Myocardial pathology induced by aldosterone is dependent on non-canonical activities of G protein-coupled receptor kinases

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Hyper-aldosteronism is associated with myocardial dysfunction including induction of cardiac fibrosis and maladaptive hypertrophy. Mechanisms of these cardiotoxicities are not fully understood. Here we show that mineralocorticoid receptor (MR) activation by aldosterone leads to pathological myocardial signalling mediated by mitochondrial G protein-coupled receptor kinase 2 (GRK2) pro-death activity and GRK5 pro-hypertrophic action. Moreover, these MR-dependent GRK2 and GRK5 non-canonical activities appear to involve cross-talk with the angiotensin II type-1 receptor (AT1R). Most importantly, we show that ventricular dysfunction caused by chronic hyper-aldosteronism in vivo is completely prevented in cardiac Grk2 knockout mice (KO) and to a lesser extent in Grk5 KO mice. However, aldosterone-induced cardiac hypertrophy is totally prevented in Grk5 KO mice. We also show human data consistent with MR activation status in heart failure influencing GRK2 levels. Therefore, our study uncovers GRKs as targets for ameliorating pathological cardiac effects associated with high-aldosterone levels.
The classic neurohormonal model of heart failure (HF) is rooted in the enhancement of molecules, such as sympathetic catecholamine neurotransmitters and over-activation of the renin–angiotensin–aldosterone system (RAAS). Aldosterone, a hormone secreted by the adrenal cortex, is directly involved in the regulation of blood pressure. Aldosterone has also been implicated in the pathogenesis of HF as patients have markedly elevated plasma aldosterone concentrations and increased aldosterone after myocardial infarction (MI) has been implicated in HF progression. Moreover, cardiac expression of aldosterone’s mineralocorticoid receptor (MR) has been shown to be elevated in HF patients. In this regard, recent evidence indicates that chronic exposure to high-aldosterone levels and persistent activation of MRs can induce myocardial tissue damage via mechanisms that are independent of blood pressure elevation. In fact, chronic infusion of aldosterone can lead to increased cardiac fibrosis and pathological hypertrophy. Accordingly, MR antagonists such as spironolactone and eplerenone have emerged as key drugs in the armamentarium against HF to combat cardiac dysfunction associated with chronic hyper-aldosteronism.

The underlying mechanisms of these deleterious effects are not completely understood and thus, there is an urgent need to uncover molecular mechanisms involved in aldosterone-mediated cardiac dysfunction to identify new molecular targets and improve HF therapy. Recently, it has been shown that, through a ‘non-genomic’ mechanism, aldosterone can activate NADPH oxidases (NOX2 and 4), thus increasing reactive oxygen species (ROS) and eliciting an apoptotic and fibrotic response. Intriguingly, some of the effects of aldosterone in the heart can be attributed to a linkage with G protein-coupled receptor (GPCR) signalling. In particular, it appears that aldosterone can activate cross-talk between the MR and the angiotensin II (AngII) type-1 receptor (AT1R), a GPCR critically involved in both hypertension and HF progression.

Since the AT1R is implicated in aldosterone-mediated cardiac dysfunction, we posited that also GPCR kinases (GRKs), as regulator of this receptor, may be involved in this deleterious mechanism. In particular, we looked at GRK2 and GRK5, the major GRKs found in the heart, since both have been linked to HF development and progression, and the levels of these kinases are elevated in human failing myocardium. Of note, GRK2 but not GRK5, has been shown to desensitize AngII responses in the heart; however, recent evidence show that these kinases can trigger pathological myocardial signalling independent of direct GPCR regulation. These non-canonical GRK activities include the unique mitochondrial localization of GRK2 promoting cell death, and the translocation of GRK5 within the nucleus of myocytes promoting pathological hypertrophic gene transcription. The latter indeed occurs in the heart downstream of AT1R activation.

In this study, we have found that these non-GPCR activities of GRK2 and GRK5 are directly involved in the pathological MR-AT1R signalling axis in the heart. By using in vitro systems and in vivo mouse models, we have uncovered a previously unknown dependence of GRK2 and GRK5 within cardiomyocytes in aldosterone-mediated cardiac dysfunction.

Results

Aldosterone activates myocyte AT1R signalling via c-Srcβ-arrestin. Aldosterone treatment of cardiomyocytes is associated with a ‘rapid’ ERK 1/2 activation response that has been suggested to depend on the cross-talk between the MR and the AT1R. Indeed, in ventricular myocytes isolated from neonatal rats (NRVMs), we found ERK activation by aldosterone peaking after 15 min (Fig. 1a). Importantly, pre-treatment of cells with spironolactone, a MR antagonist, or losartan, an AT1R antagonist, both could inhibit aldosterone-mediated ERK activation (Fig. 1a), indicating MR-AT1R cross-talk. ERK activation via GPCRs, and in particular the AT1R, can occur via both G protein-dependent and G protein-independent pathways. The latter occurs via the combined action of, GRKs and β-ARRESTINS. We therefore performed experiments to establish whether aldosterone causes AT1R-mediated β-arrestin recruitment and interestingly, found to be the case (Fig. 1b). Membrane β-ARRESTIN localization stimulated by aldosterone was attenuated with losartan pre-treatment, suggesting aldosterone-mediated activation of the AT1R endocytic machinery (Fig. 1b). Next, since C-SRC is involved in aldosterone-mediated ERK activation and is crucial for the β-ARRESTIN-mediated process of internalization and signalling transduction of the AT1R, even in the absence of agonist, we assessed whether c-Src might play a role in this mechanism. To test this, we used the Src family kinase inhibitor, PP2 and we found that PP2 pre-treatment of cells could inhibit aldosterone-mediated AT1R activation in myocytes (Fig. 1c).

Further, using an adenovirus (Ad) carrying a HA-tagged AT1R (Ad-HA-AT1R) cDNA we performed a co-immunoprecipitation (Co-IP) assay to evaluate whether c-Src inhibition can affect the β-ARRESTIN recruitment to the AT1R. In line with previous results, β-ARRESTIN recruitment was not affected in the presence of PP2 (Fig. 1d). Moreover, using an Ad-HA-AT1R and an Ad encoding for the MR to infect NRVMs, we found that aldosterone can induce the internalization of the AT1R similar to the receptor internalization induced by AngII (Fig. 1e). Interestingly, spironolactone, losartan as well as PP2 blocked aldosterone-mediated AT1R internalization (Fig. 1e). Thus, both AT1R internalization and ERK activation induced by aldosterone is C-SRC-dependent and appears to involve β-arrestins. To further support this mechanism, we used the βARKct, a peptide inhibitor of GRK2 activation via Gβγ, sequestration. βARKct expression reduced the activation of ERK by both MR and AT1R (Supplementary Fig. 1a).

