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André Filipe Mendes Leite Moreira
Cardiac response to acute stretch:
Literature review and a
new diastolic paradigm

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Assinatura conforme cartão de identificação:

André Filipe Mendes Leite Moreira

NOME

André Filipe Mendes Leite Moreira

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Cardiac response to acute stretch: Literature review and a new diastolic paradigm

ORIENTADOR

André Pedro Leite Martins Lourenço

COORDENADOR (se aplicável)

Inês Maria Falcão Sousa Pires Marques

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DE ACORDO COM A LEGISLAÇÃO EM VIGOR, (INDICAR, CASO TAL SEJA NECESSÁRIO, Nº MÁXIMO DE PÁGINAS, ILUSTRAÇÕES, GRÁFICOS, ETC.) NÃO É PERMITIDA A REPRODUÇÃO DE QUALQUER PARTE DESTES TRABALHOS.	☒

Faculdade de Medicina da Universidade do Porto, 21/03/2016

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André Filipe Mendes Leite Moreira

Para a minha mãe, por me ter tornado no homem que sou hoje.

Para a Mariana, por me tornar no homem que serei amanhã.

Cardiac Response to Acute Stretch:

Literature Review and a New Diastolic Paradigm

Abstract

Myocardial stretch, as result of acute hemodynamic overload, is one of the most frequent challenges to the heart and the ability of the heart to intrinsically adapt to it is essential to prevent circulatory congestion. In a literature review, the historical background, the currently known mechanisms, as well as the gaps in the understanding of this physiological response are highlighted. The systolic adaptation to stretch is well known for over 100 years, being dependent on an immediate increase in contractility – known as the Frank-Starling mechanism – and a further progressive potentiation – the slow force response. On the other hand, its diastolic counterpart remains largely unstudied. cGMP-related pathways are activated upon stretch and its downstream effector PKG is known to phosphorylate titin, a major determinant of myocardial stiffness. Therefore, we aimed to investigate the role of these pathways on decreasing stiffness during acute loading via titin phosphorylation in healthy and diseased hearts. Left ventricle (LV) of intact rat hearts, strips dissected from the LV or right atrium of cardiac surgery patients and rabbit papillary muscles were acutely stretched. In cardiac surgery patients, LV pressure and volume were recorded before and 15 minutes after volume overload (VO). Effect of VO was also assessed in sham and TAC rats. After an immediate increase in response to stretch/VO, diastolic pressure and passive tension significantly decreased ($\approx 30\text{--}40\%$) over the subsequent 15 min in all animal species and experimental preparations. This effect was significantly blunted by PKG inhibition and combined natriuretic peptide receptor inhibition and NO scavenging and was not observed in TAC hearts. Stretching skinned cardiomyocytes did not decrease passive tension but it was $\approx 60\%$ lower in those extracted from stretched hearts, in which it increased with protein phosphatase but not PKG incubation. Titin phosphorylation increased markedly after acute myocardial stretch in human and rabbit myocardium. Hypophosphorylation and blunted response to VO was measured in TAC myocardium. These results point to a novel mechanism of diastolic adaptation to acute overload associated with increased myocardial compliance that is abolished in the hypertrophic heart. Decreased stiffness is associated with titin phosphorylation, in which PKG plays a central role.

Keywords: cardiac function, myocardial stretch, neurohumoral adaptation, titin, diastole, heart failure, frank-starling mechanism, slow force response.

PART ONE: LITERATURE REVIEW

Introduction

The heart has a central role in the maintenance of cardiovascular homeostasis, which requires the ability to continuously adapt its function to different hemodynamic conditions.

One of the most frequent challenges to myocardium is acute stretch, as result of acute hemodynamic overload. Acute myocardial stretch can be observed in various physiological and pathophysiological conditions (*e.g.* exercise, myocardial ischemia, hypertensive crises, valvular diseases and heart failure). For example, at the start of aerobic exercise (figure 1), the pumping action of skeletal muscle contraction increases the venous return, leading to cardiac chamber dilation and acute myocardial stretch (Nobrega et al., 1995). This increase in end-diastolic volume leads to an increase in end diastolic pressure that, in the absence of adequate response, would lead to pulmonary and systemic congestion.

Being such a common challenge, one can expect the heart to have adequate intrinsic physiologic mechanisms to respond to this stimulus. The adequate physiological response to the acute increase in end-diastolic pressure must include: activation of stretch sensing molecules (the sensing organ), intermediary mechanisms (afferent, integrator and efferent pathways) and effector mechanisms that would ultimately allow the heart to reduce its increased end-diastolic pressure (figure 1). This could, in theory, be achieved by two different mechanisms:

- A systolic adaptation: an increase in contractility, leading to an increased ejection volume and, consequently, a decreased end diastolic volume.
- A diastolic adaptation: an increase in cardiac compliance, which would allow accommodating more blood at inferior end diastolic pressures.

Both adaptations would increase cardiac output, which would also be appropriate to offset the increase in venous return.

The first described mechanism of cardiac adaptation to an acute hemodynamic overload has been known for about a century (Katz, 2002). An increase in either venous return or aortic resistance leads to an increased end-diastolic volume and to an immediate increase in contractility and stroke volume. This response is presently known as Frank-Starling Mechanism (FSM) due to the contributes of Ernest H. Starling and Otto Frank to its description and is mainly attributed to enhanced myofilamental responsiveness to Ca^{2+} , a phenomenon highly dependent on the tension developed by titin (Patterson and Starling, 1914; Frank, 1959; Castro-Ferreira et al., 2011). Given its relevance for the cardiovascular homeostasis it is also known as the “law of the heart”.

In 1912, von Anrep described that, after the initial change in muscle length and contractility induced by clamping the heart outflow, it is observed a progressive and time-dependent increase in force development that goes beyond the force immediately achieved after stretch and is responsible for the return of end-diastolic volume back to its original value (von Anrep, 1912). This second progressive increase in force development, was demonstrated *in vitro* by Parmley and Chuck in 1973, being since then synonymously called slow force response (SFR) (Parmley and Chuck, 1973).

Both mechanisms highlight a highly effective systolic adaptation to an acute hemodynamic overload. A diastolic adaptation *per se*, although theoretically appropriate to the underlying challenge, remains largely unknown.

In this paper, about 100 years after the initial publish of E. Starling about the relevance of stretch to heart function, we intend to review the great progress in understanding of the myocardial adaptation to acute stretch, as well as highlight the gaps in the understanding of this mechanism.

Myocardial mechanosensing

Mechanosensors are structures capable of perceiving mechanical signals and activating pathways that allow transduction of the signal into biochemical responses. The external forces exerted on the myocardium are transmitted through the sarcolemma (and associated adhesion structures: intercalated disks and costameres), cardiomyocyte cytoskeleton and sarcomeric proteins. Concurrently, the sarcomeres generate forces that propagate in the opposite direction. Several mechanosensor molecules in this mechanical chain have been identified, being the best characterized ones located in Z line, titin molecule, sarcolemma, intercalated disks and costameres.

Recent reviews (Takahashi et al., 2013;Buyandelger et al., 2014;Lyon et al., 2015) addressed cardiac mechanotransduction in detail, particularly its relation to remodeling and chronic adaptation. The precise role of mechanosensors in the acute response to stretch is still mostly unknown, as few studies have directly addressed this issue. Therefore, in this topic, we intend to emphasize the great diversity of mechanosensors in the heart and highlight cellular components that, given their structure, mechanism of action and activated signaling pathways, may potentially contribute to the response to acute stretch (figure 2).

The Z line divides two adjacent sarcomeres and is traditionally regarded as an essential element to the normal structural arrangement of the sarcomeres. However, its position confers to the Z disk the distinct ability to sense the muscular tension, during either the diastolic or the systolic period (Hoshijima, 2006). The Z line is composed not only of overlapping actin filaments of adjacent sarcomeres tightly linked by α -actinin, but also of numerous proteins bearing diverse signaling functions (Luther, 2009). Many of these proteins may be relevant for response to acute stretch, including: Protein Kinase C (PKC) ϵ (Robia et al., 2005), PKC δ (Disatnik et al., 1994), calcineurin (Frey et al., 2000) and phosphodiesterase 5 (Senzaki et al., 2001).

Titin is a giant protein that spans the sarcomere from the Z-line to the M-line. Its molecular structure makes this protein work as a bidirectional spring that determines the myocardial passive tension (PT) at different lengths (Castro-Ferreira et al., 2011). In addition to its structural and elastic function, titin also functions as a biomechanical sensor sensing myocardial tension as well as the sarcomeric length. The central role of titin in mechanosensing and its ability to trigger downstream signaling cascades is related to its interactions with numerous structural proteins and signaling proteins at its M-band, Z line, N2B, N2A and PEVK-domains (Linke and Kruger, 2010). Titin also features a kinase domain near the carboxyl-terminal (located in the M-line region) (Mayans et al., 1998), which may phosphorylate target proteins at specific sites, according to myocardial

stretching (Puchner and Gaub, 2010). Moreover, titin presents various phosphorylation sites of its own at PEVK and N2B domains (Krüger and Linke, 2011), whose phosphorylation alters PT conferred by titin, either decreasing or increasing it (Ahmed and Lindsey, 2009). This mechanism confers titin a special role in mechanosensing, allowing it to simultaneously function as a mechanosensor and a molecular target. Therefore, it is tempting to hypothesize that upon an increase in acute PT, titin is capable of initiating a compensatory mechanism that, ultimately, would lead to decreased titin's PT with consequent normalization of sarcomeric PT. It is important to highlight that, as the main determinant of myocardial PT, modifications of titin phosphorylation status will also modify the activity of remaining mechanosensors.

The sarcolemma separates the intracellular and extracellular environments. This structure is teeming with different receptors subject to activation by extracellular mediators that set different intracellular pathways in motion. However, several sarcolemma proteins can also be activated by cellular stretch, initiating intracellular pathways without binding of extracellular mediators (Storch et al., 2012). Stretch activated channels (SACs) are a paradigmatic example of this type of activation in cytoplasmic membranes (Reed et al., 2014). In cardiomyocytes, upon stretch, SACs are permeable to sodium, potassium and calcium, thereby modulating electrical and mechanical properties of myocardium. The AT1 receptor, a G protein-coupled receptor, can also be directly activated by stretch without the involvement of angiotensin II (Zou et al., 2004). This agonist-independent activation may have a determinant role in the myocardial response to acute stretch. The blockade of this receptor has been associated with a significantly blunted SFR (Heidkamp et al., 2003).

