Piezo1 is the cardiac mechanosensor that initiates the hypertrophic response to pressure overload

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Article

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Abstract

Pressure overload-induced cardiac hypertrophy is a maladaptive response with poor outcomes and limited treatment options. The transient receptor potential melastatin 4 (TRPM4) ion channel is key to activation of a Ca^{2+} -calmodulin kinase II (CaMKII)-dependent hypertrophic signalling pathway after pressure overload, but TRPM4 is neither stretch-activated nor Ca^{2+} -permeable. Here we show that Piezo1, which is both stretch-activated and Ca^{2+} -permeable, is the mechanosensor that transduces increased myocardial forces into the chemical signal that initiates hypertrophic signalling via TRPM4. Cardiomyocyte-specific deletion of Piezo1 in adult mice prevented activation of CaMKII and inhibited the hypertrophic response: residual hypertrophy was associated with calcineurin activation in the absence of its usual inhibition by activated CaMKII. Piezo1 deletion prevented upregulation of the sodium-calcium exchanger and downregulation of the T-type calcium channel after pressure overload. These findings establish Piezo1 as the cardiomyocyte mechanosensor that instigates the maladaptive hypertrophic response to pressure overload, opening an avenue to novel therapies.

Introduction

Despite significant advances in cardiovascular medicine over the last 30 years, pathological left ventricular hypertrophy (LVH) secondary to pressure overload resulting from hypertension or aortic stenosis remains a powerful independent predictor of cardiovascular mortality and morbidity^{1, 2, 3, 4}. Thus far, the only treatment available for this condition is blood pressure reduction with anti-hypertensive medications or replacement of a stenotic aortic valve. These strategies do not fully reverse the pathological remodelling that occurs once LVH is established. Understanding the molecular mechanisms that drive LVH in response to pressure overload may open avenues to novel anti-hypertrophic therapies.

The two principal stimuli involved in LVH development are neuroendocrine hormones that activate Gq receptors (e.g. angiotensin II) and mechanical forces (e.g. pressure overload)⁵. These stimuli are thought to activate two distinct Ca^{2+} -dependent signaling cascades that ultimately result in LVH: the calcineurin-nuclear factor of activated T-cells (NFAT)-GATA4 pathway and the calmodulin-dependent protein kinase II (CaMKII)-histone deacetylase 4 (HDAC4)-myocyte enhancer factor 2 (MEF2) pathway. Our previous experimental work has demonstrated that the development of LVH in response to transverse aortic constriction (TAC), the most common surgical model of pressure overload, is dependent on activation of the CaMKII-HDAC4-MEF2 pathway but does not require Gq receptor or calcineurin activation⁶.

Moreover, we have shown recently that the Ca^{2+} -activated transient receptor potential melastatin 4 (TRPM4) ion channel acts as a positive regulator of pressure overload-induced cardiac hypertrophy, playing a key role in the activation of the CaMKII-HDAC4-MEF2 pathway⁷. Given that TRPM4 is not activated by membrane stretch⁸ and that Gq receptor activation is not required, the question remains as to the identity of the molecule at the start of the hypertrophic signaling cascade that senses changes in mechanical load within the myocardium and transduces that mechanical signal into a chemical signal that activates TRPM4 and in turn the CaMKII-HDAC4-MEF2 pathway.

A prime candidate to act upstream of TRPM4 within this mechanosensory signaling cascade that drives LVH is the Ca^{2+} -permeable mechanosensitive ion channel, Piezo1. Since the discovery and cloning of Piezo channels in 2010⁹, this family of structurally distinct ion channels has emerged as key sensors of biomechanical forces in the cardiovascular system¹⁰. Despite the significant evidence for a key role of Piezo1 channels in vascular physiology and pathophysiology^{11, 12, 13}, little is known about the role of Piezo1 in cardiac biology. A recent study has identified that Piezo1 is expressed at low levels in adult murine cardiomyocytes and is calcium permeable, increasing intracellular Ca^{2+} ¹⁴. Genetic ablation of Piezo1 using a cardiomyocyte-specific Cre driver during early cardiac development resulted in a mild dilated cardiomyopathy that worsened as the mice aged¹⁴. However, whether Piezo1 expressed in cardiomyocytes is the mechanosensor responsible for

initiating the CaMKII-HDAC4-MEF2 hypertrophic signaling cascade to induce LVH in response to pressure overload remains to be determined.

Here, using mice expressing a Piezo1 fusion protein (Piezo1-TdTomato) and a cardiomyocyte-specific inducible knockout (Piezo1 KO) mouse model, we investigate the role of Piezo1 in LVH induced by pressure overload. We demonstrate that in response to TAC-induced pressure overload, cardiomyocyte expression of Piezo1 increases, and that deletion of cardiomyocyte Piezo1 completely prevents activation of the CaMKII-HDAC4-MEF2 pathway and inhibits the LVH observed in response to TAC. Moreover, we show that the loss of Piezo1 prevents the altered expression of a number of critical Ca^{2+} handling proteins, including TRPM4 and the sodium-calcium exchanger (NCX1), that are associated with pressure overload-induced LVH. Taken together, our new findings demonstrate that Piezo1 is the mechanosensor that transduces the increased myocardial forces caused by pressure overload into a chemical signal that activates the hypertrophic signaling cascade resulting in pathological LVH.

Results

TAC induces identical levels of LVH in WT and Piezo1^{P1-tdT/P1-tdT} mice

We hypothesized that Piezo1 is involved in the signalling cascade that drives pathological LVH in response to pressure overload. To test this hypothesis, we employed *Piezo1^{P1-tdT/P1-tdT}* mice that expressed a Piezo1-tdTomato fusion protein from the *Piezo1* locus¹². This reporter mouse allowed us to use Piezo1 fusion proteins to probe Piezo1 expression using a specific mCherry antibody. We took this approach because there are no specific mouse anti-Piezo1 antibodies available commercially. We performed transverse aortic constriction (TAC) on *Piezo1^{P1-tdT/P1-tdT}* mice and their wild type littermates (WTLs).

TAC increased LV systolic pressure (LVSP) by ~57 mmHg in both WTLs and *Piezo1^{P1-tdT/P1-tdT}* mice, when compared with their respective sham-operated controls at 14 days (Table 1, both $p < 0.001$). This significant pressure overload with TAC resulted in enlarged hearts (Table 1, Supplementary Fig. 1A) and significant LVH after 14 days in both groups, when compared with their sham-operated controls. The degree of LVH did not differ between WTLs and *Piezo1^{P1-tdT/P1-tdT}* mice, whether LVH was assessed by echocardiographically-determined LV mass, wall thickness (h), or wall thickness to chamber radius ratio (h/r), or by postmortem LV weight (LVW), whether normalized to body weight (LVW/BW) or tibial length (LVW/TL) (Table 1). Consistent with the development of pathological LVH, TAC was associated with increased cardiac fibrosis ($p < 0.001$, Table 1, Supplementary Fig. 1B) and enhanced collagen III (*Col3a1*) expression ($p < 0.001$, Table 1) in both WTLs and *Piezo1^{P1-tdT/P1-tdT}* mice.

Notably, TAC-induced LVH at 14 days was not associated with any evidence of LV dysfunction in either group: there were no significant changes in heart rate (HR), echocardiographically-determined LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV), LV ejection fraction (LVEF), cardiac output (CO), or $\text{dP/dt}_{\text{max}}$, $\text{dP/dt}_{\text{min}}$, lung weight or lung weight to body weight ratio (Table 1), indicating that our TAC model is a model of pressure overload LVH without ventricular decompensation or heart failure, as reported previously^{6, 7}.

