

Dissertation

Cardiomyocyte Dysfunction in a Model of Heart Failure with Preserved Ejection Fraction

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Declaration

"I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

Disclosures

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All co-authors have explicitly agreed to the use of their data in this thesis.

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1. Developing study design – together with Prof. Heinzel, PhD
2. Subtotal nephrectomy in rats and sham operations
3. Continuous monitoring of animals during observation period
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Zusammenfassung

Einleitung: Die Prävalenz der Herzinsuffizienz mit erhaltener linksventrikulärer Auswurfleistung (HFpEF) nimmt in den letzten Jahren kontinuierlich zu. Bis dato gibt es jedoch keine spezifische Therapie, da man die zugrundeliegenden pathophysiologischen Mechanismen nicht vollständig verstanden hat. In der vorliegenden Studie soll die Funktion von Kardiomyozyten, sowie deren Beeinflussung durch SEA0400, einem spezifischen Natrium/Kalzium Kanal Blocker in einem HFpEF Rattenmodel, basierend auf einer chronischen Nierenschädigung untersucht werden.

Material und Methoden: Hierfür wurde an männlichen Ratten eine subtotale Nephrektomie durchgeführt. Im Anschluss wurde nach 8 oder 24 Wochen eine vollständige Charakterisierung der Tiere mittels Echokardiographie und Druck-Volumen-Katheter Messungen in vivo sowie Messung der Kardiomyozytenfunktion und der Kalziumhomöostase an isolierten linksventrikulären Kardiomyozyten in vitro durchgeführt. Zusätzlich wurde die Wirkung von SEA0400 nach 24 Wochen in NXT and Sham Ratten sowohl akut in vitro als auch akut in vivo untersucht. Eine Subgruppe von Tieren wurde zwischen der 8. und der 24. Woche mit SEA0400 oder einer Placebo Lösung (1 mg/kg/KG) behandelt und ebenfalls vollständig in vivo und in vitro charakterisiert.

Ergebnisse: Subtotale Nephrektomie bei Ratten führte zu einer kompensierten chronischen Niereninsuffizienz sowie zu einer Herzinsuffizienz mit erhaltener Pumpfunktion. Die Kardiomyozyten von diesen Ratten zeigten eine deutlich verlangsamte Relaxation sowie einen verlängerten Kalzium-Transienten. Die eingeschränkte Relaxation steht in einer engen Beziehung zur Verschlechterung der diastolischen Funktion der Tiere in vivo. Eine akute Behandlung in vitro und in vivo mit dem Natrium/Kalzium Austausch (SEA0400) konnte sowohl die Kardiomyozytenfunktion als auch die linksventrikuläre Funktion verbessern. Durch die Langzeit-Applikation von SEA0400 konnte ein Reverse-Remodeling bei den HFpEF Ratten beobachtet werden.

Zusammenfassung: In einem Rattenmodell der Herzinsuffizienz mit erhaltener Auswurfleistung auf dem Boden einer subtotalen Nephrektomie konnte zum ersten Mal gezeigt werden, dass es sowohl zu einer Verschlechterung der linksventrikulären diastolischen Funktion in vivo als auch der Relaxation von einzelnen Kardiomyozyten in vitro kommt. Durch die Beeinflussung des Natrium/Kalzium Austauschers mittels SEA0400 konnte eine signifikante Verbesserung des klinischen Phänotyps und der Zellfunktion hervorgerufen werden. Dies könnte ein entscheidender Schritt in der zukünftigen Behandlung von HFpEF Patientinnen und Patienten sein.

Abstract

Introduction: Heart failure with preserved ejection fraction (HFpEF) is increasingly common but there is no special treatment available yet, most likely because underlying pathomechanisms are not well understood. To investigate cardiomyocyte function and calcium handling we established an animal model of chronic kidney disease (CKD) and HFpEF. Furthermore, the role of SEA0400, a $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) inhibitor was evaluated in this animal model.

Material and Methods: Young male Wistar rats were subjected to subtotal nephrectomy (NXT) or sham operation (Sham). In vivo (hemodynamics, echocardiography) and in vitro function (left ventricular (LV) cardiomyocyte cell shortening (CS) and Ca^{2+} transients (CaT)) were determined after 8 and 24 weeks. Furthermore, SEA0400 was investigated after 24 weeks acutely in vivo and in vitro. In a subgroup of rats SEA0400 or vehicle was given p.o. (1 mg/kg b.w.) between week 8 and 24.

Results: Subtotal nephrectomy in rats resulted in stable chronic kidney disease and the clinically relevant HFpEF phenotype with LV hypertrophy, a prolongation of the isovolumetric relaxation constant TAU (IVRc TAU), an elevation in end diastolic pressure (EDP), an increase in lung weight (pulmonary congestion) and a preserved LV systolic function (EF). Cardiomyocytes amplitude of cell shortening and calcium transients were unchanged in NXT vs. Sham at 8 and 24 weeks. Relaxation and CaT decay in NXT cardiomyocytes were progressively prolonged at 8 and 24 weeks vs. Sham, individually correlating with diastolic dysfunction in vivo. In NXT rats the sodium/calcium exchanger was modulated. While NCX protein expression was upregulated at 24 weeks, NCX forward mode activity – measured via caffeine induced calcium response was progressively reduced suggesting an increased NCX reverse mode activity in the subtotal nephrectomy heart failure rats. The NCX inhibitor, SEA0400, acutely improved LV relaxation in vivo and cardiomyocyte relaxation in vitro in NXT rats. Furthermore, chronic treatment with SEA0400 reversed cardiac remodeling and improved diastolic function in NXT rats.

Conclusions: Subtotal nephrectomy in rats induced the clinically relevant HFpEF phenotype and stable compensated CKD. Impaired LV relaxation was associated with slowed relaxation in cardiomyocytes from NXT. In NXT rats, the NCX was shifted to increased reverse mode activity and the treatment with the NCX inhibitor SEA0400 improved LV relaxation and cardiomyocyte relaxation acutely. Chronical treatment with SEA0400 reversed cardiac remodeling and diastolic dysfunction in HFpEF rats and therefore could be an interesting treatment option in the future for HFpEF patients with associated renal impairment (194).

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Abbreviations and Definitions

[Ca ²⁺]	calcium concentration
AB	aortic banding
ACE	angiotensin-converting enzyme
AF	atrial fibrillation
ANP	atrial natriuretic peptide
AP	action potential
ARB	angiotensin receptor blockers
AT/ ATII	angiotensin
ATP	adenosine triphosphate
AV	atrioventricular
BDM	butanedione monoxime
BNP	brain natriuretic peptide
BSA	bovine serum albumin
BW	body weight
Ca ²⁺	calcium ion
CaCl ²	calcium chloride
CaM	calmodulin
CaMKII	calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CaN	calcineurin
CaT	calcium transients
CaT TAU	time constant of Ca ²⁺ decay from a calcium Ca ²⁺ - transients
CCD	charge-coupled device
CICR	calcium induced calcium release
CKD	chronic kidney disease
CM	cardiac myocyte

CNP	C-type natriuretic peptide
CO	cardiac output
COPD	chronic obstructive pulmonary disease
CRS	cardio-renal syndrome
CRT	cardiac resynchronization therapy
CS	cell shortening
Cs ⁺	caesium
CsAsp	caesium aspartate
CsCl	caesium chloride
CsOH	caesiumhydroxid
CVD	cardiovascular disease
DADs	delayed afterdepolarizations
DAG	diacylglycerol
DBP	diastolic blood pressure
DCM	dilated cardiomyopathy
DHPRs	dihidropyridine receptors
DMSO	dimethyl sulfoxide
$dP/dt_{\max}$	maximal slope of LV systolic pressure increment
$dP/dt_{\min}$	maximal slope of diastolic pressure decrement
dV/dP	LV end-diastolic volume at a given change in LV end- diastolic pressure
E/e'	parameter for LV diastolic function
Ea	arterial Elastance
ECC	excitation-contraction coupling
ECG	electrocardiogram

EDP	end-diastolic pressure
EDPVR	end-diastolic pressure volume relationship
EF	ejection fraction
EGTA	ethylene glycol tetraacetic acid
ESC	european society of cardiology
ESPVR	end-systolic pressure volume relationship
ET	endothelin
F/F0	ratio, which describes the change of the calcium in comparison to the unstimulated control
Fluo-4AM	calcium sensitive dye for confocal measurements
Fmax	fluorescence at saturating free calcium concentration
Fmin	fluorescence at zero free calcium concentration
Fpeak	maximum fluorescence in the systole
Frest	fluorescence at resting free calcium concentration
FS	fractional shortening
Furo-2AM	calcium sensitive dye for epifluorescence measurements
GAPDH	glyceraldehyd-3- phosphat-Dehydrogenase
GFR	glomerular filtration rate
GPCR	G protein-coupled receptor

HDAC	histone-deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	heart failure
HFmrEF	heart failure with mid-ranged ejection fraction
HFpEF	heart failure with preserved ejection fraction
HFrEF	heart failure with reduced ejection fraction
HR	heart rate
HW	heart weight
Hz	hertz
ICD	implantable cardioverter-defibrillator
ICa ²⁺	Ca ²⁺ influx
INa ⁺	Na ⁺ influx
IP3	inositol-1,4,5-trisphosphate
IP3R	inositol-1,4,5-trisphosphate receptors
IVS	intraventricular septum
IVRc TAU	isovolumetric relaxation constant TAU
KB-R7943	2-[4-[(4-nitrophenyl)methoxy]phenyl]ethyl ester carbamimidothioic acid methanesulfonate
KCl	potassium chloride
Kd	calcium dissociation constant of the indicator

Kd-app	apparent calcium dissociation constant of the indicator
LA	left atrium
LCZ696	combination of ARB (valsartan) and a neprilysin (NEP) inhibitor (sacubitril)
LiCl	lithium chloride
LiOH	lithiumhydroxid
LSM	laser scanning microscope
LTCC	voltage dependent L-Type Ca^{2+} channels
LV	left ventricle
LVEDD	left ventricular end-diastolic diameter
LVEDP	LV end-diastolic pressures
LVEDV	LV end-diastolic volume
LVEF	LV ejection fraction
LVESD	LV end-systolic diameter
LVESP	LV end-systolic pressure
LVESV	LV end-systolic volume
LVH	left ventricular hypertrophy
LV mass	left ventricular mass
LVPW	left ventricular posterior wall
LW	lung weight
MCU	mitochondrial Ca^{2+} uniporter
MgATP	magnesium adenosine triphosphate
MgCl_2	magnesium chloride
MRA	mineralocorticoid receptor antagonists
Na^+	sodium ion
NaCl	sodium chloride

NaH ² PO ⁴	monosodium phosphate
NaOH	sodium hydroxide
NCX	sodium-calcium exchanger
NP	natriuretic peptides
NT	normal Tyrode's solution
NT-proBNP	N-terminal of the prohormone brain natriuretic peptide
NXT	subtotal nephrectomy animals
NYHA	NYHA New York Heart Association
PBS	phosphate buffered saline
PDE	phosphodiesterase--5-inhibition
PIP2	phosphatidylinositol-4,5-bisphosphate
PKA and PKC,	protein kinase A and C
PKG	protein kinase G
PLB	phospholamban
PLC	phospholipase C
PMCA	plasma membrane ATPase
PRSW	preload recruitable stroke work (slope of stroke work – EDV relationship)
PV	pressure volume
PW	posterior wall
R	fluorescence signal normalized to Frest
RAAS	renin-angiotensin-aldosterone system
Rf	dynamic range
Rmax	fluorescence ratio at saturating free calcium concentration
Rmin	fluorescence ratio at zero free calcium concentration
RT50	time point at which 50% of the Ca ²⁺ has been removed from the cytosol
RT90	time point at which 90% of the Ca ²⁺ has been removed from the cytosol
RyR	ryanodine receptor
SBP	systolic blood pressure

SEA0400	2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline
SERCA	sarco-endoplasmic reticulum calcium-ATPase
Sham	sham operated animals
SNS	sympathetic nervous system
SR	sarcoplasmic reticulum
SV	stroke volume
SW	stroke work
TAUcaff	time constant of caffeine-induced Ca^{2+} transient
TTP	time to peak, which gives information about cytosolic calcium increase in the systole
VA	Ventricular arrhythmias
VT	Ventricular tachycardia

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1 Introduction

1.1 Definition of heart failure

Heart failure (HF) is characterized by typical signs and symptoms like breathlessness, orthopnoea, ankle swelling, elevated jugular venous pressure, pulmonary crackles, peripheral oedema and fatigue caused by structural and/or functional cardiac abnormalities resulting in a reduced cardiac output and/or an elevated intracardiac pressures (1,2). Patients without typical signs and symptoms of HF can show asymptomatic structural or functional cardiac abnormalities, which are precursors of the clinical syndrome. Recognition of these precursors is important because they are related to poor outcomes and starting treatment at the precursor stage may reduce mortality in these patients (1). There are different underlying myocardial abnormalities resulting in systolic ventricular dysfunction like ischaemic cardiomyopathy, coronary artery disease or myocardial infarction. However, abnormalities can also relate to myocardial diastolic function, cardiac anatomy (valvular, pericardial or endocardial disease, and congenital defects), excitation formation (supraventricular or ventricular tachy- and bradyarrhythmia) or excitation conduction (AV-blocks and branch blocks) and lead to HF (1). For patients it is crucial to identify the underlying cardiac problem for therapeutic reasons, as the precise pathology determines the specific treatment used. For example, percutaneous coronary angiography and intervention or coronary bypass surgery in ischemic heart disease. Cardiac valve repair or replacement for valvular disease or reduction of heart rate in tach-cardiomyopathy (1).

1.2 Terminology of HF

The main terminology used to describe HF is based on measurement of the left ventricular (LV) ejection fraction (EF). HF comprises a wide range of patients, from those with normal LVEF ($\geq 50\%$; HF with preserved EF (HFpEF)) to those with reduced LVEF ($< 40\%$; HF with reduced EF (HFrEF)). Additionally, patients with a LVEF in the range of 40–49% are now define as HFmrEF (HF with mid-range EF) (1). Furthermore, there is a differentiation of patients with HF based on underlying

aetiologies, demographics, co-morbidities and response to therapies. Moreover, it is important to distinguish between acute onset of heart failure and chronic heart failure. The diagnosis of HFpEF is more challenging than the diagnosis of HFrEF, because underlying pathophysiological mechanisms are not fully understood for HFpEF. Patients with HFpEF generally do not have a dilated LV, but often have an increase in LV wall thickness and/or an increased left atrial (LA) size as a sign of increased LV filling pressures. Most patients with HFpEF have additional evidence of impaired LV filling or suction capacity, also classified as diastolic dysfunction, which is generally accepted as the likely mechanism limiting cardiac output in these patients. Patients with HFmrEF have a LVEF that ranges from 40 to 49% and are not well studied at the moment in clinical trials, but potentially more closely related to HFrEF than to HFpEF (1). In figure 1.1 the different types of heart failure are summarized.

Type of HF	HFrEF	HFmrEF	HFpEF
CRITERIA	1 Symptoms ± Signs ^a	Symptoms ± Signs ^a	Symptoms ± Signs ^a
	2 LVEF <40%	LVEF 40–49%	LVEF ≥50%
	3 –	1. Elevated levels of natriuretic peptides ^b ; 2. At least one additional criterion: a. relevant structural heart disease (LVH and/or LAE), b. diastolic dysfunction (for details see Section 4.3.2).	1. Elevated levels of natriuretic peptides ^b ; 2. At least one additional criterion: a. relevant structural heart disease (LVH and/or LAE), b. diastolic dysfunction (for details see Section 4.3.2).

BNP = B-type natriuretic peptide; HF = heart failure; HFmrEF = heart failure with mid-range ejection fraction; HFpEF = heart failure with preserved ejection fraction; HFrEF = heart failure with reduced ejection fraction; LAE = left atrial enlargement; LVEF = left ventricular ejection fraction; LVH = left ventricular hypertrophy; NT-proBNP = N-terminal pro-B type natriuretic peptide.
^aSigns may not be present in the early stages of HF (especially in HFpEF) and in patients treated with diuretics.
^bBNP > 35 pg/ml and/or NT-proBNP > 125 pg/mL.

Figure 1.1 Definition of heart failure with preserved (HFpEF), mid-range (HFmrEF) and reduced ejection fraction (HFrEF) (1)

The following section will mainly focus on HFrEF and HFpEF and the most important differences between the two phenotypes.

1.2.1 HFrEF versus HFpEF

In the past experts discussed, whether HFrEF and HFpEF represent two different types of diseases or whether they define one whole heart failure spectrum with intermediate phenotypes showing overlapping aspects of both entities (3,4). But nowadays there is strong evidence and broad consent that HFrEF and HFpEF are two separate diseases, mostly based on the fact that etiological factors seem to

diverge between them. While lots of various causes contribute to HFrEF (including viral infection, toxic and idiopathic cardiomyopathy), the presence of coronary artery disease is most common (1). In HFpEF, risk factors such as arterial hypertension, diabetes, obesity or chronic kidney disease play a major role in the origin and progression of the disease (1,5). The typical HFpEF patients are older, female, obese, hypertensive and suffer from chronic kidney injury. HFrEF was named “systolic heart failure” while HFpEF was called “diastolic heart failure” in the past. These terms may be confusing and also not completely accurate because diastolic function is impaired frequently also in systolic heart failure patients (HFrEF) (6), and systolic function is abnormal in most diastolic heart failure patients (HFpEF) (7). Therefore, HFrEF and HFpEF characterize these different heart failure phenotypes better than “systolic” and “diastolic” heart failure.

To differentiate between both entities, the assessment of LVEF is important in the diagnostic process of heart failure, besides clarifying cardiac abnormality (1). The LVEF has strong prognostic (low EF correlates with poor survival (1,8)) and therapeutic implications. The ESC guidelines distinguish between HFrEF and HFpEF because of their different response to treatment. Furthermore, clinical trials which include and exclude patients based on their EF show completely different results in HFrEF and HFpEF patients. While trials in HFrEF patients with an EF of less than 30% (9,10), 35% (11-18) or 40% (19-22) has shown a successful reduction in mortality by pharmacological or device therapy; all trials including patients with an EF above 40-45% failed to prove such a survival benefit (22-24). Typical morphological and functional characteristics for HFrEF and HFpEF are shown in figure 1.2. HFrEF patients indicate, besides typical signs and symptoms of HF and reduced EF, a dilated LV with thin walls. Whereas HFpEF patients display, besides typical signs and symptoms of HF and preserved EF, a concentric LV hypertrophy with a smaller chamber size, thick walls, an enlargement of the left atrium (LA) and diastolic dysfunction (1).

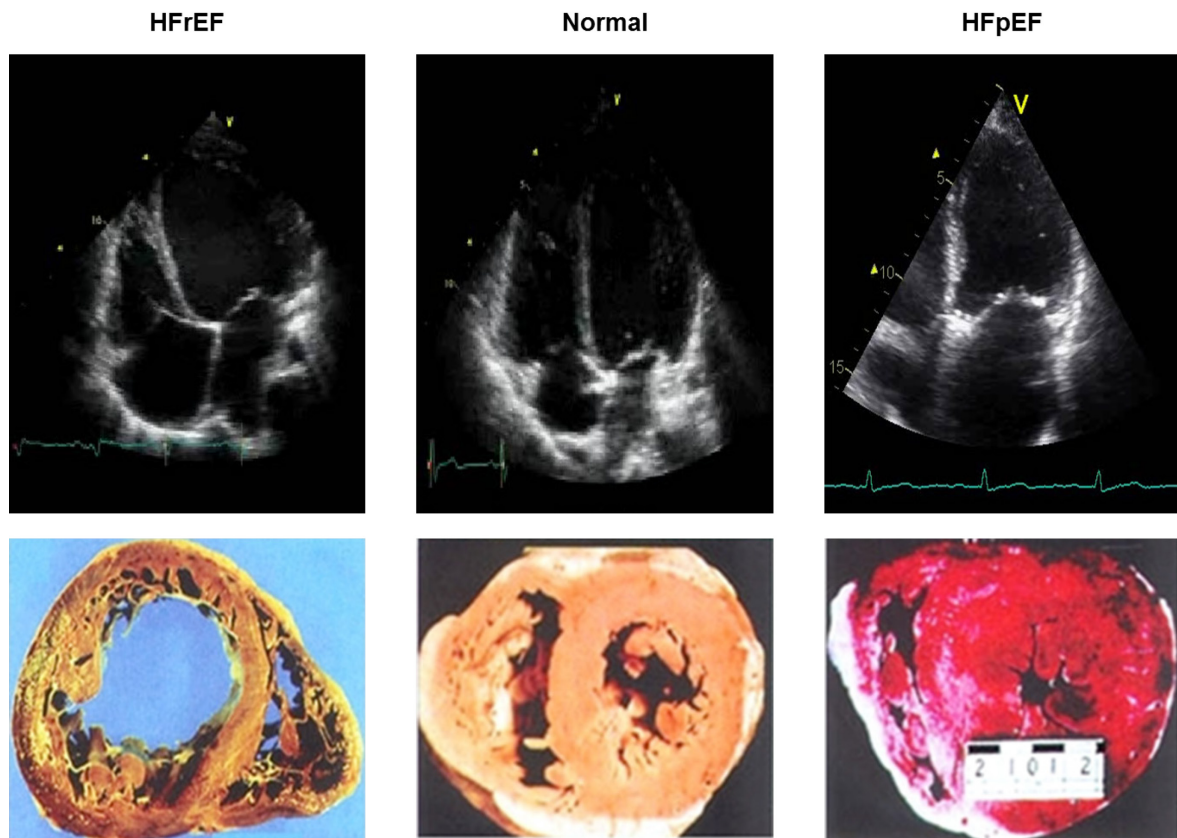


Figure 1.2 Heart failure phenotypes

Chatterjee K. et al., J Card Fail. 2007(195)

1.3 Epidemiology of HF

Heart failure is a common disease with increasing prevalence and high morbidity and mortality. Clinical trials showed one-year mortality rates between 26% to 41% (25,27-31), and 5-year mortality rates between 60% to 75% (25-31). Moreover, mortality of heart failure patients increases with age and comorbidities and is comparably or even higher with most types of cancer, despite improved heart failure therapy (25-32). The overall heart failure prevalence is around 5% (33,34) and therefore heart failure is a huge socio-economic burden (35).

1.3.1 Epidemiology of HFpEF

The prevalence of HFpEF patients in the general population is between 1% and 3% (34,36,40). In clinical trials between 43-59% (34,36-40) of HF patients have preserved ejection fraction. Particularly other diseases like severe lung disease, severe valvular disease or acute myocardial ischaemia have to be excluded before HFpEF is

diagnosed (41). Patients with HFpEF have a wide range of cardiovascular risk factors. Especially arterial hypertension, obesity, chronic kidney disease (31,42,43,46,47) and diabetes are highly present (31,37-47). Furthermore, HFpEF patients have more often a history of atrial fibrillation but less often a history of coronary artery disease. Most clinical studies which compared outcome in HF patients report a slightly favourable survival in HFpEF patients compared to HFrEF patients (31,33,38,8,47).

1.4 Pathophysiology of HF

The pathophysiological understanding of heart failure has largely advanced in recent years for HFrEF. However, today up to 50% of patients with signs and symptoms of heart failure present preserved ejection fraction. Unfortunately, the pathophysiological understanding of HFpEF is much less than for HFrEF and therapeutic options are sparse (1). Heart failure is a progressive disease caused by different risk factors (age, hypertension) and in addition some special trigger factors (atrial fibrillation, renal failure) where an initial pathophysiological stimulus (e.g. hemodynamic overload) initiates maladaptive remodelling processes that eventually lead to overt symptomatic heart failure (1). There is recent evidence that Ca^{2+} could be part of the early triggering events for myocardial remodeling, through Ca^{2+} -mediated regulation of gene transcription. It is known that this process also affects the function and regulatory pathways of Ca^{2+} and Na^{+} handling proteins which eventually dysregulates the Ca^{2+} balance of the cell. Disturbed Ca^{2+} and Na^{+} handling are hallmarks of the systolic failing phenotype (48) and are considered a major cause of contractile dysfunction and high arrhythmia risk. While considerable efforts have been done to understand the pathogenesis of HFrEF, very little is known about the cellular mechanisms of HFpEF. Recent evidence indicates a role for impaired cardiomyocyte ion homeostasis (48-50).

1.4.1 Pathophysiology in HFpEF versus HFrEF

The pathways for the development of HFpEF and HFrEF are different. In HFpEF various comorbidities induced a systemic proinflammation which leads to myocardial structural and functional alterations. Whereas in HFrEF, especially ischemia, infections and toxic agents occurs oxidative stress in the cardiomyocytes. Oxidative stress triggers autophagy, apoptosis, or necrosis and leads to dead cardiomyocytes which are replaced by fibrous tissue in the end (51).

The high prevalence of comorbidities such as chronic kidney disease, obesity, diabetes mellitus, chronic obstructive pulmonary disease, and salt-sensitive hypertension in HFpEF patients induce a systemic proinflammation. The proinflammatory state initiates microvascular endothelial inflammation, reduces nitric oxide bioavailability, cyclic guanosine monophosphate content and protein kinase G (PKG) activity in adjacent cardiomyocytes. Low PKG activity effects the development of hypertrophy and an increase in resting tension. Furthermore, stiff cardiomyocytes and interstitial fibrosis contribute to high diastolic LV stiffness and heart failure development with concentric LV hypertrophy in these patients (51).

Myocardial and functional remodeling in HFpEF differs from HFrEF, in which remodeling is driven by progressive loss of cardiomyocytes (52). This loss of cardiomyocytes results from various modalities of cell death such as exaggerated autophagy, apoptosis, or necrosis, all of which are triggered by oxidative stress present within the cardiomyocyte usually results from ischemia, infection, or toxicity (53-55). As a consequence of the loss of cardiomyocytes, excessive wall stress shifts the balance in the extracellular matrix between collagen deposition and degradation (56). These alterations within the extracellular matrix importantly contribute to LV dilation and eccentric LV remodeling (56). In HFrEF, replacement of dead cardiomyocytes by collagen creates patchy areas of fibrosis. A comparative analysis of endomyocardial biopsy samples from HFpEF and HFrEF (57,58) indeed showed the presence of replacement fibrosis in HFrEF but not in HFpEF. Figure 1.3 shows myocardial remodeling in HFpEF and HFrEF.

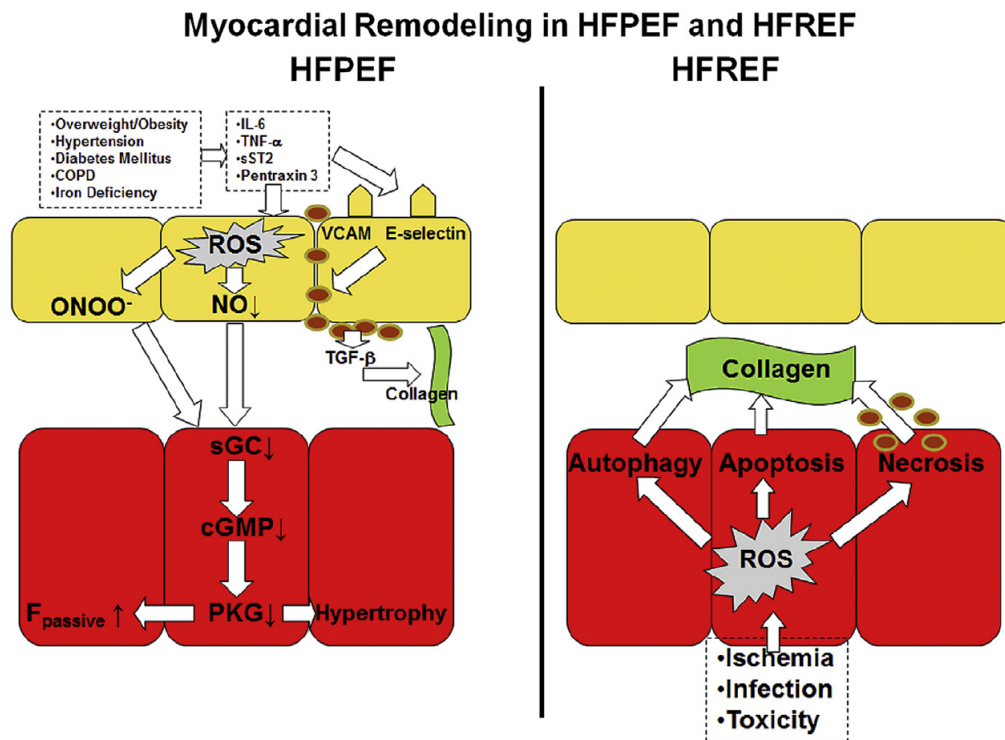


Figure 1.3 Pathophysiological myocardial remodeling in HFpEF and HFrEF (51)

1.5 Therapy of HF

The choice of the appropriate HF therapy is mostly designed due to the underlying cause of the disease. We can distinguish between surgical therapy (coronary bypass surgery or surgical valve replacement), non-surgical device therapy (implantable cardioverter-defibrillator (ICD) or cardiac resynchronization therapy (CRT)), pharmacological interventions (like angiotensin-converting enzyme inhibitors or beta-blockers) and management of arrhythmias, bradycardias, atrioventricular block and other primarily non-cardiac associated comorbidities (anaemia, diabetes, renal dysfunction etc.). Additionally, a healthy life style behaviour and moderate, regular, aerobic exercise training would be beneficial to prevent the development of heart failure or could at least improve symptoms and functional capacity in any heart failure patient (1). The three major goals of treatment in patients with established HF are to relieve signs (oedema) and symptoms (dyspnoea), prevent hospital admission and improve survival. Although the focus of most large clinical trials was previously mortality, it is nowadays recognized that preventing HF hospitalisation as well as reliving individual

symptoms and improving exercise capacity are more and more recognized as important therapeutic goals for patients and healthcare systems (1,59,60).

1.5.1 Therapy of patients with HFrEF

The primary therapeutic focus in HFrEF is to eliminate potential well-known underlying causes of heart failure (for instance coronary revascularisation therapy in coronary artery disease or aortic valve replacement in aortic stenosis) (1). Furthermore, the ESC guidelines recommend a treatment strategy for the use of drugs and devices in HFrEF patients. Clinical trials showed in patients with HFrEF a significant reduction in mortality by pharmacological interventions with angiotensin converting enzyme (ACE) inhibitors (9,11,61), β -blockers (10,19,62,63) and mineralocorticoid receptor antagonists (MRA) (13,14), which are all first-class recommendations in the treatment of HFrEF (1). For specific subgroups of heart failure patients with reduced ejection fraction the treatment with the If-blocker ivabradine (15) further demonstrated a reduction in HF hospitalisation. Furthermore, the alternative use of angiotensin receptor blockers (ARB) is recommended for patients with HFrEF and intolerance against ACIs (20,21). A new compound (LCZ696) that combines the moieties of an ARB (valsartan) and a neprilysin (NEP) inhibitor (sacubitril) has recently been shown to be superior to an ACEI (enalapril) in reducing the risk of death and of hospitalization for HF in a single trial with strict inclusion/exclusion criteria (64). Sacubitril/valsartan is therefore recommended to replace ACEIs in ambulatory HFrEF patients who remain symptomatic despite optimal therapy. Moreover, the above medications should be used in conjunction with diuretics in patients with symptoms and/or signs of congestion (1). On top of the described pharmacological treatment of HFrEF patients, additional use of different devices for cardiac resynchronisation therapy (CRT) (10,18,66) or implantable cardioverter-defibrillator (ICD) (16,17) can further improve survival and physical capacity. The success of the different treatment modalities for patients with heart failure and reduced ejection fraction is reflected by a significant reduction of mortality and mobility in the last years (31).

1.5.2 Therapy of patients with HFpEF

At the moment, there is no specific treatment available to convincingly reduce morbidity and mortality in patients with HFpEF. Diuretics are used to control sodium and water retention and relieve breathlessness and oedema as in HFrEF. Adequate treatment of hypertension and myocardial ischaemia is also considered to be important, as is control of the ventricular rate in patients with AF (1). In contrast to the beneficial effects of ACEIs and ARBs in HFrEF, randomized, placebo-controlled trials focusing on outcome in HFpEF failed to demonstrate improved survival. Moreover, the treatment with β -blocker showed no significant benefit in terms of improved survival in HFpEF patients (66). Actually, further large clinical trials focusing on the outcome of HFpEF patients with β -blockers (71) and ARBs (72) are in progress. The Aldo-DHF randomized controlled trial investigated the MRA spironolactone in HFpEF patients and failed in terms of improvement of functional capacity in these patients while echocardiographic parameters of diastolic function were significantly improved (67). Sildenafil, a phosphodiesterase-(PDE)-5-inhibitor completely failed in HFpEF patients to improve echocardiographic parameters or exercise capacity in a large placebo controlled clinical trial (68). A phase 2 double-blind randomised controlled trial with the novel neprilysin-inhibitor LCZ-696 in patients with HFpEF enhanced New York Heart Association (NYHA) class and further lowered NTproBNP levels at 12 weeks but not after 36 weeks of treatment compared to valsartan (69). Exercise training improves exercise capacity and diastolic function in patients with heart failure and preserved ejection fraction in a pilot study and is therefore the only intervention so far that is beneficial in HFpEF patients (70). Concluding current guidelines of the European Society of Cardiology do not recommend any specific therapy in HFpEF. Preventing risk factors and managing exacerbating factors such as atrial fibrillation and other comorbidities remain the only suggested interventions (1).