The interaction of cardiomyocytes with neighboring cells (at intercalated discs) and with the extracellular matrix (at costameres) is key to the maintenance of the structural integrity of the myocardium, as well as the transmission of forces between cells (Kresh and Chopra, 2011). Beyond this function, some components of intercalated discs and costameres are known to be involved in mechanosensing (Samarel, 2005). At the level of intercalated discs, the main structures capable of contributing to mechanosensing are fascia adherens junctions (constituted by N-cadherin and associated multi-molecular complex anchoring cytoskeletal actin) and desmosomes (composed of the cell adhesion proteins desmoglein and desmocollin and associated proteins internally linked to the intermediate filament desmin) (Sheikh et al., 2009). Both structures are known to contribute to long-term cytoskeletal adaptive responses to different pathophysiological forces (Lyon et al., 2015), but their importance in the acute response remains unknown. Regarding costameres, one important component contributing to mechanosensing are integrins. These heterodimeric transmembrane receptors located at costameres contribute to the connection between extracellular matrix and the intracellular cytoskeleton, and therefore to the transmission of mechanical signals to the cytoskeleton (Israeli-Rosenberg et al., 2014). One well-known mediator of integrin mechanosensing is focal adhesion kinase (FAK) (Domingos et al., 2002b). The signaling resultant of FAK activation is proportional to the magnitude and duration of mechanical stretch (Katz et al., 2002). FAK activity is also related to various signaling pathways potentially relevant to the acute response to stretch, including ERK1/2 (Domingos et al., 2002a) and PKC ϵ (Heidkamp et al., 2003). Another mediator that may be particularly important for the acute transduction of integrin signaling is integrin-linked kinase (ILK). ILK appears to mediate cardiomyocyte force transduction via regulation of the SERCA2A activity and

phosphorylation of phospholamban (Traister et al., 2014), which may contribute both to the systolic and diastolic adaptation to acute stretch.

Although several potential mechanosensing molecules have been identified, the connection between these molecules and stretch activated pathways remains elusive. This lack of knowledge is even more pronounced when it comes to the acute activation of these pathways by these molecules.

Furthermore, the interrelation between those sensors is complex, and the spread synergism and crosstalk between the pathways activated downstream of those sensors lead to a broad activation of signaling pathways that acutely modulate heart function, which is probably crucial to the ability of myocardium to adapt to acute hemodynamic overload.

Calcium-dependent Pathways

Calcium (Ca^{2+}) is the central player in the biphasic systolic adaptation to myocardial stretch. Besides its involvement in the FSM, an increase in the intracellular concentration of Ca^{2+} underlies the SFR (figure 3).

Angiotensin-II (ANG-II) and endothelin (ET) are two peptide hormones (8 and 21 amino acids long, respectively) that share some interesting features. Via binding to receptors coupled to protein G_q and subsequent release of Ca^{2+} from intracellular stores and activation of protein kinase C (PKC), they increase cardiac inotropism and have a pressor effect on blood vessels (figure 3). Though they normally play a role in the physiological regulation of blood pressure by altering salt and water balance and vasomotor tone (especially in renal vessels), they have been implicated in the pathophysiology of pulmonary and systemic hypertension and heart failure (Brunner et al., 2006; Mehta and Griendling, 2007).

More recently, the study of the role of local angiotensin and endothelin systems in cardiac function and pathophysiology has been prolific. These have been implicated in the process of cardiac remodelling that takes place as a result of neurohumoral activation in congestive heart failure (Brunner et al., 2006; De Mello and Frohlich, 2011) but they also seem to be central in the modulation of the acute myocardial response to stretch, contributing to the genesis of the FSM and the development of the SFR (Cingolani et al., 2011). In fact, Sadoshima et al. first demonstrated that cultured neonatal cardiomyocytes directly released ANG-II from intracellular vesicles in response to an acute 10 or 30 minute stretch, generating a concentration of around 1 nM in the culture medium (Sadoshima et al., 1993). The positive inotropic response to acute stretch is similar to that elicited by addition of exogenous ANG-II in amounts of the same order of magnitude and can be blocked by inhibition of AT_1 but not AT_2 receptors (Caldiz et al., 2007). However, this effect seems to depend on downstream release of ET, as inhibition of ET receptors, as well as the ET converting enzyme, ablated both the SFR and the increase in contractility that accompanies ANG-II administration but AT_1 receptor blocking did not prevent the positive inotropic effect of exogenous ET (Perez et al., 2003). Accordingly, Anderson et al. reported that ET synthesis in myocytes increases following myocardial stretch (Anderson et al., 2004).

Taking these results into account, along with their own, Cingolani's group has since described a complex autocrine/paracrine pathway involving changes in membrane ion currents mediated by ANG-II and ET that sheds some light on the role played by these molecules in the response to myocardial stretch (Cingolani et al., 2011). They propose that the initial step in generating the SFR is an ANG-II dependent release of endogenous ET by poorly described mechanisms that may involve activation of the mineralocorticoid receptor by aldosterone or another non-identified ligand.

Although all three endothelin isoforms have equal positive inotropic potency when exogenously administered, ET-3 is the most likely to be responsible for SFR in a physiological setting (Ennis et al., 2005). The ET receptor then transactivates the epidermal growth factor receptor (EGFR) through various pathways. The best described one involves activation of matrix metalloproteinases and subsequent release of heparin-binding epidermal growth factor (HBEGF), which binds to and activates EGFR. This supposedly triggers an intracellular signalling pathway leading to increased production and release of reactive oxygen species (ROS) by mitochondria through opening of mitochondrial K_{ATP} channels (Anderson et al., 2004; Villa-Abrille et al., 2010). Redox-sensitive kinases ERK1/2 and $p90^{rsk}$ therefore increase their activity in response to stretch and phosphorylate the regulatory domain of the sodium-proton exchanger 1 (NHE1), increasing its activity and cytoplasmic $[Na^+]$.

This transporter has long been implicated in the development of the SFR and this is thought to occur through modulation of ion electrochemical gradients that the increased $[Na^+]$ provokes. In this setting, the sodium-calcium exchanger (NCX), which usually extrudes calcium, reverses direction of transport, increasing the amplitude of Ca^{2+} transients and, consequently, contractile force (figure 3).

On the other hand, the increased Ca^{2+} transient, through favored actin-myosin interaction, could result in alarming levels of muscle stiffness. Thus, it is not surprising that other stretch activated mechanisms promote muscle relaxation by improving Ca^{2+} extrusion from cytosol after myocardial contraction. In fact, there is a well described acute increase in SERCA expression following hemodynamic overload (Kögler et al., 2006). Besides, Ca^{2+} load following stretch binds to the several Ca^{2+} binding proteins in the heart, with S100A1 being the most abundant member of the calcium-binding S100 protein family in myocardial tissue (Duarte-Costa et al., 2014). S100A1- Ca^{2+} complex frees the actin from titin, reducing the PT imposed by titin-actin interaction (Yamasaki et al., 2001). The relative importance of these mechanisms in the passive properties of the heart following stretch has not been studied in detail.

The complex loop involving ANG-II and ET still has gaps to be filled and seemingly contradictory results exist in the literature. Our own results confirm the major role of AT_1 receptors and the NHE-1 and NCX transporters in the development of SFR and the positive inotropic effect of the external addition of ANG-II on myocardium in normal conditions, which subsequently blunts SFR, presumably by saturating the contractile reserve of the pathway in the absence of stretch. In addition, it points to a possible role of PKC in maintaining the SFR during the late phase of acute stretch (Neves et al., 2013). Interestingly, our group has also described the role of these peptides on the acute modulation of diastolic function through direct effects on both relaxation velocity and myocardial stiffness. Activation of ET_A and AT_1 receptors results in positive lusitropism and decreased stiffness, while ET_B and AT_2 receptors promote negative lusitropism. As

in its systolic counterpart, AT₁ receptor activation seems upstream to endothelin release in the pathway that leads to faster myocardial relaxation, as this effect is dependent on ET_A receptor activity and an intact endocardial endothelium (Bras-Silva and Leite-Moreira, 2006; Castro-Chaves et al., 2006). Concurrently, decreasing stiffness depends on the activity of both NHE1 and PKC and on the integrity of the endocardial endothelium (Leite-Moreira et al., 2003; Bras-Silva and Leite-Moreira, 2006; Leite-Moreira et al., 2006).

Pathways involving Nitric Oxide and Cardiac Natriuretic Hormones

Cardiac stretch stimulates both endothelial cells and cardiomyocytes to produce nitric oxide (NO) (Casadei and Sears, 2003). In endothelial cells, large amounts of NO are produced by the endothelial nitric oxide synthase (NOS), after which it rapidly diffuses to neighboring cardiomyocytes (figure 3). In the cardiomyocyte, NO is produced in relatively low levels by both endothelial NOS and neuronal NOS (nNOS) (Petroff et al., 2001; Khan et al., 2003).

As NO produced by endothelial cells diffuses through the cardiomyocyte, soluble guanylate cyclase present in the cytoplasm is activated, leading to an increase in cGMP concentration in the cytosolic compartment. The effect of cGMP is mediated through protein kinase G (PKG), which is able to phosphorylate troponin I (TnI), rendering a reduction of myofilamentary sensitivity to calcium, therefore increasing the rate of Ca²⁺ dissociation (Layland et al., 2005). NO produced by nNOS, which is located in the sarcoplasmic reticulum membrane, simultaneously modulates the diastolic properties of the heart through PKA-dependent phosphorylation of phospholamban (PLB) in a cGMP-independent pathway (Zhang et al., 2008). The phosphorylation of PLB disinhibits the sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA), which rapidly reuptakes free cytoplasmic Ca²⁺. Taken together, NO mediated pathways, both cGMP-dependent and – independent, hasten myocardial relaxation (positive lusitropism), prolonging the diastolic time interval while reducing the PT of cardiomyocytes. These changes in myocardial properties are crucial for the heart to accommodate increasing preloads, without substantial changes in the intracardiac filling pressures (figure 3).

Besides altering the diastolic properties of the heart, NO also modulates its contractility. NO-derived cGMP coexists in the cytoplasm with several members of the phosphodiesterase (PDE) family. Proteins of this family are metallophosphohydrolases that cleave cGMP and/or cAMP to 5'-GMP and 5'-AMP, inhibiting the activation of PKG and Protein Kinase A (PKA), respectively (Zaccolo and Movsesian, 2007). Therefore, although stretch induces an increase in the production of NO, the cytoplasmic concentration of NO-derived cGMP is kept relatively low due to continuous hydrolysis mediated by PDE5, located preferentially in Z-bands, in a cGMP-activated negative feedback mechanism. An important crosstalk between cGMP and cAMP pathways occurs, as low concentrations of cGMP inhibit PDE3, thus preventing the hydrolysis of cAMP (Francis et al., 2010). Accumulated cytoplasmic cAMP activates PKA, which is then responsible for the phosphorylation of several proteins involved in cardiac contraction (figure 3). These include the sarcolemmal voltage-gated L-Type Ca²⁺ channels, SR Ca²⁺ release channel (ryanodine receptor, RyR2), PLB, the phosphatase 1 inhibitor, TnI and myosin binding protein C, all aiming to increase myocardial contractility (Fischmeister et al., 2006). Interestingly, a prolonged activation of RyR2 could ultimately lead to SR Ca²⁺ depletion, and consequently to a paradoxal negative

inotropism; however, activation of SERCA, as previously stated, is able to replenish the SR Ca^{2+} pool and maintain this cycle. RyR2 can also be opened via cyclic adenosine diphosphate ribose, which is produced by a PKG mediated phosphorylation of adenosine diphosphate ribosylcyclase (Willmott et al., 1996). NO can also exert an important effect on the systolic properties of the myocardium *per se* in a cGMP-independent pathway. NO produced by nNOS directly S-nitrosylates reactive thiol residues within RyR2, which also contributes to an increased Ca^{2+} release (Wang et al., 2010). Taken together, NO mediated pathways, both cGMP-dependent and -independent, enhance myocardial contractility (positive inotropism) in response to stretch, allowing the heart to pump more vigorously the increasing blood volume arriving after each heartbeat (figure 3). Accordingly, we recently demonstrated in rabbit papillary muscles that acute myocardial stretch in the presence of a PKG inhibitor leads to a significant attenuation of the SFR (Castro-Ferreira et al., 2014).

