Inhibition of G-protein-coupled receptor kinase 2 prevents the dysfunctional cardiac substrate metabolism in fatty acid synthase-transgenic mice

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Abstract: Impairment of myocardial fatty acid substrate metabolism is characteristic of late-stage heart failure and has limited treatment options. Here we investigated whether inhibition of G-protein-coupled receptor kinase 2 (GRK2) could counteract the disturbed substrate metabolism of late-stage heart failure. The heart failure-like substrate metabolism was reproduced in a novel transgenic model of myocardium-specific expression of fatty acid synthase (FASN), the major palmitate-synthesizing enzyme. The increased fatty acid utilization of FASN-transgenic neonatal cardiomyocytes rapidly switched to a heart failure phenotype in an adult-like lipogenic milieu. Similarly, adult FASN-transgenic mice developed signs of heart failure. The development of disturbed substrate utilization of FASN-transgenic cardiomyocytes and signs of heart failure were retarded by the transgenic expression of GRKInh, a peptide inhibitor of GRK2. Cardioprotective GRK2 inhibition required an intact ERK (extracellular signal-regulated kinase) axis, which blunted the induction of cardiotoxic transcripts, in part by enhanced serine-273 phosphorylation of Pparg (peroxisome proliferator-activated receptor γ). Conversely, the dual-specific GRK2 and ERK cascade inhibitor, RKIP (raf kinase inhibitor protein), triggered dysfunctional cardiomyocyte energetics and the expression of heart failure-promoting Pparg-regulated genes. Thus, GRK2 inhibition is a novel approach that targets the dysfunctional substrate metabolism of the failing heart.

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Inhibition of G-protein-coupled Receptor Kinase 2 Prevents the Dysfunctional Cardiac Substrate Metabolism in Fatty Acid Synthase Transgenic Mice*•

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Impairment of myocardial fatty acid substrate metabolism is characteristic of late-stage heart failure and has limited treatment options. Here, we investigated whether inhibition of G-protein-coupled receptor kinase 2 (GRK2) could counteract the disturbed substrate metabolism of late-stage heart failure. The heart failure-like substrate metabolism was reproduced in a novel transgenic model of myocardium-specific expression of fatty acid synthase (FASN), the major palmitate-synthesizing enzyme. The increased fatty acid utilization of FASN transgenic neonatal cardiomyocytes rapidly switched to a heart failure phenotype in an adult-like lipogenic milieu. Similarly, adult FASN transgenic mice developed signs of heart failure. The development of disturbed substrate utilization of FASN transgenic cardiomyocytes and signs of heart failure were retarded by the transgenic expression of GRKInh, a peptide inhibitor of GRK2. Cardioprotective GRK2 inhibition required an intact ERK axis, which blunted the induction of cardiotoxic transcripts, in part by enhanced serine 273 phosphorylation of Pparg (peroxisome proliferator-activated receptor γ). Conversely, the dual-specific GRK2 and ERK cascade inhibitor, RKIP (Raf kinase inhibitor protein), triggered dysfunctional cardiomyocyte energetics and the expression of heart failure-promoting Pparg-regulated genes. Thus, GRK2 inhibition is a novel approach that targets the dysfunctional substrate metabolism of the failing heart.

Heart failure is a debilitating syndrome that involves insufficient cardiac performance. Multiple pathomechanisms have been elucidated, but treatment options remain insufficient, and hence the mortality of heart failure is high (1). The causes of heart failure are complex with ischemic heart disease being the most frequently associated condition (2). Co-existing disorders such as diabetes, hypertension, and obesity further deteriorate symptoms (3). Despite having a different etiology, late-stage heart failure is commonly characterized by severe changes in myocardial substrate metabolism, with a switch from fatty acid oxidation toward predominant glycolysis (4–6). Conflicting evidence exists as to whether this substrate switch is beneficial or detrimental (7), but several previous studies have indicated that an increased availability of lipid substrates that counteract the substrate switch could improve cardiac function (7, 8). Moreover, treatment options, which improve substrate availability, are attractive because the failing heart is often considered to be “an engine running out of fuel” (9).

Following this concept, we aimed to investigate the impact of improved cardiac substrate availability by generating transgenic mice with myocardium-specific expression of fatty acid synthase (FASN), the major palmitate-synthesizing enzyme. Such an approach is also supported by data obtained for myocardium-specific Fasn deficiency, which have revealed the cardioprotective potential of Fasn (10). Moreover, hearts from patients with heart failure showed an increased expression and protein level of FASN2 (10, 11). By generation of transgenic mice, we found that FASN transgenic mice developed a heart failure-like phenotype with impaired cardiomyocyte substrate use. In search for a treatment approach for the disturbed cardiac substrate metabolism, we focused on the role of GRK2 inhibition because GRK2 inhibition could counteract cardiotoxicity through promotion of a cardiomyocyte survival program (12, 13) and desensitization of the cardioprotective adiponectin receptor 1 (14–16). For GRK2 inhibition in vivo, we used GRKInh, a small peptide inhibitor derived from the first intracellular loop of the β2-adrenergic receptor (12, 17). Our data with GRKInh show that GRK2 inhibition counteracts the heart failure-related cardiac metabolic dysfunction and signs of heart failure of FASN transgenic mice.

Experimental Procedures

Generation of Transgenic Mice and Animal Experiments—Transgenic mice were generated as described (12) with minor modifications. Briefly, for the generation of transgenic mice with myocardium-specific expression of FASN, we constructed a transgene that placed the FASN cDNA under the control of the α-myosin heavy chain (α-MHC) promoter (12). For the generation of transgenic mice with myocardium-specific expression of UCP1 (uncoupling protein-1), PPARG, and

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‡ The abbreviations used are: FASN, fatty acid synthase; AAC, abdominal aortic constriction; B6 mice, C57BL/6J mice; DAG, diacylglycerol; ECAR, extracellular acidification rate; FFA, free fatty acid; α-MHC, α-myosin heavy chain; OCR, oxygen consumption rate.

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PPARG-S273A (isoform-1, which lacks amino acids 1–28 of isoform-2; serine 273 refers to the numbering of isoform-2), a similar approach was used. The plasmid sequence was removed by NotI digestion, and the purified linear DNA (2 ng/μl) was injected into fertilized oocytes of superovulated B6 (C57BL/6) mice. For generation of transgenic Tg-PPARG and Tg-PPARG-S273A mice, fertilized oocytes from non-transgenic and Tg-GRKInh mice (transgenic mice with myocardium-specific expression of GRKInh, a GRK2-specific peptide inhibitor with the peptide sequence MAKFRLQTVTNFITSE) were used. Transgenic mice with myocardium-specific expression of GRKInh or human RKIP (PEBP1) were generated and characterized previously (12). Mouse lines in the study were deposited into the JAX repository (The Jackson Laboratory) and have the following strain ID numbers: 911818 (C57BL/6Tg(MHCPEBP1)1 Sjaa); 911822 (C57BL/6Tg(MHCGRK-Inh)1 Sjaa); 911826 (C57BL/6Tg(MHCFASN)1 Sjaa); and 911830 (C57BL/6Tg(MHUCP1)1 Sjaa).

The effect of rosiglitazone-induced Pparg activation was analyzed with 8-month-old male Apoe−/− mice, which had received 30 mg/kg/day rosiglitazone for 2 months. Untreated, age-matched Apoe−/−, and non-transgenic B6 mice served as control groups. Abdominal aortic constriction (AAC) was performed in 4-month-old male B6 mice to trigger pressure overload-induced cardiac hypertrophy and signs of heart failure (11). Age-matched control mice underwent the identical surgical procedure except for ligation of the aorta (sham-operated mice). All of the mice were kept on a 12-h light/12-h dark regime and had free access to food and water. The Apoe−/− mice were fed a rodent chow that contained 7% fat and 0.15% cholesterol (AIN-93-based diet), whereas B6 mice were fed a standard rodent chow containing 4.5% fat.

