Katholieke Universiteit Leuven
Group Biomedical Sciences
Department of Cardiovascular Sciences
Research Unit Experimental Cardiac Surgery

The effect of type II diabetes and the metabolic syndrome on cardiac second window of preconditioning

Gerry VAN DER MIEREN

Doctoral thesis in Biomedical Sciences
Leuven, 2013

Katholieke Universiteit Leuven
Group Biomedical Sciences
Department of Cardiovascular Sciences
Research Unit Experimental Cardiac Surgery

The effect of type II diabetes and the metabolic syndrome on cardiac second window of preconditioning

Gerry VAN DER MIEREN

Jury:

Promotor: Prof. dr. P. Herijgers

Co-promotor: Prof. dr. W. Flameng

Chair: Prof. dr. B. Meyns

Secretary: Prof. dr. J. D'hooge

Jurymembers: Prof. dr. D. De Ridder
Prof. dr. R. Klautz
Prof. dr. Ph. Kolh
Prof. dr. J. D'hooge
Prof. dr. V. Petrov

Leuven, 09.03.2013

Doctoral thesis in Biomedical Sciences

Dankwoord

Dit proefschrift wordt voorgesteld aan Prof. M. Waer, rector van de Katholieke Universiteit Leuven, Prof. M. Casteels, vice-rector van de groep Biomedische Wetenschappen en Prof. J. Goffin, decaan van de Faculteit Geneeskunde.

Allereerst wil ik mijn oprechte dank betuigen aan mijn promotor, Prof. Herijgers. Paul, de eerste keer dat ik je ontmoette was als co-assistent, ongeveer 11 jaar geleden. Je enthousiasme, gedrevenheid en expertise hebben de inspiratie en motivatie in mij losgemaakt om aan dit wetenschappelijk werk te beginnen. Je hebt me nadien alle vertrouwen en vrijheid gegeven en geleerd om kritisch, volhardend en met een nultolerantie voor fouten te werken. Het blijft me na al die jaren nog steeds verbazen hoe je erin slaagt zoveel academische, wetenschappelijke en klinische taken te combineren. Ik heb de voorbije jaren een deel van dit parcours kunnen meemaken. Professioneel bent u ongetwijfeld degene die mij het meest gevormd heeft, het meeste geduld en energie in mij gestoken heeft. Bedankt hiervoor!

Vervolgens wil ik mijn co-promotor Prof. Flameng bedanken. Uw wetenschappelijk en klinisch inzicht, ervaring en chirurgische capaciteiten hebben een diepe en blijvende indruk op mij gemaakt. Bedankt dat u mij de kans en vrijheid hebt gegeven om in het Labo Experimentele Cardiale Heelkunde te werken.

I respectfully thank the members of the jury, Prof. Klautz, Prof. Kolh, Prof. D'hooge, Prof. Petrov and Prof. De Ridder, for their valuable suggestions and criticism, which clearly improved this work. I know that this is a work-intensive job.

Ik dank de (zeer) lange lijst van collega's en medewerkers van het labo waarmee ik samengewerkt heb, die ik ontmoet heb of die me op een of andere manier hebben bijgestaan. De gedreven maar toch gemoedelijke sfeer in het Provisorium zal ik altijd herinneren. Ines, bedankt voor de vele uren van goede en aangename samenwerking voor de muizenkweek en luisteren naar alle frustraties, An voor het leren van de techniek van de PV-loops en alle adviezen, Annelies, Wouter, Nele en Leen, voor alle toffe momenten en babbels. Prof. Buyse en Prof. Vennekens wil ik bedanken voor de aangename samenwerkingen en publicaties.

Oprechte dank aan mijn vele leermeesters tijdens mijn chirurgische opleiding. Speciale dank gaat uit naar prof. Broos als coördinator van mijn opleiding tot chirurg. U was een uniek en onevenaarbaar leermeester.
Prof. Meyns wil ik bedanken als juryvoorzitter van deze thesis en coördinator van mijn opleiding cardiale heelkunde.

Verder wil ik alle collega's assistenten en residenten, de vele verpleegkundigen en secretaressen bedanken waarmee ik in de voorbije jaren vaak intensief heb samengewerkt.

Sofie, het vergt oneindig veel liefde en begrip om iemand de vrijheid te gunnen die jij me tijdens de verwezenlijking van dit project hebt gegeven. Ik was jarenlang ontelbare uren fysiek en geestelijk afwezig om dit project tot een goed einde te brengen. Zonder jouw steun en grenzeloze geduld zou dit thesismanuscript nooit tot

stand gekomen zijn.

Mijn moeder en vader wil ik bedanken voor mijn opvoeding, de vrijheid en vooral alle steun die ze mij geboden hebben. Mijn vader tevens voor het bricoleren van een aantal instrumenten voor dit werk.

Mijn schoonouders, schoonbroers en –zussen, alle neefjes en nichtjes, alsook al onze vrienden wil ik bedanken voor de hartelijke bijeenkomsten, hun luisterend oor en steun bij de moeilijkere momenten.

Tenslotte, draag ik dit werk op aan mijn oogappel Amélie. Jouw ongelooflijk enthousiasme, levenslust en onvoorwaardelijke liefde doen alle grote en kleine zorgen compleet verbleken. Jouw langverwachte komst in ons gezin is het mooiste wat ons is overkomen en hebben vele bijkomende prioriteiten tot leven gebracht. In de toekomst zullen we nog vaker met elkaar kunnen spelen, ravotten, lachen, boekjes bestuderen en naar Bumba kijken.

Table of contents

Dankwoord		2
Table of contents		4
List of abbreviations		5
Chapter 1	**General introduction**	7
	1.1. Clinical relevance of the project	8
	1.2. Preconditioning	10
	1.3. Mechanism of delayed preconditioning	11
	1.4. Diabetes and the metabolic syndrome	16
Chapter 2	**Objectives**	23
	2.1. General aim	24
	2.2. Specific aims	25
Chapter 3	**Materials and methods**	27
	3.1. Mouse models	28
	3.2. Preconditioning stimulus	29
	3.3. Treatments	30
	3.4. Biochemical analysis	30
	3.5. Ischemia/reperfusion	30
	3.6. Outcome parameters	31
	3.7. Experimental protocol	38
	3.8. Data management and statistical analysis	40
Chapter 4	**Hypoxic preconditioning preserves cardiac contractility and reduces infarct size in vivo**	41
Chapter 5	**Angiotensin-converting enzyme inhibition and food restriction in diabetic mice do not correct the increased sensitivity for ischemia-reperfusion injury**	48
Chapter 6	**Angiotensin-converting enzyme inhibition and food restriction restore delayed preconditioning in diabetic mice**	59
Chapter 7	**General discussion and future perspectives**	73
Reference list		88
Summary		96
Samenvatting		98
Curriculum vitae		100
Publications and abstracts		100

List of abbreviations

ACE	angiotensin-converting enzyme
ACE-I	angiotensin-converting enzyme inhibitors
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CO	cardiac output
COX-2	cyclo-oxygenase 2
CRP	C-reactive protein
DKO	double knock-out mice
dP/dt_{max}	maximal rate of pressure development during isovolumetric contraction
dP/dt_{min}	peak rate of pressure fall during isovolumetric relaxation
E_a	arterial elastance
EDPVR	end-diastolic pressure-volume relationship
E_{es}	end-systolic elastance
EF	ejection fraction
eNOS	endothelial nitric oxygen synthase
ESPVR	end-systolic pressure-volume relationship
FWOP	first window of preconditioning
G_p	parallel conductance
G_t	time varying conductance
HbA1c	glycosylated hemoglobin A1c
HDL	high-density lipoprotein
HO-1	heme oxygenase 1
HR	heart rate
iNOS	inducible nitric oxide synthase
IR	ischemia - reperfusion
K_{ATP}	potassium adenosine triphosphate channel
LAD	left anterior descending (coronary) artery
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LV	left ventricular
mPTP	mitochondrial permeability transition pore
MS	metabolic syndrome
NAD^+	nicotinamide adenine dinucleotide
NEFA	non-esterified fatty acids
NF-κB	nuclear factor-kappa B
NO	nitric oxide
Non P + I	non-preconditioned and ischemia/reperfusion
Non P + non I	non-preconditioned without ischemia/reperfusion
ob/ob	leptin-deficient mouse model

P + I	preconditioned and ischemia/reperfusion
P + non I	preconditioned without ischemia/reperfusion
PAI-1	plasma plasminogen activator inhibitor-1
P_{ed}	end-diastolic pressure
P_{es}	end-systolic pressure
PGE2	prostaglandin E2
PGI2	prostaglandin I2
PKC	protein kinase C
P_{max}	maximal pressure
P_{min}	minimal pressure
PRSW	preload-recruitable stroke work
PV	pressure-volume
ROS	reactive oxygen species
RVU	relative volume units
SERCA	sarco-endoplasmic reticulum Calcium^{2+}-ATPase
SH	sulfhydryl
Sham + NP	sham procedure (no ischemia-reperfusion) and non-preconditioned
Sham + P	sham procedure (no ischemia-reperfusion) and preconditioned
SOD	super oxide dismutase
STAT 1 and 3	signal transduction and transcription factor 1 and 3
ρ	resistivity
SV	stroke volume
SW	stroke work
SWOP	Second window of preconditioning
T2D	type II diabetes
TNFα	Tumor necrosis factor alfa
TTC	tri-phenyl tetrazolium chloride
V	volume
V_{ed}	end-diastolic volume
V_{es}	end-systolic volume
V_p	parallel volume
WT	wild type mice

Chapter 1

General introduction

General introduction

1.1. Clinical relevance of the project

The number of patients with diabetes and the metabolic syndrome increases in Western societies and reaches epidemic proportions [1-2]. At present, diabetes affects approximately 250 million people worldwide and by 2025 this is expected to increase to over 380 million, with type II diabetes accounting for 90-95% of them. Prevalence is expected to increase most in Asia and Africa with the majority of patients being found there in 2030 [2]. The prevalence of the metabolic syndrome currently exceeds 20% of individuals who are over 20 years of age and 40% of the population older than 40 years [1]. Heart failure is the leading cause of mortality in this patient group. The incidence of myocardial infarction is twice that of the general population and infarct size, for a given ischemic insult, is larger [3-4]. This, on top of their diabetic cardiomyopathy, increases the incidence and severity of heart failure [3-4]. Large-scale clinical trials showed that intensive glycemic control failed to reduce cardiovascular mortality and macrovascular incidents including myocardial infarction, in comparison with standard glucose control in diabetic patients [5]. Therefore, new or additional techniques to protect the diabetic heart against ischemic damage are eagerly awaited.

Tissues and organs can be protected from ensuing longer ischemia-reperfusion (IR) periods by short preceding episodes of 'preconditioning' ischemia [6]. The same effect can be obtained by administration of drugs, such as adenosine or inhalational anesthetic gasses, before the start of a period of IR [6]. This phenomenon is called preconditioning and is the most powerful endogenous protection mechanism against IR damage. In many experimental models, preconditioning can reduce infarct size as much as 80-90% [6]. Furthermore, preconditioning has been consistently observed over species and experimental models. However, after 26 years of research, it did not enter routine clinical practice yet [6]. One of the underlying reasons for this, is the uncertainty whether this technique can effectively salvage myocardium in patients with co-morbidities, such as type II diabetes or the metabolic syndrome. Most of our knowledge concerning preconditioning however has been gathered from experiments in normal healthy animal models [6]. These models are not optimal to study preconditioning for the clinical population that might benefit most of it, i.e. patients with type II diabetes or the metabolic syndrome.

After a preconditioning stimulus, the protected state is present for 1 to 2 hours, called 'classical preconditioning' or 'first window preconditioning', followed by a 'second window of protection' from 12 to 96 hours, the so-

called delayed preconditioning [6]. Clinical studies report that type II diabetic myocardium is resistant or has a higher threshold to classical preconditioning [7-8].

However, the second window of preconditioning or delayed preconditioning is the most interesting technique for myocardial protection in type II diabetic and metabolic syndrome patients, since it is capable to induce cardioprotection in a controled way for longer periods. This protection can therefore be planned more readily in clinical practice and even chronically induced when ischemia is anticipated in the following days, but not exactly predictable, e.g. in patients with cardiovascular disease undergoing non–cardiac surgery. If effective, this technique would add important therapeutic possibilities to our armamentarium. The effect of delayed preconditioning on cardiac IR injury in mouse models of type II diabetes and the metabolic syndrome has never been investigated and is the focus of this study.

Most patients with type II diabetes or the metabolic syndrome are treated [9]. These treatments might additionally influence the preconditioning potential. Despite the fact that hypocaloric diet is a standard treatment in type II diabetes and the metabolic syndrome [9], its cardiovascular effects are incompletely understood. Chronic and short-term food restriction reduce IR injury in wild type rodent models [10-12]. A study with obese rats showed that food restriction partially restores classical preconditioning [13]. The effects of long-term food restriction on delayed preconditioning was never investigated.

Angiotensin-converting enzyme (ACE)-inhibition is also a standard therapy in the treatment of hypertension in diabetic patients [9]. In addition to their blood pressure lowering properties, anti-ischemic and anti-atherogenic effects are described, as well as a reduction in oxyradical formation and cardiovascular remodeling [14]. In patients with type II diabetes, angiotensin-converting enzyme inhibitors (ACE-I) improve glucose control and insulin sensitivity, reduce proteinuria, and slow the progression of diabetic nephropathy [14]. It was previously shown that ACE-inhibition is capable to augment classical [15-16] and delayed preconditioning [17-18] in wild type animal models, by lowering the stimulus threshold. There are no data concerning the effect of ACE-I on delayed preconditioning in type II diabetes and metabolic syndrome models. Therefore, the effects of food restriction and ACE-inhibition on delayed preconditioning in mouse models of type II diabetes and the metabolic syndrome will be investigated in this study.

1.2. Preconditioning

Preconditioning is an extreme powerful and easily inducible endogenous mechanism against IR injury. Preconditioning was described for the first time in 1986 by Murry, Jennings and Reimer in their landmark study [19]. It describes the phenomenon that a short episode of preceding ischemia induces improved tolerance in an organ or tissue to the deleterious effects of a subsequent longer period of ischemia. A mild ischemic stress can thus enhance the ability of the heart to cope with another similar or greater stress [6]. The authors made this paradoxal observation after they exposed open chest dogs to a sequence of 4 brief ischemic episodes (5 min coronary occlusions, interspersed with 5 min reperfusion) and then subjected them to a prolonged, more severe ischemic insult (40 min coronary occlusion followed by 4 days of reperfusion). Control dogs underwent the 40 min occlusion without preceding exposure to ischemia. According to the conventional wisdom at that time, one would have expected that the dogs that received the 4 brief coronary occlusions would exhibit greater infarct size, because they had been exposed to longer cumulative ischemia. Surprisingly, they found that in this group of dogs infarct size was markedly reduced with controls and that this effect was independent of differences in coronary collateral flow. They coined the term “ischemic preconditioning” [6].

Although initially most studies were experimental, a growing interest in the phenomenon of preconditioning also resulted in the publication of clinical trials. There are 2 major reasons for this explosion of interest in preconditioning. First, it was clear that this cardioprotective mechanism was extremely powerful (in many studies infarct size could be reduced as much as 80% for a given ischemic insult, which has never been observed with other, existing cardioprotective mechanisms). Second, it was a remarkably general mechanism. It was found in several animal models and in humans and could be initiated by a wide range of triggers, such as local or remote ischemia, drugs, temperature changes,… [6]. Although preconditioning was first described in the heart, it became clear that also other organs can be protected by this mechanism, as described for example for lungs, kidneys, liver, peripheral muscle and central nervous system [20].

The discovery of preconditioning contributed also to the conceptual idea that the heart can sense and adapt to the environment. When exposed to a stress, it can change its phenotype in a way that promotes self-preservation [6].

After the discovery of the phenomenon of classical preconditioning in 1986, the observation that this phenomenon is biphasical (figure 1.1) was published in 1993 by 2 independent groups [21-22]. After an early protected phase, which develops within a few minutes and lasts for 1 to 2 hours, a late phase of precondition which lasts 3 to 4 days was demonstrated after a latent phase of 12 to 24 hours. This biphasic phenomenon was

subdivided as a “first window of preconditioning” (FWOP) or “classical preconditioning” and a “second window of preconditioning” (SWOP) or “delayed preconditioning” [23].

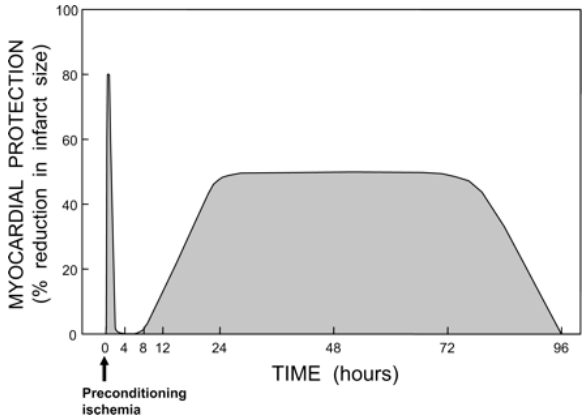


Figure 1.1: Preconditioning is a biphasic phenomenon (from [23])

The mechanism for these 2 phases is different. The underlying mechanism of FWOP is post-translational modification, most frequently phosphorylation, of preexisting proteins which are constitutively present in the cell and therefore induces instant protection. Subsequently, after a period of 12 to 24 hours, a SWOP develops and lasts for 3 to 4 days. This mechanism is due to the expression of stress-responsive cardioprotective genes, which are transcribed and code for the mediators of preconditioning. This explains the time-course of SWOP [6].

Ischemic preconditioning has to be distinguished from direct cardioprotection, that both protect the heart against IR injury. With preconditioning, the protection persists after the therapeutic intervention has dissipated, implying that the heart has a kind of “memory”. In contrast, with direct cardioprotection the enhanced resistance against IR injury occurs only while the therapeutic intervention is being applied and requires the continuous presence of this intervention. Preconditioning can thus be viewed as a “vaccination” against myocardial IR injury [6].

1.3 Mechanism of delayed preconditioning

A non-lethal cellular stress (stimulus) causes the release of chemical signals that trigger the development of delayed preconditioning.

The first discovered stimulus was ischemic stress, which is frequently used to induce both windows of preconditioning. Murry et al. described that four cycles of 5 minutes coronary occlusion and 5 minutes of reperfusion, limited the infarct size with 25 % during a subsequent 40 min occlusion [19]. This technique is also

effective in mice to induce both classical and delayed preconditioning [24].

Virtually all stimuli that cause a sublethal stress to the myocardium are capable to induce preconditioning: global hypoxia, heat stress, ventricular pacing and exercise [6,20,25-26]. Furthermore, several pharmacological agents are known to induce preconditioning: bradykinin, adenosine agonists, diazoxide, nitric oxide (NO) donors, K_{ATP}-channel openers, opioid agonists and TNFα (figure 1.2) [26].

Remote preconditioning is the phenomenon that ischemia in a remote organ, induces a preconditioned state in other organs. For example, repeated occlusion of the iliac artery can improve lung gas exchange after coronary artery occlusion or repeated occlusion of a forearm artery can protect against myocardial infarction [20].

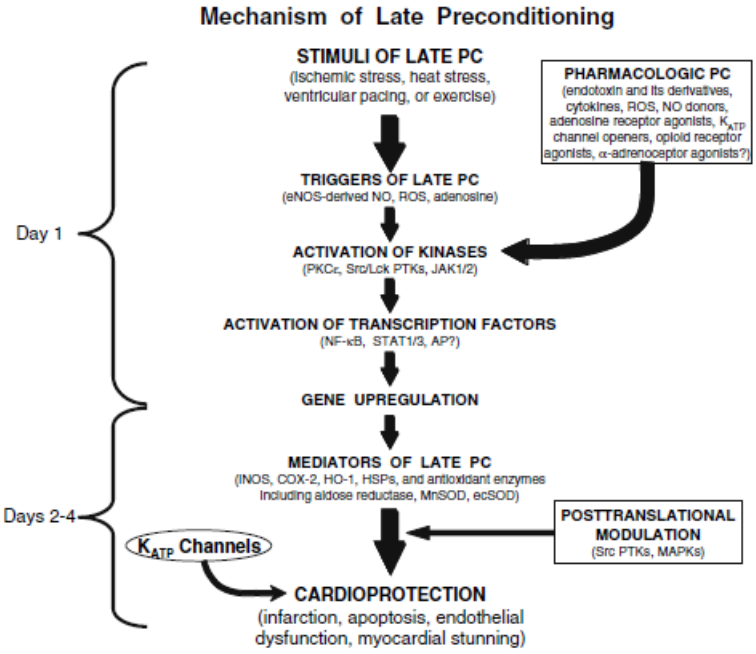


Figure 1.2: Mechanism of delayed preconditioning (from [6])

The preconditioning stimuli described cause a release of chemical triggers, such as NO derived from endothelial nitric oxide synthase (eNOS), reactive oxygen species (ROS), adenosine or stimulate opioid receptors (figure 1.2 and 1.3) [6].

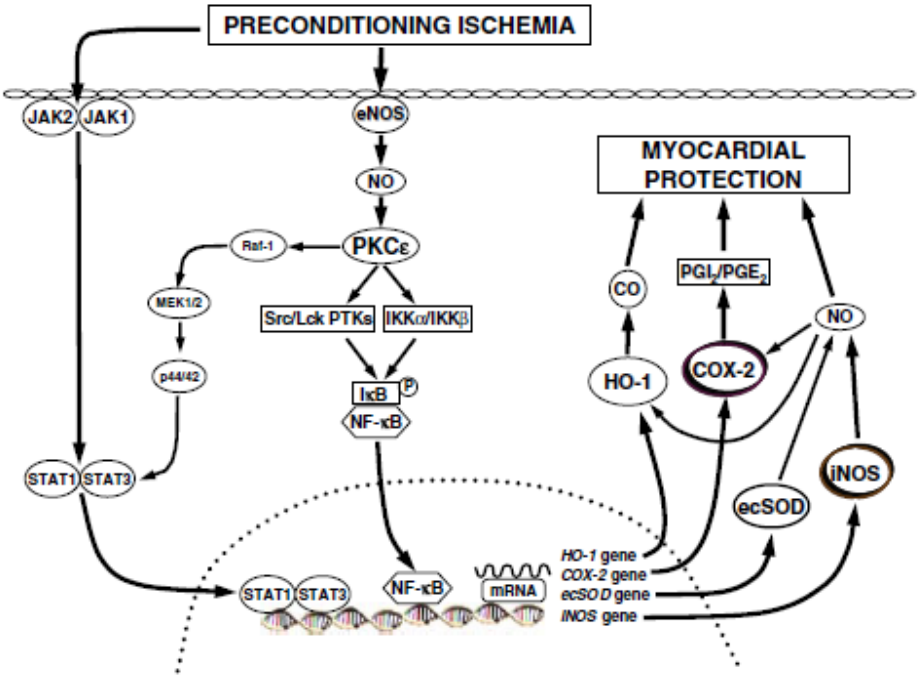


Figure 1.3: Pathway of delayed preconditioning (from [6])

The release of these triggers causes activation of a complex and yet incompletely understood, signal transduction cascade, that includes epsilon-isoform of protein kinase C (PKC), Src/lck-isoform of tyrosine kinase, Janus kinases 1 and 2 and probably other kinases as well (figure 1.2 and 1.3) [6].

These kinases result in the activation of cytoplasmatic and normally dormant stress-responsive transcription factors, such as nuclear factor kappa-B (NF-κB) and/or signal transduction and transcription factor 1 and 3 (STAT-1 and STAT-3) [6].

These transcription factors migrate to the nucleus of the cell and culminate in the up-regulation of cardioprotective genes, leading to the synthesis of new proteins that mediate the protection afforded by delayed preconditioning. This process requires time, explaining why delayed preconditioning develops only after 12 to 24 hours.

There is a plethora of genes which are involved and up-regulated during delayed preconditioning. Examples of involved genes are inducible nitric oxide synthase (iNOS), cyclo-oxygenase 2 (COX-2), heme oxygenase 1 (HO-1) and super oxide dismutase (SOD) [6].

Despite this plethora of up-regulated genes, iNOS plays an obligatory role in this pathway. The activity of COX-

2 protein requires iNOS-dependent NO-generation whereas the activity of iNOS does not require COX-2-dependent prostanoid generation [6]. COX-2 is thus downstream of iNOS in the cascade of delayed preconditioning. Targeted deletion of the iNOS gene abrogates delayed preconditioning induced by a variety of stimuli, including ischemia, adenosine A1-agonists, opioid δ1-agonists, endotoxin derivates and exercise, suggesting that iNOS is the final common mediator of cardioprotection [6]. iNOS produces NO which directly protects the myocardium. In addition, NO activates COX-2, leading to the synthesis of cardioprotective prostanoids, mainly prostaglandin I2 (PGI2) and prostaglandin E2 (PGE2). Furthermore, NO activates the HO-1 gene and HO-1 generates carbon oxide and biliverdin. Finally, delayed preconditioning induces also up-regulation of SOD-genes, which generate SOD (figure 1.2 and 1.3) [6].

The end products of this preconditioning cascade are NO, HO-1, carbon oxide, SOD and prostanoids (PGI2 and PGE2), which are the effectors of cardioprotection.

NO plays a critical and central role in the cardioprotection by delayed preconditioning by several mechanisms.

NO stimulates soluble guanylate cyclase and thus reduces the cellular calcium concentration, partly through activation of cGMP-dependent protein kinase which causes coronary vasodilatation [27].

NO terminates chain-propagating lipid radical reactions caused by oxidative stress and protects against the toxic effects on platelets and liposomes of exogenously supplied superoxide in the coronary circulation, which is anti-apoptotic [27].

NO inhibits the activity of platelets and neutrophils and the endothelial damage by their adhesion to the endothelial surface [27]. NO produced by this endothelial surface is a crucial trigger in the delayed preconditioning pathway.

Furthermore, the last years, more insight is gained in the important role of NO on mitochondrial targets. The respiratory chain releases small amounts of ROS predominantly by complex I under physiological conditions. Partial uncoupling of the respiratory chain protects against IR injury, supporting the importance of mitochondrial ROS for cardioprotection. During early reperfusion, ROS formation from various sources, including the respiratory chain, is largely augmented. Reversible inhibition of complex I by S-nitrosation (NO-mediated modification of thiols) is cardioprotective by limiting excess ROS formation during reperfusion [27].

The K_{ATP}-channel is a channel in the inner mitochondrial membrane. Opening induces potassium and

accompanying water flux along the electrochemical gradient, resulting in mitochondrial matrix swelling. The influx of potassium also triggers modest ROS formation by respiratory chain complex I, activating in turn PKC and p38 mitogen-activated protein kinase. NO induces opening of this channel by an as yet undefined mechanism [7,27].

The mitochondrial permeability transition pore (mPTP) is a large-conductance mega-channel, putatively constituted by the voltage-dependent anion channel in the outer membrane, the adenine nucleotide transporter in the inner membrane and cyclophilin D in the matrix. Under physiological conditions, mPTP is predominantly in a closed state but opens during IR. During the early period of reperfusion, opening of the mPTP causes all ions, metabolites and co-factors (such as NAD^+) smaller than 1.5 kDa to diffuse out of the matrix to the surrounding cytosol. Consequently, the cell will undergo massive cell necrosis and apoptosis [28].

It is widely accepted that preconditioning probably exerts its protective effect largely by inhibiting the opening of the mPTP at early reperfusion as final effector [28-29]. Several different signaling pathways are linked to this inhibition [29]. NO inhibits the opening of the mPTP [28]. As previously described, NO is also an inhibitor of ROS formation, which is in turn an activator of mPTP opening [28]. Hence, inhibiting mPTP transition is cardioprotective and can salvage the heart from IR injury.

The extracellular form of SOD causes an antioxidant defense: it catalyzes the reaction of superoxide to H_2O_2 and oxygen. The prostanoids, PGE2 en PGI2 protect the heart against IR injury. PGE2-induced protection is initiated by its receptor subtype EP4, but the exact underlying mechanism is incompletely understood [30].

HO-1 reduces apoptosis and cell death. The possible mechanism is unknown, but it was suggested that its action on heme generates carbon oxide, bilirubin and iron, which could act directly to prevent IR injury. The antioxidant action of bilirubin is sufficient to reduce the levels of ROS, generated during myocardium IR injury and thus prevents the cytotoxic effect associated with their generation [31].

In vitro preconditioning increases the post-ischemic developed tension in isolated atrial trabeculae muscles following simulated ischemia [32] and increases the resistance of isolated myocytes to hypoxic injury [33], as well as simulated ischemia [34].

In vivo, preconditioning can reduce infarct size up to 80%, reduces unloaded myocardial oxygen consumption [22], has endothelial protective effects [35-36], reduces reperfusion arrhythmias [37] and slows energy

metabolism during the early stages of ischemia [38]. Furthermore, it improves post-ischemic recovery of function [39] and reduces apoptosis [40].

1.4. Diabetes mellitus and the metabolic syndrome

1.4.1. Definition

Diabetes mellitus is a group of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin (type I), or because cells do not respond to the insulin that is produced (type II). Type I diabetes is an autoimmune disorder which destructs beta-cells of the pancreas. This results in a failure to produce sufficient insulin, and requires the patient to inject insulin (also referred to as insulin-dependent diabetes mellitus and juvenile diabetes).

Type II diabetes is the most common type of diabetes with 90% of the cases. It results from insulin resistance, a condition in which cells fail to use insulin properly, sometimes combined with an absolute insulin deficiency. Obesity is a frequent cause of the development of insulin resistance. Initially, insulin resistance is compensated by enhanced secretion of insulin to maintain normal glucose levels. Subsequently, the pancreatic beta-cells are unable to compensate, which results in hyperglycemia.

The third type of diabetes is gestational diabetes: pregnant women, who never had diabetes before, with an elevated blood glucose level during pregnancy. It may precede development of type 2 diabetes.

The diagnosis of diabetes mellitus is made when fasting plasma glucose is more than 126 mg/dl or with a disturbed glucose tolerance test [9].

The ***metabolic syndrome*** is a multiplex risk factor for cardiovascular disease [41]. In 1988, Raeven noted that several risk factors (dyslipidemia, hypertension, hyperglycemia) commonly cluster together and he called it Syndrome X [42]. He recognized it as a multiplex risk factor for cardiovascular disease and postulated that insulin resistance underlies Syndrome X. Nowadays, the co-incidence of several cardiovascular risk factors is described as the metabolic syndrome. The American Heart Association identified 6 components: abdominal obesity, atherogenic dyslipidemia, raised blood pressure, insulin resistance, pro-inflammatory state and pro-thrombotic state. A diagnosis of metabolic syndrome can be made, when 3 of these components are presents [41]. The World Health Organization views insulin resistance as a required component for diagnosis. In addition to insulin resistance, 2 other components are sufficient for a diagnosis of the metabolic syndrome. More recently,

a consensus was made about the clinical criteria for diagnosis of the metabolic syndrome. Presence of 3 of these criteria (table 1) constitutes the diagnosis of metabolic syndrome [43].

Measure	**Definition**
Elevated waist circumference *	Population and country-specific definitions (ex. European >102 cm men, >88 cm women)
Elevated triglycerides *	>150 mg/dl (<1.7 mmol/l)
Reduced HDL-C *	<40 mg/dl (1.0 mmol/l) in males <50mg/dl (1.3 mmol/l) in females
Elevated blood pressure *	Systolic >130 and/or diastolic > 85 mmHg
Elevated fasting glucose *	> 100 mg/dl

*(or drug treatment for this reason)

Table 1: Clinical criteria for the diagnosis of metabolic syndrome [43].

Abdominal obesity is the form of obesity associated most with the metabolic syndrome and is clinically determined by using the waist circumference. Abdominal obesity is considered as the most important determinant for the rising prevalence of the metabolic syndrome [41-43]. Obesity contributes to hypertension, high serum cholesterol and hyperglycemia. Excess adipose tissue releases several products that apparently exacerbate these risk factors: non-esterified fatty acids (NEFA), cytokines, plasma plasminogen activator inhibitor-1 (PAI-1) and adiponectin. A high plasma NEFA-level overloads muscle and liver with lipid and enhances insulin resistance. High C-reactive protein (CRP) levels accompanying obesity may signify cytokine excess and a pro-inflammatory state. An elevated PAI-1 contributes to a pro-thrombotic state, whereas low adiponectin levels that accompany obesity, correlate with worsening of metabolic risk factors [41].

Atherogenic dyslipidemia includes raised triglycerides, low concentrations high density lipoprotein (HDL) and high concentrations of low density lipoprotein (LDL) cholesterol (table 1). It can be detected in a routine blood analysis and all of these abnormalities have been implicated as independent atherogenic factors [43].

Elevated blood pressure is multifactorial in origin and strongly associates with obesity and commonly occurs in insulin resistant persons [43].

Insulin resistance strongly associates with the other metabolic risk factors but correlates univariately with cardiovascular risk. Patients with longstanding insulin resistance frequently manifest glucose intolerance. When

glucose intolerance evolves into diabetes-level hyperglycemia, elevated glucose constitutes a major independent risk factor for cardiovascular disease [43]. Many investigators advocate that insulin resistance is more important than obesity in pathogenesis [42,44]. They argue that insulin resistance and hyperglycemia directly induce other metabolic risk factors. Identifying a unique role for insulin resistance is complicated by the fact that it is linked to obesity. Insulin resistance generally rises with increasing body fat content.

Pro-inflammatory state is clinically recognized by elevations of CRP and multiple mechanisms underlie this. One cause is obesity, because excess adipose tissue releases inflammatory cytokines that may elicit higher CRP levels. Also fibrinogen can rise in response to high cytokine state [43].

Pro-thrombotic state is characterized by increased plasma PAI-1 and fibrinogen [43].

1.4.2. Current treatment of diabetes and the metabolic syndrome and potential interactions with the preconditioning mechanism

1.4.2.1. Hypocaloric diet

Weight reduction in obese persons will reduce the cardiovascular risk factors associated with type II diabetes and will improve hyperglycemia [9]. No long-term, large-scale study of lifestyle intervention or intentional weight loss has been adequately powered to examine cardiovascular disease end points in patients with diabetes mellitus. However, in a mouse model of the metabolic syndrome was it shown that food restriction induces an up-regulation of peroxisome proliferator-activated receptor α and γ and changes the expression of genes regulating glucose transport and insulin sensitivity, lipid metabolism, oxidative stress and inflammation [45]. In vivo studies have shown that food restriction partially restores classical preconditioning in rats [46]. Food restriction associated with exercise training was able to completely restore classical preconditioning in sedentary free fed rats [13]. The effect of food restriction on the second window of preconditioning is unknown and will be studied.

1.4.2.2. Blood pressure management

Epidemiological analyses and randomized clinical trials have demonstrated the impact of elevated blood pressure as a risk factor for both microvascular and macrovascular disease in diabetes and the metabolic syndrome [9,47]. As a result, many have argued that blood pressure management is the most critical aspect of the care of the patient with diabetes. Epidemiological analyses show that higher risk for cardiovascular events and mortality starts at a blood pressure higher than 115/75 mmHg in the general population and doubles for every 20 mmHg systolic or 10 mmHg diastolic increase. Patients with diabetes should be treated to a systolic blood pressure

lower than 130 mmHg and a diastolic blood pressure lower than 80 mmHg. If this goal cannot be achieved by the initiation of lifestyle modification within 3 months, pharmacological agents should be started [9,47].

Multiple studies that have used thiazide diuretics, ACE-I, angiotensin-receptor blockers, beta-blockers and calcium channel blockers have demonstrated benefits on microvascular and combined vascular end points [9]. Several trials suggest that ACE-I may be associated with better cardiovascular outcomes than dihydropyridine calcium channel blockers. Furthermore, many recent studies with ACE-I and angiotensin receptor blockers suggest benefits that cannot be fully attributed to blood pressure lowering in preventing and delaying the progression of advanced kidney disease. For this reasons, the current guidelines state that ACE-I are the drugs of choice in the initial treatment of hypertension in people with diabetes [9].

ACE-I have in addition to their blood pressure lowering properties, well documented anti-ischemic and anti-atherogenic effects, reduce oxyradical formation and has an effect on cardiovascular remodeling. In case of diabetes, ACE-I have also favorable effects on glucose control and insulin sensitivity and proven effectiveness in reducing proteinuria and slowing the progression of diabetic nephropathy [14].