Critical role for GRK2 in MR-mediated myocardial pathology. Since the above data clearly show a myocyte MR-signalling dependence on AT1R and implicates GRK2 in this mechanism, we next examined whether this receptor cross-talk is involved in aldosterone-mediated pathology in myocytes and its potential downstream signal transduction. We examined a NOX4-dependent ROS mechanism as a potential inducer of deleterious myocyte effects of aldosterone since that appears important in HF pathogenesis. We found induction of NOX4 expression after aldosterone treatment of myocytes and this was blocked by both spironolactone and losartan (Fig. 2a). Aldosterone-mediated NOX4 upregulation was also blocked by βARKct expression (Supplementary Fig. 1b). Next, we assessed ROS formation in myocytes using MitoSOX Red as an indicator of NOX4 activity since it can detect superoxide production. As shown in Fig. 2b, aldosterone (30 min of exposure) increased mitochondrial ROS generation and this was blocked by antagonizing both the MR and AT1R as well as GRK2 via the βARKct. Further, we observed increased mitochondrial dysfunction, in response to 24 h of aldosterone using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and, this was blocked by MR and AT1R antagonism and also by GRK2 inhibition (Fig. 2c).

Importantly, treatment of myocytes with aldosterone (24 h) induced a significant apoptotic response that was MR, AT1R and GRK2 dependent (Fig. 2d), consistent with the above signalling mechanisms. Since βARKct could block aldosterone-mediated NOX4 induction, ROS generation and myocyte apoptosis, we explored...
whether GRK2 played a direct role in this MR-dependent pathological signalling. Interestingly, we found that aldosterone treatment of myocytes for 12 h causes significant upregulation of GRK2 that was dependent on both MR and AT1R activation (Fig. 2e). To link GRK2 induction to aldosterone-mediated myocyte pathology, we overexpressed GRK2 in NRVMs and examined apoptosis, which we found to be significantly enhanced with GRK2 overexpression (Fig. 3a). Aldosterone can also induce hypertrophy of cardiomyocytes\(^2\) and thus, we also examined this pathological stress condition. Indeed, in control NRVMs treated with Ad encoding the green fluorescent protein (Ad-GFP), aldosterone induced increased cell size, as expected, however, unlike enhancement of apoptosis with Ad-GRK2 treatment, overexpression of GRK2 had no effect on cardiomyocyte hypertrophy in response to aldosterone (Fig. 3b).

The above data suggest that GRK2, downstream of MR activation, specifically regulates aldosterone-mediated ROS and cell death signalling. To better assess the absolute requirement of GRK2 in mitochondrial dysfunction and ROS generation, we treated cultured NRVMs with siRNA against GRK2 and as shown in Supplementary Fig. 2a,b, specific GRK2 silencing abolished the effects of aldosterone on ROS generation.

In this regard, our laboratory has shown that secondarily to ROS generation, GRK2 becomes phosphorylated by ERK at Ser670 (S670) and this causes mitochondrial localization that is dependent on the chaperone, heart shock 90 (Hsp90) protein, leading to an induction of myocyte apoptosis\(^29\). Accordingly, we investigated S670 phosphorylation (pS670) of GRK2 following aldosterone treatment and found that MR activation leads to GRK2 phosphorylation that is ERK dependent (Fig. 3c;

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**Figure 1 | Aldosterone-mediated cross-talk between the MR and the AT\(_1\)R.** (a) Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) of multiple (\(n = 3\)) independent experiments to evaluate ERK 1/2 phosphorylation (pERK) as a ratio of activated ERK to total ERK (tERK) in neonatal rat ventricular myocytes (NRVMs) either unstimulated (Ns) or stimulated with aldosterone (Aldo 1 \(\mu M\)) for 15 min. Before Aldo treatment, myocytes were pre-treated with spironolactone (Spiro 10 \(\mu M\)) or losartan (Los 10 \(\mu M\)) for 30 min; *\(P < 0.05\) versus Ns. (b) Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) of multiple (\(n = 3\)) independent experiments to evaluate \(\beta\)-arrestin membrane recruitment in crude plasma membrane preparations from NRVMs. Shown is a time course (0–30 min) of Aldo (1 \(\mu M\)) treatment alone or with 30 min pre-treatment with Los (10 \(\mu M\)). \(\beta\)-ACTIN was used as loading control; *\(P < 0.05\) versus Ns. (c) Representative immunoblots (upper panels) and densitometric analysis (lower panel) of multiple (\(n = 3\)) independent experiments to evaluate pERK in NRVMs Ns or stimulated with Aldo (1 \(\mu M\)) for 15 min. Before Aldo, a group of cells was pre-treated with PP2 (10 \(\mu M\)) for 30 min. tERK was used as loading control; *\(P < 0.05\) versus Ns. (d) Representative panels (upper panels) and densitometric analysis (lower panel) of multiple (\(n = 3\)) independent experiments of Co-IP assay in total lysates from NRVMs infected with HA-tagged AT\(_1\)R, Cells were Ns or stimulated with Aldo (1 \(\mu M\)) for 30 min. Before stimulation, a group of cells was pre-treated with the c-Src inhibitor PP2 (10 \(\mu M\)). Immunoprecipitated proteins (IP) for HA-tag were blotted with an antibody anti-\(\beta\)-arrestin 1/2 antibody; *\(P < 0.05\) versus Ns. (e) Representative immunofluorescence images of NRVMs infected with HA-tagged AT1R (red, lower panels). DAPI-stained myocyte nuclei are shown in the upper panel of images. Shown are cells treated for 30 min with Aldo (1 \(\mu M\)) or AngII (1 \(\mu M\)) or before Aldo, pre-treated for 30 min with Spiro (10 \(\mu M\)), Los (10 \(\mu M\)) or PP2 (10 \(\mu M\)). Arrows indicate receptors that are internalized. Scale bar, 20 \(\mu M\). (a–d) Statistical significance between groups was determined by one-way ANOVA with Bonferroni post hoc correction. All data are shown as mean ± s.e.m.
Supplementary Fig. 2c). To assess whether the aldosterone-MR-ERK signalling axis could induce GRK2 translocation to mitochondria, we purified mitochondrial fractions from NRVMs treated with aldosterone and found significant mitochondrial GRK2 accumulation (Fig. 3d). Interestingly, blocking MR activation or AT1R activation inhibited mitochondrial translocation of GRK2 (Fig. 3d). To establish the role of GRK2 phosphorylation on ROS generation, we analysed the effects of elevated wild-type (WT) GRK2 in NRVMs versus a mutant GRK2 at Ser670 (GRK2-S670A) that cannot translocate to the mitochondria (Fig. 3e,f). As expected, following aldosterone treatment, WT GRK2 accumulated in mitochondria (Fig. 3e,f) and this was accompanied to an augmented ROS generation (Fig. 3g). Importantly, when GRK2 cannot be phosphorylated at S670, aldosterone did not lead to mitochondrial translocation of GRK2 and subsequent ROS generation was suppressed (Fig. 3g).

