

CARDIOVASCULAR RISK AND STRESS ADAPTATION IN METABOLIC DISEASES

Ph.D. Thesis

Márta Julianna Sárközy MD

Cardiovascular Research Group

Department of Biochemistry

Faculty of Medicine

University of Szeged

Supervisor: Tamás Csont, MD, Ph.D.

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2 LIST OF PUBLICATIONS

1. The subject of the thesis is based on the following full papers:

- I. Kocsis GF, Sárközy M, Bencsik P, Pipicz M, Varga ZV, Pálóczi J, Csonka C, Ferdinandy P, Csont T. *Preconditioning protects the heart in a prolonged uremic condition. Am J Physiol Heart Circ Physiol.* 2012, **303**:H1229-1236, **IF: 3.629**
- II. Sárközy M, Zvara Á, Gyémánt N, Fekete V, Kocsis GF, Pipis J, Szűcs G, Csonka C, Puskás LG, Ferdinandy P, Csont T. *Metabolic syndrome influences cardiac gene expression pattern at the transcript level in male ZDF rats. Cardiovasc Diabetol.* 2013, **12**:16, **IF: 4.21**

Cumulative impact factor of papers subjected in the thesis: 7.839

2. Other full papers published during the Ph.D. fellowship:

- I. Monostori P, Kocsis GF, Okrös Z, Bencsik P, Czétényi O, Kiss Z, Gellén B, Bereczki C, Ocsovszki I, Pipis J, Pálóczi J, Sárközy M, Török S, Varga IS, Kiss I, Fodor E, Csont T, Ferdinandy P, Túri S. *Different administration schedules of darbepoetin alfa affect oxidized and reduced glutathione levels to a similar extent in 5/6 nephrectomized rats. Clin Exp Nephrol.* 2013, **17**:569-574, **IF: 1.246**
- II. Fekete V, Murlasits Z, Aypar E, Bencsik P, Sárközy M, Szénási G, Ferdinandy P, Csont T. *Myocardial postconditioning is lost in vascular nitrate tolerance. J Cardiovasc Pharmacol.* 2013, **62**:298-303, **IF: 2.290**
- III. Csont T, Sárközy M, Szűcs G, Szűcs C, Bárkányi J, Bencsik P, Gáspár R, Csonka C, Kónya C, Ferdinandy P. *Effect of a multivitamin preparation supplemented with phytosterol on serum lipids and infarct size in rats fed with normal and high cholesterol diet. Lipids Health Dis.* 2013, **12**:138, **IF: 2.19**

Cumulative impact factor of papers published during the Ph.D. fellowship: 5.726

Total impact factor: 13.565

3 LIST OF ABBREVIATIONS

ANOVA	analysis of variance
Akt	protein kinase B
ATP	adenosine triphosphate
CKD	chronic kidney disease
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-regulated kinases
GFR	glomerular filtration rate
GSK3 β	glycogen synthase kinase 3 beta
HOMA-IR	homeostasis model assessment-estimated insulin resistance
IHD	ischemic heart disease
IPost	ischemic preconditioning
IPre	ischemic postconditioning
I/R	ischemia/reperfusion
JAK	Janus kinase
K _{ATP}	ATP-sensitive potassium channel
LDL	low density lipoprotein
MEK	mitogen activated protein kinase
mPTP	mitochondrial permeability transition pore
OGTT	oral glucose tolerance test
PI3K	phosphatidylinositol 3-kinase
qRT-PCR	quantitative real time polymerase chain reaction
RISK	reperfusion injury salvage kinase
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAFE	survivor activating factor enhancement
STAT3	signal transducer and activator of transcription 3 factor
STZ	streptozotocin
T2DM	type 2 diabetes mellitus
TNF α	tumor necrosis factor alpha
ZDF	Zucker Diabetic Fatty

4 SUMMARY

Ischemic heart disease (IHD) is the leading cause of death in developed countries. It is well known that IHD is a multifactorial pathological condition developing as a consequence of both genetic and environmental factors as well as various interactions between them. Furthermore, IHD generally coexists with other metabolic risk factors and comorbidities including hypercholesterolemia, metabolic syndrome, diabetes mellitus, etc. Moreover, increasing prevalence of hypercholesterolemia and diabetes mellitus in the aging population results in a dramatic rise in the prevalence of chronic kidney disease (CKD) characterized by severe metabolic changes generally termed as uremia. In addition, uremia has been shown to increase the risk of cardiovascular morbidity and mortality.

Brief periods of ischemia applied before or immediately after a potentially lethal myocardial ischemia confer remarkable cardioprotection. These endogenous cardioprotective mechanisms, termed as ischemic preconditioning (IPre) and postconditioning (IPost) have been demonstrated to be abolished by several metabolic risk factors and comorbidities e.g. hypercholesterolemia, metabolic syndrome, and diabetes mellitus.

Although, the key cellular events of IPre and IPost are extensively studied in normal and comorbid states, the exact mechanisms of myocardial changes in chronic metabolic disorders as well as loss of IPre and IPost are still unknown. Beside the traditional biochemical and pharmacological approaches, genomics may provide new potentials to investigate key molecular events in the mechanisms of myocardial changes in chronic metabolic disorders as well as in the cellular events of IPre and IPost both in normal and comorbid states.

Using DNA microarray, we have previously shown in separate studies that both IPre and IPost modify myocardial ischemia/reperfusion (I/R)–induced gene expression pattern in rats in the absence of any metabolic risk factor. Similarly, our group has previously shown that a cardiovascular risk factor, hypercholesterolemia alters cardiac gene expression profile in rats. Other cardiovascular risk factors including type 2 diabetes mellitus (T2DM) and CKD have been shown to influence cardiac gene expression profile. Therefore, in the present thesis we investigated the effect of another cardiovascular risk factor and comorbid state, the metabolic syndrome on cardiac gene expression pattern in order to examine the basic differences in cardiac metabolic changes in a complex metabolic disorder. We have found genes with significantly altered cardiac gene expression due to metabolic syndrome including functional clusters of metabolism, structural proteins, signal transduction, stress response, ion channels and receptors. Moreover, some other genes with no definite functional clusters were also changed. In conclusion, metabolic syndrome

significantly alters myocardial gene expression profile which may be involved in the development of cardiac pathologies in the presence of metabolic syndrome.

We and others have previously shown that cardiovascular risk factors and comorbid states including hypercholesterolemia as well as T2DM attenuate the cardioprotective effect of IPre and IPost. However, it has been demonstrated previously that cardioprotective effect of both IPre and IPost are preserved in experimental subacute renal failure. Therefore, in the present thesis, we examined whether another cardiovascular risk factor and comorbid state, prolonged uremia, affects the I/R injury and cardioprotection by IPre. We have found that although prolonged experimental uremia leads to severe metabolic changes and the development of a mild myocardial dysfunction, the cardioprotective effect of IPre is still preserved. In conclusion, our present study suggests that patients suffering from long-term uremia may also benefit from cardioprotection by IPre. Since uremic patients are regularly excluded from clinical trials, there is a need for clinical studies to investigate the cardioprotective effect of conditioning techniques in patients with chronic renal failure suffering from IHD.

5 INTRODUCTION

5.1 Epidemiology and metabolic risk factors for ischemic heart diseases

Diseases related to the heart and circulatory system are termed as cardiovascular diseases (CVD) which are the major cause of morbidity and mortality in the industrialized world. In the USA, CVD accounted for 17 million of the total 57 million deaths in 2008 [1]. By 2030, the number of CVD deaths is expected to rise to 23.6 million in the USA [2]. There are different forms of CVD conditions, among which IHD is the most predominant form of all cases and deaths [1]. IHD develops due to atherosclerosis leading to different clinical phenotypes from a less severe angina to more severe forms including acute myocardial infarction, chronic ischemia or even sudden cardiac death [1]. IHD is a multifactorial pathological condition developing as a consequence of both genetic and environmental factors as well as interaction between them [1]. It is well known that IHD generally coexists with other metabolic risk factors and comorbidities [1, 3] including hyperlipidemia, metabolic syndrome (defined as the coexistence of visceral obesity, dyslipidemia, hyperglycemia, and systemic arterial hypertension), atherosclerosis, insulin resistance as well as diabetes mellitus, hypertension-related left ventricular hypertrophy, heart failure, and aging [3]. Moreover, increasing prevalence of hyperlipidemia and diabetes mellitus in the aging population results in a dramatic rise in the prevalence of chronic kidney disease characterized by severe metabolic changes generally termed as uremia [4]. In addition, uremia and especially end-stage renal failure have been shown to increase the risk of cardiovascular morbidity and mortality [5, 6].

Hypercholesterolemia

Hypercholesterolemia is defined as the presence of elevated serum cholesterol levels including total cholesterol (>5.2 mmol/L) and LDL-cholesterol (>2.5 mmol/L) [7, 8]. Large clinical studies showed that a significant population of adults is affected by hypercholesterolemia in the developed countries [9]. In the USA, approximately 100 million people (44.4%) suffered from hypercholesterolemia (>5.2 mmol/L for total cholesterol level) in 2008 [2]. The role of an increased serum LDL-cholesterol level as a predictor of CVD is of great importance since every 1.0 mmol/L decrease in LDL-cholesterol level has been reported to be associated with a corresponding 22% reduction in CVD morbidity and mortality [8]. Moreover, several experimental studies have demonstrated that in addition to the well-known pro-atherogenic effect in the vasculature, hyperlipidemia may directly affect the heart causing contractile dysfunction [10, 11].

Type 2 diabetes mellitus

Type 2 diabetes mellitus is defined as a metabolic disorder characterized by elevated serum glucose level (>6.1 mmol/L fasting glucose concentration or >11.1 mmol/L glucose concentration 2 hours after OGTT) with insulin resistance and relative insulin deficiency [12]. The rapid increase in the prevalence of T2DM across the world gives diabetes the status of an epidemic in the 21st century [13]. In the last decades, there was an explosive increase in the number of people diagnosed with T2DM worldwide due to aging as well as increasing prevalence of obesity and physical inactivity [13-15]. In the USA, an estimated 18 million people had diagnosed T2DM, representing 8.0% of the adult population in 2008 [2]. In addition, 36.8% of the adult population had pre-diabetes, with abnormal fasting glucose levels in 2008 [2]. Worldwide, the total number of people with diabetes is projected to rise from 347 million in 2008 [16] to 552 million in 2030 [17]. Epidemiological studies and clinical trials [3, 18-20] have shown that T2DM patients are more prone to developing CVD (2-4 fold relative risk) [21], including acute myocardial infarction (7 fold relative risk) [21].

Metabolic syndrome

Metabolic syndrome is defined as the coexistence of visceral obesity, dyslipidemia, hyperglycemia, and hypertension [22, 23]. Most individuals with metabolic syndrome have abdominal obesity and develop insulin resistance, therefore the prevalence of metabolic syndrome and pre-diabetes overlap [24, 25]. In addition, metabolic syndrome can be considered as a direct precursor state of T2DM [24] and CVD [24, 26]. Metabolic syndrome affects a large population including all ages from children to elderly and both sexes worldwide [27-29]. According to the Third National Health and Nutrition Examination Survey (NHANES III) criteria, about 47 million people (approximately 24% of the US adult population) had metabolic syndrome in the USA in 2002 [30]. Its prevalence is raising both in developed [27, 30], and in developing countries [27, 30]. In addition, patients suffering from metabolic syndrome have an approximate 5-fold increase in diabetes [24, 31] and 2.5 fold increase in chronic renal failure [32] risk compared with persons without metabolic syndrome. It is well established that metabolic syndrome is a major risk factor for cardiovascular diseases [33-36]. Several epidemiological studies have demonstrated that metabolic syndrome was associated with an approximately 1.5-3 times greater risk of CVD and IHD mortality [37, 38].

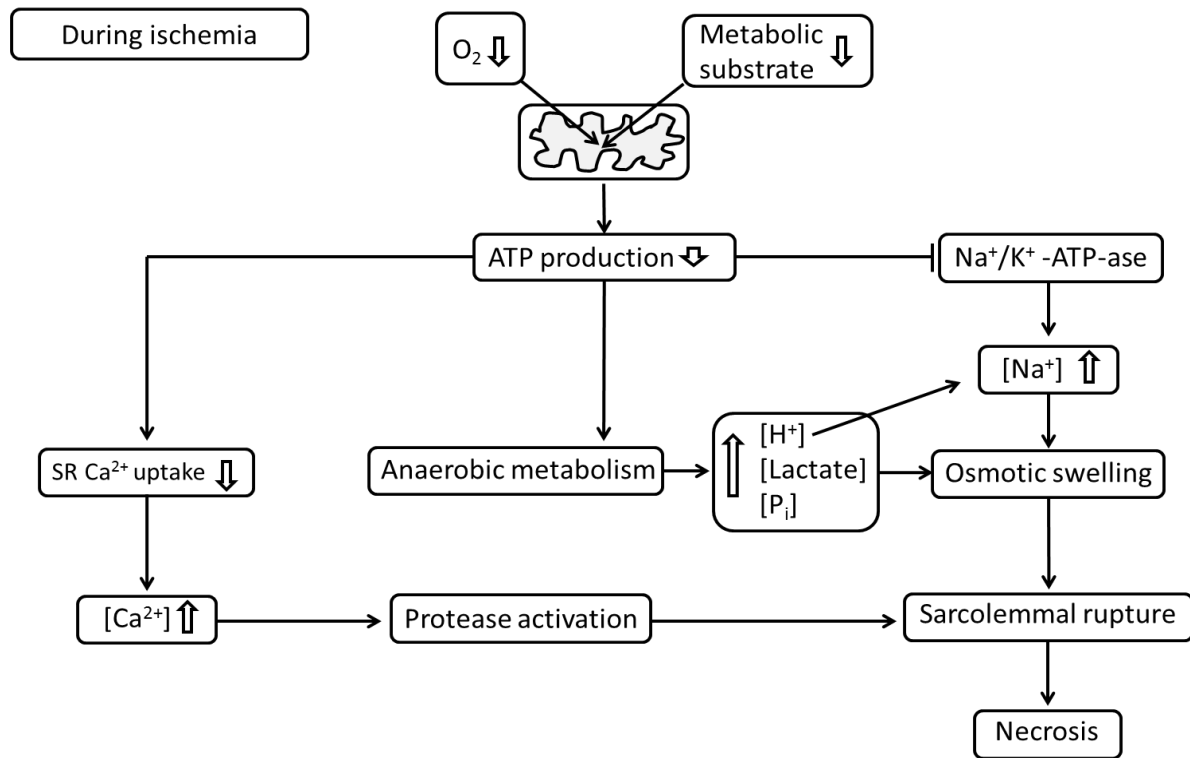
Chronic kidney disease

Chronic kidney disease is defined as either kidney damage for more than 3 months confirmed by kidney biopsy or markers of kidney damage (e.g. albuminuria), with or without a decrease in glomerular filtration rate (GFR), or $\text{GFR} < 60 \text{ mL} \cdot \text{min}^{-1}/1.73 \text{ m}^2$ for more than 3 months, with or without kidney damage [4, 39]. CKD is a worldwide public health problem due to the increasing prevalence of T2DM, hyperlipidemia and hypertension [4]. According to a meta-analysis, the prevalence of CKD was approximately 7.2% in people aged 30-64 years and varied from 23.4% to 35.8% in patients 64 years or older worldwide [40]. In the USA, prevalence of CKD was 13.1% in 1999-2004 [41]. Despite the significant number of people with CKD, the majority of them remain unaware of their condition for several years until reaching late stages of CKD [4, 42]. According to the US National Kidney Foundation's Kidney Early Evaluation Program, only 9% of patients with CKD were aware of their diagnosis from 2000 to 2009 [43]. CKD and especially end-stage renal failure have been shown to increase the risk of cardiovascular morbidity and mortality [5, 44]. The prevalence of IHD at the start of dialysis is approximately 40% [45]. In fact, CVD is the main cause of death in patients with chronic renal failure [39, 44]. Indeed, CVD mortality is approximately 10 to 30 times higher in CKD patients on dialysis than in patients in the general population irrespectively for age [39]. After stratification for age, CVD mortality remains approximately 5-fold higher in dialysis patients than in the general population [39]. The incidence of myocardial infarction is high in dialysis patients and the outcome after myocardial infarction is poor [46]. In dialysis patients, one and two year mortality rate after myocardial infarction was 59% and 73%, respectively, which is much higher than after acute myocardial infarction in the general population, even in subjects with other comorbid states e.g. diabetes mellitus [39]. In addition, left ventricular hypertrophy and dysfunction are notable in a significant number of patients on chronic dialysis [39, 46-48].