The effect of ACE-inhibition on infarct size in experimental models of acute myocardial infarction is uncertain [48]. Some studies in dogs and pigs report a significantly reduced myocardial injury after treatment with captopril. Other studies in canine models, didn't find a reduction of the extent of necrosis [15,48]. It was previously shown that ACE-inhibition is capable to augment classical [15-16] and delayed preconditioning [17-18] in wild type animal models, by lowering the stimulus threshold. The effect of ACE-I on preconditioning in diabetic models is not yet investigated.

1.4.2.3. Lipids

In patients with type II diabetes mellitus and the metabolic syndrome, triglycerides are often elevated, HDL is generally decreased and LDL level may be elevated, borderline or normal [9,43]. LDL particles are small and dense, carrying less cholesterol per particle. These small dense particles are more atherogenic, even more than suspected by their concentration alone, because they may be more readily oxidized and glycated. Although an elevated LDL-level generally is not recognized as the major lipid abnormality in patients with type II diabetes, clinical trials amply demonstrated that LDL-lowering with statins reduces risk for major coronary events, regardless of diabetic status. Triglyceride-rich lipoproteins represent a secondary target of lipid-lowering therapy with fibrates as drugs of choice [9].

Hyperlipidemia is regarded as an independent risk factor in the development of ischemic heart disease. Epidemiological studies showed that there is a strong relationship between the elevation of serum total cholesterol concentration and the morbidity and mortality of myocardial infarction. Previously, this was attributed solely to the development of coronary atherosclerosis due to hypercholesterolemia but in the last decade, the possibility of hypercholesterolemia-induced deterioration of endogenous adaptive mechanisms against myocardial IR injury in hypercholesterolemic patients was also considered [7].

Despite intensive research, the controversy still remains on whether experimental hyperlipidemia influences the severity of myocardial IR injury and whether it interferes with the cellular mechanisms of cardioprotection. Especially in mouse models of dyslipidemia conflicting data were published. It was shown that 2 weeks of a high-cholesterol diet increased infarct size after 30 min of ischemia and 120 min of reperfusion in low-density lipoprotein receptor knock-out (LDLR-/-) mice compared with wild type mice. However, a high-cholesterol diet for 12 weeks resulted in a significant decrease in infarct size in both wild type and LDLR-/- mice [7].

The 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors (statins) inhibit the synthesis of mevalonate, a rate-limiting step in cholesterol biosynthesis. A number of large clinical trials have shown that chronic administration of statins has potent cholesterol-lowering effects and reduces cardiovascular morbidity and mortality [7]. Because cardioprotection by statins was also observed in patients with normal cholesterol levels, it has been proposed that statins may exert a broad spectrum of cholesterol-independent protective effects including plaque stabilization, preservation of endothelial function, and scavenging of free radicals, as well as anti-proliferative, anti-ischemic, anti-inflammatory and anti-apoptotic effects. Some studies report a dose dependent protection or exacerbation of statins in IR injury [7]. Few data are available concerning the effects of statins on preconditioning. Pravastatin restores the infarct size-limiting effect of ischemic preconditioning, blunted by cholesterol diet-induced hypercholesterolemia in a rabbit model of myocardial infarction. However, other studies have shown that statins attenuate the infarct size limiting effect of classical preconditioning in rat hearts. The mechanism by which statins may interfere with cardioprotective mechanisms is unclear. Both mevalonate pathway dependent and independent mechanisms can be involved, including alterations of oxidative and nitrosative stress [7].

1.4.2.4. Glucose management

Glycemic control clearly reduces microvascular complications in patients with diabetes; however, one of the most hotly debated clinical questions in diabetes is whether better glycemic control is associated with a reduction

in cardiovascular diseases and how low we should go in pursuing glycemic targets [5,9]. The American Diabetes Association recommends a glycosylated hemoglobin A1c (HbA1c) target of <7%. However, targeting an HbA1c as close to normal (<6%) as possible without causing significant hypoglycemia in individual patients failed to reduce cardiovascular mortality and macrovascular incidents including myocardial infarction, in comparison with standard glucose control in diabetic patients [5].

There are several drugs available for the treatment of diabetes. Insulin is mainly used in the treatment of type I diabetes and in type II diabetes if HbA1c levels cannot be sufficiently reduced with oral anti-diabetics. Beyond the possible detrimental effect of diabetes on preconditioning phenomena, the picture is further colored by the influence of this anti-diabetic drug treatment on IR injury and cardioprotection. It was shown that insulin can induce a preconditioning effect [49]. Furthermore, insulin exerts cardioprotection when administered at the time of myocardial reperfusion through the activation of the PI3K-Akt component of the reperfusion injury and salvage kinase (RISK) pathway and the recruitment of downstream targets including the phosphorylation of p70S6K and eNOS [49]. eNOS is a key player in the SWOP pathway (figure 1.3) [6].

Besides insulin therapy, there are several oral anti-diabetics available. Sulfonureas (glibenclamide, glicazide, glimepiride, glipizide and gliquidon) and glinides (repaglinide and nateglinide) stimulate the beta-cells of the pancreas by inhibition of the K_{ATP}-channels [7]. Furthermore, K_{ATP}-channels have also important regulatory functions in the heart. First, K_{ATP}-channels in coronary smooth muscle cells contribute to the control of coronary blood flow at rest and in hypoxia. Second, K_{ATP}-channels in the sarcolemma of cardiomyocytes may contribute to the adaptation of the heart to stress. In addition, the opening of mitochondrial K_{ATP}-channels plays a central role in cardioprotective mechanisms. It has been well established that classic and late preconditioning in several species, including humans, is abolished by K_{ATP} -channel blockers. Mitochondrial K_{ATP}-dysfunction has been proposed to be involved in the loss of preconditioning in human atrial sections ex vivo [50]. Therefore, inhibition of cardiovascular K_{ATP}-channels by insulin secretagogues is considered to increase cardiovascular risk [7].

Biguanides (metformin) lower blood glucose levels primarily by decreasing the amount of glucose produced by the liver. They make muscle tissue more sensitive to insulin and increase glucose uptake. Metformin reduces infarct size in non-diabetic animals by activation of cAMP-activated protein kinase and Akt. However, clinical and experimental studies in diabetic patients and models report a significantly higher cardiovascular risk and controversial effects on the effect on IR injury in subjects treated with metformin [8].

Thiazolidinediones or peroxisome proliferator-activated receptor-γ agonists (rosiglitazone and pioglitazone) stimulate insulin action in muscle and fat and reduce glucose production in the liver. They possess lipid-lowering and anti-inflammatory properties, and reduce IR injury in wild type and hypercholesterolemic models. Nevertheless, recent meta-analysis suggest that there is an increase in the risk of myocardial infarction [51].

Chapter 2

Objectives

Objectives

2.1. General aim

Heart failure is the leading cause of mortality in people with type II diabetes. The incidence of myocardial infarction in diabetic patients is twice that of the general population [3-4]. Infarct size for a given ischemic insult is larger in diabetic mice than in controls [52-53]. This, on top of diabetic cardiomyopathy, contributes to progressive heart failure. So, new techniques to protect these diabetic hearts from IR injury are very welcome.

Preconditioning is a powerful and reproducible endogenous protection mechanism against ischemic damage. It is not known whether delayed preconditioning is inducible in patients with co-morbidities, such as type II diabetes and the metabolic syndrome.

Classical preconditioning is either attenuated or absent in the presence of type I and II diabetes [7-8,54-55]. Delayed preconditioning is therapeutically a more attractive window of preconditioning because it can be planned more adequately and because of its long standing effect from 12 till 96 hours after the preconditioning stimulus. Since the mechanism of delayed and classical preconditioning is different, it is possible that delayed preconditioning bypasses the defective mechanism of classical preconditioning. No comprehensive study was performed yet to study the efficacy of delayed preconditioning in type II diabetes and the metabolic syndrome. Therefore, the aim of this study is to investigate the efficacy of delayed preconditioning in vivo in mouse models of type II diabetes and the metabolic syndrome.

Furthermore, as previously described, most of these patients are treated. It is thus possible that these treatments and drugs interact with IR injury and the cardioprotective mechanisms. Therefore, the effect of frequent therapies in diabetes and the metabolic syndrome, i.e. food restriction and ACE-inhibition, were studied because of their potential effects on IR injury and delayed preconditioning in these populations.

2.2. Specific aims

1. To investigate contractility, the effect of IR injury and hypoxic preconditioning in vivo in wild type mice (chapter 4)

The first aim is to characterize our wild type mouse model. Left ventricular contractility was investigated. Secondly, the impact of IR injury on left ventricular contractility and infarct size was determined. Furthermore, the effect of hypoxic delayed preconditioning on the impact of IR injury was investigated.

It was hypothesized that hypoxic preconditioning induces a SWOP in vivo, measured by a better preserved left ventricular contractility and reduced infarct size.

2. To investigate contractility and the effect of IR injury in vivo in mouse models of type II diabetes and the metabolic syndrome (chapter 5)

The second goal of this study is to investigate contractility and the impact of IR injury in our diabetic and metabolic syndrome mouse models.

It was hypothesized that left ventricular contractility is worse in the models of type II diabetes and the metabolic syndrome. Furthermore, it was hypothesized that the impact of IR injury is larger in these mouse model than in wild type mice.

3. To investigate the impact of interventions (food restriction and ACE-inhibition) on in vivo contractility and IR injury in mouse models of type II diabetes and the metabolic syndrome (chapter 5)

Patients with type II diabetes and metabolic syndrome are usually treated with several drugs, to treat the combination of risk factors. This might influence contractility and the impact of IR injury. Food restriction and ACE-I are standard therapies in the treatment of type II diabetes and the metabolic syndrome [9]. The impact of 12 weeks treatment with food restriction or ACE-I on in vivo contractility and IR injury were studied in mouse models of type II diabetes and the metabolic syndrome.

It was hypothesized that food restriction and ACE-inhibition ameliorate left ventricular contractility and reduce the increased sensitivity for IR injury.

4. To investigate the efficacy of hypoxic delayed preconditioning in vivo in mouse models of type II diabetes and the metabolic syndrome (chapter 6)

Clinical studies report that type I and type II diabetic myocardium is resistant or has a higher threshold to classical preconditioning [7-8]. Delayed preconditioning does not protect the hearts in type I diabetes [56]. The effect of delayed preconditioning on cardiac IR in mouse models of type II diabetes and the metabolic syndrome has never been investigated and is the focus of this chapter.

It was hypothesized that our mouse models of type II diabetes and the metabolic syndrome are resistant or have an higher threshold to delayed preconditioning.

5. To investigate the impact of interventions (food restriction and ACE-inhibition) on the efficacy of hypoxic delayed preconditioning in vivo in mouse models of type II diabetes and the metabolic syndrome (chapter 6)

The effects of hypocaloric diet on preconditioning are incompletely understood. A study with obese rats showed that food restriction partially restores classical preconditioning [13]. The effects of long-term food restriction on delayed preconditioning was never investigated. Food restriction increases eNOS-levels in a mouse model of the metabolic syndrome [45], NOS being a determinant trigger in the delayed preconditioning pathway [6]. It is therefore conceivable that food restriction restores the delayed preconditioning potential and induces cardioprotection by this mechanism.

ACE-inhibition augments classical [15-16] and delayed preconditioning [17-18] in wild type animal models, by lowering the stimulus threshold. There are no data concerning the effect of ACE-I on the delayed preconditioning potential in type II diabetic and metabolic syndrome models. ACE-I might however impact on the delayed preconditioning potential since they are capable to block the degradation of bradykinin into inactive peptides and to increase eNOS and NO-levels [14-18]. In the delayed preconditioning pathway, bradykinin and eNOS are triggers and NO is a trigger and end-effector [6]. Therefore, the aim of this study is to investigate the effects of food restriction and ACE-inhibition on delayed cardiac preconditioning in diabetic or metabolic syndrome models.

It was hypothesized that food restriction and ACE-inhibition ameliorate or restore the preconditioning potential in our mouse models of type II diabetes and the metabolic syndrome.

Chapter 3

Materials and methods

Materials and methods

3.1. Mouse models

Experiments were conducted in wild type (WT) mice with C57Bl6/J genetic background. C57Bl6/J is the most widely used inbred strain and the first to have its genome sequenced.

Homozygous leptin deficient mice (ob/ob) were used as model for type II diabetes. Leptin is produced by adipocytes and acts through hypothalamic receptors to inhibit food intake and increase energy expenditure. The ob/ob mouse is one of the most widely used models for type II diabetes, featuring abdominal obesity, insulin resistance and hyperglycemia. Unlike db/db, ob/ob with a C57BL/6J background, show moderate features of type II diabetes with mild hyperglycemia. Therefore, ob/ob, expressed on C57Bl6/J background is an excellent mouse model to study type II diabetes, insulin resistance and obesity (table 3.1) [57]. They also develop diabetic cardiomyopathy as seen in diabetic patients [58].

Double knock-out mice (DKO) for low density lipoprotein receptor (LDLR-/-) and leptin (ob/ob) were used as model for the metabolic syndrome (figure 3.1). DKO with combined leptin and LDL-receptor deficiency feature many characteristics of the metabolic syndrome, i.e. obesity, dyslipidemia, hypertension, insulin resistance, and impaired glucose tolerance and/or diabetes (table 3.1) [45,58]. DKO mice develop left ventricular diastolic and systolic dysfunction comparable with the diabetic cardiomyopathy seen in patients [58].

WT, LDLR-/- and ob/ob and heterozygous ob/+ mice were purchased form Jackson Laboratories (Bar Harbour, Maine, USA). Mice were backcrossed for at least 10 generations into the C57Bl6/J background and mice had 98.4% C57Bl6/J background. To obtain DKO mice with combined leptin and LDLR deficiency, LDLR-/- mice were crossed with ob/+ mice as previously described [45,58]. All offspring were genotyped by polymerase chain reaction techniques. Mice were housed at 22°C on a fixed 12 hour light-dark cycle. Groups were sex-matched and investigated at the age of 24 weeks. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental protocols were approved by the Institutional Animal Care Commission and Ethical Committee.

	C57Bl6/J	ob/ob	DKO (LDLR-/-; ob/ob)
Obesity	0	+	+
Type II diabetes	0	+	+
Dyslipidemia	0	0	+
Atherosclerosis	0	0	++

Table 3.1: General characteristics of the used mouse models



Figure 3.1: Left: lean WT mouse – Right: obese DKO mouse

3.2. Preconditioning stimulus

The majority of preconditioning studies, use intermittent coronary occlusion as preconditioning stimulus [24]. However, this method needs cardiothoracic surgery with its inherent mortality in obese mouse models. Secondly, anesthesia is needed to perform ischemic preconditioning, which can interfere with the preconditioning stimulus. Intermittent hypoxia is an attractive method to induce SWOP in small animal models, such as mice. Hypoxia induces SWOP in vitro [25,59].

Mice were placed in a plexiglas container (Agnthos, Sweden) of 0.8 liter. A portable oxymeter (Analox O_2 EII, North Yorkshire, UK) was calibrated at 20.7 % for room air and was subsequently placed in this plexiglas container for continuous registration of the oxygen content (figure 3.2). A mixture of 100 % nitrogen and compressed air was blown through this container via 2 separate flowmeters. Within 30 seconds, the oxygen content was lowered to 6 % oxygen. Five cycles of 6 minutes of 6 % oxygen, interspersed with 6 minutes of room air were used [25]. In the non-preconditioned group, mice were placed in the plexiglas container, with only compressed air (without 100% nitrogen) blown through the container. After this hypoxic preconditioning protocol, mice were placed in their regular cage with food and water ad libitum for 24 hours.



Figure 3.2: Setup for hypoxic preconditioning of mice

3.3. Treatments

3.3.1. Diet (food restriction)

All mice were fed standard chow containing 9 % fat (Ssniff, Soest, Germany).

Food intake of free-fed DKO mice and lean WT-mice are respectively 5.7 g/d and 2.5 g/d [45,60].

Food intake of diet-restricted ob/ob and DKO mice was restricted to the amount of lean mice, 2.5 g/d, between 12 and 24 weeks of age [45,60].

3.3.2. ACE-inhibition

Captopril (Acros, Geel, Belgium) was injected intraperitoneally (10 mg/kg/day) between 12 and 24 weeks of age in WT, ob/ob and DKO mice [61].

3.4. Biochemical analysis

Blood of conscious mice was collected by tail bleeding into EDTA tubes after an overnight fast at 12 and 24 weeks of age. Plasma was obtained by centrifugation. Triglycerides and total cholesterol was determined with a diagnostic reagent kit (Roche) and glucose with a glucometer (Menarini Diagnostics).

3.5. Ischemia / reperfusion

Twenty-four hours after hypoxic preconditioning, anesthesia was induced with urethane (1.2 g/kg) and alfa-chloralose (50 mg/kg) intraperitoneally injected [59,61]. Mice were placed on a heating pad and rectal temperature maintained at 37.0 ± 0.5 °C. Surgery was performed under a surgical microscope. Through a midline neck incision, a tracheostomy was performed and mechanical ventilation started with room air (Minivent 845; Hugo Sachs/Harvard Apparatus, March-Hugstetten, Germany). A left anterolateral thoracotomy was

performed in the 3th intercostal space. The left lower limb was positioned to the right to facilitate visualization of the left anterior descending coronary artery (LAD). Two millimeter below the tip of the left auricle, a 8/0 polypropylene suture was passed under the LAD. The snare was tightened on a small plastic tubing piece of 1.5 mm in order to perform a non-traumatic occlusion of the LAD. Successful coronary occlusion was visually verified by observing the distal part turning pale (figure 3.3). Occlusion was maintained for 30 minutes. Afterwards, the snare was released, reperfusion visually checked by blushing of the area, the chest closed and a reperfusion period of 1 hour allowed before the left ventricular pressure-conductance measurements were started.

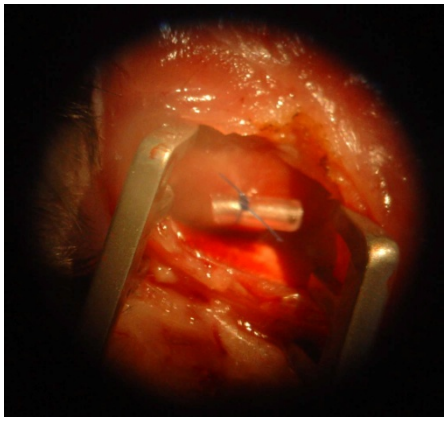


Figure 3.3: Non-traumatic occlusion of the LAD with blanching of the distal myocardium

3.6. Outcome parameters

3.6.1. Infarct size determination

To employ regional ischemia, it was necessary to delineate the field of the occluded artery (LAD). This field is referred to as the "risk zone" as it is at risk of infarction. The risk zone was marked by infusing Evans blue dye (0.8 ml, 1 % solution) into the coronary tree while the manipulated branch is occluded at exactly the same place. All the tissue that colored blue, belonged to the region *not* at risk. The tissue that didn't turn blue was situated in the distribution area, distally of the occlusion site at the LAD. Inside the area that didn't color blue (area at risk), we had to distinguish the infarcted tissue from the survived or living tissue. Tri-phenyl tetrazolium chloride (TTC) staining has emerged as the most popular and reliable method [24,55,62]. This technique relies on the ability of dehydrogenase enzymes and co-factors in the tissue to react with tetrazolium salts to form a formazan pigment. It can be argued that tissue lacking either of these would not be able to survive and is therefore dead or destined to die. To permit a washout of dehydrogenase enzymes out of the dead cells, a reperfusion period of 1 hour was respected.

Ischemic myocardium is also exquisitely sensitive to temperature. It has been reported that cooling salvages 7% of the risk zone per degree of cooling [62]. Therefore, continuous temperature monitoring and control was performed and kept between 37.0±0.5°C. The tetrazolium powder was diluted in a 2-part phosphate buffer system of Na_2HPO_4 (0.1 M, high pH) and NaH_2PO_4 (0.1 M, low pH) to a 1% solution with an pH of 7.4 [62]. Temperature of 37°C was achieved by carefully heating the TTC over a hot plate on a low setting and carefully monitoring the temperature with a thermometer.

After the pressure-conductance measurements, Evans blue (0.8 ml, 1 % solution) was given intravenously via an exchanged catheter in the right jugular vein to determine the left ventricular perfusion area at risk. After the administration of Evans blue, there was a cardiac arrest within 30 seconds, due to the direct toxicity of Evans blue and the sudden volume load (0.8 ml added in a few seconds to a total blood volume of 2 to 3 ml). The heart was excised and placed in Tissue Freezing Medium (Tissue Tek, Sakura Finetek, Torrance, USA) in a – 20°C freezer for at least 1 hour. With a custom-made razor blade system, the heart was cut in slices of 1 mm thickness. These slices were placed for 20 minutes in TTC (1% solution, 37°C, phosphate buffer pH 7.4). The slices were agitated at least once a minute, because any area constantly touching the bottom or sides of the beaker would not be stained. The surviving tissue turns into a deep red color and the infarcted tissue is a pale color (figure 3.4). To fix the slices, they were placed during 10 minutes in paraformaldehyde (4% solution, 20°C). The formalin step increases the contrast. All slices were weighed and photographed with a digital camera under microscopic magnification. Analysis of the slices was determined by the number of pixels in each zone (total slice, risk zone, infarct zone) for all the slices of each heart with Adobe Photoshop 8.0 (Adobe System Inc.). After correction for the weight of the respective slices, the risk zone and infarct zone were calculated for the entire heart.

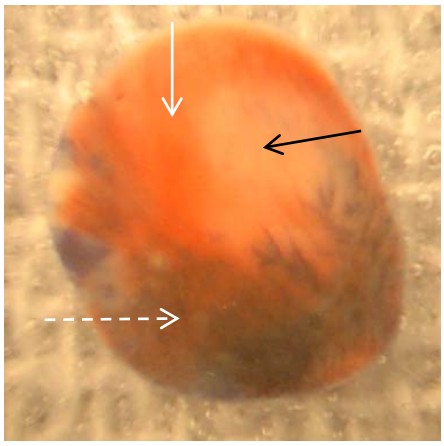


Figure 3.4: Heart slice: deep red area represents the survived tissue (white arrow). Pale area is infarcted tissue (black arrow). The blue area (white dotted arrow) represents the area which was not at risk.

3.6.2. In vivo left ventricular contractility

3.6.2.1. Measuring heart function in mice

The small size of the mouse hearts and the very high heart rates (500 - 600 beats/min) have demanded extensive modifications to techniques that have been used for analysis of heart function in larger animals. A number of techniques have been miniaturized and validated so that cardiac function can be assessed *in vivo* with an intact mouse.

Conductance micromanometry provides a continuous left ventricular (LV) pressure-volume (PV) signal that is accurate, despite the rapid heart rate of the mouse. The PV-loop method is regarded as the gold standard for assessment of intrinsic myocardial function in large animals. The development of miniaturized PV-catheters has made this methodology applicable to small animals such as mice [58,60,63-66].

The catheter is composed of 2 pairs of platinum electrodes with an intervening pressure sensor (figure 3.5). The electrodes generate an electric field and measure instantaneous segmental conductance from which cardiac volumes are determined. It offers the only method to obtain continuous volume signal in patients or intact animals to study ventricular mechanics. When coupled with pressure, the generation of ventricular PV relationships allows precise hemodynamic characterization of ventricular systolic and diastolic function and loading conditions, and therefore offers a unique method to study pathophysiology of cardiac diseases [65-66].

Because conductance is measured, transfer into volume data is needed. The formula to transfer conductance data into volume data is $V = (1/\alpha)\ \rho\ L^2\ [G(t) - G_p]$. In this formula is V the absolute volume, α is related to the current field non-linearity, ρ is the blood resistivity, L is the interelectrode distance and G(t) the time-varying conductance. G_p is parallel conductance from the tissues surrounding the ventricular cavity [66].

So, besides instantaneous conductance G(t) measurements, 3 other factors of this formula, namely α, ρ and G_p have to be determined in each experiment. Because of the difficulty to measure α and ρ in mice, the output is presented as relative volume units (RVU) during the experiments. Afterwards, a calibration needs to be performed to establish a relationship that converts RVU into absolute volumes. Therefore, cuvettes with known volume are filled with blood at the end of each experiment. The conductance catheter is suspended in the cuvettes and specific conductance is measured. Regression of the known volumes of the cuvettes against the corresponding conductance is determined and its slope and offset term are used in the analysis to convert RVU into absolute volumes [64].

Subsequently, parallel conductance G_p has to be determined. Ideally, the current applied over the excitation electrodes on the catheter should go through the blood only. However, some of the current goes through the surrounding tissues, causing an overestimation of the blood volume. The technique used to determine this parallel conductance or parallel volume is the hypertonic saline technique, as described by Baan et al [66]. A small bolus of hypertonic saline (30 %) is injected. The goal is to change the conductivity of the blood without significantly changing the volume or pressure within the ventricle. The saline will cause an offset in the volume data (cfr. infra). The end-diastolic volume (V_{ed}) is plotted against the end-systolic volume (V_{es}) and a regression line is constructed. This regression line is then extrapolated to the point of identity (V_{ed}=V_{es}), thus a left ventricle empty of blood (figure 3.6). As a result the conductance values at this point is due to parallel conductance. The source of the parallel conductance in mice arises primarily from the LV myocardium and to a lesser degree from the other structures of the chest [64,66].

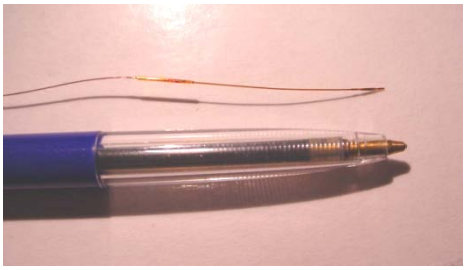


Figure 3.5: Miniaturized pressure-conductance catheter.

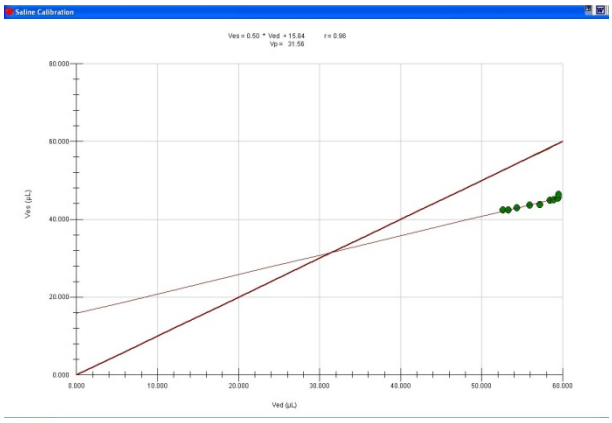


Figure 3.6: Regression line from the end-diastolic volume, plotted against the end-systolic volume.

3.6.2.2. The pressure-volume loop

Most parameters that determine cardiac performance can be deduced from recordings of LV pressure and/or volume in function of time. PV-loops can easily be constructed from these recordings if LV pressure and volume is recorded simultaneously (figure 3.7). The filling phase is initiated with opening of the mitral valve (point A,

figure 3.7) and increase in ventricular volume. At end-diastole, when the mitral valve closes (point B, figure 3.7), the systolic phase starts with pressure build up without volume ejection, i.e. the isovolumetric contraction phase (phase between point B and C, figure 3.7). Subsequently, blood is ejected from the ventricle into the aorta after opening of the aortic valve (point C in figure 3.7). This causes a decrease in left ventricular volume. The end of ejection is defined by aortic closure (point D, figure 3.7) and is followed by myocardial relaxation characterized by pressure decrease without volume change, i.e. the isovolumetric relaxation phase (phase between point D and A, figure 3.7) [64].

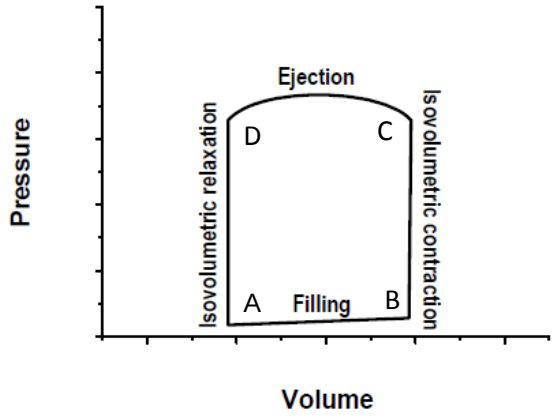


Figure 3.7: Pressure-volume loop

3.6.2.3. Load-dependent hemodynamic parameters

The dynamics of PV-loops is the representation of Starling's law. During isovolumetric contraction, tension is increased at constant length. The initial length is determined by V_{ed}, the preload. During ejection, the muscle shortens while overcoming an opposing force, the afterload. Preload is defined as the load present before contraction has started and is determined by the left ventricular volume at the end of diastole (V_{ed}). Afterload, the sum of all the forces counteracting the ejection of blood, is influenced by peripheral vascular resistance, arterial compliance, aortic characteristic impedance, and systolic and diastolic time intervals [64].

Arterial elastance (E_a), a measure for afterload, is defined as the end-systolic pressure to stroke volume ratio, and can be derived from the PV-loop.

Stroke volume (SV) can be easily determined from the PV-loop as the difference in end-diastolic (V_{ed}, point C) and end-systolic volume (V_{es}, point D).

Ejection fraction (EF), the percentage of V_{ed} that is ejected in a single beat, is in addition to contractility, highly

dependent upon preload and afterload [63]. In many instances, loading conditions vary, leading to significant misinterpretations of contractility when only EF is used as parameter.

Cardiac output (CO) can be calculated as the product of heart rate and stroke volume.

Stroke work (SW) is the mechanical energy the heart develops during the cardiac cycle. It is represented as the area enclosed by the PV-loop.

Another frequently used parameter as index of contractility is the maximal rate of pressure development in the LV during isovolumetric contraction *(*dP/dt_{max}*)*. Preload dependence of this parameter is well known and established in larger mammals. This dependence appears to be even larger in the mouse [64].

The parameters of diastolic function can be organized into measures of isovolumetric relaxation and measures of passive LV characteristics. Isovolumetric relaxation can be quantified by measuring the time-course of the fall in LV-pressure, which is quantitatively described by the peak rate of pressure fall (dP/dt_{min}) and the time constant of an exponential fit of the time course of isovolumetric pressure decline (Tau). Tau and dP/dt_{min} are influenced both by loading conditions [64]. The passive characteristics of the LV (distensibility) can be described by the end-diastolic PV-relationship (EDPVR, cfr infra).

3.6.2.4. Load-independent hemodynamic parameters

In many instances, loading conditions vary, leading to significant misinterpretations of contractility. This is especially the case in experiments in mice. Their circulation contains extremely small volumes of blood and several controlled and uncontrolled conditions (overnight fast, heat, previous anesthesia,…) can alter loading conditions. Therefore, it is extremely important in these experiments, to determine parameters which are independent of loading conditions [63-64]. The preload is varied and the functional response of the ventricle on this preload change is captured. During experiments with mice, preload can be varied by compression of the inferior caval vein between liver and diaphragm with a cotton swab, while PV-loops are recorded.

A first load-independent parameter is the slope of the end-systolic pressure-volume relationship (ESPVR), the end-systolic elastance (E_{es}) (figure 3.8). This reflects LV chamber end-systolic stiffness, and is used as an index of contractility. Increased myocardial contractility is reflected in a higher E_{es} and a leftward shift on the volume axis. The parameter E_{es} is known to reflect changes in contractility, provided that heart rate (HR) is constant. A disadvantage is that E_{es} is limited by dependence on cardiac size (if the heart increases in volume, ESPVR will

decrease regardless of contractility) and that the ESPVR is often curvilinear in the mouse, making it more difficult to use [64].

A parameter which is load-independent and represents the compliance of the ventricular myocardium at the end of the diastole is the slope of the end-diastolic PV-relationship (EDPVR) (figure 3.8). Augmented stiffness of the ventricular wall increases the slope of the EDPVR. The opposite occurs in case of increased compliance [64].

Finally, preload-recruitable stroke work (PRSW) is the slope of the relationship between V_{ed} and SW performed by the ventricle, and is a representation of the ventricular performance curves. The contractile state of the LV is reflected by the steepness of this relationship. With increasing contractility, a constant increment in end-diastolic volume leads to a larger increase in stroke work performed by the heart. Because SW is measured, the PRSW integrates both systolic and diastolic function, and changes in diastolic function alone can also influence its slope. It is a particularly useful parameter since it is chamber size independent and robust [63].
In addition, a weak point in determination of contractility by pressure-volume loops is the necessity to determine blood resistivity (ρ) and parallel volume (V_p) in order to convert conductance and RVU into absolute volumes. PRSW, when derived from invasive pressure-conductance measurements, is also independent of the calibration factors ρ and V_p, which obviates potential technical and extrapolation inaccuracies, especially important in mice studies. Indeed, the methods for determination of the calibration factor ρ and of V_p are not as precise in the mouse as in larger species. Consequently, PRSW is the most excellent parameter to characterize LV contractile function since it does not rely on the precision of determination of these calibration factors, in contrast with e.g. E_{es} [63-64].

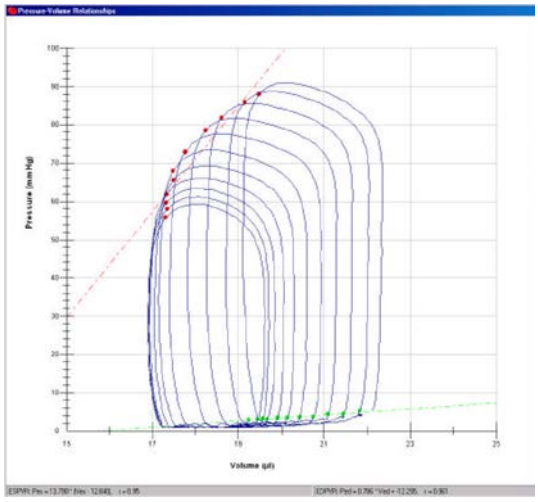


Figure 3.8: Pressure-volume loops after caval vein occlusion. Red line: ESPVR (E_{es} = slope of ESPVR). Green line: EDPVR

3.7. Experimental protocol

Hypoxic preconditioning and ischemia were induced as described in chapter 3.2 and 3.5.

A 1.4 Fr high fidelity pressure-conductance catheter (SPR-839; Millar Instruments, Houston, Texas) was calibrated to 0 mmHg, positioned with his pressure sensor at the water surface in a goblet, filled with sodium chloride 0.9% at 37°C.

Towards the end of the 1 hour reperfusion period, the right carotid artery and jugular vein were prepared to allow the starting of measurements after exactly 60 min of reperfusion. The 1.4 Fr high fidelity pressure-conductance catheter was inserted through the right carotid artery into the left ventricle (figure 3.9). After stabilization of the hemodynamic situation, baseline PV-loops were recorded (Powerlab/4SP AD Instruments, Castle Hill, Australia) while the ventilation was momentarily turned off to avoid respiratory fluctuation of cardiac signals (figure 3.10).

A small laparotomy was performed in the midline of the upper abdomen. The inferior caval vein was compressed between liver and diaphragm with a cotton swab, while PV-loops were recorded (figure 3.11). This was performed at least 5 times.

Afterwards a 24 G catheter was introduced in the right jugular vein and V_p was determined by a bolus injection of 3 µl of 30 % sodium chloride solution (figure 3.12). This was performed 3 times.

The pressure-conductance catheter was removed and the carotid artery ligated. The chest was reopened and the LAD was re-occluded with a 8/0 polypropylene suture at the initial occlusion site. The laparotomy was extended and the inferior caval vein was exposed infrahepatically. With a 24 G needle, 0.3 ml blood was retrieved from the inferior caval vein to measure specific conductivity in 3 calibrated cuvettes. Subsequently, Evans blue was given via an exchanged catheter (cfr supra), the heart was excised and prepared (cfr chapter 3.6.1).

