Response of non-failing hypertrophic rat hearts to prostaglandin F2α

Anna Maria Krstic, Sarbjot Kaur, Marie-Louise Ward [*]

Department of Physiology, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand

A B S T R A C T

Background: Prostaglandin F2α (PGF2α) has a positively inotropic effect on right ventricular (RV) trabeculae from healthy adult rat hearts, and may therefore be therapeutically useful as a non-catecholaminergic inotrope. These provide additional contractile support for the heart without the added energetic demand of increased heart rate, and are also suitable for patients with reduced β adrenergic receptor (β-AR) responsiveness, or impaired mitochondrial energy supply. However, the response of hypertrophied rat hearts to PGF2α has not previously been examined. Our aim was therefore to determine the effect of PGF2α on isolated perfused rat hearts with RV hypertrophy following induction of pulmonary artery hypertension.
Methods: Male Wistar rats (300 g) were injected with either 60 mg kg^{-1} of monocrotaline (MCT, n = 10) or sterile saline as control (CON, n = 11). Four weeks post injection; hearts were isolated and Langendorff-perfused in sinus rhythm. Measurement of left ventricular (LV) pressure and the electrocardiogram were made and the response to 0.3 μM PGF2α was determined.
Results: PGF2α increased LV developed pressure in CON and in 60% MCT hearts, with no change in heart rate. However, 40% of MCT hearts developed arrhythmias during the peak inotropic response. For comparison, the response to 0.03 μM isoproterenol (ISO) was also investigated. Peak LV pressure developed sooner in response to ISO compared to PGF2α in both rat groups, although the inotropic response to ISO was reduced in MCT hearts. Analysis of fixed ventricular tissue confirmed that only RV myocytes were hypertrophied in MCT hearts. Our study showed that PGF2α was positively inotropic for healthy hearts, but found it generated arrhythmias in 40% of MCT hearts at the dose investigated. However, a more physiological dose of PGF2α may be a useful alternative without the added energetic cost of catecholaminergic inotropes.

1. Introduction

Remodelling of the myocardium, which occurs during adaptive hypertrophy, is associated with defective electrical and mechanical functioning of the heart (Maier et al., 1998). In cardiac muscle, force is developed through excitation-contraction (EC) coupling in response to the cardiac action potential (for review see Eisner et al. (2017)). EC coupling is reportedly impaired in ventricular hypertrophy prior to heart failure (Gómez et al., 1997) due to changes in: i) ultrastructural organization (Lamberts et al., 2007), ii) ion channel expression (Benoist et al., 2011; Tanaka et al., 2013; Umar et al., 2011), and iii) intracellular Ca^{2+} handling (Miura et al., 2011; White and Saint, 2012; Benoist et al., 2014). Collectively, these alterations can affect electrical conduction and Ca^{2+} homeostasis, which is required for the heart to contract in a synchronised manner. Ultimately these changes can lead to arrhythmias and/or decreased force development, which is the hallmark of heart failure (Suzuki et al., 2006).

Inotropic agents are applied to hearts that require additional contractile support in order to achieve sufficient cardiac output. At present, contractile function is most often improved through treatment with catecholamine-based positive inotropes (i.e. β-adrenergic stimulation) (Nieminen et al., 2005; Dickstein et al., 2008). The β-adrenergic response requires increased mitochondrial energy supply to fuel the increase in the number of heart-beats per minute. However, the energetic demand of faster heart rates can be excessive in situations where mitochondrial energy supply is compromised (Neubauer, 2007). Catecholamines may also be ineffective in patients treated with β-blockers, which are commonly administered for the treatment of many heart diseases (Bristow, 2000). Therefore, there would be undeniable utility for an inotropic agent with an alternative mechanism of action, which exclusively provides contractile support without additional effects such as excessive energy expenditure (Nieminen et al., 2005).

Prostaglandin F2α (PGF2α) has been shown to be positively inotropic when exogenously applied to isolated rat myocardium (Karmazyn and

Dhalla, 1982; Otani et al., 1988; Fong Yew et al., 1998; Shen et al., 2016). PGF2α acts on a $G_{\alpha q}$ protein (via a prostaglandin F receptor) (Yuhki et al., 2011), resulting in activation of a downstream signalling pathway that increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) independent of β-AR simulation (Shen et al., 2016; Metsa Ketela, 1981). There have been no studies to date comparing the effects of PGF2α on healthy and diseased myocardium. Therefore, the aims of this study were: i) to determine the relative changes in pressure from pre-drug baseline to maximum PGF2α response of healthy and hypertrophic rat hearts; and ii) to compare the response to PGF2α with the well-studied inotropic response to β-AR stimulation. In addition to this, the changes in electrical activity from pre-drug baseline to maximum drug response were measured across the surface of whole hearts. These measurements were made to investigate the impact of RV hypertrophy on the whole heart. To carry out our aims we used the monocrotaline (MCT) rat model of pulmonary artery hypertension (PAH) with right ventricular (RV) hypertrophy (Gomez-Arroyo et al., 2012), prior to progression to heart failure. RV hypertrophy initially develops as a compensatory mechanism, which rapidly progresses to irreversible biventricular heart failure in the MCT rat (Suzuki et al., 2006). We therefore investigated the response of Langendorff-perfused MCT hearts in sinus rhythm to PGF2α immediately prior to the development of overt heart failure, since this is when therapeutic interventions would be most effective.

2. Methods

2.1. Animal model

Male Wistar rats of 305 ± 4 g (mean $\pm$ SEM, n = 10) were subcutaneously injected with $60 \, \text{mg} \, \text{kg}^{-1}$ (body weight) monocrotaline (MCT, Sigma Aldrich, Australia) at 57 ± 1 days of age. Aged-matched control rats (CON) of 310 ± 3 g, n = 11, were injected with the same volume of sterile saline. Animals were fed normal rat chow and water ad libitum for 4 weeks and monitored regularly. At 28–32 days post injection, rats were anaesthetized using 2% isoflurane in 100% O_2 as a carrier gas and *in vivo* electrocardiogram recordings performed. Approval for this research was provided by the University of Auckland Animal Ethics Committee (AEC: 001807), in accordance with the Code of Ethical Conduct of The University of Auckland, and the New Zealand Animal Welfare Act 1999.

2.2. Langendorff-perfused heart experiments

Following ECG recordings, the anaesthetized animals were euthanized and the hearts removed and placed in modified Tyrode's solution

comprised of (mM): NaCl (141), KCl (6), $MgSO_47H_2O$ (1.2), Na_2HPO_4 (1.2), HEPES (10), $CaCl_2$ (1.5) and glucose (10) at 4 °C. Isolated hearts were cannulated via the aorta, and perfused with Tyrode's buffer, bubbled with 100% O_2 at 37 °C. A peristaltic pump (Masterflex, Cole-Parmer Instruments., IL, USA) supplied buffer at a flow rate of $10–11 \, \text{mL} \, \text{min}^{-1}$. Pressure was recorded via a fluid-filled balloon inserted into the LV and connected via a thin cannula to a pressure transducer (SP844, Memscap Inc., Durham, NC, USA). The balloon volume was increased until LV developed pressure was maximal. All hearts were investigated in sinus rhythm, with the cardiac electrogram recorded from electrodes placed on each ventricular surface, with a ground electrode above the aorta. Pressure and electrical signals were simultaneously recorded using Labchart software (v.8.0.7) and coronary flow rate was determined from timed collections of the coronary effluent. All chemicals and pharmacological agents were from Sigma Aldrich Australia, unless otherwise specified.

2.3. Experimental protocol

Baseline LV pressure and electrocardiogram recordings were made prior to application of pharmacological interventions. Drugs were subsequently injected at a rate of $0.1 \, \text{mL} \, \text{s}^{-1}$ above the aortic cannula, reaching the heart approximately 6 s following application. Hearts were first exposed to a 0.5 mL bolus of 0.3 μM prostaglandin F2α (PGF2α) (Cayman Chemical Company, Michigan USA) diluted in Tyrode's. After the effects of PGF2α were completely diminished (approx. 5 min), hearts were treated with a 0.5 mL bolus of 0.03 μM isoproterenol also diluted in Tyrode's. At the end of each experiment, hearts were removed from the cannula, blotted and weighed for subsequent morphometric analysis.

