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Original Article

Changes in vascular reactivity of the coronary artery and thoracic aorta in the delta sarcoglycan null mutant mice

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Abstract

Introduction: Mutations in the delta sarcoglycan gene (d-SG) cause limb-girdle muscular dystrophy type 2F with structural and functional alterations in cardiac, smooth and skeletal muscle. The objective of the present study was to improve information about changes in vascular reactivity of the thoracic aorta and the coronary artery in the perfused heart of the d-SG-null mutant mouse model.

Methods: Female knockout (KO) and wild-type (WT) mice (5 months old) with and without nitric oxid and prostanoids antagonist were used. Curves doses response to phenylephrine, angiotensin II and acetylcholine were constructed.

Results: The results shows an increment in the contractile response to angiotensin II in the aorta and the isolated heart from the KO mice, and it seems due to a major participation of prostanoids. On the other hand the relaxant effect of acetylcholine is less in the KO than in the WT mice.

Conclusion: Changes in vascular reactivity in KO mice seems due to the participation of prostanoids instead of nitric oxide.

Keywords:
Delta sarcoglycan gene;
Vascular reactivity;
Aorta;
Heart

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Introduction

The participation of the sarcoglycan (SG) complex in the development of dilated cardiomyophaty is well documented for patients with mutations related to limb-girdle muscular dystrophy (LGMD-2F) (Ramírez-Sánchez et al., 2005; Ramirez-Sanchez et al., 2007). Apparently, the sarcoglycan-sarcospan (SG-SSPN) complex in vascular smooth muscle plays an important role in the regulation of the contractile-relaxant mechanisms of blood vessels. However, there is scant information about the SG-SSPN complex in relation to vascular smooth muscle and the endothelium of blood vessels (Ramírez-Sánchez et al., 2005; Straub et al., 1999; Wheeler et al., 2002). The deletion of the delta (d) -SG gene in a mouse experimental model of LGMD-2F is characterized by a complete loss of the SG-SSPN complex in skeletal muscle and cardiac muscle tissue, as well as structural changes in the coronary artery. This shows the importance of the SG-SSPN complex in blood vessels. Few studies have analyzed the participation of this complex in smooth muscle and endothelium tissue. Since it is difficult to evaluate cardiovascular and physiopathological alterations associated with these gene deletions in patients, experimental animal models are employed (Coral-Vazquez et al., 1999; Sandona and Betto, 2009).

Previous studies on d-SG-null mutant mice have

reported that the absence of this gene in vascular smooth muscle results in spontaneous vasospasms in the coronary artery, which leads to cardiomyophaty (Coral-Vazquez et al., 1999). However, the functional and physiological changes that arise in vascular smooth muscle and endothelial tissue in this experimental model are still not completely clear. Ramirez et al. suggest that the SG-SSPN complex in vascular smooth muscle of the umbilical cord forms a complex with caveolin-1 (Ramírez-Sánchez et al., 2005; Shin et al., 2006), which regulates the activity of diverse molecules. One such molecule is endothelial nitric oxide synthase (eNOS), which in turn modulates muscular tone (Yoshida et al., 2000).

It has been reported the relation between dilated cardiomyophaty and sarcoglycanopathies (d-SGs and g-SGs). In 24-week-old mice mutants in subunit g or d-SG, there is no difference in the expression of neuronal NOS between the heart and skeletal muscle. Nevertheless, they found an increased local expression of eNOS in damaged zones of these mutant mouse species. They associated this elevated expression with the formation of a complex of the SGs and eNOS, favoring a greater expression of the latter, which in turn would lead to an enhanced release of NO and the development of cardiomyophaty (Heydemann et al., 2004; Moncada et al., 1991).

It is known that various systems participate in the regulation of vascular tone under normal physiological and stress conditions such as vascular endothelium, the autonomous nervous system (activation of adrenergic receptors) as well as the renin-angiotensin system, which carry out their regulatory role through the release of vasoconstrictor substances (e.g., angiotensin, endothelin and noradrenaline) and vasodilator substances (e.g., NO and prostacyclin).

