Loss of PI3Kγ Enhances cAMP-Dependent MMP Remodeling of the Myocardial N-Cadherin Adhesion Complexes and Extracellular Matrix in Response to Early Biomechanical Stress


Rationale: Mechano-transduction and the response to biomechanical stress is a fundamental response in heart disease. Loss of phosphoinositide 3-kinase (PI3K)γ, the isoform linked to G protein–coupled receptor signaling, results in increased myocardial contractility, but the response to pressure overload is controversial.

Objective: To characterize molecular and cellular responses of the PI3Kγ knockout (KO) mice to biomechanical stress.

Methods and Results: In response to pressure overload, PI3KγKO mice deteriorated at an accelerated rate compared with wild-type mice despite increased basal myocardial contractility. These functional responses were associated with compromised phosphorylation of Akt and GSK-3β. In contrast, isolated single cardiomyocytes from banded PI3KγKO mice maintained their hypercontractility, suggesting compromised interaction with the extracellular matrix as the primary defect in the banded PI3KγKO mice. β-Adrenergic stimulation increased cAMP levels with increased phosphorylation of CREB, leading to increased expression of cAMP-responsive matrix metalloproteinases (MMPs), MMP2, MT1-MMP, and MMP13 in cardiomyocytes and cardiofibroblasts. Loss of PI3Kγ resulted in increased cAMP levels with increased expression of MMP2, MT1-MMP, and MMP13 and increased MMP2 activation and collagenase activity in response to biomechanical stress. Selective loss of N-cadherin from the adhesion complexes in the PI3KγKO mice resulted in reduced cell adhesion. The β-blocker propranolol prevented the upregulation of MMPs, whereas MMP inhibition prevented the adverse remodeling with both therapies, preventing the functional deterioration in banded PI3KγKO mice. In banded wild-type mice, long-term propranolol prevented the adverse remodeling and systolic dysfunction with preservation of the N-cadherin levels.

Conclusions: The enhanced propensity to develop heart failure in the PI3KγKO mice is attributable to a cAMP-dependent upregulation of MMP expression and activity and disorganization of the N-cadherin/β-catenin cell adhesion complex. β-Blocker therapy prevents these changes thereby providing a novel mechanism of action for these drugs. (Circ Res. 2010;107:1275-1289.)

Key Words: myocardial contractility ■ cardiomyopathy ■ signaling pathways ■ heart failure ■ hypertrophy

The phosphoinositide 3-kinase (PI3K) system has a fundamental role in cell signaling and is involved in cell survival and growth and modulates myocardial contractility.1-5 In the heart, both PI3Kα3-5 and PI3Kγ3,6 controls distinct aspects of cardiac structure and function. Mechano-transduction plays a fundamental role in cardiac (and vascular) function and it appears to involve interactions between extracellular matrix and intracellular cytoskeletal proteins via cell adhesion complexes which are modulated by both class Iα and Iβ PI3Ks.1,7 Although PI3Ks and lipid phosphatases can modulate cytoskeletal interactions, stretch can in turn activate Akt/PKB and GSK-3β activity in both cardiomyocytes and Langendorff-perfused hearts.8 In cardiac muscle, cell adhesion and the cardiomyocyte stretch sensor machinery play key roles in the complex mechanism leading to human DCM and associated heart failures.9 Indeed, dilated cardio-

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myopathy in humans is associated with marked remodeling of extracellular matrix and cell adhesion complexes. Loss of p110γ, the catalytic subunit of the PI3Kγ complex, leads to enhanced cAMP generation resulting from a loss of phosphodiesterase activity, resulting in enhanced Ca\(^{2+}\) cycling and attributable primarily to increased phosphorylation (and inhibition) of phospholamban (PLN) and increased sarcoplasmic reticulum Ca\(^{2+}\) stores.\(^3,6,10\) Using the aortic banding (AB) model, we showed that despite these biochemical changes, PI3KγKO mice show enhanced susceptibility to early biomechanical stress. Our results provide a crucial link between PI3Kγ and the cellular responses to biomechanical stress, with a loss of p110γ resulting in elevated matrix metalloproteinase (MMP) expression and activity, and correlates with a selective loss of N-cadherin from cell adhesion complexes. Importantly, these adverse cellular changes are not observed in the PI3KδDN and in PI3Kγ kinase dead (kDa) mutant mice in response to pressure overload. Inhibition of cAMP levels with propranolol and MMP inhibition provided significant rescue of the dilated cardiomyopathy in banded PI3KγKO mice. Chronic β-blocker therapy also resulted in prevention of adverse myocardial remodeling in wild-type (WT) mice. These results illustrate the importance of cell adhesion and extracellular matrix in the response to biomechanical stress even in the setting of enhanced Ca\(^{2+}\) cycling and increased myocardial contractility.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Experimental Animals**

PI3KγKO, PI3KδDN, and PI3KγKD mice in C57BL/6 background have been described previously.\(^3,6,11\) All experiments were performed in accordance with institutional guidelines and Canadian Council on Animal Care. Only male mice of 8 to 9 weeks of age and littermate C57BL/6 WT controls were used. For the propranolol experiments, mice were treated with propranolol in their drinking water (0.2 g/L) to deliver 15 mg/kg per day; propranolol was withdrawn 1 day before the echocardiographic and hemodynamic measurements. The broad-spectrum MMP inhibitor PD166793 (Pfizer Inc) was administered orally by daily gavage as described previously.\(^12\) Because of the rapid onset of ventricular dilation in PI3KγKO mice, PD166793 treatment (30 mg/kg per day) began 3 days before AB and continued until mice were euthanized.

**Aortic Banding**

The AB protocol was used as a means of pressure overload and biomechanical stress as described previously.\(^12,13\) Briefly, the descending aortic arch was accessed via a left thoracotomy and the descending thoracic aorta was surgically constricted to generate a transstenotic pressure gradient of 50 to 60 mm Hg.

**Histology, Hydroxyproline, and TUNEL Assays**

For heart morphometry, hearts were arrested with KCl, fixed with 10\% buffered formalin, and embedded in paraffin. Myocardial interstitial fibrosis was determined as collagen volume fraction using confocal microscopy on picrosirius red stained 10 μm thick sections pretreated with PMA. Collagen content was also determined using the hydroxyproline assay as previously reported.\(^14\) In situ DNA fragmentation was labeled using the TUNEL assay (ApopTag Plus kit; Oncor, Gaithersburg, Md).

**Echocardiography and Hemodynamic Measurements**

Echocardiographic assessments and invasive hemodynamic measurements were carried out as described previously.\(^3,12,13\)

**Isolated Cardiomyocyte Contractility**

Left ventricular (LV) cardiomyocyte contractility was carried out as described previously.\(^3\)

**Isolation and Culture of Adult Cardiomyocytes and Fibroblasts**

The protocol used for isolation and culture of adult mouse cardiomyocytes and fibroblasts was modified from O’Connell et al (see Online Methods).\(^15\)

**Measurement of cAMP Levels**

The cAMP levels in isolated adult ventricular cardiomyocytes and LV myocardial tissue were measured using the cAMP competitive enzyme immunoassay system (GE Healthcare Amersham Biosciences).

**Cell Adhesion Assay**

The ECM-based cell adhesion assay was carried out using a colorimetric-based assay (CytoSelect 48-Well Cell Adhesion Assay; Cell Biolabs Inc).

