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ROCK1 plays an essential role in the transition from cardiac hypertrophy to failure in mice

Jianjian Shi¹, Yi-Wei Zhang¹, Yu Yang¹, Lumin Zhang¹, and Lei Wei^{1,2}

¹Riley Heart Research Center, Wells Center for Pediatric Research, Indiana University, School of Medicine, Indianapolis, Indiana, USA

²Department of Cellular and Integrative Physiology, Indiana University, School of Medicine, Indianapolis, Indiana, USA

Abstract

Pathological cardiac hypertrophy caused by diverse etiologies eventually leads to cardiac dilation and functional decompensation. We have recently reported that genetic deletion of Rho-associated coiled-coil containing protein kinase 1 (ROCK1) inhibited several pathological events including cardiomyocyte apoptosis in compensated hypertrophic hearts. The present study investigated whether ROCK1 deficiency can prevent the transition from hypertrophy to heart failure. Transgenic mice with cardiac-restricted overexpression of *Gαq* develop compensated cardiac hypertrophy at young ages, but progress into lethal cardiomyopathy accompanied by increased apoptosis after pregnancy or at old ages. The studies were first carried out using age- and pregnancy-matched wild-type (WT), *Gαq*, *ROCK1*^{-/-}, and *Gαq/ROCK1*^{-/-} mice. The potent beneficial effect of ROCK1 deletion is demonstrated by abolishment of peripartum mortality, and significant attenuation of left ventricular (LV) dilation, wall thinning, and contractile dysfunction in the peripartum *Gαq* transgenic mice. Increase in cardiomyocyte apoptosis was suppressed by ROCK1 deletion, associated with increased extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) activation and inhibition of mitochondrial translocation of Bax. In addition, ROCK1 deficiency also improved survival, inhibited cardiomyocyte apoptosis, and preserved LV dimension and function in old *Gαq* mice at 12 months. Furthermore, transgenic overexpression of ROCK1 increased cardiomyocyte apoptosis and accelerated hypertrophic decompensation in *Gαq* hearts in the absence of pregnancy stress. The present study provides for the first time in vivo evidence for the long-term beneficial effects of ROCK1 deficiency in hypertrophic decompensation and suggests that ROCK1 may be an attractive therapeutic target to limit heart failure progression.

Keywords

Rho kinase; ROCK1; apoptosis; *Gαq*; ERK/MAPK; Bax; heart failure

Heart failure is a leading cause for human morbidity and mortality, and the incidence of heart failure has been constantly increasing during the past decades. Cardiac hypertrophy is initially

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Correspondence To: Lei Wei, Ph.D. Riley Heart Research Center Wells Center for Pediatric Research Indiana University School of Medicine 1044 West Walnut Street, R4-370 Indianapolis, IN 46202-5225 Ph: (317) 274-7894 Fax: (317) 278-9298 lewei@iupui.edu.

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Conflict of Interest None declared.

a compensatory response to diverse etiologies, but it eventually leads to heart failure or sudden death due to decompensation [1–3]. Identifying the signaling mechanisms underlying the development of cardiac hypertrophy and the transition to heart failure will be helpful for the design of effective therapeutics. Rho-associated coiled-coil containing protein kinase (ROCK) is a downstream mediator of RhoA, and plays a critical role in mediating the effects of RhoA on stress fiber formation, smooth muscle contraction, cell adhesion, membrane ruffling, cell motility and apoptosis [4–6]. Studies using pharmacological inhibitors, Y27632 and fasudil, suggest an *in vivo* role for ROCK in the pathogenesis of cardiac hypertrophy and remodeling [7–9]. However, these inhibitors do not distinguish between ROCK1 and ROCK2, the two isoforms of ROCK family, and could also have non-selective effects [10]. Recent genetic studies by our laboratory and others support the concept that ROCK1 and ROCK2 have distinct non-redundant functions in cardiac hypertrophy and remodeling [11–14].

We showed that ROCK1 deletion did not impair compensatory hypertrophic response, but significantly reduced cardiomyocyte apoptosis and fibrosis in response to pressure overload induced by transverse aortic constriction [11,12]. In addition, ROCK1 deletion did not affect the development of cardiac hypertrophy in *Gαq* transgenic mice, but prevented chamber dilation and contractile dysfunction at young ages (12 weeks) [13]. The *Gq* class of heterotrimeric G proteins is an important transducer of humoral (i.e., α 1-adrenergic agonists, angiotensin II, endothelin and prostaglandin F₂ α) and mechanical stimuli that are important in cardiac hypertrophy. Transgenic expression of *Gαq* in the myocardium elicits cardiac hypertrophy and contractile dysfunction, but without significant increase in cardiomyocyte apoptosis at young ages [15,16]. These results indicate that ROCK1 does not play a significant role in compensatory hypertrophic responses, and raise the possibility that ROCK1 plays a critical role in the maladaptive response which contributes to the transition from compensatory cardiac hypertrophy to heart failure.

To explore this concept and determine long-term impact of ROCK1 deficiency in the setting of cardiomyopathy, the present study examined the effects of ROCK1 deletion on decompensation of the hypertrophic *Gαq* hearts under two different stress conditions: multiple pregnancy and at 12-month-old age. Previous reports have validated this decompensation model as hypertrophic *Gαq* hearts progress into heart failure after additional stresses such as pregnancy, aging or pressure overload [15,17–19]. Our results show that ROCK1 deletion strikingly improved animal survival and prevented the development of heart failure under both conditions by preserving chamber dimension and contractile function, suppressing increase in cardiomyocyte apoptosis and cardiac fibrosis. In addition, transgenic overexpression of ROCK1 alone did not cause significant changes in heart structure/function, but increased cardiomyocyte apoptosis and accelerated hypertrophic decompensation in *Gαq* hearts in the absence of pregnancy stress. These results provide the first *in vivo* evidence for an essential role for ROCK1 in cardiac decompensation.

Methods

All animal experiments were conducted in accordance with the National Institutes Health “Guide for the Care and Use of Laboratory Animals” (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee at Indiana University School of Medicine.

