS can be modulated via its binding to the other proteins. The activation of eNOS occurs after interaction with calmodulin or heat shock protein 90 (Förstermann et al., 1991; Garcia et al., 1998). In contrast, binding to caveolin-1, NOS interacting protein or NOS traffic inducer promote decrease of eNOS activity (Smart et al., 1999; Dedio et al., 2001; Zimmermann et al., 2002).

Accordingly, the normal function of cardiovascular system requires optimal availability of endothelium-derived NO, which is determined not only by expression of eNOS, but is strictly dependent on posttranslational machinery of enzyme activity regulation.

NITRIC OXIDE AVAILABILITY IN VASCULAR DYSFUNCTION AND MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

Several risk factors contribute to the vascular dysfunction and the development of coronary artery disease (CAD), which includes angina pectoris, sudden cardiac death and myocardial infarction (Wong, 2014). Those risk factors affect the cardiovascular system via substantially impaired endothelial function due to reduction of NO availability (Su, 2015).

Factors generating endothelial dysfunction

Atherosclerosis is the underlying cause of the majority of CAD cases. This chronic disease is characterized by hypercholesterolemia, impaired vasodilation and progressive formation of atheromatous plaques in arteries, culminating in ischemic events caused by atherothrombosis. Hypercholesterolemia stimulates leukocytes adherence and adhesion of activated platelets, resulting in endothelial dysfunction. High concentrations of cholesterol in blood promote activation of oxidases and production of reactive oxygen species (ROS), which react with NO, decreasing its availability and forming reactive nitrogen species, which in turn contribute to uncoupling of eNOS and lack of NO production and release (Su, 2015). Diabetes mellitus, described as one of the most prominent risk factor for CAD, is accompanied by profound endothelial dysfunction driven by hyperglycemia-dependent generation of ROS, uncoupling of eNOS, platelet aggregation and increased expression of adhesion molecules and high incidence of apoptosis among endothelial cells (Su, 2015). Another risk factor of CAD is hypertension, which also involves uncoupling of eNOS, increased ROS production and low bioavailability of NO, leading to violated endothelium-dependent vasorelaxation (Landmesser et al., 2003). It is well-known that CAD has higher incidence among smokers and aged people. Tobacco smoke contains various reactive components which cause vascular endothelium-dependent dysfunction via increased adherence of platelets and leukocytes, inflammatory response, oxidation, scavenging of NO by ROS and downregulation of eNOS activity through its uncoupling and altered phosphorylation (Messner and Bernhard, 2014). Ageing causes functional and structural changes in the vasculature including endothelial cells, which become sensitive toward apoptotic stimuli (Hoffmann et al., 2001). In addition, aged endothelial cells are characterized by low eNOS phosphorylation, uncoupling of eNOS and increased ROS production (Su, 2015).

Thus, all major risk factors which contribute to the development of CAD and particularly myocardial infarction, are associated with decreased availability of NO and endothelial dysfunction. As mentioned previously, ischemia of the heart in subjects with myocardial infarction, followed by restoration of blood supply to the ischemic region, contribute together to the myocardial IR injury. Myocardial ischemia stimulates endothelial dysfunction and further reduction of available NO (Giraldez et al., 1997). Myocardial ischemia downregulates eNOS expression and leads to tissue acidosis, attenuating the activity of eNOS (Giraldez et al., 1997). Later on, successful reperfusion overloads previously ischemic myocardium with oxygen, which causes considerable generation of ROS, cell injury, uncoupling of eNOS and decrease of its activity below basal physiological level (Amrani et al., 1995). Reduced endothelial NO availability triggers platelet aggregation, expression of cytokines, activation of adhesion molecules and adherence of the leukocytes to the endothelium, increased vascular permeability and migration of the various inflammatory cells into the myocardium and activation of iNOS, which synthesizes high amounts of toxic NO, all contributing to further tissue damage (Darra

et al., 2010; Turer and Hill, 2010). Myocardial vasculature is deprived of basal NO production in endothelial cells, and microvascular tone shifts, accordingly, to vasoconstriction impairing perfusion (Pagliaro et al., 2003).

Clinical data indicate that the diabetic heart compared to the non-diabetic is more sensitive to IR injury. Patients with diabetes have an increased risk of ischemic events (Galiñanes and Fowler, 2004). Diabetes is an important risk factor for unfavorable outcomes after coronary revascularization (Galiñanes and Fowler, 2004). The negative influence on cardiovascular function in diabetes is caused by hyperglycemia, oxidative stress, inflammation, dyslipidemia and reduced NO bioavailability (Guerci et al., 2001; Creager et al., 2003). Diabetes alters neutrophil adhesion, promotes dysfunction of endothelial cells and myocytes, impairs function of eNOS and affects metabolic processes in the myocardium, thereby augmenting the degree of injury following myocardial IR (Galiñanes and Fowler, 2004).

Alterations of endothelial function during myocardial IR injury complicated by diabetes, as well as in patients without diabetes, emphasize the importance of treatment approaches which aim to recover the availability of NO in order to minimize the damage of myocardium. It has been proposed that maintenance of NO levels close to the normal homeostatic range can be fruitful for the heart facing the challenge of IR injury (Darra et al., 2010). It is considered that therapeutic strategies that are able to reverse eNOS uncoupling can consequently exert beneficial effects on NO availability and improve cardiovascular function (Förstermann and Sessa, 2012).
It was noted previously, that optimal cellular concentrations of the substrate L-arginine and the co-factor BH_4 are important for eNOS coupling. Therefore, mechanisms of their deficiency and approaches to eliminate their limitations in the setting of myocardial IR should be considered.

The role of arginase and RhoA/Rho associated kinase for nitric oxide availability

L-arginine is not only a substrate for all the NOS isoforms, but is also a substrate for the enzyme arginase which hydrolyzes L-arginine to L-ornithine and urea (Durante et al., 2007) and thereby competes with NOS which converts L-arginine to NO and L-citrulline (**Figure 1**). Arginase is expressed in two isoforms: cytosolic arginase 1 and mitochondrial arginase 2 (Ash et al., 2000). Arginase 1 plays an important role in the hepatic urea cycle for the disposal of ammonia. Both arginase 1 and arginase 2 are expressed in endothelial cells, vascular smooth muscle cells and cardiomyocytes in different species (Pernow and Jung, 2013). Possible differences in the functioning of the two isoforms of arginase in the cardiovascular system remain unclear due to the absence of isoform-specific arginase inhibitors. Activation of arginase leads to reduced availability of L-arginine which results in decreased NO production and contributes to endothelial dysfunction. Decreased availability of L-arginine induced by arginase leads to uncoupling of eNOS resulting to production of O_2^- instead of NO by NOS and formation of peroxynitrite ($ONOO^-$), which in turn can contribute to oxidation of the eNOS co-factor BH_4 (Förstermann and Sessa, 2012).

Factors which provoke increased expression of arginase include bacterial endotoxins (lipopolysaccharides), cytokines, oxidized low-density lipoproteins, glucose, angiotensin II, hypoxia, ROS and thrombin (Pernow and Jung, 2013) (**Figure 1**). These factors activate various intracellular kinases (**Figure 1**), including protein kinase C/RhoA/Rho kinase (ROCK)

pathway, cyclic adenosine monophosphate/protein kinase A pathway, mitogen-activated protein kinase and tyrosine kinase (Pernow and Jung, 2013). However, as for other enzymes, another functional determinant of the arginase along with expression is its activity. The activity of endothelial arginase is increased via the ROCK pathway by oxidized low-density lipoproteins (Ryoo et al., 2011; Pandey et al., 2014) or by thrombin (Ming et al., 2004). Moreover, it was shown that ROS increase arginase activity in endothelial cells through the ROCK pathway (Chandra et al., 2011) (**Figure 1**).

Numerous studies demonstrated that arginase expression and activity are increased in conditions associated with endothelial dysfunction and decreased availability of NO, including atherosclerosis (Ryoo et al., 2011; Thengchaisri et al., 2006), hypertension (Demougeot et al., 2007; Zhang et al., 2004), ageing (Berkowitz et al., 2003), smoking (Sikka et al., 2013) and in myocardial IR (Hein et al., 2003; Jung et al., 2010). Diabetes is also an important risk factor for complications in myocardial IR which is accompanied by upregulated expression and activity of arginase in the aorta (Romero et al., 2008; Grönros et al., 2011), myocardium (Grönros et al., 2011), corpora cavernosa (Toque et al., 2011), retinal endothelium (Elms et al., 2013) and the endothelium of coronary arterioles (Beleznai et al., 2011). In addition, the enhancement of the ROCK pathway is involved in endothelial dysfunction in aorta from the diabetic rats (Cicek et al., 2013). The importance of ROCK for upregulation of arginase in diabetes was supported by deletion of ROCK in diabetic knock-out mice, which attenuated aortic endothelial dysfunction and prevented increased arginase activity and expression, restoring availability of NO (Yao et al., 2013).

Therefore, arginase inhibition and ROCK inhibition seem to be effective targets to attenuate uncoupling of eNOS, increase NO availability and prevent the formation of O_2^- and $ONOO^-$. Indeed, arginase inhibition reverses uncoupling of NOS and improves endothelial function in atherosclerosis (Ryoo et al., 2008), hypertension (Bagnost et al., 2010) and in ageing (Kim et al., 2009). It has been shown that the inhibition of arginase reverses impaired endothelium-dependent vasodilation in coronary arteries from rats with type 1 diabetes (Romero et al., 2008). A clinical study in patients with CAD and type 2 diabetes demonstrated improved endothelium-dependent vasodilatation in the forearm after local infusion of the arginase inhibitor N-omega-hydroxy-nor-L-arginine (nor-NOHA) (Shemyakin et al., 2012). Administration of nor-NOHA restored coronary microvascular function in diabetic rats via increased NO availability (Grönros et al., 2011), suggesting a potential cardioprotective ability of arginase inhibition in the setting of myocardial IR, especially in a condition complicated by co-morbidity such as diabetes. Previous studies showed that pretreatment of non-diabetic rats with nor-NOHA before the coronary artery occlusion resulted in significant reduction in IS after 30 min of ischemia and 2 h of reperfusion (Jung et al., 2010). Local infusion of nor-NOHA into the coronary artery of pigs during late ischemia and early reperfusion also reduced IS (Gonon et al., 2012). However, the mechanisms underlying the cardioprotective effect of arginase inhibition in a clinically relevant setting in connection with the onset of reperfusion, as well as the possibility to obtain protective effects of arginase inhibition in myocardial IR complicated by diabetes have remained unexplored. In addition, it was reported that ROCK inhibition significantly decreased IS in the non-diabetic rats via downregulation of the ROCK pathway (Bao et al., 2004; Li et al., 2012) and effectively improved coronary vascular function in the type 1 diabetic rats in vivo (Pearson et al., 2013). These observations along with the recently described activation of arginase in endothelial cells via the ROCK pathway suggest

an intriguing possibility to increase NO availability in ischemic-reperfused myocardium in diabetes by downregulation of arginase via ROCK inhibition.

The role of tetrahydrobiopterin for nitric oxide availability

Another mechanism for the uncoupling of NOS and impaired cardiovascular function is a lack of the essential co-factor BH_4 (Crabtree and Channon, 2011). Low availability of BH_4 is associated with impaired endothelial function and decreased production of NO in atherosclerosis (Tiefenbacher et al., 2000), CAD (Maier et al., 2000), diabetes (Shinozaki et al., 2000) and smoking (Heitzer et al., 2000). Oxidative stress associated with myocardial IR (Amrani et al., 1995) leads to BH_4 oxidation by ROS and consequent formation of trihydrobiopterin (BH_3) and dihydrobiopterin (BH_2), resulting in eNOS uncoupling (Crabtree and Channon, 2011) (**Figure 1**). It is remarkable that not only the depletion of BH_4, but also the concurrence between BH_2 and BH_4 for binding with eNOS contributes to its uncoupling (Crabtree and Channon, 2011). In a setting of myocardial IR, BH_4 deficiency contributes to myocardial injury and impaired cardiac contractility (Moens et al., 2011). Exogenous administration of the BH_4 precursor sepiapterin or a synthetic analogue of BH_4 restored the response to endothelium-dependent vasodilators in pig coronary arterioles isolated from hearts subjected to IR (Tiefenbacher et al., 1996). The diminished NO production in the ischemic heart *ex vivo* is associated with depletion of BH_4 in the myocardium, and administration of exogenous BH_4 restored NO production and improved recovery of coronary flow (Dumitrescu et al., 2007). Intravenous administration of BH_4 prior to ischemia significantly reduced myocardial IS in rats subjected to IR (Wajima et al., 2006). Moreover, the administration of BH_4 or sepiapterin resulted in protection against IR injury in the liver (Hara et al., 2006), skeletal muscle (Wang et al., 2007) and kidney (Legrand et al., 2011). Importantly, BH_4 attenuated liver IR injury when administered iv during ischemia before the onset of reperfusion (Hara et al., 2006). However, it is not known whether BH_4 exerts cardioprotective effects in a clinically relevant model with administration during ischemia shortly before reperfusion. Of additional interest is the interaction between L-arginine and BH_4 in the regulation of proper NOS function. Besides the role of BH_4 in binding of L-arginine to NOS, L-arginine has proved to increase the levels of BH_4 bound to NOS indicating that L-arginine supply is also crucial for NOS coupling (Gorren et al., 1996). This suggests a synergistic effect of L-arginine and BH_4 in the setting of the reduced NO availability. Accordingly, the combination of L-arginine and BH_4 decreased endothelial dysfunction induced by forearm IR in patients with type 2 diabetes mellitus and CAD (Settergren et al., 2009). However, the efficiency of combined administration of BH_4 and L-arginine in a setting of myocardial IR injury remains to be assessed.

REMOTE ISCHEMIC CONDITIONING AND NITRIC OXIDE AVAILABILITY IN MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

Importantly, not only pharmacological approaches can cause favorable effects on the heart challenged by IR. Thirty years ago Murry and co-authors (Murry et al., 1986) described the existence of endogenous cardioprotective mechanisms activated by means of short periods of myocardial ischemia and reperfusion prior to the long lethal ischemic episode of the heart. This phenomenon is known as local ischemic preconditioning and is characterized by a remarkable reduction in IS. However, this procedure is not feasible in the clinical setting since the patients arrive to hospital with ongoing myocardial ischemia. Further preclinical and clinical studies (Zhao et al., 2003; Thibault et al., 2008) revealed cardioprotective efficacy by short cycles of

ischemia and reperfusion within risk area of the heart during ongoing ischemia (local ischemic postconditioning). Interestingly, not only local ischemic conditioning can protect the heart from IR injury. The phenomenon of remote ischemic conditioning (RIC) which involves short episodes of ischemia and reperfusion in a remote vascular bed resulting in resistance to IR injury in the heart was further discovered. This was initially described as an intracardiac phenomenon of remote ischemic preconditioning (RIPrec) (Przyklenk et al., 1993). It was subsequently demonstrated that RIPrec in the mesenterium (Gho et al., 1996), kidney (Gho et al., 1996; Pell et al., 1998) and limb (Kharbanda et al., 2002) protected the heart from IR injury. However, RIPrec is not clinically feasible since it is initiated before the onset of ischemia. Therefore, protocols of the intermittent limb ischemia during ongoing myocardial ischemia (perconditioning, RIPerc) or after the onset of reperfusion (remote ischemic postconditioning, RIPostc) of previously ischemic myocardium were investigated. These techniques of RIC seem to be the most optimal in a clinical setting due to the simplicity of application which can be started before and/or in association with reperfusion without affecting the primary PCI procedure. Both RIPerc (Schmidt et al., 2007) and RIPostc (Andreka et al., 2007) significantly reduced IS in pigs. The first randomized clinical trial established that RIC started before primary PCI increased myocardial salvage in patients with acute ST elevation myocardial infarction (Bøtker et al., 2010). The mechanisms behind the transfer of the cardioprotective signals from a remote organ to the heart have generated much attention and include neuronal and humoral components. In addition, intracardiac transduction plays an important role in cardioprotection (Heusch et al., 2015). Several candidates mediating this transfer have been proposed. NO and eNOS are involved in the transfer and transduction of the cardioprotection evoked by RIC (Heusch et al., 2015). RIPrec by occlusion of the femoral artery in mice resulted in shear stress-dependent release of eNOS-derived NO, its oxidation to nitrite, which reached the myocardium via the circulation was suggested to mediate the cardioprotective effect (Rassaf et al, 2014). In addition, the deletion of eNOS in knock-out mice or by pharmacological inhibition abolished the cardioprotective effects of RIPrec (Rassaf et al, 2014). RIPostc, in turn, attenuated myocardial IR injury in rabbits via an eNOS-dependent mechanism (Tanq et al., 2011). Interestingly, RIPerc reduced myocardial no-reflow after IR via downregulation of ROCK activity (Zhao et al., 2009). Considering that ROCK pathway is a potent activator of arginase, the possible involvement of arginase downregulation and further increase of NO availability in the cardioprotective effects of RIPerc may be suggested. Moreover, the efficiency of RIPerc to attenuate myocardial IR injury in the presence of diabetes is of importance considering the confounding effect of diabetes on upregulation of ROCK and arginase.

AIMS

The present project was focused on the novel strategies of protection against myocardial IR injury via increased NO bioavailability.

The following specific aims were set:

1. To investigate the mechanism behind the protection against myocardial IR injury induced by arginase inhibition.
2. To investigate whether administration of L-arginine and BH_4 before the onset and during reperfusion reduces IS.
3. To investigate whether the ROCK signaling pathway contributes to arginase upregulation during myocardial IR, and the involvement of this pathway in the cardioprotective mechanism of RIPerc.
4. To investigate whether inhibition of ROCK, inhibition of arginase and RIPerc protect from myocardial IR injury in type 1 diabetes and to identify the mechanisms behind these effects.

MATERIAL AND METHODS

ANIMAL MODELS OF MYOCARDIAL ISCHEMIA-REPERFUSION

Rat model of myocardial ischemia-reperfusion (Studies I-IV)

The experiments were performed on male Sprague-Dawley rats (Charles-River, Sulzfeld, Germany). Animals were allowed to acclimate in the animal facility with food and water ad libitum for a minimum of 1 week after arrival before any experimentation. The rats (260-400 g) were anesthetized with sodium pentobarbital (50 mg/kg, ip followed by continuous infusion of 5 mg/kg/h, iv) and fentanyl (30-100 µg/kg/h, iv, studies I and II). Animals were tracheotomized, intubated and ventilated with air by a rodent ventilator (55 strokes/min, 9 mL/kg tidal volume). Rectal temperature was maintained at 37.5-38.5 °C by a heated operating table. The right carotid artery was cannulated and connected to a pressure transducer for the measurement of mean arterial pressure (MAP). Heart rate (HR) was determined from the arterial pressure curve. Hemodynamic parameters were continuously recorded on a personal computer equipped with PharmLab V5.0 software (AstraZeneca R&D, Mölndal, Sweden). The left jugular vein was cannulated for drug administration. The heart was exposed via a left thoracotomy and a ligature was placed around the left coronary artery in order to induce reversible occlusion of the vessel. After completion of the surgical preparation, the rats were stabilized for at least 15 min before randomized for inclusion into study groups. Myocardial ischemia was induced by tightening of the ligature around the left coronary artery. Successful occlusion was associated with cyanosis of the myocardial area at risk (AAR). Reperfusion was initiated after 30 min of ischemia by removal of the snare and was maintained for 2 h. Reperfusion was associated with disappearance of the cyanotic colour of the myocardium and appearance of arrhythmias.

Pig model of myocardial ischemia-reperfusion (Study II)

Farm pigs (27–38 kg) were premedicated with tiletamine (1.5 mg/kg, im), zolazepam (1.5 mg/kg, im) and medetomidine hydrochloride (0.06 mg/kg, im). Anesthesia was induced by sodium pentobarbital (20 mg/kg, iv bolus, following 2-4 mg/kg/h, iv and fentanyl 0.05 mg/h, iv). Heparin (5000 IU/h, iv) was given continuously. Animals were intubated and mechanically ventilated with air and oxygen. Respiratory rate and tidal volume were adjusted to keep arterial blood pH, oxygen and carbon dioxide levels within the physiological range. Rectal temperature was kept at 39.0±0.2°C by means of a heated operating table. A central venous catheter was inserted into the right external jugular vein for drug and fluid administration. Another catheter was placed in the descending aorta via the right femoral artery for the determination of blood gases and measurement of arterial pressure via a pressure transducer. HR was determined from the arterial pressure curve. All variables were continuously recorded on a personal computer equipped with PharmLab V3.0 software (AstraZeneca R&D, Mölndal, Sweden). The heart was exposed via a sternotomy. A ligature was placed around the left anterior descending artery (LAD) at a position from which the distal third of the artery is occluded via tightening of the ligature. 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. The flow probe was connected to a Transonic 208 blood flow meter.

An additional catheter was introduced into the right femoral artery and a guide wire was advanced to the aortic arch under fluoroscopic control. A coronary angiography catheter was slid over the wire and placed into the left main coronary for local drug administration. After a post-surgery stabilization period of 30 min the pigs were subjected to myocardial ischemia by tightening the ligature around the LAD. Ischemia was maintained for 40 min followed by reperfusion for 4 h.

RAT MODEL OF TYPE 1 DIABETES MELLITUS

Male Sprague-Dawley rats of age 5-6 weeks were used in **studies III and IV** for the induction of type 1 diabetes mellitus. Type 1 diabetes was induced by a single injection of streptozotocin (STZ, 55 mg/kg) dissolved in sterile Dulbecco's phosphate buffered saline in the tail vein under anesthesia with isoflurane mixed with oxygen. Only those rats with blood glucose levels >15 mmol/L 72 h after the STZ injection were considered to be diabetic and were included in the study. Healthy age matched rats received an iv injection of vehicle (Dulbecco's phosphate buffered saline) and served as a non-diabetic control group. Blood glucose concentration was measured weekly by FreeStyle Lite blood glucose monitoring system (Abbot Diabetes Care Inc., CA, USA). The rats were provided with food and water ad libitum until used for further experiments four to five weeks after the injection of STZ.

EXPERIMENTAL PROTOCOLS

Study I

In **study I**, two separate experimental protocols were used. In protocol 1 rats were randomized to one of the following treatment groups: 1) saline (400 µL for IS determination, n=10); 2) saline (400 µL, for determination of arginase activity, n=9); 3) the arginase inhibitor nor-NOHA (100 mg/kg, n=8); 4) the NOS inhibitor N^G-monomethyl-L-arginine monoacetate (L-NMMA, 10 mg/kg) followed by nor-NOHA (n=6) or 5) the mitoK$_{ATP}$ channel blocker 5-hydroxydecanoic acid sodium (5-HD, 10 mg/kg) followed by nor-NOHA (n=6).
In protocol 2 additional rats were randomized to one of the following treatment groups: 1) saline (n=13); 2) the arginase inhibitor nor-NOHA (100 mg/kg, n=10); 3) the specific PKCε inhibitor ε-V1-2 (3 mg/kg) followed by nor-NOHA (n=6); 4) ε-V1-2 only (n=4) or 5) sham group (the coronary ligature was not tightened and no compounds were administered; n=5). Saline and all substances were given iv as bolus injections at 15 min of ischemia except 5-HD and ε-V1-2 which were injected iv at 10 min of ischemia. The dosages were based on previous studies (Deuse et al., 2010; Gourine et al., 2005; Jung et al., 2010). L-NMMA and 5-HD have previously been shown not to affect IS *per se* (Fryer et al., 2001; Gonon et al. 2000). Since the effect of ε-V1-2 on IS *per se* was less documented, it was administered to a separate group of animals subjected to myocardial IR.

Study II

Also in **study II**, two separate experimental protocols were used. In protocol 1, rats were randomized to one of the following treatment groups: 1) saline (400 µL, n=8); 2) the NOS co-factor BH_4 (10 mg/kg) followed by L-arginine (100 mg/kg, n=6); 3) BH_4 followed by saline (200 µL, n=6); 4) saline (200 µL) followed by L-arginine (n=6); and 5) the NOS inhibitor L-NMMA (10 mg/kg) followed by BH_4 and L-arginine (n=6). All substances were given iv as bolus injections at 25 min of ischemia.

In protocol 2 pigs were randomized to receive a local intracoronary infusion into the left main stem of either: 1) saline (n=6); 2) BH_4 (0.03 mg/kg/min, n=6); 3) L-arginine (3.0 mg/kg/min, n=6) or 4) the combination of L-arginine and BH_4 (n=6). In addition a fifth group received a systemic infusion of the same doses of L-arginine and BH_4 via the jugular vein (n=5). All infusions were started at 30 min of ischemia and were given for 30 min, i.e. until 20 min of reperfusion, at a rate of 2 mL/min. After 40 min of ischemia LAD was reperfused for 4 h by removal of the ligature. The dosages were based on previous studies (Dumitrescu et al., 2007; Hara et al., 2006; Jung et al., 2010; Nakanishi et al., 1992; Pernow et al, 1994; Wajima et al., 2006).

Study III

Also in **study III**, two separate experimental protocols were used. In protocol 1, rats were randomized to one of the following groups: 1) control myocardial IR (no intervention during IR; n=10), 2) myocardial IR + RIPerc induced by bilateral femoral artery occlusion using vessels clamps during the last 15 min of myocardial ischemia (n=10), 3) administration of the NOS inhibitor L-NMMA just prior to RIPerc (10 mg/kg, iv n=6), 4) administration of the ROCK inhibitor hydroxyfasudil (0.5 mg/kg, iv, n=7) 20 min prior to myocardial ischemia, or 5) administration of the $ONOO^-$ decomposition catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III) chloride (FeTPPS, 10 mg/kg, iv, n=6) 10 min prior to reperfusion.
In protocol 2, type 1 diabetic or age-matched non-diabetic rats were randomized to one of the following groups: 1) non-diabetic controls (myocardial IR with no intervention, n=6), 2) non-diabetic rats subjected to myocardial IR + RIPerc (n=6), 3) diabetic rats with no intervention during myocardial IR (n=7) and 4) diabetic rats subjected to myocardial IR + RIPerc (n=6).
The dosage of drugs and duration of the femoral artery occlusion were based on previous studies (Basalay et al., 2012; Jung et al., 2010; Loukili et al., 2011; Utsunomiya et al., 2001). NOS inhibition has previously been demonstrated not to affect IS per se (Gonon et al. 2000).

Study IV

Also in **study IV**, two separate experimental protocols were used. In protocol 1, type 1 diabetic or age-matched non-diabetic rats were randomized to one of to the following treatment groups given iv 15 min prior to myocardial ischemia: 1) saline (n=5, non-diabetic rats; n=5, diabetic rats); 2) the ROCK inhibitor hydroxyfasudil (0.5 mg/kg; n=5, non-diabetic rats; n=5, diabetic rats); 3) the NOS inhibitor L-NMMA (10 mg/kg) followed by hydroxyfasudil (n=5, diabetic rats) or 4) L-NMMA only (n=4, diabetic rats).
In protocol 2, type 1 diabetic or age-matched non-diabetic rats were randomized to one of the following treatment groups given iv 15 min prior to myocardial ischemia: 1) saline (n=7, non-diabetic rats; n=14, diabetic rats); 2) the arginase inhibitor nor-NOHA (100 mg/kg, n=8, non-diabetic rats; n=11, diabetic rats); 3) the NOS inhibitor L-NMMA (10 mg/kg) followed by nor-NOHA (n=7, diabetic rats).

DETERMINATION OF AREA AT RISK AND INFARCT SIZE

After 2 h of reperfusion in rats and 4 h of reperfusion in pigs the left coronary artery was re-occluded and 2% Evans blue solution was injected iv (1.5 mL for rat and 50 mL for pig) to stain non-ischemic myocardium for the determination of the AAR. The rats were euthanized

by exsanguination and the pigs were sacrificed by iv injection of potassium chloride. The hearts were extracted. The atria and the right ventricle were removed. The left ventricle of the rat was frozen for 20 min (−20°C) and cut into ~1.5 mm thick slices perpendicular to the base–apex axis. The left ventricle of the pig was cut into ~1 cm thick slices perpendicular to the heart base–apex axis without prior freezing. The third slice (counting from the apex) was cut into the ischemic and non-ischemic parts and frozen and stored at -80°C until further analysis. All the other slices were weighed, scanned from both sides for the determination of the AAR and put in 1% triphenyltetrazolium chloride (TTC) solution for 15 min (slices from rat) or 10 min (slices from pig) at 37°C to distinguish viable myocardium from necrotic. After 24 h of incubation in 4% formaldehyde slices from rats were scanned again from both sides, IS and AAR were determined by planimetry of computer images (Adobe Photoshop CS 2, Adobe Systems, San Jose, CA, USA). Left ventricular slices from pigs were scanned directly after incubation in 1% TTC solution and IS and AAR were determined by planimetry. IS was expressed in percent of AAR.