1.6 Hemodynamic mechanisms in HFpEF

LV systolic and diastolic function could be altered in HFpEF. Important for hemodynamic changes in diastole are an impaired relaxation, a reduced suction and an increased stiffness. Additionally, other factors like chronotropic incompetence, atrial fibrillation, pulmonary hypertension or volume overload might aggravate in HFpEF patients at rest and in particular during exercise (73).

1.6.1 Impaired relaxation in early diastole in HFpEF

The isovolumetric relaxation time is described as the time period between aortic valve closure and mitral valve opening. LV volume remains constant but LV pressure declines rapidly (isovolumetric relaxation). The decay of the pressure can be fit with a monoexponential equation (74,75) which includes the isovolumetric relaxation constant TAU (IVRc TAU). Increased values of IVRc TAU represent a slower LV pressure decay indicating slowed LV relaxation and therefore contribute to elevated LV end-diastolic pressures (LVEDP). For example, during exercise (increase in heart rate) diastolic duration shortens and in diseased hearts may lead to incomplete relaxation, which then elevates the LVEDP and consequently could explain why symptoms in HFpEF patients often occur during physical activity, whereas they are minor or even absent at rest (reduced exercise capacity) (76).

1.6.2 Reduced suction in mid diastole contribute to compromised LV filling in HFpEF

LV filling is enhanced because of an additional suction in mid diastole which occurs from an intraventricular mitral to apical pressure gradient and results in a mitral to apical blood flow (77,78). In HFpEF, the pressure gradient from mitral to apical might be diminished due to impaired relaxation and changed LV geometry with reduced untwist and vortex formation resulting in a reduced suction in mid diastole and potentially contribute to impaired LV filling in HFpEF patients (79).

1.6.3 Increased end-diastolic stiffness in HFPEF

The end-diastolic pressure volume relationship (EDPVR) describes the passive filling curve for the LV and thus the passive elastic properties of the myocardial tissue. Under physiological conditions, the LV volume and pressure constantly change during the cardiac cycle (80). Furthermore, it is relevant to discriminate between LV capacitance, compliance and stiffness (81). Capacitance of the LV is the end-diastolic volume at a given end-diastolic pressure and an up- or leftward-shift of the EDPVR slope indicates a loss of capacitance. Compliance of the LV is the change of end-diastolic volume at a given change in end-diastolic pressure (dV/dP). LV Stiffness is the reciprocal value of compliance (dP/dV). The slope of the EDPVR and thus the LV's stiffness is a function of volume using an exponential fit. A flat EDPVR always indicating high compliance and low stiffness and conversely a steeper EDPVR describe a low compliance and a high stiffness. In HFpEF a stiffer EDPVR and a loss of LV end-diastolic capacitance have been reported (82-84).

1.6.4 Altered LV systolic function

In HFpEF not only LV diastolic function is altered, also systolic function of the LV is often impaired (7,85-87), which may play a minor role at rest but become more important during exercise and might be one cause for limited exercise capacity in HFpEF patients (79,88).

1.7 Cellular mechanisms in HFpEF

1.7.1 Intracellular Calcium handling – Excitation-contraction coupling (ECC)

The main function of the human heart is to pump oxygen depleted blood into the lungs and blood, rich in oxygen, into the periphery. This happens as a sequence of contractions and relaxations of single cardiomyocytes. Atrial and ventricular beats are the result of a concerted contraction of cardiomyocytes, which comprise about 20 % of all cells within the human myocardium (89). The process that links electrical excitation with myocyte contraction is termed excitation-contraction coupling (ECC) (50, 90) and Ca^{2+} plays the main role in this process. During ECC on a single cell level, voltage dependent L-Type Ca^{2+} channels (LTCC), located in the sarcolemmal membrane, serve as the initial trigger for the translation of the electrical action-potential (AP) into mechanical force. Electric activation triggers voltage-dependent Na^{+} channels, which generate a high Na^{+} influx (I_{Na}) (90). This high I_{Na} depolarizes the cell membrane, which immediately opens L-type Ca^{2+} channels, located at sarcolemmal-sarcoplasmic reticulum junctions (couplons), also known as the dihydropyridine receptors (DHPRs), and Ca^{2+} enters the cell (I_{Ca}). Ca^{2+} influx is limited by Ca^{2+} -dependent inactivation mediated by calmodulin, a part of the DHPRs complex, bound to its cytosolic side. Ca^{2+} ions entering the cytosol through LTCC bind to ryanodine receptors (RyR), located in the membrane of the sarcoplasmic reticulum (SR) and actively lead to release of Ca^{2+} through RyR from intracellular Ca^{2+} stores, by a process termed Ca^{2+} -induced Ca^{2+} release (CICR) (50). The release of Ca^{2+} from the SR, results in a cytosolic Ca^{2+} transient (CaT) that increases free $[\text{Ca}^{2+}]_i$ to concentrations as high as 1 μM or more, allowing significant amounts of Ca^{2+} to bind troponin C, moving the tropomyosin complex from the actin binding site. This leads to the interaction of myosin and actin filaments and ultimately the generation of force. The SR is the cells most potent Ca^{2+} reservoir (50). The amount of Ca^{2+} that is stored in the SR has an influence on the opening probability of the RyRs. If SR Ca^{2+} content is high, a low I_{Ca} will be sufficient to trigger an opening. Higher SR content also allows for more Ca^{2+} to be released. In contrast a low SR Ca^{2+} content can prevent

RyRs from opening at a given I_{Ca} -trigger, thus allowing the SR to reload (50). After CICR mediated contraction of the cell, Ca^{2+} removal allows for its diastolic relaxation: Ca^{2+} is transported back into its cytosolic SR by the SR Ca^{2+} ATPase (SERCA) and extruded from the cell via the Na^+/Ca^{2+} exchanger (NCX) or to a smaller extent through the plasma membrane Ca^{2+} ATPase (PMCA) and buffered by mitochondria (uptake via the mitochondrial Ca^{2+} uniporter, MCU). Figure 1.4 shows the main Ca^{2+} transport pathways in ventricular cardiomyocytes during ECC (50,196).

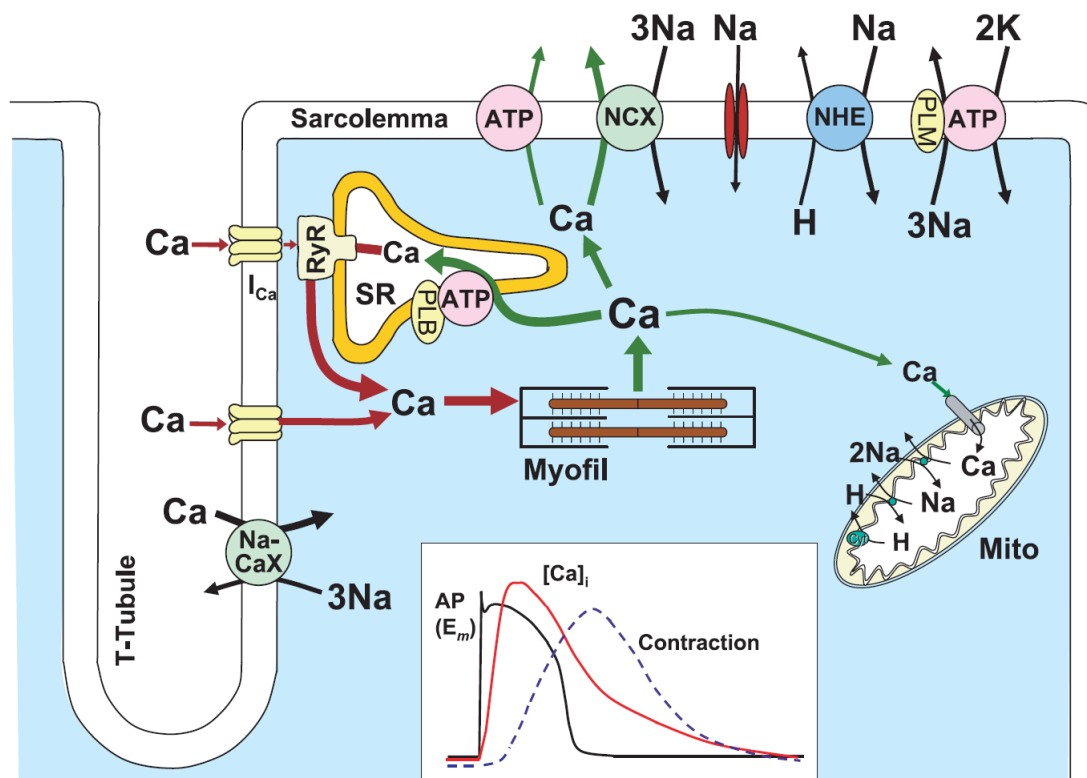


Figure 1.4 Ca^{2+} Transport in a ventricular myocyte (196)

The SR Ca^{2+} -ATPase pump (SERCA2a) induces Ca^{2+} reuptake into the SR and removes around 70% from Ca^{2+} located in the cytosol in humans and is therefore the most effective mechanism for calcium removal during diastolic relaxation. The Na^+/Ca^{2+} exchanger (NCX), located in the sarcolemma, removes 28% of intracellular Ca^{2+} . Additionally, the last 2% are being eliminated by the mitochondrial Ca^{2+} -uniporter and the sarcolemmal Ca^{2+} -ATPase. The amount of each transporter to the reuptake of calcium from the cytosol is different between species. For example, in rats the SERCA2a manages to remove 92% while the NCX remove only 7% of the intracellular Ca^{2+} .

Furthermore, the excitation-contraction coupling process is influenced by many different regulatory proteins like calmodulin, calsequestrin and phospholamban (PLB), an inhibitor of SERCA2a. For example, an ameliorated relaxation in cardiomyocytes is mediated by phospholamban. The phosphorylation of PBL results in a faster SERCA2a activity and an increased dissociation of Ca^{2+} from the myofilaments (50,89).

SR Ca^{2+} release, following influx of trigger Ca^{2+} through LTCC, on a subcellular level is mainly mediated by concerted release of Ca^{2+} from RyR clusters located in the SR membrane. The reported size of these clusters varies between 15 and >100 RyRs (91-95). The RyR mediated Ca^{2+} release event from RyR clusters is termed Ca^{2+} spark (96). Ca^{2+} sparks occur randomly in resting cells and have a spatial spread of around 2 μm and have been reported to raise local $[\text{Ca}^{2+}]_i$ up to ~ 200 nM within 10 ms (97-100). The principle of CICR, followed by cytosolic Ca^{2+} removal mainly via SERCA and NCX is similar in ventricular and atrial cells.

1.7.2 Ca^{2+} cycling in heart failure

On a molecular level, cardiomyocytes undergo a variety of changes during the course of heart failure. Most of these changes are of compensatory nature to cope with the hemodynamic demands in an acute setting of cardiac dysfunction and they occur in ventricular and atrial cells alike. However, in the long run, these changes are paralleled by maladaptive structural and functional remodeling processes that negatively affect cardiac contractility. The most profound features of heart failure are dysfunctions of cardiac contractility and arrhythmias, with defective Ca^{2+} signalling during ECC and altered Ca^{2+} homeostasis as decisive factors for the development and maintenance of the disease. This is mirrored by slower and less synchronous onset of SR Ca^{2+} release, decreased cytosolic CaT amplitudes, prolonged Ca^{2+} transient decay time, reduced SR Ca^{2+} content and slower and less synchronous cytosolic Ca^{2+} removal (90,101). Those changes in Ca^{2+} cycling become obvious especially at high heart rates, demonstrating the impaired force frequency relation of the cardiomyocyte (37,102,103). Many proteins that regulate the SR Ca^{2+} content are not functioning properly in heart failure cells. It has been observed that the RyRs are in a hyperphosphorylated

state leading to increased diastolic Ca^{2+} leak. This leak is partially responsible for the reduced SR Ca^{2+} -content and can lead to a higher $[\text{Ca}^{2+}]_i$ in diastole, thus extending relaxation time in heart failure cardiomyocytes. A high $[\text{Ca}^{2+}]_i$ combined with diastolic Ca^{2+} leakage of the RyRs can also trigger delayed afterdepolarizations (DADs), which can result in arrhythmias and sudden cardiac death (104). Another important mechanism which causes RyRs Ca^{2+} leak is the continuous β -adrenergic stimulation present in patients with heart failure (101). Elevated $[\text{Ca}^{2+}]_i$ is also a consequence of overexpression and increased activity of the NCX. Intracellular Na^+ concentration physiologically rises with higher heart rates, thus allowing NCX to work in reverse-mode and supplying the SR with Ca^{2+} . In heart failure, however, the Na^+ concentrations are considerably higher, probably due to an impaired function of the Na^+/K^+ ATPase, which does not manage to eliminate Na^+ sufficiently. These high Na^+ concentrations produce a higher Ca^{2+} influx via the NCX and can again lead to a diastolic overload of calcium in heart failure (101,102). Other factors that contribute to a decreased SR Ca^{2+} -content and increased diastolic $[\text{Ca}^{2+}]_i$ are reduced expression of SERCA2a and increased inhibition of the pump by phospholamban. All conducted studies researching the presence of SERCA2a in animal and human failing hearts have confirmed that its expression is reduced when compared with normal hearts. Furthermore, a connection between different types of SERCA2a proteins and the progression of the disease is known (102). Phospholamban levels seem to stay the same thus reducing the ratio of SERCA2a to phospholamban protein expression. A lower ratio indicates a higher inhibition of the pump (102,104).

Due to lack of animal models, which would provide a good analogy to the disease in humans, Ca^{2+} cycling in HFpEF has not yet been studied in detail. However, it seems that Ca^{2+} changes that occur are different from those in HFrEF. Selby et al (105) investigated human cells obtained from biopsies in patients with preserved EF and left ventricular hypertrophy (LVH) and observed, that even though diastolic $[\text{Ca}^{2+}]_i$ concentrations are elevated, systolic $[\text{Ca}^{2+}]_i$ are unchanged when compared to controls. Furthermore, SERCA2a levels seemed to be unaltered and NCX activity was decreased (105,106).

1.7.3 Na⁺/ Ca²⁺ exchanger (NCX) in heart failure

NCX is a plasma-membrane protein counterbalancing the influx of Ca²⁺ through LTCC during each cardiac cycle. It extrudes 1 Ca²⁺ ion in exchange for 3 Na⁺ ions and therefore carries one net charge into the cell per cycle (forward mode). Increased diastolic [Ca²⁺]_i and impaired cytosolic Ca²⁺ removal by SERCA exposes NCX to increased levels of Ca²⁺ favouring Na⁺ influx. This Na⁺ influx can activate voltage-dependent channels, eventually leading to premature AP induced SR Ca²⁺ release (DADs). DADs may lead to a disturbed cardiac rhythm and contractility and increased NCX activity has been linked with heart failure (109). Moreover, NCX can extrude Na⁺ in exchange for Ca²⁺ (reverse mode). In human heart failure, cytosolic levels of Na⁺ have been shown to be increased and NCX might play a role in the maintenance of high diastolic [Ca²⁺]_i and by that impaired contractility and relaxation in the disease (90,108,109). In conclusion, both decreased SERCA and increased NCX function might contribute to increased diastolic [Ca²⁺]_i and impaired cardiac contractility, relaxation or rhythm in heart failure.

1.7.4 SEA0400

SEA0400, with its chemical formula 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline, is a novel and selective inhibitor of the Na⁺/Ca²⁺ exchanger (NCX) with IC₅₀ value of 5-33 nM. SEA0400, as a potent inhibitor of NCX has limited impact on L-type Ca²⁺ channels (110,111). SEA0400 concentration-dependently inhibited the NCX current with a 10-fold higher potency than that of KB-R7943, another commercially available NCX blocker; 1 μM SEA0400 and 10 μM KB-R7943 inhibited the NCX current by more than 80%. KB-R7943, at 10 μM, inhibited the sodium current, L-type calcium current, delayed potassium current and inwardly rectifying potassium current by more than 50%, but SEA0400 (1 μM) had no significant effect on these currents (110,111). These results indicate that SEA0400 is a highly potent selective inhibitor of NCX (111). In experimental models, SEA0400 prevents cytoplasmic and mitochondrial Ca²⁺ overload during ischemia (112-114) and results in improved recovery of ventricular function after

reperfusion (114,115) associated with mitochondrial preservation (112,114). In figure 1.5 chemical structure of SEA0400 is shown.

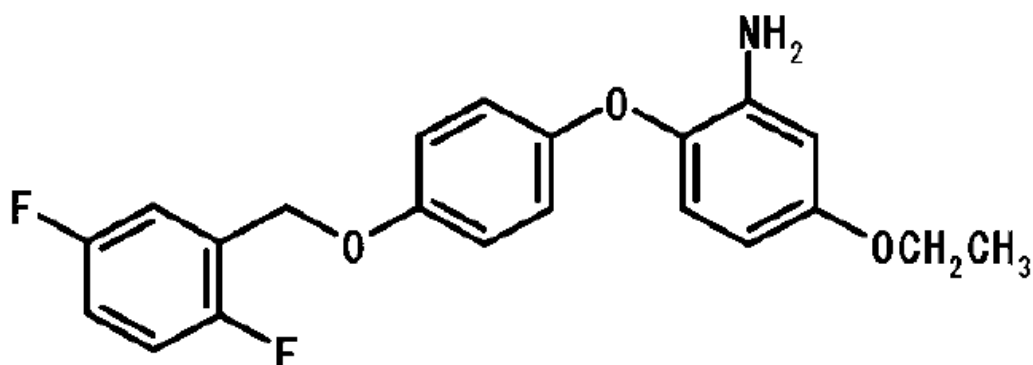


Figure 1.5 Chemical structure of SEA0400:

(2-[4-[(2,5 difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline) (197)

1.8 Cardiorenal syndrome

There is an important and close connection between the heart, the kidneys and the vascular system, which is described as “cardio-renal axis”. It is well established that a large number of hospitalized patients present various degrees of heart and kidney dysfunction. Primary disease of the heart or kidney often involves dysfunction or injury to the other. The “cardio-renal-axis” includes three different neurohormonal pathways which are closely connected to each other (116).

First, the natriuretic peptides (NP), including atrial natriuretic peptide (ANP), brain-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). While ANP and BNP are being synthesized in the heart, CNP is synthesized in the kidneys and endothelium. NPs have various functions, for example they suppress cellular proliferation, inhibit platelet activation and preserve myocardial function and structure through activation of the pGC-cGMP pathway in the cells. Moreover, natriuretic peptides promote natriuresis, inhibit aldosterone production and promote vasodilatation (116). Second, the sympathetic nervous system (SNS), especially the catecholamines are potent antagonists by inducing systemic vasoconstriction. This leads to a reduction in renal blood flow and consequently activating the renin-angiotensin-aldosterone system (RAAS) as third neurohormonal pathway (116). The activation of aldosterone, increases sodium and water retention in the nephron, leading to volume overload. The induction of pro-inflammatory and pro-fibrotic processes as well as additional cell loss through apoptosis is a result of the induced volume overload through the activation of the RAAS (116). The combination of volume overload paired with systemic vasoconstriction is responsible for the manifestation of heart failure symptoms (116). The close interactions between the heart and the kidney, in which the primary failing organ can be either one of them, have been described as the cardiorenal syndrome (CRS) (116).

1.8.1 Definition and classification of the cardio-renal syndrome (CRS)

The relationship between cardiac and renal dysfunction is described as cardio-renal syndrome (CRS). Cardiac and renal disease have similar risk factors (arterial hypertension, diabetes and obesity) and both, heart failure and renal disease are associated with poor outcome and hence a greater burden on our healthcare system and patient quality of life (117).

Since the consensus conference in 2008 there are 5 subtypes of this syndrome (118, 120). In figure 1.6 the different subtypes are shown.

CRS type 1 or acute CRS describes a sudden decrease in cardiac function which leads to acute renal dysfunction (example: cardiogenic shock → acute kidney injury).

CRS type 2 or chronic CRS describes chronic worsening of the cardiac function which leads to continuous impairment of renal function (example: congestive HF → chronic kidney disease)

CRS type 3 or acute renocardiac CRS describes acute kidney failure which consequently leads to cardiac dysfunction (example: glomerulonephritis → HF)

CRS type 4 or chronic renocardiac CRS describes chronic renal dysfunction which leads to progressive cardiac failure (chronic kidney disease → diastolic dysfunction and heart failure)

CRS type 5 or secondary CRS describes a combined renal and cardiac dysfunction due to a different primary systemic disease

Type	Denomination	Description	Example
1	Acute cardiorenal	Heart failure leading to AKI	Acute coronary syndrome leading to acute heart and kidney failure
2	Chronic cardiorenal	Chronic heart failure leading to kidney failure	Chronic heart failure
3	Acute nephrocardiac	AKI leading to acute heart failure	Uremic cardiomyopathy AKI related
4	Chronic nephrocardiac	CKD leading to heart failure	Left ventricular hypertrophy and diastolic heart failure due to kidney failure
5	Secondary	Systemic disease leading to heart and kidney failure	Sepsis, vasculitis, diabetes mellitus

Figure 1.6 Classification of cardiorenal syndrome based on a system proposed by Ronco et al (119)

1.8.2 Cardio-renal syndrome and HFpEF

The cardiorenal syndrome type 4 is characterized by cardiovascular impairment in patients with chronic kidney disease. Renal dysfunction is an independent risk factor for cardiovascular disease with a higher mortality risk and sudden cardiac death (119). In patients with CKD pressure and volume overload can directly induce left ventricular hypertrophy and therefore contribute to diastolic dysfunction and furthermore to the development of heart disease (124). Particularly, in patients with HFpEF renal dysfunction is present in up to 60% (121). Furthermore, HFpEF patients are older and have more comorbidities, which can also affect the kidney. CRS Type 4 could therefore represent a possible cause in the development of the disease (123). In an animal model unilateral nephrectomy leads to a mild impairment of the renal function and promotes LV myocardial fibrosis, increases myocardial apoptosis and results in diastolic dysfunction with preserved EF (122).

In patients with CKD, starting haemodialysis, left ventricular hypertrophy and diastolic dysfunction is highly prevalent and it is accountable for subsequent hospitalizations for heart failure (124). Almost half of cardiovascular deaths in the end-stage kidney disease population are related to cardiac arrhythmia or sudden death (125). The increased risk for sudden death seems to be particularly related to longer dialytic intervals in subjects undergoing thrice weekly haemodialysis treatment because of extreme shifts of electrolytes and fluids (125).

1.9 Description and aims of the thesis

Cumulating evidence suggests that the structural myocardial changes in HFpEF are distinctively different from HFrEF (51). However, function of beating cardiomyocytes has not been studied in HFpEF, mainly due to the lack of animal models with a stable chronic HFpEF phenotype (126, 194).

The main aims of the thesis project were

- 1.) To establish an animal model which reflects main signs and symptoms of the clinical HFpEF phenotype
- 2.) To identify (sub)cellular mechanisms of ventricular remodeling leading to HFpEF
- 3.) To establish a relationship between *in vivo* dysfunction and cellular changes *in vitro*
- 4.) To investigate intracellular Ca^{2+} disturbances as an early trigger for maladaptive remodeling and characterize defective Ca^{2+} handling as a cause of contractile dysfunction and increased arrhythmia risk at more advanced stages of the disease.
- 5.) To evaluate new therapeutic targets reversing remodeling processes, improving diastolic function, and preventing arrhythmias in HFpEF

The first part of the thesis describes the rat model of subtotal nephrectomy which developed a clinical stable phenotype of HF with preserved ejection fraction for up to 52 weeks. *In vivo* systolic and diastolic function was assessed using echocardiography and pressure-volume recordings. Additionally, organ morphometry was assessed after final experiments.

In the second part *in vitro*, global changes in Ca^{2+} at the cellular level (single cardiomyocytes), are presented at different stages of the disease. Cardiomyocytes were isolated from the ventricle, loaded with fluorescent probes for Ca^{2+} (e.g. Fura-2 AM, Fluo-4 AM) and electrically stimulated for measurements of Ca^{2+} transients and force development. Relevant Ca^{2+} and Na^{+} dependent ion currents

were studied with appropriate pharmacological tools targeting RyR2, NCX and SERCA. Ratiometric Ca^{2+} measurements were combined with confocal imaging for spatial and temporal resolution of subcellular Ca^{2+} events. Functional studies were complemented with molecular biology (Western blot) for expression levels and subcellular localization of Ca^{2+} and Na^{+} handling proteins. Spontaneous Ca^{2+} sparks were investigated as cellular equivalents of arrhythmias.

In the third part of the thesis a new potential therapeutic target in the management of HFpEF was evaluated in reversing remodeling processes, improving diastolic function, and preventing arrhythmias. Therefore, a new specific NCX inhibitor – SEA0400 – was investigated acutely in vitro in cardiomyocytes, in vivo in LV hemodynamic measurements and chronically with long-term treatment in the HFpEF animal model.

Part of this thesis has been published in a peer-reviewed journal by “*Primessnig et al, Eur J Heart Fail. 2016 (194).*”

2 Material and methods

2.1 Animal model

All investigations in this project were undertaken in fully accordance with the Guidelines for the Care and Use of Laboratory Animals (National Institute of Health, U.S.A.) and were approved by the local Ethics Committee (GZ: BMWF-66.010/0040-II/3b/2012). Experimental animals were housed in a 12-hour light/dark cycle under conventional conditions in the local animal facility with water and standard chow provided ad libitum (194).

2.1.1 Sample size

Sample size calculation was based on the discrimination of a 50% change in left ventricular end diastolic pressure (LVEDP) as an important parameter for diastolic dysfunction in HFpEF-NXT rats vs. Sham. In the literature, reported mean LVEDP in control animals varies between 4 and 11 mmHg (127-129). The initial assumption was an LVEDP of 11 mmHg with a variance of 5 mmHg, yielding an effect size of 1.1. Additionally, assuming increased mortality in NXT we planned an allocation ratio of 1.5. For an $\alpha=0.05$ and a Power of 0.8 this resulted in a sample size of N=18 for NXT and N=12 for Sham (194).

2.1.2 Subtotal nephrectomy (5/6) rat model

Subtotal (5/6) nephrectomy (NXT) or sham operation (Sham) was performed in young male 8 weeks old Wistar rats (HsdRCCHan, Harlan, Italy) with a body weight between 250 and 275 grams. NXT and Sham rats were anesthetized with 5 % isoflurane (Abbott, Germany) and 1.6 l/min oxygen in an induction chamber for 1 minute and afterwards placed on a heated table to maintain body temperature at 37.5°C with a rectal probe. Afterwards, anesthesia was maintained by injection of 400 μ l/kg/BW ketamine/xylazine i.p. and reduction to 2 % isoflurane with same oxygen flow rate via an anesthetic mask. Rats were breathing spontaneously during the whole procedure and were monitored via tail pulse oximetry and regular

control of their reflexes (194). The subtotal nephrectomy surgical procedure was performed in a one-time intervention (130,131), meaning that in a first step, the left kidney was exposed through a flank incision and decapsulated. Renal vessels of the left kidney were ligated and the kidney was carefully removed. In a second step, the right kidney was exposed and the two poles and cortex were quickly removed. The surgical procedure in Sham animals was performed in a similar way. Both kidneys were exposed for the same time but of course left intact. All animals (NXT and Sham) received as post-operative analgesia metamizole (40 mg/kg/BW) subcutaneously. After the surgical procedure, the animals were closely monitored for 24 hours (194).

2.2 Experimental procedure and monitoring of biomarkers

Experimental groups

- 8 weeks NXT/Sham group
- 24 weeks NXT/Sham group
- 52 weeks NXT/Sham group – mortality statistics
- 24 weeks NXT/Sham group with acute SEA0400 application in vivo (hemodynamics) and in vitro (cardiomyocytes)
- 24 weeks NXT/Sham group with chronical SEA0400 treatment (16 weeks SEA0400 treatment, start at week 8)

After subtotal nephrectomy (NXT) or sham operation (Sham) rats were observed up to 52 weeks and during the whole observation time period body weight was measured weekly. Furthermore, blood (lingual vein) and urine samples (8h diuresis/ metabolic cages) as well as non-invasive blood pressure measurements and echocardiography were performed before the operation, 1, 4, 8, 16 and 24 weeks after it. Additionally, after 8 and 24 weeks invasive-hemodynamic measurements (PV-loops), organ morphometry (heart, lungs), histology (fibrosis), molecular biology (protein expression levels of sodium and calcium handling proteins) as well as the isolation of single cardiomyocytes from the same animals were performed to measure cell size, cell shortening and calcium homeostasis (194). In a subgroup of animals at 24 weeks, in vitro and in vivo measurements

were performed in the presence of a $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) inhibitor, SEA0400 (in vitro: 300nM, 5 min; in vivo: 1mg/kg/BW i.v., 30 min) (132,133,194). Moreover, a chronic treatment group (NXT/Sham) was performed - figure 2.1 - where per os treatment started 8 weeks after NXT or Sham operation and all animals were treated till the 24th week with SEA0400 (1mg/kg/BW/twice per day via gavage). Afterwards, all animals were again fully characterized with non-invasive blood pressure measurements, echocardiography, invasive pressure-volume measurements, organ morphometry and isolation of single LV cardiomyocytes from the same animals to measure cell size, cell shortening and calcium homeostasis (194). In an additional experimental group, NXT and Sham animals were observed for up to 52 weeks to investigate mortality rate in these animals (Kaplan-Meier mortality analysis). The health status of the animals has continuously been controlled and the body weight was measured weekly. Rats in poor general health and nutritional conditions with a loss in body weight from more than 15% in one week were sacrificed. Furthermore, in the surviving animals' blood and urine samples as well as organ morphometry, echocardiography and invasive hemodynamics were determined again (194).

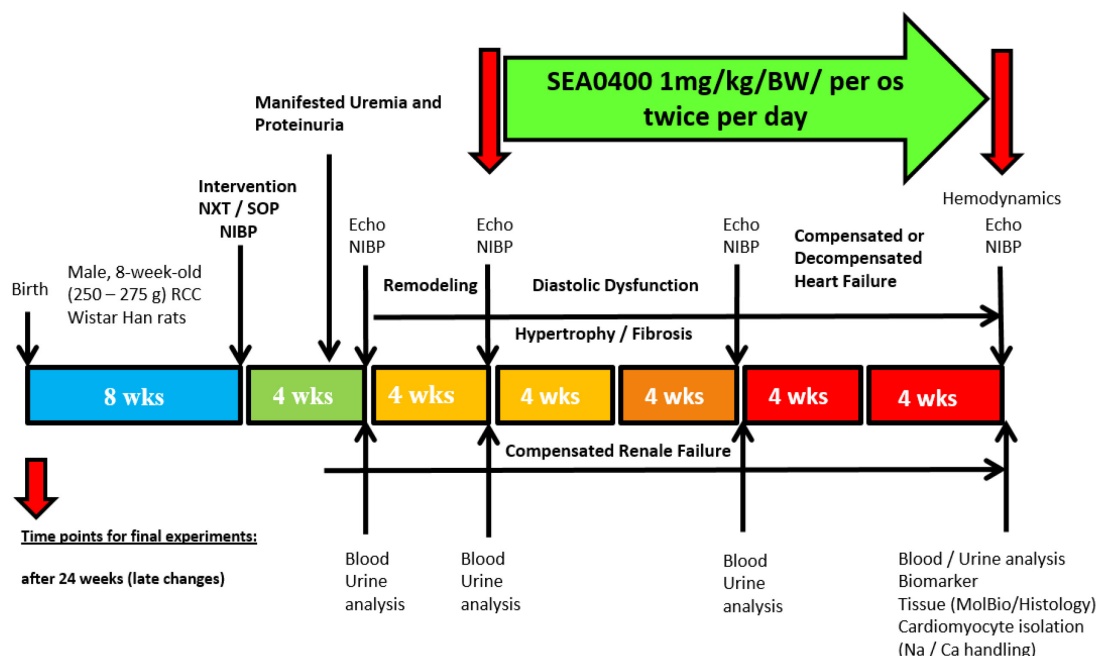


Figure 2.1 Experimental protocol

2.3 Biochemical determinations (blood/urine)

For the determination of serum creatinine, urea, albumin, total protein and electrolytes blood samples were obtained from lingual vein at week 8 and 24 in NXT and Sham animals. Furthermore, analysis of urine volume, GFR, creatinine, urea, albumin, proteinuria and electrolytes in urine samples from 8h metabolic cages were performed after 8 and 24 weeks in all animals. The parameters were measured by routine laboratory techniques. Moreover, NTproBNP levels were quantified using a commercially available enzyme-linked immunosorbent assay (Uscn Life Sciences Inc., Wuhan, China) (194).

2.4 Non-invasive blood pressure measurements

Non-invasive systemic blood pressure measurements were recorded in conscious animals by the tail-cuff method using the CODA™ system (Kent Scientific Corporation, USA (134)) before NXT/Sham operation, at week 1, 4, 8, 16 and 24. Experimental animals were awake and placed in a holder on a heated platform. All recordings were performed in steady state condition. Systolic, mean and diastolic blood pressure as well as heart rate was measured in the NXT and Sham rats (194).