Mouse models

Transgenic FVB mice overexpressing *Gαq* in cardiomyocytes (*Gαq*, 25-copy line) have been characterized previously [15,16]. Generation of ROCK1^{-/-} mice and *Gαq*/ROCK1^{-/-} mice was as previously described [12,13]. Transgenic FVB mice overexpressing ROCK1 in cardiomyocytes were generated using the 5.5-kb murine α MHC promoter kindly provided by

Dr. Jeffrey Robbins [20] and a full-length human ROCK1 cDNA (4.7 kb) kindly provided by Dr. Shuh Narumiya (Kyoto University, Kyoto, Japan).

Echocardiography

Cardiac dimension and contractile performance were evaluated by noninvasive transthoracic echocardiography with a Vevo 770 high resolution Imaging System (VisualSonics Inc, Toronto) (Rodent Ultrasound Imaging Core, Department of Cellular & Integrative Physiology, Indiana University School of Medicine) as previously described [13].

Histology

Assessment of cardiac hypertrophy was performed as previously described [13,21]. Tissue sections were stained with hematoxylin/eosin for initial evaluation, picrosirius red/Fast green to identify collagen fibers, immunostaining for laminin to measure myocyte size, and TUNEL staining to monitor cardiomyocyte apoptosis as previously described [11,13,21]. Triple staining with an anti-sarcomeric α -actinin antibody (red signal), TUNEL assay (green signal), and DAPI (blue signal) was used to identify nuclei in apoptotic cardiomyocytes.

Protein analysis

Protein samples were prepared as previously described [13,21]. Primary antibodies used include rabbit polyclonal antibodies to ROCK1, G α q (E-17), adenylyl cyclase V/VI (AC 5/6), PKC α (C-20) (Santa Cruz), active caspase 3, Akt, phospho-Akt (Ser473), phospho-PTEN (Ser380/Thr382/Thr383), Bax, Bcl-2, Bcl-xL, ERK/MAPK, SAPK/JNK, p38 MAPK, phospho-ERK/MAPK (Thr202/Tyr204), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558), ERM (Cell Signaling), mouse monoclonal antibodies to ROCK2 (BD Transduction Laboratories).

Subcellular Fractionation

Hearts were homogenized in ice cold mitochondria buffer (5 mM MOPS pH 7.0, 225 mM mannitol, 1 mM EGTA, 75 mM sucrose, 1 mM DTT) supplemented with proteinase inhibitors and phosphatase inhibitors (Roche), centrifuged twice at 700 $\times$ g at 4 °C for 10 min to pellet cell debris and nuclei. The supernatant was again centrifuged at 10,000 $\times$ g at 4 °C for 20 min, and the pellet was saved as mitochondrial fraction. The supernatant was again centrifuged at 100,000 $\times$ g at 4 °C for 1 h, and the pellet was saved as light membrane fraction, the supernatant was saved as cytosolic fraction. After measurement of the protein concentration, samples with equal amounts of protein were analyzed by Western blot with specific antibodies. The purity of the subcellular fractionations was assessed by Western blot analysis with anti-GAPDH (a cytosolic protein; Fitzgerald Industries International) and anti-cytochrome *c* oxidase subunit IV (COX IV) (a mitochondrial protein; Molecular Probes) antibodies.

Gene expression analysis

Gene expression analyses by Affymetrix mouse genomic 430 2.0 arrays and by real-time RT-PCR were performed as previously described [12]. TaqMan primers and probes for mouse GAPDH, ROCK1, ROCK2, α -myosin heavy chain (MHC), β MHC, skeletal α -actin (α SK), atrial natriuretic factor (ANP), B-type natriuretic peptide (BNP) were purchased from Applied Biosystems (Foster City, CA).

Statistical analysis

Data are reported as mean $\pm$ SE. Comparisons between groups were analyzed by Student's *t*-test or ANOVA as appropriate, with *P* < 0.05 considered as significant.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

ROCK1 deletion abolished peripartum mortality in the peripartum Gαq mice

Previous study has reported about 30% to 50% of female Gαq mice develop lethal failure in the peripartum period [15,18,19]. To determine whether ROCK1 deletion decreases the mortality of the peripartum Gαq mice, 8-week-old female wild-type (WT), ROCK1^{-/-}, Gαq and Gαq/ROCK1^{-/-} mice were mated with WT males. Gαq mice exhibited 30% lethality after first pregnancy and 100% lethality after the fourth pregnancy (Fig. 1A) as predicted. Strikingly, Gαq-induced peripartum lethality was completely suppressed in Gαq/ROCK1^{-/-} mice following 4 consecutive pregnancies as Gαq/ROCK1^{-/-} mice exhibited 100% survival as observed with WT and ROCK1^{-/-} mice (Fig. 1A).

Western blot analyses confirmed that ROCK1 deficiency did not change the level of Gαq transgene expression (supplemental Fig. 1). The mRNA level of ROCK1 in the Gαq hearts was increased (about 2-fold) (Table 1) associated with a slight increase in the protein level (supplemental Fig. 1). Phosphorylation of ERM proteins (known substrates of ROCK) was increased in the Gαq hearts indicating increased ROCK activity (supplemental Fig. 1). On the other hand, ROCK2 expression remained similar in all four groups (supplemental Fig. 1 and Table 1).

ROCK1 deletion delayed the progression of LV dilation and dysfunction in the peripartum Gαq mice induced by multiple pregnancies

To determine the degree to which ROCK1 deletion prevents LV dilation and contractile dysfunction, serial echocardiographic analyses were performed in surviving mice at postpartum day 7 following three consecutive pregnancies (Fig. 1 and supplemental Table 1). The Gαq mice exhibited an increase in LV end-diastolic dimension (LVEDD) (Fig. 1B) and LV end-systolic dimension (LVESD) (Fig. 1C) associated with a decrease in LV fractional shortening (LVFS) (Fig. 1D) when compared with WT mice at baseline. These differences between Gαq and WT mice progressively increased following consecutive pregnancies (Fig. 1B–D), indicating exacerbated cardiac dilation and contractile dysfunction in Gαq mice. In Gαq/ROCK1^{-/-} compound mice, cardiac dimension and contractile function were preserved at baseline as compared with WT and ROCK1^{-/-} mice (Fig. 1B–D). A trend toward increased cardiac dimension and decreased cardiac function was noticed in these mice after the first and second pregnancies, which became significant only after the third pregnancy (Fig. 1B–D), indicating a marked delay in heart failure progression. In addition, end-systolic LV posterior wall thickness (LWPWs) was progressively reduced in Gαq hearts, but fully preserved in Gαq/ROCK1^{-/-} mice throughout pregnancies (supplemental Table 1). The mean heart rate of Gαq/ROCK1^{-/-} mice was higher than that of Gαq mice, but was still significantly lower than that of WT and ROCK1^{-/-} mice throughout consecutive pregnancies (supplemental Table 1), suggesting that changes in heart rate unlikely contribute to the marked beneficial effects of ROCK1 deletion on cardiac decompensation.

