Essential role of stress hormone signaling in cardiomyocytes for the prevention of heart disease

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Heart failure is a leading cause of death in humans, and stress is increasingly associated with adverse cardiac outcomes. Glucocorticoids are primary stress hormones, but their direct role in cardiovascular health and disease is poorly understood. To determine the in vivo function of glucocorticoid signaling in the heart, we generated mice with cardiomyocyte-specific deletion of the glucocorticoid receptor (GR). These mice are born at the expected Mendelian ratio, but die prematurely from spontaneous cardiovascular disease. By 3 mo of age, mice deficient in cardiomyocyte GR display a marked reduction in left ventricular systolic function, as evidenced by decreases in ejection fraction and fractional shortening. Heart weight and left ventricular mass are elevated, and histology revealed cardiac hypertrophy without fibrosis. Removal of endogenous glucocorticoids and mineralocorticoids neither augmented nor lessened the hypertrophic response. Global gene expression analysis of knockout hearts before pathology onset revealed aberrant regulation of a large cohort of genes associated with cardiovascular disease as well as unique disease genes associated with inflammatory processes. Genes important for maintaining cardiac contractility, repressing cardiac hypertrophy, promoting cardiomyocyte survival, and inhibiting inflammation had decreased expression in the GR-deficient hearts. These findings demonstrate that a deficiency in cardiomyocyte glucocorticoid signaling leads to spontaneous cardiac hypertrophy, heart failure, and death, revealing an obligate role for GR in maintaining normal cardiovascular function. Moreover, our findings suggest that selective activation of cardiomyocyte GR may represent an approach for the prevention of heart disease.

Heart failure is one of the leading causes of morbidity and mortality in developed countries. Well-known risk factors for cardiovascular disease include hypertension, diabetes, obesity, physical inactivity, and smoking. In addition to these conventional factors, stress is increasingly recognized for its important contribution to the development and progression of cardiac disease (1, 2). One of the major biological systems activated during the stress response is the hypothalamic–pituitary–adrenal axis (HPA). Activation of the HPA axis results in the release of glucocorticoids from the adrenal gland, and these hormones function to maintain homeostasis in the face of internal or external challenges. As the primary stress hormone in humans, glucocorticoids act on numerous target tissues to regulate a plethora of biological processes. Because of their potent anti-inflammatory and immunosuppressive actions, synthetic glucocorticoids are also widely used in the clinic to treat inflammation, autoimmune diseases, and hematological cancers. Despite a wealth of information regarding the function of glucocorticoids and the widespread therapeutic use of these steroids, the direct biological actions of glucocorticoids in the heart remain poorly understood.

The physiological and pharmacological actions of glucocorticoids are mediated by the glucocorticoid receptor (GR; NR3C1), a member of the nuclear receptor superfamily of ligand-dependent transcription factors. Upon glucocorticoid occupancy, GR regulates the expression of thousands of genes by direct binding to DNA and/or by interactions with other chromatin-bound transcription factors (3). Global inactivation of the GR gene in mice leads to perinatal lethality (4). Therefore, to elucidate the role of glucocorticoid signaling in the heart, we generated mice specifically lacking GR in cardiomyocytes. These mice exhibit profound alterations in gene expression profiles that lead to left ventricular (LV) systolic dysfunction, cardiac hypertrophy, heart failure, and death, revealing a crucial role for cardiomyocyte GR signaling in the function of the heart.

Results

Floxed GR mice ($GR^{loxP/loxP}$) were generated by standard gene-targeting techniques and contain loxP sites flanking exons 3 and 4 of the GR gene (Fig. 1*A*). These mice were crossed with mice expressing Cre recombinase under the control of the alpha myosin heavy chain promoter ($\alpha MHC^{Cre/+}$) to specifically delete GR in cardiomyocytes (5). Cre-mediated deletion of exons 3 and 4, which encode the DNA-binding domain of GR, not only generates a null GR allele but also results in a frameshift mutation and premature stop codon if splicing occurs between exons 2 and 5. CardioGRKO mice ($GR^{loxP/loxP}\alpha MHC^{Cre/+}$) were born at the expected Mendelian ratio, and Cre-mediated recombination was detected only in heart tissue (Fig. 1*A*). Cre-negative littermates ($GR^{loxP/loxP}\alpha MHC^{+/+}$) were used as controls in our studies. Compared with control mice, GR mRNA was significantly decreased in the hearts from cardioGRKO mice (Fig. 1*B*). The residual level of GR mRNA likely reflects its expression in other cell types of the heart. Immunoblots of whole-heart lysates showed a marked reduction in the level of GR protein from the

Significance

Stress is increasingly associated with heart disease. Glucocorticoids are primary stress hormones, yet their direct role in the heart is poorly understood. Mice lacking the glucocorticoid receptor specifically in cardiomyocytes die prematurely from heart failure. The deficiency in glucocorticoid signaling leads to the aberrant regulation of a large cohort of genes strongly associated with both cardiovascular and inflammatory disease processes. These findings reveal an obligate role for cardiomyocyte glucocorticoid receptors in maintaining normal heart function and define a paradigm for stress in cardiovascular disease.

