Brazilian Journal of Medical and Biological Research (2014) 47(9): 789-798, http://dx.doi.org/10.1590/1414-431X20143073
ISSN 1414-431X

Inhibition of PKC-dependent extracellular Ca^{2+} entry contributes to the depression of contractile activity in long-term pressure-overloaded endothelium-denuded rat aortas

J. Padilla, R.M. López, P. López, M.C. Castillo, E. Querejeta, A. Ruiz and E.F. Castillo

Sección de Estudios de Posgrado e Investigación, Escuela Superior de Medicina, Instituto Politécnico Nacional, México, DF, México

Abstract

We examined the contractile responsiveness of rat thoracic aortas under pressure overload after long-term suprarenal abdominal aortic coarctation (lt-Srac). Endothelium-dependent angiotensin II (ANG II) type 2 receptor (AT_2R)-mediated depression of contractions to ANG II has been reported in short-term (1 week) pressure-overloaded rat aortas. Contractility was evaluated in the aortic rings of rats subjected to lt-Srac or sham surgery (Sham) for 8 weeks. ANG I and II levels and AT_2R protein expression in the aortas of lt-Srac and Sham rats were also evaluated. lt-Srac attenuated the contractions of ANG II and phenylephrine in the aortas in an endothelium-independent manner. However, lt-Srac did not influence the transient contractions induced in endothelium-denuded aortic rings by ANG II, phenylephrine, or caffeine in Ca^{2+}-free medium or the subsequent tonic constrictions induced by the addition of Ca^{2+} in the absence of agonists. Thus, the contractions induced by Ca^{2+} release from intracellular stores and Ca^{2+} influx through stored-operated channels were not inhibited in the aortas of lt-Srac rats. Potassium-elicited contractions in endothelium-denuded aortic rings of lt-Srac rats remained unaltered compared with control tissues. Consequently, the contractile depression observed in aortic tissues of lt-Srac rats cannot be explained by direct inhibition of voltage-operated Ca^{2+} channels. Interestingly, 12-*O*-tetradecanoylphorbol-13-acetate-induced contractions in endothelium-denuded aortic rings of lt-Srac rats were depressed in the presence but not in the absence of extracellular Ca^{2+}. Neither levels of angiotensins nor of AT_2R were modified in the aortas after lt-Srac. The results suggest that, in rat thoracic aortas, lt-Srac selectively inhibited protein kinase C-mediated activation of contraction that is dependent on extracellular Ca^{2+} entry.

Key words: Long-term pressure overload; Rat aorta; Protein kinase C

Introduction

Recent reports have indicated that angiotensin II (ANG II) type 2 receptors (AT_2Rs) are upregulated in the thoracic aorta under conditions associated with tissue damage caused by pressure overloading (1-5). These studies have shown that, in rats (1) and mice (2,3) with suprarenal abdominal aortic coarctation and mice with two-kidney one-clip (2K1C) Goldblatt hypertension (4), thoracic aortic tissues concurrently present upregulation of AT_2R mRNA, depression of the contractile responses to ANG II that are mediated by upregulated AT_2R, and an endothelium-dependent increase in the production of cyclic guanosine monophosphate (cGMP). Together, these seminal works (1-4) support the proposition that the AT_2R-dependent functional inhibition of vascular tone increases in pressure-overloaded aortic walls, which aim to restore basal levels of tensile stress. Remarkably, in both models of hypertension (and both species), upregulation of AT_2R mRNA in thoracic aortas was completely inhibited by the administration of AT_1R antagonists; thus, increased AT_2R mRNA expression was related to the previous activation of AT_1R by endogenous ANG II (1-4). In contrast, the temporal course of AT_2R upregulation in aortas of mice and rats was different.