ROCK1 deletion reversed LV dilation induced by pregnancy in Gαq mice

Volume overload in pregnancy induces eccentric hypertrophy characterized by chamber enlargement and this physiological cardiac hypertrophy is reversible. Serial echocardiographic analyses were performed before pregnancy (at 8 weeks) and at postpartum day 3, 7 and 14 in surviving mice to follow this process. In Gαq mice, compared to pre-pregnant levels, pregnancy induced a significant increase in LVEDD and LVESD at postpartum days 3 and 7, which were maintained until postpartum day 14 (Supplemental Fig. 2). In Gαq/ROCK1^{-/-} mice, pregnancy

also induced a significant increase in LVEDD and LVESD at postpartum days 3 and 7, but these parameters subsequently decreased at postpartum day 14 closely to the pre-pregnant levels (Supplemental Fig. 2), indicating reversible chamber dilation. This reversible chamber dilation was also observed in WT and ROCK1^{-/-} mice. These results indicate that combination of the genetic and physiological stresses induces irreversible chamber dilation in Gαq hearts but reversible chamber dilation in Gαq/ROCK1^{-/-} hearts as well as in WT and ROCK1^{-/-} hearts.

Morphological analysis also supported a dilated phenotype in Gαq but not in Gαq/ROCK1^{-/-} mice compared with WT mice after one pregnancy (Fig. 2). Morphometric analysis indicates that ROCK1 deletion did not prevent Gαq-induced cardiac hypertrophy (Fig. 2A, Table 2). LV cardiomyocyte cross sectional area in the postpartum Gαq and Gαq/ROCK1^{-/-} groups after first pregnancy showed significant increases compared with WT and ROCK1^{-/-} mice (Fig. 2B). The levels of induction of the hypertrophic markers such as ANF, BNP, αSK and β-MHC were similar in the postpartum Gαq and Gαq/ROCK1^{-/-} groups (Table 1). Cardiac fibrosis was significantly increased in postpartum Gαq and Gαq/ROCK1^{-/-} hearts compared with WT and ROCK1^{-/-} mice (Fig. 2C, D). However, this increased fibrosis was significantly less in postpartum Gαq/ROCK1^{-/-} hearts compared to Gαq hearts. In addition Gαq mice exhibited significant increase in lung weight at baseline, which was further increased following consecutive pregnancies, indicating progression of lung congestion due to deterioration of cardiac function. In contrast, no noticeable change in lung weight was observed in Gαq/ROCK1^{-/-} mice until the third pregnancy consistent with a marked delay in the development of cardiac dysfunction in Gαq/ROCK1^{-/-} mice throughout pregnancies (Table 2). Together, these results indicate that ROCK1 deletion did not prevent development of cardiac hypertrophy, but markedly attenuated progression of cardiac decompensation induced by combination of overexpression of a key hypertrophic mediator (Gαq) and persistent physiological stress such as multiple pregnancies.

ROCK1 deletion inhibited cardiomyocyte apoptosis and up-regulation of Bax in the postpartum Gαq hearts

Since previous studies have reported that peripartum Gαq hearts exhibited marked increase in cardiomyocyte apoptosis [15,18,19], we assessed cardiomyocyte apoptosis by TUNEL staining (Fig. 3A, B). Prior to pregnancy, there was no difference in cardiac TUNEL positivity in all groups of mice. However, the number of TUNEL positive cardiomyocytes significantly increased in hearts from Gαq mice at postpartum day 7 after the first pregnancy (Fig. 3B) as previously reported [15,18,19]. Importantly, there was no increase in the number of TUNEL positive cardiomyocytes in hearts from Gαq/ROCK1^{-/-} mice (Fig. 3B).

To determine the molecular mechanisms underlying inhibitory effects of ROCK1 deletion on cardiomyocyte apoptosis, we have examined several molecular events which have previously been shown to be critical for increased cardiomyocyte apoptosis in peripartum Gαq hearts. One important molecule identified in mediating Gαq-induced cardiomyocyte apoptosis is Nix, a BH3-only pro-apoptotic protein, which is up-regulated in Gαq hearts and in pressure overload hypertrophy [18,22]. Our microarray analysis revealed a significant increase (1.93-fold) in the mRNA level of Nix in postpartum Gαq hearts compared to WT hearts, and also a similar increase (1.87-fold) in Gαq/ROCK1^{-/-} hearts. These results indicate that ROCK1 deletion did not prevent up-regulation of Nix expression induced by Gαq.

Interestingly, Western blot analysis detected a significant increase (2.62-fold) of Bax expression in postpartum Gαq hearts but not in Gαq/ROCK1^{-/-} hearts, indicating that ROCK1 deletion prevented up-regulation of Bax (Fig. 3C). We observed that mRNA level of Bax was not affected by Gαq overexpression or ROCK1 deletion (Table 1), indicating that up-regulation of Bax by Gαq is a post-transcriptional event. Furthermore, mitochondrial Bax level was

significantly increased in *Gαq* hearts compared with other groups (Fig. 3C), suggesting that increased mitochondrial translocation of Bax contributes to the increased expression level by increasing protein stability in *Gαq* hearts. Additional Western blot analysis did not detect significant change for the expression levels of several other apoptotic (Bad, Bim) and anti-apoptotic Bcl2 family proteins (Bcl2 and Bcl-xL) in the ventricular tissues of these four groups of mice (Fig. 3C).