Cardiac stretch also stimulates cardiomyocytes to release cardiac natriuretic hormones (CNH), namely atrial natriuretic peptide and brain natriuretic peptide. Both exert their cardiac effect by activating cell surface-associated particulate guanylate cyclase A, which in turn increases the concentration of cGMP in the subsarcolemmal compartment (Francis, 2010) (figure 3). However, in opposition to NO-derived cGMP, whose concentration is kept low through a PDE5 mediated negative feedback mechanism, CNH-derived cGMP triggers a feed-forward mechanism that increases cGMP concentration even more (Castro et al., 2010). While this important difference is not reflected in the relaxation properties of the myocardium, as similarly to NO, CNH exert a positive lusitropic effect through phosphorylation of PLB and TnI, high levels of cGMP stimulate PDE2, reducing cAMP levels, which explains the absence of a positive inotropic effect following CNH release (Potter et al., 2006).

Both NO and CNH can exert their effects through cGMP; however their physiological role may be quite different. The production/degradation of NO is finely tuned and occurs at a very high pace, meaning that NO must probably be related to adaptations to acute stretch on a beat-to-beat basis (e.g. in inspiration, changing from orthostatic to lying position). Contrastingly, and taking into account their diuretic effects in the kidney and longer half-life than NO, CNH could be more important in hypervolemic, hypertensive states, where stretch is prolonged in time, which explains the importance in relaxing the myocardium without increasing contractility, which would be deleterious.

Effects of ischemia on the myocardial response to acute stretch

Myocardial ischemia is a paradigmatic situation of acute hemodynamic overload in which abnormal myocardial loading activates a variety of cellular responses. During an ischemic event, several neurohumoral agents are released, which contribute to the overall physiologic process of cardiac adaptation to hemodynamic overload. Though significant advances in knowledge have been made in the last three decades regarding the processes involved in the pathophysiological consequences of ischemia *per se*, the cardiac response to stretch under those conditions is just taking its first steps.

As previously stated, the myocardial response to stretch is a two-step adaptive response. The contractile response following acute stretch under ischemic conditions has been less studied. In a recent study carried out by our group, it was clearly demonstrated that this response is profoundly affected, as shown not only by a blunted FSM but also by the

abolishment of the SFR (Neves et al., 2013), thus supporting some previous indirect observations (Goto et al., 1988;Owen et al., 1993). In the human heart, after an occlusion of a coronary artery, pH falls within 15 seconds (Poole-Wilson, 1989), possibly contributing to the loss of contractility (Steenbergen et al., 1977;Patangay et al., 2009;Decker et al., 2012) due to a reduced sensitivity of the myofilaments to calcium (Kihara et al., 1989;Salas et al., 2006), consequently leading to a diminished Ca^{2+} availability to bind to troponin C. Metabolic abnormalities should not be excluded, as they lead to insufficient energy supply to support cardiac work due to MgADP accumulation, which decreases the sliding velocity in the vicinity ATPase, slowing down the contraction cycle (Sata et al., 1996) and impairing the FSM (Robinson et al., 2002). These mechanisms are probably responsible for the observed ablation of the SFR in these conditions. A possible explanation for this was given recently by our group, demonstrating that this effect was AT1R-dependent and partially AT2R-dependent: blocking AT1R, under ischemic conditions, leads to a soft decline of contraction capacity in response to stretch (Neves et al., 2013). This effect may also be mediated by AT2R activation through bradykinin, PKC and cGMP (Jugdutt and Balghith, 2001).

Little is known about the role of the PKC under ischemic conditions. As previously stated, PKC activity is more significant during the late phase of the SFR, preventing the development of a slow force decline. Also, under ischemic conditions, PKC was not capable of modifying contractile performance (Neves et al., 2013). This is consistent with its inability to alter the systolic adaptation during early phase of the response to stretch and does not exclude that its downstream mediators may be compromised in such conditions. However, PKC has several isoforms, some of them present within the same cell and activated by the same stimuli but showing different and, at times, opposite effects. For example, in ischemic heart disease, PKC δ induced decreased ATP generation (Inagaki et al., 2003), and therefore, insufficient energy supply may play a role. As such, it would be interesting to study how each one modulates the contractile response to stretch under ischemic conditions.

Usually, in the setting of myocardial infarction, the PKG signaling pathway through NO and CNH is modulated pharmacologically by nitrates as NO donors. Also, administration of PDE5 inhibitors may be promising therapeutic targets (Kukreja, 2013). However, only recently the specific role of the PKG signaling pathway in ischemic conditions was shown. Intriguingly, activation of PKG pathway did not alter the SFR in ischemic conditions, while PDE5 inhibition significantly mitigated the contractile decline after stretch in ischemia (Castro-Ferreira et al., 2014). This can be explained by NO/cGMP-independent cardioprotective effects (Elrod et al., 2007). However, the mechanisms responsible for the protective action of the sildenafil remain to be fully elucidated.

Conclusion

The myocardial response to acute stretch represents a fundamental adaptive capacity of the heart. Although this functional response to hemodynamic overload has been known for long time, the structures responsible for mechanosensing, as well as the pathways involved in the response, are still only partially clarified. The possibility of concomitant diastolic adaptation, although theoretically appropriate, has never been directly assessed. Despite the relevant knowledge accumulated on the cardiac response to acute hemodynamic overload, there is much that we still do not know. The quest for further understanding of the mechanosensing involved in this mechanism, the pathways

responsible for the response and the characterization of the diastolic adaptation may provide new insights, not only in its physiological importance, but also in the prevention and treatment of cardiovascular diseases, such as heart failure with reduced ejection fraction, heart failure with preserved ejection fraction, hypertensive crises or ischemic heart disease.

PART TWO: A NEW DIASTOLIC PARADIGM

Introduction

Acute hemodynamic load variation represents a permanent challenge to the heart, warranting continuous adaptation to face such changes in cardiac demands. When preload increases, the resulting stretch of chamber walls triggers a classic biphasic response consisting of an immediate (FSM) and sustained (SFR) increase in contractility that is central to cardiac function (Patterson and Starling, 1914; Parmley and Chuck, 1973; Kockskamper et al., 2008).

As stated in the previous section, this response is stimulated by factors produced or released at the onset of acute cardiac stretch, namely NO (Casadei and Sears, 2003) and B-type natriuretic peptide (BNP), both binding to receptors with guanylate cyclase domains and increasing sarcoplasmic cGMP levels (Potter et al., 2006; Francis, 2010). The systolic potentiation this second messenger produces is attributed, at these concentrations, to pathways including PKG-mediated phosphorylation of several key proteins regulating contraction, including PLB and TNI (Layland et al., 2005; Zhang et al., 2008; Castro-Ferreira et al., 2014), and also of adenosine diphosphate ribosylcyclase, increasing levels of cyclic ADP-ribose, which prolongs opening of sarcoplasmic reticulum Ca^{2+} channels (Willmott et al., 1996).

Keeping up with this, the availability and viability of the pathways through which these molecules act appear dysregulated in heart failure (HF), of which decreased preload reserve is a hallmark and a central mechanism of symptomatic congestion (van Heerebeek et al., 2012). The associated oxidative stress scavenges NO into peroxynitrite species, which further impair cardiac function, and the plasmatic concentration of the active fragment of BNP is decreased, blunting natriuretic signalling despite increased levels of its inactive precursor, a commonly used biomarker of this disease (Zois et al., 2014).

Interestingly, some of these factors acutely modulate diastolic function as well (Paulus et al., 1994; Shah and MacCarthy, 2000). An important target of PKG is titin, the giant sarcomeric protein accounting for most PT within the physiological range of sarcomere length (SL). By phosphorylating its N2B spring element, PKG shifts titin's resting length downward, making it more distensible and reducing cardiomyocyte stiffness for the same SL (Kruger et al., 2009; Bishu et al., 2011).

Therefore, there is reason to assume that similar mechanisms could play a role in a diastolic component in the adaptation to acute stretch, which remains unexplored. This hypothesis is further enlightened by evidence that low bioavailability of NO and cGMP, PKG hypoactivity (van Heerebeek et al., 2012; Franssen et al., 2015) and titin hypophosphorylation (Hamdani et al., 2013a) all importantly contribute to the pathophysiology of diastolic dysfunction observed in patients presenting with HF with

preserved ejection fraction (HFpEF). HFpEF represents more than 50% of HF cases, is associated with a poor prognosis and lacks therapeutic agents effective in reducing mortality and morbidity associated with it (Owan et al., 2006; Borlaug and Paulus, 2011).

Given the grand implications of such a mechanism in cardiovascular physiology and potential impact in understanding the pathophysiology of HF, we aimed to describe a novel evolutionarily conserved mechanism of acute diastolic adaptation to hemodynamic overload in healthy and failing hearts and assess the contribution of PKG-related pathways and titin phosphorylation across several models, including the human heart.

Material and Methods

Isolated heart preparation: Wistar rat hearts (n=10) were perfused according to the Langendorff technique at constant pressure (Radnoti LLC, ADInstruments) and electrically paced. Pressure was monitored using a pressure tip catheter (Millar, Inc.) inside a fluid-filled balloon inserted into the left ventricle (LV) and volume was determined and controlled using a syringe pump (New Era Pump Systems). Balloon volume was set to a preload yielding a mean diastolic pressure of 3 mmHg and, after stabilization, inflated to a diastolic pressure of 10 mmHg for 15 minutes. After this period, the left ventricular free wall was rapidly dissected and frozen in liquid nitrogen for posterior analysis.

Isolated muscle strip preparation: Force measurements were performed in papillary muscles dissected from the right ventricle of male New Zealand white rabbits and human atrial trabeculae (n=14) and human ventricular strips (n=10) strips from patients subject to cardiac surgery. The muscles were vertically mounted in an organ bath containing a modified Krebs-Ringer solution and the upper tendinous end was attached to an electromagnetic length-tension transducer (University of Antwerp, Belgium). The muscle was electrically stimulated to contract isometrically and the length at which active force development was maximal (L_{max}) was determined. After stabilization at 92% L_{max} , the rabbit papillary muscles were quickly stretched to 100% of L_{max} with different groups of muscles being either control (n=11) or incubated with Rp-8-Br-PET-cGMPS [inhibitor of PKG, 10^{-6} M, n=7], L-nitro-arginine [LNA, inhibitor of NO synthase, 10^{-5} M, n=8], A-71915 [natriuretic peptide receptor-A antagonist, 10^{-6} M, n=7], hydroxocobalamin (HCB, NO scavenger, 10^{-3} M, n=8) and LNA, HCB and A-71915 and combined (n=8). At the end of the experiment, the active portion of the muscle was quickly frozen in liquid nitrogen for posterior analysis. Additionally, in a second set of experiments, after 15 minutes of stretch, muscles were again set to 92% L_{max} over 15 minutes and subjected to another 15 minutes period of stretch to 100% of L_{max} plus 92% L_{max} over 15 minutes.

