Molecular Mechanisms of Myocardial Hypertrophy and Heart Failure

Experimental Studies on Cardiac G Protein-Coupled Receptor Signaling with Emphasis on Endothelin-1

by

Thomas G. von Lueder

Institute for Surgical Research, Faculty Division Rikshospitalet

University of Oslo

Rikshospitalet University Hospital

Oslo, Norway

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Contents

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Oslo, December 2008

Thomas G. von Lueder

2. List of Papers

Paper I:

von Lueder TG, Kjekshus H, Edvardsen T, Øie E, Urheim S, Vinge LE, Ahmed MS, Smiseth OA, Attramadal H. Mechanisms of elevated plasma endothelin-1 in CHF: congestion increases pulmonary synthesis and secretion of endothelin-1. *Cardiovasc Res.* 2004; 63: 41-50

Paper II:

von Lueder TG, Øie E, Ahmed MS, Edvardsen T, Smiseth OA, Attramadal H. Macrophage depletion in heart failure attenuates cardiac remodeling by mechanism involving reduced secretion of endothelin-1 and pro-inflammatory mediators. *Submitted.*

Paper III:

Vinge LE, von Lueder TG, Aasum E, Qvigstad E, Gravning J, How OJ, Bjørnerheim R, Ahmed MS, Mikkelsen B, Øie E, Attramadal T, Skomedal T, Smiseth OA, Koch WJ, Larsen T, Attramadal H. Cardiac-restricted expression of the carboxyl-terminal fragment of GRK3 uncovers distinct functions of GRK3 in regulation of cardiac contractility and growth – GRK3 controls cardiac α1-adrenergic receptor responsiveness. *J Biol Chem*. 2008;18;283(16):601-610.

Paper IV:

von Lueder TG, Gravning J, How OJ, Vinge LE, Ahmed MS, Larsen T, Smiseth OA, Aasum E, Attramadal H. Cardiomyocyte-restricted inhibition of GRK3 rescues cardiac dysfunction after chronic pressure overload. *Submitted.*

3. Abbreviations

β1-AR	β1-adrenergic receptors
AB	aortic banding
ACE	angiotensin-converting enzyme
Ang II	angiotensin II
EC	endothelial cells
ECE	endothelin-converting enzyme
ET	endothelin
$GdCl_3$	gadolinium chloride
GPCR	G protein-coupled receptors
GRK	G protein-coupled receptor kinase
HF	heart failure
IL-12	interleukin-12
LV	left ventricle
LVEDP	left ventricular end-diastolic pressure
LVH	left ventricular hypertrophy
MCP-1	monocyte chemoattractant peptide-1
MI	myocardial infarction
NEP	neutral endopeptidase
PAH	pulmonary arterial hypertension
PCWP	pulmonary capillary wedge pressure
PVAN	pressure-volume analysis
RAAS	renin-angiotensin-aldosterone system
RCT	randomised controlled trial
RV	right ventricle
TNF-α	tumor necrosis factor-α
VSMC	vascular smooth muscle cells

4. Introduction

Heart failure (HF) is the common endpoint of heart disease of various etiologies, and is a major cause of hospitalizations and death worldwide(1-3). Essentially, HF represents a pathophysiologic state of impaired cardiac function in which the heart is unable to maintain cardiac output sufficient for adequate perfusion of organs and tissues (4; 5). Coronary artery disease, hypertension, cardiomyopathies and valvular disease represent major causes of HF. Projections are that the prevalence likely will increase as a consequence of increasing mean age of the population (6; 7). Furthermore, increased survival from myocardial infarction (MI) will leave more patients living with HF(8). Even with the best treatment currently available, the overall 5-years mortality of HF is still over 50% and thus, hardly better than that of many types of cancer, reflecting the fact that the pathogenetic mechanisms underlying HF are still incompletely understood. In addition to reduced capacity of the heart to pump blood, HF is associated with activation of pro-inflammatory responses and mediators which itself can lead to progressive deterioration of cardiac function(9-11). More detailed knowledge of molecular mechanisms of HF has become a subject of intensive research. Chronic alterations in structure and geometry of the cardiac muscle, so-called remodeling, are widely found in heart failure patients(12; 13). Left ventricular (LV) remodeling itself is a progressive process which often is initiated by stress events or biomechanical loading such as myocardial infarction and poorly controlled hypertension(12; 13). As a paradigm, remodeling can either be predominantly eccentric (i.e. the heart is dilating), concentric (muscle mass is increasing) or a combination of both(13; 14). Basic research and translation of its results into clinical trials has seen major recent advances and led to the establishment of new treatment modalities targeting β–adrenergic signaling, Ang II and aldosterone activation(15-18).

Cardiac function is controlled by the autonomic nervous system, hormones, and diverse autocrine, endocrine or paracrine factors. G protein-coupled receptors (GPCR) comprise a

major class of receptors, and are in fact one of the largest known protein families(19). GPCRs are involved in most fundamental biological signaling processes, and in essence in most of mammalian tisssues(20; 21). The vital importance of GPCR signaling in cardiac disease is illustrated by the fact that the vast majority of current cardiovascular drugs target specific GPCRs, such as β1-AR, angiotensin type-II receptors, aldosterone or endothelin (ET) receptors(17; 18; 22; 23).

Increasing knowledge of signaling mechanisms in cardiac physiological and pathological states will be crucial for improving treatment of the HF and its precursing disease entities. Furthermore, targeted interaction with key pathophysiological signaling mechanisms holds the potential of preventing evolution to cardiac hypertrophy and HF upon given stress signals, as has been shown in numerous experimental studies. The present work aims to explore novel molecular mechanisms in hypertrophy and HF, as well as to investigate potential therapeutic principles.