Early markers of LVH induction in WT and Piezo1^{P1-tdT/P1-tdT} mice

Consistent with the early induction of hypertrophic signalling after TAC^{6, 7}, gene expression of atrial natriuretic peptide (ANP, *Nppa*), brain natriuretic peptide (BNP, *Nppb*) and α -skeletal actin (α -SA, *Acta1*) was increased significantly 48 hours after TAC, preceding the development of significant LVH (supplementary Table 1), in both whole LV tissue and isolated LV cardiomyocytes, with no significant differences between WTLs and *Piezo1^{P1-tdT/P1-tdT}* hearts (Table 2). The increased expression of these genes in whole LV tissue and isolated LV cardiomyocytes persisted 14 days after TAC in WTLs and *Piezo1^{P1-tdT/P1-tdT}* mice (Table 2).

Pressure overload induces Piezo1 upregulation

Under baseline conditions, *Piezo1* mRNA levels were low in isolated LV cardiomyocytes when compared to whole heart tissue, brain, aorta and lung (Fig. 1A). This is consistent with previous reports^{9, 11}. We used the Piezo1-tdTomato fusion protein to measure Piezo1 protein levels. Western blots demonstrated a clear band at ~320 kDa, indicative of the Piezo1-tdTomato fusion protein, in both whole heart tissue and isolated cardiomyocytes (Fig. 1B). Under baseline conditions, Piezo1 protein levels were low in isolated cardiomyocytes, particularly when compared with the aorta and lung (Fig. 1C).

Two days after TAC, however, *Piezo1* mRNA expression increased significantly, ~2-fold in LV tissue ($p < 0.01$) and ~6-fold in isolated LV cardiomyocytes ($p < 0.001$, Fig. 1D), when compared with sham-operated hearts. The upregulation of *Piezo1* mRNA expression was not maintained 14 days after TAC, by which time mRNA expression was similar to that in sham-operated controls in both LV tissue and isolated cardiomyocytes (Fig. 1D). In contrast, Piezo1 protein levels did not increase significantly in either LV tissue or isolated cardiomyocytes 2 days after TAC (Fig. 1E and F), but did increase significantly 14 days after TAC in both LV tissue (~1.6-fold, $p < 0.001$) and isolated cardiomyocytes (~1.4-fold, $p < 0.01$) (Fig. 1E and F).

Targeted deletion of Piezo1 from cardiomyocytes in the adult heart

To further investigate whether Piezo1 plays an important role in the induction of LVH secondary to pressure overload, we generated an inducible cardiomyocyte-specific Piezo1 knockout mouse (Piezo1 KO) that permitted targeted deletion of Piezo1 from cardiomyocytes in adult mice (8 weeks old) (Fig. 2A, B). Tamoxifen inducible α -MHC-MerCreMer transgenic mice (Cre transgene under control of the α -myosin heavy-chain (*Myh6*) promoter¹⁵) were crossed with *Piezo1*^{fl/fl} mice, and offspring backcrossed until *P1*^{fl/fl}*MCM*^{+/-} were generated (see Methods and Fig. 2B, C). To account for the potential of nonspecific Cre-recombinase mediated cardiotoxicity¹⁶, *P1*^{wt/wt}*MCM*^{+/-} mice were designated as controls for Cre. *P1*^{fl/fl}*MCM*^{+/-} male mice aged 8-10 weeks were indistinguishable in cardiac function and anatomical parameters from age- and sex-matched *P1*^{wt/wt}*MCM*^{+/-} and *P1*^{wt/wt}*MCM*^{-/-} control mice (Supplementary Fig. 2).

To establish a dosing regimen of tamoxifen that maximized Cre-recombinase activity at the Piezo1 locus but minimized tamoxifen-induced cardiotoxicity^{16, 17}, we injected different concentrations of tamoxifen into 8-week-old male *P1*^{fl/fl}*MCM*^{+/-} and *P1*^{wt/wt}*MCM*^{+/-} mice on 3 consecutive days, and allowed 10 days for them to recover. We injected the same volume of peanut oil into *P1*^{fl/fl}*MCM*^{+/-} mice as the treatment control. We observed that tamoxifen induced Cre recombinase activity in a dose-dependent manner (Supplementary Fig. 3A - C). Piezo1 deletion was observed in the cardiomyocytes of mice injected with tamoxifen but not peanut oil. *P1*^{fl/fl}*MCM*^{+/-} mice treated with tamoxifen at 30 mg/kg/d exhibited normal heart structure and function when compared with mice treated with peanut oil (Supplementary Table 2). Higher doses of tamoxifen, 50 mg/kg/d or 100 mg/kg/d, caused LV dilatation with impaired contraction (Supplementary Table 2). Consequently, a tamoxifen dose of 30 mg/kg/d was used in subsequent experiments.

As treatment for three consecutive days did not result in a satisfactory level of *Piezo1* excision we increased the number of consecutive days of tamoxifen dosing. Ultimately, tamoxifen at 30 mg/kg/d for six consecutive days produced efficient inducible deletion of *Piezo1* in *P1*^{fl/fl}*MCM*^{+/-} mice when measured ten days after the last injection with no impact on cardiac function (Fig 2). We confirmed successful Cre-recombination using PCR amplification of cardiac genomic DNA¹⁸. As shown in Figure 2C, Cre-mediated recombination of *Piezo1* was detected only in the heart, with no excision identified in liver or lung tissues. A stronger signal was identified in isolated cardiomyocytes when compared to whole heart tissue from tamoxifen-treated *P1*^{fl/fl}*MCM*^{+/-} mice, and no excision was evident in cardiomyocytes isolated from control mice treated with peanut oil (Fig.2 C).

Congruent with these results, and consistent with previous findings of the relative efficiency for the α MHC-MerCreMer construct^{15, 19}, expression of Piezo1 mRNA was reduced by approximately 78% in isolated cardiomyocytes from tamoxifen

treated $P1^{fl/fl}MCM^{+/-}$ mice (Piezo1 KO mice, $p < 0.001$) when compared with mice injected with peanut oil (Fig. 2C, D). These findings confirm that tamoxifen 30 mg/kg/d for 6 days induced specific deletion of Piezo1 from cardiomyocytes.

Next, we assessed whether tamoxifen-induced deletion of Piezo1 in cardiomyocytes had any impact on baseline cardiac function and structure. To do this, we treated $P1^{fl/fl}MCM^{+/-}$ mice with the same tamoxifen dose (30 mg/kg/d) as a control for Cre expression (termed α -MHC-MCM $^{+/-}$ mice). We performed echocardiographic measurements 10 days after mice received their last injection of tamoxifen for Piezo1 KO mice, α -MHC-MCM $^{+/-}$ mice and $P1^{fl/fl}MCM^{+/-}$ control mice (peanut oil injected). The results from this analysis showed that there were no significant differences in body weight (Fig. 2E), heart rate (Fig. 2F), cardiac function (Fig. 2G-J) or left ventricular morphology between any of the three groups (Fig. 2K, L).

Cardiomyocyte-specific deletion of Piezo1 inhibits the hypertrophic response to pressure overload

TAC or sham surgery was performed on Piezo1 KO mice, α -MHC-MCM $^{+/-}$ mice and $P1^{fl/fl}MCM^{+/-}$ control mice. After 14 days, body weight, heart rate, LV end-diastolic and end-systolic volumes, ejection fraction and dP/dt were not significantly different between TAC or sham-operated animals, or between the three genotypes (Fig. 3, Supplementary Table 3).

Importantly, TAC induced the same increase in LV systolic pressure in all three genotypes (Fig. 3C). Echocardiographic indices of LVH - LV wall thickness to chamber radius ratio (h/r) and LV mass - increased significantly (both $p < 0.001$) after TAC in both α -MHC-MCM $^{+/-}$ mice and $P1^{fl/fl}MCM^{+/-}$ control mice (Fig. 3E, F), but these changes were absent in Piezo1 KO mice after TAC (Fig. 3E, F). Similarly, post mortem indices of LVH - heart weight and LV weight, whether normalised to body weight or tibial length - increased very significantly (all $p < 0.001$) 14 days after TAC in both α -MHC-MCM $^{+/-}$ mice and $P1^{fl/fl}MCM^{+/-}$ control mice, but Piezo1 KO mice exhibited significant inhibition of the hypertrophic response to TAC (Fig. 3G-K): for example, the average increase in the LV weight/tibial length ratio after TAC was 62% lower in Piezo1 KO mice ($p < 0.001$).