MYOCARDIAL ARGINASE ACTIVITY

Arginase activity was determined by using a colorimetric assay. The assay measures the urea production using α-isonitrosopropiophenone. Following homogenization and centrifugation of tissue samples, 50 μL of the supernatant was added to 75 μL of Tris-HCl (50 mmol/L, pH=7.5) containing 10 mmol/L $MnCl_2$. The mixture was activated by heating for 10 min at 56 °C. Each sample was then incubated at 37°C for 1 h with L-arginine (50 μL, 0.5 M, in Tris-HCl, pH=9.7). 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 α-isonitrosopropiophenone (9% in ethanol) was added to the mixture and then heated at 100°C for 60 min. Arginase activity was calculated as μmol urea/mg protein/h and expressed as fold change from the control group.

MYOCARDIAL ROCK ACTIVITY

Activity of ROCK in myocardium was determined in **studies III and IV** by analyzing the extent of phosphorylation of ezrin (Thr567) by immunoblotting.

PEROXYNITRITE FORMATION IN MYOCARDIUM

Formation of $ONOO^-$ in myocardium was measured by the evaluation of the expression of 3-nitrotyrosine – a specific marker of oxidation mediated by $ONOO^-$. Immunoblotting was used to detect 3-nitrotyrosine expression.

IMMUNOBLOTTING

Protein extraction from the left ventricular myocardial tissue samples was performed in ice cold lysis buffer containing 20 mM Tris (pH=7.8), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1% TritonX-100, 10% (w/v) glycerol, 10 mM NaF, 1 mM ethylenediaminetetraacetic acid, 5 mM Na-pyrophosphate, 0.5 mM Na_3VO_4, 1 mg/mL leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride, 1 mg/mL aprotinin and 1 mM benzamidine (**study I and III**) or RIPA buffer (**study IV**). The homogenates were centrifuged at 5000g (**study I**) or 10000g **(study III and IV**) 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 (10, 40 or 50 μg per lane) were loaded onto a 7.5% or

10 % SDS gels and separated by electrophoresis. Proteins were transferred to polyvinylidine fluoride or nitrocellulose membranes and Ponceau staining was used to confirm the efficiency of transfer and to visualize protein loading. In **study I**, membranes were blocked overnight at 4°C, and then probed with antibodies against PKCε (BD Transduction Laboratories, Franklin Lakes, NJ, USA). Proteins were visualized by enhanced chemiluminescence with ECL advance Western blotting detection kit (Amersham Biosciences, Amersham, UK) and quantified using densitometry and Quantity One 4.5.1 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). In **studies III and IV**, membranes were blocked for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies against arginase 1 and 2 (1:1000, Sigma Prestige Antibody, USA); phoshorylated eNOS (anti-phosphorylated Ser-1177; 1:1000; BD Pharmingen, USA), total eNOS (1:1000; ABR Affinity BioReagents, USA); 3-nitrotyrosine (1:1000; Abcam, UK) or phosphorylated ezrin (anti-phosphorylated Thr567, 1:1000, BD Pharmingen, USA). IRDye 800-conjugated goat anti-mouse IgG (1:12000, LI-COR Biosciences, USA), IRDye 800-conjugated goat anti-rabbit IgG (1:12000, 1:15000, LI-COR Biosciences, Lincoln, NE, USA) and IRDye 680 (1:10000, LI-COR Biosciences, USA) were used as secondary antibody and bands were visualized using infrared fluorescence scanner (IR-Odyssey, LI-COR Biosciences, USA). Equal loading was confirmed by expression of GAPDH (1:5000, Sigma Aldrich, USA) or, for 3-nitrotyrosine, staining with Coomassie brilliant blue. Band densities were analyzed with Image Studio Lite Version 3.1 (LI-COR Biosciences, USA).

DETERMINATION OF TETRAHYDROBIOPTERIN LEVELS IN MYOCARDIUM

Myocardial BH_4 levels in left ventricular tissue samples from pigs (**study II**) were determined by HPLC followed by electrochemical and fluorescent detection. Tissue lysates were resuspended in phosphate-buffered saline (50 mM), pH 7.4, containing dithioerythritol (1 mM) and ethylenediaminetetraacetic acid (100 μM) and subjected to three freeze–thaw cycles. After centrifugation (15 min at 13000 rpm and 4°C), samples were transferred to new, cooled micro tubes and precipitated with cold phosphoric acid (1 M), trichloroacetic acid (2 M) and dithioerythritol (1 mM). Samples were vigorously mixed and then centrifuged for 15 min at 13000 rpm and 4°C. Samples were injected onto an isocratic high performance liquid chromatography system and quantified using sequential electrochemical detection (Coulochem III, ESA, Inc., North Sioux City, SD, USA). High performance liquid chromatography separation was performed using a 250 mm ACE C-18 column (Hichrom, Berkshire, UK) and a mobile phase comprised of sodium acetate (50 mM), citric acid (5 mM), ethylenediaminetetraacetic acid (48 μM), and dithioerythritol (160 μM, pH 5.2) (all ultrapure electrochemical high performance liquid chromatography grade) at a flow rate of 1.3 mL/min. Background currents of +500 μA and −50 μA were used for the detection of BH_4 on electrochemical cells E1 and E2, respectively. Quantification of BH_4 was done by comparison with authentic external standards and normalized to sample protein content.

DETERMINATION OF SUPEROXIDE PRODUCTION IN MYOCARDIUM

Left ventricular myocardial tissue samples (size: 0.5×0.5×2.0 cm) were excised from the AAR following IR in pigs included into **study II**, and O_2^- production was determined using dihydroethidium (Sigma-Aldrich, St. Louis, MO, USA) fluorescence staining. The tissue blocks were embedded into cutting compound in optimal temperature and 20 μm

cryosections were produced in cryostat Leica CM3050 S (Leica Microsystems, Wetzlar, Germany), stained with dihydroethidium (1 μM), dissolved in phosphate-buffered saline solution (pH 7.4) and incubated at 37°C for 30 min in a dark humidified chamber. A negative control was obtained by blocking the reaction with N-acetyl-L-cysteine (100 mM, Sigma-Aldrich, St. Louis, MO, USA). The fluorescent image was obtained using a fluorescence microscope Zeiss Axio Scope A1 (Carl Zeiss Microscopy GmbH, Jena, Germany) with a 585 nm long-pass filter. The fluorescence intensities were determined using Image J software (NIH, Bethesda, MD, USA).

STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism 4.00 or 5.00 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as mean±SEM. P-value <0.05 was considered statistically significant. In **study I** groups were compared using one-way ANOVA followed by Newman-Keuls multiple comparison post-hoc test. Unpaired t-test was used for comparisons of only two groups. In **study II** groups were compared using Kruskal–Wallis test with Dunn's multiple comparison test. In **studies III-IV** one-way ANOVA followed by Bonferroni post hoc test was used for multiple comparisons between several groups. Mann Whitney U-test was used for comparisons of only two groups where appropriate. Repeated-measures two-way ANOVA with Bonferroni post-hoc test was used for the multiple comparisons of hemodynamic parameters.

RESULTS

MECHANISMS OF THE CARDIOPROTECTION INDUCED BY ARGINASE INHIBITION (STUDY I)

Hemodynamics

In comparison with the control group, there were no significant differences in MAP or HR except a lower HR in the group given 5-HD and nor-NOHA at 30 and 60 min of reperfusion and higher MAP at 60 min of reperfusion in the sham operated group.

Area at risk and infarct size

There were no significant differences in the AAR between any of the groups in protocol 1 and in protocol 2. Among animals included in protocol 1, IS in the control group was 61±3 % (**Figure 2**). Arginase inhibition by nor-NOHA significantly reduced IS to 47±3 % ($P<0.01$). Administration of the NOS inhibitor L-NMMA or the mitoK$_{ATP}$ channel blocker 5-HD completely abolished the cardioprotective effect of nor-NOHA (**Figure 2**). In protocol 2, it was established that administration of the PKCε inhibitor ε-V1-2 did not affect IS *per se* but it abolished the cardioprotective effect of the arginase inhibitor nor-NOHA (**Figure 3**).

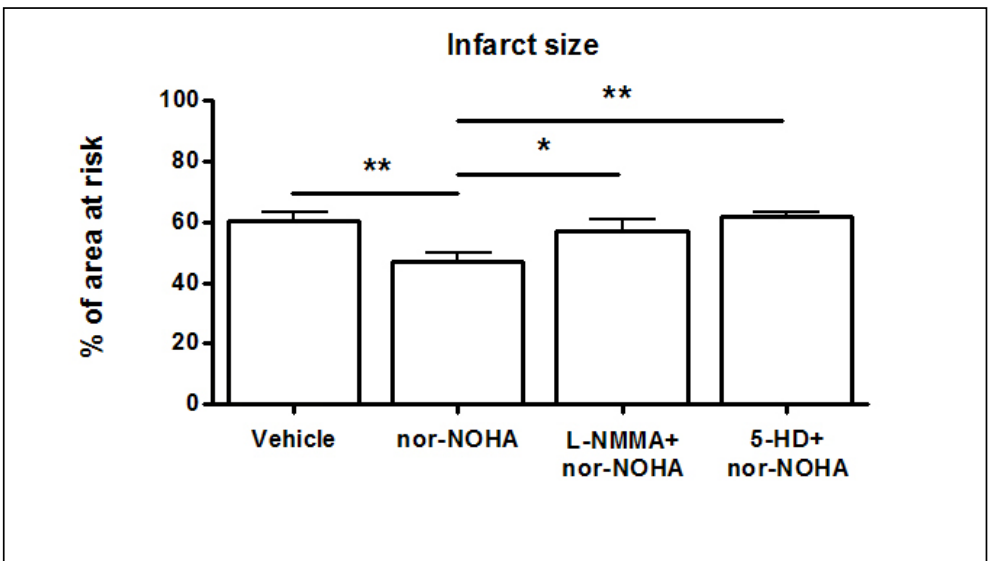

Figure 2. Infarct size in rats subjected to 30 min of ischemia and 2 h of reperfusion. The rats were given either saline (vehicle, n=10), the arginase inhibitor nor-NOHA (n=8), nor-NOHA together with the NOS inhibitor L-NMMA (n=6), nor-NOHA together with the selective mitoK$_{ATP}$ channel blocker 5-HD (n=6). Data are presented as mean ± SEM; *P<0.05; **P<0.01.

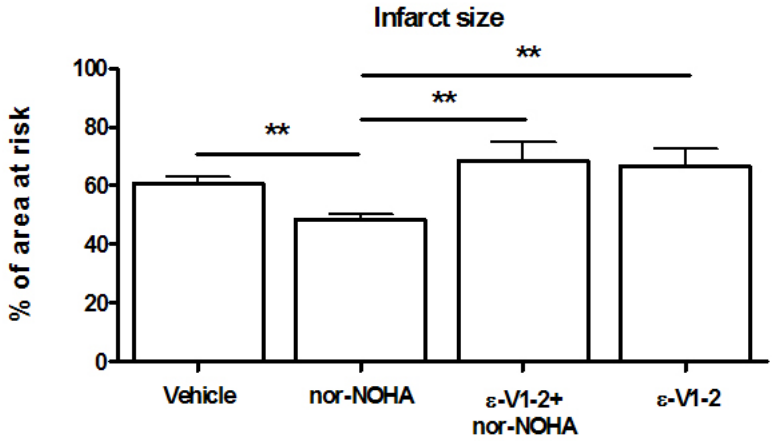

Figure 3. Infarct size in rats subjected to 30 min of ischemia and 2 h of reperfusion. The rats were given either saline (vehicle, n=13), the arginase inhibitor nor-NOHA (n=10), nor-NOHA together with the PKCε specific inhibitor ε-V1-2 (n=6) or ε-V1-2 only (n=4). Data are presented as mean ± SEM; **P<0.01

Effect of ischemia-reperfusion on myocardial arginase activity

In order to evaluate the effect of IR on myocardial arginase activity, it was measured in ischemic and non-ischemic myocardium of animals given saline. Arginase activity in ischemic-reperfused myocardium was more than 2-fold higher than that in non-ischemic myocardium (**Figure 4**).

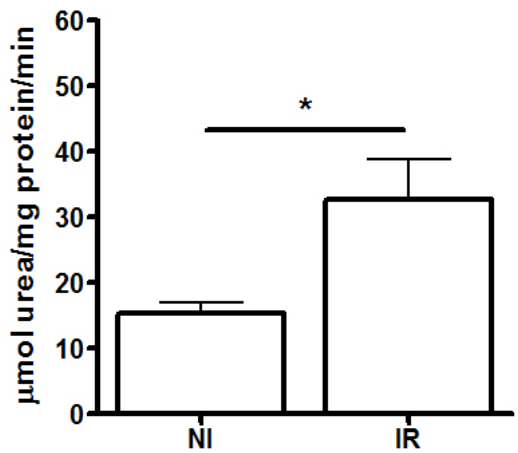

Figure 4. Arginase activity in non-ischemic (NI) and ischemic-reperfused (IR) myocardium of rats subjected to 30 min of ischemia and 2 h of reperfusion (n=9). Data are presented as mean ± SEM; *P<0.05.

Effect of arginase inhibition on protein kinase C epsilon expression in ischemic-reperfused myocardium

Expression of PKCε was markedly reduced in ischemic-reperfused myocardium compared to non-ischemic myocardium of sham-operated animals. The expression of PKCε was 7-fold higher ($P<0.01$) in ischemic-reperfused myocardium of the rats treated with nor-NOHA than in those given vehicle (**Figure 5**).

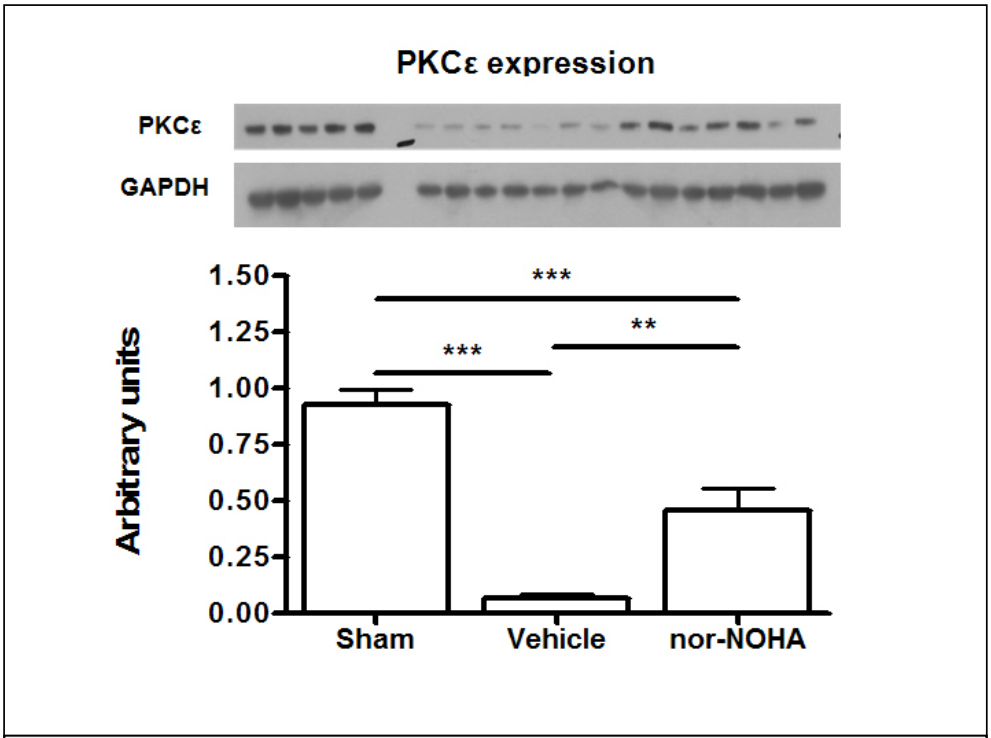

Figure 5. Representative immunoblots and quantification of the expression of protein kinase C epsilon (PKC_{ε}) in myocardium of sham-operated animals (n=5) and in ischemic-reperfused myocardium of rats subjected to 30 min of ischemia and 2 h of reperfusion, treated with vehicle (n=7) or nor-NOHA (n=7). Data are presented as mean ± SEM; **P<0.01; ***P<0.001.

CARDIOPROTECTIVE EFFECTS OF L-ARGININE AND TETRAHYDROBIOPTERIN (STUDY II)

Hemodynamics

There were no significant differences in pre-ischemic hemodynamic parameters between the groups of rats or pigs. In rats HR was significantly higher before the onset and at the end of reperfusion in the group given L-arginine + BH_4 than in the vehicle group. There were no statistical differences in hemodynamic parameters between the different groups of pigs during ischemia and reperfusion.

Area at risk and infarct size

Both for rats and pigs there were no significant differences in AAR between the groups. In rats IS was 65±3 % in the vehicle group (**Figure 6**). IS was not significantly affected by administration of L-arginine (64±3 %) or BH_4 (56±3 %) given separately. However, administration of the combination of L-arginine and BH_4 resulted in a significant reduction of IS to 50±2 % ($P<0.05$ vs. the vehicle group). Administration of the NOS inhibitor L-NMMA abolished the protective effect of L-arginine and BH_4 (IS 66±4 %).
In pigs IS was 86±5 % in the vehicle group (**Figure 7**). Neither infusion of L-arginine or BH_4 alone affected IS significantly (77±5 % and 70±5 %, respectively). IS was significantly reduced by intracoronary administration of the combination of L-arginine and BH_4 to 54±5 % ($P<0.05$ vs. the vehicle group). By contrast, systemic infusion of the same dose of L-arginine + BH_4 did not influence IS (83±6 %).

Myocardial tetrahydrobiopterin levels and superoxide production

BH_4 was determined in the myocardial AAR of pigs given intracoronary infusion of vehicle, BH_4 or the combination of BH_4 and L-arginine (**Figure 8**). Myocardial BH_4 levels were significantly increased in animals given BH_4 (5.4±2.6 pmol/mg) and BH_4 + L-arginine (3.5±1.1 pmol/mg) in comparison with the vehicle group (0.9±0.3 pmol/mg).
Formation of O_2^- in the ischemic-reperfused myocardium of pigs determined by dihydroethidium staining was significantly lower in pigs given the combination of BH_4 and L-arginine than in vehicle treated pigs (**Figure 9**). L-arginine or BH_4 alone did not significantly affect O_2^- formation.

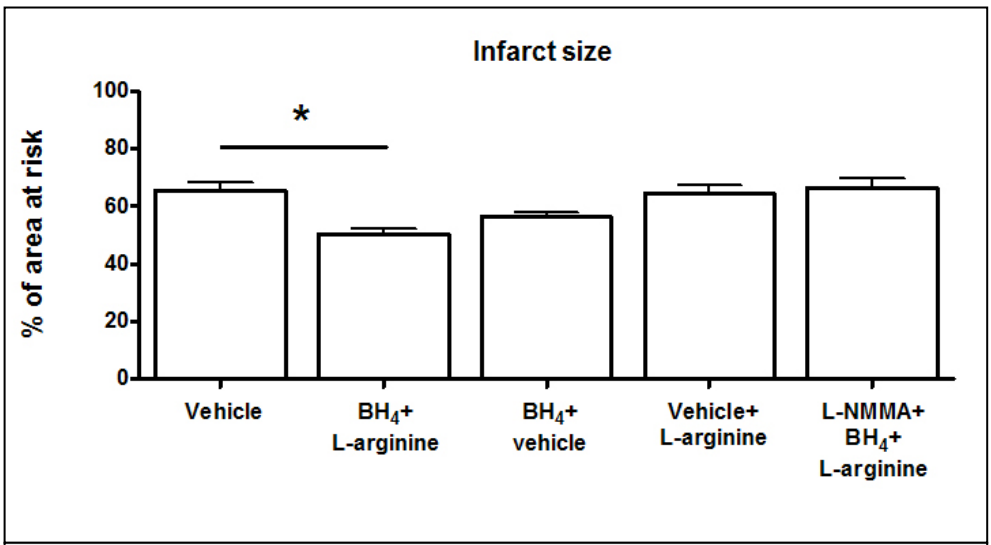

Figure 6. Infarct size in rats subjected to 30 min of ischemia and 2 h of reperfusion. The rats were given either saline (vehicle, n=8), BH_4 with L-arginine (n=6), BH_4 with vehicle (n=6), vehicle with L-arginine (n=6), the NOS inhibitor L-NMMA with BH_4 and L-arginine (n=6). Data are presented as mean ± SEM; *$P<0.05$.

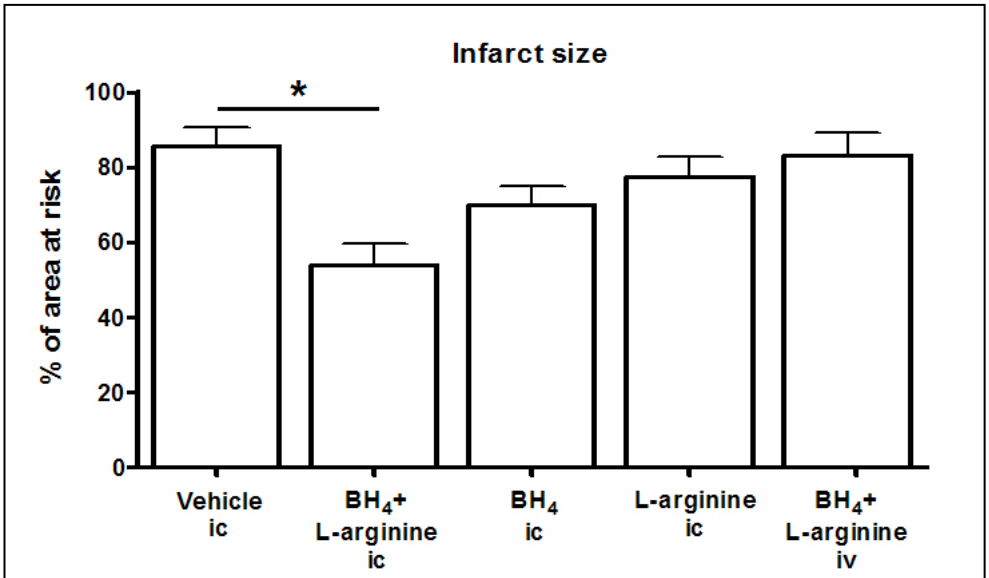

Figure 7. Infarct size in pigs subjected to 40 min of ischemia and 4 h of reperfusion. The pigs were given either saline (vehicle, n=6), BH_4 with L-arginine (n=6), BH_4 alone (n=6) or L-arginine alone (n=6) by intracoronary (ic) infusion or BH_4 with L-arginine by iv infusion (n=5). Data are presented as mean ± SEM; *P<0.05.

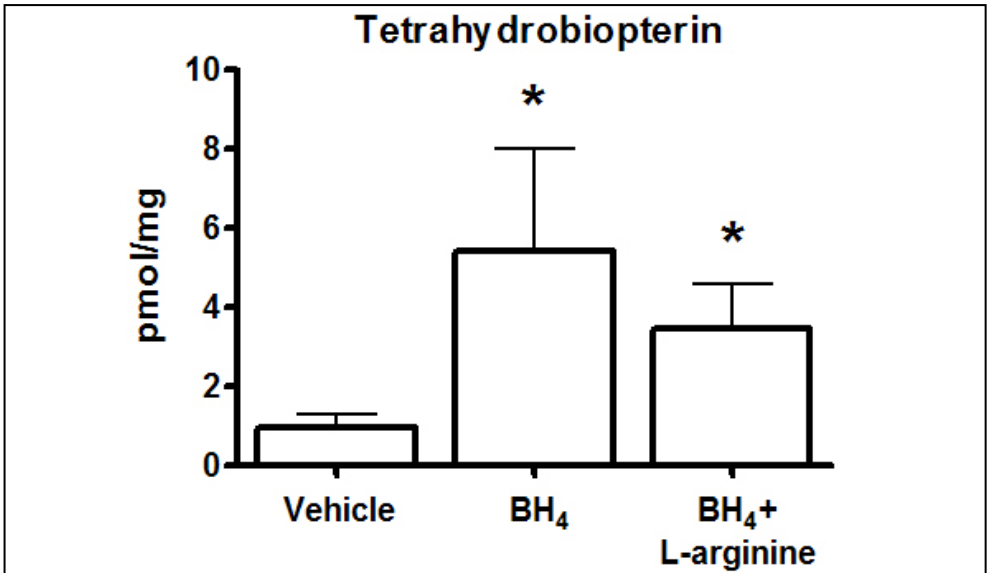

Figure 8. Tetrahydrobiopterin (BH_4) levels in the ischemic-reperfused myocardium of pigs after 40 min of ischemia and 4 h of reperfusion. The pigs were given either saline (vehicle, n=4), BH_4 (n=6) or BH_4 with L-arginine (n=4) by intracoronary infusion. Data are presented as mean ± SEM; *P<0.05. Significant differences versus the vehicle group are shown.

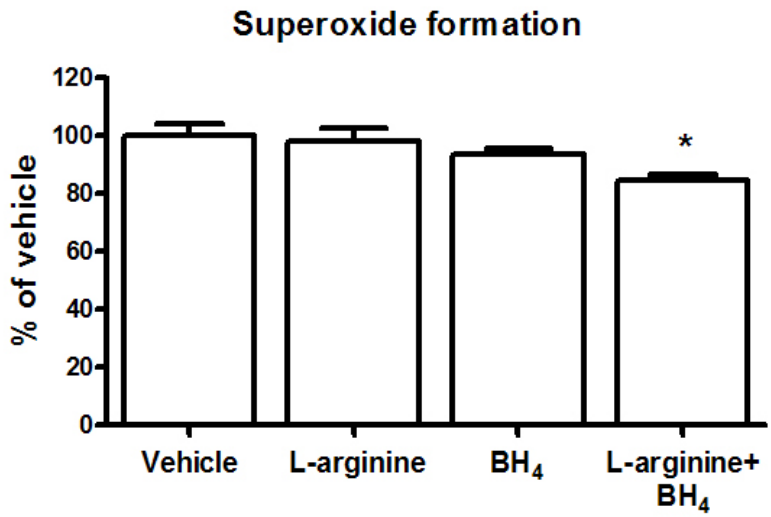

Figure 9. Superoxide formation in the ischemic-reperfused myocardium of pigs after 40 min of ischemia and 4 h of reperfusion. The pigs were given either saline (vehicle, n=4), L-arginine (n=5), BH_4 (n=4) or BH_4 with L-arginine (n=4) by intracoronary infusion. Data are presented as mean ± SEM; *P<0.05.

ROCK SIGNALING AND ARGINASE UPREGULATION FOLLOWING MYOCARDIAL ISCHEMIA-REPERFUSION: EFFECTS OF REMOTE ISCHEMIC PERCONDITIONING, ROCK INHIBITION AND ARGINASE INHIBITION ON INFARCT SIZE (STUDY III)

Area at risk and infarct size

There was no significant difference in the AAR between groups in protocol 1 of **Study III**. IS was significantly reduced by RIPerc, FeTPPS and hydroxyfasudil (42±4 %; 51±4 % and 49±3 %, respectively) in comparison to the control group (69±2%, **Figure 10A**). The effect of RIPerc on IS was completely abolished by the NOS inhibitor L-NMMA (**Figure 10A**).

Effects of remote ischemic perconditioning, peroxynitrite decomposition and ROCK inhibition on arginase expression and activity in ischemic-reperfused myocardium

RIPerc, the peroxynitrite decomposition catalyst FeTPPS and the ROCK inhibitor hydroxyfasudil markedly suppressed arginase activity in ischemic-reperfused myocardium in comparison with the control group (**Figure 10B**, P<0.001). However, expression of arginase 1 and arginase 2 was not affected by any of the interventions. Arginase activity in non-ischemic myocardium of rats subjected to myocardial IR was not affected by RIPerc, FeTTPS or hydroxyfasudil.

Effects of remote ischemic perconditioning, peroxynitrite decomposition and ROCK inhibition on ROCK activity in ischemic-reperfused myocardium

RIPerc, FeTPPS and hydroxyfasudil suppressed the expression of phosphorylated ezrin in ischemic-reperfused myocardium, indicating significant reduction of ROCK activity in comparison with the control group following IR by a magnitude similar to that induced by the ROCK inhibitor (**Figure 10C**, P<0.001). The effect of RIPerc on ROCK activity was completely abolished by the administration of L-NMMA (**Figure 10C**).