Although unpublished results from our lab suggest abnormal vascular reactivity of d-SG-null mutant mice, the causes are unknown. Hence, the aim of the current study was to explore alterations in the vascular reactivity of the thoracic aorta and the coronary artery in the perfused heart of the d-SG-null mutant mouse model, and in this manner try to identify what type of mechanism, vasocontractile or vasodilator, could be involved in such changes (Contra et al., 2008; Guimaraes and Moura, 2001; Pérez-Diaz et al., 2006; Toda and Okamura, 2003).

Materials and methods

Animals

All procedures and handling of the animals were in accordance with Mexican Federal Regulations for Animal Experimentation and Care (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico City, Mexico) and approved by the Institutional Laboratory Animal Use and Care Committee (CICUAL) of the Escuela Superior de Medicina. Female cardiopathic knockout (KO) mice mutant in the *sgcd* gene (B6.129 *Sgcdtm1Kcam*) and wild-type (WT) mice were purchased from Mutant Mouse Reginal Resource Centers (MMRRC), University of Missouri (Columbia, MO, USA). The genotype of the delta-SG locus was verified with a protocol provided by MMRRC to differentiate WT (B6.129-Sgcd) and KO. Mice from 5 months old were used herein and kept individually in acrylic cages on a 12/12 light/dark cycle at room temperature (22 ± 2 °C), with food and water available *ad libitum* until the moment of sacrifice. After anesthesia with pentobarbital (50mg/kg body weight, ip administration) (anesthesia, Pfizer), the heart and thoracic aorta were extracted from each animal.

Vascular reactivity

The extracted hearts and thoracic aorta were placed in cold Krebs solution and the adjacent connective tissue was removed. Each segment of a thoracic aorta, taken from the diaphragm to the aortic arch, was cut into rings (4-5 mm long) and placed in an isolated organ chamber containing 10 ml of Krebs-bicarbonate solution with the following composition (mM): NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4 \cdot 7H_2O$ (1.2), $CaCl_2 \cdot 2H_2O$ (2.5), $NaHCO_3$ (25), dextrose (11.7) and calcium disodium EDTA (0.026).

The solution of the isolated organ chamber was kept at 37 °C and pH 7.4, and was bubbled with a mixture of 95% O_2 and 5% CO_2. Aortic rings were mounted on two stainless steel hooks to record the isometric tension. One hook was fixed to the bottom of the chamber and the other to a Grass FTO3 tension transductor connected to a 7D Grass Polygraph (Grass Instrument Co., Quincy, MA, USA). The rings were given an initial tension of 1.5 g and then left to stabilize for 2 h. During this time, they were exposed to phenylephrine (10^{-6} M) every 30 min to test the

viability of the contractile response of the tissue. Afterwards, the tissue was washed three times with Krebs solution to recover the basal level of tension.

The Langendorff technique

Once the anesthesia had taken effect, the animal was placed in a supine decubitus position on the dissection table and immobilized. The ribs were sectioned by making two parallel cuts along the major axis of the sternum at the level of the anterior axillary line. The rib cage was lifted and clamped in a cephalic direction, thus exposing the heart and lungs. The pericardium was immediately removed and the connective tissue of the ascending aorta dissected, tied with 3-0 silk thread and cut. The heart was submerged in cold Krebs solution, cannulated through the aorta and connected to a perfusion system with constant flow. The latter consisted of an isolated chamber and a coil to which the pressure transductor was connected to register the coronary perfusion pressure. The perfusion solution, in a reservoir at a controlled temperature of 37 °C with pH 7.4, was bubbled with a mixture of 95% O_2 and 5% CO_2 supplied to the heart at a constant flow by a peristaltic pump.

At this point, the experiment was initiated by electrically stimulating the heart with square pulses of 2 milliseconds at a frequency of 4.5 Hz. Two small stainless steel vascular clamps soldered to a thin flexible cable were placed on the right atrium approximately 2 mm apart and used as stimulation electrodes.