2.5 Echocardiography

Echocardiography was performed in isoflurane-anaesthetized, spontaneously breathing rats placed on a heated plate to maintain body temperature at 37.5°C. Heart rate was continuously measured by ECG electrodes. Transthoracic echocardiography measurements were performed to assess changes in cardiac function and dimensions from at least three consecutive cardiac cycles under stable conditions using a high-resolution micro-imaging system equipped with a 17.5-Mhz linear array transducer (Vevo770™ Imaging System, VisualSonics, USA). During the examination the intraventricular septum (IVS), the posterior wall (PW) thickness, the left ventricle end-diastolic diameter (LVEDD) and the left atrium (LA) size were recorded directly. Furthermore, fractional shortening (FS),

ejection fraction (EF), LV mass and LV relative wall thickness were calculated using standard formulas (135). Figure 2.2 shows M-mode and B-mode measurements from rat left ventricle (194).

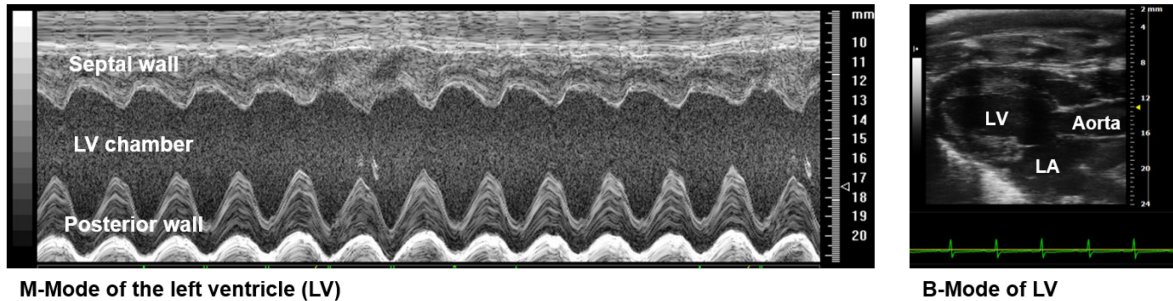


Figure 2.2 Echocardiography left ventricle

2.6 Pressure volume measurements (PV-Loops)

Invasive pressure-volume measurements (PV loops – figure 2.3) were performed as terminal procedure in isoflurane-anaesthetized, orotracheal intubated rats using a volume-controlled ventilation (rodent ventilator, SAR-1000 Small Animal Ventilator, CWE, USA). Furthermore, animals were placed on a heating platform to maintain body temperature at 37.5°C and heart rate was continuously measured by surface ECG electrodes. Two polyethylene catheters were inserted - one into the left external jugular vein for fluid administration (0.9% NaCl 10ml/kg/h) and another one, into the right external jugular vein for saline calibration (10% NaCl) at the end of the experiment. Moreover, a 2.0-Fr pressure-volume conductance catheter (SPR-838, Millar, USA) was inserted into the right carotid artery and advanced into the ascending aorta. After recording the aortic blood pressure and HR, the PV-catheter was advanced into the LV under pressure control through the aortic valve. Hemodynamic measurements of preload dependent and independent pressure and volume changes in the LV were started after stable steady state conditions occurred using the pressure-volume conductance system (MPVS Ultra, Millar Instruments, USA connected to the PowerLab 8/35 data acquisition system, UK and analyzed using LabChart7pro software system, UK). Parameters of LV systolic and diastolic function including LV end-systolic pressure (LVESP), LV end-diastolic pressure (LVEDP), LVESV, LVEDV, SV, CO, arterial Elastance (E_a), EF, Stroke work (SW), maximal slope of LV systolic pressure increment (dP/dt_{max}),

maximal slope of diastolic pressure decrement ($dP/dt_{\min}$) and isovolumetric relaxation constant Tau (IVRc Tau) were measured and calculated according to standard formulas (136) (194).

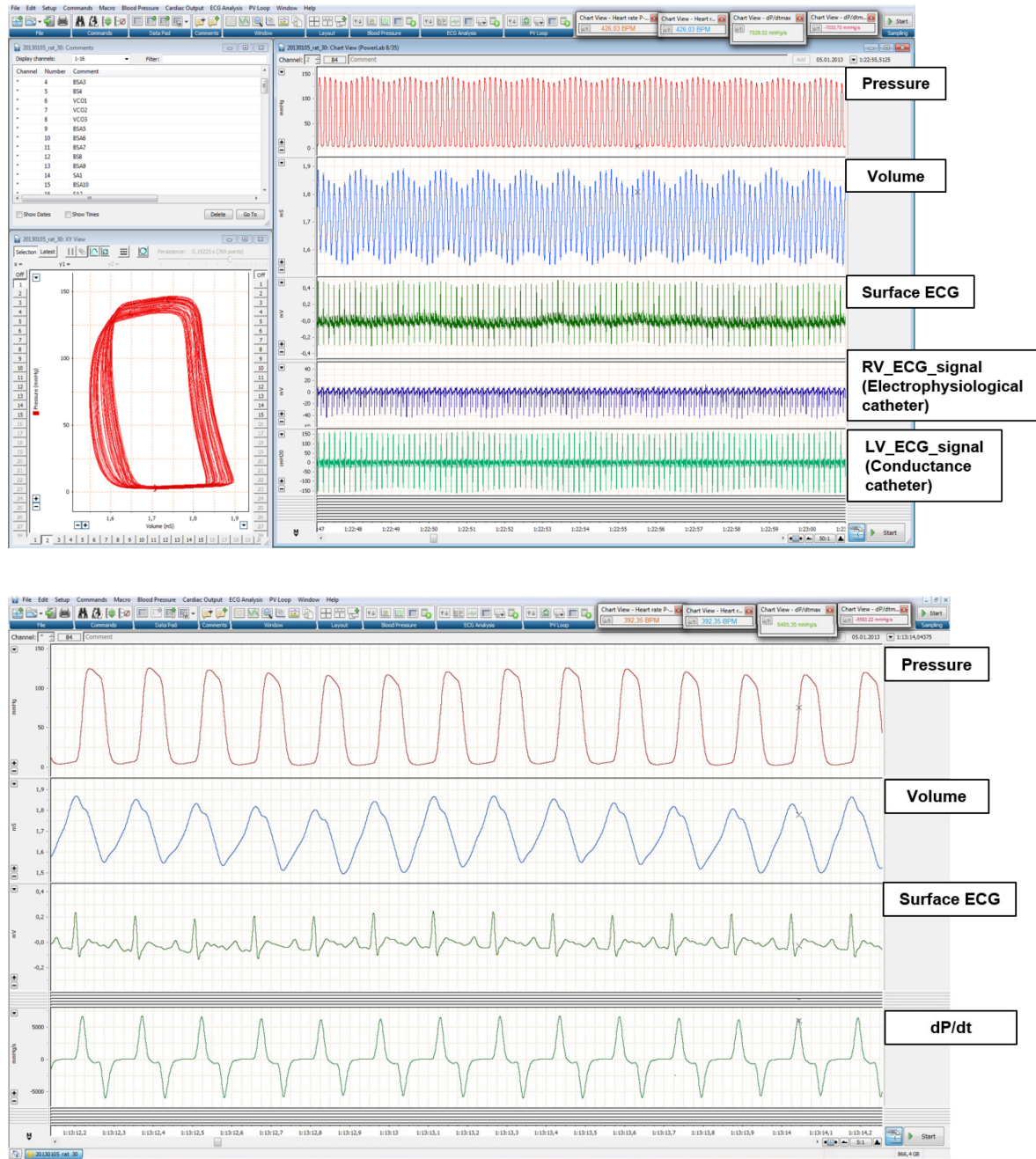


Figure 2.3 Invasive pressure-volume measurements (PV-loops)

2.7 Morphometry and histology

At the end of the final invasive hemodynamic measurements, all animals were sacrificed and organs were removed for further analysis. Besides cellular and molecular investigations (see below) morphological and histological studies were determined. Either, hearts were excised, weighed and transferred for isolation of single LV cardiomyocytes (see below) or hearts were used for further molecular and histological analysis. For histological studies, LV tissue sections of hearts were fixed in 4% neutral buffered formalin and then processed and embedded in paraffin. Transversal sections (5 μ m) at the level of the papillary muscles were produced using a sliding microtome equipped with a stretching bath. Cardiac fibrosis was visualized using picrosirius red staining for collagen in all NXT and sham rat hearts. The fibrotic area of LV was quantified as manually set threshold intensity of red-stained collagen from four sections using ImageJ analysis (NIH, USA) Additionally, lungs from NXT and Sham animals were excised and their weight was measured to calculate organ weight to body weight ratios (194).

2.8 Western blotting

To investigate different sodium and calcium handling proteins in subtotal nephrectomised (NXT) and sham-operated (Sham) rats, western blot experiments were performed on homogenates prepared from the LV and assessed by standard immunoblotting procedure (137,138) using commercially available antibodies. The expression of the different proteins was normalized to GAPDH and for the quantification of the western blot bands intensity Quantity One (Bio-Rad) was used (194).

2.9 Cardiomyocyte cell isolation and calcium measurements

2.9.1 Cardiomyocyte cell isolation

Cardiomyocytes from NXT and Sham animals were enzymatically isolated directly after final hemodynamic experiments. LV cardiomyocytes were obtained as

previously described (139). Briefly, excised hearts were quickly transferred to a Langendorff apparatus - figure 2.4 and perfused with Perfusion Solution (in mmol/L: 130 NaCl, 5 KCl, 0.5 MgCl_2 , 0.33 NaH_2PO_4 , 22 Glucose, 5 Glutamine, 25 HEPES, 0.01 U/ml Insulin; pH adjusted to 7.4 with NaOH) for 3 min followed by Enzyme Solution (Perfusion Solution with collagenase type 2 (Worthington) 285 U/mg and 0.1 mmol/L CaCl_2 added) for 15-18 min. Afterwards, left ventricle was excised and cardiomyocytes were dispersed in Perfusion Solution with 0.1 mmol/L CaCl_2 which was increased stepwise to 1 mmol/L CaCl_2 . The procedure yielded about 60 - 80% quiescent cardiomyocytes. Cardiomyocytes were then transferred to a cell bath of either a ratiometric fluorescence microscope setup (PTI Fluorescence Master Systems, UK and Zeiss Axiovert, Germany) additionally equipped with a CCD camera for edge detection (cell shortening) or to a confocal line scanning microscope (LSM 510 Meta, Zeiss, Germany) (194).

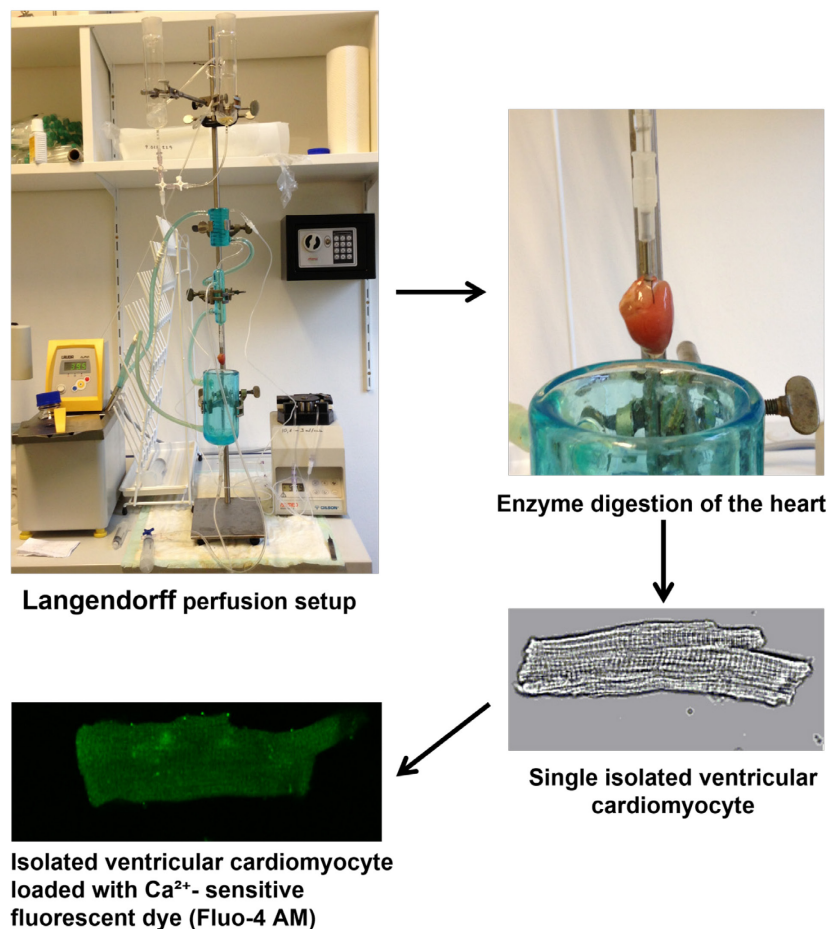


Figure 2.4 Isolation of ventricular cardiomyocytes

2.9.2 Calcium sensitive fluorescent dyes

LV cardiomyocytes were loaded with Ca^{2+} -sensitive fluorescent dyes Fluo-4 AM (140) for confocal microscope measurements or Fura-2 AM (141) for ratiometric microscope measurements. Fluo-4 and Fura-2 were negatively charged and therefore were unable to pass the cell membrane easily. To overcome this problem Fluo-4 AM and Fura-2 AM were created, where the AM stands for an acetoxymethyl group, which transforms acid into an ester, enabling the dye to enter the cell (142). Inside the cell the whole process is being reversed by cellular esterase, activating the indicator to bind Ca^{2+} and limiting dye leakage.

Cells were incubated either with Fluo-4 AM (20 $\mu\text{mol/L}$ for 30 minutes) or Fura-2 AM (1 $\mu\text{mol/L}$ for 30 minutes) in normal Tyrode's (NT) solution (rat cells: 130 mM NaCl, 5.4 mM KCl, 1.5 mM CaCl_2 , 0.5 mM MgCl_2 , 22 mM glucose, 10 mM HEPES, 5 mM glutamine and 0.01 U/ml insulin).

2.9.3 Calcium measurements with confocal line scan microscopy

Fluo-4 AM loaded ventricular cardiomyocytes were transferred to a cell bath and fast, two dimensional (2D) confocal $[\text{Ca}^{2+}]$ imaging was performed as described previously (143) using a Zeiss LSM 510 Meta confocal laser point scanning system (Carl Zeiss, Jena, Germany) equipped with a $\times 40$ oil-immersion objective lens and an argon-ion laser. For Fluo-4, excitation and emission wavelengths were 488 nm and >515 nm, respectively. A 512-pixel scan line was drawn along the longitudinal axis of the cell. Consecutive scan lines were stacked over time and visualized as 2D image. Cardiomyocytes loaded with the indicator were placed on the stage of the microscope and were continuously superfused with Superfusion Solution (in mmol/L: 130 NaCl, 5 KCl, 0.5 MgCl_2 , 0.33 NaH_2PO_4 , 22 Glucose, 5 Glutamine, 25 HEPES, 0.01 U/ml Insulin, 1 CaCl_2 ; pH adjusted to 7.4 with NaOH) delivered by a gravity-driven superfusion pipette and field-stimulated via two platinum electrodes at 1 Hz. Ca^{2+} -transients and contraction were measured at steady state (>30 s). Line scan images were recorded at 1.56 ms/line along the longitudinal axis of the cell and analyzed using custom made algorithms coded in IDL (140,144). $[\text{Ca}^{2+}]$ dependent fluorescence intensity (F) was normalized to the

lowest end-diastolic fluorescence (F_0) of the same cell (F/F_0) (194). Normalized Ca^{2+} -transients were analyzed for F_{peak} , TTP, RT50, RT90 and TAU, as shown in figure 2.5.

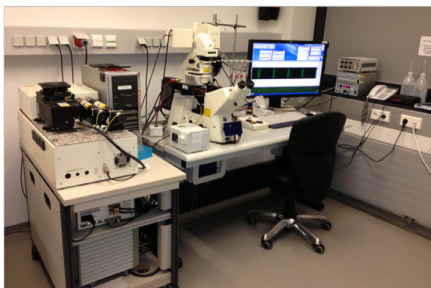
F_{peak} ... maximum $[\text{Ca}^{2+}]$ -dependent fluorescence

TTP ... time to peak: duration from start of rise to peak intracellular $[\text{Ca}^{2+}]$

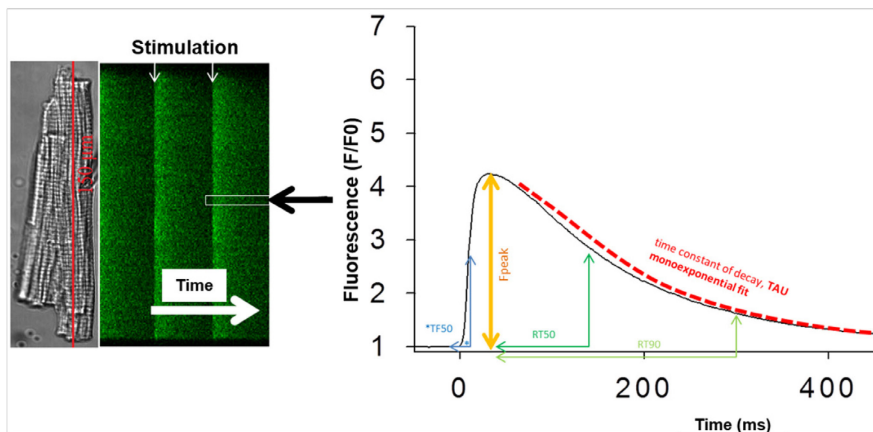
RT50 ... duration from peak to half-maximal decay of $[\text{Ca}^{2+}]_i$

RT90 ... duration from peak to 90% decay of $[\text{Ca}^{2+}]_i$

TAU ... time constant of a monoexponential fit to $[\text{Ca}^{2+}]$ decay (starting from 10% decay to end of cycle, Figure 2.5, red dotted line).



Confocal laser scanning microscope



Recording intracellular Ca^{2+} signals in living cardiomyocytes using the fluorescent indicator fluo-4 acetoxymethyl (AM) and laser scanning confocal microscopy.

Figure 2.5 Confocal calcium measurements in Fluo-4 AM loaded cardiomyocytes

For measurement of Ca^{2+} -sparks stimulation was stopped and $0 \text{ Na}^+ / 0 \text{ Ca}^{2+}$ Solution (containing in mmol/L: 91 LiCl, 21 LiOH, 4 KCl, 1 MgCl_2 , 20 HEPES, 10 EGTA, 10 Glucose; pH adjusted to 7.4 with LiOH) was washed in via a rapid perfusion system to inhibit transsarcolemmal Ca^{2+} exchange via the NCX. Ca^{2+}

sparks were quantified for 10 seconds, followed by reintroduction of Superfusion solution (2 sec) and Superfusion Solution with 30 mmol/L caffeine (10 sec) (140,144). SR Ca^{2+} content was quantified as the amplitude of the caffeine-induced Ca^{2+} transient. The time constant of Ca^{2+} decay in the presence of caffeine (TAU_{caff}) was quantified as a measure of NCX forward mode activity (140,144) (194). Figure 2.6 shows regular Ca^{2+} transients, Ca^{2+} release event (Ca^{2+} sparks and waves) and caffeine-induced Ca^{2+} transients.

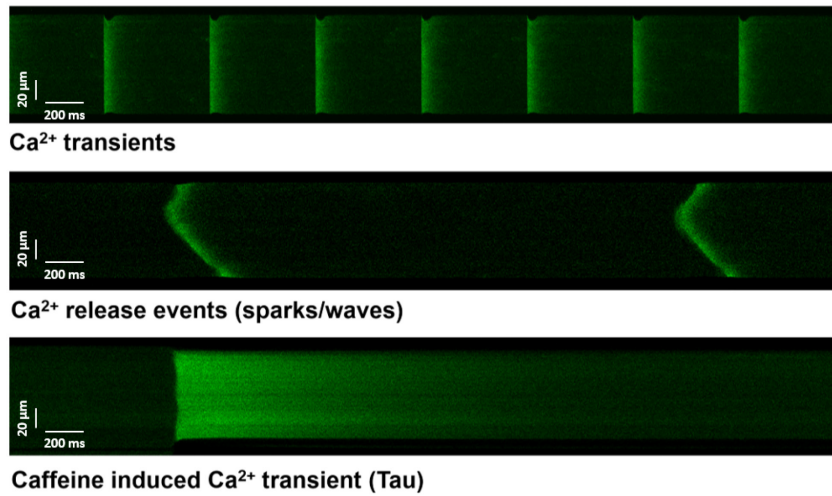


Figure 2.6 Line scan images of cardiomyocytes

The decay constant of the Ca^{2+} transient during steady-state electrical stimulation attributed to SERCA activity ($\text{TAU}_{\text{SERCA}}$) was calculated from the decay constant of the Ca^{2+} -transient (TAU_{STIM}) and TAU_{caff} obtained from the same cell by the following formula (145) (194).

$$\text{TAU}_{\text{SERCA}} = \frac{1}{\left(\frac{1}{\text{TAU}_{\text{STIM}}} - \frac{1}{\text{TAU}_{\text{caff}}} \right)}$$

2.9.4 Ca^{2+} measurements and cell shortening using Fura-2 AM and ratiometric microscopy

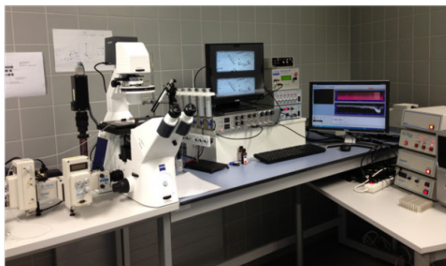
To compare diastolic $[\text{Ca}^{2+}]$ between cardiomyocytes, Fura-2 AM was used (141). LV cardiomyocytes were loaded with Fura-2 AM (1 $\mu\text{mol/L}$ for 30 min with wash-out) and placed in a cell bath on the stage of an inverted ratiometric fluorescence

microscope setup (PTI Fluorescence Master Systems, UK and Zeiss Axiovert, Germany) additionally equipped with a CCD camera for edge detection (cell shortening). Shown in figure 2.7.

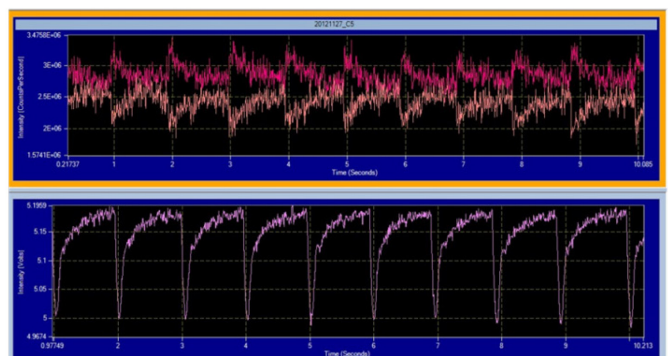
Fura-2 has a high affinity to calcium and has two peak absorbances, the first being at 340 nm, and the second at 380 nm. Emitted light of Fura-2 is collected > 510 nm for both excitation wavelengths (F340 nm and F380 nm). The ratio of emitted signals by Fura-2 at both excited wavelengths changes upon binding of the dye to $[Ca^{2+}]$. The background-corrected signal ratio (F340/F380) correlates directly with the calcium content in the cell. By using the ratio, artifacts such as cell thickness, uneven dye distribution and leakage of dye can be minimized. Background fluorescence was measured at a nearby position in the cell bath free of cells. Cell length-dependent autofluorescence was measured in cardiomyocytes not loaded with Ca^{2+} dye from the same hearts. Following correction for background- and autofluorescence, Ca^{2+} -dependent fluorescence was calculated as the ratio F340/F380. In a subset of experiments fluorescence ratios were calibrated to obtain absolute $[Ca^{2+}]_i$ using the formula (145,194):

$$[Ca^{2+}]_i = K_d * \beta * \frac{R - R_{min}}{(R_{max} - R)}$$

R was the ratio F340/F380. Fluorescence intensities in the absence and with saturating Ca^{2+} for calculation of β , R_{min} and R_{max} were determined at our setup as described in Sipido et al (145), the dissociation constant of Fura-2 was 286 nM (194).



Ratiometric setup



Ca^{2+} transients and Cell shortening

Figure 2.7 Epifluorescence ratioimetric setup

To investigate calcium homeostasis and contractility of cardiomyocytes, cells were stimulated with different frequencies (1,2,3,5,6 Hz) until the Ca^{2+} transient amplitude reached a steady state. Afterwards, caffeine response was induced immediately to measure cytosolic $[\text{Ca}^{2+}]$ elimination from the cytosol, mainly by the NCX pump, located in the sarcolemmal membrane. Calcium transients and cell shortening (contractility) was shown in figure 2.8. Furthermore, absolute systolic and diastolic calcium concentrations were calculated from emitted epifluorescence signals as described above (194).

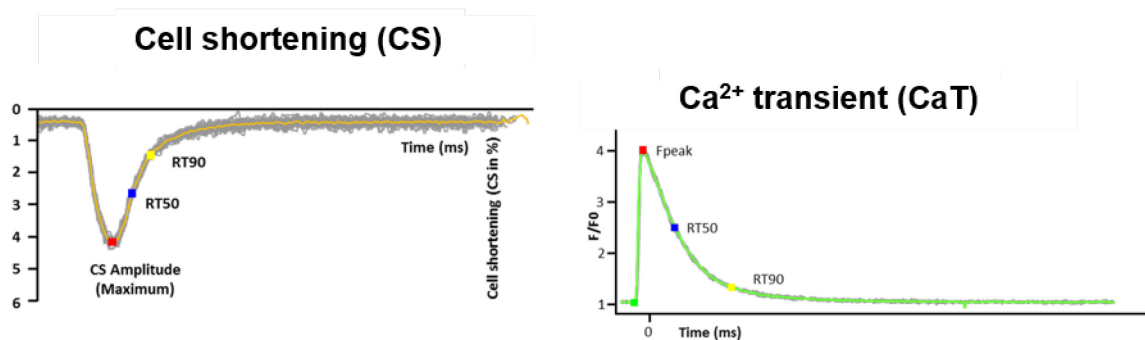


Figure 2.8 Ca^{2+} transient and cell shortening

2.10 Whole-cell patch clamping

In LV cardiomyocytes from NXT and Sham animals membrane currents were measured using an Axon 200B amplifier in the whole-cell voltage-clamp mode. Patch-clamp pipettes had a resistance of 1-3 M Ω . Measured current recordings were filtered at 2 kHz and sampled and digitized at 4 kHz using a Digidata 1440 AD converter and pCLAMP 10 software (Axon Instruments). Right after rupturing the cell membrane, external solution was switched to a K^+ -free, Cs^+ -based Tyrode solution containing, in mM: NaCl 130, CsCl 10, Hepes 11.8, MgCl_2 0.5, CaCl_2 1.8, glucose 10; pH 7.4 adjusted with NaOH. Pipette solution contained, in mM: CsAsp 120, TEACl 10, NaCl 10, Hepes 10, MgCl_2 0.5, MgATP 5, pH 7.2 adjusted with CsOH. There was a measured offset potential of -6 mV for which all recordings were corrected between these two solutions. For simultaneous measurements of ion currents and Ca^{2+} transients during confocal imaging with Zeiss LSM 700, Fluo-4 (50 μmol) was added to the pipette solution. Cells were stimulated at 1 Hz steady-state using 50 ms depolarizing steps from -60 to +20 mV to elicit inward

LTCC and NCX currents (194). LTCC was measured as the inward peak current amplitude evoked by depolarization. NCX inward current (forward mode) was quantified as the tail current amplitude 12 ms after repolarization and normalized to corresponding values of Ca fluorescence. NCX outward current (reverse mode) was measured as the Ni^{2+} -sensitive current (2.5 mM Ni^{2+}) during a 6s depolarizing step to +60 mV (194). All measured currents were normalized to cell capacitance (146). Values were 149.6 ± 14.80 pF in Sham (n=6) and 186.3 ± 11.44 in NXT (n=9, p=0.07) (194). SEA0400 (synthesized as previously described (147)) was applied (300nM; 5 min preincubation) in a subset of LV cardiomyocytes from 24 weeks post NXT/Sham rats, and following the baseline experiments cardiomyocyte function was assessed as above. In a first step, external sodium level was lowered to 70 mM in a subset of electrical field stimulated NXT cardiomyocytes, altering the transsarcolemmal electrochemical gradient and shift NCX in reverse mode by exchanging the superfusion solution from normal tyrode to low sodium solution (in mmol/L: 70 NaCl, 5 KCl, 0.5 MgCl_2 , 0.33 NaH_2PO_4 , 22 Glucose, 5 Glutamine, 25 HEPES, 0.01 U/ml Insulin, 1 CaCl_2 ; pH adjusted to 7.4 with NaOH). Furthermore, SEA0400 was applied to the low sodium solution. Finally, calcium transients and caffeine induced calcium transients in normal tyrode, low sodium solution and low sodium solution containing SEA0400 were measured (194).

2.11 Statistics

Statistical analysis was done using two-way ANOVAs or two-way repeated measures ANOVAs followed by pairwise comparisons with Bonferroni-Holm correction. A non-parametric equivalent was used, if the assumptions of normality or equality of variance were violated (148). The normality assumption was checked by using the Shapiro-Wilk-Test and the variance-covariance structure was tested by applying a Likelihood-Ratio-Test instead (148). Correlations were determined using Pearson Product Moment Correlation. An alpha level for all tests (P) of 5% was used. N=number of hearts or animals, n=number of cardiomyocytes, error bars/numerical = standard deviation. For data analysis SAS “Statistical Analysis Software”, Version 9.2 (SAS Institute Inc., Cary, NC, USA) or SigmaPlot. 13 (Systat Software, San Jose, CA) was used (194).

3 Results

3.1 Heart failure with preserved ejection fraction in a rat model with chronic stable kidney disease based on subtotal nephrectomy

Following subtotal nephrectomy (NXT) rats developed stable chronic kidney disease (CKD) and the clinical phenotype of heart failure with preserved ejection fraction, including LV hypertrophy, cardiac remodeling, LV diastolic dysfunction, preserved LVEF, lung congestion and increased biomarkers' like NTproBNP.

3.1.1 Subtotal nephrectomy rats

Young male Wistar rats underwent subtotal nephrectomy (NXT) or sham operation (Sham). Both, Sham and NXT increased in body weight continuously during observation period up to 52 weeks. Body weight (BW) of Sham rats was significantly higher at 24 and even at 52 weeks, most likely because of the simultaneous development and progression of heart failure in NXT animals (Figure 3.1, Table 3.1 and Table 3.4).