Isolated skinned cardiomyocyte preparation: Force measurements were performed in single and mechanically isolated cardiomyocytes as previously described (Borbély et al., 2005; Falcao-Pires et al., 2011) from rat hearts subjected to the perfusion protocol described above. PT was measured at SL ranging between 1.8 μ m to 2.3 μ m (0.1 μ m step increases) at 15°C. After PT stabilized at 2.2 μ m, cardiomyocytes were incubated for 40 minutes at 20°C in relaxing solution containing either purified PKG1 α , cGMP and dithiothreitol (n=25 and n=21 from stretch and control, respectively); or with protein phosphatase-1, and protein phosphatase lambda (n=25 and n=26 from stretch and control, respectively). After that, the same set of experiments was performed to measure PT at the same SL ranges.

Rat Transverse Aortic Constriction (TAC) banding model: A 3–0 non-absorbable silk suture was placed around the aortic arch of Wistar-Han rats (n=16) with 7 weeks of age, constricting it to the diameter of a 25G needle. Sham-treated animals (n=14), which underwent surgery with placement of a loose non-constrictive suture, were used as controls. After 21 weeks, echocardiographic images (VIVID 7 dimension system; General Electric-Vingmed Ultrasound) were obtained according to standards of American Society of Echocardiography (Lang et al., 2015) under anesthesia with ketamine and xylazine. Afterwards, a 1.4Fr microtip pressure-volume catheter (SPR-838, Millar Instruments) was inserted through an apical puncture wound into the LV cavity. The animal preparation was allowed to stabilize for 15 minutes, after which ventilation was suspended and a basal measurement performed at end-expiration. An acute volume load was performed by intravenous administration of 15% of total blood volume of hydroxyethyl starch (Voluven®) during 5 minutes. New recordings were made after loading with suspension of the ventilation each 5 minutes until 15 minutes. At the end, the animal was euthanized by exsanguination, and samples were collected.

Cardiac surgery patients: Hemodynamic evaluation was performed intraoperatively on patients with aortic stenosis and no evidence of LV hypertrophy on transthoracic echocardiogram (n=9) subjected to aortic valve replacement. After weaning cardiopulmonary bypass and in a hemodynamically stable period, pressure and volume were continuously measured by a 5-Fr combined catheter with 1 cm electrode spacing (SPC-551, Millar Instruments, Houston, TX) inserted through the ascending aorta. Once a basal acquisition was obtained, a volume overload was imposed through intravenous bolus of hydroxyethyl starch (Voluven®) and Trendelenburg positioning and values were registered at that moment and 15 minutes after. Four patients were male and five were female, ranging from 49 to 85 years old, and the surgery was performed by the same surgeon.

Protein kinase activity: The phosphorylation of VASP, a myocardial PKG target, was measured using immunoblots; densitometry (over GAPDH expression) was performed and presented as previously described (van Heerebeek et al., 2012).

Titin phosphorylation: After 1.8% SDS-PAGE, gels were stained with Pro-Q Diamond (phosphoprotein) for 1 hour and subsequently with Sypro Ruby (total protein) overnight. Phosphorylation signals on Pro-Q Diamond-stained gels were indexed to Sypro Ruby-stained titin signals as previously described (Hamdani et al., 2013a).

Statistical methods: Values are means $\pm$ SEM. Within the same group, the immediate effects of muscle stretch on the various experimental parameters were analysed with a paired Student's t-test. Comparison of groups at discrete points (length, volume, time) was tested with unpaired Student's t-test, being the time-dependent effects analysed by repeated measures two-way ANOVA. A $p < 0.05$ was considered statistically significant.

Results

Effects of acute cardiac stretch in myocardial stiffness

Plotting tension of acutely stretched papillary muscles from 92% to 100% L_{max} over time clearly illustrates three phenomena (Fig. 4): (1) the Frank-Starling mechanism, with active tension increasing immediately upon stretch; (2) the slow force response, with

active tension further increasing sustainably over time; (3) the diastolic response, characterized by an immediate increase in PT, followed by a progressive and time-dependent fall. Regarding this Stretch-Induced Compliance (SIC), acute stretch resulted in an immediate increase of PT from 1.5 ± 0.3 to 6.3 ± 1.2 mN.mm⁻² in rabbit papillary muscles, from 3.0 ± 0.5 to 8.1 ± 1.2 mN.mm⁻² in human atrial trabeculae, from 2.4 ± 0.2 to 8.2 ± 0.6 mN.mm⁻² in human ventricular strips. Subsequently, over the course of 15 minutes, PT of rabbit papillary muscles decreased by $43 \pm 2\%$ (Fig. 5B), human atrial trabeculae (n=14) by $27 \pm 8\%$ and human ventricular strips (n=10) by $27 \pm 6\%$ (Fig. 5C). Similarly, left ventricular diastolic pressure of isolated perfused rat hearts fell $43 \pm 2\%$ after acute inflation of the LV balloon by 38 ± 2 μ L to increase diastolic pressure from 3 to 10 mmHg (Fig. 5A).

At the end of this time period, rabbit papillary muscles were returned to their original conditions by reducing length back to 92% L_{max}. At this point, PT was significantly lower than that observed before stretch (0.8 ± 0.2 mN.mm⁻²) but rose for the following 10 minutes (1.1 ± 0.3 mN.mm⁻²), becoming significantly higher than immediately after stretch but lower than before stretch (Fig. 6). The length-PT relationship in papillary muscles delineated by these “stretch” and “unstretch” interventions clearly illustrates the SIC and its partial persistence over the following 10 minutes (Fig. 7B) and analogous results are obtained in diastolic pressure-volume relationships of isolated rat hearts (Fig. 7A).

To evaluate whether stress relaxation played a role in SIC, we stretched isolated skinned cardiomyocytes but no significant drop in PT was observed in this setting (Fig. 5D and Fig. 7C). Nonetheless, cardiomyocytes extracted from the LV free wall of five stretched isolated hearts presented with a markedly reduced PT at SL ranging from 1.8 to 2.3 μ m compared to those extracted from other five hearts not submitted to the stretch protocol (Fig. 7D), confirming the adaptation occurs only in intact tissue at the myofillamental level.

Lastly, in cardiac surgery patients evaluated *in vivo*, volume overload raised end-diastolic volume from 122.5 ± 21.8 to 150.3 ± 21.2 mL. Baseline end-diastolic pressure (EDP) was 11.0 ± 2.1 mmHg and, with overload, increased to 17.7 ± 5.5 mmHg. After 15 minutes, EDP measured in cardiac cycles with the same end-diastolic volume of the previous situation, was clearly reduced, at 13.5 ± 1.7 mmHg (Fig. 8).

TAC banding compromises the decrease in stiffness in response to stretch

Next, we used a model of cardiac hypertrophy and diastolic dysfunction, rats subjected to transverse aortic constriction (TAC) banding, to test SIC in a pathological context. Compared with sham-operated rats, TAC animals presented a significant increase in cardiac mass ($63 \pm 16\%$) and echocardiographic studies revealed significantly higher left ventricular posterior wall dimension in systole and diastole. Furthermore, they presented a significant increase of LV EDP and time constant *tau*, indicating diastolic dysfunction. LV peak systolic pressure was also significantly higher when compared to Sham, but no significant change was detected in ejection fraction (Table 1).

In response to acute myocardial stretch induced by volume overload, left ventricular EDP significantly increased in both groups (Sham $+60 \pm 4\%$; TAC $+79 \pm 37\%$). After 15 minutes, EDP significantly decreased in Sham (15 ± 1 vs. 9 ± 1 mmHg), but not in TAC

animals (19 ± 4 vs. 16 ± 3 mmHg), even if end-diastolic LV volume remained unchanged (Figure 9).

PKG-mediated pathways in the response to acute myocardial stretch

We hypothesized that PKG-mediated pathways were involved in priming myocardial stiffness to preload elevation. Because both NO and BNP are released upon myocardial stretch and increase cGMP levels, which activate PKG, we confirmed these observations by measuring PKG activity in the LV free wall of rats subjected to Langendorff perfusion, and found that myocardial stretch led to an increase in PKG activity (Fig. 10).

To assess the individual contributions of these agents to the mechanism, we investigated whether blocking them would interfere with this effect. Incubation with LNA, HCB or A-71915 alone did not significantly alter the myocardial response to stretch and the muscles exhibited a similar behaviour to that observed under basal conditions. However, simultaneous incubation with all three drugs significantly blunted the decrease in PT after acute stretch (30 ± 3 vs. $43 \pm 2\%$), as did the direct antagonism of PKG by Rp-8-Br-PET-cGMPS (26 ± 1 vs. $43 \pm 2\%$) (Figure 11).

In order to functionally assess the contribution of phosphorylation of myofilamental elements to SIC, we incubated skinned cardiomyocytes extracted from stretched or control isolated rat hearts with protein phosphatases. After 40 minutes, we observed a significant upward dislocation of length-PT relationships in both groups, more prominent in the previously stretched group, approaching control levels (Fig. 12B). On the other hand, incubation of myocytes with PKG for 40 minutes reduced stiffness in the control group, as previously shown (Paulus et al., 1994; van Heerebeek et al., 2012; Hamdani et al., 2013a), but not in the stretched group (Fig. 12A).

Titin phosphorylation in response to acute myocardial stretch

Lastly, we compared total titin phosphorylation between groups of muscles subjected to acute stretch for 15 minutes and ones that were kept at constant length throughout the protocol. As illustrated in figure 13, a significant increase in the levels of phosphorylated titin was observed in rabbit papillary muscles (23 ± 3 vs. $13 \pm 2\%$), as well as in human right atrial trabeculae (41 ± 8 vs. $11 \pm 1\%$) and left ventricular strips (71 ± 21 vs. $27 \pm 8\%$), suggesting this as a potential mechanism for the active modulation of myocardial stiffness following stretch. Interestingly, in the hearts from TAC rats not only was titin hypophosphorylated at baseline ($73 \pm 6\%$ of sham levels), but also the $51 \pm 12\%$ increase in titin phosphorylation upon VO observed in sham was blunted to $30 \pm 14\%$.

Discussion

With this work, we intended to explore the response to hemodynamic overload in detail, well characterized from a contractile standpoint for decades, but of which a diastolic component had never been described. We observed that the latter, in the form of SIC, allows optimization of filling capacity at lower diastolic pressures and, therefore, potentiation of increased cardiac output triggered by acute myocardial stretch and prevention of pulmonary and systemic congestion. This mechanism augments mechanical distensibility in a potent and reproducible way in various mammalian models, being evolutionarily conserved up to the human species. Additionally, it is evident in

experimental preparations ranging from the isolated cardiomyocyte to *in vivo* hemodynamic analysis and including *in vitro* isolated intact hearts and muscle strips from ventricle and atrium.

Acutely stretching healthy myocardium *in vitro* is followed by a drop in left ventricular EDP of intact hearts and PT of human and animal isolated muscle strips over time. After this period, EDP-volume and length-PT relationships, respectively, were shifted inferiorly, indicating decreased stiffness acquired after this stimulus. Furthermore, after its establishment, returning to baseline levels of preload reverses the effect incompletely, pointing to a role in both dynamic adaptation on the fly and more durable load conditioning.

Decreased EDP is also seen after *in vivo* volume overload both in rats and in patients undergoing aortic valve replacement surgery. Although in this setting central neural reflexes and systemic humoral agents with myocardial effects likely contribute to the effect and cannot be distinguished from an intrinsic myocardial adaptation, its significance in this context still stands.

