

Modulation of Myocardial Stiffness by β -Adrenergic Stimulation - Its Role in Normal and Failing Heart

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Summary

The acute effects of β -adrenergic stimulation on myocardial stiffness were evaluated. New-Zealand white rabbits were treated with saline (control group) or doxorubicin to induce heart failure (HF) (DOXO-HF group). Effects of isoprenaline (10^{-10} - 10^{-5} M), a non-selective β -adrenergic agonist, were tested in papillary muscles from both groups. In the control group, the effects of isoprenaline were also evaluated in the presence of a damaged endocardial endothelium, atenolol (β_1 -adrenoceptor antagonist), ICI-118551 (β_2 -adrenoceptor antagonist), KT-5720 (PKA inhibitor), L-NNA (NO-synthase inhibitor), or indomethacin (cyclooxygenase inhibitor). Passive length-tension relations were constructed before and after adding isoprenaline (10^{-5} M). In the control group, isoprenaline increased resting muscle length up to 1.017 ± 0.006 L/L_{max}. Correction of resting muscle length to its initial value resulted in a 28.5 ± 3.1 % decrease of resting tension, indicating decreased muscle stiffness, as confirmed by the isoprenaline-induced right-downward shift of the passive length-tension relation. These effects were modulated by β_1 - and β_2 -adrenoceptors and PKA. In DOXO-HF group, the effect on myocardial stiffness was significantly decreased. We conclude that β -adrenergic stimulation is a relevant mechanism of acute neurohumoral modulation of the diastolic function. Furthermore, this study clarifies the mechanisms by which myocardial stiffness is decreased.

Key words

β -adrenergic stimulation • Diastolic function • Myocardial stiffness
• Heart failure

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Introduction

Although the evaluation of the myocardial function of the heart is usually focused on its chronotropic and inotropic state, the assessment of the diastolic response to pharmacological intervention is presently recognized as one of great clinical relevance. The most important mechanisms that increase resistance to left ventricular (LV) filling and consequently, lead to diastolic dysfunction and diastolic heart failure (HF), are impaired cardiac relaxation and increased stiffness (Leite-Moreira 2006).

Beta-adrenergic stimulation is an important physiological mechanism for enhancing cardiac performance during increased circulatory demands. The activation of these receptors on cardiac myocytes initiates signalling pathways that increase contractility and accelerate relaxation. Nowadays, three β -adrenoceptor subtypes have been identified, β_1 -, β_2 -, and β_3 -adrenoceptor. Mammalian cardiac myocytes express predominantly β_1 -adrenoceptor, in a range from 60-80 % depending on the species, and in a less extent β_2 -adrenoceptor. These receptors modulate systolic and

diastolic functions in very different ways (for review see Brodde *et al.* 2006). The effects of β -adrenergic stimulation are partially mediated by cAMP-dependent protein kinase A (PKA) that subsequently phosphorylates several intracellular substrates, including membrane channels and myofilamentary proteins such as actin and myosin. Fast changes in intracellular Ca^{2+} -handling are thought to be largely responsible for the positive inotropy and lusitropy. Some of the mechanisms underlying Ca^{2+} homeostasis and responsible for increasing lusitropy are the phosphorylation of: 1) phospholamban, enhancing Ca^{2+} reuptake into the sarcoplasmic reticulum (Bers and Guo 2005), 2) troponin I (TnI), decreasing myocardial calcium (Ca^{2+}) sensitivity on the thin filaments by increasing the rate at which Ca^{2+} dissociates from troponin C (TnC) (Robertson *et al.* 1982, Wattanapermpool *et al.* 1995, Zhang *et al.* 1995, Johns *et al.* 1997, Fentzke *et al.* 1999) and 3) myosin binding protein-C (MyBP-C), accelerating crossbridge cycling and increasing myofibrillar ATPase activity (Gruen *et al.* 1999, Kunst *et al.* 2000). These mechanisms, which can be modulated by β -adrenergic stimulation, may lead to a faster rate of myofibrillar relaxation thereby shortening twitch duration.

Besides relaxation, myocardial stiffness is a major determinant of diastolic function (Leite-Moreira 2006). We previously demonstrated acute changes of myocardial stiffness after myocardial exposure to several neurohumoral agents like endothelin-1 (Leite-Moreira *et al.* 2003), angiotensin II (Leite-Moreira *et al.* 2006), urotensin II (Fontes-Sousa *et al.* 2007) and adrenomedullin (Fontes-Sousa *et al.* 2009). In the same way, nitric oxide (NO) decreases myocardial stiffness (Paulus *et al.* 1994, Shah and MacCarthy 2000). Furthermore, diastolic dysfunction induced by excessive afterload was attenuated by β -adrenergic stimulation, highlighting the lusitropic effects of this neurohumoral system (Leite-Moreira *et al.* 2001). However, the underlying mechanisms remain unexplored.

In this context, the present study aims at exploring the effects of β -adrenergic stimulation on myocardial passive properties, investigating: 1) the effects on myocardial stiffness in healthy rabbits and its underlying mechanisms, and 2) whether this effect is preserved in an animal model of HF.

Methods

This study was performed in New-Zealand

White rabbits (*Oryctolagus cuniculus*) and complied with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication N° 85-23, Revised 1996).

