Metabolic interventions in heart failure

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Chapter 1
Introduction

Introduction

Heart failure (HF) is a major public health problem with a lifetime risk of almost 1 in 3 to develop this disease.[1,2] HF is the condition where the heart's blood supply does not suffice the body's demands. Despite improved treatment, the mortality rate for HF exceeds that of most malignancies.[3] As the most energy-consuming organ in the body, failing energy metabolism is thought to play an important role in the development of HF.[4] In this thesis, we investigated several aspects of metabolic interventions in HF. In the first part we investigated clinical aspects, including the position of β-blockers that have various metabolic effects. In this part we also describe the effect of coronary artery bypass grafting (CABG) in diabetic patients on HF development. In the second part we investigated the effects of A kinase interacting protein 1 (AKIP1) on cardiac remodelling and metabolism. This potential mitochondrial target for HF treatment was investigated using a transgenic mouse model.

Metabolic interventions after revascularization

Several established therapies also affect cardiac metabolism. We focused on β-blockers, that are known to have several metabolic "side-effects", and it has been suggested that new onset diabetes and dyslipidaemia are possibly related to the use of β-blockers.[5,6] Nevertheless, β-blockers have been recommended as first line therapy for stable coronary artery disease (CAD) for over 5 decades, based on their potent anti-anginal effects and on extrapolation of the prognostic benefits that has been demonstrated after myocardial infarction (MI) and in patients with HF.[7] CAD is a leading cause of morbidity and mortality worldwide and is the main cause of systolic HF.[8] The lifetime risk of developing CAD is 30-50%.[9] While mortality rates are decreasing by continued refinements in the treatment of acute coronary syndromes, innovation in the treatment of stable CAD has been limited. The studies supporting the efficacy of β-blockers in patients with CAD predate the current era of urgent coronary revascularisation and were specifically designed to evaluate their effects on angina. Of note, there is no evidence that β-blockers provide superior angina relief compared to calcium channel antagonists, nitrates or ivabradine. Furthermore, the evidence for the efficacy of β-blockers after revascularization in patients with stable CAD is sparse.[7] Nevertheless, β-blockers are often continued in these patients, even when left ventricular function is preserved and there are no other indications for their continued use.[10] In **chapter 2** we therefore aimed to evaluate whether β-blocker therapy is associated with a reduced incidence of angina or cardiovascular events when continued after revascularization. For this purpose we performed a post-hoc analysis of the IMAGINE (Ischaemia Management with Acupril post bypass Graft via Inhibition of angiotensin coNverting Enzyme) trial database which comprised of low-risk patients with normal cardiac function, randomized to quinapril or placebo early

after elective coronary artery bypass grafting (CABG) surgery for CAD. This trail allowed us to study the low risk subgroup where we do not have evidence to support the routine use of β-blockers after CABG.

In **chapter 3**, we sought to determine whether CABG reduces the propensity to develop HF in diabetic patients with CAD and preserved cardiac function. Patients with diabetes have a two-fold higher lifetime risk to develop HF.[11,12] This is often linked to the propensity of diabetic patients to develop CAD and MI. Diabetic patients are also more prone to HF in the absence of CAD. The underlying mechanism is not completely clarified but is suggested to include increased oxidative stress and glycosylation leading to the activation of detrimental signal transduction pathways (Figure 1).[13] Diabetes is an important risk factor for CAD and the extent of CAD is more severe in diabetic patients. This results in a higher frequency of MI and contributes to the two-fold higher lifetime risk to develop HF in diabetic patients (Figure1). Accordingly, CAD is treated aggressively in diabetic patients and the threshold for choosing CABG surgery over PCI is reduced.[14,15] However, whether CABG also reduces the propensity to develop HF in diabetic patients is unknown.

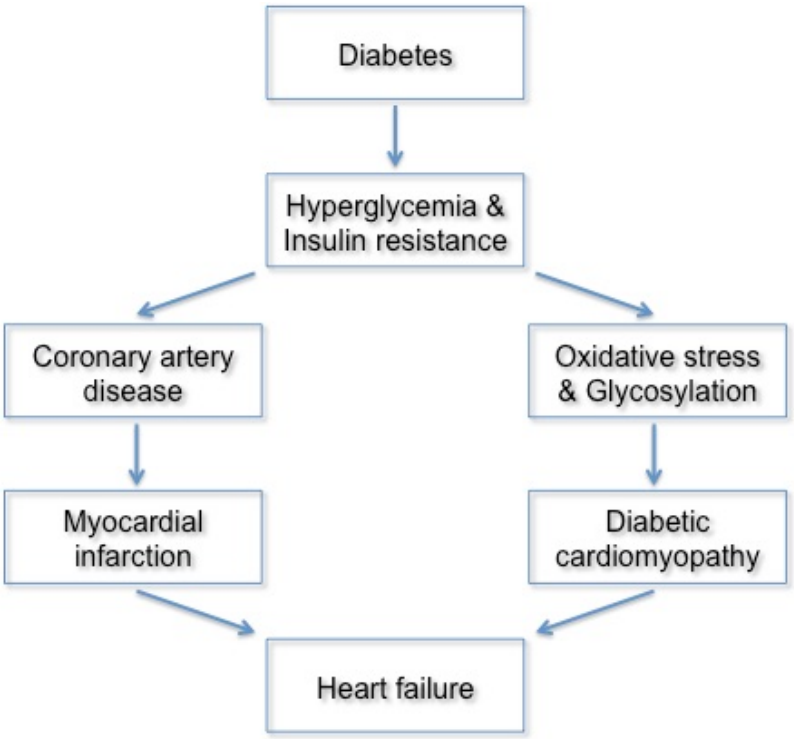

Figure 1 Pathophysiological links between diabetes and heart failure, adjusted from Dei Cas et al.[13]

AKIP1 in cardiac stress

Myocardial hypertrophy is the compensatory cardiac reaction to improve ventricular ejection performance after hemodynamic overload or injury. While initially myocardial hypertrophy reduces wall stress by restoring the ratio

between intracavitary pressure and wall thickness, it eventually decompensates and leads to HF.[16,17] This pathological hypertrophy is accompanied by a gene expression profile that resembles the embryonic heart.[18] Since it would be beneficial to only retain the adaptive aspects of physiological hypertrophy and prevent the deleterious collateral effects, we aim to determine what signaling pathways lead to hypertrophic decompensation. We previously performed genome wide transcription studies in a set of HF and hypertrophy models. We compared gene expression levels in 2 *in vivo* models (hypertension and post-MI) and 3 *in vitro* models in order to control for collateral differences in gene expression that are related to changes in hemodynamics rather than hypertrophic growth. One of the genes consistently upregulated was AKIP1.[19]

AKIP1 is a 21 kDa protein that was first identified as Breast Cancer Associatated gene 3 (BCA3). In the initial studies, AKIP1 was found to be upregulated in several cancer cell lines, to tweak NF-κB and PKA activity,[20,21] and promote the induction of apoptosis.[22,23] In contrast, AKIP1 stimulates neovascularisation and tumor growth in other malignancies.[24,25] Therefore, AKIP1 may have different roles, depending on cell type and clinical condition. We previously performed several gain- and loss of function experiments of AKIP1 in cultured cardiomyocytes and found that AKIP1 promoted physiological growth in these cells.[26] AKT-activation served as a mediator of AKIP1-induced physiological hypertrophy. Furthermore, we found that AKIP1 stimulates mitochondrial respiration while reducing mitochondrial ROS productions, arguably making respiration more efficient.[27] In **chapter 4** we will review in more detail how different aspects of metabolic dysfunction, like decreased energy supply and exacerbation of oxidative stress, lead to deteriorating HF. Another group found that AKIP1 attenuates ischemia / reperfusion (I/R) injury in *ex vivo* perfused hearts.[28] Together, this suggests that AKIP1 has a vital role in both acute and chronic cardiac stress and that interventions targeting AKIP1 could offer a viable strategy to treat patients with heart disease. To investigate this hypothesis, we generated a transgenic mouse line with cardiomyocyte specific overexpression of AKIP1. In **chapter 5 & 6** we describe our studies exploring whether cardiac overexpression of AKIP1 translates into beneficial effects on both acute and chronic cardiac insults *in vivo* and whether it modulates physiological hypertrophy (Figure2).

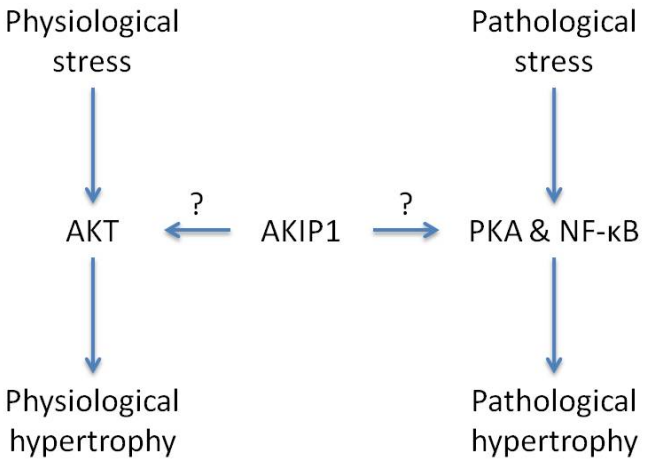

Figure 2 AKIP1 might influence cardiac hypertrophy development in response to different types of cardiac stress.

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Part 1
Metabolic interventions after revascularization

Chapter 2

β-blocker therapy is not associated with reductions in angina or cardiovascular events after Coronary Artery Bypass Graft Surgery: Insights from the IMAGINE trial

Harmen G. Booij, Kevin Damman, J. Wayne Warnica, Jean L. Rouleau, Wiek H. van Gilst and B. Daan Westenbrink

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Abstract

Purpose

To evaluate whether β-blockers were associated with a reduction in cardiovascular events or angina after Coronary Artery Bypass Graft (CABG) surgery, in otherwise stable low-risk patients during a mid-term follow-up.

Methods

We performed a post-hoc analysis of the IMAGINE (Ischemia Management with Accupril post–bypass Graft via Inhibition of angiotensin coNverting Enzyme) trial, which tested the effect of Quinapril in 2553 hemodynamically stable patients with left ventricular ejection fraction (LVEF) >40%, after scheduled CABG. The association between β-blocker therapy and the incidence of cardiovascular events (death, cardiac arrest, myocardial infarction, revascularizations, angina requiring hospitalization, stroke or hospitalization for heart failure) or angina that was documented to be due to underlying ischemia was tested with Cox regression and propensity adjusted analyses.

Results

In total, 1709 patients (76.5%) were using a β-blocker. Patients had excellent control of risk factors; with mean systolic blood pressure being 121±14 mmHg, mean LDL cholesterol of 2.8 mmol/l, 59% of patients received statins and 92% of patients received antiplatelet therapy. During a median follow-up of 33 months, β-blocker therapy was not associated with a reduction in cardiovascular events (hazard ratio 0.97; 95% confidence interval 0.74-1.27), documented angina (hazard ratio 0.85; 95% confidence interval 0.61-1.19) or any of the individual components of the combined endpoint. There were no relevant interactions for demographics, comorbidities or surgical characteristics. Propensity matched and time-dependent analyses revealed similar results.

Conclusions

β-blocker therapy after CABG is not associated with reductions in angina or cardiovascular events in low-risk patients with preserved LVEF, and may not be systematically indicated in such patients.

Introduction

Beta adrenoreceptor blockers (β-blockers) are among the most commonly prescribed cardiovascular drugs that are used to treat hypertension, arrhythmias, coronary artery disease (CAD) and heart failure. In patients with stable CAD, β-blockers are recommended as first line therapy based on their potent anti-anginal effects and on extrapolation of the prognostic benefits that has been demonstrated after myocardial infarction (MI) and in patients with heart failure.[1,2]

Most of the studies supporting the efficacy of β-blockers in patients with CAD predate the current era of coronary revascularisation, more intense anti-platelet therapy, the use of statins and more stringent blood pressure goals and were specifically designed to evaluate their effects on angina. While β-blockers are often continued in patients after coronary artery bypass grafting (CABG) surgery, even in patients with preserved left ventricular (LV) function, evidence for their efficacy in this setting is sparse.[1,3,4] This issue is particularly relevant since β-blockers may cause counterproductive side effects such as new onset diabetes and dyslipidemia.[5,6]

We therefore aimed to evaluate whether β-blocker therapy was associated with a reduced incidence of angina or cardiovascular events when continued after CABG. Therefore, we performed a post-hoc analysis of the IMAGINE (Ischaemia Management with Acupril post bypass Graft via Inhibition of angiotensin coNverting Enzyme) trial database which comprised of low-risk patients with normal cardiac function, randomized to quinapril or placebo early after elective CABG surgery.

Methods

A detailed description of the IMAGINE-trial protocol has been published previously.[7] In brief, it was a double-blinded, placebo-controlled, randomized, international, multicentre trial that tested whether the ACE-inhibitor quinapril when compared to placebo reduced symptoms of angina or cardiovascular events in patients with preserved LV function post-CABG surgery during a mid-term follow-up for a maximum of 43 months. Patients were included between November 1999 and September 2004. Written informed consent was obtained from all patients. The study was in compliance with the Declaration of Helsinki and the ethics committees from all participating institutions provided approval of the research protocol. In this trial, ACE-inhibition with quinapril did not improve outcome when started early after CABG in low-risk patients with preserved LV function, while adverse events were increased with quinapril during the first 3 months after randomization.

Patients

In total, 2553 patients were randomized to quinapril or placebo within 7 days after scheduled CABG, except in France where randomization was possible until 10 days post-CABG. If tolerated, the ACE inhibitor quinapril was uptitrated to 40 mg daily or its placebo equivalent. Patients were eligible for participation when hemodynamically stable and if left ventricular ejection fraction (LVEF) was >40%. Serum creatinine >2.26mg/dL (200 μmol/L) was an exclusion criterium as were suspicion of renal artery stenosis, a single kidney or a transplanted kidney. During the study, type II diabetes with microalbinuria and insulin-dependent diabetes became exclusion criteria due to increasing evidence of benefit of ACE inhibitors in these patients.

β-blocker treatment

Of the 2553 patients included in the IMAGINE trial, 320 patients were using sotalol. Because sotalol has class 3 anti-arrhythmic effects which may modulate event rate through pro-arrythmic effects, patients using sotalol were excluded from this analysis. Thus, 2233 patients were available for analysis. Patients were divided in two groups, according to β-blocker therapy. β-blocker dose was expressed as a percentage of the maximum recommended dose. For the time-dependent analysis β-blocker use was scored at randomization, 50 days, 90 days, 1, 2 and 3 years after randomization.

Endpoints

The primary IMAGINE endpoint consisted of the composite of cardiovascular death, resuscitated cardiac arrest, nonfatal MI, coronary revascularization, unstable angina requiring hospitalization, documented angina not requiring hospitalization, congestive heart failure which required hospitalization and stroke. The secondary IMAGINE endpoint consisted of the primary endpoint with the addition of transient ischemic attack and other cardiovascular events requiring hospitalization. One of the unique features of the IMAGINE trial was the meticulous verification of myocardial ischemia in patients with suspected recurrence of angina. An episode of angina was considered valid if typical symptoms of angina were associated with one of the following conditions: temporary ST deviations on electrocardiogram; a stress test with reversible wall motion abnormalities on echocardiography or reversible nuclear scan defects; coronary angiography demonstrating compatible lesions which could not be explained by incomplete revascularization or any episode of angina requiring hospitalization. Finally, we defined major adverse cardiovascular events (MACE) as the composite of angina, cardiovascular death, resuscitated cardiac arrest, nonfatal MI and coronary revascularization.

Statistical analysis

The baseline characteristics were compared according to presence of β-blocker therapy using students T-, Mann-Whitney U-, χ2- or Fisher exact tests, as appropriate. Time to first event was calculated by the Kaplan–Meier method and displayed graphically. Differences in event rate according to β-blocker therapy were calculated from a Cox proportional hazards regression model and expressed as an adjusted hazard ratio with two-sided confidence interval of 95%. Cox regression analysis was adjusted for the effects of age, gender, ethnicity, history of MI, revascularization, non-cardiac vascular event, hypertension, diabetes, hypercholesterolemia, the number of days after surgery that the patient was randomized, beating heart surgery, nr of grafted vessels, complete revascularization, LVEF and concomitant medication. Propensity matched analysis was performed as a sensitivity analysis and as an additional effort to adjust for residual confounding. We calculated a propensity score for β-blocker-use with multivariable logistic regression, using all available variables. Covariates were selected when associated with β-blocker therapy or when they were independently associated with the outcome. Patients were then matched based upon β-blocker treatment and similar propensity score based on 1 to 1 nearest neighbor matching without replacement. Pre-match imbalance and post-match balance were estimated with standardized differences for each covariate. Since approximately 20-25% of patients switched groups (started or discontinued a β-blocker) over time either permanent or temporary, we performed an additional Cox proportional hazards regression analysis with β-blocker as the time-dependent covariate. The software packages used for these analyses were SPSS 20.0 and STATA (version 12.0).

Results

Baseline characteristics

Of the 2233 patients analyzed, 1709 (76.5%) used a β-blocker. At 1 yr, 1174 patients (62.0%) used a β-blocker and 801 (58.9%) 2 years after randomization. Average β-blocker dose was 41.2%, 41.8% and 40.9% of the maximal recommended dose at these time points respectively. The maximal recommended dose was given in 128, 103 and 67 patients respectively (7.5%, 8.8% and 8.4% respectively). Baseline characteristics are shown in Table 1. Overall, these were low-risk patients with mean age of 61±10 years, mean LVEF 60±10%, low prevalence of diabetes (219 patients, 9.8%), and good renal function (eGFR 69±24 ml/min/1,73m2). Patients treated with β-blockers had a history of hypertension more often (50% vs 42%) while other medical history was comparable between groups. Mean arterial pressure, heart rate and LVEF were also similar between groups. The majority of patients received statin therapy (53% and 60% for no β-blocker and β-blocker patients

Table 1. Baseline characteristics stratified for use of β-blocker at randomization

Variable	no β-blocker (n=524)	β-blocker (n=1709)	p-value
General characteristics			
Age (yrs)	62 ± 10	61 ± 10	0.052
Male, n (%)	459 (88)	1495 (88)	1.000
Caucasian, n (%)	503 (96)	1645 (96)	0.169
Days post CABG	4.0 ± 1.7	4.3 ± 1.7	<0.001
Body mass index (kg/m^2)	27.9 ± 5.0	27.6 ± 5.5	0.350
Medical history			
Myocardial infarction, n (%)	213 (41)	668 (39)	0.540
Coronary revascularization, n (%)	105 (20)	19.4 (19)	0.753
Non-cardiac vascular event, n (%)	60 (12)	182 (11)	0.630
Diabetes, n (%)	50 (10)	169 (10)	0.867
Hypertension, n (%)	222 (42)	854 (50)	0.002
Hypercholesterolemia, n (%)	411 (78)	1343 (79)	0.951
Current smoker, n (%)	100 (19)	349 (20)	0.260
Surgical characteristics			
Beating heart surgery, n (%)	90 (17)	318 (19)	0.478
Three vessel disease, n (%)	340 (65)	1090 (64)	0.845
Nr of anastomoses, n (%)	3.3 ± 1.1	3.2 ± 1.1	0.335
Complete revascularization, n (%)	470 (90)	1490 (87)	0.128
Hemodynamics			
Systolic blood pressure (mmHg)	122 ± 14	121 ± 13	0.042
Diastolic blood pressure (mmHg)	70 ± 9	70 ± 9	0.257
LVEF (%)	60 ± 10	60 ± 10	0.830
Heart rate (bpm)	83 ± 13	82 ± 13	0.207
eGFR (ml/min/1,73m^2)	68 ± 26	69 ± 23	0.258
Medication			
Quinapril, n (%)	263 (50)	856 (50)	1.000
Anti-arrhytmic drug, n (%)	87 (17)	134 (8)	<0.001
Calcium-channel blocker, n (%)	198 (38)	372 (22)	<0.001
Cardiac glycoside, n (%)	29 (6)	97 (6)	1.000
Diuretic, n (%)	175 (33)	589 (35)	0.674
Coumarine derivate, n (%)	49 (9)	70 (4)	<0.001
Antiplatelet, n (%)	453 (87)	1610 (94)	<0.001
Other lipid-lowering drugs, n (%)	11 (2)	46 (3)	0.528
Statin, n (%)	280 (53)	1032 (60)	0.005
Nitrate, n (%)	39 (7)	100 (6)	0.752

Values shown are means ± SD or n (%). CABG, Coronary Artery Bypass Grafting; eGFR, estimated Glomerular Filtration Rate; LVEF, Left Ventricular Ejection Fraction;

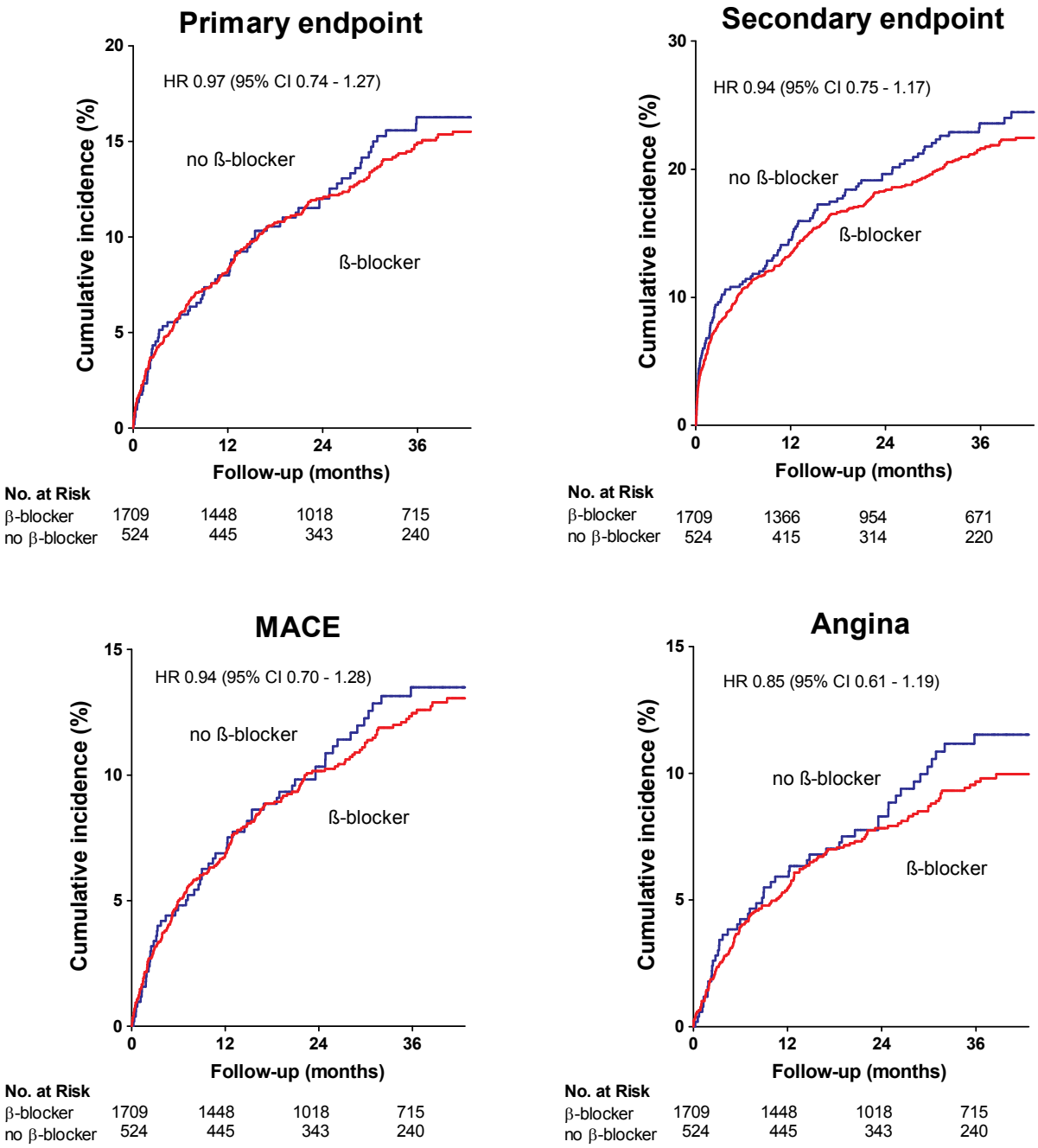

Figure 1 Outcome according to β-blocker therapy – Cumulative event rates for composite endpoints stratified for β-blocker therapy. Hazard ratios are adjusted for age, gender, ethnicity, history of myocardial infarction, revascularization, non-cardiac vascular event, hypertension, diabetes, hypercholesterolemia, days after CABG (coronary artery bypass grafting), beating heart surgery, nr of vessel disease, complete revascularization, left ventricular ejection fraction and concomitant medication. MACE, Major Adverse Cardiovascular Event.

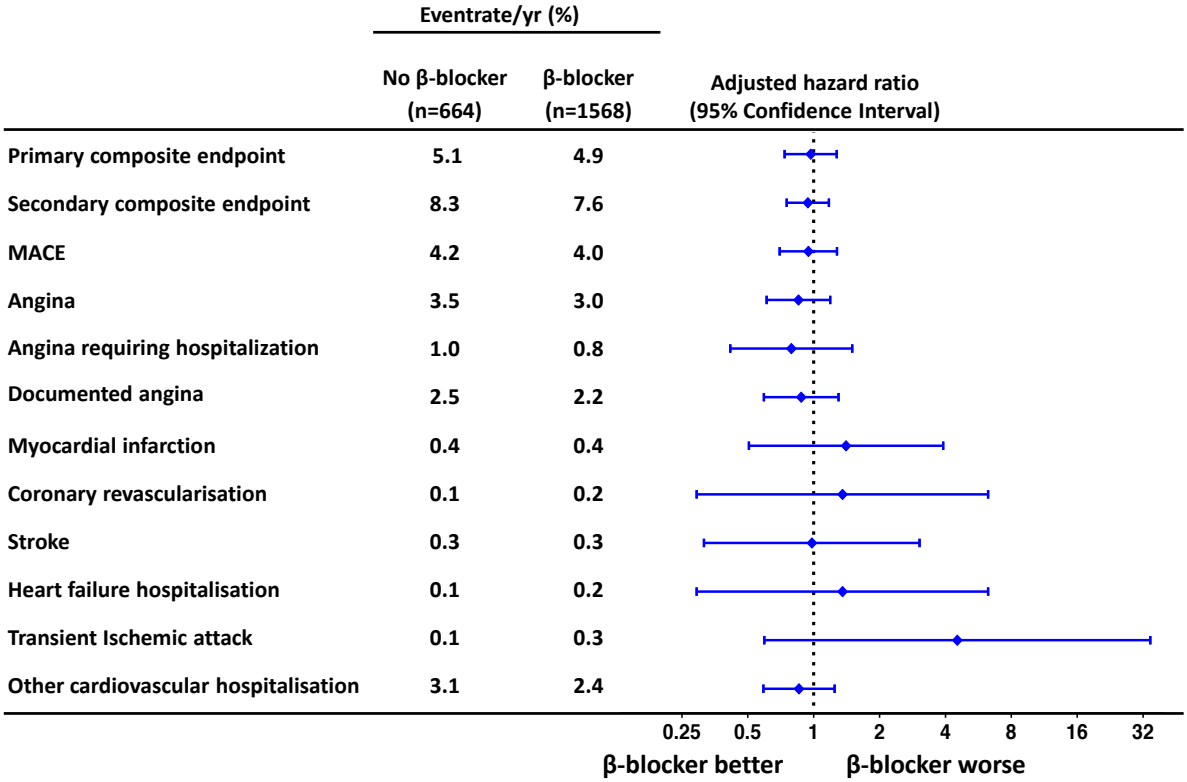

Figure 2 Cox regression – Hazard ratios and 95% confidence intervals for composite endpoints and individual components after adjustment for same variables as in Figure 1. MACE, Major Adverse Cardiovascular Event.

respectively), while other lipid-lowering therapy was given in 3% of patients. Patients on β-blocker therapy were more frequently receiving antiplatelet therapy and statins, but less frequently using anti-arrhythmic drugs or calcium channel blockers.

Mean LDL values were slightly higher at randomization in patients without β-blocker (3.0±1.0 vs 2.8±1.0, p=0.008) but did not differ during follow-up (2.6±0.8 vs 2.6±0.8 mmol/l, p=0.50 at 1 year follow-up and 2.5±0.8 vs 2.5±0.8 mmol/l, p=0.72 at study closure). Mean systolic blood pressure was 121±14 mmHg and slightly lower in patients on β-blockers at randomization, but it remained ≤131 mmHg with no significant difference between groups throughout follow-up. In the majority of patients (88%) revascularization was complete (defined as bypass of all stenosis of >70% in vessels with a diameter > 1 mm). Surgical characteristics were comparable.