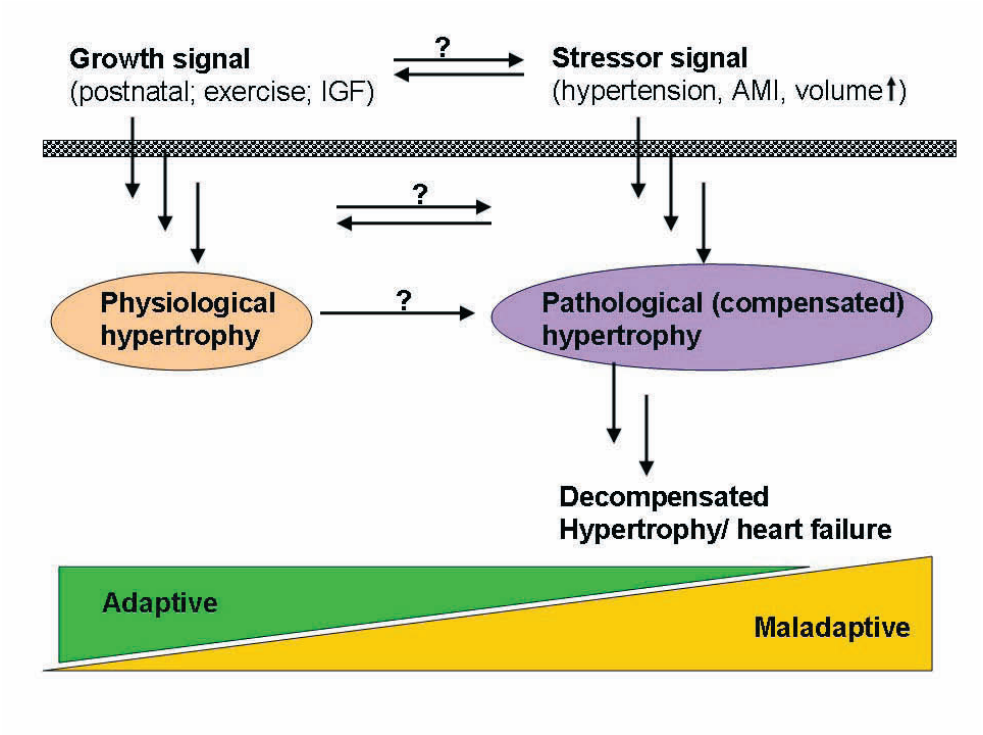

Fig. 1 Adaptive and maladaptive cardiac hypertrophy (modified from Frey et. al. (24))

Cardiac hypertrophy and remodeling in heart failure

Cardiac hypertrophy, i.e. excessive growth of the heart, can initially occur by cardiomyocyte hyperplasia, but primarily by increase of cell mass. Postnatal cardiac growth is a normal physiological phenomenon aiming at increasing heart size, i.e. to maintain cardiac output in the growing organism or to meet increased bodily demands during exercise training. Pathological stimuli such as catecholamine excess or increased afterload can lead to maladaptive cardiac hypertrophy, as seen in hypertension or aortic stenosis. Multiple signaling and transcription pathways are involved in this process, leading to hypertrophic remodeling of the LV (fig. 1)(24; 25). This type of LV hypertrophy (LVH) is an independent risk factor for cardiac morbidity and mortality(26). In HF patients, elevated levels of catecholamines are frequently seen(27; 28). Sustained adverse stimulation will increase

cardiomyoctes and thus, cardiac mass, both through increased afterload or direct cardiac effects. Among the adrenergic receptors, α1-AR couple to the hetrotrimeric G-protein $G_{\alpha q}$. Upon agonist activation, the $G_{\alpha q}$ unit activates phospholipase C, which increases inositol-1,4,5-triphosphate and diacylglycerol. The former increases intracellular calcium, while the latter leads to further activation of PKC isozymes(29). The $G_{\alpha q}$ pathway has been extensively studied for its importance in cardiac hypertrophy and HF(30). Based on a body of largely experimental evidence; one may suggest that factors leading to hyperactive $G_{\alpha q}$ signaling predispose to cardiac hypertrophy, and potentially, transition to decompensated HF(31-35). Neuroendocrine factors and cytokines such as ET-1 and AT-II promote activation of important downstream signaling cascades including MAPK, calcineurin, NFAT/GATA4, PKC, CaMK, and IGF-1 pathway constituents(36; 37). One important issue relating to GPCR-mediated hypertrophy and HF is to delineate specific signaling complexes in order to ascertain critical intracellular events regulating the hypertrophic response and transition to HF(38).

Endothelin system

Endothelin (ET) is a 21-amino acid peptide first isolated from porcine endothelial cells.(39) Three isoforms encoded by separate genes exist; ET-1, ET-2 and ET-3(39; 40). ET-1, the major isoform of the endothelin peptide family in the cardiovascular system, is among the most potent vasoconstrictors (~100 x norepinephrine) known to date, and possesses as positive inotropic and chronotropic effects (41-44), mitogenic effects on smooth muscle cells (45), influence on salt and water homeostasis, and stimulation of the renin-angiotensin-aldosterone (RAAS) and sympathetic nervous systems (for reviews, see (46; 47) (fig. 2).

