

Role and possible mechanisms of clenbuterol in enhancing reverse remodelling during mechanical unloading in murine heart failure

Gopal K.R. Soppa, Joon Lee, Mark A. Stagg, Leanne E. Felkin, Paul J.R. Barton, Urszula Siedlecka, Samuel Youssef, Magdi H. Yacoub, and Cesare M.N. Terracciano*

Heart Science Centre, Imperial College London, National Heart and Lung Institute, Laboratory of Cellular Electrophysiology, Harefield Hospital, Harefield, Middlesex UB9 6JH, UK

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Aims Combined left ventricular assist device (LVAD) and pharmacological therapy has been proposed to favour myocardial recovery in patients with end-stage heart failure (HF). Clenbuterol (Clen), a β_2 -adrenoceptor (β_2 -AR) agonist, has been used as a part of this strategy. In this study, we investigated the direct effects of clenbuterol on unloaded myocardium in HF.

Methods and results Left coronary artery ligation or sham operation was performed in male Lewis rats. After 4–6 weeks, heterotopic abdominal transplantation of the failing hearts into normal recipients was performed to induce LV unloading (UN). Recipient rats were treated with saline (Sal) or clenbuterol (2 mg/kg/day) via osmotic minipumps (HF + UN + Sal or HF + UN + Clen) for 7 days. Non-transplanted HF animals were treated with Sal (Sham + Sal, HF + Sal) or clenbuterol (HF + Clen). LV myocytes were isolated and studied using optical, fluorescence, and electrophysiological techniques. Clenbuterol treatment improved *in vivo* LV function measured with echocardiography (LVEF (%): HF 35.9 \pm 2 [16], HF + Clen 52.1 \pm 1.4 [16]; $P < 0.001$; mean \pm SEM [n]). In combination with unloading, clenbuterol increased sarcomere shortening (amplitude (μm): HF + UN + Clen 0.1 \pm 0.01 [50], HF + UN + Sal 0.07 \pm 0.01 [38]; $P < 0.001$) by normalizing the depressed myofilament sensitivity to Ca^{2+} (slope of the linear relationship between Ca^{2+} transient and sarcomere shortening hysteresis loop during relaxation (μm /ratio unit): HF + UN + Clen 2.13 \pm 0.2 [52], HF + UN + Sal 1.42 \pm 0.13 [38]; $P < 0.05$).

Conclusion Clenbuterol treatment of failing rat hearts, alone or in combination with mechanical unloading, improves LV function at the whole-heart and cellular levels by affecting cell morphology, excitation–contraction coupling, and myofilament sensitivity to calcium. This study supports the use of this drug in the strategy to enhance recovery in HF patients treated with LVADs and also begins to elucidate some of the possible cellular mechanisms responsible for the improvement in LV function.

1. Introduction

End-stage heart failure (HF) has a high incidence in the community with a mortality of 45% after 1 year.¹ Heart transplantation is the most effective treatment for this disease but is hindered due to an inadequate availability of donor organs. Among other treatments, left ventricular assist devices (LVADs) have been shown to be a suitable alternative.² LVAD support is primarily used as a 'bridge to transplantation' where the failing heart can be supported by mechanical circulatory assistance until a suitable donor organ becomes available.³ In few patients, the LVAD also acts as 'bridge to recovery' since it induces substantial functional improvement that allows explantation of the device

without the need of transplantation.⁴ However, the rate for explantation of the LVAD is low with an incidence of only 4–24%.^{5–7} In addition, the functional improvement observed during LVAD treatment seems to be a transient phenomenon.⁷

The main consequence of the use of LVAD on the heart is mechanical unloading leading to functional, structural, signalling, and molecular changes in HF.⁸ One important effect is the development of myocardial atrophy with possible detrimental consequences. Mechanical unloading in animal studies shows time-dependent changes in myocyte contractile function and excitation–contraction (EC) coupling⁹ that can impair cardiac performance after prolonged treatment with LVADs.

We have recently shown that using a combination of mechanical unloading and pharmacological therapy leads

* Corresponding author. Tel: +44 1895 453 874; fax: +44 1895 828 900.
E-mail address: c.terracciano@imperial.ac.uk

to a substantially improved recovery rate in patients with dilated cardiomyopathy.^{10,11} Together with other drugs, the β_2 -adrenoceptor (β_2 -AR) agonist clenbuterol has been included in the protocol with the aim to prevent unloading-induced atrophy and consequent myocardial dysfunction. Clenbuterol is known to produce skeletal¹² and cardiac hypertrophy, to affect EC coupling and cellular metabolism,¹³ gene expression and myocardial function^{14–16} in normal hearts. Clenbuterol has been shown to affect myocardial structure, function, metabolism, gene and protein expression at whole-heart and cellular level in normal,^{13–18} failing,^{12,19} and unloaded hearts.^{12,20} In the setting of the successful combination of mechanical unloading and pharmacological therapy for the treatment of patients with HF, the specific role of clenbuterol in the recovery process remains controversial.

The pathological process involved in the development of HF has been termed 'ventricular remodelling', a concept relating to a complex of anatomic, functional, cellular, and molecular changes that the myocardium undergoes in response to the injury and increased wall stress.¹ LVAD support has been shown to affect and sometimes reverse the remodelling process ('reverse remodelling').²¹ Whether clenbuterol enhances the process of reverse remodelling in unloaded failing hearts is unknown.

In the present study, we have investigated the role and possible mechanisms of clenbuterol in enhancing reverse remodelling during mechanical unloading in a murine chronic HF model. Specifically, we have investigated the effects of clenbuterol on whole-heart function, cell size, EC coupling, myofilament sensitivity to Ca^{2+} , and electrophysiological properties in a rat model of HF, induced by left coronary artery (LCA) ligation, and unloading, using abdominal heart transplantation.

2. Methods

Detailed description of methods can be found in Supplementary material.

2.1 Animal models

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Rat model of chronic post-infarction cardiomyopathy was used as it is a well established reproducible model of dysfunction.^{22,23} Syngeneic rat strains avoid the need for immunosuppression after abdominal transplantation. Therefore, male Lewis rats (200–300 g) were used for all experiments. HF was induced by permanent LCA ligation as previously described.^{22,23} Sham operated animals underwent left thoracotomy and the needle was passed under the LCA and removed without ligation. Change in LV function was assessed by transthoracic echocardiography (Acuson Sequoia™ 256; Acuson, USA) over a period of 4–6 weeks, as previously described.²⁴

To test the effect of clenbuterol in HF, Sham and HF animals received an osmotic minipump (Model 2001, Alzet) containing clenbuterol (2 mg/kg body weight/day) or normal saline (Sal) for 7 days (Sham + Sal, HF + Sal or HF + Clen), implanted subcutaneously.

To test the effects of mechanical unloading (UN), the failing hearts were heterotopically transplanted²⁵ into the abdomen of control Lewis rats and the recipients then received an osmotic minipump containing clenbuterol or Sal for 7 days (HF + UN + Sal or HF + UN + Clen). Briefly the failing heart was removed from the donor rat following cardioplegic arrest and the donor aorta was

anastomosed to the recipient abdominal aorta and the donor pulmonary artery to the recipient inferior vena cava.

2.2 Ex vivo left ventricular function assessment

Ex vivo LV function of the transplanted heart was assessed by measuring the pressure–volume relationship using an intraventricular balloon as described previously.²⁶ Hearts were perfused with normal Tyrode (NT) solution at 37°C on a Langendorff apparatus and paced with platinum electrodes on the right ventricle to maintain a heart rate of ~300 bpm.

2.3 Cardiomyocyte studies

LV myocytes were isolated following a previously described method²⁷ using collagenase (1 mg/mL, Worthington) and hyaluronidase (0.6 mg/mL, Sigma) for 8–10 min. All cellular experiments were performed at 37°C, within 7–8 h of cell isolation. Cell size was assessed from digital images of the cardiomyocytes. The projected two-dimensional area for each cell was measured using ImageJ software (NIH, USA).