Heart Failure Model

A well-documented regimen was used for the induction of HF secondary to doxorubicin toxicity (Arnolda *et al.* 1985). Adult male New Zealand White rabbits received doxorubicin (DOXO-HF) *via* a marginal ear vein by bolus injection (1 mg/kg) twice a week during 8 weeks (n=9) followed by a washout period of one week. This model culminates with a depressed myocardial function compatible with dilated cardiomyopathy, as we previously demonstrated by echocardiography (Bras-Silva *et al.* 2006). Control rabbits (n=39) received vehicle (0.9 % saline) in equivolumetric doses during the same period.

Experimental preparation

Isometric and isotonic contractions were analyzed in papillary muscles isolated from the right ventricle of control (n=73) and DOXO-HF (n=9) rabbits, one week after the last administration of doxorubicin or saline. Male rabbits (2.3±0.1 kg, n=48) were anesthetized with intravenous sodium pentobarbital (25 mg.kg⁻¹). A left thoracotomy was performed, beating hearts were quickly excised and immersed in a modified Krebs-Ringer solution at 35 °C, with 5 % Newborn Calf Serum and with cardioplegic 2,3-butanedione monoxime (BDM, 3 %), a selective inhibitor of cross-bridge cycling to stop mechanical activity and preserve myocardial metabolism. The modified Krebs-Ringer solutions contained (in mM): 98 NaCl, 4.7 KCl, 2.4 MgSO₄.7H₂O, 1.2 KH₂PO₄, 4.5 glucose, 1.8 CaCl₂.2H₂O, 17 NaHCO₃, 15 C₃H₃O₃Na, 5 CH₃COONa and 0.003 prazosin). Prazosin, an α -adrenergic antagonist, was used to prevent α -adrenergic mediated effects. The solutions were in equilibrium with 95 % O₂ and 5 % CO₂, to obtain a pH between 7.38-7.42.

The right ventricle was opened and papillary muscles were isolated by first dividing the chordae tendinae at the muscle tip and then freeing the muscle base and a small amount of surrounding myocardium from the ventricular wall. The time from thoracotomy to dissection was ~3 min and only long, thin, uniformly cylindrical muscles were used.

After dissection, papillary muscles (n=82, length: 3.8±0.1 mm, weight: 3.2±0.2 mg, preload: 3.6±0.1 mN) were mounted vertically in a 10 ml plexi

glass organ bath containing the aforementioned Krebs-Ringer solution. The lower muscular end was fixed in a phosphorbronze clip and the upper tendinous end was attached to an electromagnetic length-tension transducer (University of Antwerp, Belgium) (Brutsaert *et al.* 1971).

Preload was initially set between 3 to 4 mN according to muscle dimensions. The preparations were stimulated at 0.6 Hz with a voltage of 10 % above threshold (typically 30-60 mV) by rectangular pulses of 5 ms duration through two platinum electrodes arranged longitudinally alongside the entire muscle. After 20 min, bathing solutions were replaced by corresponding Krebs-Ringer solutions without BDM and the muscle started to contract. One hour later, bathing solution was replaced by corresponding serum-free Krebs-Ringer solution. During the next 2 hours the muscles stabilized. Finally, the muscles were stretched to a muscle length at which active force development was maximal (L_{max}). During the experiment, changes in diastolic muscle length and muscle shortening were measured by the isotonic transducer. Protocols were initiated after obtaining two similar isotonic and isometric control twitches separated by a 10 min interval. Throughout the entire experiment, the temperature was set at 35 °C.

At the end of the experiment the muscles were removed, lightly blotted and then weighed. Muscle cross-sectional area was calculated by dividing the weight of the muscle by its length at L_{max} . A cylindrical shape and a specific density of 1.0 were assumed. Muscle tension was then expressed as force normalized per cross-sectional area (mN/mm²).

Experimental protocols

Effects of increasing concentrations of isoprenaline (ISO, 10^{-10} to 10^{-5} M), a non-selective β -adrenergic agonist, were studied in papillary muscles from the control (n=13) and DOXO-HF (n=9) groups. In another set of papillary muscles from the control group, myocardial effects of increasing concentrations of isoprenaline (10^{-10} to 10^{-5} M) were evaluated in the presence of (i) atenolol (selective β_1 -adrenoceptor antagonist, 2×10^{-5} M, n=8), (ii) ICI 118,551 hydrochloride (ICI, selective β_2 -adrenoceptor antagonist, 10^{-6} M, n=8) or (iii) KT-5720 (KT, inhibitor of protein kinase A (PKA), 10^{-6} M, n=6). Considering that the modulation of myocardial stiffness by other neurohumoral agents require an intact endothelial endothelium (EE) (Bras-Silva and Leite-Moreira 2006) and that DOXO-HF model presents functional evidences

of EE dysfunction (Bras-Silva *et al.* 2006), the same range of isoprenaline concentrations were studied in papillary muscles from control rabbits after (iv) damaging the EE (TRX, n=12) or in the presence of (v) NG-nitro-L-arginine (L-NNA, nitric oxide synthase inhibitor, 10^{-5} M, n=7) or (vi) indomethacin (INDO, cyclooxygenase inhibitor, 10^{-5} M, n=7), two important EE mediators. EE was damaged by briefly (1 s) exposing the isolated papillary muscle to a weak solution (0.5 %) of the detergent Triton X-100 (Brutsaert *et al.* 1988, Leite-Moreira and Bras-Silva 2004).