**TaqMan Real-Time PCR, Western Blot Analyses, and Membrane Fractionation, Gelatin Zymography, and Collagenase Activity Assay**

RNA expression levels were quantified with TaqMan RT-PCR using ABI Prism 7700 sequence detection system as described previously (see Online Table I for primers and probes).\(^16,17\) Western blot analyses, gelatin zymography, and collagenase activity were performed as described previously (see Online Methods).\(^16,17\) For the in vitro analysis of N-cadherin cleavage, 100 ng of human recombinant N-cadherin Fc Chimera protein (R&D) was incubated with 0, 1, 10, 100 nM/mL MMP2 (R&D) or MT1-MMP (R&D).
Confocal Fluorescence Microscopy

Formalin-fixed sections were permeabilized with ice-cold methanol for 10 minutes, blocked with goat serum and incubated with primary antibodies (anti–N-cadherin [rabbit] and anti–β-catenin [mouse], 1:500, Abcam) overnight at 4°C in a humidified chamber. After several washes, Texas red–conjugated (for cadherin) or FITC-conjugated (for catenin) secondary antibodies were incubated for 30-minute at room temperature. The sections were evaluated with a Zeiss LSM510 confocal microscope and associated software.

Statistical Analysis

One-way ANOVA was used to test for overall significance, followed by the Student–Newman–Keuls test for multiple comparison testing between the various groups. All statistical analyses were performed with the SPSS software (version 10.1). Data are presented as means±SEM; n refers to the sample size.

Results

Accelerated Development of Ventricular Dilation and Pathological Hypertrophy in PI3KγKO Mice in Response to Biomechanical Stress

In response to pressure overload, WT mice developed concentric remodeling with increased wall thickness and reduction in chamber size at 1 and 3 weeks following AB (Figure 1A). In contrast, LV size dilated rapidly in PI3KγKO mice characteristic of eccentric remodeling at 1 and 3 weeks following AB as shown by trichrome-stained four-chamber views (Figure 1B). The early development of dilated cardiomyopathy in the banded PI3KγKO mice was associated with increased interstitial myocardial fibrosis at 3 weeks after AB as illustrated by picrosirius red staining (Figure 1C) and quantified as percentage of collagen volume fraction (CVF) (Figure 1D) and quantified as percentage of apoptotic nuclei (Figure 1F) with lack of an increase in apoptosis based on TUNEL staining (Figure 1E, left) and quantified as percentage of apoptotic nuclei (Figure 1F, right).

Expression profile of hypertrophy disease markers showing early and markedly increased expression of ANF (Figure 1G), BNP (Figure 1H), and βMHC (Figure 1I) in banded PI3KγKO mice compared with WT mice. n=5 for sham groups and n=7 for AB groups. AU indicates arbitrary unit. *P<0.05 compared with the WT group.

Figure 1. Development of early-onset dilated cardiomyopathy in response to biomechanical stress in PI3KγKO mice. A and B, Four-chamber trichrome-stained heart sections showing concentric hypertrophy in WT mice (A) with ventricular dilation and eccentric remodeling in PI3KγKO mice (B) in response to after AB. C through F, Increased interstitial fibrosis shown using picrosirius red staining (C, left) and quantification of the collagen volume fraction (CVF) (D) with lack of an increase in apoptosis based on TUNEL staining (E, left) and quantified as percentage of apoptotic nuclei (F, right) (P=0.341; n=5 per group). G through I, Expression profile of hypertrophy disease markers showing early and markedly increased expression of ANF (G), BNP (H), and βMHC (I) in banded PI3KγKO mice compared with WT mice. n=5 for sham groups and n=7 for AB groups. AU indicates arbitrary unit. *P<0.05 compared with the WT group.
signaling, phosphorylated (phospho)–GSK-3α (Figure 2C) and phospho–GSK-3β (Figure 2D) were not affected initially at 1 week and then showed a drastic reduction at 3 weeks after AB in PI3Kγ/KO mice. Interestingly, baseline level of phospho–GSK-3β was significantly greater in PI3Kγ/KO compared with WT mice (Figure 2D). The phosphorylation of focal adhesion kinase (FAK) is widely accepted as a fundamental response in myocardial mechanical stretch and hypertrophy.21 In the banded PI3Kγ/KO mice, increased phosphorylation of FAK occurred at an earlier stage compared with WT mice consistent with an aberrant response to biomechanical stress (Figure 2E). These results show that PI3Kγ/KO mice develop...
oped a rapid onset of adverse myocardial remodeling and pathological hypertrophy in response to biomechanical stress with resultant alteration in the activation of mechanosensitive and Akt-dependent signaling cascades.

Uncoupling Between In Vivo Myocardial Contractility and Single-Cardiomyocyte Contractility in Pressure-Overloaded PI3K<sup>γ</sup>KO Mice

To examine whether these differences in myocardial remodeling in WT and PI3K<sup>γ</sup>KO mice result in functional alteration, we used transthoracic echocardiography to assess heart function. LV size rapidly dilated with a marked and progressive decrement in systolic function at 1 and 3 weeks in PI3K<sup>γ</sup>KO mice (Figure 3A through 3E; Online Table II). In marked contrast, banded WT mice developed increased wall thickness with reduced ventricular size and preserved systolic function (Figure 3A through 3D; Online Table II). We used invasive hemodynamic measurements to further characterize these functional alterations which showed greater elevation in LV end diastolic pressure (Online Table II) and reduced myocardial contractility as assessed by +dP/dt<sub>max</sub> (Figure 3D; Online Table II) and −dP/dt<sub>min</sub> (Figure 3E; Online Table II) in banded PI3K<sup>γ</sup>KO mice. The early and marked decoupling in banded PI3K<sup>γ</sup>KO mice despite enhanced Ca<sup>2+</sup> cycling and increased basal myocardial contractility (Figure 3; Online Table II)<sup>3,6,10</sup> suggests that myocardial cell adhesion is compromised.

As such, we hypothesize that isolated cardiomyocyte contractility would remain elevated in banded PI3K<sup>γ</sup>KO mice. Increased phosphorylation of PLN is a critical target of the elevated cAMP levels in PI3K<sup>γ</sup>KO mice.<sup>3,10,22</sup> Western blot analyses confirmed increased serine-16 phosphorylation of PLN in PI3K<sup>γ</sup>KO compared with WT mice, which was preserved in response to pressure overload (Figure 3F). Analysis of cAMP levels in the LV from PI3K<sup>γ</sup>KO mice confirmed elevated basal levels of cAMP which were maintained in response to 1 week of after AB (Figure 3G). Indeed, isolated cardiomyocytes from banded PI3K<sup>γ</sup>KO mice showed increased cell shortening (Figure 3H) and rate of contraction (Figure 3I) compared with cardiomyocytes obtained from banded WT mice. Whereas ECM-based cell adhesion was intact in the PI3K<sup>γ</sup>KO cardiomyocytes under baseline conditions (sham), there was a drastic loss of adhesion properties to collagen IV and laminin in response to pressure overload (Figure 3J). In contrast, cardiomyocyte adhesion from WT mice remained intact following pressure overload (Figure 3J). These results show that isolated cardiomyocytes maintain an increased contractility despite the early deterioration in whole heart systolic function providing strong evidence that the primary defect in banded PI3K<sup>γ</sup>KO mice is a disorganized extracellular matrix and/or cell adhesion.