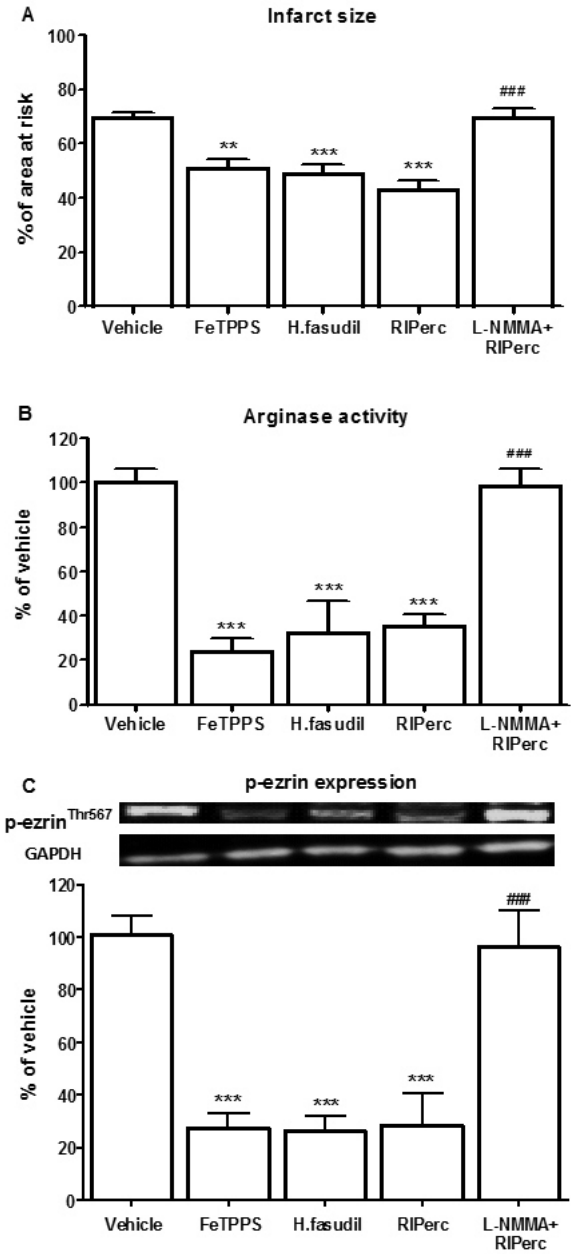

Figure 10. Infarct size (A), arginase activity (B) and ROCK activity (C) in ischemic-reperfused myocardium of the rats subjected to 30 min of ischemia and 2 h of reperfusion. ROCK activity is expressed as phosphorylation of ezrin, representative immunoblots are shown. The rats were given either saline (vehicle, A: n=10, B: n=10, C: n=7), peroxynitrite decomposition catalyst FeTPPS (A: n=10, B: n=6, C: n=5), ROCK inhibitor hydroxyfasudil (H.fasudil, A: n=7, B: n=5, C: n=4), remote ischemic perconditioning (RIPerc, A: n=6, B: n=5, C: n=6), the NOS inhibitor L-NMMA with RIPerc (A: n=6, B: n=5, C: n=5). Data are presented as mean ± SEM; **P<0.01, ***P<0.001 versus the vehicle group; ###P<0.001 versus the RIPerc group.

Effect of remote ischemic perconditioning on myocardial peroxynitrite formation

The level of 3-nitrotyrosine, a marker of $ONOO^-$ formation, in ischemic-reperfused myocardium was markedly attenuated by RIPerc (**Figure 11**).

Effect of remote ischemic perconditioning on myocardial eNOS expression

Total eNOS and phosphorylated (Ser1177) eNOS were determined in the ischemic myocardium and the ratio of phosphorylated to total eNOS was calculated. RIPerc enhanced phosphorylation of eNOS at Ser1177 in ischemic-reperfused myocardium (**Figure 12A**).

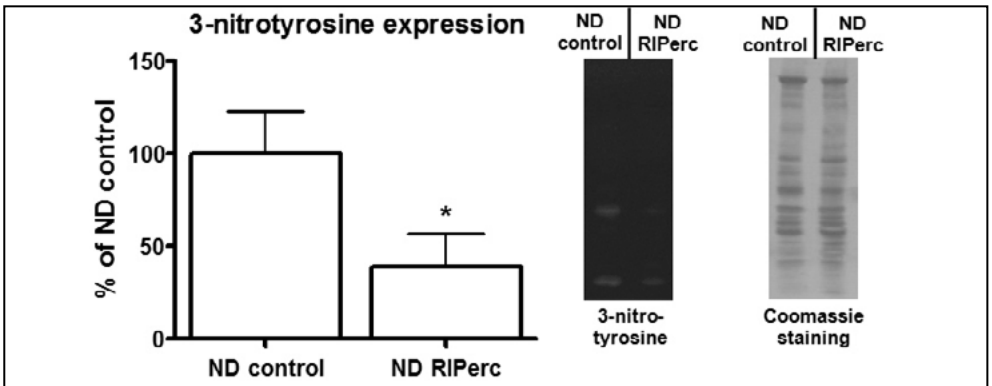

Figure 11. Peroxynitrite formation expressed as expression of 3-nitrotyrosine in ischemic-reperfused myocardium in rats subjected to 30 min of ischemia and 2 h of reperfusion. The non-diabetic (ND) rats were exposed to control myocardial ischemia-reperfusion (ND-control, n=8) or myocardial ischemia-reperfusion with remote ischemic perconditioning (ND-RIPerc, n=6). Representative immunoblots are shown. Data are presented as mean ± SEM; *P<0.05.

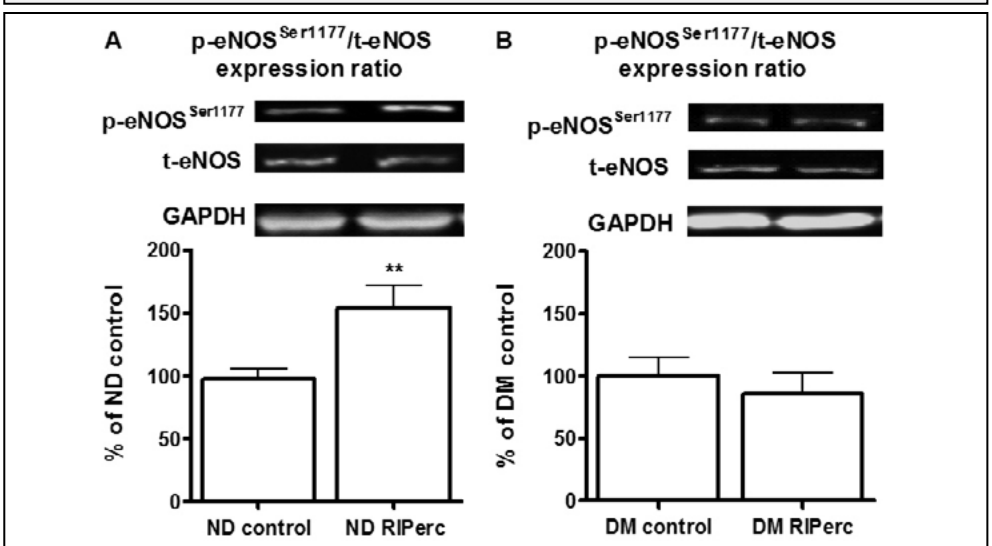

Figure 12. Representative immunoblots and ratio of the phosphorylated eNOS (p-eNOS^{Ser1177}) expression to total eNOS (t-eNOS) expression in myocardium of non-diabetic (A) and diabetic (B) rats subjected to 30 min of ischemia and 2 h of reperfusion. The non-diabetic (ND) and diabetic (DM) rats were exposed to control myocardial ischemia-reperfusion (ND-control, n=6; DM-control, n=6) or myocardial ischemia-reperfusion with remote ischemic perconditioning (ND-RIPerc n=6; DM-RIPerc, n=6). Data are presented as mean ± SEM; **P<0.01.

INFLUENCE OF DIABETES ON CARDIOPROTECTION INDUCED BY ARGINASE INHIBITION, ROCK INHIBITION AND REMOTE ISCHEMIC PERCONDITIONING (STUDIES III-IV)

Matching of diabetic and non-diabetic rats

In **Study III** blood glucose level was 27.3±1.0 mmol/L four weeks after the administration of STZ compared to 4.9±0.2 mmol/L in the control group (P<0.001). Body weight was significantly lower in the group of rats with type 1 diabetes (291±12 g) than in the control group (394±9 g, P<0.001).

In the first part of the **Study IV** blood glucose was 28.8±0.4 mmol/L four weeks after the administration of STZ, compared to 4.5±0.1 mmol/L in the control group (P<0.001). Body weight was significantly lower in the diabetic group (278±8 g) than in the control group (386±6 g, P<0.001).

In the second part of the **Study IV** blood glucose was 26.6±0.4 mmol/L four weeks after the administration of STZ, compared to 4.7±0.1 mmol/L in the control group (P<0.001). Body weight was significantly lower in the diabetic group (295±7 g) than in the control group (408±6 g, P<0.001).

There were no differences in blood glucose or body weight between the groups within the non-diabetic or diabetic rats, respectively.

Hemodynamics

HR was significantly lower in rats with type 1 diabetes in comparison with the non-diabetic controls (P<0.05). There were no significant differences in HR or MAP among non-diabetic or diabetic rats between the two parts of the study.

Effect of diabetes on myocardial ROCK activity, arginase expression and activity

The effect of diabetes on the activity of ROCK and arginase as well as expression of arginase isoforms was determined in the non-ischemic myocardium from diabetic and non-diabetic control groups in the **Study IV**. Both ROCK and arginase activity were significantly elevated in rats with diabetes in comparison with non-diabetic animals (**Figure 13 A-B**). There was no difference in arginase 1 expression between the two groups (**Figure 13 C**), whereas myocardial expression of arginase 2 was significantly increased in diabetic rats (**Figure 13 D**).

Effect of remote ischemic perconditioning, arginase inhibition and ROCK inhibition on infarct size in diabetes

In protocol 2 of **Study III**, RIPerc reduced IS in non-diabetic rats from 68±2 % to 46±4 % (P<0.001). IS in rats with type 1 diabetes subjected to myocardial IR was 68±1 %. In contrast to non-diabetic rats, RIPerc failed to evoke cardioprotective effect in type 1 diabetic rats resulting in an unchanged IS (63±2 %) (**Figure 14 A**).

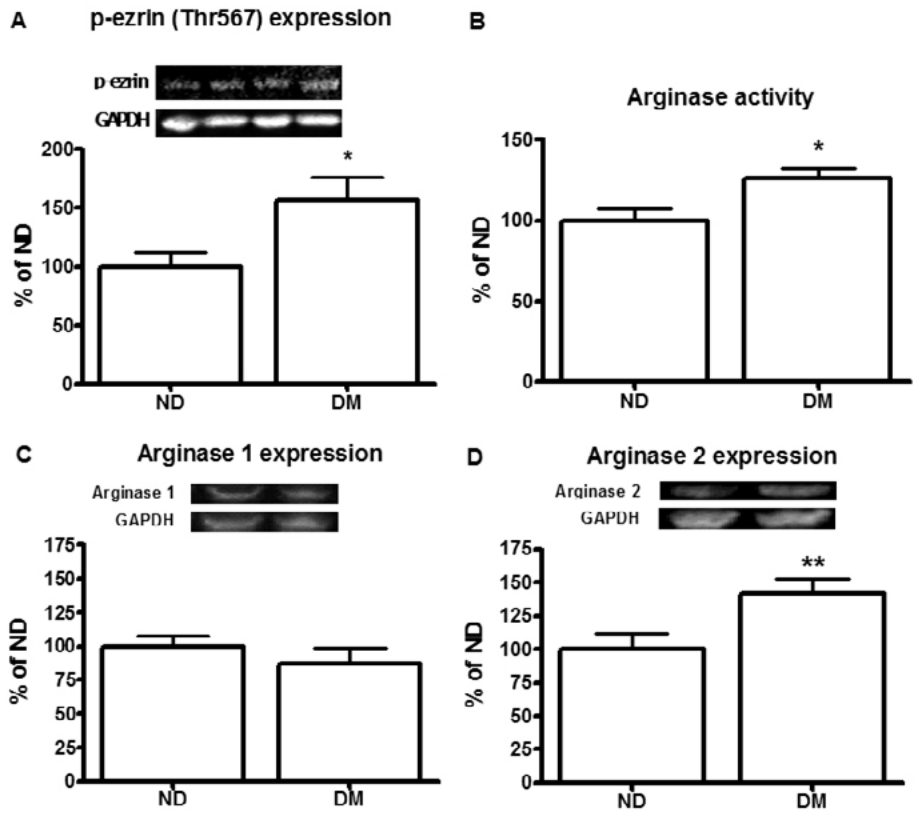

Figure 13. The effect of diabetes on activity of ROCK and arginase and the expression of arginase isoforms. (A) Expression of phosphorylated ezrin (p-ezrin) as a marker of ROCK activity, (B) arginase activity, (C) expression of arginase 1 and (D) arginase 2 in non-ischemic myocardium from non-diabetic (ND) and diabetic rats (DM) including representative immunoblots. Data are shown as mean±SEM; n=5-10; *P<0.05; **P<0.01.

In **Study IV** there were no significant differences in AAR between any of the groups. IS was comparable in the two parts of the study and it did not differ between diabetic control rats and non-diabetic controls. The ROCK inhibitor hydroxyfasudil significantly reduced IS by comparable magnitudes in diabetic and non-diabetic rats from 66±2% and 66±2% to 48±4% and 46±2%, respectively (P<0.001, **Figure 15 A**). Administration of the NOS inhibitor L-NMMA did not influence IS *per se* but abolished the cardioprotective effect of ROCK inhibition in diabetic rats (**Figure 15 A**).

Administration of nor-NOHA significantly reduced IS both in diabetic and non-diabetic rats from 67±3% and 68±3% to 51±3% and 45±4%, respectively (P<0.001, **Figure 15 B**). The NOS inhibitor L-NMMA abolished the cardioprotective effect of nor-NOHA in diabetic rats (**Figure 15 B**).

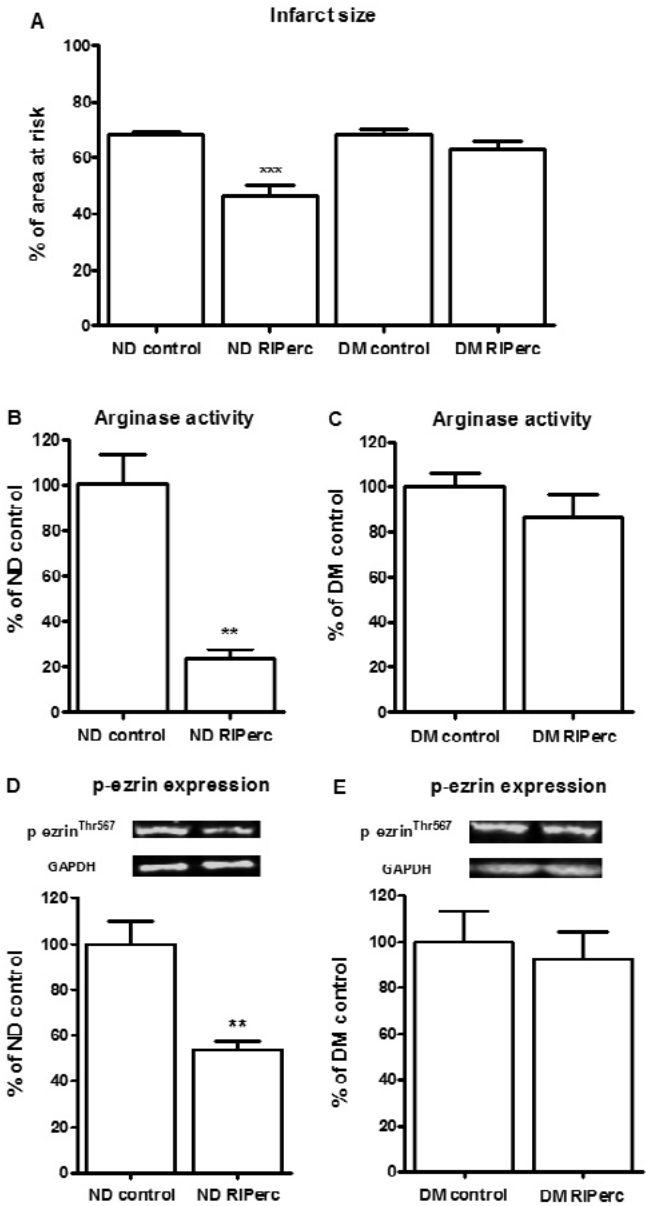

Figure 14. Infarct size (A), arginase activity (B, C) and ROCK activity (D, E) in ischemic-reperfused myocardium of non-diabetic (ND) rats and rats with type 1 diabetes mellitus (DM) subjected to 30 min of ischemia and 2 h of reperfusion. ROCK activity is expressed as phosphorylation of ezrin, representative immunoblots are shown. The rats were exposed to control myocardial ischemia-reperfusion (ND control, A: n=6, B: n=6, D: n=7; DM control, A: n=7, C: n=6, E: n=5) or myocardial ischemia-reperfusion with remote ischemic perconditioning (ND RIPerc, A: n=6, B: n=6, D: n=6; DM RIPerc, A: n=6, C: n=6, E: n=5). Data are presented as mean ± SEM; ***P<0.001 versus ND-control group; **P<0.01.

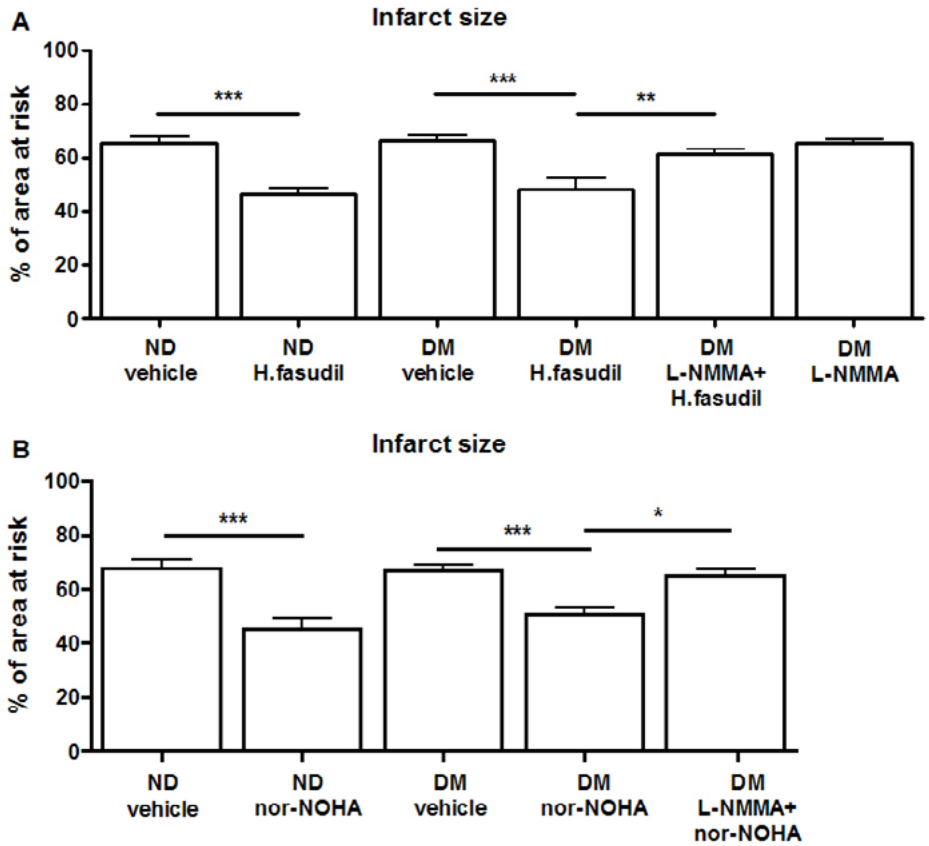

Figure 15. Infarct size in rats subjected to 30 min of myocardial ischemia and 2 h of reperfusion. (A) The non-diabetic (ND) and diabetic (DM) rats were given either saline (vehicle; n=5, DM; n=5, ND); ROCK inhibitor hydroxyfasudil (H.fasudil; n=5, DM; n=5, ND); NO synthase inhibitor L-NMMA and hydroxyfasudil (n=5, DM) or L-NMMA only (n=4, DM).
(B) The non-diabetic (ND) and diabetic (DM) rats were given either saline (vehicle, n=14, DM; n=7, ND); arginase inhibitor nor-NOHA (n=11, DM; n=8, ND) or the NO synthase inhibitor L-NMMA and nor-NOHA (n=7, DM). Data are presented as mean±SEM; *P<0.05; **P<0.01; ***P<0.001.

Effects of remote ischemic perconditioning on expression and activity of arginase, ROCK activity and phosphorylation of eNOS in ischemic-reperfused myocardium

Arginase activity in non-ischemic myocardium was increased by 48±11% (P<0.05) in rats with type 1 diabetes compared to non-diabetic controls. Arginase activity was increased by 70±20 % and 87±11 % in ischemic myocardium in comparison with non-ischemic myocardium of rats with and without type 1 diabetes, respectively (P<0.01). RIPerc significantly reduced arginase activity in ischemic-reperfused myocardium of non-diabetic rats, but not in rats with type 1 diabetes (**Figure 14 B-C**). The expression of arginase isoforms was not affected by RIPerc in non-diabetic rats or in diabetic animals.

RIPerc failed to reduce ROCK activity in ischemic-reperfused myocardium of rats with diabetes in contrast to what was observed in non-diabetic rats (**Figure 14 D-E**).

In contrast to the observation in non-diabetic rats, RIPerc failed to increase the expression of phosphorylated eNOS at Ser1177 in ischemic-reperfused myocardium of rats with type 1 diabetes (**Figure 12 B**).

Effect of ROCK inhibition on arginase activity in ischemic-reperfused myocardium in diabetes

ROCK inhibition significantly attenuated myocardial arginase activity in diabetic rats following IR in the **Study IV**. The effect of ROCK inhibition on arginase activity was reversed by NOS inhibition (**Figure 16**).

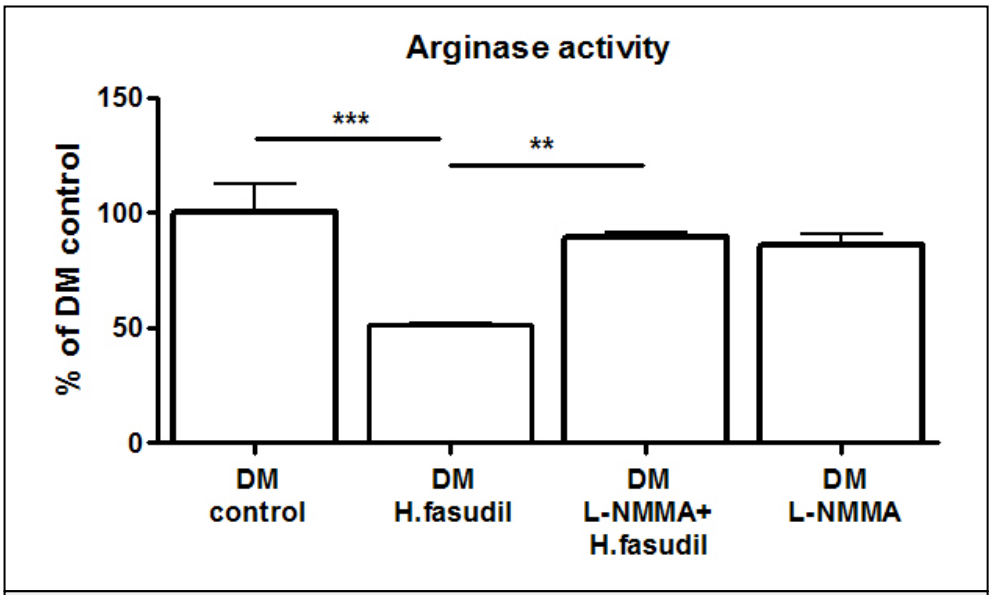

Figure 16. The effect of ROCK inhibition on arginase activity following ischemia-reperfusion. Myocardial arginase activity in rats with diabetes mellitus subjected to control ischemia-reperfusion (DM control), ROCK inhibition by hydroxyfasudil (DM H.fasudil), the combination of NOS inhibition and ROCK inhibition (DM L-NMMA+H.fasudil), and NOS inhibition alone (DM L-NMMA). Data are presented as mean±SEM; n=4-5; **P<0.01; ***P<0.001.

General Discussion

MECHANISMS OF PROTECTION AGAINST MYOCARDIAL ISCHEMIA-REPERFUSION INJURY INDUCED BY ARGINASE INHIBITION

It is known that attenuated NO bioavailability related to endothelial dysfunction is an important mechanism behind the development of IR injury (Cohen et al., 2006) and that NO exerts cardioprotective effect (Sanada et al., 2011). L-arginine is a substrate not only for NOS, but also for arginase which converts L-arginine to L-ornithine and urea (Durante et al., 2007). Increased arginase activity may thereby result in reduced formation of NO due to the competition between NOS and arginase for L-arginine. In addition, high activity of arginase is reported as a trigger of eNOS uncoupling and increased O_2^- production (Kim et al., 2009) – factors which contribute to IR injury (**Figure 17 A**).

The results of **study I** demonstrate in agreement with previous studies (Gonon et al., 2012; Jung et al., 2010) that arginase activity is increased in the myocardium following IR. Importantly, inhibition of arginase by nor-NOHA after the onset of ischemia but before the onset of reperfusion limits the extent of myocardial injury via a mechanism that involves production of NO. In agreement with previous studies (Gonon et al., 2012; Jung et al., 2010), the involvement of NOS and its product NO in cardioprotection induced by arginase inhibition is confirmed by the suppression of cardioprotective effect by pretreatment with the NOS-inhibitor L-NMMA, which does not affect IS *per se* (Gonon et al., 2000). Although L-NMMA inhibits all the isoforms of NOS, the present data indicate that arginase inhibition increases the production of eNOS-derived NO since nNOS is not involved in the development of myocardial IR injury (Jones et al., 2000) and iNOS is not influenced in the brief protocol used in the study (Bulhak et al., 2006).

Previous studies have indicated that the cardioprotective effect of NO includes stimulation of cGMP, activation of PKG, PKCε and subsequent opening of the mitoK$_{ATP}$ channel (Chai et al., 2011; Costa and Garlid, 2008; Sanada et al., 2001). **Study I** shows that arginase inhibition mediates cardioprotection via this signaling pathway. Thus, participation of PKCε in the mechanisms of the myocardial salvage was confirmed by the abolition of the cardioprotective effect of nor-NOHA by the PKCε specific inhibitor ε-V1-2. The inhibition of PKCε did not influence IS *per se*. In addition, **study I** showed remarkable downregulation of PKCε expression after IR. Interestingly, the administration of nor-NOHA maintained high expression of PKCε in the ischemic-reperfused myocardium. We further demonstrated that the selective mitoK$_{ATP}$ channel blocker 5-HD completely blocked the cardioprotective effect of arginase inhibition by nor-NOHA indicating that its mechanism depends on the activation of mitoK$_{ATP}$ channels. Previous studies established that 5-HD does not affect IS *per se* (Fryer et al., 2001). Collectively, the data provided in the present study indicate that arginase inhibition protects the myocardium from IR injury by increasing NO production, activation of PKCε followed by the activation of the mitoK$_{ATP}$ channels (**Figure 17 B**).

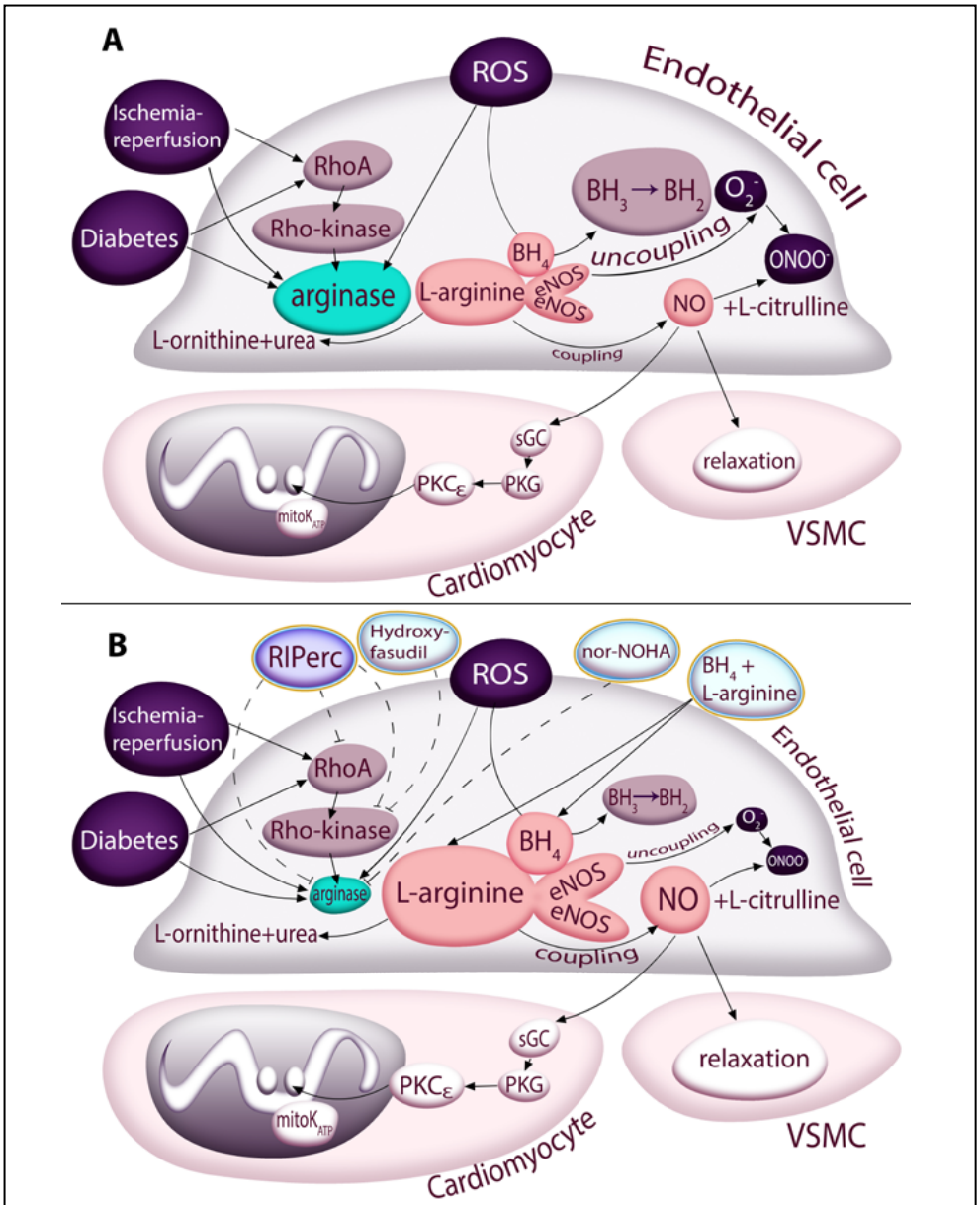

Figure 17. (A) Decrease in endothelial NO availability induced by myocardial ischemia-reperfusion and diabetes. (B) Restoration of endothelial NO availability by arginase inhibition, ROCK inhibition, supplementation of BH_4 and L-arginine or remote ischemic perconditioning (RIPerc). BH_2 – dihydrobiopterin; BH_3 – trihydrobiopterin; BH_4 – tetrahydrobiopterin; eNOS – endothelial nitric oxide synthase; $mitoK_{ATP}$ – mitochondrial adenosine triphosphate dependent potassium channel; NO – nitric oxide; O_2^- – superoxide; $ONOO^-$ – peroxynitrite; PKCε – protein kinase C epsilon; PKG – protein kinase G; ROS – reactive oxygen species; sGC – soluble guanylyl cyclase; VSMC – vascular smooth muscle cell. Arrows with solid lines – stimulation or production; dashed lines with perpendicular on the edge – inhibition.