Opposite to these situations, stretching isolated skinned cardiomyocytes does not evoke the same response *per se*. This suggests that the effect requires preserved subcellular compartmentalization to become manifest and, thus, depends on signaling pathways. Different stiffness is found only when comparing cardiomyocytes extracted from stretched and non-stretched hearts, i.e. when the intervention is performed on the intact organ. Taken together, these observations confirm that the effect is, to a grand extent, independent from the passive properties of cardiac extracellular matrix and that the effector players in these pathways must target myofillamentary elements.

To translate these findings to a pathological context, we used a classic animal model of diastolic dysfunction induced by thoracic aortic constriction. Inducing *in vivo* acute hemodynamic overload in these animals revealed that, contrarily to sham individuals, EDP, already elevated at baseline, does not decline significantly over time. This difference is relevant, as inability to increase filling capacity in the face of a hemodynamic challenge can lead to congestion. Not only does this underline the importance of the diastolic component of the response to stretch, but also represents the first description that diastolic dysfunction is attributable to both increased myocardial stiffness and compromised modulation after acute load.

We verified that PKG-related pathways were activated upon myocardial stretch and led to titin phosphorylation, representing the molecular basis explaining SIC and their dysregulation in the hypertrophied heart highlights their pathophysiological relevancy. In our study, the diastolic response to load is highly dependent on the activity of this enzyme, evident by the marked blunting of the effect observed upon its inhibition.

A similar phenomenon was observed when NO production was inhibited, its intracellular stores were scavenged and the natriuretic peptide receptor A was inhibited, even though these interventions, by themselves, exerted no effect. This suggests a compensatory or redundant interaction between NO and BNP, both activating PKG through increase in intracellular cGMP concentration, although the subcellular compartment whereby this occurs may differ between the two mediators (Castro et al., 2006; Castro et al., 2010), as discussed above in the literature review. In spite of our establishment of the importance

of this pathway in SIC, future study is necessary to ascertain the most relevant NO synthase isoform and cellular cGMP pool in this context.

At the sarcomeric level, our results corroborate previous studies showing that incubation of isolated cardiomyocytes with PKG increased their distensibility (Kruger et al., 2009; van Heerebeek et al., 2012; Hamdani et al., 2013a). Intriguingly, incubation of myocytes of previously stretched hearts, which already show decreased levels of PT, does not alter their stiffness profile. This points to a degree of saturation of kinase signalling, its target sites probably already phosphorylated by PKG concomitantly to its stretch-induced activation. On the flipside, incubation of cardiomyocytes with protein phosphatases shifted both groups' length-PT curves upward and brought them very close to each other, thus uncovering the phosphorylation-dependent component of SIC.

Some known targets of PKG include TNI (Layland et al., 2002), increasing cross bridge cycling, and PLB (Zhang et al., 2005), reducing its affinity for SERCA and boosting Ca^{2+} reuptake during diastole, but none of these actions link acute cardiac loading to decreased myocardial stiffness. We propose that this effect is in fact mediated by titin, phosphorylated by PKG on its N2B elastic element, increasing electric repulsion between its negatively charged amino acids and making it more distensible (Kruger et al., 2009; Kotter et al., 2013).

Keeping up with this, higher titin phosphorylation levels were measured in stretched myocardial samples from human atria and ventricles and rabbit ventricles. In the latter case, samples analyzed after a period of "unstretch" reveal an incomplete reversal of phosphorylation levels, mirroring the persisting decrease in stiffness observed during the protocol. Furthermore, *in vivo* hemodynamic overload in rats induced titin phosphorylation in healthy animals but not in the diastolic dysfunction group, which presented baseline hypophosphorylation, confirming the central role of this mechanism in SIC and diastolic function.

Our line of study may contribute to better understanding of mechanisms implicated in HFpEF-associated diastolic dysfunction. Firstly, it shows that the role of the cGMP/PKG pathway and titin phosphorylation in this context is not limited to modulation of ventricular stiffness but also encompasses the response to hemodynamic stress in different cardiac chambers. Since HFpEF is frequently characterized by exercise intolerance with fatigue, cough and dyspnea and underlying hypoactivity of the previously mentioned molecular mechanisms (Borlaug and Paulus, 2011; van Heerebeek et al., 2012), impaired SIC may be an additional pathophysiological link, leading to inability to optimize diastolic filling pressures after overload. Therefore, its potential diagnostic and therapeutic applications in this condition, which urgently needs new management options to reverse its tendency towards an unfavorable outcome, should be avidly studied.

As such, the decline in EDP after acute load could be an early disease marker manifesting prior to symptom onset. Noninvasive indices of diastolic dysfunction such as the E/E' ratio on transthoracic echocardiogram before, during and after preload increasing provocative maneuvers (e.g. exercise, limb elevation or Trendelenburg positioning) might be of diagnostic value. Poor adaptation to this stimulus could additionally be used as a disease classification criterion, which is not yet consensual (McMurray et al., 2012;

Yancy et al., 2013). Our group has begun recruitment of patients with unexplained shortness of breath for a pilot study of this technique for early detection of HFpEF.

Lastly, restoring the molecular pathway represents a tempting pharmacological strategy and our results root in its favor. After the establishment of its significance in disease, it has been tested by the administration of sildenafil, a phosphodiesterase-5 inhibitor that abolishes myocardial cGMP hydrolysis (Takimoto et al., 2005; Bishu et al., 2011; Borlaug et al., 2015). Unfortunately, the large-scale randomized RELAX trial did not garner results supporting this treatment in HFpEF (Redfield et al., 2013) and, despite some flaws in its study design, it is likely that this intervention, by itself, does not suffice to alter clinical course. Indeed, waning action of upstream mediators may generate cGMP levels that, even with its breakdown inhibited, are inadequate to properly activate the pathway. Hence, riociguat, directly activating soluble guanylate cyclase (Bonderman et al., 2014), may reveal more effective. Moreover, other strategies might stem from targeted activation of other enzymes phosphorylating titin, such as PKA or Ca^{2+} /calmodulin-dependent protein kinase (van Heerebeek et al., 2006; Hamdani et al., 2013b). Finally, since our data point to a durable reduction in myocardial stiffness even after the original mechanical stimulus subsides, non-pharmacological interventions promoting cardiac stretch may be useful, including physical exercise.

Conclusion

Our study identifies a novel mechanism in cardiac physiology that bridges adaptation to loading into the realm of diastolic function. Through increased compliance, healthy hearts of various species, including humans, are able to accommodate load at lower pressures and further optimize cardiac output in this context. PKG-mediated pathways, culminating on titin phosphorylation, are essential to its development and time-dependent sustained effect. SIC is impaired in diastolic dysfunction, and this observation is a harbinger of its diagnostic and therapeutic implications.

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Figures 1-3 use templates from Servier Medical Art.

Disclosures

None.

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Figures and Tables

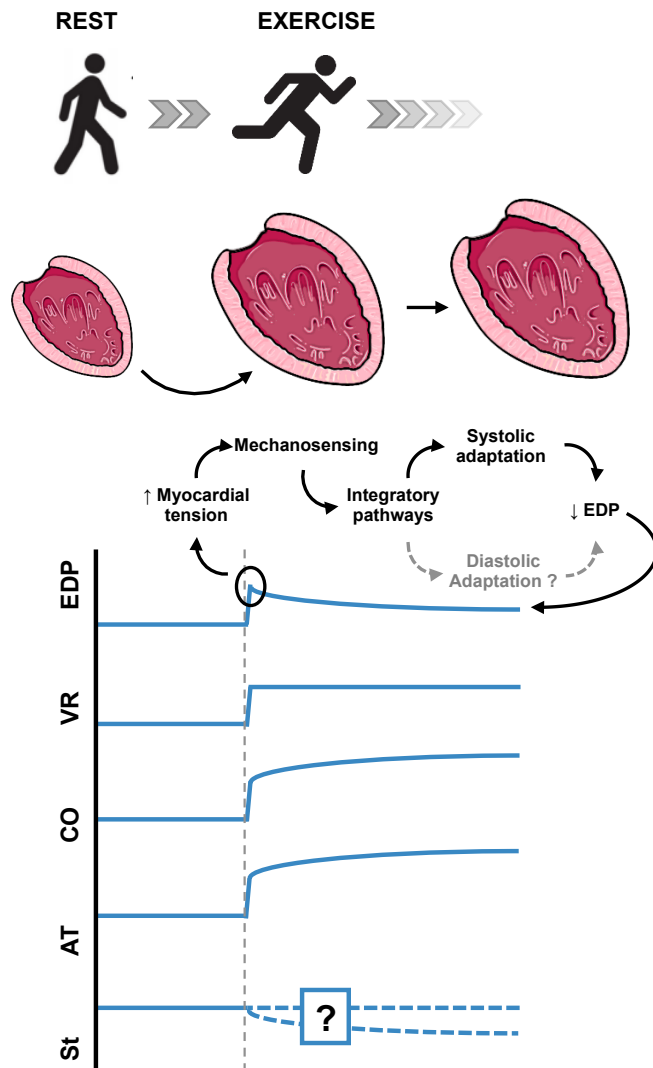


Figure 1: Acute cardiac overload. Schematic example of the normal intrinsic adaptation of the left ventricle to acute increase in preload. In this example, the additional modulation by autonomic nervous system is ignored, in order to highlight the intrinsic myocardial response to acute stretch. Myocardial stiffness changes in response to acute myocardial stretch have not yet been adequately evaluated. EDP: End Diastolic Pressure; VR: Venous Return; CO: Cardiac Output; AT: Active Tension; St: Myocardial Stiffness.

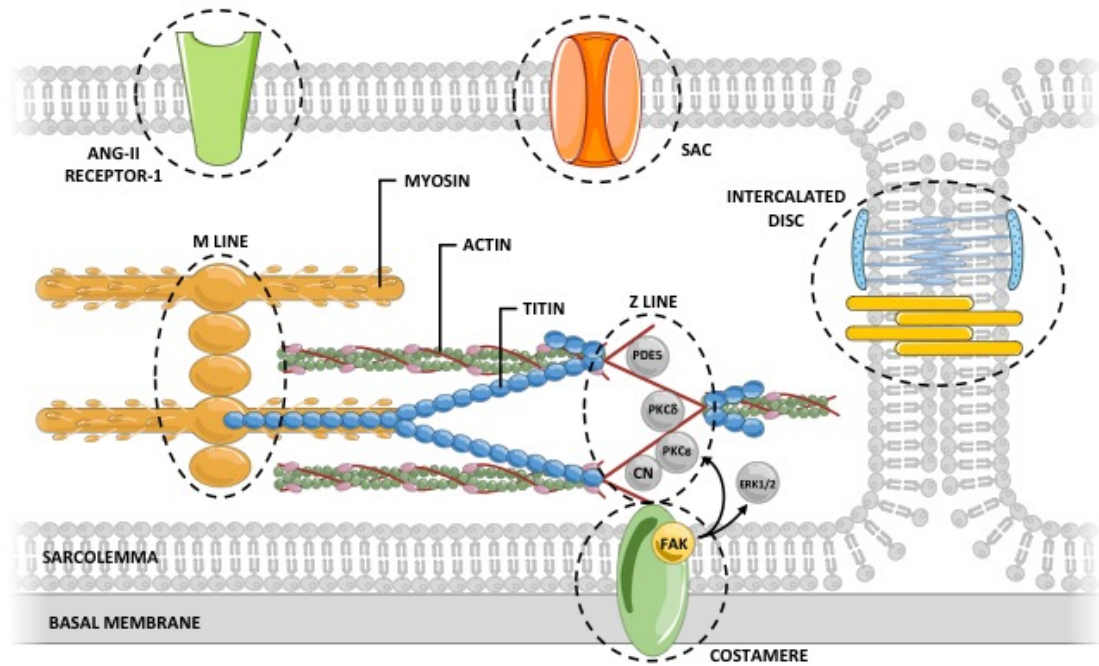


Figure 2: Myocardial mechanosensing. Acute myocardial stretch induces an increased myocardial tension that can be sensed by several potential mechanosensors. The connections between mechanosensors and activated signaling pathways are not yet fully understood. CN: calcineurin; ERK: extracellular signal-regulated kinase; FAK: focal adhesion kinase; PDE: phosphodiesterase; PKC: protein kinase C; SAC: stretch-activated channel.