Selective Upregulation of MMP2 and MT1-MMP and MMP Inhibition Mediated Rescue of Pressure-Overloaded PI3K<sup>γ</sup>KO Mice

Adverse remodeling of the extracellular matrix by MMPs is a key determinant of the response to biomechanical stress.<sup>23</sup> MMP2 (gelatinase A) and MMP13 (collagenase-3) genes contain promoter regions encoding a cAMP-response element (CRE), which binds CRE binding protein (CREB)<sup>24</sup> and mediates cAMP-dependent increase in the synthesis of MMP2<sup>25</sup> and MMP13<sup>26</sup> whereas MT1-MMP expression is also positively regulated by cAMP.<sup>27</sup> We hypothesize that elevated cAMP levels in the setting of increased biomechanical stress synergistically increase MMP expression and/or activity in banded PI3K<sup>γ</sup>KO mice. Indeed, both MMP2 (Figure 4A) and MT1-MMP (Figure 4B) myocardial mRNA expression increased within 1 week of after AB and persisted at 3 weeks in PI3K<sup>γ</sup>KO compared with WT mice, whereas MMP13 expression was drastically increased in PI3K<sup>γ</sup>KO mice at 3 weeks after AB (Figure 4C). In contrast, non-cAMP-responsive MMPs such as MMP9 (gelatinase B) (Figure 4D) and MMP8 (collagenase-2) (data not shown) showed no differential change in expression in response to AB. To provide a more definitive connection between cAMP and MMP expression/activity, we also examined the expression of these MMPs in banded PI3K<sup>α</sup>ΔN mice which also develop an early dilated cardiomyopathy<sup>4</sup> and in banded PI3K<sup>γ</sup>KD mice, which lack PI3K<sup>γ</sup> signaling without elevation in cAMP levels.<sup>6</sup> Consistent with our hypothesis, myocardial expression levels of MMP2 (Figure 4A), MT1-MMP (Figure 4B), and MMP13 (Figure 4C) were not increased in PI3K<sup>α</sup>ΔN and PI3K<sup>γ</sup>KD mice at 1 and 3 weeks after AB.

Next, we examined the changes in MMP expression in cultured adult ventricular cardiomyocyte and cardiofibroblast fractions in response to the activation of cAMP signaling using the β-adrenergic agonist, isoproterenol. The levels of cAMP in cardiomyocytes increase significantly in response to isoproterenol stimulation preventable by β-adrenergic blockade using propranolol (25 μmol/L) and specific adenylate cyclase inhibition using 2′,5′-dideoxyadenosine (DIDA) (30 μmol/L; Figure 4E). Western blot analysis showed an early and marked increase in serine-133 CREB phosphorylation in cultured cardiomyocytes and cardiofibroblasts (Figure 4F) and shown quantitatively (Figure 4G) in response to β-adrenergic receptor stimulation. Consistent with activation of CREB, mRNA levels of cAMP-responsive MMPs following 24 hours of stimulation with 100 nmol/L isoproterenol showed that MMP2 (Figure 4H), MT1-MMP (Figure 4I), and MMP13 (Figure 4J) all showed a significant rise in mRNA expression in cultured adult cardiomyocytes. Although the basal expressions of MMP2 and MT1-MMP were greater in cultured adult cardiofibroblasts, MMPs also showed a similar increase in response to β-adrenergic stimulation in these cells (Figure 4H through 4J). Importantly, the corresponding increase in mRNA expression of MMP2, MT1-MMP, and MMP13 in both cardiomyocytes and cardiofibroblasts were suppressed by both propranolol and DIDA (Figure 4H through 4J).

We next assessed for direct biochemical evidence for increased MMP activity in the PI3K<sup>γ</sup>KO mice in response to pressure overload. Myocardial collagenase activity showed a significant increase at 1 and 3 weeks after AB in the PI3K<sup>γ</sup>KO mice compared with banded WT mice, which was suppressible by the MMP inhibitor PD166793 (Figure 5A), whereas gelatin zymography showed selective activation of...
Figure 3. Uncoupling between in vivo myocardial contractility and isolated single cardiomyocyte contractility in PI3K-βKO mice in response to early biomechanical stress. A through E, M-mode echocardiograms (A) and in vivo quantitative assessment of heart function showing baseline hypercontractility followed by rapid and marked ventricular dilation (B) and reduction in systolic function based on fractional shortening (C) and invasive hemodynamic measurement of +dP/dt max (D) and −dP/dt min (E) at 1 week after AB. LVEDD indicates LV end-diastolic diameter. n=8 for sham groups; n=12 for AB groups. F through G, Western blot analysis (left) and quantification (right) using myocardial membrane fractions showing increased phospho-PLN at baseline and in banded PI3K-βKO mice (F) with elevated myocardial cAMP levels in PI3K-βKO hearts at baseline and at 1 week after AB (G). n=5 for all groups. H and I, Single cardiomyocyte contractility measurements showing basal hypercontractility based on percentage (H) and rate of change (+dL/dt) (I) of cell shortening in PI3K-βKO mice, which persists in response to 1 week of pressure overload. n=8 for sham groups; n=12 for AB groups. J, ECM-based cell adhesion of isolated adult LV cardiomyocytes following 1 week of after AB showing an increase in fibronectin adhesion in WT cardiomyocytes and a marked decrease in adhesion to collagen IV and laminin in PI3K-βKO cardiomyocytes. AU indicates arbitrary unit; Col I, collagen I; Col IV, collagen IV; FN, fibronectin; LN, laminin. n=4 for WT and PI3K-βKO. *P<0.05 compared with corresponding WT group.
MMP2 in banded PI3KγKO compared with WT mice (Figure 5B). MMP9 protein levels did not change in banded WT hearts, whereas at 3 weeks, post-AB PI3KγKO hearts showed an increase (Online Figure III) despite minimal increase in mRNA levels (Figure 4D), suggesting a posttranscriptional mechanism for the increased MMP9 levels. Given the lack of an increased MMP expression in banded PI3KγKD mice, myocardial collagenase activity was not elevated in these hearts.
Figure 5. Increased collagenase activity, MMP2 activation, and MT1-MMP levels with prevention of the dilated cardiomyopathy by broad-spectrum MMP inhibition in banded PI3Kγ/H9253 KO mice. A, Increased myocardial collagenase activity in banded PI3Kγ/H9253 KO mice suppressible by the MMP inhibitor PD166793 (30 mg/kg per day). B and C, Gelatin zymography showed increased pro-MMP2 and active MMP2 levels in the banded PI3Kγ/H9253 KO mice and Western blot analysis showing increased expression of active MMP2 (64 kDa) and pro-MMP2 (72 kDa) and membrane-fractionated MT1-MMP (C) in banded PI3Kγ/H9253 KO at 1 and 3 weeks after AB. n=4 for sham group and AB groups. *P<0.05 compared with the sham group. D, Expression profile of tissue inhibitors of metalloproteinases (TIMP) showing equivalent upregulation in TIMP1 without alteration in TIMP2, whereas TIMP3 and TIMP4 levels were increased in banded PI3Kγ/H9253 KO mice. n=6 for sham group and n=8 for AB groups. #P<0.05 compared with the sham group; *P<0.05 compared with WT group. E and F, Treatment with the broad-spectrum MMP inhibitor PD166793 (30 mg/kg per day) prevented the dilated cardiomyopathy and disruption of the extracellular collagen network shown as picrosirius red staining (E) while reversing the upregulation of disease markers, ANF and BNP, ventricular dilation at end-diastole and end-systole, and reduction in systolic performance (F) in banded PI3Kγ/H9253 KO mice. FS indicates fractional shortening; LVEDD, LV end diastolic diameter; LVESD, LV end systolic diameter. n=8 for each group. *P<0.05 compared with all other groups; #P<0.05 compared with WT group. AU indicates arbitrary unit.
mice (Figure 5A). Consistent with gelatin zymography, Western blot analysis and quantification (Figure 5B) revealed increased myocardial expression of active MMP2 (64 kDa) and pro-MMP2 (72 kDa) in banded PI3KγKO mice at 1 and 3 weeks after AB. Similarly, membrane fractionation and analysis of MT1-MMP levels showed an earlier and greater increase in MT1-MMP levels in banded PI3KγKO mice (Figure 5C). The activity of MMPs are inhibited by tissue inhibitor of matrix metalloproteinases (TIMPs), TIMP1, TIMP2, TIMP3, and TIMP4 with TIMP3 playing a key role in the myocardial response to biomechanical stress.12,28 Interestingly, TIMP1 levels increased similarly, whereas the expression of TIMP2 did not change in banded WT and PI3KγKO mice (Figure 5D). By contrast, increase in TIMP3 and TIMP4 expression occurs at 3-weeks after AB in PI3KγKO mice possibly attributable to a negative-feedback response to the increased MMP expression and activities (Figure 5D). Our data provide a crucial link between the adverse myocardial remodeling in pressure-overloaded PI3KγKO mice and the increase in activation and activity of MMPs. Human recombinant MMP2 rather than MT1-MMP cleaves human recombinant N-cadherin Fc chimera and was inhibited by the recombinant MMP2 rather than MT1-MMP cleaves human recombinant N-cadherin Fc chimera and was inhibited by the recombinant MMP2 rather than MT1-MMP cleaves human recombinant N-cadherin Fc chimera and was inhibited by the recombinant MMP2 rather than MT1-MMP cleaves human recombinant N-cadherin Fc chimera and was inhibited by the MMP inhibitor, PD166793 (Online Figure II). We hypothesize that MMP inhibition will lead to a marked protection in banded PI3KγKO mice. Daily treatment of banded PI3KγKO mice with the broad-spectrum MMP inhibitor PD166793 (30 mg/kg per day)12 prevented the dilated cardiomyopathy, fibrosis, and disruption of the extracellular collagen network (Figure 5E). This also reversed the upregulation of disease markers, ANF and BNP, and ventricular dilation at end-diastole and end-systole, resulting in improved systolic performance in banded PI3KγKO mice (Figure 5F). These results illustrate a key role of cAMP-mediated upregulation of MMP2 and MT1-MMP expressions in mediating the adverse myocardial remodeling in pressure-overloaded PI3KγKO mice.