In **study I** nor-NOHA significantly reduced IS when administered systemically before the onset of reperfusion simulating a clinically relevant scenario. Therefore, this treatment may be effective when given in the clinical setting by iv administration in combination with revascularization in patients with ST-elevation myocardial infarction. The results obtained in a large animal study of myocardial IR and arginase inhibition before and during reperfusion show that local intracoronary administration of nor-NOHA significantly reduced IS in pigs (Gonon et al., 2012). Collectively, the available data suggest that both iv and intracoronary administration of an arginase inhibitor before the onset of reperfusion exerts clear cardioprotective effects in the setting of myocardial IR. These observations may form the basis for clinical studies to investigate the efficiency of arginase inhibition as a therapeutic strategy in patients with ST-elevation myocardial infarction.

ROLE OF L-ARGININE AND TETRAHYDROBIOPTERIN FOR THE PROTECTION AGAINST MYOCARDIAL ISCHEMIA-REPERFUSION INJURY.

Decreased availability of the NOS co-factor BH_4 and the substrate L-arginine contribute to NOS uncoupling, resulting in low NO production and increased O_2^- formation (Gorren et al., 1996; Roe and Ren, 2012) (**Figure 17 A**). Previous studies have demonstrated that myocardial BH_4 levels are reduced during ischemia due to oxidation which contributes to post-ischemic endothelial dysfunction (Dumitrescu et al., 2007) and that systemic pretreatment of rats with BH_4 prior to ischemia results in cardioprotective effect *in vivo* (Wajima et al., 2006).

Study II shows in two animal models, that combined administration of L-arginine and BH_4 before and during reperfusion results in significant reduction of IS following myocardial IR. BH_4 co-administered with L-arginine systemically to rats shortly before reperfusion evoked cardioprotective effect. In contrast, individual administration of either BH_4 or L-arginine failed to reduce IS. The pig was included as a second experimental model in the **study II** since its cardiac anatomy and physiology is similar to the human heart (Hughes et al., 2003). Intracoronary infusion of substances via a catheter in the left main coronary artery resembles a clinical situation in patients with ST-elevation myocardial infarction undergoing primary PCI. Intracoronary infusion of the combination of BH_4 and L-arginine starting before reperfusion significantly reduced IS. On the other hand, the same doses of BH_4 and L-arginine infused systemically did not induce cardioprotection. This observation suggests that the cardioprotective effect of intracoronary infusion was mediated via a local effect in the ischemic-reperfused myocardium. In agreement with the data obtained in rats, individual administration of BH_4 or L-arginine did not exert a significant cardioprotective effect in pigs. In contrast to previous data (Weyrich et al., 1992; Pernow et al., 2003), **study II** demonstrated that L-arginine administered proximally to the point of occlusion did not protect from IR injury. However, the results of the **study II** are in agreement with an earlier study in pigs in which systemic administration of L-arginine alone did not reduce IS (Gonon et al., 2011). The reason for the inefficiency of L-arginine in reducing IS may be due to increased metabolism by arginase, which is activated during IR (**study I**), and/or due to a decreased cellular uptake of L-arginine (Venardos et al., 2009). Administration of BH_4 alone did not reduce IS either when administered iv in rats or by intracoronary infusion in pigs, despite a clear increase in myocardial BH_4 levels in pigs. In addition, only the combined administration of BH_4 and L-arginine reduced myocardial O_2^- accumulation in the pig study.

This observation indicates that the cardioprotective effect of BH_4 and L-arginine was not achieved due to known antioxidant properties of BH_4 (Mayahi, et al., 2007), but was attributed to the reversal of NOS uncoupling and the increase of NO availability achieved by the combined administration of BH_4 and L-arginine (**Figure 17 B**). The involvement of NOS in the cardioprotective mechanisms of BH_4 combined with L-arginine was confirmed by the finding that the NOS inhibitor L-NMMA abolished the reduction of IS in rats and pigs.

THE INTERACTION BETWEEN THE ROCK PATHWAY AND ARGINASE IN MYOCARDIAL ISCHEMIA-REPERFUSION

Activation of arginase during myocardial IR and the cardioprotective effect of arginase inhibition in the setting of IR have been reported using different protocols and animal models (Gonon et al., 2012; Jung et al., 2010; **study I**). These studies not only provided evidence for the cardioprotective efficacy of arginase inhibition, but also established its mechanisms using a diversity of pharmacological tools (inhibition of NOS and PKCε, NO scavenging, selective blocking of mitoK_{ATP} channel) and analysis of protein expression and enzyme activity. However, the upstream signaling regulating arginase in the ischemic-reperfused myocardium has remained unknown.

Study III demonstrated that $ONOO^-$ is an activator of ROCK which, in turn, stimulates the activity of arginase during myocardial IR. This was confirmed by the administration of the $ONOO^-$ decomposition catalyst FeTPPS which significantly reduced both ROCK and arginase activity as well as IS. The role of ROCK pathway in arginase activation was additionally confirmed by inhibition of ROCK with hydroxyfasudil (**Figure 17 B**) which reduced IS and myocardial arginase activity following IR. These results are in agreement with the previously reported activation of ROCK by $ONOO^-$ in endothelial cells and activation of endothelial arginase via the ROCK pathway (Chandra et al., 2012; Ming et al., 2004). In contrast to the activity of arginase, the expression of its isoforms was not changed significantly either after $ONOO^-$ decomposition or after ROCK inhibition, indicating that alterations in myocardial arginase activity are not dependent on its protein levels which is in accordance with previous observations (Gonon et al., 2012).

THE ROLE OF ROCK AND ARGINASE IN CARDIOPROTECTIVE EFFECT OF REMOTE ISCHEMIC PERCONDITIONING

The additional aim of **study III** was demonstration of the cardioprotective effect of RIPerc and the involvement of ROCK and arginase for NO availability after RIPerc. Several experimental and clinical studies suggest that RIPerc is an effective therapeutic strategy to protect from IR injury (Schmidt et al., 2007; Bøtker et al., 2010). However, the mechanisms behind the transfer of the protection from the remote organ and the cardioprotective signaling within the myocardium are a matter of debate. **Study III** clearly showed the reduction of IS induced by RIPerc and that this effect was associated with decreased myocardial ROCK activity in accordance with the previous observation (Zhao et al., 2009). We further demonstrated diminished content of 3-nitrotyrosine in the ischemic-reperfused myocardium following RIPerc suggesting attenuated formation of the ROCK activator $ONOO^-$. Of further importance, RIPerc evoked significant reduction of arginase activity in the myocardium at risk, which is in accordance with the established regulation of arginase activity via $ONOO^-$

and ROCK in a setting of myocardial IR as described above. Finally, the involvement of increased NO availability in the cardioprotection, triggered by RIPerc was confirmed by the finding that pharmacological inhibition of NOS activity abolished the cardioprotective effect of RIPerc. The key role of eNOS in protection against IR injury by RIPerc was supported by the increased phosphorylation of eNOS after RIPerc. Collectively, the presented data suggest that RIPerc protects from IR injury via a mechanism that is dependent on production of eNOS-derived NO via a decrease in ROCK activity and arginase activity (**Figure 17 B**).

THE IMPORTANCE OF DIABETES FOR CARDIOPROTECTIVE EFFECTS IN MYOCARDIAL ISCHEMIA-REPERFUSION

Since patients with diabetes have an increased risk of ischemic events and high probability for unfavorable outcomes after coronary revascularization (Galiñanes and Fowler, 2004), it is of importance to investigate the therapeutic effects of cardioprotective strategies in diabetes. Based on the observations that diabetes is associated with elevated arginase activity and low availability of NO resulting in reduced endothelial function (Guerci et al., 2001; Romero et al., 2008), and that arginase is activated via the ROCK pathway in diabetes (Yao et al., 2013), we hypothesized that targeting of ROCK may be beneficial in myocardial IR in a model of diabetes as co-morbidity. Indeed, **studies III and IV** collectively demonstrated that induction of type 1 diabetes leads to elevated in ROCK and arginase activity, and increased expression of arginase 2 in non-ischemic myocardium. Similar to the observations in non-diabetic rats in **study III**, ROCK inhibition reduced IS in a magnitude comparable to the control animals without diabetes. The cardioprotective effect of the ROCK inhibitor was associated with a significant reduction of arginase activity in the ischemic-reperfused myocardium, supporting the role of ROCK in the regulation of arginase (**Figure 17 B**). Pharmacological inhibition of arginase by nor-NOHA in diabetic animals evoked significant decrease in IS to an extent comparable to that observed in rats without diabetes. The reduction of IS by both ROCK and arginase inhibition in diabetic rats was abolished by administration of the NOS inhibitor L-NMMA, confirming the involvement of increased NO availability in the mechanisms of cardioprotection induced by hydroxyfasudil or nor-NOHA. In addition, NOS inhibition prevented the decrease in myocardial arginase activity induced by the ROCK inhibitor in ischemic-reperfused myocardium of diabetic rats, indicating a shift of L-arginine consumption from NOS to arginase. A possible modification of arginase activity by L-NMMA *per se* is unlikely since administration of L-NMMA to diabetic rats subjected to myocardial IR did not affect arginase activity.

Study III demonstrated that the cardioprotection induced by RIPerc observed in non-diabetic animals was completely lost in animals with type 1 diabetes. These observations, which are in agreement with those previously reported for local preconditioning and postconditioning (Przyklenk, 2011), suggest that the diabetic heart is resistant to endogenous cardioprotective stimuli. It is established that diabetes impairs regulation of NOS signaling pathway (Tsang et al., 2005; Przyklenk, 2011) and that vascular dysfunction in diabetes is associated with elevated vascular and endothelial cell ROCK and arginase activity (Romero et al., 2008; Zhou and Li, 2012). In accordance with these observations, data from **study III** show that RIPerc did not phosphorylate myocardial eNOS and did not reduce myocardial ROCK activity and arginase activity during myocardial ischemia in diabetic rats. Thus, these data demonstrate that RIPerc, which induced marked reduction in IS in non-diabetic rats, failed

to reduce IS and to activate the cardioprotective signaling mechanisms in the presence of type 1 diabetes. This is in contrast to the successful cardioprotection against myocardial IR injury in type 1 diabetes obtained in **study IV** by pharmacological inhibition of either ROCK or arginase. Therefore, pharmacological inhibition of arginase or ROCK seems to be a more promising therapeutic strategy than RIPerc to reduce IS in the setting of diabetes.

LIMITATIONS

Certain limitations should be acknowledged regarding the studies included in this thesis. In all the studies open chest animal model of myocardial IR was used which, of course, could affect the results due to the inflammatory response following tissue damage. However, it is unknown whether effects of applied interventions differ between a clinically more relevant setting of closed chest from those obtained using the open chest model. The advantage with the open chest model is that coronary blood flow can be continuously monitored for confirmation of complete coronary artery ligation and successful reperfusion.

Most of the experiments were performed on relatively homogenous and young populations of laboratory animals. Most patients with ST-elevation myocardial infarction have one or several co-morbidities that may interfere with the cardioprotective treatment. In **studies III and IV** we therefore used animals with pathophysiological alterations caused by induction of type 1 diabetes and were able to demonstrate that this co-morbidity affects the results. More pronounced heterogeneity of the patients in clinical setting is expected to cause distinctions from the preclinical studies. There was no significant difference in the vulnerability to myocardial IR between type 1 diabetes and non-diabetic rats. This may appear surprising but is in agreement with several studies which did not detect changes in IS in the presence of diabetes, although other studies have described both smaller and larger IS in various animal models of diabetes (Miki et al., 2012). The reasons for the apparent inconsistency regarding the extent of myocardial IR injury in diabetes remain unclear. It is of importance that we investigated the efficacy of cardioprotective interventions which clearly were influenced by the presence of diabetes as demonstrated by the lack of cardioprotection by RIPerc in the diabetic animals.

In our studies we were not able to reveal whether the activity of any specific arginase isoform is of particular importance during myocardial IR injury since specific arginase inhibitors are not available. Partially this can be investigated in the future studies using arginase 2 knockout mice. Deletion of the gene encoding for arginase 1 results in a lethal phenotype due to ammonia toxicity. However, endothelial cell arginase 1 conditional knockout models may be useful for the understanding of the arginase 1 isoform.

We demonstrated that the reduction in arginase and ROCK activity is associated with the cardioprotective effect of RIPerc. Additional studies are needed to confirm whether this mechanism is a mediator of cardioprotection by RIPerc or not, and to clarify the lack of protection against myocardial IR following RIPerc in type 1 diabetes.

The results of the studies obtained in a model of type 1 diabetes cannot be translated to type 2 diabetes *a priori*. Therefore further experiments are needed in order to validate the effects of the reported cardioprotective interventions in type 2 diabetes.

FUTURE PERSPECTIVES

Several issues remain to be solved in future. Some of them were already mentioned in the previous chapter discussing limitations of the studies and include disclosure of arginase isoforms role in myocardial IR, relevance of the observed cardioprotective effects in type 2

diabetes, further clarification of the mechanisms behind RIPerc. In addition, supplementation of the co-factor BH_4 and L-arginine is of interest to be tested in diabetes.

Novel therapeutic strategies, suggested in the thesis, should be verified in clinical trials of myocardial IR in patients with ST-elevation myocardial infarction. It is known that many previous clinical studies were unable to reproduce cardioprotective effects obtained in animal experiments. The present study underlines the importance of co-morbidities for effective cardioprotective therapy. RIPerc evoked clear reduction in IS in non-diabetic animals but was ineffective in a model of diabetes. Interestingly, it was shown that arginase inhibition reduced IS not only in healthy animals, but also in the presence of diabetes, indicating that this strategy may be effective also in a clinical setting of myocardial infarction in patients with co-morbidities. The influence of other co-morbidities and confounding factors including atherosclerosis, hyperlipidemia, age, smoking and medication on cardioprotective treatment in IR need to be carefully investigated. Ideally, more sophisticated experimental models which combine several such factors in one animal would be an attractive tool to simulate the clinically relevant situation when myocardial infarction occurs in aged subjects with co-morbidities and background medication.

In addition, optimal way of drug administration should be considered in patients. Systemic iv administration of cardioprotective compounds may not reach the myocardium at risk unless collateral flow is adequate. Local intracoronary infusion in connection with reperfusion as employed in **Study II** seems promising and may be applied together with primary PCI in clinical studies.

CONCLUSIONS

1. Inhibition of arginase before the onset of reperfusion reduces IS via a mechanism dependent on NOS activity, PKCε expression and activation of mitoK_{ATP} channels.

2. Combined administration of L-arginine and BH_4 during late ischemia and reperfusion reduces IS via a NOS-dependent pathway and reduced generation of superoxide.

3. Peroxynitrite and ROCK signaling pathways are involved in the upregulation of arginase activity during myocardial IR. Reduction in arginase activity mediated by reduced formation of peroxynitrite and ROCK activity is of importance for the NOS-dependent cardioprotective effect of RIPerc.

4. Inhibition of either ROCK or arginase protects against myocardial IR injury via a NOS–dependent mechanism both in the presence and absence of type 1 diabetes. Cardioprotective effect of RIPerc and associated signaling effects on arginase, ROCK and NOS are abolished in type 1 diabetes.

Collectively the results demonstrate the importance of the ROCK-arginase pathway and availability of BH_4 and L-arginine for protection against myocardial IR injury mediated by NO. Arginase inhibition, supplementation of BH_4 and L-arginine, inhibition of ROCK and RIPerc are potentially effective therapeutic tools for the limitation of myocardial injury in the treatment of acute myocardial infarction. Diabetes is a co-morbidity that importantly limits the cardioprotective efficacy of molecular signaling induced by RIPerc.

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Arginase inhibition reduces infarct size via nitric oxide, protein kinase C epsilon and mitochondrial ATP-dependent K^+ channels

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ABSTRACT

Reduced bioavailability of nitric oxide (NO) contributes to the development of myocardial ischemia-reperfusion (I/R) injury. Increased activity of arginase is a potential factor that reduces NO bioavailability by competing for the substrate L-arginine. The aim of the study was to test the hypothesis that inhibition of arginase after coronary artery occlusion protects from I/R injury and to explore possible mechanisms behind this effect.

Male Sprague-Dawley rats subjected to 30 min of coronary artery ligation and 2 h reperfusion were given i.v. before the reperfusion: 1) saline; 2) the arginase inhibitor N-omega-hydroxy-nor-L-arginine (nor-NOHA); 3) nor-NOHA with the NO synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA); 4) nor-NOHA with the mitochondrial ATP-dependent K^+ ($mitoK_{ATP}$) channel blocker 5-hydroxydecanoic acid (5-HD); 5) nor-NOHA with the protein kinase C epsilon (PKCε) inhibitor ε-V1-2 or 6) ε-V1-2 alone.

Infarct size in the control groups was $61 \pm 3\%$ and it was reduced to $47 \pm 3\%$ ($P < 0.01$) by nor-NOHA. The cardioprotective effect was blocked by the NOS inhibitor L-NMMA. PKCε expression was reduced by I/R and this reduction was attenuated by nor-NOHA. Furthermore, the PKCε inhibitor ε-V1-2 abolished the protective effect of nor-NOHA (infarct size $69 \pm 6\%$). In addition, the cardioprotective effect of nor-NOHA was also abolished following blockade of the $mitoK_{ATP}$ channel (infarct size $62 \pm 1\%$).

Inhibition of arginase before reperfusion protects the heart from I/R injury via a NOS-dependent pathway, increased expression of PKCε and activation of $mitoK_{ATP}$ channels.

1. Introduction

Current treatment of acute myocardial infarction due to coronary artery occlusion is aimed at initiating reperfusion to minimize myocardial damage. Early revascularization rescues the myocardium at risk and limits infarct size, which improves the outcome for the patient. Even though the blood supply is restored, reperfusion itself initiates alterations that are deleterious and enhance cellular injury, a phenomenon referred to as ischemia-reperfusion (I/R) injury which is characterized by oxidative stress, activation of inflammatory processes, and dysfunction of the microvascular endothelium (Lemasters et al., 1996; Turer and Hill, 2010). A key mechanism behind endothelial dysfunction is reduction of nitric oxide (NO) bioavailability (Cohen et al., 2006) NO produced from the substrate L-arginine by NO synthase (NOS) is a potent vasodilator (Johnson et al., 1991), inhibits apoptosis (Li et al., 1999) and neutrophil adherence to vascular endothelium (Ma et al., 1993), prevents platelet aggregation (Walter and Gambaryan, 2009) and is a scavenger of superoxide (McCall et al., 1989). One of the intracellular pathway via which NO may mediate protection from I/R injury is by activation of guanylate cyclase which in turn activates protein kinases G and C which leads to opening of the mitochondrial ATP-dependent K^+ ($mitoK_{ATP}$) channels (Heusch et al., 2008).

An alternative metabolic pathway for L-arginine that has attracted recent interest is the arginase-dependent dissociation to ornithine and urea (Durante et al., 2007, Ryoo et al., 2008). Upregulation of arginase activity may lead to reduction of L-arginine available for NO production and decreased endothelial function (Vanhoutte, 2008). It has been demonstrated that arginase expression and activity are increased in various pathophysiological cardiovascular situations including oxidative stress, atherosclerosis and ischemia (Gonon et al., 2012; Hein et al., 2003; Ryoo et al., 2011; Thengchaisri et al., 2006). It has recently been demonstrated that increased myocardial arginase expression

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and activity are involved in the development of I/R injury supporting an important functional role of arginase in this condition (Gonon et al., 2012; Jung et al., 2010). However, these previous studies have not addressed the mechanisms underlying the cardioprotective effects following arginase inhibition during I/R injury. Thus, the signaling pathway remains to be clarified. Therefore, the aim of the present study was to explore the mechanism behind the protection against I/R injury induced by arginase inhibition *in vivo*. The study was designed to test the hypothesis that arginase inhibition mediates protection against myocardial I/R injury via increased formation of NO that results in activation of protein kinase C epsilon (PKCε) and opening of the mitoK$_{ATP}$ channels.

2. Materials and methods

2.1. Experimental protocol

The study was 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).

Male Sprague-Dawley rats (Charles-River, Sulzfeld, Germany, 260–400 g body weight) were anesthetized with pentobarbital (50 mg/kg) i.p. followed by an i.v. infusion of 5 mg/kg/h pentobarbital and 30 μg/kg/h fentanyl. The absence of the hind paw withdrawal reflex was used as a reference level of anesthesia. Rectal temperature was maintained at 37.5–38.5 °C by a heated operation table. Animals were tracheotomized and ventilated with air by a rodent pressure controlled ventilator (50–55 strokes per min, 9 mL/kg tidal volume). The right carotid artery was cannulated and connected to a Statham blood pressure transducer for measurement of mean arterial pressure. The heart rate was determined from the arterial pressure curve. The left jugular vein was cannulated for administration of drugs. The heart was exposed via a left thoracotomy. A ligature was placed around the left coronary artery. After completion of the surgical preparation, the rats were allowed to stabilize for 15 min and randomized into different groups. Ischemia was induced by tightening of the ligature around the left coronary artery. Successful occlusion was associated with cyanosis of the myocardial area at risk and arrhythmias after 5–15 min of ischemia. Reperfusion was initiated after 30 min of ischemia by removal of the snare and was maintained for 2 h.

In an initial part of the study rats were randomized to the following groups given: 1) saline (400 μL for infarct size determination, $n=10$); 2) saline (400 μL, for determination of arginase activity, $n=9$); 3) the arginase inhibitor N-omega-hydroxy-nor-L-arginine (nor-NOHA, 100 mg/kg, $n=8$); 4) the NOS inhibitor N^G-monomethyl-L-arginine monoacetate (L-NMMA, 10 mg/kg) followed by nor-NOHA ($n=6$) or 5) the mitoK$_{ATP}$ channel blocker 5-hydroxydecanoic acid sodium (5-HD, 10 mg/kg) followed by nor-NOHA ($n=6$).

In a second part of the study, additional rats were randomized to vehicle ($n=13$) or nor-NOHA ($n=10$) as above and to the administration of 1) the specific PKCε inhibitor ε-V1-2 (3 mg/kg) followed by nor-NOHA ($n=6$) or (2) ε-V1-2 only (3 mg/kg, $n=4$). A separate sham group ($n=5$) was subjected to the same procedures except that the ligature placed around the left coronary artery was not tightened and compounds were not administered.

Saline and all substances were given i.v. as bolus injections at 15 min of ischemia except 5-HD and ε-V1-2 which were injected i.v. at 10 min of ischemia. The dosages were based on previous studies (Deuse et al., 2010; Gourine et al., 2005; Jung et al., 2010). L-NMMA and 5-HD have previously been shown not to affect infarct size *per se* (Fryer et al., 2001; Gonon et al. 2000). Since the effect of ε-V1-2 on infarct size *per se* is less documented, it was administered to separate group of animals subjected to I/R.

2.2. Determination of infarct size and collection of myocardium samples

Infarct size was measured as previously described (Bulhak et al., 2007). After 2 h of reperfusion, the coronary artery was reoccluded and 1.5 mL of 2% Evans blue solution was injected via the left jugular vein to stain non-ischemic myocardium for determination of the area at risk. Evans blue was not administered to sham-operated animals. The rats were euthanized by exsanguination and the heart was extracted. The right atrium, the right ventricle and the left atrium were removed, the left ventricle was frozen and cut into 7–9 slices perpendicular to the base-apex axis. The third slice (counting from the apex) was weighed, the ischemic and non-ischemic parts separated, frozen on dry ice and kept in freezer (−80 °C) until analyzed. All other slices were weighed, scanned from both sides for the determination of the area at risk and put in 1% triphenyltetrazolium chloride solution for 15 min at 37 °C to distinguish viable myocardium from necrotic. After 24 h of incubation in 4% formaldehyde slices were scanned again from both sides, and the extent of myocardial necrosis and the area at risk were determined by planimetry of computer images (Adobe Photoshop CS 2; Adobe Systems). The left ventricle obtained from sham-operated animals was also cut into slices which were frozen on dry ice and stored frozen (−80 °C) until further analyses.

2.3. Arginase activity determination

Arginase activity was determined by using a colorimetric assay previously described (Berkowitz et al., 2003). The assay measures the urea content using α-isonitrosopropiophenone. In order to detect arginase activity only the inhibitable fraction of urea was used in the analysis. A lysis buffer consisting of PBS, 2 mM EDTA, Triton X (0.1%) and protease inhibitors (Roche, Basel, Switzerland) was freshly prepared. Lysates of homogenized myocardial tissue was centrifuged for 15 min at 10,000 g at 4 °C. 50 μL of the supernatant was 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 with: 1) L-arginine (50 μL, 0.05 M, in Tris–HCl pH 9.7), 2) only Tris–HCl (50 μL, pH 9.7) and 3) L-arginine (50 μL, 0.05 m, in Tris–HCl pH 9.7) and 30 min preincubation with the arginase inhibitor 2(S)-amino-6-boronohexanoic acid (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 α-sonitrosopropiophenone (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 540 nm using spectrophotometry. The urea inhibitable fraction was then calculated and used in statistical analyses.

2.4. Immunoblotting

Frozen samples of ischemic-reperfused myocardium of vehicle and nor-NOHA treated rats and frozen samples of myocardium obtained from sham-operated animals were used for the evaluation of PKCε by immunoblotting. 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 5.000 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 per lane) were loaded onto a 10% SDS gel and separated by electrophoresis. Extracts were loaded on one gel and the amount of protein was accordingly compared pairwise. Proteins were transferred to PVDF membranes, and Ponceau staining was used to confirm efficiency of transfer and to visualize protein loading. Membranes were blocked overnight at 4 °C, and then probed with antibodies against PKCε (BD Transduction Laboratories, Franklin Lakes, NJ, USA). Proteins were visualized by enhanced chemiluminescence with ECL advance Western blotting detection kit (Amersham Biosciences, Amersham, UK) and quantified using densitometry and Quantity One 4.5.1 software (Bio-Rad Laboratories, Inc., Hercules, *CA*, USA). Finally membranes were stripped and immunoblotted for GAPDH (Sigma-Aldrich, St. Louis, MO, USA) as loading control.

2.5. Chemicals

Pentobarbital was purchased from Apoteksbolaget (Stockholm, Sweden); fentanyl from B. Braun Meslungen AG (Meslungen, Germany); nor-NOHA from Bachem (Bubendorf, Switzerland); L-NMMA from Alexis Biochemicals (Lausen, Switzerland); 5-HD, Evans blue, triphenyltetrazolium chloride, Trizma base and Trizma hydrochloride from Sigma-Aldrich (St. Louis, MO, USA); ε-V1-2 from AnaSpec, Inc (Fremont, CA, USA).

2.6. Statistical analysis

Data were analyzed using GraphPad Prism 4.00 (GraphPad Software, Inc). Data are presented as mean ± S.E.M. or mean and individual measured data points. Groups were compared using one-way ANOVA together with Newman-Keuls multiple comparison post test. Unpaired t-test was used for comparisons of only two groups. $P < 0.05$ was considered statistically significant.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed on the manuscript as written.