2.3.1 Sarcomere shortening and cytoplasmic calcium measurement

Cells were superfused with a NT solution containing (in mM): NaCl 140, KCl 6, MgCl_2 1, glucose 10, HEPES 10, CaCl_2 1; pH 7.4, field-stimulated at 1 Hz and illuminated by red light (wavelength of >600 nm), enabling the cardiomyocyte in the field of view to be visualized for simultaneous measurement of Indo-1 fluorescence (Indo-1 AM) and sarcomere shortening using a Ionoptix system (Ionoptix Corporation, USA) as described in data supplement. Sarcoplasmic reticulum (SR) Ca^{2+} content was assessed by rapid application of 20 mM caffeine and amplitude of caffeine-induced Indo-1 transient taken as an index of SR Ca^{2+} content (see Supplementary material for protocol). Time constant (τ) of the transient was assessed by fitting a mono-exponential curve on the decay phase to assess the contribution of sodium–calcium exchanger (NCX) to Ca^{2+} extrusion.

2.3.2 Assessment of myofilament sensitivity to calcium

Cardiomyocyte myofilament sensitivity to Ca^{2+} was assessed using a technique described by Bailey *et al.*²⁸ Hysteresis loops were generated for all cells studied and a constant region of the declining phase of the loop (the region between 20 and 80% from the peak of the sarcomere shortening) was selected and the slope value of this line was calculated by fitting a linear regression line. Only slope values of the regression lines with a goodness of fit (r^2) greater than 0.8 were used for this analysis and data from each treatment group were compared to assess changes in myofilament sensitivity to Ca^{2+} .

2.3.3 Electrophysiological studies

Cardiomyocytes were superfused with NT solution. Action potentials (APs) were measured in current-clamp mode using ~30 M Ω resistance pipettes (containing (in mM) KCl 2000, HEPES 5, EGTA 0.1; pH 7.2) using a 1 ms, 1.2–1.4 nA pulse. For the measurement of NCX current (I_{NCX}), cells were superfused with a solution containing (in mM) NaCl 140, HEPES 10, glucose 10, MgCl_2 1, CaCl_2 1, CsCl 6; 0.01 strophanthidin; 0.01 nifedipine; pH 7.4. The pipette-filling solution contained (in mM): CsCl 45, HEPES 20, MgCl_2 11, Na_2ATP 10, CsOH 100, EGTA 50, CaCO_3 25; pH 7.2. Pipette resistance was 2.3–3.5 M Ω . A 3 s descending ramp was applied from +80 to –120 mV at 0.1 Hz, from a holding potential of –40 mV. The I_{NCX} was taken as the 5 mM Ni^{2+} -sensitive component. For the measurement of L-type calcium current ($I_{\text{Ca,L}}$), the same hardware configuration and pipette-filling solution, as for AP were used, but in voltage-clamp mode as previously reported.^{29,30} Current–voltage (I–V) relationships for $I_{\text{Ca,L}}$ were built using 450 ms depolarization steps from a holding potential of –40 mV (range –45 to +50 mV,

in 5 mV increments). The measured current is nifedipine-sensitive and 4-aminopyridine-insensitive, hence attributable to $I_{Ca,L}$. To study time-dependent inactivation, a bi-exponential of the $I_{Ca,L}$ measured at 0 mV was fitted to the decay, and fast and slow components of $I_{Ca,L}$ inactivation identified (τ_{fast} and τ_{slow}). The voltage-dependent steady-state activation profile (G/G_{MAX}) and steady-state inactivation (F/F_{MAX}) of the $I_{Ca,L}$ was studied as previously described.³¹ All data were analysed using Clampfit[®] software (Axon Instruments, CA, USA).

2.4 Gene expression analysis

RT-PCR was performed as described previously³² (see Supplementary material). Primers and standard Taqman probes for α - and β -myosin heavy chain (MHC) were designed in-house using Primer Express v.1.5 (α -MHC: Forward primer: 5'-caaacatgagccactcttct-3', reverse primer: 5'-ggaaggatgagccttcttcttg-3', probe: 5'-FAM-ccactgt-caccggtatcagcagaagcata-3'. β -MHC: Forward primer: 5'-agctcctaag-taatctgt ttgcca-3', reverse primer: 5'-aaaggatgagccttcttcttgct-3', probe 5'-FAM-ctgtctacaggtgcatcagctccagcat-3'). Data were analysed using the comparative Ct method with target gene expression normalized to 18S ribosomal RNA levels.

2.5 Statistical analysis

Statistical comparison of data was performed using one-way analysis of variance followed by Newman-Keuls post hoc test for individual significant differences or Student's *t*-test where appropriate. All statistical analyses were performed using Prism 4 software (GraphPad Software, Inc.) and $P < 0.05$ was considered significant. Data are expressed as mean \pm SEM [*n*], where *n* is the number of cells unless otherwise specified.

3. Results

3.1 The effects of clenbuterol on left ventricular function of failing hearts *in vivo*

LV function of failing and sham operated animals, treated with clenbuterol or saline, was assessed *in vivo* by echocardiography. LCA ligation produced LV dysfunction after 4–6 weeks at whole-heart level characterized by reduced LVEF, reduced LV fractional shortening, LV wall thinning, and LV chamber dilation on echocardiography (Figure 1B and D, Table 1). Clenbuterol treatment of failing hearts increased LVEF by $\sim 16\%$, increased LV fractional shortening by $\sim 6\%$, and improved thickening of the viable posterior wall by $\sim 20\%$ (Figure 1C and D, Table 1). These results indicate that clenbuterol affects LV function of failing hearts.

3.2 The effects of clenbuterol on left ventricular function *ex vivo*

LV function of normally loaded and mechanically unloaded failing hearts, treated with clenbuterol or saline, was assessed by *ex vivo* pressure–volume relationship studies. LCA ligation reduced LV developed pressure and dP/dT_{max} (Figure 1E and F). Clenbuterol treatment or mechanical unloading alone did not affect these parameters but the combination of both clenbuterol treatment and mechanical unloading of failing hearts improved LV dP/dT_{max} (Figure 1G and H). These results indicate that the combination of clenbuterol and mechanical unloading enhances LV functional recovery of failing hearts.

3.3 The effects of clenbuterol on the unloading-induced cell size reduction

Two-dimensional cell surface area, length, and width of LV cardiomyocytes from all experimental groups were assessed by planimetry. After LCA ligation, cardiomyocyte hypertrophy developed, as shown by an increase in cell surface area (μm^2) Sham + Sal 3251 ± 64 [173], HF + Sal 4129 ± 92 [106]; $P < 0.001$), increase in length (μm): Sham + Sal 128 ± 2 [173], HF + Sal 147 ± 2 [106]; $P < 0.001$) and increase in width (μm): Sham + Sal 35 ± 1 [173], HF + Sal 40 ± 1 [106]; $P < 0.001$). As shown in Figure 2, clenbuterol treatment alone did not further increase cell size. Mechanical unloading alone reduced cell size to normal values (cell area (μm^2): Sham + Sal 3251 ± 64 [173] vs. HF + UN + Sal 3276 ± 63 [181]; $P = \text{NS}$) but clenbuterol treatment during unloading limited this effect (cell area (μm^2): HF + UN + Sal 3276 ± 63 [181], HF + UN + Clen 3547 ± 59 [232]; $P < 0.01$; HF + Sal vs. HF + UN + Clen; $P < 0.001$). These results indicate that clenbuterol limits unloading-induced cell size reduction and supports the rationale of its use to limit unloading-induced myocardial atrophy.