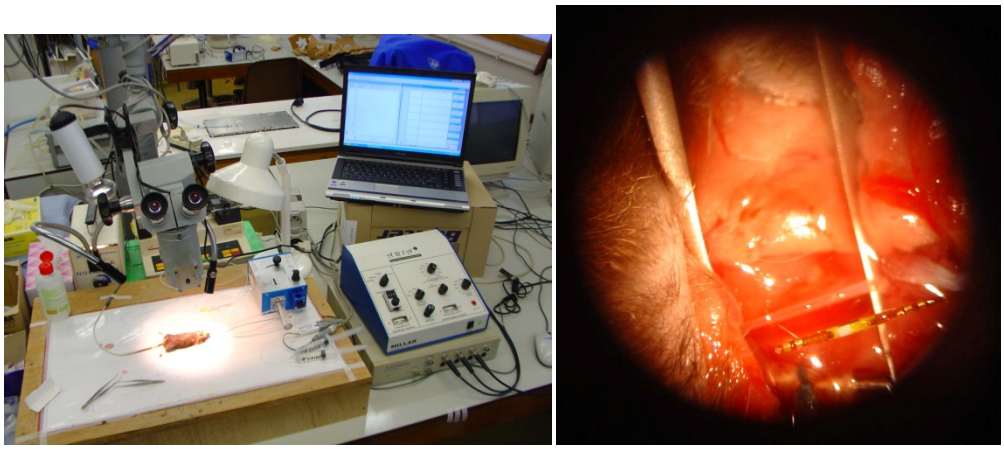


Figure 3.9: Left: Overview of the experimental setup. Right: introduction of pressure-conductance catheter into right carotid artery, view under microscopic magnification

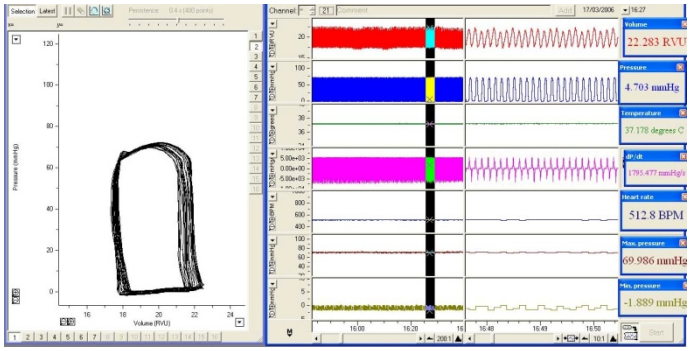


Figure 3.10: Pressure-volume loops after stabilization (baseline)

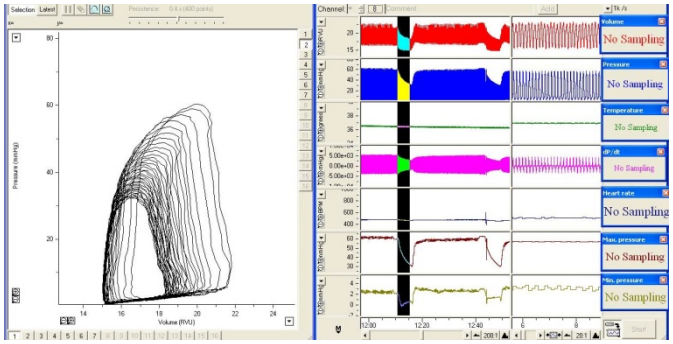


Figure 3.11: Pressure-volume loops during reduced preload (caval vein occlusion)

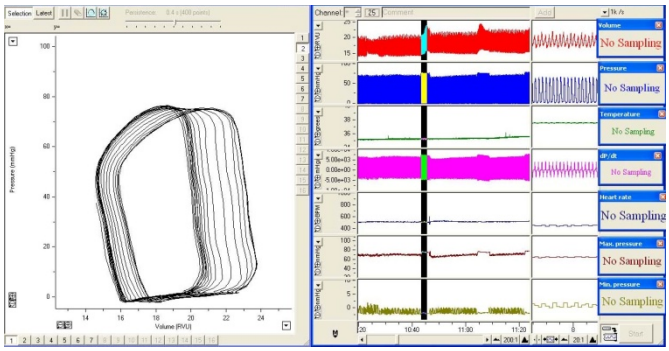


Figure 3.12: Shift of pressure-volume loops to the right during sodium chloride bolus (determination V_p)

3.8. Data management and statistical analysis

Analysis of the pressure-conductance data was performed using PVAN 3.2 software (Millar Instruments, Houston). All data were corrected for V_p and expressed in absolute volumes, as previously described. Only technically acceptable loops were included in the analysis for each experiment [58,60].

All statistical analyses were performed using statistical software (Statistica 7.1, StatSoft, Tulsa, USA). Data are expressed as mean ± standard deviation. Differences between groups were analyzed for statistical significance by student's t-test or one-way ANOVA followed by a Fisher's LSD post hoc test. Factorial ANOVA was used to determine the interaction term between preconditioning and treatments, followed by a Fisher's LSD post hoc test. A value of $p<0.05$ was considered significant.

Chapter 4

Hypoxic preconditioning preserves cardiac contractility and reduces infarct size *in vivo*

Gerry Van der Mieren, An Van den Bergh, Ines Nevelsteen, Annelies Vanderper, Willem Flameng and Paul Herijgers*

Department of Cardiovascular Diseases, Laboratory of Experimental Cardiac Surgery, K.U. Leuven, Leuven, Belgium

Published in The Open Surgery Journal 2008; 2: 24-29

Hypoxic Preconditioning Preserves Cardiac Contractility and Reduces Infarct Size *In Vivo*

Gerry Van der Mieren, An Van den Bergh, Ines Nevelsteen, Annelies Vanderper, Willem Flameng and Paul Herijgers*

Department of Cardiovascular Diseases, Laboratory of Experimental Cardiac Surgery, K.U. Leuven, Leuven, Belgium

Abstract: *Background*: Preconditioning is a powerful endogenous mechanism to protect the heart against ischemic damage. The second window of preconditioning (SWOP) is therapeutically the most attractive, but is hard to achieve by local cardiac ischemia 24 hrs before the index ischemia in the many mice models for cardiovascular pathology, because of excessive mortality during the two procedures or the period in between. Hypoxic preconditioning is an attractive alternative preconditioning stimulus. To date, the SWOP after hypoxic preconditioning has not been studied in mice concerning infarct size reduction and preservation of left ventricular contractility *in vivo*.

Aim: To determine whether transient hypoxia can induce a SWOP *in vivo* in mice.

Methods: Hypoxic preconditioning was induced by 5 cycles of 6 minutes of 6% oxygen in 24-week-old wild type mice. Twenty-four hours later, a 30 minutes coronary occlusion was performed. After 1 hour of reperfusion, *in vivo* cardiac pressure-conductance catheterization was performed with determination of the load-dependent and load-independent parameters. Infarct size was determined by TTC-staining. Sham procedures were used to obtain non-preconditioned controls.

Results: There was no mortality with the hypoxic preconditioning protocol. The left ventricular contractile parameters ejection fraction, end-systolic elastance and preload recruitable stroke work were significantly better preserved after ischemia in the preconditioned group. Diastolic relaxation (tau) was also significantly better preserved. Infarct size was reduced to half that of the non-preconditioned group.

Conclusion: Hypoxic preconditioning is a feasible stimulus to induce *in vivo* a second window of preconditioning in mice. Infarct size is reduced and cardiac contractility better preserved after 30 min regional ischemia *in vivo* by hypoxic preconditioning 24 hrs earlier.

INTRODUCTION

Preconditioning describes the phenomenon that a short episode of preceding ischemia induces improved tolerance in an organ or tissue to the deleterious effects of a subsequent longer period of ischemia [1-12]. Ischemic preconditioning is a powerful endogenous mechanism to protect the heart against ischemic damage. The second window of preconditioning (SWOP) is therapeutically the most attractive with its long standing effect from 24 hours till 72 hours. This protection can therefore be planned more readily in clinical practice and even chronically induced when ischemia is anticipated in the following days, but not exactly predictable, e.g. in patients with cardiovascular disease undergoing non–cardiac surgery.

In the past decade, numerous transgenic mouse models have been developed. They often serve as animal models to study cardiovascular diseases, for example heart failure, diabetes and cardiac hypertrophy. It is important to study preconditioning in these transgenic mice for 2 reasons: 1) it gives us the opportunity to investigate whether preconditioning can be induced in pathological conditions that would benefit most from its protective effects, and 2) it gives us the opportunity to further unravel the mechanism of preconditioning. Therefore a well-defined model of SWOP in mice is very welcome.

Intermittent hypoxia is an attractive method to try to induce SWOP in small animal models like mice. The method avoids cardiothoracic surgery and its inherent mortality in mouse models that are often expensive and difficult to breed. Secondly no anaesthesia, which can interfere with the preconditioning stimulus, is needed to perform hypoxic preconditioning.

In previous studies, hypoxia was shown to induce SWOP with cardiac protection against ischemic damage, but these studies all used *in vitro* techniques [13, 14].

The aim of this study is to examine whether intermittent hypoxia is capable to induce preconditioning in mice *in vivo* that is active 24 hrs later. We wanted to study infarct size and *in vivo* load-independent left ventricular contractility to accurately define the model and the preconditioning effect.

METHODS AND MATERIALS

Animal Models

Wild type mice with C57Bl6/J genetic background were purchased form Jackson Laboratories (Bar Harbour, Maine,

*Address correspondence to this author at the Department of Cardiovascular Diseases, Laboratory of Experimental Cardiac Surgery, K.U. Leuven, Provisorium I, Minderbroedersstraat 19, B-3000 Leuven, Belgium; Tel: +32 16 344260; Fax: +32 16 344616; E-mail: paul.herijgers@med.kuleuven.be

USA) and housed at 22°C on a fixed 12 hour light-dark cycle. Groups were sex-matched and investigated at the age of 24 weeks. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental protocols were approved by the Institutional Animal Care Commission and Ethical Committee.

Four groups of each 6 animals were used : ischemia after preconditioning (I+P), ischemia without preconditioning (I+NP), sham procedure after preconditioning (sham+P) and sham procedure without preconditioning. (sham+NP).

Intermittent Hypoxia

Mice were placed in a Plexiglas container (Agnthos, Sweden) of 0.8 liter. A portable oxymeter (Analox O_2 EII, North Yorkshire, UK) was calibrated at 20.7% for room air and was subsequently placed in this Plexiglas container for continuous registration of the oxygen content. A mixture of 100% nitrogen and compressed air was blown through this container *via* 2 separate flowmeters. Within 30 seconds, the oxygen content was lowered to 6% oxygen. Five cycles of 6 minutes of 6% oxygen, interspersed with 6 minutes of room air were used [13]. In the non-preconditioned group, mice were placed in the plexiglas container, with only compressed air (without 100% nitrogen) blown through the container.

After this hypoxic preconditioning protocol, mice were placed in their regular cage with food and water ad libitum for 24 hours.

Coronary Occlusion

Twenty-four hours later, anesthesia was induced with urethane (1.2 g/kg) and alfa-chloralose (50 mg/kg) intraperitoneally injected. Mice were placed on a heating pad, and rectal temperature kept between 36.5 and 37.5°C. Surgery was performed under a surgical microscope. Through a midline neck incision a tracheostomy was performed and mechanical ventilation started with room air (Minivent 845; Hugo Sachs/Harvard Apparatus, March-Hugstetten, Germany).

A left anterolateral thoracotomy was performed in the 3th intercostal space. The left lower limb was positioned to the right to facilitate visualization of the left coronary artery (LAD). Two millimeter below the tip of the left auricle, a 8/0 polypropylene suture was passed under the LAD. The snare was tightened on a small plastic tubing piece of 1.5 mm in order to perform an atraumatic occlusion of the LAD. Successful coronary occlusion was visually verified by observing the distal part turning pale. Occlusion was maintained for 30 minutes. Afterwards, the snare was released, reperfusion visually checked by blushing of the area, the chest closed and a reperfusion period of 1 hour allowed before the left ventricular pressure-conductance measurements were started.

Outcome Parameters: Infarct Size Determination and *In Vivo* Left Ventricular Contractility

Towards the end of the 1 hr reperfusion period, the right carotid artery and jugular vein were prepared to allow the starting of measurements after exactly 60 min of reperfusion. A 1.4 Fr high fidelity pressure-conductance catheter (1.4 Fr, SPR-839; Millar Instruments, Houston, TX) was inserted through the right carotid artery into the left ventricle. After stabilization of the hemodynamic situation, baseline pressure-volume (PV) loops were recorded (Powerlab/4SP ADInstruments, Castle Hill, Australia) while the ventilation was momentarily turned off to avoid respiratory fluctuation of cardiac signals.

A small laparotomy was performed in the midline of the upper abdomen. The inferior caval vein was compressed between liver and diaphragm with a cotton swab, while PV-loops were recorded (occlusion loops). This was performed at least 5 times. Afterwards a 24 G catheter was introduced in the right jugular vein and parallel volume was determined by a bolus injection of 3 µl of 30% sodium chloride solution. This was performed 3 times. The pressure-conductance catheter was removed and the carotid artery ligated. The chest was reopened and the LAD was re-occluded with a 8/0 polypropylene suture at the initial occlusion site. The laparotomy was extended and the inferior caval vein was exposed infrahepatically. With a 24G needle, 0.3 ml blood was retrieved from the inferior caval vein to measure specific conductivity in 3 precalibrated cuvets.

Evans blue (0.8 ml, 1% solution) was given intravenously *via* an exchanged catheter in the right jugular vein to determine the left ventricular perfusion area at risk. After the administration of Evans blue, there was a cardiac arrest within 30 seconds. The heart was excised and placed in tissue freezing medium (Tissue-Tek, Sakura Finetek, Torrance, USA) in a – 20° C freezer for at least one hour. With a custom-made razorblade system, the heart was cut in slices of 1 mm thickness. These slices were placed for 20 minutes in triphenyl tetrazolium chloride solution (1%, 37°C, phosphate buffer pH 7.4). Afterwards they were placed for 10 minutes in paraformaldehyde (4% solution, 20 °C). All slices were weighed and photographed with a digital camera under microscopic magnification. The tibial length of each animal was measured to calculate the heart weigth/tibial length ratio.

Data Management and Statistical Analysis

Analysis of the pressure-conductance data was performed using PVAN 3.2 software (Millar Instruments, Houston, TX). A conductance-volume calibration line was constructed with the cuvette data. All data were corrected for parallel volume and expressed in absolute volumes. Only technically acceptable loops were included in the analysis for each experiment [15].

Analysis of the morphological data was performed for each experiment. The number of pixels in each zone (total slice, risk zone, infarcted zone) was determined for all the slices of each heart with Adobe Photoshop 8.0 (Adobe System Inc.). After correction for the weight of the respective slices, the risk zone and infarcted zone were calculated for the entire heart.

Data are expressed as mean ± Standard Deviation. Differences between groups were analyzed for statistical significance by factorial ANOVA followed by a LSD post hoc test. Statistical software (Statistica 7.1, StatSoft) was used. A value of $p<0.05$ was considered significant.

RESULTS

Weight, heart weight and heart weight corrected for the tibial length were equal in the 4 groups.

Acute Effects of the Intermittent Hypoxia

All 24 mice survived the hypoxic preconditioning procedure. After initial agitation during lowering the oxygen content in the container, the mice calmed down when the oxygen content reached 6% after 30 seconds with however persistent tachypnea. After 15 seconds of exposure to room air, they demonstrated their normal behaviour pattern.

Mortality During Cardiac Ischemia and Reperfusion

In the I + NP group, 1 mouse died during the reperfusion phase after LAD occlusion. There was no mortality in the other groups.

Infarct Size (Table 1)

In both sham groups with and without the preconditioning procedure but without cardiac ischemia, there was no detectable infarct after the experimental protocol. Second window of hypoxic preconditioning was able to reduce infarct size to half that of the non-preconditioned group (49.9 ± 7.3% to 24.7 ± 7.3% of the risk zone, P<0.001).

Left Ventricular Contractility (Table 1)

The hypoxic preconditioning protocol in the sham groups not undergoing 30 min index ischemia 24 hrs later did not change left ventricular contractility parameters.

Load-Dependent Parameters

Heart rate was comparable in all groups. The risk zone of the heart was in both ischemia groups approximately 15%.

Ejection fraction, a conventional but load-dependent parameter for systolic function, was better preserved when ischemia was preceded by hypoxic preconditioning 24 hrs earlier (56.1 ± 5.4% *vs* 48.6 ± 5.9%, P=0.02).

Table 1. Phenotypic Characteristics, Infarct Size and Hemodynamic Parameters

	Group Sham+NP	Group Sham+P	Group I +NP	Group I + P
Phenotypic Characteristics				
Weight (g)	30.6 ± 5.8	27.7 ± 2.2	30.8 ± 5.9	26.4 ± 4.3
Heart weight (mg)	161.5 ± 23.4	145.3 ± 17.5	151.9 ± 16	140.3 ± 21.4
Heart weight/Tibial length (mg/cm)	79.7 ± 11.1	71.2 ± 7.6	74.6 ± 7.3	68.6 ± 9.8
Infart Size				
% Infarct of risk zone (%)	0 ± 0	0 ± 0	49.9 ± 7.3	24.7 ± 7.3 *
Risk zone of the heart (%)	0 ± 0	0 ± 0	14.4 ± 8.8	15 ± 7.4
Hemodynamic Parameters in Steady State (Load-Dependent)				
Vp (µl)	30.2 ± 2.7	32.5 ± 2.1	34.6 ± 3.2	30.2 ± 6.9
Heart rate (bpm)	576 ± 43	537 ± 55	565 ± 62	569 ± 50
Ves (µl)	11.6 ± 5.7	14.9 ± 7.5	9.9 ± 7	8.5 ± 2.5
Ved (µl)	26.1 ± 10.5	28.4 ± 13.3	15.3 ± 9.3	15.9 ± 5.3
Pes (mmHg)	67.7 ± 15	61.7 ± 13.3	54.9 ± 5.5	57.2 ± 13.7
Ped (mmHg)	2.8 ± 0.8	4.4 ± 1 #	3.6 ± 1	2.5 ± 0.7
Pmax (mmHg)	77.5 ± 13	69.4 ± 13.1	60.2 ± 6	62.6 ± 13.3
Stroke volume (µl)	17.8 ± 5.3	17.1 ± 5.5	7.5 ± 4	9.5 ± 3
Ejection fraction (%)	65.5 ± 10.6	57.7 ± 7.1	48.6 ± 5.9	56.1 ± 5.4 *
Cardiac output (µl/min)	10343 ± 3281	9210 ± 3194	4220 ± 2358	5436 ± 2041
Stroke work (mmHg*µl)	1145.6 ± 420.9	1021.3 ± 550.3	323.2 ± 193.8	405.5 ± 131.3
Ea (mmHg/µl)	4.1 ± 1.5	3.7 ± 0.5	10.5 ± 8.2	6.6 ± 2.4
dP/dt max (mmHg/s)	6648 ± 2142	5971 ± 2896	4366 ± 935	4603 ± 725
dP/dt min (mmHg/s)	-5454 ± 1882	-4803 ± 1659	- 3557 ± 882	- 4336 ± 1495
Tau G (ms)	10.7 ± 2.6	10 ± 1.6	12.5 ± 2	8.5 ± 2 *
Hemodynamic Parameters After Temporary Preload Reduction (Load-Independent)				
Ees (mmHg/µl)	8.2 ± 3	7.4 ± 1.9	6.3 ± 1.6	9 ± 1.7 *
PRSW (mmHg)	87.5 ± 5.8	84.3 ± 5.3	61.4 ± 12.3	76.6 ± 6.5 *
EDPVR (mmHg/µl)	0.2 ± 0.2	0.5 ± 0.5	0.4 ± 0.2	0.3 ± 0.2

*p < 0.05 *vs* "Ischemia without preconditioning".
#p < 0.05 *vs* "Sham procedure without preconditioning".

The other conventional load-dependent parameters (end-systolic pressure, stroke volume, cardiac output, stroke work and dP/dt_{max}) tended to be better preserved after ischemia with the preconditioning protocol, but this did not reach statistical significance.

Tau, as parameter for early diastolic relaxation was better preserved after ischemia preceded by the hypoxic preconditioning protocol (8.5 ± 2 ms *vs* 12.5 ± 2 ms, P=0.05). The other parameter for relaxation dP/dt_{min} tended to be more negative after preconditioning. No differences in left ventricular stiffness, as determined by the exponential fit of the end-diastolic pressure-volume relationship (EDPVR) could be demonstrated.

The effective arterial elastance (Ea) is defined as the ratio of the end-systolic pressure/stroke volume and is a parameter for afterload. Ea tended to be lower after preconditioning, but this didn't reach statistical significance.

Load-Independent Parameters

End-systolic elastance (Ees) is a relatively load-independent parameter which reflects the left ventricle end-systolic stiffness and is determined as the slope of the end-systolic pressure-volume relationship. Ees was better preserved after ischemia with the preconditioning protocol (9 ± 1.7 mmHg/tl *vs* 6.3 ± 1.6 mmHg/tl, P=0.04) (Fig. **1**).

Preload Recruitable Stroke Work (PRSW) is the slope of the relationship between end-diastolic volume and stroke work performed by the ventricle. Hypoxic preconditioning 24 hrs before 30 min regional cardiac ischemia led to a better preserved PRSW (76.6 ± 6.5 mmHg *vs* 61.4 ± 12.3 mmHg, P=0.03) (Fig. **2**).

DISCUSSION

Preconditioning of the heart was first described by Murry *et al.* in 1986 [1]. Since then, numerous papers have been published concerning this endogenous protection mechanism to unravel the underlying mechanisms [3]. In the past decade, numerous transgenic mouse models of cardiovascular diseases have been developed. The study of preconditioning in these models gives us the opportunity to investigate whether preconditioning can be induced in pathological conditions that would benefit most from its protective effects, and to further unravel the mechanism of preconditioning. Therefore, we wanted to characterize a feasible protocol of SWOP induction in mice.

Methods to precondition the myocardium include local ischemia, hypoxia, transient ischemia in a remote organ or pharmacologically [2-4, 8-14, 16-21]. Local ischemia is the oldest and most frequently used method to induce an early and late phase of preconditioning in mice. Guo *et al.* [16] described that a sequence of six cycles of 4 min coronary occlusion and 4 min reperfusion was protective in mice. With this technique the magnitude of protection afforded by the early phase was 75% reduction in infarct size and 48-55% by the late phase of preconditioning. Similar, this protocol was highly effective in inducing SWOP in rabbits [20-21].

The major disadvantage of regional cardiac ischemia as SWOP stimulus is the need to perform surgery to deliver it.

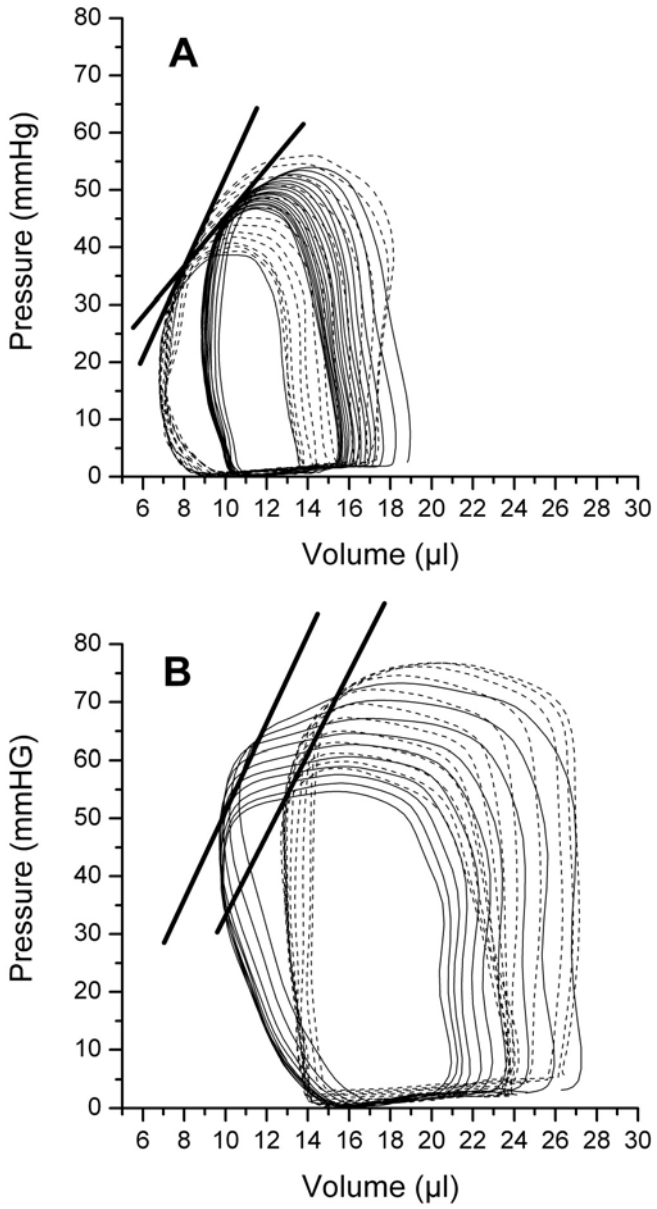


Fig. (1). (**A**) Representative example of occlusion loops and Ees-curves of the ischemia groups. Group I + P: dash loops and Group I + NP: solid loops. (**B**) Representative example of occlusion loops and Ees-curves of the sham groups. Group sham + P: dash loops and Group sham + NP: solid loops.

The required survival period between the stimulus and the index ischemia, which often requires a second cardiovascular surgery in mice, often limits the applicability. Although this is a feasible technique in lean mice, we found in a preliminary study unacceptable mortality rates of more than 50% in the obese ob/ob mouse model. Not only the limited survival after surgery to induce regional cardiac ischemia is problematic, also the fact that anesthesia is necessary for it. This anesthesia can interfere in an uncontrolled way with the preconditioning stimulus, especially if volatile anesthetics or opioid agonists are used [2, 10, 12].

Acute systemic and/or intermittent hypoxia was described in the early years 2000 as an effective preconditioning stimulus. Cai *et al.* [13] described for the first time the protocol of 5 cycles of 6 minutes, in which the oxygen con-

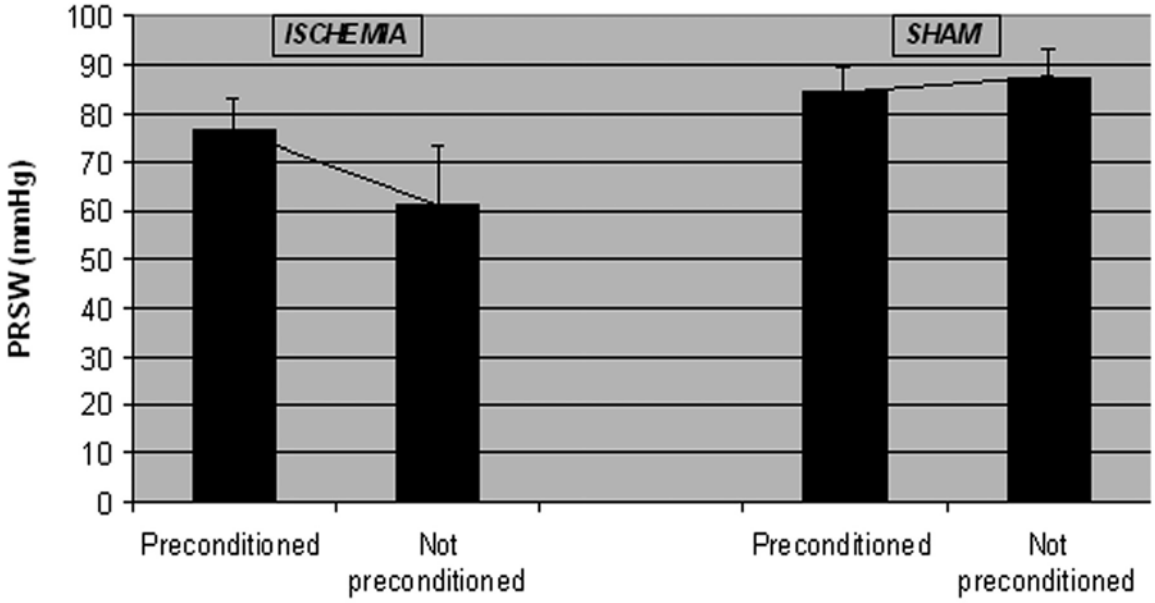


Fig. (2). PRSW is significantly (p=0.03) better preserved after the preconditioning protocol in the ischemia group. There were no statistical significant differences among the sham groups.

centration was rapidly reduced to 6%. With this method he could induce myocardial protection in mice hearts studied 24 hours later in a Langendorff system. Ischemia was induced by a perfusion stop during 30 minutes. Left ventricular developed pressure improved and infarct size was reduced [13]. With this hypoxic preconditioning protocol, no right ventricular hypertrophy and pulmonary hypertension is seen [18]. Xi *et al.* [14] studied delayed preconditioning induced by one or two cycles of 10% hypoxia for various durations (30 min, 2 h, 4 h). Infarct size was reduced only in mice pretreated with one or two cycles of 4 hour hypoxia.

To examine the *in vivo* effect of this stimulus, we performed cardiac pressure-conductance catheterization to determine left ventricular contractility with load-dependent and load-independent parameters [15, 22-24]. Infarct size measurements were used to identify the morphological damage after ischemia. In our series, there was no mortality with the described hypoxic preconditioning protocol.

Infarct size of the risk zone was significantly reduced from 49.9 ± 7.3% to 24.7 ± 7.3% after preconditioning. This reduction to about half the infarct size is comparable with the data of Guo *et al*. [16] who used local cardiac ischemia as preconditioning stimulus and the *in vitro* data of Cai *et al.* [13] with hypoxia as preconditioning stimulus.

Systolic function and active diastolic relaxation, as measured by ejection fraction and Tau were significantly better preserved. Ees and PRSW are considered as the gold standards to study ventricular contractility [24]. Accurate measurement of them requires the invasive procedure of pressure-volume measurements with different preload conditions. This parameters Ees and PRSW were respectively 42% and 25% better preserved by preconditioning after ischemia. This was never shown before.

The underlying mechanisms of the second window of preconditioning following hypoxic preconditioning in mice are not known. Cai *et al.* [13] showed that erythropoietin (EPO) mRNA expression was induced in kidneys of wild-type mice subjected to intermittent hypoxia, resulting in increased plasma EPO levels. No other data are available concerning the second window of preconditioning after hypoxic preconditioning.

In rat models, the role of NO and mitochondrial K_{ATP} channels in the hypoxic induced cardioprotection has been shown by Beguin *et al.* [25]. Rats, exposed to 4 hours of intermittent hypoxia had an infarct size reduction of 33.5% to 21.5%. This effect was abolished by L-NAME, a NOS inhibitor and the K_{ATP} channel blocker 5-HD. More recently, Beguin *et al*. [26] showed that delayed preconditioning in rats is mediated by protein kinase C and triggered by p38 MAPK and Erk1/2. Another mechanism for this protection in rats is the formation of reactive oxygen species. Kolar *et al.* [27] showed that the protective effect of chronic intermittent hypoxia in rats hearts was abolished by treatment with the antioxidant N-acetylcysteine.

In mice models following an ischemic preconditioning protocol (6 cycles of 4 min occlusion/4 min reperfusion), iNOS plays an important role. Guo *et al.* [28] showed that iNOS was increased with 40% in the ischemic/reperfused region. Our model provides a comparable degree of cardiac protection. It is thus tempting to suggest that iNOS is a potential mechanism in hypoxic preconditioning in mice.

Also COX-2 plays an important role. COX-2 activity was upregulated following ischemic preconditioning in mice, but, in contrast, the absence of iNOS prevented the activity of COX-2 protein. In the same study, it was found that iNOS and COX-2 coprecipitated 24 hours after ischemic preconditioning in the myocardium, indicating a physical interaction between these proteins. The upregulation of COX-2 protein expression after ischemic preconditioning was mediated by a JAK1/2-STAT1/3-signaling cascade [29].

In conclusion, this study shows that hypoxic preconditioning is a feasible stimulus to induce *in vivo* a second win-

dow of preconditioning in mice. Cardiac contractility can significantly be preserved by this technique. The degree of cardiac protection induced by the intermittent hypoxia protocol seems to be comparable with regional cardiac ischemia as preconditioning stimulus.

REFERENCES

[1] Murry CE, Jennings RB, Reimer KA. Preconditioning with ische- mia: a delay of lethal cell injury in ischemic myocardium. Circula- tion 1986; 74(5): 1124-36.
[2] Bolli R. The late phase of preconditioning. Circ Res 2000; 87 (11): 972-83.
[3] Bolli R, Li QH, Tang XL, *et al.* The late phase of preconditioning and its natural clinical application – gene therapy. Heart Fail Rev 2007; 12 (3-4): 189-99.
[4] Baxter GF. Role of adenosine in delayed preconditioning of the myocardium. Cardiovasc Res 2002; 55 (3): 483-94.
[5] Gross GJ, Peart JN. Katp channels and myocardial preconditioning: an update. Am J Physiol 2003; 285 (3): H921-30.
[6] Hendrikx M, Toshima Y, Mubagwa K, Flameng W. Improved functional recovery after ischemic preconditioning in the globally ischemic rabbit heart is not mediated by adenosine A1 receptor ac- tivation. Basic Res Cardiol 1993; 88 (6): 576-93.
[7] Laude K, Beauchamp P, Thuillez C, Richard V. Endothelial protective effects of preconditioning. Cardiovasc Res 2002; 55 (3): 466-73.
[8] Tanoué Y, Herijgers P, Meuris B, *et al.* Ischemic preconditioning reduces unloaded myocardial oxygen consumption in an *in vivo* sheep model. Cardiovasc Res 2002; 55 (3): 633-41.
[9] Valen G. Cellular signalling mechanisms in adaptation to ischemia-induced myocardial damage. Ann Med 2003; 35 (5): 300-7.
[10] Wakeno-Takahashi M, Otani H, Nakao S, Imamura H, Shingu K. Isoflurane induces second window of preconditioning through upregulation of inducible nitric oxide synthase in rat heart. Am J Physiol Heart Circ Physiol 2005; 289 (6): H2585-91.
[11] Xia Z, Herijgers P, Nishida T, *et al.* Remote preconditioning lessens the deterioration of pulmonary function after repeated coronary artery occlusion and reperfusion. Can J Anaesth 2003; 50 (5): 481-8.
[12] Yellon DM, Downey JM. Preconditioning the myocardium : from cellular physiology to clinical cardiology. Physiol Rev 2003; 83 (4): 1113-51.
[13] Cai Z, Manalo DJ, Wei G, *et al.* Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ische- mia-reperfusion injury. Circulation 2003; 108 (1): 79-85.
[14] Xi L, Tekin D, Gursoy E, *et al.* Evidence that NOS2 acts as a trigger and mediator of late preconditioning induced by acute systemic hypoxia. Am J Physiol 2002; 283 (1): H5-H12.
[15] Van den Bergh A, Vanderper A, Vangheluwe P, *et al.* Dyslipide- mia in type II diabetic mice does not aggravate contractile impair- ment but increases ventricular stiffness. Cardiovasc Res 2008; 77 (2): 371-9.
[16] Guo Y, Wu WJ, Qiu Y, *et al.* Demonstration of an early and a late phase of ischemic preconditioning in mice. Am J Physiol 1998; 275 (4Pt2): H1375-H1387.
[17] Kharbanda RK, Mortensen UM, White PA, *et al.* Transient limb ischemia reduces remote ischemic preconditioning *in vivo*. Circula- tion 2002; 106 (23): 2881-3.
[18] Ostadal B, Ostadalova I, Dhalla NS. Development of cardiac sensitivity to oxygen deficiency: comparative and ontogenetic aspects. Physiol Rev 1999; 79 (3): 635-659.
[19] Patel HH, Moore J, Hsu AK, Gross GJ. Cardioprotection at a distance: mesenteric artery occlusion protects the myocardium *via* an opioid sensitive mechanism. J Mol Cell Cardiol 2002; 34 (10): 1317-23.
[20] Qiu Y, Rizvi A, Tang XL, *et al.* Nitric oxide triggers late preconditioning against myocardial infarction in conscious rabbits. Am J Physiol 1997; 273 (6 Pt 2): H2931-H2936.
[21] Takano H, Manchikalapudi S, Tang XL, *et al.* Nitric oxide synthase is the mediator of late preconditioning against myocardial infarc- tion in conscious rabbits. Circulation 1998; 98 (5): 441-449.
[22] Georgakopoulos D, Mitzner WA, Chen CH, *et al. In vivo* murine left ventricular pressure-volume relations by miniaturized conduc- tance micromanometry. Am J Physiol 1998; 274 (4 Pt 2): H1416-22.
[23] Van den Bergh A, Flameng W, Herijgers P. Type II diabetic mice exihibit contractile dysfunction but maintain cardiac output by fa- vourable loading conditions. Eur J Heart Fail 2006; 8 (8): 777-83.
[24] Van den Bergh A, Flameng W, Herijgers P. Parameters of ventricular contractility in mice: influence of load and sensitivity to changes in inotropic state. Pflugers Arch 2008; 455 (6): 987-94.
[25] Beguin PC, Joyeux-Faure M, Godin-Ribuot D, Levy P, Ribuot C. Acute intermittent hypoxia improves rat myocardium tolerance to ischemia. J Appl Physiol 2005; 99 (3): 1064-1069.
[26] Beguin PC, Belaidi E, Godin-Ribuot D, Levy P, Ribuot C. Intermittent hypoxia-induced delayed cardioprotection is mediated by PKC and triggered by p38 MAP kinase and Erk. J Mol Cell Car- diol 2007 (2); 42: 343-51.
[27] Kolar F, Jezkova J, Balkova P, *et al.* Role of oxidative stress in PKC-ö upregulation and cardioprotection induced by chronic in- termittent hypoxia. Am J Physiol 2007; 292 (1): H224-H230.
[28] Guo Y, Jones WK, Xuan YT, *et al.* The late phase of ischemic preconditioning is abrogated by targeted disruption of the inducible NO synthase gene. Proc Natl Acad Sci USA 1999; 96 (20): 11507–11512.
[29] Xuan YT, Guo Y, Zhu Y, *et al.* Mechanism of cyclooxygenase-2 upregulation in late preconditioning. J Mol Cell Cardiol 2003; 35 (5): 525–537.