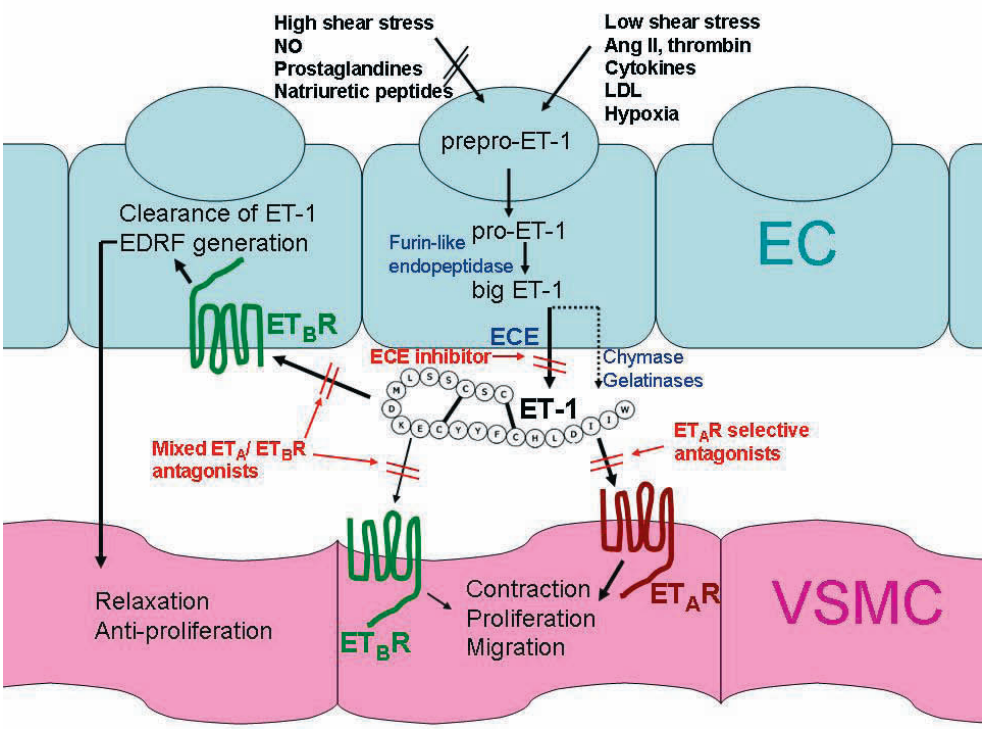

Fig. 2 The vascular endothelin (ET) system (modified from Kirkby et al (48)**)**

ET-1 is essential for normal embryonic development (49; 50). The biosynthesis of ET-1 occurs through several proteolytic steps to form the prohormone prepro-ET, the inactive intermediate big ET-1, which is subsequently processed by endothelin-converting enzyme (ECE) into biologically active ET-1 (Fig. 2)(51). Two isoforms of ECE with distinct pH-optima, ECE-1 and ECE-2, with 4 and 2 subtypes, respectively, have been characterized(51-55). In vivo, the activity of ECE-1 appears to be the rate-limiting step in ET-1 biosynthesis(56; 57).

Table 1. Function of ET receptors in the cardiovascular system.

Cell type	ET_A receptors	ET_B receptors
Cardiomyocytes	Hypertrophy(58) Positive inotropy(41) Protection from apoptosis(59)	Positive chronotropy(42) Hypertrophy?
Cardiac fibroblasts	Growth, fibrosis(60-62)	Growth, fibrosis(63; 63; 64)
Endothelial cells		Vasodilation through the release of NO and prostacyclin(65) and adrenomedullin(66) ET-1 clearance/reuptake(67) Increased ET-1 gene expression(68)
Vascular smooth muscle cells	Vasoconstriction, growth (45; 69)	Vasoconstriction(70)

ET-1 is mainly produced by endothelial, vascular smooth muscle cells, and macrophages and acts through binding to Gq-protein-coupled ET_A and ET_B receptors(71). In the cardiovascular system, ET_AR and ET_B R signaling produces distinct effects (table 1). Within the vasculature, ET-1 is secreted predominantly abluminally, i.e. on the basal side of endothelial cells to act on vascular smooth muscle cells (VSMC)(72), resulting in substantially higher concentrations within the vascular wall compared to plasma levels. Under normal physiological conditions, ET-1 plasma levels are low, with ET-1 acting rather as a paracrine factor(73). In cardiac disease such as HF, ET-1 levels are elevated and thought to derive primarily from spillover in the vasculature(74-79). Several reports have shown that the pulmonary circulation contributes to circulating plasma ET-1 levels in HF(80; 81). The synthesis and secretion of ET-1 by endothelial cells is increased by various growth factors, cytokines and vasoactive factors, such as Ang II, vasopressin, bradykinin, norepinephrine and ET-1 itself (82). Low shear stress increases ET-1 mRNA, while high shear stress decreases it (83; 84). The clearance of ETs from plasma may occur through cleavage by neutral endopeptidase EC3.4.24.11 (85), and through binding to ET_BR, which especially in the lung acts as a clearance receptor (86; 87). Due to effective clearance, the plasma half life of infused ET-1 is only one minute (47).

Importantly, ET-1 contributes in the pathogenesis of post-MI remodeling and HF, and plasma levels strongly predict mortality and morbidity(75; 76; 88-90). In this condition, ET-1 increases afterload by peripheral ET_AR mediated vasoconstriction(91-93). Moreover, ET-1 levels are increased in relation with the severity of pulmonary arterial hypertension (PAH) in HF; likewise, ETR inhibition ameliorate the degree of PAH in animals and patients with HF.(94-98). On the contrary, short-term therapy aimed at lowering afterload and elevated filling pressures in HF patients rapidly reduced ET-1 and neurohormonal activation (99). Based on encouraging experimental data (100) and human hemodynamic studies, several randomised controlled trials (RCT) have explored the putative benefit of ETR blockade in HF patients (table 2). Both dual or non-selective receptor blockers (targeting ET_AR and ET_BR) and selective ET_AR blockers have been employed. To date, the vast majority of these trials have failed to show improved outcome. However, in patients with isolated PAH, an infrequent yet rapid progressive and incurable cardiovascular disease leading to right-sided HF, non-selective ET receptor blockade has consistently demonstrated favourable outcomes(101). For this type of patients, ET-1 receptor blockers Bosentan, and more recently, Sixtasentan and Ambrisentan, have been added to the list of efficient pharmacotherapy(102). Despite recent major advances in ET research, many aspects of ET biology and in particular, origin, role and fate of elevated plasma ET-1 in HF are still poorly understood.

Table 2. Randomized controlled trials of ET antagonism in human HF.