Cox regression analysis

Out of the 2233 patients analysed, 299 (13.4%) patients had experienced a primary event, while 451 (20.2%) patients had experienced a secondary event during a median follow-up of 33 months (IQR 16-43). Total event count for MACE and Angina was 245 (11.0%) and 191 (8.6%) respectively. β-blocker treatment was not associated with a difference in cumulative incidence of any of the composite endpoints (primary endpoint, secondary endpoint, MACE,

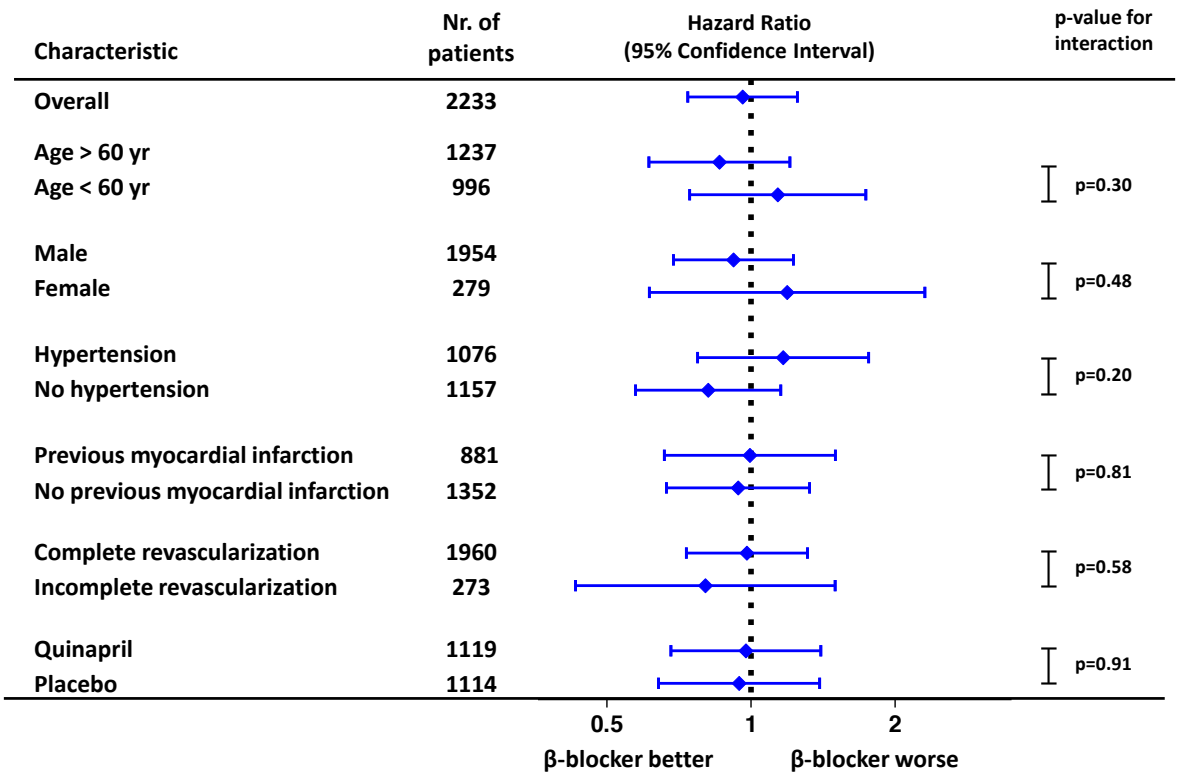

Figure 3 Interaction analysis for β-blocker – Hazard ratios for β-blocker therapy in relevant subgroups.

angina, (Figure 1)). Multivariate regression did not reveal any association between β-blocker treatment and the primary endpoint (hazard ratio (HR) 0.97; 95% confidence interval (CI) 0.74-1.27), documented angina (HR 0.85; 95%CI 0.61-1.19) or any of the other composite endpoints and their individual components (Figure 2). The neutral effects of β-blocker therapy were consistent among several relevant subgroups including age, gender, hypertension, previous MI, completeness of revascularization and treatment allocation (Figure 3).

Propensity matched and time-dependent analysis

The propensity matched population consisted of 424 patients per group. Absolute standardized differences for all baseline-characteristics were <10%, indicating an adequate match (Figure 4A). There was no association between β-blocker therapy and the occurrence of the primary IMAGINE endpoint when adjusting for propensity score and its covariates in the unmatched population nor when the propensity matched population was considered separately (Figure 4B, Figure 5). Similar results were obtained for the secondary endpoint, MACE and angina (data not shown). To account for differences in treatment over time, we analysed β-blocker therapy as a time-dependent covariate in our Cox-regression models. Again, no association was found for β-blocker therapy and outcome (Figure 5).

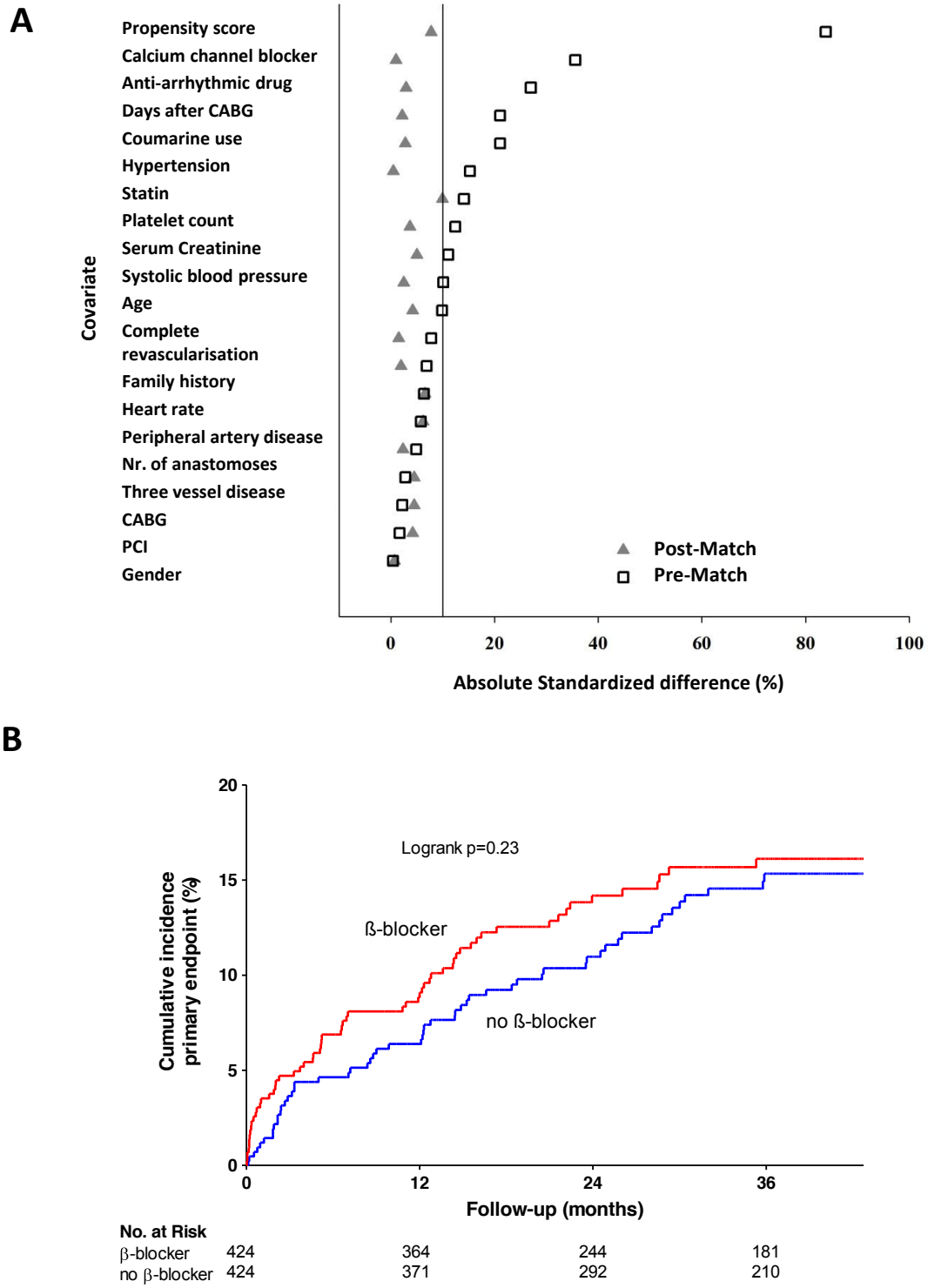

Figure 4 Propensity matched analysis – (A) standardized differences between baseline characteristics before and after matching. (B) Cumulative event rate for the primary endpoint in the propensity matched population. CABG, Coronary Artery Bypass Grafting; PCI, Percutaneous Coronary Intervention.

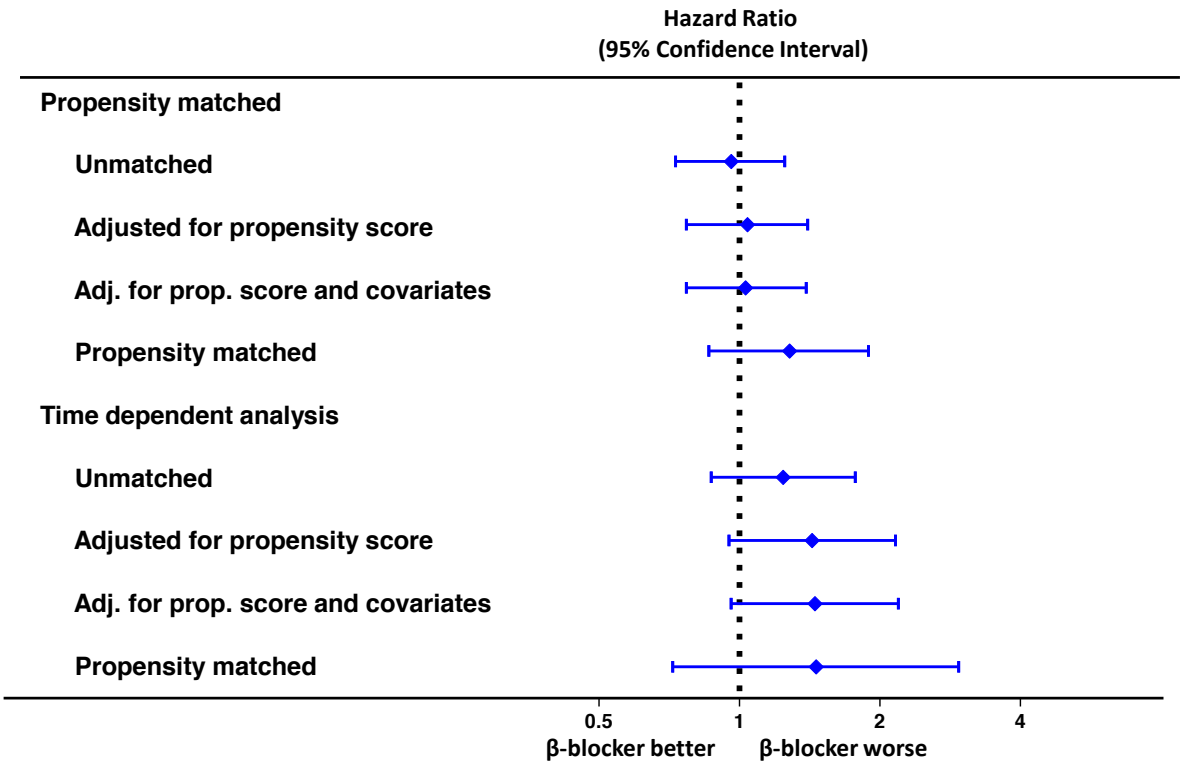

Figure 5 Risk for primary endpoint with propensity score and time-dependent analysis of β-blocker therapy - Analysis performed with β-blocker therapy at randomization and β-blocker as a time-dependent covariate.

Discussion

β-blocker therapy has been the cornerstone of pharmacotherapy of CAD for decades, but recent evidence suggests that this central role may not be justified in patients that are at relatively low risk, have good control of their cardiovascular risk factors and are receiving evidence-based therapy.[5,8,9] In patients receiving CABG, pre-operative β-blocker therapy has been reported to be as high as 80-93% over the last few years.[9] However, it is unknown if β-blockers should be continued after CABG. We therefore performed an explorative analysis to determine if β-blocker therapy was associated with reductions in the incidence of angina or cardiovascular events after CABG surgery in stable patients without heart failure or LV dysfunction. Additionally, we investigated the incidence of individual components of the composite outcome.

In our current retrospective analysis of low-risk patients, we show that β-blockers were frequently continued after CABG for a mid-term follow-up to 43 months. β-blocker use was, however, not associated with a decreased risk of recurrent angina or cardiovascular events, nor any of the individual components of the composite outcomes. Our results were consistent across different types of analyses, including propensity matched and time-dependent

analyses, suggesting that the lack of association between β-blocker therapy and clinical outcome is robust. This patient population was initially selected to test whether ACE-inhibition in patients with a low event-rate would have additional benefit on top of standard medical therapy. This low event-rate did naturally reduce the power of our analysis, but also indicates the potential benefit of β-blockers in this population is limited. While the results may be different in the general CABG population, our current data fuel the hypothesis that these agents should not be continued indiscriminately.

β-blockers in patients with CAD

β-blockers reduce heart rate, blood pressure and stroke volume, three key determinants of myocardial oxygen demand. In addition, β-blockers increase coronary blood flow through prolongation of the diastole. In concert, these mechanisms are deemed responsible for the beneficial effects of β-blockers. Indeed, several studies have convincingly demonstrated that β-blockers reduce the burden of angina in patients with obstructive CAD.[2,10] The prescription of β-blockers in patients with CAD is, however, more generally advocated based on the extrapolation of prognostic benefit observed in patients after MI and in heart failure patients.[2] Our study suggests that in low-risk patients after CABG surgery, these extrapolations may need additional investigation.

β-blocker therapy after revascularization

Several potential explanations may underlie the neutral effects of β-blocker therapy in our population. First, most patients were fully revascularized, thereby effectively removing the substrate for angina and potentially for cardiovascular events as well. Therefore the potential benefit of β-blockers seems less relevant for the occurrence of these events. Our findings are in line with an earlier study which showed no benefit of metoprolol on exercise capacity or myocardial ischemia in patients revascularized with CABG.[11]

Second, this study purposely selected CAD patients with low risk for cardiovascular events. Indeed, cardiovascular mortality was <1.4% over the median follow-up of 3 years, and the incidence of MACE was only 9.4%. This is similar to patient populations with cardiovascular risk factors, but without established CAD, underscoring the low incidence of cardiovascular events in the present population.[5,12] In fact, the recently published Study assessInG the morbidity-mortality beNefits of the If inhibitor ivabradine in patients with coronarY artery disease (SIGNIFY) which randomized 19 102 patients with normal cardiac function to the selective sinus node inhibitor ivabradine, failed to show a benefit on outcome.[13] Heart rate reduction is considered to be the most important mode of action of β-blockers in CAD. The neutral results of SIGNIFY therefore provide an additional line of evidence supporting that concept that modulation of the sympathetic tone is not generally effective after revascularization in low-risk patients. Of note, patients using β-blockers have

significantly lower heart rates compared to the reference group. β-blockers are particularly effective in patients with LV dysfunction,[14] which was previously common after MI. A recent study reported a mean LVEF of 54.8% in STEMI-patients who had received primary percutaneous coronary intervention.[15] In an analysis of contemporary patients with history of MI, β-blockers were not associated with a reduction in cardiovascular events.[5] Other studies only found a favorable association between β-blockers and cardiovascular events in patients with recent MI.[16-18] Together these findings suggest that the protective effects of β-blockers are confined to patients with a recent MI, ongoing myocardial ischemia or significant LV dysfunction.

Clinical implications

The results of this analysis of a low-risk population with normal cardiac function suggest that β-blockers do not have additional beneficial effects after CABG. This has not been studied in a prospective randomized trial. Therefore, there are no data supporting indiscriminate use of β-blockers in patients who are asymptomatic, are receiving evidence-based therapy for CAD and with good LV function after successful revascularization. This is reflected in the most recent AHA guidelines for management of stable ischemic heart disease with a class IIb recommendation for these patients.[19] The ESC guidelines do not mention a specific recommendation for β-blocker use in these low-risk asymptomatic patients.[1] Although β-blockers are still important drugs for the treatment of angina, recent MI and patients with LV dysfunction,[10,14,16-18, 20-22] their efficacy in other indications is under scrutiny. Controversy has risen about the effectiveness of β-blockers during non-cardiac surgery,[23] although it is still strongly recommended (class I) to continue β-blockers in patients who are already receiving these drugs.[24]

Limitations

The current analysis is essentially a retrospective analysis of prospectively collected data and, despite multivariate adjustments and propensity matching, residual confounding can never be fully eliminated. One might argue that the lack of benefit was partly caused through bias by indication. Patients on β-blockers might have been patients with a higher cardiovascular risk as these agents are often prescribed for residual angina, hypertension, atrial fibrillation or MI. On the contrary, patients not using β-blockers were treated with other anti-anginal drugs and had a slightly higher cardiovascular risk with higher LDL-cholesterol levels and a little less often anti-platelet therapy. Despite this apparently higher cardiovascular risk in the reference group, β-blockers were not associated with benefit in our analysis. Moreover, our analysis was adjusted for cardiovascular risk as rigorously as possible, including propensity matching. Another potential limitation of our analysis is that the sample size might be too low. Although this may true, we did not observe a trend towards

an effect. In addition, the adequately powered SIGNIFY trial, which tested a drug with a similar mechanism, did not demonstrate any beneficial effect despite a large sample size of 19 102 patients.[13] Considering the very low event rate in our analysis, a trial twice the size of SIGNIFY would be required to answer this question. Even if β-blockers would appear effective, the high number needed to treat would most likely not outweigh the risks and side effects.

Our results should be regarded as hypothesis generating. Consequently, firm treatment recommendations based only on the current analysis should be avoided. In addition, we investigated a low-risk population, with good control of their risk factors, and receiving evidence-based therapy for CAD. The results for a similar analysis in the general CABG population may be different. Nevertheless, as evidence to support the continuation of β-blockers after revascularization is currently absent, our analysis generates the hypothesis that general application of β-blockers to patients after CABG might not be justified.

Conclusions

β-blocker treatment after CABG in low-risk patients with preserved LV systolic function, good control of risk factors, and receiving evidence-based therapy, was not associated with a reduced incidence of cardiovascular events or angina during a median follow-up of 32 months.

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Chapter 3

Coronary revascularization in diabetic patients does not reduce their propensity to develop heart failure

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Submitted

Abstract

Aims

Diabetes predisposes to heart failure (HF), which is often caused by coronary artery disease (CAD). Coronary artery bypass graft surgery (CABG) prevents coronary events in these patients, but whether this also reduces their propensity to develop HF is unknown. We sought to determine whether CABG reduces the propensity to develop HF in diabetic patients with CAD and preserved cardiac function.

Methods

We analysed the IMAGINE trial database, which tested whether treatment with quinapril could reduce cardiovascular events after CABG in 2553 stable low risk patients without left ventricular dysfunction. Cox regression analysis was used to determine whether diabetes was associated with an increased incidence of HF hospitalisations and whether HF in diabetic patients could be explained by the recurrence of coronary ischemia.

Results

Diabetes was present in 253 (10%) patients (average HBA1c 6.8 ± 1.2%). Median follow-up was 2.95 years. The incidence of major adverse coronary events or a composite endpoint of all cause cardiac and cerebrovascular events was comparable between patients with and without diabetes. Diabetes was, however, associated with a 3-fold higher incidence in acute HF (adjusted hazard ratio 3.13; 95% confidence interval 1.36-7.19; p=0.007). Interestingly, acute HF in diabetic patients was never preceded by coronary events. In fact, the recurrence of documented ischemia was lower in patients with diabetes as compared to those without diabetes (adjusted hazard ratio 0.48; 95% confidence interval 0.25-0.95; p=0.034). The incidence of HF was comparable between patients randomised to quinapril or placebo.

Conclusions

Diabetes is associated with an increased incidence of acute HF in patients with preserved cardiac function after CABG. HF developed without the recurrence of epicardial CAD, suggesting that diabetes causes HF through other mechanisms such as a specific diabetic cardiomyopathy.

Introduction

Diabetes is associated with a two-fold higher lifetime risk of heart failure (HF).[1-5] Coronary artery disease (CAD) is a major contributor to HF in these patients, because diabetes increases the incidence, complexity and the propensity for recurrence of CAD.[6] Accordingly, CAD is treated aggressively in diabetic patients and often involves coronary artery bypass graft surgery (CABG). While CABG prevents future coronary events,[7] it is unknown whether CABG also influences the propensity to develop HF.

To test whether diabetic patients were more prone to develop heart failure after CABG, we used the IMAGINE (Ischemia Management with Accupril post bypass Graft via Inhibition of the coNverting Enzyme) trial database, which evaluated the impact of adding the angiotensin converting enzyme inhibitor (ACEi) quinapril to low risk patients with normal cardiac function after scheduled CABG. Our aim was to investigate whether CABG reduces the propensity to develop HF in patients with diabetes and preserved cardiac function. Due to the known beneficial effects of ACEi therapy in patients with established HF, one might expect that these agents would prevent new HF after CABG as well. The IMAGINE trial allowed us to test this hypothesis as well.

Methods

The design and results of the IMAGINE trial have been described in detail previously.[8-10] In brief, the IMAGINE study was a double-blind, placebo-controlled, parallel-group, randomized, multicenter international trial, which tested whether early initiation of an ACEi after CABG (initiated within the hospital phase) would reduce the rate of cardiovascular events in patients at relatively low risk. Patients provided written informed consent and were included between November 1999 and September 2004. The ethics committees of all participating institutions approved the research protocol.

Patients

In total, 2553 patients were included and subsequently randomized within 7 days after CABG to the ACEi quinapril or matching placebo. Due to increasing evidence for the benefit of ACEi therapy in patients with diabetes, insulin dependent patients or diabetic patients with significant micro albuminuria were no longer eligible for the study starting November 2001.

Endpoints

We used the same primary endpoint of the IMAGINE trial, as well as an additional composite endpoint of major adverse coronary events (MACE), defined as cardiovascular death or resuscitated cardiac arrest, nonfatal myocardial infarction, coronary revascularization or unstable angina that

required hospitalization, and considered all individual endpoints separately. An episode of documented ischemia was considered valid when typical symptoms were associated with temporary ST deviations on electrocardiogram; a stress test with reversible wall motion abnormalities on echocardiography or reversible nuclear scan defects; coronary angiography demonstrating new compatible lesions. The primary endpoint included the same endpoints as MACE with the addition of documented ischemia that did not require hospitalization, acute HF that required hospitalization and stroke. All individual endpoints were recorded continuously, so each patient could be scored for multiple endpoints. All endpoints, including acute HF hospitalizations were adjudicated by an endpoint committee.

Statistical analysis

Data are given as means ± standard deviation when normally distributed, as median and interquartile range in case of skewed distribution and as frequencies and percentages for categorical variables. Differences in variables between groups were compared with student T-test, Mann Whitney-U test, $\chi 2$ test or Fishers exact test, where appropriate. Differences between the diabetic and the non-diabetic groups were estimated as a hazard ratio with associated adjusted two-sided 95% confidence interval from a Cox proportional hazards regression model and we investigated whether ACEi therapy prevents new HF in diabetic patients. Cumulative event rates were calculated by the Kaplan–Meier method and displayed graphically. In an exploratory analysis, we investigated the incidence and sequence of coronary events and HF in patients with diabetes. All statistical analysis was performed using SPSS, Chicago version 18.0.

Results

Demographics of the study population

Of the 2553 patients included in the IMAGINE trial, 253 (10%) had diabetes, of which 96 (38%) were treated with metformin, 104 (41%) with sulphonylurea derivatives and 64 (25%) with insulin. Demographics of the population are given in *table 1*. Patients with diabetes were significantly older, more often female, less often completely revascularized and had a history of hypertension and higher systolic blood pressures more often than non-diabetic patients. All other demographics were comparable between diabetic and non-diabetic patients.

Effect of diabetes on the incidence of cardiovascular events after CABG

The median follow-up duration was 2.95 years. Diabetes was not associated with an increased incidence of the primary IMAGINE endpoint (Hazard ratio 1.06; 95% confidence interval 0.75-1.51; p=0.75) or MACE (Hazard ratio

Table 1. Demographics of the study population according to the presence of diabetes

Variable	Non-Diabetic (n=2300)	Diabetic (n=253)	p-value
Age	61 ± 10	62 ± 10	0.037
Female, n (% of patients)	283 (12)	41 (16)	0.035
White, n (% of patients)	2214(96)	239 (95)	0.691
Quinapril group, n (% of patients)	1159 (50)	121 (48)	0.097
Medical History, n (% of patients)			
Previous MI	897 (39)	104 (41)	0.487
Previous Stroke	47 (2)	7 (3)	0.632
Previous CABG	56 (2)	8(3.2)	0.382
Previous PCI	407 (18)	48 (19)	0.535
Hypercholesterolaemia	110 (9)	102 (11)	0.742
History of hypertension	1055 (45)	146 (57)	<0.0001
Current smoker	469 (20)	39 (15)	0.084
Laboratory values, Mean ± SD			
HBA1C	5.6 ± 1.2	6.8 ± 1.2	--
Haemoglobin (mg/dL)	75 ± 42	74 ± 43	0.802
LDL cholesterol (mmol/L)	2.8 ± 1	2.9 ± 1	0.094
HDL cholesterol (mmol/L)	1.1 ± 0.4	1.1 ± 0.3	0.191
Creatinine (µmol/L)	87 ± 18	88 ± 21	0.496
Hemodynamic measurements, Mean ± SD			
LVEF (%)	60 ± 9.7	59 ± 9.5	0.211
Systolic blood pressure (mmHg)	121 ± 14	124± 14	0.002
Diastolic blood pressure (mmHg)	70 ± 9	70 ± 9	0.865
Operative characteristics			
Beating heart surgery, n (% of patients)	427 (19)	49 (19)	0.723
Number of distal anastomosis, Mean ± SD	3.2 ± 1.1	3.2 ± 1.1	0.719
Triple vessel disease, n (% of patients)	1470 (64)	170 (67)	0.136
Complete revascularization, n (% of patients)	2044 (99)	211 (88)	0.031
Baseline medications , n (% of patients)			
Metformin	-	96 (38)	-
Sulphonylurea	-	104 (41)	-
Insulin	-	64 (25)	-
Beta blocker	1803 (78)	203 (80)	0.057
Calcium channel inhibitor	828 (36)	104 (41)	0.833
Angiotensin receptor blocker	58 (2.5)	14 (3.5)	1.000
ACE inhibitor	454 (20)	59 (23)	0.933
Platelet inhibitor	1693 (74)	198(78)	0.104
Statin	1490(65)	160(63)	0.394
Diuretic	202 (8.8)	32 (13)	0.092

SD, standard deviation; MI, myocardial infarction; CABG, coronary artery bypass graft surgery; PCI, percutaneous coronary intervention; LDL, low density lipoprotein; HDL, High density lipoprotein.

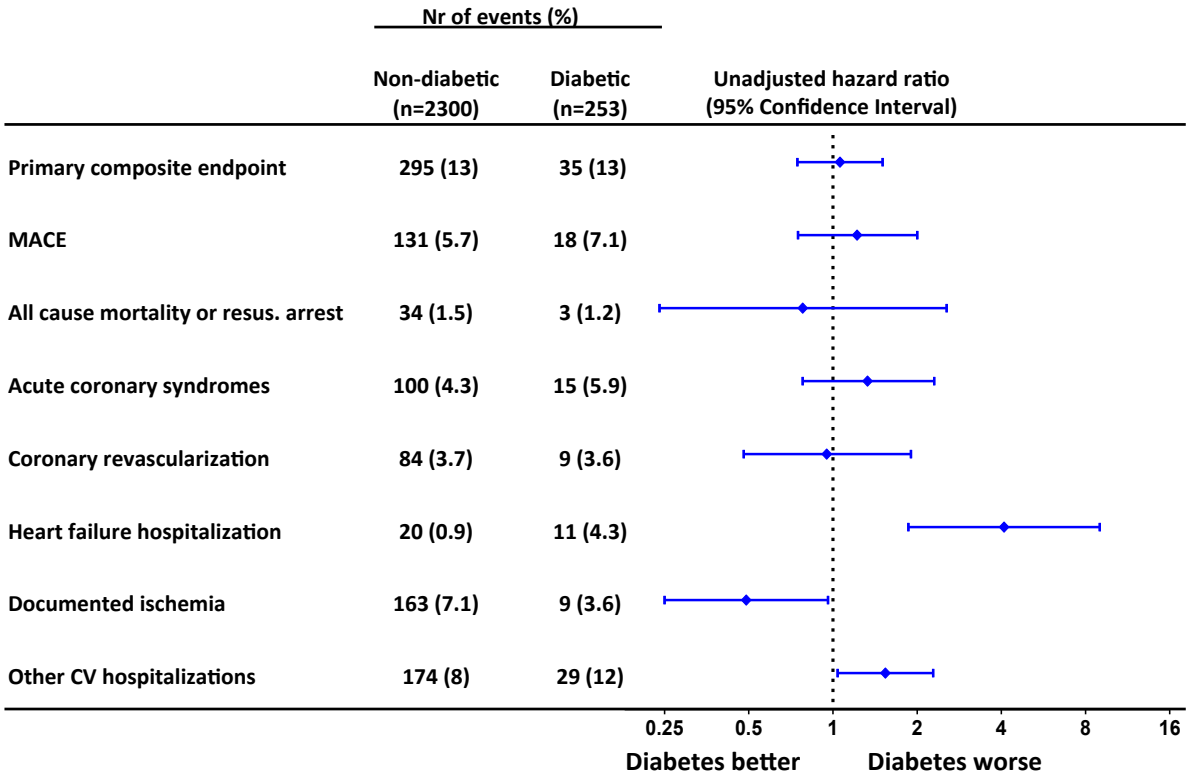

Figure 1 Hazard ratios for the composite endpoints and their components in diabetic compared to non-diabetic patients. MACE, Major Adverse Coronary Event; CV, Cardiovascular.