Received: April 21, 2008 Revised: May 6, 2008 Accepted: May 16, 2008

© Van der Mieren *et al.*; Licensee *Bentham Open*.
This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.5/), which permits unrestrictive use, distribution, and reproduction in any medium, provided the original work is properly cited.

Chapter 5

Angiotensin-converting enzyme inhibition and food restriction in diabetic mice do not correct the increased sensitivity for ischemia-reperfusion injury

Gerry Van der Mieren, MD, Ines Nevelsteen, MD, Annelies Vanderper, PhD, Wouter Oosterlinck, MD, Willem Flameng, MD, PhD, Paul Herijgers, MD, PhD

Department of Cardiovascular Sciences, Research Unit Experimental Cardiac Surgery, K.U. Leuven, Herestraat 49, B-3000 Leuven, Belgium

Published in Cardiovascular Diabetology 2012; 11: 89.

Van der Mieren et al. Cardiovascular Diabetology 2012, 11:89
http://www.cardiab.com/content/11/1/89

CARDIO VASCULAR DIABETOLOGY

Angiotensin-converting enzyme inhibition and food restriction in diabetic mice do not correct the increased sensitivity for ischemia-reperfusion injury

Gerry Van der Mieren, Ines Nevelsteen, Annelies Vanderper, Wouter Oosterlinck, Willem Flameng and Paul Herijgers*

Abstract

Background: The number of patients with diabetes or the metabolic syndrome reaches epidemic proportions. On top of their diabetic cardiomyopathy, these patients experience frequent and severe cardiac ischemia-reperfusion (IR) insults, which further aggravate their degree of heart failure. Food restriction and angiotensin-converting enzyme inhibition (ACE-I) are standard therapies in these patients but the effects on cardiac IR injury have never been investigated. In this study, we tested the hypothesis that 1° food restriction and 2° ACE-I reduce infarct size and preserve cardiac contractility after IR injury in mouse models of diabetes and the metabolic syndrome.

Methods: C57Bl6/J wild type (WT) mice, leptin deficient ob/ob (model for type II diabetes) and double knock-out (LDLR-/-;ob/ob, further called DKO) mice with combined leptin and LDL-receptor deficiency (model for metabolic syndrome) were used. The effects of 12 weeks food restriction or ACE-I on infarct size and load-independent left ventricular contractility after 30 min regional cardiac ischemia were investigated. Differences between groups were analyzed for statistical significance by Student's t-test or factorial ANOVA followed by a Fisher's LSD post hoc test.

Results: Infarct size was larger in ob/ob and DKO versus WT. Twelve weeks of ACE-I improved pre-ischemic left ventricular contractility in ob/ob and DKO. Twelve weeks of food restriction, with a weight reduction of 35-40%, or ACE-I did not reduce the effect of IR.

Conclusion: ACE-I and food restriction do not correct the increased sensitivity for cardiac IR-injury in mouse models of type II diabetes and the metabolic syndrome.

Keywords: Ischemia/reperfusion, Diabetes mellitus, Metabolic syndrome, In vivo contractility, Infarct size

Background

The number of patients with diabetes and the metabolic syndrome increases in Western societies and reaches epidemic proportions [1,2]. At present, diabetes affects approximately 250 million people worldwide and by 2025 this is expected to increase to over 380 million, with type II diabetes accounting for 90-95% of them. Prevalence is expected to increase most in Asia and Africa with the majority of patients in 2030 being found there [2]. The prevalence of the metabolic syndrome currently exceeds 20% of individuals who are over 20 years of age and 40% of the population older than 40 years [1]. Heart failure is the leading cause of mortality in people with type II diabetes. The incidence of myocardial infarction in diabetic patients is twice that of the general population [3,4]. They are at increased risk for mortality and post-ischemic complications [3,4]. Infarct size for a given ischemic insult is larger in diabetic mice than in controls [5-7]. This, on top of diabetic cardiomyopathy [8,9], contributes to progressive heart failure. Therefore, new or additional techniques to protect the diabetic heart against this ischemia/reperfusion (IR) damage are eagerly awaited.

* Correspondence: paul.herijgers@uzleuven.be
Department of Cardiovascular Sciences, Research Unit Experimental Cardiac Surgery, K.U. Leuven, Herestraat 49, B-3000, Leuven, Belgium

Food restriction and ACE-inhibition (ACE-I) are frequently used therapies in type II diabetes [10].

Although food restriction is a standard therapy in type II diabetic patients, no long-term large-scale study of intentional weight loss has been adequately powered to examine cardiovascular disease end points in patients with diabetes [10]. Conflicting data were reported concerning the effect of food restriction on cardiac contractility in experimental models. Some studies in diabetic mice report a reduced mean 24-hour blood pressure and heart rate, restored circadian variations of blood pressure and heart rate, and increased ejection fraction [11]. In other studies with obese rats, stroke volume, left ventricular work and cardiac output decreased significantly after food restriction [12]. Similarly, the effect of food restriction on the impact of IR injury was barely investigated. In a study with wild type rats, ex vivo cardiac contractility was better preserved after IR injury after 8-months of food restriction [13]. The effect of food restriction on IR injury in diabetic or metabolic syndrome models was never investigated.

Current guidelines advocate ACE-inhibitors as the drugs of choice in the initial treatment of hypertension in diabetic patients [10]. ACE-I is favored because, in addition to the blood pressure lowering properties, it has well documented anti-ischemic and anti-atherogenic effects, reduces oxyradical formation and has an effect on cardiovascular remodeling. ACE-I improves glucose control and insulin sensitivity and slows the progression of diabetic nephropathy [14]. In a large meta-analysis of patients with acute myocardial infarction, ACE-I reduced 30-day mortality from 7.6 to 7.1% and in the subgroup of diabetic patients from 12.0 to 10.3% [15].

The effect of ACE-I in experimental models of acute IR injury is uncertain [16-21]. Experiments with captopril in a canine, porcine and rat model report a reduced myocardial injury although other experiments in canine models do not show any effect [16,17]. Studies with other ACE-inhibitors report also heterogeneous data. Some report a reduction in infarct size [18,19] while others see no effect [20]. In Langendorff-perfused mouse hearts, captopril has no beneficial effect on post-ischemic contractile recovery or cardiac enzyme release [21]. Nevertheless, when cardiac p90 ribosomal S6 kinase is over-expressed in mice, captopril induces a better post-ischemic contractility and reduced enzyme release [21]. Since p90 ribosomal S6 kinase is activated in hyperglycemic mice, it might be possible that captopril induces protection in diabetic mice models.

Therefore, the aim of this study is to investigate the effects of food restriction and ACE-I on IR injury in diabetic or metabolic syndrome models. We tested the hypotheses that 1° food restriction and 2° ACE-I do correct the increased sensitivity for cardiac IR injury.

Methods

Animal models

Experiments were conducted in C57BL/6J WT-mice, leptin deficient ob/ob, and double knock-out (DKO) mice with combined leptin and LDL-receptor deficiency (total number = 145). The ob/ob mouse is a model for type II diabetes, featuring abdominal obesity and insulin resistance. DKO mice feature many characteristics of the metabolic syndrome, i.e. obesity, dyslipidemia, hypertension, insulin resistance and impaired glucose tolerance and/or diabetes [8,11]. These models develop left ventricular diastolic and systolic dysfunction comparable with the diabetic cardiomyopathy seen in patients [8].

Ob/ob and C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). DKO were generated as described previously [8,11]. IR was induced at 24 weeks of age. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 1996). All experimental protocols were approved by the K.U.Leuven Institutional Animal Care Commission and Ethical Committee.

Treatments

Food intake of diet-restricted ob/ob and DKO mice was restricted to 2.5 g/day, which is the normal daily intake of lean WT mice, between 12 and 24 weeks of age [11]. ACE-inhibition was obtained with captopril (10 mg/kg/day) intraperitoneally from 12 until 24 weeks of age in WT, ob/ob and DKO [22].

Biochemical analysis

Blood of conscious mice was collected by tail bleeding into EDTA tubes after a 24 h fast at 12 and 24 weeks of age. Plasma was obtained by centrifugation. Triglycerides and total cholesterol was determined with a diagnostic reagent kit (Roche) and glucose with a glucometer (Menarini Diagnostics).

Ischemia/reperfusion

The experimental technique was previously described [23]. Briefly, anesthesia was induced with urethane (1.2 g/kg) and alfa-chloralose (50 mg/kg). Mice were ventilated with room air, with rectal temperature kept at 37 ± 0.5°C. Via left thoracotomy, the left anterior descending artery (LAD), was non-traumatically occluded, 2 mm below the tip of the left auricle for 30 min. Afterwards, a reperfusion period of 1 hour was allowed. Successful coronary occlusion and reperfusion was visually verified by observing the myocardium distal to the coronary occlusion turning pale respectively blushing. In the groups without ischemia (sham), a thoracotomy and time-matched procedure was performed.

Outcome parameters: infarct size and in vivo left ventricular contractility

The technique was previously described [23]. At the end of 1 hour reperfusion, a pressure-conductance catheter (1.4-Fr, SPR-839; Millar Instruments, Houston, TX) was inserted through the right carotid artery into the left ventricle. Baseline pressure-volume (PV) loops were recorded (Powerlab/AD Instruments, Castle Hill, Australia). Parallel volume and specific blood conductance were determined [8,23]. The inferior caval vein was compressed to obtain left ventricular PV-loops under varying loading conditions. Heart rate, systolic and end-diastolic pressure were measured. Stroke volume was determined as the difference in end-diastolic and end-systolic volume. Stroke work (SW) is the mechanical energy which the heart develops during the cardiac cycle and is calculated as the area enclosed by the PV-loop.

Preload recruitable stroke work (PRSW) is the slope of the relationship between end-diastolic volume and SW performed by the ventricle. PRSW is the most reliable and useful parameter for general contractility since it is chamber size independent and robust [24]. The slope of the end-systolic pressure-volume relationship, end-systolic elastance (E_{es}), reflects left ventricular chamber end-systolic stiffness and is used as an index of contractility. Tau is the time constant of left ventricular relaxation during isovolumetric diastole. The end-diastolic PV-relationship (EDPVR) represents the compliance of the ventricular myocardium at the end of the diastole. Augmented stiffness of the ventricular wall increases the slope of the EDPVR. Arterial elastance (E_a), a measure for afterload, is defined as the end-systolic pressure to stroke volume ratio [23,24].

Before excision of the heart, Evans blue (0.8 ml, 1% solution) was injected intravenously after re-occlusion of the LAD, to determine the left ventricular perfusion area at risk. The heart was cut in 1 mm-slices and the slices were stained with triphenyl-tetrazolium-chloride solution (TTC, 20 min, 1%, 37°C, pH 7.4) and fixed in paraformaldehyde (10 min, 4% solution, 20°C). All slices were weighed and photographed with a digital camera under magnification.

Data management and statistical analysis

Analysis of the pressure-conductance data was performed using PVAN 3.2 software (Millar Instruments, Houston) as previously described [8,23].

Infarct size and area at risk were determined by the number of pixels in each zone with Adobe Photoshop 8.0 (Adobe System Inc.) multiplied by the weight of the respective slices.

All statistical analyses were performed using Statistical software (Statistica 7.1, StatSoft, Tulsa, USA). Data are expressed as mean ± standard deviation. Differences between groups were analyzed for statistical significance by Student's t-test or factorial ANOVA followed by a Fisher's LSD post hoc test. A value of $p < 0.05$ was considered significant.

Results

Biochemical parameters and contractility in untreated mice

Untreated ob/ob and DKO have a significantly higher weight and exhibit hyperglycemia (Table 1). Cholesterol levels are elevated in ob/ob and even higher in DKO. DKO also have increased triglycerides levels. Left ventricular contractility, expressed as PRSW, is worse in ob/ob than in WT and even worse in DKO (Figure 1). Diastolic function, represented by end-diastolic pressure and EDPVR, is significantly worse in DKO than in WT (Table 2). Ejection fraction is not reported since it is highly dependent upon preload and afterload, leading to

Table 1 Metabolic parameters

Genotype	Age (weeks)	Treatment	Weight (g)	Heart weight / tibial lenght (mg/cm)	Glucose (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
Wild type	12	Untreated	21.1±3.1		56.2±6.5	104±70	115±42
	24	Untreated	28.5±4.4	74.1±9.0	71.2±11.8	76±14	60±14
Ob/ob	12	Untreated	49.5±3.5[a]		163±73[a]	125±20	58±11[a]
	24	Untreated	62.7±3.7[a]	76.6±7.7	166±41[a]	139±35[a]	68±12
	24	Diet	38.5±4.4[ac]	71.8±7.1[c]	142±63[a]	74±24[c]	69±15
	24	ACE-I	64.1±9.4[ad]	74.8±9.1	84±19[acd]	145±34[ad]	76±13[a]
DKO	12	Untreated	47.0±2.4[a]		155±47[a]	912±188[ab]	591±181[ab]
	24	Untreated	59.4±9.3[a]	79.3±13.4	152±108[a]	749±114[ab]	245±80[ab]
	24	Diet	38.2±3.6[ac]	68.0±8.5[ac]	141±62[a]	754±123[ab]	252±110[ab]
	24	ACE-I	64.0±8.0[ad]	70.3±7.2[c]	124±36[ab]	658±166[ab]	202±67[ab]

"a" $p < 0.05$ versus WT, same age, untreated; "b" $p < 0.05$ versus ob/ob, same age, same treatment; "c" $p < 0.05$ versus same untreated genotype , same age;
"d" $p < 0.05$ versus diet, same genotype, same age.

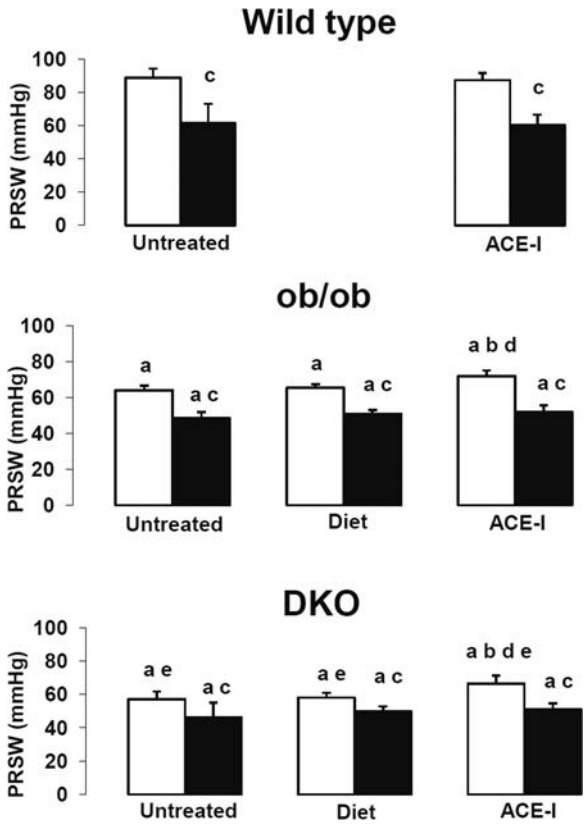


Figure 1 Preload recruitable stroke work in wild type, ob/ob and double knock-out mice. "a" $p < 0.05$ versus WT same treatment, same age; "b" $p < 0.05$ versus same untreated genotype, same age; "c" $p < 0.05$ versus sham, same genotype, same treatment; "d" $p < 0.05$ versus diet, same genotype, same age "e" $p < 0.05$ versus ob/ob same treatment, same age.

significant misinterpretations of contractility in diabetic mice [9].

Effect of IR

The area at risk after coronary occlusion is comparable in all groups (Table 3). As expected, none of the sham groups showed an infarct. After IR, infarct size was larger in DKO and ob/ob versus WT (Figure 2). After IR, PRSW decreased significantly in all groups and was significantly lower in DKO and ob/ob versus WT (Figure 1).

Effect of food restriction on biochemical parameters and contractility

Food restriction resulted in lower body and heart weight in both ob/ob and DKO (Table 1). Heart weight was even lower in food restricted DKO than in WT. Food restriction had little effect on biochemical parameters. Food restriction did not improve the reduced PRSW in ob/ob and DKO mice (Figure 1).

Effect of food restriction on IR injury

Food restriction did not reduce infarct size in DKO nor ob/ob versus the untreated groups (Figure 2). The impact of IR on PRSW was not influenced by food restriction (Figure 1).

Effect of ACE-I on biochemical parameters and contractility

ACE-I reduced glycemia levels in ob/ob but much less in DKO (Table 1). Heart weight, was lower in DKO but not in ob/ob after ACE-I compared with the untreated. PRSW was significantly better after 12 weeks of ACE-I in ob/ob and DKO versus diet (Figure 1, Table 2).

Effect of ACE-I on IR injury

ACE-I did not reduce infarct size in any genotype versus the untreated groups (Figure 2). Furthermore, ACE-I did not reduce the impact of IR on PRSW in any genotype (Figure 1).

Discussion

The number of patients with diabetes and the metabolic syndrome reaches epidemic proportions [1,2]. The incidence of myocardial infarction in diabetic patients is twice that of the general population [3,4]. Infarct size for a given ischemic insult is larger in diabetic patients than in non-diabetic patients [3,4]. This, on top of diabetic cardiomyopathy [8,9], contributes to progressive heart failure. Therefore, new or more potent strategies to protect them against cardiovascular complications are needed. Our mice models of type II diabetes and the metabolic syndrome are, similar to the clinical reality, more vulnerable for IR injury. In other experimental diabetic models, disagreement exists about the sensitivity to IR injury [25-28]. Depending on the reports, infarct size in animal models of diabetes was larger, smaller or similar to non-diabetic controls [26]. There are numerous differences in experimental protocols and a single factor cannot entirely explain the discrepancies. Duration of the diabetic state and plasma level of insulin (type I versus type II) seems to influence the myocardial susceptibility to IR injury [26]. Although type II diabetes represents 90% of the diabetic patients, the majority of the experimental studies are performed in type I diabetic rat models, induced by streptozotocin [5,25-27]. Most of them report a similar or reduced myocardial infarct area after IR injury [25,26]. Rodrigues et al. [25] reported that 3 months after IR injury, also ventricular dysfunction was attenuated and the profile of calcium handling proteins was better. Nevertheless, the mortality was higher in the type I diabetic group, due to increased autonomic dysfunction [25]. The sensitivity to IR injury in this model seems to be time-dependent. Some studies report a resistance to IR early after diabetes induction by

Table 2 Hemodynamic parameters

	Condition	Wild type		Ob/ob		DKO	
		Sham	*IR*	*Sham*	*IR*	*Sham*	*IR*
Heart rate (bpm)	Untreated	575±40	560±62	526±44[a]	540±41	523±29[a]	513±38
	Diet			504±30[a]	500±31	503±34[a]	516±51
	ACE-I	548±33	524±36	505±29	492±33	499±51	509±68
Stroke volume (µl)	Untreated	16.2±5.3	6.8±3.2[c]	14.8±3.1	12.0±4.3[a]	10.1±5.1[a]	9.6±4.1
	Diet			13.6±6.0	14.2±7.0[a]	11.8±5.1	12.7±6.1[a]
	ACE-I	16.5±4.9	8.1±1.6[c]	14.0±3.2	12.6±4.2[a]	15.1±6.1	12.4±3.0[a]
Stroke work (mmHg*µl)	Untreated	1256±328	263±167[c]	835±189[a]	509±181[ac]	606±269[a]	409±199
	Diet			804±546	667±336[a]	627±272[a]	676±332[a]
	ACE-I	877±399	369±158[c]	788±251	581±230	780±408	563±170
P_{sys} (mmHg)	Untreated	80.5±18.2	55.1±10.6[c]	68.7±7.5	62.5±2.7	69.5±11.1	62.7±10.6
	Diet			68.4±10.8	60.4±5.9	67.1±12.6	65.0±5.5[a]
	ACE-I	62.9±11.9	61.9±19.9	67.9±6.9	59.2±7.5[c]	61.4±6.2	56.1±8.1[d]
P_{ed} (mmHg)	Untreated	3.0±0.7	2.9±1.2	3.6±0.7	6.6±3.3[ac]	4.8±2.3[a]	6.6±2.4[a]
	Diet			3.0±1.1	4.3±1.2[a]	3.8±1.3	4.3±1.5[ab]
	ACE-I	3.6±2.0	2.9±2.3	4.5±0.9[d]	5.0±1.5	3.7±0.9	3.4±1.5[b]
PRSW (mmHg)	Untreated	88.8±5.5	61.5±11.6[c]	64±2.6[a]	48.5±3.3[ac]	57.1±4.6[ae]	46.2±8.8[ac]
	Diet			65.4±1.9[a]	51±1.9[ac]	58±2.8[ae]	49.7±3[ac]
	ACE-I	87.4±4.4	60.2±6.4[c]	71.8±3.2[abd]	52.1±3.4[ac]	66.3±5[abde]	51.2±3.4[ac]
E_{es} (mmHg/µl)	Untreated	8.3±2.5	6.4±1.5	5.1±0.4[a]	4.7±1.5	6.5±2.4	4.5±0.8[a]
	Diet			6.6±2.1	4.5±1.1[ac]	5.1±1.2[a]	5.3±1.2
	ACE-I	6.0±1.0	6.8±2.0	5.5±1.7	4.6±1.7	5.6±1.3	4.4±1.3[a]
Tau (ms)	Untreated	6.3±1.2	7.6±2.2	6.2±0.5	6.4±1.0	6.9±0.9	7.2±0.7
	Diet			6.9±0.5[b]	7.7±0.8[bc]	6.8±0.6	7.3±1.2
	ACE-I	6.6±1.5	7.4±1.5	8.0±1.1[bd]	8.8±1.5[b]	7.4±0.8	7.1±1.1[e]
EDPVR (mmHg/µl)	Untreated	0.2±0.2	0.4±0.2	0.4±0.3	0.5±0.3	0.6±0.4[a]	0.3±0.1
	Diet			0.2±0.2	0.2±0.1	0.2±0.2	0.2±0.1[ab]
	ACE-I	0.2±0.1	0.4±0.3	0.4±0.1	0.3±0.1	0.3±0.1[be]	0.3±0.1[d]
E_a (mmHg/µl)	Untreated	4.8±1.5	8.1±2.4[c]	4.0±0.9	5.0±2.0[a]	6.8±3.5	5.6±1.8[a]
	Diet			4.9±1.8	4.8±2.7[a]	5.2±2.2	5.4±2.1[a]
	ACE-I	3.3±0.6[b]	6.9±1.3[c]	4.6±1.5	4.5±1.5[a]	3.4±1.3[b]	3.8±1.0[ab]

ACE-I: angiotensin-converting enzyme inhibition; DKO: double knock-out (ob/ob; LDLR-/-); E_a: arterial elastance; EDPVR: end-diastolic pressure-volume relationship; E_{es}: end-systolic elastance; IR: ischemia/reperfusion; P_{ed}: end-diastolic pressure; P_{sys}: systolic pressure "a" $p < 0.05$ versus WT same treatment, same condition (sham or IR); "b" $p < 0.05$ versus same untreated genotype, same condition (sham or IR); "c" $p < 0.05$ versus sham, same genotype, same treatment; "d" $p < 0.05$ versus diet, same genotype, same condition (sham or IR); "e" $p < 0.05$ versus ob/ob same treatment, same condition (sham or IR).

streptozotocin, which disappears after 2 months [26]. Others however demonstrated an increased infarct size after coronary occlusion as early as 8 days after streptozotocin injection [26], indicating that duration of diabetes is not the only factor responsible for the heterogeneous results. Disagreement also exists about the effect of acute hyperglycemia to IR injury. Although some studies report a larger or similar susceptibility to IR injury in a hyperglycemic state, other reports demonstrate a significant protection, related to a better profile of calcium handling proteins [25,26].

The difference in presence or absence of hyperinsulinemia may also be responsible for the heterogeneous data. With the exceptions of a few studies in type II diabetic Zucker and Goto-Kakizaki rats, all type II diabetic models with obesity and hyperinsulinemia showed increased myocardial susceptibility to IR injury [26]. In the past decade, transgenic mouse models of type II diabetes and the metabolic syndrome have been developed. These models develop left ventricular diastolic and systolic dysfunction comparable with the diabetic cardiomyopathy seen in patients [8]. We demonstrated in this

Table 3 Numbers and infarct size

	Condition	Wild type		Ob/ob		DKO	
		Sham	*IR*	*Sham*	*IR*	*Sham*	*IR*
N experiments (survivors)	Untreated	9 (9)	12 (10)	7 (7)	11 (6)	7 (7)	9 (7)
	Diet			9 (7)	11 (7)	9 (7)	9 (8)
	ACE-I	6 (6)	8 (6)	10 (8)	10 (8)	8 (8)	10 (8)
Area at risk (% of heart)	Untreated	0±0	14.7±7.5	0±0	14.0±6.7	0±0	18.2±7.2
	Diet			0±0	17.8±3.4	0±0	16.4±8.6
	ACE-I	0±0	17.0±7.1	0±0	17.1±3.4	0±0	15.3±5.6
Infarct size (% of area at risk)	Untreated	0±0	49.3±6.8[b]	0±0	72.4±7.2[ab]	0±0	67.2±6.1[ab]
	Diet			0±0	68.4±9.8[ab]	0±0	70±11.9[ab]
	ACE-I	0±0	55.1±5.7[b]	0±0	69±8.1[ab]	0±0	68.8±9.9[ab]

ACE-I: angiotensin-converting enzyme inhibition; DKO: double knock-out (ob/ob; LDLR-/-); [a] $p < 0.05$ versus WT same treatment, same condition (sham or IR); [b] $p < 0.05$ versus sham, same genotype, same treatment.

study that these mice models of type II diabetes and the metabolic syndrome are more vulnerable for IR injury than their WT littermates. This correlates well with data from patient studies and previous studies in transgenic mice with type II diabetes [6,7,26]. Nevertheless, the underlying mechanisms by which type II diabetic and metabolic syndrome myocardium is more vulnerable to IR injury remains incompletely understood. Several hypotheses were suggested.

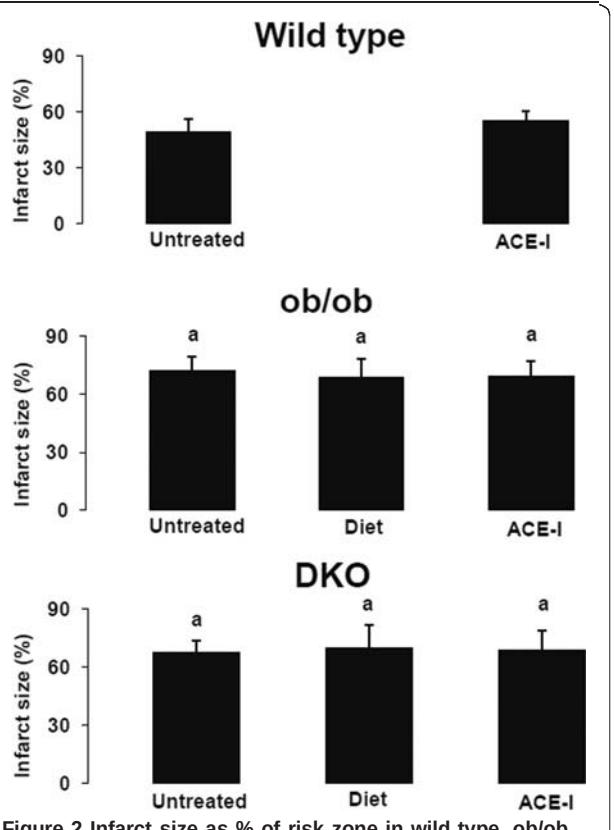


Figure 2 Infarct size as % of risk zone in wild type, ob/ob and double knock-out mice. [a] $p < 0.05$ versus WT same treatment, same age; [b] $p < 0.05$ versus same untreated genotype, same age; [c] $p < 0.05$ versus sham, same genotype, same treatment; [d] $p < 0.05$ versus diet, same genotype, same age; [e] $p < 0.05$ versus ob/ob same treatment, same age.

First, the diabetic myocardium exhibits abnormal cardiac substrate utilization and impaired energetic efficiency. In non-ischemic conditions, the myocardium uses preferentially fatty acids as energy substrate. This is even more the case in type II diabetes and the metabolic syndrome. The use of fatty acids requires 12% more oxygen per ATP generated than glucose. During ischemia, the normal myocardium switches to glucose as preferred energy substrate. This possibility is lost in case of diabetes, because oxidation of fatty acids inhibits the catabolism of glucose [29].

Second, endothelial dysfunction and reduced nitric oxide (NO) bioavailability is present in type II diabetes and the metabolic syndrome [11,14]. The beneficial or detrimental effect of NO depends on its concentration and cellular origin [5]. Although constitutive production of NO by endothelial cells (eNOS) is thought to be cytoprotective, NO generated by the inducible form (iNOS) is generally regarded as pro-inflammatory and cytotoxic. In diabetic myocardium, basal iNOS-levels are higher and the increase of iNOS-levels after IR injury is larger than in non-diabetic myocardium [30].

Third, $SERCA_{2a}$ is crucial in postischemic myocardium by significantly decreasing intracellular Ca^{2+}-overload [31]. It was shown before that $SERCA_{2a}$-activity is depressed in ob/ob and DKO mice [8]. In the streptozotocin type I diabetic model, $SERCA_{2a}$-expression after IR injury is increased versus WT animals. This is a possible explanation for the better preserved contractility after IR injury in this model [25].

Fourth, diabetic and metabolic syndrome models exhibit increased production of reactive oxygen species (ROS) [32]. ROS likely plays a central role in impairing mitochondrial energy metabolism, through

mitochondrial uncoupling, but also through direct damage of mitochondrial components. This can lead to apoptosis [32].

Fifth, the activity of aldose reductase, a NADPH-dependent enzyme, increases during IR injury [33]. Since aldose reductase activity is already increased in diabetes [34], IR injury further reduces myocardial glycolysis and glucose oxidation and increases the glycosylation of advanced end-products. This leads to more extensive cross-linking of key structures within tissues and increased IR injury [34].

Sixth, chronic hyperglycemia leads to cardiac hypertrophy and decreased microvasculature density in myocardium. This microcirculation dysfunction causes increased vulnerability to IR [28].

Seventh, the exposure to abnormal substrates and cytokines makes the diabetic heart more prone to IR. It was recently shown that IL-33 is decreased in type I diabetic mice [28]. It is known that IL-33 prevents apoptosis, attenuates myocardial infarction and improves cardiac function. In this study was also shown that chronic activation of protein kinase C-β in diabetes contributes to myocardial dysfunction and infarct [28].

Finally, leptin deficiency leads to ventricular hypertrophy and heart failure [35,36]. McGaffin et al. reported that leptin has a protective role after IR [36]. Leptin deficient mice had worse survival, worse cardiac function and increased apoptosis, 4 weeks after IR injury. Leptin induces activation of signal transducer and activator of transcription-3, which induces transcription of several cardioprotective genes. These, in turn, inhibit reverse remodeling and apoptosis [35,36]. It is thus possible that leptin deficiency leads to reduced contractility and larger infarct size versus WT, independently from the other risk factors. Nevertheless, the effect of leptin deficiency during the acute phase of IR, as in our experiments, is not known. It is unlikely that STAT-3 activation plays a role during the first hours after IR injury, since transcription of new cardioprotective genes is required.

In hypothesis 1 and 2, we postulated that food restriction respectively ACE-I could reduce the effects of IR injury in mouse models of type II diabetes and the metabolic syndrome. In our study, 12 weeks food restriction or ACE-I, was not capable to preserve in vivo cardiac contractility, nor limit infarct size after IR injury. Nevertheless, ACE-I significantly improved load-independent contractility, as measured by PRSW, in the non-ischemic group.

In food restricted mice, the daily intake was limited to the amount of 'normal' free fed wild type mice, measured to be 2.5 g/day. After 12 weeks, the weight loss of ob/ob and DKO mice was 35 à 40% of initial body weight. We did find a slight but non-significant restoration of contractility after this treatment. In a previous study by our group [37], we found that food restriction with body weight reduction of 50 à 55%, partly restored contractility. So, it is possible that weight reduction of 35 to 40% is insufficient to significantly improve contractility in our mice models. Nevertheless, heart weight was reduced in ob/ob and even more in DKO, below the level of WT, demonstrating already the impact of the food restriction. A further explanation for the difference between the present and earlier studies is that the experimental conditions in the present study differed from the previous. In the study of Van den Bergh et al. [37], hemodynamic measurements were performed in closed chest mice. In this study, an IR protocol was investigated. This implicates that also in the control groups, left ventricular contractility was measured in the same experimental conditions as the ischemia-reperfusion group, i.e. with an open thorax and time matched.

Food restriction has been shown to reduce IR injury in wild type rodent models [13,38,39]. In renal IR injury, 30% food reduction leads to significant protection. The length of restriction, required for the onset of these renal benefits, is not known but also food restrictions, as little as 2 weeks, lead to significant protection. Interestingly, there is no increase in protection between 2 and 4 weeks of food restriction, suggesting that the maximal protection afforded by these short treatments was already reached after 2 weeks [38]. Also in the heart, different short-term (2 weeks) food restriction protocols, ranging from 10% to 70%, improve cardiac function following IR injury in wild type rats [39]. Yamagishi et al. [39] reported that 70% food restriction during 11 days, resulting in a weight reduction of 38% compared to free-fed rats, has a different effect on pre-ischemic contractility and sensitivity to IR injury. In this study, pre-ischemic cardiac function was reduced but cardiac recovery after IR injury was improved [39]. An improved ischemic tolerance via altered expression of functional proteins induced by low serum T3 levels, decreased coronary flow rate and change in metabolic flux, were suggested. Anaerobic glycolysis was more activated during ischemia in food restricted animals. This activation of glycolytic flux is fundamental to effective recovery of cardiac function during reperfusion after ischemia [39]. Nevertheless, the boundary between food restriction and under nutrition is weak. More than 70% food restriction results in depressed cardiac function [39]. The effect of food restriction on IR injury was never studied in the intended group, the obese type II diabetic patients and animal models. In our type II diabetic and metabolic syndrome mouse models, food restriction did not induce significant protection against IR injury.

The effects of food restriction and ACE-I on the previously described mechanisms of increased vulnerability of type II diabetic myocardium to IR injury are incompletely

known. Data concerning the effects of these treatments on cardiac substrate utilization, energetics or aldose reductase activity are missing. Dyslipidemia seems to be a less important factor during IR injury, since infarct size and left ventricular contractility were not significantly different in DKO mice versus ob/ob. Although cholesterol-levels were normal after food restriction in ob/ob mice, this could not correct the increased sensitivity to IR injury.

ACE-I prevents downregulation of $SERCA_{2a}$ and phospholamban protein expression in heart failure [40]. Furthermore, ACE-I prevents the decrease in capillary density in type I diabetic rats [41]. This might explain the improved contractility in ob/ob and DKO mice with ACE-I.