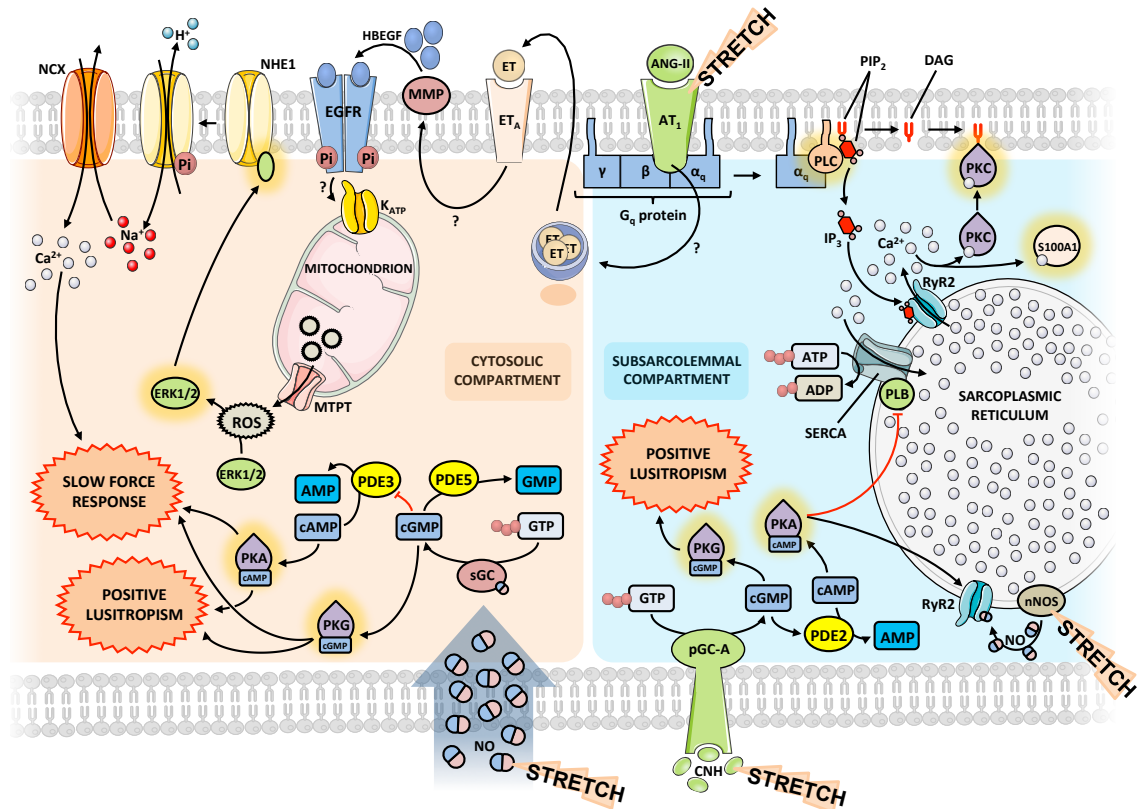


Figure 3: Integrated signaling pathways. Diagram representing the molecular interplay between the various players in the acute response to stretch. The same mediators may exert different effects depending on the subcellular compartment. Question marks indicate yet unknown mechanisms. The proposed model is based on data obtained from different species and experimental models. Some signaling pathways may differ between species. ANG-II: angiotensin-II; CNH: cardiac natriuretic hormone; EGFR: epidermal growth factor receptor; ET: endothelin; ERK: extracellular signal-regulated kinase; HBEGF: heparin-binding epidermal growth factor; MMP: matrix metalloproteinase; NCX: sodium-calcium exchanger; NHE: sodium-proton exchanger; nNOS: neuronal NO synthase; PDE: phosphodiesterase; pGC-A: particulate guanylate cyclase A PK: protein kinase; PLB: phospholamban; PLC: phospholipase C ROS: reactive oxygen species; Ryr2: ryanodine receptor 2; sGC: soluble guanylate cyclase.

Cardiac response to acute stretch

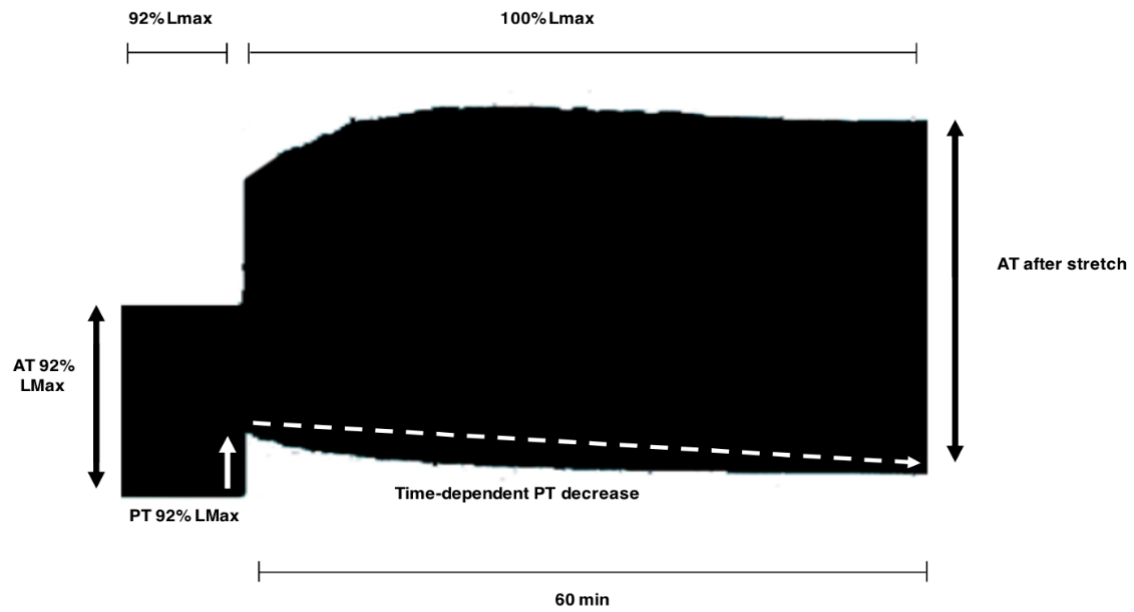


Figure 4. Effects of acute cardiac stretch in myocardial force and stiffness. After stretch of a cardiac muscle to a length in which develops maximal force (L_{max}), active tension increases, firstly through the FSM and later by SFR. There is also a progressive and time-dependent decrease in PT.

Cardiac response to acute stretch

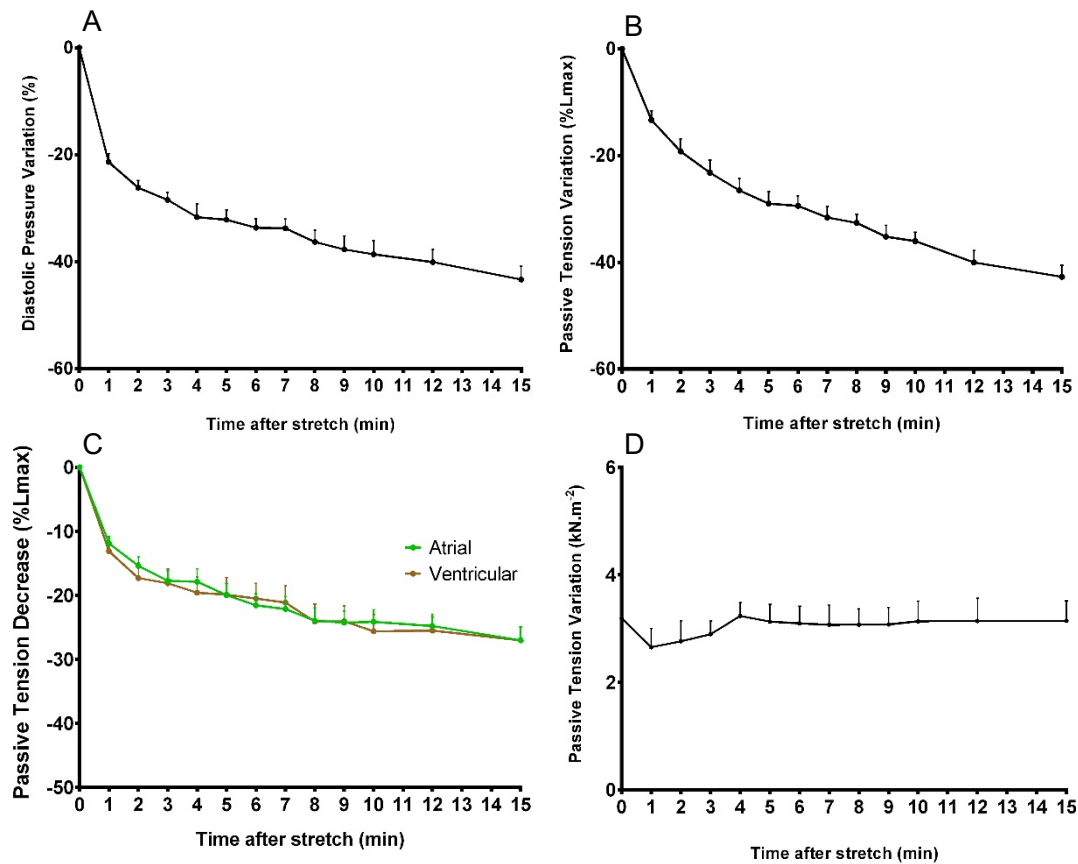


Figure 5. The variation of passive tension/diastolic pressure of rat heart (A), isolated papillary muscles from rabbit (B), human myocardial strips (C) and single skinned cardiomyocytes (D) over 15 minutes after acute myocardial stretch. In both isolated rat heart and isolated papillary muscles from rabbit right ventricle there is a decrease in PT over 15 minutes. The magnitude of the drops of the PT of papillary muscles and the end-diastolic pressure from rat hearts were quite similar, as well as the behaviour of the human myocardial strips between atria and ventricle. Stretching isolated skinned cardiomyocytes did not lead to the same response.