The concentrations of atenolol, ICI 118,551, KT, NG-nitro-L-arginine and indomethacin were selected on the basis of several studies showing that their physiological effects on myocardial tissue or whole heart preparations are exerted by concentrations in the micromolar range (Mohan *et al.* 1995, Haikala *et al.* 1997, Varma *et al.* 1999, Bras-Silva *et al.* 2008, Faucher *et al.* 2008).

Most of the substances were dissolved in a Krebs-Ringer solution bath before the addition of isoprenaline, except for atenolol, which was added to the initial Krebs-Ringer solution at the final concentration. Muscle twitches were recorded after a stable response was obtained, typically 20 minutes following addition of the antagonists/inhibitors to the muscle preparation. After that, isoprenaline was added cumulatively without any washout in between, with a maximal effect occurring approximately 3-5 min after the latest addition.

In a last set of papillary muscles from control rabbits, passive length-tension relations were constructed before and after a single concentration of isoprenaline (10^{-5} M, n=6). It consisted in decreasing the passive tension of the muscle in a stepwise manner (10 %), with an interval between each reduction of ~4 min until reaching 40 % of its initial passive tension. After restoring passive tension to its initial value, isoprenaline was added to the bath. Five minutes later, another passive tension reduction protocol was performed.

Since all the experiments were performed in the presence of prazosin (3 μ M), as described above, we evaluated its effects on myocardial function in papillary muscles from control animals (n=6, included in the total number given above). Prazosin did not change myocardial performance (data not shown).

Papillary muscles obtained from the same rabbit were used for different experimental protocols. All chemicals were obtained from Sigma Chemical Co, St Louis, Mo, except ICI 118,551 hydrochloride that was

obtained from Tocris Bioscience, Missouri, USA.

Most of the stock solutions, including isoprenaline, were prepared in distilled water and stored as frozen aliquots at $-20\text{ }^{\circ}\text{C}$ until use. KT ester was dissolved in DMSO (less than 0.1 % in the bath) and water. No statistically significant differences were observed between control experiments, made in the absence or in the presence of the solvent at the maximal concentrations used (0.5 %, v/v). The pH of the superfusion solution did not change following addition of the drugs to the muscle preparations.

Data analysis

Isotonic and isometric twitches were converted online to digital data with a sampling frequency of 1000 Hz (Daqbook/120, IOTech Inc. Cleveland, Ohio, USA) and analyzed with specific software (University of Antwerp, Belgium).

Selected parameters included: active tension (AT, mN/mm^2), maximum velocities of tension rise (dT/dt_{max} , $\text{mN/mm}^2/\text{s}$) and decline (dT/dt_{min} , $\text{mN/mm}^2/\text{s}$), peak isotonic shortening (PS, % L_{max}), maximum velocities of shortening (dL/dt_{max} , $L_{\text{max}}\text{s}^{-1}$) and lengthening (dL/dt_{min} , $L_{\text{max}}\text{s}^{-1}$), time to half-relaxation (tHR, ms), and time to active tension (tAT, ms), resting tension (RT, mN/mm^2), and muscle length (L, L/L_{max}).

In the various protocols, results are given as percent change from baseline. For the parameters that are expressed as negative values (e.g. dT/dt_{min}), such percent change refers to the absolute values. When a pharmacological inhibitor was used or the EE damaged, the term baseline refers to the experimental condition in presence of those inhibitors or after EE damaging, before the addition of isoprenaline.

An exponential curve was fitted to passive length-tension relations either before or after isoprenaline administration (10^{-5} M) to calculate muscle stiffness constant (K_c).

Statistical methods

Values are means \pm S.E.M and n represents the number of experiments. Statistical significance was determined using analysis of variances (ANOVA) and Student-Newman-Keuls for pairwise multiple comparisons. $P < 0.05$ was accepted as significant.

Results

Mean values of the morphological and

contractile parameters in papillary muscles from the control group ($n=73$) and from the DOXO-HF group ($n=9$) are shown in Table 1. Morphometric characteristics and baseline performance of rabbit papillary muscles from control group were similar within all the experimental protocols. Compared with control group, papillary muscles from DOXO-HF rabbits showed lower baseline performance, indicating contractile dysfunction.

Table 1. Mean values of the morphologic and contractile parameters in papillary muscles from the control and doxorubicin-induced heart failure (DOXO-HF).