It is widely accepted that food restriction and ACE-I reduce oxidative stress [13,14]. Nevertheless, nor food restriction nor ACE-I were capable to reduce IR injury in our mice models. This casts doubt on the role of oxidative stress in the increased vulnerability of diabetic myocardium to IR injury. No data are available concerning the effects of food restriction on capillary density, abnormal substrate utilisation or cytokinelevels in diabetic models.

Captopril has been reported to reduce infarct size in animal models, such as canine, porcine or rat models [16,17]. In the study of Itoh et al. [21], captopril did not protect WT mice against post-ischemic dysfunction and myocardial enzyme release. In contrast to this, a significant protection by captopril was found in mice with overexpression of p90 ribosomal S6 kinase. Since p90 ribosomal S6 kinase expression and activity are increased in diabetic mice, a protective effect of captopril was anticipated by Itoh et al. [21]. Nevertheless, we did not find infarct size reduction in our models of type II diabetes and the metabolic syndrome.

In this study, we investigated the effects of food restriction and ACE-I, because these treatments are standard therapies in type II diabetic and metabolic syndrome patients. Several other drugs with possible cardioprotective effects during IR injury have been developed. Peroxisome proliferator-activated receptor-γ agonists, such as rosiglitazone, have been shown to possess insulin-sensitizing and lipid-lowering and anti-inflammatory properties [42]. It has been demonstrated that peroxisome proliferator-activated receptor-γ agonists reduce IR injury in wild type and hypercholesterolemic models [42]. Nevertheless, it was shown by Ren et al. that the vasculoprotective effects of rosiglitazone are at least partially mediated by a reduction in local angiotensin II concentration [42]. Since ACE-inhibitors have similar effects on angiotensin II concentration and recent meta-analysis suggest that there is an increase in the risk of myocardial infarction, rosiglitazone was not investigated in this study [42].

Sulfonureas, such as metformin, are insulin-sensitizing agents, which reduce infarct size in non-diabetic animals by activation of cAMP-activated protein kinase and Akt. However, clinical and experimental studies in diabetic patients and models report a significantly higher cardiovascular risk and controversial effects on the effect on IR injury [26].

Recent evidence has indicated a possible importance of the endothelin system in the pathogenesis of diabetic complications [27]. Endothelin receptor blockade limits myocardial contractile depression in type I diabetic models [27]. The effect on IR injury was not studied. Since angiotensin II stimulates and bradykinin decreases endothelin-1 formation, it is conceivable that the majority of endothelin-1 formation is blocked by ACE-I. Furthermore, endothelin-receptor antagonism additional to ACE-I has no added benefit against cardiac dysfunction in type I diabetic rats [27].

Recently, Ichinomiya et al. demonstrated that high-dose fasudil, a Rho-kinase inhibitor, preserves postconditioning under acute hyperglycemic state in rats [43]. Rho-kinase activity is involved in diabetic endothelial dysfunction and regulation of Rho-kinase signaling is important for cellular function, such as contraction, mortality, proliferation and apoptosis. Correspondingly, Rho-kinase is known to be activated in ischemic myocardium. Inhibition of Rho-kinase activity is a matter of interest on myocardial protection against IR injury since it activates the ATP-sensitive potassium channels [43]. The effect of fasudil on postconditioning and IR injury in chronic hyperglycemic states, such as diabetes, has to be determined.

Limitations

We used Captopril as ACE-I. Other ACE-inhibitors, with different lipid solubility and/or tissue specificity, might have affected the impact of IR injury. In this study, we investigated the acute effects of IR injury after an ischemia/reperfusion time of respectively 30 and 60 minutes. It is possible that the beneficial effects of these treatments appear in a later phase, for example during the remodeling phase.

The combination of food restriction and ACE-I was not investigated. In ob/ob mice, food restriction reduces weight and cholesterol levels and ACE-I reduces glycemia. It is possible that the combination of these effects improves myocardial damage. In DKO mice the additional effect is unlikely, since there was no reduction of cholesterol after diet and only moderate reduction of glycemia after ACE-I. Further studies have to be conducted to study these effects.

Conclusion

Our mice models of type II diabetes and the metabolic syndrome are more sensitive to IR injury than their WT littermates, as they develop a significantly larger infarct size and worse in vivo cardiac contractility after 30 min ischemia followed by 60 min reperfusion. This mimics the clinical situation of patients with type II diabetes or

the metabolic syndrome.

Twelve weeks of food restriction, with a weight reduction of 35 à 40%, did not alter left ventricular pre-ischemic contractility. Nevertheless, twelve weeks of ACE-I ameliorated contractility in the pre-ischemic ob/ob and DKO group significantly.

Food restriction and ACE-I did not correct the increased sensitivity for IR injury in our mice models of type II diabetes and the metabolic syndrome.

Abbreviations

ACE: Angiotensin-converting enzyme; ACE-I: Angiotensin-converting enzyme inhibition; DKO: Double knock-out; E_a: Arterial elastance; EDPVR: End-diastolic pressure volume relationship; Ees: End-systolic elastance; eNOS: Endothelial nitric oxide synthase; iNOS: Inducible nitric oxide synthase; IR: Ischemia-reperfusion; LAD: Left anterior descending artery; LDL: Low density lipoprotein; LDLR: Low density lipoprotein receptor; NO: Nitric oxide; Ob/ob: Leptin deficient mouse model for type II diabetes; P_{ed}: End-diastolic pressure; PRSW: Preload recruitable stroke work; P_{sys}: Systolic pressure; PV: Pressure-volume; ROS: Reactive oxygen species; SW: Stroke work; TTC: Triphenyl-tetrazolium chloride; WT: Wild type.

Competing interest

The authors declare that they have no competing interests.

Authors' contribution

GVDM carried out the mice breeding, treatments, biochemical analysis, PV-loop experiments and infarct size determination, data and statistical analysis, and drafted the manuscript. IN, AV and WO contributed to mice breeding, treatments and biochemical analysis. WF participated in the design of the study and general supervision. PH designed the study, obtained funding, did supervision of the analysis and interpretation of data, and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by a grant of the Research Fund KU Leuven – Bijzonder Onderzoeksfonds (OT 04/39 and OT 05/55) and a grant of the FWO-Vlaanderen (G 0966.11).

Received: 21 May 2012 Accepted: 18 July 2012
Published: 1 August 2012

References

1. Kereiakes DJ, Willerson JT: **Metabolic syndrome epidemic**. *Circulation* 2003, 108:1552–1553.
2. Wild S, Roglic G, Green A, Sicree R, King H: **Global prevalence of diabetes: estimates for the year 2000 and projections for 2030.** *Diabetes Care* 2004, 27:1047–1053.
3. Dohi T, Miyauchi K, Kasai T, Kajimoto K, Kubota N, Tamura H, Yokoyama T, Kojima T, Yokoyama K, Kurata T, Daida H: **Impact of metabolic syndrome on 10-year clinical outcomes among patients with acute coronary syndrome.** *Circ J* 2009, 73:1454–1458.
4. Haffner SM, Lehto S, Rönnemaa T, Pyörälä K, Laakso M: **Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction**. *N Engl J Med* 1998, 339:229–234.
5. Marfella R, Di Filippo C, Esposito K, Nappo F, Piegari E, Cuzzocrea S, Berrino L, Rossi F, Giugliano D, D'Amico M: **Absence of inducible nitric oxide synthase reduces myocardial damage during ischemia reperfusion in streptozotocin-induced hyperglycemic mice.** *Diabetes* 2004, 53:454–462.
6. Aasum E, Hafstad AD, Severson DL, Larsen TS: **Age-dependent changes in metabolism, contractile function and ischemic sensitivity in hearts from db/db mice.** *Diabetes* 2003, 52:434–441.
7. Bouhidel O, Pons S, Souktani R, Zini R, Berdeaux A, Ghaleb B: **Myocardial ischemic postconditioning against ischemia-reperfusion is impaired in ob/ob mice.** *Am J Physiol Heart Circ Physiol* 2008, 295:H1580–H1586.
8. Van den Bergh A, Vanderper A, Vangheluwe P, Desjardins F, Nevelsteen I, Verreth W, Wuytack F, Holvoet P, Flameng W, Balligand JL, Herijgers P: **Dyslipidemia in type II diabetic mice does not aggravate contractile impairment but increases ventricular stiffness.** *Cardiovasc Res* 2008, 77:371–379.
9. Van den Bergh A, Flameng W, Herijgers P: **Type II diabetic mice exhibit contractile dysfunction but maintain cardiac output by favourable loading conditions**. *Eur J Heart Fail* 2006, 8:777–783.
10. Buse JB, Ginsberg HN, Bakris GL, Clark NG, Costa F, Eckel R, Fonseca V, Gerstein HC, Grundy S, Nesto RW, Pignone MP, Plutzky J, Porte D, Redberg R, Stitzel KF, Stone NJ, American Heart Association, American Diabetes Association: **Primary prevention of cardiovascular disease in people with diabetes mellitus. A Scientific statement from the American Heart Association and the American Diabetes Association.** *Circulation* 2007, 115:114–126.
11. Verreth W, De Keyzer D, Pelat M, Verhamme P, Ganame J, Bielicki JK, Mertens A, Quarck R, Benhabiles N, Marguerie G, Mackness B, Mackness M, Ninio E, Herregods MC, Balligand JL, Holvoet P: **Weight-loss-associated induction of peroxisome proliferator-activated receptor-alpha and peroxisome proliferator-activated receptor-gamma correlate with reduced atherosclerosis and improved cardiovascular function in obese insulin-resistant mice**. *Circulation* 2004, 110:3259–3269.
12. Crandall DL, Goldstein BM, Gabel RA, Cervoni P: **Hemodynamic effects of weight reduction in the obese rat.** *Am J Physiol* 1984, 247:R266–R271.
13. Broderick TL, Belke T, Driedzic WR**: Effects of chronic caloric restriction on mitochondrial respiration in the ischemic reperfused rat heart**. *Mol Cell Biochem* 2002, 233:119–125.
14. Velasquez MT, Bhathena SJ, Striffler JS, Thibault N, Scalbert E: **Role of angiotensin-converting enzyme inhibition in glucose metabolism and renal injury in diabetes.** *Metabolism* 1998, 47:7–11.
15. ACE Inhibitor Myocardial Infarction Collaborative Group: **Indications for ACE inhibitors in the early treatment of acute myocardial infarction: systematic overview of individual data from 100,000 patients in randomized trials**. *Circulation* 1998, 97:2202–2212.
16. Mehta PM, Przyklenk K, Kloner RA: **Cardioprotective effects of captopril in myocardial ischemia, ischemia/reperfusion and infarction**. *Eur Heart J* 1990, 11:94–99.
17. Parlakpinar H, Ozer MK, Acet A: **Effects of captopril and angiotensin II receptor blockers (AT1, AT2) on myocardial ischemia-reperfusion induced infarct size.** *Cytokine* 2011, 56:688–694.
18. Yang X-P, Liu Y-H, Shesely EG, Bulagannawar M, Liu F, Carretero OA: **Endothelial nitric oxide gene knock-out mice: cardiac phenotypes and the effect of angiotensin-converting enzyme inhibitor on myocardial ischemia/reperfusion injury.** *Hypertension* 1999, 34:24–30.
19. Liu YH, Yang XP, Sharov VG, Sigmon DH, Sabbath HN, Carretero OA: **Paracrine systems in the cardioprotective effect of angiotensin- converting enzyme inhibitors on myocardial ischemia/reperfusion injury in rats.** *Hypertension* 1996, 27:7–13.
20. Nozawa Y, Miura T, Tsuchida A, Kita H, Fukuma T, Shimamoto K: **Chronic treatment with an ACE inhibitor, temocapril, lowers the threshold for the infarct size-limiting effect of ischemic preconditioning.** *Cardiovac Drugs Ther* 1999, 13:151–157.
21. Itoh S, Ding B, Shishido T, Lerner-Marmarosh N, Wang N, Maekawa N, Berk BC, Takeishi Y, Yan C, Blaxall BC, Abe J: **Role of p90 ribosomal S6 kinase-mediated prorenin-converting enzyme in ischemic and diabetic myocardium.** *Circulation* 2006, 113:1787–1798.
22. Wichi R, Farah V, Chen Y, Irigoyen MC, Morris M: **Deficiency in angiotensin AT1a receptors prevents diabetes-induced hypertension.** *Am J Physiol Regul Integr Comp Physiol* 2007, 292:R1184–R1189.
23. Van der Mieren G, Van den Bergh A, Nevelsteen I, Vanderper A, Flameng W, Herijgers P: **Hypoxic preconditioning preserves cardiac contractility and reduces infarct size in vivo.** *The Open Surgery Journal* 2008, 2:24–29.
24. Van den Bergh A, Flameng W, Herijgers P: **Parameters of ventricular contractility in mice: influence of load and sensitivity to changes in inotropic state.** *Pflugers Arch* 2008, 455:987–994.

25. Rodrigues B, Rosa KT, Medeiros A, Schaan BD, Brum PC, De Angelis K, Irigoyen MC: **Hyperglycemia can delay left ventricular dysfunction but not autonomic damage after myocardial infarction in rodents.** *Cardiovasc Diabetol* 2011, 10:26.
26. Miki T, Itoh T, Sunaga D, Miura T: **Effects of diabetes on myocardial infarct size and cardioprotection by preconditioning and postconditioning**. *Cardiovasc Diabetol* 2012, 11:67.
27. Wölkart G, Pang X, Stessel H, Kirchengast M, Brunner F: **Chronic endothelin-A receptor antagonism is as protective as angiotensin converting enzyme inhibition against cardiac dysfunction in diabetic rats.** *Br J Pharmacol* 2007, 151:1187–1197.
28. Rui T, Zhang J, Xu X, Yao Y, Kao R, Martin M: **Reduction of IL-33 expression exaggerates ischemia/reperfusion-induced myocardial injury in mice with diabetes mellitus.** *Cardiovasc Res* 2012, 94:370–378.
29. Abozguia K, Clarke K, Lee L, Frenneaux M: **Modification of myocardial substrate use as a therapy for heart failure.** *Nat Clin Pract Cardiovasc Med* 2006, 3:490–498.
30. Marfella R, Esposito K, Nappo F, Siniscalchi M, Sasso FC, Portoghese M, Di Marino MP, Baldi A, Cuzzocrea S, Di Filippo C, Barboso G, Baldi F, Rossi F, D'Amico M, Giugliano D: **Expression of angiogenic factors during acute coronary syndromes in human type 2 diabetes**. *Diabetes* 2004, 53:2383–2391.
31. Talukder MA, Kalyanasundaram A, Zuo L, Velayutham M, Nishijima Y, Periasamy M, Zweier JL: **Is reduced SERCA2a expression detrimental or beneficial to postischemic cardiac function and injury? Evidence from heterozygous SERCA2a knockout mice**. *Am J Physiol Heart Circ Physiol* 2008, 294:H1426–H1434.
32. Bugger H, Abel ED: **Molecular mechanisms for myocardial mitochondrial dysfunction in the metabolic syndrome**. *Clin Sci* 2008, 114:195–210.
33. Hwang YC, Sato S, Tsai JY, Yan S, Bakr S, Zhang H, Oates PJ, Ramasamy R: **Aldose reductase activation is a key component of myocardial response to ischemia.** *FASEB J* 2002, 16:243–245.
34. Kaneko M, Bucciarelli L, Hwang YC, Lee L, Yan SF, Schmidt AM, Ramasamy R: **Aldose reductase and AGE-RAGE pathways: key players in myocardial ischemic injury.** *Ann N Y Acad Sci* 2005, 1043:702–709.
35. McGaffin KR, Sun CK, Rager JJ, Romano LC, Zou B, Mathier MA, O'Doherty RM, McTiernan CF, O'Donnell CP: **Leptin signalling reduces the severity of cardiac dysfunction and remodelling after chronic ischaemic injury.** *Cardiovasc Res* 2008, 77:54–63.
36. McGaffin KR, Zou B, McTiernan CF, O'Donnell CP: **Leptin attenuates cardiac apoptosis after chronic ischaemic injury.** *Cardiovasc Res* 2009, 83:313–324.
37. Van den Bergh A, Vangheluwe P, Vanderper A, Carmeliet P, Wuytack F, Janssens S, Flameng W, Holvoet P, Herijgers P**: Food-restriction in obese dyslipidaemic diabetic mice partially restores basal contractility but not contractile reserve.** *Eur J Heart Fail* 2009, 11:1118–1125.
38. Mitchell JR, Verweij M, Brand K, van de Ven M, Goemaere N, van den Engel S, Chu T, Forrer F, Müller C, de Jong M, van IJcken W, IJzermans JN, Hoeijmakers JH, de Bruin RW: **Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice.** *Aging Cell* 2010, 9:40–53.
39. Yamagishi T, Bessho M, Yanagida S, Nishizawa K, Kusuhara M, Ohsuzu F, Tamai S: **Severe, short-term food restriction improves cardiac function following ischemia/reperfusion in perfused rat hearts**. *Heart Vessels* 2010, 25:417–425.
40. Takeishi Y, Bhagwat A, Ball NA, Kirkpatrick DL, Periasamy M, Walsh RA: **Effect of angiotensin-converting enzyme inhibition on protein kinase C and SR proteins in heart failure.** *Am J Physiol Heart Circ Physiol* 1999, 276:H53–H62.
41. Gross ML, Heiss N, Weckbach M, Hansen A, El-Shakmak A, Szabo A, Münter K, Ritz E, Amann K: **ACE-inhibition is superior to endothelin A receptor blockade in preventing abnormal capillary supply and fibrosis of the heart in experimental diabetes.** *Diabetologia* 2004, 47:316–324.
42. Ren L, Liu N, Zhi H, Li Y, Li Y, Tang R, Sheng Z: **Vasculoprotective effects of rosiglitazone through modulating renin-angiotensin system in vivo and vitro.** *Cardiovasc Diabetol* 2011, 10:10.
43. Ichinomiya T, Cho S, Higashijima U, Matsumoto S, Maekawa T, Sumikawa K: **High-dose fasudil preserves postconditioning against myocardial infarction under hyperglycemia in rats: role of mitochondrial KATP channels.** *Cardiovasc Diabetol* 2012, 11:28.

doi:10.1186/1475-2840-11-89
Cite this article as: Van der Mieren et al.: **Angiotensin-converting enzyme inhibition and food restriction in diabetic mice do not correct the increased sensitivity for ischemia-reperfusion injury.** Cardiovascular Diabetology 2012 11:89.

Chapter 6

Angiotensin-converting enzyme inhibition and food restriction restore delayed preconditioning in diabetic mice

Gerry Van der Mieren, MD, Ines Nevelsteen, MD, Annelies Vanderper, PhD, Wouter Oosterlinck, MD, Willem Flameng, MD, PhD, Paul Herijgers, MD, PhD

Department of Cardiovascular Sciences, Research Unit Experimental Cardiac Surgery, K.U. Leuven, Herestraat 49, B-3000 Leuven, Belgium

Published in Cardiovascular Diabetology 2013; 12: 36.

Van der Mieren et al. Cardiovascular Diabetology 2013, 12:36
http://www.cardiab.com/content/12/1/36

CARDIO VASCULAR DIABETOLOGY

Angiotensin-converting enzyme inhibition and food restriction restore delayed preconditioning in diabetic mice

Gerry Van der Mieren, Ines Nevelsteen, Annelies Vanderper, Wouter Oosterlinck, Willem Flameng and Paul Herijgers*

Abstract

Background: Classical and delayed preconditioning are powerful endogenous protection mechanisms against ischemia-reperfusion damage. However, it is still uncertain whether delayed preconditioning can effectively salvage myocardium in patients with co-morbidities, such as diabetes and the metabolic syndrome. We investigated delayed preconditioning in mice models of type II diabetes and the metabolic syndrome and investigated interventions to optimize the preconditioning potential.

Methods: Hypoxic preconditioning was induced in C57Bl6-mice (WT), leptin deficient ob/ob (model for type II diabetes) and double knock-out (DKO) mice with combined leptin and LDL-receptor deficiency (model for metabolic syndrome). Twenty-four hours later, 30 min of regional ischemia was followed by 60 min reperfusion. Left ventricular contractility and infarct size were studied. The effect of 12 weeks food restriction or angiotensin-converting enzyme inhibition (ACE-I) on this was investigated. Differences between groups were analyzed for statistical significance by student's t-test or one-way ANOVA followed by a Fisher's LSD post hoc test. Factorial ANOVA was used to determine the interaction term between preconditioning and treatments, followed by a Fisher's LSD post hoc test. Two-way ANOVA was used to determine the relationship between infarct size and contractility (PRSW). A value of $p<0.05$ was considered significant.

Results: Left ventricular contractility is reduced in ob/ob compared with WT and even further reduced in DKO. ACE-I improved contractility in ob/ob and DKO mice. After ischemia/reperfusion without preconditioning, infarct size was larger in DKO and ob/ob versus WT. Hypoxic preconditioning induced a strong protection in WT and a partial protection in ob/ob mice. The preconditioning potential was lost in DKO. Twelve weeks of food restriction or ACE-I restored the preconditioning potential in DKO and improved it in ob/ob.

Conclusion: Delayed preconditioning is restored by food restriction and ACE-I in case of type II diabetes and the metabolic syndrome.

Keywords: Myocardial protection, Preconditioning, Ischemia/reperfusion injury, Diabetes mellitus, Metabolic syndrome

* Correspondence: paul.herijgers@uzleuven.be
Department of Cardiovascular Sciences, Research Unit Experimental Cardiac Surgery, K.U. Leuven, Herestraat 49, Leuven B-3000, Belgium

BioMed Central

© 2013 Van der Mieren et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Background

Tissues and organs can be protected from ensuing longer ischemia-reperfusion (IR) periods by short preceding episodes of 'preconditioning' ischemia [1]. However, after 26 years of research and the consistent and significant protection of cardiac preconditioning found in most experimental studies, none of these results has been translated into clinical therapies [1-5]. There are several reasons. The stimulus to induce preconditioning, such as repeated temporary coronary occlusion or aortic cross-clamping, can be harmful itself and prolongs the procedure. It was further debated whether standard maneuvers, such as cardiopulmonary bypass during cardiac surgery, themselves precondition the patient [4]. Another important issue in this failure, is the uncertainty whether this technique can effectively salvage myocardium in patients with co-morbidities, such as type 2 diabetes (T2D) or the metabolic syndrome (MS), that are frequent in the intended patient population [1,5].

The number of patients with diabetes and the metabolic syndrome increases in Western societies and reaches epidemic proportions [6-9]. The incidence of myocardial infarction is twice that of the general population and infarct size, for a given ischemic insult, is larger [7,8]. Large-scale clinical trials showed that intensive glycemic control failed to reduce cardiovascular mortality and macrovascular incidents including myocardial infarction, in comparison with standard glucose control in diabetic patients [9]. Therefore, new or additional techniques to protect the diabetic heart against ischemic damage are eagerly awaited.

Delayed preconditioning might be an interesting technique for this, since it is capable to induce cardioprotection in a controlled way for longer periods. Most of our knowledge concerning preconditioning however has been gathered from experiments in normal healthy animal models [1]. These models are not optimal to study preconditioning for the clinical population that might benefit most of it, i.e. patients with T2D or the MS. Delayed preconditioning reduces infarct size and preserves cardiac contractility in wild type (WT)-mice after ischemia-reperfusion [10], but not in models of type I diabetes [11]. Clinical studies report that type II diabetic myocardium is resistant or has a higher threshold to classical preconditioning [2,3]. The effect of delayed preconditioning on cardiac IR in mouse models of T2D and the MS has never been investigated and is the focus of this study.

Most patients with T2D or the MS are treated [12]. These treatments might additionally influence the preconditioning potential. Despite the fact that hypocaloric diet is a standard treatment in T2D and the MS [12], its cardiovascular effects are incompletely understood. A study with obese rats showed that food restriction partially restores classical preconditioning [13]. The effects of long-term food restriction on delayed preconditioning was never investigated. Food restriction increases endothelial nitric oxide synthase (eNOS)-levels in a mouse model of the metabolic syndrome [14], NO being a determinant trigger in the delayed preconditioning pathway [1]. It is therefore conceivable that food restriction restores the delayed preconditioning potential and induces cardioprotection by this mechanism.

Angiotensin-converting enzyme inhibition (ACE-I) is also a standard therapy in the treatment of hypertension in diabetic patients [12]. It was previously shown that ACE-I is capable to augment classical [15,16] and delayed preconditioning [17,18] in WT animal models, by lowering the stimulus threshold. There are no data concerning the effect of ACE-I on the delayed preconditioning in T2D and MS models. ACE-I might however impact on the delayed preconditioning potential since it is capable to block the degradation of bradykinin into inactive peptides and to increase eNOS and nitric oxide (NO)-levels [15,16,19].

Therefore, the aim of this study is to investigate the effects of food restriction and ACE-I on delayed cardiac preconditioning in diabetic or metabolic syndrome models. We tested the hypotheses that 1° mice with T2D or the MS are resistant to hypoxic delayed preconditioning; and 2° the interventions food restriction or ACE-I restore the preconditioning potential in these mouse models.

Methods and materials

Animal models

Experiments were conducted in 49 C57BL/6J WT-mice, 71 leptin deficient ob/ob, and 68 double knock-out (DKO)-mice with combined leptin and LDL-receptor deficiency. The ob/ob mouse is a model for T2D, featuring abdominal obesity and insulin resistance. DKO feature many characteristics of the metabolic syndrome, i.e. obesity, dyslipidemia, hypertension, insulin resistance and impaired glucose tolerance and/or diabetes [14]. These models develop left ventricular diastolic and systolic dysfunction comparable with the diabetic cardiomyopathy seen in patients [20]. Ob/ob and C57BL/6J-mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). DKO were generated as described previously [14,20]. IR was induced at 24 weeks of age. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 1996). All experimental protocols were approved by the K.U. Leuven Institutional Animal Care Commission and Ethical Committee.

Delayed preconditioning stimulus

The experimental technique was previously described [10]. Briefly, mice were placed in a plexiglas container, in which controlled oxygen levels of 6% could be induced within 30 seconds using a mixture of nitrogen and compressed air. Five cycles of 6 minutes of 6% oxygen,

interspersed with 6 minutes of room air were used. In the non-preconditioned groups, only compressed air was used without nitrogen. Afterwards, mice were placed in their regular cage with food and water ad libitum for 24 hours.

Treatments

Food intake (Ssniff, Soest, Germany) of diet-restricted ob/ob (n=7 sham, n=9 non-preconditioned, n=8 preconditioned) and DKO (n=7 sham, n=7 non-preconditioned, n=8 preconditioned) mice was restricted to 2.5 g/day, which is the normal daily intake of lean WT-mice, between 12 and 24 weeks of age [14,21]. ACE-inhibition was obtained with captopril (10 mg/kg/day) intraperitoneally from 12 until 24 weeks of age in WT (n=6 sham, n=7 non-preconditioned, n=8 preconditioned), ob/ob (n=7 sham, n=9 non-preconditioned, n=8 preconditioned) and DKO (n=7 sham, n=9 non-preconditioned, n=8 preconditioned) [22].

Experiments in untreated WT (n=8 sham, n=11 non-preconditioned, n=9 preconditioned), ob/ob (n=6 sham, n=9 non-preconditioned, n=8 preconditioned) and DKO (n=7 sham, n=7 non-preconditioned, n=8 preconditioned) mice served as controls.

Ischemia/reperfusion

The experimental technique was previously described [10,20]. Briefly, anesthesia was induced with urethane (1.2 g/kg) and alfa-chloralose (50 mg/kg). Mice were ventilated with room air, with rectal temperature kept at 37±0.5°C. Via left thoracotomy, the left anterior descending artery (LAD), was non-traumatically occluded, 2 mm below the tip of the left auricle for 30 min. Afterwards, a reperfusion period of 1 hour was allowed. Successful coronary occlusion and reperfusion was visually verified by observing the myocardium distal to the coronary occlusion turning pale respectively blushing. In the groups without ischemia (sham), a thoracotomy and time-matched procedure was performed.

Outcome parameters: infarct size and in vivo left ventricular contractility

The technique was previously described [10,20]. At the end of 1 hour reperfusion, a pressure-conductance catheter (1.4-Fr, SPR-839; Millar Instruments, Houston, TX) was inserted through the right carotid artery into the left ventricle. Baseline pressure-volume (PV) loops were recorded (Powerlab/ADInstruments, Castle Hill, Australia). Parallel volume and specific blood conductance were determined [10,20,21]. The inferior caval vein was compressed to obtain left ventricular PV-loops under varying loading conditions. Heart rate, systolic and end-diastolic pressure were measured. Stroke volume was determined as the difference in end-diastolic and end-systolic volume. Stroke work (SW) is the mechanical energy which the heart develops during the cardiac cycle and is calculated as the area enclosed by the PV-loop.

Preload recruitable stroke work (PRSW) is the slope of the relationship between end-diastolic volume and SW performed by the ventricle. PRSW is the most reliable and useful parameter for general contractility since it is chamber size independent and robust [23]. The slope of the end-systolic pressure-volume relationship, end-systolic elastance (E_{es}), reflects left ventricular chamber end-systolic stiffness and is used as an index of contractility. Tau is the time constant of left ventricular relaxation during isovolumetric diastole. The end-diastolic PV-relationship (EDPVR) represents the compliance of the ventricular myocardium at the end of the diastole. Augmented stiffness of the ventricular wall increases the slope of the EDPVR. Arterial elastance (E_a), a measure for afterload, is defined as the end-systolic pressure to stroke volume ratio [23].

Before excision of the heart, Evans blue (0.8 ml, 1% solution) was injected intravenously after re-occlusion of the LAD, to determine the left ventricular perfusion area at risk. The heart was cut in 1 mm-slices and the slices were stained with triphenyl-tetrazolium-chloride solution (TTC, 20 min, 1%, 37°C, pH 7.4) and fixed in paraformaldehyde (10 min, 4% solution, 20°C). All slices were weighed and photographed with a digital camera under magnification.

Data management and statistical analysis

Analysis of the pressure-conductance data was performed using PVAN 3.2 software (Millar Instruments, Houston) as previously described [10,20,21,23].

Infarct size and area at risk were determined by the number of pixels in each zone with Adobe Photoshop 8.0 (Adobe System Inc.) multiplied by the weight of the respective slices.

All statistical analyses were performed using statistical software (Statistica 7.1, StatSoft, Tulsa, USA). Data are expressed as mean ± standard deviation. Differences between groups were analyzed for statistical significance by student's t-test or one-way ANOVA followed by a Fisher's LSD post hoc test. Factorial ANOVA was used to determine the interaction term between preconditioning and treatments, followed by a Fisher's LSD post hoc test. Two-way ANOVA was used to determine the relationship between infarct size and contractility (PRSW). A value of $p<0.05$ was considered significant.

Results

Effect of preconditioning in untreated mice

Preconditioning without subsequent ischemia, was investigated in WT (n=8 sham preconditioned; n=8 sham non-preconditioned) and DKO (n=7 sham preconditioned; n=7 sham non-preconditioned) mice. As expected, this

had no effect on the contractility parameters. These results can be found in Additional file 1.

Left ventricular contractility, expressed as PRSW (Figure 1), is reduced in ob/ob compared with WT and even further reduced in DKO (Table 1). The area at risk after coronary occlusion is comparable in all groups undergoing IR. After IR without preconditioning, infarct size was larger in DKO (Table 2) and ob/ob versus WT (Figure 2). IR impaired PRSW significantly in all groups, but to a significantly lower level in DKO and ob/ob than in WT (Table 1).

Preconditioning induced a strong protection in WT mice. Infarct size, stroke volume, E_{es}, E_a and PRSW were significantly better preserved after preconditioning (Table 1, 2 and Additional file 2). In ob/ob, delayed preconditioning failed to significantly reduce infarct size ($p = 0.06$). Nevertheless, load-independent left ventricular contractility, as measured by PRSW, was better preserved after preconditioning. The preconditioning potential was lost in DKO mice: infarct size was not reduced and none of the contractility parameters were significantly better preserved after preconditioning. The relationship between infarct limitation and improved contractility was significantly different in DKO and WT mice. In DKO, preconditioning induced a proportional smaller improved PRSW per reduced infarct area versus WT mice (Additional file 3).

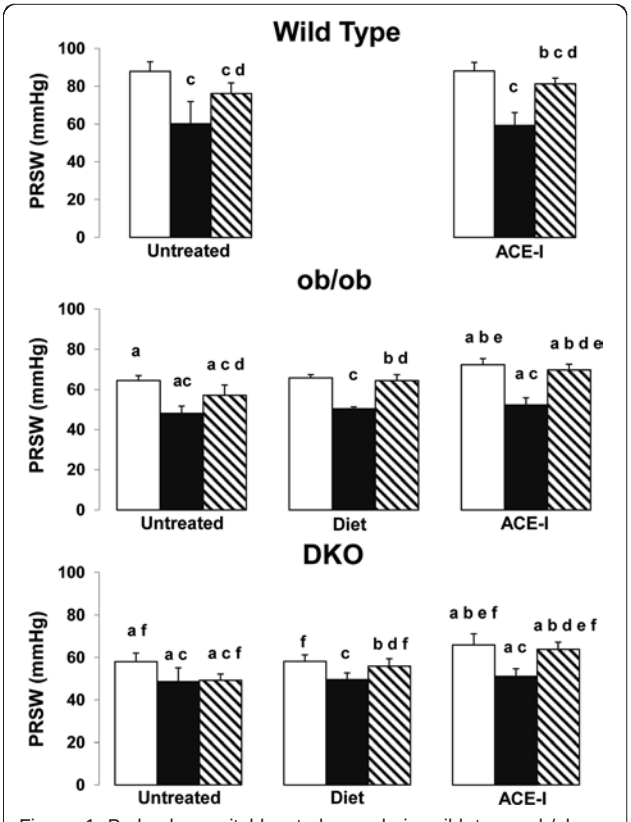


Figure 1 Preload recruitable stroke work in wild type, ob/ob and double knock-out mice. White bar: Non ischemic (sham); Black bar: Non-preconditioned and IR injury; Dashed bar: Preconditioned and IR injury "a" $p<0.05$ versus WT same treatment, same condition (sham; non-preconditioned; preconditioned); "b" $p<0.05$ versus same untreated genotype, same condition; "c" $p<0.05$ versus sham, same genotype, same treatment; "d" $p<0.05$ versus non-preconditioned, same genotype, same treatment; "e" $p<0.05$ versus diet, same genotype, same condition; "f" $p<0.05$ versus ob/ob same treatment, same condition.

Effect of food restriction on preconditioning

Food restriction did not improve the reduced PRSW in sham ob/ob and DKO mice (Figure 1). Food restriction did not influence infarct size and PRSW in non-preconditioned DKO and ob/ob versus the untreated groups (Table 1 and 2) [10].

After food restriction, delayed preconditioning was restored in ob/ob mice, as seen by the significantly reduced infarct size, and significantly better preserved E_{es} and PRSW. These load-independent contractility parameters reached values comparable to the non-ischemic sham group.

Furthermore, food restriction restored the preconditioning potential in DKO mice: infarct size and PRSW were significantly better preserved versus the non-preconditioned group. Left ventricular contractility, expressed as PRSW, remained worse versus food restricted and preconditioned ob/ob mice.

Effect of ACE-I on preconditioning

ACE-I improved contractility in sham ob/ob and DKO mice, not in WT. Furthermore, PRSW was significantly better after 12 weeks of ACE-I in ob/ob and DKO versus diet (Figure 1). Without preconditioning, ACE-I did not reduce infarct size and the impact of IR injury on PRSW in any genotype versus the untreated groups (Table 1 and 2) [10].

After ACE-I treatment, the protective effect of preconditioning on PRSW and SW was increased in WT-mice versus the untreated group (Table 1 and Additional file 2).

After ACE-I treatment, delayed preconditioning was fully restored in ob/ob mice concerning stroke work, Tau, E_{es} and PRSW. Infarct size after preconditioning was even smaller than the already beneficial effect that could be obtained with diet (Figure 2).

Also in DKO mice, ACE-I treatment restored delayed preconditioning. Infarct size and PRSW were significantly better preserved than the non-preconditioned group. After ACE-I treatment and preconditioning in DKO mice, PRSW was better preserved versus food restricted preconditioned DKO and reached the level of the sham group without ischemia. After ACE-I and preconditioning, the proportional effect on infarct limitation and improved contractility was restored to the WT-level (Additional file 3).