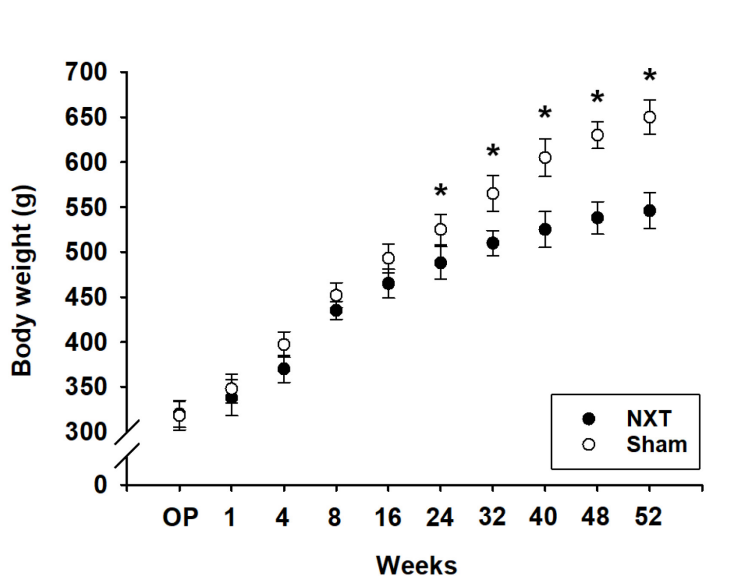
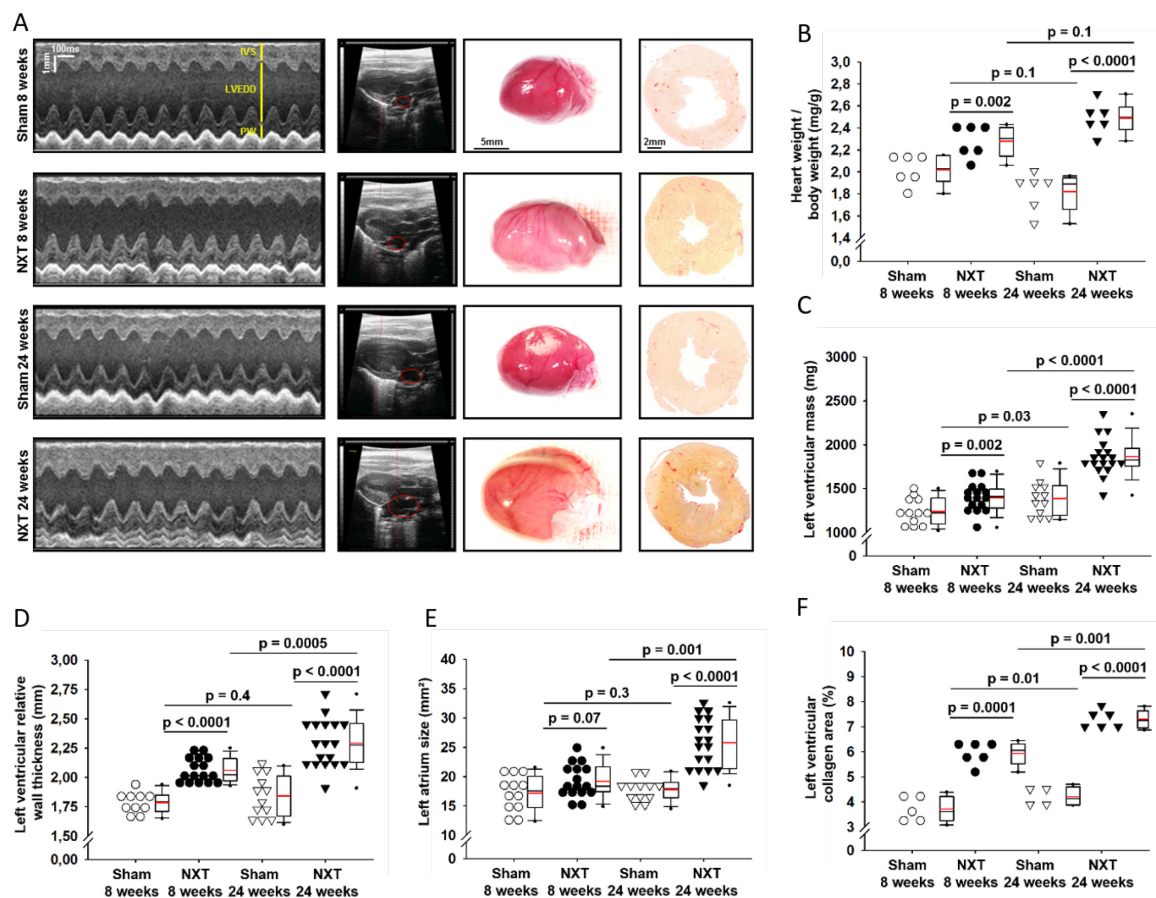


Figure 3.1 Body weight in Sham vs. NXT rats up to 52 weeks: BW was significantly different in Sham vs. NXT at week 24 up to week 52. Symbols=mean body weight per group and time point. Data are mean $\pm$ S.D. p – values. * p < 0.05 between Sham/NXT. 24 weeks group (Sham N=12/NXT N=19). 52 weeks group (Sham N=8/NXT N=8). Analysis using ANOVA.

3.1.2 LV hypertrophy and cardiac remodeling

In NXT rats, hearts were enlarged with concentric LV hypertrophy (LVH) at 8 weeks and further increase in LV hypertrophy at 24 weeks. Heart weight/body weight ratio, LV mass and LV relative wall thickness were significantly increased in NXT vs. Sham animals at 8 weeks, with an even more pronounced effect after 24 weeks. (Figure 3.2 A column 1 and 4, B, C, D, Table 3.1). Furthermore, LV fibrosis was increased in NXT at 8 weeks with a progressive increase at 24 weeks (Figure 3.2 A column 4, and F). Moreover, left atrium (LA) enlargement was observed at 24 weeks (Figure 3.2 A column 2 and E).



Modified from Primessnig et al, *Eur J Heart Fail.* 2016 (194)

Figure 3.2 LV hypertrophy and cardiac remodeling: A) Echocardiographic M-mode images of LV (column 1) and echocardiographic B-mode images of LV and left atrial (LA) size (column 2). Photomicrographs of hearts (column 3) and cross sections of LV with picrosirius red staining of the LV (column 4) at 8 and 24 weeks post-nephrectomy (NXT) or sham operation (Sham). NXT animals showed indices of hypertrophic remodeling with B) increased HW/BW ratio (Sham/NXT at 8 (N=6/6) and 24 (N=6/6) weeks), C) increased LV mass and D) increased LV relative wall thickness (Sham/NXT at 8 (N=12/16) and 24 (N=12/18) weeks). Moreover, cardiac remodeling was reflected as E) LA enlargement (Sham/NXT at 8 (N=12/16) and 24 N=12/18) weeks) and F) increase in LV fibrosis (Sham/NXT at 8 (N=5/6) and 24 (N=4/6) weeks). Symbols=animals. Data is shown as individual datapoints/box plots; p – values. Analysis using two-way ANOVA. Groups are Sham/NXT at 8 and 24 weeks.

More parameters for cardiac remodeling and function are summarized in table 3.1.

Table 3.1
Morphometric and echocardiographic characteristics

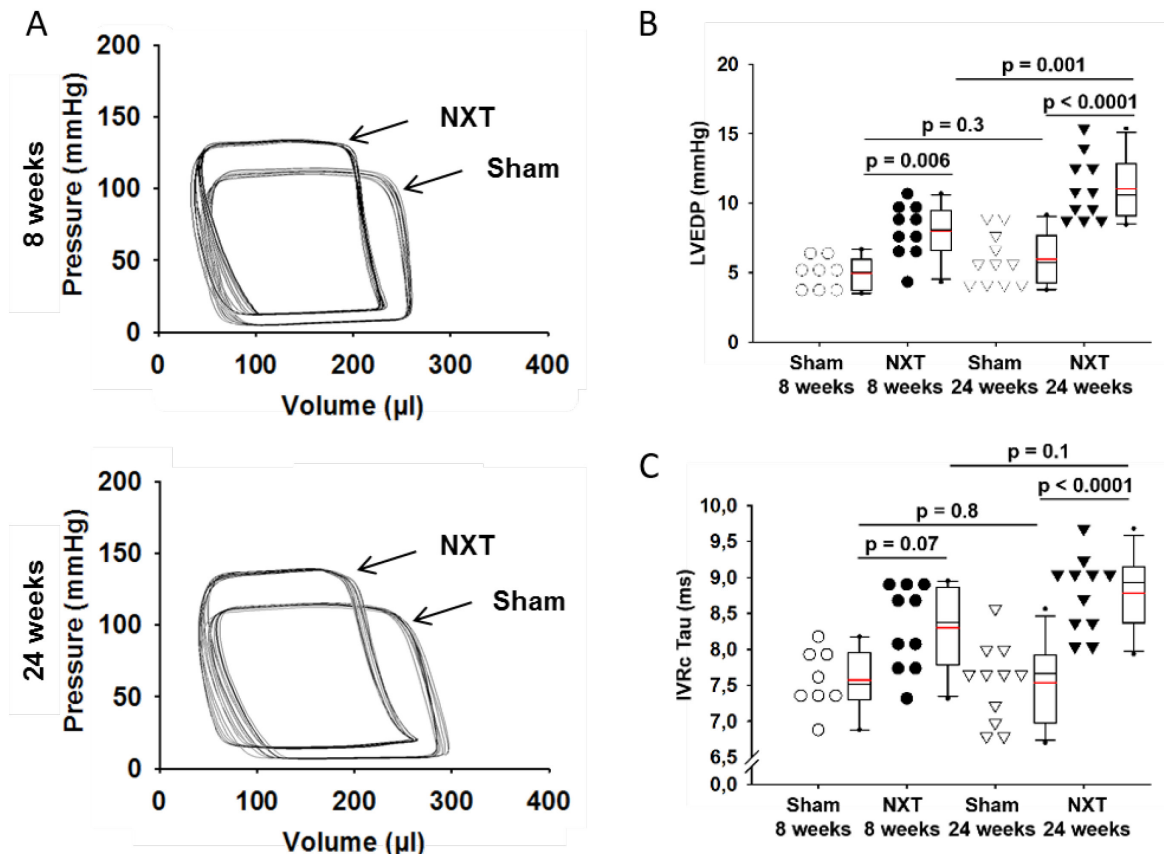
Parameters	Sham 8 weeks	NXT 8 weeks	Sham 24 weeks	NXT 24 weeks	8 weeks / 24 weeks / Sham / NXT Sham vs. NXT 8 vs. 24 weeks
Morphometry	mean±SD				p-values
BW (g)	452 ± 29 (N=12)	435 ± 28 (N=19)	525 ± 45 (N=12)	488 ± 39 (N=19)	0.09/0.02/<0.0001/0.0002
HW/BW	2.03 ± 0.14 (N=6)	2.34 ± 0.08 (N=6)	1.94 ± 0.12 (N=6)	2.49 ± 0.14 (N=6)	0.0002/<0.0001/0.1/0.1
LW/BW	2.77 ± 0.33 (N=11)	3.15 ± 0.23 (N=12)	2.80 ± 0.13 (N=10)	3.76 ± 0.33 (N=13)	0.0002/<0.0001/0.5/0.1
Echocardiography	mean±SD				p-values
HR (BPM)	397 ± 20 (N=12)	393 ± 15 (N=16)	380 ± 14 (N=12)	373 ± 23 (N=18)	0.6/0.5/0.09/0.01
FS (%)	50 ± 4 (N=12)	49 ± 5 (N=16)	50 ± 4 (N=12)	47 ± 4 (N=18)	0.8/0.1/0.9/0.1
IVS (mm)	1.72 ± 0.13 (N=12)	2.06 ± 0.09 (N=16)	1.76 ± 0.16 (N=12)	2.41 ± 0.2 (N=18)	<0.0001/<0.0001/0.6/<0.0001
LVEDD (mm)	7.01 ± 0.26 (N=12)	6.36 ± 0.40 (N=16)	7.66 ± 0.27 (N=12)	6.79 ± 0.49 (N=18)	0.0001/<0.0001/0.0002/0.001
PW (mm)	1.97 ± 0.18 (N=12)	2.54 ± 0.18 (N=16)	2.03 ± 0.24 (N=12)	2.88 ± 0.4 (N=18)	0.0001/<0.0001/0.6/0.002
LV relative wall thickness (mm)	1.80 ± 0.08 (N=12)	2.06 ± 0.11 (N=16)	1.86 ± 0.18 (N=12)	2.29 ± 0.20 (N=18)	<0.0001/<0.0001/0.4/0.0005
LV mass (mg)	1239 ± 111 (N=12)	1394 ± 161 (N=16)	1388 ± 196 (N=12)	1886 ± 284 (N=18)	0.002/<0.0001/0.03/<0.0001
LA size (mm²)	17 ± 3.0 (N=12)	19 ± 2.0 (N=16)	18 ± 2.0 (N=12)	26 ± 5.4 (N=18)	0.07/<0.0001/0.3/0.001
EF (%)	79 ± 3.8 (N=12)	80 ± 4.6 (N=16)	74 ± 7.4 (N=12)	77 ± 5.1 (N=18)	0.6/0.4/0.1/0.04

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Table 3.1 Morphometric and echocardiographic characteristics: BW – Body weight; HW – Heart weight; LW – Lung weight; HR – Heart rate; FS – Fractional shortening; IVS – Intraventricular septum; LVEDD – LV end diastolic diameter; PW – Posterior wall; LV – Left ventricle; LA – Left atrium; EF – Ejection fraction. Data are mean ± S.D., p – values between Sham 8 weeks vs. NXT 8 weeks/ Sham 24 weeks vs. NXT 24 weeks/ Sham 8 weeks vs. Sham 24 weeks/ NXT 8 weeks vs. NXT 24 weeks. N=animals. Analysis using two-way ANOVA. Groups are Sham/NXT at 8 and 24 weeks.

3.1.3 LV diastolic dysfunction, preserved LVEF, congestion and elevation of NTproBNP

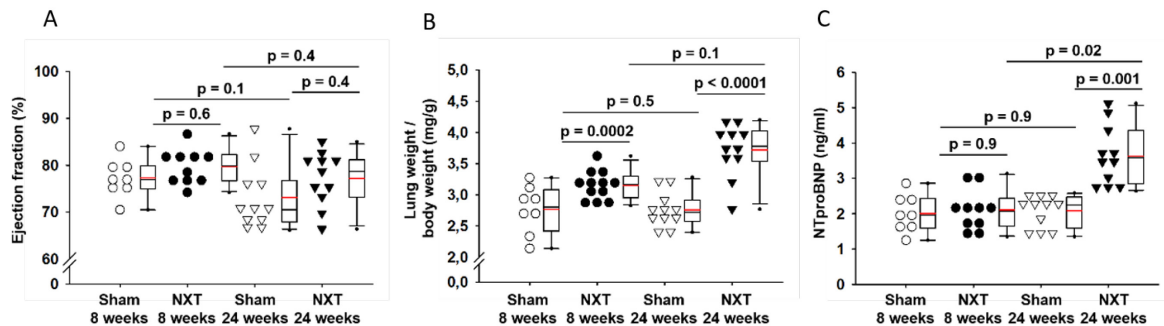
Hemodynamics revealed advanced diastolic dysfunction with increased LV end-diastolic pressure (LVEDP) and LV isovolumetric relaxation constant TAU (IVRc TAU). In NXT rats there was a significant increase in LVEDP at 8 weeks (Sham(N=8)/NXT(N=10); 5 ± 1 vs. 8 ± 2 , mmHg; $p=0.006$), with a further increase at 24 weeks (Sham(N=11)/NXT(N=11); 6 ± 2 vs. 11 ± 2 , mmHg; $p<0.0001$). Similarly, IVRc TAU was prolonged in NXT after 24 weeks (Sham(N=11)/NXT(N=11); 7.65 ± 0.57 vs. 8.78 ± 0.53 , ms; $p<0.0001$) while after 8 weeks there was just a trend (Figure 3.3 and Table 3.2).



Modified from Primessnig et al, *Eur J Heart Fail.* 2016 (194)

Figure 3.3 Diastolic dysfunction: A) Examples of invasive measured pressure-volume loops from Sham and NXT animals at 8 and 24 weeks. B) Elevated LVEDP in NXT vs. Sham animals at 8 weeks, with further increase at 24 weeks. C) Prolonged isovolumetric relaxation of the LV (IVRc TAU) in NXT vs. Sham animals at 24 weeks. Symbols=animals. Data is shown as individual datapoints/box plots; p – values. Analysis using two-way ANOVA. Groups are Sham/NXT at 8 (N=8/10) and 24 (N=11/11) weeks.

Subtotal nephrectomised rats showed severe LV hypertrophy, diastolic dysfunction and cardiac remodeling in terms of LA enlargement and LV fibrosis while ejection fraction was preserved at 8 and 24 weeks. Lung weight/body weight ratio was significantly elevated in NXT rats after 8 and 24 weeks as clinically relevant sign of congestion. Moreover, NTproBNP levels as a specific heart failure biomarker was significantly increased in NXT rats after 24 weeks (Figure 3.4).



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Figure 3.4 Preserved LVEF, congestions and NTproBNP: A) Preserved LV ejection fraction in Sham/NXT rats and 8 (N=8/10) and 24 (N=11/11) weeks. B) Elevated lung weight/body weight ratio as manifestation of congestion in NXT rats at 8 and 24 weeks. Groups are Sham/NXT at 8 (N=11/12) and 24 (N=10/13) weeks. C) Significant increased NTproBNP in NXT vs. Sham rats after 24 weeks. Groups are Sham/NXT at 8 (N=8/10) and 24 (N=11/11) weeks. Symbols=animals. Data is shown as individual datapoints/box plots; p – values. Analysis using two-way ANOVA. Groups are Sham/NXT at 8 and 24 weeks.

Invasive pressure volume measurements were performed at similar heart rates. While LV end-systolic pressure, arterial elastance and maximum rate of increase in LV pressure were significantly increased in NXT rats after 8 and 24 weeks; LV end-diastolic volume, stroke volume and maximum rate of decrease in LV pressure were significantly decreased in NXT rats after 8 and 24 weeks. Furthermore, LV end-systolic volume and cardiac output were significantly reduced after 24 weeks in NXT rats compared to Sham. More data from invasive pressure volume measurements are summarized in table 3.2.

Table 3.2 Invasive hemodynamic measurements

Parameters	Sham 8 weeks	NXT 8 weeks	Sham 24 weeks	NXT 24 weeks	8 weeks / 24 weeks / Sham / NXT Sham vs. NXT 8 vs. 24 weeks
Pressure volume measurements			mean±SD		p-values
HR (BPM)	399 ± 35 (N=8)	415 ± 33 (N=10)	401 ± 29 (N=11)	415 ± 25 (N=11)	0.2/0.3/0.8/0.9
ESP (mmHg)	105 ± 15.1 (N=8)	124 ± 14.3 (N=10)	101 ± 8.7 (N=11)	130 ± 10.3 (N=11)	0.005/<0.0001/0.5/0.4
EDP (mmHg)	5 ± 0.9 (N=8)	8 ± 1.9 (N=10)	6 ± 1.8 (N=11)	11 ± 2.3 (N=11)	0.006/<0.0001/0.3/0.001
ESV (μl)	60 ± 14.1 (N=8)	45 ± 13.7 (N=10)	84 ± 26.1 (N=11)	56 ± 19.1 (N=11)	0.06/0.03/0.03/0.05
EDV (μl)	261 ± 24.4 (N=8)	217 ± 31.8 (N=10)	309 ± 28.3 (N=11)	243 ± 37.7 (N=11)	0.0002/<0.0001/0.0002/0.02
SV (μl)	201 ± 17.3 (N=8)	172 ± 20.6 (N=10)	224 ± 13.2 (N=11)	187 ± 26.3 (N=11)	<0.0001/0.002/0.003/0.03
CO (ml/min)	80 ± 11.5 (N=8)	71 ± 10.2 (N=10)	90 ± 9.7 (N=11)	77 ± 15.5 (N=11)	0.1/0.03/0.09/0.2
Ea (mmHg/μL)	0.52 ± 0.03 (N=8)	0.73 ± 0.02 (N=10)	0.45 ± 0.05 (N=11)	0.72 ± 0.03 (N=11)	0.002/<0.0001/0.03/0.8
EF (%)	77 ± 4 (N=8)	80 ± 4 (N=10)	73 ± 7 (N=11)	77 ± 6 (N=11)	0.6/0.4/0.1/0.4
SW (mmHg*μL)	10121 ± 2180 (N=8)	10423 ± 1980 (N=10)	10415 ± 1224 (N=11)	11684 ± 2332 (N=11)	0.7/0.1/0.7/0.1
PRSW (mmHg)	39 ± 6.3 (N=8)	48 ± 5.8 (N=10)	34 ± 5.1 (N=11)	48 ± 5.2 (N=11)	0.004/0.0001/0.2/0.9
dP/dt max (mmHg/s)	6398 ± 648 (N=8)	7492 ± 1039 (N=10)	6345 ± 515 (N=11)	8337 ± 522 (N=11)	0.01/<0.0001/0.8/0.02
IVRc TAU (ms)	7.70 ± 0.47 (N=8)	8.30 ± 0.59 (N=10)	7.65 ± 0.57 (N=11)	8.78 ± 0.53 (N=11)	0.07/<0.0001/0.8/0.1
dP/dt min (mmHg/s)	-5656 ± 794 (N=8)	-6684 ± 933 (N=10)	-5664 ± 555 (N=11)	-7118 ± 658 (N=11)	0.03/0.0001/0.9/0.3

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Table 3.2 Invasive hemodynamic measurements: HR – heart rate; ESP – end-systolic pressure; EDP – end-diastolic pressure; ESV – end-systolic volume; EDV – end-diastolic volume; SV – stroke volume; CO – cardiac output; Ea – arterial elastance (measure of ventricular afterload); EF – ejection fraction; SW – stroke work; PRSW – preload recruitable stroke work (slope of stroke work – EDV relationship); dP/dt_{max} – maximum rate of increase in LV pressure; IVRc TAU – LV isovolumetric relaxation constant (relaxation time constant calculated by the Weiss method); dP/dt_{min} – maximum rate of decrease in LV pressure. Data are mean ± S.D., p – values between Sham 8 weeks vs. NXT 8 weeks/ Sham 24 weeks vs. NXT 24 weeks/ Sham 8 weeks vs. Sham 24 weeks/ NXT 8 weeks vs. NXT 24 weeks. N=animals. Analysis using two-way ANOVA. Groups are Sham/NXT at 8 and 24 weeks.

3.1.4 Chronical stable renal impairment and arterial hypertension in HFpEF rats

After subtotal nephrectomy in rats, renal function was significantly impaired after 8 and 24 weeks compared to Sham but there was no further significant decrease in renal function over time in nephrectomised rats. Glomerular filtration rate (GFR) was significantly lower in NXT rats at 8 (Sham(N=12)/NXT(N=19); 32.75 ± 6.25 vs. 20.77 ± 2.95 ; ml/min/m²; $p < 0.0001$) and 24 (Sham(N=12)/NXT(N=19); 28.84 ± 8.89 vs. 18.80 ± 3.32 ; ml/min/m²; $p < 0.007$) weeks, but there was no further decrease of GFR in NXT from week 8 to week 24 ($p = 0.1$). Consequently, after subtotal nephrectomy, rats developed a stable compensated renal dysfunction. Furthermore, other renal parameters, like serum creatine, urea and urine total proteine were measured in NXT rats and were significantly elevated after 24 weeks (Table 3.3).

In all animals, non-invasive blood pressure measurements were performed at different time points (OP, 1 week, 4 weeks, 8 weeks, 16 weeks and 24 weeks) to describe the development of blood pressure changes during the observation period in NXT and Sham animals. Systolic and diastolic blood pressure were increased significantly at 8 and 24 weeks in NXT rats compared to Sham (Figure 3.5 and Table 3.3).

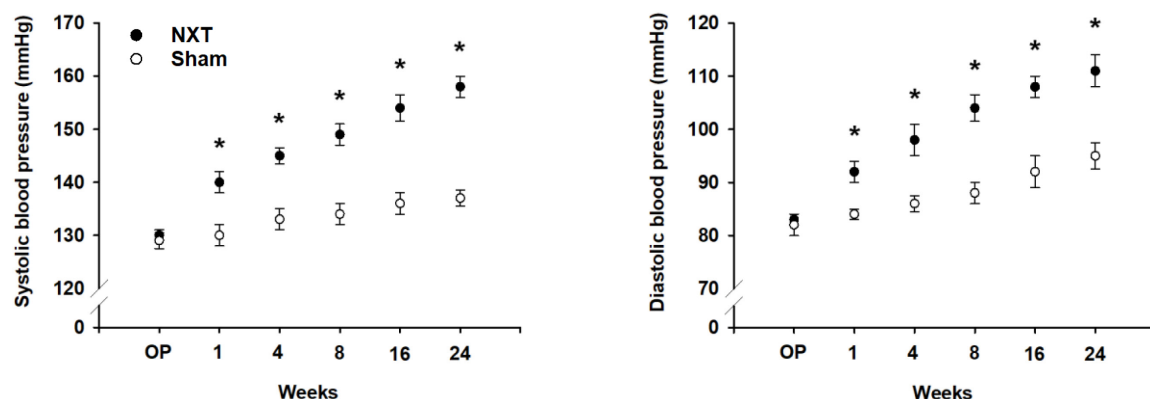


Figure 3.5 Non-invasive blood pressure measurements: Systolic blood pressure (SBP) and diastolic blood pressure (DBP) development in NXT and Sham rats. Symbols=mean blood pressure per group and time point. Data is shown as individual datapoints/box plots; p – values. * $p < 0.05$ between Sham/NXT. 8 weeks group (Sham N=8/NXT N=12). 24 weeks group (Sham n=8/NXT n=12). Analysis using ANOVA.

Table 3.3 Renal function and blood pressure

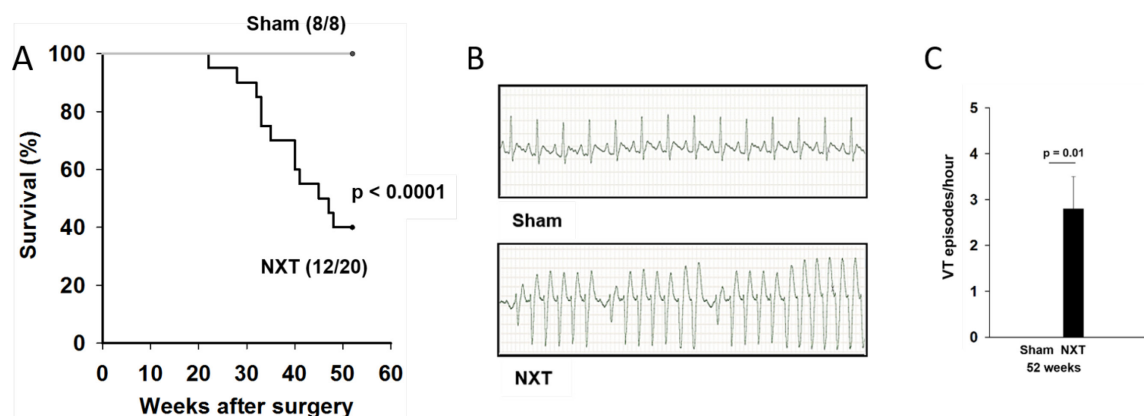
Parameters	Sham 8 weeks	NXT 8 weeks	Sham 24 weeks	NXT 24 weeks	8 weeks / 24 weeks / Sham / NXT Sham vs. NXT 8 vs. 24 weeks
Renal function	mean±SD				p-values
GFR (ml/min/m²)	32.75 ± 6.25 (N=12)	20.77 ± 2.95 (N=19)	28.84 ± 8.89 (N=12)	18.80 ± 3.32 (N=19)	<0.0001/0.007/0.2 /0.1
Serum creatinine (mg/dl)	0.41 ± 0.03 (N=12)	0.62 ± 0.07 (N=19)	0.64 ± 0.25 (N=12)	0.90 ± 0.22 (N=19)	<0.0001/<0.0001/0.0005/<0.0001
Serum urea (mg/dl)	45.42 ± 5.35 (N=12)	70.05 ± 5.63 (N=19)	55.67 ± 7.45 (N=12)	76.05 ± 13.65 (N=19)	<0.0001/<0.0001/0.0007/0.2
Serum proteine (g/dl)	6.72 ± 0.30 (N=12)	6.38 ± 0.19 (N=19)	5.41 ± 0.82 (N=12)	5.02 ± 0.72 (N=19)	0.07/0.3 /<0.0001/<0.0001
Urine proteine (mg/ml)	263.92 ± 113 (N=12)	476.74 ± 238 (N=19)	337.58 ± 149 (N=12)	604.63 ± 203 (N=19)	0.01/0.001/0.3/0.09
Non-invasive blood pressure					
SBP (mmHg)	134 ± 6.2 (N=8)	149 ± 7.5 (N=12)	137 ± 5.4 (N=8)	156 ± 7.2 (N=12)	<0.0001/<0.0001/0.5/0.06
DBP (mmHg)	88 ± 3.6 (N=8)	107 ± 4.7 (N=12)	98 ± 9.5 (N=8)	109 ± 3.6 (N=12)	0.001/0.04/0.2/0.2

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Table 3.3 Renal function and blood pressure: GFR – glomerular filtration rate; Serum creatine; Serum urea; Serum proteine; Urine proteine; SBP – systolic blood pressure; DBP – diastolic blood pressure. Data are mean ± S.D., p – values between Sham 8 weeks vs. NXT 8 weeks/ Sham 24 weeks vs. NXT 24 weeks/ Sham 8 weeks vs. Sham 24 weeks/ NXT 8 weeks vs. NXT 24 weeks. N=animals. Analysis using two-way ANOVA. Groups are Sham/NXT at 8 and 24 weeks.

3.1.5 HFpEF rats with increased mortality and arrhythmias

Subtotal nephrectomy and the associated clinically relevant HFpEF phenotype were associated with an increased mortality – starting around week 20 post nephrectomy (Figure 3.6) in NXT rats. Sham and NXT rats were observed for 52 weeks to study mortality. Kaplan Meier statistical analysis indicated a 40% mortality in NXT rats during one-year observation period while no animals died in the Sham group. Interestingly, all NXT rats immediately died over night without a decrease in body weight as an indicator for chronic cachexia. Consequently, sudden cardiac death with life-threatening ventricular arrhythmias could be a potential cause of death in HFpEF NXT rats. This theory was supported by the fact that in NXT rats ventricular arrhythmias were observed during final invasive hemodynamic measurements after 52 weeks while in Sham rats no arrhythmias were observed (Figure 3.6).



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Figure 3.6 Mortality and arrhythmias in NXT: A) Significant increased mortality in NXT (N=20) vs. Sham (N=8) rats, starting at week 20 post nephrectomy. B) Ventricular arrhythmia episodes per hour during the final in vivo hemodynamic measurements in Sham/NXT after 52 weeks (Sham/NXT 52 weeks, N = 8/8). Data are mean $\pm$ S.D., p – values. Analysis using ANOVA.

Additionally, findings in the 52 weeks survival group are summarized in table 3.4. While BW was significantly lower in NXT rats compared to Sham animals, HW/BW, LW/BW, LV mass and LV relative wall thickness were significantly higher in NXT rats. FS and LVEF were preserved even at week 52 in Sham and NXT rats supporting the theory of chronic stable heart failure with preserved ejection fraction in subtotal nephrectomy rats. Furthermore, urine excretion was preserved in Sham and NXT rats after 52 weeks.

Table 3.4 Survival group (52 weeks)

Parameters	Sham 52 weeks	NXT 52 weeks	52 weeks Sham vs. NXT
	mean±SD		p-values
BW (g)	650 ± 63 (N=8)	546 ± 35 (N=8)	0.001
HW/BW	1.91 ± 0.23 (N=8)	2.81 ± 0.43 (N=8)	0.0001
LW/BW	2.81 ± 0.18 (N=8)	3.93 ± 0.49 (N=8)	<0.0001
HR (BPM)	386 ± 18 (N=8)	382 ± 12 (N=8)	0.6
FS (%)	49 ± 4.05 (N=8)	49 ± 5.36 (N=8)	0.7
EF (%)	74 ± 4 (N=8)	72 ± 3 (N=8)	0.3
LV mass d (mm)	1731 ± 193 (N=8)	2275 ± 349 (N=8)	0.001
LV relative wall thickness (mm)	2.26 ± 0.17 (N=8)	2.69 ± 0.32 (N=8)	0.005
Urine volume (ml/8h)	6.9 ± 1.99 (N=8)	6.5 ± 1.77 (N=8)	0.7

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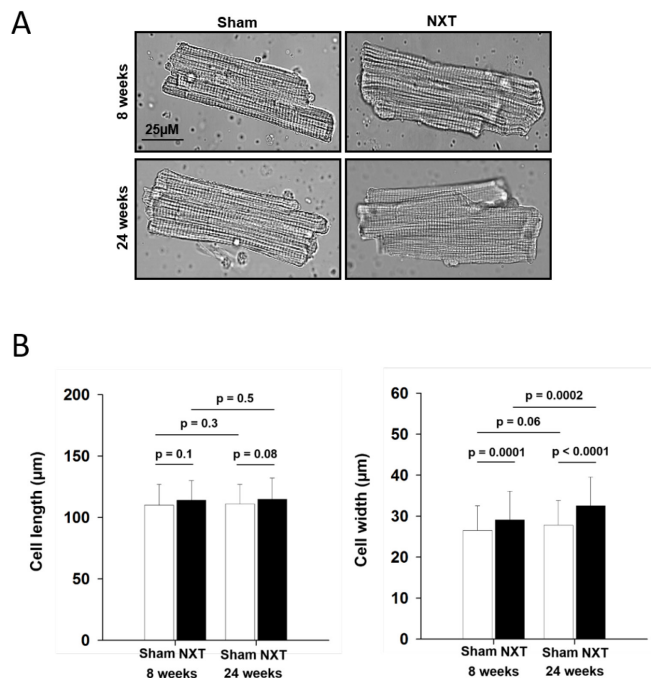
Table 3.4 Survival group (52 weeks): BW – Body weight; HW – Heart weight; LW – Lung weight; HR – Heart rate; FS – Fractional shortening; EF – Ejection fraction. LV mass – Left ventricular mass; LV relative wall thickness; Urine volume; Data are mean ± S.D., p – values; N=animals. Analysis using ANOVA. Groups are Sham (N=8)/ NXT (N=8) at 52 weeks.

3.2 Cardiomyocyte remodelling, dysfunction and impaired calcium handling in HFpEF rats

Cardiomyocytes remodeling, dysfunction and calcium handling was investigated from NXT and Sham rats after 8 and 24 weeks. LV cardiomyocytes were enzymatically isolated from the same animals which underwent final invasive hemodynamic measurements to compare in-vivo and in-vitro function of the hearts and cardiomyocytes.

3.2.1 Cardiomyocyte hypertrophy

LV cardiomyocytes from NXT rats were significantly hypertrophied at 8 weeks, with a further increase at 24 weeks compared to Sham rats (Figure 3.7 A). Especially cell width was significantly increased in NXT rats at 8 and even more at 24 weeks (Figure 3.7 B). Consequently, subtotal nephrectomy in rats lead to relevant LV hypertrophy in NXT hearts and LV cardiomyocyte remodeling with a significant increase in LV cell size (LV cardiomyocyte hypertrophy).