2.4. Fixation of tissue and immunohistochemistry

In separate MCT animal, structural analyses investigating the degree of myocyte hypertrophy was performed. Left and right ventricular tissue was fixed in 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 1 h at 4 °C. Tissue blocks were sequentially transferred in 10%, 20% and 30% sucrose with 0.1% sodium azide in PBS. Fixed LV and RV blocks were then separately snap-frozen in 2-methylbutane cooled with liquid nitrogen and transverse sections of 10 μm thickness were taken on poly-L-lysine coated coverslips using a cryostat (Leica, Wetzlar, Germany). All sections were labelled with an extracellular matrix and sarcolemma marker (wheat germ agglutinin, WGA) by incubating for 2 h at room temperature with Alexa 488-WGA (1:200, Thermo Fisher Scientific, Life Technologies NZ, catalogue no. W11261). Sections were washed three times in PBS and mounted with Prolong Gold (Thermo Fisher Scientific, Life Technologies NZ). Images were obtained with a laser scanning confocal microscope (LSM 710, Zeiss, Oberkochen, Germany) using a Zeiss x63 oil-immersion objective lens (NA 1.40). Images had a 0.1 μm pixel resolution and were captured with a 488 nm laser at 2% laser power. Using Image J software, cross-sectional images of ventricular myocytes were traced along the sarcolemma to determine cross-sectional area of individual cells. To ensure consistent analysis of cross-sectional area, only myocytes with visible, circular, nuclei were preferentially selected for analysis.

2.5. Data analysis

In vitro ECG and LV pressure data were acquired and analysed using Labchart (v.8.0.7, AD Instruments, New Zealand). HR and the corrected QT interval (QTc) based on Bazett's formula (Bazett, 1920) (to remove the dependence of HR on the action potential duration) were calculated. The following variables were determined from the pressure traces: LV developed pressure, minimum diastolic pressure, peak systolic pressure, pressure-time integral (over a fixed, 5 s periods), time-to-peak pressure, and time-to-50% relaxation. The isolated heart sinus rhythm was determined from the cardiac electrogram by measuring the mean R - R

interval. Morphometric data including body, heart, lung and liver weights, along with RV and LV thicknesses were also recorded. Statistical analyses of *in vivo* data, morphometric data and confocal images were carried out by unpaired, two tailed, t-tests (GraphPad Prism 7). Isolated heart data was analysed using two-way ANOVA for multiple comparisons between groups and interventions, with repeated measures for baseline comparisons (independent of interventions). Statistical significance was assigned as a P value of <0.05.

3. Results

3.1. Electrocardiogram and morphometric data

At four weeks post injection, CON animals had greater body weights in comparison to MCT animals (P < 0.0001, Table 1 & Supplementary Fig. 1). Recording of electrocardiogram (ECG) in anaesthetized animals showed a higher *in vivo* heart rate (HR) in CON relative to MCT (P < 0.01, Table 1). The QTc intervals, which are an indication of action potential duration (Bazett, 1920) were not different between MCT and CON. However, MCT animals had shorter tibial lengths (P < 0.05), greater RV thickness to body weight ratio (RV: BW %, P < 0.0001) and RV thickness to tibial length ratio (RV: Tibial length %, P < 0.0001) in comparison to CON. MCT animals also had heavier lung weights (P < 0.01) and lung weight to body weight ratio (LW: BW %, P < 0.001) compared to CON, which confirmed RV hypertrophy in MCT hearts.

Table 1
Morphometric data.

	CON	MCT
Body Weight (g)	444 ± 9	386 ± 5 ****
RV: BW (%)	0.36 ± 0.03	0.61 ± 0.02 ****
LV: BW (%)	0.72 ± 0.09	0.89 ± 0.09
Tibial length (mm)	54.51 ± 0.87	51.98 ± 0.54 *
RV: Tibial length (%)	2.95 ± 0.22	4.58 ± 0.15 ****
LV: Tibial length (%)	6.93 ± 0.30	7.12 ± 0.37
Lung weight (g)	1.80 ± 0.1	2.2 ± 0.1 **
Liver weight (g)	15.40 ± 0.48	14.5 ± 0.03
LW: BW (%)	0.39 ± 0.02	0.56 ± 0.03 ***
HR (min^{-1})	367 ± 23	340 ± 13 **
QTc interval (s)	0.14 ± 0.01	0.15 ± 0.02

Table 1 Morphometric data obtained from both CON (n = 11) and MCT (n = 10) animals four weeks post injection. Results are expressed as mean ± SEM. RV/LV: BW (%) = right ventricular/left ventricular thickness: body weight, LW: BW = lung weight: body weight, HR = heart rate, QTc = corrected QT interval. P values were determined using unpaired, two-tailed t-tests, which indicates significance between groups, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.2. Heart rate in isolated, perfused hearts

Fig. 1 shows HR at pre-drug baseline (−) and during the maximum drug effect (+) for Langendorff-perfused hearts in sinus rhythm. Panel A shows no effect of PGF2α on HR for either CON or MCT hearts. Panel B shows β-AR stimulation with isoproterenol (ISO) increased HR relative to baseline for CON (P < 0.01), but not for MCT hearts. No difference was observed in the rate of isolated hearts between groups.

3.3. Response to prostaglandin F2 in isolated, perfused hearts

Application of 0.3 μM PGF2α as a bolus injection into the aortic cannula showed a biphasic response: an initial, negative inotropic response, followed by a larger positive inotropic response. Mean ± SEM responses to PGF2α are shown in Table 2 for isolated hearts from CON (n = 9) and MCT (n = 6). There was no difference in the initial % decrease in left ventricular (LV) pressure between CON and MCT, however, the initial response was established sooner in MCT (16.3 ± 1.0 s post application) in comparison to CON (21.1 ± 1.1 s post application, P < 0.001). No difference in the time to reach maximum PGF2α effect or in the % increase in LV pressure from pre-drug baseline (120.4 ± 21.0% in CON and 80.5 ± 14.6% in MCT) was observed between groups. Similarly, the change in pressure-time integral from pre-drug baseline to maximum PGF2α was also not different between groups (Table 2). Representative data from CON and MCT are shown in response to PGF2α

Table 2
Response to PGF2α.

0.3 μM PGF2α	CON	MCT
Time to initial drug effect (s)	21.1 ± 1.1	16.3 ± 1.0 ***
% decrease in LV pressure	28.1 ± 4.5	27.8 ± 6.8
Time to max. drug effect (s)	66.2 ± 4.8	55.8 ± 3.6
% increase in LV pressure	120.4 ± 21.0	80.5 ± 14.6
Duration of the positive inotropic response (s)	199.3 ± 20.8	176.4 ± 10.6
% change in HR during peak response	7.7 ± 2.6	8.3 ± 9.5
Δ Pressure-time integral (kPa s^{-1})	10.5 ± 1.6	10.9 ± 2.2
Time to initiation of arrhythmic activity (s)	N/A	38.7 ± 6.4

Table 2 Time course and contractile response to 0.3 μM prostaglandin F2α (PGF2α) in CON (n = 9) and MCT (n = 6) isolated hearts. Table shows the time taken for PGF2α to induce a maximal effect starting from the point at which a bolus injected into the aortic cannula reached the hearts. The % increase in LV pressure and heart rate from pre-drug baseline was recorded, along with total duration of the positive inotropic response until LV pressure recovered to baseline. The Δpressure-time integral shows the change in LV developed pressure over a period of 5 s from pre-drug baseline and maximum PGF2α response, independent of heart rate. In (n = 4) MCT hearts, the time to initiation of arrhythmic activity was also reported. Results are expressed as mean ± SEM.
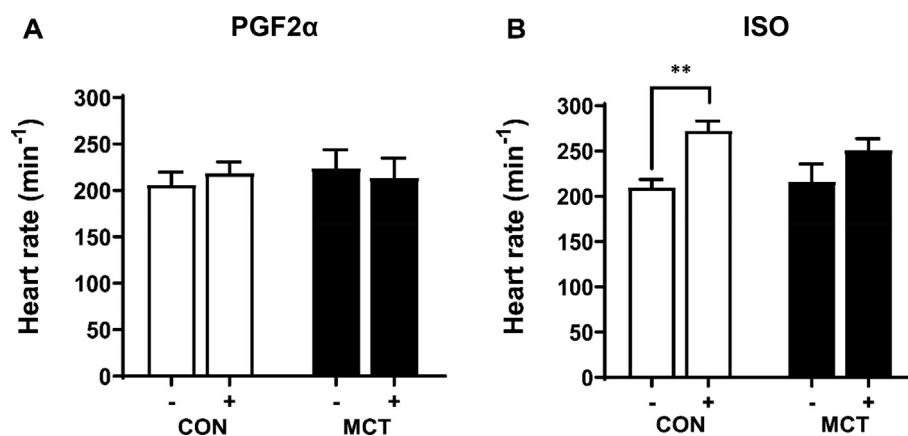

Fig. 1. Heart rates of CON (white) and MCT (black) isolated, Langendorff-perfused, hearts in sinus rhythm before (−) and during (+) exposure to drugs. Panel A shows the response to 0.3 μM prostaglandin F2α (PGF2α), and panel B shows the response to 0.03 μM isoproterenol (ISO). MCT hearts that displayed arrhythmic activity (n = 4) were excluded from the analysis shown in Panel A. Results are expressed as mean ± SEM, n = 11 (CON) and n = 10 (MCT). **P < 0.01.