ROCK1 deletion did not prevent suppression of Akt activation, but increased ERK/MAPK activation in the postpartum *Gαq* hearts

To further determine the molecular mechanisms underlying protective effects of ROCK1 deletion against cardiac decompensation, we have examined the effects of ROCK1 deletion on several molecular events which have previously been shown to be affected in *Gαq* hearts. Previous study has shown that Akt-mediated cardiomyocyte survival pathways were compromised in the peripartum *Gαq* hearts due to the reduced level of phosphatidylinositol 4,5-bisphosphate (PIP2), which is a common substrate for PI3 kinase and phospholipase C (PLC) [23]. Increased *Gαq* activity in the peripartum *Gαq* hearts resulted in PLC activation, which in turn reduced the availability of PIP2 for PI3 kinase, thereby leading to reduced PIP3 generation and decreased Akt activation. Western blot analysis indicates that prior to pregnancy there was no difference in the level of phospho-Akt in all groups of mice (Fig. 4A). However, similar reduction in Akt phosphorylation was observed in the postpartum *Gαq* and *Gαq*/ROCK1^{-/-} hearts (Fig. 4A), indicating impaired Akt-mediated cardiomyocyte survival pathways. On the other hand, this reduced Akt activation was apparently not due to increased PTEN activity/expression which is a PI3 kinase upstream negative regulator, as there was no significant change in phospho-PTEN detected in the ventricular tissues of all groups of mice (Fig. 4A).

We have previously shown that ROCK1 deletion prevented down-regulation of the cardiac expression of AC5/6 in *Gαq* overexpressed hearts at 12 weeks of age [13]. This molecular defect is a major mechanism underlying impaired ventricular contractile function and β-adrenergic signaling in *Gαq* overexpressed hearts [24]. We observed that the down-regulation of AC5/6 was persisted in the postpartum *Gαq* hearts, and this molecular defect was rescued in the postpartum *Gαq*/ROCK1^{-/-} hearts (Fig. 4B).

We have previously shown that ROCK1 deletion could not prevent activation of JNK and ERK/MAPK at 3 weeks [13]. Interestingly, ERK/MAPK phosphorylation was significantly increased in the postpartum *Gαq*/ROCK1^{-/-} hearts compared to *Gαq* hearts (Fig. 4C). Moreover, this enhanced ERK/MAPK activation in the *Gαq*/ROCK1^{-/-} hearts was also observed at 8 weeks prior to the pregnancy stress (Fig. 4C). Since a large body of evidence supports a protective anti-apoptotic function for ERK/MAPK in cardiac decompensation [25–27], the enhanced ERK/MAPK activation observed in postpartum *Gαq*/ROCK1^{-/-} hearts may contribute to the protective phenotypes. In addition, a decrease in phosphorylation of JNK was observed in the postpartum *Gαq*/ROCK1^{-/-} hearts compared to *Gαq* hearts, which may also contribute to the protective effects of ROCK1 deletion (Supplemental Fig. 3). Since PKCα expression was up-regulated and activation of p38 MAPK was reduced in both postpartum *Gαq* and *Gαq*/ROCK1^{-/-} hearts compared to the WT hearts (Supplemental Fig. 3), these pathways unlikely mediate protective effects of ROCK1 deletion.

ROCK1 deletion prevented LV dilation and dysfunction, and preserved ERK/MAPK and Akt-mediated survival pathway in old *Gαq* mice at 12 months

To further evaluate long-term impact of ROCK1 deficiency on cardiac decompensation, we have determined to what extent ROCK1 deletion could attenuate cardiac decompensation in old *Gαq* mice at 12 months. Male mice were used for this study, as male group provides

complementary information to the pregnancy study in female group described above. Death started to occur in *Gαq* mice around 10 months of age with signs of overt heart failure and the mortality reached 31.25% (5 out of 16 mice) at 12 months of age. In contrast, *Gαq/ROCK1^{-/-}* mice exhibited significant improved survival with 100% survival rate ($n = 16$) at 12 months of age as observed with WT and *ROCK1^{-/-}* mice. Morphometric and echocardiographic analyses revealed that *ROCK1* deficiency did not affect cardiac hypertrophy at both 3 and 12 months (Fig. 5A), but prevented progression of cardiac decompensation in *Gαq* from 3 to 12 months (Fig. 5B–C, supplemental Fig. 4). No trend toward mild impairment in *Gαq/ROCK1^{-/-}* mice was observed at 12 months. In addition, the number of TUNEL positive cardiomyocytes significantly increased in hearts from *Gαq* mice, but no increase was observed in hearts from *Gαq/ROCK1^{-/-}* mice at 12 months (Fig. 5D).

To compare molecular events associated with *ROCK1* deletion in old *Gαq* mice at 12 months with those at postpartum condition, we have examined the effects of *ROCK1* deletion on Akt phosphorylation, AC5/6 expression and ERK/MAPK phosphorylation (Fig. 4A–C). The level of phospho-Akt was markedly reduced in 12-month old *Gαq* hearts compared with WT hearts (Fig. 4A) as observed in postpartum condition. However, Akt phosphorylation was partially preserved in the postpartum *Gαq/ROCK1^{-/-}* hearts (about 30% reduction in *Gαq/ROCK1^{-/-}* hearts vs. 80% reduction in *Gαq* hearts compared with WT hearts) (Fig. 4A). This reduced Akt activation in *Gαq* hearts was not due to increased PTEN activity/expression as no significant change for phospho-PTEN was detected in the ventricular tissues of these four groups of mice (Fig. 4A).

In addition, down-regulation of AC5/6 was persisted in *Gαq* hearts at 12 months of age (Fig. 4B) and this molecular defect was also rescued in *Gαq/ROCK1^{-/-}* hearts (Fig. 4B). Finally, ERK/MAPK phosphorylation was greatly reduced in *Gαq* hearts at 12 months, however it was persistently enhanced in the *Gαq/ROCK1^{-/-}* hearts; their difference has reached to 7.5-fold at 12 months of age (Fig. 4C). Together, these results suggest that preservation of ERK/MAPK and Akt mediated survival pathways and AC5/6 expression may contribute to the long-term beneficial effects of *ROCK1* deletion in cardiomyopathy.