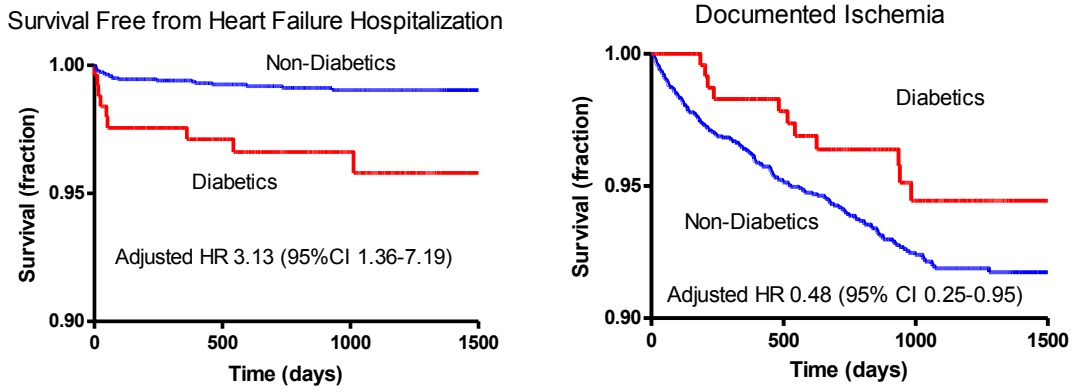

Figure 2 Survival rate free from heart failure hospitalizations HR, hazard ratio; CI, confidence interval. Adjusted for, age, gender, treatment assignment, days after CABG-surgery, baseline medications, left ventricular ejection fraction, systolic and diastolic blood pressure, creatinine, history of hypertension / percutaneous coronary interventions / myocardial infarction / previous CABG surgery / peripheral vascular disease / stroke, number of distal anastomosis, completeness of revascularization, and beating heart (off pump) surgery.

1.22; Confidence interval 0.75-2.00; p=0.43 *figure 1*). Patients with diabetes displayed a markedly higher incidence of new HF as evidenced by a 3-fold higher frequency of hospitalizations for acute HF (*figure 1, 2*). In contrast, patients with diabetes had a lower incidence of documented ischemia (*figure 1, 2*). The other components of the composite endpoints were comparable between patients with or without diabetes (*figure 1)*.

Exploration of heart failure and cardiovascular events in diabetic patients

In patients with diabetes, a hospitalization for acute HF was never preceded by a MACE or documented ischemia and only one patient with diabetes developed a MACE after the initial acute HF hospitalization. In contrast, 25% of the non-diabetic patients that developed acute HF experienced a MACE or documented ischemia during follow up. Randomization to quinapril in patients with diabetes did not affect the incidence of acute HF hospitalizations (Hazard ratio 1.11; Confidence interval 0.45-2.73; p=0.82, test for interaction p=0.778) or other components of the individual endpoints.

Discussion

In the current analysis, diabetes was associated with a markedly higher incidence of acute HF in low risk patients with a normal cardiac function during an average of 2.95 years of follow-up after scheduled CABG. Interestingly, acute HF in these patients was not preceded by evidence of acute or clinically worsening chronic myocardial ischemia, suggesting that mechanisms beyond epicardial CAD were responsible. Finally, quinapril did not influence the propensity for acute HF in these patients.

It is known that diabetes is associated with a worse prognosis, a higher incidence of CAD and an increased risk of developing ischemic HF.[11-14] Additionally, it has been suggested that diabetes causes a distinct, "diabetic" cardiomyopathy, through increased oxidative stress and activation of detrimental signal transduction pathways by glycosylation.[6,15-17] These specific processes underlying diabetic cardiomyopathy are not influenced by revascularization and will therefore continue to exert their detrimental effects on the heart. Accordingly, these pathways could cause HF to develop after adequate revascularization. Our finding that, over a mean 2.95 year follow-up period, revascularization does not prevent HF in diabetic patients with CAD supports the importance of non-ischemic mechanisms as drivers of HF in patients with CAD. However, because of the limited follow-up period, it does not exclude an eventual contribution of worsening CAD to the development of HF in some diabetic patients.

We studied patients with preserved cardiac function, and patients with diabetes had several risk factors for HF with preserved ejection fraction

(HFpEF), including older age, female sex and a history of hypertension.[18] Although ventricular function was not systematically evaluated when HF did develop, it is probable that in many cases HFpEF was the underlying structural abnormality, because no acute coronary event occurred and patients with diabetes had risk factors consistent with HFpEF.

Consistently with the main IMAGINE analysis,[9] we did not find evidence that ACEi therapy prevented HF development in our patients with diabetes. However, our analysis was not powered for this analysis and we can therefore not exclude the possibility that ACEi therapy prevents HF in diabetic patients at low risk, particularly over a long follow-up period. Indeed, several mechanisms, including preservation of renal function, likely contribute over time to reduce the risk of the development of HF in some patients with diabetes.

Despite extensive multivariable adjustments, a retrospective analysis cannot exclude all biases, particularly considering the limited number of events. Although increase in HF in diabetics was impressive, our results may actually reflect an underestimation of the relationship between diabetes and the development of HF because patients with severe diabetes were excluded from the trial. Also, because the IMAGINE study purposely selected low risk patients, should patients with more underlying cardiac disease had been included, the results may have been different.

Conclusions

Diabetes is associated with an increased incidence of acute HF in patients with preserved cardiac function after CABG. HF developed without the recurrence of epicardial CAD, suggesting that diabetes can contribute to HF through other mechanisms. Over a mean of 2.95 years, quinapril did not prevent the development of acute HF in patients with diabetes.

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Part 2

AKIP1 in cardiac stress

Chapter 4
Selecting heart failure patients for metabolic interventions

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Abstract

Introduction

Heart failure (HF) has become the cardiovascular epidemic of the century and now imposes an immense burden on health care systems. While our understanding of the pathophysiology of HF has increased dramatically, the translation of knowledge into clinical practice has been disappointing. Metabolic dysfunction in HF has been studied for eight decades, but these efforts have not translated into successful therapeutic interventions. This paucity probably results from the variable contribution of metabolic dysfunction to the underlying heart disease. A major unmet need in cardiac drug development is therefore the ability to identify a homogeneous subset of patients in whom HF is driven by a specific mechanism that can be targeted.

Areas covered

The available literature was evaluated to describe maladaptive metabolic perturbations that occur in failing hearts and may cause metabolic inflexibility, oxidative stress and cardiac energy depletion. Furthermore, the potential utility of various biomarkers and molecular imaging techniques to detect and quantify specific metabolic dysfunctions in HF were compared. Finally, we propose ways to utilize these techniques to select patients for specific metabolic interventions.

Expert commentary

Metabolic dysfunction is among the most promising therapeutic targets in HF. Meticulous patient-selection with molecular imaging techniques and specific biomarkers appears indispensable for the effective translation of decades of scientific knowledge into clinical therapeutics.

Heart failure: the scope of the problem

Over the past four decades, heart failure (HF) patients have derived substantial benefits from the advances in our understanding of the pathophysiology of this devastating disease. Multiple fundamental discoveries in cardiovascular biology have fueled novel treatment paradigms that have successfully been translated into clinical applications. As a result, HF has transformed from a simple descriptive entity treated with palliative measures into a well-defined syndrome that can be treated with a vast array of life saving drugs, interventions and devices.[1] Paradoxically, these advances have done little to reduce the population disease burden, including its economic impact. For instance, the rate of hospital admissions for acute HF in the United States has remained around 1 million per year since the beginning of the century. While the prognosis of HF has improved, the 5-year mortality rate is still worse than for most types of cancer.[2] In fact, HF is currently responsible for one in nine deaths in the US and Europe.[3] In 2012, HF affected 2% of the US adult population and 9% of those over 60 years of age, and the associated health care costs were estimated at 30.7 billion per year.[3] While health care systems are barely coping with the immense health care burden of HF as it is, the prevalence of HF and the associated costs are projected to increase by 50% within the next 15 years.[3] HF may thus be regarded as the most pressing cardiovascular epidemic of this century. New strategies to treat or prevent HF are therefore urgently needed.

Heart Failure therapy: is it time to transcend neurohormonal blockade?

The evolution of HF treatment is regarded as the epitome of evidence based medicine because current pharmacological regimens have all survived the scrutiny of randomized controlled trials in relatively unselected patient populations. With some exceptions, all HF patients are treated with the same drugs and devices irrespective of the underlying disease mechanism. The ability to treat such a heterogeneous disorder in such a homogeneous fashion may be explained by the fact that these drugs essentially target the systemic response to cardiac failure. Indeed, while HF drugs target very specific components of the sympathetic nervous system, the renin-angiotensin system or natriuretic peptide biology, they can all be regarded as interventions intended to restore the systemic neurohormonal balance. After 15 years without any progress in HF therapy, the PARADIGM-HF trial recently demonstrated that the combination of a neutral endopeptidase inhibitor with an angiotensin-receptor blocker was superior to the well-established angiotensin converting enzyme-inhibitor enalapril.[4] As both of these drugs target the neurohumoral system, the PARADIGM-HF trial clearly indicates that there are still viable treatment targets left within the neurohumoral system. However,

the increasing time-window between subsequent pharmacological innovations does suggest that we are approaching the asymptote for neurohormonal blockade.[2] Accordingly, there is a broad precedent to refocus drug development efforts from non-selective blockade of the systemic consequences of HF, towards interventions that target the specific underlying disease mechanisms within the heart.[5]

Over the years, multiple perturbations in the cardiac muscle have been identified that can cause HF or contribute to disease progression, including but not limited to altered cardiac excitation-contraction (EC) coupling, aberrant activation of signal transduction pathways, and dysfunctional myocardial metabolism.[6-11] While the role of these mechanisms in HF-development has been firmly established, their contribution to the underlying disease may vary among HF patients, as well as during different stages of disease progression within an individual patient. For instance, the expression and activity of the key calcium cycling molecule sarcoplasmic reticulum calcium ATPase (SERCA2a) is not uniformly reduced in myocardial tissue samples from HF patients and the degree of metabolic dysfunction (as specified below) also varies.[11,12] An intervention that targets a specific myocardial defect will probably not be effective or may even be harmful in patients in whom that particular mechanism is not causal. This may be one of the explanations for the lack off effect of SERCA2a gene transfer in the CUPID-II trial, as a reduction in myocardial SERCA2a expression was not an entry criterion for participation in this study.[13] Whereas routine interrogation of myocardial SERCA2a expression levels may cross ethical and practical boundaries, there are ample opportunities to evaluate metabolic dysfunction in HF that could routinely be incorporated in trial design for metabolic therapies. In the following sections we will therefore discuss major metabolic perturbations in HF and the potential utility of biomarkers and molecular imaging techniques to pinpoint underlying disease mechanisms and thereby select patients for specific metabolic interventions.

Metabolic dysfunction in heart failure

The heart pumps 10 tons of blood around the body each day for which it requires up to 30 times its own weight in adenosine triphosphate (ATP). The cardiac ATP stores are, however, only sufficient to sustain three heart beats. Cardiac function and myocardial ATP production are therefore heavily intertwined and small perturbations in the provision of carbon-based fuels and the efficiency of mitochondrial respiration can have major consequences for myocardial performance. The first indication that energy reserves are reduced in HF dates back more than eight decades, when Herrmann and Decherd discovered that creatine levels in failing hearts are reduced.[14] Based on these observations, it was hypothesized that energy deprivation leads to cardiac

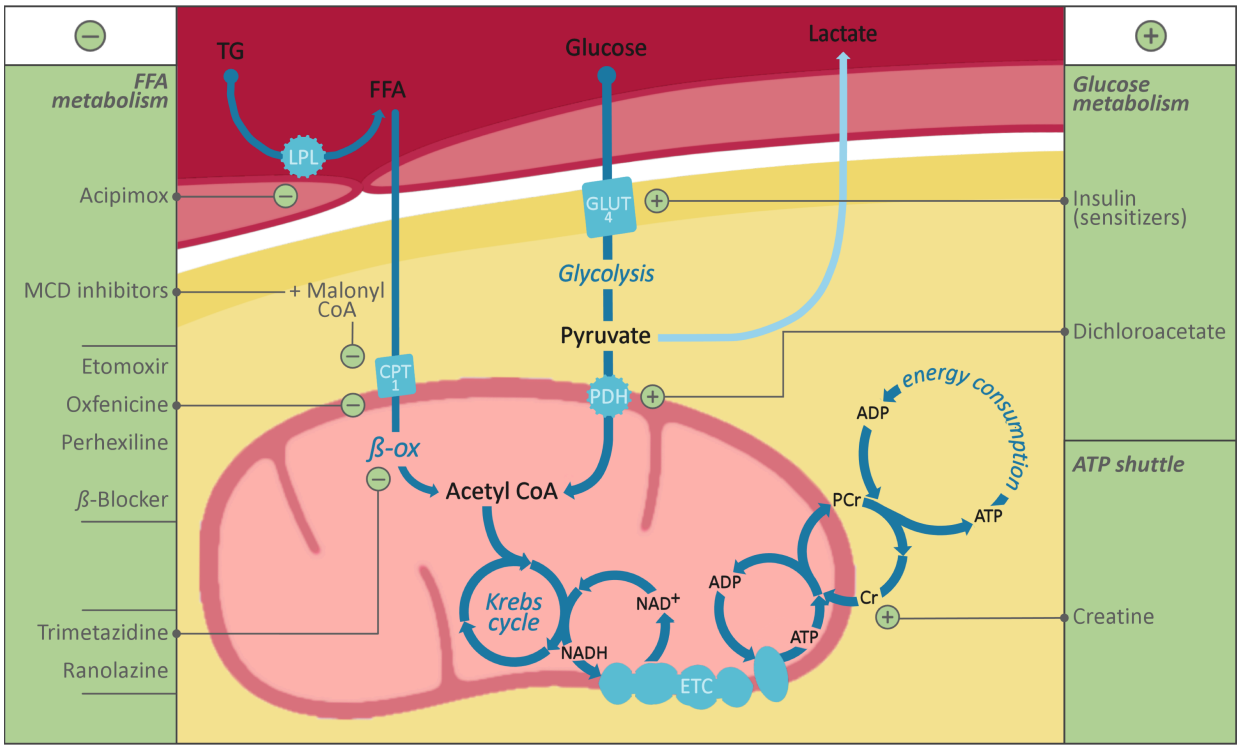

Figure 1 Therapeutic concepts to restore metabolic dysfunction in heart failure. Simplified schematic depicting cellular energy metabolism from substrate utilization to oxidative phosphorylation and energy transfer to the sites of energy consumption. Current therapeutic concepts to improve energetic efficiency in heart failure either target proportional substrate use by decreasing fatty acid metabolism (left panel), increasing glucose oxidation (right upper panel), or augmenting energy transfer (right lower panel). ADP, adenosine diphosphate; ATP, adenosine triphosphate; CPT, carnitine palmitoyltransferase; Cr, free creatine; FFA, free fatty acids; GLUT4, glucose transporter 4; PCr, phosphocreatine; PDH, pyruvate dehydrogenase; MCD, malonyl-CoA decarboxylase; NAD^+, NADH, oxidized and reduced forms of nicotinamide adenine dinucleotide; TG, triglycerides; β-ox, beta oxidation. Adapted from Doehner et al.[17]

dysfunction, equivalent to an engine running out of fuel.[11] With few exceptions, the majority of the evidence gathered since then has supported this hypothesis. In the following paragraphs we will provide an overview of the major metabolic changes that occur in the failing heart and briefly mention metabolic therapies that are in various stages of clinical development. Current therapeutic concepts to restore cardiac metabolism are summarized in figure 1.

Cardiac substrate preference

The heart has considerable metabolic flexibility as it can transform chemical energy stored in various carbon-based substrates, including fatty acids, glucose, lactate, ketones and amino acids, into ATP through oxidative phosphorylation.[15] In healthy resting myocardium 60-90% of the total ATP production is derived from β-oxidation of fatty acids. The second major fuel is pyruvate, which can be generated by glycolysis or lactate oxidation. During exercise as well as the initial stages of most cardiac pathologies, the heart

shifts from fatty acids to glucose as the primary source of energy. This shift in substrate preference is generally considered to be protective as it improves the stoichiometric ratio of oxygen consumption to ATP production.[16] β-oxidation of fatty acids also promotes uncoupling of the proton motive force generated by the mitochondrial respiratory chain and ATP synthesis, thereby reducing oxygen utilization-efficiency even further.[10-17] While the amount of energy generated per gram of substrate is higher for fatty acids compared to carbohydrates, the oxygen utilization-efficiency is much more favorable for pyruvate- than for β-oxidation.[17]

An additional aspect favoring carbohydrate metabolism in failing hearts is that 2 ATP molecules are generated anaerobically within the cytosol during glycolysis, i.e. the transformation of glucose into pyruvate. Glycolysis is the only source of ATP production under hypoxic conditions, but it is very inefficient as the net ATP yield is about 8% of that obtained from oxidative phosphorylation of pyruvate. In advanced HF, glycolytic enzymes and glycolytic activity are markedly induced while the mitochondrial capacity to oxidize pyruvate and other major fuels becomes impaired. This phenomenon results in uncoupling of glycolysis and glucose oxidation and promotes anaerobic glycolysis as the primary cardiac route of ATP production.[18] Because anaerobic glycolysis is an inefficient pathway for energy production, augmentation of glycolysis further aggravates the myocardial energy deficit. Accordingly, cardiac ATP depletion typically develops when glucose oxidation diminishes, and increased levels of circulating pyruvate and lactate can be detected in plasma samples of HF patients at this point.[19-21]

The reductions in glucose oxidation in advanced HF are at least partially caused by mitochondrial damage or dysfunction (discussed below). In addition, a functional block at the level of the pyruvate dehydrogenase (PDH) complex has also emerged as a central driver of this process.[17] PDH is considered the rate limiting step in glucose oxidation as it transforms pyruvate into acetyl-CoA, which subsequently enters the Krebs cycle. The negative regulator of PDH, PDH kinase, is strongly activated in failing hearts essentially blocking the cardiac capacity to oxidize carbohydrates.[22] It must be stressed, however, that these metabolic adaptations are not exclusive to cardiomyocytes or HF models as they occur in virtually all cell types subjected to hypoxic or metabolic stress.[23] Furthermore, while the data from animal experiments are more or less consistent, there is a large variation in substrate utilization in patients with cardiac hypertrophy or HF.[19,20] Finally, comprehensive metabolomics profiling recently indicated that the failing heart relies more on ketone bodies than on pyruvate, suggesting that advances in biochemical techniques may further refine contemporary paradigms.[24] In summary, the initial stages of pathological cardiac stress are accompanied by a marked augmentation of glycolysis and glucose oxidation, while advanced HF is

associated with a reduced capacity to metabolize all major fuels and increased dependence on anaerobic glycolysis.

Oxidative stress in heart failure

Oxidative stress is another key element of the pathophysiology of HF. Since the metabolic changes in HF are inextricably linked to the production of reactive oxygen species (ROS) by mitochondria, oxidative stress can be a cause as well as a consequence of HF. Even under physiological conditions, mitochondria produce superoxide (O_2^-) as a by-product of respiration.[25] Preferably, this free radical is broken down to water by manganese-dependent superoxide dismutase (MnSOD) and nicotinamide adenine dinucleotide phosphate (NADPH) dependent enzymatic reduction.[25] In HF, defective EC coupling and mitochondrial ion homeostasis impede activation of the Krebs cycle.[26] This not only disturbs regeneration of NADH to supply the electron transport chain (ETC) with electrons, but also that of NADPH required for adequate antioxidant capacity.[26] The consequent oxidative stress leads to a vicious circle in which oxidative damage to mitochondrial DNA causes increased electron leakage from the ETC and oxidation of proteins contributes to further derangement of EC coupling.[26,27] Adaptive changes associated with HF, including activation of the sympathetic nervous system and the renin-angiotensin-aldosterone system, aggravate this process by increasing cardiac energy supply-and-demand mismatch as well as direct stimulation of ROS production.[28,29] Mitochondrial ROS (mtROS) trigger increased mitochondrial permeability by activation of the mitochondrial permeability transition pore, among other pores and channels, allowing ROS to escape to the cytosol.[30] On emission, mtROS not only induce oxidative damage to non-mitochondrial structures, but also stimulate ROS production by other sources, including NADPH oxidase (Nox), uncoupled NO synthase (NOS), and xanthine oxidase (XO).[30] Reciprocally, ROS produced by these sources promote the production of mtROS.[30] ROS production in HF is summarized in figure 2.

Mitochondrial dysfunction and myocardial energy depletion

Mitochondria from failing hearts exhibit major structural and functional defects that diminish their capacity to generate ATP and increase superoxide release from the respiratory chain. The activity of respiratory chain complexes and ATP-synthase is significantly decreased in HF and the sensitivity to endogenous regulators of oxidative phosphorylation becomes diminished.[32] The contribution of mitochondria to other cellular processes such as cardiac calcium handling, cellular signaling and the regulation of cell death also becomes severely perturbed.[33] Mitochondrial defects are generally considered to result from ROS-mediated damage to mitochondrial DNA and proteins. However, uncontrolled activation of mitochondrial signaling pathways, transcriptional mitochondrial reprogramming and reduced elimination of damaged mitochondria by defective

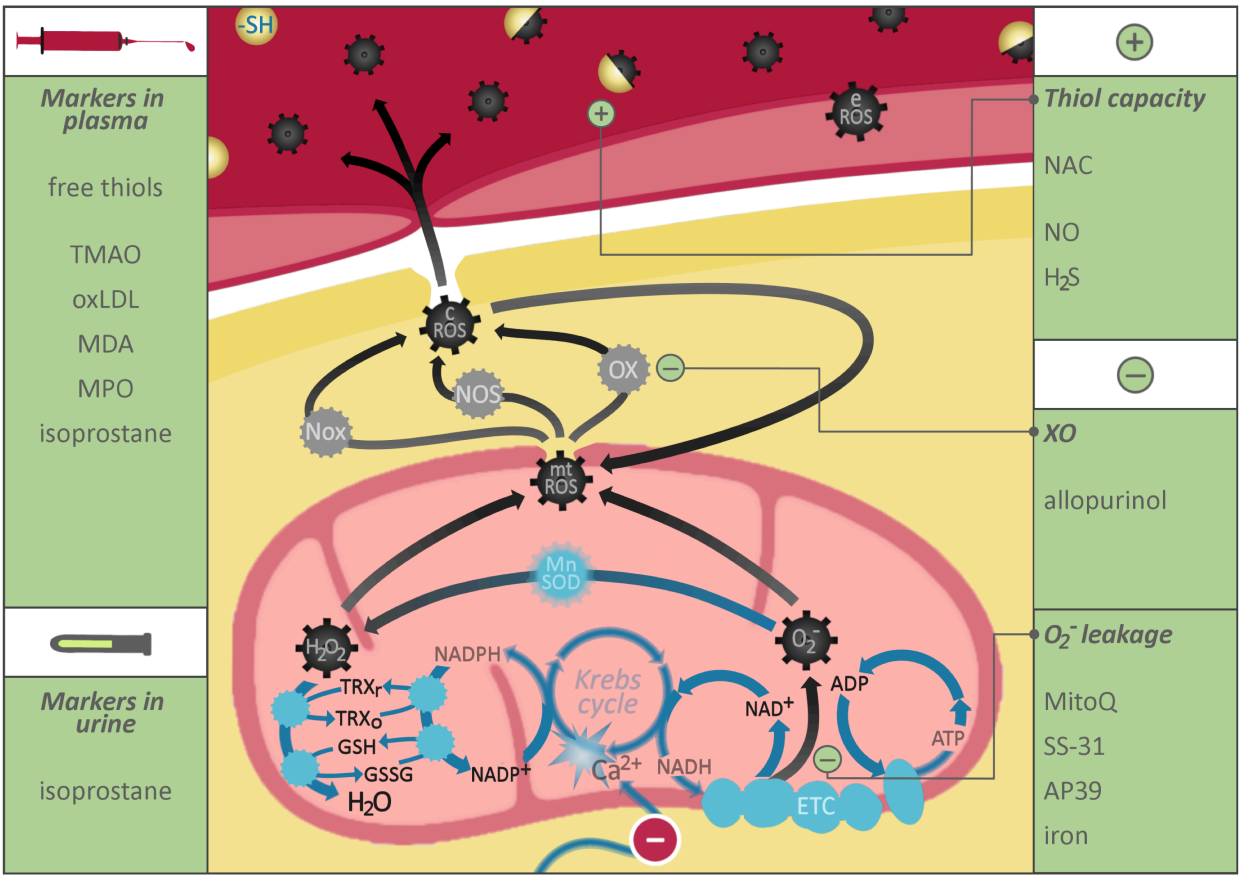

Figure 2 Oxidative stress in heart failure: mechanisms, markers and therapeutic concepts.

Simplified representation of the mechanisms causing oxidative stress in HF. MtROS production is linked to metabolic dysfunction through defective mitochondrial Ca^{2+} homeostasis. This impedes activation of the Krebs cycle, which in turn disturbs regeneration of NADH to supply the ETC with electrons and NADPH required for adequate antioxidant capacity. The consequent oxidative stress leads to a vicious circle of increased O_2^- leakage from the ETC and aggravated metabolic dysfunction. MtROS stimulates ROS production by cytosolic sources (cROS) and vice versa. The contribution of specific cell types, such as endothelial cells (producing eROS) may vary based on underlying disease mechanisms. Plasma and urine markers of oxidative stress are listed in the left panels. Therapeutic targets include O_2^- leakage (lower right panel), XO (middle right panel) and thiol capacity (upper right panel). ADP = adenosine diphosphate; ATP = adenosine triphosphate; Ca^{2+}, calcium ions; cROS, cytosolic ROS; eROS, endothelial ROS; ETC, electron transport chain; GSH, GSSG, reduced and oxidized forms of glutathione; H_2O, water; H_2O_2, hydrogen peroxide; H_2S, hydrogen sulfide; MDA, malondialdehyde; MnSOD, manganese-dependent superoxide dismutase; MPO, myeloperoxidase; mtROS, mitochondrial ROS; NAC, N-acetylcysteine; NAD^+, NADH, oxidized and reduced forms of nicotinamide adenine dinucleotide; $NADP^+$, NADPH, oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, NO synthase; Nox, NADPH oxidase; oxLDL, oxidized low-density lipoprotein; ROS, reactive oxygen species; TMAO, trimethylamine-N-oxide; TRX_r, TRX_o, reduced and oxidized forms of thioredoxin; O_2^-, superoxide; XO, xanthine oxidase; -SH, thiol.

mitophagy are now increasingly recognized as additional causal factors.[10,34,35] Nevertheless, current pharmacological strategies to alleviate mitochondrial dysfunction are primarily designed to attenuate mitochondrial oxidative stress with mitochondria-targeted anti-oxidants, such as Szeto-Schiller peptide (SS-31) and MitoQ.[36,37] Further advances in our understanding of mitochondrial regulatory mechanisms in HF may provide novel treatment paradigms that will allow us to specifically target the underlying disease mechanisms rather than its consequences. For instance, the endogenous regulation of ATP-synthase is still poorly understood and several key regulatory mechanisms have only recently been discovered.[38-40]

The transport of ATP from mitochondria to the sites of ATP-consumption within the cell is facilitated through the creatine kinase (CK) shuttle. To allow effective diffusion of energy throughout the cell, one phosphate group is transferred from ATP to creatine by mitochondrial CK to generate phosphocreatine. Phosphocreatine rapidly diffuses throughout the cell and ATP is subsequently regenerated from phosphocreatine by CK at the sites of ATP consumption. In addition to the catalysis of ATP transfer, the CK shuttle also acts as a cellular energy buffer. Whereas cardiac ATP levels only drop in the more advanced stages of HF, an energy deficit expressed as the phosphocreatine/creatine ratio, already becomes apparent before overt cardiac dysfunction develops.[41] Accordingly, the energy status of the heart can be expressed as the phosphocreatine/creatine ratio. As described above, the energy depletion hypothesis in HF was based on creatine depletion in failing hearts, perhaps making this the best-studied metabolic defect in HF. Despite eight decades of research and several clinical trials there are no therapies that can specifically restore cardiac energy levels or ATP transfer.[42] For instance, creatine supplementation failed to alleviate HF development in multiple clinical scenario's.[43,44] The importance of the cardiac CK shuttle was recently scrutinized in a mouse model of systemic creatine knockdown. While the hearts of these mice were fully depleted of creatine, cardiac function and exercise performance were not altered.[45] Despite the lack of successful interventions in ATP transfer mechanisms, and the debate surrounding its physiological relevance, the phosphocreatine/creatine ratio may still prove valuable as a biomarker to select patients for metabolic interventions.