Cardiomyocyte-specific deletion of Piezo1 inhibits myocardial fibrosis in response to pressure overload

Pathological LVH is associated with cardiac fibrosis, with upregulated collagen expression and deposition. We evaluated interstitial cardiac fibrosis in response to pressure overload 14 days after TAC in Piezo1 KO hearts and α -MHC-MCM $^{+/-}$ hearts using Masson's trichrome staining (Fig. 3L). There was increased interstitial cardiac fibrosis in α -MHC-MCM $^{+/-}$ -TAC hearts when compared with their sham controls ($p < 0.001$), but there was no increase in cardiac fibrosis in Piezo1 KO hearts after TAC (Fig. 3L, M). Consistent with these findings, Collagen III (*Col3a1*) mRNA expression increased ~6-fold in α -MHC-MCM $^{+/-}$ -TAC hearts when compared with their sham controls ($p < 0.001$), but a more attenuated response was observed in Piezo1 KO hearts after TAC when compared with sham-operated hearts (~2-fold; $p < 0.05$, Fig. 3N) and with α -MHC-MCM $^{+/-}$ -TAC-operated hearts ($p < 0.001$, Fig. 3N).

Cardiomyocyte-specific deletion of Piezo 1 inhibits early markers of induction of hypertrophy in response to pressure overload

Although LVH had not yet developed 2 days after TAC (Supplementary Fig. 4), induction of hypertrophic signalling in α -MHC-MCM $^{+/-}$ hearts was already evident at this time, as was apparent from 5 to 10-fold increases in gene expression of ANP (*Nppa*), BNP (*Nppb*) and α -SA (*Acta1*) (all $p < 0.001$). However, this early response of markers of hypertrophic signalling was completely absent in Piezo1 KO hearts 2 days after TAC (Fig. 4A). The increased expression of these hypertrophy-associated genes was maintained 14 days after TAC in α -MHC-MCM $^{+/-}$ hearts (~4 to 10-fold, all $p < 0.001$), while an attenuated late response was observed in Piezo 1 KO mice 14 days after TAC (~2 to 5-fold, $p < 0.001$ for both *Nppa* and *Nppb*, not significant for *Acta1*, Fig. 4B).

Cardiomyocyte-specific deletion of Piezo1 prevents activation of the CaMKII-HDAC4-MEF2 hypertrophic signalling pathway in response to pressure overload

The cytoplasmic and the nuclear fractions of LV tissue were separated as described in the Materials and Methods. Purity of the isolated fractions was confirmed by western blot analysis using antibodies against marker proteins specific for cytoplasmic (glyceraldehyde 3-phosphate dehydrogenase, GAPDH) and nuclear (Histone H2B) fractions (Supplementary Fig. 5).

As expected, α -MHC-MCM^{+/-} mice exhibited strong activation of the CaMKII-HDAC4-MEF2 hypertrophic signalling pathway 2 days after TAC (Fig. 5), consistent with our previous findings in TAC-induced hypertrophy^{6,7}. The hallmark of this activation is increased levels of both total and activated CaMKII, which is auto-phosphorylated at threonine 287 (p-CaMKII)^{20,21}, and the resultant increase in the cytoplasmic to nuclear ratio of HDAC4 ($p < 0.01$), indicating nuclear export of HDAC4, with consequent de-repression of MEF2A ($p < 0.001$, Fig. 5).

Remarkably, Piezo1 KO mice failed to exhibit any evidence of activation of the CaMKII-HDAC4-MEF2 hypertrophic signalling pathway 2 days after TAC: the findings in Piezo1 KO mice 2 days after TAC were indistinguishable from those in their sham-operated controls (Fig. 5). These data indicate that Piezo1 is essential for activation of the CaMKII-HDAC4-MEF2 hypertrophic signalling pathway in response to pressure overload-induced by TAC.

Cardiomyocyte-specific deletion of Piezo1 is associated with activation of the calcineurin-NFAT hypertrophic signaling pathway in response to pressure overload

As expected, α -MHC-MCM^{+/-} mice exhibited no evidence of activation of the calcineurin-NFAT hypertrophic signalling pathway 2 days after TAC (Fig. 6), consistent with our previous findings in TAC-induced hypertrophy^{6,7}. One explanation for this finding is that pressure overload activates CaMKII and activated CaMKII inhibits calcineurin activation²². Because Piezo1 KO mice exhibited no evidence of CaMKII activation in response to pressure overload, yet they exhibited *incomplete* inhibition of LVH, we postulated that the residual LVH was driven by calcineurin activation in the absence of its inhibition by CaMKII. The results obtained in Piezo1 KO mice 2 days after TAC supported this hypothesis: the hallmark of calcineurin activation, an increase in the nuclear to cytoplasmic NFAT ratio, indicating translocation of NFAT to the nucleus due to dephosphorylation by activated calcineurin, was clearly evident in Piezo1 KO mice 2 days after TAC ($p < 0.01$, Fig. 6A, B) but absent in both sham-operated controls and α -MHC-MCM^{+/-} mice subjected to TAC.

Piezo1 is upstream of changes in Ca²⁺ handling proteins in response to pressure overload

We have demonstrated recently that the Ca²⁺-activated ion channel TRPM4 plays an important role in the activation of the Ca²⁺-calmodulin dependent kinase, CaMKII, and thus the CaMKII-HDAC4-MEF2 hypertrophic signalling pathway⁷, but TRPM4 is neither stretch-activated nor Ca²⁺-permeable, whereas Piezo1 is both stretch-activated and Ca²⁺-permeable. To investigate whether Piezo1 can modify the expression of proteins important in cardiomyocyte Ca²⁺ handling, we probed the expression of TRPM4, the sodium-calcium exchanger (NCX1) and the T-type Ca²⁺ channel (Ca_{v3.2}) in response to LV pressure overload.

α -MHC-MCM^{+/-} hearts showed significantly reduced expression of *Trpm4* mRNA and TRPM4 protein 2 days after TAC (Fig. 7A, E and F, both $p < 0.01$), replicating our previous findings in wild type mice⁷, but Piezo1 KO mice exhibited no change in *TRPM4* mRNA 2 days after TAC (Fig. 7A, E and F), indicating that Piezo1 is upstream of TRPM4, and mediates the response of TRPM4 to pressure overload.

Conversely, the upregulation of Piezo1 mRNA expression 2 days after TAC in wild type hearts ($p < 0.001$, Fig. 7B) was not abolished in TRPM4 KO hearts ($p < 0.01$) 2 days after TAC, confirming that TRPM4 is downstream of Piezo1 in the response to pressure overload. Nevertheless, the magnitude of upregulation of Piezo1 mRNA 2 days after TAC was diminished significantly in TRPM4 KO mice ($p < 0.001$, Fig. 7B), suggesting that TRPM4 plays a significant role in the feedback regulation of Piezo1 in response to pressure overload.

α -MHC-MCM^{+/-} hearts exhibited significant increases in both NCX1 mRNA (*Slc8a1*) (Fig. 7C) and protein expression 2 days after TAC (Fig. 7E and G, both $p < 0.01$). In contrast, Piezo1 KO hearts exhibited no change in NCX1 mRNA or protein levels 2 days after TAC (Fig. 7C, E and G), indicating that Piezo1 is upstream of NCX1, and mediates the response of NCX1 to pressure overload.