Cardiac-specific *ROCK1* overexpression increased cardiomyocyte apoptosis and accelerated heart failure progression in *Gαq* mice

To further define a role for *ROCK1* in hypertrophic decompensation, we generated α MHC-driven *ROCK1* transgenic mice and examined if increased *ROCK1* expression could accelerate hypertrophic decompensation in *Gαq* mice. *ROCK1*-expressing mice from three independent lines with transgenic *ROCK1* expression of 25-, 40- and 55-fold over the endogenous level were viable and normal in histological appearance and ventricular function up to 8 months of age (data not shown). Western blot analyses also confirmed that *ROCK1* overexpression did not change the level of *Gαq* transgene expression and *ROCK2* expression in ventricular tissue (Fig. 6A). The majority of the subsequent characterization was then carried out using the 40-fold overexpressing line, shown as TG-RK1 in Fig. 6 and supplemental Fig. 5. A trend toward increased number of TUNEL positive cardiomyocytes was noticed in TG-RK1 mice (Fig. 6B), but this increase was not significant ($p = 0.089$). As described above, in the absence of pregnancy stress, female *Gαq* mice exhibited mild ventricular remodeling without increased cardiomyocyte apoptosis compared to the WT mice at 12 weeks (Fig. 6 and supplemental Fig. 5). Strikingly, combined cardiac overexpression of *ROCK1* and *Gαq* (*Gαq/TG-RK1* mice at 12 weeks of age) resulted in a significant increase in the number of TUNEL positive cardiomyocytes (Fig. 6B) associated with increased Bax expression and reduced ERK/MAPK phosphorylation (Fig. 6A). This greater rate of cardiomyocyte apoptosis in *Gαq/TG-RK1* mice was associated with increased fibrosis (Fig. 6C) and lung weight (Supplemental Fig. 5B) as an indicator of progression of lung congestion due to deterioration of cardiac function. Moreover,

female *Gαq*/TG-RK1 mice exhibited reduced survival rate with 100% lethality within 8 months while TG-RK1 or *Gαq* mice remained 100% viable during this time window (Fig. 6D). These results indicate that increased expression of ROCK1 alone did not cause significant changes in heart structure/function, but accelerated *Gαq*-mediated hypertrophic decompensation at least in part through increasing cardiomyocyte apoptosis.

Discussion

The present study examined the effects of ROCK1 deficiency on hypertrophy decompensation in the context of *Gαq*-induced cardiomyopathy under two conditions associated with lethal dilated cardiomyopathy such as pregnancy (single or multiple pregnancies) and aging (12 months). Our results provide multiple new insights into the role of ROCK1 in the development of heart failure. First, ROCK1 deficiency abolished *Gαq*-induced animal death after single or multiple pregnancies as well as at 12 months of age. Second, ROCK1 deficiency attenuated progression into heart failure by preserving chamber dimension and contractile function. By serial echocardiography following consecutive pregnancy in *Gαq* mice or following aging from 3 to 12 months, the progressive nature of heart failure was clearly demonstrated by LV dilation, LV wall thinning and contractile dysfunction, leading to heart failure and animal death. These processes were markedly blunted by ROCK1 deficiency. Third, ROCK1 deficiency suppressed increase in cardiomyocyte apoptosis and cardiac fibrosis while preserving cardiomyocyte hypertrophy, consistent with our previous report that ROCK1 deletion did not prevent the development of myocardial and cardiomyocyte hypertrophy induced by pressure overload [12] or by *Gαq* overexpression at young age [13]. This is the first demonstration of long-term protective effects of ROCK1 deficiency in murine cardiomyopathy.

The current study also showed that overexpression of ROCK1 produced opposite effects compared to ROCK1 deletion on cardiac decompensation in the context of *Gαq*-mediated hypertrophy. Overexpression of ROCK1 alone was not sufficient to cause significant LV remodeling and cardiac dysfunction, but markedly increased animal death of *Gαq* mice under unstressed condition (without pregnancy) associated with increased cardiomyocyte apoptosis, cardiac fibrosis, and contractile dysfunction. Together, these results support an essential role for ROCK1 in hypertrophic decompensation and suggest that inhibiting ROCK1 activation can limit progression to heart failure.

The remarkable beneficial effect of ROCK1 deletion in slowing down *Gαq*-mediated heart failure progression most likely involves inhibition of cardiomyocyte apoptosis. One critical pro-apoptotic signaling event occurring in *Gαq* transgenic hearts is up-regulation of Nix, a proapoptotic factor of the Bcl2 family [18]. Our present study suggests that increased mitochondrial translocation of Bax, a pro-apoptotic factor, in the postpartum *Gαq* hearts may synergistically act with Nix to increase cardiomyocyte apoptosis. This concept is supported by shared cellular function of Nix and Bax as both are involved in mitochondrial/intrinsic apoptotic pathway. Although induction of Nix was not prevented, ROCK1 deletion suppressed increased mitochondrial translocation and up-regulation of Bax, which likely contributed to the inhibition of apoptosis. In contrast to ROCK1 deletion, ROCK1 overexpression increased Bax expression in pre-pregnant *Gαq* hearts associated with increased cardiomyocyte apoptosis, providing additional evidence for a role of ROCK1-mediated apoptotic cell death in hypertrophic decompensation. A recent study has shown that activated RhoA/ROCK up-regulates Bax at transcriptional level through a p53-dependent mechanism in rat neonatal cardiomyocytes [28]. However, this mechanism is unlikely involved in the current context as Bax mRNA level was not affected by *Gαq* overexpression, ROCK1 deletion and/or ROCK1 overexpression, and no change in p53 expression level could be detected (data not shown). Increased protein stability has been observed for mitochondrial Bax [29], which may contribute to its up-regulation in the current context.

The current study has identified several favorable and long lasting molecular events associated with ROCK1 deletion in *Gαq* mouse hearts, including enhanced activation of ERK/MAPK and preservation of AC5/6 under both postpartum condition and at 12 months of age, and partial preservation of Akt activation at 12-month age (Fig. 4). Their contribution to the protective effects of ROCK1 deletion on cardiac dilation and dysfunction, and cardiomyocyte apoptosis remains to be determined. Based on the known functions of these molecules in the context of cardiac decompensation, these molecular changes associated with ROCK1 deletion likely reduce the susceptibility of the hypertrophic cardiomyocyte to apoptotic stimuli thereby attenuating hypertrophic decompensation. This view is further supported by association of reduced ERK/MAPK activation with increased sensitivity to apoptosis caused by increased ROCK1 expression in *Gαq*-mediated hypertrophy. Given the complexity of ROCK cellular functions due to a large and growing number of ROCK substrates [4–6], the impressive protective effects of ROCK1 deletion in hypertrophic decompensation are probably mediated by multiple molecular mechanisms. Future studies are required to understand the molecular mechanisms through which ROCK1 regulates these molecular events in *Gαq* transgenic hearts. None of these molecules (ERK/MAPK, AC5/6 and Akt) is a direct substrate of ROCK1 and other molecules are involved in the interplays between ROCK1 and these signal pathways.