(Fig. 2). CON hearts displayed no sign of arrhythmic activity in response to PGF2α. However, in a subgroup of MCT hearts, PGF2α triggered arrhythmic activity, which was initiated 38.7 ± 6.4 s after administration (Table 2 & Fig. 2C). Arrhythmic MCT hearts (n = 4) returned to baseline sinus rhythm 85.5 ± 11.6 s after PGF2α reached the heart. Separate experiments showed it took approximately 330 s for a drug to completely wash out of the coronary circulation. There was no difference between LV pressure prior to PGF2α application (10.9 ± 2.1 kPa) and LV pressure after PGF2α washout (11.9 ± 1.3 kPa).

Superimposed data of the electrical and mechanical responses to PGF2α in representative MCT hearts are shown in Fig. 3. Normally, a single electrical impulse results in corresponding mechanical activity

across the whole heart (Fig. 3A). However, the representative arrhythmic MCT heart showed a clear mismatch between the electrical and mechanical activity during peak PGF2α stimulation (Fig. 3B). Fig. 4 presents mean ± SEM LV pressure measurements for a single cardiac cycle in CON and MCT hearts at pre-drug baseline (−) and during (+) the maximum PGF2α response (Fig. 4A, B). Hearts that displayed arrhythmic activity (n = 4 MCT), were excluded from the analysis presented in panels 4C, 4E and 4F (refer to Fig. 2C & Table 2). In panels 4A-C, PGF2α increased LV developed pressure in comparison to baseline pressure for both CON (P < 0.001) and MCT (P < 0.01) hearts, with no difference in baseline LV developed pressure between groups (Fig. 4C & Supplementary Fig. 2). Furthermore, the pressure-time integral (Panel 4D), which is an index of
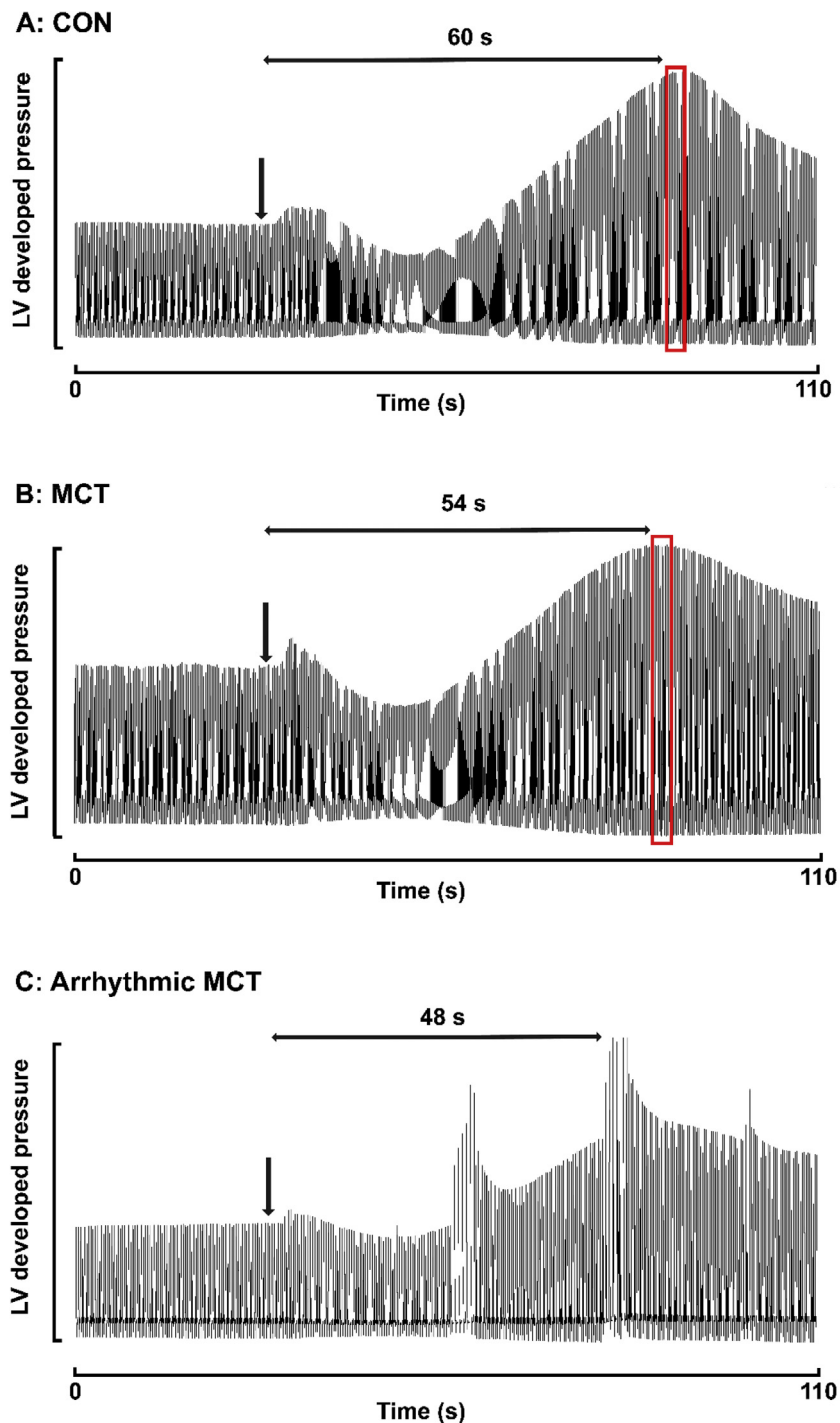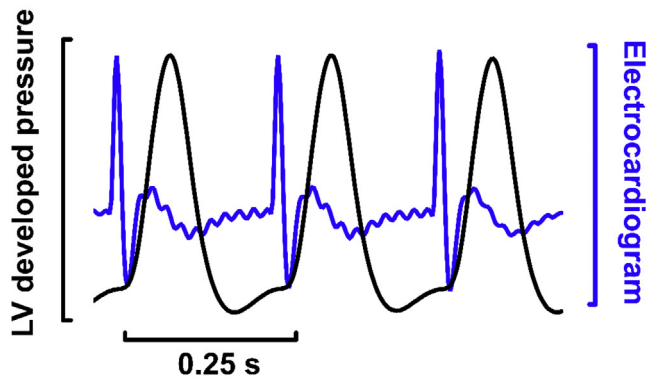

Fig. 2. Representative left ventricular (LV) pressure traces showing the effect of 0.3 μM prostaglandin F2α (PGF2α) in CON (Panel A) and MCT (Panel B) hearts. In a subgroup of MCT hearts (n = 4), PGF2α triggered arrhythmic activity after 38.7 ± 6.4 s. Panel C shows an example of the arrhythmic response to PGF2α in a representative MCT heart. Vertical arrows indicate the time at which PGF2α was added via the aortic cannula. Red rectangles show the regions used for mean calculations presented in Fig. 4.

A: MCT in sinus rhythm
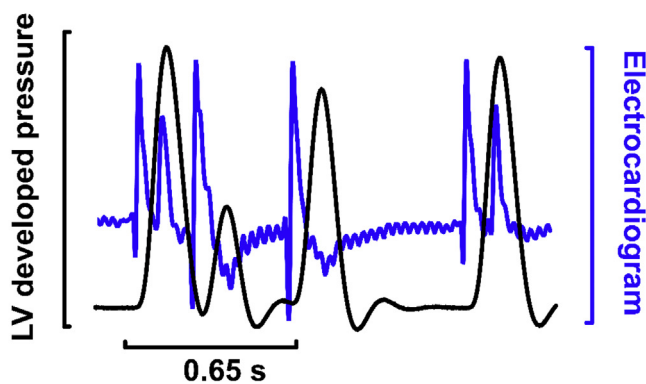

B: Arrhythmic MCT during peak PGF2α

Fig. 3. Representative traces of the electrocardiogram superimposed with left ventricular pressure recorded from a non-arrhythmic MCT heart in sinus rhythm (Panel A) and an arrhythmic MCT heart during peak PGF2α stimulation (Panel B).

contractile activity independent of heart rate, was also increased for both CON (P < 0.001) and MCT (P < 0.01) hearts in response to PGF2α. In Panels 4E and 4F, time course of LV pressure development is presented as mean ± SEM for a single cardiac cycle. PGF2α decreased the time-to peak pressure in CON (P < 0.05), but not MCT hearts (Panel 4E). There was also a reduction in the time-to-50% relaxation with PGF2α (Panel 4F) in CON (P < 0.001), but not MCT hearts (n = 6).