In mice with suprarenal abdominal aortic coarctation, the plasma renin concentration increased coincidentally with aortic AT_2R mRNA upregulation and contractile

Correspondence: E.F. Castillo, Sección de Estudios de Posgrado e Investigación, Escuela Superior de Medicina, Instituto Politécnico Nacional, Plan de San Luis y Díaz Mirón, s/n, Casco de Santo Tomás, CP 11340, México, DF, México. Fax: +52-5-341-2726. E-mail: ecastilloh@ipn.mx

Received March 4, 2013. Accepted May 22, 2014. First published online August 1, 2014.

depression within 4 days after banding; renin, AT_2R mRNA, and contractility returned to control levels by day 28 (2). These results are equivalent to the reported observations in mice with 2K1C hypertension, but the episodic changes in renin, AT_2R mRNA, and contractility lasted for 6 weeks rather than 4 weeks (4). Thus, plasma renin-ANG II-dependent AT_2R upregulation in these two mouse models of hypertension may be considered a transient adaptive aortic response to increased mechanical stress (5).

In rats, however, the elevation of plasma renin activity was significant 2 days after aortic coarctation and returned to control levels at 7 days; nevertheless, aortic AT_2R mRNA expression significantly increased within 4 days but remained elevated for at least 4 weeks. Therefore, the rapid increase in AT_2R mRNA within 4 days seemed to depend on a transient elevation of plasma renin (1), but the sustained increase in AT_2R mRNA required further explanation.

Yayama et al. (1) proposed that the increase in AT_2R mRNA for 4 weeks is most likely caused by locally generated ANG II in pressure-overloaded rat aortas, but this hypothesis was not tested. Moreover, the contractile activity of the pressure-overloaded rat thoracic aortas was markedly inhibited 7 days after coarctation (1); however, the study did not evaluate whether that decrease continued in parallel with upregulated AT_2R 4 weeks after aortic constriction. Thus, we wondered whether locally generated ANG II in the pressure-overloaded rat aorta may be responsible for AT_2R upregulation and contractile depression on a long-term basis (over a longer period than the previously cited works reported). Nonetheless, considering the potential for blood vessel damage, we made the assumption that pressure overload per se should also elicit additional long-term compensatory responses (independent of AT_2R upregulation) in the rat thoracic aorta to prevent injury. In principle, several possible mechanisms involved in the activation of smooth muscle contractility might be altered by long-term pressure overload.

Evidence has shown that, in smooth muscle of rat aorta, the contractile response elicited by activation of G-protein-coupled phospholipase C-linked receptors is composed of two phases: an initial spike supported primarily by the inositol 1,4,5-trisphosphate (IP_3)-induced release of Ca^{2+} from intracellular stores and a plateau phase sustained by Ca^{2+} influx from the extracellular medium (6-8). This transmembrane Ca^{2+} entry mechanism, responsible for sustained contractile activation, is considered to be mediated via either voltage-dependent Ca^{2+} channels (VDCC) or via receptor-operated Ca^{2+} channels (6-9). In addition, the depleted Ca^{2+} stores sensitive to G-protein-coupled receptor (GPCR) agonists (e.g., noradrenaline) are the signal for entry of extracellular Ca^{2+} to refill the stores according to the store-operated Ca^{2+} entry model and to activate contractile proteins (6-11). Among other mechanisms, protein kinase C (PKC) plays an important role in regulating aortic contractility (12,13). Under physiological conditions, PKC activity is increased by agonist binding to GPCR (12). PKC activity contributes to vasomotor tone by enhancing the sensitivity to intracellular Ca^{2+} of the arterial smooth muscle contractile apparatus, and by depolarizing the sarcolemma to activate Ca^{2+} influx via VDCC (12-14).

The present study was conducted to investigate the contractile responsiveness of rat thoracic aortas under pressure-overload conditions after long-term (8 weeks) suprarenal abdominal aortic coarctation (lt-Srac). Various mechanisms involved in the activation of smooth muscle contractility were investigated. Additionally, ANG I and II levels and AT_2R protein expression regulation in long-term pressure-overloaded rat thoracic aortas were also investigated using radioimmunoassay and Western blot analysis, respectively. Considering the technical difficulty of reliably measuring the local production of ANG I and II, we decided to determine the concentrations of both peptides in pressure-overloaded aortas as a first approach, so that we could use those values to indicate either increased production or accumulation.