3.4 The effects of clenbuterol on cardiomyocyte contractility

Cardiomyocyte contractility was assessed during field-stimulation by real time changes of the sarcomere length. Sarcomere shortening and relaxation were impaired after LCA ligation (Figure 3A, Table 2) and were unaffected by mechanical unloading alone (Figure 3C, Table 2). Clenbuterol treatment alone or in combination with mechanical unloading improved sarcomere shortening and relaxation (Figure 3B and D, Table 2). These results indicate that mechanical unloading alone did not affect the depressed cardiomyocyte contractility but clenbuterol combined with mechanical unloading enhanced functional recovery.

3.5 The effects of clenbuterol on calcium cycling

Cytoplasmic $[\text{Ca}^{2+}]$ was assessed by measuring Indo-1 fluorescence ratio. Cardiomyocyte Ca^{2+} cycling, following LCA ligation, was characterized by delayed Ca^{2+} release and extrusion. However, the Ca^{2+} transient amplitude was paradoxically increased (Figure 3A, Table 2). The increased Ca^{2+} transient amplitude was accompanied by increased SR Ca^{2+} content with delayed removal in caffeine-induced Ca^{2+} transient (Table 2) suggesting a reduced NCX contribution to Ca^{2+} extrusion from the cytoplasm. Clenbuterol treatment alone did not affect Ca^{2+} transient amplitude or SR Ca^{2+} content but normalized the delayed stimulation-induced and caffeine-induced Ca^{2+} transient decline (Figure 3B, Table 2). Mechanical unloading alone did not affect the Ca^{2+} transient kinetics or SR Ca^{2+} content but induced a faster decline of the caffeine-induced Ca^{2+} transient (Table 2). The combination of clenbuterol treatment and mechanical unloading of HF normalized Ca^{2+} handling at cytoplasmic and SR levels (Figure 3D, Table 2). These results indicate that the combination of clenbuterol and mechanical unloading normalized the deranged cardiomyocyte Ca^{2+} cycling.

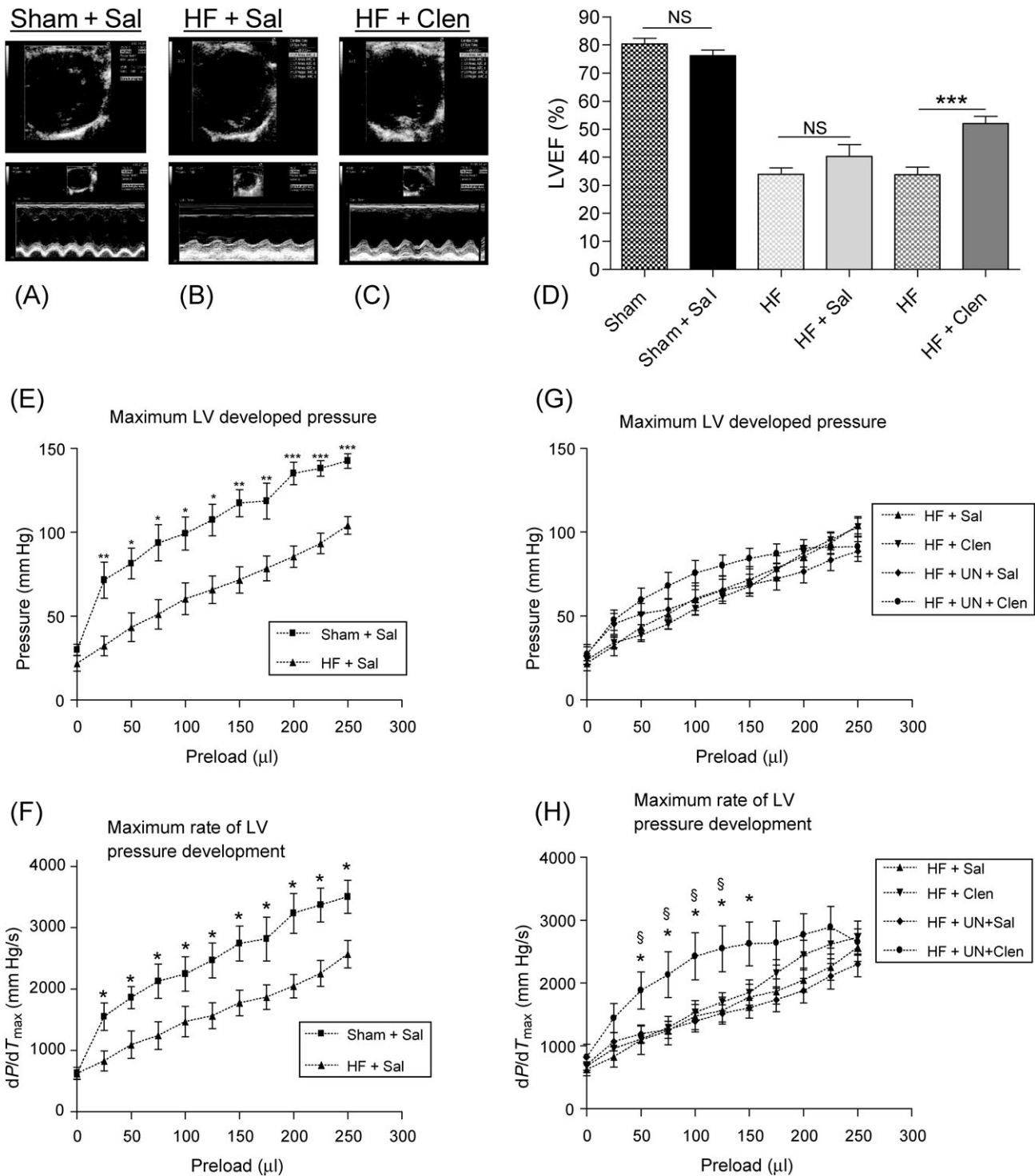


Figure 1 Representative two-dimensional (top) and M-mode (bottom) echocardiography traces at mid-papillary level of a normal control heart (A), a failing heart treated with saline showing a kinetic anterior scar and reduced contractility and thinning of posterior wall (B). A failing heart treated with clenbuterol shows improved posterior wall contractility with unchanged anterior scar movement (C). Sham operated and heart failure animals treated with saline showed no change in LVEF but clenbuterol treatment of failing hearts improved LVEF by ~18% (D) (data shown in Table 1). (*** $P < 0.001$ HF vs. HF + Clen). Pressure-volume relationship of sham operated and heart failure groups showing a reduced LV developed pressure (E) and dP/dT_{max} (F) of failing hearts treated with saline. The combination of clenbuterol treatment and mechanical unloading improved dP/dT_{max} (H) but did not affect the LV developed pressure (G). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ HF + Sal vs. Sham + Sal; $^{\S}P < 0.05$ HF + UN + Sal vs. HF + UN + Clen).

3.6 The effects of clenbuterol on myofilament sensitivity to calcium

Myofilament sensitivity to Ca^{2+} was assessed by sarcomere length- $[Ca^{2+}]_i$ relationship from simultaneously recorded

Indo-1 fluorescence changes and sarcomere shortening of LV cardiomyocytes. The sarcomere shortening- Ca^{2+} transient hysteresis loops showed that less contractility was produced for the same $[Ca^{2+}]_i$ following LCA ligation,