3.4. Response to β-adrenergic stimulation in isolated, perfused hearts

Table 3 shows mean ± SEM responses of isolated hearts to 0.03 μM isoproterenol. MCT hearts had a reduced % increase in LV pressure development (101.0 ± 17.5%) relative to CON hearts (248.0 ± 34.4%, P < 0.01). Similarly, MCT hearts showed a smaller change in PTI $(14.2 \pm 2.0 \text{ kPa}^{s-1})$ from pre-drug baseline to maximum ISO response in comparison to CON hearts $(22.2 \pm 2.7 \text{ kPa}^{s-1}, P < 0.05)$. However, the time to reach maximum response was much faster with ISO in both CON (17.2 ± 0.9) and MCT $(17.7 \pm 0.9 \text{ s})$ in comparison to PGF2α $(66.2 \pm 4.8 \text{ s in CON and } 55.8 \pm 3.6 \text{ s in MCT, Table 2})$ while the duration of the positive inotropic response to ISO was much shorter in comparison to PGF2α (P < 0.0001). The change in the maximum rate of rise (Δ dP/ dT) was also greater with ISO in comparison to PGF2α in CON hearts (P < 0.01), though MCT hearts showed a trend towards decreased Δ dP/ dT from pre-drug baseline to maximum ISO response (Supplementary Fig. 3). There was no difference between LV pressure prior to ISO application (9.0 ± 1.0 kPa) and after washout (7.8 ± 0.8 kPa). Representative data from CON and MCT in response to ISO are shown in Fig. 5.

Fig. 6 presents mean ± SEM LV pressure measurements before (−) and during (+) the maximum 0.03 μM ISO response in both CON (n = 9) and MCT (n = 9) hearts. Panels 6A-C show ISO increased LV developed pressure in both CON (P < 0.0001) and MCT (P < 0.001) hearts. Application of ISO also increased the pressure-time integral (Panel 6D) in both CON (P < 0.0001) and MCT (P < 0.01) hearts. Panel 6E shows ISO reduced the time-to-peak pressure for both groups (P < 0.0001), however, the time-to-50% relaxation (Panel 6F), was only decreased in response to ISO in CON hearts (P < 0.001), and not MCT hearts.

3.5. Analysis of cross-sectional area in fixed ventricular tissue

To determine the degree of hypertrophy in the MCT heart, immunohistochemistry and confocal imaging was carried out on 10 μm thick sections of ventricular tissue labelled with wheat germ agglutinin (WGA). Fig. 7 shows representative CON LV (7A), CON RV (7B), MCT LV (7C) and MCT RV (7D) tissue. Analyses of cross-sectional area are shown in Panel E. Within group comparisons (#P < 0.0001) shows MCT RV myocytes had a larger cross-sectional area (P < 0.0001) in comparison to MCT LV myocytes while CON LV myocytes had larger cross-sectional area compared to CON RV myocytes. Furthermore, when comparing ventricles between groups, MCT RV myocytes were larger than both LV and RV CON myocytes, while MCT LV myocytes had a smaller cross-sectional area in comparison to CON LV myocytes (P < 0.0001).

4. Discussion

4.1. Morphometric data

In this study, the use of PGF2α as a positive inotrope was investigated using the MCT animal model of PAH (Chesney et al., 1974; Tanaka et al., 1996). Four weeks post injection, HR from the *in vivo* ECG recording was reduced in MCT, with no difference in QTc intervals between groups (Table 1). In addition to this, the MCT rats had shorter tibial lengths, greater RV: BW (%), heavier lung weights and greater LW: BW (%) relative to the CON rats, which is characteristic of RV hypertrophy (Hessel et al., 2006; Jones et al., 2002). The morphometric data suggests that at 4 weeks post-injection, the usual signs of overt heart failure (i.e. prolonged QTc interval and hepatomegaly) were absent in the MCT rats used in this study (Benoist et al., 2012). However, there was evidence of progression towards heart failure (Werchan et al., 1989) as seen by the body weight of the MCT rats, which reached a plateau at around 3 weeks post injection in comparison to CON animals (refer to Supplementary Fig. 1). Alongside body weight, ventricular wall thickness is also an indicator of disease progression (Krayenbuehl et al., 1978). In this study, an increase in myocyte cross-sectional area was observed in MCT RV tissue relative to both CON RV and LV tissue and MCT LV tissue (Fig. 7). This increase in cell size was evidence of disease progression exclusively within the RV in the MCT rat hearts. Furthermore, previous *in vivo* studies of the MCT rat with RV hypertrophy reported enhanced ventricular function (Chesney et al., 1974; Jones et al., 2002; Werchan et al., 1989), with no decline in HR, such as that seen in the present study. The slower HR in MCT may have resulted from a desensitisation to sympathetic nerve input at the SA node, which would decrease the chronotropic response to endogenous β-AR activation. This has been previously reported in MCT rats with heart failure, but not in this model during RV hypertrophies (Lourenηo et al., 2006; Leineweber et al., 2002; Correia-Pinto et al., 2009).

4.2. Heart rate of isolated, perfused hearts

As expected, the HR recorded from isolated hearts in sinus rhythm was slower in comparison to *in vivo* ECG measurements from both CON and MCT rats (Table 1, Fig. 1A, B). This is generally attributed to the removal of the heart from the neuro-hormonal influence of the body (Bell et al., 2011). Application of PGF2α and isoproterenol into the aortic
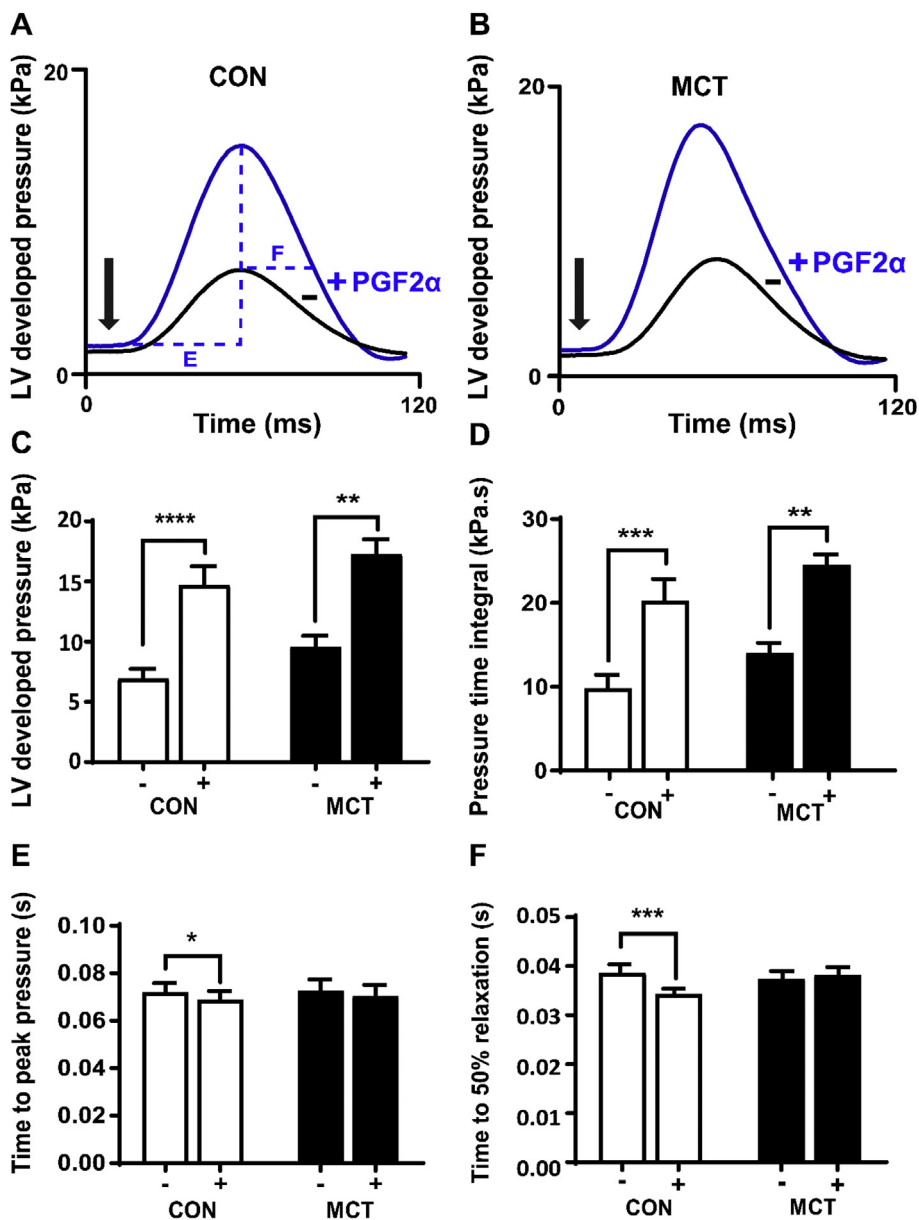
Fig. 4. Left ventricular pressure and time course of pressure before and at the peak of the response to 0.3 μM prostaglandin F2α (+) in CON (white) and MCT (black) isolated hearts. Panels A & B show overlays of LV developed pressure synchronised to the peak of the QRS at pre-drug baseline (- black) and during maximum PGF2α response (+blue) throughout a single cardiac cycle from representative CON and MCT hearts. The blue dashes on Panel A represent how the time to peak pressure (in Panel E) and the time to 50% relaxation (in Panel F) were calculated. Results are expressed as mean ± SEM for a single cardiac cycle, n = 11 (CON) and n = 6 (MCT). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Table 3
Response to isoproterenol.