Parameter	Control group (n=73)	DOXO-HF group (n=9)
Length (mm)	3.9 ± 0.2	3.9 ± 0.2
Weight (mg)	3.1 ± 0.2	$4.2 \pm 0.6^*$
Preload (mN)	3.8 ± 0.1	2.8 ± 0.4
AT (mN/mm^2)	27.6 ± 2.0	$7.2 \pm 1.9^*$
dT/dt_{max} ($\text{mN/mm}^2/\text{s}$)	197.4 ± 14.5	$61.6 \pm 11.2^*$
dT/dt_{min} ($\text{mN/mm}^2/\text{s}$)	-149.2 ± 9.2	$-54.9 \pm 9.2^*$
tHR (ms)	382.9 ± 10.1	$265.4 \pm 28.6^*$
tAT (ms)	239.1 ± 5.6	$175.9 \pm 17.8^*$
PS (% L_{max})	12.9 ± 0.6	$5.6 \pm 0.7^*$
dL/dt_{max} (L_{max}/s)	1.0 ± 0.05	$0.5 \pm 0.1^*$
dL/dt_{min} (L_{max}/s)	-3.7 ± 0.2	$-1.3 \pm 0.2^*$

AT: active tension; dT/dt_{max} , dT/dt_{min} : maximum velocities of tension rise and decline, respectively; tHR: time to half relaxation; tAT: time to active tension; PS: peak isotonic shortening; dL/dt_{max} , dL/dt_{min} : maximum velocities of shortening and lengthening, respectively. Values are means \pm S.E.M. $P < 0.05$: * vs control group baseline values.

Effects of increasing concentration of isoprenaline on the systolic and diastolic properties of isolated papillary muscles are illustrated in Figures 1 and 2. In the control group with intact EE, increasing concentrations of isoprenaline enhanced both contractility (AT and dT/dt_{max}) and lusitropy (dT/dt_{min} , tAT and tHR). The highest concentration of isoprenaline (10^{-5} M) increased AT by $107.4 \pm 7.9\%$, dT/dt_{max} by $276.2 \pm 27.6\%$ and dT/dt_{min} by $182.1 \pm 16.1\%$ ($p < 0.05$, Fig. 1a). On the contrary, tAT and tHR decreased by $36.8 \pm 3.5\%$ and $33.0 \pm 3.2\%$, respectively ($p < 0.05$, Fig. 1b). Concerning the diastolic properties of the myocardium, besides increasing relaxation rate (dT/dt_{min}), decreasing time to half relaxation (tHR) and promoting an earlier onset of relaxation (tAT), isoprenaline progressively increased resting muscle length, at a constant resting tension, up to

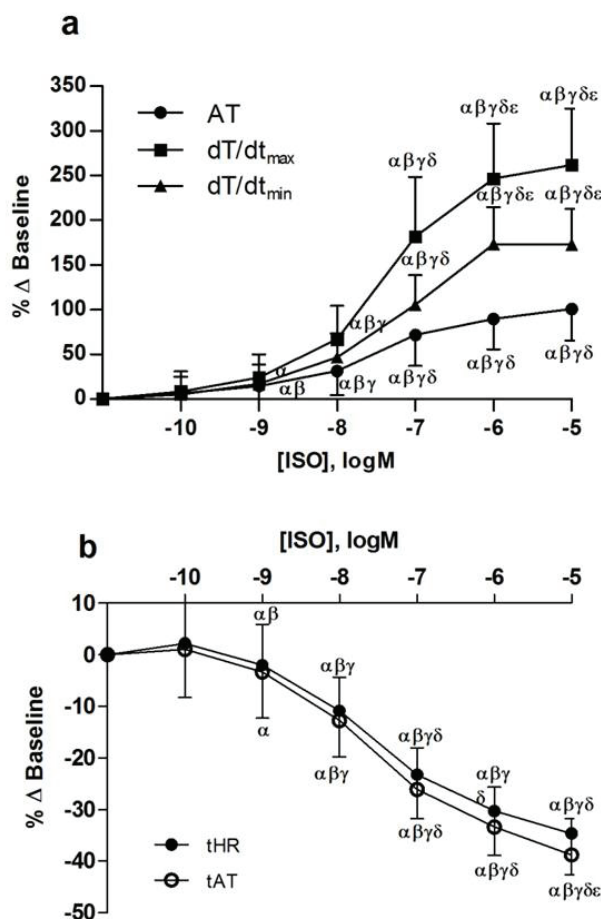


Fig. 1. Effects of increasing concentrations of isoprenaline (ISO, 10^{-10} to 10^{-5} M; $n=13$) on: (a) active tension (AT), maximum velocities of tension rise (dT/dt_{max}) and decline (dT/dt_{min}); and (b) time to half-relaxation (tHR) and time to active tension (tAT). Data are means \pm S.E.M. $P < 0.05$: α vs baseline, β vs 10^{-10} M ISO, γ vs 10^{-9} M ISO, δ vs 10^{-8} M ISO, ϵ vs 10^{-7} M ISO.

1.017 ± 0.006 L/L_{max} (Fig. 2). Correcting final muscle length to its initial value resulted in a 28.5 ± 3.1 % decrease of RT, without altering other contractile parameters. All these results indicate a decrease in muscle stiffness or, in other words, an increase in muscle distensibility.

This aspect is further explored in Figure 3 where passive length-tension relations at baseline and in the presence of isoprenaline (10^{-5} M) are presented. Muscle stiffness constant (K_c) acutely decreased from 23.1 ± 4.1 to 20.9 ± 4.0 mN/mm² ($p=0.048$) while no significant changes were observed in the intercept of the stiffness curve (baseline: $4.6 \cdot 10^{-10} \pm 1.7 \cdot 10^{-9}$ mN/mm², ISO: $3.1 \cdot 10^{-9} \pm 2.3 \cdot 10^{-9}$ mN/mm², $p=0.44$).