5.2 Myocardial ischemia/reperfusion injury

IHD means different clinical manifestations of myocardial ischemia including acute myocardial infarction [1]. Myocardial ischemia develops when coronary flow to the myocardium is relatively or absolutely reduced which results in myocardial ischemic injury [3]. During ischemia, oxygen supply to the mitochondria is inadequate to support oxidative phosphorylation and the related ATP synthesis [3]. The imbalance of intracellular oxygen and energy supply leads to intracellular accumulation of inorganic phosphate, lactate, H^+ , Na^+ and Ca^{2+} [3, 49] (Figure 1). The increasing intracellular concentrations of solutes result in osmotic swelling with increased sarcolemmal

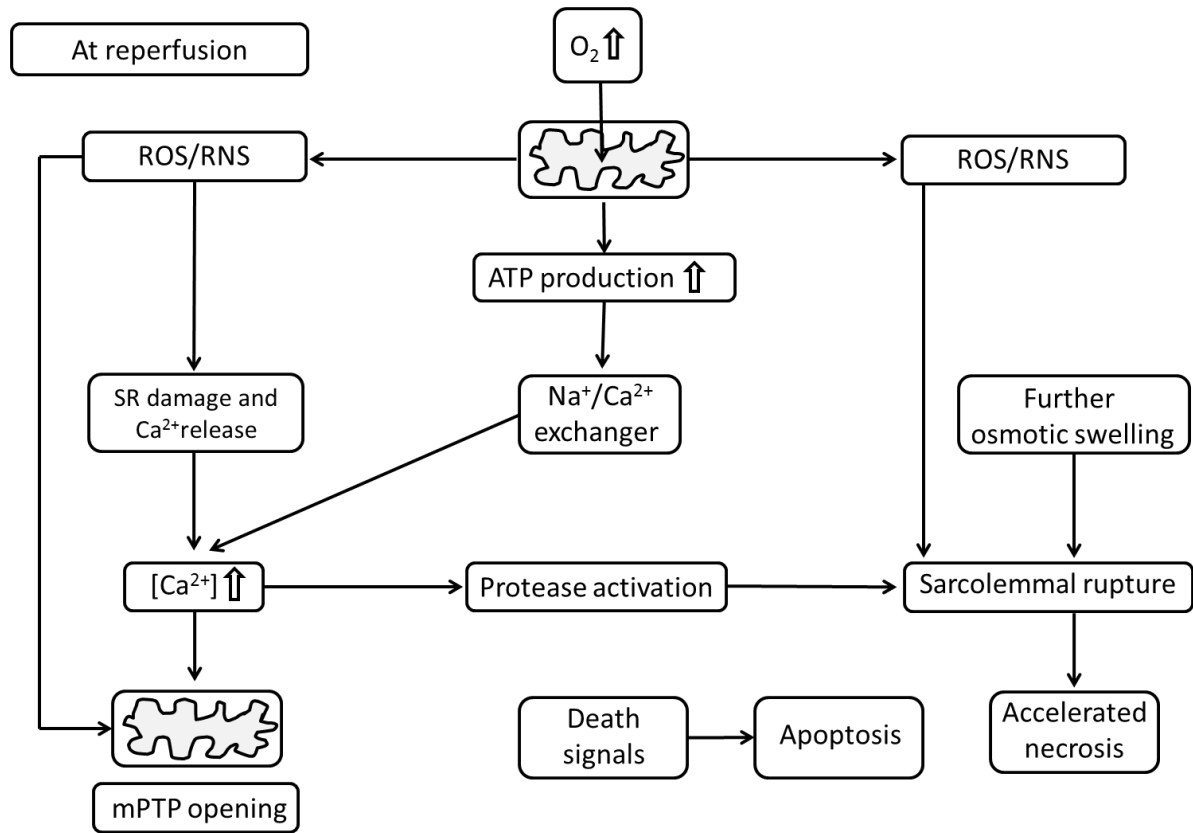
fragility and cell disruption as well as activation of Ca^{2+} -dependent nucleases, proteases and phospholipases (Figure 1) resulting in the destruction of membranes and leading to cell death [3, 49]. The process of irreversible cell injury is time-dependent and results in the pathological features of necrosis (Figure 1).



1. Figure. Myocardial injury during ischemia (modified from Ferdinandy et al., Pharmacol Rev. 2007).

Under experimental circumstances and in clinical situations, ischemia may be followed by reperfusion, that is, the readmission of oxygen and metabolic substrates with washout of ischemic metabolites [3] (Figure 2). Although, reperfusion is essential to salvage ischemic tissue, it has the potential to cause further irreversible cell damage termed as reperfusion injury [3]. Paradoxically, the sudden readmission of metabolic substrates and oxygen contributes to reperfusion injury [3]. Reperfusion leads to increasing intracellular pH due to H^+ washout and inadequately restored ATP synthesis [3], Ca^{2+} overload and reduced contractility, coronary endothelial dysfunction, low production of nitric oxide and oxidative stress, migration of inflammatory cells, disruption of membranes and induction of apoptotic and necrotic signals in the myocardium [49, 50] (Figure 2). The recovery of pH, oxidative/nitrative stress and Ca^{2+} accumulation can induce the opening of the

mitochondrial permeability transition pores (mPTP) which strongly contributes to myocardial apoptosis and necrosis [49] (Figure 2).



2. Figure Myocardial reperfusion injury (modified from Ferdinandy et al., Pharmacol Rev. 2007).

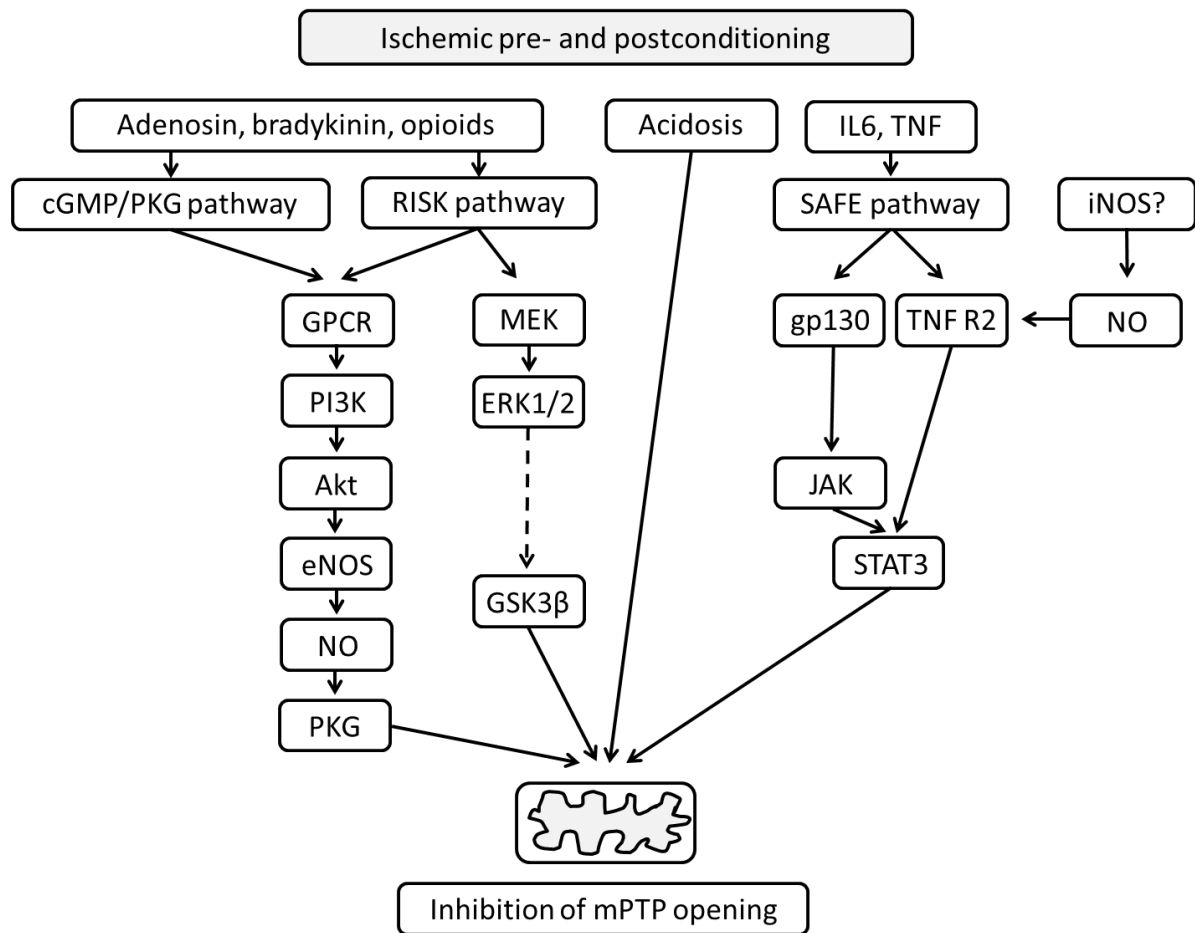
In summary, myocardial ischemia and reperfusion leads to “ischemia/reperfusion injury” which might result in the development of myocardial infarction, contractile dysfunction and arrhythmias.

5.3 Endogenous cardioprotective mechanisms of the myocardium

Coronary interventions and thrombolytic therapies are the most widely used clinical interventions to reduce infarct size by reopening the occluded arteries. Pharmacologic agents (e.g., nitrates and beta blockers) reducing energy demand and/or increasing coronary flow are also common strategies to ameliorate infarct size. An alternative approach to minimize the development of myocardial infarction is the triggering of endogenous adaptive mechanisms of the heart.

Experimentally, the most powerful endogenous adaptive cardioprotective mechanisms are the ischemic preconditioning and postconditioning (Figure 3). IPre is a well described endogenous adaptive mechanism in which brief exposure to I/R markedly enhances the ability of the heart to withstand a subsequent, potentially lethal I/R injury [3, 51]. Brief cycles of I/R applied after a

longer period of ischemia also confer cardioprotection against myocardial I/R injury. This phenomenon is termed as IPost [3, 52]. The cardioprotective effect of IPre and IPost results in attenuation of I/R injury characterized by improvement of post-ischemic contractile function, a decrease in the occurrence and severity of arrhythmias, and a reduction in infarct size [3].



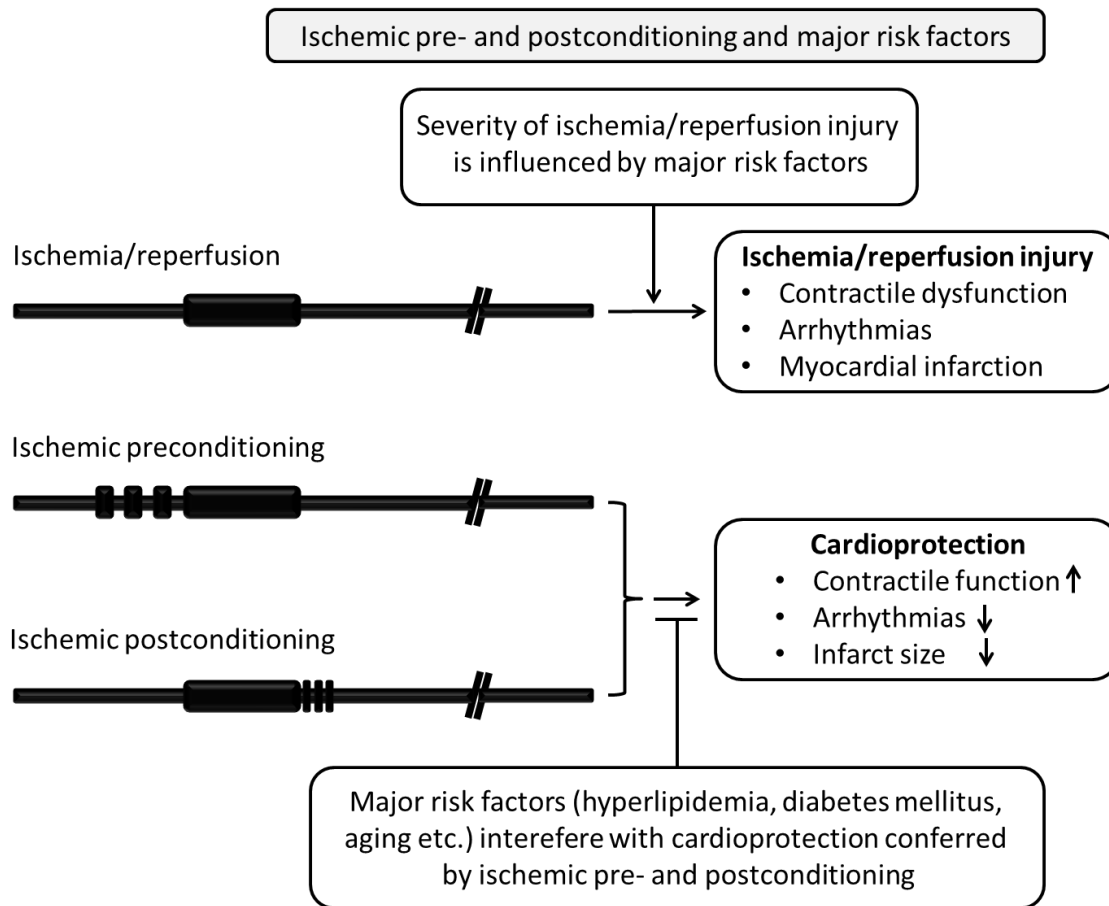
3. Figure Potential mechanism of ischemic pre- and postconditioning (modified from Perrelli MJ et al, World J Cardiol, 2011).

The exact mechanisms responsible for the cardioprotective mechanisms of IPre and IPost, are still not fully understood. Briefly, both IPre [3] and IPost [53] are considered to be triggered by a number of endogenous factors, including adenosine, bradykinin, and opioids (Figure 3). Two major intracellular signaling cascades have been proposed as mediators both of the preconditioning and postconditioning stimulus, the reperfusion injury salvage kinase (RISK) [54, 55] pathway and the survivor activating factor enhancement (SAFE) [55, 56] pathway (Figure 3). The RISK pathway is considered to consist of two arms, one of which involves mitogen activated protein kinase 1/2 (MEK1/2) and extracellular signal-regulated kinases 1/2 (ERK1/2) and the other involves

phosphatidylinositol 3-kinase (PI3K) and Protein kinase B (Akt) (Figure 3) [55]. Both arms are thought to be triggered by the binding of specific ligands with G-protein–coupled receptors and terminate on glycogen synthase kinase 3 beta (GSK3 β) to reduce the likelihood of mitochondrial permeability transition pore (mPTP) opening and thus attenuate reperfusion injury [54, 55] (Figure 3). The activation of the SAFE pathway including the activation of the cytokine tumor necrosis factor alpha (TNF α) [56] and the transcription factor signal transducer and activator of transcription-3 (STAT-3) [57] is now recognized as a “RISK-free” pathway that can confer cardioprotection by both in IPre and IPost (Figure 3). As with the RISK pathway, inhibition of the mPTP is thought to be the end effector of this pathway [56] (Figure 3). In addition, mitochondrial ATP-sensitive potassium channel (K_{ATP})-mediated inhibition of mPTP opening also thought to play an important role both in the mechanism of IPre and IPost [3, 58]. Moreover, another consequence of mitochondrial K_{ATP} opening, the enhanced generation of reactive oxygen (ROS) and nitrogen species (RNS) seems to be an obligatory part of the signaling cascade [3, 59]. It is likely that ROS/RNS signal activates distal kinases which may include MEK, JAK and STAT [3].