Transthoracic echocardiography was performed with a Vivid 7 echocardiograph (GE Healthcare) with a 12 MHz linear array transducer similarly as described previously (11). The left ventricular ejection fraction was calculated in the M-mode of the transducer similarly as described previously (11). Transthoracic echocardiography was performed with a Vivid 7 echocardiograph (GE Healthcare) with a 12 MHz linear array transducer similarly as described previously (11). The left ventricular ejection fraction was calculated in the M-mode of the transducer similarly as described previously (11).

Animal experiments were performed in accordance with National Institutes of Health guidelines, and they were reviewed and approved by the local committee on animal care and use (University of Zurich).

Whole Genome Microarray Gene Expression Analysis—Whole genome microarray gene expression analysis of cardiac tissue was performed using Affymetrix GeneChip Mouse genome MG430 2.0 arrays essentially as described previously (18). Gene ontology analyses of microarray data were performed with GCOS and/or RNA-processed data using GeneSpring GX software (Agilent). Probe sets, which were significantly up-regulated in failing hearts (fold change ≥2 relative to the respective control group and p ≤ 0.01) were used for gene ontology classification. Microarray gene expression data are available at the NCBI GEO database accession numbers GSE25765-8 (GSE25765, GSE25766, GSE25767, and GSE25768), GSE28031, and GSE49351.

Gene expression of selected genes was also analyzed by real time quantitative (q) RT-PCR with a LightCycler 480 (Roche Diagnostics). Sequences of the forward and reverse primers were as follows: Acaca forward 5′-GGCTTGGTCAGCTAC-3′ and Acaca reverse 5′-GACCCAGCAGGATAGTCTG-3′; Adipqk forward 5′-ACTGCAATCTCCGGACT-3′ and Adipqk reverse 5′-GAGGCTGTTGGACACTTTCT-3′; Fasn forward 5′-GGCCCTCTGTTAATTTGCT-3′ and Fasn reverse 5′-GCCTTGTGGTGAGACACTTG-3′; Fasn forward 5′-TCGTGTTGACTCTCCTGCC-3′ and Fasn reverse 5′-AACGGCTAGTCTGCTGTC-3′; PPARG forward 5′-GTCCTGGTGATCTCTCCGTA-3′ and PPARG reverse 5′-AGCTTTATCCTCAGACAGCA-3′; Retn forward 5′-GTCCTGCTAAGTGCTTGCAC-3′ and Retn reverse 5′-GGCTGCTGTCCAGTCTATCCTTG-3′; and Ucp1 forward 5′-CACTGCGAAAGTCCGCTTCAGA-3′ and Ucp1 reverse 5′-GCAGGCGAGCCGCTACGTTT-3′.
antibodies, raised in rabbit against an antigen encompassing amino acids 8–106 of PPARG (Santa Cruz Biotechnology Inc.) or synthetic phosphopeptides derived from PPARG around the phosphorylation site of Ser-273 or Ser-112 (BIOSS antibodies; Abcam); and Ucp1/UCP1 antibodies raised in rabbit against an antigen encompassing amino acids 288–302 of mouse/human Ucp1/UCP1 (11). For the immunohistological and immunoblot detection of activated phospho-ERK1/2, phospho-ERK1/2-specific antibodies were used detecting activated ERK1/2 phosphorylated at Thr-202 + Tyr-204 of ERK1 and Thr-185 + Tyr-187 of ERK2 (E10 mouse mAb, Cell Signaling). For immunoblot detection of ERK1/2, the ERK1/2-specific antibodies raised in rabbits (Cell Signaling) were used, and immunofluorescence detection of p38 MAPK on cardiac sections was performed with anti-p38 antibodies (Cell Signaling). The immunoblot detection of activated AMPKα (Prkaa1/2; protein kinase, AMP-activated α1/2 catalytic subunit) phosphorylated on Thr-183/172 was detected with antibodies raised in rabbit against a synthetic peptide corresponding to residues that surrounded Thr-172 (40H9, Cell Signaling). Immunoblotting and immunohistochemistry were routinely used to determine and confirm the cross-reactivity of the antibodies with the respective mouse and human proteins.

Immunohistology Analyses and Immunofluorescence—For immunohistology, we used paraffin sections or cryosections of mouse heart specimens. Immunohistological detection of Fasn (FASN) was performed with affinity-purified polyclonal antibodies as described (11). Methods describing Oil Red O staining, immunofluorescence detection of proteins, and immunohistology for activated phospho-ERK1/2 in paraffin sections or cryosections have been described previously (11, 12). Immunohistology sections were imaged with a Leica DMi6000 microscope equipped with a DFC420 camera, and immunofluorescence imaging was performed with a Leica (TCS) confocal laser microscope.

Immunoblot Detection of Proteins—For immunoblot detection of proteins, cardiac tissue was pulverized in liquid nitrogen and extracted with RIPA buffer supplemented with protease/phosphatase inhibitor mixture, as described previously (20). Detection of proteins was performed with affinity-purified antibodies or F(ab)_2 fragments of the respective antibodies (11, 12) after separation of proteins by SDS-PAGE and subsequent electrophoretic protein transfer to PVDF membranes. Bound antibody was visualized with F(ab)_2 fragments of enzyme-coupled secondary antibodies (Dianova) or by enzyme-coupled protein A (Merck Millipore) as applicable and was followed by enhanced chemiluminescent detection (ECL Prime, Amersham Biosciences).

Functional Assays—Mouse or rat neonatal cardiomyocytes were isolated and transfected as described (12, 21). Fibroblasts were removed by preplating for 1 h at 37 °C. Cardiomyocytes were collected and cultivated in minimum essential medium supplemented with 5% FCS and 25 mg/liter BrdU (5-bromo-2′-deoxyuridine). For knockdown of Fasn and Ucp1, neonatal cardiomyocytes were transfected with stealth RNAi targeting the coding sequence of rat or mouse Fasn (nucleotides 428–452 and 1990–2014; Invitrogen) and Ucp1 (nucleotides 289–313 and 401–425; Invitrogen). For cardiomyocyte expression of PPARG and PPARG-S273A, the human cDNAs encoding PPARG and PPARG-S273A were inserted into the KpnI/XbaI sites of pcDNA3 (Invitrogen). All of the mutants and constructs that were generated by PCR were sequenced entirely. DNA strand breaks were determined in situ by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) technique (Roche Applied Science) (12).

Measurement of the [ATP] of cardiac tissue extracts was performed as described (11), and Pparg transcription factor DNA binding activity was determined with a Pparg transcription factor assay kit (Abcam). Cellular AMP, total cardiac free fatty acids (FFA), and triacylglycerol (TAG) contents were analyzed as detailed previously (11, 21). Cardiac contents of diacylglycerol (DAG) and ceramides were determined with the DAG kinase assay method as described (22).

Measurement of Cardiomyocyte Substrate Metabolism—We used a Seahorse XF24 extracellular flux analyzer (Seahorse Bioscience) to determine the cardiomyocyte substrate metabolism. The oxygen consumption rates (OCR) (pmol/min) and extracellular acidification rates (ECAR) (the H⁺ production rate, pH/min), of neonatal cardiomyocytes (10 000 cells/well) plated on Cell-Tak-coated plates (Discovery Labware Inc., Bedford, MA) were measured in assay medium (i.e. unbuffered DMEM supplemented with 5.5 mM glucose and 0.5 mM carnitine) according to the Installation and Operation Manual from Seahorse Bioscience. The oxidation of endogenous fatty acids (without exogenously added palmitate, to detect the function of transgenic FASN expression) was determined by measurement of the absolute and relative OCR that was inhibited by the CPT-1 (carnitine palmitoyltransferase 1) inhibitor, Etomoxir (50 μM). The extent of glycolysis was determined by measurement of the absolute and relative ECAR, which was inhibited by 50 mM 2-deoxyglucose. As indicated, we also determined the effect of an adult-like lipogenic milieu by the cultivation of cardiomyocytes for 10 days with a 3F protocol that consisted of insulin (5 μg/ml), 3-isobutylmethylxanthine (0.25 mM), and dexamethasone (0.5 μM) (23), which were added as supplements to the standard medium. As a control for the 3F protocol, we used cardiomyocytes that were cultivated for 10 days under standard conditions. Long term cultivation of neonatal cardiomyocytes is a model of in vitro senescence characterized by metabolic deficiencies (24), which could account for the overall low β-oxidation rate of 5–25% in our experiments. Metabolic flux experiments were performed on 6 wells of a 24-well plate (technical replicates) and were reproduced at least three times (biological replicates). The oligomycin-insensitive OCR (a measure of mitochondrial uncoupling) was determined after the addition of 2.5 μM oligomycin. The non-mitochondrial OCR that remained after the addition of rotenone/antimycin A (2 μM) was subtracted.