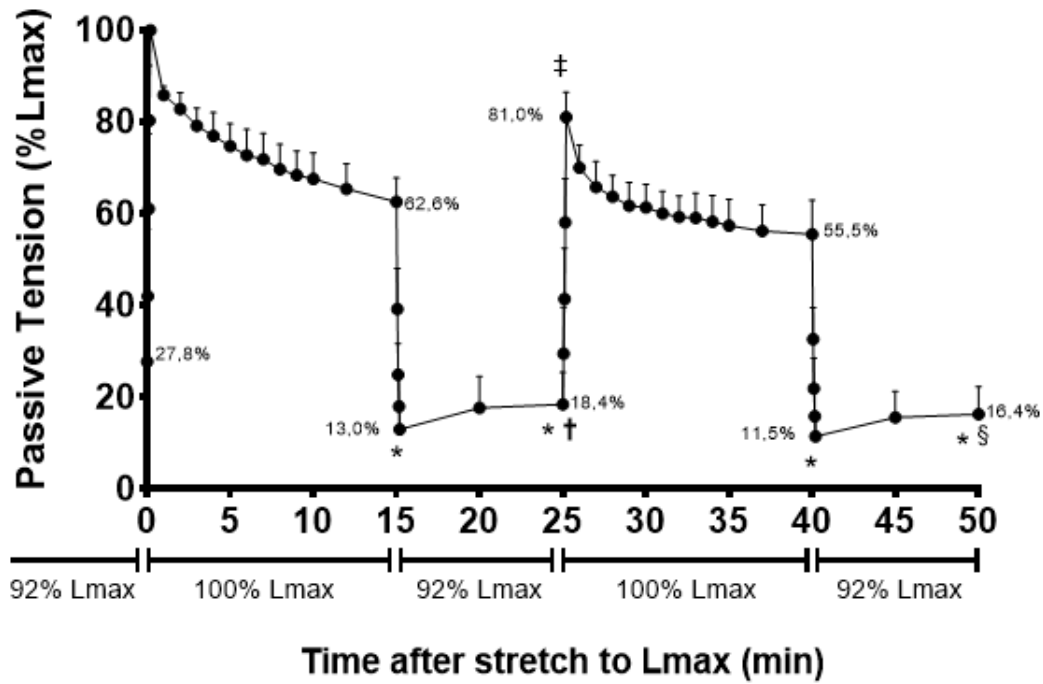


Figure 6. Effects of acute cardiac stretch and unstretch in rabbit right ventricle papillary muscles. After acute myocardial stretch, the unstretch to 92% L_{max} led to a significantly decrease in passive tension at this length. A subsequent stretch led to a significantly lower passive tension increase regarding the first stretch as well to a lesser passive tension after 15 minutes of stretch.

Cardiac response to acute stretch

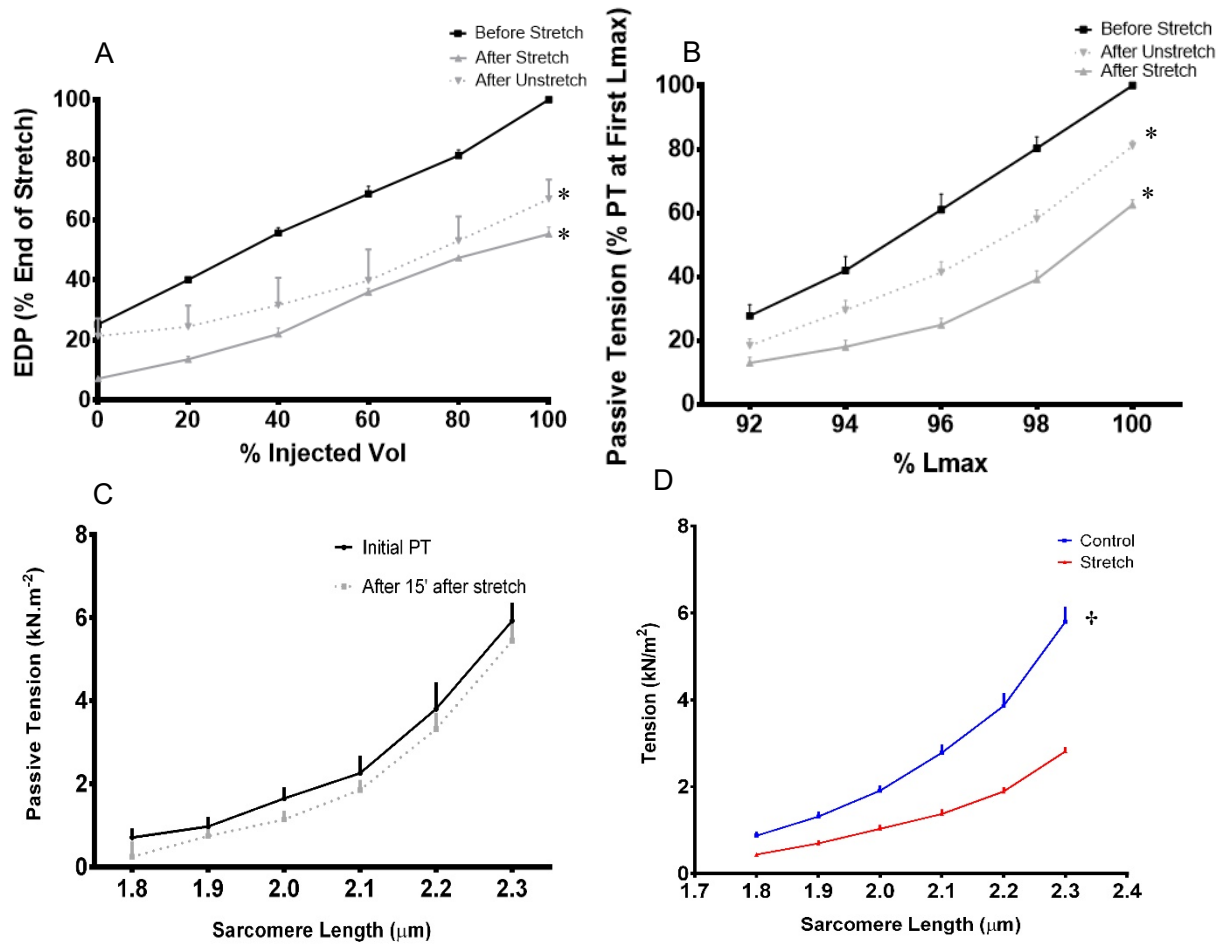


Figure 7. Passive length-tension relationships before and after stretch (A-C), after unstretch. (A-B) and stretched versus control rat hearts (D). Relationships from rabbit papillary muscles (A), diastolic pressure-volume relationship in isolated rat hearts (B). Panel C and D refer to passive length-tension relationship in single skinned cardiomyocytes: C refers to before and after 15 minutes of stretch while D compares hearts previously or not stretched. * p < 0.05 vs. before stretch, and † p < 0.05 vs. Stretch.

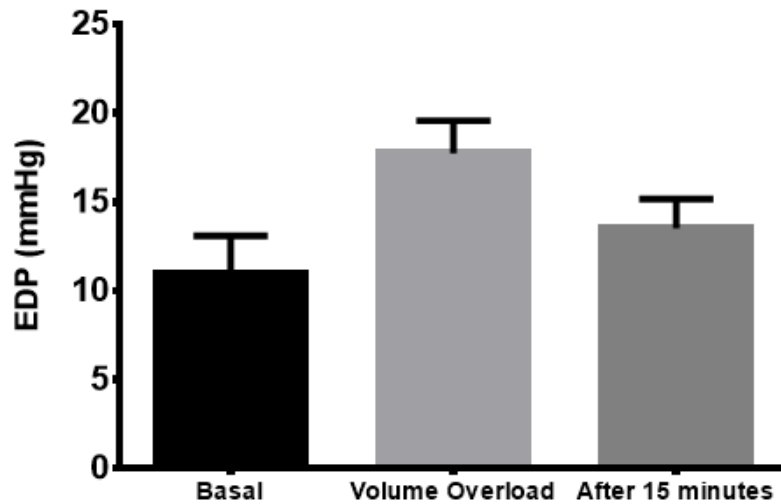


Figure 8. End-diastolic pressure (EDP) in cardiac surgery patients before (basal), immediately after (Volume Overload) and 15 minute after (After 15 minutes) volume overload. There is a remarkable increase in EDP after volume overload, which returns closer to the baseline after 15 minutes. Pressures were measured in the last two situations identical values of EDP. * $p < 0.05$. vs. volume overload.

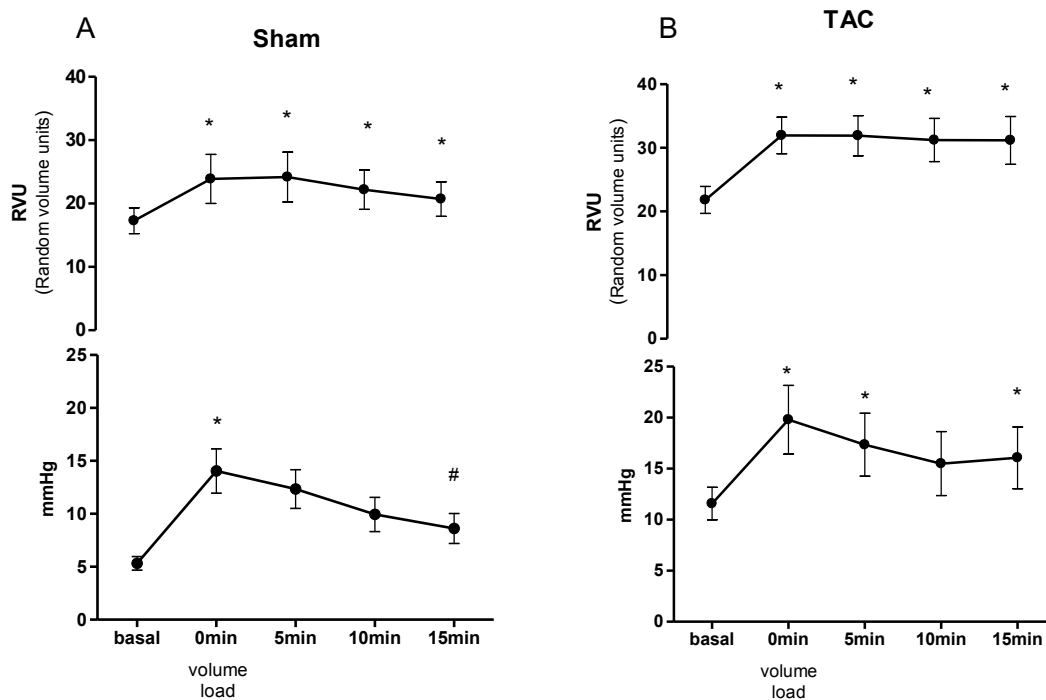


Figure 9. Hemodynamic response to the acute stretch induced by volume overload in sham (A) and TAC (B) rats. The acute myocardial stretch induced an increase in end-diastolic pressure in both sham and TAC rats. After 15 minutes of stretch end-diastolic pressure significantly decreased only in sham group. * $p < 0.05$ vs. basal and # $p < 0.05$ vs. 0min.