Table 1 Load-independent hemodynamic parameters

		Wild type			Ob/ob			DKO		
	Condition	*Sham*	*NP*	*P*	*Sham*	*NP*	*P*	*Sham*	*NP*	*P*
PRSW (mmHg)	Untreated	87.9±5.0	60.2±11.8[c]	76.3±5.4 [cd]	64.5±2.5 [a]	48.1±3.6 [ac]	57.1±5.2 [acd]	58.0±4.1 [af]	48.6±6.5 [ac]	49.2±3.0 [acf]
	Diet				65.8±1.7	50.4±1.0 [c]	64.4±3.1 [bd]	58.2±3.0 [f]	49.5±3.2 [c]	55.9±3.5 [bdf]
	ACE-I	88.0±4.6	59.3±6.8 [c]	81.2±3.1 [bcd]	72.4±3.1 [abe]	52.3±3.6 [ac]	69.8±2.9 [abde]	65.9±5.3 [abef]	51.1±3.6 [ac]	63.9±3.3 [abdef]
Ees (mmHg/µl)	Untreated	8.3±2.5	6.4±1.5	8.5±1.7 [d]	5.0±0.4 [a]	4.8±1.7	5.6±1.2 [a]	6.0±2.2	4.5±0.8 [a]	4.8±2.3 [a]
	Diet				6.3±2.2	4.4±1.2	7.3±2.2[d]	5.1±1.3	5.4±1.2	5.6±1.1
	ACE-I	6.0±1.1	6.2±1.6	8.7±1.7 [cd]	5.3±1.7	4.2±1.3	6.4±1.6 [ad]	5.6±1.4	4.3±1.4	5.2±3.0 [a]
EDPVR(mmHg/µl)	Untreated	0.2±0.2	0.4±0.2	0.3±0.2	0.4±0.3	0.4±0.3	0.5±0.3 [a]	0.5±0.4	0.3±0.1	0.3±0.2
	Diet				0.3±0.2	0.2±0.1	0.2±0.1 [b]	0.3±0.1	0.2±0.1 [b]	0.3±0.2
	ACE-I	0.2±0.2	0.4±0.4	0.2±0.1	0.4±0.1	0.3±0.1	0.3±0.1 [bc]	0.3±0.1 [f]	0.3±0.1	0.2±0.1

ACE-I: angiotensin-converting enzyme inhibition; DKO: double knock-out (ob/ob; LDLR-/-); EDPVR: end-diastolic pressure-volume relationship; E_{es}: end-systolic elastance; IR: ischemia/reperfusion; NP: Non-Preconditioned and ischemia/reperfusion; P: Preconditioned and ischemia/reperfusion; PRSW: preload recruitable stroke work; sham: group without ischemia/reperfusion. "a" p<0.05 versus WT same treatment, same condition (sham; non-preconditioned; preconditioned); "b" p<0.05 versus same untreated genotype, same condition; "c" p<0.05 versus sham, same genotype, same treatment; "d" p<0.05 versus non-preconditioned, same genotype, same treatment; "e" p<0.05 versus diet, same genotype, same condition; "f" p<0.05 versus ob/ob same treatment, same condition.

Discussion

Hypoxic preconditioning was capable to induce a strong protection in WT mice, but not in the DKO model of MS. Furthermore, the relationship between infarct limitation and improved contractility after preconditioning was different in DKO than in WT mice. In DKO, preconditioning induced a proportionally smaller improved PRSW per reduced infarct area versus WT mice. In ob/ob, preconditioning induced only a partial preservation of left ventricular contractility. The degree of protection was inferior to that in WT.

Since we have not directly investigated the mechanisms underlying the reduced or abolished delayed preconditioning potential, we can only hypothesize on them. This should further be investigated in depth to allow specific, additional therapeutic interventions. Potential underlying mechanisms are numerous.

First, our models are known with raised concentrations of non-esterified fatty acids, insulin resistance and hyperglycemia [14,20,21]. These metabolic anomalies induce atypical isoforms of protein-kinase C [24], which is crucial in the pathway of delayed preconditioning [1].

Second, insulin induces a preconditioning effect [5]. It was shown that at 24 weeks, both ob/ob and DKO mice have increased insulin levels [14,20]. It is therefore possible that mouse models with hyperinsulinemia are in a permanent preconditioned state or require a higher threshold to preconditioning [5,25].

Table 2 Numbers and infarct size

		Wild type			Ob/ob			DKO		
	Condition	*Sham*	*NP*	*P*	*Sham*	*NP*	*P*	*Sham*	*NP*	*P*
N experiments (survivors)	Untreated	8 (8)	11 (9)	9 (9)	6 (6)	9 (6)	8 (7)	7 (6)	7 (6)	8 (7)
	Diet				7 (6)	9 (6)	8 (7)	7 (6)	7 (7)	8 (8)
	ACE-I	6 (6)	7 (6)	8 (8)	7 (7)	9 (7)	8 (7)	7 (7)	9 (7)	8 (7)
Weight (g)	Untreated		28.3±4.6			62.4±3.8 [a]			59.1±9.5 [a]	
	Diet					38.3±4.7 [b]			37.9±3.4 [b]	
	ACE-I		27.1±3.4			62.9±9.4 [ae]			63.6±8.5 [ae]	
Area at risk (% of heart)	Untreated	0	15.4±7.6 [c]	16.6±6.9 [c]	0	15.6±6.1[c]	12.4±5.5 [c]	0	18.8±7.6 [c]	12.9±3.5 [c]
	Diet				0	18.7±2.6 [c]	19.4±6.6 [c]	0	17.5±8.7 [c]	17.9±10.7 [c]
	ACE-I	0	19.0±5.8 [c]	18.8±7.4 [c]	0	16.4±3.0 [c]	17.5±8.8 [c]	0	14.4±5.3 [c]	16.1±7.1 [c]
Infarct size (% of area at risk)	Untreated	0	50.2±6.5 [c]	28.9±12.3 [cd]	0	72.7±8.0 [ac]	62.9±7.8 [ac]	0	67.4±6.6 [ac]	65.1±7.1 [ac]
	Diet				0	66.8±9.7 [c]	56.5±6.4 [cd]	0	71.2±12.3 [c]	57.7±7.6 [cd]
	ACE-I	0	53.2±3.5 [c]	23.6±6.1 [cd]	0	69.6±8.6 [ac]	49.7±5.5 [abcde]	0	66.4±7.8 [ac]	53.3±10.4 [abcd]

ACE-I: angiotensin-converting enzyme inhibition; DKO: double knock-out (ob/ob; LDLR-/-); NP: ischemia/reperfusion and Non-Preconditioned; P: ischemia/reperfusion and Preconditioned. "a" p<0.05 versus WT same treatment, same condition (sham; non-preconditioned; preconditioned); "b" p<0.05 versus same untreated genotype, same condition; "c" p<0.05 versus sham, same genotype, same treatment; "d" p<0.05 versus non-preconditioned, same genotype, same treatment; "e" p<0.05 versus diet, same genotype, same condition.

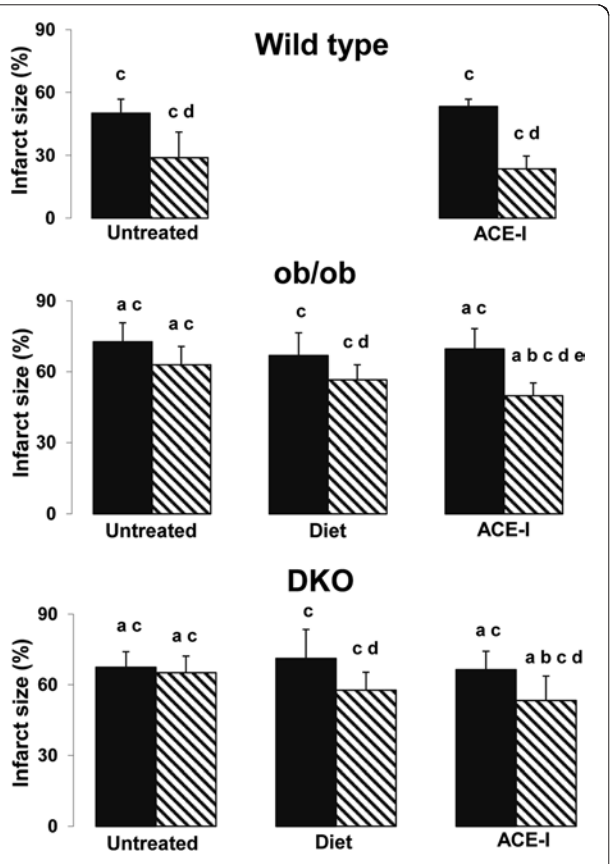


Figure 2 Infarct size as % of risk zone in wild type, ob/ob and double knock-out mice. Black bar: Non-preconditioned and IR injury; Dashed bar: Preconditioned and IR injury "a" p<0.05 versus WT same treatment, same condition (sham; non-preconditioned; preconditioned); "b" p<0.05 versus same untreated genotype, same condition; "c" p<0.05 versus sham, same genotype, same treatment; "d" p<0.05 versus non preconditioned, same genotype, same treatment; "e" p<0.05 versus diet, same genotype, same condition.

Third, previous studies demonstrated the importance of mitochondrial reactive oxygen species (ROS), generated in response to a preconditioning stimulus [1,2]. Increased oxidative stress is demonstrated in our mouse models [14,20]. It is therefore possible that increased ROS-levels induce a permanent preconditioned state or induce a higher threshold to preconditioning.

Fourth, the role of NO in IR injury and preconditioning is complex and depends on its concentration and cellular origin [1,26]. eNOS is a trigger in the delayed preconditioning pathway. Endothelial dysfunction and reduced nitric oxide (NO) bioavailability is present in T2D and the MS [27]. During IR injury, iNOS has a beneficial role in normal myocardium since iNOS knock-out non-diabetic mice show a larger infarct size versus WT-controls [28]. The small magnitude of increase in iNOS levels in this situation is important, because the up regulation of iNOS has a pronounced dose-dependent effect: beneficial at low levels but toxic at high levels [1,26]. In diabetic myocardium, basal iNOS-levels are 3 times higher than in non-diabetic myocardium [29]. Furthermore, the increase of iNOS-levels in ischemic areas after IR injury is 2.6 times larger than in the ischemic areas of non-diabetic myocardium [29]. The detrimental role of these high iNOS-levels during IR injury in diabetic myocardium was confirmed in another study [28], in which iNOS knock-out diabetic mice showed a smaller infarct size and reduced caspase-3 activity versus control diabetic mice. This might explain the increased sensitivity of our diabetic mice to IR injury. In the delayed preconditioning pathway, iNOS has a critical beneficial role [1]. Targeted deletion of the iNOS-gene abrogates delayed preconditioning, suggesting that iNOS is a common effector of cardioprotection [26]. Since basal iNOS levels are higher, it is conceivable that diabetic mice have a disturbed threshold or are even already in a permanent maximally protected or preconditioned state.

Fifth, delayed preconditioning is regulated through an up regulation of several other cardioprotective proteins [1]. Previous studies focused mainly on the gene expression profile after delayed preconditioning in healthy subjects [30] and the effect of diabetes or the metabolic syndrome on this has never been investigated.

Finally, although a plethora of mechanisms are involved in preconditioning, it is widely accepted that all these mechanisms converge at the inhibition of the mitochondrial permeability transition pore (mPTP) opening at early reperfusion [31]. Inhibiting mPTP opening will prevent the cardiomyocytes to undergo cellular necrosis and apoptosis. No data are available about mPTP-inhibition in our mouse models of T2D and the MS.

In an attempt to restore delayed preconditioning in ob/ob and DKO mice, therapeutic strategies were investigated. After food restriction or ACE-I, hypoxic preconditioning reduced infarct size and preserved PRSW up to the level without ischemia in both ob/ob and DKO. Furthermore, ACE-I restored the proportional effect of preconditioning on infarct limitation and contractility improvement to the WT-level. Comparable with our previous study [21], we found that diet or ACE-I without preconditioning did not reduce the impact of IR injury in these mice models, in contrast with the regained protection by delayed preconditioning potential in this study. The regained preconditioning potential is thus independent from the direct effects of ACE-I on IR injury. A possible explanation is that food restriction and ACE-I are not capable to influence insulin levels, glycemia, cholesterol, ROS, eNOS and iNOS sufficiently to see an effect on IR injury without previous preconditioning. Nevertheless, these parameters might have been restored sufficiently to induce again a threshold for delayed preconditioning.

It is already shown that food restriction reduces insulin levels and ACE-I improves insulin sensitivity [14,19].

Furthermore, ACE-I reduces glycemia in ob/ob and DKO mice and food restriction lowers cholesterol levels in ob/ob mice [21]. This can have lowered the threshold to delayed preconditioning since insulin has a preconditioning effect [5,25], and hyperglycemia and hypercholesterolemia are key player in the induction of atypical isoforms of protein-kinase C [24], which is crucial in the delayed preconditioning pathway [1]. In the aging heart, an impaired protein-kinase C translocation is one of the reasons responsible for the impaired preconditioning potential [32].

Food restriction partially restores classical preconditioning in senescent animals, but in combination with exercise, this restoration becomes complete. A restored norepinephrine release was suggested as the underlying mechanism [32]. Furthermore, food restriction induces upregulation of eNOS in DKO mice [14]. ACE-I is capable to block the degradation of bradykinin into inactive peptides and to increase eNOS-levels [15,16,19,33]. eNOS is a trigger in the delayed preconditioning pathway [1], which might explain the regained preconditioning potential.

Food restriction prevents ROS production [34] and also ACE-I has well known anti-oxidative activity [15,16,19]. Since an increase in ROS formation is an important trigger of delayed preconditioning [1], it is possible that these treatments reduce the increased baseline ROS-level in diabetic myocardium and restore the threshold for delayed preconditioning [1].

Another possible explanation why the treatments induced cardioprotection after preconditioning but did not have a direct effect during IR injury, is the different role and levels of iNOS in both pathways. As described earlier, basal iNOS-levels are higher and the increase of iNOS-levels after IR injury is larger than in non-diabetic myocardium [29] with a deleterious role in diabetic myocardium during IR injury [28]. In contrast with this, a small increase in iNOS-levels is critical to induce its effects as common end-effector in the delayed preconditioning pathway [1]. No studies were conducted to study the effect of food restriction on iNOS-levels in myocardium, but it was shown in a normotensive rat model that ACE-I reduces iNOS-levels with 24% [33]. It is tempting to speculate that ACE-I did not reduce iNOS-levels sufficiently to reduce its toxic high-dose level during IR injury but enough to restore the threshold to induce delayed precondition (by a small iNOS increase). Further investigations need to be performed to elucidate the role of these underlying mechanisms.

Conclusion

Hypoxic preconditioning induces a strong protection in WT and a partial protection in ob/ob mice. The preconditioning potential is lost in DKO. Twelve weeks of food restriction or ACE-I restores the preconditioning potential in DKO and improves it in ob/ob. After preconditioning, PRSW is preserved to the level without ischemia, after 12 weeks food restriction or ACE-I. As previously shown, food restriction and ACE-I did not protect the diabetic myocardium against IR injury. The regained preconditioning potential appears to be independent from the direct effects of food restriction ACE-I on IR injury.

Additional files

Additional file 1: Hemodynamic parameters and infarct size of the non-preconditioned and preconditioned wild type and DKO, without subsequent ischemia (shams).

Additional file 2: Hemodynamic parameters.

Additional file 3: Relationship between infarct limitation and improved contractility.

Abbreviations

ACE: Angiotensin-converting enzyme; ACE-I: Angiotensin-converting enzyme inhibition; DKO: Double knock-out; E_a: Arterial elastance; EDPVR: End-diastolic pressure volume relationship; Ees: End-systolic elastance; eNOS: Endothelial nitric oxide synthase; iNOS: Inducible nitric oxide synthase; IR: Ischemia-reperfusion; LAD: Left anterior descending artery; LDL: Low density lipoprotein; LDLR: Low density lipoprotein receptor; mPTP: Mitochondrial permeability transition pore; NO: Nitric oxide; NP: Non-Preconditioned and ischemia/reperfusion; Ob/ob: Leptin deficient mouse model for type II diabetes; P: Preconditioned and ischemia/reperfusion; P_{ed}: End-diastolic pressure; PRSW: Preload recruitable stroke work; P_{sys}: Systolic pressure; PV: Pressure-volume; ROS: Reactive oxygen species; STAT-3: Signal transducer and activator of transcription-3; SW: Stroke work; TTC: Triphenyl-tetrazolium chloride; WT: Wild type.

Competing interest

The authors declare that they have no competing interest.

Authors' contributions

GVDM carried out the mice breeding, treatments, preconditioning, PV-loop experiments and infarct size determination, data and statistical analysis, and drafted the manuscript. IN, AV and WO contributed to the mice breeding, treatments and preconditioning. WF participated in the design of the study and general supervision. PH designed the study, obtained funding, did supervision of the analysis and interpretation of data, and revised the manuscript for important intellectual content. All authors have read and approved the final manuscript.

Acknowledgements

This work was supported by a grant of the Research Fund KU Leuven – Bijzonder Onderzoeksfonds (OT 04/39 and OT 05/55) and a grant of the FWO-Vlaanderen (G 0966.11).

Received: 7 November 2012 Accepted: 19 February 2013
Published: 23 February 2013

References

1. Bolli R, Li QH, Tang XL, Guo Y, Xuan YT, Rokosh G, Dawn B: **The late phase of preconditioning and its natural clinical application–gene therapy**. *Heart Fail Rev* 2007, 12:189–199.
2. Ferdinandy P, Schulz R, Baxter GF**: Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning and postconditioning.** *Pharmacol Rev* 2007, 59:418–458.
3. Miki T, Itoh T, Sunaga D, Miura T: **Effects of diabetes on myocardial infarct size and cardioprotection by preconditioning and postconditioning**. *Cardiovasc Diabetol* 2012, 11:67.
4. Vaage J, Valen G: **Preconditioning and cardiac surgery.** *Ann Thorac Surg* 2003, 75:S709–714.

5. Tsang A, Hausenloy DJ, Mocanu MM, Carr RD, Yellon DM: **Preconditioning the diabetic heart: the importance of Akt phosphorylation**. *Diabetes* 2005, 54:2360–2364.
6. Wild S, Roglic G, Green A, Sicree R, King H: **Global prevalence of diabetes: estimates for the year 2000 and projections for 2030.** *Diabetes Care* 2004, 27:1047–1053.
7. Dohi T, Miyauchi K, Kasai T, Kajimoto K, Kubota N, Tamura H, Yokoyama T, Kojima T, Yokoyama K, Kurata T, Daida H: **Impact of metabolic syndrome on 10-year clinical outcomes among patients with acute coronary syndrome.** *Circ J* 2009, 73:1454–1458.
8. Haffner SM, Lehto S, Rönnemaa T, Pyörälä K, Laakso M: **Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction.** *N Engl J Med* 1998, 339:229–234.
9. ADVANCE Collaborative Group: **Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes.** *N Engl J Med* 2008, 358:2560–2572.
10. Van der Mieren G, Van den Bergh A, Nevelsteen I, Vanderper A, Flameng W, Herijgers P: **Hypoxic preconditioning preserves cardiac contractility and reduces infarct size in vivo.** *The Open Surgery Journal* 2008, 2:24–29.
11. Ebel D, Müllenheim J, Frässdorf J, Heinen A, Huhn R, Bohlen T, Ferrari J, Südkamp H, Preckel B, Schlack W, Thämer V: **Effect of acute hyperglycaemia and diabetes mellitus with and without short-term insulin treatment on myocardial ischaemic late preconditioning in the rabbit heart in vivo.** *Pflugers Arch* 2003, 446:175–182.
12. Buse JB, Ginsberg HN, Bakris GL, Clark NG, Costa F, Eckel R, Fonseca V, Gerstein HC, Grundy S, Nesto RW, Pignone MP, Plutzky J, Porte D, Redberg R, Stitzel KF, Stone NJ, American Heart Association; American Diabetes Association: **Primary prevention of cardiovascular disease in people with diabetes mellitus. A Scientific statement from the American Heart Association and the American Diabetes Association.** *Circulation* 2007, 115:114–126.
13. Abete P, Testa G, Galizia G, Mazzella F, Della Morte D, de Santis D, Calabrese C, Cacciatore F, Gargiulo G, Ferrara N, Rengo G, Sica V, Napoli C, Rengo F**: Tandem action of exercise training and food restriction completely preserves ischemic preconditioning in the aging heart.** *Exp Gerontol* 2005, 40:43–50.
14. Verreth W, De Keyzer D, Pelat M, Verhamme P, Ganame J, Bielicki JK, Mertens A, Quarck R, Benhabilès N, Marguerie G, Mackness B, Mackness M, Ninio E, Herregods MC, Balligand JL, Holvoet P: **Weight-loss-associated induction of peroxisome proliferator-activated receptor-alpha and peroxisome proliferator-activated receptor-gamma correlate with reduced atherosclerosis and improved cardiovascular function in obese insulin-resistant mice.** *Circulation* 2004, 110:3259–3269.
15. Miki T, Miura T, Ura N, Ogawa T, Suzuki K, Shimamoto K, Limura O: **Captopril potentiates the myocardial infarct size-limiting effect of ischemic preconditioning through bradykinin B2 receptor activation.** *J Am Coll Cardiol* 1996, 28:1616–1622.
16. Nozawa Y, Miura T, Tsuchida A, Kita H, Fukuma T, Shimamoto K: **Chronic treatment with an ACE inhibitor, temocapril, lowers the threshold for the infarct size-limiting effect of ischemic preconditioning.** *Cardiovac Drugs Ther* 1999, 13:151–157.
17. Jaberansari MT, Baxter GF, Muller CA, Latouf SE, Röth E, Opie LH, Yellon DM: **Angiotensin-converting enzyme inhibition enhances a subthreshold stimulus to elicit delayed preconditioning in pig myocardium.** *J Am Coll Cardiol* 2001, 37:1996–2001.
18. Marktanner R, Nacke P, Feindt P, Hohlfeld T, Schipke JD, Gams E**: Delayed preconditioning via Angiotensin-converting enzyme inhibition: pros and cons from an experimental study.** *Clin Exp Pharmacol Physiol* 2006, 33:787–792.
19. Velasquez MT, Bhathena SJ, Striffler JS, Thibault N, Scalbert E**: Role of angiotensin-converting enzyme inhibition in glucose metabolism and renal injury in diabetes**. *Metabolism* 1998, 47:7–11.
20. Van den Bergh A, Vanderper A, Vangheluwe P, Desjardins F, Nevelsteen I, Verreth W, Wuytack F, Holvoet P, Flameng W, Balligand JL, Herijgers P: **Dyslipidemia in type II diabetic mice does not aggravate contractile impairment but increases ventricular stiffness.** *Cardiovasc Res* 2008, 77:371–379.
21. Van der Mieren G, Nevelsteen I, Vanderper A, Oosterlinck W, Flameng W, Herijgers P: **Angiotensin-converting enzyme inhibition and food restriction in diabetic mice do not correct the increased sensitivity for ischemia-reperfusion injury**. *Cardiovasc Diabetol* 2012, 11:89.
22. Wichi R, Farah V, Chen Y, Irigoyen MC, Morris M: **Deficiency in angiotensin AT1a receptors prevents diabetes-induced hypertension.** *Am J Physiol Regul Integr Comp Physiol* 2007, 292:R1184–1189.
23. Van den Bergh A, Flameng W, Herijgers P**: Parameters of ventricular contractility in mice: influence of load and sensitivity to changes in inotropic state**. *Pflugers Arch* 2008, 455:987–994.
24. Poornima IG, Parikh P, Shannon RP**: Diabetic cardiomyopathy: the search for a unifying hypothesis**. *Circ Res* 2006, 98:596–605.
25. Hausenloy DJ, Yellon DM: **Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection.** *Heart Fail Rev* 2007, 12:217–234.
26. Guo Y, Sanganalmath SK, Wu W, Zhu X, Huang Y, Tan W, Ildstad ST, Li Q, Bolli R: **Identification of inducible nitric oxide synthase in peripheral blood cells as a mediator of myocardial ischemia/reperfusion injury**. *Basic Res Cardiol* 2012, 107:1–8.
27. Natali A, Ferrannini E: **Endothelial dysfunction in type 2 diabetes**. *Diabetologia* 2012, 55:1559–1563.
28. Marfella R, Di Filippo C, Esposito K, Nappo F, Piegari E, Cuzzocrea S, Berrino L, Rossi F, Giugliano D, D'Amico M**: Absence of inducible nitric oxide synthase reduces myocardial damage during ischemia-reperfusion in streptozotocin-induced hyperglycemic mice.** *Diabetes* 2004, 53:454–462.
29. Marfella R, Esposito K, Nappo F, Siniscalchi M, Sasso FC, Portoghese M, Di Marino MP, Baldi A, Cuzzocrea S, Di Filippo C, Barboso G, Baldi F, Rossi F, D'Amico M, Giugliano D: **Expression of angiogenic factors during acute coronary syndromes in human type 2 diabetes.** *Diabetes* 2004, 53:2383–2391.
30. Das DK, Maulik N: **Cardiac genomic response following preconditioning stimulus**. *Cardiovasc Res* 2006, 70:254–263.
31. Halestrap AP, Clarke SJ, Javadov A: **Mitochondrial permeability transition pore opening during myocardial reperfusion- a target for cardioprotection.** *Cardiovasc Res* 2004, 61:372–385.
32. Abete P, Cacciatore F, Testa G, Della-Morte D, Galizia G, de Santis D, Calabrese C, Cioppa A, Ferrara N, Rengo F: **Ischemic preconditioning in the aging heart: from bench to bedside**. *Ageing Res Rev* 2010, 9:153–162.
33. Bachetti T, Comini L, Pasini E, Cargnoni A, Curello S, Ferrari R: **Ace-inhibition with quinapril modulates the nitric oxide pathway in normotensive rats**. *J Mol Cell Cardiol* 2001, 33:395–403.
34. Sohal RS, Ku HH, Agarwal S, Forster MJ, Lal H: **Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse.** *Mech Ageing Dev* 1994, 74:121–133.

doi:10.1186/1475-2840-12-36
Cite this article as: Van der Mieren et al.: Angiotensin-converting enzyme inhibition and food restriction restore delayed preconditioning in diabetic mice. Cardiovascular Diabetology 2013 12:36.

<u>Additional file 1:</u> Hemodynamic parameters and infarct size of the non-preconditioned and preconditioned wild type and DKO, without subsequent ischemia (shams)

	Wild Type		DKO	
	Sham non preconditioned	**Sham preconditioned**	**Sham non preconditioned**	**Sham preconditioned**
Numbers (survivors)	8 (8)	8 (8)	7(6)	7 (7)
Area at risk (% of heart)	0	0	0	0
Infarct size (% of area at risk)	0	0	0	0
Heart rate (bpm)	570±40	549±51	525±31	522±48
Stroke volume (µl)	16.0±5.6	18.8±6.9	10.9±5.2	14.2±6
Stroke work (mmHg*µl)	1197±315	1206±658	656±269	852±494
Psys (mmHg)	75.6±11.6	73.4±13.5	65.6±4.9	70.1±8.5
Ped (mmHg)	2.9±0.7	4.2±0.9 [a]	4.1±1.1	3.7±1.5
PRSW (mmHg)	87.9±5.0	86±5.6	58.0±4.1	57.5±2.1
Ees (mmHg/µl)	8.3±2.5	7.6±1.7	6.0±2.2	4.9±1
Tau (ms)	6.5±1.2	6.4±1.6	6.8±0.9	6.2±0.7
EDPVR (mmHg/µl)	0.2±0.2	0.5±0.5	0.5±0.4	0.4±0.3
Ea (mmHg/µl)	4.6±1.5	3.7±0.6	5.8±2.6	4.4±1.2

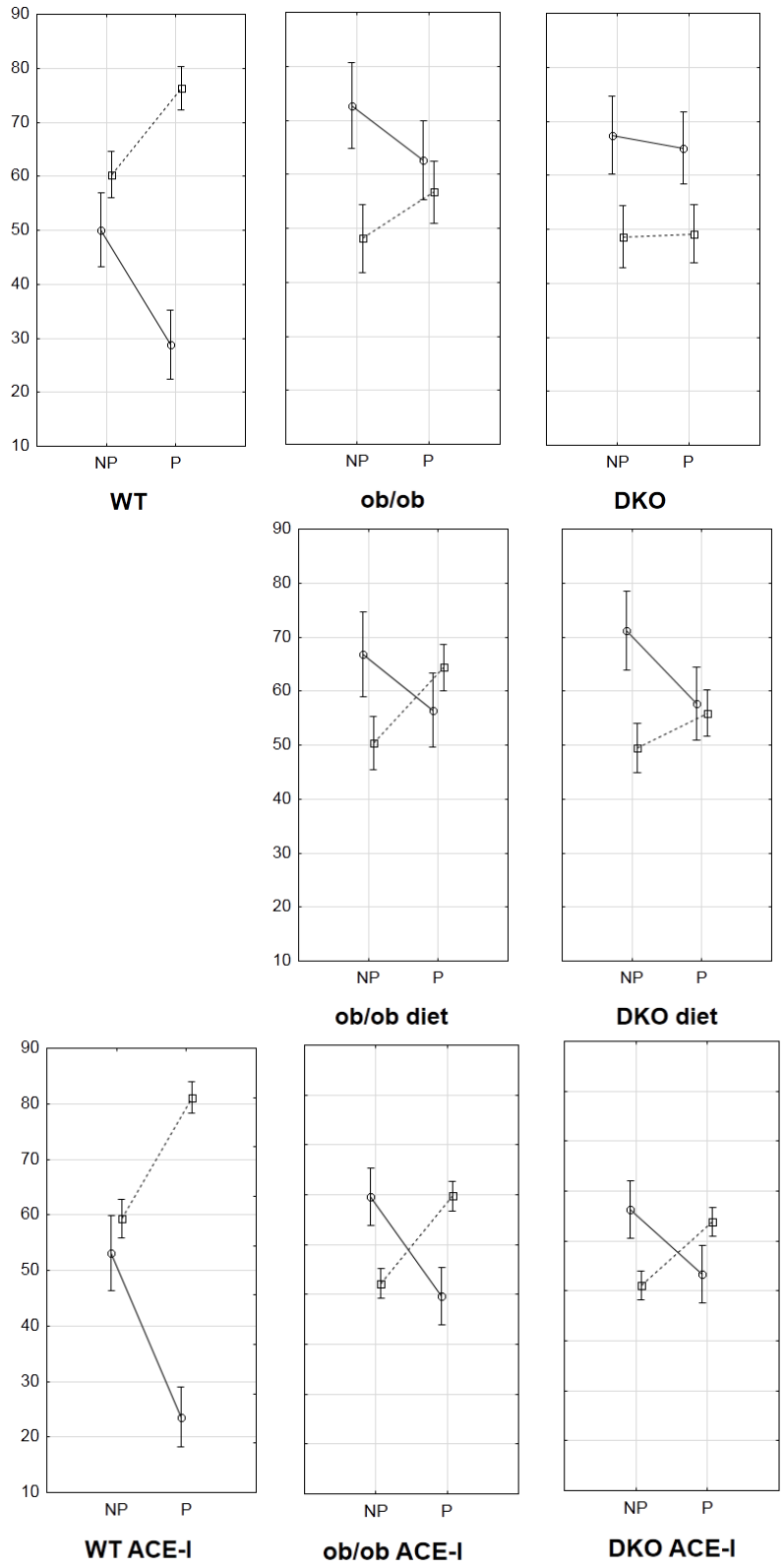
DKO: double knock-out (ob/ob; LDLR-/-); "[a]" $p<0.05$ versus non preconditioned sham group, same genotype

Additional file 2: Hemodynamic parameters

		Wild Type			Ob/ob			DKO		
	Condition	***Sham***	***NP***	***P***	***Sham***	***NP***	***P***	***Sham***	***NP***	***P***
Heart rate (bpm)	Untreated	570±40	567±61	561±49	532±45	552±31	542±59	525±31	505±35 [f]	496±18 [a]
	Diet				511±26	494±32	516±40	503±34	519±54	536±32 [b]
	ACE-I	546±36	518±36	547±77	503±35	493±38	529±45	512±37	501±70	528±69
Stroke volume (µl)	Untreated	16.0±5.6	6.9±3.3 [c]	10.2±2.7 [cd]	15.4±3.0	12.4±4.7 [a]	15.5±5.9 [a]	10.9±5.2	10.4±3.9	10.2±4.7
	Diet				14.2±6.3	14.6±7.5	13.9±6.6	12.1±5.5	11.4±5.1	10.0±3.0
	ACE-I	17.5±4.8	7.9±1.6 [c]	13.0±3.4 [d]	14.2±3.4	13.3±4 [a]	16.5±2.8	15.3±6.5	12.5±3.2 [a]	12.8±4.8
Stroke work (mmHg*µl)	Untreated	1197±315	277±171 [c]	417±107 [c]	861±192 [a]	524±206 [ac]	732±282 [a]	656±269 [a]	458±178	469±230
	Diet				857±578	689±362	735±364	668±273	617±306	573±229
	ACE-I	963±378	359±175 [c]	670±23 [bd]	798±269	622±216 [a]	940 ± 302 [d]	781±441	566±183	659±298
Psys (mmHg)	Untreated	75.6±11.6	56.8±9.6 [c]	58.9±12 [c]	68.2±8.1	62.9±2.8	61.5±3.1	65.6±4.9	65.6±8.1	58.2±3.5 [c]
	Diet				69.7±11.3	60.5±6.5	69.0±8.3 [b]	70.0±11	65.5±5.7	74.9±19.6
	ACE-I	65.5±11.2	60.5±21.9	64.1±6.7	66.7±6.4	59.4±8.0	67.1±11.0	60.6±6.2	56.3±8.7 [e]	63.2±2.7 [b]
Ped (mmHg)	Untreated	2.9±0.7	3.0±1.2	2.5±0.5	3.6±0.7	6.6±3.6 [a]	6.6±0.9 [ac]	4.1±1.1 [a]	7.2±2.1 [ac]	5.6±3.5 [a]
	Diet				3.1±1.1	4.5±1.2	4.3±1.5 [b]	3.8±1.4	4.4±1.6 [b]	4.9±2.3
	ACE-I	3.6±2.2	3.1±2.5	3.7±1.0 [b]	4.5±1.0 [e]	5.0±1.6	5.0±2.0	3.6±1.0	3.7±1.4 [be]	3.9±1.2
Tau (ms)	Untreated	6.5±1.2	7.2±1.9	6.0±0.8	6.1±0.5	6.2±0.8	6.8±0.6	6.8±0.9	7.3±0.8 [f]	7.4±0.6 [a]
	Diet				6.9±0.6 [b]	7.7±0.8 [b]	6.9±1.4	6.7±0.6	7.3±1.3	7.6±0.9
	ACE-I	6.8±1.6	7.6±1.6	5.9±0.9 [d]	7.9±1.2 [b]	8.8±1.6 [b]	6.4±1.0 [cd]	7.3±0.7	7.1±1.1 [f]	7.2±1.4 [a]
Ea (mmHg/µl)	Untreated	4.6±1.5	8.4±2.5 [c]	5.7±2.3 [d]	3.8±0.7	5.0±2.2 [a]	3.6±1.1 [a]	5.8±2.6	5.3±1.7 [a]	5.3±2.3
	Diet				4.8±1.9	4.8±2.9	5.2±2.0	5.4±2.4	5.8±1.9	7.5±3.8
	ACE-I	3.2±0.7	6.9±1.4 [c]	4.4±0.7 [cd]	4.3±1.5	4.1±1 [a]	3.3±1.1 [ae]	3.3±1.4 [b]	3.8±1.1 [ae]	4.7±1.6

ACE-I: angiotensin-converting enzyme inhibition; DKO: double knock-out (ob/ob; LDLR-/-); E_a: arterial elastance; IR: ischemia/reperfusion; NP: Non Preconditioned and ischemia/reperfusion; P: Preconditioned and ischemia/reperfusion; P_{ed}: end-diastolic pressure; P_{sys}: systolic pressure; sham: group without ischemia/reperfusion. "a" $p<0.05$ versus WT same treatment, same condition (sham-non preconditioned-preconditioned); "b" $p<0.05$ versus same untreated genotype, same condition; "c" $p<0.05$ versus sham, same genotype, same treatment; "d" $p<0.05$ versus non preconditioned, same genotype, same treatment; "e" $p<0.05$ versus diet, same genotype, same condition; "f" $p<0.05$ versus ob/ob same treatment, same condition

<u>Additional file 3</u>: Relationship between infarct limitation and improved contractility



Solid lines: Infarct of risk area (%); Dotted lines: PRSW

ACE-I: angiotensin-converting enzyme inhibition; DKO: double knock-out (ob/ob; LDLR-/-); NP: non

preconditioned; P: preconditioned; WT: wild type

The relationship between infarct limitation and improved contractility was significantly different in DKO and WT mice, but not in ob/ob. In DKO, preconditioning induced a proportional smaller improved PRSW per reduced infarct area versus WT mice. An infarct size reduction of 1%, corresponds with an increased PRSW of 1.25% in WT and only 0.53% in DKO. After diet and preconditioning in DKO, this relationship was partially restored (increase PRSW of 0.95% per 1% reduced infarct size, p=0.1 versus untreated DKO) but significantly after ACE-I (increase PRSW of 1.91% per 1% reduced infarct size, p=0.003 versus untreated DKO).