3. Results

3.1. Hemodynamics

Mean arterial pressure and heart rate during the experimental procedure in all groups are presented in Tables 1 and 2. In comparison with the control group there was no significant difference in mean arterial pressure or heart rate except a lower heart rate in the group given 5-HD and nor-NOHA at 30 and 60 min of reperfusion and higher mean arterial pressure at 60 min of reperfusion in the sham operated group.

3.2. Infarct size

The two parts of the study are represented in Figs. 1 and 2, respectively. There were no significant differences in the area at risk between any of the groups (Figs. 1 and 2A). In the first part of the study, infarct size in the control group was 61 ± 3% (Fig. 1B). Administration of nor-NOHA significantly reduced infarct size to 47 ± 3% ($P < 0.01$). Administration of the NOS inhibitor L-NMMA or the mitoK_{ATP} channel blocker 5-HD completely abolished the cardioprotective effect of nor-NOHA (Fig. 1B). In the second part of the study the effect of the PKCε inhibitor ε-V1-2 was investigated. Administration of ε-V1-2 did not affect infarct size *per se* but it abolished the cardioprotective effect of nor-NOHA (Fig. 2B).

Table 1
Hemodynamics.

Group	Parameter	15 min before ischemia	Before reperfusion	30 min reperfusion	60 min reperfusion	120 min reperfusion
Vehicle	MAP	129 ± 3	89 ± 5	83 ± 5	75 ± 3	70 ± 5
	HR	464 ± 18	453 ± 6	443 ± 9	417 ± 10	400 ± 9
nor-NOHA	MAP	123 ± 6	80 ± 5	74 ± 4	67 ± 3	66 ± 5
	HR	491 ± 13	478 ± 14	451 ± 10	438 ± 11	408 ± 15
L-NMMA+nor- NOHA	MAP	132 ± 5	93 ± 10	78 ± 7	71 ± 5	68 ± 11
	HR	479 ± 8	448 ± 15	428 ± 15	423 ± 11	395 ± 6
5-HD+nor-NOHA	MAP	105 ± 10	83 ± 7	71 ± 5	63 ± 4	61 ± 3
	HR	415 ± 19	433 ± 19	398 ± 15[a]	372 ± 10[a]	359 ± 13
Sham operated	MAP	110 ± 11	105 ± 9	94 ± 9	91 ± 6[a]	86 ± 6
	HR	415 ± 23	432 ± 15	419 ± 12	407 ± 14	404 ± 14

MAP: Mean arterial pressure (mmHg); HR: Heart rate (bpm). Significant differences from the vehicle group are indicated. Data are mean ± S.E.M. Significant differences from vehicle are indicated.

[a] $P < 0.05$.

Table 2
Hemodynamics.

Group	Parameter	15 min before ischemia	Before reperfusion	30 min reperfusion	60 min reperfusion	120 min reperfusion
Vehicle	MAP	132 ± 3	95 ± 5	83 ± 4	76 ± 3	72 ± 5
	HR	467 ± 14	459 ± 6	441 ± 8	417 ± 9	400 ± 9
nor-NOHA	MAP	119 ± 6	87 ± 6	79 ± 4	69 ± 4	68 ± 4
	HR	478 ± 13	475 ± 11	448 ± 8	437 ± 9	409 ± 12
ε-V1-2+nor-NOHA	MAP	114 ± 9	79 ± 7	77 ± 6	70 ± 7	66 ± 3
	HR	470 ± 14	451 ± 19	424 ± 27	399 ± 22	377 ± 23
ε-V1-2	MAP	131 ± 3	100 ± 4	91 ± 4	86 ± 9	79 ± 4
	HR	476 ± 16	453 ± 16	435 ± 14	407 ± 12	388 ± 11

MAP: Mean arterial pressure (mmHg); HR: Heart rate (bpm). Data are mean ± S.E.M.

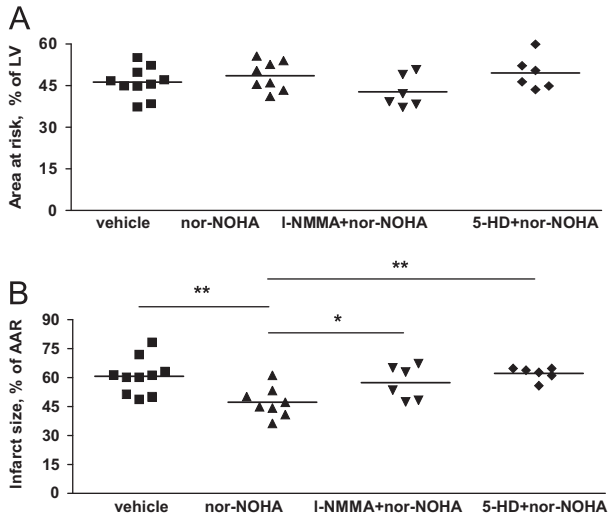

Fig. 1. (A) Area at risk (AAR) expressed as % of the left ventricle (LV) and (B) infarct size expressed as % of AAR after 30 min of ischemia and 2 h of reperfusion. The rats were given either saline (vehicle, $n=10$), the arginase inhibitor nor-NOHA ($n=8$), nor-NOHA together with the NOS inhibitor L-NMMA ($n=6$), nor-NOHA together with the selective mitoK$_{ATP}$ channel antagonist 5-HD ($n=6$). Date are presented as mean and individual data points; $^{*}P<0.05$; $^{**}P<0.01$.

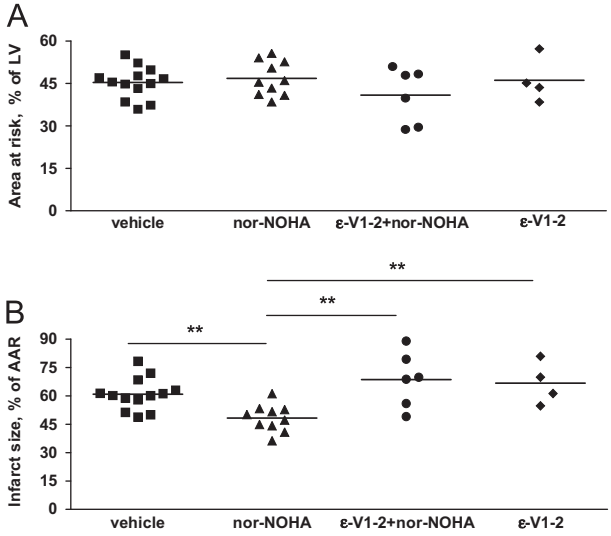

Fig. 2. (A) Area at risk (AAR) expressed as % of the left ventricle (LV) and (B) infarct size expressed as % of AAR after 30 min of ischemia and 2 h of reperfusion. The rats were given either saline (vehicle, $n=13$), the arginase inhibitor nor-NOHA ($n=10$), nor-NOHA together with the PKCε specific inhibitor ε-V1-2 ($n=6$) or ε-V1-2 only ($n=4$). Data are presented as mean and individual data points; $^{**}P<0.01$.

3.3. Myocardial arginase activity

In order to evaluate the effect of I/R on arginase activity, it was measured in ischemic and non-ischemic myocardium of animals given saline. Arginase activity in ischemic-reperfused myocardium was more than 2 times higher than in non-ischemic myocardium (Fig. 3).

3.4. Myocardial protein kinase C epsilon expression

Expression of PKCε was markedly reduced in ischemic-reperfused myocardium compared to non-ischemic myocardium of sham-operated animals. The expression of PKCε was 7 times higher ($P<0.01$) in ischemic-reperfused myocardium of the rats treated with nor-NOHA than in those given vehicle (Fig. 4).

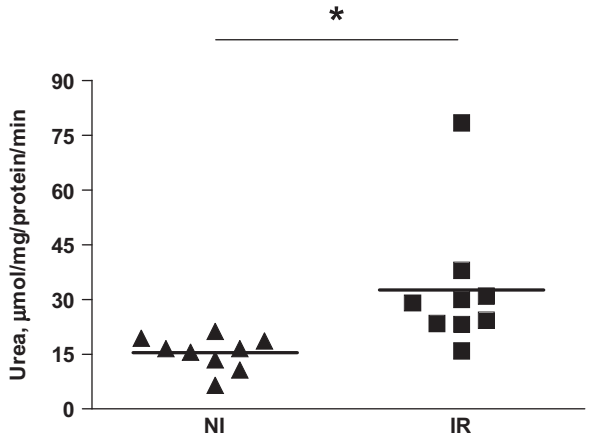

Fig. 3. Arginase activity in ischemic-reperfused (I/R) and in non-ischemic (NI) myocardium of rats subjected to i.v. injection of vehicle ($n=9$). Data are presented as mean and individual data points; $^{*}P<0.05$.

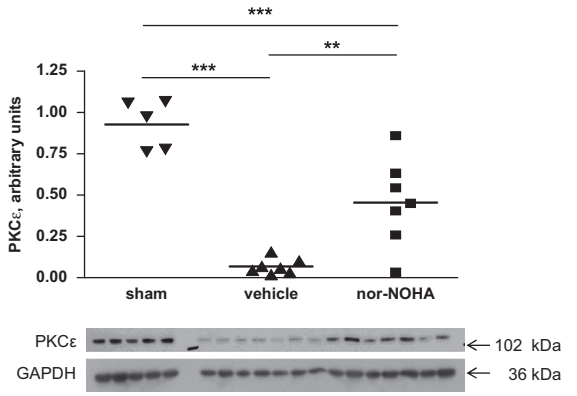

Fig. 4. Quantification and immunoblots showing the expression of protein kinase C epsilon (PKCε) in ischemic-reperfused myocardium of rats treated with nor-NOHA, vehicle and in myocardium of sham-operated animals. Data are presented as mean and individual data points; $^{**}P<0.01$; $^{***}P<0.001$.

4. Discussion and conclusions

In this study we show that arginase activity is upregulated in the myocardium during I/R and that inhibition of arginase by nor-NOHA after the onset of ischemia, but before reperfusion protects from myocardial I/R injury. The protective effect is abolished by the NOS-inhibitor L-NMMA, inhibition of PKCε and the mitoK$_{ATP}$ channel blocker 5-HD. Furthermore, the I/R-induced reduction of PKCε expression was attenuated by arginase inhibition. These observations indicate that arginase inhibition protects from reperfusion injury via a mechanism involving NOS activity, upregulation of PKCε and activation of mitoK$_{ATP}$ channels.

Endothelial dysfunction with diminished NO bioavailability is a prominent feature of I/R injury (Cohen et al., 2006). Previous studies employing pre- and postconditioning have demonstrated that NO provides cardioprotection as a signal mediator and an effector, both immediately following the ischemic insult and at later time points (Sanada et al., 2011). Attenuation of NO bioavailability is therefore a central mechanism behind development of I/R injury. L-arginine is a substrate not only for NOS but also for arginase which converts L-arginine into L-ornithine and urea (Durante et al., 2007). Increased arginase activity may result in reduced formation of NO due to the competition between NOS and arginase for their common substrate L-arginine. In addition, it has been shown that the phenomenon of NOS uncoupling, a situation when eNOS produces superoxide instead of of NO, is provoked by arginase upregulation and that inhibition of arginase restores the

nitroso-redox balance (Kim et al., 2009). The mechanism behind this effect may be reduced availability of the eNOS substrate L-arginine due to consumption by arginase (Romero et al., 2008). Furthermore, reactive oxygen species, that are known to contribute to the development of I/R injury, may further increase arginase production (Pernow and Jung, 2013). Accordingly, previous studies (Gonon et al., 2012; Jung et al., 2010) have demonstrated that arginase inhibition results in reduction in infarct size via a mechanism that involves production of NO.

Furthermore, the cardioprotective effect was abolished by pre-treatment with the NOS-inhibitor L-NMMA, which does not affect infarct size *per se* (Gonon et al., 2000), demonstrating that the cardioprotective effect of arginase inhibition is dependent on NO production. L-NMMA blocks all isoforms of NOS but neuronal NOS has been shown not to be involved in the development of myocardial injury (Jones et al., 2000) and inducible NOS is not influenced in the brief protocol used (Bulhak et al., 2006). The present findings therefore support the notion that endothelial NOS is responsible for the cardioprotective effect of arginase inhibition. Previous studies have suggested that the cardioprotective effect of NO includes stimulation of cyclic guanosine monophosphate, formation of PKCε, protein kinase G and subsequent opening of the mitoK$_{ATP}$ channel (Chai et al., 2011; Costa and Garlid, 2008; Sanada et al., 2001). Our hypothesis was therefore that arginase inhibition mediates cardioprotection via this signaling pathway induced by increased NO production. Participation of PKCε in the cardioprotective mechanism was tested by the administration of the PKCε specific inhibitor ε-V1-2 before the treatment with nor-NOHA. This resulted in abolition of the cardioprotective effect of nor-NOHA. Inhibition of PKCε did not influence infarct size *per se*. This latter observation can be expected since PKCε was markedly downregulated by I/R. Moreover, we demonstrate that the expression of PKCε was maintained in the ischemic-reperfused myocardium after administration of nor-NOHA. We further demonstrate that the selective mitoK$_{ATP}$ channel blocker 5-HD completely blocked the cardioprotective effect of arginase inhibition by nor-NOHA suggesting that its mechanism depends on the activation of mitoK$_{ATP}$ channels. Previous studies have established that 5-HD does not affect infarct size *per se* (Fryer et al., 2001). The involvement of mitoK$_{ATP}$ channels is of importance due to the central role of mitochondrial function in the regulation of I/R injury (Heusch et al., 2008). Collectively, the data provided in the present study indicate that arginase inhibition protects the myocardium by increasing NO production, activation of PKCε followed by activation of the mitoK$_{ATP}$ channels.

In the present study nor-NOHA was administered systemically during late ischemia (before the onset of reperfusion) as an attempt to evaluate the feasibility to afford protection in clinically relevant scenario. We were able to demonstrate that nor-NOHA significantly reduced infarct size when given systemically after the onset of ischemia. This clinically important observation adds significantly to previous knowledge since it demonstrates that the arginase inhibitor affords protection also when given systemically following the onset of ischemia. Therefore this treatment may be effective when given in the clinical setting by i.v. administration in connection with revascularization in patients with acute myocardial infarction.

A limitation of this study is that it is not known whether arginase 1 or 2 is of importance during I/R injury since no arginase isoform specific inhibitors are available. Previous data suggest that arginase 1 is upregulated in the rat myocardium during I/R injury which would indicate that this isoform is involved (Jung et al., 2010). However, it is not possible to determine whether this isoform accounts for the increase in arginase activity observed in the present study.

In conclusion, the present study demonstrates that arginase activity is induced by I/R injury, and that inhibition of arginase immediately before the onset of reperfusion reduces infarct size via a mechanism dependent on NOS activity, PKCε expression and activation of mitoK$_{ATP}$ channels. The findings suggest that arginase inhibition is a promising therapeutic target for limitation of myocardial injury in the treatment of acute myocardial infarction.

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Myocardial protection by co-administration of L-arginine and tetrahydrobiopterin during ischemia and reperfusion ☆,☆☆

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ABSTRACT

Background: Reduced bioavailability of nitric oxide (NO) is a key factor contributing to myocardial ischemia and reperfusion injury. The mechanism behind the reduction of NO is related to deficiency of the NO synthase (NOS) substrate L-arginine and cofactor tetrahydrobiopterin (BH_4) resulting in NOS uncoupling. The aim of the study was to investigate if the combination of L-arginine and BH_4 given iv or intracoronary before reperfusion protects from reperfusion injury.

Methods: Sprague–Dawley rats and pigs were subjected to myocardial ischemia and reperfusion. Rats received vehicle, L-arginine, BH_4, L-arginine + BH_4 with or without the NOS-inhibitor L-NMMA iv 5 min before reperfusion. Pigs received infusion of vehicle, L-arginine, BH_4 or L-arginine + BH_4 into the left main coronary artery for 30 min starting 10 min before reperfusion.

Results: Infarct size was significantly smaller in the rats (50 ± 2%) and pigs (54 ± 5%) given L-arginine + BH_4 in comparison with the vehicle groups (rats 65 ± 3% and pigs 86 ± 5%, $P < 0.05$). Neither L-arginine nor BH_4 alone significantly reduced infarct size. Administration of L-NMMA abrogated the cardioprotective effect of L-arginine + BH_4. Myocardial BH_4 levels were 3.5- to 5-fold higher in pigs given L-arginine + BH_4 and BH_4 alone. The generation of superoxide in the ischemic-reperfused myocardium was reduced in pigs treated with intracoronary L-arginine + BH_4 versus the vehicle group ($P < 0.05$).

Conclusion: Administration of L-arginine + BH_4 before reperfusion protects the heart from ischemia–reperfusion injury. The cardioprotective effect is mediated via NOS-dependent pathway resulting in diminished superoxide generation.

1. Introduction

Acute ST-elevation myocardial infarction demands rapid restoration of coronary blood flow to the jeopardized myocardium by either primary coronary intervention or thrombolysis to limit the extent of necrosis [1]. However, acute reperfusion of the ischemic myocardium results in a cascade of harmful events, referred to as reperfusion injury. Factors contributing to the reperfusion injury include endothelial dysfunction, activation of pro-inflammatory cascades, intracellular calcium overload and generation of reactive oxygen species [2,3]. Endothelium-derived nitric oxide (NO) plays an important role as a regulator of reperfusion injury by maintaining the vascular integrity, inhibiting inflammatory processes and as a scavenger of superoxide [4]. NO production is dependent on the availability of the substrate L-arginine [5] and the function of nitric oxide synthase (NOS) [6]. During ischemia and reperfusion restriction of NO production is attributed to reduced availability of the NO substrate L-arginine by increased activity of arginase [7,8] and by reduced transport of L-arginine into the cell [9]. Earlier studies demonstrated that administration of L-arginine or inhibition of arginase attenuated ischemia and reperfusion injury via an NO-mediated mechanism [7–12].

Another reason for decreased production of NO is uncoupling of NOS [13,14], a situation when NOS produces superoxide instead of NO. One mechanism is lack of the essential cofactor tetrahydrobiopterin (BH_4) that leads to instability and uncoupling of NOS, reduced production of NO and increased generation of superoxide resulting in oxidative stress, myocardial injury and impaired contractility [15–17]. Exogenous

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administration of the BH_4 precursor sepiapterin or a synthetic analog of BH_4 restored the response to endothelium-dependent vasodilators in pig coronary arterioles isolated from the hearts subjected to ischemia and reperfusion [18]. Diminished NO production in the ischemic heart ex vivo is associated with depletion of BH_4 in myocardium and administration of exogenous BH_4 restored NO production and improved recovery of coronary flow [19]. Intravenous pretreatment by BH_4 prior to ischemia significantly reduced the myocardial infarct size in rats subjected to ischemia and reperfusion [20]. Moreover, administration of BH_4 or dihydrobiopterin resulted in protection against ischemia and reperfusion injury in the liver [21], skeletal muscle [22] and kidney [23]. However, it is not known whether BH_4 exerts cardioprotective effects when given shortly before reperfusion. Of additional interest is the interaction between L-arginine and BH_4 in the regulation of proper NOS function. Besides the role of BH_4 for binding of L-arginine to NOS, L-arginine has been shown to increase the levels of BH_4 bound to NOS indicating that also L-arginine supply is crucial for NOS coupling [24]. This may suggest synergistic effects of L-arginine and BH_4 in the setting of reduced bioavailability of NO production. In support of this, we have previously shown that the combination of L-arginine and BH_4 protects against forearm endothelial dysfunction induced by ischemia–reperfusion in patients with coronary artery disease and type 2 diabetes mellitus [25].

Based on the assumption that NO production is dependent on availability of both L-arginine and BH_4, we hypothesized that the combination of L-arginine and BH_4 will provide protection from reperfusion injury beyond that evoked by either compound alone. Therefore, the aim of the present study was to investigate if administration of L-arginine and BH_4 before the onset and during reperfusion reduces infarct size in two different species and using a protocol resembling the clinical setting of ST-elevation myocardial infarction.

2. Methods

The study was approved by the regional Ethics Committee for laboratory animal experiments in Stockholm and conformed 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).

2.1. Experimental protocol

2.1.1. Rat

Fifty-three male Sprague–Dawley rats (260–400 g body weight, Charles River Sulzfeld, Germany) were anesthetized with pentobarbital sodium ip (50 mg/kg) followed by continuous iv infusion of 5 mg/kg/h pentobarbital and 30 μg/kg/h fentanyl. Rectal temperature was maintained at 38 ± 0.5 °C by a heated operation table. Animals were tracheotomized, intubated, and ventilated with air by a rodent ventilator (54 strokes/min, 9 ml/kg tidal volume). The right carotid artery was cannulated and connected to a Statham blood pressure transducer for the measurement of mean arterial pressure. The heart rate was determined from the arterial pressure curve. Hemodynamic parameters were continuously recorded on a personal computer equipped with PharmLab V4.0 (AstraZeneca R&D, Mölndal, Sweden). The left 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. A ligature was placed around the left coronary artery.

After the surgical preparation, the rats were allowed to stabilize for 15 min and randomized into five groups. Ischemia was induced by tightening of the ligature around the left coronary artery. Successful occlusion was associated with cyanosis of the myocardial area at risk and arrhythmias after 5–15 min of ischemia. The different groups were given: 1) saline (400 μl, n = 8); 2) BH_4 (5,6,7,8-tetrahydro-L-biopterin, 10 mg/kg) followed by L-arginine (100 mg/kg, n = 6); 3) BH_4 (10 mg/kg) followed by saline (200 μl, n = 6); 4) saline (200 μl) followed by L-arginine (100 mg/kg, n = 6); and 5) the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA, 10 mg/kg) followed by BH_4 (10 mg/kg) and L-arginine (100 mg/kg, n = 6). All substances were given iv as bolus injections at 25 min of ischemia. Reperfusion was initiated after 30 min of ischemia by removal of the snare and was maintained for 2 h. The reperfusion was associated with vanishing of the cyanotic color of the myocardium and arrhythmias.

2.1.2. Pig

Twenty-nine farm pigs (27–38 kg) were premedicated with tiletamine (1.5 mg/kg, im), zolazepam (1.5 mg/kg, im) and medetomidine hydrochloride (0.06 mg/kg, im). Anesthesia was induced by sodium pentobarbital bolus (20 mg/kg, iv) and maintained with infusion of pentobarbital (2–4 mg/kg/h, iv) and fentanyl (0.05 mg/h, iv). The animals received heparin 5000 IU/h, iv. The animals were intubated and mechanically ventilated with air and oxygen. Respiratory rate and tidal volume were adjusted to keep arterial blood pH, pO2 and pCO2 within the physiological range. Rectal temperature was kept at 39.0 ± 0.2 °C by means of a heated operating table. A central venous catheter was inserted in the right external jugular vein for drug and fluid administration. Another catheter was positioned in the descending aorta via the right femoral artery for determination of blood gases and measurement of arterial pressure via a pressure transducer. Heart rate was determined from the arterial pressure curve. All variables were continuously recorded on personal computer equipped with PharmLab V3.0 (AstraZeneca R&D, Mölndal, Sweden). The heart was exposed via a sternotomy. A ligature was placed around the left anterior descending artery (LAD) at a position from which the distal third of the artery is occluded when tightening the ligature. 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. The flow probe was connected to a Transonic 208 blood flow meter. Finally, a catheter was introduced into the right femoral artery and a guide wire was advanced to the aortic arch under control with fluoroscopy. A 5F coronary angiography catheter was slid over the wire and placed into the left main coronary for drug administration.

After a post-surgery stabilization period of 30 min the pigs were subjected to myocardial ischemia by tightening the ligature around the LAD. Ischemia was induced by tightening of the ligature around LAD. The animals were randomized to receive a local infusion into the left main stem of either: 1) saline (n = 6); 2) BH_4 (0.03 mg/kg/min, n = 6); 3) L-arginine (3.0 mg/kg/min, n = 6) or 4) the combination of L-arginine and BH_4 (n = 6). In addition the fifth group received a systemic infusion of the same doses of L-arginine and BH_4 via the jugular vein (n = 5). All infusions were started at 30 min of ischemia and were given for 30 min, i.e. until 20 min of reperfusion, at a rate of 2 ml/min. After 40 min of ischemia LAD was reperfused for 4 h by removal of the ligature.

2.2. Determination of infarct size and collection of myocardium samples

After 2 h of reperfusion in rats and 4 h of reperfusion in pigs the coronary artery was reoccluded. An injection of 2% Evans blue solution was given iv (1.5 ml and 50 ml for rat and pig respectively) to stain non-ischemic myocardium for determination of the area at risk. The rats were euthanized by exsanguination and the pigs were sacrificed by iv injection of potassium chloride. The heart was extracted. The atria and the right ventricle were removed. The left ventricle of the rat was frozen for 20 min (−20 °C) and cut into 7–9 slices perpendicular to the base–apex axis. The left ventricle of the pig was cut into 5–6 slices perpendicular to the heart base–apex axis without prior freezing. The third slice (counting from the apex) was cut into the ischemic and non-ischemic parts which were used for further analyses. All other slices were weighed, scanned from both sides for the determination of the area at risk and put in 1% triphenyltetrazolium chloride solution for 15 min (slices from rat) or 10 min (slices from pig) at 37 °C to distinguish viable myocardium from necrotic. After 24 h of incubation in 4% formaldehyde slices from rats were scanned again from both sides, and the extent of myocardial necrosis and the area at risk were determined by planimetry of computer images (Adobe Photoshop CS 2, Adobe Systems, San Jose, CA, USA). Left ventricular slices from pigs were stained in triphenyltetrazolium chloride solution and the extent of myocardial necrosis and the area at risk were determined by planimetry. Infarct size was expressed in percent of the area at risk.

2.3. Tetrahydrobiopterin quantification

Myocardial BH_4 levels were determined by HPLC followed by electrochemical and fluorescent detection, as previously described [26]. Tissue lysates were resuspended in phosphate-buffered saline (50 mM), pH 7.4, containing dithioerythritol (1 mM) and EDTA (100 μM) and subjected to three freeze–thaw cycles. After centrifugation (15 min at 13000 rpm and 4 °C), samples were transferred to new, cooled micro tubes and precipitated with cold phosphoric acid (1 M), trichloroacetic acid (2 M) and dithioerythritol (1 mM). Samples were vigorously mixed and then centrifuged for 15 min at 13000 rpm and 4 °C. Samples were injected onto an isocratic HPLC system and quantified using sequential electrochemical (Coulochem III, ESA, Inc., North Sioux City, SD, USA). HPLC separation was performed using a 250-mm ACE C-18 column (Hichrom, Berkshire, UK) and a mobile phase comprised of sodium acetate (50 mM), citric acid (5 mM), EDTA (48 μM), and dithioerythritol (160 μM, pH 5.2) (all ultrapure electrochemical HPLC grade) at a flow rate of 1.3 ml/min. Background currents of +500 μA and −50 μA were used for the detection of BH_4 on electrochemical cells E1 and E2, respectively. Quantification of BH_4 was done by comparison with authentic external standards and normalized to sample protein content.

2.4. Determination of superoxide production in ischemic myocardium

Left ventricle myocardial tissue samples (size: 0.5 × 0.5 × 2.0 cm) were excised from the area at risk following ischemia–reperfusion, and superoxide production was determined using dihydroethidium (DHE; Sigma-Aldrich, St. Louis, MO, USA) fluorescence staining [27]. In brief, the tissue blocks were embedded in optimal cutting temperature compounds and cryosections (20 μm) were produced in cryostat Leica CM3050 S (Leica Microsystems, Wetzlar, Germany), stained with DHE (1 μM) dissolved in phosphate buffer solution (pH 7.4) and incubated at 37 °C for 30 min in a dark humidified chamber. A negative control was obtained by blocking the reaction with N-acetyl-L-cysteine (100 mM, Sigma-Aldrich, St. Louis, MO, USA). The fluorescent image was obtained using a fluorescence microscope Zeiss Axio Scope A1 (Carl Zeiss Microscopy GmbH, Jena, Germany) with a 585 nm long-pass filter. The fluorescence intensity was determined

using Image J software (NIH, Bethesda, MD, USA), and expressed in percentage of the control group.

2.5. Chemicals

Pentobarbital sodium was purchased from Apoteksbolaget (Stockholm, Sweden); Fentanyl from B. Braun Melsungen AG (Melsungen, Germany); BH_4 and L-NMMA from Alexis Biochemicals (Lausen, Switzerland); L-arginine hydrochloride, Evans blue, triphenyltetrazolium chloride, Trizma base and Trizma hydrochloride from Sigma-Aldrich (St. Louis, MO, USA). BH_4 was dissolved in distilled deoxygenated water containing 1 mM dithioerythritol as antioxidant and kept in −80 °C freezer until usage.

2.6. Statistical analysis

Data are presented as mean ± SE. Groups were compared using Kruskal–Wallis test with Dunn's multiple comparison test. $P < 0.05$ was considered statistically significant.