Acute HF	Intervention	N pat.	Outcome	Comment	Reference
Pilot study	Tezosentan 20 or 50 mg/h IV for 24 h	14	Improved systemic and pulmonary hemodynamics	Safety-trial. Control group received dobutamine.	(103)
Pilot study	Tezosentan 5-100 mg/h IV for 6 h	61	Improved systemic and pulmonary hemodynamics	Hemodynamics and safety-trial. No serious adverse events	(104)
RITZ-1	Tezosentan 25 mg/h IV for 1 h, then 50 mg/h for 24-72 h	669	No differences in end points	More renal failure and hypotension in Tezosentan group	(105)
RITZ-2	Tezosentan 50 or 100 mg/h IV	240	Improved systemic and pulmonary hemodynamics at 6 h	No serious adverse events	(106; 107)
RITZ-4	Tezosentan 25 mg/h IV for 1h, then 50 mg/h for 24-48 h	193	No differences in end points	HF patients with ACS. More symptomatic hypotension in Tezosentan group.	(108; 108)
RITZ-5	Tezosentan 50-100 mg/h IV for 24 h	84	No differences in end points	Pat with fulminant PE. Better outcome with 50 mg dose. More side effects with higher doses	(109)
VERITAS-1 and 2	Tezosentan 5 mg/h for 30 min, so 1mg/h for 24-72 h	730	Non-significant benefit	More hypotension in Tezosentan group. Discontinued early for presumed lack of benefit.	(110; 111)
Chronic HF	**Intervention**	**N pat.**	**Outcome**	**Comment**	**Reference**
Pilot study	Bosentan 1000 mg PO BID for 2 weeks	36	Improved systemic and pulmonary hemodynamics	N=24 Bosentan vs N=12 placebo	(112)
REACH-1	Bosentan 250 mg PO BID	370	No differences in end points; trend to lower mortality	Trial stopped early. Toxic effects. Unpublished.	(113)
ENABLE-1 and 2	Bosentan 125 mg PO BID for 9 months	1613	No differences in end points	9 months follow-up. Early worsening of HF in Bosentan group. Unpublished.	(114; 115)
HEAT	Darusentan 3 different doses PO	157	Improvements in CI by Darusentan	More side effects at higher doses	(116)
ENCOR	Enrasentan (unpublished)	419	No differences in end points	Trend for increased rate of hospitalization. Unpublished.	(117)
EARTH	Darusentan 10-300 mg PO for 24 weeks	642	No differences in end points (LV volumes by MRI)	Increased adverse effects	(118)

ACS, acute coronary syndromes; CI, cardiac index; PCWP, pulmonary capillary wedge pressure; BID, twice daily; PE, pulmonary edema;

Inflammation in heart failure

Among multiple compensatory processes being activated as beforementioned, chronic HF is characterized by inflammatory reponses and activation of the innate immunity. Recently it has been shown that patients with HF have increased plasma and myocardial levels of inflammatory cytokines(9; 10; 119-123). Among proposed mechanisms for this immune activation, which are not mutually exclusive, are neurohormonal activation, hemodynamic overload, and activation of the innate immune system secondary to cardiac stress events, i.e. myocardial infarction. Experimental data have demonstrated a role for inflammatory and vasoactive cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), ET-1, and monocyte chemoattractant peptide-1 (MCP-1), all of which may contribute to the development and progression of HF by promoting myocardial hypertrophy or dysfunction, extracellular matrix remodeling, inducing apoptosis(9; 10; 119-124). Uncertainties exist as to the organ and cellular source of many of these cytokines, but the clinical significance is illustrated by a consistent and significant correlation of plasma levels and clinical outcomes(122; 125-128). Both TNF-α and ET-1 are such proinflammatory cytokines which can be produced by macrophages. Importantly, cardiac overexpresion of TNF-α (129) or ET-1 (130) in mice leads to similar phenotype of inflammatory cardiomyopathy. Currently, only few experimental and human studies have addressed the question whether targeting of an overactivated immunity in HF may carry benefit. “Single target” approaches such as blockade of TNF-α receptor in patients with HF have not demonstrated outcomes superior to conventional treatment, while more broad-based anti-inflammatory strategies demonstrated clinical improvements (131-135). More research in this area is needed to precisely identify important mechanisms in the immunopathogenesis of chronic HF which then could be counteracted pharmacologically.

G protein-coupled receptor signaling and desensitization in HF

Cardiac output is normally regulated by the autonomic nervous system and can be increased through release of stress hormones such as catecholamines. Catecholamines such as norepinephrine transmit their "message" through G protein-coupled receptors (GPCR), whose common feature is a seven-transmembrane span and their ability to activate heterotrimeric G-proteins. Activation of G-proteins initiates transduction of the extracellular signal to intracellular effector molecules(136). In the heart, GPCRs signaling may regulate function by modulating heart rate and contractility, or structure by inducing events such as cell growth or death (apoptosis). As a typical example for the GPCR-related signaling cascades, agonist binding to β1-adrenergic receptors (β1-AR) in the heart will activate the G protein $G\alpha s_s$ (s, stimulatory). Activated $G\alpha s_s$ will then activate adenylate cyclase (AC), leading to AC-catalyzed synthesis of cAMP. Functioning as a second messenger, cAMP then activates protein kinase A (PKA), leading to positive chronotropy (increased heart rate), inotropy (increased contractile force) and lusitropy (quicker relaxation)(137-140). β1-adrenergic receptors (β1-AR) are the predominant cardiac GPCRs activated by endogenous norepinephrine and epinephrine, with minor contributions by β2-AR and, at least in some mammalian species, α1-AR(141; 142). In HF irrespective of the initiating event, compensatory mechanisms such as augmentation of β1-AR signaling are rapidly activated, in order to maintain sufficient cardiac output. This is afforded at the cost of increased heart rate and myocardial oxygen consumption. Prolonged activation of β1-AR, moreover, can induce programmed cell death of heart muscle cells (cardiomyocyte apoptosis), reduced number as well as reduced response of receptors, and ultimately worsening of cardiac function(142; 143). The deleterious consequences of chronic neurohormonal overactivation suggest an important protective role for mechanisms which desensitize neurohormone-mediated GPCR responses in HF. Based on these fundamental molecular events, β1-AR blockade has