Table 1 Echocardiographic parameters measured in experimental groups

	Sham	Sham + Sal	HF	HF + Sal	HF	HF + Clen
LV ejection fraction (%)	79.0 ± 1.4 [12]	81.1 ± 1.9 [11]	35.9 ± 2.4 [11]**	41.7 ± 1.8 [10]**	35.9 ± 2 [16]**	52.1 ± 1.4 [16]***†††
LV fractional shortening (%)	44.7 ± 1.8 [12]	48.3 ± 2.1 [10]	18.9 ± 1.6 [11]**	18.0 ± 1.3 [9]**	18.9 ± 0.9 [15]**	24.7 ± 1.1 [16]***††
LV diameter (cm)						
Diastole	0.71 ± 0.02 [12]	0.73 ± 0.02 [11]	0.98 ± 0.02 [11]**	0.98 ± 0.01 [9]**	0.96 ± 0.02 [15]**	1.02 ± 0.03 [16]**
Systole	0.39 ± 0.02 [12]	0.37 ± 0.02 [11]	0.79 ± 0.02 [11]**	0.81 ± 0.02 [9]**	0.78 ± 0.02 [15]**	0.78 ± 0.03 [16]**
LV posterior wall thickness (cm)						
Diastole	0.23 ± 0.02 [12]	0.23 ± 0.01 [11]	0.18 ± 0.01 [11]*	0.21 ± 0.02 [9]	0.18 ± 0.01 [15]*	0.20 ± 0.01 [16]
Systole	0.31 ± 0.01 [12]	0.32 ± 0.01 [11]	0.25 ± 0.01 [11]**	0.27 ± 0.02 [9]	0.25 ± 0.01 [15]**	0.30 ± 0.01 [16]†††
LV anterior wall thickness (cm)						
Diastole	0.17 ± 0.01 [12]	0.16 ± 0.01 [11]	0.10 ± 0.01 [11]**	0.10 ± 0.01 [9]**	0.13 ± 0.01 [15]**	0.12 ± 0.01 [16]
Systole	0.29 ± 0.01 [12]	0.30 ± 0.01 [11]	0.12 ± 0.01 [11]**	0.11 ± 0.01 [9]**	0.14 ± 0.01 [15]**	0.15 ± 0.01 [16]**

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Sham; †† $P < 0.001$ HF vs. HF + Clen. (data in [] indicates number of hearts studied).

suggesting reduced myofilament sensitivity to Ca^{2+} (Figure 4E, Table 2). Clenbuterol treatment alone or in combination with mechanical unloading normalized this parameter. Mechanical unloading did not affect the depressed myofilament sensitivity to Ca^{2+} (Figure 4E, Table 2).

3.7 The effects of clenbuterol on action potential prolongation

APD was assessed in current-clamp mode as described above. Following LCA ligation, electrophysiological studies demonstrated increased AP duration at all frequencies compared to sham-operated controls (Figure 5A). Clenbuterol treatment did not affect the prolonged AP duration. Mechanical unloading alone further prolonged the AP duration at higher stimulation frequency (APD_{90} at 5 Hz (ms): HF + Sal 44.7 ± 3.0 [51], HF + UN + Sal 63.0 ± 4.8 [26]; $P < 0.01$) (Figure 5A). The combination of clenbuterol treatment and mechanical unloading normalized the prolonged AP duration in HF at all frequencies (Figure 5A). These results indicate that clenbuterol combined with mechanical unloading enhances recovery of deranged AP properties.

3.8 The effects of clenbuterol on sodium–calcium exchanger current

Clenbuterol normalized the delayed stimulation-induced and caffeine-induced Ca^{2+} transient decline suggesting an improved NCX contribution to Ca^{2+} extrusion from the cytoplasm. Therefore, I_{NCX} was measured to test if clenbuterol affected the biophysical properties of NCX. The I_{NCX} was unchanged at all voltages tested in cardiomyocytes from hearts following LCA ligation and unaffected by clenbuterol treatment alone (Figure 5B). Mechanical unloading reduced I_{NCX} but this current was partially restored by clenbuterol treatment (at +80 mV (pA/pF): HF + Sal 1.7 ± 0.2 [30], HF + UN + Sal 0.9 ± 0.1 [25], HF + UN + Clen 1.6 ± 0.3 [21]; HF + Sal vs. HF + UN + Sal, $P < 0.01$; HF + Sal vs. HF + UN + Clen, $P = NS$) (Figure 5B). These results indicate that clenbuterol combined with mechanical unloading improves NCX activity.

3.9 The effects of clenbuterol on L-type calcium current

HF reduced peak $I_{Ca,L}$ (Figure 6B) and resulted in slower inactivation times (Figure 6E and F) which may partially account for delayed Ca^{2+} release of the Ca^{2+} transient and the prolonged APD_{90} , respectively. Clenbuterol treatment alone or in combination with the mechanical unloading did not affect the $I_{Ca,L}$ properties. Mechanical unloading alone of failing hearts did not affect the peak $I_{Ca,L}$ but normalized slower $I_{Ca,L}$ inactivation times seen in HF (τ_{fast} (ms): Sham + Sal 7.2 ± 0.6 [43], HF + UN + Sal 5.5 ± 0.4 [23]; $P = NS$; τ_{slow} (ms): Sham + Sal 31.2 ± 2.0 [43], HF + UN + Sal 27.3 ± 2.0 [23]; $P = NS$) (Figure 6E and F). These results indicate that clenbuterol does not affect L-type calcium current.

3.10 The effects of clenbuterol on myosin heavy chain isoform changes

HF³³ and mechanical unloading³⁴ are known to induce a shift in the expression of MHC isoforms. This can be a reason for