3. Results

Twenty-one rats were excluded from the study; 10 rats due to irreversible ventricular fibrillation during ischemia (1 animal in the vehicle group, 2 animals in the BH_4 + L-arginine group, 1 animal in the BH_4 group, 3 animals in the L-arginine group, and 3 animals in the L-NMMA + BH_4 + L-arginine group), and 11 rats due to continuously low mean arterial pressure (exclusion criteria was mean arterial pressure lower than 60 mm Hg) during the ischemic or reperfusion period (5 animals in the vehicle group, 2 animals in the BH_4 + L-arginine group, 3 animals in the L-arginine group, and 1 animal in the L-NMMA + BH_4 + L-arginine group).

Three pigs were excluded due to irreversible ventricular fibrillation occurring during the ischemic or reperfusion periods (2 pigs randomized to the vehicle group and 1 pig in the L-arginine group).

3.1. Hemodynamics

There were no significant differences in pre-ischemic hemodynamic parameters between the groups of rats or pigs (Tables 1 and 2, respectively). In rats heart rate was significantly higher before the onset and at the end of reperfusion in the group given L-arginine + BH_4 than in the vehicle group. There was no statistical difference in hemodynamic parameters between the different groups of pigs during ischemia and reperfusion.

3.2. Area at risk and infarct size

Figs. 1A and 2A depict the area at risk in the rats and pigs, respectively. For each species there were no significant differences between the groups.

In rats infarct size was 65 ± 3% of the area at risk in the vehicle group (Fig. 1B). Infarct size was not significantly affected by administration of L-arginine (64 ± 3%) or BH_4 (56 ± 3%). However, iv administration of the combination of L-arginine and BH_4 resulted in a significant reduction of infarct size to 50 ± 2% of the area at risk ($P < 0.05$ vs. vehicle). Administration of the NOS inhibitor L-NMMA abolished the protective effect of L-arginine + BH_4 (66 ± 4% of the area at risk).

In pigs infarct size was 86 ± 5% of the area at risk in the vehicle group (Fig. 2B). Neither infusion of L-arginine nor BH_4 alone significantly affected infarct size (77 ± 5% and 70 ± 5% of the area at risk, respectively). Infarct size was significantly reduced by intracoronary administration of the combination of L-arginine and BH_4 to 54 ± 5% of the area at risk ($P < 0.05$ vs. vehicle). On the other hand, systemic infusion of the same dose of L-arginine + BH_4 did not influence infarct size (83 ± 6% of the area at risk).

Table 2
Hemodynamics in pigs.

Group	Parameter	Before ischemia	30 min ischemia	60 min reperfusion	240 min reperfusion
Vehicle	MAP	98 ± 6	80 ± 14	59 ± 14	69 ± 15
	HR	88 ± 6	106 ± 12	115 ± 16	150 ± 9
	CF	16.5 ± 1.0	0	26.1 ± 5.8	7.3 ± 2.8
BH_4 + L-arginine	MAP	95 ± 7	87 ± 8	65 ± 4	67 ± 4
	HR	79 ± 4	79 ± 5	99 ± 5	120 ± 7
	CF	19.2 ± 2.7	0	27.8 ± 4.1	11.5 ± 3.0
BH_4	MAP	96 ± 5	94 ± 6	93 ± 7	73 ± 3
	HR	87 ± 12	82 ± 6	119 ± 8	121 ± 6
	CF	18.6 ± 1.7	0	44.5 ± 5.0	8.5 ± 0.7
L-arginine	MAP	93 ± 4	92 ± 3	81 ± 8	67 ± 4
	HR	87 ± 6	119 ± 29	119 ± 6	119 ± 4
	CF	19.4 ± 3.1	0	25.6 ± 3.5	8.9 ± 2.7
Systemic BH_4 + L-arginine	MAP	97 ± 3	76 ± 11	72 ± 7	64 ± 10
	HR	86 ± 5	73 ± 12	101 ± 6	120 ± 7
	CF	14.3 ± 0.7	0	21.5 ± 3.3	7.0 ± 1.6

MAP: mean arterial pressure; HR: heart rate; CF: coronary flow. Data are mean ± SE. There are no significant differences between groups.

3.3. Myocardial tetrahydrobiopterin levels

BH_4 was determined in the myocardial area at risk of pigs given intracoronary infusion of vehicle, BH_4 or the combination of BH_4 and L-arginine (Fig. 3). Myocardial BH_4 levels were significantly increased in animals given BH_4 (5.4 ± 2.6 pmol/mg) and BH_4 + L-arginine (3.5 ± 1.1 pmol/mg) in comparison with the vehicle group (0.9 ± 0.3 pmol/mg).

3.4. Superoxide production

Superoxide formation in the ischemic/reperfused myocardium determined by DHE was significantly lower in pigs given BH_4 + L-arginine than in vehicle treated pigs (Fig. 4). L-arginine or BH_4 alone did not significantly affect superoxide formation.

4. Discussion

The novel finding of this study is that the combined administration of L-arginine and BH_4 during late ischemia and reperfusion reduces

Table 1
Hemodynamics in rats.

Group	Parameter	Before ischemia	30 min ischemia	30 min reperfusion	60 min reperfusion	120 min reperfusion
Vehicle	MAP	123 ± 6	78 ± 8	74 ± 7	70 ± 6	65 ± 6
	HR	455 ± 13	438 ± 10	417 ± 10	408 ± 9	373 ± 7
BH_4 + L-arginine	MAP	137 ± 3	107 ± 5	93 ± 5	91 ± 9	84 ± 6
	HR	510 ± 7	489 ± 6 *	464 ± 16	448 ± 10	407 ± 9
BH_4 + vehicle	MAP	131 ± 6	87 ± 8	78 ± 8	75 ± 7	66 ± 8
	HR	486 ± 17	472 ± 15	445 ± 21	422 ± 16	385 ± 7
Vehicle + L-arginine	MAP	122 ± 12	100 ± 6	81 ± 4	78 ± 5	81 ± 6
	HR	476 ± 24	469 ± 14	443 ± 15	426 ± 12	406 ± 12
L-NMMA + BH_4 + L-arginine	MAP	124 ± 6	91 ± 7	71 ± 4	65 ± 3	61 ± 3
	HR	461 ± 14	442 ± 14	412 ± 13	402 ± 4	393 ± 6

MAP: mean arterial pressure; HR: heart rate. Data are mean ± SE. Significant difference from vehicle is indicated (*$P < 0.05$).

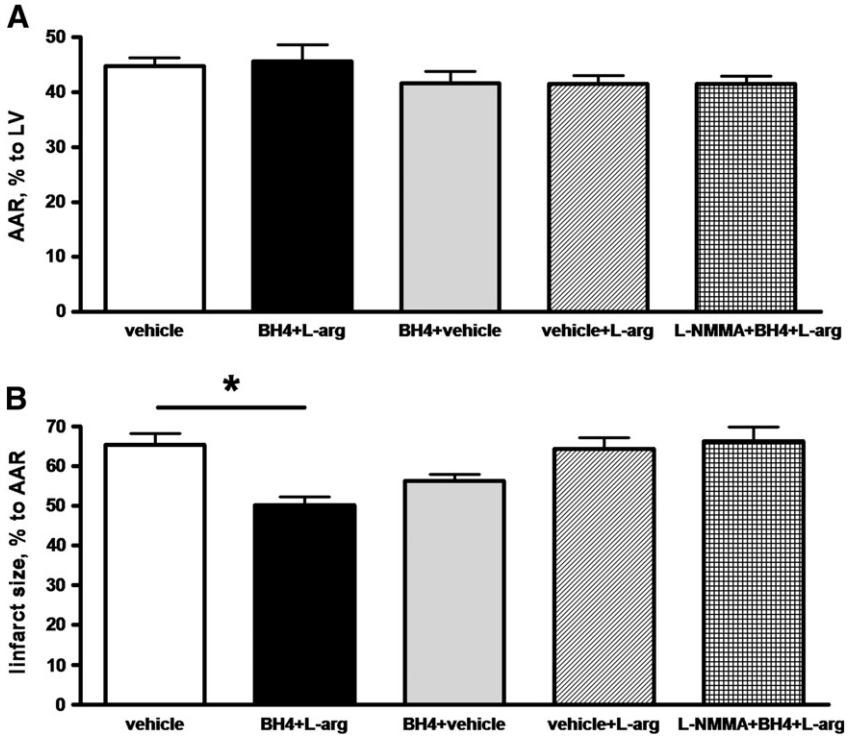

Fig. 1. (A) Area at risk (AAR) expressed as % of the left ventricle (LV) and (B) infarct size expressed as % of AAR after 30 min of ischemia and 2 h of reperfusion. The rats were given either saline (vehicle), BH_4 with L-arginine (L-arg) or vehicle, vehicle with L-arginine, the NOS inhibitor L-NMMA with BH_4 and L-arginine. Significant difference versus vehicle group is indicated (*$P < 0.05$).

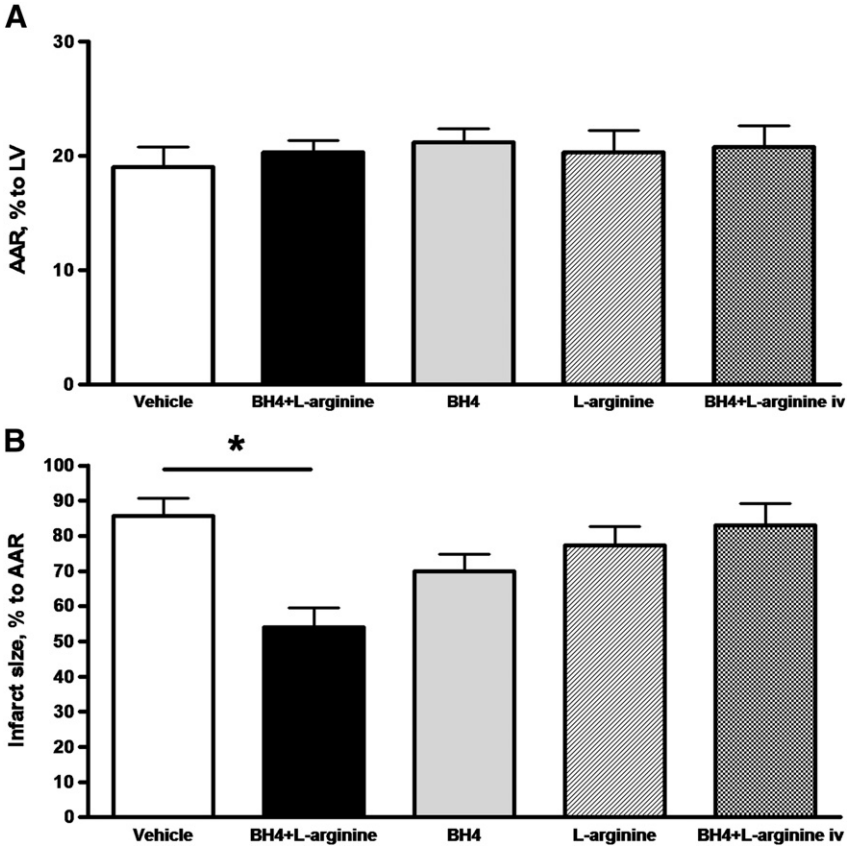

Fig. 2. (A) Area at risk (AAR) expressed as % of the left ventricle (LV) and (B) infarct size expressed as % of AAR after 40 min of ischemia and 4 h of reperfusion. The pigs were given either saline (vehicle), BH_4 with L-arginine, BH_4 alone or L-arginine alone by intracoronary (ic) infusion or BH_4 with L-arginine by iv infusion. Significant difference versus vehicle group is indicated (*$P < 0.05$).

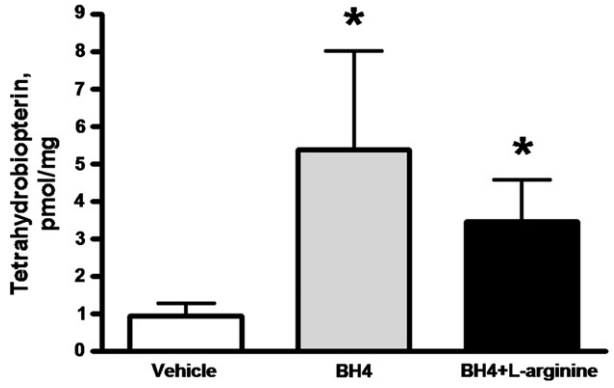

Fig. 3. Tetrahydrobiopterin (BH_4) levels in the ischemic-reperfused myocardium of pigs after 40 min of ischemia and 4 h of reperfusion. The pigs were given either saline (vehicle), BH_4 or BH_4 with L-arginine by intracoronary infusion. Significant differences from vehicle are indicated (*$P < 0.05$).

infarct size in two different animal species using two different experimental protocols, whereas either compound given alone failed to induce significant cardioprotection. The cardioprotective effect of L-arginine + BH_4 is dependent on production of NO and attenuated superoxide generation.

Production of NO by NOS is an indisputable requirement for cardioprotection during ischemia and reperfusion [13,14]. The present study was designed to test the hypothesis that co-administration of the substrate L-arginine and the co-factor for NOS (BH_4) results in protection against reperfusion injury. This was sequentially tested in two animal models. Rats were used initially to investigate the cardioprotective properties of BH_4 and L-arginine administered systemically before the onset of reperfusion. Only the combination of BH_4 and L-arginine but neither the cofactor nor the substrate for NOS given alone induced significant reduction in infarct size. The cardioprotective effect of BH_4 + L-arginine was abolished by the NOS inhibitor L-NMMA demonstrating that it was dependent on NOS activity and production of NO. In the second part of the study a pig model was used since this species has a cardiac anatomy and physiology that resemble the human heart [28]. Intracoronary infusion of substances via a catheter in the left main coronary artery could therefore be performed to resemble a clinical situation in patients with ST-elevation myocardial infarction undergoing percutaneous coronary intervention. Intracoronary infusion of the combination of BH_4 and L-arginine starting before reperfusion resulted in significant reduction in infarct size. On the other hand, systemic

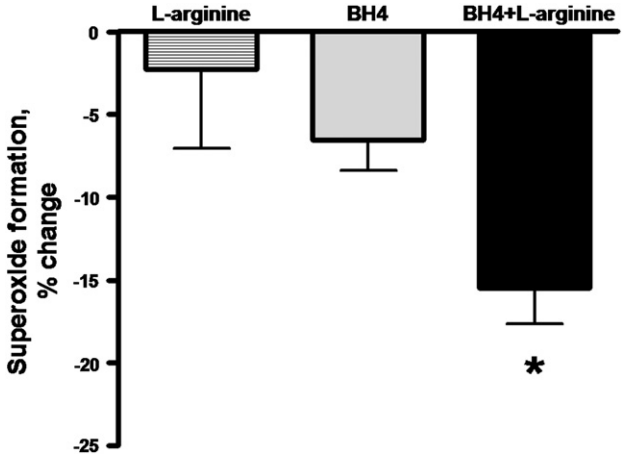

Fig. 4. Change in superoxide generation in the ischemic-reperfused myocardium of pigs expressed as % change from the vehicle group after 40 min of ischemia and 4 h of reperfusion. The pigs were given either saline (vehicle), L-arginine, BH_4 or BH_4 with L-arginine by intracoronary infusion. Significant difference from vehicle is indicated (*$P < 0.05$).

infusion of BH_4 and L-arginine in doses that were similar to those given by intracoronary infusion did not induce cardioprotection. This observation suggests that the cardioprotective effect of intracoronary infusion was mediated via a local effect in the ischemic-reperfused myocardium. In agreement with the data obtained in rats, intracoronary administration of BH_4 or L-arginine alone did not induce a significant cardioprotective effect in pigs. In contrast to previous data [10,11] our study demonstrated that L-arginine administered proximally to the point of occlusion did not reduce infarct size. The current data are, however, in agreement with an earlier study in pigs showing that systemic administration of L-arginine alone did not reduce infarct size [12]. The reason why administration of L-arginine alone might not be sufficient to significantly reduce infarct size may be that it is competitively metabolized by arginase, the activity of which is upregulated during ischemia–reperfusion [7,8], and/or decreased uptake of L-arginine into the cells [9].

Diminished availability of the NOS cofactor BH_4 leads to uncoupling of the enzyme, resulting in decreased NO production and increased superoxide formation [15–17]. Previous studies have demonstrated that myocardial BH_4 levels are reduced during ischemia due to oxidation which contributes to post-ischemic endothelial dysfunction [19] and that systemic pretreatment of rats with BH_4 prior to ischemia results in cardioprotective effect in vivo [20]. We demonstrate that BH_4 administered alone prior to reperfusion does not provide significant reduction in infarct size neither when administered iv in rats nor by intracoronary infusion in pigs, despite a clear increase in myocardial BH_4 levels. On the other hand, the combined administration of BH_4 and L-arginine resulted in significant infarct size reduction in both the rat and pig models. This effect was achieved with an increase in myocardial BH_4 that were comparable to that achieved with administration of BH_4 alone. The cardioprotective effect of L-arginine + BH_4 was blocked by the NOS inhibitor L-NMMA indicating that it was mediated via production of NO. These observations suggest that the combined administration of L-arginine and BH_4 enhances NO production causing a protection against reperfusion injury.

Several mechanistic possibilities exist that may explain the effect of L-arginine and BH_4 during reperfusion. Earlier observations suggest that both L-arginine and BH_4 are needed for coupling of NOS [29]. Furthermore, it has been shown that availability of L-arginine is crucial for NOS coupling [24]. Relative deficiency of L-arginine during ischemia–reperfusion may be due to upregulation of arginase. Thus, the combination of BH_4 and L-arginine may be needed to achieve fully coupled NOS in the setting of ischemia–reperfusion. In support of this, we show that superoxide formation was significantly reduced in pigs only when given of the combination of BH_4 and L-arginine. Previously it was speculated that the improvement of endothelial function after the treatment with BH_4 can be attributed to its antioxidant properties [30]. However, we did not observe significant decrease of superoxide formation or cardioprotective effect in animals given BH_4 alone. Furthermore, the cardioprotective effect of BH_4 + L-arginine was blocked by NOS inhibition. Collectively, these findings indicate that the effect of BH_4 + L-arginine was mediated enzymatically via restored NOS coupling and NOS-dependent reduction of infarct size rather than via an antioxidative effect of BH_4. The cardioprotective effect of L-arginine and BH_4 achieved by systemic administration to rats 5 min before the onset of reperfusion may be explained by the relatively long biological half-life of L-arginine [31] and BH_4 [32] that allows the compounds to remain in blood and reach the area at risk at the onset of reperfusion. In addition rats have 6.1 ± 0.7% collateral flow of non-ischemic flow which is 10-fold higher than 0.6 ± 0.2% collateral flow in pigs [33]. Accordingly, since the rat has a distinguishable collateral blood flow, blood enriched with L-arginine and BH_4 may be distributed to the area at risk during coronary artery occlusion.

Of clinical interest is the novel demonstration that it is possible to achieve significant reduction in infarct size by administration of pharmacological compounds via the guide catheter in the left main coronary

artery during coronary artery occlusion and early reperfusion. The guide catheter was introduced and positioned in a fashion similar to that used in clinical practice during primary coronary interventions of patients with ST elevation myocardial infarction. Thus, the results of the present study suggest that it may be feasible to use the same route of administration in the clinical situation.

This study has certain limitations that need commenting. Only one dose of each compound was tested. The doses of L-arginine and BH_4 were based on previous studies employing local infusions demonstrating either cardioprotective effect or improvement of coronary endothelial function [19,34,35]. The doses used in our study are well within the range used in these previous studies. A group given only the NOS inhibitor L-NMMA was not included and therefore its effect on infarct size per se was not determined. However, several previous studies using similar protocols have demonstrated that NOS blockade does not affect infarct size per se [36,37].

In conclusion, the combined administration of BH_4 and L-arginine during late ischemia and early reperfusion reduced infarct size in two different experimental models and two different species. The cardioprotection was mediated via a NOS-dependent pathway and involving reduced generation of superoxide. The finding that the combination of BH_4 and L-arginine provides extensive cardioprotection compared to each individual compound makes it a promising therapeutic tool for the limitation of reperfusion injury in acute myocardial infarction. The novel route of administration of drugs through a clinically used guide catheter placed in the left main coronary artery to mimic the clinical situation of ST-elevation myocardial infarction proved to be a method that induces cardioprotection in the pig. Therefore, this method could be feasible also in clinical studies aiming for intra-coronary infusions in patients.

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III

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The Role of Arginase and Rho Kinase in Cardioprotection from Remote Ischemic Perconditioning in Non-Diabetic and Diabetic Rat *In Vivo*

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Abstract

Background: Pharmacological inhibition of arginase and remote ischemic perconditioning (RIPerc) are known to protect the heart against ischemia/reperfusion (IR) injury.

Purpose: The objective of this study was to investigate whether (1) peroxynitrite-mediated RhoA/Rho associated kinase (ROCK) signaling pathway contributes to arginase upregulation following myocardial IR; (2) the inhibition of this pathway is involved as a cardioprotective mechanism of remote ischemic perconditioning and (3) the influence of diabetes on these mechanisms.

Methods: Anesthetized rats were subjected to 30 min left coronary artery ligation followed by 2 h reperfusion and included in two protocols. In protocol 1 rats were randomized to 1) control IR, 2) RIPerc induced by bilateral femoral artery occlusion for 15 min during myocardial ischemia, 3) RIPerc and administration of the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA), 4) administration of the ROCK inhibitor hydroxyfasudil or 5) the peroxynitrite decomposition catalyst FeTPPS. In protocol 2 non-diabetic and type 1 diabetic rats were randomosed to IR or RIPerc as described above.

Results: Infarct size was significantly reduced in rats treated with FeTPPS, hydroxyfasudil and RIPerc compared to controls ($P<0.001$). FeTPPS attenuated both ROCK and arginase activity ($P<0.001$ vs. control). Similarly, RIPerc reduced arginase and ROCK activity, peroxynitrite formation and enhanced phospho-eNOS expression ($P<0.05$ vs. control). The cardioprotective effect of RIPerc was abolished by L-NMMA. The protective effect of RIPerc and its associated changes in arginase and ROCK activity were abolished in diabetes.

Conclusion: Arginase is activated by peroxynitrite/ROCK signaling cascade in myocardial IR. RIPerc protects against IR injury via a mechanism involving inhibition of this pathway and enhanced eNOS activation. The beneficial effect and associated molecular changes of RIPerc is abolished in type 1 diabetes.

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Introduction

Emerging evidence suggested a pathophysiological role of arginase in several diseases related to endothelial dysfunction such as atherosclerosis [1], diabetes mellitus (DM) [2] and myocardial ischemia/reperfusion (IR) injury [3,4,5] by reducing bioavailability of nitric oxide (NO). Arginase regulates NO bioavailability by competing with endothelial NO synthase (eNOS) for their common substrate L-arginine. Arginase exists in two isoforms (1 and 2) and both are widely expressed in the cardiovascular system [6,7]. Recently, our group has demonstrated that inhibition of arginase either prior to ischemia or reperfusion protects the heart against IR injury through NOS- and NO-dependent mechanisms [3,4,5]. These studies also confirmed that arginase activity is increased within the myocardium at risk after myocardial IR. Cell culture experiments have revealed that the expression and activity of arginase are regulated by several factors including pro-inflammatory cytokines, hypoxia, reactive oxygen and nitrogen species and RhoA/Rho associated kinase (ROCK) [7,8,9].

However, the mechanism underlying the upregulation of arginase activity in myocardial IR still remains to be identified.

It is well known that ischemic preconditioning provides a robust intrinsic cardioprotection in the experimental setting of myocardial IR [10]. The clinical application of preconditioning is limited, however, due to the unpredictable occurrence of myocardial infarction. More recently, it was described that remote ischemic perconditioning (RIPerc), defined as brief cycle(s) of IR of a remote organ applied during sustained myocardial ischemia also reduces myocardial infarct size [11,12]. The magnitude of cardioprotection induced by RIPerc is comparable to preconditioning [12] but has the advantage of being feasible in the clinical setting on patients with ST elevation myocardial infarction [13]. It is still unclear how the protective signal translates from the remote organ to the heart and which signaling pathways within the myocardium that are involved as a mediator of the protection. Substantial evidence suggests that the attenuation of the deleterious effect of ROCK and the activation of eNOS are involved in the cardioprotective effect of RIPerc [14,15]. Based on the link between ROCK and arginase activity described above, we hypothesized that the cardioprotective effect of RIPerc is associated with down-regulation of ROCK activity and arginase activity.

Recent studies have suggested that co-morbidities such as diabetes attenuate the cardioprotective effect of local pre- and postconditioning, at least in part, via attenuation of the eNOS signaling pathway in the myocardium [16,17]. However, it is unknown, whether the molecular signaling and the cardioprotective effect to RIPerc is affected by co-morbidities.

Therefore, the present study was design to investigate (1) the contribution of oxidative/nitrosative stress and ROCK signaling pathway to IR-induced arginase upregulation and (2) whether inhibition of this pathway is involved in the cardioprotective mechanism of RIPerc in the absence and presence of diabetes.

Materials and Methods

2.1 Ethics Statement

The study was approved by the regional Ethics Committee (Stockholm Norra Djurförsöksetiska Nämnd, approval number N192/12) 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).

2.2 Surgical procedures

The surgical interventions were similar as described previously [3,5]. Male Sprague–Dawley rats (Charles-River, Sulzfeld, Germany) were anaesthetized with sodium pentobarbital (50 mg/kg ip, followed by 3–5 mg/kg/h iv throughout the experiment), tracheotomized, intubated and ventilated with air by a rodent ventilator (54 strokes/min, 9 ml/kg tidal volume). Rectal temperature was maintained at 37.5–38.5°C by a heated operating table. The right carotid artery was cannulated and connected to a pressure transducer for measurement of mean arterial pressure (MAP). Heart rate (HR) was determined from the arterial pressure curve. Hemodynamic parameters were continuously recorded on a personal computer equipped with PharmLab V5.0 (AstraZeneca R&D, Mölndal, Sweden). The left jugular vein was cannulated for drug administration. The heart was exposed via a left thoracotomy and a ligature was placed around the left coronary artery. Myocardial ischemia was induced by tightening of the ligature and successful occlusion was associated with cyanosis of the myocardial area at risk. Reperfusion was initiated after 30 min of ischemia by removal of the snare and was maintained for 2 h. The reperfusion was associated with disappearance of the cyanotic color of the myocardium. All efforts were made to minimize animals' suffering.

2.3 Induction of type 1 diabetes mellitus

Type 1 diabetes mellitus was induced by a single iv (tail) injection of streptozotocin (55 mg/kg, Sigma Aldrich, USA) [18]. Three days later, only rats with blood glucose levels >15.0 mM were considered to be diabetic. The rats were given unlimited food and water and were not supplemented with insulin or anti-hyperglycaemic agents. Four to five weeks following streptozotocin injection, the rats were used for IR experiments as described above. Age matched rats served as a non-diabetic control group.

2.4 Experimental protocols

2.4.1 Protocol 1. Protocol 1 was designed to investigate whether the peroxynitrite/ROCK signaling pathway play role in arginase activation and whether the inhibition of this pathway is involved in the cardioprotective mechanism of RIPerc.

After the surgical preparation rats were allowed to stabilize for 15 min and randomized into one of the following groups: (1) control IR (CIR; no intervention during IR; n = 10), (2) RIPerc induced by bilateral femoral artery occlusion using vessels clamps during the last 15 min of coronary artery occlusion (n = 10), (3) administration of the NO synthase inhibitor N^G-monomethyl-L-arginine inhibitor (L-NMMA, Alexis Biochemicals, Switzerland; 10 mg/kg, iv n = 6) just prior to RIPerc, (4) administration of the ROCK inhibitor hydroxyfasudil (Tocris Biosience, UK; 0.5 mg/kg, iv n = 7) 20 min prior to ischemia, or (5) administration of the peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III), chloride (FeTPPS, Calbiochem, USA; 10 mg/kg, iv n = 6) were given 10 min prior to reperfusion. The dosage of drugs and the length of time the femoral artery occlusion were based on previous studies [5,12,19,20]. NOS blockade has previously been demonstrated not to affect infarct size per se in our laboratory [21,22,23].

2.4.2 Protocol 2. Protocol 2 aimed to assess the influence of diabetes on the cardioprotective effect and molecular signaling induced by RIPerc. After the surgical preparation as described above, type 1 diabetic or age-matched non-diabetic rats were allowed to stabilize for 15 min and randomized into four groups. These groups were: (1) non-diabetic controls (n = 6) with no intervention during IR (ND-CIR), (2) non-diabetic+RIPerc (ND-RIPerc; n = 6); (3) diabetic with no intervention during myocardial IR (DM-CIR; n = 7) and (4) diabetic+RIPerc (DM-RIPerc; n = 6).