α -MHC-MCM^{+/-} hearts exhibited significant decreases in the gene expression of the T-type calcium channel (*Cacna1h*) 2 days after TAC ($p < 0.01$), which was abolished in Piezo1 KO hearts (Fig. 7D), but the absence of any change at the protein level (Ca_{v3.2}) 2 days after TAC in α -MHC-MCM^{+/-} hearts (Fig. 7H) casts some doubt on the functional significance of the T-type calcium channel in mediating the response to pressure overload governed by Piezo1.

Discussion

Recently, we reported that the TRPM4 ion channel is a positive regulator of LVH induced by pressure overload, playing a key role in the activation of the CaMKII-HDAC4-MEF2 hypertrophic signaling pathway⁷. Activation of CaMKII and downstream hypertrophic signaling were inhibited after TAC in a mouse model in which TRPM4 was deleted in cardiomyocytes (TRPM4 KO), and this was associated with significant inhibition of the hypertrophic response to pressure overload. Since TRPM4 is not a stretch-activated⁸ channel but is Ca²⁺-activated, we postulated that TRPM4 must be downstream of a stretch-activated source of Ca²⁺ in the hypertrophic signaling cascade triggered by pressure overload. The primary aim of the current study, therefore, was to identify that stretch-activated Ca²⁺ source. Piezo1 was our prime candidate for this role because it is both stretch-activated^{9, 23, 24} and Ca²⁺-permeable^{9, 25}. In addition, because CaMKII is a Ca²⁺-calmodulin dependent kinase and TRPM4 is not Ca²⁺-permeable, it was apparent that TRPM4's role in CaMKII activation must depend on one or more downstream sources of the Ca²⁺ that activates CaMKII via calmodulin.

This study identified the Ca²⁺-permeable Piezo1 mechanosensitive ion channel as the primary mechanotransducer that initiates the hypertrophic response to pressure overload via TRPM4 and the CaMKII-HDAC4-MEF2 signalling pathway. Using a conditional, cardiomyocyte-specific Piezo1 KO mouse model, we showed that deletion of Piezo1 completely prevented activation of the CaMKII-HDAC4-MEF2 hypertrophic signalling pathway after TAC, and this was associated with significant inhibition of the hypertrophic response to pressure overload. We then demonstrated in the Piezo1 KO mouse that Piezo1 was upstream of TRPM4 in the hypertrophic signalling cascade after TAC, and that Piezo 1 controlled TRPM4 gene and protein expression early after TAC. We also showed in a TRPM4 KO mouse that TRPM4 contributed to the positive feedback that increased Piezo1 expression after TAC.

In the Piezo1 KO mouse, we also demonstrated that Piezo1 controls the expression of two Ca²⁺ handling proteins after TAC, positively in the case of NCX1 and negatively in the case of the T-type calcium channel. Because Piezo1 activation by TAC reduced gene expression of the T-type calcium channel early after TAC and because the fall in protein expression of the channel was not observed until after LVH was established, it seems more likely that the downregulation of the T-type calcium channel was a secondary negative feedback response to the high local [Ca²⁺] induced by NCX1. NCX1 could explain increased [Ca²⁺], however, only if operating in reverse mode in response to high local Na²⁺. Given that TRPM4 is Na²⁺-permeable, its activation by Piezo1 would account for the high intracellular Na²⁺ necessary to drive NCX1 in reverse mode, exporting Na²⁺ in exchange for increased cell entry of Ca²⁺, which has been demonstrated elsewhere^{26, 27, 28}.

These new observations support the hypertrophic signalling pathway outlined schematically in Figure 8. Stretch activation of Piezo1 in cardiomyocytes by the increased myocardial forces associated with pressure overload results in Ca²⁺ entry into the cell, increasing local [Ca²⁺] that then activates the Na²⁺-permeable TRPM4 channel. The resultant increase in local intracellular Na²⁺ is postulated here to drive NCX1 in reverse mode²⁹, thus amplifying the increase in local [Ca²⁺] initiated by Piezo1. CaMKII is preferentially activated in response to high-frequency, high-amplitude calcium oscillations³⁰, and it is known that aortic constriction provides this type of calcium signal³¹. In contrast, calcineurin activation requires a sustained

increase in the resting intracellular calcium level³². There is evidence that calmodulin can distinguish high-amplitude versus low-amplitude calcium signals via the differential calcium sensitivity of its N- and C-lobes^{33,34}, and may thus provide a gatekeeper role in the differential activation of the CaMKII-dependent and calcineurin-dependent hypertrophic signalling pathways (Fig. 8). In addition, once activated, CaMKII inhibits calcineurin activation²².

We reported previously that calcineurin activation was not required for the hypertrophic response to pressure overload with TAC, which was associated with activation of the CaMKII-HDAC4-MEF2 pathway⁶. In the present study, we found that cardiomyocyte-specific deletion of Piezo1 prevented any activation of the CaMKII-HDAC4-MEF2 signalling pathway secondary to TAC, and reduced the amount of LVH developed after TAC by approximately 60%. While this observation strengthens the causal link between activation of the CaMKII-HDAC4-MEF2 signalling pathway and the induction of LVH secondary to pressure overload²², it might reasonably have been expected that the complete absence of CaMKII activation secondary to pressure overload in Piezo1 KO hearts would prevent any hypertrophic response. Our findings support the conclusion, however, that the residual LVH in Piezo1 KO hearts after TAC is due to the absence of the usual inhibition of calcineurin activation by activated CaMKII. We observed no evidence of calcineurin activation after TAC in the α -MHC-MCM^{+/-} (control) hearts, consistent with our previous report⁶, but in Piezo1 KO hearts in which CaMKII activation after TAC was abolished, calcineurin activation was clearly evident. It is notable, however, that calcineurin activation in Piezo1 KO hearts after TAC produced significantly less LVH than observed with CaMKII activation when Piezo1 was present and calcineurin activation was absent, emphasising the importance of the CaMKII-HDAC4-MEF2 signalling pathway in pressure overload LVH.

In the same previous report⁶, we also demonstrated that Gq receptor activation was not required for the hypertrophic response to pressure overload with TAC since we found the same amount of LVH after TAC in the presence and absence of functioning Gq receptors. This raises the question: how is calcineurin activated after TAC in Piezo1 KO mice? It is important to note that TAC increases diacylglycerol (DAG), a downstream marker of Gq activation³⁵. The implication of the present results, therefore, is that Gq is indeed activated directly by TAC, but that activation has no impact on the hypertrophic response in the presence of Piezo1 activation because Piezo1 activation results in CaMKII activation, which inhibits Gq-dependent calcineurin activation. When Piezo1 is deleted, CaMKII is not activated and Gq-dependent calcineurin activation in response to TAC is no longer inhibited. We observed the same phenomenon of calcineurin activation associated with residual LVH after TAC in the TRPM4 KO mouse, in which CaMKII activation was strongly inhibited⁷.

Although pressure overload resulted in a significant increase in *Piezo1* mRNA 2 days after TAC, Piezo1 protein was not increased at this early time point when evidence of hypertrophic signalling is already abundant. Since we have shown that Piezo1 is the instigator of the hypertrophic signalling cascade after TAC, it would seem that the normal very low expression of Piezo1 protein is sufficient for this purpose due to the amplification of the calcium signal by TRPM4. This raises an interesting question regarding the late increase in Piezo1 protein observed 14 days after TAC, a time when the hypertrophic response to TAC has already plateaued⁶. We speculate that stretch-activated Ca²⁺ entry via Piezo1 may be maximal when Piezo1 protein expression is low, and that increased Piezo1 protein production may act to reduce stretch activation and Ca²⁺ entry by cooperative load sharing between the increased Piezo1 molecules, and this is the subject of our ongoing research.