Statistical Analyses—The results are presented as the means ± S.D. unless otherwise specified. The p values were calculated with Student’s t test. Analysis of variance was performed for comparisons between more than two groups followed by a post test (Tukey’s multiple comparison test unless otherwise specified), and statistical significance was set at a p value of <0.05 unless otherwise stated.
Results

FASN Transgenic Cardiomyocytes Developed a Dysfunctional Cardiac Substrate Metabolism—A dysfunctional cardiac substrate metabolism is a common feature of late-stage heart failure with limited treatment options. To reproduce the energy substrate use of heart failure patients and experimental models, which commonly show up-regulation of the major palmitate-synthesizing enzyme, FASN (10, 11), we generated a transgenic model with myocardium-specific FASN expression under the control of the α-MHC promoter (Fig. 1, A and B). Immunoblot detection of the FASN protein confirmed transgenic protein expression in hearts from mice with stable genomic integration of the FASN transgene, whereas the Fasn protein was barely detectable in non-transgenic B6 hearts (Fig. 1C). Two different transgenic lines (derived from founders numbers 3 and 9) were established, which showed comparable FASN protein levels (Fig. 1C). All of the experiments were independently performed with these two transgenic lines.

After the generation of the transgenic mouse lines, the metabolic energetics of isolated neonatal FASN transgenic (Tg-FASN) cardiomyocytes was determined with a Seahorse Bioscience XF24 Extracellular Flux Analyzer. We measured the α-MHC-oxidation of endogenous fatty acids by the Etomoxir-sensitive fraction of the OCR, and glycolysis by the 2-deoxyglucose-sensitive ECAR (Fig. 1, D and E). Compared with non-transgenic controls, FASN transgenic cardiomyocytes showed significantly more α-MHC-oxidation under basal conditions (22.7 ± 1.5% versus 5.3 ± 0.9%; Fig. 1, F and G). This finding indicated that FASN increases the substrate availability of cardiomyocytes for α-MHC-oxidation.
We next determined the effect of an adult–like lipogenic milieu on neonatal cardiomyocytes by treatment with a 3F protocol consisting of insulin, 3-isobutylmethylxanthine, and dexamethasone for 10 days (23). In control cardiomyocytes, the 3F protocol switched the embryo-like metabolism dominated by glycolysis (25) to an adult-like metabolism, which was characterized by an increased baseline OCR (Fig. 1E), and more fatty acid β-oxidation (Fig. 1G). In contrast, FASN transgenic cardiomyocytes developed a heart failure-like phenotype with an overall depressed substrate metabolism (Fig. 1E), and predominant glycolysis (Fig. 1F). Thus, the increased availability of the lipid substrate, palmitate, did not protect neonatal cardiomyocytes from a substrate switch to a heart failure-like phenotype that had depressed bioenergetics dominated by glycolysis.

**FASN Transgenic Mice Developed Signs of Heart Failure and Cardiac Lipid Load**—The extracellular flux analysis detected a heart failure-like metabolic substrate use in isolated cardiomyocytes from Tg-FASN mice. But the method employs unloaded cardiomyocytes and does not represent the condition in the loaded myocardium, which functions with real heart rates. Therefore, we analyzed the cardiac phenotype in vivo. Our data show that adult FASN transgenic mice developed signs of heart failure as early as 6 months of age, as evidenced by a significantly decreased left ventricular ejection fraction, cardiac hypertrophy with dilatation, and increased cardiomyocyte apoptosis (Fig. 2, A–E). As a control, the body weight of 6 month-old Tg-FASN mice was not different from B6 control mice (i.e. 34.28 ± 1.02 g and 33.45 ± 0.98 g, respectively; ± S.D.; n = 4; p = 0.5909).

The cardiac FASN protein in FASN-expressing mice was detected by immunoblotting and immunohistochemistry (Fig. 2, F–H). The FASN protein level in Tg-FASN hearts was increased ~2.4-fold (Fig. 2, F and G), which is comparable with the up-regulated FASN level of failing human hearts (10, 11).

Concomitantly to the increased FASN protein, cardiac FFA and triacylglycerol contents of Tg-FASN mice with signs of heart failure were elevated 2.2- and 2.1-fold, respectively (Fig. 2, I and J). The cardiac contents of DAG and ceramides, which can be induced by palmitate, i.e. the major lipid synthesized by FASN, were also significantly increased (Fig. 2, K and L). These lipids could be involved in the heart failure phenotype of Tg-FASN mice because they trigger a wide spectrum of cardiotoxic mechanisms, which involves, for example, the excessive formation of reactive oxygen species, an increased endoplasmic reticulum stress, enhanced apoptosis, and mitochondrial dysfunction (22, 26). Additionally, increased cardiac contents of DAG and ceramide could mediate the activation of protein kinase C (PKC), which further decreases heart function (22, 27, 28).

Thus, FASN transgenic hearts developed signs of heart failure with cardiotoxic lipid load in addition to the dysfunctional energy substrate metabolism, which was detected in isolated cardiomyocytes.

**Up-regulation of the Heart Failure-related Cardiac Lipid Metabolic Process in FASN Transgenic Mice**—Whole genome microarray gene expression profiling further confirmed the heart failure phenotype of Tg-FASN hearts by demonstrating the significant up-regulation of the heart failure-related cardiac lipid metabolic process (Fig. 3A). A similar induction of those adipogenic genes was also observed when signs of heart failure were triggered by 6 months of pressure overload (Fig. 3B) (11). In contrast, cardiac hypertrophy, without signs of heart failure (11) and induced by 4 weeks of pressure overload, did not up-regulate the heart failure-related adipogenic gene expression signature (Fig. 3C). Moreover, cardiac hypertrophy in the absence of heart failure signs promoted a significantly decreased expression of two probe sets that detect two enzymes of fatty acid biosynthesis, i.e. Scd1 (stearoyl-CoA desaturase-1) and Acly (ATP citrate lyase) (Fig. 3C). Thus, a heart failure-related adipogenic gene expression signature accompanied the onset of heart failure signs in FASN transgenic mice.

**Heart Failure-related Adipogenic Genes Triggered by FASN Are Pparg Targets**—In search of FASN-induced pathomechanisms, we focused on the adipogenic and heart failure-promoting transcription factor, Pparg (29, 30), because (i) palmitate, the major lipid synthesized by FASN, enhances the activity of Pparg (31), and (ii) adipogenic genes induced by FASN are Pparg targets (32), which are similarly up-regulated by Pparg.
Next, we investigated the impact of Fasn and Pparg activation in promoting cardiac hypertrophy induced by 1 month of ACC (cardiac hypertrophy) in 8-month-old ApoE–/– mice. Genes were not significantly up-regulated in 5-month-old B6 mice with cardiac hypertrophy compared with untreated ApoE–/– mice. Fasn and Pparg activation with rosiglitazone (Fig. 4, panel A) also triggered by direct delivery of miFasn decreased the expression of Fasn in hearts of ApoE–/– mice with 2 months of Pparg activation by rosiglitazone; (±S.D.; n = 3; **, p = 0.0029). D and E, immunohistological detection of Fasn (D) with anti-Fasn antibodies (anti-Fasn), and total lipids by Oil Red O staining (E) in cardiac sections of ApoE–/– mice after 2 months of Pparg activation and transduction of a control lentivirus (miCon) or lentiviral delivery of miFasn (bar, 40 μm). F, lentivirus-mediated delivery of miFasn normalized the enhanced cardiomyocyte apoptosis of ApoE–/– mice that was triggered by Pparg activation (±S.D.; n = 3; **, p = 0.0031). The right panels show representative immunohistology images of TUNEL staining (bar, 20 μm). G, viral transduction of ApoE–/– mice with a lentivirus that targets Fasn by RNAi (miFasn) retarded the increase in the heart weight-to-body weight ratio that was induced by 2 months of Pparg activation with rosiglitazone (±S.D.; n = 6; **, p = 0.0011). H, histological analysis of hearts from 8-month-old ApoE–/– mice isolated after 2 months of Pparg activation and transduction of a control lentivirus (miCon) or miFasn (bar, 2 mm). I, left ventricular ejection fraction of ApoE–/– mice after 2 months of Pparg activation and transduction of a control lentivirus (miCon) or miFasn (±S.D.; n = 6; ***, p = 0.0002). Histology experiments are representative of four different mice/group (D, E, and H).