emerged as a major therapeutic principle in heart failure during the last decade. Activation of the renin-angiotensin-aldosterone system (RAAS) is another important mechanism activated in HF, leading to increased circulating and myocardial levels of the vasoconstrictor peptides renin and angiotensin II as well as the mineralocorticoid hormone aldosterone. All of these three components of the RAAS are established or emerging drug targets in heart failure(15-18). In HF, loss of response due to prolonged or augmented activation of GPCRs such as the β-AR has been identified, a phenomenon termed receptor desensitization (138; 144-146). Receptor desensitization can occur quickly, experimentally even after a few seconds or minutes. Also, desensitization can either be limited to agonists acting at a particular GPCR subtype, referred to as homologous desensitization, or represent a more general loss of agonist responsiveness involving several GPCR even in the absence of agonist occupation of these receptors. The former usually involves changes at the level of the GPCR itself, while the latter may involve adaptive changes in downstream signaling components. Importantly, desensitization is a process distinct from GPCR downregulation, which involves lysosomal degradation of GPCRs. Even excessive GPCR desensitization does not necessarily lead to downregulation, but both can occur simultaneously, adding to loss of functional response upon agonist stimulation. An important mechanism in the ´classical´ model of agonist-induced desensitization is phosphorylation of the GPCR(147). Phosphorylation is catalyzed by a family of kinases termed G protein coupled receptor kinases (GRK). GRK have been demonstrated to play a key role in agonist-induced phosphorylation and desensitization of numerous GPCR mediated responses. The classical model for agonist-occupied desensitization of GPCR involves phosphorylation of serine or threonine residues on the 3rd intracellular loop or COOH- terminus of the GPCR(148). Arrestins, members of another family of regulatory proteins, then bind to the GRK-phosphorylated GPCR with high affinity,

uncoupling it from further G-protein activation, thus inducing desensitization of the GPCR.

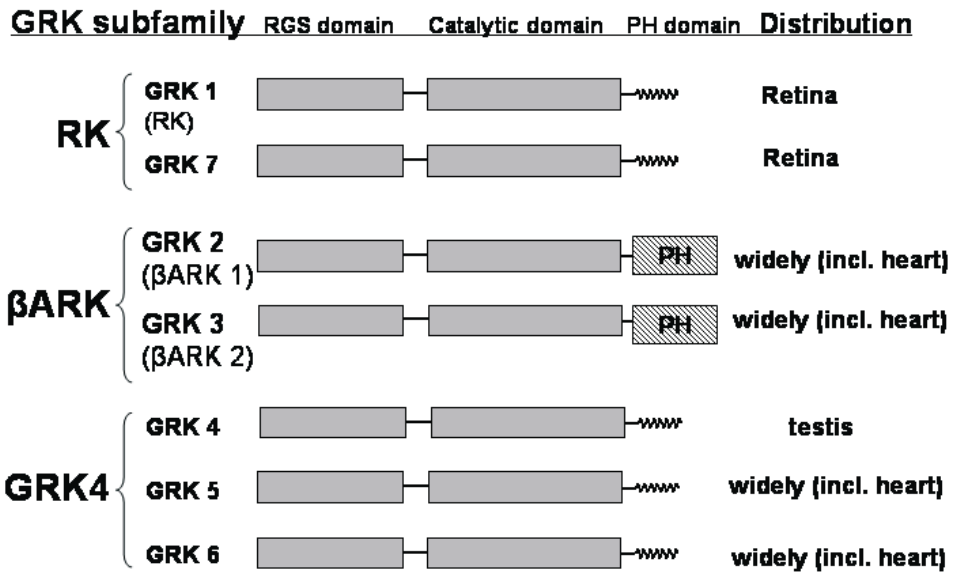

Fig. 3 The G protein-coupled receptor kinase (GRK) family

G protein-coupled receptor kinases (GRKs)

GRKs constitute a family of seven serine/threonine protein kinases which are further subdivided into three main subgroups, i.e. visual GRKs or the rhodopsin kinase subfamily (GRK1 and GRK7), the βARK kinase subfamily including GRK2 (βARK1) and GRK3 (βARK2), and the GRK4 family (GRK4, GRK5, and GRK6) (fig. 3)(149).

In myocardial tissue, 4 different GRKs have been found, GRK2, 3, 5 and 6. Of these, GRK2 and GRK3 share important structural similarities. In contrast to the other GRKs, GRK2 and GRK3 possess a carboxy-terminal (CT) pleckstrin-homology (PH) domain important for membrane targeting and binding to G-protein subunits(149). GRK2, initially termed βARK1,

has been shown to mediate desensitization of myocardial β-AR(150; 151). In experimental and human HF upregulation of myocardial GRK2 is found(152; 153). Inhibition of GRK2 in genetically engineered mouse models of heart failure, such as the muscle *lim* protein–knockout model and cardiac-specific overexpression of calsequestrin, and in a number of experimental settings has provided robust evidence of improving cardiac function and survival(150; 154-159). Although similar overall structure, GRK3 has distinct substrate spesificities determined by the CT domain. While GRK2 regulates cardiac β-AR and Ang II-R, α1-ARs are not touched by it(150; 160; 161). Vice versa, GRK3 strongly modulates cardiac α1-AR, ET-R and thrombin receptor mediated responses without altering β1-AR mediated responses or receptor internalization(160-162). Accordingly, GRK2 and GRK3 seem to have distinct substrate specificities at least within the cardiovascular system (table 3). The role of myocardial GRK3 is little studied, as is its potential involvement in cardiac disease states, and the role of GRK5 and GRK6 are almost unknown. Several powerful molecular strategies have emerged during the last decade and proven valuable tools to study GRK isozyme function in vitro and in vivo.

Table 3. Substrate preferences of GRK2 and GRK3 in cardiovascular tissues.

Receptor	GRK2	GRK3	References
β1	+	-	(150; 160; 162)
β2	+	-	(163)
α1	-	+	(160-162)
ET	-	+	(162)
Angiotensin II	+	-	(160; 164)
Thrombin	-	+	(160; 165; 166)
Muscarinic	?	+(?)	(167)

5. Aims of the Study

This work aimed to elucidate molecular mechanisms involved in the pathophysiology of cardiac hypertrophy and heart failure.