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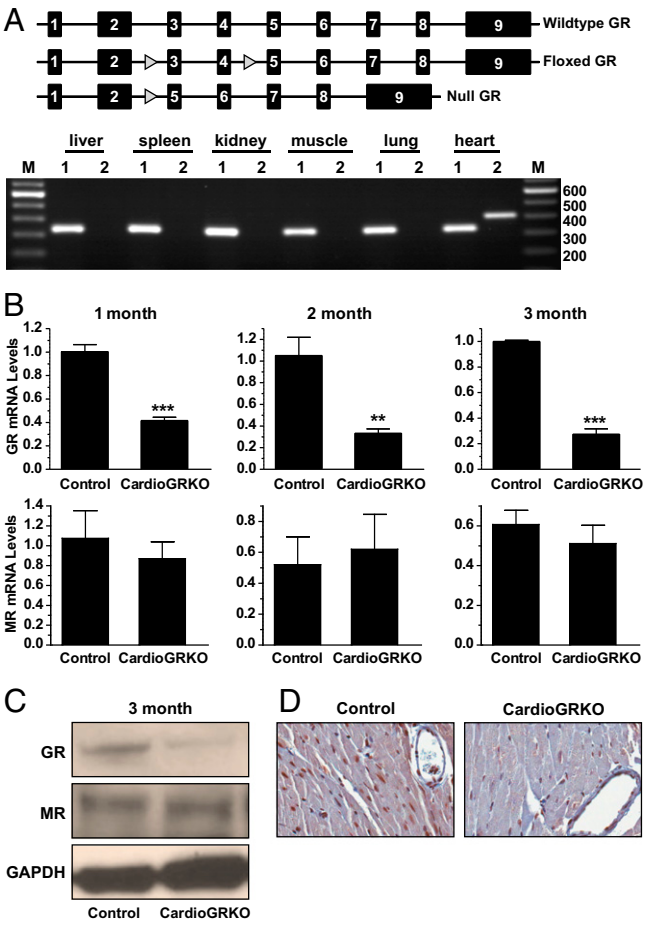


Fig. 1. Generation of mice with conditional knockout of GR in cardiomyocytes. (*A*) (*Upper*) Mice deficient in GR specifically in cardiomyocytes (cardioGRKO) were generated by crossing mice with a floxed GR allele ($GR^{loxP,loxP}$) with mice expressing Cre recombinase only in cardiomyocytes ($\alpha MHC^{Cre/+}$). Cre-mediated deletion of exons 3 and 4 results in a null GR allele. A frameshift mutation and premature stop codon result if splicing occurs between exons 2 and 5. (*Lower*) Cre-mediated recombination is specific to the hearts of cardioGRKO mice. DNA from various tissues of cardioGRKO mice was subjected to PCR using primers that detect the floxed GR allele (lane 1) and the null GR allele (lane 2). M, size marker. (*B*) RT-PCR of GR and MR mRNA from hearts of control and cardioGRKO mice. Data are mean ± SEM (n = 3–4 mice per group). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control mice. (*C*) Representative immunoblot of GR and MR protein from hearts of control and cardioGRKO mice. (*D*) Representative immunofluorescence staining of heart sections from control and cardioGRKO mice with anti-GR antibody.

cardioGRKO mice (Fig. 1*C*), and immunohistochemistry revealed that the decrease in GR expression was confined to cardiomyocytes (Fig. 1*D*). In contrast to GR, no differences were observed in the expression level of the closely related mineralocorticoid receptor (MR) (Fig. 1 *B* and *C*). To determine whether glucocorticoid responsiveness was diminished, we treated cardioGRKO mice with the synthetic glucocorticoid dexamethasone for 6 h and evaluated the regulation of three genes classically induced by activated GR. FK506-binding protein 5 (Fkbp5) functions to modulate GR activity, and both lipocalin 2 (Lcn2) and zinc finger protein 36 (Zfp36) inhibit the inflammatory response. Compared with littermate controls, the dexamethasone-dependent induction of these three genes was greatly impaired in the cardioGRKO mice (Fig. 2). The small glucocorticoid-dependent increase in Lcn2 and Zfp36 expression in the cardioGRKO mice did not reach statistical significance, and likely reflects glucocorticoid signaling in other cells of the heart where GR expression remains intact.

The cardioGRKO mice appeared normal early in life, but by ∼5 mo of age began exhibiting increased morbidity and mortality. Kaplan–Meier survival curves reveal a reduced life span for the cardioGRKO mice, as their median survival age was only 7 mo (Fig. 3). Male mice had a median survival age of 8 mo, and female mice had a median survival age of 6 mo (Fig. 3). A trend (P = 0.0689, female vs. male mice) for the shorter life span in females suggests that the susceptibility to the detrimental effects of inactivation of GR signaling in cardiomyocytes may exhibit some degree of sexual dimorphism. Of the 41 cardioGRKO mice that died prematurely, 17 were found dead in their cage and 24 were euthanized after showing symptoms of respiratory distress. Nearly all of the necropsied mice had enlarged hearts, and the majority had excess fluid in the thoracic cavity (Fig. 4*A*). Heart sections from cardioGRKO mice euthanized at 6 mo of age due to labored breathing showed marked dilation of the left ventricle (Fig. 4*B*, *Left*). Atrial thrombosis characterized by lamellation of fibrin and inflammatory cells, chronic inflammation, neovascularization, and fibrosis was also observed, and is consistent with atrial blood stasis and chronic heart failure (Fig. 4*B*, *Left* and *SI Appendix*, Fig. S1*A*). Moreover, lungs from these mice exhibited free erythrocytes in alveoli and hemosiderin-laden macrophages (heart failure cells) within alveolar spaces (Fig. 4*B*, *Right*). These findings suggest that the cardioGRKO mice are dying from heart failure.