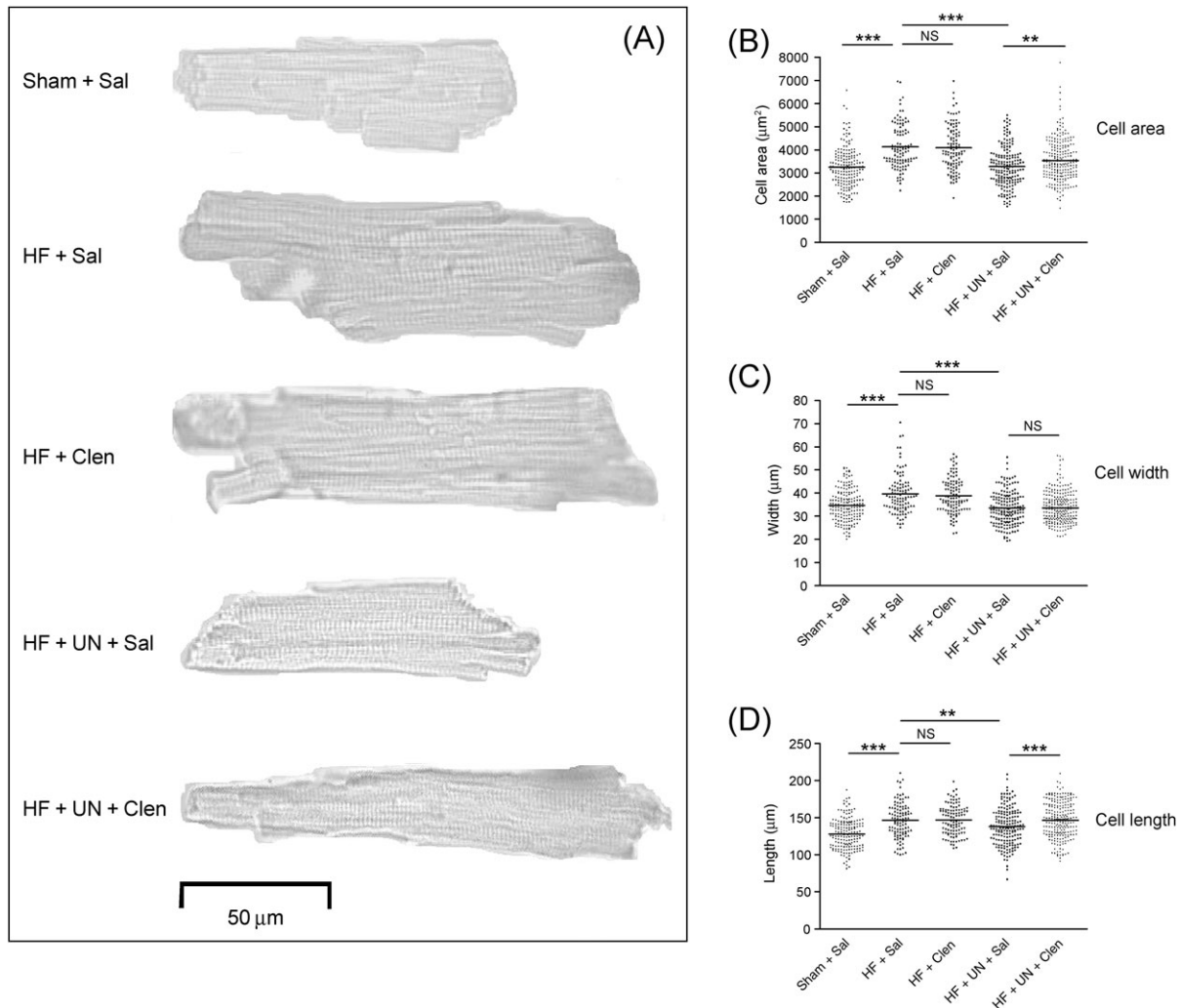


Figure 2 Representative pictures of left ventricular myocytes showing changes induced by heart failure, mechanical unloading and clenbuterol treatment (A). Heart failure induced myocyte hypertrophy, indicated by an increased cell area (B), width (C) and length (D). Clenbuterol treatment limited unloading-induced normalization of cell area by retaining cell length. (** $P < 0.01$, *** $P < 0.001$).

changes in the myofilament sensitivity to Ca^{2+} . In this study, we show that clenbuterol normalizes the depressed myofilament sensitivity to Ca^{2+} (Figure 4E). To clarify this point, gene expression of α - and β -MHC were measured by RT-PCR. There was a reduction in α -MHC RNA expression in HF, which was further reduced by mechanical unloading ((relative mRNA expression) Sham + Sal 1.58 ± 0.14 ,⁶ HF + Sal 0.93 ± 0.06 ;⁶ $P < 0.01$; HF + UN + Sal 0.31 ± 0.13 ⁵ vs. HF + Sal; $P < 0.001$). β -MHC expression was unchanged in HF and mechanical unloading. The ratio of α -MHC/ β -MHC confirmed a reduction in relative α -MHC expression in HF and mechanical unloading ((relative mRNA expression) Sham + Sal 2.05 ± 0.31 ,⁶ HF + Sal 0.83 ± 0.25 ;⁶ $P < 0.05$; HF + UN + Sal 0.14 ± 0.05 ⁵ vs. HF + Sal; $P < 0.05$). Clenbuterol treatment had no further effect on the relative α -MHC isoform expression changes ((relative mRNA expression) HF + Sal vs. HF + Clen 0.63 ± 0.12 ;⁵ $P = \text{NS}$; HF + UN + Sal vs. HF + UN + Clen 0.15 ± 0.05 ;⁴ $P = \text{NS}$). This suggests that the improved myofilament sensitivity to Ca^{2+} seen after clenbuterol treatment is unlikely to be due to changes in MHC isoform expression.

4. Discussion

This study demonstrates that the depressed whole-heart and cellular contractile function observed in HF can be improved by chronic administration of β_2 -AR agonist clenbuterol. At whole-heart level *in vivo* clenbuterol improves LVEF and, when combined with mechanical unloading, it also improves dP/dT_{max} *ex vivo*. At a cellular level, depressed sarcomere contractility is restored to normal values in HF by clenbuterol treatment, with or without mechanical unloading. Clenbuterol affects cell size, EC coupling, ion channel function and AP properties but the cellular functional improvement can be ascribed particularly to the normalization of myofilament sensitivity to Ca^{2+} . Mechanical unloading alone has no effects on the depressed sarcomere shortening or whole-heart function.

4.1 β_2 -AR agonists and heart failure

HF causes derangements of β -AR signalling pathways.³⁵ There is evidence that the β_2 -AR- G_i subtype specific

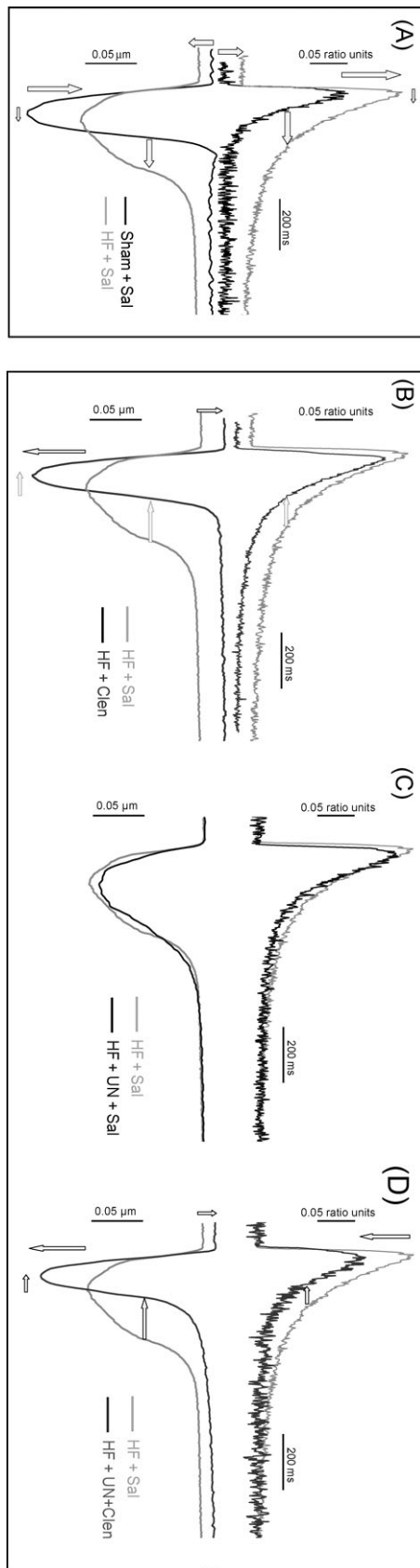


Figure 3 Simultaneously acquired sarcomere shortening and Indo-1 transients showing defective EC coupling in heart failure (A). Clenbuterol treatment, with and without mechanical unloading, improved myocyte contractility and Ca^{2+} transient kinetics (B-D). Arrows highlight statistically significant differences between groups (data shown in Table 2).

stimulation has cardioprotective effects and beneficial functional consequences in rodent ischaemic cardiomyopathy and may also be enhanced when combined with β_1 -AR blockade.^{19,23,36} In the present study, we demonstrate whole-heart and cellular function improvement with the β_2 -AR agonist clenbuterol without mechanical unloading. Xydas *et al.*¹⁹ have shown an improvement in *ex vivo* end diastolic pressure-volume relationship in failing hearts treated for 9 weeks with clenbuterol compared to β_1 blocker metoprolol. In a preliminary study, we have found that clenbuterol predominantly activates the β_2 -AR- G_i pathway³⁷ and this may explain the cardioprotective effects observed. More studies are required to elucidate this point. The role of β_2 -AR stimulation during mechanical unloading is also complex. Although mechanical unloading is known to restore β -AR responsiveness and density in HF³⁸ and increase β_2 -AR mRNA expression,³⁹ it has been shown that adenoviral gene transfer of a β_2 -AR transgene in unloaded rabbit hearts further improved myocardial function.⁴⁰ In the present study, the combination of β_2 -AR agonist clenbuterol and mechanical unloading improved whole-heart and cardiomyocyte contractile function, affected cell morphology, normalized deranged Ca^{2+} cycling, myofilament sensitivity to Ca^{2+} , AP duration and NCX current density. Our study further supports the use of β_2 -AR stimulation during mechanical unloading to improve myocardial function.