Experimental design

The contractile mechanism was explored in two important systems regulating vascular tone, the renin-angiotensin system and the autonomous nervous system. For this purpose, angiotensin II and phenylephrine were applied to the thoracic aorta and the coronary arteries of the perfused hearts from KO and WT mice, and concentration-response curves were constructed. Changes in the endothelium-dependent response were examined by treating these same two tissues with acetylcholine (ACh) and constructing a concentration-response curve for each compound. Finally, to determine whether the resulting modifications were mediated by NO or contractile or relaxant prostanoids, concentration-response curves were constructed for angiotensin and ACh in the presence and absence of L-NAME (an inhibitor of the three isoforms of NOS) and indomethacin.

Drugs

Acethylcholine chloride (SIGMA, A6625, was diluted in water), angiotensin II (SIGMA, A9525, was diluted in water), phenylephrine hydrochloride (SIGMA, P1250000, was diluted in water), indomethacin (SIGMA, 57413, was diluted in ethanol) and L-NAME (SIGMA, N5751, was diluted in water) were used.

Data analysis and statistics

Data represent mean±SEM. Each experimental condition had an “n” value of 6 mice. Comparisons between groups and among treatments were performed using an ANOVA and Bonferroni post-test for individual differences. Differences were considered statistically significant where $P<0.05$.

Results

Angiotensin II applied to the thoracic aorta and the coronary artery

When angiotensin II (10^{-9} to 10^{-6} M) was administered to thoracic aortic rings (Fig. 1), it induced a greater contraction in d-SG-null mutant than WT mice. Interestingly, the response of the coronary artery of the perfused heart was similar (Fig. 2) to that of the thoracic aorta, suggesting that the mechanism of action is similar. Hence, it is likely that the increase in the contractile response observed in KO mice could be due to an alteration in the regulation and relaxant response of endothelium tissue.

On the other hand, the contractile response to phenylephrine showed no significant difference between KO and WT mice, which indicates that the changes in the contractile response in the former species are mediated by the renin-angiotensin system (Fig. 3).

The participation of endothelium-dependent factors

To evaluate whether endothelium-dependent relaxation was affected, and to explore a possible modification of the contractile effect of angiotensin, ACh was used at increasing concentrations (1×10^{-9} to 1×10^{-5} M) to explore a possible alteration of endothelium-independent relaxation (in relation to the contractile effect of angiotensin).

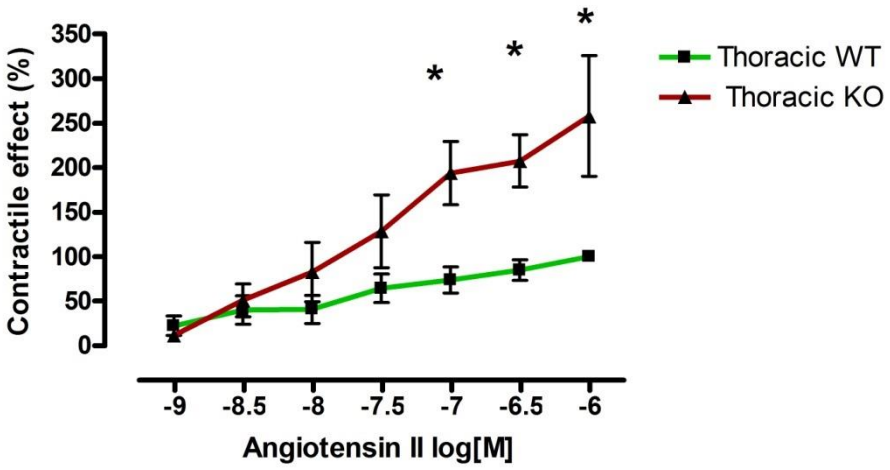

Fig.1. Contractile effect of angiotensin II (1×10^{-9} to 1×10^{-6}M) in thoracic aorta from WT vs KO mice. Each point represent the mean±SEM, n=5, *$P\leq0.05$.