Previously, the ROS-, ERK- and Hsp90-dependent mitochondrial targeting of GRK2 was found to be inhibited by βARKct, since this peptide also contains S670 and ERK phosphorylation of βARKct, which competes with endogenous GRK2 for Hsp90 binding29. We found that aldosterone-mediated GRK2 mitochondrial translocation also appears consistent with this model as βARKct expression blocked aldosterone-mediated mitochondrial GRK2 accumulation (Supplementary Fig. 2d). As expected, βARKct expression did not inhibit mitochondrial translocation of Hsp90 downstream of aldosterone (Supplementary Fig. 2d). Further, we found that this intracellular targeting of GRK2 is dependent on the actions of AT1R transactivation-mediated β- arrestin function, since knockdown of β-arrestin 1/2 in NRVMs abolished GRK2 mitochondrial accumulation after aldosterone stimulation (Supplementary Fig. 2e).

Our data above suggest that aldosterone through a MR-AT1R-dependent pathway, induces a rapid response that increases ERK with a consequent increase in ROS generation. However, previous reports have shown that aldosterone can directly activate the oestrogen receptor (GPER) that is responsible for the rapid ERK activation observed following aldosterone stimulation39.
To clarify whether aldosterone effects in myocytes are due to a GPER or MR-AT\(_1\)R pathway, we used the GPER agonist E2 and the GPER antagonist G36. As expected, E2 stimulation resulted in a significant ERK activation that was efficiently blocked by G36 (Supplementary Fig. 3a). Notably, pre-treatment of cells with G36 resulted in a significant but incomplete inhibition of aldosterone-mediated ERK phosphorylation that was much less effective at blocking aldosterone signalling compared to spironolactone and losartan suggests that GPERs can be modestly involved in acute ERK activation mediated by aldosterone (Supplementary Fig. 3b). Since a recent report has shown that genetic silencing of GPER further increased aldosterone-induced ROS production with mechanisms that may suggest GPER-dependent inhibition of deleterious AT\(_1\)R or MR signalling\(^{40}\), we explored the effects of GPER activation/inhibition on aldosterone-dependent GRK2 phosphorylation and ROS generation. Surprisingly, we found that E2 treatment of NRV\(\text{M}\)s resulted in a consistent reduction of basal phosphorylation of GRK2, at S670, that was restored by G36 (Supplementary Fig. 3c). Most importantly, we found that GPER inhibition did not affect aldosterone-dependent GRK2 phosphorylation (Supplementary Fig. 3d). In line with these results, we found that GPER activation was not able to increase ROS generation, and its inhibition with G36 also did not affect aldosterone-dependent ROS generation (Supplementary Fig. 3e). However, treatment of cells with both E2 and aldosterone resulted in an impaired cardiotoxicity compared to aldosterone alone-treated cells (Supplementary Fig. 3e). Taken together, these data suggest that GPER activation downstream of high aldosterone are not a major player in myocyte pathology as previously suggested\(^{39}\), and it appears to be due to MR-AT\(_1\)R signalling.

**GRK5 is a regulator of MR/AT\(_1\)R-mediated cardiomyocyte hypertrophy.** The above results show that, although GRK2’s non-canonical actions on mitochondrion-mediated cell death appear activated by MR-AT\(_1\)R signalling, there was no effect on cardiac hypertrophy, prompted us to investigate another GRK involved in myocardial pathology, GRK5. The role of GRK5 in maladaptive hypertrophy has been shown to also entail non-canonical activity as a class II histone deacetylase (HDAC) kinase and nuclear factor

![Image of Figure 3](https://example.com/figure3.png)

**Figure 3** | Aldosterone-mediated GRK2 mitochondrial localization in myocytes. (a,b) Representative images and quantitative data from (n = 3) independent experiments showing (a) apoptotic (TUNEL staining; scale bar, 100 μm) NRV\(\text{M}\)s (~1,000 cells analysed for the group for each experiment) or (b) hypertrophic (α-sarcomeric actin staining; scale bar, 50 μm) NRV\(\text{M}\)s (~200 cells analysed for the group for each experiment) infected with either Ad-GFP or Ad-GRK2 to determine the effect of Aldo with GRK2 overexpression. Myocytes were unstimulated (Ns) or stimulated with Aldo (1 μM) for 24 (a) or 48 h (b). *P < 0.05 versus GFP Ns; \#P < 0.05 versus GFP Aldo. (c) Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) of multiple (n = 3) independent experiments to evaluate GRK2 phosphorylation (ser670) or ERK 1/2 phosphorylation (pERK) levels in NRV\(\text{M}\)s Ns or stimulated with Aldo (1 μM) for 15 min. Before Aldo, a group of cells was pre-treated with U0126 (3 μM) for 30 min. Total ERK (tERK) and GRK2 are shown as loading controls; *P < 0.05 versus Ns. (d) Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) of multiple (n = 3) independent experiments to evaluate GRK2 levels in mitochondrial fractions purified from NRV\(\text{M}\)s that were either Ns or stimulated with Aldo (1 μM) for 30 min. Before Aldo, a group of cells was pre-treated with Spiro (10 μM) or Los (10 μM) for 30 min. Dvac was used as loading and mitochondrial purity control; *P < 0.05 versus Ns. (e,f) Representative immunoblots (e) and densitometric quantitative analysis (f) of multiple (n = 3) independent experiments to evaluate GRK2 levels in mitochondrial fractions purified from NRV\(\text{M}\)s infected with Ad-GRK2 and Ad-GRK2-S670A that were either Ns or stimulated with Aldo (1 μM) for 30 min. Dvac was used as loading and mitochondrial purity control; *P < 0.05 versus GRK2 Ns. (g) Cumulative fluorescence data from (n = 3) independent experiments of MitoSOX Red staining of NRV\(\text{M}\)s (~200 cells analysed for the group for each experiment) infected with adenoviruses encoding for either GRK2 or GRK2-S670A. Myocytes were then Ns or stimulated for 30 min with Aldo. *P < 0.05 versus GRK2 Ns; \#P < 0.05 versus GRK2 Aldo. (a-d,f) Statistical significance between groups was determined by one-way ANOVA with Bonferroni post hoc correction. All data are shown as mean ± s.e.m.
of activated T-cells (NFAT) activator/co-factor where increased nuclear accumulation of GRK5 induces pathological gene transcription.\(^\text{27,31}\). In fact, AngII, which is a potent inducer of myocardial hypertrophy, can induce GRK5 translocation to the nucleus and interestingly, the AT\(_R\); R is not a target of GRK5-mediated desensitization.\(^\text{27}\). Importantly, it has been previously shown that high-aldosterone levels can increase, in the heart, transcription of the myocyte enhancer factor-2 (MEF2) through the activation of Ca\(^{2+}\)/calmodulin kinase II (CAMKII).\(^\text{32}\). Further, aldosterone can activate the calcineurin-NFAT pathway, in an AT\(_R\); R-dependent manner.\(^\text{4}\). Notably, these mechanisms are similar to the proposed model of pathological cardiac hypertrophy induced by the AT\(_R\) that is dependent on GRK5 (ref. 27). To test the hypothesis that aldosterone might induce this nuclear activity of GRK5, we first looked at NRVMs and found that aldosterone was indeed an inducer of GRK5's nuclear accumulation similar to AngII (Fig. 4a). Moreover, in adult murine ventricular myocytes we found similar induction of nuclear translocation of GRK5 via immunofluorescence (Fig. 4b). GRK5's nuclear localization after aldosterone was blocked by spironolactone treatment as well as losartan (Fig. 4c). GRK5 nuclear localization was dependent on GRK5 activity as GRK5 knockdown assay (Fig. 4e), we confirmed the absolute requirement of GRK5 in MEF2 activation (Fig. 4f) and in cardiac hypertrophy (Fig. 4g) downstream of aldosterone. Consistent with this, we found that overexpression of GRK5 enhanced NRVM cell size after 48 h of aldosterone treatment compared to the normal hypertrophy induced by aldosterone treatment without elevated GRK5 levels (GFP;control cells; Fig. 5a).