Material and Methods

All experimental procedures were approved by the Animal Care and Use Committee of our institution and complied with the guidelines of the National Health and Medical Research Council of Mexico. The animals were maintained on a 12:12-h light-dark cycle in a special room at a constant temperature ($22 \pm 2^{\circ}C$) with food and water freely available in their home cages.

Surgical procedures

Groups of male Wistar rats (weighing 80-100 g, 4-6 weeks of age) were subjected to either lt-Srac or sham surgery (Sham). To determine whether the surgical procedure produced a hemodynamic (or contractile) change, age-matched untreated (Unt) rats were also used as controls in selective experiments. The rats were anesthetized with pentobarbital sodium (60 mg/kg, *ip*), allowed to breathe room air spontaneously, and placed on heated pads to maintain a temperature of $37^{\circ}C$, as measured with a rectal probe. Later, the animals were subjected to midline laparotomy. Suprarenal aortic coarctation was produced by tying a silk ligature around a blunted needle (external diameter 0.7 mm) and the abdominal aorta (between the superior mesenteric artery and right renal artery) and then removing the needle. The Sham rats underwent the same surgical procedure, except for the placement of the ligature. At the end of 8 weeks, the animals from the experimental groups were used for all or some of the following experiments.

Blood pressure measurements

The animals were anesthetized and prepared as for

the surgical procedures. The right carotid arteries were catheterized with PE-50 tubing. To measure systemic pressure, the carotid cannulae were filled with heparinized saline (50 U/mL) and connected to pressure transducers (TSD 104, Biopac Systems Inc., USA). After a stable hemodynamic condition had been observed for 20 min, mean arterial pressure (MAP) values were recorded on a computer with the AcqKnowledge software (MP100WSW, Biopac Systems, Inc.). Heart rate (beats/min) was computed from the carotid blood pressure signal.

Tension measurements

After the blood pressure was measured, the anesthetized rats were exsanguinated. The thoracic aortas were removed, cleaned of fat and connective tissue, and cut into ring segments (4-5 mm in length). In some preparations, the endothelium was damaged by gently abrading the intimae of the aortic rings with the tip of small forceps. The isolated arteries were placed in 10-mL tissue chambers filled with a Krebs-bicarbonate solution (KBS) with the following composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11.7 mM dextrose. The Ca^{2+}-free solution had the same composition, except that $CaCl_2$ was omitted and 0.5 mM EGTA was added. High K^+ (40 and 80 mM) depolarizing solutions were prepared with the equimolar replacement of NaCl with KCl. The medium was maintained at 37°C and pH 7.4 and gassed continuously with 95% O_2 and 5% CO_2. Each tissue sample was placed under an initial resting tension of a 2-g weight and equilibrated for 60 min prior to the execution of the experimental protocols. Contractions were measured isometrically and recorded on a computer with the AcqKnowledge software (MP100WSW, Biopac Systems, Inc.). The tissues were primed by the addition of 1 μM phenylephrine (PHE) to the organ bath. Functional endothelium was determined by the presence of at least 80% relaxation in response to acetylcholine (1 μM) after the tissues were preconstricted with PHE (1 μM). Otherwise, successful endothelial denudation was confirmed by the presence of small (less than 10%) relaxations or the complete absence of relaxations in response to acetylcholine.

Aortic responsiveness

In this series of experiments, contractile activity was elicited in thoracic aortic tissues using four different stimuli. First, in the endothelium-intact and endothelium-denuded thoracic aortic rings, cumulative concentration-response curves were constructed by exposing the tissues to increasing concentrations of ANG II until maximum responses were observed. To examine the effects of AT_2R antagonism and nitric oxide synthase (NOS) inhibition, endothelium-intact aortic rings were incubated for 30 min with PD123319 (1 μM) or N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM), respectively, before cumulative exposure to ANG II. Because these studies indicated that the observed effect of suprarenal aortic coarctation on ANG II-induced contractions in aortic rings was endothelium independent (see Results), the following experiments were developed in aortic tissues without endothelium. Second, cumulative concentration-response curves to the G-protein-coupled α_1-adrenergic receptor (α_1-AR) agonist PHE were obtained in endothelium-denuded aortic rings. Third, high K^+ depolarizing solutions were used to activate Ca^{2+} entry from the extracellular space. Contractile responses to K^+ (40 or 80 mM) in endothelium-denuded aortic rings were recorded for 30 min. Fourth, the PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was used to activate contractile responses in the absence and presence of extracellular Ca^{2+}. After the stabilization period, the endothelium-denuded aortic rings were washed three times in either Ca^{2+}-free medium or standard KBS for 30 min. TPA (1 μM) was then added to the Ca^{2+}-free solution and KBS. TPA induced slow-developing and sustained contractile responses. Each contractile substance studied was tested in separated aortic rings.