2.5 Assessment of myocardial infarct size

Infarct size was measured as described previously [5]. Briefly, after 2 h of reperfusion, the coronary artery was reoccluded and 1.5 ml of 2% Evans Blue was injected in the right atrium via the left jugular vein to demarcated the area at risk (ischemic myocardium). Then rats were euthanized rapidly by exsanguination and the heart was rapidly excised. The atria and the right ventricle were removed. The left ventricle of hearts were frozen for 20 minutes (−20°C) and cut into 5–7 slices perpendicular to the base-apex axis. The third slice (counting from the apex) was cut into the ischemic and non-ischemic parts and frozen at −80°C for further analyses [5]. The remaining slices were scanned from both sides for determination of the area at risk weighed, and put in 1% 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. Viable myocardium is stained red by TTC, whereas necrotic myocardium appears pale yellow. The area at risk and the

Table 1. Hemodynamic changes in Protocol 1.

GROUP	Parameters	30 min before ischemia	Ischemia start	Ischemia 30 min	Reperfusion 30 min	Reperfusion 60 min	Reperfusion 120 min
CIR	**MABP**	90±5	92±5	79±3	80±6	86±6	71±5
	HR	420±8	420±11	412±10	410±13	403±11	389±10
RIPerc	**MABP**	88±4	93±5	76±4	75±4	76±3	72±2
	HR	419±7	421±10	406±10	399±8	390±9	375±7
FeTPPS	**MABP**	89±3	97±4	77±4	87±5	79±3	70±3
	HR	422±21	418±23	398±10	420±14	411±13	391±12
H.fasudil	**MABP**	94±6	90±4	77±2	89±8	84±8	77±3
	HR	409±10	406±7	397±13	377±4	377±3	366±5
L-NMMA+RIPerc	**MABP**	96±5	100±5	89±3	86±6	80±5	74±4
	HR	415±13	429±7	412±8	397±16	391±10	372±6

Values are mean ± SEM; n = 6–10. Abbreviations: MABP (mm Hg), mean arterial blood pressure; HR (beats/min), heart rate; CIR, control ischemia/reperfusion; RIPerc, remote ischemic perconditioning; FeTPPS, 5,10,15,20-Tetrakis(4-sulfonatophenyl)porphyrinato iron (III), chloride; H.fasudil, hydroxyfasudil and L-NMMA, N^G-monomethyl-L-arginine.

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necrotic area were determined by computerized planimetry images (Photoshop 6.0; Adobe Systems, USA), normalized to the weight of each slice, with the degree of necrosis expressed as the percentage of area at risk.

2.6 Western blotting

Tissue samples from the left ventricle were extracted in lysate buffer (containing 20 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_3VO_4, glycerol, 0.05 M NaF, 100 mM Na-pyrophosphate, Tris pH 7.8, Triton-X (0.1%), protease inhibitor (Roche), PBS and 1 mM of EDTA), homogenized and centrifuged for 20 min at 10000 g at 4°C. Total protein content of the extracts was quantified by using bicinchoninic acid protein assay kit (Pierce Biotechnology, USA). The proteins were separated on 7.5% SDS gel (10 or 40 µg/lane) and transferred onto either polyvinylidine fluoride (Millipore, USA) or nitrocellulose membranes (Amersham Biosciences, USA). Membranes were blocked for 1 h at room temperature and incubated overnight at 4°C with primary antibodies against arginase 1 and 2 (1:1000, Sigma Prestige Antibody, USA); phoshorylated eNOS (p-eNOS; anti-phosphorylated Ser-1177; 1:1000; BD Pharmigen, USA), total eNOS (1:1000; ABR Affinity BioReagents, USA); 3-nitrotyrosine (3NT, 1:1000; Abcam) or phosphorylated ezrin (p-ezrin; anti-phosphorylated Thr567, 1:1000, BD Pharmingen, USA). IRDye 800-conjugated goat anti-mouse IgG (1:12000, LI-COR), IRDye 800-conjugated goat anti-rabbit IgG (1:12000, LI-COR) and IRDye 680 (1:10000) used as secondary antibody and bands visualized using infrared fluorescence scanner (IR-Odyssey, LI-COR Biosciences, USA). Equal loading was confirmed by expression of GAPDH (1:5000, Sigma Aldrich, USA) or, for 3NT, staining with Coomassie brilliant blue. Band densities were analyzed with Image Studio Lit Version 3.1 (LI-COR Biosciences, USA) and expressed percent of controls.

2.7 Determination of ROCK and arginase activity

Activity of ROCK was determined similar to described previously [14]. Briefly, left ventricular tissue samples were prepeared as described above. To evaluate Rho-kinase activity, the extent of phosphorylation of ezrin (Thr567) was determined by Western blotting.

Arginase activity was determined by using a modified colorimetric assay previously described [24]. The assay measures the urea content using α-isonitrosopropiophenone. Tissue samples from the area at risk and non-risk of the left ventricle were homogenized and centrifuged (see above), 50 µl of the supernatant was 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 with: (1) L-arginine (50 µl, 0.05 M, in Tris-HCl pH 9.7) and (2) L-arginine with 30 min preincubation with the arginase inhibitor 2 (S)-amino-6-boronohexanoic acid (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 α-isonitrosopropiophenone (9% in ethanol), and the mixture was then heated at 100°C for 60 min. Arginase activity was calculated as urea (µmol/mg protein/min) production and expressed in percent of control.

2.8 Statistical analysis

Data are presented as mean ± SEM. For multiple comparisons between several groups one-way ANOVA followed by Bonferroni post hoc test was used (Prism 5 software, GraphPad Inc., USA). Mann Whitney U-test was used for comparisons of only two

Table 2. Hemodynamic changes in Protocol 2.

GROUP	Parameters	Baseline	Ischemia 30 min	Reperfusion 30 min	Reperfusion 60 min	Reperfusion 120 min
ND- CIR	**MABP**	92±2	77±6	85±6	82±6	77±2
	HR	422±11	391±8	384±9	385±7	379±6
ND- RIPerc	**MABP**	98±3	82±7	82±3	85±5	75±3
	HR	414±9	388±10	387±15	382±9	374±11
DM- CIR	**MAB**	97±8	82±2	82±2	77±6	72±5
	HR	363±6*	334±7*	336±6*	328±7*	320±2*
DM- RIPerc	**MABP**	97±4	80±4	83±3	79±5	77±2
	HR	360±4*	340±6*	347±13*	345±8*	328±10*

Values are mean ± SEM; n = 6–7. Abbreviations: MABP (mm Hg), mean arterial blood pressure; HR (beats/min), heart rate; ND-CIR, non-diabetic control ischemia/reperfusion; ND-RIPerc, non-diabeic remote ischemic perconditioning; DM-CIR: diabetes mellitus control ischemia/reperfusion; DM-RIPerc, diabetes mellitus remote perconditioning.
*$P<0.05$ vs. ND-CIR.
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groups where appropriate. The P-value<0.05 was considered to be significant.

Results

3.1 Hemodynamics

Hemodynamic parameters of animals included in protocol 1 and 2 are presented in Table 1 and 2. There was no significant difference in MAP or HR between the groups in protocol 1 (Table 1). In protocol 2, baseline HR was significantly lower in rats with diabetes in comparison with the non-diabetic controls (Table 2).

3.2 Results of protocol 1

3.2.1 Effect of peroxynitrite and ROCK on arginase and ROCK activity in IR. The effect of peroxynitrite and ROCK activity were determined by the peroxynitrite decomposition catalyst FeTPPS and the ROCK inhibitor hydroxyfasudil, respectively. There was no difference in the area at risk between groups (Fig. 1A). Infarct size was significantly reduced by both FeTPPS and hydroxyfasudil (51±4% and 49±3% of the area at risk, respectively) in comparison with the CIR group (69±2%, Fig. 1C). FeTPPS and hydroxyfasudil markedly suppressed arginase activity in tissue samples taken from the area at risk of the left ventricle following IR (Fig. 2A, $P<0.001$ vs. CIR) without affecting expressions of either arginase 1 or 2 protein (Fig. 2B and

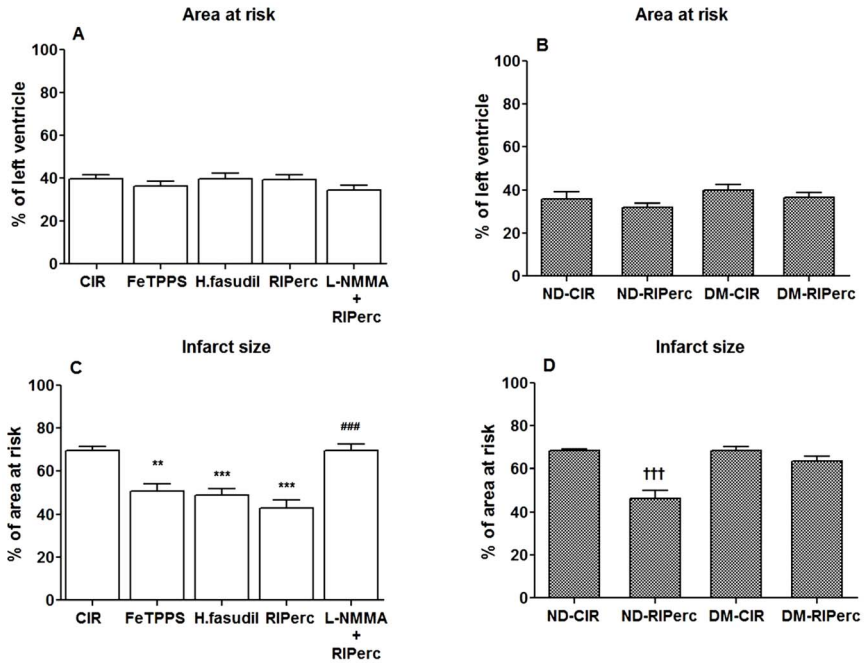

Figure 1. Myocardial area at risk and infarct size. Area at risk (A and B) expressed as % of left ventricle and infarct size (C and D) expressed as % of the area at risk following 30 min ischemia and 2 hrs reperfusion in rats included in protocol 1 (A and C) and in non-diabetic and diabetic rats included in protocol 2 (B and D). Values are means ± SEM; n = 6–10. **$P<0.01$, ***$P<0.001$ vs. CIR; $^{\#\#\#}P<0.001$ vs. RIPerc and $^{\dagger\dagger\dagger}P<0.001$ vs. ND-CIR.
doi:10.1371/journal.pone.0104731.g001

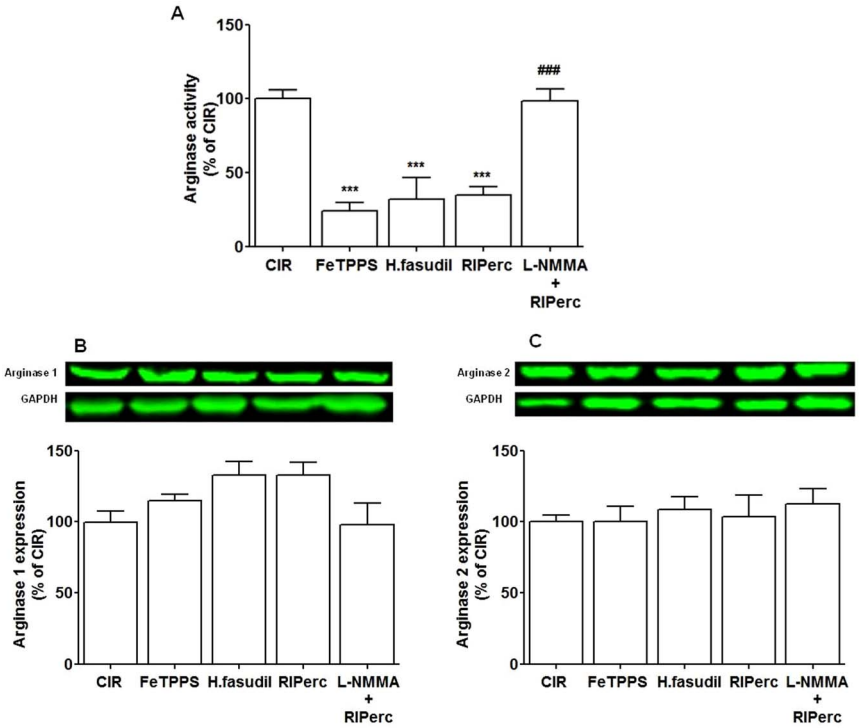

Figure 2. Arginase activity and protein expression in protocol 1. (A) Administration of the peroxynitrite decomposition catalyst FeTPPS, the ROCK inhibitor hydroxyfasudil (H.fasudil), remote ischemic preconditioning (RIPerc) and RIPerc+L-NMMA on arginase activity following ischemia/reperfusion. (B and C) Arginase 1 and 2 protein expression is depicted as representative blots. Values are means ± SEM n = 5–10. ***$P<0.001$ vs. CIR; ###$P<0.001$ vs. RIPerc.
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2C). By contrast, arginase activity in non-ischemic myocardium was unaffected by either FeTTPS or hydroxyfasudil (data not shown). In addition, FeTPPS suppressed the phosphorylated form of ezrin as a marker of ROCK activity in compared to controls following IR by a magnitude similar to that induced by the ROCK inhibitor (Fig. 3A, $P<0.001$).

3.2.2 Effect of RIPerc on infarct size and ROCK–arginase activity. RIPerc reduced infarct size by a magnitude comparable to that induced by FeTTPS and hydroxyfasudil (Fig. 1C). In addition, RIPerc markedly reduced arginase activity (Fig. 2A) and ROCK activity (Fig. 3A). These effects of RIPerc were completely abolished by administration of L-NMMA (Figs. 1C, 2A and 3A).

3.2.3 Effect of RIPerc on eNOS phoshorylation and peroxynitrite formation. Total and phosphorylated (Ser1177) eNOS was determined in left ventricle tissue samples taken from the area at risk from CIR and RIPerc groups. RIPerc enhanced phoshorylation of eNOS at Ser1177 following IR (Fig. 4A). In addition, the level of 3NT, a marker of peroxynitrite formation, was markedly attenuated by RIPerc (Fig. 4C).

3.3 Results of protocol 2

3.3.1 Characteristics of type 1 diabetes. Blood glucose level was 27.3±1.0 mmol/l four weeks after the administration of streptozotocin compared to 4.9±0.2 mmol/l in the control group ($P<0.001$). Body weight was significantly lower in the diabetic group (291±12 g) than in the control group (394±9 g, $P<0.001$).

3.3.2 Infarct size in protocol 2. Infarct size was similar in non-diabetic and diabetic animals subjected to IR (Fig. 1D). As in animals included in protocol 1, RIPerc resulted in a significant reduction of infarct size in the non-diabetic group (Fig. 1D). In contrast, RIPerc failed to reduce infarct size in rats with type 1 diabetes in comparison with controls (68±2% of area at risk in DM-CIR: vs. 63±2% in DM-RIPerc; Fig. 1D).

3.3.3 Arginase expression and ROCK–arginase activity following IR in diabetes. Arginase activity in non-ischemic myocardium was increased by 48±11% ($P<0.05$) in rats with type 1 diabetes compared to non-diabetic controls. Furthermore, arginase activity was increased in myocardium from the area at risk (ischemic) of rats with diabetes (by 70±20%) and in non-diabetic control rats (by 87±11%), ($P<0.01$) in comparison with non-ischemic myocardium. In tissue samples from the area at risk of the left ventricle arginase activity was significantly reduced by RIPerc in the non-diabetic control rats (Fig. 5A). By contrast, RIPerc did not affect arginase activity rats with diabetes (Fig. 5B). The expressions of arginase 1 or 2 protein were unaffected by RIPerc (Fig. 5C–F). Moreover, in contrast to the marked changes observed in non-diabetic rats, RIPerc failed to reduce ROCK activity or to increase the expression of phosphorylated eNOS at Ser1177 in rats with diabetes (Figs. 3 and 4).

Discussion

The main findings of the current study are that (1) upregulation of arginase activity by IR is mediated by peroxynitrite and ROCK activity in the myocardium, (2) RIPerc induced by bilateral femoral artery occlusion during sustained myocardial ischemia protects the heart against IR injury via a mechanism involving attenuated peroxynitrite formation and ROCK signaling, (3) the inhibition of this pathway results in downstream attenuation of arginase and increased NOS activity, and (4) the cardioprotective effect as well as the associated down-regulation of ROCK and

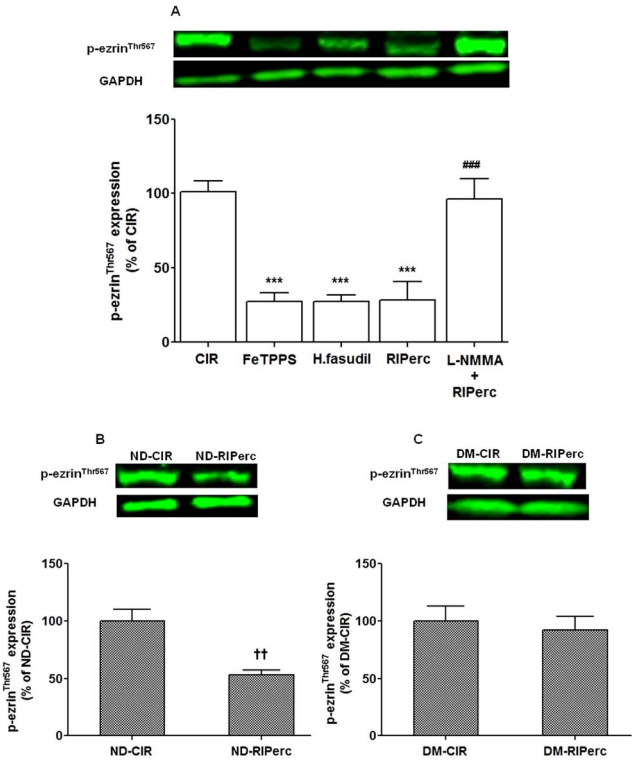

Figure 3. ROCK activity expressed as a phosphorylation of ezrin following ischemia/reperfusion. (A) Effect of the peroxynitrite decomposition catalyst FeTPPS, the ROCK inhibitor hydroxyfasudil (H.fasudil), remote ischemic preconditioning (RIPerc) and RIPerc+the NOS inhibitor L-NMMA in protocol 1. (B and C) Effect of RIPerC in non-diabetic and diabetic rats of protocol 2. Values are means ± SEM; n = 5–7. ***$P<0.001$ vs. CIR; ###$P<0.001$ vs. RIPerc and ††$P<0.01$ vs. ND-CIR.
doi:10.1371/journal.pone.0104731.g003

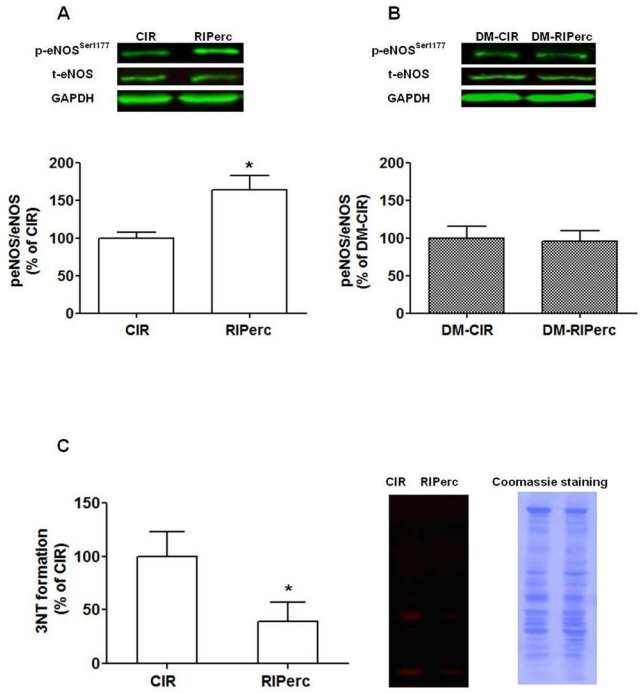

Figure 4. The effect of RIPerc on eNOS expression and peroxynitrite formation. (A and C) Effect of remote ischemic perconditioning (RIPerc) on phosphorylated of eNOS at Ser1177 (p-eNOS), total eNOS and nitrotyrosine (3NT) in protocol 1. (B) Effect of RIPerc on NOS expression in non-diabetic and diabetic rats of protocol 2. Values are means ± SEM; n = 5–8. *$P<0.05$ vs. CIR.
doi:10.1371/journal.pone.0104731.g004

arginase activity induced by RIPerc is abolished in a model of type 1 diabetes.

There is strong evidence that myocardial IR-injury is critically dependent on enhanced arginase activity resulting in reduced NO production and bioavailability [3,4,5]. In addition, available evidence suggests that arginase activity is significantly increased in the myocardium at risk already within 20 min after the onset of reperfusion [25]. Although the protective effect of arginase inhibition under IR has been confirmed, the exact mechanism regarding the upregulation of arginase activity in the myocardium is still unknown. Data obtained in cultured endothelial cells suggest that peroxynitrite is an upstream mediator of ROCK and arginase activation [8,9]. Since both peroxynitrite and ROCK activity are increased upon reperfusion and contribute to myocardial IR injury [26,27], we hypothesized that peroxynitrite and ROCK are possible upstream activators of arginase in the myocardium during IR *in vivo*. We demonstrate that administration of the peroxynitrite decomposition catalyst FeTPPS prior to the onset of reperfusion significantly reduced both ROCK and arginase activity, indicating that peroxynitrite is an activator of ROCK and arginase during myocardial IR. Furthermore, inhibition of ROCK suppressed arginase activity during myocardial IR. These findings are agreement with previous data from cell culture studies [8] and suggest that peroxynitrite-induced ROCK signaling stimulates arginase activity in the myocardium during IR. Although FeTPPS and hydroxyfasudil attenuated arginase activity, expression of arginase 1 and 2 protein was unaffected. In accordance with previous results [4] this indicates that alterations in myocardial arginase activity are not critically dependent on changes in arginase protein levels.

Next we investigated whether the peroxynitrite-ROCK-arginase-NO pathway is involved in the cardioprotective effect mediated by RIPerc. The reduction in infarct size induced by RIPerc was associated with a down-regulation of ROCK activity, which is in line with a previous observation [14]. In addition, we demonstrate that RIPerc reduced the 3NT-levels reflecting attenuated peroxynitrite formation. Based on the finding that the peroxynitrite-ROCK pathway activate arginase in IR, we hypothesized that arginase may be down-regulated by RIPerc. Accordingly, the endogenous cadioprotective mechanism triggered by RIPerc reduces myocardial arginase activity following IR. Since both inhibition of ROCK and arginase is known to result in increased eNOS activity [3,28,29] and enhanced eNOS substrate availability, we explored the effect of RIPerc following NOS inhibition and its effect on eNOS phosphorylation. In accordance with previous studies demonstrating the protective effect of eNOS and NO against myocardial IR injury [30,31], we found that RIPerc increased eNOS phosphorylation at Ser1177. Furthermore, the cardioprotective effect of RIPerc was completely abolished by the NOS inhibitor L-NMMA, confirming involvement of NOS. Since previous studies have shown that arginase inhibition protects from myocardial IR injury by a mechanism dependent on NOS activity and bioavailability of NO [3,4,5], it is tempting to speculate that down regulation of arginase is involved in the cardioprotective effect mediated by RIPerc. This needs to be established in future studies, however.

In this study, we demonstrate that RIPerc failed to induce cardioprotection in a rat model of type 1 diabetes. It is well

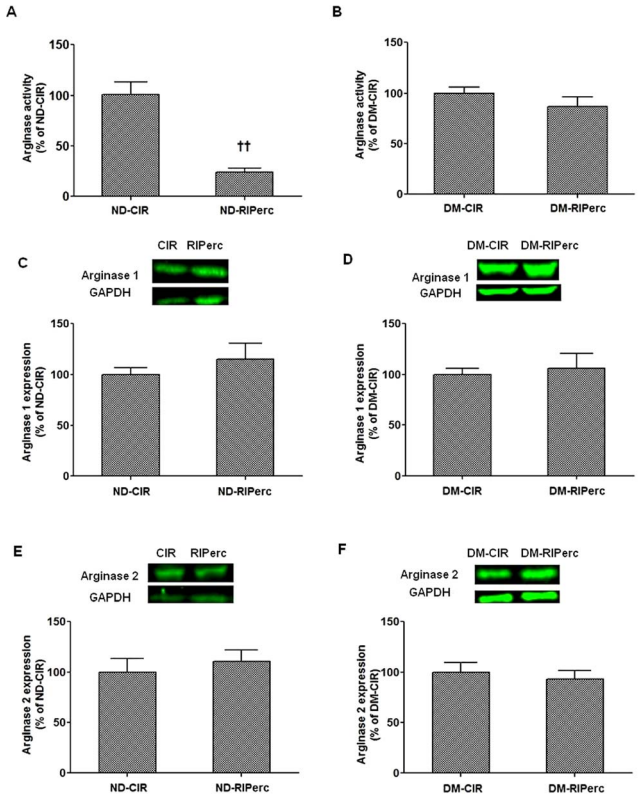

Figure 5. Arginase activity and protein expression in protocol 2. (A and B) Arginase activity and (C–F) expression of arginase 1 and 2 in non-diabetic rats (ND) and rats with type 1 diabetes mellitus (DM). Values are means ± SEM; n = 5–7 $^{\dagger\dagger}P<0.01$ vs. ND-CIR.
doi:10.1371/journal.pone.0104731.g005

established that the sensitivity of the diabetic heart to local pre- and postconditioning is impaired [32], but this is to the best of our knowledge the first demonstration that the cardioprotective effect of remote ischemic perconditioning is lost in diabetes. This may, at least in part, be associated with the dysregulation of signaling pathway activating eNOS [16,32]. In accordance, we found that myocardial eNOS (Ser1177) phosphorylation was increased in non-diabetic rats but unaffected by RIPerc in diabetic rats. Previous studies showed that increased arginase and ROCK activity are involved in diabetes-induced vascular dysfunction via mechanisms, at least in part, associated with reduction in NO bioavailability [2,33]. It has further been demonstrated that the Rho/ROCK signaling pathway is enhanced in the diabetic myocardium [34]. In the present study we show that myocardial arginase activity is increased rats with type 1 diabetes compared to non-diabetic controls. Interestingly, both arginase and ROCK activity was unchanged by RIPerc in the diabetic group, which is in sharp contrast to the down-regulation of arginase and ROCK activity by of RIPerc in non-diabetic rats. It remains to be established whether diabetes-induced upregulation of ROCK and arginase activity contribute to failure of RIPerc to induce cardioprotection in type 1 diabetes.

There are some limitations with the present study that deserve consideration. First, L-NMMA completely abolished the cardioprotective effect of RIPerc. We did not include a group receiving L-NMMA alone since it previously has been shown that L-NMMA and other NOS inhibitors do not influence IR injury *per se* [21,22,23]. Second, the current study demonstrates that reduced arginase and ROCK activity is associated with the cardioprotective effect of RIPerc, but the data do not provide evidence regarding a causative effect. Additional studies are needed to confirm whether this mechanism is a mediator of cardioprotection by RIPerc. Third, although our data demonstrate loss of cardioprotection induced by RIPerc in a model of type 1 diabetes, further studies are needed to establish whether similar effect are obtained in other models of diabetes including type 2 diabetes. Fourth, further studies are needed to clarify if the loss of the cardioprotective effect of RIPerc in type 1 in diabetes is due to impairment of signal transduction from the remote organ to the heart and/or impairment of protective signaling pathways in heart and their relation to arginase and ROCK activity.

In conclusion, the present study demonstrates that peroxynitrite and ROCK signaling pathways are involved in the upregulation of arginase activity during myocardial IR. In addition, we propose that reduction in arginase activity mediated by reduced formation of peroxynitrite and ROCK activity is of importance for the NOS-dependent cardioprotective effect of RIPerc. Importantly, our study also demonstrates that the cardioprotective effect and the associated signaling effects on arginase, ROCK and NOS by RIPerc are abolished in a model of type 1 diabetes.

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Author Contributions

Conceived and designed the experiments: AK YT ATG JP. Performed the experiments: AK YT OF. Analyzed the data: AK YT ATG OF JTL DCA JY JP. Contributed reagents/materials/analysis tools: AK YT OF JTL DCA JP. Contributed to the writing of the manuscript: AK YT ATG OF JTL DCA JY JP.

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IV

Inhibition of Rho kinase protects from ischemia-reperfusion injury via regulation of arginase activity and nitric oxide synthase in type 1 diabetes

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Abstract

Aim:
RhoA/Rho associated kinase (ROCK) and arginase are implicated in vascular complications in diabetes. This study investigated whether ROCK and arginase inhibition protect from myocardial ischemia-reperfusion injury in type 1 diabetes and the mechanisms behind these effects.

Methods:
Rats with streptozotocin-induced type 1 diabetes and non-diabetic rats were subjected to 30 min myocardial ischemia and 2 h reperfusion after being randomized to treatment with: 1) saline, 2) ROCK inhibitor hydroxyfasudil, 3) NO synthase (NOS) inhibitor N^G-monomethyl-L-arginine monoacetate (L-NMMA) followed by hydroxyfasudil, 4) arginase inhibitor N-omega-hydroxy-nor-L-arginine (nor-NOHA), 5) L-NMMA followed by nor-NOHA or 6) L-NMMA given iv before ischemia.