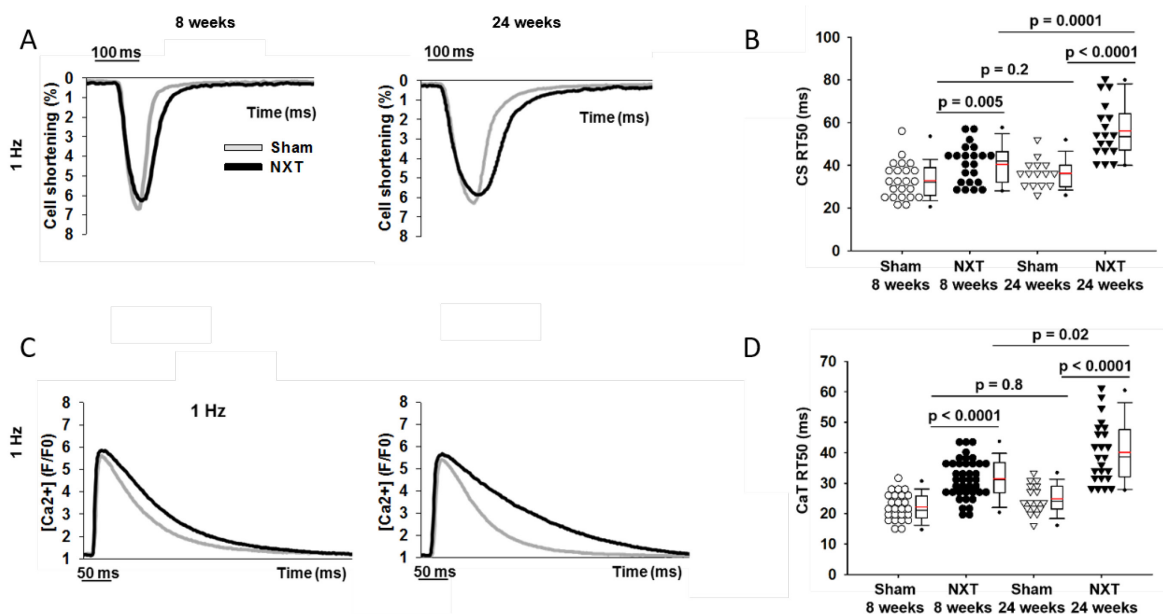


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Figure 3.7 LV cardiomyocyte hypertrophy: A) Cardiomyocytes 8 and 24 weeks post NXT/Sham. B) Increased cell width with unchanged cell length (Sham/NXT 8 weeks, N/n (hearts=6/6 and cells=184/202) and Sham/NXT 24 weeks, N/n (hearts=6/6 and cells=129/147)). Data are mean ± S.D., p – values. Analysis using ANOVA.

3.2.2 Cardiomyocyte contractility (cell shortening), calcium transients and mechanisms of slowed active cardiomyocyte relaxation

Cell shortening amplitude (CS,%) from enzymatically isolated cardiomyocytes was unchanged in NXT and Sham at 8 and 24 weeks. Time to peak cardiomyocyte (TTP,ms) shortening (1 Hz) was also unchanged in NXT and Sham at both timepoints. Cell relengthening (relaxation) was significantly prolonged in NXT vs. Sham cardiomyocytes already at 8 weeks and significantly more at 24 weeks (Figure 3.8 A, B). Amplitudes and time to peak of intracellular Ca^{2+} transients were unchanged in NXT vs. Sham at 8 and 24 weeks. $[\text{Ca}^{2+}]_i$ decay slowed with age in both groups. In addition, $[\text{Ca}^{2+}]_i$ decay was markedly slower in NXT vs. Sham (by 42% at 8 weeks and 77% at 24 weeks) (Figure 3.8 C, D).



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Figure 3.8 Slowed active relaxation in LV cardiomyocytes: A) Example of LV cardiomyocyte CS amplitude at 8 and 24 weeks (NXT/Sham). B) Progressive prolongation of CS relaxation (CSRT50,ms) at 8 (NXT/Sham hearts=6/6 and cells=23/22) and significantly more at 24 weeks (NXT/Sham hearts=6/6 and cells=15/18). C) Example of LV cardiomyocyte Ca^{2+} transient amplitude (1 Hz) at 8 and 24 weeks (NXT/Sham). D) Significant prolongation of Ca^{2+} decay (CaT RT50,ms) in NXT at 8 (NXT/Sham hearts=6/6 and cells=26/40) and 24 weeks (NXT/Sham hearts=6/6 and cells=18/24). Data is shown as individual datapoints/box plots; p – values. 6 hearts/group; symbol=cell. Analysis using ANOVA.

In table 3.5 the basic characteristics of LV cardiomyocyte cell shortening, calcium transients and mechanisms of slowed active relaxation are summarized.

Table 3.5 Basic characteristics of cardiomyocytes

Parameter	Sham 8 weeks	NXT 8 weeks	Sham 24 weeks	NXT 24 weeks	8 weeks/24 weeks/Sham/NXT Sham vs. NXT 8 vs. 24 weeks
Cell shortening (CS)					
CS amplitude (%)	6.8 ± 3.0 (n=23)	6.1 ± 2.6 (n=22)	6.2 ± 2.0 (n=15)	5.6 ± 1.5 (n=18)	0.5/0.2/0.6/0.3
CS TTP (ms)	85.0 ± 14.4 (n=23)	80.0 ± 17.0 (n=22)	87.7 ± 16.4 (n=15)	79.5 ± 18.3 (n=18)	0.2/0.1/0.5/0.9
CS RT50 (ms)	32.8 ± 8.4 (n=23)	40.5 ± 9.0 (n=22)	36.2 ± 6.4 (n=15)	54.1 ± 9.3 (n=18)	0.005/<0.0001/0.2/0.0001
CS RT90 (ms)	56.6 ± 10.3 (n=23)	67.4 ± 17.3 (n=22)	60.3 ± 14.2 (n=15)	77.2 ± 18.9 (n=18)	0.1/0.02/0.5/0.2
Calcium homeostasis					
CaT amplitude (F/F0)	4.3 ± 0.9 (n=26)	4.4 ± 1.0 (n=40)	4.0 ± 0.5 (n=18)	4.2 ± 0.9 (n=24)	0.8/0.3/0.2/0.5
CaT TTP (ms)	32.1 ± 6.1 (n=26)	32.8 ± 6.5 (n=40)	26.6 ± 4.9 (n=18)	29.4 ± 6.5 (n=24)	0.6/0.2/0.01/0.1
CaT RT50 (ms)	22.2 ± 4.4 (n=26)	31.6 ± 6.3 (n=40)	22.5 ± 6.3 (n=18)	39.9 ± 5.4 (n=24)	<0.0001/<0.0001/0.8/0.02
CaT RT90 (ms)	121.1 ± 27.1 (n=26)	132.0 ± 17.6 (n=40)	122.4 ± 16.7 (n=18)	146.7 ± 20.2 (n=24)	0.1/0.004/0.8/0.06

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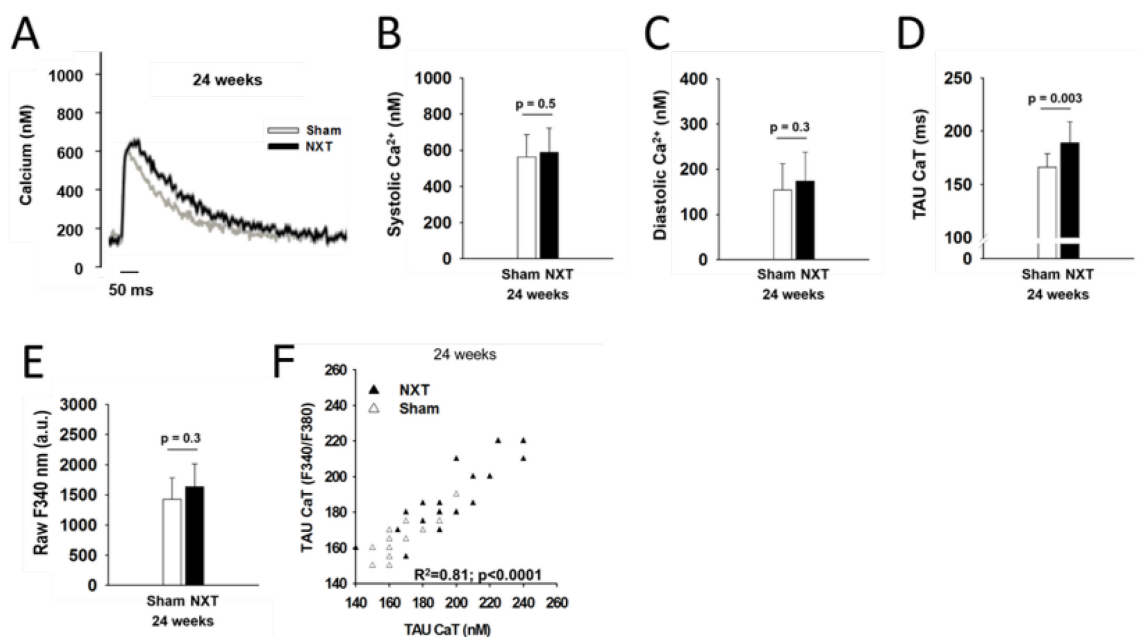
Table 3.5 Basic characteristics of cardiomyocytes: CS – Cell shortening; TTP – Time to peak; CS RT50 – 50% of the relaxation time; CS RT90 – 90% of the relaxation time; CaT – Calcium transient; CaT RT50 – 50 % of the calcium transient decay; CaT RT90 – 90% of the calcium transient decay; Data are mean ± S.D., p – values between Sham 8 weeks vs. NXT 8 weeks/ Sham 24 weeks vs. NXT 24 weeks/ Sham 8 weeks vs. Sham 24 weeks/ NXT 8 weeks vs. NXT 24 weeks. N/n (hearts/cells). Analysis using ANOVA.

3.2.3 Cytosolic Ca²⁺ measurements in cardiomyocytes

Ca²⁺ transients using ratiometric fluorescence measurements were investigated. Slowed relaxation was confirmed in Fura-2-loaded cardiomyocytes with the fluorescence signal calibrated to absolute [Ca²⁺] concentration. No differences in absolute systolic and diastolic [Ca²⁺] between NXT and Sham were found at 24

weeks. (Figure 3.9 A-C). Prolonged decay of the Ca^{2+} transient in NXT (24 weeks) was confirmed using Fura-2 as Ca^{2+} indicator (Figure 3.9 D).

In order to exclude differences in Fura-2 dye loading between groups that may lead to differences in the degree of Ca^{2+} buffering (and kinetics), absolute (raw) Fura-2 fluorescence (emission > 510 nm) at excitation 340 nm in Sham and NXT at diastole (1 Hz) were compared. Ratiometric measurements indicated similar $[\text{Ca}^{2+}]$ at diastole (Figure 3.9 C). Mean background-corrected Fura-2 fluorescence at 340 nm was comparable between groups (Figure 3.9 E), suggesting that average dye loading was comparable in cardiomyocytes of either group. Fluorescence ratios (F340/F380) correlated linearly with the derived $[\text{Ca}^{2+}]$ values within the dynamic range of the measured Ca^{2+} signals (Figure 3.9 F) and were used for further analyses.

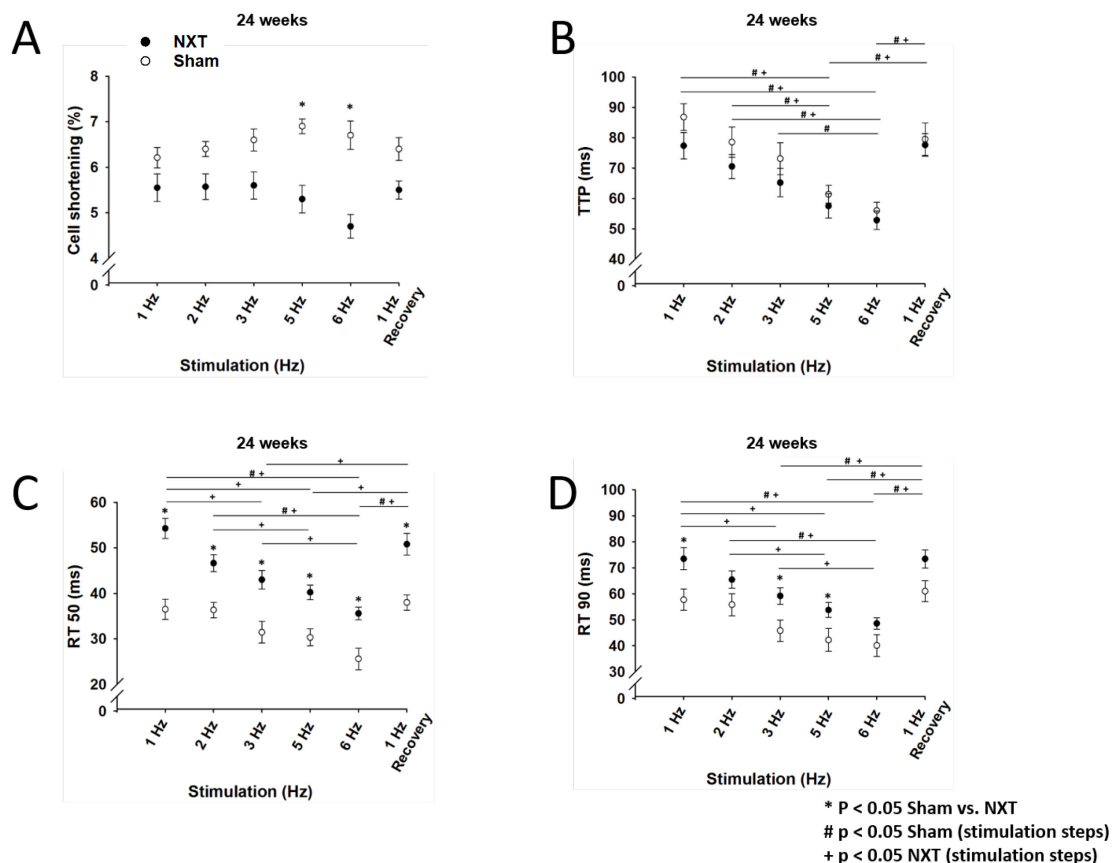


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Figure 3.9 Cytosolic Ca^{2+} measurements in cardiomyocytes: Examples (A) and mean systolic (B) and diastolic (C) values of calibrated Ca^{2+} transients (1 Hz) measured by ratiometry (Fura-2) after 24 weeks in Sham/NXT. Prolonged decay of the Ca^{2+} transient in NXT (24 weeks) was confirmed using Fura-2 as Ca^{2+} indicator (D). Similar raw (background-corrected) fluorescence values at excitation 340 nm (F340) suggest similar dye-loading in Sham vs. NXT (E). Panel F shows a close linear correlation between the time constants of Ca^{2+} decay quantified in ratiometry and calibrated transients. Data are mean $\pm$ S.D., p – values. NXT/Sham hearts=6/6 and cells=17/12. Analysis using ANOVA.

3.2.4 Frequency dependence of cardiomyocyte contractility in HFpEF

LV cardiomyocytes isolated from Sham and NXT rats after 24 weeks were stimulated at different frequencies (1-6 Hz) to investigate frequency dependence cell contractility in cardiomyocyte. In Sham, cardiomyocyte cell shortening amplitude increased with higher stimulation frequency (2-6 Hz), whereas in NXT cell contraction amplitude decreased at higher frequencies. At 5 and 6 Hz cell shortening amplitude was significantly different between Sham and NXT. Time to peak cardiomyocyte (TTP, ms) shortening decreased with higher stimulation frequency (3-6 Hz) in both but was unchanged between Sham and NXT. In NXT animals, significantly prolonged cardiomyocyte relaxation time persisted at higher frequencies. (Figure 3.10).

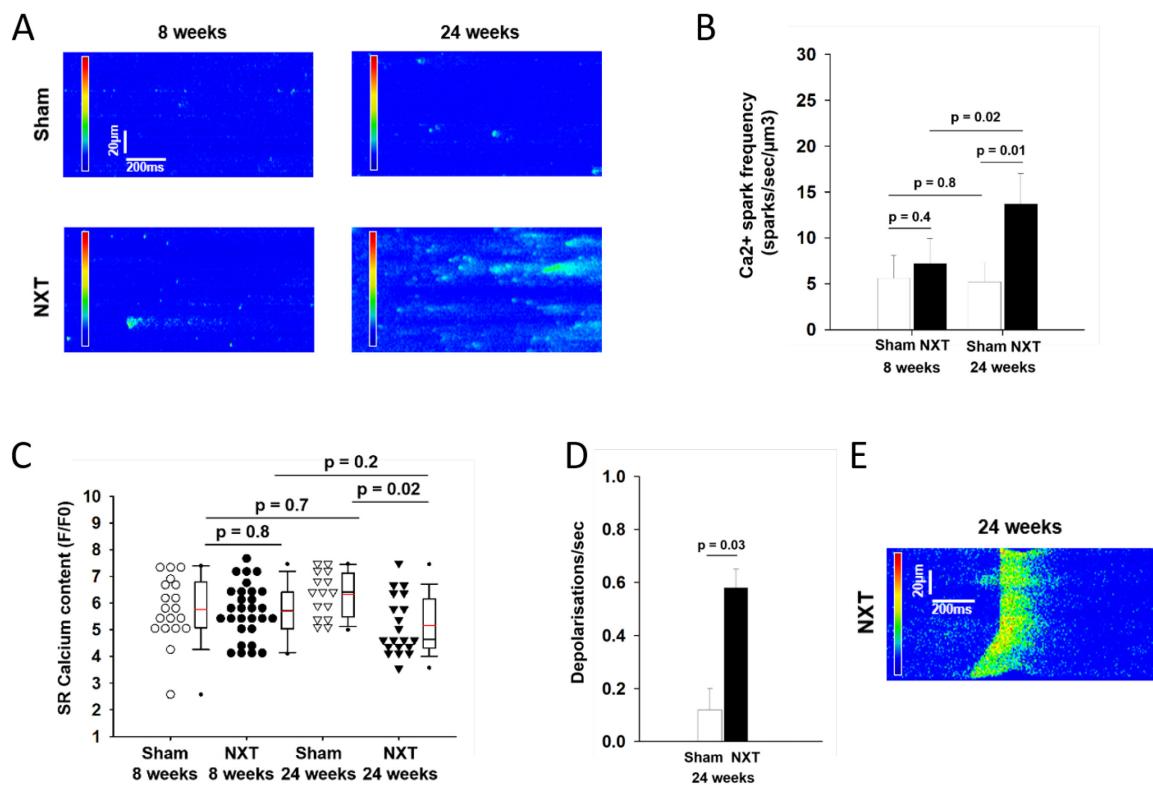


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Figure 3.10 Frequency dependence of cardiomyocyte contractility: A) Cell shortening amplitude (CS, %), B) Time to peak (TTP, ms), C) 50% of relaxation time (RT50, ms) and D) 90% of relaxation time (RT90, ms) at 1 Hz (Sham/NXT n=14/18), 2 Hz (Sham/NXT n=12/16), 3 Hz (Sham/NXT n=12/12), 5 Hz (Sham/NXT n=10/12), 6 Hz (Sham/NXT n=9/10) and 1 Hz Recovery (Sham/NXT n=9/10) period after 24 weeks. Data are mean $\pm$ S.D., p – values. * p < 0.05 between Sham/NXT; # p < 0.05 in Sham at different frequencies; + p < 0.05 in NXT at different frequencies. Symbols=mean cell per group/time point. Analysis using ANOVA.

3.2.5 Increased sarcoplasmic reticulum (SR) Ca²⁺ leak and spontaneous depolarisations in cardiomyocytes

SR Ca²⁺ content and SR Ca²⁺ leak (Ca²⁺ sparks) were unchanged in NXT vs. Sham at 8 weeks, whereas at 24 weeks SR Ca²⁺ content (NXT (n=20)/ Sham (n=16); 5.20 ± 0.92 vs. 6.36 ± 0.78, F/F₀; p=0.02) was significantly reduced and SR Ca²⁺ leak - Ca²⁺ spark frequency (NXT (n=27)/ Sham (n=21); 13.7 ± 5.9 vs. 5.2 ± 3.2, sparks/sec/μm³; p=0.01) was significantly increased in NXT vs. Sham animals (Figure 3.11 A-C). Moreover, spontaneously induced depolarisations were significantly more frequent in NXT vs. Sham at 24 weeks (Figure 3.11 D-E). This data supported the theory that in LV cardiomyocytes from HFpEF rats (24 weeks NXT) more pro-arrhythmic potential was observed compared to Sham rats.

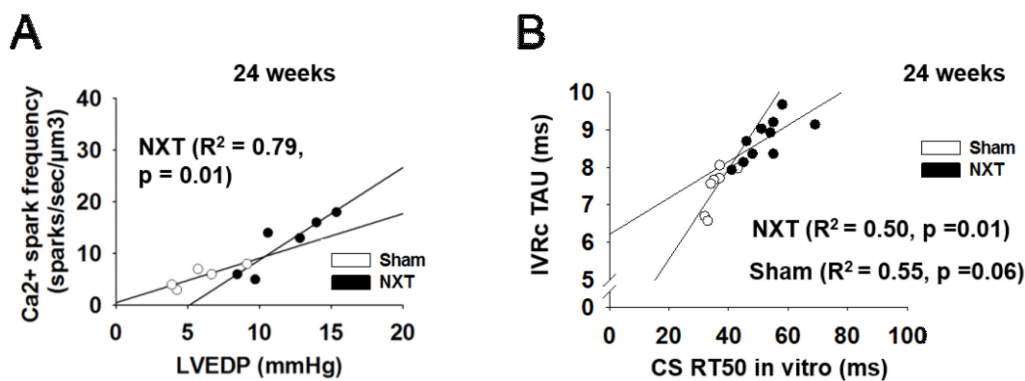


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Figure 3.11 Increased sarcoplasmic reticulum (SR) Ca²⁺ leak and spontaneous depolarisations in cardiomyocytes: A) Line scan images from cardiomyocytes showed an increase in B) Ca²⁺ spark frequency, sparks/sec/μm³ only at 24 weeks (Sham/NXT 8 weeks, N/n (hearts=6/6 and cells=30/31) and Sham/NXT 24 weeks, N/n (hearts=6/6 and cells=21/27)). C) SR Ca²⁺ content was decreased only after 24 weeks in NXT vs. Sham. D and E) Line scan images from cardiomyocytes at 24 weeks post Sham/NXT with significantly increased spontaneous induced depolarisations in NXT (Sham/NXT 24 weeks, N/n (hearts=6/6 and cells=21/27)). Data are mean ± S.D. or shown as individual datapoints/box plots; p – values. Analysis using ANOVA.

3.3 LV diastolic dysfunction in vivo is related to slowed cardiomyocyte relaxation and increased Ca^{2+} leak in vitro in HFpEF rats

To establish a correlation between in vivo dysfunction and cellular changes in vitro in HFpEF rats based on chronic renal failure induced by subtotal nephrectomy, the hearts were excised after final invasive hemodynamic experiments and were enzymatically digested to obtain single cardiomyocytes. Based on that, in vivo systolic and diastolic function measured with echocardiography and pressure volume loops could be compared to in vitro cardiomyocyte measurements with ratiometric and laser scanning microscopy. There was a significant correlation between SR Ca^{2+} leak and LVEDP in NXT rats after 24 weeks. The SR Ca^{2+} leak, represented by the SR Ca^{2+} spark frequency rate, was significantly increased in HFpEF rat hearts after 24 weeks and correlated significantly with elevated LVEDP in NXT rats after 24 weeks (Figure 3.12 A). Moreover, slowed relaxation of isolated cardiomyocytes from NXT rat hearts *in vitro* (CS RT50, ms) correlated significantly with increased LV isovolumetric relaxation constant TAU (IVRc TAU) *in vivo* (Figure 3.12 B). Both findings demonstrated that altered LV diastolic dysfunction in HFpEF rats induced by subtotal nephrectomy was significantly correlated to impaired cardiomyocyte function.



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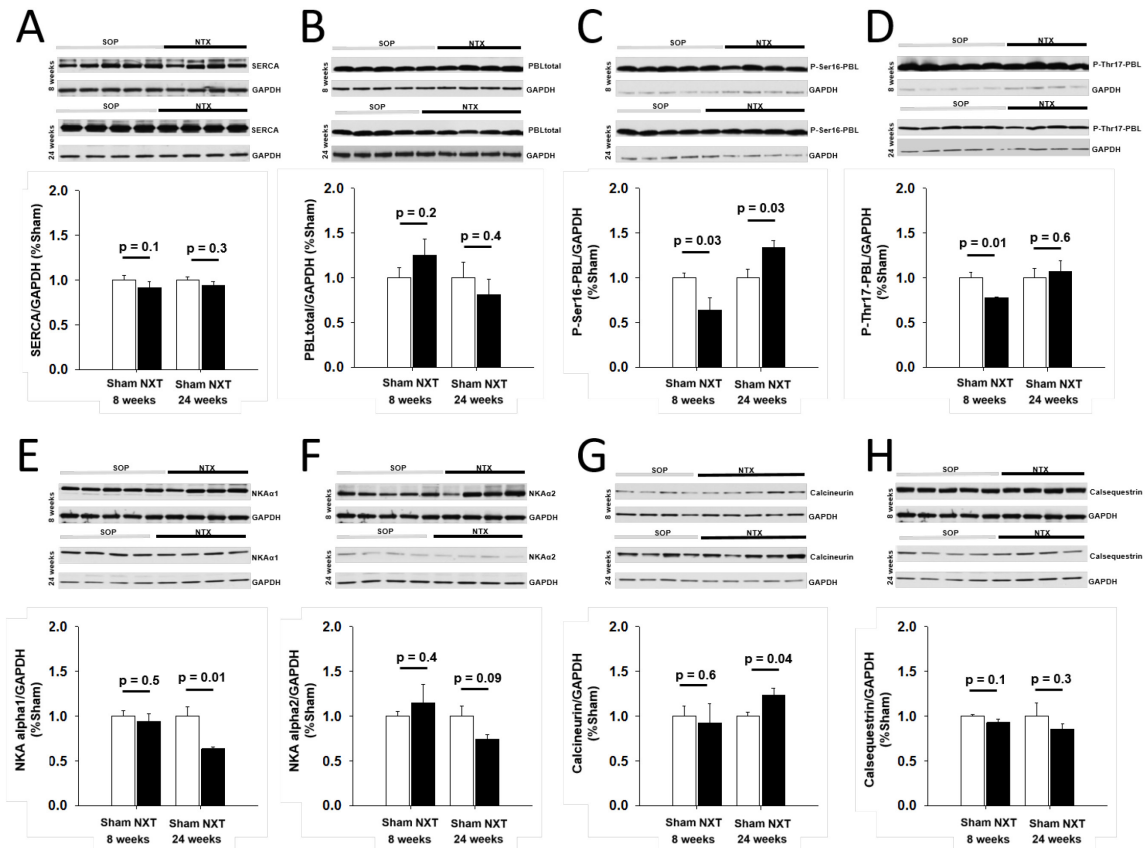
Figure 3.12 In vivo / in vitro correlations in HFpEF: A) Ca^{2+} spark frequency in vitro was significantly correlated to the LVEDP in vivo at 24 weeks (Sham N=5, $R^2=0.76$, $p=0.05$ and NXT N=6, $R^2=0.79$, $p=0.01$). Data points indicate mean spark frequency of all measured cardiomyocytes (3-12) of that heart. B) Cardiomyocyte relaxation time (CS RT50, ms) in vitro was significantly correlated to LV isovolumetric relaxation constant (IVRc TAU) in vivo at 24 weeks (Sham N=6, $R^2=0.55$, $p=0.06$ and NXT N=10, $R^2=0.50$, $p=0.01$). Symbol=cell average/heart.

3.4 Protein expression levels of Ca²⁺- and Na⁺-handling proteins

Protein expression levels of calcium and sodium handling proteins involved in heart failure were analyzed in subgroups from NXT and Sham animals at 8 and 24 weeks to explore underlying molecular pathways of cellular remodeling. Analyzed proteins were sodium-calcium exchanger (NCX), SR Ca²⁺ ATPase (SERCA), phospholamban (PBLtotal), phospholamban phosphorylated at serine 16 (P-Ser16-PLB), phospholamban phosphorylated at threonine 17 (P-Thr17-PLB), sarcolemmal Na⁺/K⁺-ATPase subunits 1 (NKAalpha1) and 2 (NKAalpha2), calcineurin and calsequestrin.

All western blot experiments were performed on homogenates prepared from the LV and assessed by standard immunoblotting procedure using commercially available antibodies. The bands intensity was quantified by Quantity One (Bio-Rad) and the expression of proteins was normalized to GAPDH.

Total expression of SERCA protein and SR Ca²⁺ store protein calsequestrin were unchanged (Figure 3.13 A, H). While reduced phosphorylation of SERCA regulator phospholamban (PLB) at Ser16 and Thr17 at 8 weeks could be associated with reduced SERCA activity, increased phosphorylation of PLB at 24 weeks would favour increased SERCA activity (Figure 3.13 B-D). Overall, the time constant of Ca²⁺ transient decay attributed to SERCA activity (as calculated from the electrically stimulated Ca²⁺ transient and caffeine-induced Ca²⁺ transient in the same cell) was not different in NXT vs Sham (at 24 weeks: 202±11 vs. 190±9 ms; p=0.2), indicating that reduced SERCA activity was not responsible for the slowed Ca²⁺ transient decay (194).



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Figure 3.13 Protein expression levels of Ca^{2+} - and Na^{+} -handling proteins:

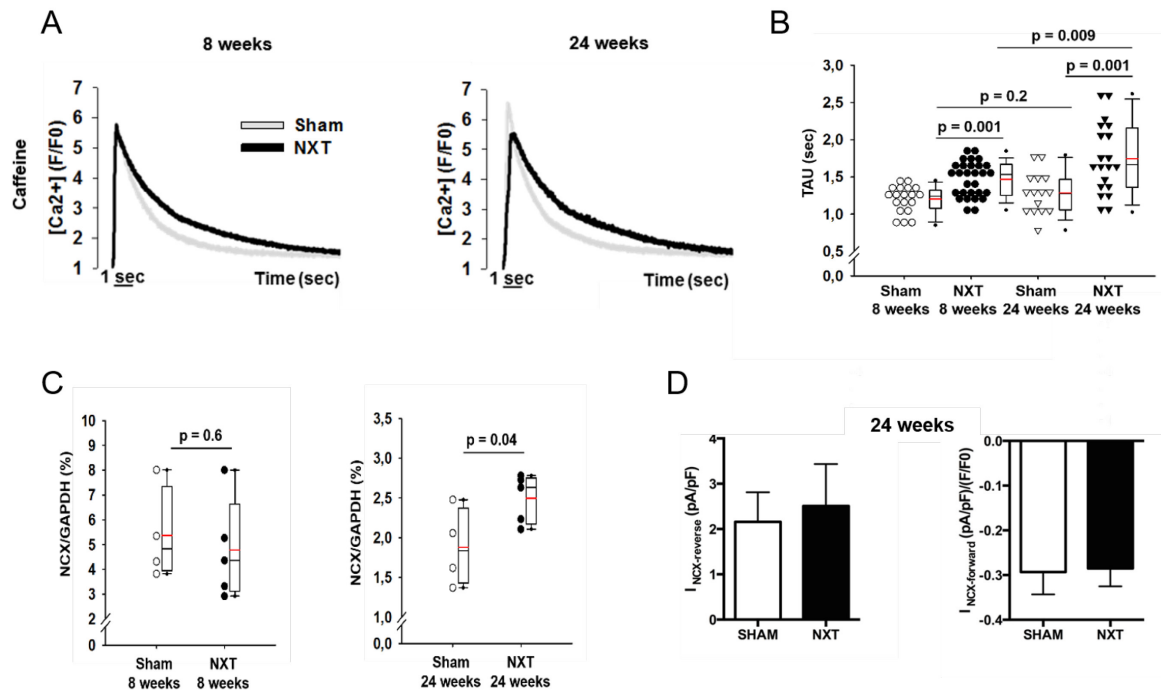
Protein expression (in myocardial homogenates) of A) SR Ca^{2+} ATPase (SERCA, Sham/NXT 8 weeks, n=5/4 and Sham/NXT 24 weeks, n=4/4). B); phospholamban (PBLtotal, Sham/NXT 8 weeks, n=5/4 and Sham/NXT 24 weeks, n=4/5); C) phospholamban phosphorylated at serine 16 (P-Ser16-PLB, Sham/NXT 8 weeks, n=5/4 and Sham/NXT 24 weeks, n=4/5); D) phospholamban phosphorylated at threonine 17 (P-Thr17-PLB, Sham/NXT 8 weeks, n=5/4 and Sham/NXT 24 weeks, n=5/4); sarcolemmal $\text{Na}^{+}/\text{K}^{+}$ -ATPase subunits 1 E) and 2 F) (NKAalpha1: Sham/NXT 8 weeks, n=5/4 and Sham/NXT 24 weeks, n=4/4; NKAalpha2: Sham/NXT 8 weeks, n=5/4 and Sham/NXT 24 weeks, n=4/4); G) calcineurin (Sham/NXT 8 weeks, n=4/5 and Sham/NXT 24 weeks, n=4/5); H) calsequestrin (Sham/NXT 8 weeks, n=5/4 and Sham/NXT 24 weeks, n=4/4). Data are mean $\pm$ S.D., p – values. Analysis using ANOVA.