overexpression in the mouse heart (29). Similarly, the treatment of ApoE–/– mice for 2 months with the Pparg agonist, rosiglitazone, also significantly up-regulated those heart failure-related adipogenic Pparg target genes, which were induced by FASN (Fig. 3D). As a control, rosiglitazone promoted signs of heart failure in ApoE–/– mice (cf. Fig. 4, F–I). Those findings demonstrate that the heart failure-related adipogenic gene signature induced by Tg-FASN and chronic pressure overload is also triggered by direct Pparg activation with rosiglitazone.

**Down-regulation of endogenous Fasn reveals a causal relationship between Fasn and Pparg activation in promoting cardiomycotoxicity and cardiac dysfunction.**

**GRK2 inhibition by GRKInh in FASN Transgenic Mice**—In view of the central role of FASN, the inhibition of FASN would be a straightforward treatment approach. However, FASN is an essential enzyme that has indispensable functions in energy homeostasis, membrane biology, and neurogenesis, which preclude long term FASN inhibition in vivo (10, 33, 34). We therefore searched for an alternative strategy to target the dysfunctional cardiac substrate metabolism. We focused on the inhibition of GRK2, which is a well established means of cardioprotection (12, 35). Furthermore, GRK2 inhibition enhances the ERK cascade (12), which promotes (partial) inac-

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**FIGURE 4. Down-regulation of endogenous Fasn reveals a causal relationship between Fasn and Pparg activation in promoting cardiac dysfunction.** A and B, stable integration of lentiviral miFasn-DNA into the genomic DNA of ApoE–/– mouse hearts (H1, H2), kidneys (K1, K2), and livers (L1, L2) isolated 2 months after lentiviral transduction. Control DNA (cont. DNA) was isolated from an ApoE–/– mouse. B, genomic integration of lentiviral DNA was quantified by quantitative PCR (±S.D.; n = 6; ***, p < 0.001; *p < 0.05 versus heart). C, lentivirus-mediated delivery of miFasn decreased the expression of Fasn in hearts of ApoE–/– mice with 2 months of Pparg activation by rosiglitazone (±S.D.; n = 3; **, p = 0.0029). D and E, immunohistological detection of Fasn (D) with anti-Fasn antibodies (anti-Fasn), and total lipids by Oil Red O staining (E) in cardiac sections of ApoE–/– mice after 2 months of Pparg activation and transduction of a control lentivirus (miCon) or lentiviral delivery of miFasn (bar, 40 μm). F, lentivirus-mediated delivery of miFasn normalized the enhanced cardiomyocyte apoptosis of ApoE–/– mice that was triggered by Pparg activation (±S.D.; n = 3; **, p = 0.0031). The right panels show representative immunohistology images of TUNEL staining (bar, 20 μm). G, viral transduction of ApoE–/– mice with a lentivirus that targets Fasn by RNAi (miFasn) retarded the increase in the heart weight-to-body weight ratio that was induced by 2 months of Pparg activation with rosiglitazone (±S.D.; n = 6; **, p = 0.0011). H, histological analysis of hearts from 8-month-old ApoE–/– mice isolated after 2 months of Pparg activation and transduction of a control lentivirus (miCon) or miFasn (bar, 2 mm). I, left ventricular ejection fraction of ApoE–/– mice after 2 months of Pparg activation and transduction of a control lentivirus (miCon) or miFasn (±S.D.; n = 6; ***, p = 0.0002). Histology experiments are representative of four different mice/group (D, E, and H).
GRK2 Inhibition Retards Cardiometabolic Dysfunction

GRK2 peptide interacted specifically with GRK2 in heart tissue extracts from Tg-GRKInh/FASN mice as demonstrated by co-enrichment, whereas the amount of GRK5 co-enriched with GRKInh-specific antibodies was below the limit of detection (Fig. 5B). Quantitative assessment of the GRK2-GRKInh interaction indicated that 83.6 ± 4.2% of the total cardiac GRK2 protein was captured by GRKInh affinity enrichment, whereas the amount of GRK5 protein bound to GRKInh was less than 20% (i.e. 18.9 ± 2.2%) of the total cardiac GRK5 content (Fig. 5C).

The functional effects of GRK2 inhibition in transgenic Tg-GRKInh/FASN hearts were analyzed by the immunofluorescence detection of Arrb1, which translocates to phosphorylated membrane-spanning receptors as a direct consequence of GRK2-mediated phosphorylation (Fig. 5D). In agreement with an increased GRK2 activity, immunofluorescence analysis detected the substantial membrane localization of Arrb1 in a cardiac section from a Tg-FASN mouse with signs of heart failure (Fig. 5D, left panel). In contrast, the double transgenic Tg-GRKInh/FASN heart section showed a largely cytoplasmic localization of Arrb1 as evidenced by co-localization with the cytosolic p38 MAPK (Fig. 5D, right panel). These findings indicate that GRKInh interacts with GRK2 in hearts from double transgenic Tg-GRKInh/FASN mice. As a consequence of the GRKInh-GRK2 interaction, the enhanced GRK2-mediated membrane translocation could be blunted.

FIGURE 5. GRK2 inhibition by GRKInh in FASN transgenic mice. A, cardiac up-regulation of the GRK2 protein level in Tg-FASN relative to B6 hearts was detected by immunoblotting (IB) with GRK2-specific antibodies (n = 4 mice/group). The lower panel is a control immunoblot detecting Gnb. The right panel shows the quantitative immunoblot evaluation (±S.D., n = 4), B, immunofluorescence enrichment of GRKInh (AP, +) with GRKInh-specific antibodies from Tg-GRKInh/FASN hearts, and immunoblot detection (IB) of co-enriched GRK2 protein (left panel) and GRK5 protein (right panel). The control experiment (AP, −) applied an affinity matrix with immobilized control IgG. C, quantitative assessment of the GRKInh-GRK2 interaction. GRK2 and GRK5 protein levels were determined by immunoblotting with GRK2-specific and GRK5-specific antibodies, respectively, of cardiac lysates from Tg-GRKInh/FASN mice before (total) and after (not bound to GRKInh) GRK2 incubation with an affinity matrix with immobilized anti-GRK2 antibodies. The left and middle panels present data evaluation from three independent experiments (±S.D., n = 3), and the right panels show representative immunoblots. D, immunofluorescence co-localization of Arrb1 with p38 MAPK in cardiac sections from Tg-FASN and Tg-GRKInh/FASN mice (bar, 20 μm). Arrb1 was detected with affinity-purified mouse anti-Arrb1 antibodies followed by F(ab), fragments of Alexa Fluor 546-labeled (red) secondary antibodies, and p38 was detected with affinity-purified rabbit anti-p38 MAPK antibodies followed by F(ab), fragments of Alexa Fluor 488-labeled (green) secondary antibodies. Cell nuclei were stained with DAPI (blue). Immunofluorescence experiments are representative of four different mice/group.

activation of Pparg (36). In agreement with heart failure patients (37), the GRK2 protein levels were significantly up-regulated (i.e. 1.81 ± 0.12-fold) in Tg-FASN hearts with signs of heart failure relative to the B6 controls (Fig. 5A).