The specific aims of the study were:

1) to identify the origins of increased plasma ET-1 levels in HF
2) to elucidate the mechanism of increased pulmonary secretion of ET-1 in experimental HF
3) to investigate whether depletion of macrophages reduces pulmonary ET-1 secretion and progressive cardiac remodeling in HF
4) to elucidate the role of GRK3 in regulation of myocardial function *in vivo*
5) to investigate to what extent inhibition of GRK3 *in vivo* alters development of pathological cardiac hypertrophy and HF after pressure-overload

6. Summary of Results

Paper I

Juvenile pigs subjected to three weeks of rapid cardiac pacing exhibited significant left ventricular dilatation and dysfunction, increased cardiac filling pressures, and over 4-fold increase of arterial plasma ET-1 levels, consistent with induction of severe HF. Repeated investigations showed an increasing trans-pulmonary gradient of plasma ET-1 during evolving HF. Single-bolus multiple indicator-dilution experiments revealed increased pulmonary synthesis and release of ET-1 in HF, with pulmonary clearance of ET-1 remained unaltered. ECE-1 isozyme activity was selectively increased in congested pulmonary tissue of HF pigs, and correlated significantly with the wet/dry weight ratios of the samples, i.e. a marker of pulmonary congestion. Furthermore, pulmonary macrophages (PM) in congested lobe segments were identified as likely sites of increased synthesis and release of ET-1.

Paper II

Two weeks (baseline) after induction of myocardial infarction by coronary ligation, rats in severe HF were randomized to treatment with the macrophage toxicant gadolinium chloride $GdCl_3$ (HF-Gad) or vehicle (HF-V) for 21 days (end-point). In HF-Gad compared to HF-V rats, massive apoptosis of PM and lower pulmonary tissue levels of the macrophage-derived cytokines IL-12A and IL-12B and ET-1 were found. Arterial plasma ET-1 levels were increased 6-fold in HF-V rats vs. sham-operated rats. Depletion of PM led to reduced arterial plasma ET-1 levels and eliminated the trans-pulmonary gradient of ET-1. Moreover, HF-Gad rats exhibited halted progression of cardiac dilatation and dysfunction and significantly reduced filling pressures.

Paper III

Cardiac function of GRK3 was investigated in transgenic mice (Tg-GRK3ct) with cardiac-specific expression of the carboxyl-terminal portion of GRK3 (GRK3ct) to inhibit its

activation through $G_{\beta\gamma}$-directed membrane translocation. Tail-cuff plethysmography of 3-9 months old Tg-GRK3ct mice revealed modest hypertension compared to non-transgenic littermate control (NLC) mice, an observation confirmed by blood pressure radiotelemetry of conscious, unrestrained mice. Heart rate, however, was similar between Tg-GRK3ct and NLC mice. Young Tg-GRK3ct mice (3 months) had normal cardiac dimensions but enhanced contractility. Moreover, Tg-GRK3ct mice displayed supersensitivity to α_1-adrenergic receptor stimulation, while response to chronic β_1-adrenergic receptor stimulation was unaltered. Pressure-volume relationships obtained in electrically paced *ex vivo*-perfused working hearts confirmed hypercontractile myocardium with elevated dP/dt_{max}, LV developed pressure, cardiac output, and stroke work in Tg-GRK3ct mice at physiological filling pressures.

Paper IV

Here we sought to elucidate the putative role of myocardial GRK3 in the development of pathological cardiac hypertrophy and HF. Tg-GRK3ct and NLC mice were subjected to chronic pressure-overload by suprarenal abdominal aortic banding (AB). Six weeks after AB, pressure-volume analysis of *ex vivo* perfused working hearts revealed substantial systolic and diastolic cardiac dysfunction in NLC mice, while cardiac function was entirely preserved in banded Tg-GRK3ct mice. Cardiac and LV mass was significantly enhanced in banded compared to their respective sham groups confirming LVH, but without significant differences between banded Tg-GRK3ct and NLC mice. At 12 weeks after AB, NLC mice displayed increased LV filling pressures, reduced cardiac output and augmented myocardial mRNA levels of BNP consistent with HF, all of which were prevented in banded Tg-GRK3ct mice.

7. Discussion

This thesis sheds light on novel molecular mechanisms involved in the pathogenesis of myocardial hypertrophy, LV remodeling, and HF. Comprehensive integrative physiology and molecular techniques were applied in a range of ischemic and non-ischemic HF models in pigs, rats and mice, to elucidate important components of GPCR signaling.

Origin and mechanisms of elevated ET-1 levels in HF

In a large animal model of tachycardia-induced HF, we studied the tissue-specific and cellular origin of increased plasma ET-1 levels. In HF, ET-1 may act in a endocrine fashion, and several reports have indicated the lungs to play a contributing role to increased plasma levels, but the relative importance of the pulmonary compared to other vascular beds had not been established(80; 81). We here not only demonstrate the pulmonary circulation to be the most important source of elevated plasma ET-1, but also show that the trans-pulmonary gradient of ET-1 increased with progression of HF. The lungs efficiently remove ET-1 from the circulation via binding to its presumed clearance receptor ET_BR(86; 87; 168). Other investigators have previously found reduced pulmonary density of ET_BR and reduced fractional extraction of ET-1 in HF models, indicating failure of the lungs to remove ET-1 in HF(80; 169). The relative contributions of altered clearance or production of ET-1 to raise plasma ET-1 levels in HF remained yet to be investigated(87; 168; 170). We found the pulmonary fractional extraction of plasma ET-1 to be about halved in HF pigs. However, fractional ET-1 extraction does not take into consideration the circulating plasma volume per time unit (cardiac output). Clearance of ET-1, i.e. the absolute amounts of ET-1 being removed from the circulation per minute, was not altered, contrasting with previous reports(80). However, that study was performed in rats with HF post-MI, and with ET-1 clearance determined in lungs *ex vivo*. Another important finding of the present study was that

pulmonary synthesis of ET-1 was enhanced and correlated significantly with markers of LV filling pressures (LVEDP and PCWP). Augmented ECE-1 activity in congested pulmonary tissue was identified as important molecular mechanism for increased pulmonary synthesis of ET-1 in HF. These novel findings clearly illustrate the relevance of pulmonary congestion and pulmonary endothelial dysfunction for ET-1 activation.