0.03 μM ISO	CON	MCT
Time to max. drug effect (s)	17.7 ± 0.9	17.2 ± 0.9
Duration of the positive inotropic response (s)	47.4 ± 5.8	31.9 ± 4.8
% increase in LV pressure	248.0 ± 34.4	101.0 ± 17.5 **
% change in HR	30.9 ± 6.2	23.2 ± 6.4
Δ Pressure-time integral (kPa s^{-1})	22.2 ± 2.7	14.2 ± 2.0 *

Table 3 Time course and contractile response to 0.03 μM isoproterenol (ISO) in CON (n = 9) and MCT (n = 9) isolated hearts. Table shows the time taken for ISO to induce a maximal effect starting from the point at which a bolus injected into the aortic cannula reached the hearts. The % increase in LV pressure and % change in heart rate (HR) from pre-drug baseline to peak ISO response is presented in this table. The duration of the positive inotropic response until LV pressure recovered to baseline was also reported. The Δ pressure-time integral shows the change in LV developed pressure over a period of 5 s from pre-drug baseline and maximum ISO response, independent of heart rate. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.01.

cannula showed PGF2α had no effect on the pacemaker cells, as there were no significant change in HR (Fig. 1A, Table 2) within and between rat groups (CON: from 206 ± 14 min^{-1} to 219 ± 12 min^{-1} during drug; and MCT: from 207 ± 19 min^{-1} to 216 ± 17 min^{-1} during drug). However, isolated MCT hearts showed a reduced chronotropic response to ISO (216 ± 19 beats min^{-1} to 251 ± 12.4 beats min^{-1}), as opposed to CON hearts, which displayed the expected positive chronotropic response to ISO (210 ± 8.9 beats min^{-1} to 273 ± 10.4 beats min^{-1}). This finding correlated with the depressed MCT HR observed *in vivo*, which suggests that the MCT hearts in this study had a desensitized pacemaker response to β-AR stimulation (Fig. 1B). Desensitisation to β-AR stimulation has previously been reported in humans and other rat models of hypertrophy and heart failure (Huang et al., 2017; Wallukat, 2002).

4.3. Inotropic response to prostaglandin F2α

Application of PGF2α increased LV developed pressure and the pressure time integral equally in CON and MCT hearts (Fig. 4C, D & Table 2). Similar findings were previously reported for healthy rat hearts
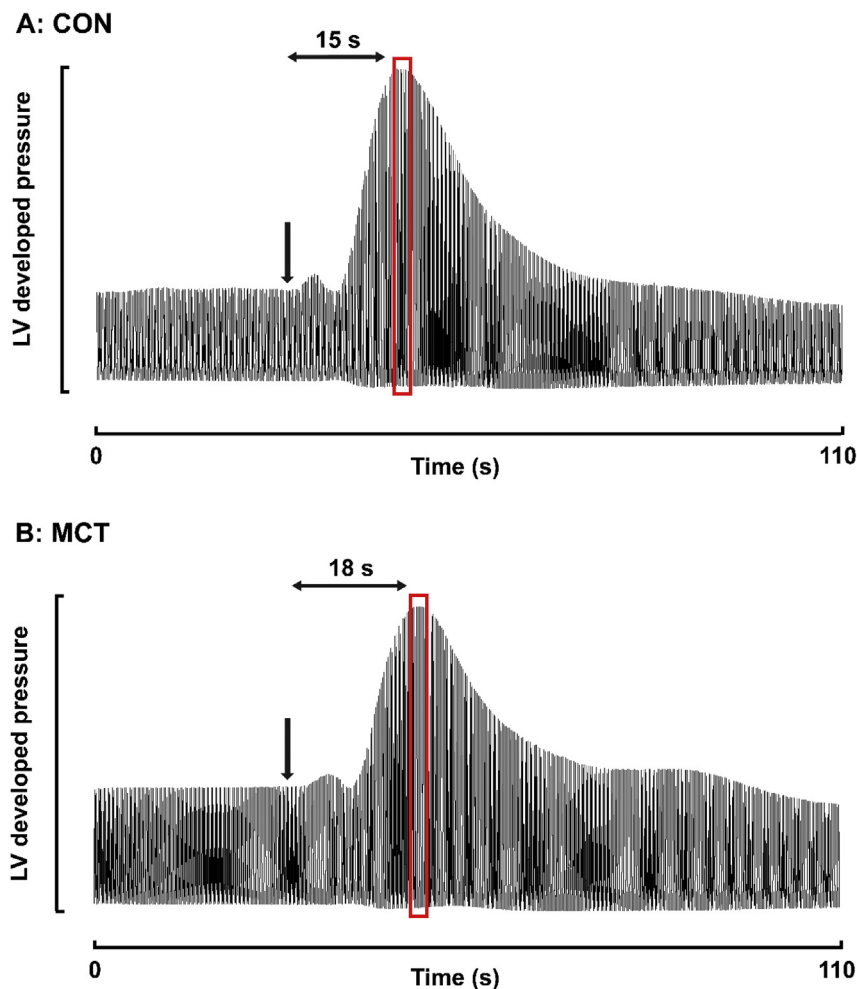
Fig. 5. Representative left ventricular (LV) pressure traces showing the response to 0.03 μM isoproterenol (ISO) recorded from isolated CON (Panel A) and MCT hearts (Panel B). Vertical arrows indicate the time at which ISO was added via the aortic cannula. Red outlines represent the regions used for mean LV pressure calculations presented in Fig. 6.

(Otani et al., 1988; Fong Yew et al., 1998; Shen et al., 2016). The administration of PGF2α to Langendorff-perfused isolated hearts, elicited a biphasic effect on contractility (see Table 2 & Fig. 2). This was characterized by an initial transient decrease in LV developed pressure, followed by a maximum positive inotropic response (44.8% increase from baseline) established 55.8 ± 3.6 s post drug administration (see Fig. 2A, Table 2). The response to PGF2α was slower to establish in comparison to that of β-AR stimulation (ISO), which increased LV developed pressure 17.2 ± 0.9 s post ISO application (refer to Table 3 & Fig. 5). Therefore, although both interventions exerted similar positively inotropic effects, they apparently did so via separate cellular mechanisms, which altered the time to establish the peak response. The biphasic response to PGF2α in the isolated perfused heart would include: i) an effect on the coronary vascular smooth muscle, and subsequently ii) the activation of downstream, signalling pathways within the cardiomyocytes. Previous findings have reported PGF2α acts as a vasoconstrictor (Karmazyn and Dhalla, 1982; Nakano, 1968), which could reduce coronary flow; potentially affecting oxygen supply and LV pressure development. However, in our study the perfusate was delivered to the hearts at constant flow, therefore we cannot attribute the distinctive inotropic response of PGF2α in comparison to ISO to changes in flow rate. Furthermore, fixed tissue sections showed cardiomyocytes from non-hypertrophied MCT LV were adjacent to several capillaries (Fig. 7C), as shown previously (Leonard et al., 2012). This suggests that the diffusion distance between capillaries and myocytes is minimal. Therefore, the slower time to reach maximum LV pressure after PGF2α, in

comparison to peak ISO response, cannot be explained by its effects on the coronary vessels. Instead, we attribute the slower onset of the peak PGF2α response in both rat groups to its downstream signalling pathway within the cardiomyocytes. Metsa (Metsa Ketela, 1981) reported that the positive inotropic response of PGF2α was independent of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) activity, such as that seen with β-AR stimulation. Previously, it was shown that PGF2α increased Ca^{2+} transient amplitude in isolated trabeculae from rat hearts via a protein kinase C (PKC) sensitive signalling pathway (Shen et al., 2016).

A major finding of this study was that PGF2α triggered sustained, slow onset arrhythmic activity in 40% of MCT hearts (see Fig. 2C). No effect of PGF2α was observed on pacemaker activity, since HR was unaffected in both control and hypertrophic groups (Fig. 1A), suggesting the arrhythmic activity observed in some MCT hearts was not initiated at the SA node. Instead, we speculate that the arrhythmias arose from exacerbation of Ca^{2+} handling defects within the hypertrophic cardiomyocytes, as previously shown in the MCT rat myocardium (Reilly et al., 2001; Vescovo et al., 1989; Power et al., 2018a).