As referred previously, the effects of isoprenaline were also tested in the presence of antagonists/inhibitors, and none of them modified *per se* baseline muscle performance. Selective antagonism of

β_1 -adrenoceptor rightward shifted the concentration-response curve of isoprenaline concerning its positive inotropic (AT) and lusitropic (dT/dt_{min}) effects. On the other hand, neither antagonism of β_2 -adrenoceptor nor PKA inhibition altered these effects (data not shown). Regarding the diastolic properties of the myocardium, interestingly, antagonism of β_1 -adrenoceptor and PKA inhibition significantly decreased isoprenaline effects on muscle length (L/L_{max}, Fig. 4a). β_2 -adrenoceptor antagonism abolished this effect as no difference in L/L_{max} was observed before and after adding the maximal concentration of isoprenaline (Fig. 4a). These findings together with the distinct EC₅₀ values for positive inotropism (0.14 ± 0.09 μ M, Figure 1a) and decreased stiffness (879 ± 6 μ M, Fig. 2b) of isoprenaline highlight the dissociation between its effects on myocardial contractility and stiffness.

In DOXO-HF group, increasing concentrations of isoprenaline promoted higher percentage of variation mostly because the baseline muscle performance was significantly lower (Table 1). Maximal concentration of isoprenaline increased AT by 380.2 ± 83.4 %, dT/dt_{max} by 513.6 ± 95.2 %, dT/dt_{min} by 558.9 ± 124.0 % and decreased tAT by 23.8 ± 4.0 % and tHR by 24.4 ± 3.8 % ($p < 0.05$). Furthermore, isoprenaline-induced increase of distensibility was attenuated in DOXO-HF group (1.004 ± 0.002 L/L_{max}, Fig. 4b).

In the control group, we additionally investigated the contribution of EE and its mediators on the increase of distensibility induced by isoprenaline. Neither its removal, nor the inhibition of prostaglandins (INDO) nor NO (L-NNA) release significantly altered the inotropic or lusitropic response to isoprenaline (TRX: increased AT by 209.4 ± 54.8 %, dT/dt_{max} by 458.2 ± 98.8 %, dT/dt_{min} by 268.1 ± 51.8 % and decreased tAT by 36.0 ± 2.9 %, and tHR by 30.4 ± 2.4 %, INDO: increased AT by 108.9 ± 16.0 %, dT/dt_{max} by 238.7 ± 31.9 %, dT/dt_{min} by 172.5 ± 18.4 % and decreased tAT by 32.7 ± 3.2 %, and tHR by 30.5 ± 3.0 %, L-NNA: increased AT by 137.6 ± 66.4 %, dT/dt_{max} by 335.3 ± 99.8 %, dT/dt_{min} by 251.2 ± 109.6 % and decreased tAT by 46.6 ± 4.6 %, and tHR by 44.8 ± 4.4 %). Additionally, none of these interventions significantly altered the effects of isoprenaline on muscle distensibility (Fig. 4c).

The response of passive muscle length and tension to the maximal concentration of isoprenaline (10^{-5} M) alone and in all experimental protocols is summarised in Figure 5. Only the selective antagonism of β_1 -adrenoceptor, β_2 -adrenoceptor or the inhibition of

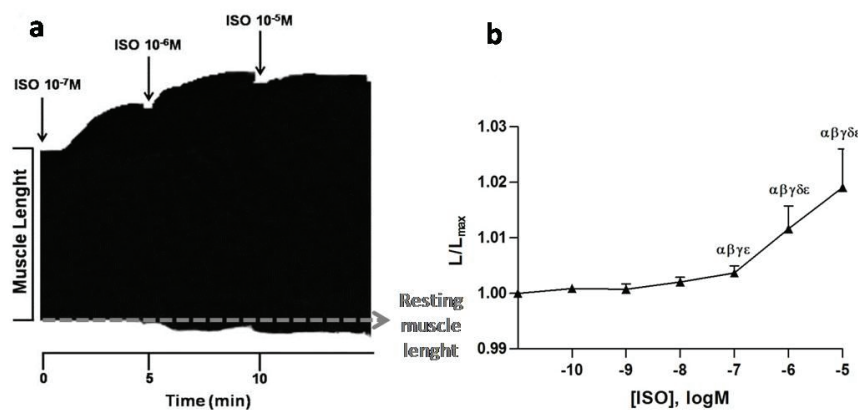


Fig. 2. (a) Representative time band of isotonic contractions of electrically paced papillary muscles contractions. Increasing concentrations of isoprenaline (ISO; 10^{-7} – 10^{-5} M, black arrows) induce a positive inotropic response (illustrated by an increase in muscle shortening with a decrease in systolic length), together with an increase in myocardial distensibility, represented by resting muscle length (L/L_{\max}), illustrated by a raise in resting muscle length from baseline, gray dotted line); (b) Effects of increasing concentrations of ISO (10^{-10} to 10^{-5} M; $n=13$) on resting muscle length (L/L_{\max}). Data are means \pm S.E.M. $P < 0.05$: α vs baseline, β vs 10^{-10} M ISO, γ vs 10^{-9} M ISO, δ vs 10^{-8} M ISO, ϵ vs 10^{-7} M ISO.