Specific Loss of N-Cadherin From Adhesion Complexes While β-Adrenergic Blocker Prevents Upregulation of MMP and Loss of N-Cadherin in Banded PI3KγKO Mice

In addition to the degradation of the ECM, N-cadherin/β-catenin cell adhesion complexes are also important targets of an activated MMP system.29,30 Western blot analysis of the myocardial membrane fraction in banded WT and PI3KαDN mice showed a modest increase in N-cadherin levels (Figure 6A and 6B), whereas in banded PI3KγKO mice, there was a 75% loss of N-cadherin levels (Figure 6A and 6B). In contrast, in the banded PI3KγKO (and PI3KαDN) mice, levels of β-catenin in the heart were significantly increased compared with banded WT mice (Figure 6C). The relative preservation of N-cadherin levels in the PI3KγKD hearts is consistent with a critical role of cAMP in mediating the loss of N-cadherin from adhesion complexes independent of the PI3Kγ lipid kinase activity per se. The quality of Western blot analysis was confirmed by absence of the membrane-specific protein (toll-like receptor 4) in the cytosolic fraction and absence of the cytosolic-specific protein (caspase-3) in the membrane fraction (Online Figure I). Immunofluorescence microscopy confirmed colocalization of N-cadherin and β-catenin in the end-to-end and side-to-side connections between cardiomyocytes in banded WT hearts (Figure 6D). Consistent with our Western blot analysis, there was a near complete loss of N-cadherin from end-to-end and side-to-side connections from pressure-overloaded PI3KγKO myocardium (Figure 6E). Consistent with the ability of PD166793 to prevent MMP2-mediated cleavage of N-cadherin (Online Figure II) and rescue the dilated cardiomyopathy in banded PI3KγKO mice (Figure 5), Western blot analysis confirmed reduced loss of membrane-associated N-cadherin (Figure 6F and 6G), resulting in more pronounced staining of N-cadherin in the myocardial cell adhesion junctions (Figure 6H) in banded PI3KγKO mice treated with PD166793. Given the key role of integrin complexes in mediating cell adhesion in the heart,31,32 we also examined changes in integrin levels in response to pressure overload. Western blot analysis of membrane fractionated β1D and α7B integrins showed increased levels in WT hearts at 1 and 3 weeks after AB with a delayed increase in the PI3KγKO hearts with a significant increase only at 3 weeks after AB (Online Figure IV).

We then tested the hypothesis that elevated cAMP plays a key in vivo role in the adverse remodeling in banded PI3KγKO mice by using the nonspecific β-adrenergic blocker propranolol at a dose that has been previously shown to normalize elevated cAMP levels.6 Propranolol treatment normalizes the mRNA expression of MMP2, MT1-MMP, and MMP13 (Figure 7A) with similar changes seen in the expression of disease markers, ANF, βMHC, and BNP (Figure 7B). Consistent with a reduction in MMP expression, elevated collagenase activity in the banded PI3KγKO mice was normalized in response to propranolol (Figure 7C) with relative preservation of N-cadherin levels in the myocardial membrane fraction (Figure 7D). These biochemical and cellular changes implies that cardiac function was rescued in banded PI3KγKO mice treated with propranolol. Indeed, echocardiographic assessment showed a near normalization of the increased LV dilation and improved fractional shortening with invasive hemodynamic parameters showing marked increase in +dP/dt max and −dP/dt min in the banded PI3KγKO mice treated with propranolol (Figure 7E). These results highlight a key reversible defect in the N-cadherin system in the PI3KγKO mice in response to biomechanical stress caused by cAMP-dependent upregulation of MMP activity.

β-Blocker Therapy Prevents the Adverse Remodeling in Response to Chronic Pressure Overload

The beneficial effects of β-blocker therapy in the banded PI3KγKO mice suggest that βblockade may have a similar protective role in a long-term (9 weeks) AB model of WT mice. Aortic banding for 9 weeks resulted in marked increase in expression of hypertrophy markers, α-skeletal actin, βMHC and BNP (Figure 8A) in association with LV dilation and reduced systolic function (Figure 8B; Online Figure V). Propranolol treatment prevented the adverse remodeling and systolic dysfunction in response to chronic biomechanical stress (Figure 8A and 8B; Online Figure V). Quantitative
assessment of cardiac systolic function using echocardiography showed a marked improvement in fractional shortening and reduction in LV end-diastolic dimension (Online Figure V). Similarly, invasive hemodynamic measurement revealed restoration of an elevated LV end-diastolic pressure and normalization of \( +\frac{dP}{dt_{\text{max}}} \) and \( -\frac{dP}{dt_{\text{min}}} \) in response to chronic \( \beta \)-blocker therapy (Figure 8C). These functional changes correlated with a reduction of MMP2 and MT1-