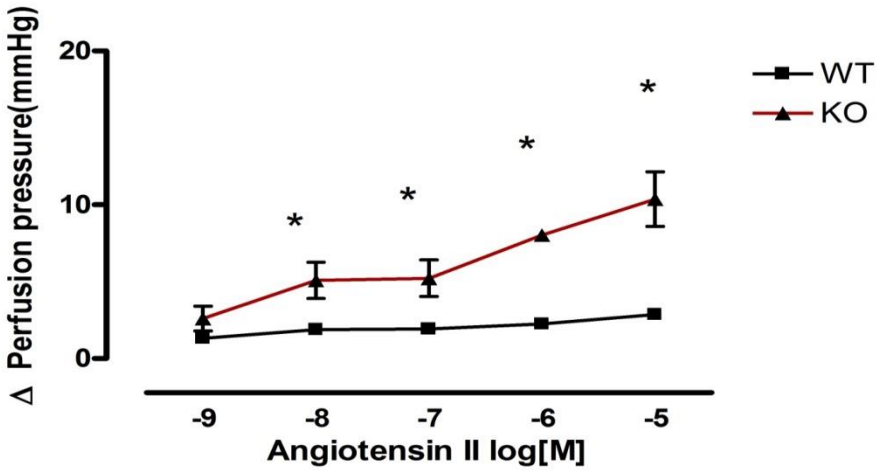

Fig.2. Perfusion pressure of angiotensin II (1×10^{-9} to 1×10^{-6}M) in isolated heart from WT vs KO mice. Each point represent the mean±SEM, n=5, *$P\leq0.05$.

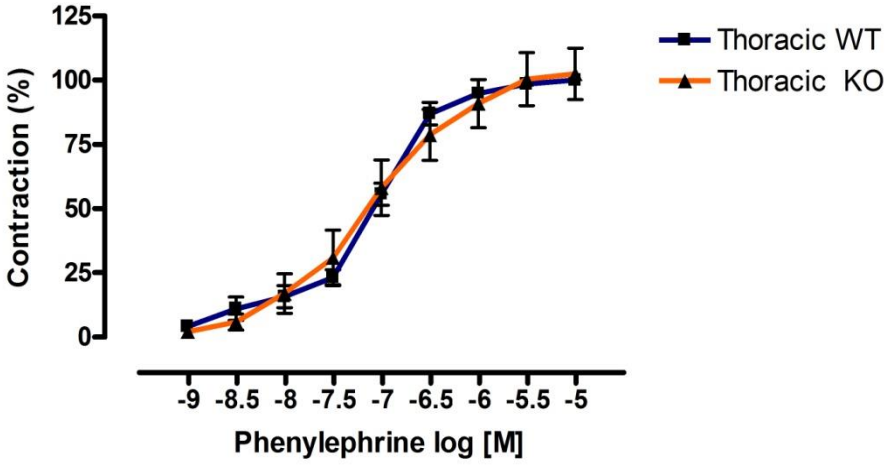

Fig.3. Contractile effect of phenylephrine (1×10^{-9} to 1×10^{-5}M) in thoracic aorta from WT vs KO mice. Each point represent the mean±SEM, n=5.

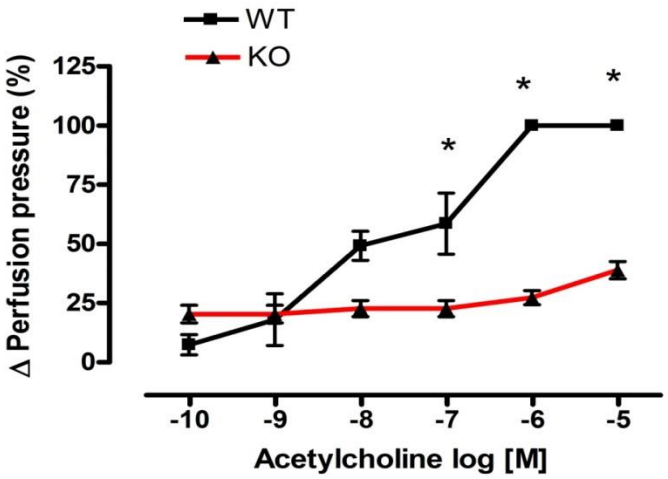

Fig.4. Perfusion pressure of acethylcholine (1×10^{-9} to 1×10^{-5}M) in isolated heart from WT vs KO mice. Each point represent the mean±SEM, n=5, *$P\leq0.05$.