To better define the role of GRK5 and its nuclear activity being involved in aldosterone-mediated myocyte hypertrophy, we tested outcomes of elevated WT GRK5 versus overexpression of a mutant GRK5 lacking its nuclear localization signal (GRK5-ΔNLS). As expected, we observed that aldosterone treatment of myocytes induced consistent GRK5 nuclear localization with a robust increase in cell size growth (Fig. 5b–d). However, when GRK5-ΔNLS was overexpressed, there was no nuclear GRK5 localization and significantly diminished aldosterone-dependent cardiac hypertrophy (Fig. 5b–d).

To better evaluate the specific mechanism involved in MR-AT\(_R\); R-dependent GRK5 nuclear translocation, we explored

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**Figure 4 | Aldosterone-mediated GRK5 nuclear localization and hypertrophic response.** (a) Representative immunoblots (upper panels) and densitometric analysis (lower panel) of multiple independent experiments (\(n=3\)) to evaluate GRK5 levels in nuclear fractions purified from NRVMs, unstimulated (Ns) or stimulated with Aldo (1 \(\mu\)M) or AngII (1 \(\mu\)M) for 30 min. Fibrillarin was used as loading control; \(*P<0.05\) versus Ns. (b) Representative panels of DAPI (upper) and GRK5 (bottom) immunofluorescence images in adult ventricular myocytes. The cells were Ns or stimulated with Aldo (1 \(\mu\)M) or AngII (1 \(\mu\)M) for 30 min; scale bar, 10 \(\mu\)m. (c) Representative immunoblots (upper panels) and densitometric analysis (lower panel) of multiple independent experiments (\(n=3\)) to evaluate GRK5 levels in nuclear fractions purified from NRVMs Ns or stimulated with Aldo (1 \(\mu\)M) for 30 min. Before Aldo, a group of cells was pre-treated with Spiro (10 \(\mu\)M) or Los (10 \(\mu\)M) for 30 min. Fibrillarin was used as loading control; \(*P<0.05\) versus Ns. (d) Bar graph showing MEF2 reporter activity in NRVMs measured using a luciferase assay system. Cells were infected with an Ad encoding for MEF2 promoter-luciferase (Ad-MEF2-Luc) reporter construct for 48 h. Following the infection, the cells were Ns or stimulated for 24 h with Aldo (1 \(\mu\)M). Before Aldo, a group of cells was pre-treated with Spiro (10 \(\mu\)M) or Los (10 \(\mu\)M) for 30 min; \(*P<0.05\) versus Ns. (e) Representative immunoblots showing total GRK5 levels in NRVMs transfected with siRNAs targeting GRK5 (siGRK5). Scrambled siRNAs (siScr) were used as control. The cells were then Ns or stimulated for 24 h with Aldo (1 \(\mu\)M). (f) Bar graph showing MEF2 reporter activity in NRVMs measured using a luciferase assay system. Cells were infected with an Ad-MEF2-Luc and transfected with siRNAs targeting GRK5 (siGRK5). siScr were used as control. The cells were then Ns or stimulated for 24 h with Aldo (1 \(\mu\)M); \(*P<0.05\) versus siScr. (g) Cumulative fluorescence data from (\(n=3\)) independent experiments of \(\alpha\)-sarcomeric actinin staining in NRVMs (~200 cells analysed for the group for each experiment) transfected with siGRK5 or siScr. The cells were Ns or stimulated with Aldo (1 \(\mu\)M) for 48 h; \(*P<0.05\) versus siScr Ns. (a c d f g) Statistical significance between groups was determined by one-way ANOVA with Bonferroni post hoc correction. All data are shown as mean \(\pm\) s.e.m.
the ability of β-arrestin recruitment to influence the nuclear translocation of GRK5. Interestingly, we found that knockdown of β-arrestin 1/2 abolished GRK5 nuclear translocation downstream of aldosterone (Supplementary Fig. 4a). Finally, we explored the ability of aldosterone to induce the activation of Ca²⁺-calmodulin (CaM) that previously has been shown to be the nodal regulator of AngII-mediated GRK5-nuclear accumulation³¹. We pre-treated NRVMs with losartan, to inhibit the AT₁R, and we stimulated the cells with aldosterone. Following this, we performed a Co-IP assay between GRK5 and CaM and importantly, we observed that while aldosterone induced a significant increase in CaM binding to GRK5, this was blocked by β-arrestin 1/2 abolished GRK5 nuclear translocation downstream of aldosterone (Supplementary Fig. 4a). Finally, we explored the ability of aldosterone to induce the activation of Ca²⁺-calmodulin (CaM) that previously has been shown to be the nodal regulator of AngII-mediated GRK5-nuclear accumulation³¹. We pre-treated NRVMs with losartan, to inhibit the AT₁R, and we stimulated the cells with aldosterone. Following this, we performed a Co-IP assay between GRK5 and CaM and importantly, we observed that while aldosterone induced a significant increase in CaM binding to GRK5, this was blocked by

**In vivo effects of aldosterone are mediated by GRK2 and GRK5.** After MI in humans, plasma aldosterone concentrations are robustly increased¹³ and the augmented concentration of this hormone can negatively affect cardiac function and HF progression¹³. To determine if any of the above in vitro results translate in vivo, we administered aldosterone to mice for 4 weeks to create a state of hyper-aldosteronism. Following chronic administration of aldosterone (2 μg per day for 4 weeks) via mini-osmotic pumps implanted subcutaneously, serum aldosterone levels were similar to the endogenous upregulation of this hormone observed in the blood serum of post-MI mice at 4 weeks (Supplementary Fig. 5a), which is consistent with reports in the literature²,¹³,⁴³,⁴⁴ of a ~2-fold increase after MI, including in humans.