To better understand the cardiac pathology underlying the spontaneous death, we evaluated the hearts of the cardioGRKO mice by conscious transthoracic echocardiography. No major differences were observed between 1-mo-old control and cardioGRKO mice (*SI Appendix*, Table S1). However, by 3 mo, a distinct cardiac phenotype emerged. Mice deficient in cardiac GR exhibited spontaneous LV systolic dysfunction, as evidenced by significant decreases in ejection fraction (87.3% control vs. 71.7% cardioGRKO) and fractional shortening (55.5% control vs. 40.5% cardioGRKO) (Table 1). Representative motion mode (M-mode) images clearly depict the altered LV end-systolic dimension in the cardioGRKO mice (Fig. 4*C*). Paralleling the observed functional deficits, we measured an increase in the size of the GR-deficient hearts. Both heart weight and LV mass adjusted to tibia length and body weight, respectively, were significantly elevated in the cardioGRKO mice (Table 1). Consistent with the age range over which the cardioGRKO mice died, variability was observed in the progression of the heart pathology. Of the nine cardioGRKO mice analyzed in Table 1, two of the 6-mo-old mice had large hearts but normal systolic function (prefailure), and seven of the mice had systolic dysfunction consistent with advanced heart failure. Separate analysis of the seven more severely affected mice revealed an even greater reduction in ejection fraction (87.3% control vs. 67.6% cardioGRKO) and fractional shortening (55.5% control vs. 36.7% cardioGRKO) (*SI Appendix*, Table S2). It has been suggested that high levels of Cre expression in cardiomyocytes can lead to cardiac toxicity in mice older than those used in our studies (6). Thus, we performed echocardiography on mice expressing Cre alone ($GR^{+/+}\alpha MHC^{Cre/+}$). Our data showed no significant effect on any of the echocardiographic indices measured in Cre-only mice up to 6 mo of age (*SI Appendix*, Table S3), indicating that the impaired cardiac function in 3- to 6-mo-old cardioGRKO mice is due to the loss of GR.

Hearts from the cardioGRKO mice were further analyzed by histology to investigate the molecular basis of the increased LV mass. A pronounced thickening was apparent in the ventricular walls of 3-mo-old cardioGRKO mice (Fig. 4*D*). No fibrosis was detected in the GR-deficient hearts (*SI Appendix*, Fig. S1*B*); however, there was an ∼140% increase in cardiomyocyte cross-sectional area (Fig. 4*E*). Interestingly, the cardiac remodeling and compromised heart function in the cardioGRKO mice 3–6 mo of age were not associated with any electrocardiogram abnormalities (*SI Appendix*, Table S4). In addition, cardioGRKO

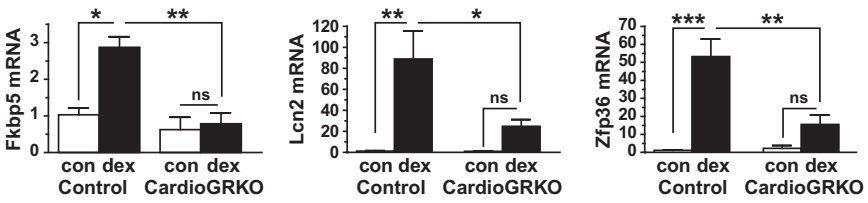


Fig. 2. Glucocorticoid regulation of target genes is impaired in cardioGRKO mice. Control and cardioGRKO mice were injected intraperitoneally with vehicle (con) or dexamethasone (dex; 1 mg/kg) for 6 h. Total RNA was isolated from whole hearts, and RT-PCR was performed to measure Fkbp5, Lcn2, and Zfp36 mRNA levels. Data are mean ± SEM (n = 3–5 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001 vs. PBS-injected controls. ns, not significant.

mice adrenalectomized at 2 mo to remove endogenous glucocorticoids and mineralocorticoids and then aged for an additional 4 mo exhibited an increase in cardiomyocyte size comparable to that observed in intact cardioGRKO mice (*SI Appendix*, Fig. S1*C*), suggesting that unabated MR signaling in the cardiomyocytes did not contribute to the pathology. Collectively, these data suggest that GR deficiency in mouse cardiomyocytes leads to spontaneous cardiac hypertrophy and concomitant LV dysfunction that is consistent with heart failure.

A hallmark of pathological cardiac hypertrophy and heart failure is the expression of certain fetal genes in the adult heart. In the hearts of the cardioGRKO mice, we found age-dependent increases in β-myosin heavy chain (βMHC) and skeletal muscle α-actin (SKA) that reached 8-fold and 12.1-fold, respectively, over control mice by 3 mo of age (*SI Appendix*, Fig. S2*A*). Other hypertrophic marker genes were also dysregulated in the GR-deficient hearts. We measured a 3.1-fold increase in brain natriuretic peptide (BNP), a 2.4-fold increase in smooth muscle α-actin (SMA), and a 40% reduction in αMHC (*SI Appendix*, Fig. S2*B*). These findings indicate that the observed cardiac hypertrophy in the cardioGRKO mice is mechanistically pathological.