Although *Gαq*-overexpressing mice represent a genetic model sharing similarities to clinical heart failure, the relevance of our findings to clinical settings needs to be determined. Our previous study supports an increase in ROCK1 activation in human heart failure as ROCK1 was found to be cleaved in failing human hearts, most likely via activated caspase 3, leading to ROCK1 activation by removal of the C-terminal auto-inhibitory domain [11]. To date, the only ROCK inhibitor employed clinically in humans is fasudil, which has been used safely in Japan since 1995 for the treatment of cerebral vasospasm [30]. A major challenge for the inhibitor studies is to determine if ROCK truly represents a viable target for the treatment of human disease and whether ROCK1 and ROCK2 mediate different cellular functions. The current study showed that ROCK1 mRNA level was induced and ROCK activity was increased in postpartum *Gαq* hearts, but ROCK2 expression remained unchanged under all the condition tested and may not play a significant role in hypertrophic decompensation. These observations further support an isoform specific role of ROCK1 in pathological remodeling.

In summary, this study shows for the first time the long-term impact of ROCK1 deficiency on the progression of heart failure in a murine congestive heart failure model. ROCK1 deletion significantly prevented apoptotic cardiomyocyte death, and preserved chamber dimension and contractile function thereby attenuating development of heart failure. In contrast, increased ROCK1 expression potentiated these remodeling events and accelerated heart failure progression. These observations strongly support the conclusion that ROCK1 plays an essential role in hypertrophic decompensation, and suggest ROCK1 as a potential target to prevent the development of heart failure. It will be of interest to determine if ROCK1 deficiency could inhibit heart failure progression in other decompensated heart failure settings, if inducible deletion of ROCK1 could reverse or attenuates cardiac remodeling in failing hearts, and if specific inhibition of ROCK1 activity in human heart failure patients represents a valid therapeutic approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nature reviews* 2006;7:589–600.
- [2]. Brown JH, Del Re DP, Sussman MA. The Rac and Rho hall of fame: a decade of hypertrophic signaling hits. *Circ Res* 2006;98:730–42. [PubMed: 16574914]
- [3]. Hill JA, Olson EN. Cardiac plasticity. *N Engl J Med* 2008;358:1370–80. [PubMed: 18367740]
- [4]. Shi J, Wei L. Rho kinase in the regulation of cell death and survival. *Arch Immunol Ther Exp (Warsz)* 2007;55:61–75. [PubMed: 17347801]
- [5]. Loirand G, Guerin P, Pacaud P. Rho kinases in cardiovascular physiology and pathophysiology. *Circ Res* 2006;98:322–34. [PubMed: 16484628]
- [6]. Noma K, Oyama N, Liao JK. Physiological role of ROCKs in the cardiovascular system. *Am J Physiol Cell Physiol* 2006;290:C661–8. [PubMed: 16469861]
- [7]. Satoh S, Ueda Y, Koyanagi M, Kadokami T, Sugano M, Yoshikawa Y, et al. Chronic inhibition of Rho kinase blunts the process of left ventricular hypertrophy leading to cardiac contractile dysfunction in hypertension-induced heart failure. *J Mol Cell Cardiol* 2003;35:59–70. [PubMed: 12623300]
- [8]. Higashi M, Shimokawa H, Hattori T, Hiroki J, Mukai Y, Morikawa K, et al. Long-term inhibition of Rho-kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats in vivo: effect on endothelial NAD(P)H oxidase system. *Circ Res* 2003;93:767–75. [PubMed: 14500337]
- [9]. Hattori T, Shimokawa H, Higashi M, Hiroki J, Mukai Y, Tsutsui H, et al. Long-term inhibition of Rho-kinase suppresses left ventricular remodeling after myocardial infarction in mice. *Circulation* 2004;109:2234–9. [PubMed: 15096457]
- [10]. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, et al. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997;389:990–4. [PubMed: 9353125]
- [11]. Chang J, Xie M, Shah VR, Schneider MD, Entman ML, Wei L, et al. Activation of Rho-associated coiled-coil protein kinase 1 (ROCK-1) by caspase-3 cleavage plays an essential role in cardiac myocyte apoptosis. *Proc Natl Acad Sci U S A* 2006;103:14495–500. [PubMed: 16983089]
- [12]. Zhang YM, Bo J, Taffet GE, Chang J, Shi J, Reddy AK, et al. Targeted deletion of ROCK1 protects the heart against pressure overload by inhibiting reactive fibrosis. *Faseb J* 2006;20:916–25. [PubMed: 16675849]
- [13]. Shi J, Zhang YW, Summers LJ, Dorn GW 2nd, Wei L. Disruption of ROCK1 gene attenuates cardiac dilation and improves contractile function in pathological cardiac hypertrophy. *J Mol Cell Cardiol* 2008;44:551–60. [PubMed: 18178218]
- [14]. Rikitake Y, Oyama N, Wang CY, Noma K, Satoh M, Kim HH, et al. Decreased perivascular fibrosis but not cardiac hypertrophy in ROCK1^{+/-} haploinsufficient mice. *Circulation* 2005;112:2959–65. [PubMed: 16260635]
- [15]. Adams JW, Sakata Y, Davis MG, Sah VP, Wang Y, Liggett SB, et al. Enhanced G α signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure. *Proc Natl Acad Sci U S A* 1998;95:10140–5. [PubMed: 9707614]
- [16]. D'Angelo DD, Sakata Y, Lorenz JN, Boivin GP, Walsh RA, Liggett SB, et al. Transgenic G α overexpression induces cardiac contractile failure in mice. *Proc Natl Acad Sci U S A* 1997;94:8121–6. [PubMed: 9223325]
- [17]. Roth DM, Bayat H, Drumm JD, Gao MH, Swaney JS, Ander A, et al. Adenylyl cyclase increases survival in cardiomyopathy. *Circulation* 2002;105:1989–94. [PubMed: 11997288]
- [18]. Yussman MG, Toyokawa T, Odley A, Lynch RA, Wu G, Colbert MC, et al. Mitochondrial death protein Nix is induced in cardiac hypertrophy and triggers apoptotic cardiomyopathy. *Nature medicine* 2002;8:725–30.
- [19]. Hayakawa Y, Chandra M, Miao W, Shirani J, Brown JH, Dorn GW 2nd, et al. Inhibition of cardiac myocyte apoptosis improves cardiac function and abolishes mortality in the peripartum