Transient and tonic contractile responses

In endothelium-denuded thoracic aortic rings, either ANG II or PHE was used to stimulate Ca^{2+} release from intracellular stores and Ca^{2+} entry from the extracellular space (6-8). Following the equilibration period, PHE (1 μM) was administered to contract the endothelium-denuded aortic rings in KBS; after the tissues were washed and their basal tones were restored, the tissues were treated with a Ca^{2+}-free solution for 15 min. Then 0.1 μM ANG II or 1 μM PHE was applied, and a transient (phasic) contraction was elicited (this transient response depends on intracellular stored Ca^{2+} sensitive to ANG II or PHE). The agonist being tested was then washed and applied and washed three more times to deplete internal Ca^{2+} stores. Subsequently, the tissues were incubated for 60 min in KBS to refill the intracellular Ca^{2+} stores (in the absence of an agonist), and a spontaneous increase in the resting tone (tonic contraction) was observed. The increases in contraction induced by Ca^{2+} restoration were measured as a function of time.

In endothelium-denuded aortic rings, caffeine was used to activate Ca^{2+}-induced Ca^{2+} release in Ca^{2+}-free solution (8). After the equilibration period, 1 μM PHE was added to contract the endothelium-free aortic rings in KBS. After the tissues were washed and their resting tone was reestablished, the tissues were immersed in Ca^{2+}-free solution for 15 min at 25°C (8). Afterward, 10 mM caffeine was administered, and a transient contraction was obtained.

To investigate the effect of thapsigargin, a selective sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor (8,11), on PHE-induced phasic

responses in Ca^{2+}-free medium, experiments similar to those described above for endothelium-denuded aortic tissues were performed. First, a reference contractile response was induced by adding 1 μM PHE in KBS. After the aortic rings were washed, they were exposed to Ca^{2+}-free medium for 15 min, followed by 1 μM thapsigargin or vehicle administration. Ten minutes later, 1 μM PHE-induced transient contractions were elicited in Ca^{2+}-free solution.

ANG I and ANG II measurements

After blood pressure was measured in the anesthetized rats, the thoracic aortas were quickly dissected, removed, cut into small pieces, transferred into liquid nitrogen, and stored at –80°C until analysis. ANG I and II were measured in the aortic tissues using Sep-Pak C_{18} cartridge (Waters Corporation, USA) extraction, reversed-phase high-performance liquid chromatography (HPLC) separation, and radioimmunoassay (15). Briefly, the frozen tissues were homogenized with a Polytron (Kinematica, AG, Switzerland) in 4 mL of ice-cold 0.1 M HCl-80% ethanol. The homogenate was centrifuged at 20,000 *g* for 10 min at 4°C, the ethanol in the supernatant was evaporated under constant air flow, and the remainder of the supernatant was diluted in 8 mL of 1% orthophosphoric acid and concentrated on Sep-Pak C_{18} cartridges. The Sep-Pak extracts were dissolved in 100 μL of HPLC elution buffer and injected into the HPLC column. The concentrations of ANG I and II in the HPLC eluate fractions were quantified by radioimmunoassay with the anti-C-terminal of ANG I and II antisera (Santa Cruz Biotechnology, USA), respectively.