An independent research group has very recently reported inhibition of hypertrophy after TAC in a Piezo1 KO mouse model based on the same Cre recombinase strategy used in our experiments³⁶. While the inhibition of pressure overload hypertrophy with Piezo1 deletion is confirmatory, the authors did not examine the mechanism of the inhibition of hypertrophy *in vivo*, and did not examine the CaMKII-HDAC4-MEF2 hypertrophic signalling pathway or the central role of TRPM4 in amplifying the initial Ca²⁺ signal provided by Piezo1. Based only on *in vitro* experiments in neonatal rat ventricular cardiomyocytes (NRVCs) subjected to Yoda1, a specific activator of Piezo1³⁷, the authors reported increased activity of calcineurin and calpain without increased expression of either protein. Stimulation of Piezo1 with Yoda1

produces a tonic increase in the calcium concentration^{37,38}, which is well known to activate calcineurin³², but this is not relevant to the high-amplitude calcium signal produced by aortic constriction and necessary for CaMKII activation^{30,31}. Given the significant differences also in maturity and terminal differentiation between NRVCs and adult cardiomyocytes and the absence of data on CaMKII activation in their study of NRVCs, it is difficult to determine the relevance of the NRVC data to our findings in adult mice. We did demonstrate in adult mice *in vivo*, however, that due to inhibition of calcineurin activation by activated CaMKII, calcineurin activation is absent in the hypertrophic response to TAC initiated by Piezo1.

In summary, our study not only identifies Piezo1 as the primary instigator of hypertrophic signalling in response to pressure overload, it provides a mechanistic explanation for the central role of TRPM4 in amplifying the initial Ca^{2+} signal via NCX1 to activate CaMKII via calmodulin, and thus activate the CaMKII-HDAC4-MEF2 hypertrophic signalling pathway. In addition, our new findings reveal the potency of the inhibition of calcineurin activation by activated CaMKII as the main explanation for the apparent segregation of the CaMKII-mediated and calcineurin-mediated hypertrophic signalling pathways, despite the fact that activation of both CaMKII and calcineurin is Ca^{2+} -calmodulin dependent. The closely cooperative role we have demonstrated between Piezo1 and TRPM4 in providing a stretch-activated biological signal seems likely to provide a universal paradigm for Piezo1's broader role in all biological tissues. These new findings may also provide targets for the development of novel therapies to prevent or reverse pathological hypertrophy and its harmful clinical sequelae.

Materials And Methods

Mice and genotyping

All experimental procedures were approved by the Animal Ethics Committee of Garvan/St Vincent's (Australia), in accordance with the guidelines of both the Australian code for the care and use of animals for scientific purposes (8th edition, National Health and Medical Research Council, AU, 2013) and the Guide for the Care and Use of Laboratory Animals (8th edition, National Research Council, USA, 2011). The homozygous Piezo1 reporter mice expressing a fusion protein of Piezo1 and the fluorophore TdTomato (*Piezo1^{P1-tdT/P1-tdT}*, The Jackson Laboratory Stock, No: 029214) were backcrossed to C57BL/6J mice to yield heterozygous *Piezo1^{P1-tdT/wt}* mice that were intercrossed to each other to obtain homozygous *Piezo1^{P1-tdT/P1-tdT}* mice for the experiments, and their wild type littermates (WTLs) served as controls.

To generate inducible cardiomyocyte-specific Piezo1 knockout (KO) mice, we crossed homozygous *Piezo1^{flox/flox}* mice (The Jackson Laboratory Stock, No: 029213) with homozygous *Myh6-MerCreMer* mice (MCM), which have a tamoxifen-inducible Cre recombinase under the control of the α -myosin heavy-chain (α MHC; *Myh6*) promoter¹⁵, to produce *Piezo1^{flox/flox}; α MHC-MCM^{+/-}* (termed *P1^{f/f}MCM^{+/-}*) mice. The age and sex-matched *Piezo1^{wt/wt}; α MHC-MCM^{+/-}* (termed *α MHC-MCM^{+/-}*) mice and *Piezo1^{wt/wt}; α MHC-MCM^{-/-}* (termed *P1^{wt/wt}MCM^{-/-}*) mice were used as controls for experiments characterizing phenotype at baseline.

To induce Cre recombinase-mediated deletion of exons 20-23 of the Piezo1 gene selectively in cardiomyocytes of adult mice, a daily intraperitoneal (i.p.) injection of tamoxifen (30mg/kg, Sigma, T5648) dissolved in 95% peanut oil (Sigma, P2144) was administered for 6 consecutive days to male *P1^{f/f}MCM^{+/-}* mice aged 8-10 weeks old (termed Piezo1 KO). The age- and sex-matched *P1^{wt/wt} α MHC-MCM^{+/-}* mice treated with tamoxifen served as Cre-only controls. *P1^{f/f}MCM^{+/-}* mice treated with peanut oil acted as flox controls. Mice were given 10 days to recover after receiving the last injection of tamoxifen before experiments. Mice were genotyped using the following primers: P1 F: CTT GAC CTG TCC CCT TCC CCA TCA AG, P1 WT/f R: CAG TCA CTG CTC TTA ACC ATT GAG CCA TCT C, P1 KO R: AGG TTG CAG GGT GGC ATG GCT CTT TTT using Phire II polymerase (Thermo Scientific, Waltham, MA) using the following cycling conditions: Initial denaturation 98°C for 30 s, followed by 31 cycles of 98°C for 5 s, 65°C for 5 s, 72°C for 5 s, followed by a final hold of 72°C for 2 min.

Reactions were separated on 2% agarose gels yielding the following band sizes: P1⁺: 160 bp, P1^f: 330 bp, P1⁻: 230 bp¹⁸. All mice were on the C57BL/6J genetic background, and male mice aged between 8-13 weeks old were used throughout.

Induction of LVH

As previously described^{6,7}, male mice were subjected to TAC to induce pressure overload. Mice were anesthetized with 5% isoflurane and ventilated at 120 breaths/min (Harvard Apparatus Rodent Ventilator). The transverse aortic arch was accessed via an incision in the second intercostal space, and constricted with a ligature tied around a 25-gauge needle, which was then removed. The TAC procedure was modified from a published paper³⁹. Sham mice underwent the same procedure but the ligature was not tied. Simultaneous direct pressure recordings (1.4 F pressure catheter, AD Instruments, P/L) from both the right carotid artery and the aorta distal to the ligature (n=20 mice) indicated a TAC pressure gradient of 60 ± 8 mmHg with this technique. Animals were sacrificed after 2 days or 14 days.

Echocardiographic measurements

As previously described⁴⁰, echocardiography was performed using an MS400 18–38 MHz transducer probe and VEVO 2,100 ultrasound system (VisualSonics Inc., Canada). The mice were anesthetized (3–5% isoflurane for induction, 1–2% isoflurane for maintenance with adjustment to maintain heart rate at ~500 bpm) and imaged at the endpoint of study to assess cardiac function. The acquisition of images and evaluation of data were performed by an operator blinded to treatment.

Invasive hemodynamic measurements

As previously described^{6,7}, after 14 days of sham or TAC, mice were anesthetized by inhalation of isoflurane (1.5%) and a 1.4F micro-tip pressure catheter (Millar Instruments Inc, Houston, Texas, USA) was inserted into the left ventricle via the right carotid artery. The heart rate, aortic systolic pressure, LV systolic pressure, +dP/dt, and –dP/dt were recorded (LabChart 6 Reader, AD Instruments, P/L). Animals were sacrificed, and the heart weight (HW) and left ventricle weight (LVW) normalized to body weight (BW) and to tibia length (TL) were measured as indicators of LVH.