Biomarkers for metabolic dysfunction in heart failure

Biomarkers for cardiac substrate preference and energy depletion

As described above, severe perturbations in cardiac glucose oxidation in advanced HF are associated with increased circulating lactate and pyruvate levels and these biomarkers have been used to monitor metabolic interventions in the past.[46] Nevertheless, such biomarkers to monitor myocardial substrate preference have limited sensitivity and specificity as they reflect a combination

of nutritional intake, gastrointestinal uptake and systemic substrate utilization in all organs. Likewise, it will be very hard to untangle cardiac and extracardiac contributions to circulating levels of markers for ATP depletion, such as circulating ADP or AMP levels. Advances in metabolomics profiling have more recently been adopted to discover biomarkers with higher specificity for cardiac energy homeostasis. For instance, long-chain acylcarnitines have emerged as very promising biomarkers for disrupted β-oxidation, but require further validation.[47,48] A more direct approach to evaluate cardiac substrate utilization is the comparison of metabolite levels in aortic and coronary sinus blood samples, which allows more detailed analysis of cardiac substrate use, including metabolic substrate flux and ATP production efficiency.[49] The obvious drawback of this technique is the invasive nature and this type of analysis is typically reserved to monitor the effect of invasive procedures such as cardiac resynchronization therapy.

Biomarkers for oxidative stress

As extensively described elsewhere, several biomarkers are commonly used to assess oxidative stress in HF. These include oxidized low-density lipoprotein, malondialdehyde, and myeloperoxidase in plasma, and isoprostane in plasma and urine.[50] The gut microbe-generated metabolite trimethylamine-N-oxide (TMAO), which is adversely associated with HF prognosis, has also been proposed to be related to oxidative stress.[51] Some of these traditional biomarkers, particularly aldehydes, have been suggested to further increase ROS formation, although studies on their role in pathophysiologiology of cardiovascular diseases are conflicting.[50] Aldehydes are formed during many processes including lipid peroxidation.[52] During HF, increased ROS production decreases the activity of aldehyde dehydrogenase 2.[53] This causes an accumulation of aldehydes, including malondialdehyde and 4-hydroxy-2-nonenal (HNE), which localize to mitochondria and further increase mitochondrial ROS formation through reactions with nucleophilic protein residues.[52,54,55] Aldehyde levels can be detected in the plasma and several studies have indicated that plasma Aldehyde levels can serve as biomarkers for oxidative stress. For instance, lipid peroxidation has been investigated as a biomarker for artherogenesis.[56] Nevertheless, it should be noted that, regardless of their pathological significance, aldehydes are products of the reaction of ROS with lipids, and thus arise secondarily to the process that initiated oxidative stress. Thus while aldehydes may serve as biomarkers, it is unlikely that they will serve as a nodal point for metabolic interventions.[50] Consequently, these biomarkers can be used as a marker for oxidative stress, but may not for the selection of patients for specific metabolic interventions.

Serum free thiols

Recently, the serum free thiol level regained interest as an indicator of oxidative stress in the context of chronic HF. Reactive species can oxidize their targets by reacting with thiols, i.e. functional groups composed of a sulfur and a hydrogen atom. Because free (or reduced) thiols are readily oxidized by reactive species, their level may be seen as a direct reflection of the balance between oxidants and antioxidant capacity, and thus the overall level of oxidative stress.[57,58] What makes free thiols different from other biomarkers is that apart from reflecting the overall level of oxidative stress, thiols are critical active components of the antioxidant defence, which may be receptive to therapeutic modulation. So far, most studies looking into antioxidant machinery have focused on low molecular weight thiols, in particular glutathione en cysteine. For example, a recent study has shown an association between higher cystine (i.e. oxidized cysteine) and lower glutathione levels and an increased mortality risk of coronary artery disease patients.[59] However, whereas these low molecular weight thiols are key actors in intracellular antioxidant defense, in the extracellular compartment they are strongly outnumbered by protein thiols.[60] In fact, the single sulfhydryl group of albumin, the most abundant serum protein, accounts for the majority of thiols in serum.[60] Hence, compared to the concentration of low molecular weight thiols, the total free thiol level may be a more relevant circulatory biomarker of oxidative stress.

Depletion of circulatory free thiols has been shown in patients with cardiovascular disease (CVD), including acute myocardial infarction.[57,61,62] Also, thiol oxidation has been linked to established CVD risk factors, such as aging, smoking and obesity.[63] Moreover, our group has recently reported a positive association between protein-adjusted serum free thiols and a favorable disease outcome in a small cohort of 101 stable chronic HF patients.[64] This finding suggests that serum free thiols provide a robust reflection of redox status and warrants confirmation in a larger cohort.

Gaseous signaling molecules NO and H_2S

Reversible oxidative modifications of protein thiols by several small molecules may protect proteins from irreversible oxidative damage and, in some cases, alter protein function.[58] Among these small molecules are gasotransmitters nitric oxide (NO) and hydrogen sulfide (H_2S).

NO, also known as endothelial derived relaxing factor, has long been acknowledged for its versatile role in cardiac physiology.[65] As the alias suggests, it is an important regulator of vascular tone, among numerous other bodily processes.[65] In HF, NO bioavailability is assumed to be reduced.[66,67] At the same time, increased levels of NO metabolites, nitrite and nitrate (NOx) have been described in HF, possibly reflecting impaired renal excretion.[68,69] More recently, H_2S has been recognized as a cardiovascular signaling molecule,

similar to NO. Like NO, H_2S is involved in the regulation of various (patho)physiological processes and features vasodilatory, antioxidant and anti-inflammatory properties.[70]

Unfortunately, reliable methods for direct detection of NO and H_2S in biological samples are lacking. Alternatively, quantification of their metabolites – i.e. NOx for NO and thiosulfate and sulfate for H_2S – may be used to evaluate changes in the metabolism of these gaseous signaling molecules. Naturally, other factors that may be of influence, such as intake and renal function, should be considered in this process. To date very few studies have looked into concentrations of NO and H_2S metabolites in relation to outcome of cardiovascular disease. One paper describes a higher plasma concentration of NOx 24 hours after onset of ST-segment elevation myocardial infarction to be associated with an increased risk of one-year all-cause mortality or rehospitalisation.[71] Another study has found both urinary thiosulfate and sulfate concentrations of renal transplant recipients to be positively associated with a favorable cardiovascular risk profile and patient survival.[72]

Molecular imaging of cardiac metabolism

Positron emission tomography

Positron Emission Tomography (PET) is a three dimensional imaging technique that can monitor biological processes *in vivo* through the use of molecules that have been labeled with positron emitting radionucleotides. PET already has a central role in clinical cardiac diagnostics, for the evaluation of myocardial perfusion and viability in ischemic heart disease, the differential diagnostics of cardiomyopathies, and the diagnosis and risk-stratification of endocarditis. PET also holds great promise for cardiac drug development as virtually every molecule can be modified to be detectable by PET.[73] The most abundantly used clinical PET-tracer, ^{18}F-FDG, was specifically designed to monitor glycolytic activity and tracers for β-oxidation have also been developed for clinical application. These tracers accurately monitor changes in glycolysis and β-oxidation in experimental animals, as well as in patients with HF.[74-77] In particular ^{18}F-FDG PET is a sensitive marker for cardiac glucose uptake, and appears to have reasonable accuracy for the prediction of a treatment response to various interventions.[78-80] More studies are, however, required to further define the utility of PET as a biomarker for cardiac substrate utilization in HF.

Monitoring and quantification of focal oxidative stress in tissues has several potential clinical applications and several PET-probes have been developed for the detection of hypoxia or ROS production, mostly for oncological or for cardiovascular applications.[81-83] Unfortunately, the cardiac uptake of these tracers has been disappointing as they provide insufficient signal intensity to detect the mild hypoxia or ROS production in HF. There are,

however, several promising novel tracers in development and these may prove more reliable for application in HF.[84,85] Of particular interest for ROS monitoring in HF is radiolabeled vitamin C ([11C]Ascorbic acid), that exhibits ROS-dependent cellular accumulation which could result in significant augmentation of signal intensity. Accordingly, PET holds great promise for the selection and monitoring of patients for metabolic interventions.

Magnetic Resonance Spectroscopy

Cardiovascular magnetic resonance imaging (MRI) has emerged as the primary method for myocardial tissue characterization in clinical cardiology, but few people realize that MRI is in fact a molecular imaging technique. Traditional MRI employs the combination of a high magnetic field to align hydrogen nuclei, the most abundant MR active nuclei in the body, and radio waves that causes these nuclei to resonate. When hydrogen nuclei relax from their resonance, they emit radio waves that can be detected. Differences in relaxation time in various tissues result in differences in resonance intensity that can be transformed to create images. These images can be used to evaluate myocardial tissue characteristics, such as the presence of fibrosis or edema, and also allows for detailed quantification of cardiac volumes and function in HF. Nuclear Magnetic Resonance Spectroscopy (MRS) utilizes the same hardware and radio waves, but rather than creating an image from the signal that it receives, it detects distortions in magnetic resonance frequency within the tissue. This so called chemical shift results from the interactions among neighboring nuclei and electrons in the tissue. As specific molecules generate a specific shift in resonance frequency, detailed analysis of the frequencies can be used to quantify the concentrations of various abundant metabolites in tissues.(figure 3) MRS can be used to measure the concentrations of molecules rich in protons (^{1}H) but also less abundant nuclei such as phosphorus (^{31}P), carbon (^{13}C), or sodium (^{23}Na). While several metabolites can be detected with MRS, for human applications, ^{1}H MRS has primarily been used to detect cardiac creatine and triglyceride content, ^{31}P MRS to detect cardiac ATP, phosphocreatine and phosphocreatine/creatine ratio's and ^{23}Na to monitor cardiac sodium content (figure 3).[86] Despite its vast potential, cardiac MRS is a challenging technique as measurements need to be adjusted for cardiac and respiratory motions. The time required to measure MRS spectra varies between 30 seconds and 20 minutes for ^{1}H MRS and 20 and 30 minutes for ^{31}P MRS, depending on the gating technique and the pulse sequences employed. Even though it can be performed on most commercially available scanners, currently, MRS is primarily used as a research tool in specialized centers. The most commonly studied MRS-derived parameters are myocardial triglyceride content and phosphocreatine/creatine ratio's.[86] The latter appears to have remarkable specificity for changes in cardiac energy stores. For instance, a study in type 2 diabetic patients was capable of detecting a transient, 12%

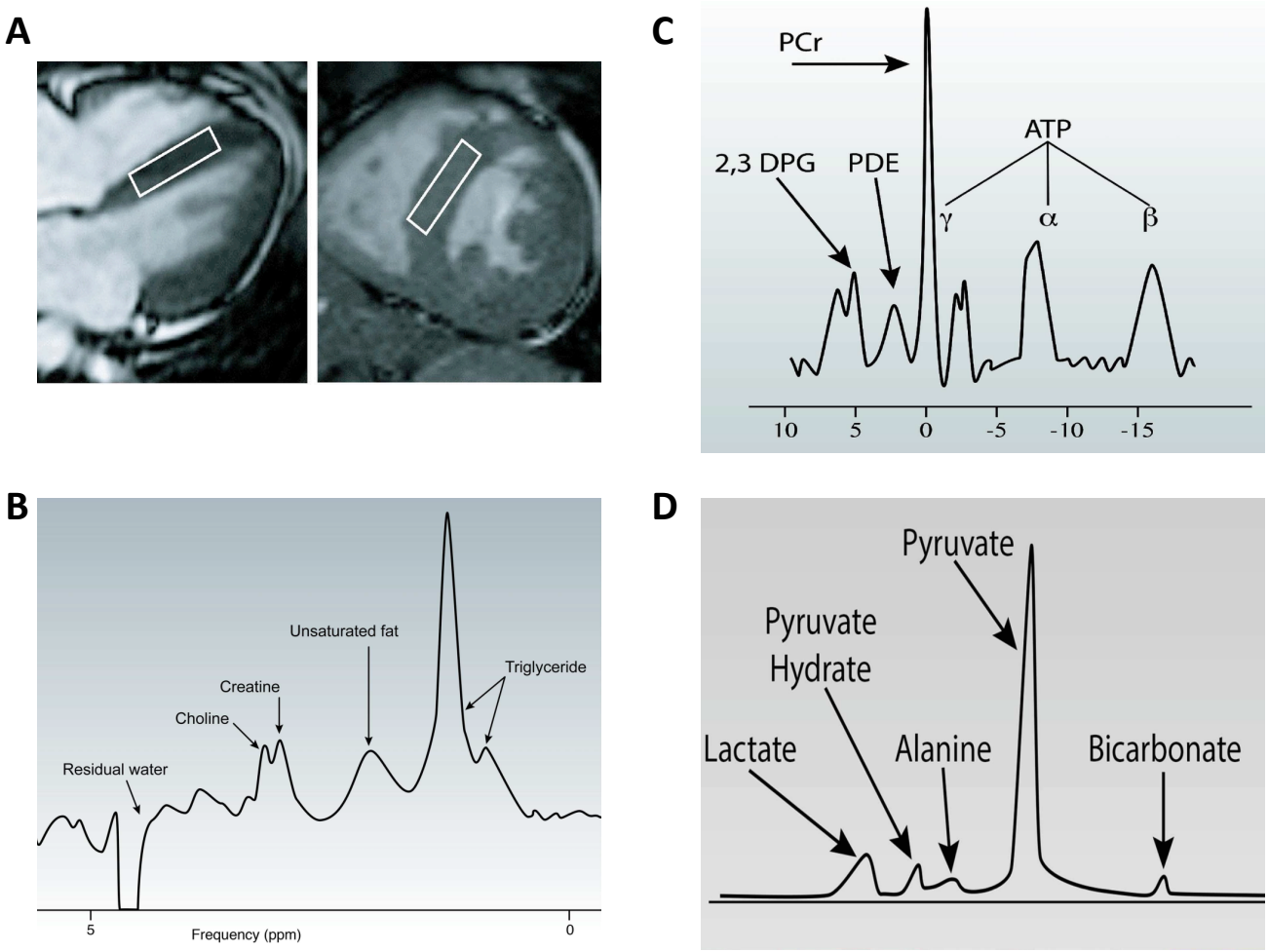

Figure 3 Cardiac magnetic resonance spectroscopy.

(A) Four chamber and short axis magnetic resonance image of a human heart. The white square depicts the spectroscopic volume that is typically placed in the intraventricular septum to reduce motion artifacts. (B) Typical ^{1}H spectrum of the intraventricular septum. Because water is the most abundant substance, the images are acquired after a water suppression pulse. (C) Typical ^{31}P magnetic resonance spectrum of the left ventricle. (D) Typical ^{13}C spectrum of the pig heart obtained after infusion of a hyperpolarized 1-^{13}C pyruvate tracer. ATP, adenosine triphosphate; 2,3 DPG, 2,3-diphosphoglycerate; PDE, phosphodiesters; PCr, phosphocreatine. Adapted from Bizino et al.[81]

reduction in cardiac phosphocreatine/creatine ratio during exercise.[87] MRS has been used to monitor cardiac energy levels for more than 25 years and can detect changes in high energy phosphate levels during treatment.[88] As such, it could be regarded as the gold standard for the evaluation of energy stores.

The application of MRS as a tool to study cardiac ROS is limited, although myocardial oxygen levels can be measured with MRI using blood oxygen level-dependent sequences.[89] In theory, ^{13}C MRS holds a vast potential for metabolic imaging because cellular energy is derived from carbon based fuels. Unfortunately, the clinical application of native ^{13}C MRS is limited by the

low abundance and low sensitivity of ^{13}C nuclei to MR pulses. To circumvent this limitation, a hyperpolarization technique has been developed that can increase the signal of specific ^{13}C-containing molecules by more than 10,000 fold, using a combination of free radicals and intense freezing under high magnetic field strengths. These hyperpolarized metabolites are subsequently injected into the bloodstream and their cardiac uptake and transformation into downstream metabolites can be monitored. Hyperpolarization has a short half-life and cannot be used to enhance native molecules within the heart. It can be used to monitor metabolic processes *in vivo*, including the PDH flux, β-oxidation and the Krebs cycle.[90] In experimental animals, hyperpolarized ^{13}C MRS can effectively monitor changes in substrate utilization during the progression of HF and the effects of interventions in cardiac substrate utilization.[20,91,92] Regulatory approval of hyperpolarized ^{13}C MRS for application in humans is pending and, considering its potential, eagerly anticipated.

In summary, PET is a well-established technique that could be regarded as the gold standard to monitor cardiac substrate utilization and is a promising technique for myocardial ROS detection. Systemic redox status can be measured with circulating and urinary biomarkers, although cardiac specificity remains limited. MRS is the gold standard for the detection of cardiac energy levels and holds great promise for the detection of cardiac substrate utilization through hyperpolarization techniques, but clinical validation is still pending. Accordingly, we propose that PET should be employed for patient selection and monitoring when interventions in cardiac substrate utilization are considered, whereas biomarkers, such as total serum free thiols, should be used to select patients for anti-oxidant treatments. Finally, MRS could be used to monitor interventions in cardiac ATP production, but may also serve to monitor the efficacy of other metabolic interventions. A scheme depicting the potential value of biomarkers, PET and MRS to select HF patients for metabolic interventions during various stages of disease progression is summarized in figure 4.

Therapeutic interventions in cardiac metabolism

Therapeutic interventions in cardiac substrate preference

As the shift from fatty acid to glucose oxidation is generally considered a physiological adaptation to stress, and several therapeutic strategies have been developed that aim to promote glucose oxidation in failing hearts.[16] These drugs either promote cellular glucose uptake, augment glucose oxidation by restoring PDH complex functionality or inhibit fatty acid β-oxidation (as summarized in figure 1 and extensively reviewed in ref. 17).[17] While there have been some encouraging improvements in symptoms and exercise performance with these drugs such as dicholoracetate and etomoxir, phase 3 clinical trials have been neutral or were stopped early due to drug toxicity or

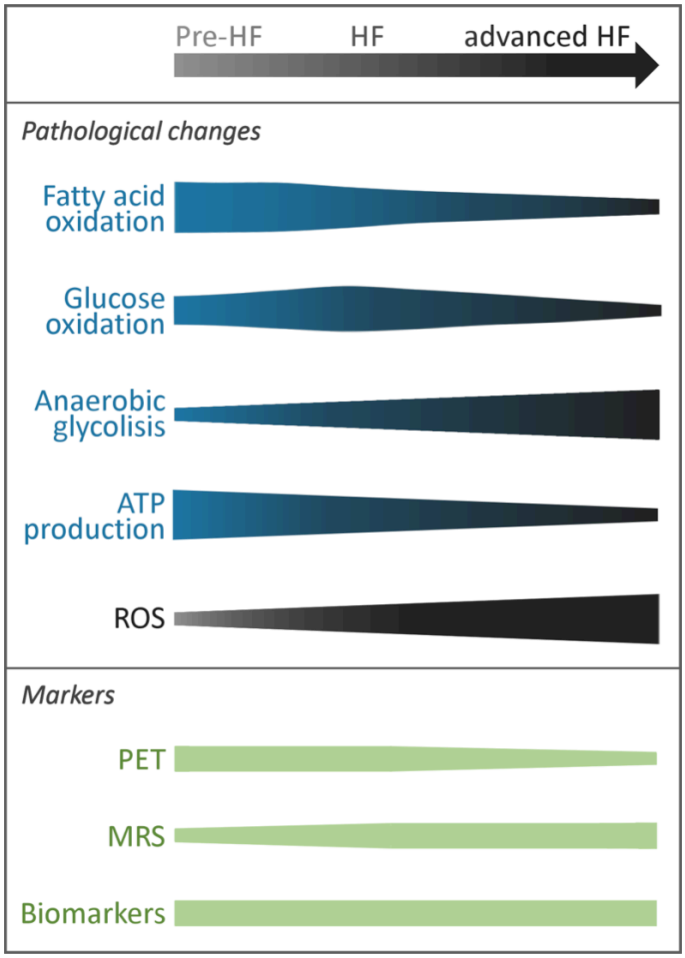

Figure 4 Pathological changes and applicability of markers during heart failure development.
Scheme illustrating the pathological changes and applicability of PET, MRS and biomarkers to select patients for metabolic interventions during various stages of HF development. ATP, adenosine triphosphate; HF, heart failure; MRS, magnetic resonance spectroscopy; PET, Positron Emission Tomography; ROS, reactive oxygen species.

increased HF events.[17,93-96] While this might be regarded as proof that reductions in glucose oxidation reflect an adaptive mechanism or that that the therapeutic interventions were poorly designed, it should be borne in mind that defective cardiac substrate utilization was not an inclusion criterion for these studies. In selected patients, the outcomes of these studies may have been positive.

Therapeutic interventions to alleviate myocardial oxidative stress
As outlined above, excess ROS production is a key factor in the pathophysiology of HF. At the same time redox signaling is an integral part of physiology and lower levels of ROS are involved in all kinds of beneficial cellular processes, including immunity, cell growth and apoptosis.[97] Besides, recurrent episodes of oxidative stress have been found to up-regulate

endogenous antioxidant mechanisms, which in turn may promote health and longevity.[97] Thus, disturbance of the physiological functions of ROS is likely to have contributed to the contradicting findings of studies evaluating uncontrolled and untargeted antioxidant therapies, including N-acetylcysteine (NAC) and vitamins, in cardiac disease.[98,99] Also, when a specific ROS producing component is disarmed, this may adversely affect other parts of the redox network. For example, this may in part explain the disappointing results of treatment with an XO inhibitor in HF, as this substance prevents XO from producing O^{2-}, but also decreases NO production.[100] Perhaps combination therapy of an XO inhibitor and an NO donor can resolve this problem. Moreover, physiological regulatory processes may prevent administered antioxidants from reaching adequate bioavailability, unless there is a deficiency to overcome.[97] Finally, oxidation of an antioxidant substance before or during ingestion may not only abate its efficacy, but in fact induce oxidative stress.[97]

Despite these challenges, in patients with aberrant endogenous antioxidant capacity, antioxidant therapies may well be beneficial. For example, restoring redox status by therapeutic modulation of free thiols may hold promise to improve disease outcome in HF. Indeed, in multiple clinical trials, the cysteine derivative NAC has been shown to both directly reduce disulfide bonds and act as a glutathione precursor.[101] Other thiol compounds, with similar modes of action, have also been described.[102] Although therapeutic results have been inconsistent, these compounds may prove to be effective in selected patients with low free thiol concentrations. Alternatively, pharmacological stimulation of molecules that induce reversible protein modifications, including NO and H_2S, may offer opportunities to influence the amount of free thiols, as well as favorably steer protein function.[58,65,70] One H_2S releasing compound, AP39, may be of particular interest as it is specifically targeted to mitochondria, one of the most important sources of ROS in HF. In experimental models AP39 has been shown to attenuate H_2O_2 induced cytotoxicity and to positively affect vascular tone and intracellular calcium homeostasis.[103] However, further research is needed to prove its clinical applicability. Other mitochondria-targeted antioxidants, including MitoQ and SS-31, have also shown promise in experimental HF. Moreover, SS-31 is currently investigated in a clinical trial focused on myocardial reperfusion injury and a trial studying this compound it the context of HF will soon begin.[36,104]

Expert commentary

Although HF patients are treated with up to 5 different classes of drugs, their mode of action is roughly similar as they can all be regarded as interventions that restore the systemic neurohormonal balance. Despite continuous refinements in this strategy, multiple lines of evidence indicate that we are approaching the asymptote for added benefit of neurohormonal interventions.

Rather than focusing on the secondary effects of HF, we believe that we should refocus drug development efforts towards interventions that target specific pathologic changes within the heart. Despite tremendous advances in our understanding of the molecular perturbations that occur within the failing heart, the translation of this knowledge into clinical practice has been disappointing. The most likely explanation is that the contribution of a given defect to the underlying heart disease is highly variable, highlighting the need to select a homogeneous subset of patients in whom HF is driven by a specific mechanism that can be targeted. This is particularly true for myocardial metabolism, which has been studied for the better part of a century, but has not proven to be amendable by specific therapeutic interventions. Perhaps the most daunting challenge for metabolic drug development is the paucity of specific tools to study cardiac metabolism in an individual patient. Metabolomic analysis of plasma lacks cardiac specificity and isolation of cardiac muscle may identify specific metabolic defects, but cannot be applied to intervention trials, as it requires ventricular biopsies. Cardiac PET is a highly versatile technique that allows virtually every molecule to be modified for detection by PET. Nevertheless, the technique is limited by variations in signal to noise, the inability to distinguish tracers from metabolites and by the fact that it exposes subjects to ionizing radiation. Another key limitation in metabolic research in HF is the fact that there is no evidence to support that the level of energy depletion is causing cardiac dysfunction. It is possible that the metabolic changes in the myocardium reflect beneficial cardiac adaptation to stress or even an epiphenomenon.

Five-year view

Eight decades of metabolic research in HF have not provided us with reliable metabolic interventions for this devastating disease. Over the next five years we anticipate that several metabolic interventions will be tested in clinical trials. One of the most promising and timely interventions is the mitochondria-targeted anti-oxidant SS-31 (also known as Bendavia), which has been established as safe and well-tolerated in patients after an acute myocardial infarction.[104] While a conference abstract indicated that Bendavia is effective in HF patients as well, appropriately sized trials have not started yet. We expect that the success of these interventions depend on the individual degree of mitochondrial dysfunction and oxidative stress. Accordingly, we believe that the biomarkers described above should be employed for patient selection and monitoring. Furthermore, we expect that hyperpolarized MRS will replace PET as the gold standard for the assessment of cardiac substrate utilization, as it can link metabolism to function, differentiate between tracers and metabolites, and avoids ionizing radiation. Whether biomarker-based patient selection will

illuminate pathways forward for metabolic interventions in HF remains to be established.

Conclusions

Metabolic dysfunction is among the most promising therapeutic targets in HF. Meticulous patient-selection with molecular imaging techniques and specific biomarkers appears indispensable for the effective translation of decades of scientific knowledge into clinical therapeutics.

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Chapter 5

Overexpression of A kinase interacting protein 1 attenuates myocardial ischemia / reperfusion injury, but does not influence heart failure development

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Abstract

Aims

A kinase interacting protein 1 (AKIP1) stimulates physiological growth in cultured cardiomyocytes and attenuates ischemia / reperfusion (I/R) injury in *ex vivo* perfused hearts. We aimed to determine whether AKIP1 modulates the cardiac response to acute and chronic cardiac stress *in vivo*.

Methods and results

Transgenic mice with cardiac-specific overexpression of AKIP1 (AKIP1-TG) were created. AKIP1-TG mice and their wild type (WT) littermates displayed similar cardiac structure and function. Likewise, cardiac remodeling in response to transverse aortic constriction or permanent coronary artery ligation was identical in AKIP1-TG and WT littermates, as evidenced by serial cardiac magnetic resonance imaging and pressure-volume loop analysis. Histological indices of remodeling, including cardiomyocyte cross-sectional diameter, capillary density and left ventricular fibrosis were also similar in AKIP1-TG mice and WT littermates. When subjected to 45 minutes of ischemia followed by 24 hours of reperfusion, AKIP1-TG mice displayed a significant 2-fold reduction in myocardial infarct size and reductions in cardiac apoptosis. In contrast to previous reports, AKIP1 did not co-immunoprecipitate with or regulate the activity of the signaling molecules NF-kB, protein kinase A or AKT. AKIP1 was, however, enriched in cardiac mitochondria and co-immunoprecipitated with a key component of the mitochondrial permeability transition (MPT) pore, ATP-synthase. Finally, mitochondria isolated from AKIP1-TG hearts displayed markedly reduced calcium induced swelling, indicative of reduced MPT pore formation.

Conclusions

In contrast to *in vitro* studies, AKIP1 overexpression does not attenuate cardiac remodeling in response to chronic cardiac stress. AKIP1 does, however, reduce myocardial I/R injury through stabilization of the MPT pore. These findings suggest that AKIP1 deserves further investigation as a putative treatment target for cardioprotection from I/R injury during acute myocardial infarction.

Introduction

Heart failure constitutes a major public health problem with a lifetime risk of 20-30% to develop this disease.[1,2] While treatment and survival of heart failure have improved over the past decades, morbidity and mortality for heart failure still exceeds that of most malignancies.[3] Novel therapies are therefore urgently needed. In an attempt to identify novel therapies that contribute to heart failure development, we performed a multilevel genome wide transcription study in several complimentary heart failure models. This analysis revealed marked upregulation of the gene encoding for A Kinase Interacting Protein 1 (AKIP1).[4]

AKIP1 was originally identified as Breast Cancer Associated gene 3 (BCA3), which is upregulated in several different types of cancer. In cancer cells, AKIP1 has been shown to modulate NF-kB and PKA activity,[5,6] which results in the induction of apoptosis and the reduction in tumour progression.[7-10] In contrast, some investigators have reported that AKIP1 rather stimulates neovascularisation and tumor growth.[11,12] This apparent discrepancy suggests that the role of AKIP1 may vary in different cell types and clinical situations.

Gain- and loss of function experiments with AKIP1 in cultured cardiomyocytes have revealed that AKIP1 promotes a physiological type of hypertrophy by activating AKT.[13] AKIP1 was also found to promote mitochondrial respiration and improve mitochondrial coupling efficiency while simultaneously attenuating mitochondrial ROS emissions.[14] Additional evidence for the involvement of AKIP1 in mitochondrial physiology comes from a recent study which showed that AKIP1-gene transfer protects cultured cardiomyocytes and isolated perfused hearts from ischemia / reperfusion (I/R).[15]

Together these findings suggest that AKIP1 may have a beneficial effect on both acute and chronic cardiac insults. It also suggests that interventions that target AKIP1 could offer a viable strategy to prevent cardiac dysfunction in various cardiac diseases. Accordingly, we hypothesized that overexpression of AKIP1 would improve cardiac resilience to acute and chronic cardiac stress. To investigate this hypothesis, we generated a mouse line with cardiomyocyte-specific overexpression of AKIP1.