To inhibit GRK2 in vivo, we used a GRKInh derived from the first intracellular loop of the β2 adrenergic receptor (12, 17). We used transgenic mice with myocardium-specific expression of GRKInh, which were established previously (12). The
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**FIGURE 6. GRK2 inhibition by GRKInh prevents the dysfunctional cardiac energetics of FASN transgenic cardiomyocytes.** A and B, development of dysfunctional cardiomyocyte energetics of FASN transgenic cardiomyocytes was retarded in double transgenic mice that co-expressed the GRK2-inhibitor, GRKInh. Real time measurements of OCR and ECAR of neonatal cardiomyocytes isolated from FASN transgenic (Tg-FASN), double transgenic Tg-GRKInh/FASN, and non-transgenic B6 mice were performed under basal conditions (left panels) and after treatment with the 3F protocol (right panels). The OCR and ECAR values normalized to the baseline (A), and the absolute values of OCR and ECAR (B) are presented. C–E, Etomoxir (Etom.)-blocked fraction of OCR, which represents fatty acid β-oxidation (C), the 2-deoxyglucose (2-DG)-blocked fraction of ECAR, which represents glycolysis (D), and the ratio of glycolysis/β-oxidation (E) were determined with cardiomyocytes isolated from Tg-FASN, Tg-GRKInh/FASN, B6, and Tg-GRKInh mice. The data are shown as the means ± S.D.; n = 6 technical replicates (A and B) and n = 3 biological replicates (C–E); * p < 0.05, ** p < 0.01 and *** p < 0.001 versus Tg-FASN. F, cardiomyocytes from Tg-GRKInh/FASN (lanes 3 and 4) showed significant adiponectin (Adipoq)-stimulated activation of Prkaa relative to Tg-FASN cardiomyocytes (lanes 1 and 2). Cardiomyocytes (3F protocol) were stimulated without (−) and with (+) globular domain adiponectin (2 μg/ml) for 60 min, and the activation of Prkaa was determined by immunoblotting (IB) with phospho-Prkaa (p-Prkaa)-specific antibodies. The left panel presents quantitative data from four independent experiments (± S.D.; n = 4; *** p < 0.0001), and the middle and right panels show representative immunoblots.

(Prkaa) could protect against palmitate-induced toxicity (15, 16).

**GRK2 Inhibition Retards the Development of Heart Failure Signs in Tg-FASN Mice—**The improved substrate metabolism upon GRK2 inhibition was also reflected in vivo, in adult murine hearts. The presence of GRKInh in double transgenic Tg-GRKInh/FASN hearts compared with single transgenic Tg-FASN hearts led to a significantly decreased total FFA and triacylglycerol load compared with single transgenic Tg-FASN hearts (Fig. 7, A and B).

The decreased lipid load of Tg-GRKInh/FASN hearts was accompanied by a significantly decreased cardiac expression of the acetyl-CoA carboxylase α (Acaca), which mediates an essential step of fatty acid synthesis by catalyzing the conversion of acetyl-CoA into malonyl-CoA (Fig. 7C). Notably, GRKInh largely prevented the up-regulation of Acaca, i.e. a gene up-regulated by hypoxia (38), which was commonly triggered at the onset of heart failure induced by Tg-FASN, pressure overload, and Pparg (Fig. 7C and cf. Fig. 3, A, B, and D).

Although the expression of the human FASN transgene was not significantly altered between single transgenic Tg-FASN and double transgenic Tg-GRKInh/FASN hearts (Fig. 7D, left panel), GRKInh led to a significantly decreased expression of the endogenous murine Fasn gene, which is also a hypoxia-induced Pparg target (39), and shows up-regulated expression in Tg-FASN hearts with signs of heart failure (Fig. 7D, right panel, and cf. Fig. 3A). Concomitantly, the total cardiac FASN/Fasn protein level of Tg-GRKInh/FASN hearts was significantly decreased relative to that in Tg-FASN hearts (Fig. 7E). Together, these experiments show that GRK2 inhibition retards the FASN-induced dysfunction of the cardiac substrate metabolism and lipid overload. Concomitantly with the
GRK2 inhibition retards cardiometabolic dysfunction. We investigated the mechanism that accounts for GRK2 inhibition-mediated protection against FASN-induced cardioprotectivity and focused on the interrelationship between GRK2 inhibition-mediated ERK axis activation and the inactivation of Pparg. Several lines of evidence support such a relationship. (i) The expression of several heart failure-related Pparg targets such as adiponectin (Adipoq), resistin (Retn), and uncoupling protein 1 (Ucp1) is down-regulated by ERK-dependent inactivation of Pparg, partially by involving serine 273 phosphorylation (36, 40). (ii) GRK2 inhibition enhances the activation of the ERK cascade (12, 41). (iii) Additionally, the reversal of palmitate toxicity can be achieved by ERK activation (cf. Fig. 6F). Conversely, excess palmitate down-regulated the ERK axis (16).
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In agreement with palmitate-mediated inhibition of ERK (16), the cardiac content of activated phospho-ERK1/2 was low in Tg-FASN hearts relative to double transgenic Tg-GRKInh/FASN hearts (Fig. 7, J and K). Notably, GRKInh enhanced the activation of ERK2 (Fig. 7K), which is cardioprotective and protects the myocardium against ischemic injury (42). Concomitantly with enhanced ERK1/2 activation, serine 273-phosphorylated Pparg was increased in double transgenic Tg-GRKInh/FASN hearts relative to Tg-FASN hearts (Fig. 7L).

In agreement with the ERK-dependent inactivation of Pparg (36, 40, 43), the enhanced phosphorylation of Pparg on serine 273 and serine 112 of double transgenic Tg-GRKInh/FASN cardiomyocytes was accompanied by a significantly decreased Pparg transcription factor DNA binding activity compared with that of Tg-FASN cardiomyocytes (Fig. 7, M and N). The decreased Pparg activity of Tg-GRKInh/FASN cardiomyocytes was dependent on an activated ERK axis because the MEK inhibitor PD0325901 blunted the phosphorylation of Pparg on serine 273 and serine 112 and led to a significant up-regulation of the Pparg transcriptional activity of Tg-GRKInh/FASN cardiomyocytes (Fig. 7, M and N).

Concomitantly with Pparg inhibition, the expression of ERK-regulated, heart failure-related Pparg targets (i.e. Ucp1 and Adip0q) was significantly lower in double transgenic Tg-GRKInh/FASN hearts compared with Tg-FASN hearts (Fig. 7O). Concordantly with decreased signs of heart failure, GRKInh also led to a significantly decreased expression of the heart failure marker and Pparg target gene, Retn (Fig. 7O). Because Adipoq and Retn are heart failure-related Pparg targets (44, 45) that are induced by serine 273 phosphorylation-deficient PPARG-S273A (40), these data are compatible with the notion that cardioprotective GRK2 inhibition could involve the suppression of Pparg-dependent cardiotoxic gene expression by enhanced ERK-mediated serine 273 phosphorylation and the inactivation of Pparg.

Inhibition of Fasn Lowers the Cardiotoxicity Induced by Serine 273 Phosphorylation-deficient PPARG-S273A—We analyzed the impact of phosphorylation-deficient PPARG-S273A on cardiomyocyte function. Our experiments showed that both cardiomyocyte FFA content and the Fasn protein were triggered by PPARG activated with the PPARG agonist rosiglitazone and by PPARG-S273A (Fig. 8, A, C, and D). These data are in agreement with those from previous studies, which have shown that PPARG serine 273 dephosphorylation can enhance the expression of Fasn (46).

Concomitantly with the FFA load, cardiomyocyte dysfunction developed as evidenced by a significantly decreased cardiomyocyte ATP content induced either by rosiglitazone-activated PPARG or PPARG-S273A, respectively (Fig. 8B). The inhibition of Fasn by RNAi (Fig. 8, C and D) led to significantly decreased cardiomyocyte FFA content and largely prevented the decrease in cardiomyocyte ATP (Fig. 8, A and B). Together, these findings provide evidence that PPARG-S273A deteriorates cardiomyocyte function by regulating Fasn.