Mouse LV cardiomyocyte isolation and purification

As previously described⁷, the mice were heparinized and euthanized according to the Animal Research Act 1985 No 123 (New South Wales, Australia). Hearts were dissected and perfused through the aorta and the coronary arteries by 10 mL pH 7.2 perfusion buffer containing 135 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 0.33 mM NaH₂PO₂, 10 mM HEPES, 10 mM Glucose, 10 mM 2,3-Butanedione 2-monoxime (BDM), and 5 mM Taurine, with a Langendorff apparatus at 37 degrees for 5 minutes. Next, 30 mL digestion buffer composed of the above solution and Collagenase B, D (dose by BW: 0.4 mg/g, Roche) and Protease Enzyme Type XIV (dose by BW: 0.07 mg/g, Sigma-Aldrich) was used to perfuse the hearts for 15 minutes. After the perfusion, the heart was removed from the setup and placed into a pH 7.4 transfer buffer containing 135 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 0.33 mM NaH₂PO₂, 10 mM HEPES, 5.5 mM Glucose, 10 mM BDM, and 5 mg/mL BSA. Both atria and the right ventricle were discarded, and the LV muscle was torn into small pieces and gently triturated in transfer buffer with a pipette to isolate cardiomyocytes. The suspension was then filtered through a 200 micro filcon cup filter (BD), and centrifuged at 20xg for 2 minutes. After that, the cardiomyocytes were purified by a method described previously⁴¹. We confirmed that rod-shaped cardiomyocytes accounted for more than 85% of the total purified cardiomyocytes. The isolated cardiomyocytes were frozen immediately in liquid nitrogen and stored at -80°C for following experiments.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Gene expression was determined by quantitative RT-PCR. Total RNA was extracted and purified from LV tissue and isolated cardiomyocytes with the RNeasy Fibrous Tissue Mini Kit (QIAGEN), following the manufacturer's protocol. RNA (500 ng)

was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen). cDNA was subjected to PCR amplification to detect ANP (*Nppa*), BNP (*Nppa*), α -SA (*Acta1*), collagen III (*Col3a1*), *Piezo1*, *Trpm4*, *Slc8a1*, *Cacna1h* gene expression, using the PCR master mix LightCycler 480 SYBR Green I Master (Invitrogen) and performed with the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Samples were run in technical triplicates and the mRNA expression levels were normalized to those of GAPDH to calculate relative gene expression using the delta-delta Ct method. Three independent experiments yielded similar results. The mouse RT-PCR primers (Sigma-Aldrich) used are shown in Supplementary Table 4.

Western blotting

For total protein extraction, LV tissue and isolated cardiomyocytes were lysed in a pH 7.4 lysis buffer containing 150 mM NaCl, 50 mM Tris-HCL, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM beta-glycerophosphate, 5 mM dithiothreitol and MiniComplete protease inhibitors (Roche). The cytoplasmic and the nuclear fractions of LV tissue were separated as described previously with confirmed high fraction purity^{6,7}. Briefly, LV tissue was lysed using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology) and Protease Inhibitor Cocktail Kit and Halt Phosphatase Inhibitor Cocktail (Pierce Biotechnology), both with an homogenizer (PRO Scientific). Protein (40 μ g for each sample) was loaded on 4%-20% Mini-PROTEAN TGX Gels (Bio-Rad) and separated by electrophoresis. Samples were transferred to PVDF membranes (Bio-Rad), blocked with 5% bovine serum albumin (BSA) then labelled overnight with primary antibodies (Supplementary Table 4): anti-mCherry (1:500, Thermo Scientific), anti-CACNA1H (1:2000, Abcam), anti-NCX1 (1:1000, Thermo Scientific), anti-TRPM4 (1:200, Alomone Labs), anti-CaMKII δ (1:1000; Abcam), anti-p-CaMKII (Thr287, 1:5000; Thermo Scientific), anti-HDAC4 (1:1500; Cell Signalling), anti-p-HDAC4 (Ser246, 1:1500; Cell Signalling), anti-MEF2A (1:3000; Cell Signalling), anti-NFATc4 (1:1500; Abcam), and anti-GATA4 (1:1000; Santa Cruz Biotechnology). Anti-GAPDH (1:10000; Cell Signaling Technology) and anti-Histone H2B (1:5000; Abcam) were used to standardize sample loading. Horseradish peroxidase-conjugated (HRP) goat anti-rabbit (1:10000) or rabbit anti-mouse (1:5000) or goat anti-rat (1:5000) secondary antibodies (Abcam) (Supplementary Table 4) were used at room temperature for one hour. Immunologic detection was accomplished using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific). Protein levels were quantified by densitometry using ImageJ (NIH) software. Protein levels were normalized to relative changes in Histone H2B for the nuclear fraction and GAPDH for the cytoplasmic fraction and expressed as fold changes relative to those of control animals. Experiments were repeated independently three times with similar results.

Histological analysis

As previously described⁷, Masson's trichrome stain was used to quantify fibrosis in the LV (collagen fibres stain blue). The hearts were excised from isoflurane-euthanized mice and washed with phosphate-buffered saline (PBS). Hearts were then cut longitudinally in the coronal plane, embedded into optimal cutting temperature (OCT) compound (Sakura Finetek), and gradually frozen in melting isopentane, precooled in liquid nitrogen to avoid tissue damage, and stored at -80°C for following experiments. Serial 6 mm sections were sliced with a cryostat (Leica) and stained using a Masson's trichrome staining kit (Sigma-Aldrich), following the manufacturer's instructions. Images of the LV were obtained with 4 to 6 fields per section⁴² using a brightfield microscope (Leica). Blue-stained areas of fibrosis within sections were determined using colour-based thresholding⁴³ and measured with ImageJ software (NIH; <http://rsbweb.nih.gov/ij/>). The percentage of total fibrotic area was calculated by taking the sum of the blue-stained areas divided by the total LV area.

Statistics

All experiments and analyses were blinded. Averaged data are presented as mean $\pm$ standard error of the mean (SEM). The statistical analyses were performed using GraphPad Prism software, version 7.04 (GraphPad). For comparisons among multiple sets of data with one factor or two factors, one-way or two-way ANOVA was used accordingly, followed by Tukey's post-hoc test. $p < 0.05$ was considered statistically significant.

Source Data

All source data are provided with this paper.

Declarations

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Tables

	14 days			
	WTLs		<i>Piezo1</i> ^{P1-tdT/P1-tdT}	
	Sham	TAC	Sham	TAC
Echocardiography				
n	6	6	6	6
HR (bpm)	472 ± 12	499 ± 9	488 ± 9	494 ± 23
LVEDV (μL)	85.26 ± 7.09	84.78 ± 3.24	86.60 ± 3.09	92.58 ± 2.54
LVESV (μL)	34.51 ± 4.85	29.77 ± 3.27	35.28 ± 2.47	34.43 ± 2.73
LVEF (%)	60.33 ± 2.84	65.17 ± 3.24	59.33 ± 2.19	62.67 ± 2.86
CO (mL/min)	23.86 ± 1.37	27.37 ± 1.28	25.13 ± 1.39	28.87 ± 2.32
h (mm)	0.78 ± 0.03	0.97 ± 0.03 ***	0.77 ± 0.02	0.98 ± 0.02 ***
r (mm)	2.06 ± 0.08	2.00 ± 0.03	2.02 ± 0.02	2.06 ± 0.02
h/r	0.38 ± 0.01	0.48 ± 0.02 ***	0.38 ± 0.01	0.47 ± 0.01 ***
LV mass (mg)	80.67 ± 6.06	106.73 ± 3.93 **	82.55 ± 3.54	113.87 ± 1.81 ***
Hemodynamic parameters				
n	7	7	7	7
HR (bpm)	497 ± 5	504 ± 7	498 ± 6	495 ± 4
LVSP (mmHg)	105.85 ± 2.63	163.86 ± 3.76 ***	104.43 ± 3.79	160.71 ± 5.02 ***
dP/dt _{max} (mmHg/s)	9522.57 ± 287.69	9698.14 ± 287.21	9591.71 ± 276.08	9620.43 ± 248.76
dP/dt _{min} (mmHg/s)	-9320.57 ± 232.18	-9402.86 ± 341.19	9227.57 ± 326.18	-9354.14 ± 300.41
Anatomical parameters				
n	7	7	7	7
BW (g)	28.79 ± 0.45	28.43 ± 0.44	28.51 ± 0.55	28.37 ± 0.41
HW (mg)	128.71 ± 2.26	161.01 ± 4.34 ***	131.25 ± 2.02	162.82 ± 4.73 ***
LVW (mg)	93.57 ± 3.87	135.71 ± 4.47 ***	94.38 ± 2.28	132.36 ± 3.13 ***
HW/BW (mg/g)	4.91 ± 0.24	6.06 ± 0.05 ***	4.86 ± 0.12	5.59 ± 0.12 ***
LVW/BW (mg/g)	3.58 ± 0.22	4.78 ± 0.10 ***	3.59 ± 0.12	4.55 ± 0.14 ***
LVW/TL (mg/mm)	5.35 ± 0.21	7.80 ± 0.26 ***	5.38 ± 0.12	7.49 ± 0.23 ***
LW/BW (mg/g)	5.06 ± 0.07	5.16 ± 0.07	5.12 ± 0.10	5.14 ± 0.07
Assessment of cardiac fibrosis				
n	5	5	5	5