Methods

Animal models

Generation of transgenic mice

To create mice with cardiac-specific overexpression of AKIP1, a construct was created which consisted of the mouse AKIP1 sequence (NM_020616.1) inserted between an α-MHC promoter and a polyA tail (*Figure 1A*). This construct was used for pronuclear injection in FVB embryos to create transgenic mice. These mice were created by the UMCG mouse transgenic facility in collaboration with

Mayo Clinics (Minnesota, USA) and backcrossed into a C57Bl6/J background, as described before.[16] Male mice were used for all experiments. AKIP1-transgenic mice (AKIP1-TG) and wild type (WT) littermates were phenotyped 4 and 12 months of age with magnetic resonance imaging (MRI), echo and histological staining as described below. Mice between the ages of 8-12 weeks were used for all the experiments. Surgery, imaging and euthanasia were performed under 2% isoflurane anaesthesia with heart rate, temperature and breathing monitoring. The animal experiments were approved by the Animal Ethics Committee from the University of Groningen (DEC6237) and performed in adherence to the guidelines from Directive 2010/63/EU of the European Parliament.

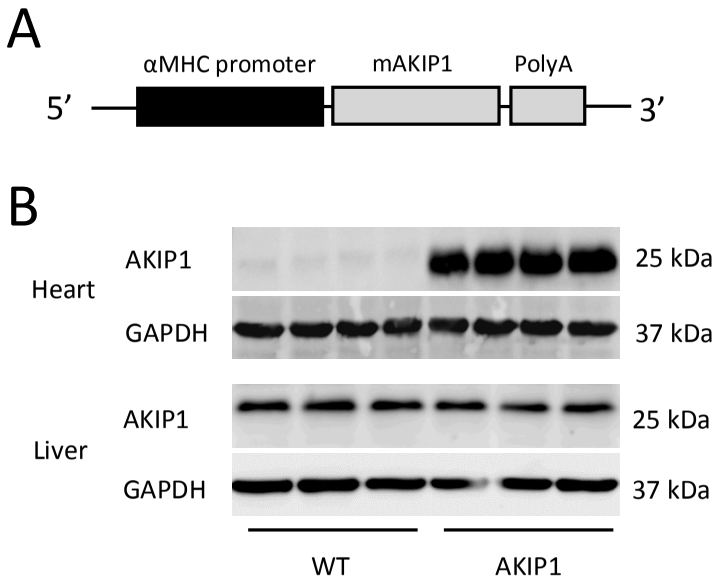
Heart failure models

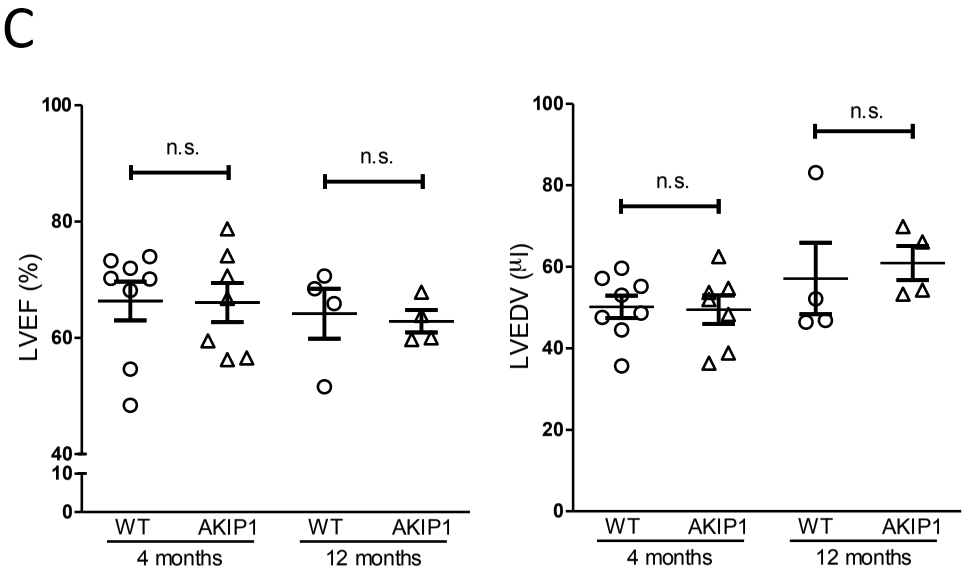
We used 2 distinct models for chronic heart failure; first, transverse aortic constriction (TAC) was performed with a 7-0 nylon suture between the carotid arteries around a 27.5G needle, as described.[17] Second, post-myocardial infarction (MI) heart failure was achieved through a permanent ligation of the left coronary artery with a premilene 6-0 suture. Cardiac function was assessed with echocardiography or MRI at consecutive time points. Finally, intracardiac pressure measurements were obtained before sacrifice, as described.[18]

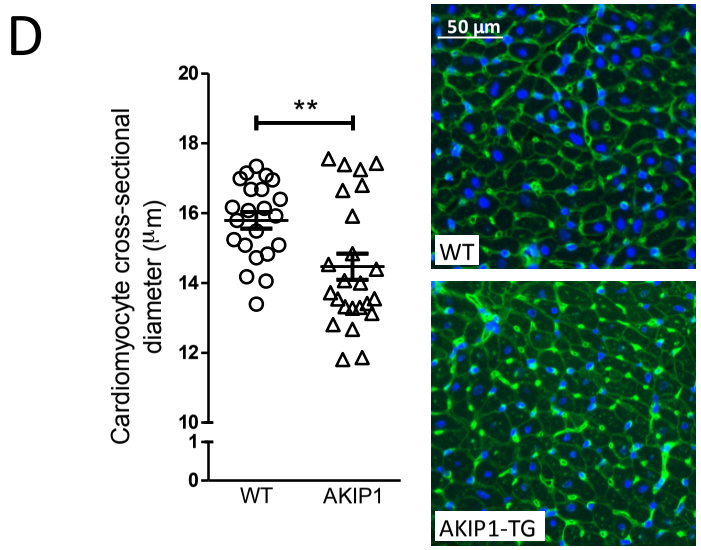
Myocardial I/R injury

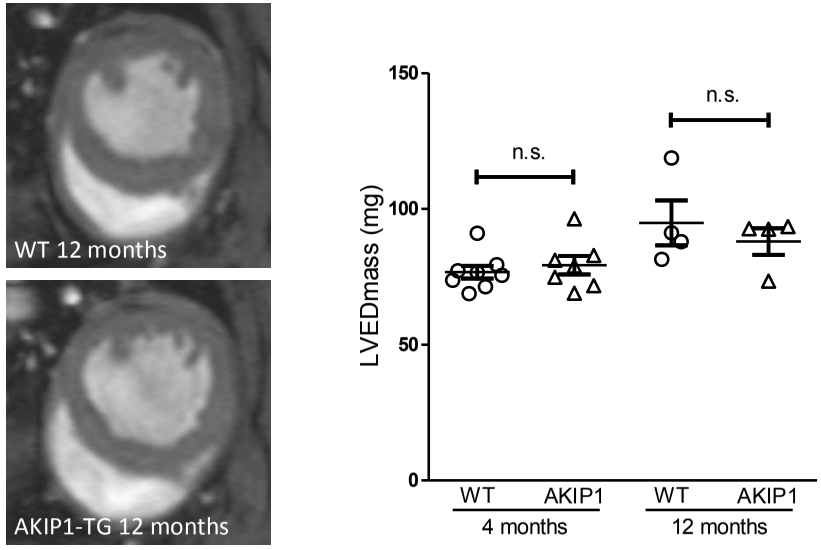
In vivo I/R injury was performed by 45min ligation of the left coronary artery, followed by 24 hours of reperfusion. After 24h, the coronary artery was re-ligated, followed by abdominal vein injection with Evans Blue (2% in 0.9% NaCl) to delineate the area at risk. The hearts were immediately excised, cut in 1 mm thick slices and stained in 1% Triphenyltetrazoliumchloride for 15 minutes at 37 ^{o}C. Slices were fixed in 4% paraformaldehyde and scanned at 4800 dpi (Cannon 900F). Area at risk and infarct size were quantified using ImageJ.

Figure 1 Changes in cardiac structure and function induced by AKIP1 overexpression. (A) Schematic picture of the transgenic construct that was inserted in mice. (B) Western blot of AKIP1 expression in hearts of AKIP1-transgenic (AKIP1) and wild-type (WT) mice. GAPDH was used as a loading control. Samples were run on the same gel and pictures were taken from the same blot and same exposure time. For quantitative measurements, see Supplementary material online, *Figure S1A*. (C) Cardiac function parameters as determined by magnetic resonance imaging at 4 and 12 months and representative end-diastolic images. Shown are Left Ventricular Ejection Fraction, LVEF; Left Ventricular End-Diastolic Volume, LVEDV and Left Ventricular End-diastolic Mass, LVEDmass. *N*=7-8 animals for the groups at 4 months and *N*=4 animals for both groups at 12 months. Error bars represent mean ± SE. Statistical testing was performed with a Student *t* test. AKIP1-TG and WT were compared at 4 months and a separate comparison was made at 12 months; n.s., not significant. (D) Cardiomyocyte size was slightly smaller in AKIP1-TG mice compared to WT mice at age 4 months. Shown are representative images of wheat germ agglutinin staining and measurements of cardiomyocyte cross-sectional diameter. *N*=22 animals for WT and *N*=24 animals for AKIP1-TG. All values are expressed as mean ± SE. Statistical analysis was performed by student *t* test; **, $p < 0.01$.


A
αMHC promoter
mAKIP1
PolyA
5'
3'
B
Heart
AKIP1
25 kDa
GAPDH
37 kDa
Liver
AKIP1
25 kDa
GAPDH
37 kDa
WT
AKIP1


C
LVEF (%)
n.s.
n.s.
WT
AKIP1
WT
AKIP1
4 months
12 months
LVEDV (µl)
n.s.
n.s.
WT
AKIP1
WT
AKIP1
4 months
12 months


D
Cardiomyocyte cross-sectional diameter (µm)
**
WT
AKIP1
50 µm
WT
AKIP1-TG


WT 12 months
AKIP1-TG 12 months
LVEDmass (mg)
n.s.
n.s.
WT
AKIP1
WT
AKIP1
4 months
12 months

Assessment of cardiac function and hemodynamics

Hemodynamic function parameters were obtained under isoflurane anaesthesia with echocardiography, MRI and intracardiac pressure measurements. MRI was performed in a 9.4T 400 MR system (Bruker BioSpin, Ellingen, Germany) as previously described.[19] ParaVision 4.0 and IntraGate software (Bruker BioSpin GmBH, Germany) were used for cine-MR acquisition and reconstruction. Short-axis slices were obtained to determine the end-systolic and end-diastolic dimensions of the left ventricle (QMass, version MR 6.1.5, Medis Medical Imaging Systems, The Netherlands). Aortic and intra-cardiac pressures were measured with a Millar catheter (Micro-Tip 1.4 French; SPR,-839, Millar instruments, Houston, TX, USA), as described.[18] Data were analysed with LabChart 7 software (Version 7.2 ADinstruments, New Zealand).

Histology

Midpapillary slices of the left ventricle were fixated for 24h in 4% paraformaldehyde. Tissues were dehydrated using an automated system (Leica, TP 1020, Germany) and embedded in paraffin. Sections of 4 µm were stained with Masson-Trichrome staining to determine fibrosis. Infarct size after MI was measured from a midpapillary slice as the percentage of the epicardial contour with transmural infarction. Apoptosis and capillary density were determined with antibodies against cleaved-caspase-3 (Cell Signalling, USA) and CD31 (Dianova, Germany) respectively with subsequent incubation of IgG secondary antibodies coupled to peroxidase followed by diaminobenzidine-staining. Cardiomyocyte diameter was determined with staining for wheat germ agglutinin (Sigma, USA). Quantification was performed with ImageJ or Aperio ImageScope v11.

Subcellular fractionation

Fresh ventricular tissue was minced and subsequently homogenised in homogenization buffer (70 mM sucrose, 190 mM Mannitol, 20 mM HEPES, 0.2 mM EDTA, proteaseinhibitor-cocktail (Roche), phosphatase inhibitor cocktail (Sigma), 1 µM sodiumorthovanadate, 1 mM PMSF) with a glass dounce mortar. The homogenate was centrifuged at increasing speed to separate enriched subcellular fractions. Protein concentration was measured and processed for western blot.

Mitochondrial swelling

Cardiac mitochondria were freshly isolated from AKIP1-TG and WT littermates with a polytron 3000, speed 3 in MSHE buffer (70 mM sucrose, 210 mM mannitol, 5 mM HEPES, 1 mM EGTA, 0.5% fatty-acid-free BSA). The solution was centrifuged at 500 G for 10 minutes at 4 ^{o}C to remove nuclei and cell debris. The supernatant was spun at 6000 G for 10 minutes at 4 ^{o}C to pellet the mitochondria. The pellet was washed again and diluted to 300 µg/ml in

swelling buffer (125 mM KCl, 5 mM KH2PO4, 20 mM HEPES, 5 mM glutamate, 5 mM malate) and pipeted in a clear-bottom microtiter-plate. Absorbance was measured at 540 nm on 37 ^{o}C. After initiation, Ca2+ (150 µM) was added.

Co-immunoprecipitation

We performed a co-immunoprecipitation study as previously described.[20] A rabbit anti mouse AKIP1 antibody and protein lysates from AKIP1-TG and WT mice were used. After co-immunoprecipitation, samples were processed for western blot.

PKA activity assay

PKA activity was measured in mitochondria isolated from the area at risk, using a colorimetric assay (MESACUP Protein Kinase Assay, MBL CO., Ltd, Nagoya, Japan). The assay was performed according to the manufacturers protocol for the specific measurement of PKA-activity.

NF-κB activity assay

NF-κB activity was measured in nuclear enriched lysates with an enzyme-linked NF-κB immunosorbent assay method (TransAM NF-κB, Active Motif, Carlsbad, CA, United States) according to the manufacturers protocol.

Culturing of primary cardiomyocytes and cell size measurement

Neonatal rat ventricular cardiomyocytes (NRVCs) were isolated and cultured as previously described.[13] 24 hours after isolation, NRVCs were infected with adenovirus virus overnight. After subsequent starvation in serum-free conditions for 48h, protein was isolated for western blot analysis or cells were fixed in paraformaldehyde for staining and cell size measurement, as previously described.[13]

Western blot and antibodies/reagents

Western blot was performed as described previously.[13] An AKIP1 antibody was made in our lab as described previously.[13] Antibodies for PARP, COX-IV, α-actinin, wheat germ agglutinin, α-tubulin (SIGMA), SERCA2ATPase (ABCAM), anti-phosphorylated-Akt^{Ser473}, anti-total-Akt (cell signaling) and GAPDH (Fitzgerald) were bought commercially.

Statistics

Values are displayed as mean ± standard error of the mean. Comparisons between 2 groups were made with Student t test or a Logrank test where applicable. Whenever the experimental design compared the response of AKIP1-TG and WT mice to an intervention, we first compared the effect of the intervention using a two-way ANNOVA. If the two-way ANNOVA revealed a significant difference between the combined intervention groups, we compared

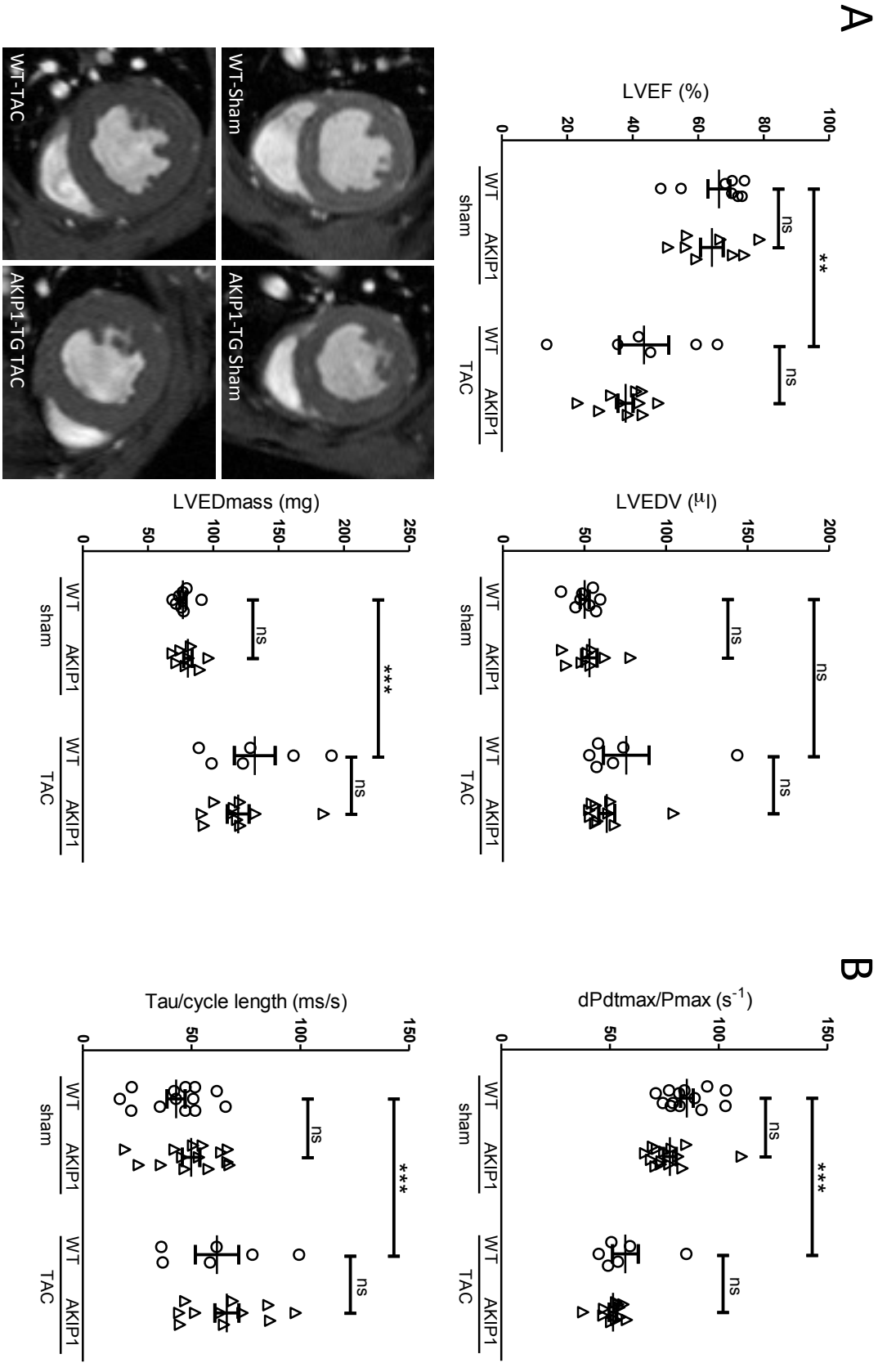
all 4 groups using the post-hoc Tukey test. A p-value <0.05 was considered significant.

Results

Baseline phenotyping of AKIP1-TG mice

To investigate the role of AKIP1 in cardiomyocytes, mice that express AKIP1 under control of the α-MHC promoter were generated (Cloning strategy is depicted in *Figure 1A*). Overexpression of AKIP1 was cardiac specific as confirmed by analysis of mRNA and protein levels (*Figure 1B*, and Suppl. mat. online, *Figure S1A*). AKIP1-TG mice were born in normal mendelian frequencies and were phenotypically normal. They displayed normal behaviour and reproductive capacity was not affected. In contrast to *in vitro* studies[13], AKIP1 overexpression did not induce cardiac hypertrophy. In fact, cardiomyocyte cross-sectional diameter was marginally smaller in AKIP1-TG mice compared to WT mice, but overall left ventricular (LV) mass was identical (*Figure 1C* and *D,* and Suppl. mat. online, *Figure S1C*). Furthermore, AKIP1-TG mice displayed normal cardiac function and LV dimensions throughout the course of 12 months (*Figure 1C,* and Supplementary material online, *Figure S1B*). Levels of AKIP1 expression were stable throughout the course of one year, and chronic cardiac stress did not affect AKIP1 expression levels (Suppl. mat. online, *Figure S1E*). The 40-fold increase in cardiac AKIP1 protein expression that was achieved in our AKIP1-TG mice may be considered to be relatively high. To exclude that the lack of spontaneous hypertrophy *in vivo* could be explained by an off-target effects resulting from the level of protein overexpression we studied the effect of multiple levels of AKIP1 overexpression in NRVCs. Interestingly, increasing AKIP1 protein expression levels up to 600-fold, resulted in a continuous dose-dependent increase in cardiomyocyte hypertrophy in NRVCs (Suppl. mat. online, *Figure S1D*). Accordingly, it is unlikely that the the level of AKIP1 protein expression is sufficient to explain the the absence of AKIP1-induced cardiac hypertrophy *in vivo*.

Figure 2 Overexpression of AKIP1 does not influence cardiac remodeling after transverse aortic constriction (TAC). (A) Cardiac function parameters as determined by magnetic resonance imaging and representative end-diastolic images of AKIP1-transgenic (AKIP1) and wild-type (WT) mice. Shown are Left Ventricular Ejection Fraction, LVEF; Left Ventricular End-Diastolic Volume, LVEDV and Left Ventricular End-diastolic Mass, LVEDmass. *N*=6-9 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; **, p<0.01; ***, p<0.001; n.s. not significant. (B) Intracardiac pressure and relaxation measurements. Shown are dPdtmax corrected for maximum Pressure, Pmax; Tau corrected for cycle length. *N*=13 for WT sham, *N*=14 animals for AKIP1-transgenic sham, *N*=6 animals for WT TAC and *N*=11 animals for AKIP1-transgenic TAC. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; ***, p<0.001; n.s., not significant.


A
WT-Sham
AKIP1-TG Sham
WT-TAC
AKIP1-TG TAC
LVEF (%)
LVEDV (µl)
LVEDmass (mg)
B
dPdtmax/Pmax (s-1)
Tau/cycle length (ms/s)
WT
AKIP1
sham
TAC
ns
**
***

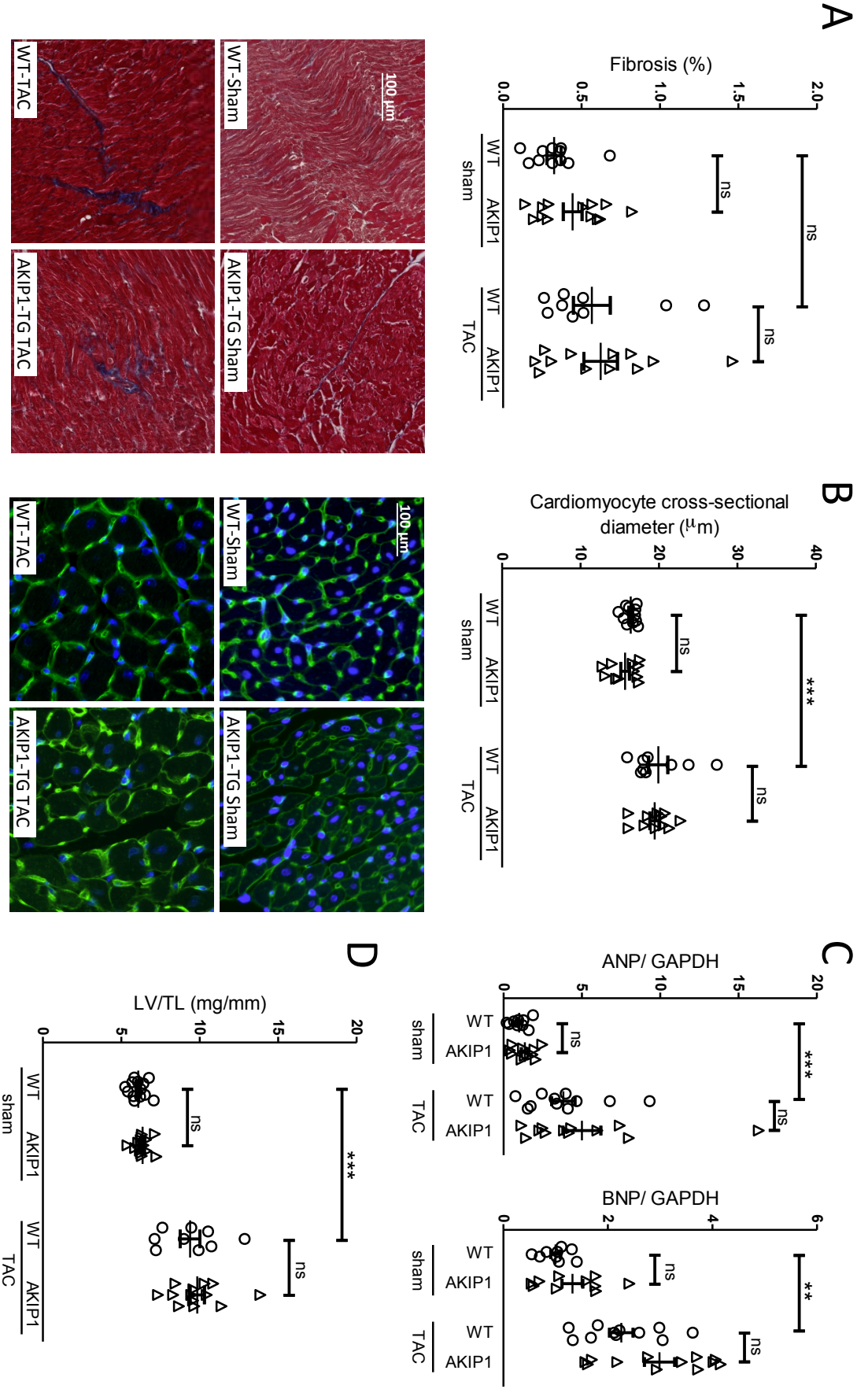
AKIP1 does not influence cardiac hypertrophy and heart failure development

To evaluate whether AKIP1 modulates hypertrophy and heart failure development, AKIP1-TG and WT mice were subjected to 2 clinically relevant models of heart failure development.

First, the mice were subjected to TAC and followed up for a total duration of 7 weeks. The degree of LV hypertrophy that developed after TAC was comparable between WT and AKIP1-TG mice, as determined by LV mass measured with MRI, LV weight divided by tibia length and cardiomyocyte cross sectional diameter (*Figure 2A, 3B and D*). TAC resulted in marked LV-dysfunction, but the reductions in LV-ejection fraction and LV-dilatation were similar in AKIP1-TG and WT mice (*Figure 2A*). Additionally, invasive measurements of contractility and relaxation were all similar in AKIP1-TG and WT mice (*Figure 2B)*. The expression of molecular markers of LV-remodeling and cardiac fibrosis were also comparable between the groups (*Figure 3A and C*).

As the TAC model described above resulted in a relatively mild form of heart failure, we also investigated the role of AKIP1 after permanent proximal LAD ligation. Approximately 60% of the mice survived the entire study duration of 6 weeks. Most deaths occurred within the first week after MI and mortality was similar in AKIP1-TG and WT mice (*Figure 4A*). Total fibrosis in the left ventricular free wall remote from the infarction at 6 weeks after MI was reduced in AKIP1-TG mice (Figure 5A, left panel) as was infarct size after MI (51 ± 4% vs. 36 ± 4%, $p<0.05$ in WT and AKIP1-TG mice respectively (*Figure 5A right panel*). Average heart weight at 6 weeks was comparable between groups, but cardiomyocyte cross-sectional diameter after MI or sham surgery was significantly lower in AKIP1-TG animals compared to WT mice (*Figure 4B,C*

Figure 3 Characterization of AKIP1-transgenic (AKIP1) and wild-type (WT) mice 7 weeks after Transverse Aortic Constriction (TAC). (A) Fibrosis measurements. Shown are percentage of fibrosis in the midpappillary slice and representative images of Masson-Trichrome staining. *N*=9-13 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; n.s., not significant. (B) Cell-size measurements. Shown are representative images of wheat germ agglutinin staining and measurements of cardiomyocyte cross-sectional diameter. *N*=9-11 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; ***, $p<0.001$; n.s., not significant. (C) mRNA expression of markers of pathological hypertrophy. Shown are Atrial Natriuretic Peptide (ANP) and Brain Natriuretic Peptide (BNP) expression corrected for 36B4. *N*=9-12 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; **, $p<0.01$; ***, $p<0.001$; n.s., not significant. (D) Left Ventricular wet weight (LV) divided by tibia length. *N*=9-14 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; ***, $p<0.001$; n.s., not significant.