5.4 The effects of cardiovascular risk factors on IPre and IPost

A number of evidence have demonstrated that IPre and IPost are powerful interventional strategies to limit I/R-induced myocardial injury [3]. Despite this fact, cardioprotection by ischemic conditioning techniques is affected in the presence of a number of cardiovascular risk factors and chronic metabolic diseases e.g. hyperlipidemia, atherosclerosis, metabolic syndrome, diabetes mellitus and insulin resistance [3]. This suggests that IPre and IPost are moderately “healthy heart protective phenomena” [3]. The mechanisms by which these cardiovascular risk factors and chronic metabolic diseases interfere with cardioprotective effects of ischemic conditioning techniques are uncertain and are not completely understood [3]. Therefore, the development of rational therapeutic strategies to protect the heart against ischemia requires preclinical studies that examine cardioprotection specifically in relation to complicating cardiovascular risk factors and chronic metabolic diseases [3].



4. Figure Cardioprotective effects of ischemic pre- and postconditioning are influenced by major cardiovascular risk factors and comorbidities (modified from Ferdinandy et al., Pharmacol Rev. 2007).

Hyperlipidemia and ischemic pre- and postconditioning

Although IPre confers remarkable cardioprotection in a variety of species [3, 53], including humans [60-62], we and others have shown that its effectiveness is attenuated by hyperlipidemia [3, 63] (Figure 4-5). Szilvassy et al. [3, 64] demonstrated for the first time in the literature that cardioprotection conferred by pacing-induced preconditioning was lost in rabbits developing hypercholesterolemia and atherosclerosis after 8 weeks of 1.5% cholesterol-enriched diet. After re-exposure to a normal diet, reduction of serum cholesterol levels recaptured the cardioprotective effect of pacing-induced preconditioning despite the presence of atherosclerosis [3, 64]. This result proved that hypercholesterolemia, independently from the development of atherosclerosis, interferes with the cardioprotective mechanisms of pacing-induced preconditioning [3, 64]. The loss of pacing-induced preconditioning was subsequently confirmed in isolated hearts from rats fed with cholesterol-enriched diet without development of atherosclerosis, since rats do not develop significant atherosclerosis due to high-cholesterol diet [3, 65]. The loss of the anti-ischemic effect of IPre (assessed by ST-segment elevation) in hyperlipidemia has been confirmed by two

independent groups in patients undergoing repeated balloon inflations during coronary angioplasty [3, 62, 66] as well. Limited literature data are available on the effect of IPost in hyperlipidemia. Iliodromitis et al. [3, 67] showed that the infarct size-limiting effect of IPost is lost in rabbits with experimental hyperlipidemia and atherosclerosis. The loss of the infarct size-limiting effect of IPost has been also confirmed by our group in hearts isolated from cholesterol-fed rats [59].

Though the precise mechanisms involved in the loss of cardioprotective effects of ischemic conditioning techniques in hypercholesterolemia are uncertain, reduced activation of Akt signals, redistribution of both sarcolemmal and mitochondrial Connexin 43, down-regulation of tetrahydrobiopterin and subsequent inactivation of NOS, impaired opening of mitochondrial-K_{ATP} channels, eNOS uncoupling, increased oxidative and nitrosative stress and high incidence of proapoptotic signaling could play pivotal roles in the loss of cardioprotective effects of ischemic conditioning techniques in hyperlipidemic myocardium [50].

Diabetes mellitus and ischemic pre- and postconditioning

A number of preclinical and clinical studies have examined the cardioprotective effect of IPre and IPost in T2DM [3]. The majority of these studies showed that diabetes interferes with cardioprotective mechanisms, attenuating the effectiveness of cardioprotective strategies (Figure 4-5) [3].

The first preclinical study to examine the IPre in experimental streptozotocin (STZ)-induced diabetes was reported by Liu et al. [3, 68]. Two weeks after STZ injection, hearts of diabetic rats were found to be more resistant to myocardial infarction in vivo as compared to normal control hearts, and IPre afforded additional cardioprotection [3, 68]. Another study by Tosaki et al. reported that brief cycles of preconditioning ischemia did not confer protection against I/R induced arrhythmias, stunning, and intracellular Na⁺ accumulation and K⁺ efflux in diabetic rats 4 or 8 weeks after the STZ-treatment in isolated rat hearts [3, 69]. In addition, IPre failed to reduce the infarct size in dogs 3 weeks after diabetes induction [3, 70]. Therefore, the duration of the diabetic state is a crucial factor in the efficacy of IPre. Some clinical observations suggest that IPre or preconditioning-like phenomena are impaired in diabetic patients with ischemic heart disease [3]. Angina, considered to be a correlation factor of IPre in patients, failed to limit infarct size, enhance recovery after myocardial function, or improve survival in diabetic patients with myocardial infarction as compared to non-diabetic patients [3, 71]. Impairment of IPre during coronary angioplasty was observed in diabetic patients as well [3, 72].

In fact, literature data on the efficacy of IPost in T2DM are controversial. Some preclinical studies have shown that the cardioprotective effect of IPost is lost in murine model of T2DM [3, 73].

Another preclinical study has shown that IPost confers infarct size limiting effect in STZ-induced diabetic mice [74]. Though the precise mechanisms involved in loss of cardioprotection by IPre and IPost in diabetes mellitus are uncertain, GSK-3 β phosphorylation and mPTP opening upon reperfusion seem to be crucial points [75]. However, multiple protective signaling pathways appear to converge on GSK-3 β regulation and inhibition of mPTP opening, T2DM-induced defects in these protective signaling pathways may be different depending on the model and/or phase of T2DM [75].

Metabolic syndrome and ischemic pre- and postconditioning

Although, the effect of metabolic syndrome on gene expression pattern in various tissue types has been investigated in a few studies (liver, skeletal muscle, adipose tissue [76] and pancreatic β -cells [77] obtained from the well-known metabolic syndrome model Zucker Diabetic Fatty (ZDF) rat, limited or no data are available on the effect of metabolic syndrome on cardiac gene expression pattern in relation to differences in basic cardiac metabolic features, susceptibility to myocardial I/R injury or endogenous cardioprotective mechanisms (Figure 5). The degree to which metabolic syndrome or its components contribute to myocardial I/R injury is unknown. However, the efficacy of cardioprotective mechanisms appears to be abolished in the presence of pathological conditions associated with metabolic syndrome such as obesity, hyperlipidemia and hyperglycemia. In hearts from ZDF rat, IPre did not afford protection against I/R injury [78]. In another well-known model of metabolic syndrome, in the ob/ob mice cardioprotection by IPost was also lost [79, 80].

Chronic kidney disease and ischemic pre- and postconditioning

One may speculate that the severe metabolic changes seen in uremia may interfere with endogenous cardioprotective mechanisms as seen with other metabolic diseases (Figure 5). However, there are limited data on the possible interaction of CKD with endogenous adaptive cardioprotective mechanisms including IPre and IPost. In a preliminary study, we have demonstrated for the first time in the literature that the infarct size limiting effect of IPost was still present 10 weeks after subtotal nephrectomy resulting in uremia in rats [81]. Moreover, an extensive study published by Byrne et al. [55] has reported that IPre, IPost, and remote ischemic conditioning are still cardioprotective after 4 weeks of subtotal nephrectomy. In addition, in the same study IPre was shown to limit infarct size in an adenine-enriched diet-induced model of uremia in rats [55]. It has been shown in this study, that IPre and IPost in a subacute renal failure model were associated with an increase in phospho-STAT3 and phospho-ERK1/2 representing key mediators of RISK and

SAFE pathways [55]. Although the uremic state seems to be too short and was not characterized thoroughly in Byrne's aforementioned studies [55], they may suggest that the uremic heart can be still protected by conditioning techniques. Indeed, an experimental model of 4 weeks of uremia probably may not properly reflect the clinical situation, since uremia may remain unexplored for a long time in patients [4, 82]. Additionally, it has been shown that 5/6 partial nephrectomy followed by a postoperative duration of 3 weeks does not lead to advanced uremia in rats [83]. However, it is not known if a substantially longer period of uremia may influence endogenous cardioprotective mechanisms. Therefore, in the present thesis we examined the influence of prolonged uremia (30 weeks) on the severity of I/R injury and the infarct size limiting effect of IPre.

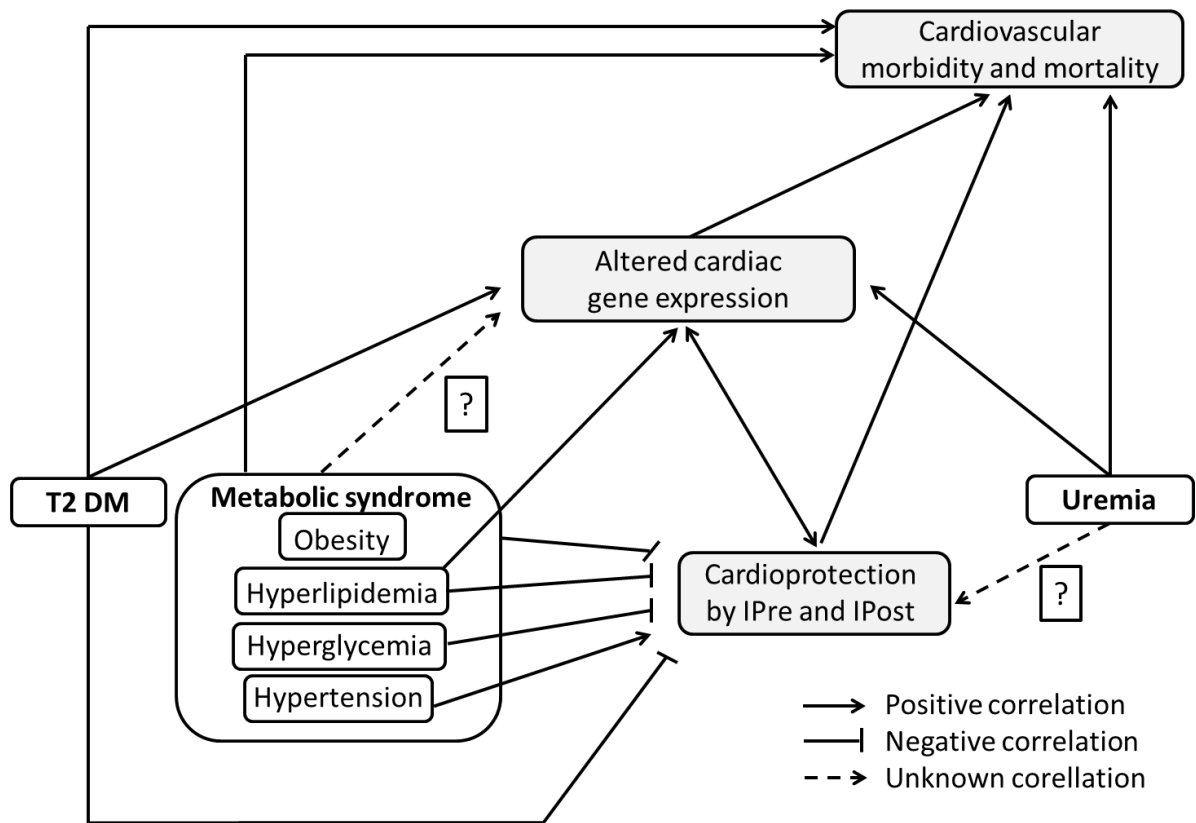
5.5 Alterations in myocardial gene expression by IPre and IPost in normal and comorbid conditions

Although, the molecular mechanisms of IPre and IPost are extensively studied, the exact signal transduction pathways of IPre and IPost are still unknown due to the complexity of these mechanisms. Beside the traditional biochemical and pharmacological approaches, genomics may provide new potentials to investigate key molecular events in the mechanisms of IPre and IPost. We and others have previously shown that IPre significantly influenced myocardial gene expression pattern as assessed by miRNA chip [84] as well as DNA microarray and qRT-PCR [85, 86]. We have previously demonstrated that hypercholesterolemia resulted in marked changes in preconditioning-induced gene expression which might lead to significant alterations of oxidative/nitrative stress signaling and to the loss of the cardioprotective effect of IPre [86] (Figure 5). However, another cardiovascular risk factors and comorbid conditions including T2DM [87, 88] and CKD [89] have been shown to influence cardiac gene expression profile (Figure 5), the alterations in myocardial gene expression by IPre and IPost in the presence of these cardiovascular risk factors and comorbid conditions are not investigated yet. Therefore, in the present thesis we investigated the effect of another cardiovascular risk factor, the metabolic syndrome on cardiac gene expression pattern in order to examine the basic differences in cardiac metabolic changes which might provide a new potential to understand the mechanisms of different susceptibility to myocardial I/R injury in metabolic syndrome.

6 AIMS

The aims of our present thesis were to

1. investigate the effect of metabolic syndrome on cardiac gene expression pattern in male ZDF rats which is a well-known model of metabolic syndrome.
2. examine the influence of another severe metabolic state, the prolonged uremia (30 weeks) on the severity of
 - a. myocardial I/R injury and
 - b. the infarct size limiting effect of IPre.



5. Figure Effects of comorbidities on cardiovascular morbidity and mortality, cardiac gene expression and cardioprotection conferred by IPre and IPost.

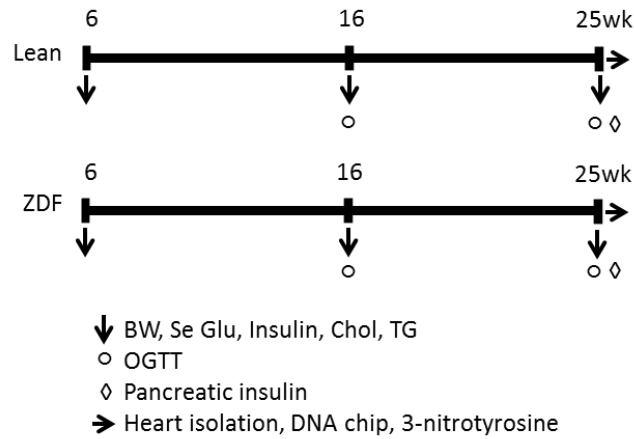
7 MATERIALS AND METHODS

These investigations conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996) and was approved by the Animal Research Ethics Committee of the University of Szeged.

7.1 Experimental setup for investigation of cardiac gene expression pattern in metabolic syndrome

Male Zucker Diabetic Fatty (ZDF/Gmi-fa/fa) rats and their lean controls were obtained from Charles River Laboratories at the age of 5 weeks and were housed at 22±2°C with a 12:12-h light-dark cycle. The rats received Purina 5008 chow and water ad libitum for 20 weeks after their arrival. The Zucker diabetic fatty rat with a point mutation in the leptin receptor is a recognized model of obesity, hyperlipidemia, hyperglycemia and hypertension [90-92]. In the present study, only male rats were used, since female ZDF rats are less prone to the development of metabolic syndrome [93, 94]. Male ZDF rats develop an age-dependent obese and hyperglycemic phenotype at 10-12 weeks of age accompanied by a metabolic state of obesity, dyslipidemia, hyperinsulinemia and insulin resistance [95, 96] which develops to a hyperglycemic insulin-deficient state [95]. The metabolic features manifested in this animal model are in many ways similar to the pathogenesis of metabolic syndrome in humans [95, 97]. Therefore, the ZDF rat is an ideal model for investigation of cardiac gene expression pattern changes related to human metabolic syndrome.

Body weight, serum glucose, insulin, cholesterol and triglyceride levels and homeostasis model assessment-estimated insulin resistance (HOMA-IR) were determined at 6, 16 and 25 weeks of age in order to monitor the basic parameters of glucose and lipid metabolism and insulin resistance in ZDF and lean rats (Figure 6). Oral glucose tolerance test (OGTT) was performed at week 16 and 25 in order to further characterize glucose homeostasis in ZDF and lean rats (Figure 6). At 25 weeks of age, rats were anaesthetized using diethyl ether. Hearts and pancreata were isolated (Figure 6), and then hearts were perfused according to Langendorff as described earlier [98]. After 10 min perfusion, ventricular tissue was frozen and stored at -80°C until DNA microarray investigation and gene expression analysis (Figure 6). To validate the well-known nitrate stress-inducing effect of metabolic syndrome on the heart, frozen ventricular tissue was used for determination of cardiac free 3-nitrotyrosine level (Figure 6).