3.5 Role of Na⁺/Ca²⁺ exchanger in heart failure

Sodium-calcium exchanger (NCX) is a plasma-membrane protein working in two different directions. NCX forward mode activity – mainly in healthy cardiomyocytes – extrudes 1 Ca²⁺ ion in exchange for 3 Na⁺ ions. Moreover, NCX can extrude Na⁺ in exchange for Ca²⁺ (reverse mode). In human heart failure, cytosolic levels of Na⁺ have been shown to be increased and NCX might play a role in the maintenance of high diastolic [Ca²⁺]_i and by that impaired contractility and relaxation in the disease (90,108,109).

Therefore, the role of the Na⁺/Ca²⁺ exchanger in LV cardiomyocytes from HFpEF rats after 8 and 24 weeks were investigated and compared to Sham animals.

Decay of the caffeine-induced Ca²⁺ transient as a measure of NCX forward mode function (Ca²⁺ extrusion) was progressively prolonged at 8 (NXT (n=29)/ Sham (n=20); 1.47 ± 0.23 vs. 1.20 ± 0.18, sec; p=0.001) and 24 (NXT (n=20)/ Sham (n=16); 1.75 ± 0.37 vs. 1.29 ± 0.28, sec; p=0.001) weeks in NXT vs. Sham (Figure 3.14 A and B). Moreover, in NXT rats the effect was significantly pronounced between week 8 and week 24 (p=0.009). Yet, protein expression of NCX was unchanged at 8 and even significantly increased at 24 weeks in NXT (Figure 3.14 C). Expression of the dominant alpha1 subunit as well as the alpha2 subunit of the Na⁺/K⁺ pump was significantly reduced at 24 weeks in NXT, suggesting reduced cytosolic Na⁺ removal capacity at an advanced stage in HFpEF in NXT (Figure 3.13 E and F). Expression of calcineurin, a downstream Ca²⁺ signalling protein involved among others in cardiac hypertrophy was increased at 24 weeks (Figure 3.13 G). In conditions of whole-cell voltage clamping (10 mM Na⁺ in pipette), transsarcolemmal NCX current in forward and reverse mode was not different in NXT vs. Sham at 24 weeks (Figure 3.14 D).



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Figure 3.14 Na⁺/Ca²⁺ exchanger: A) Example of LV cardiomyocyte caffeine-induced Ca²⁺ transient in Sham and NXT at 8 and 24 wks. B) Decay of the Caffeine induced calcium transient TAU (sec) as a measure of NCX-mediated Ca²⁺ extrusion was prolonged already at 8 weeks (Sham n=20 / NXT n=29) and significantly more at 24 weeks (Sham n=16 / NXT n=20) in NXT (symbol=cell; 6 hearts/group). C) Increased NCX protein expression at 24 weeks in NXT (N=5) vs. Sham (N=4), while at 8 weeks NCX protein expression was unchanged (NXT N=5 and Sham N=4) (symbol=animal). D) In conditions of whole-cell voltage clamping (10 mM Na⁺ in pipette), transsarcolemmal NCX current in forward A) and reverse B) mode was not different in NXT vs. Sham (24 weeks). N ≥ 6 cells from 4 hearts per group. TAU – Time constant of the caffeine induced CaT (reflected NCX1-forward mode activity). Data are mean ± S.D. or shown as individual datapoints/box plots; p – values. Analysis using ANOVA.

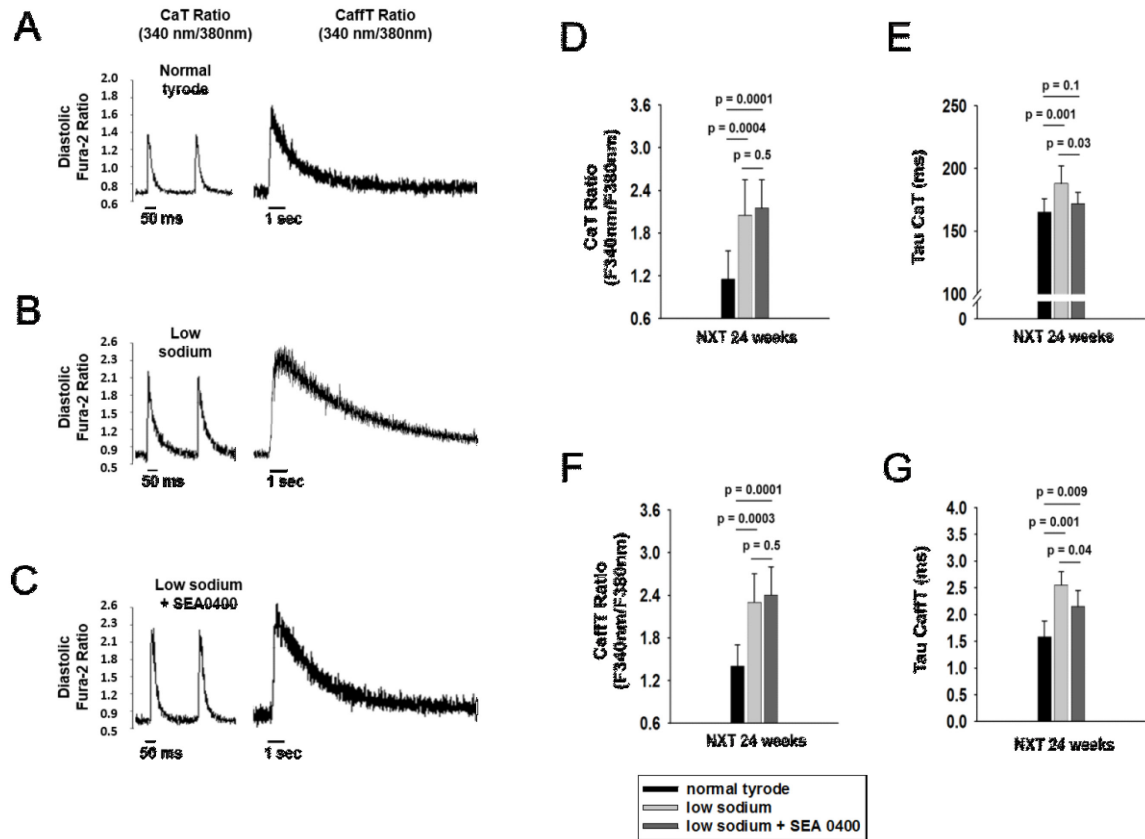
3.5.1 Increased NCX reverse mode activity in HFpEF

As NCX protein expression was increased in NXT despite its reduced capacity for Ca²⁺ extrusion we hypothesized that the exchanger may be shifted towards increased activity in reverse mode (Ca²⁺ import). During steady-state stimulation in voltage-clamp mode, NCX current density was comparable between Sham and NXT at 24 weeks (Figure 3.14 D). Under these experimental conditions, controlling the duration of the depolarizing step and clamping [Na⁺]_i at 10 mM via the pipette solution, the slower decay of Ca²⁺ transients were no longer observed in NXT cells.

Together, this data indicated functional inhibition of NCX forward mode activity secondary to AP morphology, and/or changes in $[Na^+]_i$. To prove this hypothesis the role of sodium as a trigger for reverse mode activity of the NCX in NXT was investigated. To evaluate the effects of functional inhibition of NCX forward mode on the Ca^{2+} transient, intact NXT cardiomyocytes were exposed to low extracellular $[Na^+]$ (Figure 3.15 A and B) which reproduced a prolongation of the electrically stimulated Ca^{2+} transients and caffeine-induced Ca^{2+} transients. This effect was inhibited by the NCX blocker SEA0400 (Figure 3.15 C). In non-dialysed, non-voltage clamped cardiomyocyte from NXT, a reduction in external $[Na^+]$ leads to prolongation of the electrically (field-) stimulated Ca^{2+} transient and the caffeine transient due to influx of Ca^{2+} through the NCX in reverse mode (Figure 3.15 A and B). SEA0400 accelerates Ca^{2+} decay by inhibition of NCX (Figure 3.15 C).

Calcium transient ratio (CaT ratio, F340nm/F380nm) measured with Fura-2 in NXT cardiomyocytes after 24 weeks (n=10) was significantly higher in low sodium condition and was unaffected by SEA0400 (Figure 3.15 D). Tau of the calcium transient (TAU CaT, ms) in NXT cardiomyocytes after 24 weeks (n=10) was significantly prolonged in low sodium solution and was ameliorated after SEA0400 application (Figure 3.15. E). Caffeine induced calcium transient ratio (CaffT ratio, F340nm/F380nm) measured with Fura-2 in NXT cardiomyocytes after 24 weeks (n=10) was significantly higher in low sodium conditions and was not altered after SEA0400 application (Figure 3.15 F). Tau of the caffeine induced calcium transient (TAU CaffT, ms) in NXT after 24 weeks (n=10) was significantly prolonged in low sodium experimental setting and after SEA0400 treatment it significantly improved (Figure 3.15 G).

Out of these experiments it could be concluded that in the experimental conditions of low sodium - NCX in NXT-HFpEF rats worked in reverse mode and SEA0400 ameliorated NCX reverse mode function.



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Figure 3.15 Role of sodium as a trigger for reverse mode activity of the NCX in NXT: A) Representative examples of two electrically stimulated Ca^{2+} transients (left) at steady state and the caffeine-induced Ca^{2+} transient (right) in intact, field stimulated cardiomyocytes. Upper trace shows NXT cardiomyocyte in normal Tyrode solution, B) middle trace with reduced external $[\text{Na}^+]$ (70 mmol/L, to shift NCX into reverse mode) and C) lower trace with reduced external $[\text{Na}^+]$ + SEA0400. In non-dialysed, non-voltage clamped cardiomyocyte from NXT, a reduction in external $[\text{Na}^+]$ leads to prolongation of the electrically (field-) stimulated Ca^{2+} transient and the caffeine transient due to influx of Ca^{2+} through the NCX in reverse mode. SEA0400 accelerates Ca^{2+} decay by inhibition of NCX. D) Calcium transient ratio (CaT ratio, F340nm/F380nm) measured with Fura-2 in NXT after 24 weeks (n=10). E) Tau of the calcium transient (TAU CaT, ms) in NXT after 24 weeks (n=10). F) Caffeine induced calcium transient ratio (CaffT ratio, F340nm/F380nm) measured with Fura-2 in NXT after 24 weeks (n=10). G) Tau of the caffeine induced calcium transient (TAU CaffT, ms) in NXT after 24 weeks (n=10). Data are mean $\pm$ S.D., p – values. Analysis using ANOVA.

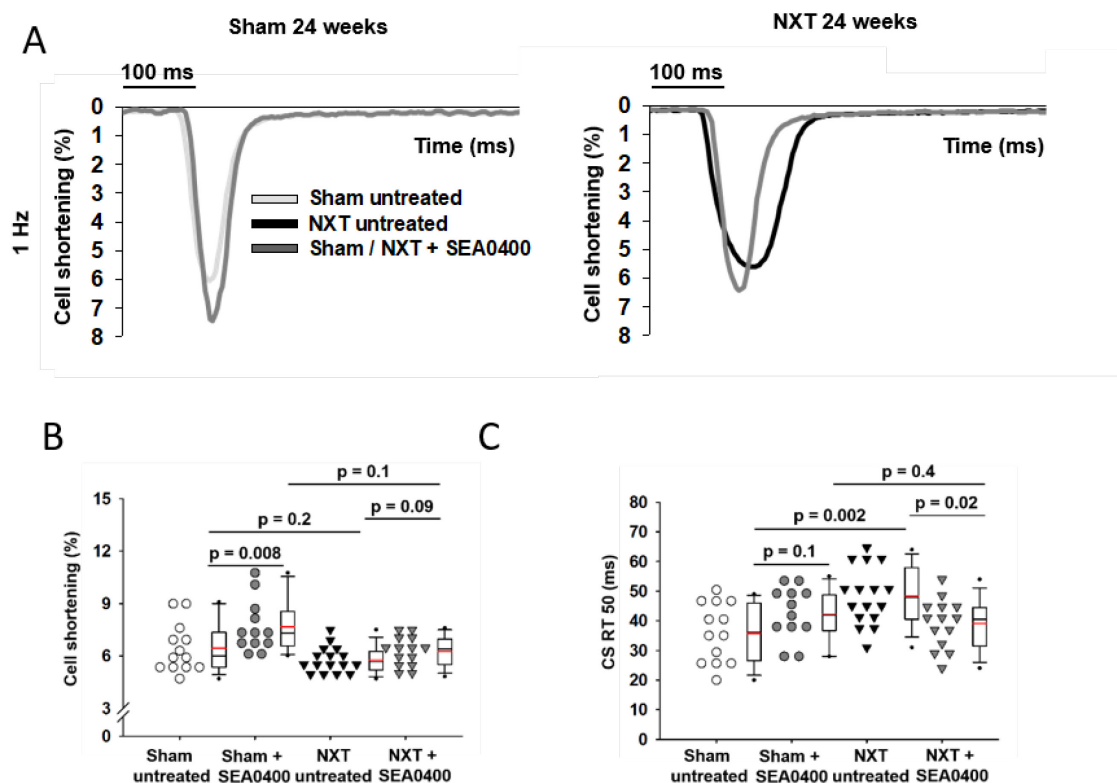
3.6 Acute effects of the NCX inhibition with SEA0400 on LV cardiomyocytes in HFpEF rats

After the establishment of an animal model with heart failure and preserved ejection fraction in a rat model with chronic stable kidney disease based on subtotal nephrectomy, cellular, subcellular and molecular mechanisms of ventricular remodeling leading to HFpEF were identified in this model. Especially, intracellular Ca^{2+} disturbances were characterized as a cause of contractile dysfunction and increased arrhythmia risk at more advanced stages of the disease. Moreover, a correlation between *in vivo* cardiac dysfunction and cellular changes *in vitro* were shown. The sodium-calcium exchanger (NCX), as an important player in heart failure and especially in HFpEF in its reverse mode were shown in this animal model of subtotal nephrectomy in rats.

To evaluate new therapeutic targets reversing remodeling processes, improving diastolic function, and preventing arrhythmias in HFpEF the effect of SEA0400 – a new, specific NCX inhibitor – was investigated acutely *in vitro* on cardiomyocytes, *in vivo* in NXT and Sham rats and as chronical treatment over 16 weeks.

3.6.1 Effects of SEA0400 on LV cardiomyocyte cell shortening

In Sham and NXT animals after 24 weeks acute in vitro measurements on contractile function were performed in the presence of the NCX inhibitor SEA0400. After baseline measurements of cell shortening at 1 Hz field stimulation, 300nM SEA0400 was applied. After an incubation time period of 5 minutes time to peak (TTP, ms) and amplitude of cell shortening (CS, %) were increased but there were no effects on relaxation (CS RT50, ms) in sham cardiomyocytes. In contrast, after 5 minutes incubation of SEA0400 in LV cardiomyocytes from 24 weeks old NXT animals, there were no effects on time to peak (TTP, ms) and cell shortening amplitude (CS, %) but SEA0400 ameliorated slowed relaxation (CS RT50, ms; NXT untreated (n=16) / NXT + SEA0400 (n=14), 48.3 ± 9.8 vs. 39.1 ± 8.3 , $p=0.02$) significantly (Figure 3.16 A-C and Table 3.6).

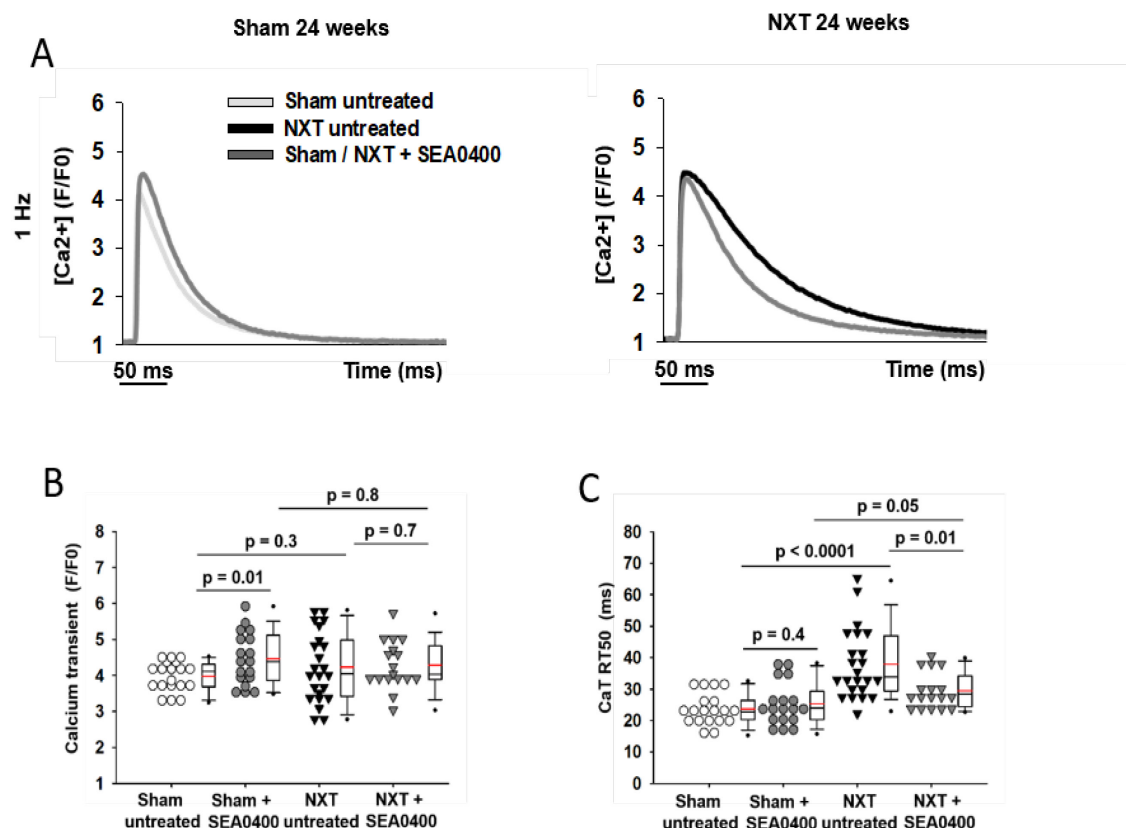


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Figure 3.16 SEA0400 improved slowed relaxation in NXT: A) Representative examples of CS transients in Sham (left) and NXT (right) cardiomyocytes at 24 weeks with 1 Hz field stimulation. B) CS amplitude (CS, %) in 24 weeks Sham/NXT cardiomyocytes incubated with 300 nM SEA0400 for 5 minutes and untreated. C) Cardiomyocyte relaxation (CS RT50, ms) in 24 weeks Sham and NXT cardiomyocytes incubated with 300 nM SEA0400 for 5 minutes and untreated. Sham (13/12) and NXT (16/14). Symbols=cells from 6 hearts per group. Data is shown as individual datapoints/box plots; p – values. Analysis using two-way ANOVA.

3.6.2 Effects of SEA0400 on LV cardiomyocyte calcium transients

Similar to the cell shortening measurements in LV cardiomyocytes from Sham and NXT animals after 24 weeks calcium transient baseline in vitro measurements were performed in the presence of the NCX inhibitor SEA0400. After baseline measurements of calcium transients at 1 Hz field stimulation, 300nM SEA0400 was applied. After an incubation time period of 5 minutes in Sham TTP of $[Ca^{2+}]_i$ and Ca^{2+} transients (CaT) were increased by SEA0400 but no effect on diastolic Ca^{2+} decay (CaT RT50, ms) was observed. In LV cardiomyocytes, from 24 weeks NXT rats, after 5 minutes incubation of 300nM SEA0400 TTP of $[Ca^{2+}]_i$ and Ca^{2+} transients (CaT) were unchanged but slowed Ca^{2+} decay in cardiomyocytes (CaT RT50, ms; NXT untreated (n=23) / NXT + SEA0400 (n=17), 39.2 ± 5.4 vs. 29.4 ± 5.8 , $p=0.01$) was significantly enhanced (Figure 3.17 A-C and Table 3.6).

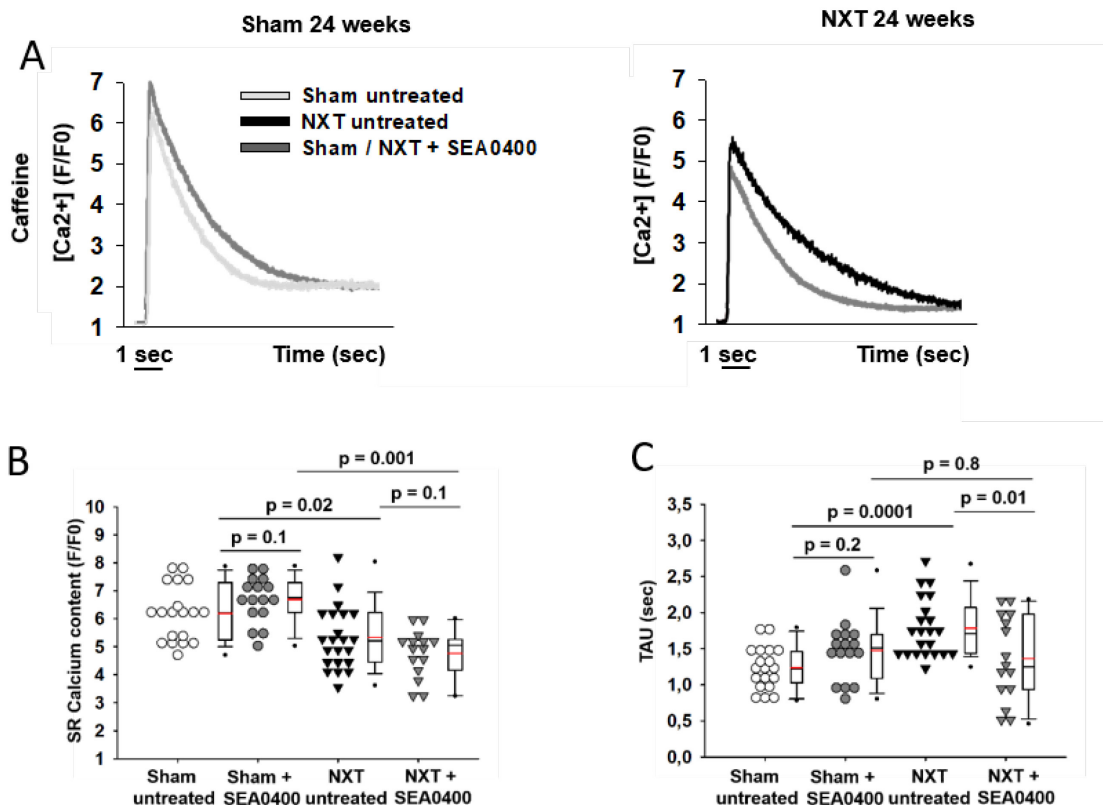


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Figure 3.17 SEA0400 ameliorated Ca^{2+} decay in NXT: A) Representative examples of CaT in Sham (left) and NXT (right) cardiomyocytes at 24 weeks with 1 Hz field stimulation. B) CaT amplitude (CaT, F/F0) in 24 weeks Sham/NXT cardiomyocytes incubated with 300 nM SEA0400 for 5 minutes and untreated. C) Cardiomyocyte calcium decay (CaT RT50, ms) in 24 weeks Sham/NXT cardiomyocytes incubated with 300 nM SEA0400 for 5 minutes and untreated. Sham (20/18) and NXT (23/17). Symbols=cells from 6 hearts per group. Data is shown as individual datapoints/box plots; p – values. Analysis using two-way ANOVA.

3.6.3 Effects of SEA0400 on calcium extrusion via NCX in LV cardiomyocytes

Caffeine-induced Ca^{2+} transient measurements were performed in 24 weeks Sham and NXT animals without and after 5 minutes incubation with 300nM SEA0400. In Sham cardiomyocytes decay of the caffeine-induced Ca^{2+} transient (TAU, sec) was not affected by SEA0400. In NXT cardiomyocytes decay of the caffeine-induced Ca^{2+} transient (TAU, sec; NXT untreated (n=22) / NXT + SEA0400 (n=15), 1.79 ± 0.41 vs. 1.38 ± 0.50 , $p=0.01$) was significantly accelerated after 5 minutes incubation of 300nM SEA0400. SR Ca^{2+} content in 24 weeks Sham and NXT cardiomyocytes was not affected after 5 minutes incubation with 300nm SEA0400 (Figure 3.18 A-C and Table 3.6).

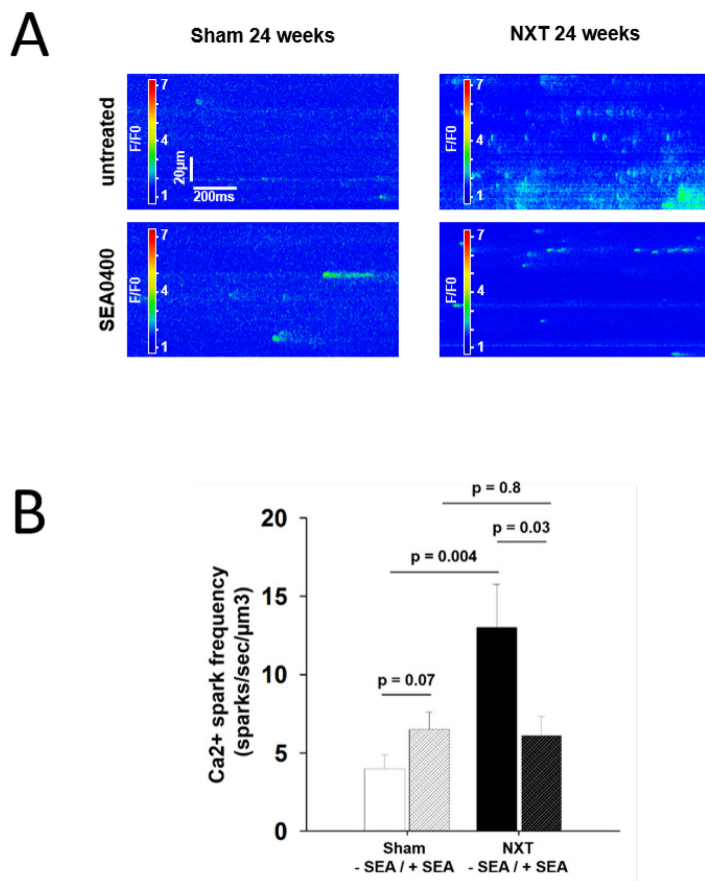


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Figure 3.18 SEA0400 accelerated Ca^{2+} extrusion in NXT: A) Representative examples of caffeine-induced Ca^{2+} transient in Sham treated with 300nM SEA0400 and untreated (left) and NXT treated with 300nM SEA0400 and untreated (right) cardiomyocytes after 24 weeks B) Sarcoplasmatic Ca^{2+} content (SR Ca^{2+} content, F/F0) in 24 weeks Sham and NXT cardiomyocytes incubated with 300 nM SEA0400 for 5 minutes and untreated. C) Cardiomyocyte caffeine-induced Ca^{2+} transient decay (TAU, sec) in 24 weeks Sham and NXT cardiomyocytes incubated with 300 nM SEA0400 for 5 minutes and untreated. Sham (20/16) and NXT (22/15). Symbols=cells from 6 hearts per group. Data is shown as individual datapoints/box plots; p – values. Analysis using two-way ANOVA.

3.6.4 Effect of SEA0400 on sarcoplasmic reticulum (SR) Ca²⁺ leak in cardiomyocytes

SR Ca²⁺ leak was measured in cardiomyocytes from Sham and NXT rats after 24 weeks due to the SR Ca²⁺ spark frequency rate. Therefore, after baselines measurements of calcium transients with 1 Hz field stimulation and normal perfusion solution the stimulation was turned off and cardiomyocytes from Sham and NXT treated or untreated with 300 nM SEA0400 for 5 minutes were perfused with 0 Na⁺/ 0 Ca²⁺ solution. Figure 3.19 showed that incubation with 300 nM SEA0400 had no effect on SR Ca²⁺ spark frequency rate in Sham cardiomyocytes but significantly reduced SR Ca²⁺ leak (Ca²⁺ spark frequency, sparks/sec/ μ m³; NXT untreated (n=25) / NXT + SEA0400 (n=23), 13.0 $\pm$ 3.3 vs. 6.1 $\pm$ 1.9, p=0.03) in 24 weeks NXT cardiomyocytes (Figure 3.19 A-B and Table 3.16).



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Figure 3.19 Effect of SEA0400 on sarcoplasmic reticulum (SR) Ca²⁺ leak in NXT: A) Line scan images from cardiomyocytes at 24 weeks post Sham/NXT showing Ca²⁺ sparks. B) SEA0400 had no significant effect on Ca²⁺ spark frequency in Sham but decreased elevated Ca²⁺ spark frequency in NXT. Sham (23/17) and NXT (25/23). 6 hearts per group. Data are mean $\pm$ S.D., p – values. Analysis using two-way ANOVA.

In table 3.6 acute effects of SEA0400 on LV cardiomyocytes from Sham and NXT rats at 24 weeks are summarized.

Table 3.6 SEA0400 acute in vitro (24 weeks group)

Parameter	Sham untreated	Sham + SEA0400	NXT untreated	NXT + SEA0400	Sham/NXT/untreated/+SEA0400 untreated vs. SEA Sham vs. NXT
Cell shortening (CS)	mean±SD				p-values
CS amplitude (%)	6.4 ± 1.4 (n=13)	7.7 ± 1.4 (n=12)	5.8 ± 0.7 (n=16)	6.2 ± 0.8 (n=14)	0.008/0.09/0.2/0.1
CS TTP (ms)	80.0 ± 14.3 (n=13)	93.6 ± 11.1 (n=12)	73.3 ± 17.0 (n=16)	82.4 ± 12.6 (n=14)	0.08/0.1/0.1/0.2
CS RT50 (ms)	35.8 ± 10.0 (n=13)	42.2 ± 8.8 (n=12)	48.3 ± 9.8 (n=16)	39.1 ± 8.3 (n=14)	0.1/0.02/0.002/0.4
CS RT90 (ms)	56.5 ± 13.7 (n=13)	60.7 ± 16.2 (n=12)	74.4 ± 12.9 (n=16)	69.6 ± 18.9 (n=14)	0.4/0.5/0.01/0.5
Calcium homeostasis					
CaT amplitude (F/F0)	4.0 ± 0.4 (n=20)	4.5 ± 0.7 (n=18)	4.2 ± 1.0 (n=23)	4.3 ± 0.7 (n=17)	0.01/0.7/0.3/0.8
CaT TTP (ms)	25.7 ± 5.6 (n=20)	36.2 ± 7.1 (n=18)	29.0 ± 5.8 (n=23)	36.1 ± 7.4 (n=17)	<0.0001/0.002/0.2/0.9
CaT RT50 (ms)	23.6 ± 5.0 (n=20)	25.2 ± 6.9 (n=18)	39.2 ± 5.4 (n=23)	29.4 ± 5.8 (n=17)	0.4/0.01/<0.0001/0.05
CaT RT90 (ms)	117.5 ± 18.3 (n=20)	130.6 ± 12.2 (n=18)	147.0 ± 27.7 (n=23)	142.9 ± 31.2 (n=17)	0.07/0.4/0.0002/0.3
SR Ca²⁺ content (F/F0)	6.19 ± 0.90 (n=20)	6.68 ± 0.83 (n=16)	5.32 ± 0.93 (n=22)	4.72 ± 0.85 (n=15)	0.1/0.1/0.02/0.001
TAU (sec)	1.22 ± 0.29 (n=20)	1.48 ± 0.43 (n=16)	1.79 ± 0.41 (n=22)	1.38 ± 0.50 (n=15)	0.2/0.01/0.0001/0.8
Ca²⁺ spark frequency (sparks/sec/μm³)	4.0 ± 2.1 (n=23)	6.5 ± 2.5 (n=17)	13.0 ± 3-3 (n=25)	6.1 ± 1.9 (n=23)	0.07/0.03/0.004/0.8

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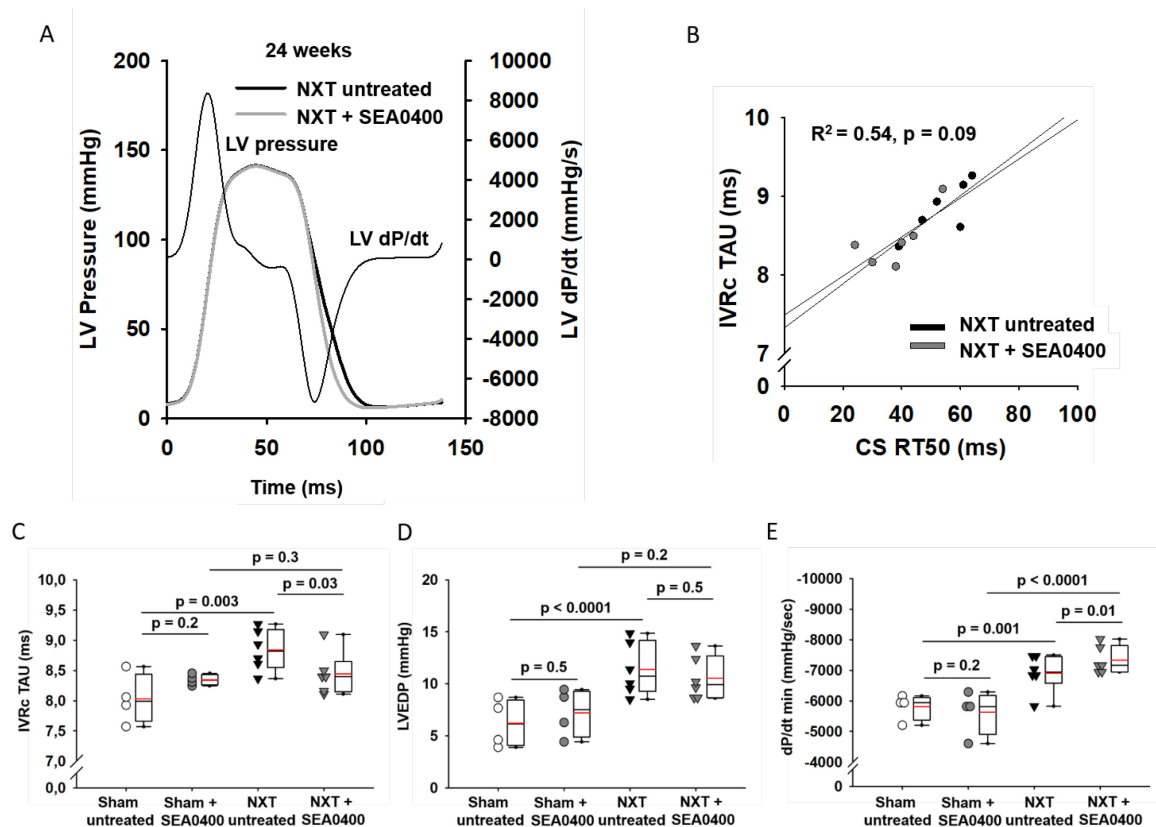
Table 3.6 Acute effects of SEA0400 on LV cardiomyocytes in NXT: CS – Cell shortening; TTP – Time to peak; CS RT50 – 50% of the relaxation time; CS RT90 – 90% of the relaxation time; CaT – Calcium transient; CaT RT50 – 50 % of the calcium transient decay; CaT RT90 – 90% of the calcium transient decay; ; SR – Sarcoplasmic reticulum Ca²⁺ content (F/F0); TAU – Time constant of the caffeine induced CaT (reflected NCX1-forward mode activity; sec); Ca²⁺ spark frequency (sparks/sec/μm³); Data are mean ± S.D., p – values between Sham 8 weeks vs. NXT 8 weeks/ Sham 24 weeks vs. NXT 24 weeks/ Sham 8 weeks vs. Sham 24 weeks/ NXT 8 weeks vs. NXT 24 weeks. 6 hearts per group. N=cells. Analysis using ANOVA.