To define the molecular events and biological pathways underlying the cardiac pathology, we performed a genome-wide microarray analysis on hearts from control and cardioGRKO mice that were 1, 2, or 3 mo of age. The younger 1- and 2-mo-old mice were chosen because they permit an assessment of the genetic changes in the heart that precede, and potentially give rise to, the cardiac dysfunction observed in 3-mo-old mice. Ablation of GR resulted in the differential expression of 302, 1,189, and 925 genes in the hearts from knockout mice that were 1, 2, or 3 mo old, respectively (Fig. 5*A* and *SI Appendix*, Table S5). The majority of genes showed decreased levels of mRNA in mice 1 or 2 mo of age, suggesting that GR signaling is necessary to maintain their expression. At the 3-mo time point, when heart dysfunction was evident, a large number of genes showed increased levels of mRNA. These later changes may be secondary to the developing pathology, and they suggest that GR signaling is important for limiting the expression of many genes in the normal heart. Analysis of the differentially expressed genes at each time point by literature-based Ingenuity Pathway Analysis (IPA) software identified "cardiovascular disease" as one of the top biological functions most significantly affected by the altered gene expression pattern in the cardioGRKO mice (Fig. 5*B*).

A total of 25, 112, and 105 genes associated with cardiovascular disease were, respectively, dysregulated in hearts from cardioGRKO mice that were 1, 2, and 3 mo of age (Fig. 5*B* and *SI Appendix*, Table S6). Among the genes with diminished expression were dystrophin (Dmd), ryanodine receptor 2 (RyR2), Kruppel-like factor 15 (Klf15), and the lipocalin-type prostaglandin D synthase (Ptgds). Dmd is a structural protein that links the sarcomere to the extracellular matrix and is important for cardiomyocyte contraction. RyR2 is a calcium channel that plays a crucial role in cardiomyocyte contraction by mediating the release of calcium from the sarcoplasmic reticulum. Klf15 is a transcriptional regulator that functions to prevent cardiac hypertrophy by repressing the activity of MEF2 and GATA4. Ptgds is an enzyme responsible for prostaglandin D2 (PGD2) biosynthesis, and Ptgds-mediated production of PGD2 promotes cardiomyocyte survival. By impairing cardiac contractility and stimulating cardiac hypertrophy, the aberrant down-regulation of these four genes may underlie and/or contribute to the observed cardiomyopathy in the cardioGRKO mice. To independently confirm the changes in these four genes, we performed RT-PCR on hearts from additional cardioGRKO mice that were 1, 2, or 3 mo old. All four genes exhibit an age-dependent reduction in expression that precedes the onset of the heart pathology in the cardioGRKO mice (Fig. 6).

Decreases in the expression and function of RyR2 have been associated with abnormal calcium handling in cardiac hypertrophy and heart failure (7). Consistent with the reduction in RyR2 mRNA (Fig. 6), RyR2 protein levels were also reduced in hearts from cardioGRKO mice (*SI Appendix*, Fig. S3*A*). To determine the functional consequence of the reduction in RyR2, we isolated cardiomyocytes from adult control and cardioGRKO mice and evaluated intracellular calcium changes following treatment with the classic RyR2 activator caffeine (*SI Appendix*, Fig. S3*B*). In response to low concentrations of caffeine (0.25 or 0.5 mM), the majority of cardiomyocytes from control mice (10/12 cells, 83.3%) exhibited a pronounced and sustained increase in intracellular calcium. In contrast, the majority of GR-deficient cardiomyocytes (18/28 cells, 64.3%) showed no sustained increase in intracellular calcium under these same conditions, but did exhibit an increase in the frequency of calcium oscillations. Both control and cardioGRKO cardiomyocytes all responded with large and sustained increases in intracellular calcium upon subsequent addition of a high concentration of caffeine (10 mM). These data indicate that GR-deficient cardiomyocytes have reduced levels of RyR2 and altered sensitivity to caffeine-induced intracellular calcium responses.

It is of great interest that the majority of the dysregulated genes in the GR-deficient hearts have not been previously associated with cardiovascular disease. This finding suggests that a deficiency in cardiomyocyte GR signaling may impact other cellular pathways with novel roles in the pathogenesis of heart failure. One of the most-established and clinically important actions of glucocorticoids is to suppress the immune system and inflammatory response. Examination of the dysregulated genes in the knockout hearts by IPA revealed a strong association with inflammation (Fig. 5*B* and *SI Appendix*, Table S7). Preceding the development of cardiac pathology, there is a large increase in the

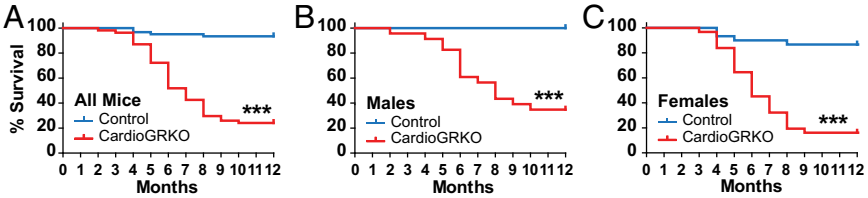


Fig. 3. GR deficiency leads to premature death in cardioGRKO mice. Kaplan–Meier analysis of survival after birth was performed for control and cardioGRKO mice. Shown are survival curves for all mice (*A*), males only (*B*), and females only (*C*). Data are from 61 control mice (31 male, 30 female) and 54 cardioGRKO mice (23 male, 31 female). ***P < 0.001 vs. control mice.