6. Figure Experimental protocol. Male ZDF rats and their lean controls were followed up from 6 weeks of age until 25 weeks of age. Body weight (BW), serum glucose (Se Glu), insulin (Insulin), cholesterol (Chol) and triglyceride (TG) levels were determined at week 6, 16 and 25. Oral glucose tolerance test (OGTT) was performed at week 16 and 25. At week 25, hearts and pancreata were isolated. Pancreata were frozen and homogenized to measure pancreatic insulin levels. Hearts were perfused according to Langendorff for 10 minutes using Krebs Henseleit buffer. Then ventricular tissue was frozen and used for DNA microarray analysis and cardiac free 3-nitrotyrosine level measurements.

Serum glucose level measurements and OGTT

Rats were fasted overnight (12 h) prior to serum glucose level measurements (week 6, 16 and 25) and OGTTs (week 16 and 25) to verify the development of hyperglycemia as a diagnostic criterion of metabolic syndrome. Blood samples were collected from the v. saphena. Blood glucose levels were measured using AccuCheck blood glucose monitoring systems (Roche Diagnostics Corporation, USA, Indianapolis). In case of OGTT, after the measurement of baseline glucose concentrations, a standard dose of glucose (1.5 g/kg body weight) was administered per os via gavage and plasma glucose levels were checked 30, 60 and 120 minutes later. Area under the curve values for OGTT was also calculated.

Measurement of serum and pancreatic insulin levels

Serum and pancreatic insulin levels were measured by an enzyme linked immunosorbent assay (Mercodia, Ultrasensitive Rat Insulin ELISA) in order to verify the development of hyperinsulinemia and decreased pancreatic insulin content as a consequence of beta cell damage in metabolic syndrome. Insulin ELISA was carried out according to the instructions of the manufacturer from either sera or homogenized pancreatic tissue samples of ZDF and lean control rats. Sera were centrifuged (4500 rpm for 10 min at 4°C) and kept at -20°C until further investigation. Pancreata were removed, trimmed free of adipose tissue and weighed. Pancreata were

homogenized in 6 ml cold acidified-ethanol (0.7 M HCl: ethanol (1:3 v/v) with an Ultraturrax homogenizer and were kept at 4°C for 24 h. Then pancreas homogenates were centrifuged (900 g for 15 min at 4°C), and the supernatants were stored at 4°C. The pellet was extracted again with 3 ml acidified ethanol for 24 h at 4°C. The supernatant obtained after centrifugation was pooled with the previous one and kept at -20°C until assayed.

HOMA-IR index

To estimate insulin resistance in ZDF or lean rats the widely used HOMA-IR index was calculated [99-101] by multiplying fasting plasma insulin ($\mu\text{IU/mL}$) with fasting plasma glucose (mmol/L), then dividing by the constant 22.5, i.e. $\text{HOMA-IR} = (\text{fasting plasma insulin concentration} \times \text{fasting plasma glucose concentration}) / 22.5$.

Measurement of serum lipid levels

Serum cholesterol and triglyceride levels were measured at week 6, 16 and 25 using a test kit supplied by Diagnosticum Zrt. (Budapest, Hungary) as described previously [11] in order to follow up the development of hyperlipidemia which is a diagnostic criterion of metabolic syndrome.

Cardiac 3-nitrotyrosine level

To verify the well-known increased oxidative/nitrative stress [102, 103] in the heart in metabolic syndrome, cardiac free 3-nitrotyrosine level, an indirect marker of nitrative stress, was measured by ELISA (Cayman Chemical) from ZDF and lean control heart tissue samples at week 25 as described earlier [11]. Briefly, supernatants of ventricular tissue homogenates were incubated overnight with anti-nitrotyrosine rabbit IgG specific to free 3-nitrotyrosine and nitrotyrosine acetylcholinesterase tracer in precoated (mouse anti-rabbit IgG) microplates followed by development with Ellman's reagent. Free nitrotyrosine content was normalized to protein content of the cardiac homogenate and expressed as nanograms per milligram protein [11].

RNA preparation, DNA microarray analysis and quantitative real-time PCR (qRT-PCR)

RNA preparation, DNA microarray analysis and qRT-PCR were performed in the Department of Functional Genomics, Biological Research Center, Szeged, Hungary as described in the article related to the subject of the thesis [104].

Statistical analysis

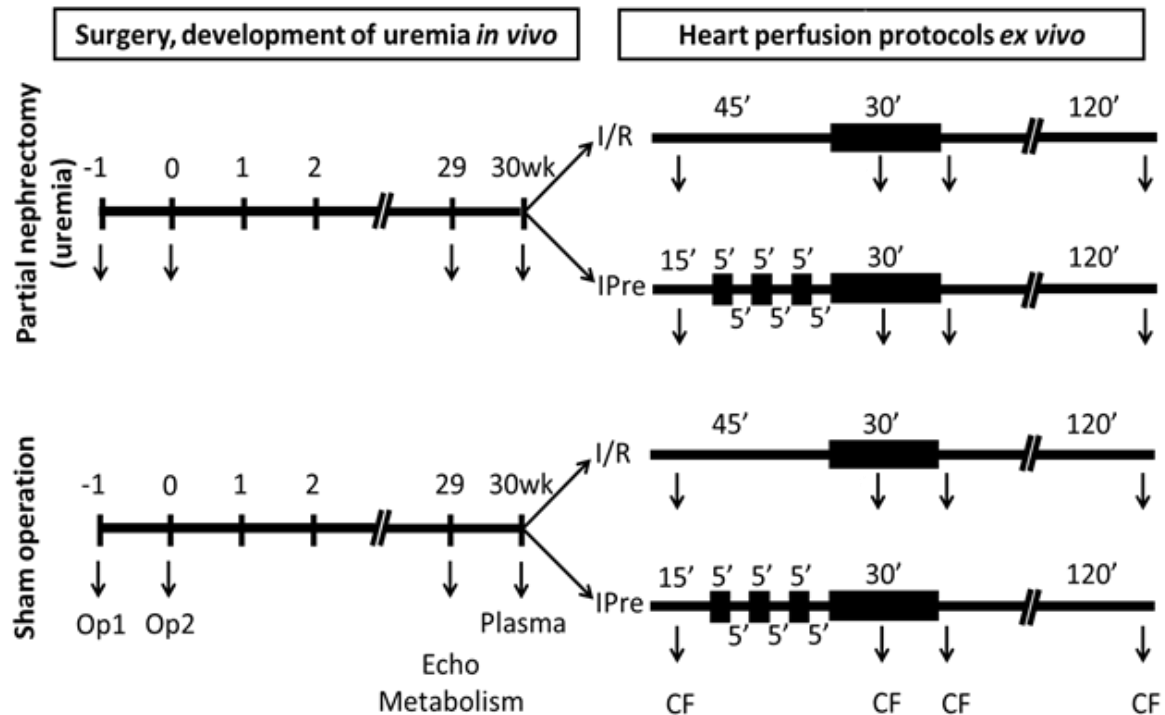
For characterization of the ZDF model and lean controls, all values (body weight, serum glucose, insulin, HOMA-IR, cholesterol and triglyceride levels, pancreas weight and insulin content, myocardial 3-nitrotyrosine levels and microarray experiments) are presented as mean \pm SEM. Significance between groups was determined with unpaired t-test. $P < 0.05$ was accepted as a statistically significant difference. Gene expression ratio with p -value < 0.05 and \log_2 ratio < -0.75 or \log_2 ratio > 0.75 (~1.7 fold change) were considered as repression or overexpression respectively in gene activity.

7.2 Experimental setup for examination of the influence of prolonged uremia on IPre

Adult male Wistar rats were used in the study. Animals were housed in pairs in individually ventilated cages (Sealsafe IVC system, Italy) and were maintained in a temperature controlled room with a 12:12 h light: dark cycles throughout the study. Standard rat chow and tap water were supplied ad libitum. Drinking water contained 1mg/100g body weight iron (II) sulfate to attenuate the development of severe anemia.

Experimental prolonged uremia was induced by partial (5/6) nephrectomy. Animals underwent sham operation or partial nephrectomy in two phases (Figure 7) [105]. There was no difference in mortality between sham operated and partially nephrectomized groups. At week 29, cardiac function was assessed by transthoracic echocardiographic examination (Figure 7). At week 29, a group of animals were placed for 24 hours in metabolic cages in order to estimate creatinine clearance and to measure urine creatinine and protein levels (Figure 7). At week 30, rats were anesthetized, hearts were isolated and perfused ex vivo by oxygenated Krebs-Henseleit solution according to Langendorff as described previously [106] (Figure 7). Immediately after excision of the heart, blood was collected from the thoracic cavity to measure plasma uric acid, carbamide and creatinine levels in order to verify the development of uremia. Some plasma was used for determination of angiotensin II as an indirect marker of hypertension and hypertrophy [107-111] and nitrotyrosine as a marker of systemic oxidative stress [98]. To assess the cardioprotective effect

of IPre in the hearts of uremic and sham operated animals, the perfused hearts were subjected to I/R with or without preconditioning protocol (Figure 7). At the end of the appropriate perfusion protocol, the coronary artery was reoccluded and the area at risk and the infarcted area were delineated using an Evans blue/triphenyltetrazolium chloride double staining method [112].



7. Figure Wistar rats underwent sham operation or partial nephrectomy in two phases. First, 2/3 of the left kidney was ligated and excised (Op1). One week later, the right kidney was removed (Op2). Corresponding time-matched sham operations were performed in the sham group. At week 29, cardiac function was assessed by transthoracic echocardiographic examination (Echo). In this week the animals (12-12 in uremia and sham groups) were placed for 24 hours in metabolic cage (Metabolism) to determine urine creatinine and protein levels. At week 30, rats were anesthetized and blood was collected from thoracic cavity to measure plasma carbamide and creatinine levels (Plasma). Hearts were then isolated and perfused according to Langendorff. After 15 minutes of aerobic perfusion, hearts were subjected to either preconditioning induced by three intermittent periods of 5 min ischemia/reperfusion cycles or a time-matched (30 min) aerobic perfusion, both followed by a 30-min coronary occlusion and 2-hour reperfusion (n=6-9 in each group). Coronary flow (CF) was measured 10 minutes after the start of perfusion, in the 15th minute of ischemia, in the first 5 minutes of reperfusion and at the end of the reperfusion.

Partial nephrectomy

Anesthesia was induced by intraperitoneal injection of sodium pentobarbital (Euthasol, 50 mg/kg, Produlab Pharma b.v., Raamsdonksveer, The Netherlands). After depilation and a ventral midline incision in the abdominal wall, the intestines were retracted laterally to expose the animal's left kidney. The kidney was freed from the perirenal adipose tissue and renal capsule. Two pieces of sutures (5-0 Mersilk, Ethicon, Sommerville, NJ, USA) were placed around both poles of the kidney approximately at their 1/3 position. The sutures were gently ligated around the kidney. The 1/3

kidney on both ends were excised right beyond the ligatures. The abdominal incision was closed with running sutures. One week after the first operation, animals were anesthetized and prepared as described above [105]. The right kidney was freed from the surrounding adipose tissue and renal capsule, and then it was pulled out of the incision gently. The adrenal gland, which is attached loosely to the anterior pole of the kidney, was gently freed and was placed back into the abdominal cavity. The renal blood vessels and the ureter were ligated and the right kidney was removed [113]. Then the incision was closed with running sutures and povidone iodide was applied on the surface of the skin. After operation, animals were placed on a warm heat-pad until they become conscious again. As a post-operative medication, 0.3 mg/kg nalbuphine hydrochloride (Nalbuphine 10 mg/mL, TEVA, Debrecen, Hungary) was administered subcutaneously. Antibiotics (Enroxil, 75 mg/L (Krka, Slovenia) and analgesics (10 mg/L of nalbuphine hydrochloride, Nalbuphine, TEVA, Debrecen, Hungary) were administered in tap water for 2 days after both surgeries.

Transthoracic echocardiography

Cardiac function was assessed by transthoracic echocardiographic examination 29 weeks after the second surgery. Echocardiography was performed as described previously [114]. The rats were anesthetized with sodium pentobarbital (Euthasol, 40 mg/kg body weight, ip.), the chest was shaved and the animal was placed in supine position onto a heating pad. Two-dimensional, M-mode and Doppler echocardiographic examinations were performed in accordance with the criteria of the American Society of Echocardiography with a Vivid 7 Dimension ultrasound system (General Electric Medical Systems, USA) using a phased array 5.5-12 MHz transducer (10S probe). Data of three consecutive heart cycles were analyzed (EchoPac Dimension software, General Electric Medical Systems, USA) by an experienced investigator in a blinded manner. The mean values of three measurements were calculated and used for statistical evaluation. Systolic and diastolic wall thickness parameters were obtained from parasternal short-axis view at the level of the papillary muscles and long-axis view at the level of the mitral valve. The left ventricle diameters were measured by means of M-mode echocardiography from long-axis and short-axis views between the endocardial borders. Functional parameters including left ventricular end-diastolic volume, left ventricular end-systolic volume, and ejection fraction were calculated on four chamber view images. Diastolic function was assessed using pulse-wave Doppler across the mitral valve from the apical four chamber view. Early (E) and atrial (A) flow velocity as well as mitral valve deceleration time and isovolumic relaxation time provide an indication of diastolic function.

Urine creatinine and total protein levels

At week 29, the animals were placed in a metabolic cage (Tecniplast, Italy) for 24 hours in order to collect urine for the measurement of urine creatinine and protein levels to verify the development of advanced uremia. Urine creatinine and urine protein levels were measured by standard laboratory methods as described previously by others [115, 116].

Plasma carbamide and creatinine levels

Blood was collected from the thoracic cavity after isolation of the heart to measure plasma carbamide and creatinine levels in order to verify the development of chronic uremia at week 30. Plasma carbamide and creatinine levels were measured in triplicate, using commercially available colorimetric assay kits (Diagnosticum Ltd., Budapest, Hungary) applying enzymatic determinations adapted to 96-well plates.

Creatinine clearance

Creatinine clearance, an indicator of renal function, was calculated according to the standard formula (urine creatinine concentration [μM] x urine volume for 24 hr [mL])/(plasma creatinine concentration [μM] X 24 X 60 min).

Hematocrit and hemoglobin level

Hematocrit and hemoglobin were measured from whole blood by means of a blood gas analyzer (Radiometer ABL 77, Radiometer Medical, Bronshøj, Denmark) at week 30 in order to verify the development of renal anemia.

Ex vivo cardiac perfusions and infarct size determination

At week 30, rats were anesthetized and hearts were isolated and perfused at 37°C according to Langendorff with oxygenated Krebs-Henseleit buffer as previously described [63, 106]. Hearts from the sham-operated and the uremic groups were further subdivided into two subgroups (n=6–9) and subjected to either a non-conditioning or a preconditioning perfusion protocol, respectively (Figure 7). Non-conditioned hearts were subjected to time-matched aerobic perfusion followed by test ischemia-reperfusion induced by a 30-min occlusion of the left descending coronary artery.

Preconditioned hearts were subjected to 3 intermittent periods of 5-min I/R followed by test I/R. A 3-0 silk suture was placed around the origin of the left descending coronary artery and passed through a plastic tube to form a snare. After stabilization of the heart, coronary occlusion was induced by pulling the ends of the suture taut and clamping the snare onto the epicardial surface. Reperfusion was achieved by releasing the snare as previously described [106]. Heart rate and coronary flow were monitored throughout the perfusion protocol (Figure 7). At the end of the 2 hour reperfusion protocol, the coronary artery was reoccluded and 5 mL of 0.1% Evans blue dye (Merk, Germany) was injected into the aorta to delineate the area at risk zone. Stained hearts were weighed, frozen, sliced and incubated at 37°C in 1% triphenyl-tetrazolium chloride (Sigma Aldrich, Germany) to delineate infarcted tissue. Slices were then fixed and quantified by planimetry using Infarctsize 2.5 software™ (Pharmahungary, Szeged, Hungary) [106]. Infarct size was expressed as a percentage of the area at risk zone [112]. The area at risk was calculated as a percentage of total ventricular area [112].