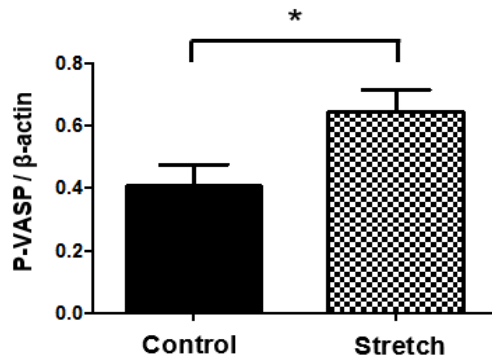


Figure 10. Enzymatic activity of PKG in myocardial tissue homogenates in control and stretched rat hearts. There is an increase in PKG activity in the hearts of rats subjected to 15 minutes of stretch. * $p < 0.05$ vs. control.

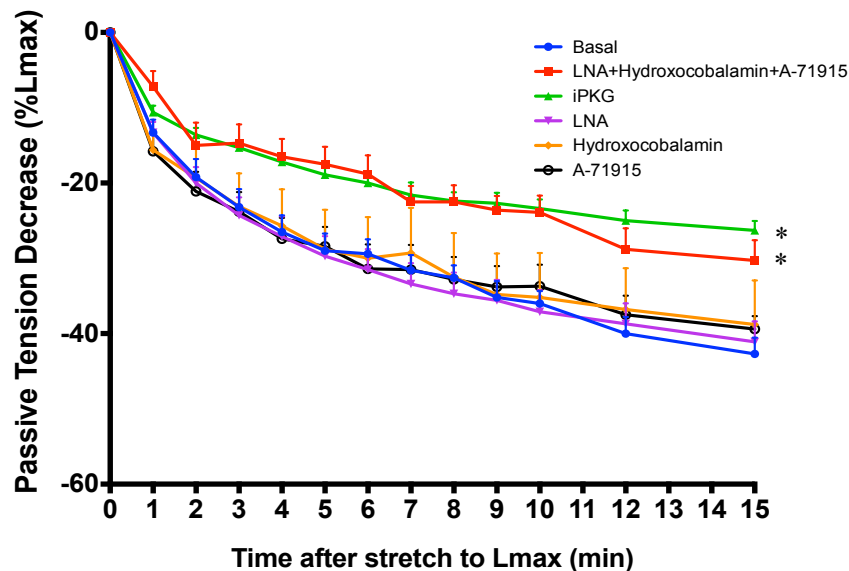


Figure 11. PKG-mediated pathways in the response to acute myocardial stretch. Incubation with LNA, hydroxocobalamin or A-71915 alone did not significantly alter the myocardial response to stretch comparing to basal conditions. Simultaneous incubation with all three drugs significantly blunted the decrease in PT after acute stretch as did the direct antagonism of PKG by Rp-8-Br-PET-cGMPS. * $p < 0.05$ vs. basal.

Cardiac response to acute stretch

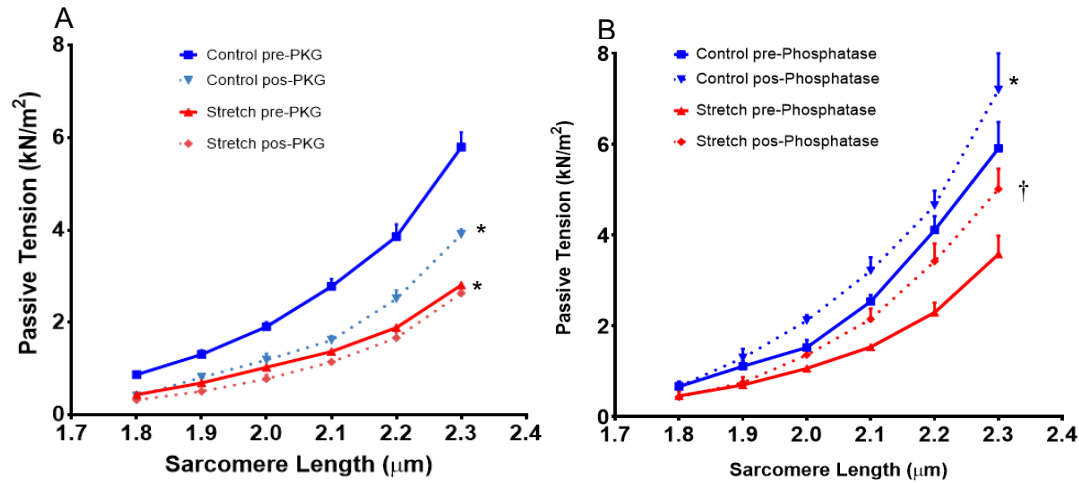


Figure 12. Effect of phosphorylation (A) and dephosphorylation (B) in single skinned cardiomyocytes from stretch or control hearts. Only the cardiomyocytes extracted from control hearts had a significant downward shift in passive length-tension relationship over the entire range of SL after incubation with PKG (A). When cardiomyocytes were incubated with phosphatases the passive length-tension relationship of both groups shifted upward (B). * $p < 0.05$ vs. control, and † $p < 0.05$ vs. stretch.

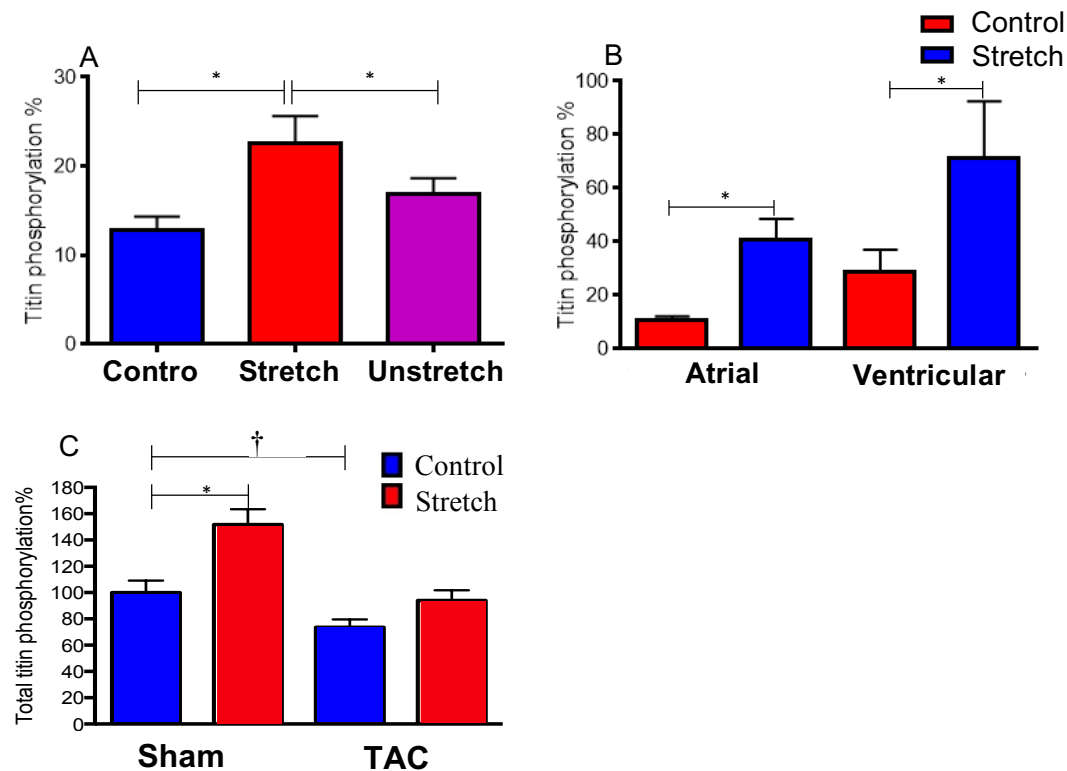


Figure 13. Titin phosphorylation levels in rabbit right ventricle papillary muscles (A), human myocardial muscles (atria and ventricle) (B) and sham and TAC banding rats (C). The acute myocardial stretch over 15 minutes led to a significant increase of the titin phosphorylation levels, which were reverted after unstretch to 92% L_{max} . Also in human myocardial stretch, titin phosphorylation levels increased after stretch. There is a blunt of the increase in titin phosphorylation levels in TAC banding rats in response to stretch. * $p < 0.05$ vs. control and † $p < 0.05$ vs. sham.

Cardiac response to acute stretch

	Sham	TAC
Body weight (g)	444±7	479±30
Heart weight (g)	1.3±0.04	2.1±0.2*
<i>Echocardiographic data</i>		
LVPWDd (mm)	1.5±0.2	1.9±0.1*
LVPWDs (mm)	2.5±0.1	3.2±0.1*
EF (%)	76±1	84±1
E/E'	9.9±0.8	16.8±1.7*
IRT	18.0±0.5	22.0±1.0*
Aortic gradient (mmHg)	2±13	61±5*
<i>Hemodynamic data</i>		
LVEDP (mmHg)	5±1	12±2*
LVPmax (mmHg)	123±5	156±9*
Tau (ms)	11±0.3	21±4

Table 1. Characteristics of sham operated (Sham) and aortic constriction (TAC) rats. LVPWDd, LVPWDs: Left ventricular posterior wall dimension in diastole and systole, respectively; LVDd: Left ventricular end diastolic diameter; EF: ejection fraction; IRT: Isovolumic relaxation time LVEDP: Left ventricular end diastolic pressure; LVPmax: peak systolic left ventricular pressure; Tau: time constant of isovolumic relaxation. * $P < 0.05$ vs Sham.

ANEXO – Normas da Revista

Revista: Frontiers in Physiology

Nome encurtado: Front Physiol

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Limite de palavras do resumo: 350

Limite de palavras do corpo do texto: 12000

Limite de figuras e tabelas: 15

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The title is written in title case, centered, and in 16 point bold Times New Roman font at the top of page.

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The body text is in 12 point normal Times New Roman. New paragraphs will be separated with a single empty line. The entire document should be single-spaced and should contain page and line numbers in order to facilitate the review process.

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- The use of abbreviations should be kept to a minimum. Non-standard abbreviations should be avoided unless they appear at least four times, and defined upon first use in the main text. Consider also giving a list of non-standard abbreviations at the end, immediately before the Acknowledgments.
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- Gene symbols should be italicized; protein products are not italicized.
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Article in an online journal: Tahimic, C.G.T., Wang, Y., Bikle, D.D. (2013). Anabolic effects of IGF-1 signaling on the skeleton. *Front. Endocrinol.* 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book: Sorenson, P. W., and Caprio, J. C. (1998). "Chemoreception," in *The Physiology of Fishes*, ed. D. H. Evans (Boca Raton, FL: CRC Press), 375-405.

Book: Cowan, W. M., Jessell, T. M., and Zipursky, S. L. (1997). *Molecular and Cellular Approaches to Neural Development*. New York: Oxford University Press.

Abstract: Hendricks, J., Applebaum, R., and Kunkel, S. (2010). A world apart? Bridging the gap between theory and applied social gerontology. *Gerontologist* 50, 284-293. Abstract retrieved from Abstracts in Social Gerontology database. (Accession No. 50360869)

Patent: Marshall, S. P. (2000). Method and apparatus for eye tracking and monitoring pupil dilation to evaluate cognitive activity. U.S. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

Data: Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. *Dryad Digital Repository*. (2015) <http://dx.doi.org/10.5061/dryad.ps837>

Theses and Dissertations: Smith, J. (2008) Post-structuralist discourse relative to phenomenological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

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