GRK2 Inhibition Retards the Up-regulation of the Heart Failure-related Ucp1 and Mitochondrial Uncoupling—In search of additional heart failure-promoting ERK-controlled Pparg target genes, we focused on Ucp1 (36, 47), which exerts mitochondrial uncoupling, a major metabolic feature of the failing heart metabolism (9). GRK2 inhibition by GRKInh led to a decreased cardiac Ucp1 expression and protein level in Tg-GRKInh/FASN mice relative to Tg-FASN mice (Fig. 9A and cf. Fig. 7O). Moreover, cardiomyocytes from Tg-GRKInh/FASN mice showed a significantly decreased oligomycin-insensitive OCR (a measure of mitochondrial uncoupling) compared with Tg-FASN cardiomyocytes (Fig. 9B). Conversely, the inhibition of the ERK axis in Tg-GRKInh/FASN cardiomyocytes significantly increased the Ucp1 protein and mitochondrial uncoupling (Fig. 9, B and C). These findings indicate that GRK2 inhibition decreased Ucp1-dependent mitochondrial uncoupling in Tg-GRKInh/FASN cardiomyocytes by enhanced activation of the ERK axis.

Transgenic Tg-Ucp1 Mice Developed Signs of Heart Failure and Increased Mitochondrial Uncoupling—To analyze whether an increased cardiac UCP1 level contributed to heart failure...
pathogenesis in vivo, we generated Tg-UCP1 mice with myocardium-specific UCP1 expression (Fig. 10, A and B). Immunoblot detection confirmed the increased cardiac UCP1 protein in Tg-UCP1 (Tg-6) mice relative to non-transgenic B6 controls (Fig. 10C). In addition to the Tg-6 line, we also used Tg-11 offspring with lower UCP1 protein levels (Fig. 10D).

Aged Tg-UCP1 mice from the Tg-6 line developed cardiac dysfunction with a significantly decreased left ventricular ejection fraction and decreased cardiac ATP content compared with non-transgenic B6 mice, whereas cardiac function parameters of the low-expressing Tg-11 line were not significantly different from B6 controls (Fig. 10, E and F). Concomitantly with cardiac dysfunction, Tg-UCP1 mice showed cardiac dilatation and loss of heart muscle, whereas the heart weight-to-body weight ratio was not significantly different from that in the B6 controls (Fig. 10, G and H). Signs of heart failure were accompanied by a significant up-regulation of the cardiac FASN protein in Tg-UCP1 mice compared with non-transgenic B6 controls (Fig. 10I). The up-regulation of the Fasn protein by UCP1 could be a consequence of the impaired cardiac function and insufficient tissue oxygen supply, which could induce Fasn up-regulation because it is a hypoxia-induced gene (39).

The ensuing increase in palmitate may enhance mitochondrial uncoupling by the transgenic UCP1 protein. In support of that notion, Tg-UCP1 cardiomyocytes showed a significantly increased oligomycin-insensitive (uncoupled) respiration compared with B6 cardiomyocytes (Fig. 10J). Together, these experiments indicate that the UCP1 protein could have a major role in the depressed substrate metabolism of a failing heart because up-regulated UCP1 could account for an enhanced palmitate-triggered uncoupled respiration upon FASN induction. Under those conditions, GRK2 inhibition by GRKInh could confer several modes of cardioprotection as follows: (i) by counteracting mitochondrial uncoupling via ERK activation-mediated down-regulation of UCP1 (36); and (ii) by decreasing the FASN-triggered lipid load, which involves, e.g. down-regulation of hypoxia-induced Pparg targets, Acaca and Fasn, and the re sensitization of Prkkaa.
get gene up-regulation, cardiac lipid load developed, and cardiac dysfunction became evident in hearts of aged RKIP transgenic mice (Fig. 11, E–H) (12). In vitro data documented the dysfunctional cardiomyocyte energetics of RKIP transgenic cardiomyocytes compared with the normal metabolism of GRKInh transgenic cardiomyocytes (Fig. 11, I–M). Taken together, our data strongly suggest that an intact ERK axis is required for GRK2 inhibition-dependent protection against dysfunctional metabolic substrate use.

GRK2 Inhibition Retards the Development of Heart Failure Signs, Cardiac Lipid Load, and Pparg Target Gene Induction in a Pressure Overload-induced Heart Failure Model—Thus, we have presented evidence for GRKInh-mediated protection of Tg-FASN hearts. However, up-regulation of the Pparg-depen-
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In agreement with previous data (12), AAC promoted cardiac hypertrophy with dilatation in non-transgenic mice, whereas Tg-GRKInh mice showed a significantly decreased cardiac hypertrophy (Fig. 12, A and B). The development of cardiac dysfunction upon AAC, as assessed by the left ventricular ejection fraction, was also significantly retarded in Tg-GRKInh mice (Fig. 12C). In addition to the improved cardiac function, the AAC-stimulated up-regulation of the cardiac Fasn protein was blunted in Tg-GRKInh mice (Fig. 12D). Com-constitutively, Oil Red O staining of cardiac sections indicated that the AAC-triggered lipid load was lower in Tg-GRKInh mice (Fig. 12E). In agreement with the decreased lipid-induced cardio-perturbation, Tg-GRKInh mice showed a significantly decreased number of AAC-induced TUNEL-positive cardiomyocytes compared with the number in non-transgenic mice with AAC (Fig. 12, F and G).

In view of the decreased AAC-induced cardioperturbation, we analyzed the potential effect of GRK2 inhibition on the Pparg-inhibitory serine 273 phosphorylation. Immunoblot detection indicated an increased cardiac content of serine 273-phosphorylated Pparg of Tg-GRKInh hearts with AAC compared with non-transgenic B6 mice with AAC (Fig. 12, H and I). The increased level of Pparg-inhibitory serine 273 phosphorylation was accompanied by a significantly lower expression of heart failure-related Pparg targets, which are blunted by ERK activation and/or ERK-dependent Pparg serine 273 phosphorylation, i.e. Ucp1, Adipoq, and Retn (Fig. 12J). Taken together, cardio-protective GRK2 inhibition with GRKInh retarded the up-regulation of heart failure-related and ERK-inhibited Pparg targets and enhanced Pparg-inhibitory serine 273 phosphorylation.

Down-regulation of Endogenous Ucp1 Retards the Development of Cardiac Dysfunction in a Pressure Overload-induced Heart Failure Model—Although previous studies have provided evidence for the involvement of Adipoq and Retn in heart failure pathogenesis of patients and animal models (44, 45, 51–53), the role of Ucp1 up-regulation in cardiac dysfunction is less clear. Notably, the onset of heart failure signs in different heart failure models was characterized by a strong cardiac Ucp1 up-regulation (cf. Fig. 3), and transgenic expression of UCPI in the heart promoted signs of heart failure (cf. Fig. 10). To investigate the effect of Ucp1 up-regulation in the AAC-induced heart failure model, we down-regulated the endogenously expressed Ucp1 by lentiviral transduction of an miRNA that targets Ucp1 by RNA interference (Fig. 13, A and B). The down-regulation of Ucp1 in the AAC-induced heart failure model led to a small but significant retardation of the development of
dent lipid metabolic process is also a characteristic feature of heart failure models that imitate major risk factors of patients such as chronic pressure overload (cf. Fig. 3B and Ref. 11). We therefore analyzed the effect of GRKInh on the cardiac lipid metabolism in a chronic pressure overload-induced heart failure model imposed by long term (6 months) AAC.
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FIGURE 12. GRK2 inhibition retards the development of heart failure signs, cardiac lipid load, and Pparγ target gene induction in a pressure overload-induced heart failure model. A, representative histological sections of hearts from a 10-month-old B6 mouse (AAC relative to an age-matched GRKInh transgenic mouse (AAC + GRKInh) after 6 months of pressure overload imposed by AAC. The lower panels show age-matched sham-operated control hearts, bar, 2 mm. B and C, heart weight-to-body weight ratio (B) and the left ventricular ejection fraction (C) of 10-month-old GRKInh transgenic mice with 6 months of AAC (AAC + GRKInh) relative to age-matched non-transgenic B6 mice with 6 months of AAC (AAC). Age-matched sham-operated non-transgenic B6 mice (Sham) and sham-operated GRKInh transgenic mice (Sham + GRKInh) served as controls. Data are shown as the means ± S.D., n = 4 (*, p < 0.05 versus AAC + GRKInh; **, p < 0.01 versus AAC; ***, p < 0.001 versus AAC). D, immunoblot detection of Fasn in cardiac tissue extracts of 10-month-old B6 mice with 6 months of AAC relative to age-matched GRKInh transgenic mice with 6 months of AAC (n = 4 hearts/group, left blot). Under the experimental conditions, the Fasn protein (lane 1, A, positive control of a 10-month-old B6 heart with 6 months of AAC) was not detectable in cardiac tissue extracts from sham-operated B6 (Sham) or GRKInh transgenic mice (n = 2; right blot). The lower panels show control immunoblots detecting Gnb. E, Oil Red O staining of cardiac sections from the different groups of mice (bar, 40 μm). F, Immunohistochemical sections of LV ventricular wall stained with TUNEL (bar, 20 μm). H and J, immunoblot (IB) detection of pS273-Pparg (upper panels) and total Pparg (lower panels) in cardiac tissue extracts from the different groups of mice (n = 4 hearts/group (H), and n = 2 hearts/group (J)). J, expression of heart failure-related Pparγ targets in hearts from 10-month-old B6 mice with 6 months of AAC (AAC) and age-matched Tg-GRKInh mice with 6 months of AAC (AAC + GRKInh) relative to sham-operated controls (± S.D.; n = 4; **, p < 0.01 versus AAC + GRKInh, Sham, and Sham + GRKInh).