Chapter 7

General discussion and future perspectives

General discussion

1. Investigation of contractility, the effect of IR injury and hypoxic preconditioning in vivo in wild type mice

The number of patients with diabetes and the metabolic syndrome reaches epidemic proportions and is still increasing worldwide [1-2]. It has been estimated that this population has a 30% increased periprocedural risk during coronary artery bypass grafting [67]. The currently applied therapies seem to have reached their limits [5]. Delayed preconditioning is a powerful protection mechanism against IR injury. Nevertheless, the potential therapeutic possibilities are not yet exploited.

There are several reasons for this.

The stimulus to induce preconditioning, such as repeated temporary coronary occlusion or aortic cross-clamping, might be harmful and prolong the procedure. It was further suggested that standard maneuvers, such as cardiopulmonary bypass during cardiac surgery, already precondition the heart of the patient [68]. Another important issue in this failure, is the uncertainty whether this technique can effectively salvage myocardium in patients with co-morbidities, such as type II diabetes or the metabolic syndrome, that occur frequently in the intended patient population [6].

Although type II diabetes accounts for 90% of the diabetic cases, most of the studies with experimental diabetic models focus on acute type I diabetes, induced by streptozotocin injection in lean wild type animals, a few days before the experiments. It was shown in these models that delayed preconditioning does not protect these hearts in type I diabetes [7-8,56]. In the past decade, numerous transgenic mouse models have been developed that give us the opportunity to study IR injury and preconditioning in models with underlying chronic co-morbidities. The ob/ob mouse is a widely used model for type II diabetes, featuring abdominal obesity and insulin resistance. DKO mice with combined leptin and LDL-receptor deficiency feature many characteristics of the metabolic syndrome, i.e. obesity, dyslipidemia, hypertension, insulin resistance, and impaired glucose tolerance and/or diabetes [45,58]. DKO mice develop left ventricular diastolic and systolic dysfunction comparable with the diabetic cardiomyopathy seen in patients [58].

Furthermore, most studies that evaluated the effects of IR injury and preconditioning, were *ex vivo* studies [7-8]. Using an *in vivo* model, all possible physiological interactions are maintained, a more reliable and realistic way to study the effects of IR injury and preconditioning.

In vivo pressure-volume measurements with determination of the load-independent parameters is the gold standard to measure cardiac function [63]. TTC-staining is the gold standard to measure the extent of necrosis early after IR injury in animal models [62].

In the first part of this study (chapter 4), we had to develop a technique to induce delayed preconditioning, which was feasible in obese mice models. The most popular method to induce preconditioning is intermittent coronary occlusion [24,55]. However, this is not an ideal technique to study delayed preconditioning in obese mice models for several reasons. First, this method needs cardiothoracic surgery with subsequently a survival period of more than 24 hours, to study the SWOP. The weight of ob/ob and DKO mice is 2,5 to 3 times larger than lean WT mice. The mortality rate of obese mice models during survival experiments is very high. In a preliminary study, we used intermittent coronary occlusion to induce preconditioning with subsequently 24 hours delay and new coronary occlusion. The mortality rate during the 24 hours period in between induction of SWOP and induction of index ischemia was 50%, which was unacceptability high. Furthermore, these mouse models are expensive and difficult to breed. Secondly, anesthesia is needed to perform intermittent coronary occlusion. The anesthesia agent itself can interfere with the preconditioning stimulus. Therefore another well-defined model of SWOP in obese mice without cardiothoracic surgery was searched.

Intermittent global hypoxia is an attractive method to induce SWOP in small animal models like mice, since it avoids cardiothoracic surgery. In previous studies, hypoxia was shown to induce SWOP in rats in vitro [25,59]. Using the hypoxia protocol of 5 times 6 minutes of 6% hypoxia, we had no mortality in WT mice. Furthermore, only 1 mouse out of 12 died during the IR protocol.

Hypoxic preconditioning without subsequent IR injury (sham groups), did not induce a detectable infarct area or change of the left ventricular contractility parameters. Our IR injury protocol induced an infarct area of 49.9% in the perfusion area. Hypoxic delayed preconditioning was able to reduce infarct size to half that of the non-preconditioned group. Furthermore, ejection fraction, Tau, E_{es} and PRSW after hypoxic preconditioning 24 hours before 30 min regional cardiac ischemia were significantly better preserved than without preconditioning.

In summary, in this first part, the efficacy and feasibility of general hypoxia to induce in vivo delayed preconditioning in mice was shown.

The hypothesis that hypoxic preconditioning induces a SWOP in vivo, measured by a better preserved left ventricular contractility and reduced infarct size, could thus be confirmed.

2. Investigation of contractility and the effect of IR injury in vivo in mice models of type II diabetes and the metabolic syndrome

In a second part we investigated contractility and the effects of IR injury in the obese ob/ob and DKO mouse models. Left ventricular contractility, expressed as PRSW, is worse in ob/ob than in WT mice and even worse in DKO mice. Left ventricular stiffness represented by end-diastolic pressure and EDPVR, is significantly worse in DKO mice than in WT mice.

Untreated ob/ob and DKO mice have a significantly higher weight and exhibit hyperglycemia. Cholesterol levels are elevated in ob/ob and even higher in DKO mice. DKO mice also have increased triglycerides levels.

Our mouse models of type II diabetes and the metabolic syndrome are, similar to clinical reality, more vulnerable for IR injury. First, the mortality rate of the investigated IR protocol was high: 45% in ob/ob, 23% in DKO mice versus 17% in WT. Second, infarct size was significantly higher in ob/ob (72.4%) and DKO (67.2%) versus WT mice. Third, after IR, PRSW decreased significantly in all groups and was significantly lower in DKO and ob/ob versus WT.

In other experimental diabetic models, the sensitivity for IR injury was not always higher [7-8]. Depending on the reports, infarct size in animal models of diabetes was larger, smaller or similar to non-diabetic controls [8]. There are numerous differences in experimental protocols and a single factor cannot entirely explain the discrepancies. Duration of the diabetic state and plasma level of insulin (type I versus type II) seems to influence the myocardial susceptibility to IR injury [8]. Although 90% of the diabetic patients present with type II diabetes, the majority of the experimental studies are performed in rat models, with type I diabetes induced by streptozotocin [8]. Most report a similar or reduced myocardial infarct area after IR injury [8]. The sensitivity to IR injury in this model seems to be time-dependent. Some studies report a resistance to IR early after diabetes induction by streptozotocin, which disappears after 2 months [8]. Others however demonstrated an increased infarct size after coronary occlusion as early as 8 days after streptozotocin injection [8], indicating that duration of diabetes is not the only factor responsible for the heterogeneous results. Disagreement also exists about the effect of acute hyperglycemia to IR injury. Although some studies report a larger or similar susceptibility to IR injury in a hyperglycemic state, other reports demonstrate a significant protection, related to a better profile of calcium handling proteins [8].

The difference in presence or absence of hyperinsulinemia may also be responsible for the heterogeneous data.

With the exceptions of a few studies in type II diabetic Zucker and Goto-Kakizaki rats, all type II diabetic models with obesity and hyperinsulinemia showed increased myocardial susceptibility to IR injury [8].

We demonstrated in this study that our mouse models of type II diabetes and the metabolic syndrome are more vulnerable for IR injury than their WT littermates. This correlates well with data from patient studies and previous studies in transgenic mice with type II diabetes [8,52-53]. Nevertheless, the underlying mechanisms by which type II diabetic and metabolic syndrome myocardium is more vulnerable to IR injury remains incompletely understood. Several hypotheses were suggested.

The diabetic myocardium exhibits abnormal cardiac substrate utilization and impaired energetic efficiency. In non-ischemic conditions, the myocardium uses preferentially fatty acids as energy substrate. This is even more the case in type II diabetes and the metabolic syndrome. The use of fatty acids requires 12% more oxygen per ATP generated than glucose. During ischemia, the normal myocardium switches to glucose as preferred energy substrate. This possibility is lost in case of diabetes, because oxidation of fatty acids inhibits the catabolism of glucose [69].

Endothelial dysfunction and reduced NO bioavailability is present in type II diabetes and the metabolic syndrome [14,45,70]. The beneficial or detrimental effect of NO depends on its concentration and cellular origin [71]. Although constitutive production of NO by endothelial cells (eNOS) is thought to be cytoprotective, NO generated by the inducible form (iNOS) is generally regarded as pro-inflammatory and cytotoxic [71]. In diabetic myocardium, basal iNOS-levels are higher and the increase of iNOS-levels after IR injury is larger than in non-diabetic myocardium [72].

$SERCA_{2a}$ is crucial in postischemic myocardium by significantly decreasing intracellular Ca^{2+}-overload [73]. It was shown before that $SERCA_{2a}$-activity is depressed in ob/ob and DKO mice [58]. In the streptozotocin type I diabetic model, $SERCA_{2a}$-expression after IR injury is increased versus WT animals. This is a possible explanation for the better preserved contractility after IR injury in this model [74].

Diabetic and metabolic syndrome models exhibit increased production of ROS [75]. ROS likely plays a central role in impairing mitochondrial energy metabolism, through mitochondrial uncoupling, but also through direct damage of mitochondrial components. This can lead to apoptosis [75].

The activity of aldose reductase, a NADPH-dependent enzyme, increases during IR injury [76]. Since aldose

reductase activity is already increased in diabetes [77], IR injury further reduces myocardial glycolysis and glucose oxidation and increases the glycosylation of advanced end-products. This leads to more extensive cross-linking of key structures within tissues and increased IR injury [78]. The exposure to abnormal substrates and cytokines makes the diabetic heart also more prone to IR [78].

Chronic hyperglycemia leads to cardiac hypertrophy and decreased microvasculature density in myocardium. This microcirculation dysfunction causes increased vulnerability to IR [78].

Finally, leptin deficiency leads to ventricular hypertrophy and heart failure [79-80]. McGaffin et al. reported that leptin has a protective role after IR [79-80]. Leptin deficient mice had worse survival, worse cardiac function and increased apoptosis, 4 weeks after IR injury. Leptin induces activation of signal transducer and activator of transcription-3, which induces transcription of several cardioprotective genes. These, in turn, inhibit reverse remodeling and apoptosis [79-80]. It is thus possible that leptin deficiency leads to reduced contractility and larger infarct size versus WT, independently from the other risk factors. Nevertheless, the effect of leptin deficiency during the acute phase of IR, as in our experiments, is not known. It is unlikely that STAT-3 activation plays a role during the first hours after IR injury, since transcription of new cardioprotective genes is required.

In conclusion, the hypotheses that left ventricular contractility is worse in the models of type II diabetes and the metabolic syndrome, and that the impact of IR injury is larger in these mouse model than in wild type mice, is confirmed.

3. Investigation of the impact of interventions (food restriction and ACE-inhibition) on contractility and IR injury in vivo in mice models of type II diabetes and the metabolic syndrome

Patients with type II diabetes and metabolic syndrome usually receive different drugs, which might interact with the impact of IR injury, as previously described. Food restriction and ACE-I are standard therapies in the treatment of type II diabetes and the metabolic syndrome. The impact of 12 weeks treatment with food restriction or ACE-inhibition on in vivo contractility and IR injury was studied in mouse models of type II diabetes and the metabolic syndrome.

Food restriction resulted in lower body and heart weight in both ob/ob and DKO. Heart weight was even lower in food restricted DKO than in WT. Food restriction had little effect on biochemical parameters. Food restriction

did not improve the reduced PRSW in ob/ob and DKO mice. Food restriction did not reduce infarct size in DKO nor ob/ob versus the untreated groups. The impact of IR on PRSW was not influenced by food restriction.

ACE-inhibition reduced glucose levels in ob/ob but much less in DKO. Heart weight, was lower in DKO but not in ob/ob after ACE-inhibition compared with the untreated. PRSW was significantly better after 12 weeks of ACE-inhibition in ob/ob and DKO versus baseline and diet. ACE-inhibition did not reduce infarct size in any genotype versus the untreated groups. Furthermore, ACE-inhibition did not reduce the impact of IR on PRSW in any genotype.

The effects of food restriction and ACE-inhibition on the previously described mechanisms of increased vulnerability of type II diabetic myocardium to IR injury are incompletely known.

Food restriction has been shown to reduce IR injury in wild type rodent models [10-12]. Short-term (2 weeks) food restriction protocols, ranging from 10% to 70%, improve cardiac function following IR injury in wild type rats [12]. Yamagishi et al. [12] reported that 70% food restriction during 11 days, resulting in a weight reduction of 38% compared to free-fed rats, has a different effect on pre-ischemic contractility and sensitivity to IR injury. In this study, pre-ischemic cardiac function was reduced but cardiac recovery after IR injury was improved [12]. An improved ischemic tolerance via altered expression of functional proteins induced by low serum T3 levels, decreased coronary flow rate and change in metabolic flux, were suggested. Anaerobic glycolysis was more activated during ischemia in food restricted animals. This activation of glycolytic flux is fundamental for effective recovery of cardiac function during reperfusion after ischemia [12]. Nevertheless, the boundary between food restriction and undernutrition is unclear. More than 70% food restriction results in depressed cardiac function [12]. The effect of food restriction on IR injury was never studied in the intended group, the obese type II diabetic patients and animal models. In our type II diabetic and metabolic syndrome mouse models, food restriction did not induce significant protection against IR injury.

Dyslipidemia seems to be a less important factor during IR injury, since infarct size and left ventricular contractility were not significantly different in DKO mice versus ob/ob. Although cholesterol-levels were normal after food restriction in ob/ob mice, this could not correct the increased sensitivity to IR injury.

ACE-inhibition prevents downregulation of $SERCA_{2a}$ and phospholamban protein expression in heart failure [81]. Furthermore, ACE-inhibition prevents the decrease in capillary density in type I diabetic rats [82]. This might explain the improved contractility in ob/ob and DKO mice with ACE-inhibition.

It is widely accepted that food restriction and ACE-inhibition reduces oxidative stress [10,14-18,45]. Nevertheless, nor food restriction nor ACE-inhibition were capable to reduce IR injury in our mice models. This casts doubt on the role of oxidative stress in the increased vulnerability of diabetic myocardium to IR injury. Data concerning the effects of these treatments on cardiac substrate utilization, energetics and aldose reductase activity are missing.

Captopril has been reported to reduce infarct size in animal models, such as canine, porcine or rat models [48,83]. In the study of Itoh et al. [84], captopril did not protect WT mice against post-ischemic dysfunction and myocardial enzyme release. In contrast to this, a significant protection by captopril was found in mice with overexpression of p90 ribosomal S6 kinase. Since p90 ribosomal S6 kinase expression and activity are increased in streptozotocin-induced type I diabetic mice, a protective effect of captopril was anticipated by Itoh et al [84]. Nevertheless, we did not find infarct size reduction in our models of type II diabetes and the metabolic syndrome. So, the role and expression of p90 ribosomal S kinase seems to be less important in type II diabetic ob/ob mice and DKO mice of the metabolic syndrome. This has to be further investigated.

In conclusion, the hypothesis that food restriction ameliorates left ventricular contractility was denied but in contrast, the hypothesis that ACE-inhibition ameliorates left ventricular contractility was confirmed. The hypotheses that food restriction and ACE-inhibition reduce the increased sensitivity for IR injury were denied.

4. Investigation of the efficacy of hypoxic delayed preconditioning in vivo in mice models of type II diabetes and the metabolic syndrome

Hypoxic preconditioning was capable to induce a strong protection against myocardial infarction and hemodynamic failure in WT, as previously shown. The preconditioning potential was completely lost in the DKO model of the metabolic syndrome. In ob/ob, preconditioning induced only a partial preservation of left ventricular contractility. The degree of protection was inferior to that in WT.

The potential underlying mechanisms are numerous.

Our models are known to exhibit raised concentrations of non-esterified fatty acids, insulin resistance and hyperglycemia [45,58]. These metabolic anomalies, especially non-esterified fatty acids, induce atypical isoforms of PKC [85]. In contrast, the activation of the epsilon-isoform of PKC is crucial [6] to recruite the genes which induce delayed preconditioning. The atypical isoforms of PKC activate nuclear factor kappa-B (NF-

κB) in a different way, which results in the activation of different genes [6,85].

Insulin induces a preconditioning effect [49]. It was shown that at 24 weeks, both ob/ob and DKO mice have increased insulin levels [45,58]. It is therefore possible that mouse models with hyperinsulinemia are in a permanent preconditioned state or require a higher treshold to preconditioning [49,54]. Because insulin not only regulates the balance of energy substrates available for the heart but also regulates metabolism and myocardial perfusion via actions on various intracellular regulatory proteins and messenger systems, it is conceivable that diabetes interferes with the biochemical pathways of preconditioning [7].

Previous studies demonstrated the importance of mitochondrial ROS, generated in response to a preconditioning stimulus [6-7,29], as a subsequent trigger for activation of the kinases involved in the delayed preconditioning pathway. Increased oxidative stress is demonstrated in our mouse models [45,58]. It is therefore possible that increased ROS-levels induce a permanent preconditioned state or induce a higher treshold to preconditioning. To elucidate the cardioprotective effect of hypoxic preconditioning at the level of the mitochondria, further research was done in our lab [86]. Under basal conditions, there are no significant differences between the protein levels and activity of the complexes in the electron transfer chain between DKO and WT mice. Hypoxic preconditioning increased the expression of complex V or ATP_{ase} in WT but not in DKO. An increase of the ATP_{ase} might be an adaptive response to maintain cellular function and protect the heart against severe IR injury [86]. Furthermore, it was seen that mitochondria of WT mice, subjected to hypoxic preconditioning, were characterized with a slightly depolarized membrane. In DKO, where the membrane potential was already less polarized, the inner mitochondrial membrane was not further depolarized by hypoxic preconditioning. This might be involved in their cardioprotective deficiency. Previous studies demonstrated the importance of mitochondrial ROS molecules generated in response to a preconditioning stimulus [6-7,29]. Subsequently, these molecules can activate several survival kinases, which were implicated in delayed preconditioning through the activation of nuclear transcription factors. Depolarization of the inner mitochondrial membrane might imply that ROS molecules produced after the stimulus, needed to initiate further gene transcription, are generated to a lesser extent in our mouse model of the metabolic syndrome. Uncoupling of mitochondria might also lead to a reduction in pathological oxidative stress, thereby preventing sustained opening of the mPTP [86].

The role of NO in IR injury and preconditioning is complex. The beneficial or detrimental effect of NO depends on its concentration and cellular origin [6,71]. eNOS is a trigger in the delayed preconditioning pathway [6]. Endothelial dysfunction and reduced NO-bioavailability is present in type II diabetes and the metabolic

syndrome [70]. During IR injury, iNOS has a beneficial role in normal myocardium since iNOS knock-out non-diabetic mice show a larger infarct size versus WT-controls [52]. The small magnitude of increase in iNOS levels in this situation is important, because the upregulation of iNOS has a pronounced dose-dependent effect: beneficial at low levels but toxic at high levels, particularly if it is upregulated in an environment or a cell type that is associated with increased ROS generation [6,71]. Marfella et al. [72] described that in diabetic myocardium, basal iNOS-levels are 3 times higher than in non-diabetic myocardium. Furthermore, the increase of iNOS-levels in ischemic areas after IR injury is 2.6 times larger than in the ischemic areas of non-diabetic myocardium [72]. The detrimental role of these high iNOS-levels during IR injury in diabetic myocardium was confirmed in another study of Marfella et al. [52], in which iNOS knock-out type I diabetic mice show a smaller infarct size and reduced caspase-3 activity versus control diabetic mice. This might explain the increased sensitivity of our diabetic mice to IR injury. In the delayed preconditioning pathway, iNOS has a critical beneficial role [6]. Targeted deletion of the iNOS-gene abrogates delayed preconditioning, suggesting that iNOS is a common effector of cardioprotection [6,71]. Since basal iNOS levels are permanent higher, it is conceivable that diabetic mice have a disturbed threshold or are even already in a permanent maximally protected or preconditioned state.

Delayed preconditioning is regulated through an up-regulation of several other cardioprotective proteins [6]. Previous studies focused mainly on the gene expression profile after delayed preconditioning in healthy subjects [87]. Investigation of gene expression profiles in DKO and ob/ob showed that expression of antioxidant genes and rate limiting components of the electron tranport chain resembled the adaptive phenotype observed in WT in response to hypoxic preconditioning. These results might indicate that this endogenous cardioprotective mechanisms are already maximally activated in these mice [86].

Although a plethora of mechanisms are involved in preconditioning, it is widely accepted that all these mechanisms converge at the inhibition of the mPTP opening at early reperfusion [28-29]. Inhibiting mPTP opening will prevent the cardiomyocytes to undergo cellular necrosis and apoptosis. It was shown in our lab that hypoxic delayed preconditioning protected the myocardium of WT mice against IR injury by inhibiting mPTP opening [86]. Mitochondrial NAD^+ content in myocardial mitochondria isolated from Langendorff perfused hearts was preserved in WT mice after preconditioning. In hearts from ob/ob and DKO mice, hypoxic preconditioning did not preserve mitochondrial NAD^+ content [86]. The exact reason why hypoxic preconditioning cannot protect these mice by inhibiting mPTP is still unknown.

Finally, our mice models exhibit leptin deficiency. Leptin has a protective role after IR [79-80]. It induces activation of ignal transducer and activator of transcription-3 (STAT-3), which induces transcription of several cardioprotective genes. STAT-3 is one of the nuclear transcription factors involved in the delayed preconditioning pathway [6]. It is thus possible that leptin deficiency leads to reduced preconditioning capacity, independently from the other risk factors.

In conclusion, the hypothesis that our mouse models of type II diabetes and the metabolic syndrome are resistant or have an higher threshold to preconditioning is confirmed. Hypoxic preconditioning was completely lost in the DKO model of the metabolic syndrome. In ob/ob, preconditioning induced only a partial preservation of left ventricular contractility. The degree of protection was inferior to that in WT.

5. Investigation of the impact of interventions (food restriction and ACE-inhibition) on the efficacy of hypoxic delayed preconditioning in vivo in mice models of type II diabetes and the metabolic syndrome

The impact of 12 weeks treatment with food restriction or ACE-inhibition on delayed hypoxic preconditioning was studied in mouse models of type II diabetes and the metabolic syndrome.

After food restriction, delayed preconditioning was restored in ob/ob mice, as seen by the reduced infarct size, and better preserved E_{es} and PRSW. These load-independent contractility parameters reached values comparable to the non-ischemic sham group. Furthermore, food restriction restored the preconditioning potential in DKO mice: infarct size and PRSW were significantly better preserved versus the non-preconditioned group. Left ventricular contractility, expressed as PRSW, remained worse versus food restricted and preconditioned ob/ob mice.

After ACE-inhibition treatment, delayed preconditioning was fully restored in ob/ob mice. This was evident from the contractile parameters stroke work, Tau, E_{es} and PRSW. Infarct size after preconditioning was even smaller than the already beneficial effect that could be obtained with diet. Also in DKO mice, ACE-inhibition treatment restored delayed preconditioning. Infarct size and PRSW were significantly better preserved than in the non-preconditioned group. After ACE-inhibition treatment and preconditioning in DKO mice, PRSW was better preserved versus food restricted preconditioned DKO and reached the level of the group without ischemia.

Despite the fact that diet or ACE-inhibition do not reduce the impact of IR injury without preconditioning in these mouse models, both treatments reintroduce the protective effect of delayed preconditioning. The regained

preconditioning potential is thus independent from the direct effects of ACE-inhibition on IR injury. A possible explanation is that food restriction and ACE-inhibition are not capable to influence insuline levels, glycemia, cholesterol, ROS, eNOS and iNOS sufficiently to see an effect on IR injury without previous preconditioning. Nevertheless, these parameters might have been restored sufficiently to induce again a threshold for delayed preconditioning. Indeed, a trigger or 'small change' in these levels is crucial to start the delayed preconditioning cascade [6]. It is thus conceivable that diet and ACE-inhibition lowered insulin, glycemia, cholesterol, ROS and iNOS-levels sufficiently, to allow this trigger.

It is already shown that food restriction reduces insulin levels and ACE-I improve insulin sensitivity [14,45,60]. Since insulin has a preconditioning effect, this reduction might have restored the threshold to preconditioning [49,54]. Furthermore, ACE-inhibition reduces glycemia in ob/ob and DKO mice and food restriction lowers cholesterol levels in ob/ob mice. This can have lowered the threshold to delayed preconditioning since hyperglycemia and hypercholesterolemia are key player in the induction of atypical isoforms of protein kinase C [85], which is crucial in the delayed preconditioning pathway [6].

Food restriction induces up-regulation of eNOS in DKO mice [45]. ACE-I are capable to block the degradation of bradykinin into inactive peptides and to increase eNOS-levels [14-18]. eNOS is a trigger in the delayed preconditioning pathway [6], which might explain the regained preconditioning potential.

Food restriction prevents ROS production [88] and also ACE-I have well known anti-oxidative activity [14-18]. Since an increase in ROS formation is an important trigger of delayed preconditioning [6], it is possible that these treatments reduce the increased baseline ROS-level in diabetic myocardium and restore the threshold for delayed preconditioning [6].

Another possible explanation why the treatments induced cardioprotection after preconditioning but did not have a direct effect during IR injury, is the different role and levels of iNOS in both pathways. As described earlier, basal iNOS-levels are higher and the increase of iNOS-levels after IR injury is larger than in non-diabetic myocardium [72] with a deleterious role in diabetic myocardium during IR injury [52]. In contrast with this, a small increase in iNOS-levels is critical to induce its effects as common end-effector in the delayed preconditioning pathway [6]. No studies were conducted to study the effect of food restriction on iNOS-levels in myocardium, but it was shown in a normotensive rat model that ACE-I reduces iNOS-protein expression with 24% [89]. It is tempting to speculate that ACE-inhibition did not reduce iNOS-levels sufficiently to reduce its

toxic high-dose level during IR injury but enough to restore the threshold to induce delayed preconditioning (by a small iNOS increase). Further investigations need to be performed to elucidate the role of these underlying mechanisms.

Finally, the specific rol of the sulfhydryl (SH)-group of Captopril is uncertain. Captopril molecules, (2S)-1-[(2S)-2-methyl-3-sulfanylpropanoyl] pyrrolidine-2-carboxylic acid, contain SH-groups, which are chemically active and may affect various processes in tissues. Westlin and Mullane [90], as well as Grover et al [91], previously showed that Captopril and other SH-group containing ACE-I, improve post-ischemic cardiac contractility in rats or dogs by a mechanism independent of the ACE-inhibition. Also, in experimental heart failure was found that SH-containing Zofenopril, but not SH-free Lisinopril potentiated vasodilatation response to nitroglycerin or sodium nitrite [92]. In vitro experiments showed that SH-containing but not SH-free ACE-I were effective scavengers of free radicals [90,93]. Contrary to this, Captopril and SH-free Lisinopril were equally effective in improving NYHA class in the treatment chronic heart failure in elderly patients, indicating that just ACE-inhibition was responsible for the positive effect [94]. Also, SH-containing Zofenopril and SH-free Lisinopril equally normalized vasodilatation induced by acetylcholine in experimental heart failure [92]. Therefore, the preconditioning potential in the mouse models of type II diabetes and the metabolic syndrome was restored via three possible mechanisms: via ACE-inhibition only, via the combined effect (ACE-inhibition and SH-group), or even via SH-group impact only. The SH-group augments endothelial NO-levels [92] and has a well known antioxidant effect [90,91]. NO, as well ROS, have an important role in the preconditioning pathway. ACE-inhibition prevents the breakdown of bradykinin, which is an trigger in the delayed preconditioning pathway. The specific contribution of these effects has to be analyzed.

In conclusion, the hypothesis that food restriction and ACE-inhibition ameliorate or restore the preconditioning potential in our mouse models of type II diabetes and the metabolic syndrome is confirmed.

Future perspectives

Despite the fact that preconditioning is an easily inducible and powerful endogenous protection mechanism and despite the extensive research in this domain, preconditioning has not been translated into clinical therapies. There are several important reasons.

First, the intended population has frequently underlying pathologies and several co-morbidities. The effect of type II diabetes and the metabolic on delayed preconditioning was the focus of this study, but the effect of several other pathologies such as hypertension, heart failure or aging are not studied yet.

Second, the impact of standard treatments for this co-morbidities on preconditioning, is incompletely known. In this study, the effect of diet and ACE-inhibition was investigated, but other treatments, such as insulin, oral anti-diabetics and lipid-lowering therapy should be investigated.

Third, the applied stimuli to induce preconditioning, such as repeated cross-clamping or temporary coronary occlusion, can be harmful because of the longer duration of the operation and the risk of tearing tissues or embolization. Studies should be undertaken without these harmful mechanical stimuli. Pharmacological preconditioning (ex. isoflurane, bradykinin, adenosine agonists,…) is an interesting method [26]. With this method, it could be possible that a drug is administrated the day before surgery to induce preconditioning. Several drugs, which can be further studied, are NO-donors, opioid agonists, TNFα and statins. Recent evidence has indicated a possible implication of the endothelin system in the pathogenesis of diabetic complications [95]. Endothelin receptor blockade limits myocardial contractile depression in type I diabetic models [95]. The effect on IR injury was not studied.
Another recent study has shown that high-dose fasudil, a Rho-kinase inhibitor, preserves postconditioning under acute hyperglycemic state in rats [96]. Rho-kinase activity is involved in diabetic endothelial dysfunction and activated in ischemic myocardium. Furthermore, inhibition of Rho-kinase activity might be of interest for myocardial protection against IR injury since it activates the ATP-sensitive potassium channels [96]. The effect of fasudil on preconditioning and IR injury in chronic hyperglycemic states, such as diabetes, has still to be determined.

Fourth, most studies on preconditioning focused on classical or first window preconditioning. Nevertheless, delayed preconditioning is therapeutically the most attractive because of its long standing effect from 24 hours

till 72 hours. This protection can therefore be planned more readily in clinical practice and even chronically induced when ischemia is anticipated in the following days, but not exactly predictable, e.g. in patients with cardiovascular disease undergoing non–cardiac surgery. The potential therapeutic field of preconditioning is large. Myocardial protection against infarction is the best known condition but also surgery with a high risk for hypotension or ischemia, for example cardiovascular and transplantation surgery could benefit from this endogenous protection mechanism. Especially patients with a high risk for peroperative morbidity and mortality (metabolic syndrome, diabetes, elderly, bad ventricular function) might benefit from it.

Fifth, the underlying mechanisms were not directly investigated in this study. The effect of type II diabetes and the metabolic syndrome and their treatments on several important mediators and end-effectors in the delayed preconditioning pathway, such as iNOS, COX-2, ROS and NO should be further investigated. This would give us the opportunity to develop specific, additional therapeutic interventions.

Sixth, the specific role of the SH-group of Captopril has to be determined [90-94]. Experiments with SH-free ACE-I and molecules with SH-groups but without ACE-activity (such as acetylcysteine), can elucidate the specific contribution to the regained preconditioning potential.

Reference list

[1] Kereiakes DJ, Willerson JT. Metabolic syndrome epidemic. *Circulation* 2003; 108: 1552.

[2] Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* 2004; 27: 1047-1053.

[3] Dohi T, Miyauchi K, Kasai T, Kajimoto K, Kubota N, Tamura H, Yokoyama T, Kojima T, Yokoyama K, Kurata T, Daida H. Impact of metabolic syndrome on 10-year clinical outcomes among patients with acute coronary syndrome. *Circ J* 2009; 73: 1454-1458.

[4] Haffner SM, Lehto S, Rönnemaa T, Pyörälä K, Laakso M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* 1998; 339: 229-234.

[5] ADVANCE Collaborative Group, Patel A, MacMahon S, Chalmers J, Neal B, Billot L, Woodward M, Marre M, Cooper M, Glasziou P, Grobbee D, Hamet P, Harrap S, Heller S, Liu L, Mancia G, Mogensen CE, Pan C, Poulter N, Rodgers A, Williams B, Bompoint S, de Galan BE, Joshi R, Travert F. Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. *N Engl J Med* 2008; 358: 2560-2572.

[6] Bolli R, Li QH, Tang XL, Guo Y, Xuan YT, Rokosh G, Dawn B. The late phase of preconditioning and its natural clinical application–gene therapy. *Heart Fail Rev* 2007; 12: 189-199.

[7] Ferdinandy P, Schulz R, Baxter GF. Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning and postconditioning. *Pharmacol Rev* 2007; 59: 418-458.

[8] Miki T, Itoh T, Sunaga D, Miura T. Effects of diabetes on myocardial infarct size and cardioprotection by preconditioning and postconditioning. *Cardiovasc Diabetol* 2012; 11: 67.

[9] Buse JB, Ginsberg HN, Bakris GL, Clark NG, Costa F, Eckel R, Fonseca V, Gerstein HC, Grundy S, Nesto RW, Pignone MP, Plutzky J, Porte D, Redberg R, Stitzel KF, Stone NJ; American Heart Association; American Diabetes Association. Primary prevention of cardiovascular disease in people with diabetes mellitus. A Scientific statement from the American Heart Association and the American Diabetes Association. *Circulation* 2007; 115: 114-126.

[10] Broderick TL, Belke T, Driedzic WR. Effects of chronic caloric restriction on mitochondrial respiration in the ischemic reperfused rat heart. *Mol Cell Biochem* 2002; 233: 119-125.

[11] Mitchell JR, Verweij M, Brand K, van de Ven M, Goemaere N, van den Engel S, Chu T, Forrer F, Müller C, de Jong M, van IJcken W, IJzermans JN, Hoeijmakers JH, de Bruin RW. Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. *Aging Cell* 2010; 9: 40-53.

[12] Yamagishi T, Bessho M, Yanagida S, Nishizawa K, Kusuhara M, Ohsuzu F, Tamai S. Severe, short-term food restriction improves cardiac function following ischemia/reperfusion in perfused rat hearts. *Heart Vessels*

2010; 25: 417-425.

[13] Abete P, Testa G, Galizia G, Mazzella F, Della Morte D, de Santis D, Calabrese C, Cacciatore F, Gargiulo G, Ferrara N, Rengo G, Sica V, Napoli C, Rengo F. Tandem action of exercise training and food restriction completely preserves ischemic preconditioning in the aging heart. *Exp Gerontol* 2005; 40: 43-50.

[14] Velasquez MT, Bhathena SJ, Striffler JS, Thibault N, Scalbert E. Role of angiotensin-converting enzyme inhibition in glucose metabolism and renal injury in diabetes. *Metabolism* 1998; 47: 7-11.

[15] Miki T, Miura T, Ura N, Ogawa T, Suzuki K, Shimamoto K, Limura O. Captopril potentiates the myocardial infarct size-limiting effect of ischemic preconditioning through bradykinin B2 receptor activation. *J Am Coll Cardiol* 1996; 28: 1616-1622.

[16] Nozawa Y, Miura T, Tsuchida A, Kita H, Fukuma T, Shimamoto K. Chronic treatment with an ACE inhibitor, temocapril, lowers the threshold for the infarct size-limiting effect of ischemic preconditioning. *Cardiovasc Drugs Ther* 1999; 13: 151-157.

[17] Jaberansari MT, Baxter GF, Muller CA, Latouf SE, Röth E, Opie LH, Yellon DM. Angiotensin-converting enzyme inhibition enhances a subthreshold stimulus to elicit delayed preconditioning in pig myocardium. *J Am Coll Cardiol* 2001; 37: 1996-2001.