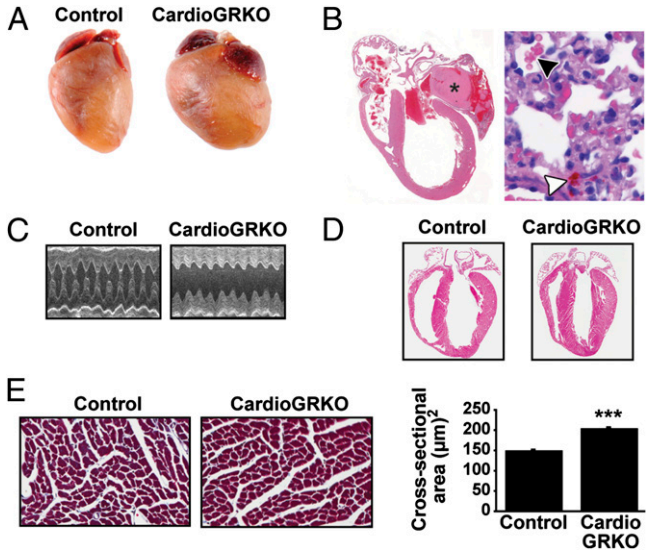


Fig. 4. Heart pathology and dysfunction in cardioGRKO mice. (*A*) Representative images of intact hearts from 6-mo-old control and cardioGRKO mice. (*B*) Representative images of H&E-stained sections of heart (*Left*) and lungs (*Right*) from a 6-mo-old cardioGRKO mouse. Indicated are left atrial thrombus with lamellated fibrin (asterisk), erythrocytes in alveoli (black arrowhead), and hemosiderin-laden macrophages (white arrowhead). (*C*) Representative M-mode images from 3-mo-old control and cardioGRKO mice. (*D*) Representative images of H&E-stained sections of 3-mo-old control and cardioGRKO hearts. (*E*) Representative images of Masson's trichrome staining of cardiomyocytes from 3-mo-old control and cardioGRKO mice and morphometric analysis of cardiomyocyte cross-sectional area. Data are mean ± SEM (n = 4 mice per group). ***P < 0.001 vs. control mice.

number of genes associated with inflammation in 2-mo-old cardioGRKO mice (*SI Appendix*, Fig. S4). A further increase in the number of dysregulated inflammatory genes is observed in 3-mo-old cardioGRKO mice, when heart dysfunction is evident (*SI Appendix*, Fig. S4). Moreover, there is a pronounced shift in the proportion of these genes that are up-regulated, consistent with an exaggerated immune response (*SI Appendix*, Fig. S4). Gene Ontology (GO) analysis of the genes differentially expressed in the cardioGRKO mice further substantiated the link to inflammation (*SI Appendix*, Table S8). Both "inflammatory response" and "immune response" were among the most significantly affected annotations in cardioGRKO mice 1–3 mo of age. In fact, inflammatory response was the top GO annotation associated with the set of genes dysregulated in the hearts of 1-mo-old cardioGRKO mice. Together, these data indicate that disruption of GR signaling in the hearts of cardioGRKO mice leads to the dysregulation of multiple genes and pathways with critical roles in cardiomyocyte biology.

Discussion

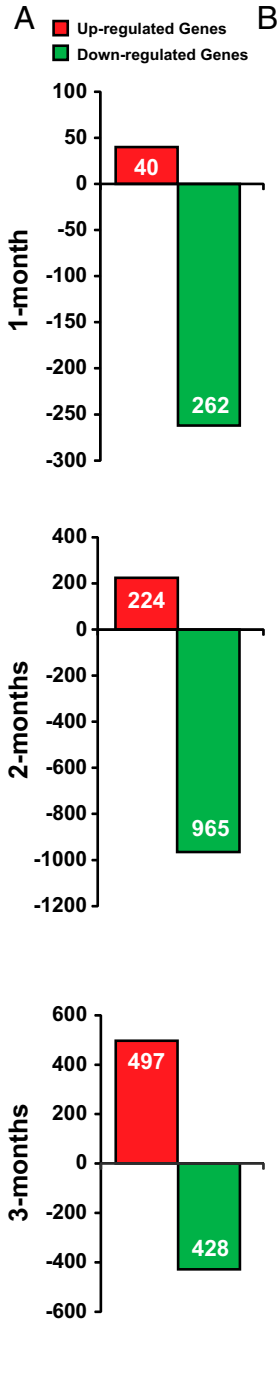
The severe pathology that develops in the hearts of cardioGRKO mice reveals an essential role for cardiomyocyte GR in normal heart homeostasis. Glucocorticoids regulate thousands of genes and impact numerous biological processes in cardiomyocytes (8). Therefore, the etiology of the heart dysfunction in the cardioGRKO mice is likely to be multifaceted and result from the combined dysregulation of many different genes. Indeed, global gene expression analysis of the cardioGRKO mouse hearts before pathology onset identified aberrant expression profiles for 130 genes known to be associated with cardiovascular disease and over 1,000 genes not previously associated with heart dysfunction. We further characterized the aberrant down-regulation of a key set of genes important for the contraction of the heart (Dmd and RyR2), for repressing the hypertrophic program (Klf15), and for promoting the survival of cardiomyocytes (Ptgds). All four genes show an age-dependent decline in expression that precedes the onset of the cardiomyopathy in the cardioGRKO mice. The reduced expression of RyR2 leads to altered calcium responses in GR-deficient cardiomyocytes. Consistent with the phenotype of the cardioGRKO mice, abnormal calcium handling, cardiac hypertrophy, and systolic dysfunction have been reported in RyR2-deficient mice (9, 10). Knockout of RyR2 can also lead to fatal arrhythmias (9), suggesting that the cardioGRKO mice may exhibit acute episodes of abnormal heart rhythms at the time of death. Changes in the expression of other genes may also play an important role in the pathogenesis of the cardiac dysfunction in the cardioGRKO mice. For example, the ubiquitin ligase muscle ring finger protein 1 (MuRF1) is a negative regulator of cardiac hypertrophy that is significantly repressed in GR knockout hearts (*SI Appendix*, Table S6). Whether the Dmd and RyR2 genes are primary or secondary targets of GR regulation is currently unknown. However, Klf15, Ptgds, and MuRF1 are bona fide glucocorticoid-responsive target genes that are induced by activated GR (11–14). Accordingly, in the absence of an intact GR signaling pathway in cardiomyocytes, the expression of these genes is significantly repressed.