Plasma angiotensin II level

Plasma angiotensin II level was determined as a marker of hypertension and left ventricular hypertrophy. The level of angiotensin II in blood plasma of uremic and sham operated rats was determined with ELISA kits that recognize rat peptides (Phoenix Pharmaceuticals) in accordance with the manufacturer's instructions.

Plasma 3-Nitrotyrosine level

Plasma 3-nitrotyrosine level was determined as a marker of systemic nitrosative stress. Free 3-nitrotyrosine level was measured by ELISA (Cayman Chemical) as described earlier [97] from sham and uremic plasma samples taken at week 30. Briefly, plasma samples were deproteinized by the addition of ice-cold ethanol. After centrifugation, the supernatants were evaporated in nitrogen flow, dissolved in phosphate buffer, and incubated overnight with anti-nitrotyrosine rabbit IgG and nitrotyrosine acetylcholinesterase tracer in precoated (mouse anti-rabbit IgG) microplates, followed by development with Ellman's reagent [98].

Plasma uric acid level

Plasma uric acid level was measured in order to further characterize our uremic model. Plasma uric acid level was measured in duplicate, using a commercially available colorimetric assay kit (Diagnosticum Ltd., Budapest, Hungary) according to the manufacturer's instructions.

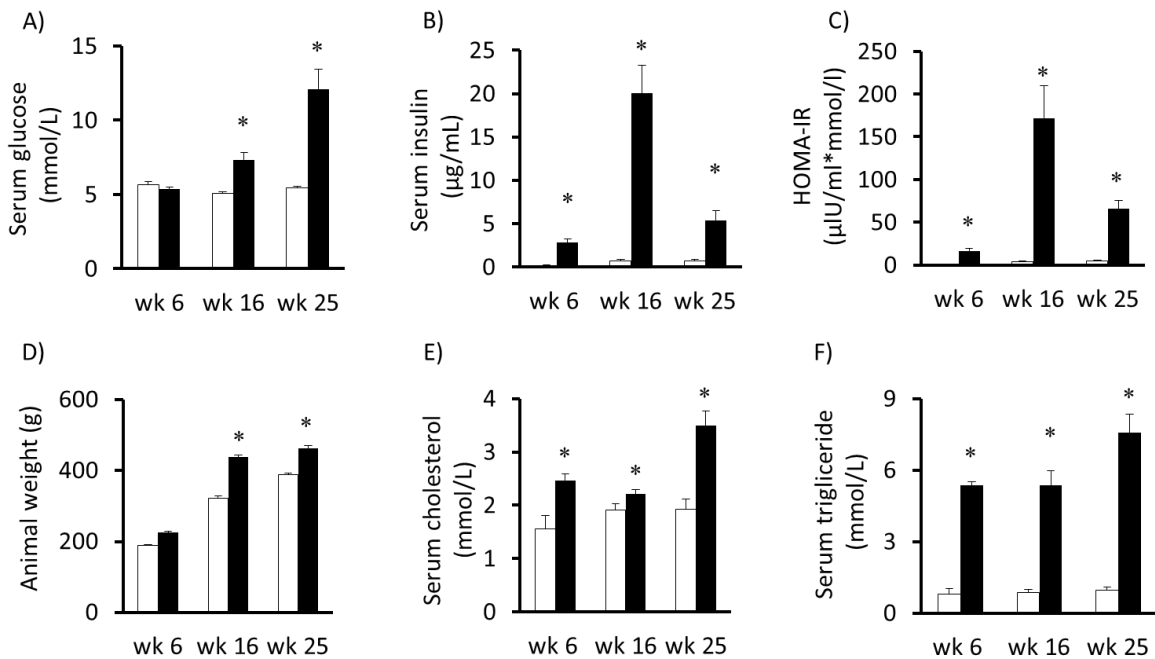
Statistical analysis

All values are presented as mean \pm SEM. Two-way ANOVA was used to determine the effect of uremia or preconditioning on infarct size, area at risk, and coronary flow. Then to analyze the effect of preconditioning on infarct size within sham as well as uremic groups an unpaired t-test was applied. All other parameters were analyzed by unpaired t-tests comparing data in the uremic groups to sham controls. $P < 0.05$ was accepted as a statistically significant difference.

8 RESULTS

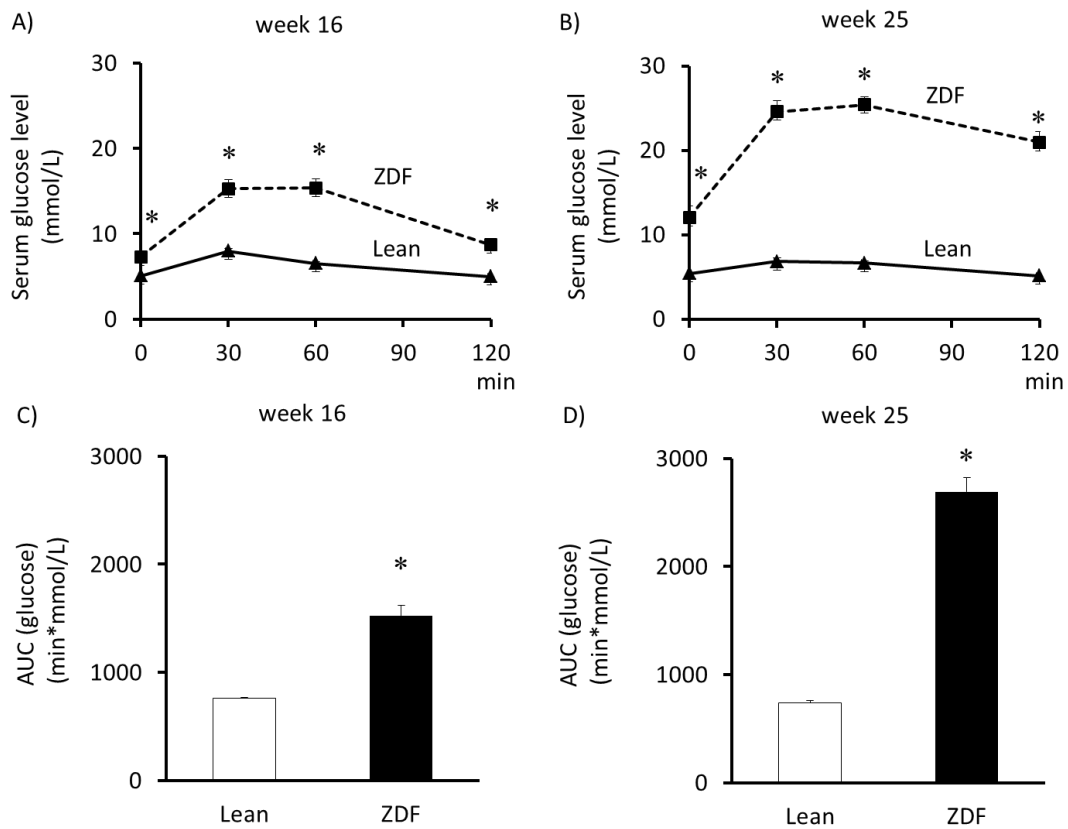
8.1 Characterization of metabolic syndrome in ZDF rats

In order to verify the development of metabolic syndrome in male ZDF rats, concentrations of several plasma metabolites and body weight were measured at week 6, 16 and 25 (Figure 8). ZDF rats showed a significant rise in serum fasting glucose level starting from week 16 as compared to lean controls (Figure 8A). Parallel with hyperglycemia, serum insulin levels were significantly increased in ZDF rats compared to lean ones during the 25 weeks showing the presence of hyperinsulinemia in ZDF animals (Figure 8B). However, serum insulin concentration in ZDF rats was significantly lower at week 25 as compared to serum insulin level measured at week 16 indicating beta-cell damage. HOMA-IR was significantly higher at week 6, 16 and 25 in ZDF rats when compared to lean controls showing insulin resistance in ZDF animals (Figure 8C). Body weight increased throughout the study and was significantly higher in ZDF animals compared to lean ones showing obesity (Figure 8D). Both serum cholesterol and triglyceride levels were significantly increased in ZDF rats as compared to lean ones throughout the study duration representing hyperlipidemia (Figure 8E and 8F).



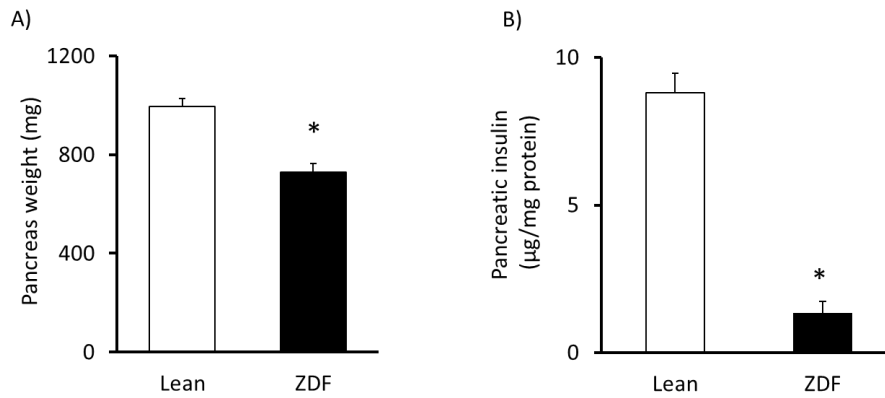
8. Figure Serum glucose (A, n=6-8) and insulin levels (B, n=6-8), HOMA-IR (C), animal weight (D), serum cholesterol (E, n=6-8) and triglyceride (F, n=6-8) shown at week 6, 16 and 25 in both lean and ZDF rats. White bars: Lean; black bars: ZDF. Values are means±SEM, *p<0.05.

Oral glucose tolerance test (OGTT) was performed at week 16 and 25 in order to verify the development of impaired glucose tolerance in ZDF rats. Glucose levels during OGTTs were markedly increased in ZDF rats in every time point of blood glucose measurements both at weeks 16 and 25 (Figure 9A-9B). Area under the curve (AUC) of blood glucose concentration during OGTTs was significantly elevated in ZDF rats at both weeks 16 and 25 representing impaired glucose tolerance (Figure 9C-9D).



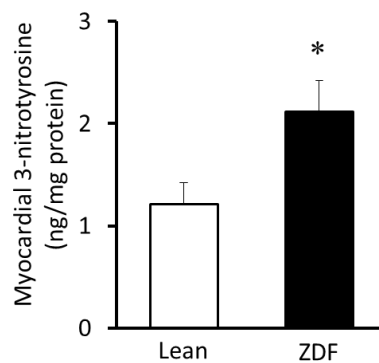
9. Figure Glucose levels during OGTT. Glucose levels during OGTT at week 16 (A) and week 25 (B) in both lean and ZDF rats. Solid line: Lean; dashed line: ZDF. Area under the curve (AUC) of blood glucose concentration during OGTT at week 16 (C) and week 25 (D) in both lean and ZDF rats. Values are means \pm SEM, n=6-8, *p<0.05.

Pancreas weight and pancreatic insulin content were measured at the end of the experiment in order to investigate the severity of diabetes mellitus in ZDF rats. Pancreas weight and pancreatic insulin concentration were significantly decreased in ZDF rats at week 25 showing impaired pancreatic function (Figure 10A and 10B).



10. Figure Pancreas weight and pancreas insulin content. Pancreas weight (A) at week 25 and pancreatic insulin content (B) in both lean and ZDF rats. Values are means±SEM, n=6-8, *p<0.05.

In order to verify the increased oxidative/nitrative stress in ZDF animals, myocardial 3-nitrotyrosine levels were determined in both groups at week 25. A marker molecule of peroxynitrite, 3-nitrotyrosine level was significantly elevated in the heart of ZDF animals showing increased cardiac oxidative/nitrative stress (Figure 11).



11. Figure Myocardial 3-nitrotyrosine level. Myocardial 3-nitrotyrosine level at week 25 in both lean and ZDF rats. Values are means±SEM, *p<0.05.

8.2 Cardiac gene expression profile in metabolic syndrome measured using cDNA microarrays and by qRT-PCR

In order to determine alterations in cardiac gene expression profile, a DNA microarray was carried out. Among the 14921 genes surveyed, 10244 genes were expressed on the cDNA microarray, and 85 genes whose expression was > ~1.7-fold up- or down-regulated (\log_2 ratio < -0.75 or \log_2 ratio > 0.75) in hearts of ZDF rats relative to levels of lean control rats showed significant change in expression. According to our results, 49 genes showed down-regulation (Table 1) and 36 genes showed up-regulation in hearts of ZDF rats (Table 2). Many of these differentially expressed genes are known to be involved in multiple cell functions, including metabolism, stress response, signal transduction, regulation of transcription, cytoskeletal structure, cell adhesion, membrane proteins, receptors and others.

1. Table Down-regulated genes on DNA microarray in the heart of ZDF rats

Gene function	Description [Gene symbol]	AVE log ₂	SD	P value	Fold change
Metabolism	Argininosuccinate synthetase [Ass]	-1.33	0.06	0.001	-2.51
	Glycine C-acetyltransferase (2-amino-3-ketobutyrate-coenzyme A ligase) [Gcat]	-1.15	0.34	0.006	-2.22
	3-hydroxybutyrate dehydrogenase, type 1 [Bdh1]	-1.14	0.44	0.014	-2.21
	Thymidylate synthase [Tyms]	-0.92	0.08	0.041	-1.89
	Dicarbonyl L-xylulose reductase [Dcxr]	-0.76	0.30	0.015	-1.70
Stress response	Heat shock 70kd protein 1A [Hspa1a]	-1.84	0.48	0.005	-3.59
	Similar to 60 kDa heat shock protein, mitochondrial precursor (Hsp60) 60 kda heat shock protein [LOC294396Hspa1a]	-1.64	0.24	0.057	-3.11
	Interleukin 18 [il18]	-1.38	0.02	0.007	-2.60
Signal transduction, regulation of transcription	Hairy/anahancer-of-split-related with YRPW motif2 [Hey2]	-1.40	0.15	0.049	-2.64
	Aryl hydrocarbon receptor nuclear translocator-like [Adra1d]	-1.33	0.07	0.001	-2.52
	Activating transcription factor 3 [Atf3]	-1.03	0.26	0.004	-2.05
	RAB7, member RAS oncogene family [Rab7]	-0.98	0.48	0.026	-1.97
	B-cell leukemia/lymphoma 2 related protein A1 [Bcl2a1]	-0.89	0.04	0.019	-1.85
	Sushi, nidogen and EGF-like domains 1 [Sned1]	-0.87	0.51	0.041	-1.83
	Spermatid perinuclear RNA binding protein [Strbp]	-0.87	0.25	0.027	-1.82
	TRAF3 interacting protein 3 [Traf3ip3]	-0.85	0.25	0.028	-1.81
	Putative bhlh transcription factor (Fragment) [Ascl3]	-0.79	0.15	0.013	-1.73
Membrane proteins, receptors	ATPase, (Na ⁺)/K ⁺ transporting, beta 4 polypeptide [Atp1b4]	-1.38	0.13	0.003	-2.60
	Nerve growth factor receptor (TNFR superfamily, member 16) [Ngfr]	-0.91	0.05	0.027	-1.88
	Cholinergic receptor, nicotinic, gamma polypeptide [Chrng]	-0.99	0.39	0.048	-1.98
	G protein-coupled receptor 37 [Gpr37]	-0.87	0.06	0.030	-1.82
	Adrenergic receptor, alpha 1d [Adra1d]	-0.79	0.43	0.036	-1.73
	Neurotensin receptor 2 [Ntsr2]	-0.77	0.32	0.018	-1.70
Structural protein, cell adhesion	Myosin IXa [myo9a]	-1.26	0.01	0.002	-2.40
	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4 [St8sia4]	-1.00	0.07	0.032	-2.01
	Similar to collagen, type XXIV, alpha 1 [RGD1565539_predicted]	-1.16	0.03	0.011	-2.24
Others	ADAM metalloproteinase with thrombospondin type 1 motif, 1 [Adamts1]	-2.84	0.90	0.123 0	-7.16
	Similar to tatd DNAase domain containing 1 [RGD1566244_predicted]	-1.86	0.16	0.038	-3.63
	Aryl hydrocarbon receptor nuclear translocator-like protein [Arntl]	-1.33	0.67	0.001	-2.52
	TAF13 RNA polymerase II, TATA box binding protein (TBP)-associated factor [Taf13_predicted]	-1.22	0.11	0.039	-2.34
	G protein-coupled receptor 107 [Gpr107_predicted]	-1.18	0.02	0.007	-2.26
	CDK5 regulatory subunit associated protein 1-like 1 [Cdkal1_predicted]	-0.92	0.06	0.041	-1.89
	Similar to chondroitin beta1,4 N-acetylgalactosaminyltransferase 2 [RGD1563660_predicted]	-0.91	0.03	0.017	-1.87
	S100 calcium binding protein A3 [S100a3]	-0.90	0.06	0.028	-1.87
	Similar to TPR repeat-containing protein KIAA1043 [LOC304558]	-0.89	0.08	0.003	-1.85
	Similar to Ten-m4 [Odz4_predicted]	-0.88	0.34	0.047	-1.84
	Kallikrein 14 [Klk14_predicted]	-0.88	0.09	0.047	-1.84
	Mucin 19 [Muc19_predicted]	-0.84	0.33	0.048	1.79
	Copine family member IX [Cpne9]	-0.84	0.11	0.006	-1.79
	Suppressor of Ty 16 homolog (S. Cerevisiae) [Supt16h_predicted]	-0.81	1.57	0.021	-1.75
	Heterogeneous nuclear ribonucleoprotein L-like [Hnrpll_predicted]	-0.81	0.01	0.008	-1.75
	cDNA clone UI-R-BJ0p-afn-b-03-0-UI 3' [Sln]	-0.81	0.30	0.013	-1.75
	Discs, large homolog 4 (Drosophila) [Dlgh4]	-0.80	0.23	0.026	-1.75
	Similar to chondroitin beta1,4 N-acetylgalactosaminyltransferase [RGD1307618_predicted]	-0.80	0.25	0.031	-1.74
	Sterile alpha motif domain containing 4 [Samd4_predicted]	-0.80	0.02	0.012	-1.74
	Similar to RIKEN cdna 1190005B03 [Cdkal1_predicted]	-0.79	0.06	0.035	-1.73
	WDNM1 homolog [LOC360228]	-0.78	0.24	0.007	-1.72
	CD300 antigen like family member E [Cd300le_predicted]	-0.78	0.362	0.023	-1.71
	Neuronatin [nnat]	-0.77	0.35	0.022	-1.71
	Connective tissue growth factor [Ctgf]	-0.77	0.47	0.049	-1.69
	Chemokine (C-X-C motif) ligand 11 [Cxcl11]	-0.75	0.17	0.003	-1.68