The ability of clenbuterol to induce structural changes to the myocardium is controversial. Burniston *et al.*⁴¹ have shown a dose-dependent, β_1 -AR mediated, clenbuterol-induced myocardial apoptosis, necrosis, and collagen content in the heart and skeletal muscle of the rat after clenbuterol administration. We did not selectively block the β_1 -AR in our study but it is possible to predict that this strategy would further improve the beneficial effects of clenbuterol during mechanical unloading in HF. On the basis of the above rationale to maximize the β_2 -AR mediated beneficial effects of clenbuterol during mechanical unloading, bisoprolol a selective β_1 blocker, is used in combination with clenbuterol in the patients treated with LVADs.¹¹ Other studies have shown that clenbuterol administration has a beneficial effect on fibrosis and apoptosis. Wong *et al.* have shown that clenbuterol administration in conjunction with pressure overload by aortic banding in rats increases LV mass with less fibrosis¹⁶ and collagen content¹⁵ than banding alone. George *et al.*¹² report no change in collagen content in HF patients treated with clenbuterol during mechanical unloading by LVAD.

4.2 Clenbuterol and unloading-induced myocyte atrophy

Myocardial atrophy is an important consequence of mechanical unloading and has been demonstrated in both human⁷ and animal⁴² studies. This is likely to be the main limiting factor to improve myocardial recovery and forms the basis for use of clenbuterol in patients treated with LVADs in order to improve explantation rate for 'bridge to recovery'.¹⁰ We have shown in a previous study that clenbuterol increases cardiomyocyte size in normal hearts¹³ and, in this study, limits cell size reduction after mechanical unloading in HF. In contrast, Tsuneyoshi *et al.*²⁰ found that clenbuterol could not prevent atrophy during mechanical unloading of a normal rat heart for 2 weeks. This may

Table 2 Contractile and calcium handling parameters in experimental groups

	Sham + Sal	HF + Sal	HF + Clen	HF + UN + Sal	HF + UN + Clen
1 Hz Sarcomere shortening					
Baseline SL (μm)	1.63 \pm 0.02 [46]	1.59 \pm 0.01 [68]*	1.64 \pm 0.01 [69] ^{††}	1.59 \pm 0.01 [38]	1.64 \pm 0.01 [50] ^{††,5}
Amplitude (μm)	0.08 \pm 0.01 [46]	0.07 \pm 0.01 [68]*	0.1 \pm 0.01 [69] ^{†††}	0.07 \pm 0.01 [38]	0.1 \pm 0.01 [50] ^{†††,555}
Time to peak (ms)	86.9 \pm 3.3 [46]	102.6 \pm 2.6 [68] ^{***}	84.9 \pm 2.3 [69] ^{†††}	97.8 \pm 2.7 [38]	71.9 \pm 1.7 [50] ^{†††,555}
Time to 50% relaxation (ms)	70.5 \pm 5.7 [42]	91.2 \pm 6.1 [65]*	53.6 \pm 2.4 [68] ^{†††}	90.0 \pm 6.2 [38]	46.0 \pm 2.2 [50] ^{†††,555}
1 Hz Ca²⁺ transient					
Baseline (Indo-1 ratio units)	0.31 \pm 0.01 [45]	0.33 \pm 0.01 [67]**	0.33 \pm 0.01 [69]	0.31 \pm 0.01 [38]	0.32 \pm 0.01 [48]
Amplitude (Indo-1 ratio units)	0.12 \pm 0.01 [45]	0.15 \pm 0.01 [67]**	0.14 \pm 0.01 [69]	0.14 \pm 0.01 [38]	0.11 \pm 0.01 [48] ^{†††,55}
Time to peak (ms)	33.3 \pm 1.1 [45]	36.4 \pm 0.6 [67]**	34.6 \pm 0.7 [69]	34.9 \pm 1.1 [38]	31.2 \pm 0.7 [47] ^{†††,55}
Time to 50% decay (ms)	70.9 \pm 1.5 [45]	75.4 \pm 1.4 [66]*	68.5 \pm 1.1 [69] ^{†††}	74.3 \pm 1.9 [38]	62.5 \pm 1.4 [48] ^{†††,555}
Indo-1 decay, τ (ms)	76.4 \pm 2.9 [45]	88.2 \pm 3.4 [66]*	65.8 \pm 2.2 [69] ^{†††}	83.1 \pm 3.8 [38]	58.5 \pm 1.3 [48] ^{†††,555}
Caffeine induced Ca²⁺ transient					
SR Ca ²⁺ content (Indo-1 ratio units)	0.11 \pm 0.01 [29]	0.14 \pm 0.01 [43]**	0.14 \pm 0.004 [41]	0.14 \pm 0.01 [26]	0.11 \pm 0.01 [34] ^{†,5}
Indo-1 decay, τ (s)	1.21 \pm 0.08 [22]	1.44 \pm 0.06 [42]*	1.21 \pm 0.07 [35] ^{††}	1.20 \pm 0.07 [23] [†]	1.14 \pm 0.07 [26] ^{††}
Myofilament sensitivity to Ca²⁺					
Slope value ($\mu\text{m}/\text{ratio unit}$)	2.23 \pm 0.27 [35]	1.65 \pm 0.12 [66]*	2.36 \pm 0.18 [69] ^{††}	1.42 \pm 0.13 [38]	2.13 \pm 0.2 [52] ^{†,5}

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ HF + Sal vs. Sham + Sal; [†] $P < 0.05$, ^{††} $P < 0.01$, ^{†††} $P < 0.001$ vs. HF + Sal; ⁵ $P < 0.05$, ⁵⁵ $P < 0.01$, ⁵⁵⁵ $P < 0.001$, HF + UN + Sal vs. HF + UN + Clen (data in [] indicates number of cells studied).

suggest that myocyte hypertrophy, induced by HF, may be a prerequisite for the effects of clenbuterol during mechanical unloading. It should be noted that we only assessed unloading after 1 week. It is known that longer duration of unloading in failing human⁷ and animal³⁴ hearts can deleteriously affect whole-heart and cardiomyocyte function, despite regression of myocyte hypertrophy. Testing whether clenbuterol administration is beneficial when used during longer periods of mechanical unloading of failing hearts would be of interest. Clenbuterol may have an

important role in providing an early phase of protection, delaying the onset or preventing unloading-induced atrophy. To elucidate these points, further studies are required.

We have shown that clenbuterol treatment during unloading (HF + UN + Clen) limits cardiomyocyte atrophy by a preferential retention of cell length than width. This may indicate a different form of hypertrophy induced by clenbuterol, which has been proposed to be more 'physiological'.^{14-17,43,44} In addition, in the unloaded myocardium,