Inflammatory processes are involved in many conditions that injure the myocardium and cause both structural and functional defects in the heart (15, 16), and glucocorticoids have well-documented anti-inflammatory actions. Examination of the genes dysregulated in the hearts of the cardioGRKO mice by both IPA and GO revealed a prominent association with the immune response and inflammation. Among the dysregulated immunomodulatory genes is Lcn2. Lcn2 is known to be induced by activated GR and can function to inhibit chemokine expression and restrain inflammation. In the hearts of the cardioGRKO mice, exogenous glucocorticoid regulation of Lcn2 is impaired and the basal expression of this gene is reduced (Fig. 2 and *SI Appendix*, Table S5). Similar findings were observed for

Table 1. Echocardiography on control and cardioGRKO mice

Parameter	Control, n = 10	CardioGRKO, n = 9
IVSTD, mm	1.09 ± 0.02	1.14 ± 0.01
LVEDD, mm	3.02 ± 0.10	3.23 ± 0.04
PWTD, mm	1.01 ± 0.02	1.03 ± 0.02
IVSTS, mm	1.72 ± 0.03	1.72 ± 0.02
LVESD, mm	1.34 ± 0.05	1.93 ± 0.04***
PWTS, mm	1.56 ± 0.04	1.49 ± 0.02
LV VolD, μL	36.11 ± 3.08	42.9 ± 1.2
LV VolS, μL	4.64 ± 0.44	12.6 ± 0.6***
EF, %	87.3 ± 0.3	71.7 ± 0.9***
FS, %	55.5 ± 0.3	40.5 ± 0.8***
LV mass, mg	112.65 ± 6.65	132.0 ± 3.0*
Tibia length, mm	17.23 ± 0.13	17.20 ± 0.03
Heart weight, mg	119.5 ± 4.5	141.5 ± 2.7***
HW/BW, mg/g	3.54 ± 0.18	4.44 ± 0.10***
HW/TL, mg/mm	6.9 ± 0.2	8.2 ± 0.2***
LV mass/BW, mg/g	3.3 ± 0.1	3.7 ± 0.1**
LV mass/TL, mg/mm	6.5 ± 0.3	7.7 ± 0.2*
HR, bpm	668 ± 13	644 ± 5
BW, g	28.3 ± 2.7	33.2 ± 0.5

Echocardiographic measurements from transthoracic M-mode tracings on conscious male mice 3–6 mo of age. Data are mean ± SEM. bpm, beats per min; BW, body weight; EF, ejection fraction; FS, fractional shortening; HR, heart rate; HW, heart weight; IVSTD, interventricular septal thickness in diastole; IVSTS, interventricular septal thickness in systole; LV VolD, LV volume in diastole; LV VolS, LV volume in systole; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; PWTD, posterior wall thickness in diastole; PWTS, posterior wall thickness in systole; TL, tibia length. A Student t test was performed to determine significance. Values without asterisks were not significantly different. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control mice.



B

Pathway	p-value	genes
Cardiovascular Disease	1.01E-04-2.55E-02	25
Organismal Injury and Abnormalities	1.63E-04-2.55E-02	16
Infectious Disease	1.55E-03-2.55E-02	11
Dermatological Diseases and Conditions	2.36E-03-2.55E-02	5
Developmental Disorder	2.36E-03-2.66E-02	18
Gastrointestinal Disease	2.36E-03-2.55E-02	14
Hereditary Disorder	2.36E-03-2.64E-02	26
Inflammatory Disease	2.36E-03-2.55E-02	5
Endocrine System Disorders	3.43E-03-2.55E-02	12
Cancer	3.86E-03-2.64E-02	11