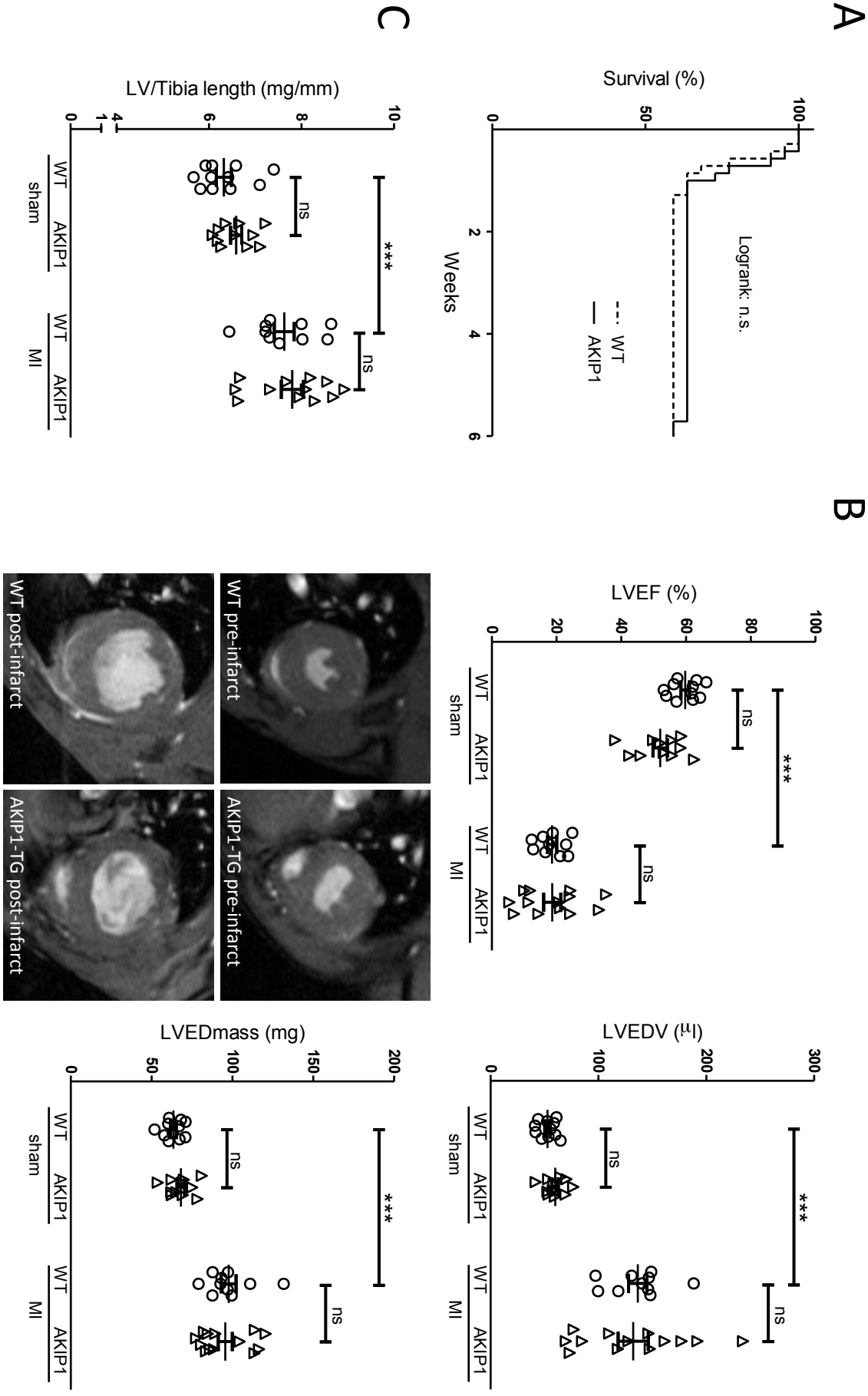

A
Fibrosis (%)
0.0
0.5
1.0
1.5
2.0
ns
ns
ns
WT
AKIP1
sham
WT
AKIP1
TAC
WT-Sham
100 µm
AKIP1-TG Sham
WT-TAC
AKIP1-TG TAC
B
Cardiomyocyte cross-sectional diameter (µm)
0
10
20
30
40
***
ns
ns
WT-Sham
100 µm
AKIP1-TG Sham
WT-TAC
AKIP1-TG TAC
C
ANP/ GAPDH
0
5
10
15
20
***
ns
ns
BNP/ GAPDH
0
2
4
6
**
ns
ns
WT
AKIP1
sham
WT
AKIP1
TAC
D
LV/TL (mg/mm)
0
5
10
15
20
***
ns
ns
WT
AKIP1
sham
WT
AKIP1
TAC

and 5B). The degree of LV dysfunction that developed after MI was more severe than in the TAC-model, but again cardiac function parameters were comparable between AKIP1-TG and WT mice (*Figure 4B*). The cardiac expression of markers for heart failure severity, ANP and BNP, were comparable between AKIP1-TG and WT mice after MI (*Figure 5C*). Furthermore, cardiac microvasculature was also comparable between the groups (Supplementary material online, *Figure S2*).

AKIP1 reduces infarct size after I/R and reduces mitochondrial swelling

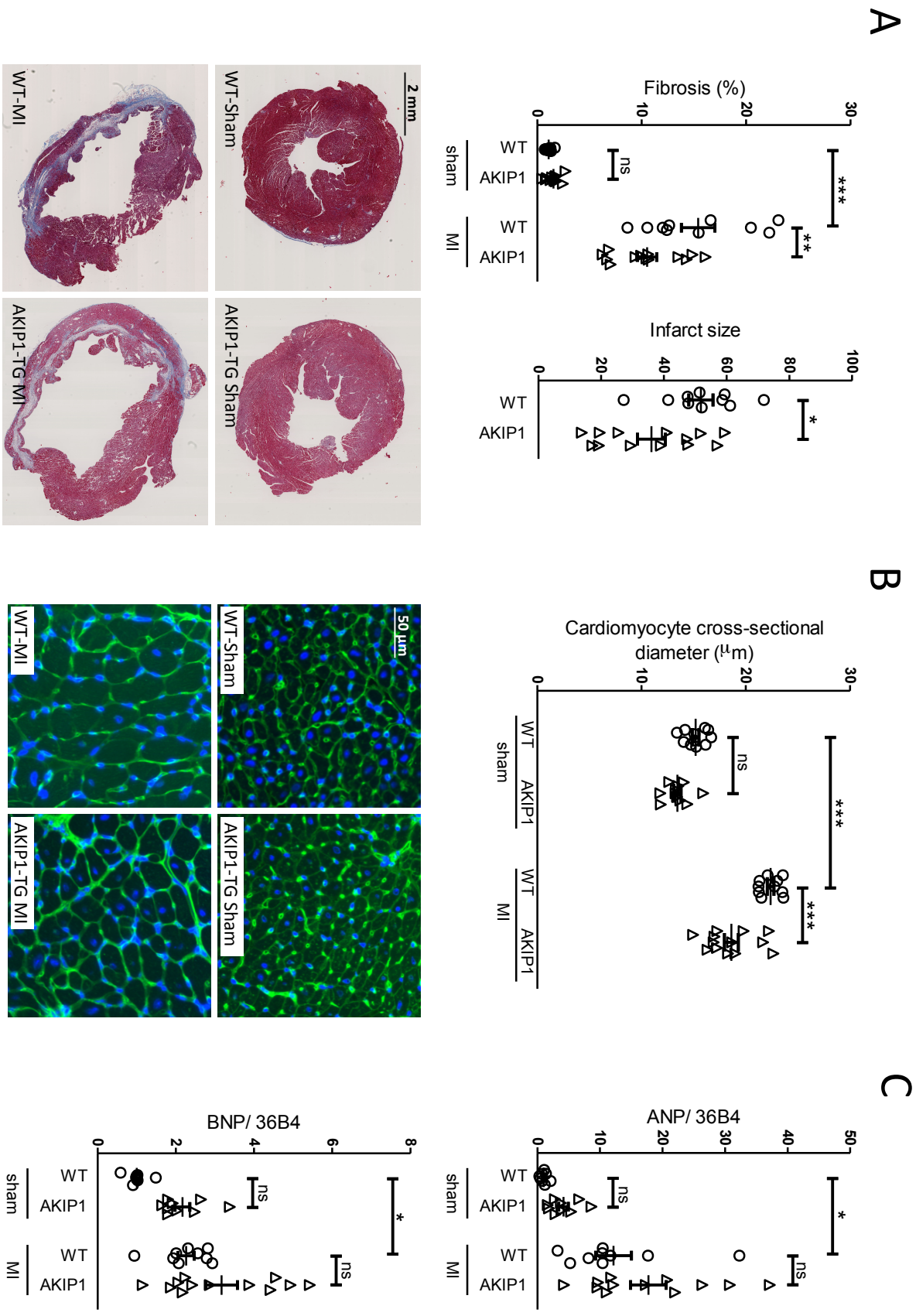
Sastri et al. previously showed that AKIP1-levels are increased in cultured cardiomyocytes upon oxidant stress and that AKIP1 exerts cardioprotective effects during *in vitro* and *ex vivo* myocardial ischemia.[15] To test whether these effects translate into protection *in vivo*, AKIP1-TG and WT littermates were subjected to cardiac I/R injury. Survival after 24h was 100% in both groups. While the area at risk was comparable between the groups, AKIP1-TG animals had a 2-fold reduction in myocardial infarct size (*Figure 6A*) accompanied by a significant reduction in apoptosis in the area at risk (*Figure 6B*). Previous studies have indicated that AKIP1 functions as a signalling modulator affecting, among others, the activity of NF-kB, PKA and AKT.[5,6,13] Furthermore, AKIP1 was shown to directly bind to NF-kB and PKA in various malignant cell lines.[5] However, the reduction in I/R injury in AKIP1-TG mice could not be explained by modulation of NF-kB, PKA and AKT that are known to influence the degree of I/R injury (*Figure 6C,D, 7B*, Supplementary material online Figure S3A, B). In addition, AKIP1 did not immunoprecipitate with NF-kB, PKA or AKT in lysates from WT and AKIP1-TG animals subjected to I/R (*Figure 7A*). Therefore, the reduction in I/R damage cannot be explained by activation of these previously indentified targets of AKIP1

Figure 4 Overexpression of AKIP1 does not influence cardiac remodeling after permanent myocardial infarction. (A) Post-MI survival curves. *N*=22 animals for both groups. Statistical testing was performed by Logrank test; n.s., not significant. (B) Cardiac function parameters as determined by magnetic resonance imaging and representative end-diastolic images of AKIP1-transgenic (AKIP1) and wild-type (WT) mice. Shown are Left Ventricular Ejection Fraction, LVEF; Left Ventricular End-Diastolic Volume, LVEDV and Left Ventricular End-diastolic Mass, LVEDmass. *N*=10-13 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; ***, $p<0.001$; n.s. not significant. (C) Left Ventricular wet weight (LV) corrected for tibia length. *N*=10-12 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; ***$p<0.001$; n.s., not significant.


A
Survival (%)
100
50
0
2
4
6
Weeks
Logrank: n.s.
WT
AKIP1
B
LVEF (%)
LVEDV (µl)
LVEDmass (mg)
sham
MI
WT
AKIP1
ns
***
WT pre-infarct
AKIP1-TG pre-infarct
WT post-infarct
AKIP1-TG post-infarct
C
LV/Tibia length (mg/mm)

Mitochondria are central mediators of I/R-injury as they modulate both apoptosis and necroptosis.[21,22] Previously it was shown that AKIP1 is expressed in mitochondria and that overexpression of AKIP1 improved mitochondrial function.[14,15] First we performed a subcellular fraction study in AKIP1-TG and WT mice, which confirmed that AKIP1 is enriched in mitochondria (Supplementary material online, *Figure S3C*). Because Sastri et al. suggested that AKIP1 activated mitochondrial PKA,[15] we evaluated PKA activity in the mitochondrial fraction after I/R. Similar to what was observed in the whole cell lysate, mitochondrial PKA activity after I/R was also not affected by AKIP1 overexpression (Figure 7B). Recent evidence indicates that a large component of I/R damage is mediated through the activation of the mitochondrial permeability transition pore (MPT pore).[22] Mitochondrial ATP-synthase has been recognised as a key component of the MPT pore.[23] It has been shown that AKIP1 influences the phosphorylation of ATP synthase by PKA, but a direct interaction between AKIP1 and ATP-synthase has not been established. In our co-immunoprecipitation analysis, we discovered that AKIP1 co-immunoprecipitated with ATP-synthase (*Figure 7A*), suggesting that the protective effects of AKIP1 result from the attenuation of MPT pore formation. To test this hypothesis, we performed a calcium-induced swelling assay in isolated cardiac mitochondria. Interestingly, the mitochondria isolated from AKIP1-TG mice displayed a significant reduction in calcium induced swelling which is indicative of attenuated MPT pore formation (*Figure 7C*).

Figure 5 Characterization of AKIP1-transgenic (AKIP1) and wild-type (WT) mice, 6 weeks after myocardial infarction (MI). (A) Fibrosis measurements and infarct size. Shown are percentage of fibrosis in the left ventricle, infarct size as a percentage of Left Ventricular epicardial contour (LV) and representative images of Masson-Trichrome staining. *N*=10-13 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; **, $p<0.01$; ***, $p<0.001$; n.s., not significant. Difference in infarct size was tested by Student *t* test; *, $p<0.05$; (B) Cell-size measurements. Shown are representative images of wheat germ agglutinin staining and measurements of cardiomyocyte cross-sectional diameter. *N*=10-13 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; ***, $p<0.001$; n.s., not significant. (C) mRNA expression of markers of pathological hypertrophy. Shown are Atrial Natriuretic Peptide (ANP) and Brain Natriuretic Peptide (BNP) expression corrected for 36B4. *N*=8-12 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; *, $p<0.05$; n.s., not significant.

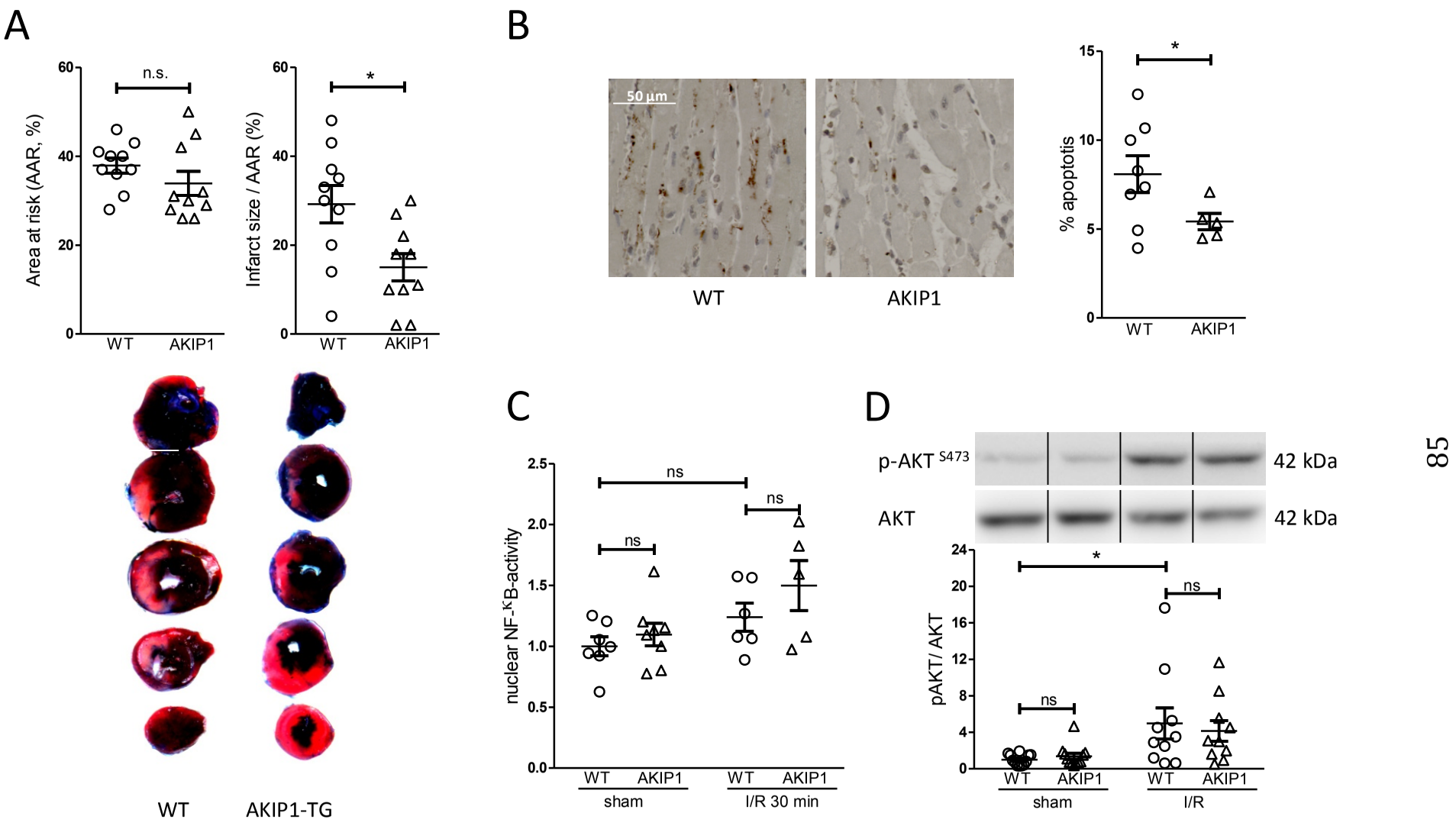

A
Fibrosis (%)
0
10
20
30
sham
MI
WT
AKIP1
ns
***
**
Infarct size
0
20
40
60
80
100
*
2 mm
WT-Sham
WT-MI
AKIP1-TG Sham
AKIP1-TG MI
B
Cardiomyocyte cross-sectional diameter (μm)
***
***
50 μm
C
ANP/ 36B4
*
BNP/ 36B4
*

Discussion

We sought to determine the role of AKIP1 in cardiac remodeling because *in vitro* evidence suggested that AKIP1 is critically involved in the development of cardiomyocyte hypertrophy and the cardiac response to oxidative stress.[4,13-15] By generating AKIP1-TG mice, we were able to determine whether these *in vitro* findings could translate into relevant protective mechanisms *in vivo* for the first time. In contrast to previous reports, however, a stable 40-fold increase in cardiac AKIP1-protein expression did not influence cardiac structure and function up to the age of 1 year. In addition, AKIP1 overexpression did not modulate reactive hypertrophy or LV remodeling induced by pressure overload or chronic MI. AKIP1-overexpression did, however, attenuate myocardial infarct formation in response to permanent and transient myocardial ischemia and reduced myocardial apoptosis. In contrast to our expectations, the reduction of I/R injury by AKIP1 could not be explained by an interaction with the signaling molecules NF-kB, PKA or AKT. Instead, we found that AKIP1 was enriched in cardiac mitochondria, where it co-immunoprecipitated with a key component of the MPT pore, ATP synthase. AKIP1 also prevented calcium-induced mitochondrial swelling, which is considered to be indicative of reduced MPT pore formation. Together, our findings indicate that cardiomyocyte-specific overexpression of AKIP1 does not influence heart failure development but does offer protection during myocardial I/R injury.

Previous findings in cultured cardiomyocytes and other cell types suggested that AKIP1 could be a key mediator of the cardiac response to acute and chronic stress.[11-13,15] However, our *in vivo* data do not fully confirm this hypothesis as AKIP1 overexpression did not induce cardiac hypertrophy, nor

Figure 6 Overexpression of AKIP1 reduces myocardial ischemia / reperfusion injury. (A) Infarct formation after 45 minutes of ischemia and 24 hours of reperfusion in hearts of AKIP1-transgenic (AKIP1) and wild-type (WT) mice. Shown are Area at risk (AAR), infarct size relative to the AAR and representative images of the Evans Bleu / TTC staining. *N*=10 animals for both groups; Error bars represent mean ± SE. Statistical testing was performed with Student *t* test; *, $p<0.05$; n.s., not significant. (B) Apoptosis after reperfusion. Shown are representative images of Caspase-3 staining in the area at risk 24 hours after reperfusion. *N*=8 animals for wild type (WT) and *N*=5 animals for AKIP1-transgenic groups; Error bars represent mean ± SE. Statistical testing was performed with Student *t* test; *, $p<0.05$. (C) Shown is the relative nuclear activity of NF-kB of AKIP1-transgenic and WT mice after reperfusion for 30 minutes. *N*=5-8 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; n.s., not significant. (D) Myocardial AKT-phosphorylation after ischemia / reperfusion. Shown are densitometric analysis and representative blots. Samples were run on the same gel and pictures were taken from the same blot and same exposure time. *N*=10-12 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; *, $p<0.05$; n.s. not significant.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% apoptotis
*
C
nuclear NF-κB-activity
ns
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I/R 30 min
D
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AKT
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did it influence cardiac remodelling or cardiac dysfunction upon chronic cardiac stress. In fact, where AKIP1 overexpression induced hypertrophic growth in NRVCs, AKIP1-TG mice displayed a modest reduction of cardiomyocyte hypertrophy. There are numerous potential explanations for this apparent discrepancy. First, the *in vitro* studies were done with immature NRVCs. In addition to the non-physiological influence of the isolation procedure and culture conditions, these cells are harvested during a postnatal period where the heart and circulatory system undergoes significant transitions that influence the cardiac response to stress. This is exemplified by the response to ischemia / reperfusion injury.[24] Second, the development of cardiac hypertrophy is strongly influenced by the activation neurohormonal systems and several other interactions between cardiomyocytes and other cell types, such as cardiac fibroblasts and endothelial cells. These interactions are lost *in vitro.* Third, the type of hypertrophy induced by AKIP1 in cultured cardiomyocytes did resemble physiological cardiac hypertrophy rather than the "pathological" hypertrophy that typically develops during heart disease.[13] Since we only used models of cardiac disease, we cannot exclude the possibility that AKIP1 is a specific mediator of physiological hypertrophy. Future studies are required to specifically address the role of AIKP1 during exercise.

Our I/R studies did confirm previous work by Sastri et al. who reported that AKIP1 was upregulated in cardiomyocytes in response to oxidative stress and that AKIP1 attenuated cardiomyocyte apoptosis *in vitro*.[15] Furthermore, they also showed that acute adenoviral gene-transfer of AKIP1 attenuated *ex vivo* I/R injury through various mitoprotective mechanisms including phosphorylation of the alpha subunit of ATP synthase.[15] ATP synthase is the key component of the MPT pore, which is a critical mediator of myocardial damage after I/R.[22,23] Our studies also showed that AKIP1 is enriched in mitochondria and that it interacts with ATP-synthase. Furthermore, we now show for the first time that AKIP1 attenuates *in vivo* I/R injury and reduces mitochondrial MPT pore formation as evidenced by reduced calcium induced mitochondrial swelling. Together these findings strongly suggest that the cardioprotective effects of AKIP1 are mediated by an interaction with ATP synthase that results in the stabilization of the MPT pore. It therefore, appears that AKIP1 is primarily involved in the regulation of acute mitochondrial stress-responses in cardiomyocytes. Of note, the changes in mitochondrial respiration observed in NRVCs,[14] could also result from modulation of ATP synthase.

The major difference between our findings and previous work using *in vitro* and *ex vivo* systems is the fact that AKIP1 did not co-immunoprecipitate with or modulate the signaling molecules NF-kB, PKA or AKT. As described above, this could be explained by differences between (immature) cell culture systems and adult animals. Another major difference is that previous studies used acute global gene transfer using adenoviral vectors, whereas our AKIP1-TG mice displayed stable constitutional and cardiomyocyte-specific

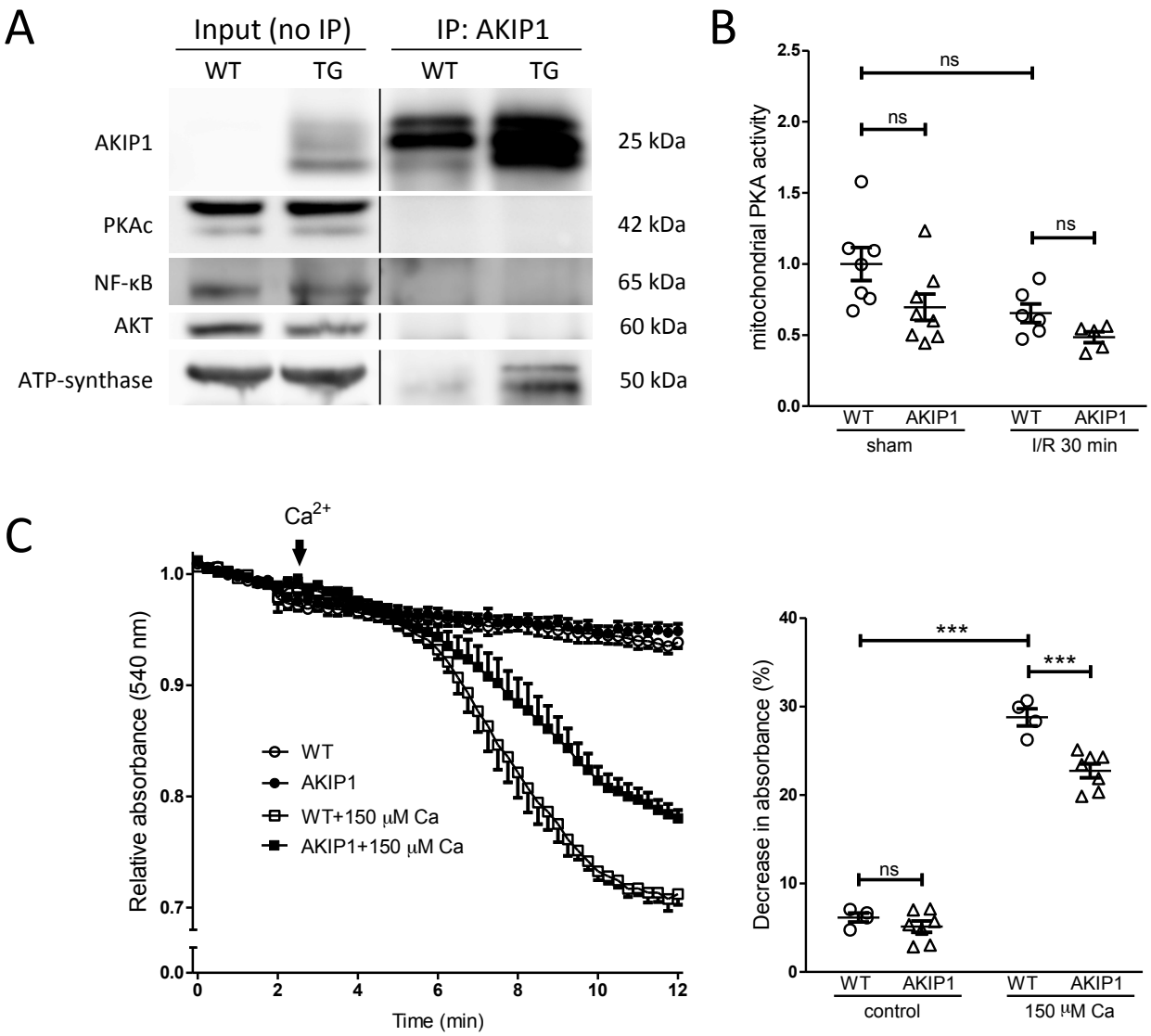

Figure 7 Activation of signal transduction pathways after ischemia / reperfusion injury. (A) Western blot of co-immunoprecipitation (IP) of AKIP1 from cardiac protein lysates (input) derived from AKIP1-transgenic (TG) and wild-type (WT) mice. Shown are blots of AKIP1, NF-kB and PKA. Samples were run on the same gel and pictures were taken from the same blot. (C) Shown is the relative mitochondrial activity of PKA of AKIP1-transgenic (AKIP1) and WT mice. *N*=5-8 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; n.s., not significant. (C) Calcium-induced swelling of cardiac mitochondria from AKIP1-transgenic and WT mice. Shown are the tracings of the average absorbance measurements over time per group (left panel) and graphic representation of the change in absorbance at 12 minutes after the addition of calcium (right panel). *N*=4 animals for both WT groups and *N*=7 animals for AKIP1-transgenic groups; Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; ***, p<0.001; n.s., not significant.

overexpression of AKIP1. One can envision that the different activation of signaling pathways could result from these methodological differences or from the expression of AKIP1 in other cardiac cells. As our model is more stable and allows us to study the specific effect of AKIP1 in cardiomyocytes, we consider our findings more robust. Finally, the protective effects of AKIP1 on cardiac tissue and cardiomyocytes is in contrast to studies in malignant cell lines where AKIP1 stimulated apoptosis.[9,10] This apparent discrepancy could be explained by the fact that the cytotoxic stressors used in experiments with malignant cells are in many ways very different from I/R injury. Additionally, the metabolic stress in malignant cells is predominantly caused by proliferation, whereas cardiomyocytes are terminally differentiated.

Several mechanisms are responsible for the ultimate development of reperfusion injury.[25,26] Many promising therapeutics are currently under development that target the production of reactive oxygen species (ROS) or specifically inhibit early MPT pore opening.[25,26] Mitochondrial swelling and MPT pore formation can lead to the activation of the apoptotic cascade and also activate the process of necroptosis.[21,22] Our finding that AKIP1 attenuates mitochondrial MPT pore formation suggests that interventions that modulate AKIP1 could be protective when given to patients with an acute MI. The apparent discrepancy between the effect of AKIP1 overexpression during acute and chronic cardiac stress could be explained by the fact that the MPT pore formation has a very distinct and critical physiologic role in the regulation of mitochondrial calcium handling and metabolism.[27] Indeed, our findings are in line with studies using the specific MPT pore inhibitor cyclosporine A, which failed to influence cardiac remodelling after TAC but does offer potent protection during MI.[28]

Conclusion

In contrast to *in vitro* studies, AKIP1 overexpression does not attenuate cardiac remodeling in response to chronic cardiac stress. AKIP1 does reduce myocardial I/R injury through stabilization of the MPT pore. These findings suggest that AKIP1 deserves further investigation as a putative treatment target for cardioprotection from I/R injury during acute myocardial infarction.

Acknowledgements

We thank Inge Vreeswijk for performing the animal surgery and cardiac pressure measurements.