2. Table Up-regulated genes on DNA microarray in the heart of ZDF rats

Gene function	Description [Gene symbol]	AVE log ₂	SD	P value	Fold change
Metabolism	Acyl-CoA thioesterase 7 [Acot7]	0.75	0.34	0.021	1.69
	Angiopoietin-like 4 [Angptl4]	0.83	0.25	0.007	1.78
	Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme C [Mgat4c_predicted]	0.87	0.29	0.037	1.82
	Carbonyl reductase 1 [Cbr1]	0.99	0.40	0.016	1.99
	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 [Hmgcs2]	1.05	0.25	0.004	2.07
	Transglutaminase 1 [tgm1]	1.35	0.13	0.044	2.55
	Cytosolic acyl-CoA thioesterase 1 [Cte1]	1.88	0.41	0.003	3.69
Stress response	Cold inducible RNA binding protein [Cirbp]	0.77	0.21	0.005	1.71
	Glutathione S-transferase Yc2 subunit [Yc2]	0.86	0.16	0.002	1.82
Signal transduction, regulation of transcription	Calcium/calmodulin-dependent protein kinase II gamma [Camk2g]	0.75	0.21	0.006	1.68
	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) [Pla2g7]	1.45	0.84	0.041	2.74
	Brain expressed X-linked 1 [Bex1]	0.92	0.57	0.048	1.90
	Fibroblast growth factor receptor substrate 3 [Frs3]	1.05	0.04	0.018	2.07
Membrane proteins, receptors	Huntingtin-associated protein 1 [Hap1]	0.77	0.29	0.043	1.71
	Membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3) [RGD1560049_predicted]	1.10	0.45	0.016	2.15
	ATPase, H ⁺ /K ⁺ transporting, nongastric, alpha polypeptide [Atp12a]	1.39	0.19	0.006	2.61
Structural protein, cell adhesion	Spectrin beta 3 [Spnb3]	0.95	0.05	0.026	1.93
	Aggrecan 1 [agc1]	1.06	0.09	0.037	2.08
Others	Similar to mucin 7, salivary [RGD1311530_predicted]	0.75	0.22	0.007	1.69
	Leukocyte tyrosine kinase [Ltk_predicted]	0.77	0.04	0.021	1.70
	Similar to RIKEN cDNA 9130022B02 [Pck2_predicted]	0.78	0.28	0.042	1.72
	Similar to hypothetical protein [Arid2_predicted]	0.79	0.20	0.004	1.73
	Iroquois related homeobox 3 (Drosophila) [Irx3_predicted]	0.809	0.41	0.031	1.74
	Hdac5 protein (Fragment) [Hdac5]	0.86	0.70	0.022	1.81
	Ribonuclease, RNAase A family, 1 (pancreatic) [Rnase1]	0.90	0.22	0.004	1.86
	Ring finger protein 24 [Rnf24_predicted]	0.92	0.22	0.037	1.89
	Amyloid beta (A4) precursor protein-binding, family A, member 1 [Apba1]	0.96	0.36	0.044	1.94
	CWF19-like 1, cell cycle control (S. Pombe) [Cwf1911_predicted]	0.97	0.09	0.044	1.95
	Similar to high density lipoprotein-binding protein [RGD1564237_predicted]	0.98	0.19	0.002	1.97
	P21 (CDKN1A)-activated kinase 6 [Pak6_predicted]	0.10	0.08	0.036	2.00
	Similar to nuclear body associated kinase 1a [Hpk2_predicted]	1.06	0.66	0.049	2.09
	Cationic trypsinogen [LOC286911]	1.08	0.07	0.031	2.11
	Chac, cation transport regulator-like 1 (E. Coli) [RGD1560049_predicted]	1.10	0.08	0.033	2.15
	NTAK alpha2 [Nrg2]	1.13	0.10	0.042	2.18
	Ubiquitin carboxy-terminal hydrolase L1 [Uchl1]	1.24	0.26	0.014	2.37
	Claudin 19 [cldn19]	1.43	0.12	0.037	2.70

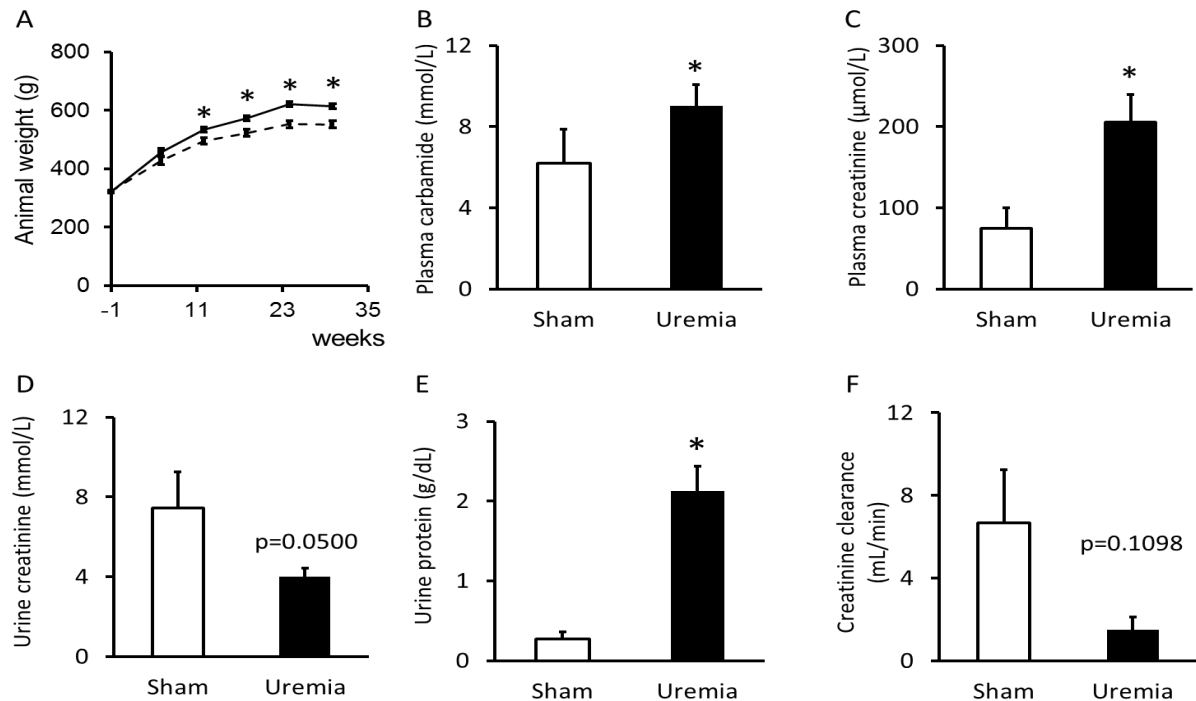
The expression change of selected 23 genes was validated by qRT-PCR (Table 3), 18 of these 23 genes have been confirmed by qRT PCR (Table 3). Most of these genes have not been shown to be involved in the development of cardiovascular complications of metabolic syndrome yet.

3. Table qRT-PCR in the heart of ZDF rats

Description [Gene symbol]	DNA MICROARRAY		qRT-PCR			confirmed
	fold change	p value	ratio (SD)	fold change	regulation	
ADAM metalloproteinase with thrombospondin type 1 motif, 1 [Adams1]	-7.16	0.1295	0.58 (0.09)	-1.72	down	<i>yes</i>
heat shock 70kD protein 1A [Hspa1a]	-3.59	0.0045	0.34 (0.06)	-2.94	down	<i>yes</i>
60 kDa heat shock protein [Hsp60]	-3.11	0.0565	0.64 (0.10)	-1.56	down	<i>yes</i>
interleukin 18 [Il18]	-2.60	0.0066	0.91 (0.15)	-1.10	no change	no
ATPase, (Na ⁺)/K ⁺ transporting, beta 4 polypeptide [Atp1b4]	-2.60	0.0031	0.37 (0.06)	-2.70	down	<i>yes</i>
argininosuccinate synthetase [Ass]	-2.51	0.0007	0.29 (0.05)	-3.42	down	<i>yes</i>
myosin IXA [Myo9a]	-2.40	0.0018	0.90 (0.15)	-1.11	no change	no
glycine C-acetyltransferase (2-amino-3-ketobutyrate-coenzyme A ligase) [Gcat]	-2.22	0.0063	0.33 (0.03)	-3.07	down	<i>yes</i>
activating transcription factor 3 [Atf3]	-2.05	0.0044	0.35 (0.06)	-2.85	down	<i>yes</i>
similar to chondroitin sulfate GalNAcT-2 [RGD1563660_predicted]	-1.87	0.0170	0.92 (0.15)	-1.09	no change	no
S100 calcium binding protein A3 [S100a3]	-1.87	0.0281	1.74 (0.28)	1.74	up	no
sushi, nidogen and EGF-like domains 1 [Sned1]	-1.83	0.0407	0.42 (0.07)	-2.36	down	<i>yes</i>
G protein-coupled receptor 37 [Gpr37]	-1.82	0.0297	1.08 (0.18)	1.08	no change	no
angiotensin-like 4 [Angptl4]	1.78	0.0073	2.90 (0.47)	2.90	up	<i>yes</i>
glutathione S-transferase Yc2 subunit [Yc2]	1.82	0.0017	2.21 (0.36)	2.21	up	<i>yes</i>
ribonuclease, RNase A family, 1 (pancreatic) [Rnase1]	1.86	0.0039	2.68 (0.44)	2.68	up	<i>yes</i>
similar to high density lipoprotein-binding protein [RGD1564237_predicted]	1.97	0.0020	2.73 (0.44)	2.73	up	<i>yes</i>
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2[Hmgcs2]	2.07	0.0036	2.42 (0.39)	2.42	up	<i>yes</i>
similar to tetracycline transporter-like protein [RGD1311900_predicted]	2.24	0.1671	1.29 (0.21)	1.29	no change	no
ubiquitin carboxy-terminal hydrolase L1 [Uch11]	2.37	0.0144	2.50 (0.41)	2.50	up	<i>yes</i>
ATPase, H ⁺ /K ⁺ transporting, nongastric, alpha polypeptide [Atp12a]	2.61	0.0061	3.06 (0.50)	3.06	up	<i>yes</i>
phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) [Pla2g7]	2.74	0.0411	4.25 (0.69)	4.25	up	<i>yes</i>
cytosolic acyl-CoA thioesterase 1 [Cte1]	3.69	0.0028	3.16 (0.51)	3.16	up	<i>yes</i>

8.3 Characterization of prolonged uremia

In order to verify the development of long term uremia induced by partial nephrectomy, body weight was monitored during the experiment and concentrations of several plasma and urine metabolites were measured at week 29. Partially nephrectomized rats showed significantly lower body weights starting from week 7 (Figure 12A). Plasma carbamide and creatinine levels were markedly increased in partially nephrectomized rats representing the uremic state of these animals (Figure 12B and 12C). Plasma glucose levels were similar in both the sham-operated and the partially nephrectomized groups (5.7 ± 0.1 vs. 5.0 ± 0.2 , $n=13-16$, ns). Urine protein concentration was significantly increased in the partially nephrectomized rats as compared to sham-operated controls (Figure 12E) showing an impaired renal function. Moreover, urine creatinine level and creatinine clearance showed a marked but statistically not significant decrease at the level of $p<0.05$ in partially nephrectomized rats (Figure 12D and 12F). Hematocrit and hemoglobin levels were significantly decreased in uremic animals when compared to sham rats showing renal anemia (Table 4). There was no difference in plasma sodium, potassium, calcium, chloride, standard bicarbonate, pH and anion gap between the groups (Table 4).



12. Figure Animal weight (Panel A) shown in every six weeks. Solid line: Sham; dashed line: uremia. Plasma carbamide (Panel B, $n=14$ in both groups) and creatinine (Panel C, $n=12-15$), urine creatinine (Panel D, $n=12$ in each group) and protein concentrations (Panel E, $n=10-12$) and creatinine clearance (Panel F, $n=9-10$) in both sham-operated and uremic rats. Values are means \pm SEM, * $p<0.05$.

4. Table Effect of uremia on haematocrite, haemoglobin and blood gas parameters at week 29.

Measurement	Sham (n=6)	Uremia (n=10)	p-value
Haematocrite (%)	54.2±1.0	44.7±2.2*	0.007
Haemoglobin (g/dL)	17.7±0.3	15.0±0.7*	0.006
Plasma sodium (mmol/L)	139±2	134±3	0.409
Plasma potassium (mmol/L)	6.50±0.50	6.30±0.25	0.694
Plasma calcium (mmol/L)	1.10±0.05	1.19±0.04	0.174
Plasma chloride (mmol/L)	107±3	102±3	0.267
Plasma standard bicarbonate (mmol/L)	25.8±0.4	24.3±0.5	0.051
pH	7.38±0.01	7.36±0.01	0.069
Anion gap (mmol/L)	11.5±0.8	12.9±0.7	0.213

Studies were performed before ex vivo Langendorff heart perfusion 30 wk after sham-operation or partial nephrectomy; Values are mean±SEM, n=6-10, *p<0.05 vs. sham, unpaired t-test.