PKA markedly reduced the effect of isoprenaline on muscle length, leading to a decrease in passive tension. The acute effect of β -adrenergic stimulation on muscle length in DOXO-HF animals was significantly decreased when compared with control group.

Discussion

The myocardial effects of β -adrenoceptor stimulation by isoprenaline on papillary muscles from healthy and DOXO-HF rabbits were evaluated in this study. Besides the demonstration of the well-documented positive inotropic and lusitropic effects (Bers 2002), the novel finding herein reported was that β -adrenergic stimulation induces a significant concentration-dependent acute decrease of myocardial stiffness, dependent on the activation of β_1 , β_2 -adrenoceptor and PKA. Both the endothelium and the evaluated endothelial mediators, NO and prostaglandins, did not interfere with this effect. Furthermore, this effect was significantly decreased in the presence of HF induced by doxorubicin.

Myocardial function was evaluated *in vitro* using papillary muscles. This model has the advantage of excluding confounding systemic variables, such as changes in preload, afterload or coronary flow. Specifically in this study, the use of a rabbit model presents many advantages as both β_1 and β_2 -adrenoceptors are present in its ventricular myocytes (Marian 2006), and the failing rabbit heart exhibits molecular changes in β -adrenergic signaling similar to those observed in human HF (Maurice *et al.* 1999). These characteristics make such species a suitable experimental model to study myocardial passive properties and performance under β -adrenergic stimulation.

Beta-adrenergic stimulation induced by the sympathetic nervous system plays a pivotal role in the

regulation of myocardial structure and function in the normal and failing heart. Several studies focusing on the effects of β -adrenergic stimulation support that crossbridge cycle and several other phosphorylation events are the major determinants of the intrinsic rate of myocardial relaxation (Bronzwaer and Paulus 2005). On the other hand, cardiac hypertrophy and failure are also characterized by an overall loss of sensitivity to β -adrenoceptor stimulation (Bristow *et al.* 1986, Steinberg 1999). However, the effects of β -adrenergic stimulation on other major determinants of diastolic function, such as the passive properties, like myocardial stiffness, remained to be clarified in both conditions.

We observed that β -adrenoceptor stimulation decreases myocardial stiffness through both β_1 and β_2 -AR activation. There has been a tendency to think of β_1 - and β_2 -adrenoceptors as being nearly equivalent, at least in terms of their cAMP-mediated effects, but there are important differences. Several studies focusing on the myocardial response to β_2 -adrenoceptor stimulation have reported that while there are similar agonist-dependent increases in tension development, the acceleration of relaxation typically seen with β_1 -adrenoceptor stimulation is attenuated or absent with β_2 -AR stimulation (Xiao *et al.* 1995). Although our results demonstrate a potential cardiovascular role for β_2 -AR as an acute modulator of myocardial stiffness, we cannot exclude an additional effect of ICI-118551 on β_1 -AR.

In the current study, by using KT, a PKA specific inhibitor (Bishopric *et al.* 1992, Haikala *et al.* 1997, Kiehn *et al.* 1998, Iwai-Kanai *et al.* 1999), we confirmed that isoprenaline-induced decrease of myocardial stiffness is dependent on the activation of PKA which is consonant with previously published data on the effects of PKA in engineered rat heart tissue (Zimmermann *et al.* 2002) and human cardiac cells (Borbely *et al.* 2005, van Heerebeek

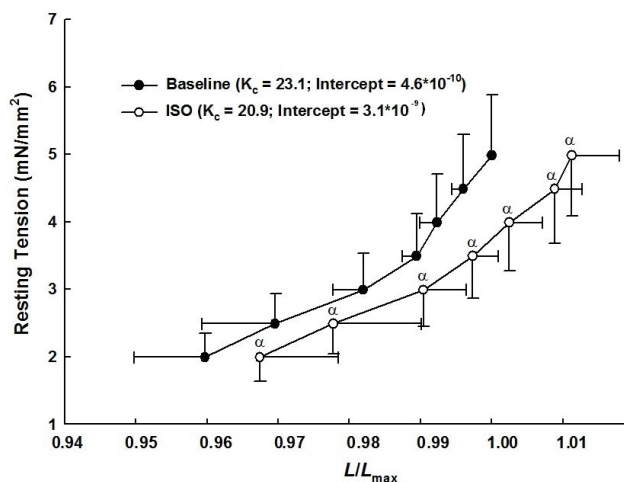


Fig. 3. Passive length-tension relations at baseline ($K_c=23.1$, Intercept= 4.6×10^{-10}) and in the presence of isoprenaline (ISO, 10^{-5} M, $n=6$; $K_c=20.9$, Intercept= 3.1×10^{-9}). Data are means \pm S.E.M. $P < 0.05$: α ISO vs baseline.

et al. 2006). Although our main goal was to study the role of isoprenaline on diastolic properties, we did not observe an antagonist activity of KT against isoprenaline effects on inotropism and lusitropism, which is in line with previous studies (Gotoh 1995, Yatani *et al.* 1999). These studies suggested that β -adrenergic stimulation increases the peak L-type Ca^{2+} current *via* PKA-independent activation of Ca^{2+} channels (Yatani *et al.* 1999) or increases calcium leak from sarcoplasmic reticulum *via* calcium/calmodulin-dependent protein kinase (Curran *et al.* 2007). The effects of sustained β_1 -adrenoceptor stimulation (inotropy, cell growth and cell death) are indeed primarily due to this latter pathway, rather than PKA signalling (Zhu *et al.* 2003, Wang *et al.* 2004). So, under certain physiological and pathological circumstances, this signaling pathway becomes more relevant (Singh *et al.* 2001, Xiao 2001).