Pathway	p-value	genes
Skeletal and Muscular Disorders	7.47E-08-9.17E-03	173
Hereditary Disorder	4.65E-07-9.17E-03	79
Cardiovascular Disease	3.9E-06-7.97E-03	112
Neurological Disease	1.13E-05-7.6E-03	125
Developmental Disorder	1.24E-05-9.17E-03	70
Renal and Urological Disease	5.58E-05-8.57E-03	45
Metabolic Disease	8.77E-05-7.71E-03	84
Infectious Disease	1.17E-04-8.22E-03	121
Organismal Injury and Abnormalities	1.76E-04-9.02E-03	82
Endocrine System Disorders	2.73E-04-9.78E-03	73

Pathway	p-value	genes
Neurological Disease	1.7E-09-7.85E-03	159
Cardiovascular Disease	1.06E-08-6.98E-03	105
Psychological Disorders	1.12E-08-6.38E-03	94
Inflammatory Response	2.24E-06-7.45E-03	79
Infectious Disease	1.98E-05-7.18E-03	34
Hereditary Disorder	2.45E-05-6.98E-03	105
Respiratory Disease	2.45E-05-7.8E-03	28
Connective Tissue Disorders	4.09E-05-6.89E-03	80
Inflammatory Disease	4.09E-05-7.85E-03	101
Skeletal and Muscular Disorders	4.09E-05-7.85E-03	131

Fig. 5. Global gene expression profile in hearts from cardioGRKO mice. Microarray analysis was performed on RNA from the hearts of 1-, 2-, and 3-mo-old control and cardioGRKO mice. (*A*) Total number of genes differentially expressed in the hearts of cardioGRKO mice compared with control littermates (ANOVA, $P < 0.01$). (*B*) Differentially expressed genes were analyzed by IPA software for each age group. Shown are the top 10 biological functions most significantly associated with the genes differentially regulated in the hearts of the cardioGRKO mice.

the Zfp36 gene, whose protein product, tristetraprolin, destabilizes the mRNA for many proinflammatory cytokines. Glucocorticoid induction of Zfp36 is abrogated and basal levels are decreased in GR-deficient hearts (Fig. 2 and *SI Appendix*, Table S5). Failure of endogenous glucocorticoids to maintain appropriate expression of these and other immunomodulatory genes may lead to excessive and persistent activation of inflammatory processes that underlie and/or exacerbate the severity of the heart pathology in the cardioGRKO mice. Gross histological evidence for increased inflammation was not observed in young cardioGRKO mice, suggesting these immune changes may become more prominent as the mice age.

Some of the genetic changes detected in the 1-, 2-, and 3-mo-old cardioGRKO mice may be secondary to the inappropriate regulation of glucocorticoid-responsive target genes at an earlier time. In an effort to capture these primary genes, we also performed a genome-wide microarray on neonatal hearts from control and cardioGRKO mice (*SI Appendix*, Fig. S5). As observed in GR knockout mice 1 or 2 mo of age (Fig. 5), the majority of dysregulated genes showed decreased expression, and IPA identified cardiovascular disease as one of the top biological functions most significantly affected by ablation of GR. Strikingly, more than one-third of the 49 genes associated with cardiovascular disease that were aberrantly expressed in hearts from neonatal cardioGRKO mice have been shown in the literature to be regulated by glucocorticoids. These GR target genes include many transcription factors and plasma membrane receptors, as well as several intracellular proteins involved in GPCR and MAPK signal transduction pathways. Reduced expression of one or more of these primary genes may lead to subsequent alterations in the expression profile of other secondary genes that contribute to the pathology in the cardioGRKO mice.

In addition to GR, cardiomyocytes express the closely related mineralocorticoid receptor that binds aldosterone and glucocorticoids with similar high affinity. Glucocorticoids typically circulate at much higher levels than aldosterone, and cardiac-specific MR is thought to be primarily occupied by glucocorticoids under normal physiological conditions. To assess whether glucocorticoid activation of MR contributes to the heart failure phenotype observed in the cardioGRKO mice, we removed circulating glucocorticoids in these mice by surgical excision of the adrenal glands. Adrenalectomy neither augmented nor lessened the hypertrophic response in the cardioGRKO mice, suggesting the cardiac dysfunction is mediated solely by a deficiency in glucocorticoid activation of GR signaling in cardiomyocytes.

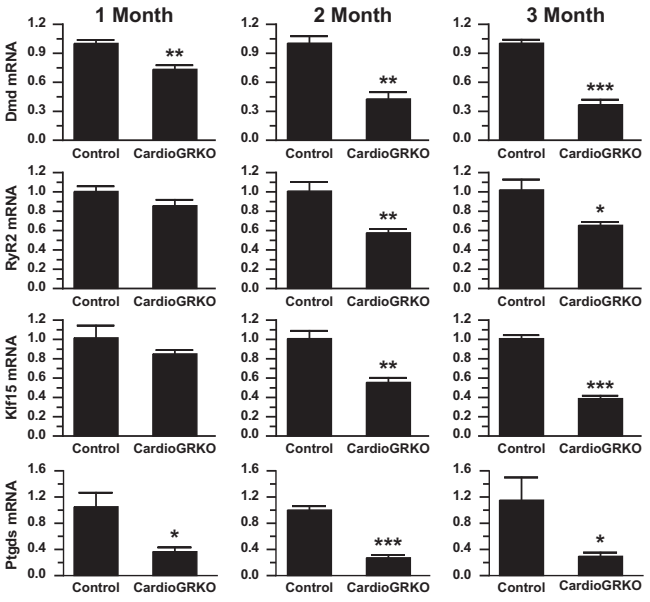


Fig. 6. Down-regulation of genes associated with cardiovascular disease in the hearts of cardioGRKO mice. Total RNA was isolated from whole hearts of control and cardioGRKO mice 1, 2, or 3 mo of age. Dmd, RyR2, Klf15, and Ptgds mRNA levels were measured by RT-PCR. Data are mean ± SEM ($n = 3$–5 mice per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control mice.