Why 60% of MCT hearts did not show arrhythmic activity in response to PGF2α is unclear, as RV thickness (data not shown) and body weight was not different between the two MCT subgroups (see Supplementary Fig. 1). However, Supplementary Fig. 2 shows a trend towards greater LV developed pressure in the arrhythmic MCT hearts relative to the non-arrhythmic MCT and CON hearts. Our data shows 0.3 μM PGF2α is too high a dose for use as an alternative inotropic agent for improving
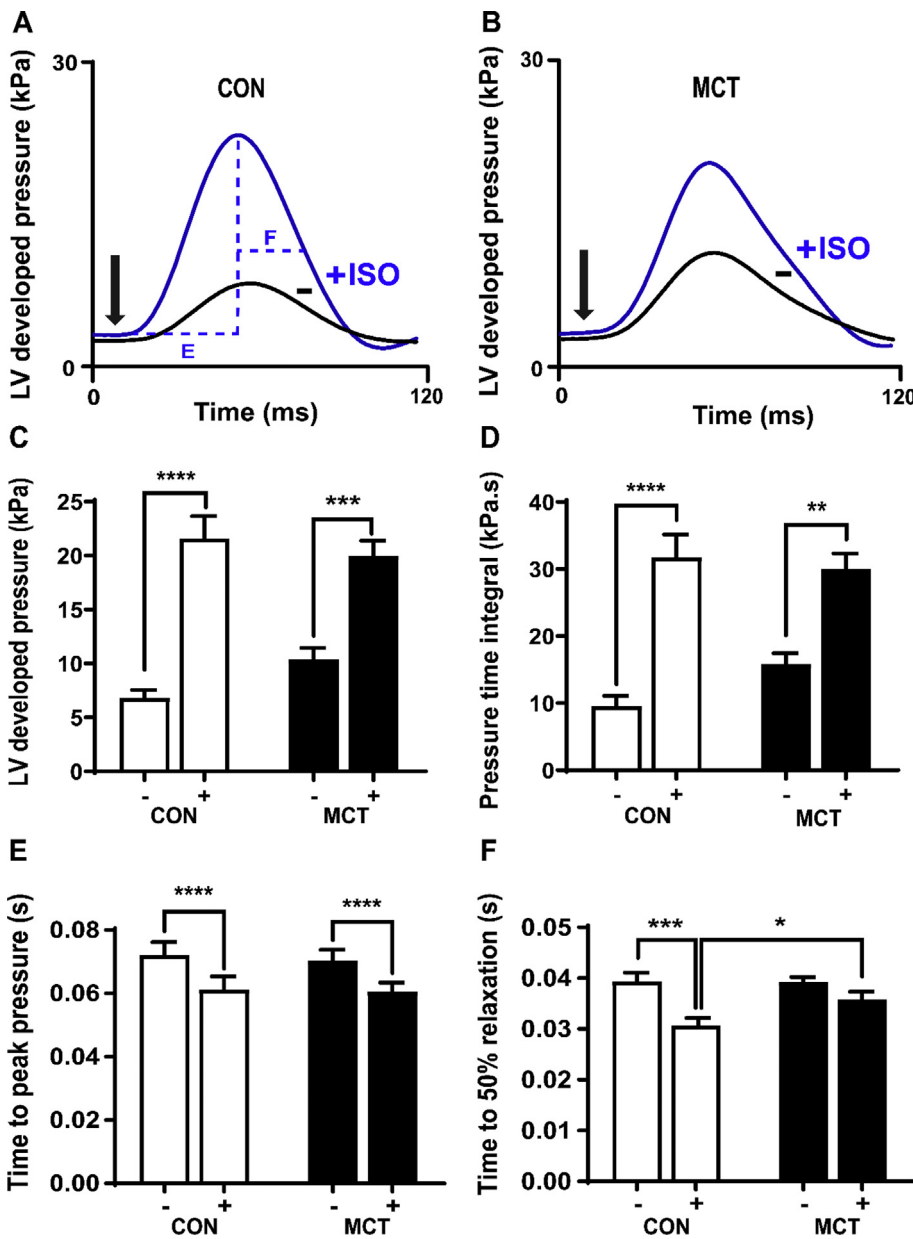
Fig. 6. Left ventricular pressure and time course of pressure before and at the peak of the response to 0.03 μM isoproterenol (+) in CON (white) and MCT (black) isolated hearts. Panels A & B show overlays of LV developed pressure synchronised to the peak of the QRS at pre-drug baseline (- black) and during maximum ISO response (+blue) throughout a single cardiac single in representative CON and MCT hearts. The blue dashes on Panel A represent how the time to peak pressure (in Panel E) and the time to 50% relaxation (in Panel F) were calculated Results are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

contractile function. Previously, the physiological release of PGF2α in response to stretch was shown to be in the nanomolar range (Shen et al., 2016).

4.4. Inotropic response to β-AR stimulation

The most common pathway upregulated in hypertrophy is β-AR receptor signalling from increased sympathetic nerve stimulation to maintain cardiac output by PKA activity. β_1-AR receptors are $G_{\alpha s}$ proteins, which are activated by the binding of catecholamines, resulting in a positive inotropic (and chronotropic) response elicited by PKA activity (Reuter et al., 1983; Tsien et al., 1986; Bers, 2007). In this study, application of 0.03 μM isoproterenol, a non-selective β-AR agonist, increased LV pressure in both CON and MCT hearts (Fig. 5A, B & 6A–C). MCT hearts also had a reduced % increase in LV pressure development and a decreased change in PTI from pre-drug baseline to maximum ISO response relative to CON hearts (Table 3). Similarly, trends towards a decreased maximum rate of rise in response to ISO were also observed in the MCT rat hearts (Supplementary Fig. 3). Collectively, our results show

that the chronotropic and lusitropic effects of β-AR stimulation were reduced but still conserved in the MCT hearts (Figs. 1B and 6F). However, when comparing the differences in PTI from baseline to maximum drug effect, the change in PTI with ISO is larger in CON than MCT hearts (Table 3). Since PTI indicates contractile activity independent of HR, a lower PTI during ISO in MCT confirms that there is a dampened inotropic response to β-AR stimulation. Since the chronotropic effects of ISO is conserved in the MCT hearts (Fig. 1), in the absence of an inotropic response, ISO is considered energetically unfavourable. Some studies have reported a reduction in inotropic response to β-AR stimulation in rat ventricular hypertrophy (Scamps et al., 1990; Kumano et al., 1983; Seyfarth et al., 2000; Foster et al., 1991; Power et al., 2018b), which has been attributed to either a down regulation of β_1-AR receptors or a reduction in cAMP activity. However, previous findings in MCT RV trabeculae have shown a similar time constant of Ca^{2+} decay to CON RV trabeculae during maximum ISO response (Kumano et al., 1983). Therefore, the reduction in inotropic response cannot simply be attributed to decreased β AR receptor expression, but potentially as a result of impaired mitochondrial function (Kumano et al., 1983). Overall, there
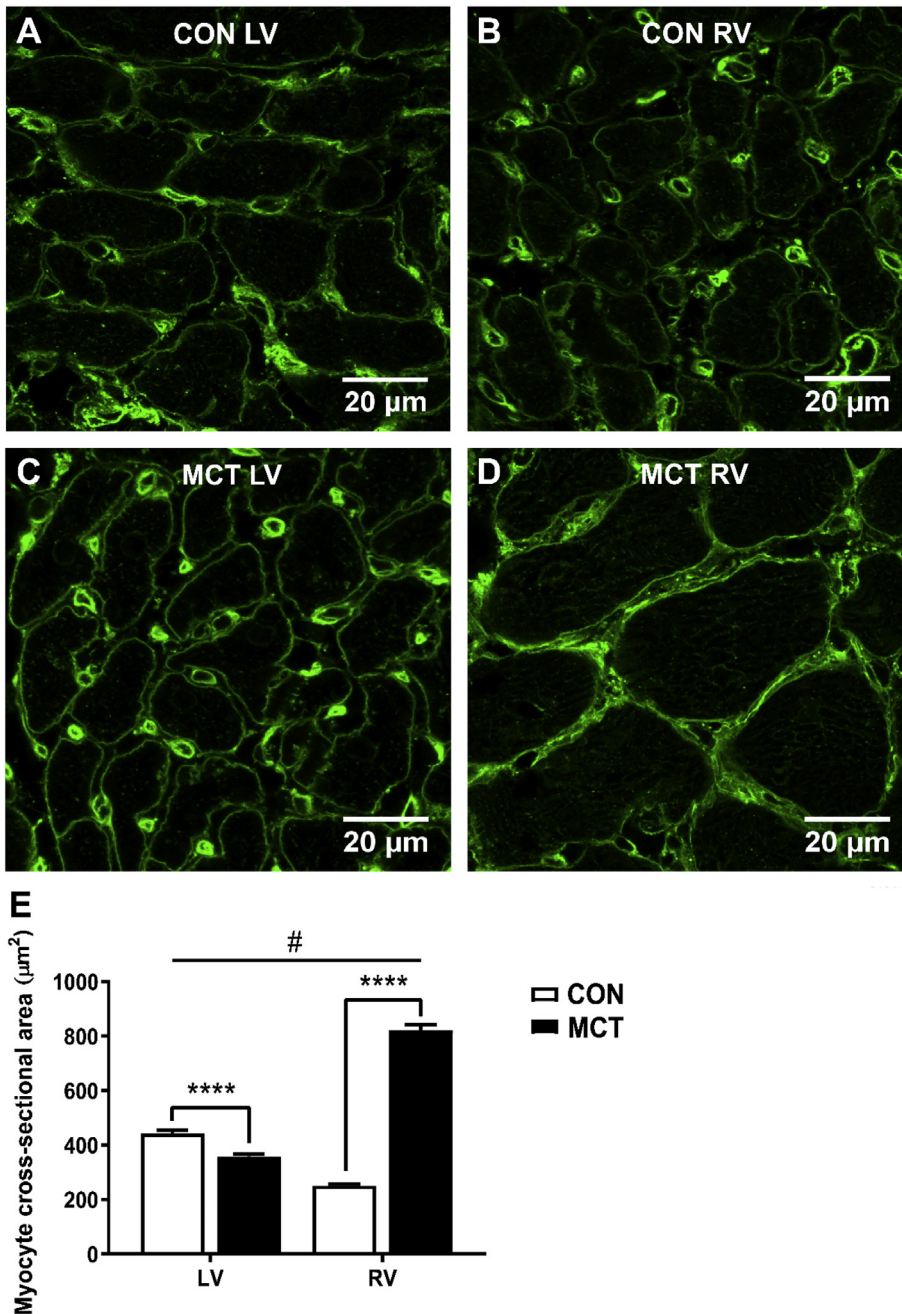
Fig. 7. Confocal images (Panels A–D) and analysis of rat ventricular tissue in cross section (Panel E) from N = 3 CON and N = 3 MCT hearts. The confocal images show fixed left ventricular (LV) and right ventricular (RV) tissue sections from CON (Panels A & B) and MCT (Panels C & D) hearts, labelled with a sarcolemma and extracellular matrix marker (wheat germ agglutinin). Results from the analyses of cross-sectional area (Panel E) are expressed as mean ± SEM. MCT RV myocytes (n = 108) had a larger cross-sectional area in comparison to MCT LV myocytes (n = 237, #P < 0.0001) and both CON LV (n = 85) and CON RV (n = 160) myocytes (****P < 0.0001). Panel E also shows CON LV myocytes had a larger cross-sectional area in comparison to both MCT LV (****P < 0.0001) and CON RV myocytes (#P < 0.0001).