Caution has, however, to be exerted when extrapolating these data in order to reach a broader understanding of HF. The pacing overdrive model employed her induces homogenous eccentric LV remodeling, generating a stable, predictable and relatively homogenous experimental HF cohort, but cardiac ultrastructural changes may differ from those observed in HF post MI(171; 172). Yet, LV dilatation and dysfunction, peripheral vasoconstriction, and neurohormonal activation including that of the ET-1 system, share important similarities with human dilated HF(173-177).

Possible implications for future management of HF

ET-1 is a multifunctional peptide governing numerous and complex biological functions in the cardiovascular system. The data presented in this study underbuild the notion that ET-1 is an important player in the pathogenesis of HF. Targeting of GPCRs in HF is of proven benefit in the case of β1-AR, but has not produced similar beneficial outcomes when targeting ETR. In the future, alternative approaches to counteract ET-1 mediated actions should be pursued, such as targeting biosynthesis at its molecular and cellular sources. ECE-1 inhibitors have been shown to produce similar acute vasodilator effects as ET_AR antagonists in HF patients already on ACE inhibitor treatment(91; 178). ECE-1 antagonists have been shown to normalize ET-1 levels; and several approaches targeting ECE-1 individually or as dual ECE-1/NEP inhibition or triple ECE-1/NEP/ACE inhibition have produced functional benefits in experimental and human HF(179-184). Besides ECE-1, also NEP and chymase may convert

bigET-1 to fully mature ET-1; thus, agents inhibiting multiple peptidases may be required. However, as both ECE-1, NEP and ACE also participate in the hydrolysis of bradykinin, putative adverse effects by accumulation of bradykinin need to be considered when triple inhibitors are employed(185; 186). Data from larger RCTs testing the concept of inhibiting ET-1 biosynthesis are lacking(48; 184).

Effects of macrophage depletion on ET-levels and LV remodeling in HF after MI

In the second paper, following up on findings in paper 1, we aimed to further explore the importance of specific components of the innate immunity, i.e. PM, for ET-1 synthesis and HF progression. Based on successful protocols of PM targeting in a model of PAH and right-sided HF, we administered $GdCl_3$ in the classical ischemic HF rat model, commencing two weeks after induction of a large MI with evidence of HF(187). Immunohistochemical and molecular analysis indicated successful PM depletion. The study provided first evidence that targeting PM significantly reduced systemic and pulmonary ET-1 levels as well as halted cardiac remodeling. There are several important considerations relating to the intervention as well as the assumed mode of action of $GdCl_3$. Neither $GdCl_3$ itself nor the chosen route of administration may provide entirely selective and specific targeting of PM. More likely, $GdCl_3$ may affect several types of actively phagocytosing cells in liver and lungs as well as other organs, i.e. liver, spleen and kidneys(188; 189). For instance, there is evidence that $GdCl_3$ interferes with hepatic Kupffer cell function, and reduces pro-inflammatory cytokines in sepsis or liver ischemia-reperfusion models(190-195). Moreover, even after first passage through the lungs, relevant amounts of $GdCl_3$ could have reached the myocardium to exert positive inotropic effects as demonstrated at least *in vitro* in a dilated cardiomyopathy HF model(196). Nevertheless, several lines of evidence support the effectiveness of the intervention: First, massive apoptosis of CD68-positive cells in lung tissue were found in

$GdCl_3$ -treated HF rats. Next, cardiac tissues showed only few CD68 positive cells, little apoptosis, and no differences between HF groups (data not shown). Last, trans-pulmonary gradients of plasma ET-1 as well as pulmonary tissue ET-1 and IL-12A and IL-12B levels were substantially reduced by the intervention. However, due to the small animal size, it was not feasible to perform experiments deciphering the relative contribution of pulmonary clearance and synthesis of ET-1, as in paper 1.

The putative importance of PM in ET-1 biosynthesis and functional progression in HF needs to be corroborated in future studies. Both application of tissue- and cell-specific drugs and genetic targeting of PM or PM-related cytokines may be valuable and technically feasible strategies.

Inhibition of myocardial GRK3 *in vivo* enhances contractility and α1-AR signaling

Specific aspects of GPCR signaling, i.e. the role of myocardial GRK3 in regulation of cardiac function *in vivo*, were addressed in papers III and IV. GRK2 (formerly βARK-1), the isozyme of GRK3 has been shown to be regulated in experimental and human HF, and inhibition of GRK2 provided rescue of cardiac function in several HF models(150; 152; 153; 155-159). GRK3, previously thought to be subservient to its isozyme GRK2, is increasingly appreciated as a novel important regulatory kinase. Unlike GRK2, GRK3 does not seem to be regulated in cardiac tissue in HF(197). However, its selective expression in cardiomyocytes may imply an important functional role(197). Previous studies, performed in transgenic mice with cardiac-restricted overexpression of GRK3, revealed specificity of GRK3 to desensitize $\alpha 1_B$-AR and thrombin receptor, while β1-AR and Ang II signaling were not altered(160; 161). To date, studies of cardiac-specific targeting of GRK3 *in vivo* are lacking. A recent report from our laboratory provided *in vitro* evidence of striking differences in receptor specificities of GRK2 and GRK3 in adult rat cardiomyocytes(162). It could be clearly shown that GRK3, but not

GRK2, regulated α1-AR and ET-R, while GRK2 or its peptide inhibitor reduced and enhanced β1-AR signaling, respectively. To study these findings *in vivo*, we generated transgenic mice expressing a peptide inhibitor of GRK3 (GRK3ct) in the myocardium, which exhibit phenotype with enhanced cardiac function and elevated blood pressure. Evidence of subtle LV diastolic dysfunction was found in the GRK3ct mice although the relevance of these *ex vivo* findings at very high filling pressures is uncertain. By *ex vivo* and *in vivo* experiments, GRK3 was identified to modulate α1-AR and ET-R, but not β1-AR. The dominant α1-AR subtypes in mice appear to be $\alpha1_A$-AR and $\alpha1_B$-AR. While cardiac-restricted overexpression of the $\alpha1_A$-AR in transgenic mice increased contractility in the absence of hypertrophy, overexpression of the $\alpha1_B$-AR induced early diastolic dysfunction and progression towards overt dilated HF later on(198-200)**.** We did not succeed in identifying which α1-AR subtype was modulated most by GRK3ct expression, but the phenotypic findings point to predominant augmentation of $\alpha1_A$-AR signaling.