- cardiomyopathy of Galpha(q) transgenic mice. *Circulation* 2003;108:3036–41. [PubMed: 14638549]
- [20]. Gulick J, Subramaniam A, Neumann J, Robbins J. Isolation and characterization of the mouse cardiac myosin heavy chain genes. *J Biol Chem* 1991;266:9180–5. [PubMed: 2026617]
- [21]. Wei L, Taffet GE, Khoury DS, Bo J, Li Y, Yatani A, et al. Disruption of Rho signaling results in progressive atrioventricular conduction defects while ventricular function remains preserved. *Faseb J* 2004;18:857–9. [PubMed: 15033930]
- [22]. Diwan A, Wansapura J, Syed FM, Matkovich SJ, Lorenz JN, Dorn GW 2nd. Nix-mediated apoptosis links myocardial fibrosis, cardiac remodeling, and hypertrophy decompensation. *Circulation* 2008;117:396–404. [PubMed: 18178777]
- [23]. Howes AL, Arthur JF, Zhang T, Miyamoto S, Adams JW, Dorn IG, et al. Akt-mediated cardiomyocyte survival pathways are compromised by G alpha q-induced phosphoinositide 4,5-bisphosphate depletion. *J Biol Chem* 2003;278:40343–51. [PubMed: 12900409]
- [24]. Dorn GW 2nd, Tepe NM, Wu G, Yatani A, Liggett SB. Mechanisms of impaired beta-adrenergic receptor signaling in G(alphaq)-mediated cardiac hypertrophy and ventricular dysfunction. *Molecular pharmacology* 2000;57:278–87. [PubMed: 10648637]
- [25]. Lorenz K, Schmitt JP, Vidal M, Lohse MJ. Cardiac hypertrophy: targeting Raf/MEK/ERK1/2-signaling. *The international journal of biochemistry & cell biology* 2009;41:2351–5.
- [26]. Purcell NH, Wilkins BJ, York A, Saba-El-Leil MK, Meloche S, Robbins J, et al. Genetic inhibition of cardiac ERK1/2 promotes stress-induced apoptosis and heart failure but has no effect on hypertrophy in vivo. *Proc Natl Acad Sci U S A* 2007;104:14074–9. [PubMed: 17709754]
- [27]. Baines CP, Molkentin JD. STRESS signaling pathways that modulate cardiac myocyte apoptosis. *J Mol Cell Cardiol* 2005;38:47–62. [PubMed: 15623421]
- [28]. Del Re DP, Miyamoto S, Brown JH. RhoA/Rho kinase up-regulate Bax to activate a mitochondrial death pathway and induce cardiomyocyte apoptosis. *J Biol Chem* 2007;282:8069–78. [PubMed: 17234627]
- [29]. Bleicken S, Zeth K. Conformational changes and protein stability of the pro-apoptotic protein Bax. *Journal of bioenergetics and biomembranes* 2009;41:29–40. [PubMed: 19255832]
- [30]. Olson MF. Applications for ROCK kinase inhibition. *Curr Opin Cell Biol* 2008;20:242–8. [PubMed: 18282695]

Figure 1. ROCK1 deletion abolished peripartum mortality and delayed the progression of cardiac dilation and dysfunction in the peripartum *Gαq* mice

Kaplan-Meier survival analysis (A) of female WT ($n = 10$), ROCK1^{-/-} ($n = 10$), *Gαq* ($n = 25$) and *Gαq*/ROCK1^{-/-} ($n = 14$) mice which were mated repeatedly. The majority of peripartum mortality of *Gαq* mice occurred between 7 days before and 14 days after delivery. WT, ROCK1^{-/-} and *Gαq*/ROCK1^{-/-} mice exhibited 100% survival. M-mode echocardiography was performed in series in four groups of female mice with indicated genotype before pregnancy and subjected to consecutive pregnancies. LVEDD (B), LVESD (C) and LVFS (D) were determined. All measurements were performed at 7 days postpartum ($n = 6-12$ in each group). * $P < 0.05$ vs. WT under same condition. # $P < 0.05$ *Gαq*/ROCK1^{-/-} vs. *Gαq* under same condition.

Figure 2. ROCK1 deletion did not prevent cardiomyocyte hypertrophy, but inhibited fibrosis in postpartum *Gαq* mice

A. Quantitative analysis of heart weight (Heart wt)/tibial length (TL) ratios from female mice at postpartum day 7 with indicated genotype ($n = 8$ in each group). **B.** Quantitative analysis of cardiac myocyte area measured from laminin stained sections. Each column represents results obtained from approximately 200 myocytes from four hearts per group. **C.** Representative heart sections of picrosirius red/Fast green staining. Picrosirius red/Fast green staining was performed in heart sections ($n = 4-6$ in each group), showing cardiac dilation and increased fibrosis in *Gαq* mice. Cardiac dilation and fibrosis were reduced in *Gαq*/ROCK1^{-/-} mice. **D.** Quantitative analysis of the collagen deposition. * $P < 0.05$ vs. WT. # $P < 0.05$ for *Gαq*/ROCK1^{-/-} vs. *Gαq*.

Figure 3. ROCK1 deletion inhibited cardiomyocyte apoptosis in postpartum Gαq hearts
A. representative image showing a TUNEL-positive cardiomyocyte (green staining) in heart sections from female Gαq mice at postpartum day 7. **B.** Apoptotic index was expressed as number of TUNEL positive cardiomyocytes per 10⁵ total nuclei in ventricular myocardium before or at postpartum day 7 with indicated genotype ($n = 4-6$ in each group). **C.** Representative images (top) of Western blot analysis of Bax, Bcl-xL, and GAPDH in ventricular homogenates from female mice at postpartum day 7. An equal amount of protein samples (50 μ g) was applied to each lane. Quantitative analysis (bottom) of immunoreactive bands of Bax ($n = 4-6$ in each group), expressed as fold change relative to WT group. **D.** Representative images (top) of Western blot analysis of Bax, COX IV and GAPDH in mitochondrial fraction. Quantitative analysis (bottom) of immunoreactive bands of Bax ($n = 4-6$ in each group), expressed as fold change relative to WT group. * $P < 0.05$ vs. WT under same condition. # $P < 0.05$ for Gαq/ROCK1^{-/-} vs. Gαq.