are two key reasons supporting the need for developing positive inotropes that are non-catecholaminergic: i) the increased energetic cost of using β-AR stimulation to improve performance; and ii) the prior desensitisation to β-AR stimulation as a result of either a downregulation in β-AR receptors, impaired mitochondrial function or continuous β-blocker treatment.

4.5. Structural analysis of ventricular tissue

PAH is characterized by sustained pressure overload in the RV, which leads to compensatory hypertrophy to the RV free wall (Fig. 7D, E). Previously, it has been shown that RV hypertrophy directly affects the mechanical output of the LV, which was attributed to LV myocyte atrophy (Han et al., 2018). We found CON RV myocytes had significantly smaller cross-sectional areas compared to CON LV myocytes (Fig. 7). On the contrary, MCT LV myocytes were not only smaller than the MCT RV

myocytes, but they were also significantly smaller than the CON LV myocytes, providing further evidence of LV atrophy in MCT hearts.

5. Conclusion

PGF2α elicited a positive inotropic response in isolated perfused hearts from healthy rats, and from 60% of MCT rats with established right ventricular hypertrophy. However, the dose of 0.3 μM PGF2α investigated triggered sustained, slow onset, arrhythmic activity in 40% of hypertrophic MCT hearts. The peak inotropic response was slower to establish in comparison to the characteristic response to β-AR stimulation, which suggests PGF2α acts via a separate signalling pathway within cardiomyocytes. Importantly, PGF2α was positively inotropic in healthy hearts, with no chronotropic effects, unlike β-AR stimulation. However, hypertrophic MCT hearts had a *reduced* response to β-AR stimulation, perhaps a result of desensitisation. This illustrates the importance of

developing non-catecholaminergic inotropes. Although the response to PGF2α was arrhythmogenic in some hearts, a decreased dose of PGF2α should be investigated because of its energetic advantage.

Author Contributions

Study conceived by MLW; experiments performed by AK, SK; data analysis: AK; drafting of the article: AK, MLW; graphical abstract: AK; critical revision of the article for important intellectual content AK, SK, MLW.

Disclosure of funding

The University of Auckland Faculty Research Development Fund.

Declaration of Competing Interest

All authors state they have no conflicts of interest.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https ://doi.org/10.1016/j.crphys.2019.12.002.

References

Bazett, H.C., 1920. An analysis of the time-relations of electrocardiograms. Heart 7, 353–370.

Bell, R.M., Mocanu, M.M., Yellon, D.M., 2011. Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion. J. Mol. Cell. Cardiol. 50 (6), 940–950.

Benoist, D., Stones, R., Drinkhill, M., Bernus, O., White, E., 2011. Arrhythmogenic substrate in hearts of rats with monocrotaline-induced pulmonary hypertension and right ventricular hypertrophy. Am. J. Physiol. 300 (6), H2230–H2237.

Benoist, D., Stones, R., Drinkhill, M.J., Benson, A.P., Yang, Z., Cassan, C., et al., 2012. Cardiac arrhythmia mechanisms in rats with heart failure induced by pulmonary hypertension. Am. J. Physiol. Heart Circ. Physiol. 302 (11), H2381–H2395.

Benoist, D., Stones, R., Benson, A.P., Fowler, E.D., Drinkhill, M.J., Hardy, M.E., et al., 2014. Systems approach to the study of stretch and arrhythmias in right ventricular failure induced in rats by monocrotaline. Prog. Biophys. Mol. Biol. 115 (2), 162–172.

Bers, D.M., 2007. Going to cAMP just got more complicated. J. Physiol. 583 (2), 415–416.

Bristow, M.R., 2000. β-Adrenergic receptor blockade in chronic heart failure. Circulation 101 (5), 558–569.

Chesney, C., Allen, J., Hsu, I., 1974. Right ventricular hypertrophy in monocrotaline pyrrole treated rats. Exp. Mol. Pathol. 20 (2), 257–268.

Correia-Pinto, J., Henriques-Coelho, T., Roncon-Albuquerque, R., Lourenço, A.P., Melo-Rocha, G., Vasques-Nóvoa, F., et al., 2009. Time course and mechanisms of left ventricular systolic and diastolic dysfunction in monocrotaline-induced pulmonary hypertension. Basic Res. Cardiol. 104 (5), 535–545.

Dickstein, K., Cohen-Solal, A., Filippatos, G., McMurray, J.J.V., Ponikowski, P., Poole-Wilson, P.A., et al., 2008. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2008‡. Eur J Fail 10 (10), 933–989.

Eisner, D.A., Caldwell, J.L., Kistamás, K., Trafford, A.W., 2017. Calcium and excitation-contraction coupling in the heart. Circ. Res. 121 (2), 181–195.

Fong Yew, S., Reeves, K.A., Woodward, B., 1998. Effects of prostaglandin F2α on intracellular pH, intracellular calcium, cell shortening and L-type calcium currents in rat myocytes. Cardiovasc. Res. 40 (3), 538–545.

Foster, K.A., Hock, C.E., Reibel, D.K., 1991. Altered responsiveness of hypertrophied rat hearts to alpha- and beta-adrenergic stimulation. J. Mol. Cell. Cardiol. 23 (1), 91–101.

Gómez, A.M., Valdivia, H.H., Cheng, H., Lederer, M.R., Santana, L.F., Cannell, M.B., et al., 1997. Defective excitation-contraction coupling in experimental cardiac hypertrophy and heart failure. Science 276 (5313), 800.

Gomez-Arroyo, J.G., Farkas, L., Alhussaini, A.A., Farkas, D., Kraskauskas, D., Voelkel, N.F., et al., 2012. The monocrotaline model of pulmonary hypertension in perspective. Am. J. Physiol. Lung Cell Mol. Physiol. 302 (4), L363–L369.

Han, J.-C., Guild, S.-J., Pham, T., Nisbet, L., Tran, K., Taberner, A.J., et al., 2018. Left-ventricular energetics in pulmonary arterial hypertension-induced right-ventricular hypertrophic failure. Front. Physiol. 8 (1115).