We assessed the effects of 4 weeks of aldosterone treatment on murine cardiac function. As shown in Table 1, aldosterone induced significant pathology indicated by ventricular dysfunction and adverse remodelling, including cardiac hypertrophy. At the tissue level, we found increased cardiac apoptosis, as shown by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (Fig. 6a), and increased myocardial fibrosis as assessed by Picro-Sirius red staining (Fig. 6b) in mice treated for 4 weeks with aldosterone. Consistent with the significant ventricular dysfunction observed in aldosterone-treated mice, GRK2 and GRK5 were upregulated in the heart (Fig. 6c,d). Interestingly, the in vivo increases in both GRKs, induced by aldosterone, resulted in the enhancement of their non-canonical pathological cellular locations as these hearts showed significantly more GRK2 localized to mitochondria (Fig. 6e) and significant GRK5 accumulation in nuclear fractions (Fig. 6f).
To determine the absolute requirement of GRK2 and GRK5 on the pathological myocardial effects of chronic hyper-aldosteronism, we used conditional Grk2 and Grk5 knockout (KO) mice where these kinases were deleted specifically in cardiomyocytes. Control mice (floxed alleles alone), or cardiac Grk2 KO or Grk5 KO mice were treated as above for 4 weeks with aldosterone and we serially assessed their cardiac function with echocardiography. We also included αMHC-Cre alone mice as an additional control for Cre expression in the heart for these experiments. Impaired cardiac function (EF) and increased left ventricular diameter (LVIDd) were similarly altered in control mice (including αMHC-Cre alone mice; Supplementary Fig. 5b,c) consistent with the above results. However, these effects were completely prevented by the loss of GRK2 in myocardies and in part attenuated by the lack of GRK5 in cardiomyocytes (Fig. 7a,b). Of interest, posterior wall (PW) thickness, a marker of ventricular growth, was increased by aldosterone in both controls and Grk2 KO mice (Fig. 7c). However, consistent with the role of GRK5 in the nucleus) in pathological cardiac hypertrophy, no PW increase was observed in Grk5 KO mice (Fig. 7c). To further examine mechanisms of aldosterone-mediated cardiac dysfunction, we analysed cardiac hypertrophy via heart weight and found that indeed after 4 weeks of aldosterone infusion there was significant cardiac hypertrophy as measured by heart weight to body weight (HW/BW) ratios with control mice have the largest hearts (Fig. 7d). Of note, both Grk2 KO and Grk5 KO had only slight increases in HW/BW ratios after 4 weeks of aldosterone compared to controls (Fig. 7d).

Table 1 | Effect of aldosterone treatment on LV function evaluated by echocardiography at 4 weeks after minipump implantation in WT mice.

| WT C57BL/6 | N = 6 | N = 6 |
|-----------|------|------|
| 4 Weeks   | Vehicle | Aldosterone |
| EF (%)    | 58.1 ± 4 | 42 ± 4.1* |
| FS (%)    | 30.4 ± 2.5 | 22.6 ± 4* |
| LVIDd     | 3.5 ± 0.14 | 3.94 ± 0.18* |
| LVIDs     | 2.5 ± 0.22 | 2.78 ± 0.3 |
| PWd       | 5.2 ± 0.04 | 8.85 ± 0.04* |
| PWs       | 5.6 ± 0.05 | 1.1 ± 0.06* |
| HR        | 415 ± 17.1 | 425 ± 21.6 |
| HW/BW     | 5.5 ± 0.2 | 6.4 ± 0.2 |

In vivo ejection fraction (EF) and fractional shortening (FS) percentage, LV internal diameter at diastole (LVIDd), LV internal diameter at systole (LVIDs), posterior wall diastolic thickness (PWd), PW systolic thickness (PWs) and heart rate (HR) were assessed in wild-type (WT) mice at diastole (LVIDd), LV internal diameter at systole (LVIDs), posterior wall diastolic thickness (PWd), PW systolic thickness (PWs) and heart rate (HR) were measured in all groups. All data are shown as mean ± s.e.m. Statistical significance between groups was determined by Mann-Whitney exact test. *P<0.05 versus vehicle.

Figure 6 | In vivo effects of chronic aldosterone treatment on murine myocardium. (a,b) Representative images and quantitative data from mice treated with vehicle (Veh-saline) or aldosterone (Aldo, 2 μg per day) for 4 weeks (n = 6 mice each group). Shown in (a) is TUNEL/DAPI staining of cardiac sections of mice treated with Veh or Aldo and arrows indicate TUNEL-positive nuclei. Shown in (b) is Picro-Sirius red staining from these sections denoting cardiac fibrosis; *P<0.05 versus Veh; scale bar, 100 μm. (c,d) Representative immunoblots and quantitative data (n = 6 mice each group) showing Grk2 (c) and Grk5 (d) protein levels in total cardiac lysates from mice treated with Aldo or Veh for 4 weeks; *P<0.05 versus Veh. (e) Representative immunoblots and quantitative data (n = 6 mice each group) showing Grk2 and HSP90 levels in mitochondrial fractions purified from mouse hearts after 4 weeks of Aldo or Veh treatments. VDAC was used as mitochondrial marker and loading control; *P<0.05 versus Veh. (f) Representative immunoblots and quantitative data (n = 6 mice each group) showing Grk5 levels in nuclear fractions purified from mouse hearts after 4 weeks of Aldo or Veh treatments with LAMIN A/C was used as loading control; *P<0.05 versus Veh. (a-f) Statistical significance between groups was determined by Mann-Whitney exact test. All data are shown as mean ± s.e.m.
To explain the differences observed between PW thickness with loss of expression of GRK2 or GRK5 in myocytes, we evaluated the effects of aldosterone on cardiac apoptosis and fibrosis. Consistent with the above results, chronic hyperaldosteronism caused significant cardiac apoptosis and fibrosis in control mice (Fig. 8a–d) and the loss of GRK2 or GRK5 significantly affected this; however, the loss of GRK2 was significantly more effective at attenuating myocyte death and replacement fibrosis compared to the loss of GRK5 (Fig. 8a–d). As a further proof of this GRK-selective mechanism, we analysed the expression of connective tissue growth factor (CTGF), a surrogate marker of pro-fibrosis shown to be modulated directly by MR. Indeed, in the hearts of aldosterone-treated control mice there was robust upregulation of Ctgf mRNA (Fig. 8e). In cardiac Grk2 KO mice, there was no Ctgf induction while a significant but smaller reduction was observed when GRK5 was deleted in cardiomyocytes compared to control mice (Fig. 8e). These results are consistent with GRK2 being selectively involved in the fibrotic response induced by aldosterone via mitochondrial cell death, while GRK5 appears significantly involved in the hypertrophic mechanisms of pathology induced by this hormone, that is, translationally significant since both GRKs have been shown to be upregulated in human HF.