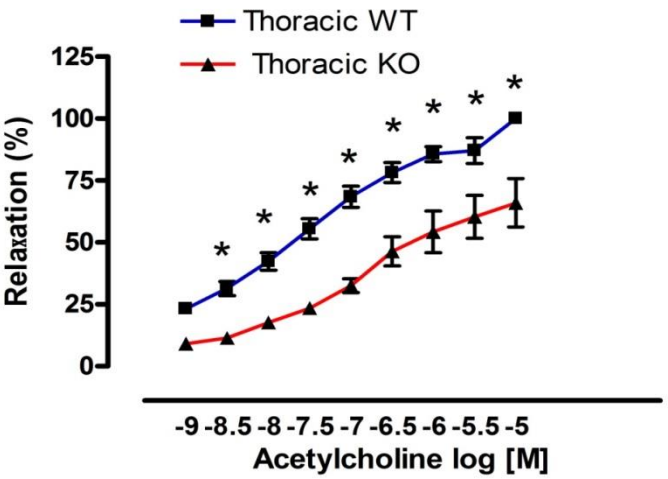

Fig.5. Relaxation effect of acetylcholine (1×10^{-9} to 1×10^{-5}M) in thoracic aorta from WT vs KO mice. Each point represent the mean±SEM, n=5, *$P\leq0.05$.

With ACh (Fig. 4), there was an inhibition of the relaxation of the coronary artery in KO and WT mice. Taking the maximum value of inhibition for WT mice as 100%, the maximum effect for KO mice was 38.8%. The 50% effective dose (ED_{50}) was 1×10^{-5} and 1×10^{-7} of ACh for the KO and WT mice, respectively.

To determine whether the changes in relaxation were similar in other regions, ACh was applied to the thoracic aorta from KO and WT mice. Compared to the maximum value found for WT mice (taken as 100%), the maximum inhibitory effect for the aorta of KO mice was 65%. The ED_{50} of ACh in thoracic aorta was 1×10^{-7} and $1\times10^{-7.5}$ for KO and WT mice, respectively. Therefore, ACh produced a much lower relaxant effect for KO mice in the thoracic aorta than the coronary artery (Fig. 5).

Angiotensin II applied to the thoracic aorta and coronary artery in the presence of L-NAME

To evaluate whether the contractile response of KO mice to angiotensin II was influenced by eNO, concentration-response curves were constructed for the application of angiotensin II in the presence and absence of L-NAME to the thoracic aorta and the coronary arteries of the perfused hearts from KO and WT mice (Fig. 6). When administering angiotensin II (10^{-9} to 10^{-6} M) to thoracic aortic rings from WT mice in the presence of L-NAME (1×10^{-4} M), a slight but statistically insignificant ($P>0.05$) inhibition of the relaxant response was found. This suggests that NO does not alter the endothelial-dependent contractile

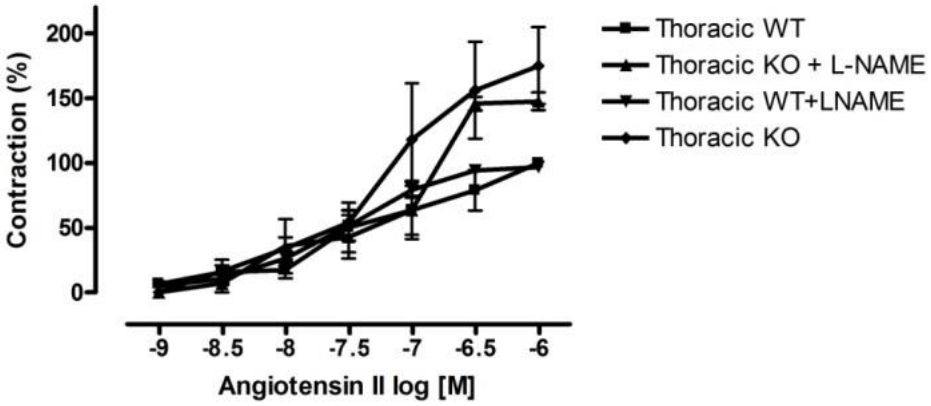

Fig.6. Contractile effect of angiotensin II (1×10^{-9} to 1×10^{-6}M) in thoracic aorta from WT vs KO mice, with vs without LNAME (1×10^{-4}M). Each point represent the mean±SEM, n=5.