Figure 4. Effects of ROCK1 deletion on Akt activation, AC 5/6 expression and ERK/MAPK activation in compensated and decompensated *Gαq* hearts

Representative images and quantitative analysis of Western blot analysis of Akt and p-Akt (A), AC5/6 (B) and ERK and p-ERK (C) in ventricular homogenates from pre-pregnant (8 weeks), 7-day postpartum after first pregnancy and 12-month old mice with indicated genotypes. An equal amount of protein samples (50 μ g) was applied to each lane. $n = 4-6$ in each group. * $P < 0.05$ vs. WT. # $P < 0.05$ for *Gαq*/ROCK1^{-/-} vs. *Gαq*.

Figure 5. ROCK1 deletion inhibited the progression of cardiac dilation and dysfunction, and cardiomyocyte apoptosis in old Gαq mice at 12 months

Quantitative analysis of heart weight (Heart wt)/tibial length (TL) ratios (A) and lung weight (Lung wt)/tibial length (TL) ratios (B) from male mice at 3 and 12 months of age with indicated genotype ($n = 8$ in each group). C. M-mode echocardiography was performed in series at 3 and 12 months of age ($n = 8$ in each group). LVFS showed progressive impairment of cardiac function in Gαq but not in Gαq/ROCK1^{-/-} mice. D. Apoptotic index was expressed as number of TUNEL positive cardiomyocytes per 10⁵ total nuclei in ventricular myocardium at 3 and 12 months with indicated genotype ($n = 4-6$ in each group). * $P < 0.05$ vs. WT under same condition. # $P < 0.05$ for Gαq/ROCK1^{-/-} vs. Gαq.

Figure 6. Cardiac-specific overexpression of ROCK1 increased cardiomyocyte apoptosis, cardiac fibrosis, and mortality rate in *Gαq* mice

A. Representative images of Western blot analysis of *Gαq*, ROCK1, ROCK2, Bax, ERK, p-ERK and GAPDH in ventricular homogenates (50 μg per lane) from 12-week old female mice. **B.** Apoptotic index was expressed as number of TUNEL positive cardiomyocytes per 10⁵ total nuclei in ventricular myocardium from 12-week old female mice with indicated genotype without pregnancy stress (n = 4–6 in each group). **C.** Quantitative analysis of the collagen deposition from picrosirius red staining of heart sections (n = 4–6 in each group). **D.** Kaplan-Meier survival analysis of female WT (n = 7), TG-RK1 (n = 10), *Gαq* (n = 7) and *Gαq*/TG-RK1 (n = 7) mice without pregnancy stress. *P < 0.05 vs. WT. # P < 0.05 *Gαq*/TG-RK1 vs. *Gαq*.

Table 1

Gene expression analysis from 7-day postpartum mice after first pregnancy.

	Microarray (Fold over WT)			Real time RT-PCR (Fold over WT)		
	Gaq n = 3	Gaq/ROCK1 ^{-/-} n = 3	ROCK1 ^{-/-} n = 3	Gaq n = 6	Gaq/ROCK1 ^{-/-} n = 6	ROCK1 ^{-/-} n = 6
BNP	1.7 [*]	1.98 [*]	-1.23	3.41 [*]	3.55 [*]	1.10
ANF	ND	ND	ND	4.22 [*]	4.83 [*]	-1.04
βMHC	8.54 [*]	7.82 [*]	-1.13	6.18 [*]	6.70 [*]	-1.09
αSK	1.65 [*]	1.79 [*]	-2.63 [*]	2.65 [*]	2.71 [*]	-2.01 [*]
αMHC	-1.91 [*]	-1.66 [*]	1.05	-1.76 [*]	-1.87 [*]	1.05
Nix	1.93 [*]	1.87 [*]	1.06	ND	ND	ND
Bax	1.12	1.08	1.03	ND	ND	ND
ROCK1	1.50 [*]	-3.86 ^{**#}	-4.05 [*]	2.05 [*]	-3.66 ^{**#}	-3.98 [*]
ROCK2	-1.12	-1.06	-1.04	-1.04	1.02	-1.11

* $P < 0.05$ vs. WT.# $P < 0.05$ for Gaq/ROCK1^{-/-} vs. Gaq. ND: not determined.

Table 2Morphometric analysis of *Gαq*, *Gαq/ROCK1^{-/-}*, *ROCK1^{-/-}* and WT mice

	WT	ROCK1 ^{-/-}	<i>Gαq</i>	<i>Gαq/ROCK1^{-/-}</i>
Pre-pregnant	n = 8	n = 8	n = 8	n = 8
Heart weight/tibial length (mg/mm)	6.43±0.20	6.39±0.20	6.24±0.33	6.73±0.54
Lung weight/tibial length (mg/mm)	9.22±0.32	9.04±0.68	10.73±0.41 [*]	8.86±0.82 [#]
7 days after 1 st delivery	n = 10	n = 10	n = 10	n = 10
Heart weight/tibial length (mg/mm)	7.86±0.29	7.99±0.33	8.96±0.54 [*]	9.22±0.58 [*]
Lung weight/tibial length (mg/mm)	9.88±0.70	9.78±0.53	12.05±0.62 [*]	9.89±0.72 [#]
7 days after 3 rd delivery	n = 9	n = 9	n = 9	n = 9
Heart weight/tibial length (mg/mm)	8.17±0.65	8.74±0.56	11.49±1.03 [*]	11.65±0.52 [*]
Lung weight/tibial length (mg/mm)	10.41±0.38	10.51±0.68	13.16±0.81 [*]	12.29±0.82 [*]

Data are presented as mean ± SE.

^{*} $P < 0.05$ vs WT under same condition.[#] $P < 0.05$ vs *Gαq* under same condition.