To further explore the specific role of GRK2 and GRK5 in the aldosterone-dependent fibrotic response, we analysed the effects in vitro, in NRVMs, of the overexpression of GRK2 and GRK5 on Ctgf expression. Importantly, we found in control myocytes that aldosterone induced significant upregulation of CTGF (Supplementary Fig. 6a). Interestingly, GRK5 overexpression had no effect on aldosterone-mediated CTGF upregulation but GRK2 overexpression significantly enhanced this response (Supplementary Fig. 6a). Consistent with GRK2 being nodal in the fibrotic response of aldosterone in myocytes, we took conditional media from aldosterone-treated myocytes and found that the media from GRK2 overexpressing myocytes significantly enhanced the migration of cultured cardiac fibroblasts more than cultured media of control (Ad-GFP) and GRK5 overexpressing myocytes (Supplementary Fig. 6b).

These data strongly suggest that aldosterone-mediated activation of GRK2 increases the secretion of the stress-induced factor CTGF from cardiomyocytes that then acts as a paracrine factor that enhances the activation of cardiac fibroblasts contributing to fibrosis.

Finally, since we have observed that spironolactone treatment blocked the aldosterone-mediated upregulation of GRK2 in myocytes (Fig. 2e), we investigated whether MR-antagonist-treated human HF patients had altered GRK2 levels in peripheral lymphocytes. Importantly, lymphocyte GRK2 levels have been shown to mirror levels in failing human myocardium and negatively correlating with haemodynamic function. Accordingly, we analysed, by immunoblot, lymphocyte GRK2 levels in human HF patients treated with spironolactone and compared these levels to a cohort of similarly diseased patients where the MR-antagonist was not used (Supplementary Table 1). Interestingly, patients treated with the MR-blocker had significantly lower GRK2 levels (Supplementary Fig. 7) consistent with this pathway having a pathophysiological influence on myocardial GRK2 levels and activity.

**Discussion**

It is well known that a primary pathogenic driver of cardiac dysfunction and HF is hyper-activation of neurohormonal signalling, which propagates and maintains a vicious cycle of injury. This involves chronic sympathetic nervous system activation through increased catecholamines and also enhanced RAAS activation. The catecholamines, norepinephrine and...
epinephrine, chronically stimulate β-adrenergic receptors (βARs) on cardiomyocytes while the RAAS promotes chronic AT1R activation via AngII and enhanced hyper-aldosteronism that leads to chronic MR activation. Adding to the importance of these systems and their hyper-activation, agents that block the chronic stimulation of these receptors are part of the current standard of care of HF patients, including MR antagonists. However, not all patients benefit from these drugs and new and innovative strategies for treating HF are desperately needed. Accordingly, a deeper understanding of the molecular mechanisms contributing to HF development and progression represents the best case scenario for finding new therapeutics. In this study, we have uncovered that GRK2 and GRK5 are critical molecules downstream of aldosterone and MR activation. In fact, they appear to be nodally involved in MR-mediated cardiac pathology as loss of GRK2 and GRK5 in myocytes significantly alleviates cardiac dysfunction due to hyper-aldosteronism.

Our data clearly indicate that some of the pathological effects of the aldosterone are not mediated solely on MR activation, but are dependent on the transactivation of AT1R. We found that aldosterone treatment elicited a robust apoptotic, fibrotic and hypertrophic response in myocytes and these effects were completely abolished by selective MR- or AT1R-antagonism, in vitro (Figs 2–4). At the molecular level, we observed that following aldosterone-MR binding that specific GPCR-dependent endocytic machinery is recruited (Gβγ/β-arrestin/c-Src) at the plasma membrane leading to AT1R internalization and to the activation of ERK 1/2 and NOX4. This increase in ROS formation consequently can cause myocardial dysfunction via mitochondrial superoxide formation and a robust apoptotic response. Importantly, our data clearly show that GRK2 is vital to this response mainly through its non-canonical mitochondrial localization, which appears nodally involved in the ‘non-genomic’ actions of aldosterone (Fig. 9).

In addition to GRK2 being involved in aldosterone-dependent pathology, we found that MR-AT1R activation leads to the nuclear translocation of GRK5, thus explaining in part the mechanism of aldosterone-mediated hypertrophic response in cardiomyocytes where this non-canonical action of GRK5 contributes to the ‘genomic’ pathway of aldosterone-mediated pathology (Fig. 9).

Importantly, we also evaluated the role of the GPER that has been widely associated to the non-genomic role of aldosterone in non-myocytes and recently also in an in vitro model of cardiomyocytes (H9c2) (ref. 39). H9c2 cells are skeletal muscle myoblasts and although they are widely used as a surrogate of cardiomyocytes they have properties that complicate the state of MR signalling, including demonstration that H9c2 cells lose functional MRs and exhibit very low levels of aldosterone-specific MR-binding31. Interestingly, in H9c2 cells the GPER was found to play an important role in aldosterone-mediated deleterious signalling that could be crucial in human HF39.

Our data, in NRVMs show that high-aldosterone levels can activate GPER signalling but this receptor appears to have a minimal effect on myocardial pathology induced by aldosterone that instead is suggested to be fully mediated by AT1R-MR signalling.
Importantly, we uncovered in myocytes that aldosterone-dependent GPER activity does not explain induction of pathology, which is consistent with previous reports\textsuperscript{49,52}, including in vascular smooth muscle cells where GPER stimulation can protect against hyper-activation of the MR\textsuperscript{52}.

The most notable findings of our study were that the aldosterone-mediated negative effects on mitochondrial superoxide formation, myocyte cell death and hypertrophy were all attenuated by MR blockade with spironolactone or AT\textsubscript{1}R antagonism with losartan and limited aldosterone’s actions by reducing the non-canonical activities of GRK2 and GRK5. Similar effects were seen using the most selective MR-antagonist eplerenone and the AT\textsubscript{1}R antagonist irbesartan (Supplementary Fig. 8a–c). Our data appear to be relevant to human HF as previously described\textsuperscript{24,48}.

Of note, previous studies have already linked GRK2 and GRK5 to HF development\textsuperscript{20,21,27,31}, but, our data demonstrate for the first time that GRK2 and GRK5, acting downstream of MR activation, can induce specific myocardial pathologies. We demonstrate in vivo that 4 weeks of aldosterone represent a trigger stimulus for GRK2 and GRK5 upregulation and the above-discussed, non-canonical activities. These activities of GRKs appear to be significantly relevant for aldosterone-mediated HF, since cardiac Grk2 and Grk5 KO mice have ameliorated myocardial dysfunction. The importance of GRK-mediated cardiac pathology following chronic hyperaldosteronism is surprising since GRKs do not regulate MR signalling. However, with the finding that MR activation transactivates AT\textsubscript{1}R in myocytes there is still a potential GPCR dependence to these pathological actions of GRK2 and GRK5. These results also demonstrate that limiting MR activation can reduce these pathological actions.