Fibrosis areas (%)	4.29 ± 0.22	12.02 ± 0.64 ***	4.47 ± 0.21	12.08 ± 0.81 ***
Collagen III mRNA expression (fold change)	1.03 ± 0.13	4.27 ± 0.36 ***	1.04 ± 0.14	4.88 ± 0.54 ***

Table 1. Echocardiographic, hemodynamic and anatomical parameters 14 days after TAC or sham surgery in WTLs and

***Piezo1^{P1-tdT/P1-tdT}* mice.** Echocardiographic and hemodynamic parameters were measured in *Piezo1^{P1-tdT/P1-}*

tdT mice and their wild type littermates (WTLs) 14 days after TAC or sham surgery (n = 6-7/group). Post-mortem analysis was performed 14 days after TAC or sham surgery. Cardiac fibrosis was evaluated by Masson's trichrome staining, and the degree of fibrosis was graded (n = 5/group). Relative Collagen III (*Col3a1*) mRNA expression was normalized with GAPDH and calculated as fold change relative to the sham group, (n = 5/group). HR: heart rate; LVEDV: left ventricular (LV) end-diastolic volume; LVDSV: LV end-systolic volume; LVEF: LV ejection fraction; CO: cardiac output; h: LV wall thickness; r: chamber radius; h/r: LV wall thickness to chamber radius ratio. LVSP: left ventricular systolic pressure; HR: heart rate; dP/dt_{max}: the peak rate of pressure increase; dP/dt_{min}: the peak rate of pressure decrease; BW: body weight; HW: heart weight; LVW: left ventricular weight; LW: HW/BW: heart weight to body weight ratio; LVW/BW: LV weight to body weight ratio; LVW/TL: LV weight to tibia length ratio; LW/BW: lung weight to body weight ratio. Data are presented as means ± SEM, Two-way ANOVA followed with Tukey's post-hoc test, ***p* < 0.01, ****p* < 0.001 vs sham-operated groups.

	2 days				14 days			
	WTLs		<i>Piezo1^{P1-tdT/P1-tdT}</i>		WTLs		<i>Piezo1^{P1-tdT/P1-tdT}</i>	
	Sham	TAC	Sham	TAC	Sham	TAC	Sham	TAC
n	5	5	3	3	5	5	4	4
LVH markers (folder change)	LV							
ANP	1.00 ± 0.13	9.54 ± 1.02 ***	1.01 ± 0.06	10.47 ± 1.32 *	1.00 ± 0.13	10.10 ± 0.67 ***	1.00 ± 0.12	9.75 ± 1.10 ***
BNP	1.00 ± 0.07	7.05 ± 0.30 ***	0.99 ± 0.13	6.16 ± 0.46 **	1.00 ± 0.08	5.56 ± 0.36 ***	1.01 ± 0.09	5.63 ± 0.75 **
a-SA	1.00 ± 0.08	5.28 ± 0.19 ***	1.00 ± 0.06	4.81 ± 0.54 *	1.00 ± 0.09	4.90 ± 0.29 ***	1.00 ± 0.08	4.45 ± 0.38 ***
LVH markers (folder change)	CMs							
ANP	1.00 ± 0.10	9.79 ± 0.39 ***	1.02 ± 0.17	10.43 ± 1.32 **	1.00 ± 0.12	9.12 ± 0.92 ***	1.00 ± 0.10	9.35 ± 1.08 ***
BNP	1.00 ± 0.07	7.27 ± 0.77 ***	1.00 ± 0.06	8.74 ± 1.61 ***	1.00 ± 0.09	7.41 ± 0.97 ***	1.01 ± 0.10	8.32 ± 0.82 **
a-SA	1.00 ± 0.10	4.39 ± 0.40 ***	1.00 ± 0.06	3.70 ± 0.32 ***	1.00 ± 0.09	4.58 ± 0.48 **	1.00 ± 0.11	3.81 ± 0.50 ***

Table 2. Early markers of LVH induction in response to left ventricular pressure overload in WTLs and *Piezo1*^{P1-tdT/P1-tdT} mice. Relative mRNA expression of ANP (*Nppa*), BNP (*Nppb*) and α -SA (*Acta1*) in LV tissue and cardiomyocytes (CMs) 2 days and 14 days after TAC compared to sham (n = 3-5/group). The relative mRNA expression was normalized with GAPDH and calculated as fold change relative to sham in 2 days and 14 days groups, respectively. Results are presented as mean $\pm$ SEM, Two-way ANOVA followed with Tukey's post-hoc test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, compared between sham- and TAC-operated groups.

Figures

Figure 1

Piezo1 expression was upregulated in response to left ventricular (LV) pressure overload in *Piezo1*^{P1-tdT/P1-tdT} mice. (A) Relative Piezo1 mRNA expression, (B) representative Western blots of Piezo1 protein expression (top panel) and (C) quantitative densitometric analysis of 320 kDa Piezo1 protein expression (bottom panel) from isolated cardiomyocytes (CMs), brain, whole heart, aorta and lung tissues collected from *Piezo1*^{P1-tdT/P1-tdT} mice under baseline conditions. Relative Piezo1 mRNA and protein expression from isolated cardiomyocytes, brain, heart, aorta, and lung were normalized to GAPDH (n = 3/group). (D) Relative Piezo1 mRNA expression in LV tissue and in LV cardiomyocytes (CMs) 2 days and 14

days after TAC or sham surgery (n = 5/group) from Piezo1^{P1}-tdT/P1-tdT mice. (E) Representative western blots of Piezo1 protein expression in LV tissue and in LV cardiomyocytes. (F) Quantitative densitometric analysis of 320 kDa Piezo1 protein expression from LV tissue and LV cardiomyocytes 2 days and 14 days after TAC from Piezo1^{P1}-tdT/P1-tdT mice (n = 4/group). Relative Piezo1 mRNA and protein expression in the LV tissue and cardiomyocytes were normalized to GAPDH and calculated as fold change relative to sham in the 2 day and 14 day of groups, respectively. Results are presented as mean ± SEM, Two-way ANOVA followed with Tukey's post-hoc test, **p < 0.01, ***p < 0.001 vs. sham-operated groups, n.s., no significant difference. Figure 1 –source data 1. Source data file (Excel) for Figure 3A, C, D and F.