[18] Marktanner R, Nacke P, Feindt P, Hohlfeld T, Schipke JD, Gams E. Delayed preconditioning via Angiotensin-converting enzyme inhibition: pros and cons from an experimental study. *Clin Exp Pharmacol Physiol* 2006; 33: 787-792.

[19] Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; 74: 1124-1136.

[20] Xia Z, Herijgers P, Nishida T, Ozaki S, Wouters P, Flameng W. Remote preconditioning lessens the deterioration of pulmonary function after repeated coronary artery occlusion and reperfusion. *Can J Anesth* 2003; 50: 481-488.

[21] Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada M. Delayed effects of sublethal ischemia on the axquisition of tolerance to ischemia. *Circ Res* 1993; 72: 1293-1299.

[22] Marber MS, Latchman DS, Walker JM, Yellon DM. Cardiac stress is associated with resistance to myocardial infarction. *Circulation* 1993; 88: 1264-1272.

[23] Bolli R. Preconditioning: a paradigm shift in the biology of myocardial ischemia. *Am J Physiol Heart Circ Physiol* 2007; 292: H19-27.

[24] Guo Y, Wu WJ, Qiu Y, Tang XL, Yang Z, Bolli R. Demonstration of an early and late phase of ischemic preconditioning in mice. *Am J Physiol* 1998; 275: H1375-1387.

[25] Cai Z, Manalo DJ, Wei G, Rodriguez ER, Fox-Talbot K, Lu H, Zweier JL, Semenza GL. Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. *Circulation* 2003; 108: 79-85.

[26] Eisen A, Fisman EZ, Rubenfire M, Freimark D, McKechnie R, Tenenbaum A, Motro M, Adler Y. Ischemic preconditioning: nearly two decades of research. A comprehensive review. *Atherosclerosis* 2004; 172: 201-210.

[27] Ferdinandy P, Schulz R. Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. *British journal of Pharmacology* 2003; 138: 532-543.

[28] West MB, Rokosh G, Obal D, Velayutham M, XuanY-T, Hill BG, Keith RJ, Schrader J, Guo Y, Conklin DJ, Prabhu SD, Zweier JL, Bolli R, Bhatnagar A. Cardiac Myocyte–Specific Expression of Inducible Nitric Oxide Synthase Protects Against Ischemia/Reperfusion Injury by Preventing Mitochondrial Permeability Transition. *Circulation* 2008; 118: 1970-1978.

[29] Halestrap AP, Clarke SJ, Khaliulin I. The role of mitochondria in protection of the heart by preconditioning. *Biochim Biophys Acta* 2007; 1767: 1007-1031.

[30] Xiao CY, Yuhki K, Hara A, Fujino T, Kuriyama S, Yamada T, Takayama K, Takahata O, Karibe H, Taniguchi T, Narumiya S, Ushikubi F. Prostaglandin E2 protects the heart from ischemia-reperfusion injury via its receptor subtype EP4. *Circulation* 2004; 109: 2462-2468.

[31] Jancso G, Cserepes B, Gasz B, Benko L, Borsiczky B, Ferenc A, Kürthy M, Racz B, Lantos J, Gal J, Arato E, Sinayc L, Weber G and Roth E. Expression and protective role of heme oxygenase-1 in delayed myocardial preconditioning. *Ann N.Y. Acad Sci* 2007; 1095: 251-261.

[32] Walker DM, Walker JM, Pugsley WB, Pattison CW, Yellon DM. Preconditioning in isolated superfused human muscle. *J Mol Cell Cardiol* 1995; 27: 1349-1357.

[33] Armstrong S, Ganote CE. Adenosine receptor specificity in preconditioning of isolated rabbit cardiomyocytes: evidence of A3 receptor involvement. *Cardiovasc Res* 1994; 28: 1049-1056.

[34] Ikonomidis JS, Tumiati LC, Weisel RD, Mickle DA, Li RK. Preconditioning human ventricular cardiomyocytes with brief periods of simulates ischemia. *Cardiovasc Res* 1994; 28: 1285-1291.

[35] Laude K, Beauchamp P, Thuillez C, Richard V. Endothelial protective effects of preconditioning. *Cardiovasc Res* 2002; 15: 466-473.

[36] Richard V, Kaeffer N, Tron C, Thuillez C. Ischemic preconditioning protects against coronary endothelial dysfunction induced by ischemia and reperfusion. *Circulation* 1994; 89: 1254-1261.

[37] Shiki K, Hearse DJ. Preconditioning of ischemic myocardium: reperfusion-induced arrhythmias. *Am J Physiol* 1987; 253: H1470-1476.

[38] Murry CE, Richard VJ, Reimer KA, Jennings RB. Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res* 1990; 66: 913-931.

[39] Cave AC, Hearse DJ. Ischemic preconditioning and contractile function : studies with normothermic and hypothermic global ischemia. *J Mol Cell Cardiol* 1992: 24; 1113-1123.

[40] Stein AB, Bolli R, Guo Y, Wang OL, Tan W, Wu WJ, Zhu X, Zhu Y, Xuan YT. The late phase of ischemic preconditioning induces a prosurvival genetic program that results in marked attenuation of apoptosis. *J Mol Cell Cardiol* 2007; 42: 1075-1085.

[41] Grundy SM, Brewer HB Jr, Cleeman JI, Smith SC Jr, Lenfant C; American Heart Association; National Heart, Lung, and Blood Institute. Definition of metabolic syndrome. *Circulation* 2004; 109 : 433-438.

[42] Raeven GM. Banting lecture 1988: role of insulin resistance in human disease. *Diabetes* 1988; 37: 1595-1607.

[43] Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC Jr; International Diabetes Federation Task Force on Epidemiology and Prevention; Hational Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; International Association for the Study of Obesity. Harmonizing the Metabolic Syndrome. A Joint Interim Statement of International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute, American Heart Association, World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* 2009; 120: 1640-1645.

[44] Ferrannini E, Haffner SM, Mitchell BD, Stern MP. Hyperinsulinaemia: the key feature of a cardiovascular and metabolic syndrome. *Diabetologia* 1991; 34: 416-422.

[45] Verreth W, De Keyzer D, Pelat M, Verhamme P, Ganame J, Bielicki JK, Mertens A, Quarck R, Benhabiles N, Marguerie G, Mackness B, Mackness M, Ninio E, Herregods MC, Balligand JL, Holvoet P. Weight-loss-associated induction of peroxisome proliferator-activated receptor-alpha and peroxisome proliferator-activated receptor-gamma correlate with reduced atherosclerosis and improved cardiovascular function in obese insulin-resistant mice. *Circulation* 2004; 110: 3259-3269.

[46] Abete P, Testa G, Ferrara N, De Santis D, Capaccio P, Viati L, Calabrese C, Cacciatore F, Longobardi G, Condorelli M, Napoli C, Rengo F. Cardioprotective effect of ischemic preconditioning is preserved in food-restricted senescent rats. *Am J Physiol Heart Circ Physiol* 2002; 282: H1978-1987.

[47] Grundy SM, Hansen B, Smith SCJr, Cleeman JI, Kahn RA. Clinical management of metabolic syndrome. *Circulation* 2004; 109: 551-556.

[48] Mehta PM, Przyklenk K, Kloner RA. Cardioprotective effects of captopril in myocardial ischemia, ischemia/reperfusion and infarction. *Eur Heart J* 1990; 11: 94-99.

[49] Hausenloy DJ, Yellon DM. Reperfusion injury salvage kinase signaling: taking a RISK for cardioprotection. *Heart Fail Rev* 2007; 12: 217-234.

[50] Ghosh S, Standen NB, Galinianes M. Failure to precondition pathological human myocardium. *J Am Coll Cardiol* 2001; 37: 711-718.

[51] Ren L, Liu N, Zhi H, Li Y, Li Y, Tang R, Sheng Z. Vasculoprotective effects of rosiglitazone through modulating renin-angiotensin system in vivo and vitro. *Cardiovasc Diabetol* 2011; 10: 10.

[52] Marfella R, Di Filippo C, Esposito K, Nappo F, Piegari E, Cuzzocrea S, Berrino L, Rossi F, Giugliano D, D'Amico M. Absence of inducible nitric oxide synthase reduces myocardial damage during ischemia reperfusion in streptozotocin-induced hyperglycemic mice. *Diabetes* 2004; 53: 454-462.

[53] Aasum E, Hafstad AD, Severson DL, Larsen TS. Age-dependent changes in metabolism, contractile function and ischemic sensitivity in hearts from db/db mice. *Diabetes* 2003; 52: 434-441.

[54] Tsang A, Hausenloy DJ, Mocanu MM, Carr RD, Yellon DM. Preconditioning the diabetic heart: the importance of Akt phosphorylation. *Diabetes* 2005; 54: 2360-2364.

[55] Kristiansen SB, Løfgren B, Støttrup NB, Khatir D, Nielsen-Kudsk JE, Nielsen TT, Bøtker HE, Flyvbjerg A. Ischaemic preconditioning does not protect the heart in obese and lean animal models of type 2 diabetes. *Diabetologia* 2004; 47: 1716-1721.

[56] Ebel D, Müllenheim J, Frässdorf J, Heinen A, Huhn R, Bohlen T, Ferrari J, Südkamp H, Preckel B, Schlack W, Thämer V. Effect of acute hyperglycaemia and diabetes mellitus with and without short-term insulin treatment on myocardial ischaemic late preconditioning in the rabbit heart in vivo. *Pflugers Arch* 2003; 446: 175–182.

[57] Srinivasan K, Ramarao P. Animal models in type 2 diabetes research: an overview. *Indian J Med Res* 2007; 125: 451-472.

[58] Van den Bergh A, Vanderper A, Vangheluwe P, Desjardins F, Nevelsteen I, Verreth W, Wuytack F, Holvoet P, Flameng W, Balligand JL, Herijgers P. Dyslipidemia in type II diabetic mice does not aggravate contractile impairment but increases ventricular stiffness. *Cardiovasc Res* 2008; 77: 371-379.

[59] Xi L, Tekin D, Gursoy E, Salloum F, Levasseur JE, Kukreja RC. Evidence that NOS2 acts as a trigger and mediator of late preconditioning induced by acute systemic hypoxia. *Am J Physiol* 2002; 283 : H5-H12.

[60] Van den Bergh A, Vangheluwe P, Vanderper A, Carmeliet P, Wuytack F, Janssens S, Flameng W, Holvoet P, Herijgers P. Food-restriction in obese dyslipidaemic diabetic mice partially restores basal contractility but not contractile reserve. *Eur J Heart Fail* 2009; 11: 1118-1125.

[61] Wichi RB, Farah V, Chen Y, Irigoyen MC, Morris M. Deficiency in angiotensin AT1a receptors prevents

diabetes-induced hypertension. *Am J Physiol Regul Integr Comp Physiol* 2007; 292: R1184-1189.

[62] Downey JM. Measuring infarct size by the tetrazolium method. Available at www.ishrworld.org

[63] Van den Bergh A, Flameng W, Herijgers P. Parameters of ventricular contractility in mice: influence of load and sensitivity to changes in inotropic state. *Pflugers Arch* 2008; 455: 987-994.

[64] An Van den Bergh. Cardiac contractility and Calcium handling in Mouse models of type II diabetes. *Acta Biomedica Lovaniensia* 2006; ISBN 978 90 5867 581 1.

[65] Baan J, Jong TT, Kerkhof PLM, Moene RJ, Van Dijk AD, Van der Velde ET, Koops J. Continuous stroke volume and cardiac output from intra-ventricular dimensions obtained with impedance catheter. *Cardiovasc Res* 1981; 15: 328-334.

[66] Baan J, van der Velde ET, de Bruin HG, Smeenk GJ, Koops J, van Dijk AD, Temmerman D, Senden J, Buis B. Continuous measurement of left ventricular volume in animals and humans by conductance catheter. *Circulation* 1984; 70: 812-823.

[67] Ferguson Jr TB, Hammill BG, Peterson ED, DeLong ER, Grover FL. A decade of change-risk profiles and outcomes for isolated coronary artery bypass grafting procedures, 1990-1999: a report from the STS national database committee and the Duke clinical research institute. *Ann Thorac Surg* 2002; 73: 480-489.

[68] Vaage J, Valen G. Preconditioning and cardiac surgery. *Ann Thorac Surg* 2003, 75: S709-714.

[69] Abozguia K, Clarke K, Lee L, Frenneaux M. Modification of myocardial substrate use as a therapy for heart failure. *Nat Clin Pract Cardiovasc Med* 2006; 3: 490-498.

[70] Natali A, Ferrannini E. Endothelial dysfunction in type 2 diabetes. *Diabetologia* 2012; 55: 1559-1563.

[71] Guo Y, Sanganalmath SK, Wu W, Zhu X, Huang Y, Tan W, Ildstad ST, Li Q, Bolli R. Identification of inducible nitric oxide synthase in peripheral blood cells as a mediator of myocardial ischemia/reperfusion injury. *Basic Res Cardiol* 2012; 107: 1-8.

[72] Marfella R, Esposito K, Nappo F, Siniscalchi M, Sasso FC, Portoghese M, Di Marino MP, Baldi A, Cuzzocrea S, Di Filippo C, Barboso G, Baldi F, Rossi F, D'Amico M, Giugliano D. Expression of angiogenic factors during acute coronary syndromes in human type 2 diabetes. *Diabetes* 2004; 53: 2383-2391.

[73] Talukder MA, Kalyanasundaram A, Zuo L, Velayutham M, Nishijima Y, Periasamy M, Zweier JL. Is reduced SERCA2a expression detrimental or beneficial to postischemic cardiac function and injury? Evidence from heterozygous SERCA2a knockout mice. *Am J Physiol Heart Circ Physiol* 2008; 294: H1426-1434.

[74] Rodrigues B, Rosa KT, Medeiros A, Schaan BD, Brum PC, De Angelis K, Irigoyen MC. Hyperglycemia can delay left ventricular dysfunction but not autonomic damage after myocardial infarction in rodents. *Cardiovasc Diabetol* 2011; 10: 26.

[75] Bugger H, Abel ED. Molecular mechanisms for myocardial mitochondrial dysfunction in the metabolic syndrome. *Clin Sci* 2008; 114: 195-210.

[76] Hwang YC, Sato S, Tsai JY, Yan S, Bakr S, Zhang H, Oates PJ, Ramasamy R. Aldose reductase activation is a key component of myocardial response to ischemia. *FASEB J* 2002; 16: 243-245.

[77] Kaneko M, Bucciarelli L, Hwang YC, Lee L, Yan SF, Schmidt AM, Ramasamy R. Aldose reductase and AGE-RAGE pathways: key players in myocardial ischemic injury. *Ann N Y Acad Sci* 2005; 1043: 702-709.

[78] Rui T, Zhang J, Xu X, Yao Y, Kao R, Martin M. Reduction of IL-33 expression exaggerates ischemia/reperfusion-induced myocardial injury in mice with diabetes mellitus. *Cardiovasc Res* 2012; 94: 370-378.

[79] McGaffin KR, Sun CK, Rager JJ, Romano LC, Zou B, Mathier MA, O'Doherty RM, McTiernan CF, O'Donnell CP. Leptin signalling reduces the severity of cardiac dysfunction and remodelling after chronic ischaemic injury. *Cardiovasc Res* 2008, 77: 54-63.

[80] McGaffin KR, Zou B, McTiernan CF, O'Donnell CP. Leptin attenuates cardiac apoptosis after chronic ischaemic injury. *Cardiovasc Res* 2009; 83: 313-324.

[81] Takeishi Y, Bhagwat A, Ball NA, Kirkpatrick DL, Periasamy M, Walsh RA. Effect of angiotensin-converting enzyme inhibition on protein kinase C and SR proteins in heart failure. *Am J Physiol Heart Circ Physiol* 1999; 276: H53-62.

[82] Gross ML, Heiss N, Weckbach M, Hansen A, El-Shakmak A, Szabo A, Münter K, Ritz E, Amann K. ACE-inhibition is superior to endothelin A receptor blockade in preventing abnormal capillary supply and fibrosis of the heart in experimental diabetes. *Diabetologia* 2004; 47: 316-324.

[83] Parlakpinar H, Ozer MK, Acet A. Effects of captopril and angiotensin II receptor blockers (AT1, AT2) on myocardial ischemia-reperfusion induced infarct size. *Cytokine* 2011; 56: 688-694.

[84] Itoh S, Ding B, Shishido T, Lerner-Marmarosh N, Wang N, Maekawa N, Berk BC, Takeishi Y, Yan C, Blaxall BC, Abe J. Role of p90 ribosomal S6 kinase-mediated prorenin-converting enzyme in ischemic and diabetic myocardium. *Circulation* 2006; 113: 1787-1798.

[85] Poornima IG, Parikh P, Shannon RP. Diabetic cardiomyopathy: the search for a unifying hypothesis. *Circ Res* 2006; 98: 596-605.

[86] Vanderper A. Failing delayed preconditioning of the heart in mouse models of type II diabetes and the metabolic syndrome: in search of underlying mechanisms. *Doctoral thesis in Biomedical Sciences* KU Leuven, 2010

[87] Das DK, Maulik N. Cardiac genomic response following preconditioning stimulus. *Cardiovasc Res* 2006; 70: 254-263.

[88] Sohal RS, Ku HH, Agarwal S, Forster MJ, Lal H. Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. *Mech Ageing Dev* 1994; 74: 121-133.

[89] Bachetti T, Comini L, Pasini E, Cargnoni A, Curello S, Ferrari R. Ace-inhibition with quinapril modulates the nitric oxide pathway in normotensive rats. *J Mol Cell Cardiol* 2001; 33: 395-403.

[90] Westlin W, Mullane K. Does captopril attenuate reperfusion-induced myocardial dysfunction by scavenging free radicals? *Circulation* 1988; 77: I30-39.

[91] Grover GJ, Sleph PG, Dzwonczyk S, Wang P, Fung W, Tobias D, Cushman DW. Effects of different angiotensin-converting enzyme (ACE) inhibitors on ischemic isolated rat hearts: relationship between cardiac ACE inhibition and cardioprotection. *J Pharmacol Exp Ther* 1991; 257: 919-929.

[92] Buikema H, Monnink SH, Tio RA, Crijns HJ, de Zeeuw D, van Gilst WH. Comparison of zofenopril and lisinopril to study the role of the sulfhydryl-group in improvement of endothelial dysfunction with ACE-inhibitors in experimental heart failure. *Br J Pharmacol* 2000; 130: 1999-2007.

[93] Chopra M, Beswick H, Clapperton M, Dargie HJ, Smith WE, McMurray J. Antioxidant effects of angiotensin-converting enzyme (ACE) inhibitors: free radical and oxidant scavenging are sulfhydryl dependent, but lipid peroxidation is inhibited by both sulfhydryl- and nonsulfhydryl-containing ACE inhibitors. *J Cardiovasc Pharmacol* 1992; 19: 330-340.

[94] Morisco C, Condorelli M, Crepaldi G, Rizzon P, Zardini P, Villa G, Argenziano L, Trimarco B. Lisinopril in the treatment of congestive heart failure in elderly patients: comparison versus captopril. *Cardiovasc Drugs Ther* 1997; 11: 63-69.

[95] Wölkart G, Pang X, Stessel H, Kirchengast M, Brunner F. Chronic endothelin-A receptor antagonism is as protective as angiotensin converting enzyme inhibition against cardiac dysfunction in diabetic rats. *Br J Pharmacol* 2007; 151: 1187-1197.

[96] Ichinomiya T, Cho S, Higashijima U, Matsumoto S, Maekawa T, Sumikawa K. High-dose fasudil preserves postconditioning against myocardial infarction under hyperglycemia in rats: role of mitochondrial KATP channels. *Cardiovasc Diabetol* 2012; 11: 28.

Summary

The number of patients with type II diabetes or the metabolic syndrome increases in Western societies and reaches epidemic proportions. These patients are at higher risk for cardiovascular complications and suffer more frequently from ischemic insults. This causes significant increased morbidity and mortality. Protective strategies to prevent these insults or reduce the consequences of it are eagerly awaited.

Tissues and organs can be protected from ensuing longer ischemia-reperfusion periods by short preceding episodes of 'preconditioning' ischemia. After a preconditioning stimulus, the protected state is present for 1 to 2 hours, called 'classical preconditioning' or 'first window preconditioning', followed by a 'second window of protection' from 12 to 96 hours, the so-called delayed preconditioning. Despite the fact that preconditioning is a powerful and easily inducible endogenous protection mechanism against ischemia-reperfusion injury in healthy persons or animal models, so far, it has not fulfilled its expectations in patients with type II diabetes and the metabolic syndrome. Clinical studies report that type II diabetic myocardium is resistant or has a higher threshold to classical preconditioning. Delayed preconditioning is the most interesting technique for myocardial protection in type II diabetic and metabolic syndrome patients, since it is capable to induce cardioprotection in a controled way for longer periods. The effect type II diabetes and the metabolic syndrome on delayed preconditioning has never been investigated and is the focus of this study.

Furthermore, most patients with type II diabetes or the metabolic syndrome are treated. These treatments might additionally influence the preconditioning potential. Despite the fact that hypocaloric diet and angiotensin-converting enzyme (ACE)-inhibition are standard treatments in type II diabetes and the metabolic syndrome, its cardiovascular effects are incompletely understood. Since these treatments have potential benefits, the effects on delayed preconditioning in mouse models of type II diabetes and the metabolic syndrome were investigated.

In the first part of this study, we focused on the development of an in vivo mouse model of hypoxic preconditioning and ischemia-reperfusion. Outcome was assessed by infarct size determination and in vivo left ventricular pressure-volume measurements. We demonstrated that hypoxic delayed preconditioning induced a strong protection in wild type mice, as seen by reduced infarct size and better preserved contractility.

In a second phase, we have shown that left ventricular contractility is reduced in hearts form mice with type II diabetes and even further reduced in mice with the metabolic syndrome. Hearts from both mice models were more vulnerable to ischemia-reperfusion injury than wild type mice. Food restriction did not influence

contractility and did not reduce the increased sensitivity for ischemia-reperfusion. Angiotensin-converting enzyme inhibition ameliorated contractility but did not protect against the increased sensitivity for ischemia-reperfusion.

In the third part, we demonstrated that hypoxic preconditioning induced a partial protection in mice with type II diabetes, but could not protect the hearts of mice with the metabolic syndrome. Food restriction restored the preconditioning potential, as seen by a reduced infarct size and preserved contractility, in both mice models of type II diabetes and the metabolic syndrome. ACE-inhibition induced even a more extensive restoration of the preconditioning potential than food restriction. After these treatments, hypoxic preconditioning preserved contractility to the level of the non-ischemic groups and significantly reduced infarct size.

Samenvatting

Patiënten met type II diabetes of het metabool syndroom hebben een verhoogd cardiovasculair risico en zijn meer gevoelig voor ischemie. Dit veroorzaakt een belangrijke morbiditeit en mortaliteit in de Westers wereld. Door de toenemende aantallen van patiënten met type II diabetes en het metabool syndroom is er nood aan protectieve strategieën om het aantal ischemische incidenten of de gevolgen ervan te reduceren.

Weefsels en organen kunnen beschermd worden tegen lange ischemie-reperfusie episodes, door op voorhand korte 'preconditionerende' ischemie toe te passen. Na deze preconditionerende stimulus, is een orgaan beschermd in de daaropvolgende 1 à 2 uur, hetgeen klassieke of eerste venster preconditionering genoemd wordt. Nadien volgt er van 12 uur tot 96 uur na deze stimulus een tweede venster van preconditionering, ook wel late preconditionering genoemd. Ondanks preconditionering een krachtig en eenvoudig induceerbaar endogeen protectief mechanisme is tegen ischemie-reperfusie schade in gezonde personen of diermodellen, heeft het tot nu toe zijn verwachtingen niet kunnen inlossen bij patiënten met type II diabetes en het metabool syndroom. Het is aangetoond dat het myocard van patiënten met type II diabetes resistent is of een grotere drempel heeft tot eerste venster preconditionering. Nochtans is het tweede venster van preconditionering klinisch het meest interessante protectiemechanisme, gezien het meer gecontroleerd en langduriger kan geïnduceerd worden. Het effect van type II diabetes en het metabool syndroom op tweede venster preconditionering werd nooit bestudeerd en is de focus van deze studie.

Bovendien worden de meeste patiënten met type II diabetes en het metabool syndroom behandeld. Deze behandelingen kunnen op hun beurt de mogelijkheid tot preconditionering beïnvloeden. Desondanks calorie-arme diëten en angiotensine-converting enzyme (ACE)-inhibitie standaard behandelingen zijn bij patiënten met type II diabetes en het metabool syndroom, zijn de cardiovasculaire effecten ervan in deze populatie amper bekend. Omwille van de potentieel gunstige effecten op tweede venster preconditionering, werden de effecten van deze behandelingen onderzocht bij muismodellen van type II diabetes en het metabool syndroom.

In het eerste deel van deze studie, hebben we ons toegespitst op de oppuntstelling van een in vivo muismodel voor hypoxische preconditionering en ischemie-reperfusie. De effecten ervan werden beoordeeld door infarctgrootte bepalingen en in vivo linker ventrikel druk-volume metingen uit te voeren. We hebben kunnen aantonen dat hypoxie een tweede venster van preconditionering kan opwekken in wild type muizen met een krachtige bescherming onder de vorm van kleinere infarctgrootte en beter bewaarde contractiliteit.

In een tweede fase, hebben we aangetoond dat de linker ventrikel contractiliteit verminderd is bij muizen met type II diabetes en zelfs in een ergere graad bij muizen van het metabool syndroom. De harten van beide muismodellen zijn gevoeliger voor ischemie-reperfusie schade dan wild type muizen. Dieet had geen effect op de contractiliteit en kon de hogere gevoeligheid voor ischemie-reperfusie schade niet reduceren. ACE-inhibitie verbeterde de contractiliteit maar kon eveneens de verhoogde gevoeligheid voor ischemie-reperfusie schade niet reduceren.

In het derde deel hebben we kunnen aantonen dat hypoxische preconditionering slechts een beperkte bescherming induceert bij muizen met type II diabetes en geen bescherming bij muizen met het metabool syndroom. Na een dieet behandeling, kon tweede venster preconditionering opnieuw geïnduceerd worden, zowel bij muizen met type II diabetes als deze met het metabool syndroom. Dit effect was nog meer uitgesproken na behandeling met ACE-inhibitie. Na deze behandelingen, kon hypoxische tweede venster preconditionering de contractiliteit behouden op het niveau van de groepen die niet blootgesteld werden aan ischemie en kon de infarctgrootte significant gereduceerd worden.

CURRICULUM VITAE

Gerry Van der Mieren

Born on September 19, 1978, Lier, Belgium

Married on October 1, 2005 with Sofie Embrechts

Father of Amélie (°October 28, 2011)

Education

July 1996: Secondary high school (Latin-Sciences-Mathematics), Damiaaninstituut Aarschot

July 2003: Medical Doctor, Katholieke Universiteit Leuven (1999-2003) – Universiteit Hasselt (1996-1999)

July 2010: General Surgeon, Katholieke Universiteit Leuven. Clinical training in General Surgery under guidance of Prof. dr. P. Broos, combined with scientific research and PhD training at the Reseach Unit of Experimental Cardiac Surgery under guidance of Prof. dr. P. Herijgers and Prof. dr. W. Flameng.

July 2012: Certificate of specific competence in Cardiac surgery, UZ Leuven under guidance of Prof. dr. B. Meyns

August 2012: Fellowship ZOL Genk, under guidance of Prof. dr. R. Dion

Papers in peer-reviewed journals

- I. Nevelsteen, V. Bito, **G. Van der Mieren**, A. Vanderper, A. Van den Bergh, K. Sipido, K. Mubagwa, P. Herijgers. ACE-Inhibition, but not weight reduction restores cardiomyocyte response to beta-adrenergic stimulation in the Metabolic Syndrome. *BMC Cardiovascular Disorders*. Submitted.

- A. Vanderper, I. Vanhorebeek, T. Van Assche, A. Carpi, B. Geeraert, **G. Van der Mieren**, I. Nevelsteen, W. Oosterlinck, G. Van den Berghe, J-L. Balligand, P. Holvoet, P. Herijgers. Failing delayed hypoxic preconditioning in a mouse model of the metabolic syndrome: in search of underlying mechanisms. *Cardiovascular Diabetology*. Submitted.

- **G. Van der Mieren**, I. Nevelsteen, A. Vanderper, W. Oosterlinck, W. Flameng, P. Herijgers. Angiotensin-converting enzyme inhibition and food restriction restore delayed preconditioning in diabetic mice. *Cardiovascular Diabetology* 2013; 12: 36.

- **G. Van der Mieren**, I. Nevelsteen, A. Vanderper, W. Oosterlinck, W. Flameng, P. Herijgers. Angiotensin-converting enzyme inhibition and food restriction in diabetic mice do not correct the increased sensitivity for ischemia-reperfusion injury. *Cardiovascular Diabetology* 2012; 11: 89.

- **G. Van der Mieren**, C. Van Kerrebroeck, H. Gutermann, R. Dion. Surgical angioplasty and unroofing technique for intramural coronary anomaly. *Interactive CardioVascular and Thoracic Surgery* 2011; 13: 424-426.

- W. Flameng, MC. Herregods, H. Hermans, **G. Van der Mieren**, M. Vercalsteren, G. Poortmans, J. Van Hemelrijck, B. Meuris. Effect of sutureless implantation of the Perceval S aortic valve bioprosthesis on intraoperative and early postoperative outcomes. *Journal of Thoracic and Cardiovascular Surgery* 2011; 142: 1453-1457.

- I. Mathar, R. Vennekens, M. Meissner, F. Kees, **G. Van der Mieren**, J. Camacho Londono, S. Uhl, B. Hummel, A. Van den Bergh, P. Herijgers, B. Nilius, V. Flockerzi, F. Schweda, M. Freichel. Increased catecholamine secretion contributes to hypertension in TRPM4-deficient mice. *Journal of Clinical Investigation* 2010; 120: 3267-3279.

- G. Buyse, **G. Van der Mieren**, M. Erb, J. D'hooge, P. Herijgers, E. Verbeken, A. Jara, A. Van den Bergh, L. Mertens, I. Courdier-Fruh, P. Barzaghi, T. Meier. Long-term blinded placebo-controlled study of SNT-MC17/idebenone in the dystrophin deficient mdx mouse: cardiac protection and improved exercise performance. *European Heart Journal* 2009; 30: 116-124.

- **G. Van der Mieren**, W. Flameng, P. Herijgers. The effect of type II diabetes and the metabolic syndrome on cardiac second window of preconditioning. *Verhandelingen Koninklijke Academie voor Geneeskunde van België* 2008; 70: 221-244.

- **G. Van der Mieren**, A. Van den Bergh, I. Nevelsteen, A. Vanderper, W. Flameng, P. Herijgers. Hypoxic preconditioning preserves cardiac contractility and reduces infarct size in vivo. *The Open Surgery Journal* 2008; 2: 24-29.

- **G. Van der Mieren**, J. Duchateau, P. Herijgers. Left atrial myxoma: presentation with acute aortic occlusion and resolution of the primary tumor. *Acta Chirurgica Belgica* 2007; 107: 687-689.

- **G. Van der Mieren**, C. de Gheldere, P. Vanclooster. Transmesosigmoid hernia : report of a case and review of the literature. *Acta Chirurgica Belgica* 2005; 105: 653-655.

- **G. Van der Mieren**, E. Hill, M-C. Herregods, W. Peetermans, J. Verhaegen, W. Flameng, P. Herijgers. Infectieuze endocarditis : diagnose en behandeling. *Tijdschrift voor Geneeskunde* 2005; 61: 1317-1327.

- **G. Van der Mieren**, J. Duchateau, Ph. De Vleeschauwer, J. De Leersnijder. The case for urgent carotid endarterectomy. *Acta Chirurgica Belgica* 2005; 105: 403-406.

- **G. Van der Mieren**, S. Willems, R. Sciot, H. Dumez, A. Van Oosterom, W. Flameng, P. Herijgers. Pericardial synovial sarcoma : 14-years survival with multimodality therapy. *Annals of Thoracic Surgery* 2004; 78: e41-42.

Presentations

- **G. Van der Mieren**, H. Gutermann, Chr. Van Kerrebroeck, R. Dion. Anomalous origin of the circumflex artery: an underestimated threat during mitral valve surgery.
  Presented: LOK cardiac surgery, Genk, 8 November 2012.

- B. Meuris, **G. Van der Mieren**, H. Hermans, M. Vercalsteren, MC. Herregods,W. Flameng. Sutureless aortic valve replacement: first experience with the perceval bioprosthesis.
  Videopresentation BACTS, 14 November 2009.

- **G. Van der Mieren**, F. Rega. Mogelijkheden in de klepchirurgie.
  Gepresenteerd: klinische lessenreeks assistenten heelkunde, UZ Leuven, 25 november 2009.

- **G. Van der Mieren**, W. Flameng, P. Herijgers. Het effect van type II diabetes en het metabool syndroom op cardiale tweede venster preconditionering.
  Gepresenteerd: definitief werkplan doctoraat, KU Leuven, 2 april 2009.

- **G. Van der Mieren**, W. Flameng, P. Herijgers. Preconditionering igv onderliggende pathologie.
  Gepresenteerd: LOK Cardiale Heelkunde, UZ Leuven, 24 maart 2009.

- **G. Van der Mieren**, W. Flameng, P. Herijgers. The effect of type II diabetes and the metabolic syndrome on cardiac second window of preconditioning.
  Gepresenteerd: departementsseminarie, KU Leuven, 6 juni 2008.

- **G. Van der Mieren**, A. Van den Bergh, W. Flameng, P. Herijgers. Second window of preconditioning in a type II diabetes mouse model.
  Posterpresentation - International Society for Heart Research, Bologna, 25 June 2007.
  Journal of Molecular and Cellular Cardiology 2007; 42: S18.

- **G. Van der Mieren**, G. Buyse, J. D'hooge, P. Herijgers. Dystrophin deficient MDX mice poorly tolerate dobutamine stress.
  Posterpresentation - Congres International Society for Heart Research, Bologna, 25 June 2007.
  Journal of Molecular and Cellular Cardiology 2007; 42: S164-165.

- G. M. Buyse, **G. Van der Mieren**, M. Erb, J. D'Hooge, P. Herijgers, E. Verbeken, A. Jara, L. Mertens, I. Courdier-Fruh, P. Barzaghi, T. Meier. A Long-term Blinded Controlled Efficacy Study of SNT-MC17/Idebenone in the Dystrophin-Deficient MDX Mouse.
  Presented: 9th Annual meeting of the American Academy of Neurology, Boston, 4 May 2007.

- **G. Van der Mieren**, P. Herijgers. Preconditionering en de mogelijke toepassingen in de chirurgie.
  Gepresenteerd: literatuurseminarie doctoraatsopleiding, KU Leuven, 11 april 2007.

- **G. Van der Mieren**, P. Herijgers. Effects of food restriction on cardiac function in type II diabetes and the metabolic syndrome.
  Gepresenteerd: Centrum voor Experimentele Heelkunde en Anesthesie, 13 maart 2007.

- **G. Van der Mieren**, P. Herijgers, B. Meyns. Experimentele Cardiale Heelkunde in kleine proefdieren.
  Gepresenteerd: lessenreeks vrij vak Experimentele Heelkunde, KU Leuven, 20 november 2006.

- **G. Van der Mieren**, J. Duchateau, Ph. De Vleeschauwer, W. Flameng, P. Herijgers. Left atrial myxoma: presentation with acute aortic occlusion and resolution of the primary tumor.
  Presented: Belgian Surgical Week, 5 May 2006.

- **G. Van der Mieren**, C. de Gheldere, P. Vanclooster. Buikwandherniae en anatomie van het lieskanaal.
  Gepresenteerd: postgraduaat onderwijs verpleging - H-Hart ziekenhuis Lier, 23 september 2004.

- **G. Van der Mieren**, J. Duchateau, Ph. De Vleeschauwer, J. De Leersnijder. The case for urgent carotid endarterectomy.
  Presented: Belgian Surgical Week, 6 May 2004.

Scientific awards

Laureaat prijs Prof. Dr. A. Lacquet ter bevordering van de Heelkunde 2004-2007.
Koninklijke Academie voor Geneeskunde, 15 november 2008.
G. Van der Mieren, W. Flameng, P. Herijgers.
Het effect van type II diabetes en het metabool syndroom op cardiale tweede venster preconditionering.