Several aspects of the study, in particular relating to the transgenic model need to be discussed as important limitations. Cardiac myocyte-restricted expression of an inhibitory peptide, i.e. GRK3ct; may not only inhibit GRK3; an argument already raised in the case of GRK2ct. Apart from GRK3, other PH domain-containing proteins might be inhibited. GRK3ct may in fact inhibit GRK2, and although data obtained in rat cardiomyocytes *ex vivo* demonstrated selectivity of GRK3ct peptides for GRK3, we cannot exclude such effects to occur in mice *in vivo*(162). However, the lack of enhanced β1-AR signaling in the present study argues against relevant inhibition of GRK2. Moreover, even though not observed in our experiments, downstream regulation by sequestration of Gβγ could have occurred, leading to altered Gβγ-mediated signaling. However, the distinct specificities of GRK3ct compared to GRK2ct at the functionally most important cardiac GPCR and the similar specificities of the corresponding kinases GRK3 and 2 argue against sequestration of Gβγ(162).

To substantiate the findings presented here, alternative routes of manipulating GRK3-mediated signaling should be pursued. For instance, targeted deletion of cardiac GRK3 (i.e. cardiac specific knock-out of GRK3, GRK3-KO) could be performed. Previously, enhanced chronotropic component of the baroreceptor reflex has been described in a general GRK3-KO model(167). However, blood pressure was not determined in that paper, making judgments on possibly altered β1-AR mediated enhancement of heart rate after nitroprusside administration difficult. Also, the lack of cardiac-selectivity in that genetic model obscures interpretation, and to date no further studies have been conducted attempting to clarify these findings. To gain more knowledge on putative dose-response effects of GRK3 manipulation on distinct GPCR signaling, supplemental functional studies in transgenic mice with different cardiac expression levels of GRK3 or an inhibitor would be needed.

GRK3 inhibition rescues pressure-overload induced cardiac dysfunction

In paper four, we extended our study on the regulatory role of GRK3 on cardiac function into a pathophysiological setting. In a pressure-overload model, inhibition of GRK3 prevented development of HF, and preserved cardiac function, while induction of pathological LVH itself was not altered. To exclude peripheral circulatory effects, comprehensive analysis of LV pressure-volume relations both *in vivo* and *ex vivo* were performed. An interesting observation is the lack of GRK3ct to augment increases of cardiac mass after pressure-overload, compared to our findings after chronic α1-AR stimulation. This behaviour resembles indeed that of GRK2ct in comparable settings(201; 202). One readily available explanation might be a concomitant GRK3ct-mediated enhancement of pro-hypertrophic pathways such as via $\alpha 1_B$-AR, being counteracted by beneficial $\alpha 1_A$-AR-mediated enhancement of cardiac function in resisting high afterload, resulting in a neutral net effect on cardiac mass. In addition, pressure-overload induced hypertrophy also occurs through activation of neurohormones such as Ang

II and ET-1; mediators which did not evoke enhanced ERK1/2 activation in cardiomyocytes of GRK3ct mice compared NLC mice (fig. 6, paper 3). Phosducin, a Gβγ-binding protein that does not resensitize β-ARs, enhances contractility of failing cardiomyocytes in a similar fashion as GRK2ct(203), and in the absence of increased β-AR-stimulated cAMP formation. Conceptually, the effects of GRK2ct could at least partially be due to inhibition of Gβγ rather than β-AR resensitization. In view of the current findings that GRK3ct mediates similar cardioprotection as GRK3ct, at least some of the beneficial effects could involve signal transduction via common downstream pathways, including Gβγ-mediated effects. Recent data showed Gβγ-dependent phosphoinositide 3-kinase (PI3K) activation in afterload-induced cardiac hypertrophy(204). The same group also demonstrated PI3K to form a cytosolic complex with GRK2, leading to GRK2-mediated translocation of PI3K to the membrane with subsequent attenuation of β-AR sequestration (205). These data were supported by evidence for preserved β-AR function and restored cardiac function through inhibition of receptor-localized PI3K in several HF models (206; 207).

In order to decisively establish a protective role for GRK3 inhibition in HF, several strategies would be applicable. MI-induced HF or volume overload models would need to be applied in order to address the impact of particular stressor stimuli on GRK3 signaling. Ongoing projects are going to evaluate cardiac function in hybrids of GRK3ct mice or mice with cardiac-specific GRK3-KO cross-bred with genetic HF mice. Findings obtained in experimental models may eventually be tested by adequate pharmaceutical interventions, i.e. application of small-molecule approaches(208). If successful, this may provide the basis for testing GRK3 modulation in human HF.

8. Conclusions

1. In a large animal model, the lungs were identified as the most important contributors to elevated plasma ET-1 levels in severe HF. Pulmonary synthesis and release of ET-1were increased, while pulmonary clearance of ET-1 remained unaltered. Increased ECE-1 isozyme activity in congested pulmonary tissue was identified as an important mechanism, with pulmonary macrophages (PM) appearing as novel cellular sites of increased synthesis of ET-1.
2. In post-MI HF in rats, treatment with the macrophage toxicant $GdCl_3$ for 21 days induced massive apoptosis of PM, lowered inflammatory cytokines and plasma levels of ET-1, and eliminated the trans-pulmonary gradient of ET-1. Importantly, macrophage depletion halted progressive cardiac dysfunction and HF after MI.
3. Cardiac-specific inhibition of GRK3 in transgenic mice induces modest cardiac hypercontractility and hypertension, with structurally normal hearts. GRK3 inhibition increased responsiveness to α_1-AR stimulation, while response to β_1-AR stimulation was unaltered.
4. Inhibition of GRK3 did not affect cardiac hypertrophy upon chronic pressure-overload. However, cardiac function was preserved in Tg-GRK3ct compared to NLC mice, indicating a protective role of GRK3 inhibition in this HF model.

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