Hessel, M.H., Steendijk, P., den Adel, B., Schutte, C.I., van der Laarse, A., 2006. Characterization of right ventricular function after monocrotaline-induced pulmonary hypertension in the intact rat. Am. J. Physiol. Heart Circ. Physiol. 291 (5), H2424–H2430.

Huang, Y., Liu, X.-L., Wen, J., Huang, L.-H., Lu, Y., Miao, R.-J., et al., 2017. Downregulation of the β1 adrenergic receptor in the myocardium results in insensitivity to metoprolol and reduces blood pressure in spontaneously hypertensive rats. Mol. Med. Rep. 15 (2), 703–711.

Jones, J.E., Mendes, L., Rudd, M.A., Russo, G., Loscalzo, J., Zhang, Y.-Y., 2002. Serial noninvasive assessment of progressive pulmonary hypertension in a rat model. Am. J. Physiol. Heart Circ. Physiol. 283 (1), H364.

Karmazyn, M., Dhalla, N.S., 1982. Effect of adenosine on the cardiac actions of prostaglandins E2, I2, and F2α. Can. J. Physiol. Pharmacol. 60 (6), 819–824.

Krayenbuehl, H.P., Turina, J., Hess, O., 1978. Left ventricular function in chronic pulmonary hypertension. Am. J. Cardiol. 41 (7), 1150–1158.

Kumano, K., Upsher, M.E., Khairallah, P.A., 1983. Beta adrenergic receptor response coupling in hypertrophied hearts. Hypertension 5 (2 Pt 2), I175.

Lamberts, R.R., Vaessen, R.J., Westerhof, N., Stienen, G.J., 2007. Right ventricular hypertrophy causes impairment of left ventricular diastolic function in the rat. Basic Res. Cardiol. 102 (1), 19–27.

Leineweber, K., Brandt, K., Wludyka, B., Beilfuí, A., Pφnicke, K., Heinroth-Hoffmann, I., et al., 2002. Ventricular hypertrophy plus neurohumoral activation is necessary to alter the cardiac β-adrenoceptor system in experimental heart failure. Circ. Res. 91 (11), 1056.

Leonard, B.L., Smaill, B.H., LeGrice, I.J., 2012. Structural remodeling and mechanical function in heart failure. Microsc. Microanal. 18 (1), 50–67.

Lourenço, A.P., Roncon-Albuquerque, R., Brás-Silva, C., Faria, B., Wieland, J., Henriques-Coelho, T., et al., 2006. Myocardial dysfunction and neurohumoral activation without remodeling in left ventricle of monocrotaline-induced pulmonary hypertensive rats. Am. J. Physiol. Heart Circ. Physiol. 291 (4), H1587.

Maier, L.S., Brandes, R., Pieske, B., Bers, D.M., 1998. Effects of left ventricular hypertrophy on force and Ca2+ handling in isolated rat myocardium. Am. J. Physiol. Heart Circ. Physiol. 274 (4), H1361–H1370.

Metsa Ketela, T., 1981. Cyclic AMP-dependent and independent effects of prostaglandins on the contraction-relaxation cycle of spontaneously beating isolated rat atria. Acta Physiol. Scand. Suppl. 112 (4), 481–485.

Miura, M., Hirose, M., Endoh, H., Wakayama, Y., Sugai, Y., Nakano, M., et al., 2011. Acceleration of Ca2+ waves in monocrotaline-induced right ventricular hypertrophy in the rat. Circ. Res. 75 (6), 1343–1349.

Nakano, J., 1968. Effects of Prostaglandins E1, A1 and F2α on the coronary and peripheral circulations. Proc. Soc. Exp. Biol. Med. 127 (4), 1160–1163.

Neubauer, S., 2007. The failing heart — an engine out of fuel. N. Engl. J. Med. 356 (11), 1140–1151.

Nieminen, M.S., Böhm, M., Cowie, M.R., Drexler, H., Filippatos, G.S., Jondeau, G., et al., 2005. Executive summary of the guidelines on the diagnosis and treatment of acute heart failure. The task force on acute heart failure of the European society of cardiology. Eur. Heart J. 26 (4), 384–416.

Otani, H., Otani, H., Das, D.K., 1988. Positive inotropic effect and phosphoinositide breakdown mediated by arachidonic acid and prostaglandin F2 alpha. J. Pharmacol. Exp. Ther. 244 (3), 844.

Power, A.S., Hickey, A.J., Crossman, D.J., Loiselle, D.S., Ward, M.-L., 2018. Calcium mishandling impairs contraction in right ventricular hypertrophy prior to overt heart failure. Pflug. Arch. Eur. J. Phy. 470 (7), 1115–1126.

Power, A.S., Hickey, A.J., Crossman, D.J., Loiselle, D.S., Ward, M.-L., 2018. Calcium mishandling impairs contraction in right ventricular hypertrophy prior to overt heart failure. Pflüg. Arch. 470 (7), 1115–1126.

Reilly, A.M., Petrou, S., Pancha, R.G., Williams, D.A., 2001. Restoration of calcium handling properties of adult cardiac myocytes from hypertrophied hearts. Cell Calcium 30 (1), 59–66.

Modulation of calcium channels in cultured cardiac cells by isoproterenol and 8-bromo-cAMP. In: Reuter, H., Cachelin, A., De Peyer, J., Kokubun, S. (Eds.), 1983. Col Spring Harb Symp Quant Biol. Cold Spring Harbor Laboratory Press.

Scamps, F., Mayoux, E., Charlemagne, D., Vassort, G., 1990. Calcium current in single cells isolated from normal and hypertrophied rat heart. Effects of beta-adrenergic stimulation. Circ. Res. 67 (1), 199–208.

Seyfarth, T., Gerbershagen, H.-P., Giessler, C., Leineweber, K., Heinroth-Hoffmann, I., Pönicke, K., et al., 2000. The Cardiac β -Adrenoceptor-G-protein(s)-adenylyl Cyclase system in monocrotaline-treated rats. J. Mol. Cell. Cardiol. 32 (12), 2315–2326.

Shen, X., Kaur, S., Power, A., Williams, L.Z., Ward, M.L., 2016. Positive inotropic effect of prostaglandin f2alpha in rat ventricular trabeculae. J. Cardiovasc. Pharmacol. 68 (1), 81–88.

Suzuki, Y.J., Gladwin, M., Denholm, E.M., Gail, D.B., Voelkel, N.F., Quaife, R.A., et al., 2006 Oct 24. Right ventricular function and failure report of a national heart, lung, and blood institute working group on. Circulation 114 (17), 1883–1891.

Tanaka, Y., Bernstein, M.L., Mecham, R.P., Patterson, G.A., Cooper, J.D., Botney, M.D., 1996. Site-specific responses to monocrotaline-induced vascular injury: evidence for two distinct mechanisms of remodeling. Am. J. Respir. Cell Mol. Biol. 15 (3), 390–397.

Tanaka, Y., Takase, B., Yao, T., Ishihara, M., 2013. Right ventricular electrical remodeling and arrhythmogenic substrate in rat pulmonary hypertension. Am. J. Respir. Cell Mol. Biol. 49 (3), 426–436.

Tsien, R.W., Bean, B.P., Hess, P., Lansman, J.B., Nilius, B., Nowycky, M.C., 1986. Mechanisms of calcium channel modulation by β-adrenergic agents and dihydropyridine calcium agonists. J. Mol. Cell. Cardiol. 18 (7), 691–710.

Umar, S., Lee, J.-H., de Lange, E., Iorga, A., Partow-Navid, R., Bapat, A., et al., 2011. Spontaneous ventricular fibrillation in right ventricular failure secondary to chronic pulmonary hypertension. Circ. Arrhythm. Electrophysiol. CIRCEP 111, 967265.

Vescovo, G., Harding, S.E., Jones, M., Dalla Libera, L., Pessina, A.C., Poole-Wilson, P.A., 1989. Contractile abnormalities of single right ventricular myocytes isolated from rats with right ventricular hypertrophy. J. Mol. Cell. Cardiol. 21 (Suppl. 5), 103–111.

Wallukat, G., 2002. The beta-adrenergic receptors. Herz 27 (7), 683–690.

Werchan, P.M., Summer, W.R., Gerdes, A.M., McDonough, K., 1989. Right ventricular performance after monocrotaline-induced pulmonary hypertension. Am. J. Physiol. Heart Circ. Physiol. 256 (5), H1328–H1336.

White, E., Saint, D.A., 2012. Increased mechanically-induced ectopy in the hypertrophied heart. Prog. Biophys. Mol. Biol. 110 (2), 331–339.

Yuhki, K-i, Kojima, F., Kashiwagi, H., Kawabe, J-i, Fujino, T., Narumiya, S., et al., 2011. Roles of prostanoids in the pathogenesis of cardiovascular diseases: novel insights from knockout mouse studies. Pharmacol. Ther. 129 (2), 195–205.