8.4 Effect of prolonged uremia on myocardial morphology and function

Transthoracic echocardiography was performed at week 29 in order to investigate whether the development of prolonged uremia leads to alteration of myocardial morphology and function. Left ventricular systolic anterior and septal wall thicknesses were increased in uremic rats as compared to sham-operated controls (Table 5). However, there was no difference in diastolic anterior and septal wall thickness between the uremic and sham operated group. In addition, left ventricular lateral and posterior wall thickness both in systole and diastole were similar in the uremic and the sham operated groups (Table 5). Uremic animals demonstrated a mild reduction both in left ventricular systolic and diastolic function at week 29. Ejection fraction was significantly reduced in the uremic group as compared to the sham-operated control group (Table 5). Stroke volume showed a tendency of decrease in uremic animals as compared to sham-operated controls (Table 5). Heart rate and left ventricular end-systolic and end-diastolic volumes were similar in both uremic and sham-operated groups (Table 5). Isovolumic relaxation time was decreased in uremic rats as compared to sham-operated controls (Table 4). Other diastolic functional parameters including early and late ventricular filling velocity, E/A ratio, deceleration time, maximal and mean left ventricular gradient were not changed in the uremic group as compared to sham-operated controls (Table 5).

5. Table Effects of uremia on various in vivo left ventricular morphological and functional parameters measured by transthoracic echocardiography.

Echocardiography				
Morphology	View/Mode	Sham (n=14)	Uremia (n=16)	p-value
Anterior wall thickness-systolic (mm)	short axis/MM	3.31±0.05	3.59±0.11*	0.032
Anterior wall thickness-diastolic (mm)	short axis/MM	1.87±0.07	1.97±0.09	0.371
Lateral wall thickness-systolic (mm)	short axis/MM	3.47±0.11	3.38±0.18	0.796
Lateral wall thickness-diastolic (mm)	short axis/MM	2.14±0.40	1.98±0.08	0.270
Posterior wall thickness-systolic (mm)	long axis/MM	3.77±0.11	3.81±0.11	0.781
Posterior wall thickness-diastolic (mm)	long axis/MM	2.18±0.07	2.30±0.08	0.287
Septal wall thickness-systolic (mm)	long axis/MM	2.70±0.12	3.04±0.13*	0.046
Septal wall thickness-diastolic (mm)	long axis/MM	2.25±0.10	2.26±0.08	0.961
Left ventricular end diastolic diameter (mm)	long axis/MM	7.25±0.17	6.95±0.14	0.176
Left ventricular end diastolic diameter (mm)	short axis/MM	7.23±0.18	7.23±0.19	0.967
Left ventricular end systolic diameter (mm)	long axis/MM	3.82±0.16	3.55±0.15	0.237
Left ventricular end systolic diameter (mm)	short axis/MM	3.77±0.20	3.53±0.19	0.400
Systolic function		Sham (n=14)	Uremia (n=16)	p-value
Ejection fraction (%)	four chamber/2D	54.9±1.7	48.9±1.4*	0.012
Stroke volume (μL)	four chamber/2D	252.2±13.8	222.9±10.1	0.093
Left ventricular end-systolic volume (μL)	four chamber/2D	219.3±23.1	240.0±18.7	0.487
Left ventricular end-diastolic volume (μL)	four chamber/2D	477.0±36.1	461.7±27.3	0.736
Heart rate (beats/min)	four chamber/2D	391.1±6.9	396.1±4.9	0.565
Diastolic function - Doppler imaging		Sham (n=13)	Uremia (n=14)	p-value
E-wave (m/s)	four chamber/PW	0.82±0.03	0.85±0.03	0.664
A-wave (m/s)	four chamber/PW	0.50±0.03	0.51±0.02	0.889
E/A ratio	four chamber/PW	1.69±0.07	1.72±0.09	0.830
Deceleration time (ms)	four chamber/PW	33.98±1.95	34.39±1.75	0.885
Isovolumic relaxation time (ms)	four chamber/PW	25.05±0.89	21.69±0.9*	0.025
Maximal left ventricular gradient (mmHg)	four chamber/PW	2.89±0.25	3.09±0.24	0.598
Mean left ventricular gradient (mmHg)	four chamber/PW	1.31±0.10	1.47±0.12	0.351

Transthoracic echocardiographic measurements were performed 29 wk after sham-operation or partial nephrectomy; Values are mean±SEM, n=13-16, *p<0.05 vs. sham, unpaired t-test. MM, M (motion) Mode; 2D, two dimensional; PW, pulse wave, E-wave, early ventricular filling velocity; A-wave, late or atrial ventricular filling velocity.

8.5 Effect of prolonged uremia on ex vivo morphological and functional parameters

Coronary flow and morphological parameters including heart weight, left kidney weight and tibia length were measured at week 30 to investigate whether advanced uremia influences ex vivo functional and morphological parameters of the heart. Coronary flow was significantly reduced in uremic groups when compared to corresponding sham-operated control groups as assessed by two-way ANOVA (Table 6).

6. Table Effects of uremia on ex vivo coronary flow data

Coronary flow			
During non-conditioning perfusion protocol			
Time points of the measurements	Sham (n=8)	Uremia (n=9)	p-value
Base line, mL/min	16.9±1.3	13.1±1.1*	0.044
15 th min of ischemia (mL/min)	8.6±0.9	7.4±0.5	0.260
First 5 min of reperfusion (mL/min)	19.0±1.3	16.9±1.6	0.367
End of reperfusion (mL/min)	13.0±0.7	9.4±1.1*	0.023
During preconditioning perfusion protocol			
Time point of the measurement	Sham (n=6)	Uremia (n=7)	p-value
Base line, mL/min	17.5±1.1	13.0±0.6*	0.007
15 th min of ischemia (mL/min)	8.6±0.7	6.4±0.7	0.071
First 5 min of reperfusion (mL/min)	22.2±1.1	15.5±1.7*	0.016
End of reperfusion (mL/min)	14.1±0.9	8.4±0.8*	0.003

Studies were performed on ex vivo Langendorff heart perfusion system 30 wk after sham-operation or partial nephrectomy; n=6-9, *p<0.05 vs. sham, unpaired t-test. Values are mean±SEM.

The ratio of heart weight to body weight showed a tendency of increase in uremic animals (Table 7), as a result of a significantly lower body weight in the uremic group (Figure 12A). However, there was no difference in heart weight and heart weight to tibia length ratio between the uremic and the sham-operated control group (Table 7). In addition, the weight of the whole left kidney in the sham-operated group and the remaining one third of the left kidney in the uremic group were similar suggesting a marked renal hypertrophy in the uremic animals (Table 7).

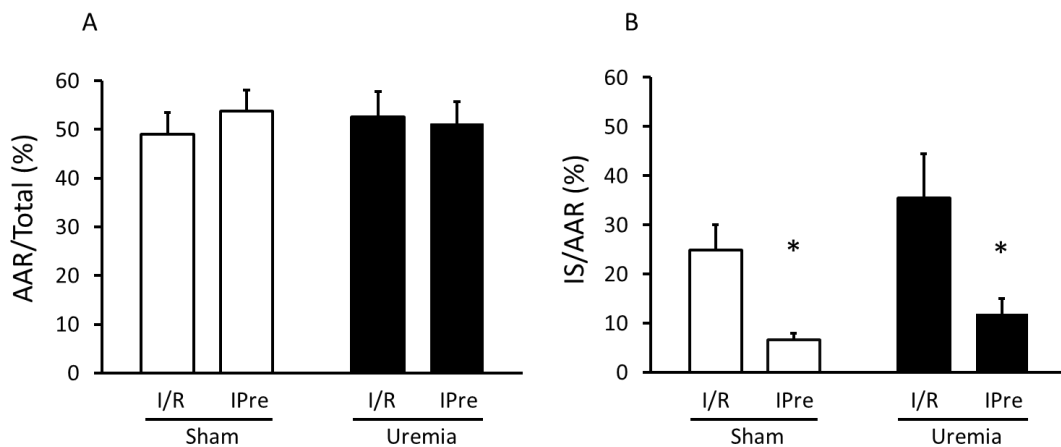
7. Table Effect of uremia on various ex vivo morphological parameters

Measurement	Sham (n=14)	Uremia (n=12)	p-value
Heart weight (g)	2.10±0.09	2.10±0.10	0.980
Heart weight/body weight × 10 ³	3.40±0.17	3.77±0.15	0.126
Heart weight/tibia length × 10 ²	4.00±0.20	4.20±0.20	0.483
Left kidney weight (g)	Full kidney weight 1.90±0.11	1/3 kidney weight 2.04±0.09	-

Measurements were performed 30 wk after sham-operation or partial nephrectomy. Values are mean±SEM, n=12-14, unpaired t-test.

8.6 Effect of ischemic preconditioning on infarct size

Infarct size was measured at week 30 to investigate the severity of ischemia/reperfusion injury and the cardioprotective effect of IPre in prolonged uremia (Figure 13A). Preconditioning significantly decreased infarct size, however, the presence of prolonged uremia did not significantly influence the size of infarction as assessed by two-way ANOVA. Additional analysis with unpaired t-tests showed a significant infarct size limiting effect of preconditioning in hearts of both uremic and sham-operated rats (Figure 13B). The area at risk zone was not affected significantly in any of the groups (Figure 13A).

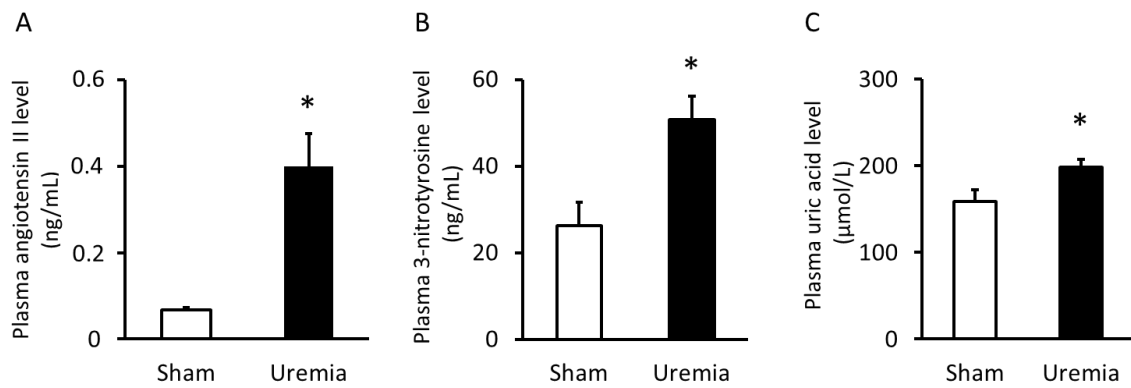


13. Figure Area at risk (Panel A) and infarct size (Panel B) after ischemia/reperfusion (I/R) or ischemic preconditioning (IPre) in both sham and uremic groups. Values are means \pm SEM, n=6-9 in each group. Two-way ANOVA on all groups showed that preconditioning significantly decreased infarct size, however, uremia did not influence infarct size. *p<0.05, * represents significance due to unpaired t-test between I/R and IPre groups.

8.7 Effect of prolonged uremia on plasma angiotensin II, 3-nitrotyrosine and uric acid levels

Partially nephrectomized rats showed significantly higher plasma angiotensin II level (Figure 14A) which is a well-known marker of hypertension and left ventricular hypertrophy. Plasma 3-nitrotyrosine level was determined as a marker of peroxynitrite and systemic nitrative stress. Plasma 3-nitrotyrosine level was markedly increased in uremic rats as compared to sham operated controls representing increased systemic nitrative stress in uremic animals (Figure 14B). Plasma level of uric acid, a well-known anti-oxidant, was significantly increased in the partially nephrectomized

rats as compared to sham-operated controls (Figure 14C) showing increased anti-oxidant capacity in uremic rats.



14. Figure Plasma angiotensin II (Panel A, n=9-10), nitrotyrosine (Panel B, n=9-10), and uric acid (Panel C, n=9-10) levels in both sham-operated and uremic rats. Values are means±SEM, *p<0.05.

9 DISCUSSION

9.1 New findings

1. Our novel finding is that metabolic syndrome is associated with profound modifications of the cardiac transcriptome. In hearts of ZDF rats, 36 genes showed significant up-regulation and 49 genes showed down-regulation as compared to lean controls. Several of the genes showing altered expression in the hearts of ZDF rats have not been implicated in metabolic syndrome previously.
2. We have also found here that 30 weeks of experimental renal failure although leads to severe metabolic changes and the development of myocardial dysfunction, prolonged uremia does not interfere with the severity of myocardial I/R and the cardioprotective effect of IPre.

9.2 Effect of metabolic syndrome on cardiac gene expression pattern in male ZDF rats

Components of metabolic syndrome and their effects on cardiac gene expression

Here we show several characteristics of metabolic syndrome in ZDF rats including obesity, fasting hyperglycemia, hyperlipidemia, hyperinsulinemia, insulin resistance, and impaired glucose tolerance as well as increased cardiac oxidative stress. Here we have shown that 25 week old male ZDF rats develop insulin resistance with hyperinsulinemia, hyperglycemia and impaired HOMA-index in line with other studies [95-97, 117-119]. Both metabolic syndrome and type 2 diabetes mellitus are associated with insulin resistance, hyperinsulinemia and hyperglycemia. Insulin resistance has been reported to be influenced by certain genetic factors and nutrients in patients suffering from metabolic syndrome [120]. Some groups have proposed that hyperinsulinemia stimulated myocardial SERCA2a overexpression plays an important role in the cardiac adaptation process at early phase of type 2 diabetes mellitus in ZDF animals [121]. Others have shown that GLUT4 content decreases along with the development of insulin resistance in the myocardium and other insulin sensitive tissues which might play a key role in the impaired glycaemic homeostasis in metabolic syndrome [122]. Interestingly, hyperglycemia has been reported to activate p53 and p53-regulated genes involving the local renin-angiotensin system which leads to increased apoptosis of cardiomyocytes [123]. Moreover, postprandial hyperglycemia has been shown to play an important

role in the onset and development of heart failure in several large-scale clinical trials [124]. Chronic hyperglycemia has been reported to enhance the vasoconstrictor response by Rho-kinase [125]. Hyperglycemia itself has been shown to increase rat aortic smooth muscle cell growth and gene expression in diabetes mellitus [126]. Some drugs e.g. statins [125] and nitrates [127] have been reported to abolish hyperglycemia induced vasoconstriction. These aforementioned studies clearly demonstrate that metabolic serum parameters could influence cardiac gene expression pattern and may lead to functional consequences. Although we have not measured blood pressure in our study, ZDF rats are well known to develop elevated blood pressure at ages similar to that of used in the present study [128-130].

Groups of altered cardiac genes in metabolic syndrome

We identified 85 genes which were differentially expressed dominantly in the myocardium of 25 weeks old ZDF rats compared to normal controls. Many of these differentially expressed genes are known to be involved in multiple cell functions, including metabolism, stress response, signal transduction, regulation of transcription, cytoskeletal structure, cell adhesion, membrane proteins, receptors and others.

Altered cardiac genes related to metabolism

It is not surprising that the expression of several genes related to metabolism were found to be affected in the hearts of ZDF rats as compared to controls in our present study. A group of these altered genes is involved in ketone body metabolism (down-regulation of 3-hydroxybutyrate dehydrogenase, type 1; up-regulation of 2-amino-3-ketobutyrate-coenzyme A ligase and 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2). Decreased rate of ketone body oxidation and decreased activity of 3-hydroxybutyrate dehydrogenase activity in streptozotocin-induced diabetic rat hearts have been shown previously [131]. Another group of differentially regulated metabolic genes in ZDF hearts in our present study plays a role in the metabolism of carbohydrates (down-regulation of dicarbonyl L-xylulose reductase and up-regulation of mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isoenzyme C, predicted) as well as lipids (up-regulation of acyl-CoA thioesterase 1 and cytosolic acyl-CoA thioesterase 1). Gene expression of cytosolic acyl-CoA thioesterase 1 has been reported to be up-regulated by high fat diet [132] or STZ-induced diabetes [130] in the rat myocardium. A third group of differentially expressed metabolic genes in our present study (down-regulation of argininosuccinate synthetase and up-regulation of angiopoietin-like 4) in ZDF hearts is potentially regulated by oxidative and nitrative stress which is

increased in metabolic diseases e.g. hyperlipidemia [133], hypertension [134], insulin resistance [135], diabetes mellitus [136] and in the heart of ZDF rats as well [103]. High TNF-alpha concentrations [137] and insulin resistance [138, 139] in endothelial cells have been reported to reduce the expression of the arginine recycling enzyme, argininosuccinate synthetase. Overexpression of hepatic angiopoietin-like 4 gene has been shown in diabetic mice [140] and up-regulation of this gene has reported to be induced by fatty acids via PPAR-gamma in muscle tissue [141]. Additionally, insulin has been shown to down-regulate angiopoietin-like 4 in adipocytes [142] and this down-regulation could be attenuated in insulin resistance [142].