FIGURE 13. Down-regulation of endogenous Ucp1 retards the development of cardiac dysfunction in a pressure overload-induced heart failure model. A and B, endogenous Ucp1 expression (A) and Ucp1 protein level (B) in hearts of B6 mice with 2 months of AAC and transduction of a control lentivirus (AAC + miCont.) or a lentivirus targeting Ucp1 by RNAi (AAC + miUcp1) relative to sham-operated B6 controls. C, down-regulation of Ucp1 retarded the AAC-triggered decrease in the left ventricular ejection fraction (AAC + miUcp1) relative to AAC-subjected B6 mice transduced with a control lentivirus (AAC + miCont.). The data are shown as the means ± S.D. (n = 4; *, p < 0.05, and ***, p < 0.001 versus Sham B6; Dunnett’s multiple comparison test). IB, immunoblot.

AAC-induced cardiac dysfunction (Fig. 13C). These findings provide further evidence (cf. Fig. 10) for the role of Ucp1 up-regulation in AAC-induced signs of heart failure.

Low Efficacy of GRKInh in Retarding the Cardiac Phenotype of PPARG-S273A Transgenic Mice—Our data provided evidence that GRK2 inhibition counteracts the dysfunctional cardiac substrate use of heart failure (at least partially) by ERK-dependent inactivation of Pparγ involving serine 273 phosphorylation. To further analyze the role of Pparγ and a serine 273 phosphorylation-deficient Pparγ mutant (PPARG-S273A) in the heart, we generated transgenic mice with myocardium-specific expression of wild-type Pparγ and mutated PPARG-S273A, respectively (Fig. 14A). Histological analysis revealed that transgenic PPARG-S273A mice developed cardiac hypertrophy with dilatation, which was already evident in newborn mice (Fig. 14B). The dilatation of the PPARG-S273A-expressing heart was greater than that of the Pparγ-expressing heart (Fig. 14B), although the cardiac Pparγ protein level was comparable between the two transgenic groups (Fig. 14C).

Next, we investigated the effect of GRK2 inhibition by GRKInh and compared single transgenic mice (PPARG-S273A and PPARG), with double transgenic PPARG-S273A + GRKInh-expressing and PPARG + GRKInh-expressing mice, respectively. All four groups of mice showed similar cardiac PPARG expression (Fig. 14D). Despite having similar PPARG expression, the GRKInh largely prevented the cardiac hypertrophy of PPARG-expressing mice, whereas the effect of GRKInh on PPARG-S273A-expressing mice was not significant (Fig. 14E). In addition, there was an increased postnatal mortality of PPARG-S273A-expressing mice compared with wild-type PPARG-expressing mice (47.8% versus 9.5%), which was not rescued by the GRK2 inhibitor (Fig. 14F).

We determined the expression of selected heart failure-related Pparγ targets, i.e. Adipoq, Retn, Fasn, and Ucp1. The genes were all significantly up-regulated in the cardiac tissue of 4-week-old PPARG-S273A transgenic mice compared with non-transgenic B6 mice (Fig. 14G), which confirms the heart failure-like phenotype of newborn PPARG-S273A transgenic mice. We found that the effect of GRKInh was not significant in reducing the PPARG-S273A-mediated up-regulation of Adipoq, Retn, Fasn, and Ucp1 (Fig. 14G). In contrast to PPARG-
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S273A transgenic hearts, the transgenic expression of PPARγ caused significantly less up-regulation of selected Ppar targets, and only the expression of cardiac Adipoq and Fasn was significantly increased in 4-week-old mice (Fig. 14G). The up-regulation of Adipoq in PPARγ transgenic mice may have contributed to the cardiac hypertrophy (cf. Fig. 14, B and E) because Adipoq is required for pro-hypertrophic signaling during pressure overload (44).

In agreement with the heart failure-like phenotype of PPARγ-S273A-expressing mice, the cardiomyocyte energetics of neonatal cardiomyocytes from PPARγ-S273A transgenic mice showed an overall depressed substrate metabolism under basal conditions with predominant glycolysis. This heart failure-like substrate use was not rescued by co-expression of GRKInh (Fig. 14H). Conversely, PPARγ-expressing cardiomyocytes showed predominant β-oxidation under basal conditions indicative of lipid load, which was retarded by GRKInh (Fig. 14H). Upon induction of an adult-like metabolism by the 3F protocol, PPARγ-expressing cardiomyocytes shifted to a heart failure-like metabolic substrate use (Fig. 14H).

In contrast to PPARγ-S273A, the development of the PPARγ-induced heart failure-like phenotype was retarded by GRKInh (Fig. 14H). Together these findings provide evidence for an enhanced cardiac deterioration of PPARγ-S273A transgenic mice compared with PPARγ-WT mice. Moreover, GRKInh was inefficient in retarding the development of the PPARγ-S273A-induced cardiometabolic dysfunction and the up-regulation of PPARγ-S273A-regulated targets.

Discussion

In this study, we investigated whether GRK2 inhibition could be a specific approach for targeting of the dysfunctional cardiac substrate metabolism, which is characteristic of late-stage heart failure (4–6). To reproduce the dysfunctional cardiac substrate use, we generated a novel transgenic model with myocardium-specific Fasn expression. The model imitated the up-regulation of Fasn, which occurs in patients with heart failure (10, 11). In the context of cardiovascular disease and heart failure, the up-regulation of Fasn could be a direct consequence of decreased cardiac output and insufficient oxygen supply because Fasn is a hypoxia-induced gene (39). Because cardiac ischemia is triggered by major cardiovascular risk factors such as pressure overload and atherosclerosis, up-regulation of Fasn could also be an early and causative event in the pathogenesis of heart failure. In agreement with this notion, we found that the sole expression of Fasn was sufficient to trigger the signs of heart failure. Additionally, models of heart failure, which imitated cardiovascular risk factors of patients such as chronic pressure overload or advanced atherosclerosis, also showed up-regulation of cardiac Fasn (11).

How could Fasn advance the symptoms of heart failure? Initially, up-regulation of Fasn might be considered to be beneficial by supplying more energy substrate to the heart muscle.