Importantly, we observed that although GRK2 clearly is a nodal determinant of aldosterone-mediated myocyte death and ultimately fibrosis in vivo, this kinase did not influence aldosterone-mediated cardiac hypertrophy, which is also a pathological feature of hyper-aldosteronism\textsuperscript{8,9}. This is an interesting finding and demonstrates that this MR-mediated hypertrophic signalling, which also may include AT\textsubscript{1}R transactivation, does not involve GRK2. This is in line with previous observation since GRK2 has not been previously shown to be important in cardiac hypertrophy\textsuperscript{49}. However, GRK5 aldosterone-MR-AT\textsubscript{1}R signalling is a nodal regulator of pathological cardiac growth in hyper-aldosteronism due to nuclear accumulation where its recent transcriptional influence has been revealed\textsuperscript{27,41}. This was clearly shown in cardiac Grk5 KO mice where ventricular wall thickness was prevented after hyper-aldosteronism.

Recently, He \textit{et al.}\textsuperscript{13} showed that aldosterone can induce direct cardiotoxic effects through the activation of the NADPH oxidases and subsequent ROS generation via actions of CAMKII acting in the nucleus after it becomes oxidized. CAMKII could become oxidized by MR activation and AT\textsubscript{1}R activation separately, but the co-dependence of the two receptor systems was not directly studied as in our experiments herein where GRK2 and GRK5 appear to also be nodal regulators of the pathological effects of aldosterone. Importantly, the mechanism proposed for CAMKII appears to be only responsible for cardiac rupture and mortality after MI\textsuperscript{13}, which was not directly, evaluated in our study. The authors in this study used aldosterone infusion after MI, which raises levels to supra-physiological concentrations\textsuperscript{13} and in our study, levels of aldosterone were equivalent to what is seen in control mice after MI. We importantly, did not see significant deaths via rupture with physiological levels of aldosterone, but
significant ventricular dysfunction and remodeling that was beneficially altered with less GRK2 and GRK5. Interestingly, although a Camki1 KO mouse was protected from rupture\(^1\), it was not protected from fibrosis, which we find in Grk2 KO mice, or by hypertrophy as seen in our Grk5 KO mice. Therefore, GRK pathways and an oxidized-CAMKII pathway appear parallel and distinct with GRK2 being a nodal regulator of aldosterone-mediated cell death and fibrosis, and GRK5 being involved in its hypertrophic response.

Over the past two decades, GRK2 inhibition has led to prevention or reversal of several animal models of HF\(^2\),\(^3\), including most recently with βARKct gene therapy in a pre-clinical large animal model of HF\(^5\). Thus, GRK2 inhibition appears to be a novel therapeutic strategy for alleviating cardiac dysfunction. New data are emerging, that part of the mechanism for the beneficial effects in the heart of limiting GRK2 activity includes non-canonical roles for GRK2, including its actions on insulin signalling\(^2\) and mitochondrial-dependent cell death\(^5\). Similarly, GRK5 is also becoming an emerging novel target for its non-canonical role in the pathogenesis of hyper-aldosteronism.

Importantly, our present study demonstrates that GRK2 and GRK5 are primary determinants of cardiac dysfunction downstream of hyper-aldosteronism via their non-GPCR activities. Further, at least for GRK2, clinical treatment in downstream of hyper-aldosteronism via their non-GPCR activities. Thus, GRK2 inhibition was beneficially altered with less GRK2 and GRK5. Interestingly, all cells were used within 2–8 h of isolation. Myocytes were plated on laminin-coated coverslips and were bathed in HEPS-buffered (20 mM, pH 7.4) medium 199 containing 1.8 mM extracellular Ca\(^2\+) to facilitate the perfusion they were injected with heparin 100 USP into the renal artery. After removal from thoracic cavity, hearts were washed in cold perfusion buffer (110 mM NaCl, 4.6 mM KCl, 1.025 mM CaCl\(_2\), and MgSO\(_4\)-7H\(_2\)O 0.8 mM, NaHCO\(_3\) 4.6 mM, butanedione monoxime (BDM) 1 mM, taurine 30 mM and glucose 5.5 mM, pH 7.0). Following cannulation on a Langendorff system, they were perfused with perfusion buffer at 37 °C for 2–3 min and then digested with digestion solution (50 mM perfusion solution with collagenase 364 U ml\(^{-1}\), 5 mg BD Difco-trypsin 250 and 20 μM l-1 CaCl\(_2\)) for 6–7 min. The atrias were removed and the ventricles were gently minced with plastic pipettes in 100 mM min dish containing 2.5 ml of digestion solution. Once the ventricles are almost completely dissolved, the digestion is stopped adding 7 ml of stopping buffer. The resulting post-digestion solutions were filtered through a 70 μm filter. The cardiomycocytes were separated from fibroblasts and smaller cells by 5-min sedimentation and followed by centrifuging at 100g for 30 s. The supernatant pellet containing cardiac myocytes was resuspended in a stopping solution (perfusion buffer contains 250 mg BSA and 125 μM l-1 CaCl\(_2\)) and allowed to rest for 10 min. Cells were spun down with the same conditions described above and then underwent three steps to increase gradient (100 mM l-1 calcium solution: 10 ml stopping buffer plus 10 μl of 1 M CaCl\(_2\); 400 μl of 1 μl l-1 calcium solution; 7 ml of medium 199 containing 1.8 mM extracellular Ca\(^2\+) to reach a final calcium concentration of 1.025 mM l-1. GRK2, GRK5 and β-arrestin 1/2 knockdown assay. NRVMs were transfected with specific siRNAs targeting Grk2 (Ambion, Thermo Fisher Scientific); Grk5 (Invitrogen-Stealth siRNA; GRK5 R33/2434; Thermo Fisher Scientific); and β-arrestin 1/2 (Arrb1 –197/273; Arrb2 –197/273; Thermo Fisher Scientific). Scrambled siRNAs were used as negative control (Thermo Fisher Scientific). Transfection was performed using 5 μl of siRNAs together with Lipofectamine 2000 (Ambion, Thermo Fisher Scientific). All cells were used within 2–8 h of isolation. Myocytes were plated on laminin-coated coverslips and were bathed in HEPS-buffered (20 mM, pH 7.4) medium 199 containing 1.8 mM extracellular Ca\(^2\+)
Cell fractionation. Membrane proteins were isolated from LV samples (0.1 mg) and NRVMs (30 mg) containing ~ 2 × 10^6 cells using a homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and protease inhibitors; cOmplete-Roche). The tissues were lysed by homogenization while the cells by scraping and pipetting. Next, both the lysates were sonicated and centrifuged for 15 min at 4 °C at 2,000 r.p.m. The supernatant was then centrifuged at 54,000 r.p.m. for 30 min at 4 °C. The pellet was washed twice using the homogenization buffer and then centrifuged again at the same speed. The washed pellet was then resuspended in PBS with 0.5% Triton X-100. The final lysate was used for western blot analysis or stored at −80 °C.