3.7 Acute hemodynamic effects of NCX inhibition with SEA0400 on in vivo LV function in HFpEF rats

During final invasive hemodynamic pressure volume measurements in Sham and NXT animals after 24 weeks acute application of SEA0400 (1 mg/kg b.w. i.v.) in vivo over 30 min was performed after baseline protocols for determination of systolic and diastolic parameters of the LV. Acute application of the NCX inhibitor SEA0400 had no significant effect on hemodynamic measured systolic or diastolic parameters in Sham rats after 24 weeks. In NXT rats after 24 weeks acute application of 1 mg/kg b.w. SEA0400 intravenously over 30 minutes had no effect on LV peak systolic pressure (LVP_{max}, mmHg) and LV end-diastolic pressure (LVEDP, mmHg). SEA0400 significantly accelerated the LV isovolumetric relaxation constant TAU (IVRc TAU, ms; NXT untreated (n=6) / NXT + SEA0400 (n=6), 8.84 ± 0.34 vs. 8.43 ± 0.31 , $p=0.03$) in NXT after acute application over 30 minutes indicating improved diastolic active relaxation (Figure 3.20 A, C, D and Table 3.7). In 24-week NXT rats faster IVRc TAU *in vivo* in the presence of SEA0400 correlated with faster relaxation of the cardiomyocytes with SEA0400 from the same hearts in vitro compared to untreated NXT hearts and cardiomyocytes (Figure 3.20 B). Maximal rate of pressure decline (dP/dt_{min}) was significantly higher in NXT vs. Sham (Figure 3.20 E and Table 3.7).

In the context of the other hemodynamic and echocardiographic parameters indicating diastolic dysfunction, we interpret this as a restrictive pattern reflecting chronic structural maladaptive remodeling as previously described (149). The modest further increase in dP/dt_{min} with acute SEA0400 may reflect accelerated active relaxation.

So, while acute application of SEA0400 had no effect on LVEDP in NXT rats after 24 weeks active relaxation (IVRc TAU) was significantly ameliorated meaning LV relaxation was improved after NCX inhibition acutely.



Modified from Primessnig et al, *Eur J Heart Fail.* 2016 (194)

Figure 3.20 SEA0400 improves LV relaxation acutely in vivo in NXT: A) Example of in vivo measured LV pressure and LV dP/ dt in NXT and NXT treated acutely with SEA0400 (1mg/kg/BW for 30 minutes); B) Correlation between cardiomyocyte relengthening in vitro (CS RT 50, ms) and isovolumetric relaxation constant (IVRc TAU, ms) in vivo (symbol=cell average/heart); C) Mean values for LV isovolumetric relaxation constant TAU (IVRc TAU, ms); D) LV end-diastolic pressure (LVEDP, mmHg) and E) maximum rate of fall in LV pressure (dP/dt min, mmHg/sec). Sham (4/4) and NXT (6/6). Data is shown as individual datapoints/box plots; p – values. Analysis using two-way ANOVA.

In table 3.7 acute effects of SEA0400 on LV hemodynamics from Sham and NXT rats at 24 weeks are summarized.

Table 3.7 SEA0400 acute in vivo (24 weeks group)

Parameter	Sham untreated	Sham + SEA0400	NXT untreated	NXT + SEA0400	Sham/NXT/untreated/+SEA0400 untreated vs. SEA Sham vs. NXT
Pressure volume measurements	mean±SD				p-values
HR (BPM)	395 ± 18 (N=4)	391 ± 23 (N=4)	420 ± 33 (N=6)	429 ± 33 (N=6)	0.3/0.3/0.3/0.1
LV Pmax (mmHg)	114 ± 3 (N=4)	113 ± 6 (N=4)	138 ± 5 (N=6)	140 ± 8 (N=6)	0.8/0.2/0.003/0.006
EDP (mmHg)	6 ± 2 (N=4)	7 ± 2 (N=4)	11 ± 2 (N=6)	11 ± 1 (N=6)	0.5/0.5/<0.0001/0.2
IVRc Tau (ms)	7.88 ± 0.28 (N=4)	8.12 ± 0.31 (N=4)	8.84 ± 0.34 (N=6)	8.43 ± 0.31 (N=6)	0.2/0.03/0.003/0.3
dP/dt max (mmHg/s)	6331 ± 418 (N=4)	6553 ± 375 (N=4)	8035 ± 399 (N=6)	8272 ± 390 (N=6)	0.03/0.02/<0.0001/<0.0001
dP/dt min (mmHg/s)	-5810 ± 385 (N=4)	-5631 ± 460 (N=4)	-6911 ± 410 (N=6)	-7446 ± 340 (N=6)	0.2/0.01/0.001/<0.0001

Modified from *Primessnig et al, Eur J Heart Fail. 2016 (194)*

Table 3.7 Acute effects of SEA0400 on LV hemodynamics in NXT: HR – Heart rate; LV Pmax – Left ventricular peak pressure; EDP – End-diastolic pressure; IVRc TAU – LV isovolumetric relaxation constant (relaxation time constant calculated by the Weiss method); dP/dt max – maximum rate of increase in LV pressure; dP/dt min – maximum rate of decrease in LV pressure; Data are mean ± S.D., p – values between Sham 24 weeks untreated vs. Sham 24 weeks + SEA0400/ NXT 24 weeks untreated vs. NXT 24 weeks + SEA0400/ Sham 24 weeks untreated vs. NXT 24 weeks untreated/ Sham 24 weeks + SEA0400 vs. NXT 24 weeks + SEA0400. Sham (4/4) and NXT (6/6). Analysis using two-way ANOVA.

3.8 Chronic treatment with the NCX inhibitor SEA0400 reverses HFpEF phenotype

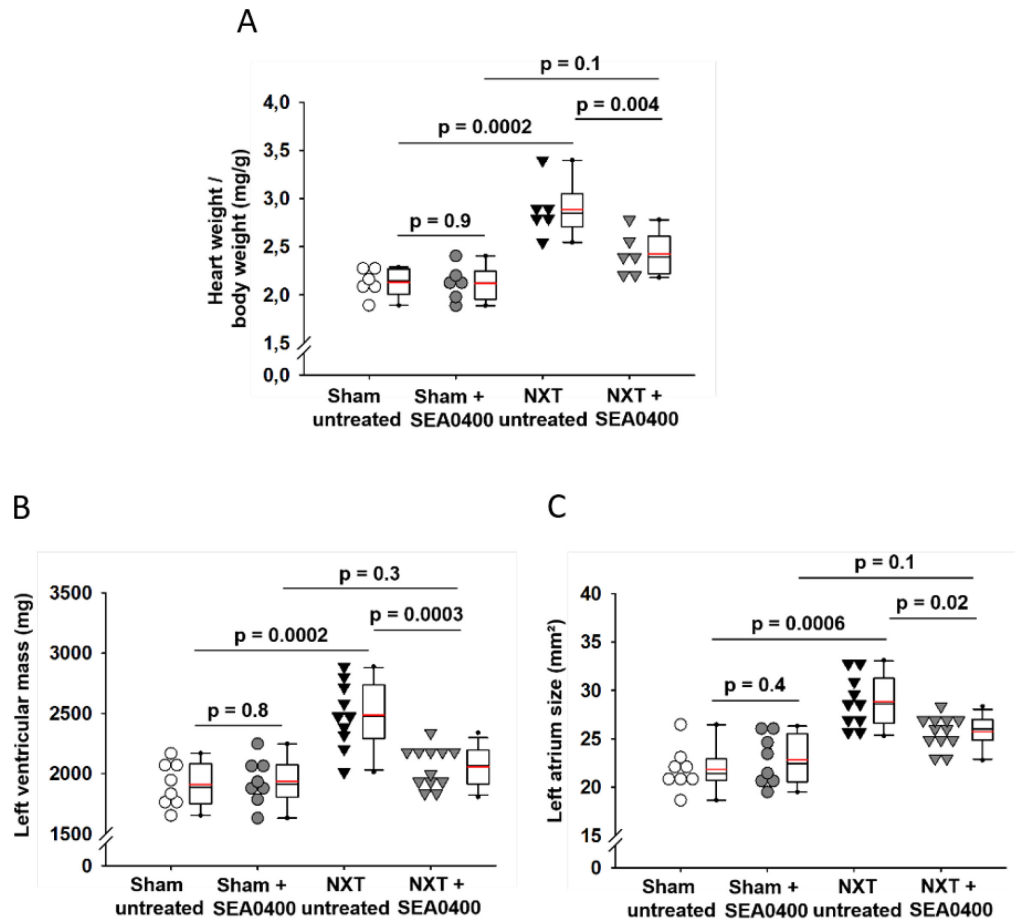
After the evaluation of the NCX inhibitor SEA0400 acutely in vitro on LV cardiomyocyte function and in vivo on LV hemodynamics in Sham and NXT rats after 24 weeks, the effect of chronical application of the new therapeutic compound SEA0400 on reversing remodeling processes, improving diastolic function, and preventing arrhythmias, was investigated in HFpEF rats.

Therefore, we determined the long-term effect of SEA0400 application for 16 weeks in Sham and NXT rats on LV hypertrophy, cardiac remodeling, diastolic dysfunction, LV systolic function, congestion, biomarkers and blood pressure changes.

Sham and NXT animals were treated with SEA0400 1 mg/ kg b.w. per os twice daily for 16 weeks started 8 weeks after initial subtotal nephrectomy or sham operation. In total 38 rats were subjected into 4 different groups: Sham untreated and treated with SEA0400 and NXT untreated and treated with SEA0400.

3.8.1 Effect of chronical treatment with SEA0400 on LV hypertrophy and cardiac remodeling in HFpEF rats

Chronical treatment with the NCX inhibitor SEA0400 1 mg/ kg b.w per os twice daily for 16 weeks in NXT rats with an established HFpEF phenotype (8 weeks after initial subtotal nephrectomy) significantly ameliorated LV hypertrophy and cardiac remodeling. In Sham rats, treated chronically with SEA0400 for 16 weeks, heart weight/body weight ratio, LV mass and LA size were unchanged compared to untreated Sham rats. NXT rats had a significantly reduced HW/BW ratio (NXT(N=6)/ NXT+SEA0400(N=6); 2.94 ± 0.15 vs. 2.41 ± 0.12 , $p=0.004$), LV mass and LA size after 16 weeks chronical treatment with the NCX inhibitor SEA0400 1 mg / kg b.w per os twice daily (Figure 3.21 and Table 3.8).



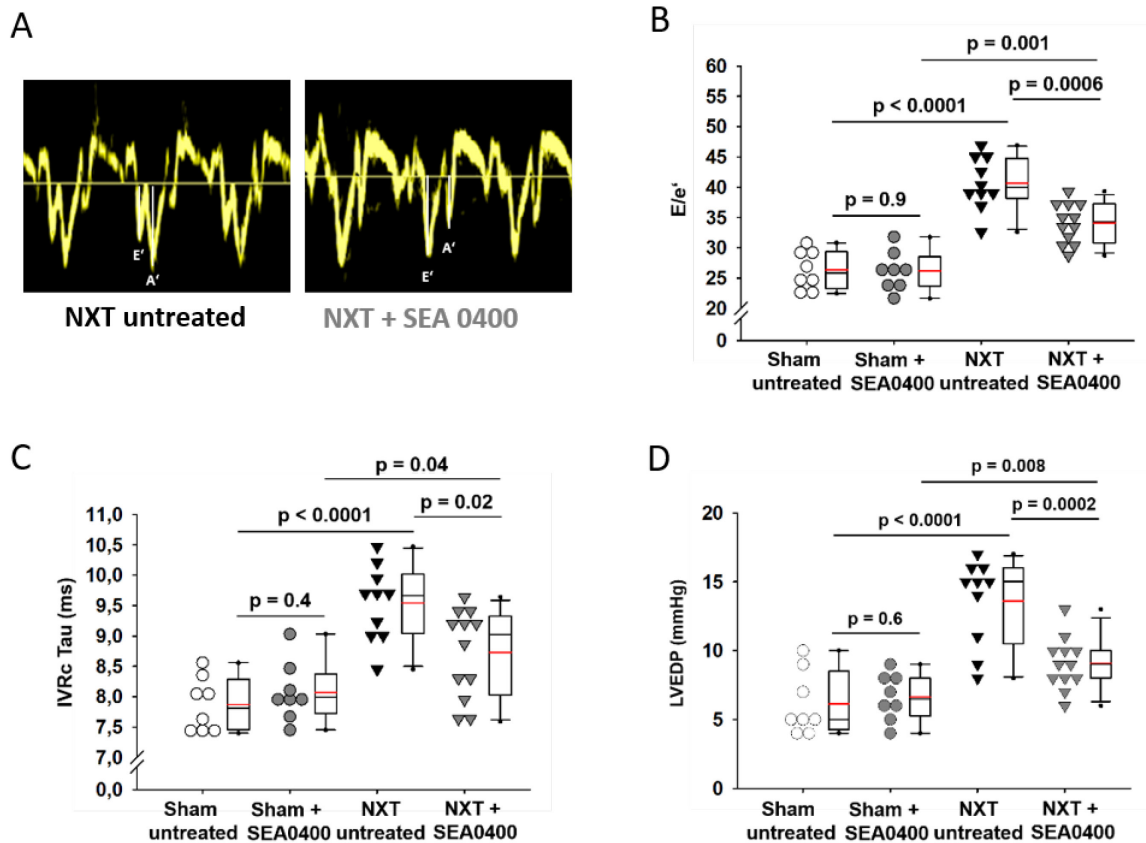
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Figure 3.21 Chronical SEA0400 attenuated LV hypertrophy and cardiac remodeling: A) Heart weight / body weight ratio in Sham/NXT treated with SEA0400 and untreated – significant reduced in NXT after SEA0400 treatment compared to NXT untreated; B) Left ventricular mass in Sham/NXT treated with SEA0400 and untreated - significant reduced in NXT after SEA0400 treatment compared to NXT untreated; C) Left atrium size in Sham/NXT treated with SEA0400 and untreated - significant reduced in NXT after SEA0400 treatment compared to NXT untreated; Sham (8/8) and NXT (10/12). Data is shown as individual datapoints/box plots; p – values. Analysis using two-way ANOVA.

3.8.2 Effect of chronical NCX inhibition with SEA0400 on diastolic dysfunction in HFpEF rats

Chronical treatment with SEA0400 1 mg/ kg b.w. per os twice daily for 16 weeks started after 8 weeks (established HFpEF phenotype in NXT) significantly improved diastolic dysfunction in NXT rats. While in Sham animals E/e' , isovolumetric LV relaxation constant TAU (IVRc TAU, ms) and LV end-diastolic pressure (LVEDP, mmHg) were unchanged after chronic SEA0400 application, in NXT rats a significant enhancement in E/e' (NXT(N=10)/ NXT+SEA0400(N=12);

40 ± 5 vs. 34 ± 3, p=0.006), isovolumetric LV relaxation constant TAU (IVRc TAU, ms) (NXT(N=10)/ NXT+SEA0400(N=12); 9.52 ± 0.29 vs. 8.73 ± 0.31, p=0.02) and LV end-diastolic pressure (LVEDP, mmHg) (NXT(N=10)/ NXT+SEA0400(N=12); 13 ± 3 vs. 9 ± 3, p=0.0002) was observed (Figure 3.22 and Table 3.8).

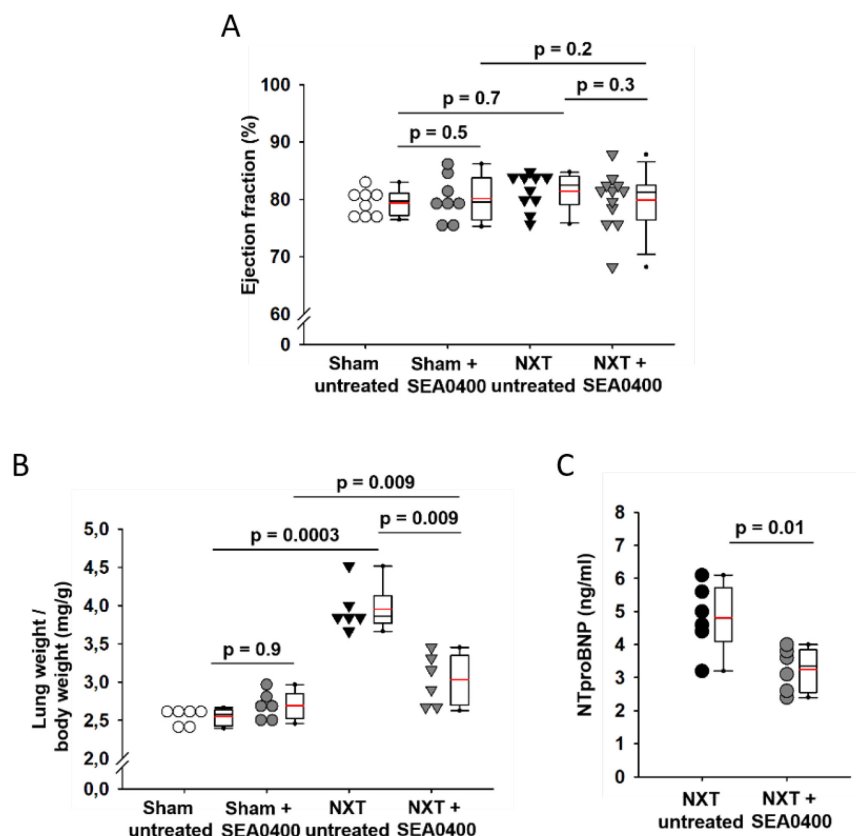


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Figure 3.22 Chronic NCX inhibition with SEA0400 ameliorated diastolic dysfunction: A) Examples of tissue doppler measured e' and a' in NXT treated with SEA0400 und untreated; B) E/e' ratio in Sham/NXT treated with SEA0400 and untreated; C) Isovolumetric LV relaxation constant TAU (IVRc TAU, ms) in Sham/NXT treated with SEA0400 and untreated; D) LV end-diastolic pressure (LVEDP, mmHg) in Sham/NXT treated with SEA0400 and untreated - significant reduced in NXT after SEA0400 treatment compared to NXT untreated; Sham (8/8) and NXT (10/12). Data is shown as individual datapoints/box plots; p – values. Analysis using two-way ANOVA.

3.8.3 Effect of chronic treatment with SEA0400 on LV function, congestion and NTproBNP in HFpEF rats

Chronical NCX inhibition with SEA0400 1 mg/ kg b.w. per os twice daily started after 8 weeks (established HFpEF in NXT) for the duration of 16 weeks significantly improved congestion and NTproBNP levels in NXT while LV ejection fraction was preserved. In Sham animals chronic SEA0400 application had no effect on LV ejection fraction (LVEF, %), lung weight / body weight as marker for congestion and NTproBNP. As mentioned, chronic SEA0400 treatment significantly enhanced congestion with a reduction in lung weight / body weight ratio. Moreover, NTproBNP levels were significantly lower in SEA0400 treated NXT rats compared to untreated NXT rats. LV ejection fraction (LVEF, %) was preserved after SEA0400 in NXT rats (Figure 3.23 and Table 3.8).



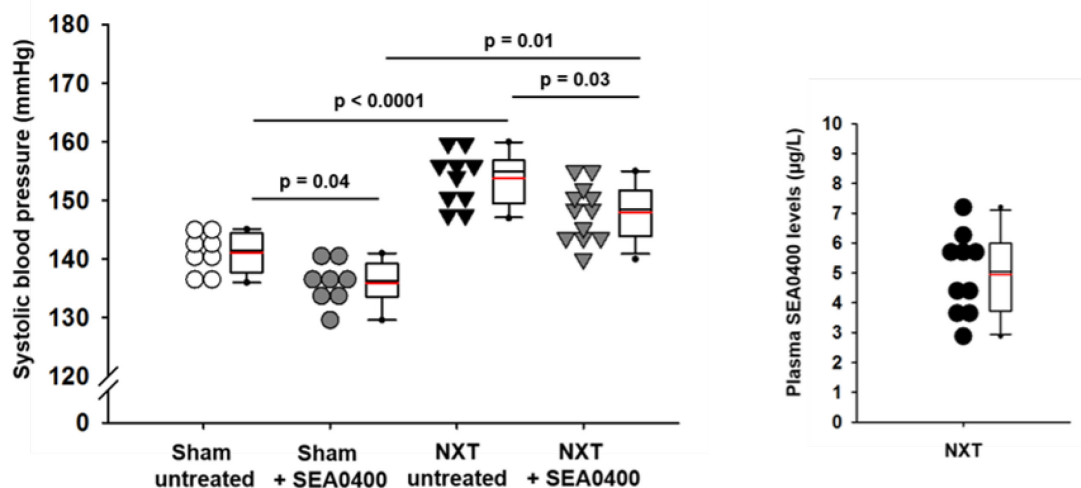
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Figure 3.23 Chronical NCX inhibition with SEA0400 improved congestion, NTproBNP and had no effect on LVEF: A) LV ejection fraction (LVEF, %) in Sham/NXT treated with SEA0400 and untreated; B) Lung weight / body weight ratio as manifestation for congestion in Sham/NXT treated with SEA0400 and untreated; C) NTproBNP in NXT treated with SEA0400 compared to untreated NXT rats - significant reduced in NXT after SEA0400 treatment compared to NXT untreated; Sham (8/8) and NXT (10/12). Data is shown as individual datapoints/box plots; p – values. Analysis using two-way ANOVA.

Summarized, chronic treatment with the NCX inhibitor SEA0400 applied following the manifestation of HFpEF (at 8 weeks) for 16 weeks partially reversed myocardial remodeling (Figure 3.21), improved diastolic function by echocardiography and invasive hemodynamics (Figure 3.22) and reduced congestion and NTproBNP levels while LV ejection fraction was preserved (Figure 3.23). Furthermore, chronic treatment with SEA0400 had a moderate but significant influence on systolic and diastolic blood pressure in NXT and Sham animals.

3.8.4 SEA0400 plasma levels and the effect of chronic NCX inhibition on blood pressure in HFpEF rats

Plasma levels of SEA0400, determined by high-performance liquid chromatography and mass spectrometry following one week of therapy, were 4.7 ± 1.7 $\mu\text{g/ml}$ (12.6 $\mu\text{mol/L}$, $N = 10$) (Figure 3.24). Chronic treatment with SEA0400 was associated with moderately decreased systolic blood pressure (SBP) in Sham and NXT rats after 24 weeks (Figure 3.24 and Table 3.8).



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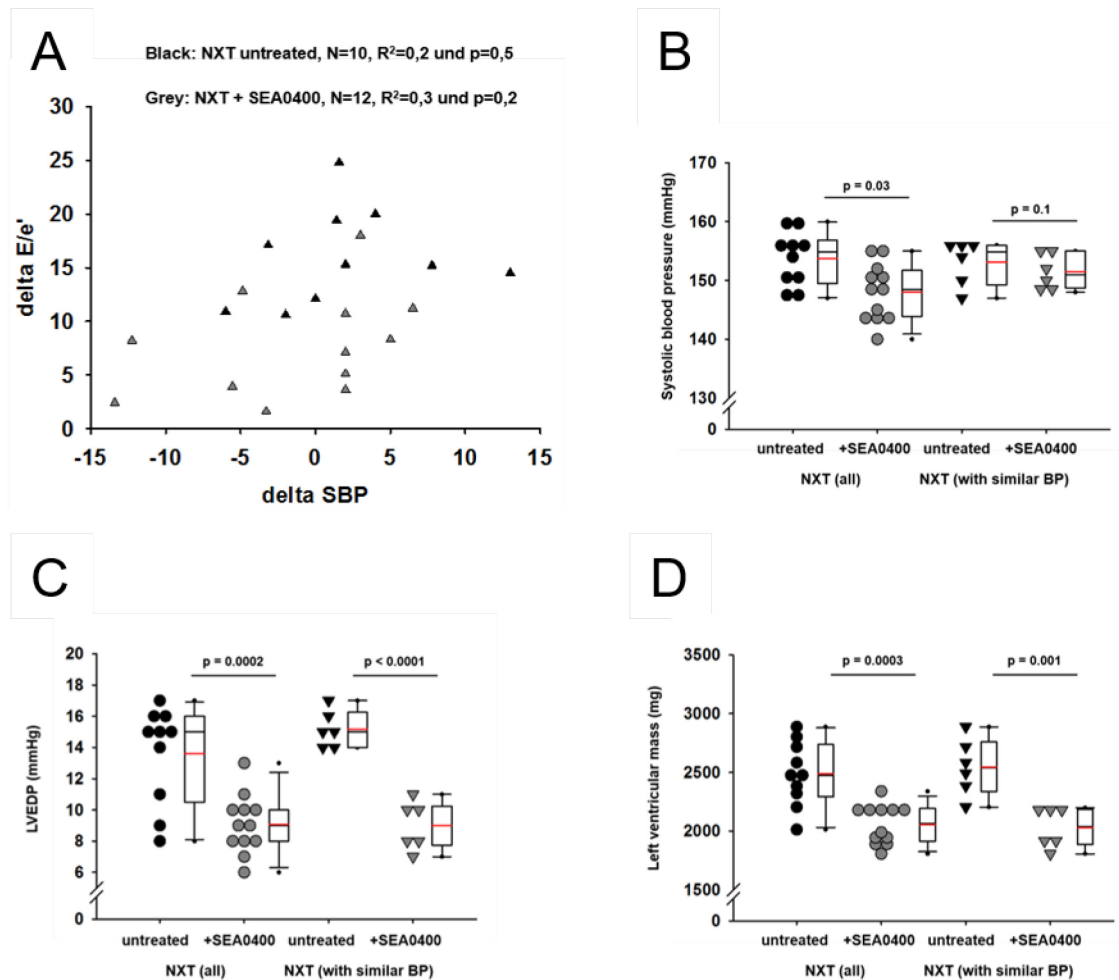
Figure 3.24 Effect of NCX inhibition on blood pressure and SEA0400 plasma levels: A) Chronic treatment with SEA0400 moderately decreased systolic blood pressure (SBP) in Sham and NXT after 24 weeks (Sham untreated/ Sham + SEA0400 $N=8/8$ and NXT untreated/ NXT + SEA0400 $N=10/12$). B) Plasma concentration of SEA0400 in NXT ($N=10$; 4.7 ± 1.7). Data is shown as individual datapoints/box plots; p – values. Analysis using two-way ANOVA.

The role of elevated blood pressure in progression of cardiac involvement in cardiorenal HFpEF plays an important role. Experimental (150) as well as clinical (151) studies suggest that blood pressure lowering per se may improve some but not all aspects of cardiac remodeling in chronic kidney disease. It has to be kept in mind that the blood pressure lowering drug classes, angiotensin-converting enzymes and angiotensin II receptor blockers, have failed to improve diastolic function and prognosis in clinical HFpEF trials.

However, to address the role of blood pressure-lowering in more detail, the change in systolic blood pressure (SBP) between 8 and 24 weeks to the change in E/e' as a measure of LV diastolic function in the same animal was compared.

As shown in figure 3.25 – while the increase in E/e' was attenuated in the NXT+SEA0400 group, there was no correlation between the change in SBP and diastolic function as measured by E/e' (Figure 3.25 A). In addition, we selected subgroups of NXT animals $\pm$ SEA0400 with matching mean systolic blood pressure (Figure 3.25 B) and compared LVEDP (Figure 3.25 C) and LV mass (Figure 3.25 D). In these subgroups, despite similar SBP at 24 weeks, LVEDP and LV mass were significantly lower with SEA0400. This suggests effects of SEA0400 on diastolic function and hypertrophic remodeling that are independent of its effects on blood pressure.

To conclude these findings - there was no correlation between lowering of SBP and change in diastolic function. Furthermore, SEA0400 significantly improved LVEDP and LV mass also in subgroups matched for similar SBP, indicating blood pressure-independent effects of SEA0400 (194).



Modified from Primessnig et al, *Eur J Heart Fail.* 2016 (194)

Figure 3.25 Effect of blood pressure on diastolic function: A) No correlation between change in blood pressure and change in diastolic function (E/e') in NXT between 8 and 24 weeks (NXT untreated/ NXT + SEA0400 N=10/12). B) Identification of subgroups of NXT and NXT+SEA0400 with comparable systolic blood pressure (BP). Despite similar systolic blood pressure, NXT animal following chronic SEA0400 treatment had significantly reduced LVEDP (C) and LV mass (D), indicating a blood-pressure independent improvement in diastolic function and remodeling. Data is shown as individual datapoints/box plots; p – values. Analysis using two-way ANOVA.

In table 3.8 data from the chronic treatment group with SEA0400 are summarized.

Table 3.8 Chronic treatment with SEA0400 (24 weeks group)

Parameter	Sham untreated	Sham + SEA0400	NXT untreated	NXT + SEA0400	Sham/NXT/untreated/+SEA0400 untreated vs. SEA Sham vs. NXT
Data	mean±SD				p-values
HW/BW	2.13 ± 0.08 (N=6)	2.21 ± 0.12 (N=6)	2.94 ± 0.15 (N=6)	2.41 ± 0.12 (N=6)	0.9/0.004/0.0002/0.1
LW/BW	2.53 ± 0.05 (N=6)	2.61 ± 0.11 (N=6)	3.95 ± 0.21 (N=6)	3.07 ± 0.13 (N=6)	0.9/0.009/0.0003/0.009
LV mass (mg)	1895 ± 105 (N=8)	1915 ± 135 (N=8)	2515 ± 180 (N=10)	2060 ± 110 (N=12)	0.8/0.0003/0.0002/0.3
LA size (mm²)	22 ± 4.0 (N=8)	23 ± 2.0 (N=8)	29 ± 3.0 (N=10)	25 ± 3 (N=12)	0.4/0.02/0.0006/0.1
E/e'	25 ± 2 (N=8)	25 ± 4 (N=8)	40 ± 5 (N=10)	34 ± 3 (N=12)	0.9/0.006/<0.0001/0.001
LVEDP (mmHg)	6 ± 2 (N=8)	7 ± 2 (N=8)	13 ± 3 (N=10)	9 ± 3 (N=12)	0.6/0.0002/<0.0001/0.008
IVRc Tau (ms)	7.90 ± 0.18 (N=8)	8.07 ± 0.26 (N=8)	9.52 ± 0.29 (N=10)	8.73 ± 0.31 (N=12)	0.4/0.02/<0.0001/0.04
EF (%)	79 ± 3 (N=8)	80 ± 4 (N=8)	82 ± 4 (N=10)	80 ± 6 (N=12)	0.5/0.3/0.7/0.2
SBP (mmHg)	141 ± 3 (N=8)	137 ± 5 (N=8)	154 ± 4 (N=10)	148 ± 4 (N=12)	0.04/0.03/<0.0001/0.01
DBP (mmHg)	89 ± 3 (N=8)	86 ± 4 (N=8)	110 ± 5 (N=10)	106 ± 3 (N=12)	0.05/0.01/<0.0001/<0.0001
HR (BPM)	399 ± 15 (N=8)	397 ± 20 (N=8)	405 ± 25 (N=10)	400 ± 20 (N=12)	0.8/0.5/0.3/0.5

Table 3.8 Chronic treatment with SEA0400 in HFpEF rats: HW – Heart weight; LW – Lung weight; LV mass – Left ventricular mass; LA size – Left atrial size; E/e'; LVEDP – Left ventricular End-diastolic pressure; IVRc TAU – LV isovolumetric relaxation constant (relaxation time constant calculated by the Weiss method); EF – Ejection fraction; SBP – Systolic blood pressure; DBP – Diastolic blood pressure; HR – Heart rate; Data are mean ± S.D., p – values between Sham 24 weeks untreated vs. Sham 24 weeks + SEA0400/ NXT 24 weeks untreated vs. NXT 24 weeks + SEA0400/ Sham 24 weeks untreated vs. NXT 24 weeks untreated/ Sham 24 weeks + SEA0400 vs. NXT 24 weeks + SEA0400. Sham (8/8) and NXT (10/12). Analysis using two-way ANOVA.