FIGURE 14. Low efficacy of GRKInh in retarding the cardiac phenotype of PPARγ-S273A transgenic mice. A, transgenic vector used for the generation of transgenic mice with myocardium-specific expression of PPARγ (and PPARγ-S273A). B, cardiac sections from newborn transgenic mice that expressed PPARγ-S273A or wild-type PPARγ relative to a non-transgenic B6 control. Histological sections were stained with hematoxylin-eosin (H&E), and are representative of three mice/groups. C, detection of PPARγ/PParg in cardiac tissue extracts of 4-week-old mice with transgenic PPARγ-S273A (S273A) expression or PPARγ expression (as indicated) relative to non-transgenic B6 controls (n = 4 hearts/group). The lower panel is a control immunoblot (IB) detecting Gnb, D and E, cardiac expression of PPARγ (D), and the heart weight-to-body weight ratio (E) of 4-week-old transgenic mice with expression of PPARγ-S273A (S273A), PPARγ-S273A + GRKInh, PPARγ, and PPARγ-S273A + GRKInh are shown relative to age-matched non-transgenic B6 mice (± S.D., n = 4, *, p < 0.05, **, p < 0.01, and ***, p < 0.001 versus B6 control, Dunnett’s multiple comparison test). F, increased postnatal mortality of mice with myocardium-specific expression of PPARγ-S273A + GRKInh compared with mice expressing PPARγ and PPARγ + GRKInh, respectively. G, gene expression analysis of heart failure-related Ppar targets in hearts from 4-week-old transgenic mice that express PPARγ-S273A, PPARγ-S273A + GRKInh, PPARγ, and PPARγ + GRKInh relative to non-transgenic B6 hearts (± S.D., n = 4, *, p < 0.05, **, p < 0.01, and ***, p < 0.001 versus B6 control, Dunnett’s multiple comparison test). H, cardiomyocyte energetics was determined with neonatal cardiomyocytes isolated from transgenic mice with myocardium-specific expression of PPARγ-S273A (S273A), PPARγ-S273A + GRKInh, PPARγ, and non-transgenic B6 mice. The etomoxir-blocked fraction of OCR, which represents fatty acid β-oxidation (left panels), the 2-deoxyglucose-blocked fraction of ECAR, which represents glycolysis (middle panels), and the ratio of glycolysis/β-oxidation (right panels) were determined under basal conditions (upper panels) and after the creation of an adult-like lipogenic milieu by the 3F protocol for 10 days (lower panels). The data are shown as the means ± S.D.; n = 3 biological replicates; *, p < 0.05, **, p < 0.01, and ***, p < 0.001 versus B6; Dunnett’s multiple comparison test.
However, in the long term, the uncontrolled accumulation of palmitate as the major lipid synthesized by FASN could activate the heart failure-promoting transcription factor Pparg, as has been documented by up-regulation of the Pparg-dependent lipid metabolic process in Tg-FASN hearts and various other models of heart failure. Because Fasn is also a Pparg target, a vicious cycle of FASN/Fasn-induced Fasn could be triggered, which finally results in cardiotoxic lipid load, dysfunctional substrate use, and mitochondrial uncoupling due to palmitate-triggered activation of Ucp1 (Fig. 15). The accumulation of palmitate further promotes pro-apoptotic signaling and inhibits the pro-survival ERK axis (16). As a result, there is an enhanced expression of heart failure-associated Pparg targets that are triggered by ERK inhibition (36, 40), such as Adipop (44, 51, 52), Retn (45, 53), and Ucp1. Ensuing cardiomyocyte death and remodeling and impaired cardiac energy generation due to mitochondrial uncoupling could further aggravate the symptoms of heart failure (Fig. 15).

When we applied the Tg-FASN mice as a model of a dysfunctional cardiac substrate metabolism and an additional pressure overload-induced heart failure model, we found that GRK2 inhibition directly interfered with the cardiac lipid accumulation and mediated a reduction in the cardiac Fasn protein. These activities could be attributed (at least partially) to several mechanisms as follows: (i) the inhibition of the endogenous Fasn up-regulation, a hypoxia-induced Pparg target (39); (ii) an interference with fatty acid synthesis by preventing Acaca up-regulation, which is also a Pparg-regulated gene induced by hypoxia (38); and (iii) the enhancement of the fatty acid metabolism by re-sensitization of Adipop and Prkaa-mediated signaling (14). As a consequence, GRK2 inhibition retarded the development of the dysfunctional cardiac substrate use characteristic of late-stage heart failure (Fig. 15).

Cardioprotective GRK2 inhibition required an intact ERK axis to preserve the cardiac energetics because RKIP as a dual-specific GRK2 and ERK cascade inhibitor promoted dysfunction of cardiomyocyte energetics, cardiac lipid load, and signs of heart failure. Concomitantly, inhibition of the ERK cascade by human RKIP was accompanied by decreased ERK-dependent phosphorylation of Pparg. A decreased ERK-dependent phosphorylation of Pparg on serine 273 and serine 112 is known to enhance Pparg activity and/or increase Pparg target gene induction (36, 40, 43). Similarly, heart failure-related Pparg targets are triggered by RKIP, resulting in development of cardiac lipid load and cardiac dysfunction (12).

Conversely, GRK2 inhibition by GRKInh led to an increased ERK activation and enhanced the ERK-mediated phosphorylation of Pparg on serine 273. ERK axis activation could be part of the cardioprotective gene expression program initiated by GRK2 inhibition (12, 13, 41). Concomitantly, the expression of heart failure-promoting Pparg targets was blunted, and the appearance of the dysfunctional cardiac substrate metabolism was retarded. In agreement with a causal role of PPARG-S273 phosphorylation in GRKInh-mediated cardioprotection, the phosphorylation-deficient PPARG-S273A mutant promoted dysfunction of the cardiomyocyte substrate metabolism and caused enhanced postnatal death, which was largely insensitive to GRKInh. In contrast, the phenotype of wild-type PPARG was less severe and could be (at least partially) rescued by GRK2 inhibition. Together, these data indicate that the ERK axis may specifically counteract the heart failure-promoting transcription factor Pparg by preventing heart failure-related Pparg target gene induction (36, 54) and/or could confer protection against palmitate-induced endoplasmic reticulum stress (55).

Several heart failure-related Pparg targets are inhibited by ERK-dependent Pparg inactivation (36, 40) with GRKInh, such as Adipop (44, 51, 52) and Retn (45, 53). By generating Tg-Ucp1 mice with myocardium-specific Ucp1 expression, our study identified UCP1 as a heart failure-related ERK-regulated Pparg target (36, 47), which was also down-regulated upon GRK2 inhibition (Fig. 15). Consequently, GRK2 inhibition could decrease excessive mitochondrial uncoupling as a key event that contributes to inefficient cardiac ATP generation and lipid-induced cardiomyocyte death in heart failure (49, 50).

Although the study was performed with experimental mouse models, the data could also be relevant for the human disease because FASN up-regulation is a characteristic feature of patients with heart failure (10, 11). Because PPARG up-regulation occurs in heart failure patients with pressure-overloaded heart and metabolic syndrome (56), GRK2 inhibition is expected to disrupt a vicious FASN/PPARG cycle in patients who suffer from multiple risk factors (Fig. 15). Such a situation was modeled with rosiglitazone-treated ApoE−/− mice because these mice are prone to the development of atherosclerosis and insulin resistance (57), and thereby mimic the risk profile of patients with enhanced PPARG activation and cardiovascular disease. In this model, Pparg activation triggered the up-regulation of Fasn and signs of heart failure within 2 months. The causal interplay between Fasn and Pparg-induced cardiotoxicity was demonstrated by RNAi-mediated inhibition of Fasn, which prevented the Pparg-induced cardio-lipotoxicity and signs of heart failure. Because GRK2 inhibition also mediated the down-regulation of FASN-dependent cardio-lipotoxicity, patients with high morbidity and multiple risk factors may benefit from GRK2 inhibition. The additional insulin sensitivity-enhancing activity of GRK2 inhibition (13, 58) may further increase the value of such a strategy.

Moreover, heart-specific GRK2 inhibition could become a cardioprotective combination partner for a novel class of insulin sensitivity-enhancing PPARG activators, which rely on the inhibition of ERK-dependent PPARG phosphorylation for antidiabetic activity (36), but they have promoted signs of heart failure in clinical trials (59).
In sum, our study provides strong evidence that cardioprotective GRK2 inhibition specifically targets the dysfunctional cardiac substrate use that is a symptom of late-stage heart failure (Fig. 15). The identified targeting approach could stimulate the development of new therapeutic strategies.

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