Figure 6. Selective loss of N-cadherin from myocardial membrane fraction and the cell adhesion complexes in the myocardium from banded PI3K\( \gamma \)-KO mice. A through C, Western blot analysis of the myocardial membrane fraction (A) and quantification (B) showing a selective loss of N-cadherin levels without alteration in \( \beta \)-catenin levels (C) in banded PI3K\( \gamma \)-KO mice, whereas the level of N-cadherin increases in banded WT and PI3K\( \alpha \)-DN mice. *P<0.05 compared with the corresponding sham group. D and E, Immunofluorescence microscopy of the myocardial \( \beta \)-catenin and N-cadherin proteins showing end-to-end connections (top) and side-to-side connections (bottom) in banded WT (D) and PI3K\( \gamma \)-KO (E) mice with a distinct loss of N-cadherin in the banded PI3K\( \gamma \)-KO mice. F through H, Western blot analysis of the myocardial membrane fraction (F) and quantification (G) and immunofluorescence microscopy of myocardial \( \beta \)-catenin and N-cadherin proteins (H) showing MMP inhibition prevents loss of N-cadherin in banded PI3K\( \gamma \)-KO mice. AU indicates arbitrary unit. n=5 for all groups. *P<0.05 compared with all other groups.
The nonspecific β-receptor blocker propranolol (15 mg/kg per day) prevents the molecular, biochemical, and functional deterioration in response to biomechanical stress in PI3Kγ-KO mice. A and B, Expression profiling showing that treatment with propranolol prevents the increased expression of MMPs including MMP2, MT1-MMP, and MMP13 (A) and disease markers such as ANF, βMHC, and BNP (B) in banded PI3Kγ-KO mice. *P<0.05 compared with all other groups. C and D, Suppression of the increased myocardial collagenase activity (C) with Western blot analysis and quantification (D) showing restoration of the membrane N-cadherin levels in banded PI3Kγ-KO mice. *P<0.05 compared with all other groups. E, Reduction in LV dilation and preservation of systolic function in response to treatment with propranolol in banded PI3Kγ-KO mice based on echocardiographic parameters, LV end diastolic diameter (LVEDD) and fractional shortening (FS), and hemodynamic assessment, +dP/dt_max and −dP/dt_min. AU indicates arbitrary unit. n=8 for all groups. *P<0.05 compared with all other groups.
Figure 8. Chronic β-blocker prevents pressure overload–induced adverse remodeling and preserves systolic function in WT mice. A, Expression profile of hypertrophy disease markers showing markedly increased expression of α-skeletal actin, β-MHC, and BNP in banded WT mice at 9 weeks, which was dramatically reduced by treatment with propranolol. n = 5 per group. B, Echocardiographic assessment of systolic function with M-mode echocardiograms showing a marked reduction in LV dilation and increase in fractional shortening in response to therapy with propranolol. C, Invasive hemodynamic assessment showing marked reduction in LVEDP and prevention of the deterioration in myocardial contractility based on dP/dt max and dP/dt min in response to chronic β-blocker therapy. n = 6 for WT sham and n = 8 for WT AB groups. D, Expression of MMPs showing a marked increase in MMP2 and MT1-MMP expression without a differential effect on MMP-13 levels and a normalization in response to chronic β-blocker therapy. AU indicates arbitrary unit. n = 5 per group. *P < 0.05 compared with all other groups; #P < 0.05 compared with WT sham group; ‡P < 0.05 compared with WT+AB 9 weeks; †P < 0.05 compared with WT sham group.
MMP expression in the absence of a differential effect on MMP13 expression (Figure 8D). Western blot analysis showed a higher level of membrane-associated N-cadherin with an equivalent increase in β-catenin levels resulting in preservation of the myocardial N-cadherin/β-catenin ratio (Figure 8E) in response to chronic β-blocker therapy. Our results highlight a novel mechanism of chronic β-blocker therapy in preventing the adverse myocardial remodelling in pressure overload–induced heart failure.

**Discussion**

The response to biomechanical stress is a fundamental response in heart disease and plays a key adaptive role in response to a pressure overload state characteristic of hypertension and valvular stenosis. Mechanotransduction plays a fundamental role in cardiac structure and function and involves a concerted interaction between extracellular matrix, intracellular cytoskeletal proteins, and cell adhesion complexes. Advanced heart failure in response to myocardial injury including biomechanical stress leads to impaired Ca$^{2+}$ cycling and strategies aimed at enhancing Ca$^{2+}$ cycling are currently being developed as therapies for heart failure. In contrast, in PI3K KO hearts, basal phospho–GSK-3β activity. Indeed, loss of PTEN prevents phosphorylation of Akt in response to G protein–coupled receptor agonists, and PI3KγKO mice are resistant to the pathological effects of β-adrenergic stimulation. Despite these biochemical changes, PI3KγKO mice show enhanced susceptibility to biomechanical stress, and we provide direct evidence that the primary defect is a compromised cell-adhesion/extracellular matrix system linked to elevated cAMP levels. In contrast, in PI3KγKD mice, which have normal myocardial cAMP levels, there is no upregulation of MMPs with preservation of N-cadherin levels consistent with the ability of these mice to maintain normal systolic function in response to early biomechanical stress.

The PI3K/PTEN system may also have a more direct role in the cardiac response to biomechanical stress. Whereas PI3K and lipid phosphatases can modulate cytoskeletal interactions, stretch can in turn activate Akt/PKB and GSK-3β activity. Indeed, loss of PTEN prevents pressure overload–induced heart failure, whereas loss of Akt1/PKBα leads to a rapid onset of ventricular dilation and systolic dysfunction in response to pressure overload. Consistent with a critical role of PI3Kγ in pathological G protein–coupled receptor signaling, phosphorylation of ERK1/2 is impaired in response to early biomechanical stress in the PI3KγKO mice. However, this differential response is lost at 3 weeks after AB, which is likely driven by severe disruption of the ECM and/or cell adhesion leading to G protein–coupled receptor–independent activation of ERK1/2 in the PI3KγKO mice. Despite equivalent basal phospho-Akt levels in WT and PI3KγKO hearts, basal phospho–GSK-3β was increased in the PI3KγKO mice, likely mediated by PKA given the chronic elevation of myocardial cAMP levels in these mice. The diverse downstream effects of elevated basal phospho–GSK-3β may have contributed to the adverse remodeling in the PI3KγKO mice.

Elevated cAMP levels in response to increased biomechanical stress leads to increased MMP expression and increased active (cleaved) MMP2 and collagenase activity, leading to adverse myocardial remodeling. Activation of pro-MMP2 occurs by proteolytic cleavage of the N-terminal propeptide and requires two MT1-MMP molecules in association with TIMP2. In addition to degrading various components of the ECM, increased MMP activity can also adversely modify cell–cell adhesion complexes. Cardiomyocyte cell adhesion complexes provide an important mechanism by which cardiomyocytes (and cardiofibroblasts) are anchored to the extracellular matrix while allowing force transmission to the intracellular cytoskeletal network. In particular, we have shown that increased MMP expression and activity can regulate N-cadherin function through proteolytic degradation. Cardiac-specific loss of N-cadherin in the heart leads to a dilated cardiomyopathy attributable to loss of the integrity of cell adhesion junctions. Because of their homophilic binding and adhesive specificities, N-cadherin/catenin complex is required for cadherin-mediated cell adhesion and linkage to the actin cytoskeleton. The delayed increase in membrane β1D and α7B integrins in the banded PI3KγKO hearts may have also contributed to the early onset of dilated cardiomyopathy. In addition, MT1-MMP is a potent collagenase that also targets other ECM components such as fibronectin, laminin, and integrins while activating MMP13 (collagenase-3), thereby amplifying the collagen-degradation process. Our results are consistent with the conclusion that elevated cAMP (and its downstream effects) is the primary driver of the adverse remodeling in banded PI3KγKO mice rather than loss of PI3Kγ signaling per se. Our findings may help to explain the cardiomyopathy in experimental models with enhanced β-adrenergic signaling (and cAMP levels) and lack of a protection against pressure overload and tumor necrosis factor–induced heart failure despite enhanced Ca$^{2+}$ cycling in the PLN knockout mice. Indeed, whereas Ca$^{2+}$ transients in cardiomyocytes were normalized in tumor necrosis factor–induced cardiomyopathy in a PLN-null background, global systolic function remained depressed and unchanged. We propose that enhancing cell–cell adhesion and cell–ECM interaction will promote the salutary effects of enhanced intracellular Ca$^{2+}$ cycling on whole heart function and boost the therapeutic potential of normalizing intracellular Ca$^{2+}$ cycling in patients with heart failure. Increased sympathetic nervous system activity and β-adrenergic receptor signalling are key aspects of the pathophysiology of heart failure and in catecholamine-mediated cardiomyopathies. β-Adrenergic receptor blockers improve clinical outcomes in patients with chronic heart failure. In contrast, agents that increase myocardial cAMP such as PDE3 inhibitors are associated with adverse outcomes and increased mortality in patients with heart failure. Our data support a novel role of β-adrenergic blocker in reducing MMP expression and/or activity and preservation of cell adhesion, thereby curtailing adverse myocardial remodeling.
Disclosures
None.