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Supplementary figure 1 Phenotyping of AKIP1-transgenic (AKIP1) and WT mice after 4 and 12 months (A) Protein and mRNA expression of AKIP1 in hearts of AKIP1-transgenic (AKIP1) and wild-type (WT) mice. Shown are values normalized for house-keeping protein GAPDH and house-keeping mRNA from 36B4 respectively. For both graphs, *N*=4 animals for per group. Error bars represent mean ± SE. Statistical testing was performed by Student *t* test; ***, p<0.001 (B) Intracardiac pressure measurements after 4 and 12 months. Shown are Mean Arterial Pressure, MAP; End-systolic Pressure, Pes; End-diastolic Pressure, Ped; dPdt-max and dPdt-min corrected for maximum pressure (Pmax). Error bars represent mean ± SE. Statistical testing was performed by Student *t* test. AKIP1-TG and WT were compared at 4 months and a separate comparison was made at 12 months; *p<0.05 compared to WT. (C) Left ventricular wet weight at 4 and 12 months of age. *N*=4 animals for both groups at 4 months and *N*=10 animals for both groups at 12 months. Error bars represent mean ± SE. Statistical analysis was performed by student *t* test. AKIP1-transgenic and WT were compared at 4 months and a separate comparison was made at 12 months; n.s., not significant. (D) Increasing levels of AKIP1 further augment hypertrophy. Shown are a representative western blot from AKIP1 expression levels with increasing dose of overexpression where alpha tubulin is used as a loading control. Western blot samples were run on the same gel and pictures were taken from the same blot and same exposure time. The graph depicts average cell size from an individual experiment. *N*=4-9 experiments per group. Error bars represent mean ± SE. Statistical analysis was performed by One-way ANOVA with post-hoc test for trend; ***, p<0.001, ****, p<0.0001. (E) Western blot of AKIP1 expression in hearts of AKIP1-transgenic mice 7 weeks after sham or TAC surgery. GAPDH was used as a loading control. Shown are values normalized for house-keeping protein GAPDH. Samples were run on the same gel and pictures were taken from the same blot and same exposure time. *N*=12-14 animals per group. Error bars represent mean ± SE. Statistical testing was performed by Student *t* test; n.s., not significant.

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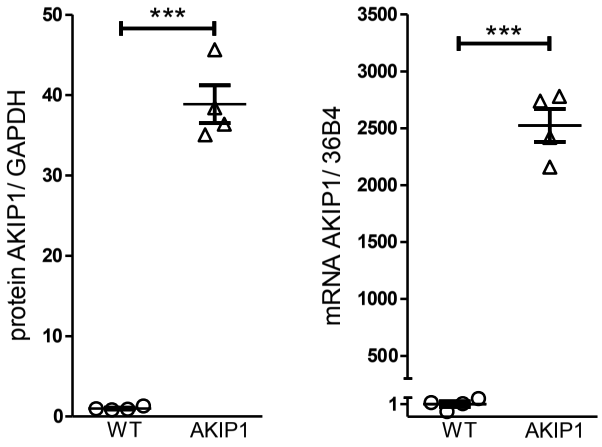
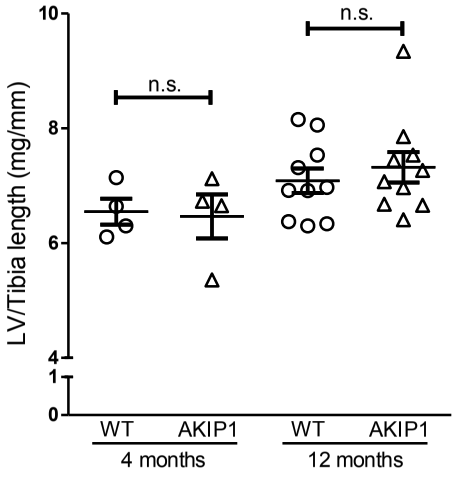


B

Parameter	WT -4m (n = 8)	AKIP1-4m (n = 5)	WT -12m (n = 10)	AKIP1 -12m (n = 9)
MAP (mmHg)	77 ± 2	78 ± 5	82 ± 4	81 ± 3
Pes (mmHg)	93 ± 2	95 ± 5	105 ± 4	102 ± 4
Ped (mmHg)	5 ± 1	8 ± 1	12 ± 1	12 ± 1
dPdtmax/Pmax	82 ± 3	76 ± 3	75 ± 4	72 ± 3
dPdtmin/Pmax	-85 ± 3	-70 ± 2*	-66 ± 5	-64 ± 4
Tau (ms) / cycle length (s)	49 ± 4	59 ± 6	66 ± 5	73 ± 8
Heart rate (min^{-1})	518 ± 14	534 ± 16	485 ± 17	512 ± 18

C



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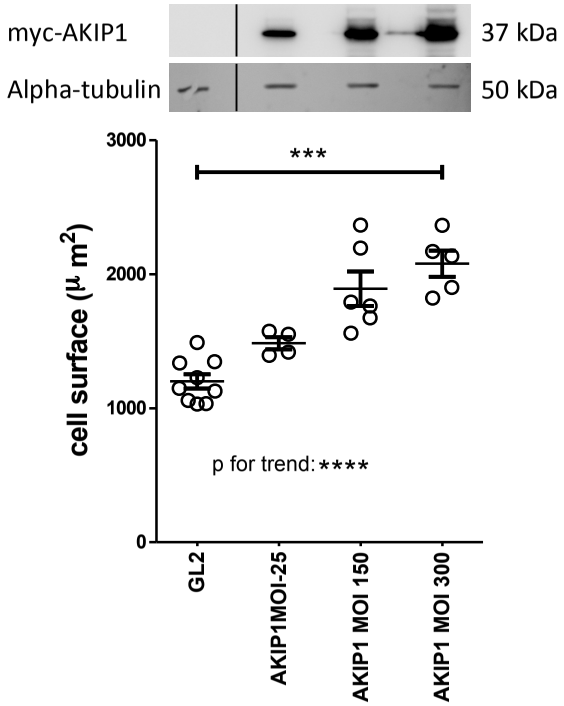

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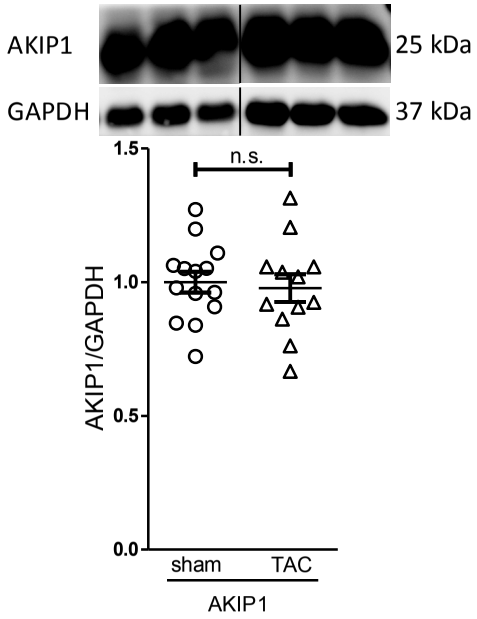
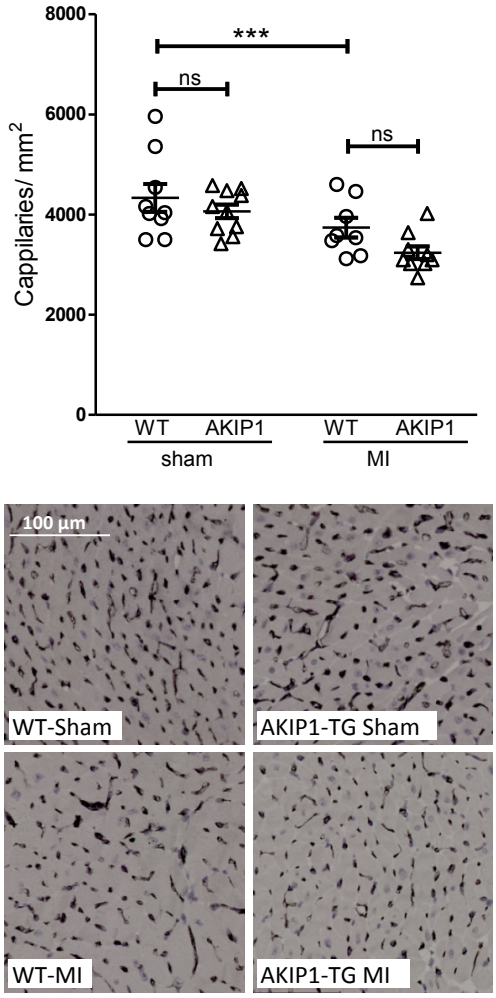
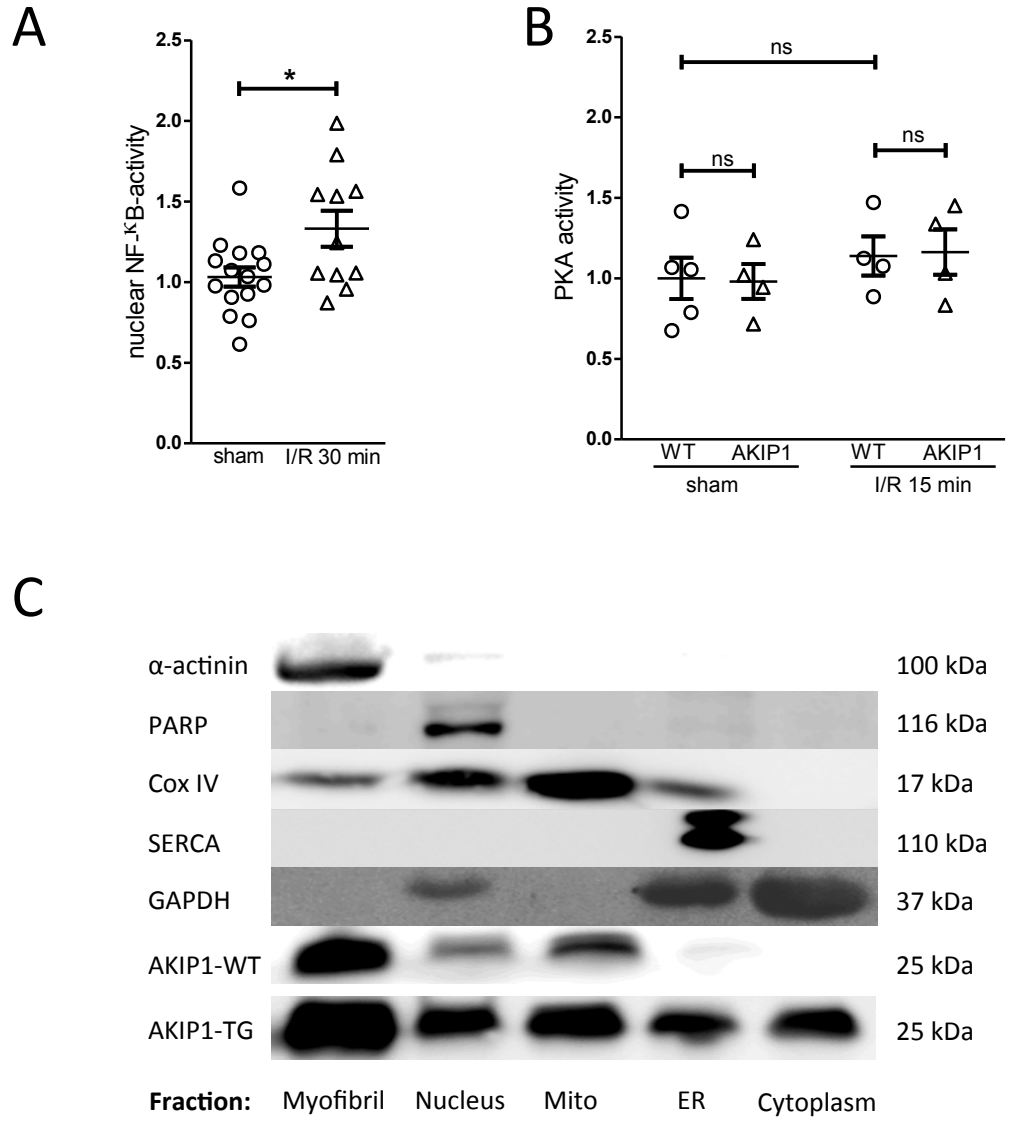




Supplementary figure 2 Capillary density 6 weeks after myocardial infarction as determined with CD-31 staining. Shown are numbers of capillaries per mm^2 and representative images of CD-31 staining in hearts of AKIP1-transgenic (AKIP1) and wild-type (WT) mice. *N*=10-11 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; ***, $p<0.001$; n.s., not significant.



Supplementary figure 3 (A) NF-kB activity in nuclear lysates from I/R and sham operated animals. Shown are measurements of NF-kB activity in nuclear fractions from mice after I/R or sham surgery where AKIP1-transgenic and wild type animals are combined. Statistical testing was performed by student *t* test; *, $p<0.05$ (B) Shown is the relative mitochondrial activity of PKA of AKIP1-transgenic (AKIP1) and WT mice. *N*=5-8 animals per group. Error bars represent mean ± SE. Statistical testing was performed by two-way ANOVA, followed by a post-hoc Tukey to compare the differences between all groups; n.s., not significant. (C) Results of sub cellular fractionation studies. Shown are (top to bottom): First 5 panels; quality control measurements in the various fractions using representative markers, 6th panel AKIP1 protein expression in these fractions obtained from a wildtype mouse, 7th panel, AKIP1 protein expression in these fractions obtained from an AKIP1-transgenic mouse. Mito, Mitochondrial fraction; ER, Endoplasmic reticulum.

Chapter 6

AKIP1 promotes physiological hypertrophy after voluntary exercise

Harmen G. Booij, Rudolf A. de Boer, Cees W.A. van de Kolk, Wiek H. van Gilst, Herman H.W. Silljé, B. Daan Westenbrink

under progress

Abstract

Background
Overexpression of A Kinase Interacting Protein 1 (AKIP1) promotes physiological hypertrophy in cultured cardiomyocytes. Whether AKIP1 stimulates physiological cardiac hypertrophy *in vivo* is unknown.

Methods and results
Mice with cardiomyocyte-specific overexpression of AKIP1 (AKIP1-TG) and their wild type (WT) littermates were subjected to 4 weeks of voluntary wheel running, whereas control mice remained sedentary. While running time and distance were comparable between AKIP1-TG and WT mice, Heart weight / tibia length was markedly increased in AKIP1-TG mice after voluntary exercise (9.5± 0,3 mg/mm in AKIP1-TG vs. 8,7± 0.2 mg/mm in WT mice, $p<0.05$). The augmentation of cardiac hypertrophy was associated with a 6-fold increase in AKT-phosphorylation upon exercise.

Conclusion
Cardiomyocyte-specific overexpression of AKIP1 promotes physiological cardiac hypertrophy after voluntary exercise.

Introduction

Heart failure (HF) is a huge burden on the health of many people. It is expected that one third of the inhabitants of the developed world will develop HF in their lifetime.[1,2] Mortality in HF is worse than in most types of malignancies.[3] To reduce this burden, we need interventions that can prevent HF or attenuate the progression of HF.

When the heart is faced with physiological and pathophysiological stress, cardiac hypertrophy develops. Hypertrophy is an adaptive mechanism that reduces wall stress and allows the heart to adapt to the increased workload. Indeed, cardiac hypertrophy that develops upon exercise or during pregnancy augments cardiac performance and is fully reversible. During sustained pathological stress, compensatory hypertrophy progresses into a pathological phenotype.[4,5] The differences in physiological versus pathological hypertrophy are recapitulated by the activation of very distinct signal transduction pathways and transcription factors. Pathological hypertrophy is characterized by fibrosis, apoptosis and fetal gene expression whereas these features are not observed in the physiological setting.[6] One strategy to alleviate pathologic cardiac remodeling is to superimpose the beneficial aspects of physiological hypertrophy upon pathologically remodeled hearts. Indeed, pathological hypertrophy can be reversed by physiological exercise in experimental HF models.[7,8] Furthermore, it has been shown that exercise training attenuates cardiovascular mortality and HF hospitalizations in HF patients.[9]

We previously performed a screen of several *in vivo* and *in vitro* models of cardiac hypertrophy and HF to determine putative molecular targets for therapy. One of the genes consistently upregulated in these models was A Kinase Interacting Protein 1 (AKIP1).[10] Several gain of function experiments demonstrated that AKIP1 could induce a physiological type of hypertrophy in cultured cardiomyocytes as evidenced by the improved mitochondrial respiration and a lack of fetal gene expression.[11] Surprisingly, cardiomyocyte-specific overexpression of AKIP1 did not cause spontaneous cardiac hypertrophy *in vivo*.[12] Also, development of pathological hypertrophy and HF remodeling was not affected by AKIP1 after pressure overload or myocardial infarction, suggesting that AIP1 does not regulated cardiac growth in heart disease. In summary, several lines of evidence suggest that AKIP1 is a specific regulator of physiological hypertrophy. To investigate this hypothesis, AKIP1-TG mice were subjected to voluntary wheel running to determine the effect on cardiac function and hypertrophy.

Methods

Animals and experimental model

AKIP1-transgenic mice (AKIP1-TG) were created as previously described.[12] These mice are phenotypically normal and have a reproduction pattern with normal mendelian ratios. AKIP1-TG mice and wild type (WT) littermates of 8-12 weeks of age were individually housed with unlimited access to a running wheel for the duration of 4 weeks. Control mice were housed similarly but in the absence of a running wheel. Running distance and running time were measured daily with a cyclometer connected to the running wheel. After 4 weeks, cardiac function was determined with MRI as previously described.[12] Left ventricular mass, left ventricular end-systolic and end-diastolic dimensions were quantified with Qmass (version MR 6.1.5, Medis Medical Imaging Systems, The Netherlands). Finally, mice were anesthetized and intra-cardiac pressures were measured with a pressure volume system (ADVantage Admittance PV system, Transonic Scisense Inc, London, ON, Canada) and analyzed with Labchart 7 (ADInstruments LtD, Dunedin, New Zealand). Hereafter, hearts were excised. Left ventricle, right ventricle and atria were separated, weighed and snap frozen in liquid nitrogen for molecular analysis.

Western Blot

Western blot was performed as described previously.[11] An AKIP1 antibody was made in our lab as described previously.[11] Antibodies for anti-phosphorylated-AKT^{Ser473} and anti-total-AKT (cell signaling) were bought commercially.

Statistical analysis.

Values are displayed as mean ± standard error of the mean. Comparisons were made with Student *t* test or one-way ANOVA followed by the Tukey post-hoc test where appropriate. A p-value <0.05 was considered significant.

Results

To investigate the effect of AKIP1-overexpression on development of physiological hypertrophy of the heart, we used transgenic mice with cardiac specific over-expression of AKIP1 as previously described.[12] AKIP1-TG mice and WT littermates were subjected to a regimen of 4 weeks of voluntary exercise or a sedentary control group. During the first week, running distance for both groups increased and then remained stable for the remainder of the experiments (Figure 1A). Running time picked up in the first few days and remained stable over the course of the 4 weeks (Figure 1B). Average daily running distance, running time and average speed were similar between both genotypes in the exercise groups (Figure 1A,B,C). Voluntary exercise resulted in a marked increase in heart weight and left ventricular weight in AKIP1-TG

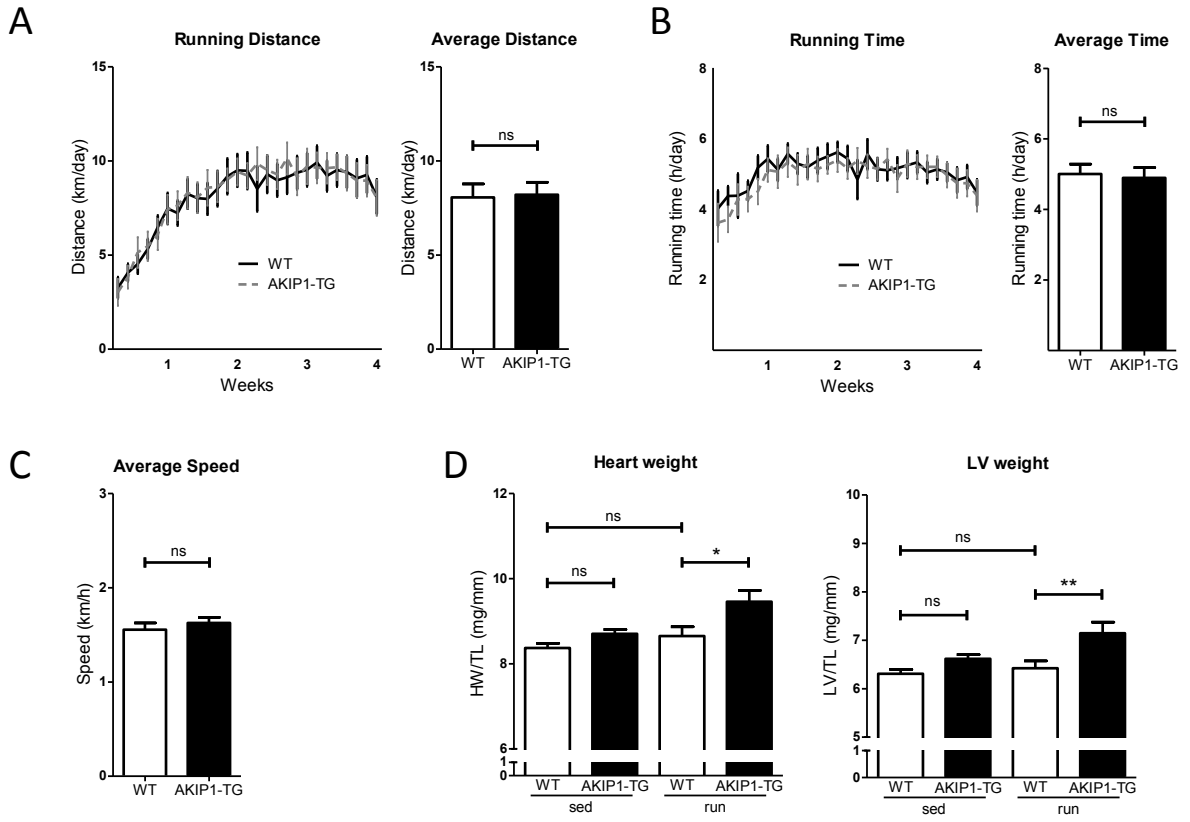


Figure 1 Running distance and heart weight after 4 weeks voluntary wheel running. Graphs depict the running distance (A), running time (B) and average running speed (C). Line graphs represent the mean ± SE for each day and bar graphs represent the average over 4 weeks with mean ± SE. *N*=9-12 animals for the groups. Statistical testing was performed with a Student *t* test. ; n.s., not significant. (D) Cardiac overexpression of AKIP1 increases heart weight and left ventricular weight corrected for tibia length after 4 weeks voluntary wheel running. Heart weight, HW; Left Ventricular weight, LV; Tibia length, TL. *N*=9-21 animals per group. Error bars represent mean ± SE. Statistical testing was performed by one-way ANOVA; *, p<0.05; **, p<0.01; n.s. not significant.

mice compared to their WT littermates (Figure 1D).

To evaluate whether the differences in cardiac weight translated into differences in cardiac function, we performed cardiac MRI and pressure-volume measurements at the end of the study. As observed before, left ventricular ejection fraction was slightly reduced in sedentary AKIP1-TG animals compared to the WT littermates (Figure 2A). This was not altered by exercise. We observed a trend to increased left ventricular end diastolic mass as determined by MRI, but this was not significant (Figure 2A). Left ventricular end-diastolic volume was similar between the experimental groups (Figure 2A). Left ventricular end-systolic volume was increased in sedentary AKIP1-TG compared to WT mice.

Indices of cardiac contractility (dP/dt max) and relaxation (dP/dt min, Tau) were determined by pressure-volume loop analysis before sacrifice. There

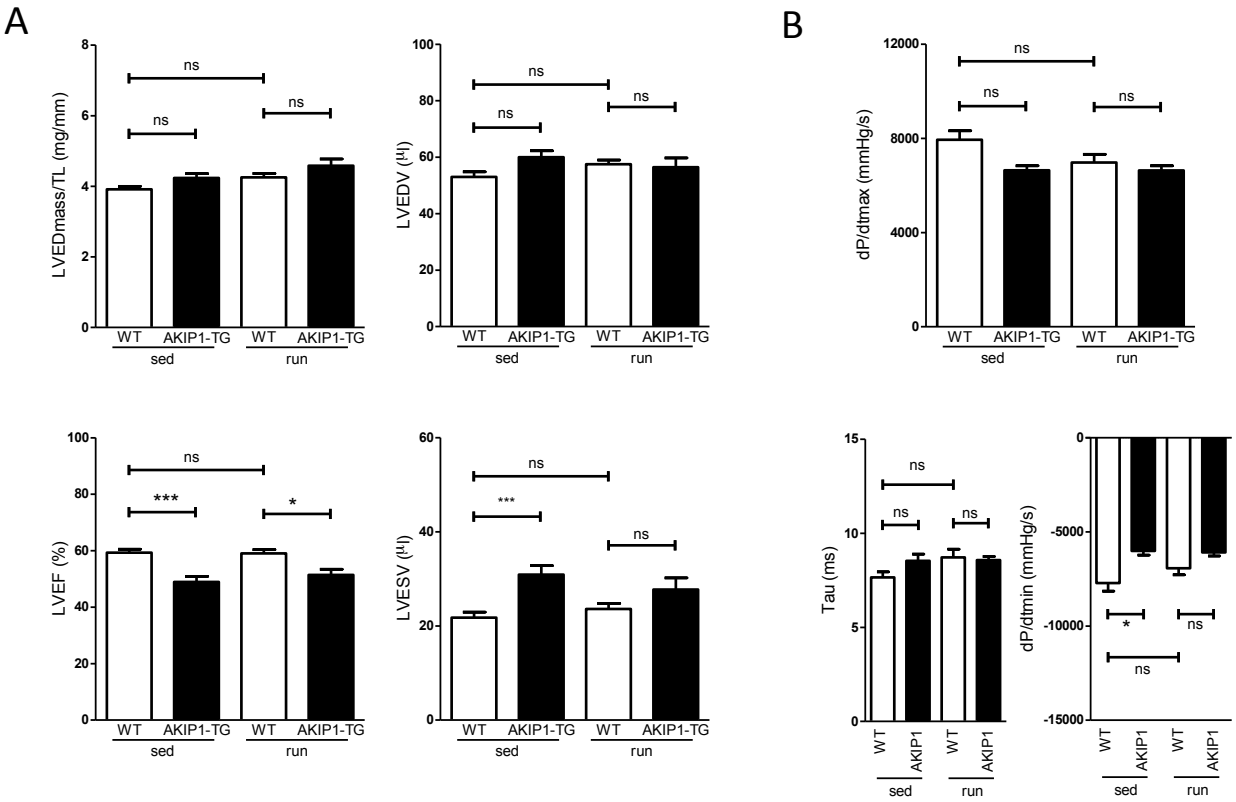


Figure 2 Overexpression of AKIP1 does not influence cardiac remodeling after transverse aortic constriction (TAC). (A) Cardiac function parameters as determined by magnetic resonance imaging and representative end-diastolic images of AKIP1-transgenic (AKIP1) and wild-type (WT) mice. Shown are Left Ventricular End-Diastolic Mass, LVEDmass corrected for Tibia Length, TL; Left Ventricular Ejection Fraction, LVEF; Left Ventricular End-Diastolic Volume, LVEDV and Left Ventricular End-Systolic Volume, LVESV. *N*=9-21 animals per group. Error bars represent mean ± SE. Statistical testing was performed by one-way ANOVA; *, $p<0.05$; ***, $p<0.001$; n.s. not significant. (B) Intracardiac pressure and relaxation measurements. Shown are dPdtmax, dPdtmin and Tau. *N*=6-12 animals per group. Error bars represent mean ± SE. Statistical testing was performed by one-way ANOVA; *, $p<0.05$; n.s. not significant.

was a slight decrease in dP/dt min in AKIP1-TG vs. WT sedentary mice, but contractility and relaxation measurements were similar between genotypes after 4 weeks of voluntary wheel running (Figure 2B).

The signal transduction molecule AKT is a central driver of physiological hypertrophy. Previous research from our group demonstrated that the pro-hypertrophic effect of AKIP1 in cultured cardiomyocytes was regulated by AKT.[11] Accordingly, we tested whether the salutary effect of AKIP1 overexpression on physiological hypertrophy was associated with activation of AKT. Voluntary wheel running was associated with a significant increase in AKT phosphorylation after exercise. While AKT phosphorylation was comparable between the genotypes in sedentary animals, AKIP1 transgenic mice displayed a significant greater augmentation of AKT phosphorylation than WT animals after exercise (Figure 3).