4 Discussion

The prevalence of heart failure is increasing in the last decades and almost half of patients with clinical symptoms (dyspnoea) and signs (congestion) of heart failure have a preserved left ventricular ejection fraction (LVEF $\geq$ 50%). Furthermore, structural heart disease such as left ventricular hypertrophy, diastolic dysfunction and left atrium enlargement without LV dilatation confirm the diagnosis of HFpEF. Patients with heart failure with preserved ejection fraction often have different comorbidities, such as diabetes, atrial fibrillation or chronic kidney disease. Particularly, in the presence of CKD, which is present in up to two thirds of HFpEF patients, mortality may be even higher than in HFrEF (123). While therapeutic strategies significantly improve mortality and morbidity in HFrEF patients, there is no specific therapy for HFpEF patients available yet, indicating distinctively different underlying pathomechanisms and myocardial structural changes (51). Cellular and molecular mechanisms of HFpEF, especially, the function of beating cardiomyocytes has not been studied yet, mainly due to the lack of animal models which represents the clinical HFpEF phenotype (126).

In a rat model of CKD that developed HFpEF for up to 52 weeks it was demonstrated for the first time that in vivo LV myocardial dysfunction is related to impaired active relaxation and slowed Ca^{2+} removal in isolated cardiomyocytes. Prolonged cellular Ca^{2+} transients in this renal HFpEF model were associated with altered function of the cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). SEA0400, an NCX inhibitor, improved diastolic dysfunction in vitro and in vivo (194).

4.1 Heart failure with preserved ejection in a rat model with chronic stable kidney disease based on subtotal nephrectomy

Chronic kidney disease is a well-established risk factor for cardiac dysfunction and heart failure (152). Approximately, 50% of HFpEF patients have moderately severe (stage 3) CKD (153) and in almost all of them, arterial hypertension is a common finding. In the present model of subtotal nephrectomy in rats, arterial hypertension and myocardial structural changes were determined. However, previous studies in similar animal models have observed structural changes of the myocardium, including increased interstitial fibrosis (135,154), cardiomyocyte hypertrophy (155) and also alterations in cardiomyocyte function (156,157) (194).

Following subtotal nephrectomy, rats developed chronic kidney disease and the clinical phenotype of heart failure with preserved ejection fraction - including LV hypertrophy, cardiac remodeling, LV diastolic dysfunction, preserved LVEF, lung congestion and increased biomarkers' like NTproBNP (194). In this rat model of subtotal nephrectomy, the activation of the neurohumoral axis and the consecutive volume overload after renal damage is associated with arterial hypertension as a potential trigger for the development of the HFpEF phenotype.

Systemic systolic and diastolic blood pressure were mildly increased after subtotal nephrectomy with a constant elevation compared to sham operated animals during the observation period (Figure 3.5). Compared to many other HFpEF animal models (158,159,160) there was just a slightly elevation of systemic blood pressure observed in NXT rats (194). However, in a mouse model, chronic infusion of low-dose angiotensin recapitulated the HFpEF phenotype (LV diastolic dysfunction and preserved LVEF), without increasing systemic arterial blood pressure and significant LV hypertrophy (161).

Renal impairment in NXT rats was observed early after subtotal nephrectomy but stayed stable for the rest of the observation period compared to sham animals (Table 3.3) (194). In metabolic syndrome rats with diabetes, hypertension and

obesity, renal function and structure were severely changed compared to subtotal nephrectomy rats (162).

LV hypertrophy – including HW/BW, LV mass and LV relative wall thickness as well as cardiac remodeling – including LV fibrosis showed a continuous progression over time up to 24 weeks in NXT rats (Figure 3.2 and Table 3.1) (194). Concentric LV hypertrophy was a common finding in different HFpEF-like models, especially in Dahl Salt-Sensitive rats (158), DOCA-Salt rats (159), aortic constriction (160) and ZSF1-obese rats (163). Furthermore, LV fibrosis was observed in some of the rodent models of HFpEF (164).

Diastolic function was impaired 8 weeks after subtotal nephrectomy with a further progression after 24 weeks in terms of elevated LVEDP, prolongation of the LV isovolumetric relaxation constant (IVRc TAU), increased E/e' and enlargement of the LA size in NXT compared to Sham (Figure 3.3 and Table 3.2). Moreover, LVEF was preserved up to 52 weeks in NXT and Sham animals (194). Active relaxation (IVRC TAU) and passive stiffness (LVEDP) as parameters for diastolic function were significantly altered in many animal models of a HFpEF-like phenotype, e.g. Dahl Salt-Sensitive rats (158), DOCA-Salt rats (159), aortic constriction (160) and ZSF1-obese rats (163). It is important to mention, that, LVEF decreased in models of aortic constriction (160) after a certain time period – therefore all animal models with aortic constriction were no “true” HFpEF models.

Lung congestion was observed in NXT animals after 8 weeks with a further increase after 24 weeks. NTproBNP, a clinically relevant biomarker for heart failure was significantly increased after 24 weeks in NXT rats compared to Sham (Figure 3.4). In NXT rats, LV fibrosis was increased after 8 and 24 weeks compared to sham animals (Figure 3.2) (194). Hamdani et al showed in a metabolic syndrome based HFpEF rat model similar changes regarding cardiac remodeling, diastolic dysfunction and lung congestion (163). In another rat model, Dahl Salt-Sensitive (DS)/obese rats developed severe salt-sensitive hypertension and LV diastolic dysfunction as well as LV hypertrophy and fibrosis at 12 weeks of age. Additionally, the survival rate of DS/obese rats was significantly lower than that of the control DS/lean rats at 15 weeks. These changes were associated with

increased cardiac oxidative stress and inflammation (165). Van Dijk et al demonstrated in the ZSF1 rats significant microvascular fibrotic responses in heart and kidneys, associated with functional impairment of both organs (166).

In the animal model of renal induced HFpEF, NXT rats showed a significantly higher mortality rate compared to sham animals. Interestingly, mortality in NXT increased in later stages of the disease, starting at week 20 post nephrectomy (Figure 3.6). Kaplan Meier statistical analysis indicated an overall 40% mortality in NXT rats during one-year observation period while no animals died in Sham group. It is interesting, that all NXT rats died immediately over night without a decrease in body weight as an indicator for chronic cachexia. Consequently, sudden cardiac death with life-threatening ventricular arrhythmias could be a potential cause of death in HFpEF rats. This theory was supported by the fact that in NXT rats, ventricular arrhythmias were observed during final invasive hemodynamic measurements after 52 weeks while in Sham rats no arrhythmias were observed (Figure 3.6) (194). In another animal model of HFpEF published by Cho et al, Dahl salt-sensitive rats were fed a high-salt diet (8% NaCl) for 7 weeks. Susceptibility to ventricular arrhythmias (VA) was markedly increased in rats with HFpEF (167).

In the 52-observation group from the subtotal nephrectomised rats, there was a further increase in LV hypertrophy and cardiac remodeling in NXT compared to Sham animals while LV ejection fraction was preserved. In terms of renal function, urine volume was not altered between Sham and NXT rats after 52 weeks indicating no further decrease of initial renal impairment over time (Table 3.4) (194).

Summarizing, the present model of subtotal nephrectomy in rats showed clinical criteria of structural myocardial remodeling with LV hypertrophy and diastolic dysfunction. Moreover, it bears other similarities to the clinical HFpEF-syndrome such as an increased left atrial size, elevated NTproBNP and increased mortality (194).

4.2 Cardiomyocyte remodelling, dysfunction and impaired calcium handling in HFpEF rats

NXT cardiomyocytes were hypertrophied at 8 weeks, with a further increase at 24 weeks compared to sham rats (Figure 3.7). Cell shortening amplitude from enzymatically isolated cardiomyocytes and amplitudes of intracellular Ca^{2+} transients were unchanged in NXT vs. Sham at 8 and 24 weeks (Figure 3.8 A, C). Cell shortening active relaxation and $[\text{Ca}^{2+}]_i$ transient decay was markedly slower in NXT vs. Sham (by 42% at 8 weeks and 77% at 24 weeks) (Figure 3.8 C, D). In addition, $[\text{Ca}^{2+}]_i$ decay slowed with age in both groups. Absolute systolic and diastolic $[\text{Ca}^{2+}]$ concentrations in Sham and NXT rats at 24 weeks were not changed. Slowed relaxation with a prolonged decay of the Ca^{2+} transient in NXT (24 weeks) was confirmed using Fura-2 as Ca^{2+} indicator (Figure 3.9) (194).

In the model of inbred hypertrophic heart rats (HHR), cardiomyocytes were markedly hypertrophied and remodeled with an increase in cardiomyocyte cell volume (cell enlargement >20% compared to control). Furthermore, in the HHR, cardiomyocytes were hypercontractile with high Ca^{2+} operational levels and markedly increased L-type Ca^{2+} channel current. Cell shortening amplitude and calcium amplitude were significantly increased (168). In contrast to the NXT rats, the Ca^{2+} transient decay time constant – TAU, was not prolonged (168). In the HHR model, cardiac remodeling and diastolic dysfunction was developed without arterial hypertension as a trigger, in contrast to many other HFpEF models like the Dahl Salt Sensitive rats (158), the ZSF1 rats (163) or even the NXT rats (194).

Røe et al subjected rats to aortic banding (AB) and experiments were performed 6 weeks after surgery using sham operated rats as controls. Left ventricular concentric hypertrophy and diastolic dysfunction following AB were indicated by thickening of the ventricular wall and higher E/e' values. Additional LVEF was preserved in AB rats at this timepoint (169). Slowed relaxation was observed in left ventricular muscle strips isolated from AB hearts, during both isometric and isotonic stimulation and accompanied by increases in passive tension, viscosity, and extracellular collagen. In contrast, isolated, unloaded cardiomyocytes

exhibited accelerated relaxation in AB compared to sham and less contracture at high pacing frequencies. Parallel enhancement of diastolic Ca^{2+} handling was observed, with augmented NCX and SERCA2 activity and lowered resting cytosolic $[\text{Ca}^{2+}]$ (169). Røe et al concluded that in the hypertrophied heart with preserved systolic function, in vivo diastolic dysfunction develops as cardiac fibrosis and alterations in titin phosphorylation compromise left ventricular compliance and despite compensatory changes in cardiomyocyte Ca^{2+} homeostasis (169).

In a translational large animal model of chronic pressure overload-induced HFpEF (aortic-banded (AB) in miniature swine) with relevance to human HFpEF, depressed Ca^{2+} transient amplitude and impaired cardiomyocyte cell shortening were observed (170). Interestingly, exercise training attenuated AB-induced decreases in cardiomyocyte Ca^{2+} transient amplitude but did not prevent impaired cell shortening in AB-induced HF compared to control (170).

In our subtotal nephrectomy induced HFpEF model, frequency dependence of cardiomyocyte contractility and relaxation was observed after 24 weeks in NXT rats. CS amplitude in Sham rats was significantly increased compared to NXT at higher frequencies while cardiomyocytes active relaxation remained significantly prolonged in NXT at all stimulation frequencies (Figure 3.10). In NXT, cell shortening amplitude decreases at higher frequencies and is significantly lower than in Sham, likely related to impaired relaxation. This indicated that impaired cardiomyocyte relaxation persists at higher frequencies (194). While in AB-induced HFpEF rats, cardiomyocytes also showed a lower Ca^{2+} transient amplitude at higher stimulation frequencies compared to control, time to peak of the transient was unchanged and time to 50% decline was reduced. Furthermore, diastolic Ca^{2+} levels were lower in AB, with less accumulation of resting $[\text{Ca}^{2+}]$ at high pacing frequencies (169).

SR Ca^{2+} content and SR Ca^{2+} leak (Ca^{2+} sparks) were unchanged in NXT vs. Sham at 8 weeks, whereas at 24 weeks SR Ca^{2+} content was reduced and SR Ca^{2+} leak was increased in NXT vs. Sham (Figure 3.11 A-C). Moreover, spontaneous induced depolarisation was significantly higher in NXT vs. Sham at

24 weeks (Figure 3.11 D-E) (194). In cardiomyocytes from aortic-banded rats, the magnitude of the caffeine-induced Ca^{2+} release was reduced compared to control rats, suggesting lower SR Ca^{2+} content. SR Ca^{2+} reuptake constant was higher in AB, indicating enhanced SERCA2 function. The sarcolemmal Ca^{2+} extrusion constant was higher in AB, indicating enhanced NCX function (169). Moreover, in HHR cardiomyocytes, spontaneous Ca^{2+} release events in non-paced cardiomyocytes, quantified over 30 seconds were significantly increased and indicated a higher risk for arrhythmias in HFpEF (168).

In subtotal nephrectomised HFpEF rats cardiomyocyte dysfunction was related to a slowed diastolic decay of the $[\text{Ca}^{2+}]$ transient already at an early timepoint. Mechanistically, slowed Ca^{2+} removal was not explained by either a reduced SERCA activity or an increased leak of Ca^{2+} from the SR as reported in HFrEF (144). SR Ca^{2+} leak was considerably increased at a later stage, suggesting that it may be a consequence rather than a cause of diastolic dysfunction, also supported by the close association between LVEDP and SR Ca^{2+} leak in NXT rats (Figure 3.12 A) (194). Moreover, cytosolic Ca^{2+} decay further slowed from 8 to 24 weeks in NXT rats. Sacherer et al reported that increased SR Ca^{2+} leak in human cardiomyocytes can contribute to diastolic dysfunction and that pharmacological reduction of SR Ca^{2+} leak can improve diastolic dysfunction in human myocardium (144). Therefore, it is conceivable that targeting SR Ca^{2+} leak may also improve diastolic function at advanced stages in the present model of cardiorenal HFpEF (194).

At the level of cardiomyocytes, Ca^{2+} waves were observed and lead to a simultaneous release of Ca^{2+} across the scan line. This synchronized Ca^{2+} release indicated Ca^{2+} release induced by L-type Ca^{2+} influx during an action potential triggered by arrhythmic membrane depolarization (after-depolarization). These “depolarizations” were quantified in cardiomyocytes from Sham and NXT and a significantly higher incidence of depolarizations in NXT vs. Sham was determined (194). Ca^{2+} -mediated arrhythmias due to increased Ca^{2+} leak in NXT at the later stage may be a cause for increased mortality, as an increased incidence of cellular arrhythmias and peri-operative ventricular tachycardias in NXT were observed. Additionally, analyzed continuous ECG recordings from animals that underwent

the final hemodynamic experiment at 24 weeks showed an increased incidence of non-sustained ventricular arrhythmias in NXT vs. Sham rats which may indicate an increased risk for arrhythmogenic death in NXT (Figure 3.6 B, C) (194). This hypothesis was supported by Cho et al (167). Dahl Salt-Sensitive rats were fed a high-salt diet (8% NaCl) for 7 weeks. Susceptibility to ventricular arrhythmias (VA) was markedly increased in rats with HFpEF. Underlying mechanisms include QT prolongation, delayed repolarization from downregulation of potassium currents and multiple reentry circuits during ventricular arrhythmias. These results were according with the hypothesis that a potassium current downregulation lead to abnormal repolarization in HFpEF, which in turn predisposes to ventricular arrhythmias and sudden cardiac death (167).

4.3 Diastolic dysfunction in vivo is related to slowed cardiomyocyte relaxation and increased Ca²⁺ leak in vitro

In HFpEF rats – induced after subtotal nephrectomy – diastolic dysfunction in vivo was significantly correlated with impaired cardiomyocyte active relaxation and increased calcium leak in vitro. The significant correlation between LV cardiomyocyte active relaxation in vitro and LV isovolumetric relaxation constant TAU in vivo in the same hearts (Figure 3.12 B) suggests an important contribution of slowed cardiomyocyte active relaxation to the development of diastolic dysfunction in HFpEF rats (194). Additionally, there was a significant correlation between SR Ca²⁺ leak and LVEDP in NXT rats after 24 weeks. The SR Ca²⁺ leak, represented by the SR Ca²⁺ spark frequency rate, was significantly increased in HFpEF rat hearts after 24 weeks and correlated significantly with elevated LVEDP (Figure 3.12 A) (194). Therefore, SR Ca²⁺ leak had an important role in the development of arrhythmias in later stages of HFpEF caused by higher LV end diastolic filling pressures (194).

This link is further corroborated by the close correlation between the acute hemodynamic response to SEA0400 in vivo and in the cardiomyocytes from the same hearts in vitro. More precisely, there was a significant correlation between LV relaxation constant TAU in vivo and cardiomyocyte active relaxation in vitro in the presence of SEA0400 and without in NXT rats after 24 weeks acutely (Figure 3.20 B) (194).

Interestingly, the amplitude of unloaded cardiomyocyte shortening in vitro remained unchanged despite progression of the HFpEF phenotype in vivo. These observations contrast with previous reports in models with early diastolic dysfunction that ultimately progress into HFrEF (171,172) and suggests a distinct cellular phenotype in stable HFpEF (194).

4.4 Role of Na⁺/Ca²⁺ exchanger

The NCX is a transsarcolemmal ion channel in cardiomyocytes which regulates sodium and calcium flux through the cell membrane. The NCX-mediated transsarcolemmal Ca²⁺ flux changes direction within the cardiac cycle (173) and therefore is important for the intracellular ion homeostasis. NCX is working mainly in forward mode under physiological conditions and contributes to calcium efflux. Under pathophysiological conditions of heart failure, it has been demonstrated that NCX can contribute to Ca²⁺ influx early (174) and late (175) during the cardiac cycle, although this effect may be too small to result in an increased Ca²⁺ transient amplitude (176).

In subtotal nephrectomy rats, the decay of the caffeine-induced Ca²⁺ transient (TAU) - as a surrogate for NCX forward mode (Ca²⁺ extrusion) activity, was progressively prolonged at 8 weeks and even more at 24 weeks (Figure 3.14) in parallel to the slowed [Ca²⁺] decay (194). Reduced NCX forward mode activity in NXT rats was associated with unchanged NCX protein expression at an early timepoint (Figure 3.14 C). These findings suggest that NCX-dependent Ca²⁺ extrusion could be explained by a shift towards increased reverse mode activity (Ca²⁺ import) in the NXT-HFpEF rats and furthermore would be expected with an increase in intracellular [Na⁺] (177) (194).

Our results indicate that in the CKD induced HFpEF model, NCX was shifted to an increased reverse mode activity and that in accordance with other studies diastolic dysfunction may be related to intracellular Na⁺ dysregulation (178,179). This hypothesis could be corroborated as we observed an amelioration of altered NCX activity and Ca²⁺ decay in cardiomyocytes dialyzed with equal [Na⁺] (194).

A potential explanation for increased cardiomyocyte [Na⁺] includes inhibition of the cardiac Na⁺/K⁺-ATPase by circulating digitalis-like endogenous cardiotonic steroids (180). Indeed, in serum samples of patients with CKD they have been elevated and consequently were able to inhibit cardiomyocyte Na⁺/K⁺-ATPase leading to slowed Ca²⁺ transient decay (181) (194).

While NCX expression was unchanged at early timepoint - at a later stage in this HFpEF – CKD model, increased expression of NCX (Figure 3.14 C) had been observed, which even may have aggravated the phenotype (194). Previous studies explored that increased SR Ca^{2+} leak (as also observed in the present model) leads to increased NCX expression (182) (194).

The relationship between chronic kidney disease and dysregulated intracellular Na^+ concentration in cardiomyocytes is of extreme importance. The relatively high cytosolic $[\text{Na}^+]$ and the short action potential duration in rodents considerably differ from humans. The assumption was, that in particular the latter favours a stronger role of NCX reverse mode in human myocardium and even more so in diseased hearts, as has been shown by Weisser-Thomas et al. in HFrEF (175). In the present model of CKD induced HFpEF there was evidence which provided altered $[\text{Na}^+]_i$. In whole-cell patch clamped cardiomyocytes no differences were found between NCX forward or NCX reverse activity currents between Sham and NXT at 24 weeks. Also, no differences in cytosolic Ca^{2+} decay between NXT and Sham were found at equal $[\text{Na}^+]$. This suggests that intracellular dialysis of the cell with identical Na^+ abrogates the differences in NCX activity (194).

In the present model, NCX expression was not increased at first manifestation of HFpEF at 8 weeks. Therefore, it had been concluded that altered protein expression of NCX is not necessary for HFpEF to develop and we have not explored upstream regulation of NCX protein expression in the present study. However, it has been shown in a previous study that increased SR Ca^{2+} leak (induced by a gain-of-function defect of the RyR2) lead to increased expression of NCX1 (182). Therefore, increased SR Ca^{2+} leak observed at the later timepoint (24 weeks) in the present model may have contributed to increased NCX1 expression, e.g. by Ca^{2+} -triggered CaMKII- or HDAC-dependent pathways (183). Consequently, SR Ca^{2+} leak could contribute as a potential mechanism for increased NCX1 expression in this HFpEF model induced by CKD (194).

4.5 Acute effects of the NCX inhibition with SEA0400 on LV cardiomyocytes

As a consequence, from the collected data in the investigated subtotal nephrectomy rat model with a clinically relevant HFpEF phenotype and the importance of the NCX reverse mode activity – acute effects of the NCX inhibition with SEA0400 (300 nM) on LV cardiomyocytes were explored.

Acute application of 300 nM SEA0400 on LV cardiomyocytes increased CS amplitude and CaT amplitude in Sham animals after 24 weeks but had no effect on cardiomyocyte relaxation or Ca^{2+} decay. In NXT rats after 24 weeks in contrast, acute SEA0400 treatment did not influence the CS or CaT amplitude but significantly improved slowed cardiomyocyte relaxation and ameliorated Ca^{2+} decay in LV cardiomyocytes (Figure 3.16 and 3.17) (194). The effect of SEA0400 on Ca^{2+} transient amplitude depends on NCX expression levels. In a murine model of NCX overexpression it was determined that NCX inhibition with SEA0400 increased Ca^{2+} transient amplitude and therefore was a measure of inotropy (184).

Moreover, SEA0400 accelerated Ca^{2+} extrusion via NCX by improving decay of the caffeine-induced Ca^{2+} transient (TAU, sec) in NXT rats after 24 weeks, while in Sham animals no effect was observed (Figure 3.18 A, C). SR Ca^{2+} content in 24 weeks Sham and NXT cardiomyocytes was not affected after 5 minutes incubation with 300nm SEA0400 (Figure 3.18 A, B). Furthermore, acute SEA0400 application significantly reduced SR Ca^{2+} leak in LV cardiomyocytes from NXT rats after 24 weeks (Figure 3.19) (194). The antiarrhythmic effect of SEA0400 in conditions of increased NCX expression promotes the therapeutic concept of NCX inhibition in heart failure and atrial fibrillation (184). In conditions of reduced NCX expression, SEA0400 suppressed the NCX function leading to adverse Ca^{2+} accumulation as reflected by an increase in Ca^{2+} transient amplitude, proarrhythmia and cell death (184). Especially, selective NCX inhibition prevents Ca^{2+} overload-induced triggered arrhythmias (185).

Following these results, it could be concluded that acute application of SEA0400 for inhibition of NCX improved cardiomyocytes diastolic function in our renal induced HFpEF model. The findings may be explained by enhanced NCX reverse mode in NXT, but negligible NCX activity during diastole in healthy rats (186). A more physiological shape of the cytosolic Ca^{2+} transient in NXT rats following acute SEA0400 does not imply a normalization of the chronic cellular phenotype but may provide the basis for attenuation of cardiac remodeling with SEA0400 (194).

With regard to the acute effects of SEA0400 in vitro, it has to be noticed, that multiple ion handling proteins are dysregulated by chronic remodeling in NXT as indicated by the protein expression/phosphorylation pattern shown in table 3.13. Still the acute effects of SEA0400 on cardiomyocyte function in NXT are striking. There is no acute normalization of chronically dysfunctional ion homeostasis by one intervention (in fact, SR Ca^{2+} content remains low despite SEA0400 in NXT). Rather, SEA0400 causes an acute deflection of altered ion homeostasis in NXT resulting in a more physiologically shaped cytosolic Ca^{2+} transient despite dysregulated ion handling proteins. While these results indicate relevant increased NCX activity in NXT that can be targeted, it remains to be determined how the resulting alterations in the Ca^{2+} transient lead to attenuation of cardiac remodeling (194).

4.6 Acute hemodynamic effects of NCX inhibition with SEA0400 on in vivo LV function

Acute hemodynamic effects of NCX inhibition with SEA0400 on LV systolic and diastolic function was investigated in NXT and Sham animals after 24 weeks. In vivo, acute application of 1 mg/kg/BW SEA0400 for 30 minutes in NXT (not in Sham) significantly accelerated the prolonged IVRc TAU, which correlated well with faster relaxation of SEA0400-treated cardiomyocytes from these hearts in vitro (Figure 3.20). Furthermore, acute SEA0400 infusion had no effect on LV systolic peak pressure or LV end-diastolic pressure, suggesting that late diastolic filling was not acutely affected (194). In acute settings parameters of late diastolic filling may be more related to structural remodeling and passive stiffness (187).

These findings do not suggest that increased NCX activity is the only relevant factor for cardiac dysfunction in this renal HFpEF model. As shown in Figure 3.20 acute infusion of SEA0400 has a statistically significant, but clinically only modest effect on relaxation in vivo. End-diastolic pressure which is not only related to active relaxation but also to passive mechanical properties of the remodeled myocardium is not significantly altered by acute infusion of SEA0400. On the other hand, the fact that a modest improvement of isovolumetric relaxation was observed indicates that reverse mode NCX does contribute to excitation-contraction coupling in this model. It is important to clearly separate late diastolic filling/EDP as potentially related to structural remodeling and passive stiffness (187) from IVRc (early filling) which moderately improved with SEA0400 (194).

4.7 Chronic treatment with the NCX inhibitor SEA0400 reverses HFpEF phenotype

Long term chronic treatment with the NCX inhibitor SEA0400 1 mg/ kg b.w per os twice daily for 16 weeks in NXT rats with an established HFpEF phenotype (8 weeks after initial subtotal nephrectomy) significantly ameliorated LV hypertrophy, cardiac remodeling and diastolic dysfunction (Figure 3.21 and 3.22). Furthermore, NCX inhibition improved congestion and NTproBNP levels in NXT while LVEF was preserved (Figure 3.23). Chronic SEA0400 treatment was associated with moderately decreased systolic blood pressure in Sham and NXT after 24 weeks (Figure 3.24) (194) .

It has been demonstrated that IVRc and LVEDP are closely related in the chronic course of cardiac remodeling in HFpEF (188). Kamimura et al. (189) reported reduced LVEDP and improved survival following chronic treatment with SEA0400 in salt-sensitive rats, which is further associated with attenuation of LV fibrosis (189). In the present model of renal induced HFpEF an improvement in diastolic function and reverse myocardial remodeling following chronic SEA0400 therapy was demonstrated (194). These effects are not only explained by SEA0400's moderate blood pressure-lowering effects. Importantly, reverse myocardial remodeling with SEA0400 is an established parameter of prognosis in experimental and clinical studies (190). Furthermore, SEA0400 has been shown to improve mortality in a model of hypertensive heart disease (189). To confirm NCX inhibition as a potential therapeutic target in cardiorenal HFpEF, further studies are needed in future (194).

5 Conclusion

Subtotal nephrectomy in rats resulted in stable compensated CKD and the clinically relevant HFpEF phenotype with a hypertrophied LV, a prolonged LV isovolumetric relaxation constant TAU (IVRc TAU), an elevated end diastolic pressure (EDP), an increased lung weight (pulmonary congestion) and preserved LV systolic function. In NXT rats, cardiomyocytes relaxation and CaT decay were progressively prolonged at 8 and 24 weeks and individually correlating with diastolic dysfunction in vivo. Increased NCX reverse mode activity in NXT was suggested by a progressively reduced NCX forward mode activity and an upregulation of NCX protein expression. NCX inhibition with SEA0400 improved relaxation in NXT in vivo and in cardiomyocytes acutely and enhanced cardiac remodeling and diastolic function when given chronically (194).

Summarizing, in this model of renal induced HFpEF the modulation of the NCX improved cardiomyocyte function, remodeling and HFpEF and therefore could be an interesting target in the future therapy of HFpEF patients (194).

6 Translational perspective

There is no established therapy for the treatment of HFpEF patients yet. The heterogeneity of the clinical syndrome with a various number of comorbidities complicates the identification of underlying mechanisms. Paulus and Tschöpe described that endothelial dysfunction and increased myocardial passive stiffness contribute to the development of HFpEF (51).

In the present study it has been demonstrated for the first time that impaired active cardiomyocyte relaxation appears to be a relevant contributor to HFpEF (194). The results suggest NCX modulation as an interesting target to treat HFpEF with CKD. In cardiomyocytes Ca^{2+} transport through NCX depends on membrane $[\text{Na}^+]$ and $[\text{Ca}^{2+}]$ and on the membrane potential, resulting in shifting directions of transport during the same cardiac cycle (194). The different cytosolic sodium concentrations between species have to be mentioned. Particularly, the higher cytosolic $[\text{Na}^+]$ in rodents as compared to larger animals may over-emphasize the role of Ca^{2+} influx through NCX in this CKD induced HFpEF model. Otherwise, action potentials are also different between murine and human cardiomyocytes. Human cardiomyocytes have much longer plateau phase of the action potential than murine cardiomyocytes, which favors Ca^{2+} influx via reverse mode NCX. A relevant Ca^{2+} influx via reverse mode NCX has been documented in human heart with advanced HFrEF (175, 194).

Current NCX inhibitors including SEA0400 have limited and species-dependent specificity, mainly related to L-type Ca^{2+} current inhibition (132,146). In our study a submaximal dosage of SEA0400 (0.3 μM) was used in vitro, reported to reduce NCX activity in rat cardiomyocytes by around 60% and have only minor effects on L-type Ca^{2+} channels in various species (132,110,111). This assumption was confirmed because no relevant negative inotropic effect of SEA0400 in cardiomyocytes or in vivo have been observed, which could be suggestive of relevant inhibition of L-type Ca^{2+} current (194). Additionally, other studies showed that similar plasma concentrations of SEA0400 were hemodynamically well tolerated (146).

In the present rat model of cardiorenal-induced HFpEF remarkable similarities with HFpEF patients in key cardiac functional and morphological parameters have been reported. Compared to the experimental setting - in the clinical routine patients with HFpEF and CKD have a high burden of co-morbidities (e.g. diabetes mellitus, or obesity) (191) which have themselves been associated with poor prognosis (192). The novel therapeutic approach with NCX modulation in HFpEF associated with CKD need to be validated in HFpEF of different etiology (194).

7 Limitations

The subtotal nephrectomy rat model based on chronic kidney disease and developing heart failure with preserved ejection fraction is a clinically highly relevant animal model. Nevertheless, further studies needed to confirm the potential mechanism of NCX modulation as a therapeutic target in HFpEF patients. It has to be mentioned that volume overload and resulting arterial hypertension are main triggers of the HFpEF phenotype in NXT rats. Importantly, NCX modulation has only minor effects on blood pressure in long-term application studies and therefore its effect on cardiac remodeling and diastolic dysfunction is mainly blood pressure independent. As mentioned before there are different cytosolic sodium concentrations between species and because of that the role of Ca^{2+} influx through NCX could be overestimated in rodent models compared to large animal models. However, in human cardiomyocytes action potential duration is longer compared to murine cardiomyocytes which favors Ca^{2+} influx via reverse mode NCX. Finally, it has to be mentioned that actual NCX inhibitors available including SEA0400 have limited and species-dependent specificity, mainly related to L-type Ca^{2+} current inhibition.

8 References

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