Results:
Myocardial arginase activity, arginase 2 expression and ROCK activity were increased in type 1 diabetes ($P<0.05$). ROCK inhibition and arginase inhibition significantly reduced infarct size in diabetic and non-diabetic rats ($P<0.001$). The cardioprotective effects of hydroxyfasudil and nor-NOHA in diabetes were abolished by NOS inhibition. ROCK inhibition attenuated myocardial arginase activity in diabetic rats via a NOS-dependent mechanism.

Conclusions:
Inhibition of either ROCK or arginase protect from ischemia-reperfusion injury in rats with type 1 diabetes via a NOS-dependent pathway. These results suggest that inhibition of ROCK and arginase constitutes a potential therapeutic strategy to protect the diabetic heart against ischemia-reperfusion injury.

Keywords: Diabetes, Rho kinase, arginase, ischemia-reperfusion.

Introduction

It is well known that nitric oxide (NO) formed by endothelial nitric oxide synthase (NOS) plays a pivotal role in cardiovascular regulation.[1] Emerging evidence suggests that a decrease in NO bioavailability is a key factor behind cardiovascular disease including ischemia-reperfusion (IR) injury associated with myocardial infarction[2] and vascular complication in diabetes[3, 4]. Although the signaling events leading to reduction in NO bioavailability remain to be fully clarified, recent data suggest that upregulation of RhoA/Rho associated kinase (ROCK)[5] and arginase[6] are involved. Growing evidence suggests a pivotal role for ROCK in the pathophysiology of cardiovascular diseases associated with endothelial dysfunction and inflammation.[5] It has also been suggested that increased ROCK signaling events contribute to myocardial IR injury.[7] Interestingly, arginase which converts arginine to citrulline is also activated during myocardial IR which results in reduced NO production due to a shift in arginine metabolism from NOS to arginase.[8-11] Two isoforms of arginase (1 and 2) are represented in the cardiovascular system,[6, 12] and inhibition of this enzyme protects the heart against IR injury through NO-dependent mechanisms[9-11]. The activity and expression of arginase are stimulated by reactive oxygen species and proinflammatory cytokines via the ROCK pathway.[6, 13, 14] We recently demonstrated that pharmacological inhibition of the ROCK pathway significantly reduced infarct size and arginase activity in the ischemic-reperfused myocardium[15], indicating an important interaction between ROCK and arginase in the setting of myocardial IR.

Both the ROCK and arginase pathways are activated in diabetes mellitus – a disease associated with cardiovascular complications and poor outcome following acute myocardial infarction.[4, 16-18] Thus, ROCK and arginase activities are enhanced in the vasculature and have been proposed to be involved in the development of vascular dysfunction in diabetes.[19-23] Considering that activated ROCK mediates up-regulation of arginase that contributes to impaired vascular function in diabetes[24], there seems to be an intriguing signaling pathway including ROCK-mediated activation of arginase leading to reduction in NO bioavailability and cardiovascular complications in diabetes. Moreover, it has been shown that the diabetic heart is more resistant to pharmacological and non-pharmacological (classical pre- and postconditioning) treatments aiming at reduction of IR injury.[25] In light of our recent observation showing that remote ischemic perconditioning was ineffective in type 1 diabetic rats[15], there is a need for identification of specific pharmacological strategies that protect from IR injury in diabetes.

Based on these observations, the aim of the present study was to investigate whether inhibition of ROCK and arginase protect from IR injury in a rat model of type 1 diabetes and to identify the mechanisms behind these effects. We hypothesized that targeting ROCK has beneficial effects in the setting of myocardial IR in diabetes via a mechanism involving downregulation of arginase activity and increased NO bioavailability.

Methods

The study was 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).

Induction of type 1 diabetes mellitus

Male Sprague-Dawley rats (Charles-River, Sulzfeld, Germany) of age 5-6 weeks were used in the study. Type 1 diabetes mellitus was induced by a single injection of streptozotocin

(STZ, 55 mg/kg) dissolved in sterile Dulbecco's phosphate buffered saline (DPBS) in the tail vein under anesthesia with isoflurane mixed with oxygen.[15] Only rats with blood glucose levels >15 mmol L^{-1} 72 h after the STZ injection were considered to be diabetic and were included in the study. Healthy age matched rats received an iv injection of vehicle (DPBS) and served as a non-diabetic control group. Blood glucose concentration was measured weekly by FreeStyle Lite blood glucose monitoring system (Abbot Diabetes Care Inc., CA, USA). The rats were used for further experiments four to five weeks after the injection of STZ or vehicle during which time they were given food and water ad libitum.

Experimental protocol

Rats were anesthetized with pentobarbital (50 mg kg^{-1}) ip followed by an iv infusion of 5 mg kg^{-1} h^{-1} pentobarbital. Rectal temperature was maintained at 37.5-38.5°C by a heated operation table. Animals were tracheotomized and ventilated with air by a rodent pressure controlled ventilator (50-55 strokes min^{-1}, 9 ml kg^{-1} tidal volume). The right carotid artery was cannulated and the catheter connected to a Statham blood pressure transducer for measurement of mean arterial pressure. Heart rate was determined from the arterial pressure curve. Hemodynamic parameters were continuously recorded on a personal computer equipped with PharmLab V5.0 (AstraZeneca R&D, Mölndal, Sweden). The left jugular vein was cannulated for administration of drugs. The heart was exposed via a left thoracotomy. A ligature was placed around the left coronary artery. After completion of the surgical preparation, the rats were allowed to stabilize for at least 15 min before being randomized into different groups (see below). Ischemia was induced by tightening of the ligature around the left coronary artery. Successful occlusion was associated with cyanosis of the myocardial area at risk. Reperfusion was initiated after 30 min of ischemia by removal of the snare and was maintained for 2 h. The reperfusion was associated with disappearance of the cyanotic color of the myocardium.

In one part of the study aimed at investigating the role of ROCK, rats with and without diabetes were randomized to the following treatments given iv 15 min prior to coronary artery ligation: 1) saline (n=5, non-diabetic rats; n=5, diabetic rats); 2) ROCK inhibitor hydroxyfasudil (0.5 mg kg^{-1}; n=5, non-diabetic rats; n=5, diabetic rats); 3) NOS inhibitor N^G-monomethyl-L-arginine monoacetate (L-NMMA, 10 mg kg^{-1}) followed by hydroxyfasudil (n=5, diabetic rats) or 4) L-NMMA only (10 mg kg^{-1}, n=4, diabetic rats).

In an additional part of the study aimed at investigating the role of arginase, separate rats with and without diabetes were randomized to the following treatments given iv 15 min prior to coronary artery ligation: 1) saline (n=7, non-diabetic rats; n=14, diabetic rats); 2) arginase inhibitor N-omega-hydroxy-nor-L-arginine (nor-NOHA, 100 mg kg^{-1}; n=8, non-diabetic rats; n=11, diabetic rats); 3) L-NMMA (10 mg kg^{-1}) followed by nor-NOHA (100 mg kg^{-1}, n=7, diabetic rats).

The doses were based on previous studies. L-NMMA has previously been shown not to affect infarct size *per se*[26] and to abolish the cardioprotective effects of ROCK inhibition[15] and arginase inhibition[9] in animals without diabetes using a similar protocol. These groups were therefore not repeated in this study.

Determination of infarct size and collection of myocardial samples

Infarct size was measured as previously described.[11] After 2 h of reperfusion, the coronary artery was reoccluded and 1.5 ml of 2% Evans blue solution was injected into the left jugular vein to stain non-ischemic myocardium for the determination of the area at risk.

The rats were euthanized by exsanguination and the heart was extracted. The right atrium, the right ventricle and the left atrium were removed, the remaining left ventricle was frozen and cut into 7-9 slices perpendicular to the base-apex axis. The third slice (counting from the apex) was weighed, and the ischemic and non-ischemic parts separated, frozen on dry ice and stored at -80°C until analyzed. All other slices were weighed, scanned from both sides for the determination of the area at risk and put in 1% triphenyltetrazolium chloride solution for 15 min at 37°C to distinguish viable myocardium from necrotic. After 24 h of incubation in 4% formaldehyde slices were scanned again from both sides, and the extent of myocardial necrosis and the area at risk were determined by planimetry of computer images (Adobe Photoshop 6.0, Adobe Photoshop CS 2; Adobe Systems, CA, USA).

Determination of arginase and ROCK activity

Arginase activity was determined by using a colorimetric assay previously described.[15] The assay measures the urea production using α-isonitrosopropiophenone. Following homogenization and centrifugation of tissue samples, 50 µl of the supernatant was added to 75 µl of Tris-HCl (50 mmol L^{-1}, pH 7.5) containing 10 mmol L^{-1} $MnCl_2$. The mixture was activated by heating for 10 min at 56°C. Each sample was then incubated at 37°C for 1 h with L-arginine (50 µL, 0.5 M, in Tris-HCl pH 9.7). 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 α-isonitrosopropiophenone (9% in ethanol) was added to the mixture and then heated at 100°C for 60 min. Arginase activity was calculated as µmol urea/mg protein/h and expressed as fold change from the control group.
Activity of ROCK was determined as described previously by analyzing the extent of phosphorylation of ezrin (Thr567) by immunoblotting.[15]

Immunoblotting

Left ventricular tissue samples were extracted in ice-cold RIPA buffer. Total protein content of the extracts was quantified by using bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The proteins were separated on a 7.5% SDS gel (40 µg $lane^{-1}$) and transferred onto nitrocellulose membranes (Amersham Biosciences, USA). Membranes were blocked for 1 h at room temperature and incubated overnight at 4°C with primary antibodies against arginase 1 and arginase 2 (1:1000, Sigma Aldrich, USA) or phosphorylated ezrin (p-ezrin; anti-phosphorylated Thr567, 1:1000, BD Pharmingen, USA). IRDye 800-conjugated goat anti-rabbit IgG (1:15000, LI-COR Biosciences, Lincoln, NE, USA) was used as secondary antibody and bands were visualized using infrared fluorescence scanner (IR-Odyssey, LI-COR Biosciences, Lincoln, NE, USA). Equal loading was confirmed by expression of GAPDH (1:5000, Sigma Aldrich). Band densities were analyzed with Image Studio Lite Version 3.1 (LI-COR Biosciences, Lincoln, NE, USA).

Chemicals

Pentobarbital was purchased from Apoteksbolaget (Stockholm, Sweden); hydroxyfasudil from Tocris Bioscience (Bristol, UK); nor-NOHA from Bachem (Bubendorf, Switzerland); L-NMMA from Alexis Biochemicals (Lausen, Switzerland); STZ, Evans blue, triphenyltetrazolium chloride, Trizma base and Trizma hydrochloride from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analysis

Data are presented as mean±SEM. Repeated-measures two-way ANOVA with Bonferroni post-hoc test was used for multiple comparisons of hemodynamic parameters. One-way ANOVA followed by Bonferroni post hoc test was used for comparisons of protein expression, enzyme activity and infarct size. Mann Whitney U-test was used for comparisons of two groups. A P-value <0.05 was considered to be significant. All analyses were performed using Prism™ 5 software (GraphPad Inc., San Diego, CA, USA).

Results

Matching of diabetic and non-diabetic rats

In the first part of the study blood glucose was 28.8±0.4 mmol L^{-1} four weeks after the administration of STZ compared to 4.5±0.1 mmol L^{-1} in the control group ($P<0.001$). Body weight was significantly lower in the diabetic group (278±8 g) than in the control group (386±6 g, $P<0.001$).

In the second part of the study blood glucose was 26.6±0.4 mmol L^{-1} four weeks after the administration of STZ compared to 4.7±0.1 mmol L^{-1} in the control group ($P<0.001$). Body weight was significantly lower in the diabetic group (295±7 g) than in the control group (408±6 g, $P<0.001$).

There were no differences in blood glucose or body weight between the groups within the non-diabetic or diabetic rats, respectively.

Hemodynamics in rats subjected to myocardial IR

Hemodynamic parameters of the rats included in the first and the second part of the study are presented in **Table 1** and **2**, respectively. In comparison with the non-diabetic animals, heart rate was significantly lower in rats with type 1 diabetes ($P<0.05$). There were no significant differences in heart rate or mean arterial pressure among non-diabetic or diabetic rats between the two parts of the study.

Infarct size

There were no significant differences in the area at risk between any of the groups (**Figure 1A and 2A**). Infarct size was comparable in the two parts of the study and it did not differ between diabetic control rats and non-diabetic controls (Figure 1B and 2B). The ROCK inhibitor hydroxyfasudil significantly reduced infarct size by comparable magnitudes in diabetic and non-diabetic rats from 66±2% and 66±2% to 48±4% and 46±2%, respectively ($P<0.001$, **Figure 1B**). Administration of the NOS inhibitor L-NMMA did not influence infarct size *per se* but abolished the cardioprotective effect of ROCK inhibition in diabetic rats (**Figure 1B**).

Administration of nor-NOHA significantly reduced infarct size both in diabetic and non-diabetic rats from 67±3% and 68±3% to 51±3% and 45±4% respectively ($P<0.001$, **Figure 2B**). The NOS inhibitor L-NMMA abolished the cardioprotective effect of nor-NOHA in diabetic rats (**Figure 2B**).

Effect of diabetes on ROCK activity, arginase expression and activity

The effect of diabetes on the activity of ROCK and arginase as well as expression of arginase isoforms was determined in the non-ischemic myocardium from diabetic and non-diabetic control groups. Both ROCK and arginase activity were significantly elevated in rats with diabetes in comparison to non-diabetic animals (**Figure 3A and B**). There was no difference in arginase 1 expression between the two groups (**Figure 3C**), whereas myocardial expression of arginase 2 was significantly increased in diabetic rats (**Figure 3D**).

Effect of ROCK inhibition on arginase activity following ischemia-reperfusion

ROCK inhibition significantly attenuated myocardial arginase activity in diabetic rats following IR. The effect of ROCK inhibition on arginase activity was reversed by NOS inhibition (**Figure 4**).

Table 1. Hemodynamic data, part 1.

Group	Parameter	Baseline	Before reperfusion	60 min reperfusion	120 min reperfusion
ND vehicle	MAP	90±3	81±2	83±5	74±3
	HR	426±7	395±12	386±7	367±5
ND hydroxyfasudil	MAP	85±4	78±3	67±3	72±4
	HR	424±9	401±9	373±14	362±5
DM vehicle	MAP	94±5	80±5	75±2	72±2
	HR	340±11	336±8**	324±9**	321±4
DM hydroxyfasudil	MAP	88±6	84±4	76±3	72±3
	HR	342±13***	325±8***	325±11**	325±5
DM L-NMMA+ hydroxyfasudil	MAP	90±8	88±7	75±5	73±3
	HR	335±8***	336±16**	317±12***	320±13
DM L-NMMA	MAP	89±3	77±5	71±4	70±4
	HR	344±7***	349±20	335±5*	331±4

Data are shown as mean±SEM. DM: diabetes mellitus rats; ND: non-diabetic rats; MAP: Mean arterial pressure, mmHg; HR: Heart rate, bpm. Significant differences from the ND vehicle group are indicated. Data are mean±SEM. * P<0.05; ** P<0.01; *** P<0.001.

Table 2. Hemodynamic data, part 2.

Group	Parameter	Baseline	Before reperfusion	60 min reperfusion	120 min reperfusion
ND vehicle	MAP	97±10	79±5	76±6	84±6
	HR	375±13	416±16	388±12	370±11
ND nor-NOHA	MAP	98±6	92±5	76±3	81±5
	HR	397±15	429±13	371±4	368±6
DM vehicle	MAP	91±5	82±4	72±3	71±3
	HR	345±10	360±12***	334±8**	330±6*
DM nor-NOHA	MAP	90±5	80±4	73±3	72±3
	HR	322±12**	340±6***	326±6***	321±7**
DM L-NMMA+ nor- NOHA	MAP	90±4	81±6	73±4	71±4
	HR	354±15	353±16**	343±10*	342±7

Data are shown as mean±SEM. DM: diabetes mellitus rats; ND: non-diabetic rats; MAP: Mean arterial pressure, mm Hg; HR: Heart rate, bpm. Significant differences from the ND vehicle group are indicated. Data are mean±SEM. * P<0.05; ** P<0.01; *** P<0.001.

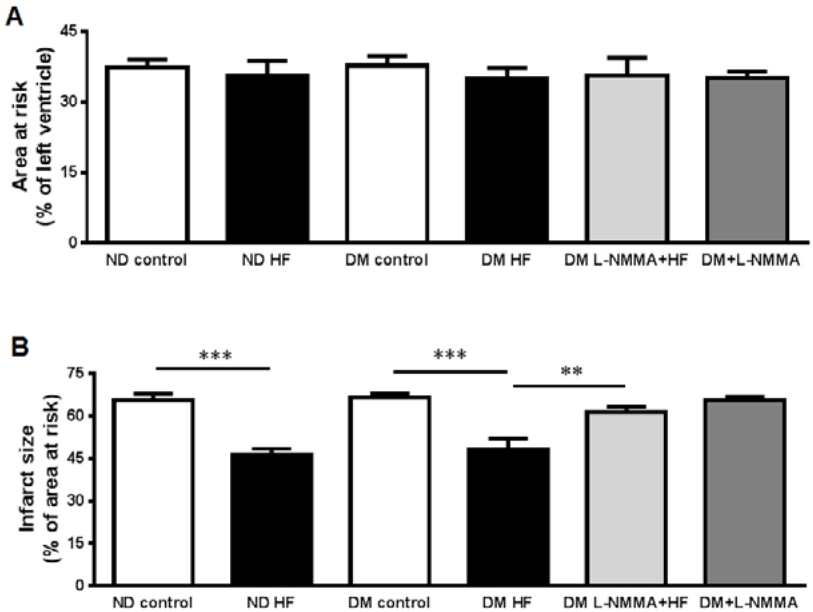

Figure 1. (A) Area at risk and (B) infarct size after 30 min of ischemia and 2 h of reperfusion. The diabetic (DM) and non-diabetic (ND) rats were given either saline (control, n=5, DM; n=5, ND); ROCK inhibitor hydroxyfasudil (HF; n=5, DM; n=5, ND); NO synthase inhibitor L-NMMA and hydroxyfasudil (n=5, DM); L-NMMA alone (n=4, DM). Data are presented as mean±SEM; ** P<0.01; *** P<0.001.

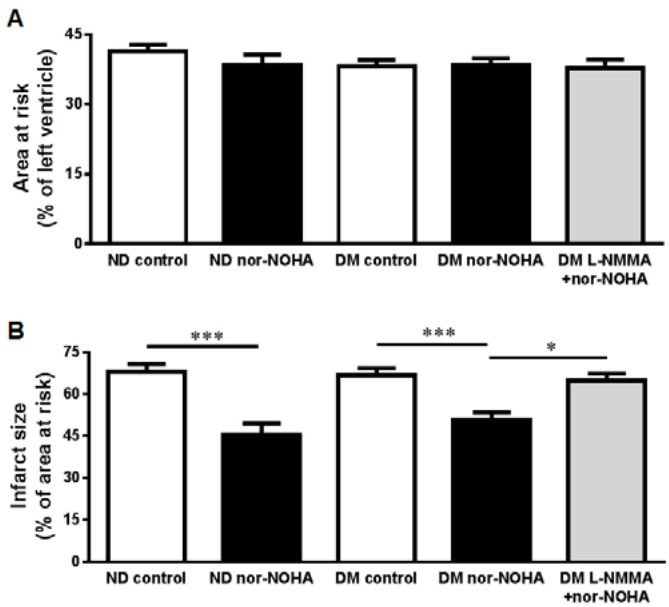

Figure 2. (A) Area at risk and (B) infarct size after 30 min of ischemia and 2 h of reperfusion. The diabetic (DM) and non-diabetic (ND) rats were given either saline (control, n=14, DM; n=7, ND); arginase inhibitor nor-NOHA (n=11, DM; n=8, ND); NO synthase inhibitor L-NMMA and nor-NOHA (n=7, DM). Data are presented as mean±SEM; * P<0.05; *** P<0.001.

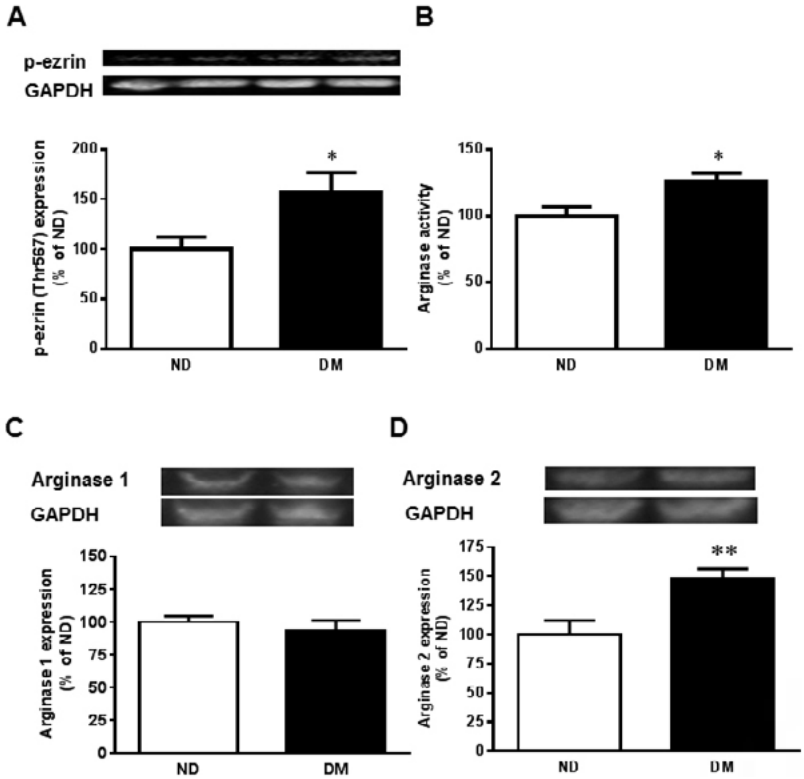

Figure 3. The effect of diabetes on activity of ROCK and arginase and the expression of arginase isoforms. (A) Expression of phosphorylated ezrin (p-ezrin) as a marker of ROCK activity, (B) arginase activity, (C) expression of arginase 1 and (D) arginase 2 in non-ischemic myocardium from non-diabetic (ND) and diabetic rats (DM) including representative immunoblots. Data are shown as mean±SEM; n=5-10; *P<0.05; **P<0.01.

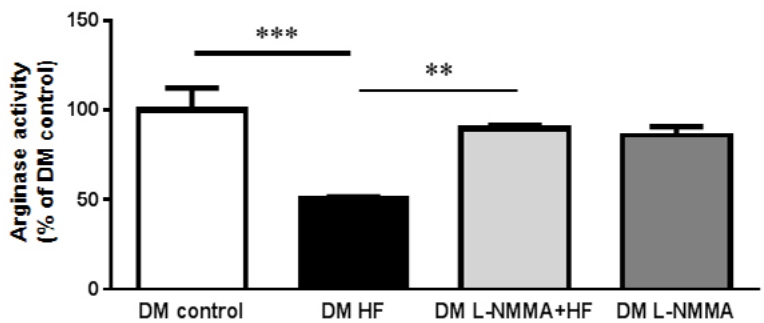

Figure 4. The effect of ROCK inhibition on arginase activity following ischemia-reperfusion. Myocardial arginase activity in rats with diabetes mellitus subjected to control ischemia-reperfusion (DM control), ROCK inhibition by hydroxyfasudil (DM HF), the combination of NOS inhibition and ROCK inhibition (DM L-NMMA+HF), and NOS inhibition alone (DM L-NMMA). Data are presented as mean±SEM; n=4-5; **P<0.01; ***P<0.001.

Discussion and conclusions

In this study we show that ROCK activity, arginase activity and arginase 2 protein expression are upregulated in the myocardium of type 1 diabetic rats. Inhibition of ROCK by hydroxyfasudil protects the diabetic heart from IR injury. The cardioprotective effect of ROCK inhibition is associated with significant decrease of arginase activity in ischemic-reperfused myocardium. In turn, inhibition of arginase by nor-NOHA protects the diabetic heart from IR injury emphasizing the importance of arginase for the deleterious consequences of IR injury in diabetes. The protective effects of both ROCK and arginase inhibition are abolished by the NOS-inhibitor L-NMMA. These observations indicate that ROCK inhibition provides protection against IR injury via a mechanism that involves downregulation of arginase and increased bioavailability of NO. Furthermore, both ROCK inhibition and arginase inhibition provide effective protection from IR injury in the setting of diabetes.

It is well established that attenuation of NO bioavailability is a dominant mechanism contributing to IR injury[27] and cardiovascular complications associated with diabetes[28]. Although the underlying mechanisms are not fully understood, recent data suggest that upregulation of ROCK[5] and arginase[6] are involved in signaling events leading to reduction in NO bioavailability. We therefore hypothesized that these signaling pathways are of importance for the development of IR injury in diabetes and targeting them would result in reduction in infarct size in a model of diabetes. Furthermore, there seems to be an intriguing link between ROCK and arginase whereby activation of arginase involves activation of ROCK.[13, 29] Whether this is of functional importance in the setting of IR has previously not been investigated.

Based on the involvement of ROCK in the signaling events contributing to IR injury[7] and its key function in the development of diabetic vascular complications, we first investigated whether targeting ROCK had beneficial effects in the setting of myocardial IR in diabetes. Accordingly, we found that myocardial ROCK activity was upregulated in rats with diabetes. Of further importance, administration of the ROCK inhibitor hydroxyfasudil attenuated infarct size in rats with type 1 diabetes. This cardioprotective effect of ROCK inhibition was abolished by NOS inhibition supporting the notion that cardioprotection from ROCK inhibition is mediated via a NOS-dependent mechanism in diabetes.

Given the previous data suggesting that ROCK may stimulate arginase activity and that arginase may be involved in the development of IR injury, we next investigated the effect of ROCK inhibition on arginase activity and the efficacy of arginase inhibition to reduce IR. Recent studies have been shown that diabetes is associated with elevation of arginase activity and impairment of vascular endothelial function characterized by low availability of NO.[17, 21] Furthermore, ROCK is reported as a trigger for increased arginase activity in diabetes.[24] It was found that the ROCK inhibitor hydroxyfasudil markedly attenuated the increase in arginase activity induced by IR demonstrating that arginase activity induced by IR is mediated via a signaling pathway involving ROCK. This observation in rats with type 1 diabetes is in line with that obtained in non-diabetic rats[15] and supports the notion that arginase activity is influenced by ROCK activity. Administration of the NOS inhibitor L-NMMA reversed the downregulation of arginase activity induced by the ROCK inhibitor hydroxyfasudil in diabetes but did not modify arginase activity *per se* in the presence of diabetes. This effect suggests that inhibition of NOS by L-NMMA shifted the utilization of L-arginine to arginase.

It was in addition demonstrated that inhibition of arginase reduced infarct size in rats with type 1 diabetes via an effect that was dependent on NOS activity. The cardioprotective effects induced by ROCK inhibition and arginase inhibition together with the observation that the ROCK inhibitor hydroxyfasudil decreased arginase activity collectively suggest that ROCK reduces infarct size via a mechanism including downregulation of arginase and increased bioavailability of NO.

Cardioprotective treatment strategies aimed at limiting IR injury have been described to be attenuated or absent in animal models of diabetes. Thus, it has been shown that the diabetic heart is more resistant to pharmacological and non-pharmacological (classical pre- and postconditioning) treatments aiming at reducing IR injury.[25] In addition, we have recently shown that remote ischemic perconditioning which markedly reduced infarct size in non-diabetic rats failed to induce cardioprotective effect in type 1 diabetic rats.[15] It is therefore an unmet need to develop treatment strategies that protect the myocardium in the presence of diabetes. The present study provides evidence that pharmacological inhibition of ROCK and arginase effectively reduce infarct size by magnitudes comparable to that obtained in non-diabetic animals. This opens up for new possibilities for treatment of diabetic disorders. Certain limitations of the study need to be acknowledged. These data are obtained in a model of type 1 diabetes and it is not known whether similar results are valid for other models of diabetes. There was no significant difference in the vulnerability to IR in type 1 diabetic hearts in comparison with non-diabetic hearts. This is in agreement with previous studies, although some studies have described both smaller and larger infarct size in various animal models of diabetes.[25] Of importance, the purpose of the present study was to investigate the efficacy of the cardioprotective interventions, not to compare the extent of IR injury *per se* between diabetes and non-diabetes. The presently used model is known to be resistant to cardioprotection induced by remote ischemic perconditioning, demonstrating a marked difference from non-diabetic rats and thereby the suitability of the model for this type of study.[15]

In conclusion, the present study demonstrates that myocardial ROCK activity, arginase activity and arginase 2 expression are increased in type 1 diabetic animals. Both ROCK and arginase inhibition reduce infarct size via a NOS–dependent mechanism. Furthermore, the cardioprotective effect of ROCK-inhibition involves downregulation of arginase. These findings suggest that ROCK and arginase are promising therapeutic targets for limitation of myocardial injury in diabetes.

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