References
Novelty and Significance

What Is Known?

- Phosphoinositide 3-kinase (PI3K)γ couples G protein–coupled receptors to downstream signaling pathways.
- Loss of PI3Kγ enhances cAMP levels and enhances basal myocardial contractility.

What New Information Does This Article Contribute?

- Loss of PI3Kγ enhances susceptibility to biomechanical stress and early-onset heart failure.
- Activation of matrix metalloproteinase (MMP) and loss of N-cadherin–mediated cell adhesion are critical mediators of this adverse remodeling.
- β-Blocker suppresses cAMP-mediated MMP upregulation and preserves N-cadherin levels with minimization of cardiac systolic dysfunction.

Altered PI3Kγ signaling plays a fundamental role in heart disease. Although loss of PI3Kγ enhances myocardial contractility, there is a paradoxical rapid development of heart failure in response to biomechanical stress. The uncoupling between basal hypercontractility and the response to pathological stimulus was driven by adverse remodeling of the extracellular matrix and N-cadherin mediated cell-adhesion. In particular, there was upregulation of the myocardial MMP2 and MT1-MMP axis. Inhibition of MMP prevented the accelerated development of dilated cardiomyopathy in pressure-overloaded PI3KγKO mice. The molecular trigger of this adverse remodeling is excess cAMP, and β-blocker therapy normalizes cAMP levels, suppresses MMP expression, and rescues the early onset of dilated cardiomyopathy in the mutant mice. These findings provide valuable insight into the phenotype of other gene-targeted animal models with elevated Ca2⁺ cycling but an inability to rescue heart failure. Our findings highlight the potential negative impact chronic elevations in cAMP can have on the heart in the setting of heart failure while highlighting a novel mechanism of chronic β-blocker therapy in preventing the adverse myocardial remodeling in pressure overload–induced heart failure.
Loss of PI3Kγ Enhances cAMP-Dependent MMP Remodeling of the Myocardial N-Cadherin Adhesion Complexes and Extracellular Matrix in Response to Early Biomechanical Stress


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ONLINE METHODS and DATA SUPPLEMENT

Loss of PI3Kγ enhances cAMP-dependent MMP remodeling of the myocardial N-cadherin adhesion complexes and extracellular matrix in response to early biomechanical stress

by

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ONLINE METHODS

Experimental Animals. PI3Kgamma knockout (PI3KγKO), PI3Kalpha dominant negative (PI3KαDN), PI3Kgamma kinase dead (PI3KγKD) mice have been previously described.1-3 All experiments were performed in accordance to institutional guidelines and Canadian Council on Animal Care. Mice were backcrossed into a pure C57Bl/6 background for at least 10 generations and only littermate male mice of 8-9 weeks of age were used. For the propranolol experiments, mice were treated with propranolol in their drinking water (0.2 g/L) in order to deliver 15 mg/kg/day; propranolol was withdrawn one day prior to the echocardiographic and hemodynamic measurements. The broad spectrum MMP inhibitor, PD166793 (Pfizer Inc.), was administered orally by daily gavage as previously described.4 Due to the rapid onset of ventricular dilation in PI3KγKO mice following pressure-overload, PD166793 treatment (30 mg/kg/day) began 3 days prior to aortic-banding and continued until mice were sacrificed.

Aortic Banding. 8-9 week old male C57Bl/6 WT, PI3KαDN, PI3KγKO and PI3KγKD mice weighing (20–25 g) were used in these experiments. Mice were anesthetized with 1.5 ketamine (100 mg/kg) and xylaxine (10 mg/kg). A topical depilatory agent was applied to the neck and chest area to
remove fur at and around the area of incision. The skin was cleaned with Germex and Betadine. One dose of penicillin (10 mg/kg, 0.1 mL i.p.) was administered prior to start of surgery. Mice were placed supine and body temperature was maintained at 37°C with a heating pad. A horizontal skin incision of 1 cm in length was made at the level of second intercostal space, once the animal was in surgical plane of anesthesia (lack of reflex or response to toe-pinching). A 6-0 silk suture was passed under the aortic arch. A bent 26-gauge needle was then placed next to the aortic arch and the suture was snugly tied around the needle and aorta between the left carotid artery and the brachiocephalic trunk. The needle was quickly removed allowing the suture to constrict the aorta. The incision was closed in layers and the mice were allowed to recover on a warming pad until they were fully awake. Immediately after the surgery, mice received one dose of buprenorphine and for the first 24 hours. The sham animals underwent the same procedure without the aortic banding. The heart tissues were snap frozen in liquid nitrogen and were kept in -80°C until being used for experiments.

**Echocardiographic Imaging.** Transthoracic M-mode and Doppler echocardiographic examination at 1 wk and 3 wks post-aortic banding were performed using an Acuson® Sequoia C256 system equipped with a 15-MHz linear transducer (15L8) (Version 4.0, Acuson Corporation, Mountain View, California) as previously described. Data were stored on the hard drive and 230 MB optical disk for image processing. Mice were placed on a heating pad and a nose cone with 0.75-1% isoflurane in 100% oxygen was applied. The temperature was maintained at 36.5 to 37.5°C. Ultrasound gel was placed on the chest of the anesthetized mouse. The ultrasound probe was placed in contact with the ultrasound gel and scanning was performed over 30 min. The temperature, heart rate (HR) and blood pressure (BP) were constantly monitored during the scanning. M-mode images were obtained for measurements of left ventricular (LV) wall thickness (LVWT), LV end-diastolic
diameter (LVEDD), and LV end-systolic diameter (LVESD). M-mode images were used to measure LV chamber sizes and wall thicknesses. Fractional shortening and velocity of circumferential shortening were calculated. For the propanolol experiments in the WT mice, the echocardiographic measurements were made using the Vevo 770 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada) as we have previously described.6

Isolated Cardiomyocyte Contractility. Ventricular cardiomyocytes were placed in a Plexiglas chamber and continuously perfused with oxygenated Tyrodes buffer (137 mM NaCl, 5.4 mM KCl, 10 mM glucose, 10 mM HEPES, 0.5 mM NaH2PO4, 1 mM MgCl2, 1.2 mM CaCl2, and pH 7.4) at 2.5 mL/min at 36°C. Cardiomyocytes were stimulated at 1 Hz with a Grass S44 stimulator (pulse duration 3 ms; 15-20 V) and a video edge detector (Crescent Electronics) was used to track myocyte contractions. Steady state contractions were recorded at 1 kHz following a 4 min equilibrium period using a Phillips 800 camera system (240 Hz) and Felix acquisition software (Photon Technologies Inc.). Cardiomyocyte length, percent fractional shortening, shortening rate (+dL/dt) and relaxation rate (-dL/dt) were determined at baseline.