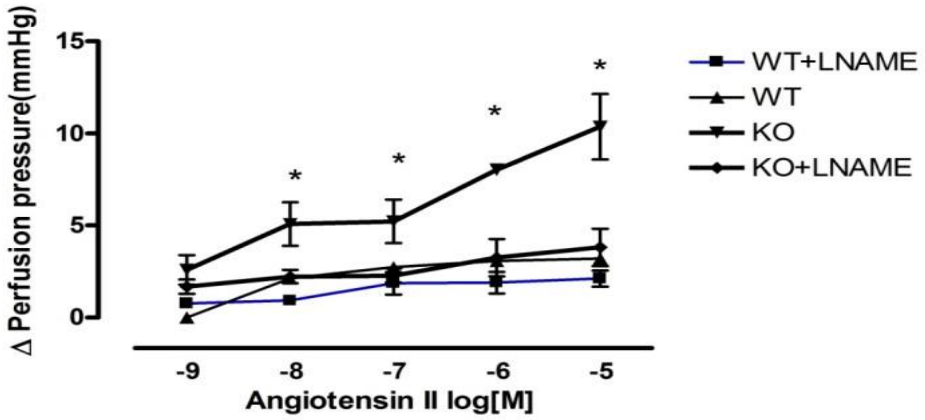

Fig.7. Perfusion pressure of angiotensin II (1×10^{-9} to 1×10^{-6}M) in thoracic aorta from WT vs KO mice, with and without LNAME (1×10^{-4}M). Each point represent the mean±SEM, n=5, *$P\leq0.05$.

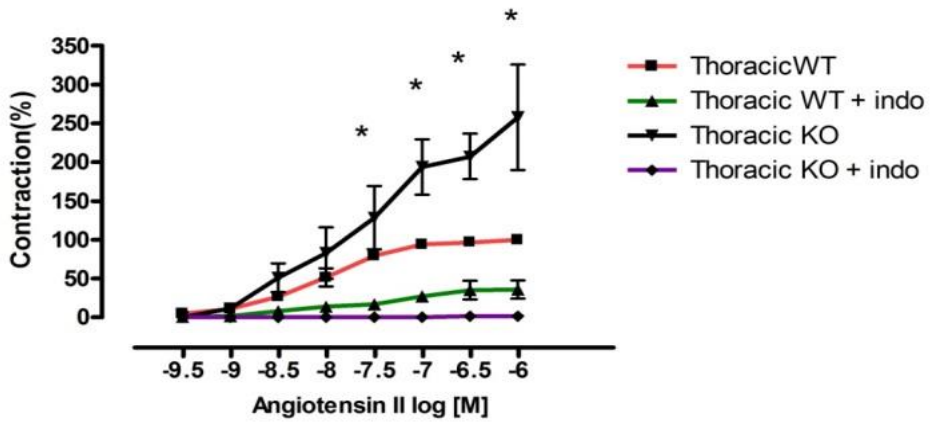

Fig.8. Contractile effect of angiotensin II (1×10^{-9} to 1×10^{-6}M) in thoracic aorta from WT vs KO mice, with and without indomethacin (1×10^{-5}M). Each point represent the mean±SEM, n=5, *$P\leq0.05$.

response of KO mice to angiotensin. Similar results were displayed with the coronary artery of the perfused heart (Fig. 7).