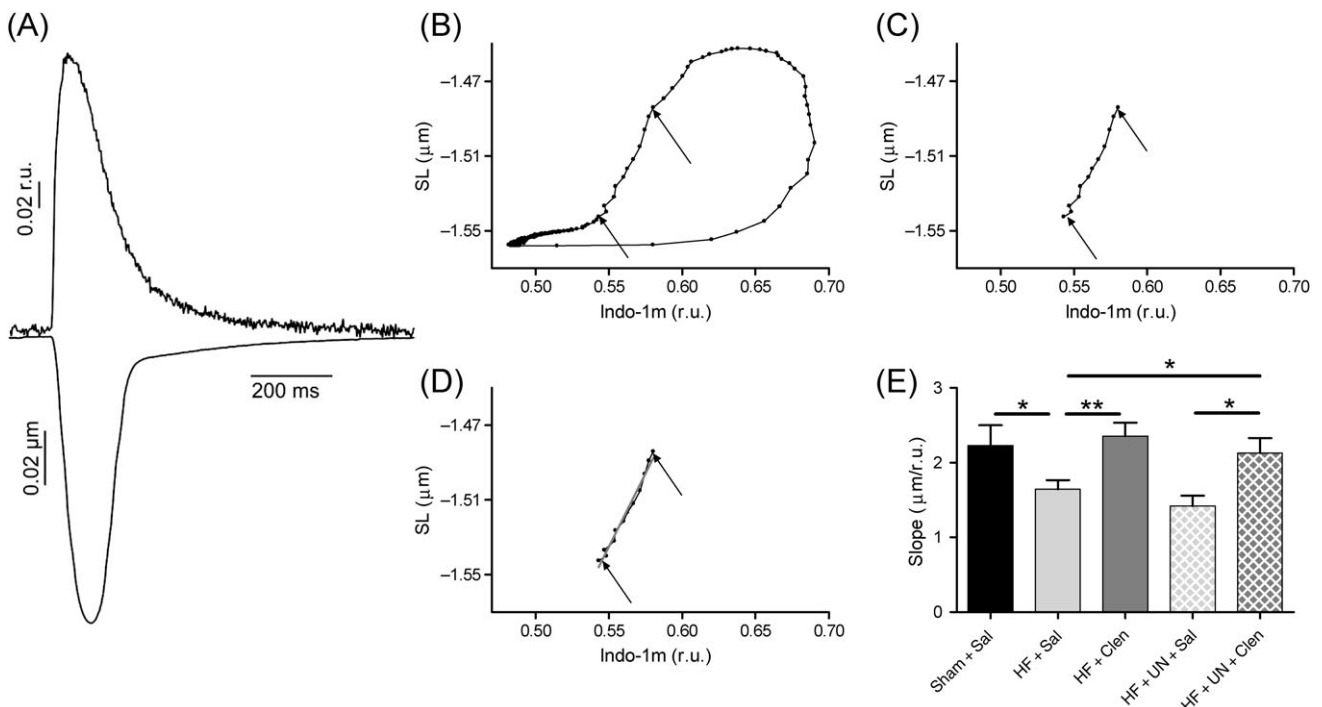


Figure 4 Simultaneously acquired sarcomere shortening and Indo-1 transient traces (A) were used to generate hysteresis loops (B) showing the relationship of sarcomere length and cytoplasmic Ca²⁺ concentration, measured by Indo-1 (r.u.–ratio units). The slope of a regression line, fitted through the linear portion of the loop (C, D), was used as a measure of myofilament sensitivity to Ca²⁺. Clenbuterol treatment improved the depressed myofilament sensitivity to Ca²⁺ in heart failure, with and without mechanical unloading (E, data shown in Table 2). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

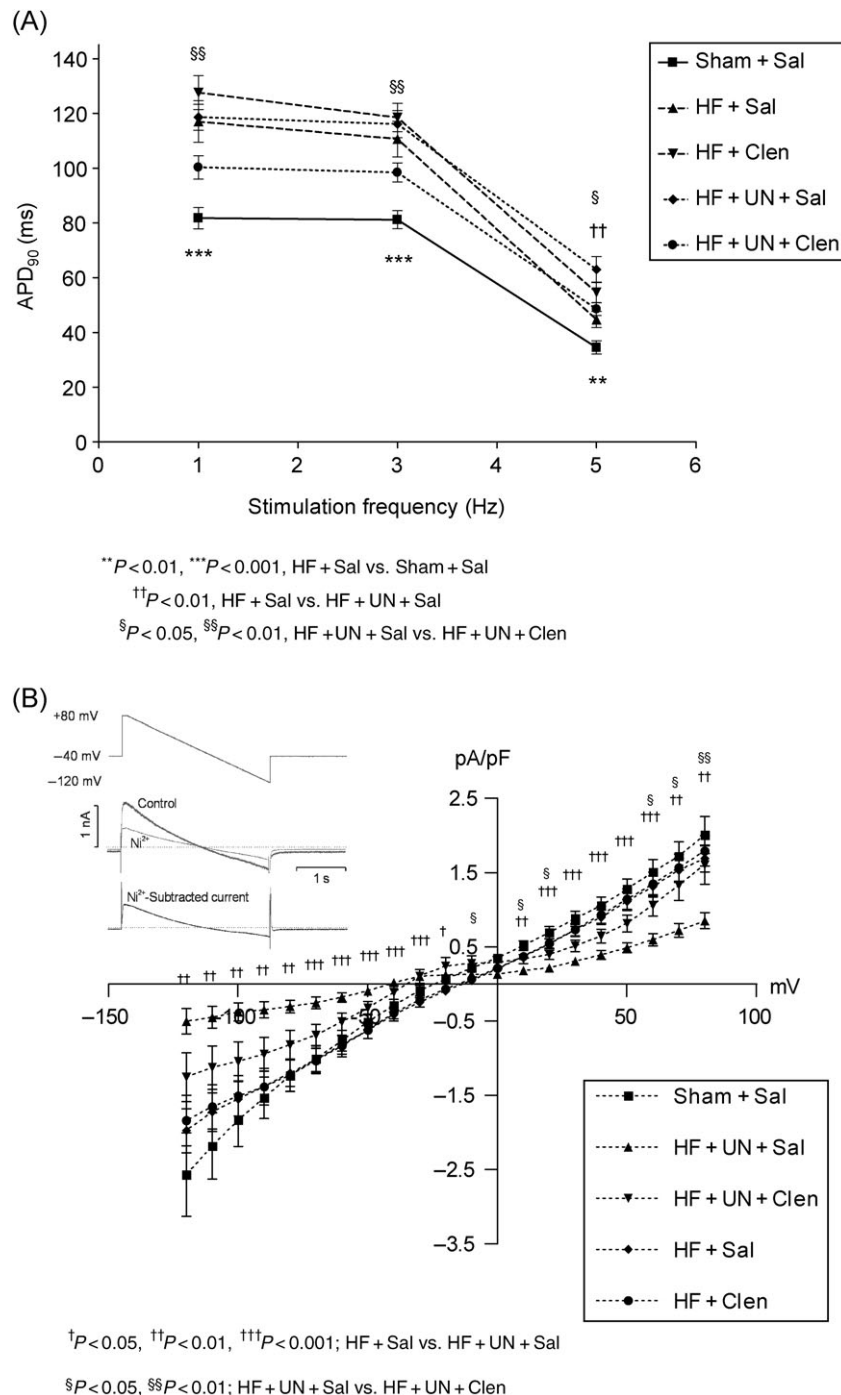


Figure 5 Action potential duration was prolonged in heart failure and unaffected by clenbuterol treatment (A). Mechanical unloading of failing hearts further prolonged action potential duration at higher stimulation frequency which was normalized by clenbuterol treatment. Clenbuterol normalized the unloading-induced reduction of I_{NCX} current density (B). Inset showing the voltage-clamp protocol used.

there is an association with the atrophic response of the myocytes and this can explain the morphological differences observed. More studies are required to investigate this point.

4.3 Clenbuterol and excitation–contraction coupling

Twitch Ca^{2+} transient amplitude and SR Ca^{2+} content are generally reduced in human⁴⁵ and animal models of HF.⁴⁶ However, some studies in animal models have also shown

unchanged⁴⁷ or increased²² Ca^{2+} transient amplitude with altered contractility. As in the present study, Loennechen *et al.*²² have shown an increased diastolic and systolic Ca^{2+} transient at 1, 4, and 13 weeks post-infarction in a rat model. Vahl *et al.*⁴⁸ have demonstrated the after-load dependence of the Ca^{2+} transient amplitude in human HF. They attribute this to an increased dissociation of Ca^{2+} from the contractile proteins, defective Ca^{2+} reuptake of the SR or an increased Ca^{2+} inflow via Ca^{2+} channels. The differing results in Ca^{2+} transient kinetics in the above studies may depend on the model of HF and experimental