Myocardial stiffness is determined both by cardiomyocytes' cytoskeleton and the extracellular matrix (Kass *et al.* 2004). Most of the elastic force of the cardiomyocytes is now thought to reside in the cytoskeletal protein, titin (Kruger and Linke 2009) which is known to be phosphorylated by PKA, PKG and PKC (Fukuda *et al.* 2005, Hidalgo *et al.* 2009, Kruger *et al.* 2009). Changes in its isoform composition and phosphorylation status have been shown to alter diastolic function and myocardial passive properties (Nagueh *et al.* 2004, Borbely *et al.* 2009). Based on this evidence, one of the possibilities that could explain our observations is that the acute decrease of stiffness induced by isoprenaline is associated with the modulation of titin's phosphorylation status by PKA, as

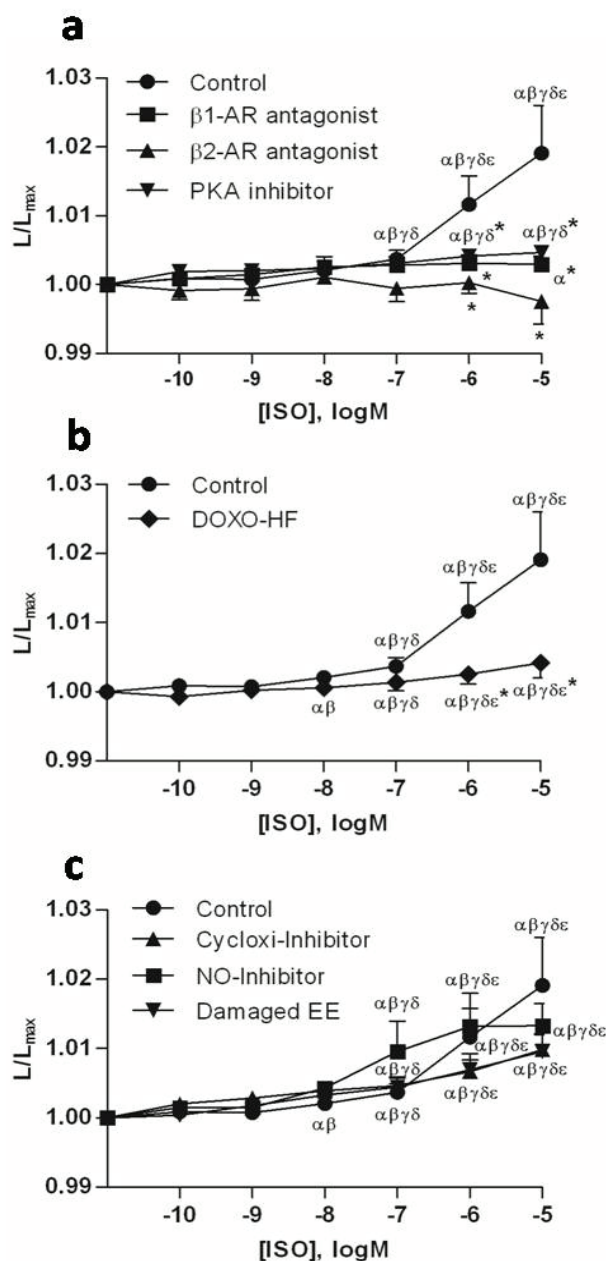


Fig. 4. Effects of increasing concentrations of isoprenaline (ISO, 10^{-10} to 10^{-5} M; $n=8$) on passive muscle length (L/L_{max}): **(a)** in the absence (control; $n=13$) or presence of: a β_1 -adrenoceptor antagonist (atenolol, 2.10^{-5} M; $n=8$); a β_2 -adrenoceptor antagonist (ICI, 10^{-6} M; $n=8$) or a PKA inhibitor (KT, 10^{-6} M; $n=6$); **(b)** in doxorubicin-induced heart failure group (DOXO-HF, $n=9$); **(c)** in the presence of: damaged endothelial endothelium (TRX; $n=12$); a NO synthase inhibitor, NG-Nitro-L-Arginine (L-NNA, 10^{-6} M; $n=7$) or a cyclooxygenase inhibitor, indomethacin (INDO, 10^{-6} M; $n=7$). Data are means \pm S.E.M., expressed as percent variation from baseline. $P < 0.05$: α vs baseline, β vs 10^{-10} M ISO, γ vs 10^{-9} M ISO, δ vs 10^{-8} M ISO, ϵ vs 10^{-7} M ISO and * vs ISO alone.

demonstrated in the present study by its inhibition by KT.