In agreement with our findings in the cardioGRKO mice are several independent lines of evidence that suggest that GR signaling is important for maintaining normal heart function in humans. For example, persons with a polymorphism (A3669G) in the GR gene resulting in increased expression of the dominant-negative receptor variant GRβ and concomitant glucocorticoid resistance have an increased risk of coronary artery disease, enlarged hearts, systolic dysfunction, and heart failure (17–19). In addition, patients with Addison disease, a life-threatening disorder characterized by decreased production of glucocorticoids, present with weak hearts (20). Although heart failure secondary to Addison disease has been described (21), cardiac hypertrophy has not been reported, perhaps reflecting the diagnosis and effective treatment of individuals before cardiac abnormalities become severe (22). Alternatively, because Addison disease operates at a systemic level and is also accompanied by insufficient mineralocorticoid signaling, the resulting heart pathology in these patients may differ from the cardiac abnormalities detected in the cardioGRKO mice, where glucocorticoid signaling is deficient only in cardiomyocytes.

Heart failure remains one of the leading causes of mortality in the Western world, and stress is increasingly recognized as a contributing factor to cardiac disease. The stress-induced rise in glucocorticoids may benefit the heart in the short term by the direct actions of glucocorticoids on cardiomyocytes that improve their contractility and promote their survival. However, sustained increases in glucocorticoids negatively impact heart function via the systemic actions of glucocorticoids on noncardiac tissues that lead to hypertension and metabolic syndrome. Progress has been made in understanding the molecular basis for heart failure and in developing therapeutic agents to combat its progression, yet the clinical outcome for heart failure patients remains poor. Using mice deleted of GR specifically in cardiomyocytes, we have discovered that a deficiency in cardiomyocyte GR signaling leads to spontaneous cardiac hypertrophy, LV dysfunction, heart failure, and death. These findings reveal an obligate role for GR in maintaining normal heart function, and suggest that activating GR signaling selectively in cardiomyocytes through local delivery of glucocorticoids to the myocardium may provide an attractive approach for treating heart disease.

Materials and Methods

Please see *SI Appendix, Materials and Methods* for details.

Generation of CardioGRKO Mice. The mouse GR (NR3C1) locus was modified by insertion of loxP sites upstream of exon 3 and downstream of exon 4 by standard gene-targeting procedures. Mice harboring the modified GR allele were derived by blastocyst (albino B6) injection. Mice homozygous for the floxed GR allele ($GR^{loxP,loxP}$) were mated with mice expressing Cre recombinase under the direction of cardiomyocyte-specific $\alpha MHC^{Cre/+}$ (5). The resulting offspring, on a mixed C57BL/6 and FVB/N background, were $GR^{loxP/loxP}\alpha MHC^{Cre/+}$ mice (designated cardioGRKO) and Cre-negative $GR^{loxP/loxP}\alpha MHC^{+/+}$ littermate mice that served as controls. All experiments were approved and performed according to the guidelines of the Animal Care and Use Committees at the University of North Carolina at Chapel Hill and the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

Real-Time PCR. Total RNA was isolated from the whole hearts of control and cardioGRKO mice, and the abundance of mRNAs was determined on a 7900HT sequence detection system (Applied Biosystems).

Immunoblotting. Hearts from control and cardioGRKO mice were lysed in RIPA buffer. Membranes with equivalent amounts of protein were incubated with primary antibodies and developed by enhanced chemiluminescence (GE Healthcare).

Histological Analysis. Hearts from control and cardioGRKO mice were perfused with PBS and fixed with paraformaldehyde. Samples were stained with H&E or Masson's trichrome or processed for immunohistochemistry.

Survival Analysis. Control and cardioGRKO mice were monitored on a daily basis for morbidity or death until 12 mo of age.

Echocardiographic Analysis. Transthoracic echocardiography was performed on conscious mice using a Vevo 770 ultrasound biomicroscopy system (VisualSonics).

Microarray Analysis. Gene expression analysis was performed on RNA from hearts of control and cardioGRKO mice using Whole Mouse Genome oligo arrays (014868) (Agilent Technologies). Significant changes in gene expression were defined on the basis of P value ($P < 0.01$). Specifically, an error-weighted ANOVA and Benjamini–Hochberg multiple test correction were performed using the Rosetta Resolver system (Rosetta Biosoftware). Significantly regulated genes were analyzed by IPA (Ingenuity Systems) and by GO. Gene enrichment P values ($P < 0.05$) for biological functions were determined by IPA using Fisher's exact test.

Statistical Analysis. A Student t test or one-way ANOVA with Tukey's post hoc analysis was used to evaluate whether differences between groups were statistically significant. For Kaplan–Meier survival curves, the log-rank test was used. Statistical significance was defined as $P < 0.05$.

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