To isolate mitochondria, NRVMs (30 mm dish containing ~ 2 × 10^6 cells) and LV specimens (0.1 mg) were lysed in an ice cold mitochondrial isolation buffer (MIB) containing 200 mmol−1 mannitol, 70 mmol−1 sucrose, 5 mmol−1 HEPES and 1 mmol−1 EGTA, pH 7.5. The MIB was supplemented with protease inhibitors (cOmplete-Roche). Cardiac specimens were first homogenized using a tissue homogenizer (PT-1200E POLYTRON), and then the lysate was subsequently homogenized on ice by passage in a 1 cc syringe with a 27/12 G needle (BD, Franklin Lakes, NJ). LV samples were homogenized using a motor-driven sonicator (Misonix). The homogenate was filtered through a 40 μm-mesh nylon filter. The pooled supernatants were transferred to a new 1.5 ml tube and centrifuged again at 14,000 g for 15 min at 4 °C. The supernatant was collected in a new tube (cytosolic fraction).

While, the pellet containing the mitochondria fraction was resuspended in fresh MIB buffer and centrifuged again at 14,000 g for 15 min at 4 °C. The washed mitochondrial pellet was resuspended in MIB and directly used for protein quantification and immunoblotting. When mitochondria were taken from NRVMs, cells were washed once with ice cold Dulbecco’s PBS, scraped into MIB buffer and processed as described above. Nuclear fractions were obtained from NRVMs (30 mm dish containing ~ 2 × 10^6 cells) and from LV samples (0.1 mg).

Confocal microscopy. NRVMs or AVMs washed three times in ice cold PBS and fixed in 3% paraformaldehyde (PFA) for 10 min. Then the cells were permeabilized with 0.2% Triton X-100 for 2 min. After three washes in PBS, the cells were incubated with 1% BSA for 30 min and then incubated with an anti-HA (Santa Cruz Biotechnology, 1:2000) and anti-Grk5 (Santa Cruz Biotechnology, 1:200), respectively, diluted in 1% BSA. Next, the cells were incubated with the respective secondary antibodies: a mouse monoclonal to reveal the HA-tag (Alexa Fluor 488; Thermofisher Scientific, 1:200) and a rabbit polyclonal to reveal Grk5 (Alexa Fluor 546; Thermofisher Scientific 1:200). The fluorescent data sets were visualized with a Zeiss 510 confocal laser scanning microscope and analysed by LSM 510 software.
Histology. Cardiac specimens were fixed in 4% formaldehyde embedded in paraffin. After de-paraffinization and re-hydration, 5-μm-thick sections were prepared and mounted on glass slides and stained with 1% Sirius red in picric acid (Sigma-Aldrich) to detect interstitial fibrosis. The percentage of fibrosis was quantified using a software (ImageJ). All sections were examined with a microscope (Nikon Eclipse E1) and images were acquired with a digital camera (Nikon).

Real-time PCR. Total RNA was isolated from NRVMs and from LV specimens with TRIzol (Thermo Fisher Scientific) according to the company's instructions. After RNA isolation, cDNA was synthesized by reverse transcription of the RNA (Script cDNA synthesis kit, Bio-Rad Laboratories). Real-time PCR was performed in duplicate on a CFX96 real-time PCR detection system (Bio-Rad Laboratories) using the SYBR Green mix (Bio-Rad Laboratories) and specific primers for mouse Ctgf as follows: forward 5'-GGAGACACATTGGCCGCAG-3' and reverse 5'-TAGGTGCGGATGCACTTT-3'. The expression levels of Ctgf were normalized to the rRNA 18S. Specificity of PCR products was confirmed by melting curve and gel electrophoresis.

Enzyme-linked immunosorbent assay. Aldosterone serum levels were measured in mice using a commercial kit (KAI8835; Abnova, Walnut, CA, USA) and the assay was performed according to manufacturer's instructions. The assay was performed on blood serum isolated from aldosterone-treated (4 weeks) mice. Blood (300 μl) was collected by puncturing the heart. Then, blood samples were centrifuged at 1,500 rpm for 15 min at room temperature and the serum was transferred to a 1.5 ml tube. Blood serum isolated from vehicle-treated mice was used as control.

Lymphocytes GRK2 protein levels in human HF patients. We studied 127 patients with an established diagnosis of HF enrolled at Federico II University (Naples, Italy) and Salvatore Mangieri Foundation, Scientific Institute of Telese Terme (Telese Terme, BN, Italy). All patients signed consent form. The patients' inclusion criteria were as follows: diagnosis of HF due to ischaemic or non-ischaemic aetiology, LV ejection fraction (LVEF) ≤ 45%, stable clinical conditions for at least 3 months before inclusion and guideline-based optimal pharmacotherapy. All subjects underwent a complete clinical examination (including New York Heart Association (NYHA) functional class assessment and echocardiography) and blood draw (3 ml) for lymphocyte isolation. The blood, collected in a tube containing EDTA (Vacutainer-BD, Franklin Lakes, NJ, USA), was diluted with an equal volume of PBS. The diluted blood was layered over 3 ml of Lympholyte-H Cell Separation Media (Cedarlane, Burlington, NC, USA) and centrifuged for 20 min at 800 g. The lymphocyte layer was transferred to a new tube using a Pasteur pipette. The transferred cells were diluted with PBS and centrifuged at 800 g for 10 min to pellet the lymphocytes. After three washes with PBS, the cells were centrifuged as above and the supernatant was removed. The resulting lymphocyte pellet was stored at − 80 °C and lyzed, and was analysed by immunoblot for GRK2 levels, as described above. Demographic data including age, sex, HF medications and cardiovascular risk factors and presence of comorbidities were also collected.

Statistics. Data are expressed as mean ± s.e. Statistical significance was determined by a Student's t-test or Mann-Whitney exact test (when sample size was < 10). For multiple comparisons, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc correction was performed. Categorical variables were expressed as proportion and compared by use of χ2 test. All data were analysed using GraphPad Prism software version 6. Statistical significance was accepted at P < 0.05.

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**Author contributions**

A.C., designed and performed experiments, analysed data and wrote the manuscript; D.Li. performed experiments and assisted with animal and human studies; A.E. assisted with immunoblotting; K.J.E. assisted in developing adenoviral constructs; C-I.T. assisted with animal studies; J.I. performed the isolation of cardiomyocytes; S.E. assisted in developing adenoviral constructs and edited the manuscript; D.Lee. and N.F. assisted with human studies and edited the manuscript. G.R. assisted with animal and human studies and edited the manuscript; W.J.K. designed experiments, analysed data, co-wrote the manuscript and supervised the project.

**Additional information**

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