The acute decrease of myocardial stiffness induced by isoprenaline was attenuated in DOX-treated rabbits, where β -adrenoceptor downregulation has been

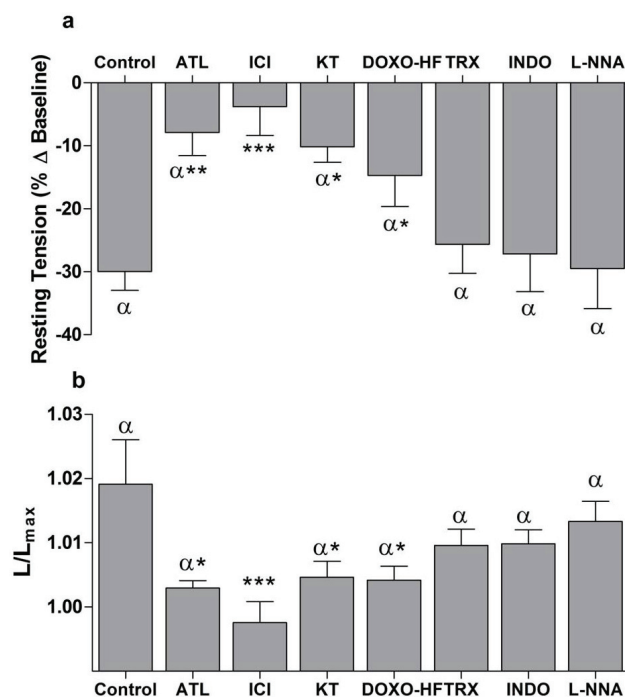


Fig. 5. Summary of the effects of isoprenaline (ISO, 10^{-5} M) on: **(a)** resting tension and **(b)** resting muscle length (L/L_{max}) in the absence (control, $n=13$) or presence of a β_1 -adrenoceptor antagonist (atenolol, ATL, 2.10^{-5} M; $n=8$); a β_2 -adrenoceptor antagonist (ICI, 10^{-6} M; $n=8$); a PKA inhibitor (KT, 10^{-6} M, $n=6$); in doxorubicin-induced heart failure animals (DOXO-HF, $n=9$); damaged endothelial endothelium (TRX, $n=12$); a NO synthase inhibitor, NG-Nitro-L-Arginine (L-NNA, 10^{-5} M; $n=7$); a cyclooxygenase inhibitor, indomethacin (INDO, 10^{-6} M, $n=7$). Data are means \pm S.E.M., expressed as percent variation from baseline. $P < 0.05$: α vs baseline, * vs control (ISO alone).

documented (Nagami *et al.* 1997, Kizaki *et al.* 2004). However, other factors can account for this effect such as a shift in titin isoform. In this regard, a compensatory shift from the stiff N₂B to the compliant N₂BA isoform was described in patients with higher LV end-diastolic wall stress induced by dilated cardiomyopathy (Nagueh *et al.* 2004). Moreover, a smaller PKA-induced RT decrease was reported when the compliant N₂BA titin isoform is phosphorylated rather than the stiff N₂B isoform (Fukuda *et al.* 2005). We and others demonstrated an increase in LV-end-diastolic pressure and dilated cardiomyopathy in DOXO-HF rabbits (Nagami *et al.* 1997, Bras-Silva *et al.* 2007). Therefore, we could speculate that in DOX-treated rabbits a similar compensatory shift from N₂B to the non-PKA sensitive N₂BA isoform could have taken place during the course of HF progression explaining the attenuation of the isoprenaline-induced decrease of myocardial stiffness in dilated DOXO-HF hearts. Even though interesting, the confirmation of this aspect is beyond the scope of the present study and needs further investigation.

Another study of our group provided functional evidence of EE dysfunction in the DOXO-HF model (Bras-Silva and Leite-Moreira 2006). With regard to diastolic function, we have recently shown, in the same animal species, that the decrease of myocardial stiffness induced by ET-1 (Bras-Silva and Leite-Moreira 2006, Bras-Silva *et al.* 2008), urotensin II (Fontes-Sousa *et al.* 2007) and adrenomedullin (Fontes-Sousa *et al.* 2009) was dependent on EE and/or its mediators (NO and prostaglandins). Therefore, we performed other series of experimental protocols in order to confirm whether EE integrity is mandatory for isoprenaline-induced decrease on myocardial stiffness. In this set of studies, we found that although there was a clear trend towards a decrease of the isoprenaline-induced decrease of myocardial stiffness after removing the EE or upon inhibition of prostaglandins' release or NO synthase, none of these interventions was able *per se* to modify this effect.

Conclusions

Besides the well-known effects of β -adrenergic stimulation on myocardial contractility, the present study reveals that it acutely lowers myocardial stiffness. This novel effect, which requires the activation of β_1 and β_2 -adrenoceptor and is mediated by PKA, broadens our knowledge with regard to the acute neurohumoral modulation of diastolic function. This effect is of potential high physiological relevance as it might acutely decrease passive myocardial tension by as much as 30 %, allowing an intact heart to reach higher filling volumes at almost one third lower filling pressures. As this effect is abolished in the failing heart it might contribute to diastolic dysfunction in heart failure and therefore constitute a potential target for therapy. In this regard, these results highlight another possible important effect of β -blocker therapy in the treatment of HF.

Conflict of Interest

There is no conflict of interest.

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