Figure 2

Cardiomyocyte-specific tamoxifen inducible Piezo1 KO. (A) Experimental design timeline for tamoxifen induction, TAC surgery and serial echocardiograms. (B) A schematic diagram showing the generation of cardiomyocyte-specific Piezo1 knockout (KO) mice. (C) Confirmation of Cre-recombination by PCR of cardiac genomic DNA extracted from isolated CMs from P1^{fl}/flMCM^{+/+} hearts treated with tamoxifen or with peanut oil (n = 3/group). (D) Piezo1 mRNA expression confirmed by RT-PCR measured from isolated CMs from P1^{fl}/flMCM^{+/+} hearts treated with tamoxifen (Piezo1 KO, n = 10/group) or

with peanut oil (n = 6) using GAPDH as an internal control. (E-L) Echocardiographic measurements following tamoxifen treatment of α MHC-MCM $^{+/-}$, Piezo1 KO mice and P1 $^{fl/fl}$ MCM $^{+/-}$ controls showing (E) BW, Body weight. (F) HR, heart rate. (G) LVEDV, LV end-diastolic volume. (H) LVESV, LV end-systolic volume. (I) LVEF, LV ejection fraction. (J) CO, cardiac output. (K) h/r, LV wall thickness to chamber radius ratio. (L) LV mass. Results are presented as mean $\pm$ SEM, Two-tailed Student's T-test and One-way ANOVA followed with Bonferroni post-hoc test, ***p < 0.001 vs. peanut oil treated cardiomyocytes. Figure 2—source data 1. Source data file (Excel) for Figure 4C, D, E, F, G, H, I, J, K and L.

Figure 3

Piezo1 KO mice exhibit less LVH in response to pressure overload. (A) BW, body weight. (B) HR, heart rate. (C) LVSP, LV systolic pressure. (D) LVEF, LV ejection fraction. (E) h/r, LV wall thickness to chamber radius ratio. (F) LV mass. (G) Representative photos indicate heart size differences 14 days after TAC or sham surgery in α MHC-MCM $^{+/-}$ mice, Piezo1 KO mice and P1 $^{fl/fl}$ MCM $^{+/-}$ controls (n = 6/group). (H-K) Anatomical analysis of (H) HW, heart weight. (I) HW/BW, heart weight to body weight ratio. (J) LVW/BW, left ventricular weight to body weight ratio. (K) LVW/TL, left ventricular weight to tibia length (n = 6-10/group). (L) Representative micrographs of Masson's trichrome staining of LV tissue. (M) Quantitation of

Masson's trichrome staining of LV tissue 14 days after TAC or sham surgery (n = 5/group), scale bar = 200 μ m in (L). (N) Relative Collagen III (Col3a1) mRNA expression 14 days after TAC or sham surgery (n = 6/group). The mRNA relative expression was normalized with GAPDH and calculated as fold change relative to sham in P1fl/fMCM+/- mice treated with peanut oil. Results are presented as mean $\pm$ SEM, Two-way ANOVA followed with Tukey's post-hoc test, *p < 0.05, ***p < 0.001 vs. sham-operated groups, ###p < 0.001, vs. TAC groups, n.s.: no significant difference. Figure 3–source data 1. Source data file (Excel) for Figure 1A, B, C, D, E, F, G, H, I, and J.

Figure 4

Comparison of gene expression of LVH markers in response to TAC-induced pressure overload in Piezo1 KO and control α MHC-MCM^{+/+} mice. (A) Relative mRNA expression of ANP (Nppa), BNP (Nppb) and α -SA (Acta1) in LV tissue (left panel) and in LV cardiomyocytes (CMs, right panel) 2 days after TAC compared to sham-operated mice (n = 3-4/group). (B) Relative mRNA expression of ANP (Nppa), BNP (Nppb) and α -SA (Acta1) in LV tissue (left panel) and in LV cardiomyocytes (CMs, right panel) 14 days after TAC compared with sham-operated mice (n = 4-5/group). The mRNA relative expression was normalized with GAPDH and calculated as fold change relative to α MHC-MCM^{+/+} sham-operated hearts in 2 days and 14 days groups, respectively. Results are presented as mean $\pm$ SEM, Two-way ANOVA followed with Tukey's post-hoc test, ***p < 0.001 vs. sham-operated groups, ###p < 0.001 vs. TAC groups, n.s., no significant difference. Figure 4–source data 1. Source data file (Excel) for Figure 2A and B.

Figure 5

TAC-induced changes in the CaMKII-HDAC4-MEF2 signalling pathway in Piezo1 KO and α MHC-MCM^{+/-} mouse hearts. (A) Representative western blots showing the expression of key proteins in the CaMKII-HDAC4-MEF2 signalling pathway in the cytoplasm (left panel) and nucleus (right panel). (B) Cytoplasmic (left panel) and nuclear (right panel) quantitative data were normalized with GAPDH and Histone H2B, respectively. Fold changes and cytoplasmic/nuclear ratios were calculated relative to sham groups for each genotype (n = 6/group). Results are presented as mean $\pm$ SEM, Two-way ANOVA followed with Tukey's post-hoc test, **p < 0.01, ***p < 0.001 vs. sham-operated group. Figure 5–source data 1. Source data file (Excel) for Figure 7B.

Figure 6

Calcineurin-NFAT signalling pathway in α MHC-MCM^{+/-} and Piezo1 KO mouse hearts 2 days post-TAC. (A) Representative Western blots showing the expression of NFAT and GATA4 in cytoplasm (left) and nucleus (right). (B) Cytoplasmic and nuclear (right) quantitative data were normalised by GAPDH and Histone H2B, respectively (n = 6/group). Fold changes and nuclear/cytoplasmic ratio were calculated relative to sham groups, in each genotype. Results are presented as mean $\pm$ SEM, Two-way ANOVA followed with Tukey's post-hoc test, **p < 0.01, ***p < 0.001 vs. sham-operated group.

Figure 7

Gene expression and protein levels of selected Ca²⁺ handling molecules 2 days after TAC in Piezo1 KO and αMHC-MCM^{+/-} mouse hearts. (A- D) Relative mRNA expression of; (A) *Trpm4*, (B) *Piezo1*, (C) *Slc8a1* and (D) *Cacna1h* in LV tissue 2 days after sham or TAC. (E) Representative western blots of TRPM4, NCX1 and Cav3.2 in LV tissue. (F-H) Western blots from LV tissue after 2 days of TAC were used to quantify the protein levels of; (F) TRPM4, (G) NCX1 and (H) Ca_{v3.2}. Relative mRNA (n = 5/group) and protein expression (n = 6/group) in the LV tissue was normalized to GAPDH and calculated as fold change relative to the 2 days post-sham group, respectively. Results are presented as mean ± SEM, Two-way ANOVA followed with Tukey's post-hoc test, **p < 0.01 vs. sham-operated groups; ### p < 0.001 vs. TAC-operated groups. Figure 7– source data 1. Source data file (Excel) for Figure 6A, B, C, D, F, G and H.

Figure 8

Schematic of the Piezo1-dependent signalling pathway that drives LVH secondary to pressure overload. Piezo1 is the cardiomyocyte mechanotransducer that converts the increased mechanical forces secondary to pressure overload into Ca^{2+} entry into the cell. This increases local $[\text{Ca}^{2+}]$, resulting in TRPM4 activation. The Na^{+} -permeable TRPM4 activity then likely induces reverse activity of the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger through local Na^{+} loading. This leads to a high-amplitude increase in local $[\text{Ca}^{2+}]$. Calmodulin responds to this high-amplitude Ca^{2+} stimulus through the lower affinity Ca^{2+} binding site at its N-lobe, which then preferentially activates CaMKII and thus stimulates the CaMKII-HDAC4-MEF2 pathway to induce LVH6. In contrast, calcineurin is activated preferentially by low-amplitude Ca^{2+} signalling via Gq-coupled receptors and calmodulin, and calcineurin activation is strongly inhibited by activated CaMKII. ECM: extracellular matrix, CaM: calmodulin.

Supplementary Files

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