Angiotensin II applied to thoracic aorta and coronary artery in the presence of indomethacin

Another important aspect to analyze is the influence of prostanoids on the contractile effect induced by angiotensin II in thoracic aortae and coronary arteries of the perfused hearts. Accordingly, concentration-

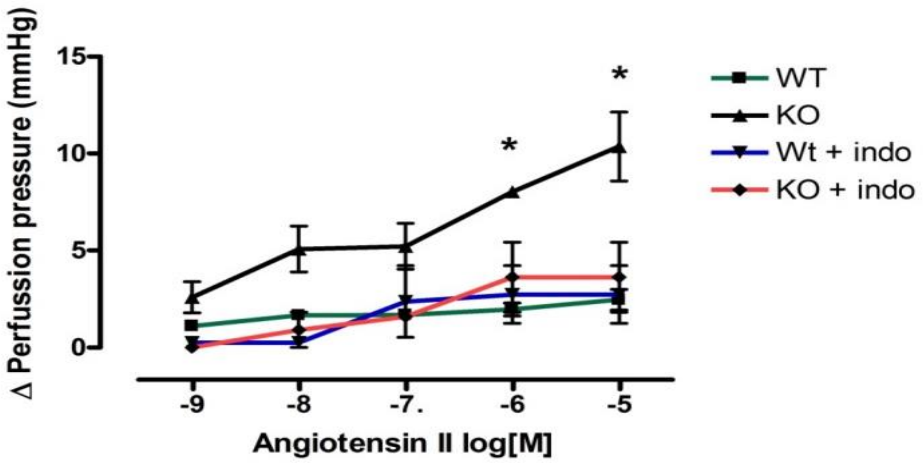

Fig.9. Perfusion pressure of angiotensin II (1×10^{-9} to 1×10^{-6}M) in thoracic aorta from WT and KO mice, with and without indomethacin (1×10^{-5}M). Each point represent the mean±SEM, n=5, *$P\leq0.05$.

response curves were constructed for angiotensin II (10^{-9} to 10^{-6} M) in the presence and absence of indomethacin (10^{-5} M) applied to thoracic aorta and coronary arteries from KO and WT mice (Figs. 8 and 9). An almost complete inhibition of the contractile effect of angiotensin II was detected in KO mice, but only a partial inhibition in WT mice, in both cases showing statistical significance. Hence, there seems to have been a greater participation of prostanoids in the contractile response of KO than WT mice.

Discussion

The disruption of the dystrophin glycoprotein complex leads cardiomyophaties (Milani-Nejad et al., 2016), involving vascular disfunction according to diverse studies. This has been demonstrated by various types of coronary vasoconstriction apparently caused by the loss of the SG-SSPN complex in smooth muscle tissue of the coronary artery (Durbeej et al., 2000). It has been reported that modifications in vascular function as well as persistent ischemic events have a significant role in the pathogenesis of cardiomyophaty in d-SG-null mutant mice (Durbeej et al, 2000; Cohn et al, 2001). On the other hand, there are no reports on variations in the response of vascular smooth muscle in the thoracic aorta or coronary artery from the perfused heart.

Due to the antecedents that exist in relation to structural changes in vascular smooth muscle during cardiomyophaty, we consider it important to analyze variations in the vascular response of the thoracic aorta and coronary artery. Initially, we explored the contractile response to phenylephrine of thoracic aorta and coronary arteries from KO and WT mice (data not shown), observing no significant differences. Therefore, we decided to compare the contractile response of thoracic aorta and the coronary arteries from KO and WT mice after the application of angiotensin II. Chadwick et al. (2017) describe a disregulation in the angiotensin-aldosterone system in dystrophic skeletal muscles, also they do not describe the alterations in the cardiovascular system, being one of the reasons that this study was made. Sabharwal et al. (2015) reports that the angiotensin dysregulation in dsg-/- mice which has been treated with AT1R blocker prevented ventricular abnormalities.

It has been reported that inhibitors of the converting enzyme or selective inhibitors of angiotensin were administered to patients with cardiomyophaty as a therapeutic aid. However, the resulting changes in the vascular response of these patients is unknown (Sandona and Betto, 2009). Interestingly, a higher angiotensin II-induced contractile response was detected in KO than WT mice, which is important when considering that we found no significant difference in the current study with phenylephrine. This increased contractile response in KO mice could owe itself to an alteration in the regulation of vascular tone with a minor participation of NO and/or prostacyclins. We know that the endothelium participates in the regulation of vascular tone by synthesizing and releasing vasodilator substances such as NO, whose effect modulates angiotensin II and other vasoconstrictors.