Isolation and culture of adult cardiomyocytes and fibroblasts. Adult murine left ventricular cardiomyocytes and cardiofibroblasts were isolated and cultured as previously described.7 Briefly, 11-week old mice were injected with 0.05 mL of 1000USP/mL heparin for 15 min and then anesthetized using 2% isoflurane (1 L/min oxygen flow rate) provided through a nose cone. After opening the chest cavity, the heart was quickly excised and perfused using a Langendorff system within 45 s. Following 3 min perfusion, the heart was then digested with 2.4 mg/mL collagenase type 2 (Worthington) for 7-8 min. After sufficient digestion, the ventricles were removed, dissociated using forceps and transfer
pipettes, and resuspended in stopping buffer (10% FBS perfusion buffer). The isolated cardiomyocytes were then exposed to increasing calcium concentrations (100 µM, 400 µM, and 900 µM) for 15 min each before being plated onto laminin coated culture dishes in plating buffer (Eagle’s MEM with 10% FBS, Sigma) and placed at 37°C in a sterile 2% CO₂ incubator. The discarded stopping buffer was set aside for cardiofibroblasts collection. One hour after plating, the plating buffer was gently aspirated and replaced with culture buffer (serum free Eagle’s MEM with 0.1% BSA) and then placed into the incubator for 18 h before treatment. The discarded stopping buffer is centrifuged at 20 g for 3 min and the resulting supernatant was collected in a 15 mL conical tube. This was then centrifuged at 1500 rpm for 5 min and the pellet was collected and washed in 10% FBS DMEM (GIBCO). The solution is once again centrifuged at 1500 rpm for 5 min and the pellet was collected and plated onto a 10 cm culture dish in 10% FBS DMEM. The cardiofibroblasts were then passaged 2 times and put into 24 h serum free DMEM prior to treatment.

**TaqMan Real-time PCR.** RNA expression levels of various genes were determined by TaqMan Real-time PCR as previously described. Total RNA was extracted from flash-frozen tissue or cardiofibroblasts using TRIzol extraction protocol, and cDNA was synthesized from 1 µg RNA by using random hexamers. For each gene, a standard curve was generated using known concentrations of cDNA (0.625, 1.25, 2.5, 5, 10 and 20 µg) as a function of cycle threshold (CT). Expression analysis of the reported genes was performed by TaqMan Real-time PCR using ABI 7900 Sequence Detection System. The SDS2.2 software (integral to ABI7900 real-time machine) fits the CT values for the experimental samples and generates values for cDNA levels. All samples were run in triplicates in 384 well plates. 18S rRNA was used as an endogenous control. The primers and probes for mRNA expression analysis by Taqman Real-time PCR are listed in Supplementary Table 1 (see below).
**Western Blot Analysis and Membrane Fractionation.** The phosphorylated and/or total proteins from cultured cells and heart tissues of mice were measured by Western blot analysis as previously described.⁸,⁹ Protein was extracted in 50 mM Tris, 120 mM NaCl, 1 mM EDTA and 1% Triton X-100 (final pH 7.4). After quantification using the BCA Protein Array Kit (Pierce, Rockford, IL, USA), protein samples were separated by 8%-12% SDS-PAGE gel electrophoresis and then transferred to nitrocellulose membrane (Millipore). The membrane was blocked with 5% milk in Tris-Buffered Saline Tween-20 (TBST) for 2 h and then incubated overnight at 4°C with primary antibody against phosphorylated and total ERK1 (44kDa), ERK2 (42kDa), AKT (60kDa), GSK-3α (51kDa), GSK-3β (46kDa) (Cell Signaling), FAK (125kDa) (Santa Cruz and BD Biosciences), PLN (25kDa) (Santa Cruz), and CREB (43kDa) (Cell Signaling). Primary antibodies against N-Cadherin (130kDa), β-Catenin (92kDa) (BD Biosciences), MMP2 (72 and 64 kDa) (Millipore), MT1-MMP (60 kDa) (Millipore), MMP9 (92 kDa) (Millipore) and β1D-integrin (116 kDa) (Millipore) antibodies were also used. The anti-α7B-integrin (26-34 kDa) was kindly provided by Dr. Stephen J. Kaufman, University of Illinois, Urbana, Illinois and used as previously described.¹⁰ After primary antibody was removed, the membrane was washed 3 times with TBST for 15 min each. The membrane was then incubated with an appropriate horseradish peroxidase (HRP) coupled secondary antibody at a 1:5000 dilution in TBST for 2 h at room temperature, then washed 3 times with TBST for 15 min each. Proteins were detected by enhanced chemiluminescence (GE) using X-ray film (Fuji) and analyzed by the ImageJ software (U.S. National Institutes of Health, Bethesda, MD). For the *in vitro* analysis of N-cadherin cleavage, 100ng of N-Cadherin Fc Chimera protein (R&D) was incubated with 0, 1, 10, 100 nM of MMP2 (R&D) or MT1-MMP (R&D) (see Online Supp. Fig. II). An additional group of 100 ng of N-Cadherin Fc Chimera and 100 nM of MMP2 or MT1-MMP was incubated with 10 μM of the MMP
inhibitor PD166793. All components were dissolved in 50 mM Tris/HCl pH 7.8, 50 mL CaCl$_2$ and 0.5 M NaCl. The mixture was incubated in 37°C for 4 h and then the proteolysis was stopped with 5X SDS sample buffer. The samples were then immediately separated in a 12% SDS-PAGE gel and blotted with an N-Cadherin specific antibody (Santa Cruz).

Membrane fraction was extracted from LV myocardium by homogenized frozen tissue in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA) with DTT (1 mM), PMSF (1 mM), protease inhibitor (Calbiochem, San Diego, USA) and phosphatase inhibitor cocktails (Sigma-Aldrich, Oakville, Canada), centrifugation at 2,900g for 20 min. The supernatant containing the cytosolic and membrane proteins was centrifuged at 29,000g for 45 min. This high speed centrifugation separated the cytosolic protein (supernatant) from the membrane protein (pellet). The pellet was resuspended in 50 μL RIPA buffer (containing DTT, PMSF, protease and phosphatase inhibitors, with 0.25% sodium deoxycholate and 1% NP40 and then centrifuged at 15,000 g for 20 min. This supernatant was stored at -80°C as the membrane fraction. Western blot analysis for N-cadherin, β-catenin and MT1-MMP were performed on the membrane fraction using a 10% SDS-PAGE gel. The nitrocellulose membrane was then stripped with a mild stripping buffer (62.5 mM Tris-HCl, pH 6.8, 69.4 mM SDS, and 0.7% β-mercaptoethanol; 30 min at 55°C), blocked with 10% skim milk and blotted for Toll-like receptor 4 (TLR4) (1:500 dilution, Santa Cruz, Santa Cruz, USA), and subsequently for caspase 3 (1:3000 dilution, Cell Signaling). The purity of the membrane fraction was confirmed by the presence of the membrane specific protein, TLR4, and the absence of the cytoplasmic protein, caspase 3.

**Cell Adhesion Assay.** The cell adhesion assay was performed in accordance with the protocol provided by CELL BIOLABS, INC. CytoSelect 48-Well Cell Adhesion Assay (ECM Array,
Colorimetric Format) as previously described. Adult murine cardiomyocytes from sham and 1 wk post-AB hearts were freshly isolated and a hemocytometer was used to determine cell concentration per mL. The concentration was adjusted to 100,000 cells/mL and the cells were plated into each well at 20,000 cells/well. The cells were then placed into a 2% CO₂ sterile 37°C incubator for 60 min for cells to adhere. The adherent cells were washed with PBS before 200 µL of the Cell Stain solution was added for 10 min. The cells were then washed 5 times with PBS to remove the staining solution and left to air dry for 10 min. 200 µL of extraction solution was then added to each well and the plate was incubated for 10 min on a rocker. Once the color of the solution stabilized, the 48-well plate was placed in a plate reader and optical density (OD) was recorded at 560 nm. The reported OD values are the raw OD values of samples minus the OD value of a blank.