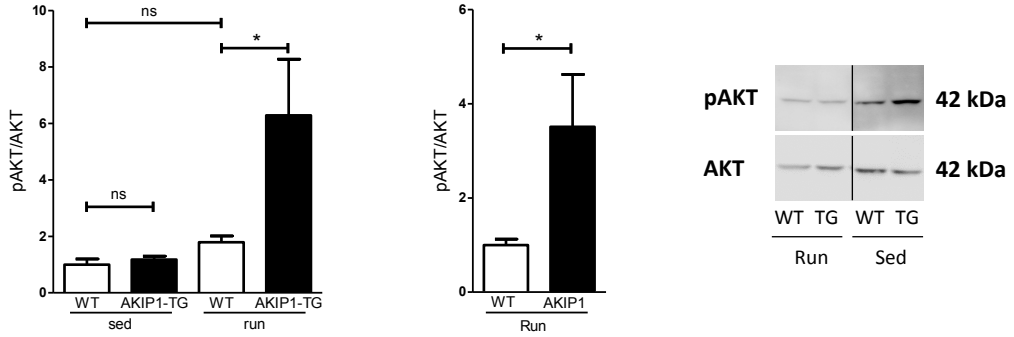


Figure 3 Myocardial AKT-phosphorylation after voluntary wheel running. Shown are densitometric analysis and representative blots. Samples were run on the same gel and pictures were taken from the same blot and same exposure time. *N*=3-4 animals per group. Error bars represent mean ± SE. Statistical testing was performed by one-way ANOVA (left panel) or Student *t* test (right panel); *, $p<0.05$; n.s. not significant.

Discussion

In the current study we demonstrate that cardiomyocyte-specific overexpression of AKIP1 results in a marked augmentation of physiological hypertrophy, as evidenced by markedly greater augmentation of heart weight and left ventricular mass than WT mice after voluntary wheel running. This difference could not be explained by differences in the amount or intensity of exercise as these parameters were comparable between genotypes. The stimulation of cardiac hypertrophy by AKIP1 was not accompanied by the development of systolic or diastolic cardiac dysfunction. Furthermore, the augmentation of cardiac hypertrophy in AKIP1-TG mice was associated with a 6-fold greater increase in the activation of AKT. Together, these findings indicate that overexpression of AKIP1 is a central and specific driver of physiological hypertrophy after exercise.

Cardiac hypertrophy is the response to increased cardiac workload and wall stress. Previous research suggests there is a beneficent, physiological type of hypertrophy and a maladaptive type of hypertrophy.[4,5] We previously showed that AKIP1 increased hypertrophy in cultured cardiomyocytes.[11] This hypertrophy occurred in the absence of several transcriptional an phenotypic signatures of pathological hypertrophy. Instead, AKIP1-overexpression was found to activate AKT and several downstream mediators of physiological hypertrophy. Importantly, cardiomyocyte hypertrophy induced by AKIP1 overexpression was blocked by the addition of an AKT-inhibitor. As AKT has been identified as a central regulator of physiological hypertrophy.[4] Our finding that cardiomyocyte-specific overexpression of AKIP1 stimulates cardiac hypertrophy and AKT phosphorylation, confirms our previous findings in cultured cardiomyocytes and reinforces the role for AKIP1 in physiological growth.

We observed that left ventricular ejection fraction was slightly decreased in AKIP1-TG animals. However, this difference was not influenced by voluntary wheel running. It should be noted, however, that our results were measured during rest. In trained human athletes it has been demonstrated that cardiac function is also slightly reduced when in rest, but becomes supra-physiological during exercise.[13,14] It is tempting to speculate that a similar phenotype exist in AKIP1-TG mice, but it would require additional experiments where cardiac function is measured during physiological or pharmacological exercise.

In the current analysis, we measured cardiac function after 4 weeks voluntary wheel running. We did not determine cardiac function before the experiment and thus could not determine the alterations in cardiac function within each experimental group. Also, this protocol consisted of voluntary exercise. The results in a forced exercise experiment might be different. Furthermore, additional measurements of biochemistry and histological measurements like cell size and fibrosis are required to claim that hypertrophy does indeed have a physiological phenotype.

In conclusion, Cardiomyocyte-specific overexpression of AKIP1 promotes physiological cardiac hypertrophy after voluntary exercise.

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Chapter 7
Discussion

Discussion

Survival for heart failure (HF) is still worse than most malignancies, despite the development of new therapeutic strategies for HF.[1] To improve the outcome of HF therapy, better insight in its pathophysiology is needed. In **part 1** of this thesis we investigated whether β-blocker therapy should be continued after successful revascularization in patients with CAD. Coronary artery disease (CAD) is still a major cause for HF, although treatment has improved with the introduction of revascularization strategies. The question remains whether traditional pharmacotherapy remains appropriate in this changing landscape. We also investigated whether revascularization for CAD reduces the propensity of diabetic patients to develop HF. **In part 2** of the thesis we studied the role of A kinase interacting protein 1 (AKIP1) in the cardiac response to stress by generating a mouse model with autonomous cardiomyocyte specific overexpression of AKIP1. First, we investigated whether AKIP1 could modulate the acute and chronic cardiac stress response, and then we also studied the role of AKIP1 in the heart in relation to physical exercise.

Metabolic interventions after revascularization

Revascularization strategies have altered the course of CAD in many patients dramatically. Previously, HF with reduced ejection fraction was common after myocardial infarction (MI). However, as treatment efficacy is becoming more and more efficient, the incidence of post-MI HF is declining. For instance, a recent trial in ST-elevation MI patients that had received a primary percutaneous coronary intervention reported that the average left ventricular ejection fraction (LVEF) after MI was close to normal (54%).[2] In this changing landscape, pharmacological therapy has been remarkably consistent. β-blocker therapy has been the cornerstone of pharmacotherapy of CAD for decades, but recent evidence suggests that this central role may not be justified in patients that are at relatively low risk, have good control of their cardiovascular risk factors and are receiving evidence-based therapy.[3-5] In patients receiving coronary artery bypass graft surgery (CABG), pre-operative β-blocker therapy has been reported to be as high as 80-93% over the last few years.[3] β-blockers are effective in reducing symptoms, but their effects on prognosis in patients with a preserved LVEF is unclear. **In chapter 2**, we performed an explorative analysis to determine if β-blocker therapy was associated with a reduction in the incidence of cardiovascular events after CABG. We showed that β-blocker therapy in fact was not related to a decreased risk of recurrent angina or cardiovascular events after CABG, in low risk patients with CAD and preserved cardiac function. Our results were consistent across different types of analyses and subgroups, including propensity matched and time-dependent analyses, suggesting that the lack of association between β-blocker therapy and clinical outcome is robust. We must stress that this study focused on low

risk patients with a low event-rate, thus the results may be different in the general CABG population which will include many patients with a previous MI and overt cardiac dysfunction. Nevertheless, our data fuel the hypothesis that these agents should not be continued indiscriminately in low risk patients. Our results are in line with the recently published Study assessInG the morbidity-mortality beNefits of the If inhibitor ivabradine in patients with coronarY artery disease (SIGNIFY) which randomized 19,102 patients with stable CAD and normal cardiac function to the selective sinus node inhibitor ivabradine. In SIGNIFY, Ivabradine also did not influence clinical outcome compared to placebo.[6] Of note, heart rate reduction is considered to be the most important mode of action of β-blockers in CAD. The neutral results of SIGNIFY therefore provide an additional line of evidence supporting that concept that modulation of the sympathetic tone is not generally effective after revascularization in low-risk patients. Although β-blockers are still important drugs for the treatment of symptomatic angina, recent MI and patients with left ventricular (LV) dysfunction,[7-9] their efficacy in other indications is under scrutiny.

HF and CAD are both common in patients with diabetes. Diabetes predisposes to severe CAD and an increased incidence of subsequent MIs, which may cause HF. Nevertheless, diabetes may also cause HF through direct toxic and / or metabolic effects on the heart that may cause a distinct diabetic cardiomyopathy (Figure 1). In **chapter 3**, we show that revascularization with CABG did not reduce the propensity of diabetic patients to develop HF. Interestingly, acute HF in these patients was not preceded by evidence of acute or clinically worsening chronic myocardial ischemia, suggesting that mechanisms beyond epicardial CAD were responsible. We should therefore be watchful of HF development in diabetic patients even if they are completely revascularized for CAD. Diabetes is believed to increase oxidative stress and activation of detrimental signal transduction pathways by glycosylation, AGEs and changes in mitochondrial metabolism.[10-13] These specific processes underlying diabetic cardiomyopathy are not influenced by revascularization and will therefore continue to exert their detrimental effects on the heart. Accordingly, these pathways could cause HF to develop after adequate revascularization (Figure 1).

In conclusion, we observed no hints that continuation of a β-blocker therapy in a-symptomatic CAD patients with preserved cardiac function is of benefit. Diabetic patients with CAD should be monitored (more) closely because they appear to develop HF, also in the absence of CAD recurrence.

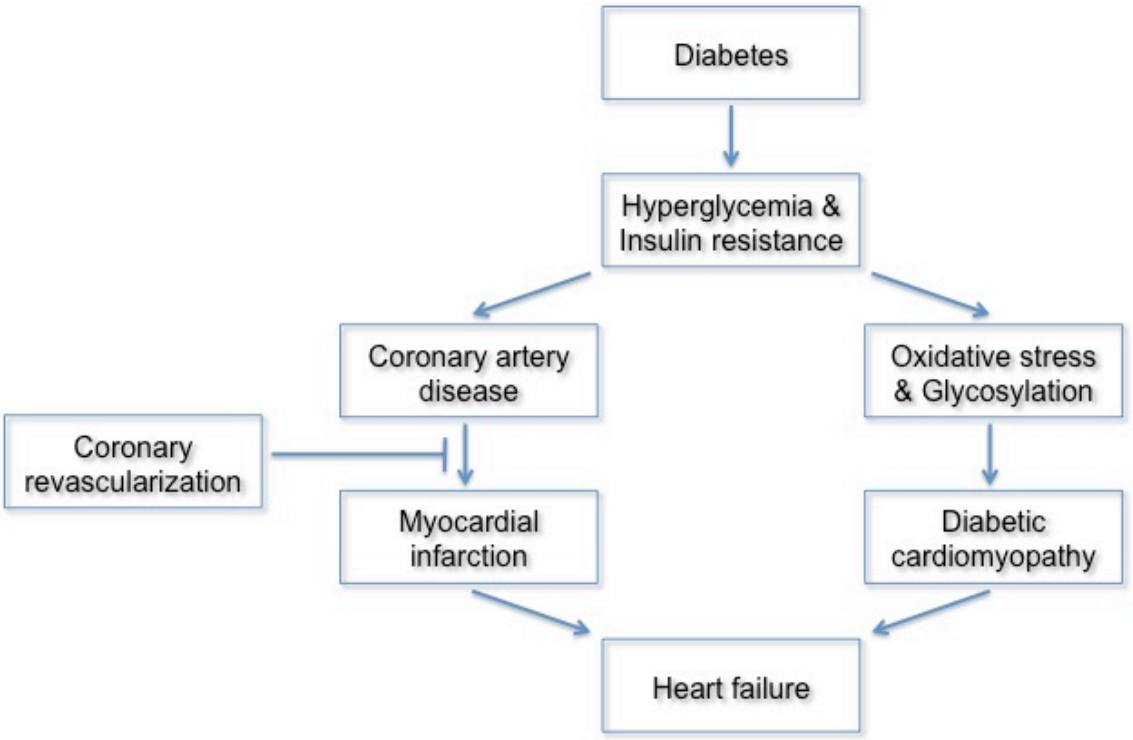

Figure 1 Pathophysiological links between diabetes and HF, adjusted and amended from Dei Cas et al.[14]

AKIP1 in cardiac stress

Myocardial hypertrophy decompensation is a main characteristic of HF development.(chapter1) Hypertrophy is a compensatory response to increased wall stress, but ultimately fails. It has long been a goal to dissect the adaptive features of hypertrophy from it's deleterious effects. We previously performed a genome wide transcription study and showed that AKIP1 was consistently upregulated in several *in vivo* and *in vitro* models of HF and pathological hypertrophy.[15] AKIP1 has previously been described in several malignant cell lines and the role of AKIP1 may vary in different cell types.[16-21] Previous findings in cultured cardiomyocytes and other cell types suggested that AKIP1 could be an important protein with protective properties under various forms of cardiac stress.[22-24] It might induce cardiomyocyte hypertrophy *in vitro* by activating AKT and protect from ischemic injury in several *in vitro* and *ex vivo* models. Furthermore, we showed that AKIP1 improves mitochondrial efficiency, by increasing mitochondrial energy production while decreasing mitochondrial reactive oxygen species (ROS) production.[22]

In **chapter 4** we describe how mitochondrial efficiency becomes compromised during advanced HF, leading to an energy deficit of the heart. Also, increased mitochondrial ROS production during HF damages structures in the mitochondrial membrane, leading to further loss of mitochondrial energy production and even more oxidative stress. To investigate whether the effects of AKIP1 on hypertrophy and efficient energy metabolism translate to

beneficial effects of AKIP1 *in vivo*, we generated a transgenic mouse line with cardiomyocyte-autonomous overexpression of AKIP1 (AKIP1-TG).

In **chapter 5** we showed that, in contrast to our *in vitro* studies, a stable 40-fold increase in cardiac AKIP1-protein expression did not cause a spontaneous cardiac phenotype *in vivo*. The only detectable difference between AKIP1-TG mice and their wild type (WT) littermates was a minor reduction in cardiomyocyte diameter after 4 months. To investigate whether overexpression of AKIP1 could be beneficial in HF, we subjected AKIP1-TG mice to two well-established HF models; transverse aortic constriction (TAC) through banding of the aorta between the carotid arteries and permanent myocardial infarction through ligation of the left coronary artery. In contrast to our expectations, AKIP1 overexpression did not affect left ventricular hypertrophy, nor did it influence left ventricular dysfunction after TAC. After permanent coronary artery ligation, AKIP1-TG mice displayed a 1/3rd reduction in myocardial infarct size and modest reduction in cardiomyocyte size. However, this did not translate into improvements in cardiac function. To investigate whether AKIP1 could reduce infarct size after ischemia/reperfusion (I/R), we subjected our mice to temporary coronary artery ligation. We found that infarct size after 45 minutes of ischemia followed by 24 h of reperfusion was significantly reduced in AKIP1-TG mice and resulted in marked reduction in myocardial infarct formation. Most studies have shown that the primary role for AKIP1 in cells is the modulation of PKA and NF-κB activity. As these proteins are key mediators of myocardial reperfusion injury, we first hypothesized that the protective effect of AKIP1 could be explained by modulation of these proteins. In contrast to studies in other cell types, AKIP1 did not bind to NF-κB or PKA and also did not influence the activity of these signaling molecules. Furthermore, in contrast to our findings in cultured cardiomyocytes,[23] AKT phosphorylation was not influenced by AKIP1 overexpression after I/R injury. AKIP1, however, did localize to mitochondria, where it was found to associate with ATP-synthase. ATP-synthase has been identified as a key component of the mitochondrial permeability transition (MPT) pore, which is a key effector of necroptosis.[25] To test whether the salutary effects of AKIP1 could be explained by reductions in MPT pore formation, we performed calcium induced mitochondrial swelling analysis. Interestingly, mitochondrial isolated from AKIP1-TG mice displayed markedly reduced calcium-induced swelling, indicative of reduced MPT pore formation. These last findings indicate that AKIP1 attenuates myocardial I/R injury by reducing MPT pore formation, which could be explained by its interaction with ATP-synthase.

While previous *in vitro* evidence suggested that AKIP1 could induce a physiological type of cardiac hypertrophy,[23] we investigated this hypothesis in more detail in **chapter 6,** where we describe the results of a voluntary wheel running exercise. AKIP1-TG and WT mice were subjected to voluntary wheel running or regular housing without running wheel. AKIP1-TG and WT mice ran

the same distance over the course of 4 weeks. We showed that AKIP1 overexpression caused an increase in heart weight during wheel running, while cardiac function was preserved. This was associated with increased AKT-activity in AKIP1-TG mice after running (Figure 2). Together, this shows that AKIP1 is not essential for development of pathological hypertrophy, but we observed it may promote physiological hypertrophy and reduce infarct size formation after an acute ischemic injury.

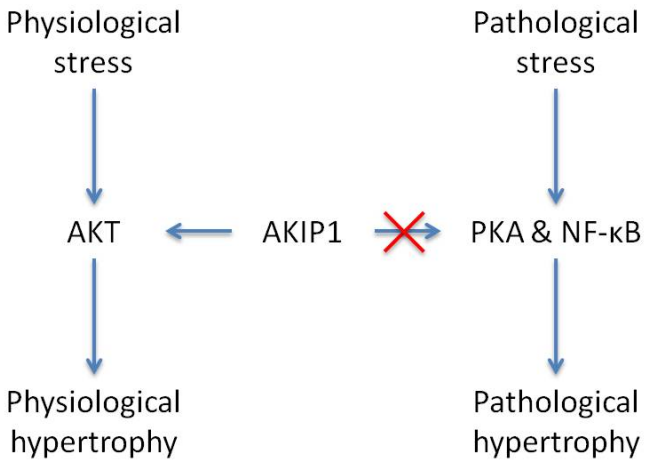

Figure 2 AKIP1 does promote physiological hypertrophy, but does not alter development of pathological hypertrophy.

In summary, we conclude that AKIP1 does not change myocardial hypertrophy decompensation or HF development, but elevated AKIP1 levels do enhance myocardial hypertrophy after exercise. Furthermore, cardiac-specific AKIP1 overexpression reduced infarct size, presumably via its interaction with ATP-synthase and inhibition of MPT pore formation.

Future perspectives.

As treatment for CAD continues to improve, the classical type of ischemic HF with reduced ejection fraction will become less prevalent. In patients with acute coronary syndromes, rapid revascularization therapy is extremely effective as it minimizes injury, reduces scar formation and preserves cardiac function. The suitability of the temporary pharmacological regimen after MI should therefore be reconsidered in patients with small infarcts. More importantly, data from studies such as **chapter 2** will hopefully change our approach to the treatment of stable CAD. Currently β-blockers are continued for decades in patients with stable CAD, without any evidence for their efficacy in the absence of angina. Furthermore, common comorbidities in patients with CAD such as hypertension and diabetes also predispose to HF through mechanisms beyond CAD and can therefore cause non-ischemic HF in patients with CAD. **Chapter 3** underscores this concept because new HF in diabetic patients was increased in the absence of CAD recurrence. Therefore we should

be mindful that patients with diabetes are at increased risk of developing non-ischemic HF. We should focus future research towards the development of therapeutic targets in non-ischemic HF.

Overexpression of AKIP1 did protect from reperfusion injury. Simulating or stimulating the effect of AKIP1 might therefore be a putative target for pharmacotherapeutics during acute MI. Our research suggests that AKIP1 reduces infarct size through inhibition of MPT pore opening. The MPT pore forms during acute stress and although its structure is not clear, it is composed of at least ATP-synthase, mitochondrial translocator TSPO and cyclophilin-D.[26] Of note, the TSPO inhibitor TRO40303 did not cause reductions in infarct size in the clinical setting.[27] We might need to target another part of the MPT pore. Therefore, we need to look further into the interaction of AKIP1 with the MPT pore to elucidate what part of AKIP1 and the MPT pore do interact. Subsequently, we could potentially design a pharmacotherapeutic or small peptide that resembles the functional domain of AKIP1 and test this in experimental setup. An important question is whether a pharmacotherapeutic would only be beneficial when given before ischemia, or that it would also be beneficial when given at reperfusion. The latter would be more promising as a future therapy. AKIP1 appears to have a specific role in mitochondria. In addition, many signaling molecules have different functions in different cellular compartments, necessitating specific mitochondrial targeting. There are several ways to improve the delivery of a pharmacotherapeutic to the right cellular compartment. For instance, we could add a molecular group including a cation to the pharmacon that targets directly to mitochondria.[28]

AKIP1 increased cardiac hypertrophy after exercise i.e. physiological hypertrophy, but pathological hypertrophy was not affected. Since both types of hypertrophy are a response to increased cardiac workload, it is an interesting question whether we could superimpose physiological hypertrophy to pathologically remodelled hearts. Physiological hypertrophy is the result of an intermittent pattern of increased cardiac workload and cardiac structure and organization remains normal.[29] As physiological hypertrophy is accompanied with improvements in cardiac performance in healthy individuals, this may suggest that physiological hypertrophy is also helpful in the failing heart. While studies show that exercise training in HF patients can improve exercise capacity,[30,31] the benefits on survival rate are limited. The HF-ACTION trial, which randomized 2331 HF patients to exercise or control, only showed a trend towards decreased mortality or hospitalizations after a thorough exercise training program during a median follow up of 2.5 years.[30] Although cardiac hypertrophy, either physiological or pathological, was not assessed in this trial, the results are supportive for the suggestion that stimulation of physiological hypertrophy of the heart has a slight benefit in patients with HF. Nevertheless, it is unlikely that AKIP1 would offer us possibilities to physiologically remodel

pathological hypertrophy because AKIP1 overexpression did not affect pathological remodeling in our model.

Several screens have been reported which compare the molecular characteristics of physiological and pathophysiological hypertrophy. It would be useful if we could identify and modulate a key factor that promotes physiological hypertrophy. The AKT-signalling pathway is implicated as such a key factor.[32] The challenge in translating the concept of physiological hypertrophy to the clinical setting might be that the line between physiological and pathological hypertrophy is blurry. Whereas physiological hypertrophy is regarded to regress after deconditioning from training, some athletes retain characteristics like enlarged left ventricular cavity dimensions.[33,34] It might therefore be difficult to hit the sweet spot for pharmacological stimulation of physiological hypertrophy in HF.

Several screens were performed that improved our understanding of physiological and pathological gene expression.[35-37] Some of these screens used up to 5 models of hypertrophy. While some used only genetic models, others used also more clinical models of HF. To improve the previously mentioned screen performed by our laboratory,[15] it would be useful to shift focus from the currently combined *in vitro/in* vivo models to *in vivo* models, include more in vivo models, add a comparison from pathologic and physiologic models and include a comparison of human samples.

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Nederlandse wetenschappelijke samenvatting

Hartfalen is een groot gezondheidsprobleem. Het kans dat iemand hartfalen ontwikkelt tijdens zijn leven is bijna 1 op 3. De afgelopen jaren is de therapie voor hartfalen verbeterd. De prognose van hartfalen patiënten is vaak slechter dan voor patiënten met kanker. Het hart is als orgaan één van de grootverbruikers van energie in het lichaam. Er wordt gedacht dat een falend energie metabolisme een belangrijke oorzaak is voor het ontwikkeling van hartfalen. In dit proefschrift onderzochten we verschillende metabole behandelingen bij patiënten met hartziekten en bij proefdiermodellen. In het eerste deel onderzochten we klinische aspecten, waaronder de plaats van β-blockers die verschillende metabole bijwerkingen hebben. In dit eerste deel beschrijven we ook het effect van bypass-chirurgie van de kranslagaders (coronary artery bypass grafting, CABG) op de ontwikkeling van hartfalen in patiënten met diabetes. In deel 2 van dit proefschrift onderzochten we de rol van A kinase interacting protein 1 (AKIP1) op structurele en functionele veranderingen die het hart ondergaat wanneer hartfalen ontstaat. AKIP1 is een mitochondrieel eiwit dat een aangrijpingspunt zou kunnen vormen voor nieuwe behandelingen van hartfalen. Het effect van AKIP1 hebben we onderzocht in een transgeen muismodel, waarbij AKIP1 tot overexpressie was gebracht in hartspiercellen.

Deel 1: Metabole interventies na revascularisaties

In **hoofdstuk 2** van dit proefschrift hebben we de rol van β-blockers onderzocht bij laag risico patiënten met behouden linker ventrikelfunctie, die behandeld werden voor coronairlijden met CABG. Behandeling met β-blockers was niet geassocieerd met een vermindering van cardiovasculaire aandoeningen, zoals recidief coronairlijden (hartinfarct of pijn op de borst door vernauwing van de kransslagaders van het hart) en ziekenhuisopnames voor hartfalen.

Diabetes is een risicofactor voor hartfalen, wat vaak komt door een hogere kans op coronairlijden. In **hoofdstuk 3** onderzochten we of een bypassoperatie bij coronairlijden de kans op hartfalen verminderde bij patiënten met diabetes. In de groep onderzochte laag-risico patiënten die een bypassoperatie hadden ondergaan, was diabetes geassocieerd met een verhoogd risico op hartfalen. Dit werd niet voorafgegaan door nieuwe coronaire events of obstructief coronairlijden. Dit suggereert dat andere effecten van diabetes, zoals diabetische cardiomyopathie, verantwoordelijk waren voor de ontwikkeling van hartfalen.

Deel 2: AKIP1 in hartziekten

In **hoofdstuk 4** beschrijven we de vermindering van de mitochondriële functie tijdens hartfalen. Dit zorgt voor een tekort aan energie in het hart en een

verhoogde productie van zuurstofradicalen (oxidatieve stress). Dit beschadigt verschillende structuren in het cellen van het hart, waaronder de mitochondriële membraan. Dit leidt tot verder verlies van mitochondriële functie en meer oxidatieve stress.

Daarna onderzochten we AKIP1 als potentieel aangrijpingspunt voor therapie tijdens de cardiale respons op acute en chronische belasting *in vivo*. AKIP1 is naar voren gekomen uit een screen waarbij expressie van genen is vergeleken bij verschillende experimentele modellen van hartfalen en pathologische hypertrofie. Hierbij zagen we dat er meer AKIP1 gemaakt werd in het hart. In cel-experimenten met hartspiercellen, zagen we dat AKIP1 gunstige (fysiologische) hypertrofie stimuleerde via activatie van de second messenger AKT. Daarnaast beschermde AKIP1 hartspiercellen tegen ischemische schade in verschillende *in* vitro en *ex vivo* modellen. Ook had overexpressie van AKIP1 een gunstig effect op de functie van mitochondriën doordat het mitochondriële energieproductie stimuleerde terwijl het de productie van zuurstofradicalen juist verminderde. In **hoofdstuk 5** onderzochten we de effecten van AKIP1 in genetisch gemodificeerde muizen waarbij AKIP1 tot overexpressie was gebracht in hartspiercellen. In tegenstelling tot *in vitro* studies, zorgde transgene hartspier-specifieke overexpressie van AKIP1 niet voor verbetering van hartfunctie na chronische belasting van het hart. We vonden wel dat AKIP1 zorgde voor een vermindering van infarct-grootte na ischemie-reperfusie schade (een model voor het acute myocardinfarct) doordat AKIP1 voorkwam dat de mitochondriële permeability transition (MPT) pore ontstond. De opening van de MPT pore is een essentiële stap in celdood na reperfusie. Deze bevindingen suggereren dat AKIP1 verder onderzocht zou moeten worden als aangrijpingspunt voor therapie bij ischemie-reperfusie schade na een hartinfarct. In **hoofdstuk 6** onderzochten we de effecten van AKIP1 na een inspanningsprotocol. Hartspier-specifieke overexpressie van AKIP1 zorgde voor toename van hartgewichten terwijl hartfunctie behouden bleef. Dit was geassocieerd met activatie van AKT. Samenvattend kunnen we dus concluderen dat AKIP1 niet zorgt voor een vermindering van de ontwikkeling van hartfalen, maar dat verhoogde AKIP1-expresssie wel zorgt voor een toename van gunstige (fysiologische) hypertrofie na inspanning. Verder zorgt AKIP1 overexpressie in hartspiercellen voor kleinere infarcten, vermoedelijk doordat AKIP1 de vorming van dat de MPT pore tegengaat.

Vooruitblik naar de toekomst

Nu de behandeling van coronairlijden steeds verder verbetert, zal ischemisch hartfalen met verminderde kamerfunctie minder vaak voorkomen. In patiënten met een acuut coronair syndroom zorgt de huidige snelle revascularisatie voor een sterke vermindering van schade en voor een behoud van kamerfunctie. De medicatie die gegeven wordt na een hartinfarct, zou daarom nog eens tegen

het licht gehouden van de huidige tijd. Studies zoals die in **hoofdstuk 2**, zullen hopelijk de benadering van patiënten met coronairlijden veranderen. Op dit moment krijgen patiënten met stabiel coronairlijden nog vaak β-blockers, terwijl de effectiviteit hiervan in de afwezigheid van angina pectoris niet bewezen is. Verder predisponeren comorbiditeiten, zoals diabetes, voor verergering van coronairlijden waardoor hartfalen kan ontstaan. Maar deze comorbiditeiten hebben ook directe effecten op het hart, waaronder toxische en / of metabole effecten. Deze comorbiditeiten kunnen hierdoor leiden tot hartfalen door andere mechanismen dan coronairlijden, zoals beschreven in **hoofdstuk 3**. We zouden toekomstig onderzoek moeten richten op therapeutische aangrijpingspunten in niet-ischemisch hartfalen.

Overexpressie van AKIP1 beschermde tegen reperfusie-schade. Ons onderzoek suggereert dat AKIP1 de infarctgrootte kan verminderen door de formatie van de MPT pore tegen te gaan. De MPT pore wordt gevormd tijdens acute ischemie-reperfusie. Hoewel de structuur van de MPT pore nog niet helemaal duidelijk is, zouden we de interactie tussen AKIP1 en de MPT pore moeten onderzoeken om te ontdekken hoe AKIP1 de formatie van de MPT pore tegengaat. Vervolgens zouden we een farmacologisch molecuul kunnen ontwerpen dat vergelijkbaar is met het functionele domein van AKIP1 en dit testen in een experimenteel model.

AKIP1 versterkt de fysiologische hypertrofie respons in het hart na een inspanningsprotocol, maar had geen effect op de pathologische hypertrofie respons. Het blijft een interessante vraag of het stimuleren van fysiologische hypertrofie zorgt voor verbetering in harten die pathologisch geremodelleerd zijn. Desalniettemin is het minder waarschijnlijk dat AKIP1 ons mogelijkheden biedt om pathologische hypertrofie te verbeteren omdat pathologische veranderingen van het hart niet verbeterden door AKIP1 overexpressie in ons *in vivo* model.