Altered cardiac genes related to stress response

Members of another functional gene cluster that is related to stress response showed altered expression in ZDF hearts as compared to controls (down-regulation of heat shock 70kD protein 1A; 60 kDa heat shock protein; interleukin 18 and up-regulation of cold inducible RNA binding protein; glutathione S-transferase Yc2 subunit) in the present study. We have previously shown that hyperlipidemia inhibits cardiac heat shock response [98]. Moreover, heat shock proteins, especially Hsp60, were found to have protective effect against cardiac oxidative and nitrative stress [143]. According to the attenuated expression of heat shock protein 60 and 70 in our present study, metabolic syndrome with well-known increased cardiovascular oxidative and nitrative stress [102, 103] due to hyperlipidemia [133], hypertension [134] and hyperglycemia [135] might interfere with cardiac heat shock response. Glutathione S-transferase catalyzes the conjugation of reduced glutathione on a wide variety of substrates [144] including reactive oxygen and nitrogen species [145]. Interestingly, we have found here the overexpression of glutathione S-transferase in metabolic syndrome similarly to the up-regulation of this gene in cholesterol diet-induced hyperlipidemia in our previous study [146]. Additionally, the absence of cardiomyopathy in diabetes has been reported to be accompanied by increased glutathione S-transferase activity in rat hearts [147]. These results suggest that up-regulation of glutathione S-transferase may be an adaptive response in metabolic syndrome to antagonize elevated oxidative/nitrative stress in the myocardium. Elevated circulating interleukin 18 levels have been reported to be associated with metabolic syndrome independent of obesity and insulin resistance [148], however, in our present study; the myocardial gene expression of interleukin 18 was down-regulated.

Altered cardiac genes related to signal transduction and regulation of transcription

In the present study, we have also shown altered expression of several genes related to signal transduction and regulation of transcription in the hearts of ZDF rats as compared to controls (e.g. down-regulation of activating transcription factor 3; sushi, nidogen and EGF-like domains 1

(insulin responsive sequence DNA binding protein-1) and up-regulation of calcium/calmodulin-dependent protein kinase II gamma; phospholipase A2, group VII). Interestingly, in our present study, an adaptive and oxidative stress-responsive transcription factor [149-151], activating transcription factor 3 showed down-regulation in the heart in metabolic syndrome. Although, enhanced expression of activating transcription factor 3 has been reported to play a role in diabetic angiopathy [149], in stress-induced beta cell dysfunction [152, 153] and hepatic LDL receptor down-regulation [154, 155], its cardiac role in metabolic syndrome has not been implicated yet. Another stress inducible and regulator gene of eicosanoid biosynthesis, the phospholipase A2, group VII gene was up-regulated in our present study in ZDF rat hearts. Increase of the expression of this gene was previously shown in ZDF rats in the liver and suggested to be a factor in the development of chronic low-grade inflammation in metabolic syndrome [156]. In our present study, a regulator gene of insulin action, the insulin responsive sequence DNA binding protein-1 showed down-regulation in metabolic syndrome in ZDF rat hearts. Down-regulation of this gene has been previously shown in the liver of diabetic [157] and obese [157] rats. However, it is unclear whether decreased expression of insulin responsive sequence DNA binding protein-1 is a consequence of insulin resistance or contributes to hyperglycemic phenotype. Calcium/calmodulin-dependent protein kinase II gamma showed up-regulation in ZDF hearts in our present study. This gene was reported to potentially mediate cardiac hypertrophy in pressure overload hypertension in mouse hearts [158].

Altered cardiac genes related to functional cluster of membrane proteins or receptors

In the present study, several genes related to the functional cluster of membrane proteins or receptors showed altered expression in ZDF hearts as compared to controls (e.g. down-regulation of ATPase, Na⁺/K⁺ transporting, beta 4 polypeptide; G protein-coupled receptor 37 and up-regulation of ATPase, H⁺/K⁺ transporting, nongastric, alpha polypeptide; Huntingtin-associated protein 1). Interestingly, here we have found gene expression changes of two members of the X,K-ATPase family due to metabolic syndrome. Surprisingly, ATPase, Na⁺/K⁺ transporting, beta 4 polypeptide showed down-regulation in obese ZDF rat hearts characterized by marked hyperlipidemia in the current study, however, this gene showed up-regulation in our previous study in cholesterol-induced hyperlipidemia in the rat myocardium [146]. Additionally, it has been shown in the heart of spontaneously hypertensive rats that the microsomal Na⁺/K⁺-ATPase activity is reduced [159].

Altered cardiac genes related to functional cluster of structural proteins

Another set of genes related to the functional cluster of structural proteins was found to be regulated differentially in hearts of ZDF rats as compared to controls (e.g. down-regulation of myosin IXA

and similar to collagen, type XXIV, alpha 1 (pred) and up-regulation of spectrin beta 3 and aggrecan 1). To our current knowledge, we are the first in the literature demonstrating cardiac gene expression changes of a novel epithelial extracellular matrix component [160], similar to collagen type XXIV; a cell migration regulator molecule [161], myosin IXA; a membrane stabilizer molecule [162], spectrin beta 3 and an extracellular matrix component proteoglycan [163], aggrecan 1, due to metabolic syndrome.

Altered cardiac genes not related to specific functional clusters

Some of the genes showing altered expression in ZDF rat hearts in the present study were not related to specific functional clusters or indicated as yet uncharacterized, predicted genes and fragments. Many of these genes are reported for the first time in the literature to show altered expression in the heart due to metabolic syndrome including down-regulation e.g. of disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif; G protein-coupled receptor 107 (predicted); S100 calcium binding protein A3; kallikrein 14 (predicted); neuronatin; connective tissue growth factor and up-regulation e.g. of amyloid beta (A4) precursor protein-binding, family A, member 1; similar to high density lipoprotein-binding protein (predicted); cationic trypsinogen; ubiquitin carboxy-terminal hydrolase L1.

9.3 Effect of subacute and chronic renal failure on endogenous cardioprotective mechanisms

The cardioprotective effect of ischemic pre- and postconditioning is inhibited by several co-morbid conditions and risk factors such as e.g. aging [164-166] hyperlipidemia [59, 62, 63, 167-169] and diabetes [69, 78, 170]. However, recent evidence suggests that the cardioprotective effect of pre- and postconditioning is maintained in subacute renal failure (4 weeks) [55] and short-term (10 weeks) [81] model of uremia. In a preliminary study, we have demonstrated for the first time in the literature that the infarct size limiting effect of postconditioning was still present 10 weeks after subtotal nephrectomy resulting in uremia in rats [81]. Moreover, an extensive study published by Byrne et al. [55] has reported that IPre, remote ischemic conditioning, and IPost are still cardioprotective after 4 weeks of subtotal nephrectomy. In addition, in the same study IPre was shown to limit infarct size in an adenine-enriched diet-induced model of uremia in rats [55]. Although the uremic state seems to be too short and was not characterized thoroughly in Byrne's aforementioned studies [55], they may suggest that the uremic heart can be still protected by conditioning techniques. Indeed, an experimental model of 4 weeks of uremia probably may not properly reflect the clinical situation, [4, 82]. Additionally, it has been shown that 5/6 partial

nephrectomy followed by a postoperative duration of 3 weeks does not lead to advanced uremia in rats [83]. Therefore, in the present study we used a prolonged (30 weeks) postoperative duration following partial nephrectomy to induce a more severe long-term uremic condition for investigating the cardioprotective effect of preconditioning.

9.4 Characteristic features and cardiac consequences of the prolonged uremic state

In this prolonged uremic model, here we have found characteristic biochemical and pathophysiological signs of prolonged uremia, including increased plasma uric acid, carbamide and creatinine levels, decreased hematocrit and hemoglobin, increased urine protein and decreased creatinine concentration, as well as diminished creatinine clearance. In patients with end-stage renal failure, uremic cardiomyopathy is a common complication and reported to be a prognostic factor of cardiovascular mortality [5, 39, 171]. In the present study, we have found increased septal and anterior wall thickness in uremic animals. Additionally, plasma angiotensin II level which is an indirect marker of hypertension and left ventricular hypertrophy [107-111] was also higher in uremic rats. These results together with literature data [172-175] suggest the development of a minimal left ventricular hypertrophy in uremic animals at week 30 in our present study. In this model, we have found here that IPre is still effective in prolonged uremic condition. This is a refreshingly interesting result in the light of the fact that several metabolic diseases including diabetes and hyperlipidemia inhibit the endogenous cardioprotective mechanisms of ischemic conditioning.

9.5 Cardioprotection by IPre in prolonged uremia with increased nitrative stress

The reason why the complex metabolic changes of uremia which significantly affect several intracellular signaling pathways in the heart and leads to myocardial dysfunction do not affect the overall efficacy of cardioprotection by IPre is unknown. It has been previously shown that 6-8 week long chronic renal failure leads to increased oxidative and nitrative stress in the rat heart [45, 173, 176, 177]. Here we have also shown increased nitrative stress in a 30-week model of prolonged uremia. It is well known that the cardioprotective effect of IPre is lost in metabolic diseases associated with increased myocardial oxidative and nitrative stress [3]. Therefore, our present findings showing that the cardioprotective effect of IPre is surprisingly still preserved in uremic animals in spite of the increased systemic nitrative stress are particularly exciting. These results suggest that even though prolonged uremia leads to some deleterious effects (e.g. mild cardiac

dysfunction and minimal hypertrophy, tendency of increased infarct size) it likely induces some complex compensatory alterations supporting maintained cardioprotection by IPre. However, further studies should be carried out to determine the precise role of oxidative/nitrative stress in the mechanism of preconditioning in uremia.

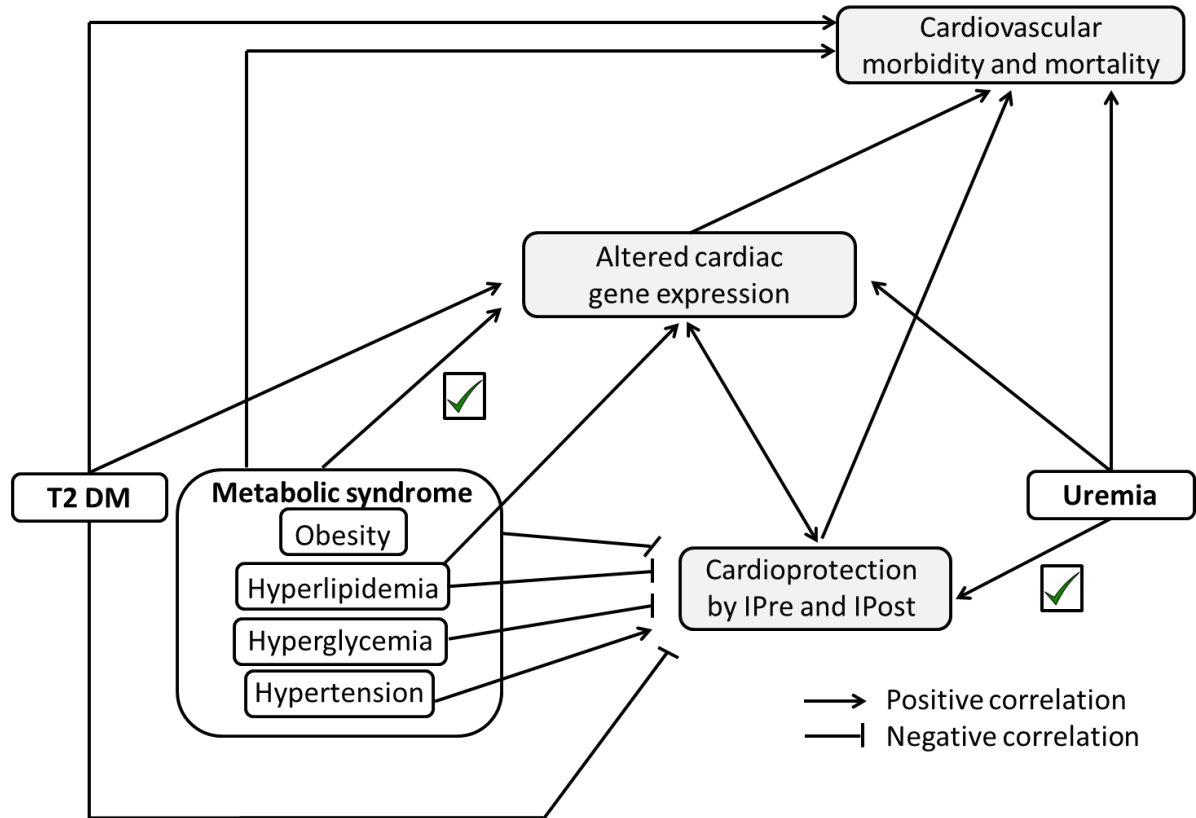
9.6 Cardioprotection by IPre in uremia-related minimal cardiac hypertrophy

Up-regulation of renal renin-angiotensin-aldosterone system was also shown in rats with partial nephrectomy in our present study similarly to findings of other research groups [172, 175, 178]. In our present study, increased plasma angiotensin II levels together with echocardiographic data suggest the development of a minimal left ventricular hypertrophy at week 30 in uremic animals. In this aspect our study is in line with other studies showing that IPre is still cardioprotective in case of left ventricular hypertrophy [179, 180] and hypertension [181, 182] associated with increased plasma angiotensin II level [3]. Therefore, we believe that the presence of a minimal cardiac hypertrophy and mild left ventricular dysfunction in uremic animals might not interfere with the cardioprotective mechanism of IPre. However, it cannot be excluded that the cardioprotection by preconditioning may be lost with the progression of the disease and development of severe heart failure in end-stage renal failure, as it has been shown that endogenous cardioprotection is lost in severe heart failure [183-185]. In addition, renal failure seems to interact with some protein kinases [172, 174] suggested to be involved in the mechanism of IPre. The overall net changes in uremia seem to preserve the cardioprotective effect of IPre, however, the exact mechanisms remain to be elucidated.

10 CONCLUSIONS

In the present thesis, we investigated the effect of a cardiovascular risk factor, the metabolic syndrome on cardiac gene expression pattern, and we examined whether another cardiovascular risk factor and comorbid state, prolonged uremia, affects the I/R injury and cardioprotection by IPre.

1. In the present thesis we have found that 25 weeks old ZDF rats develop severe metabolic syndrome (dyslipidemia, obesity, hyperglycemia, hyperinsulinemia, impaired glucose tolerance and insulin resistance). We have demonstrated for the first time that metabolic syndrome is associated with profound modifications of the cardiac transcriptom. Several of the genes showing altered expression in the hearts of ZDF rats have not been implicated in metabolic syndrome previously. We conclude that metabolic syndrome alters the gene expression pattern of the myocardium which may be involved in the development of cardiac pathologies in the presence of metabolic syndrome (Figure 15). The precise role of these genes in the cardiac consequences of metabolic syndrome needs to be further investigated in future studies.
2. We have also found here that another metabolic disease, chronic renal failure although leads to severe metabolic changes and the development of myocardial dysfunction as well as minimal cardiac hypertrophy, the cardioprotective effect of IPre is still observed. In conclusion, our present study suggests that patients suffering from long-term uremia may also benefit from cardioprotection by IPre. This is particularly important as acute myocardial infarction frequently occurs in patients with late stages of renal failure. Since uremic patients are regularly excluded from clinical trials, there is a need for clinical studies to investigate the cardioprotective effect of conditioning techniques in patients with chronic renal failure suffering from acute myocardial infarction (Figure 15).



15. Figure Conclusion of the present thesis: Metabolic syndrome alters cardiac gene expression pattern and cardioprotection is preserved by ischemic preconditioning (IPre) in prolonged uremia. (Ischemic postconditioning (IPost), T2DM type 2 diabetes mellitus).

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13 ANNEX