**Measurement of cAMP levels.** The cAMP assay was performed following the protocol provided with the GE Healthcare Amersham cAMP Biotrak Enzyme immunoassay System. Isolated ventricular cells and LV myocardial tissue were homogenized with the provided lysis reagent and centrifuged to remove debris. The homogenate was placed into the appropriate wells in the 96-well pre-made plate and incubated with antiserum for 2 h at 4°C. After the incubation, cAMP-peroxidase conjugate was pipetted into all the wells except blanks and the plate was then incubated at 4°C for 1 h before the wells were aspirated, washed and dried. Enzyme substrate was then added to each well for 30 min at room temperature before collecting the OD values at 630 nm. The OD values were converted to concentrations using a standard curve prepared from provided standards. The effects of β-adrenergic blockade using propranolol (25 µM) (Sigma) and specific adenylate cyclase inhibition using 2′,5′-dideoxyadenosine (DIDA; 30 µM) (Sigma) on isoproterenol-induced increase in cAMP were also examined.
**Confocal Fluorescence Microscopy.** Formalin-fixed sections were permeabilized with ice-cold methanol for 10 min, blocked with goat serum and incubated with primary antibodies (N-cadherin (rabbit) and β-catenin (mouse), 1:500, Abcam®) overnight at 4°C in a humidified chamber. After several washes, Texas Red-conjugated (for N-cadherin) or FITC-conjugated (for β-catenin) secondary antibodies were added and the sections were incubated for 30 min at room temperature. The sections were evaluated with a Zeiss LSM510 confocal microscope and associated software.

**References**


### SUPPLEMENTAL TABLES

**Supplemental Table I. Taqman primers and probes**

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<thead>
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<th>Gene</th>
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### Supplemental Table II. Echocardiographic, hemodynamic and morphometric parameters following aortic banding

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<td>549±12</td>
<td>561±14</td>
<td>548±15</td>
<td>539±16</td>
<td>568±17</td>
</tr>
<tr>
<td>PWT (mm)</td>
<td>0.68±0.06</td>
<td>0.67±0.05</td>
<td>0.74±0.09</td>
<td>0.73±0.11</td>
<td>0.86±0.12*</td>
<td>0.84±0.14*</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.81±0.11</td>
<td>3.79±0.12</td>
<td>3.78±0.14</td>
<td>4.41±0.2*</td>
<td>3.65±0.17</td>
<td>5.53±0.23*</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.71±0.09</td>
<td>1.52±0.1*</td>
<td>1.69±0.16</td>
<td>2.63±0.18*</td>
<td>1.69±0.21</td>
<td>4.58±0.19*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>55.1±1.8</td>
<td>59.9±1.9*</td>
<td>55.3±2.5</td>
<td>40.3±2.8*</td>
<td>53.8±3.1</td>
<td>17.2±4.2*</td>
</tr>
<tr>
<td>VCFc (circ/s)</td>
<td>10.8±0.32</td>
<td>12.94±0.4*</td>
<td>10.9±0.38</td>
<td>7.48±0.31*</td>
<td>10.9±0.45</td>
<td>4.52±0.361*</td>
</tr>
<tr>
<td>+dP/dt&lt;sub&gt;max&lt;/sub&gt; (mmHg/s)</td>
<td>1013±301</td>
<td>1289±276*</td>
<td>9935±378</td>
<td>7138±330*</td>
<td>9984±423</td>
<td>5012±359*</td>
</tr>
<tr>
<td>-dP/dt&lt;sub&gt;min&lt;/sub&gt; (mmHg/s)</td>
<td>9945±252</td>
<td>12671±311</td>
<td>9471±283</td>
<td>6902±363*</td>
<td>6145±369</td>
<td>4875±408*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>5.23±1.96</td>
<td>6.56±2.7</td>
<td>7.71±2.8</td>
<td>12.8±2.2*</td>
<td>9.23±4.1</td>
<td>19.3±3.9*</td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>3.75±0.13</td>
<td>3.71±0.17</td>
<td>4.17±0.21</td>
<td>4.22±0.24</td>
<td>5.63±0.22*</td>
<td>5.69±0.29*</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>4.9±0.15</td>
<td>4.88±0.15</td>
<td>5.21±0.23</td>
<td>5.28±0.27</td>
<td>6.27±0.41*</td>
<td>6.31±0.38*</td>
</tr>
</tbody>
</table>

AB, aortic banded; SHAM, sham-operated; HR, heart rate; PWT, posterior left ventricular wall thickness; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; FS, fractional shortening; VCFc, velocity of circumferential shortening corrected for heart rate; +dP/dt<sub>max</sub>, maximum first derivative of the change in left ventricular pressure; and -dP/dt<sub>min</sub>, minimum first derivative of the change in left ventricular pressure; LVEDP, left ventricular end-diastolic pressure. *p<0.05 compared with the corresponding WT+AB group; #p<0.05 compared with corresponding SHAM group.
SUPPLEMENTAL FIGURES

Online Figure I

The purity of protein fractionation was confirmed using western blot for caspase-3 (a membrane bound protein) and toll-like receptor 4 (a cytosolic protein). Caspase-3 protein was only found in the cytosolic fraction and not the membrane fraction whereas toll-like receptor 4 protein was present in the membrane fraction but not in the cytosolic fraction.

Online Supplemental Figure I. The purity of protein fractionation was confirmed using western blot for caspase-3 (a membrane bound protein) and toll-like receptor 4 (a cytosolic protein). Caspase-3 protein was only found in the cytosolic fraction and not the membrane fraction whereas toll-like receptor 4 protein was present in the membrane fraction but not in the cytosolic fraction.
Online Figure II

Online Supplemental Figure II. MMP2, but not MT1-MMP, cleaves human recombinant N-cadherin Fc Chimera. N-cadherin Fc Chimera (NCAD, 100 ng) was incubated with 3 different concentrations of human recombinant MMP2 or MT1-MMP and the MMP inhibitor (MMPi, PD166793 (10 μM)) for 4 hours at 37°C. Western blotting detected bands at 65, 60, and 40kDa representing fragments of the N-cadherin Fc Chimera.
Online Figure III

Online Supplemental Figure III. Western blot analysis of MMP9 protein levels showing an increase in the PI3KγKO hearts at 3 weeks after AB but not at 1 week after AB whereas WT hearts showed no changes in MMP9 protein levels. n=4 for all groups. *p<0.05 compared with sham and 1 week AB groups.
Online Figure IV

Western blot analysis of membrane β1D and α7B integrin protein showing increased levels in WT hearts at 1 and 3 weeks after AB. Both integrins increased in the PI3Kγ-KO at 3 weeks after AB. n=4 for all groups. *p<0.05 compared with corresponding sham group; #p<0.05 compared with the PI3Kγ-KO group.

Online Supplemental Figure IV. Western blot analysis of membrane β1D and α7B integrin protein showing increased levels in WT hearts at 1 and 3 weeks after AB. Both integrins increased in the PI3Kγ-KO at 3 weeks after AB. n=4 for all groups. *p<0.05 compared with corresponding sham group; #p<0.05 compared with the PI3Kγ-KO group.
Online Figure V

Online Supplemental Figure V. Echocardiography shows that 9 weeks of AB in WT mice induces LV systolic dysfunction characterized by a reduction in percentage of fractional shortening (A) and an increase in left ventricular end diastolic diameter (LVEDD) (B). These changes are reversed with a daily intake of the beta-blocker, propranolol (15 mg/kg/day) (A-B). *p<0.05 compared with all other groups.