According to Dikalov and Nazarewicz, angiotensin II seems to regulate eNOS in the AT1 receptor pathway, causing a low expression. It is not known if the inhibitory modulation of the contractile agent owes itself to the basal release of NO or to the stimulation of endothelial cells by angiotensin II (Dikalov and Nazarewicz, 2013). There are receptors for angiotensin II in the endothelium and their activation leads to the release of vasoactive factors that could mediates the effect of angiotensin II on smooth cells and regulate fibrinilysis (Pueyo and Michel, 1997).

To explore a possible modification in the endothelial-dependent relaxant mechanisms, concentration-response curves were constructed for ACh, finding that the endothelial-dependent relaxant response was lower in KO than WT mice. Hence, the alteration of the contractile response of KO mice was probably related to changes in the relaxant mechanisms of smooth muscle forming part of the coronary artery and thoracic aorta.

On the other hand, Heydemann A et al. (2004) reported elevated levels of eNOS in the heart of mice with a disruption in g-SG and d-SG. Based on an immunohistochemical analysis, this increase was found to be significantly greater in the focal point of damage to cardiomyocytes. Additionally, a higher concentration of NO was observed in regions of damaged tissue and the permeability of the membrane was affected. In relation to these factors, to date there are still no studies published, to our knowledge, about changes in the vascular response of blood vessels. We consider that eNOS forms a complex with g- and d-SG in normal hearts.

Regarding the increase in NO described by some researchers, we considered it important to examine whether NO participated in the greater vascular contractile response herein exhibited in KO mice. Therefore, a concentration-response curve was constructed for the response of WT mice to angiotensin II when pretreated with L-NAME. Based on this data, it could be appreciated if pretreatment with a NOS inhibitor caused changes in the contractile response induced by angiotensin II in KO and WT mice (Cohn et al., 2001; Contra et al., 2008; Dikalov and Nazarewicz, 2013; Durbeej et al., 2000).

When comparing the presence and absence of L-NAME pretreatment of WT mice, there was no significant difference in the vascular contractile response produced by angiotensin II. Hence, in these mice the contractile response to angiotensin II in thoracic aorta is not regulated by endothelial NO. However, the contractile response to angiotensin II was indeed altered in KO mice when compared to WT mice.

With the aforementioned results, it can be appreciated that an alteration was found in KO mice (compared to WT mice) in regard to the contractile response to angiotensin II and the relaxant response to ACh. Furthermore, the contractile response to angiotensin II in the thoracic aorta of WT mice does not seem to be regulated by the release of NO in the endothelium. The remaining issue in the present study is in relation to the possible participation of contractile or relaxant prostanoids in the modification of the contractile response to angiotensin II in KO mice. Thus, concentration-response curves were constructed for angiotensin II in the presence and absence of indomethacin (an inhibitor non-selective for COX). An almost complete inhibition of the contractile response to angiotensin II was shown in KO mice, but only a small (but significant) partial inhibition in WT mice, which seems to indicate the greater participation of a contractile prostanoid in KO than WT mice (Cohn et al, 2001; Dikalov and Nazarewicz, 2013).

In previous research by our group, we found the participation of contractile prostanoids in vascular smooth muscle cells during the contractile response to angiotensin II in thoracic aorta. This contractile action of prostanoids is inhibited by eNO (Castillo-Hernandez et al., 2010).

Conclusion

This experimental model of muscular dystrophy shows an increment in the contractile response to angiotensin II in the aorta and the isolated heart from the KO mice that it could be to a major participation of prostanoids. The increment in the participation of prostanoids affects the contractile and relaxant vascular responses and with the time developing cardiovascular pathologies.

Acknowledgments

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Conflict of interest

The authors have no conflicts of interest and declare that this article has not been published nor is being considered for publication elsewhere.

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