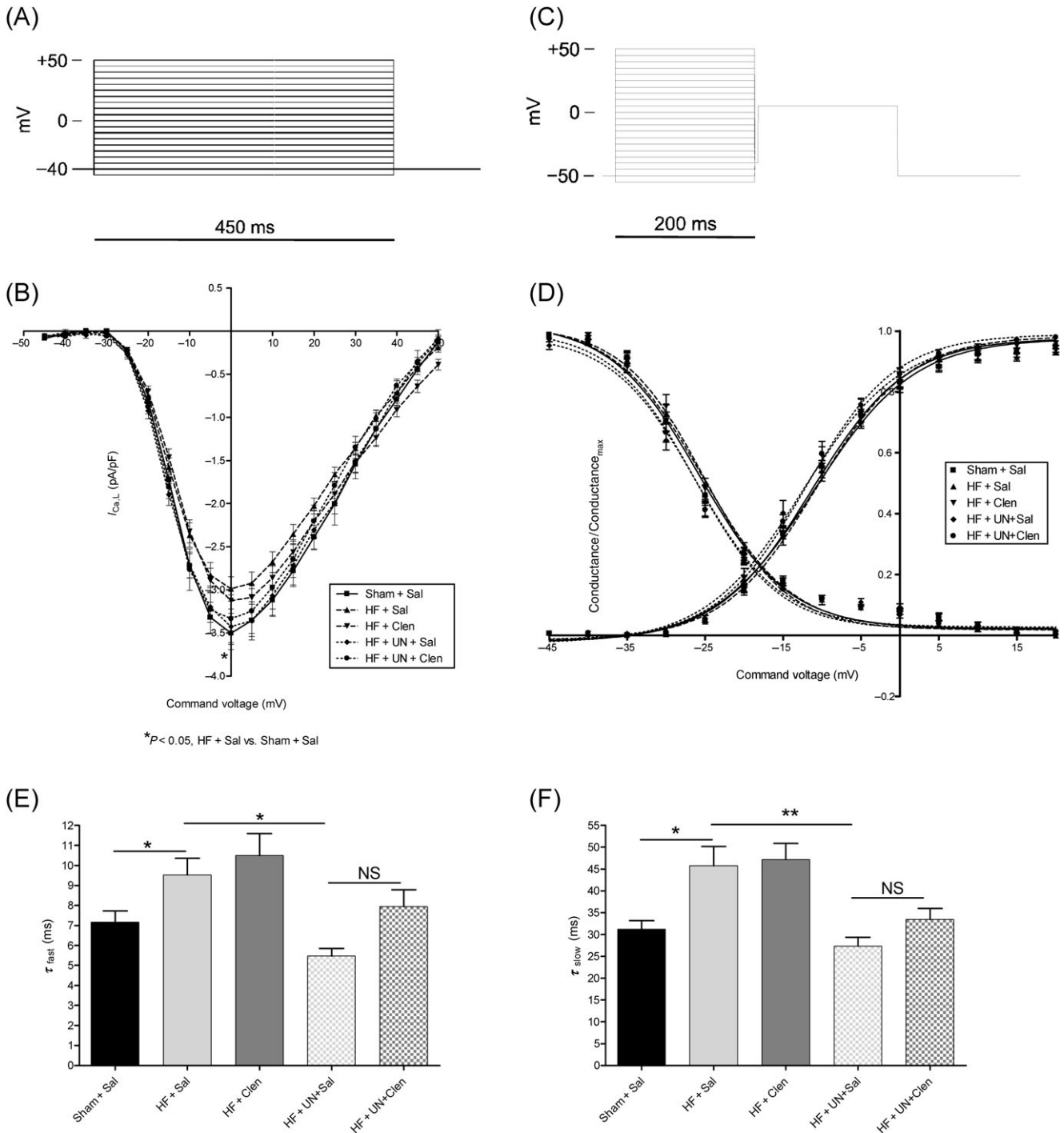


Figure 6 $I_{Ca,L}$ activation and inactivation properties were studied using the protocols A and C, respectively. Heart failure reduced peak $I_{Ca,L}$ as shown by I-V relationship (B) prolonged inactivation times (E, F) which were unaffected by clenbuterol treatment. Mechanical unloading normalized the prolonged inactivation times with no additional affect by clenbuterol treatment. The conductance/conductance_{max} relationship of all groups was unchanged (D). (* $P < 0.05$, ** $P < 0.01$).

conditions. The paradoxical increased Ca^{2+} transient amplitude and SR Ca^{2+} content with reduced sarcomere shortening amplitude seen in our model of post-infarction cardiomyopathy may indicate an intermediate stage in the development of HF with pathological hypertrophy, reduced contractile function and defective myofilament sensitivity to Ca^{2+} .

An increased Ca^{2+} transient may result from a reduced function of NCX and consequent increased Ca^{2+} load of the

SR in the presence of maintained SR Ca^{2+} uptake. The mechanisms by which NCX is involved in the improvement are unclear. It is possible that NCX has a compensatory rather than a causal role in the improvement observed. Other Ca^{2+} regulatory mechanisms, such as SR Ca^{2+} uptake, and factors that regulate NCX activity, such as $[Na^+]_i$ ^{49,50} and phosphorylation,⁵¹ can be involved. NCX mediated- Ca^{2+} extrusion was faster in unloaded myocytes with or without clenbuterol. In the clenbuterol-treated group, the

biophysical properties of NCX (assessed as I_{NCX}) were directly affected. More studies are required to investigate this point.

The prolongation of the AP in HF may also contribute to the increased SR Ca^{2+} loading and increase the twitch Ca^{2+} transient amplitude³⁰ as seen in our study. Mechanical unloading alone did not affect the deranged cardiomyocyte contractility or Ca^{2+} cycling of failing hearts in our study. This might be due to the short duration of unloading as suggested by Ito *et al.*⁹

We found a delayed time course of both twitch and caffeine-induced Ca^{2+} removal in myocytes from failing hearts which was improved by clenbuterol treatment alone and in combination with mechanical unloading. A number of factors can explain this behaviour. In the group treated with clenbuterol alone, the NCX contribution to Ca^{2+} removal may be increased due to the changes in intracellular $[Na^+]$ as discussed above. In the group with clenbuterol and mechanical unloading, the shortening of the AP and increased NCX current density could also be important. Finally the contribution of the SR Ca^{2+} uptake, which has not been addressed in this study, may also play an important role.

4.4 Clenbuterol and myofilament sensitivity to calcium

Abnormal myofilament function contributes significantly to contractile dysfunction in chronic HF although its relative importance compared to altered Ca^{2+} homeostasis is currently debated.⁵² In the model used in this study, we have demonstrated depressed myofilament Ca^{2+} sensitivity. Clenbuterol treatment normalized this depressed myofilament Ca^{2+} sensitivity irrespective of whether it was associated to mechanical unloading. This may be a key mechanism involved in the ability of clenbuterol to improve contractile function at cellular level. Our results suggest that the improved myofilament sensitivity to Ca^{2+} seen after clenbuterol treatment is not due to the effects of clenbuterol on MHC expression. It is possible that clenbuterol affects other key mechanisms like the phosphorylation states of troponin-I and myosin binding protein-C.

One possible limitation in our study is that we have estimated myofilament sensitivity to Ca^{2+} by assessing the relationship between sarcomere shortening and Indo-1 fluorescence. We did not calibrate the fluorescence signals and quantify cytoplasmic $[Ca^{2+}]$. Although this can be a possible source of error, the relationship between Indo-1 fluorescence ratio and cytoplasmic $[Ca^{2+}]$ is sufficiently linear in the range of measurements reported (20–80% of Ca transients)^{53,54} to allow comparisons between groups.

4.5 Conclusions

In our study, clenbuterol affects cell size, EC coupling, ion channel function, and AP properties and normalizes myofilament sensitivity to Ca^{2+} . The combination of all the above changes, rather than changes in a single parameter, may lead to the improved cellular and whole-heart function. The temporal and causal relationship of the changes in relation to contractile functional improvement is a challenging point that requires further studies.

In conclusion, the treatment of failing hearts with clenbuterol, alone or in combination with mechanical unloading, improves LV function at whole-heart and cellular level.

This study supports the use of this drug in the strategy to enhance recovery in HF patients treated with LVADs and also begins to elucidate some of the possible cellular mechanisms responsible for the improvement in LV function.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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