A known amount of ^{125}I-labeled ANG I was added to the tissues as an internal standard before homogenization. ^{125}I-labeled ANG I recovery after HPLC separation was used to correct for losses (recovery was better than 70%) that occurred during extraction and separation, and concentrations of ^{125}I-labeled ANG I in the HPLC fractions were measured using a gamma counter.

Western blot analysis

Western blotting was performed as described previously (16). Tissue samples were prepared from a collection of 12 aortas per group. The thoracic aortas were immersed in liquid nitrogen and stored at –80°C until analysis. The frozen tissues were thawed, minced into small pieces, and homogenized with a Polytron (Kinematica, AG) in Tris-HCl, pH 7.4, with a protease cocktail (cOmplete, Roche, Germany). The homogenate was centrifuged at 900 *g* for 10 min at 4°C, and the supernatant was used for analysis. The concentration was determined using the Lowry method. The solubilized samples were subjected to SDS-PAGE (10% polyacrylamide gel). To compare AT_2R protein expression levels of the pressure-overloaded and control aortas, exactly 50 μg of protein was loaded per well. After electrophoresis, the proteins were electrotransferred onto a polyvinylidene fluoride membrane (Hybond-P PVDF, Amersham Biosciences, USA) at 15 V for 45 min (Transblot SD, Bio-Rad Laboratories, Inc., USA). The membrane was soaked in Tris-buffered saline (TBS: 10 mM Tris-HCl, 150 mM NaCl) containing 5% nonfat dry milk and 0.1% polyoxyethylene-sorbitan monolaurate (Tween 20) for 2 h at room temperature and then incubated with the AT_2R receptor antiserum (1:500 dilution in TBS with 5% nonfat dry milk and 0.1% Tween 20; Santa Cruz Biotechnology) overnight at 4°C. The membrane was then washed and reacted with a peroxidase-conjugated donkey anti-rabbit secondary antibody (1:10,000 dilution) for 1 h at room temperature (Zymed Laboratories, Inc., USA). Immunoreactivity was visualized with an enhanced chemiluminescence Western blotting detection luminol reagent (Santa Cruz Biotechnology). The blots were stripped and re-proved with a β-actin polyclonal antibody as a control. Images were digitally acquired from films, and a densitometric analysis was performed using the Quantity One Image Acquisition and Analysis Software (Bio-Rad Laboratories, Inc.). Data are reported as normalized absorbance.

Drugs

The following drugs were used: ANG II, L-NAME, PD123319, l-phenylephrine hydrochloride, acetylcholine chloride, anhydrous caffeine, thapsigargin, and TPA (Sigma Chemical Company, USA). The drugs were dissolved in distilled water or dimethyl sulfoxide, and subsequent dilutions were made using assay buffer.

Data analysis

Data are reported as means ± SE for the number of aortic rings (n) or whole thoracic aortas obtained from 4-12 different animals. Comparisons between two independent groups were made using an unpaired Student *t*-test and between multiple groups using one-way analysis of variance (ANOVA). Two-way ANOVA was used to compare two or more concentration-response curves. Where one-way or two-way ANOVA showed significant differences, the results were analyzed further using the Tukey or the Bonferroni *post hoc* test, respectively (Prism version 4.0, Graph Pad Software, USA). In all comparisons, a value of $P<0.05$ was considered to be statistically significant.

Results

Increases in blood pressure

In the carotid arteries of lt-Srac anesthetized rats, significant increases in MAP (138 ± 2 mmHg) were observed compared with the corresponding values measured in Sham (108 ± 3 mmHg; $P<0.05$) and Unt rats (103 ± 5 mmHg; $P<0.05$). However, when the MAP of Sham and Unt anesthetized rats were compared, no

significant differences were found. In addition, there were no differences in heart rate between lt-Srac (414 ± 7 beats/min) Sham (408 ± 8 beats/min), and Unt anesthetized rats (401 ± 13 beats/min). The study was performed with 76 lt-Srac, 76 Sham, and 8 Unt rats.

Depression of ANG II-induced contractions

After long-term suprarenal aortic coarctation, ANG II-induced concentration-dependent contractions in the aortic rings with and without endothelium were significantly depressed compared to the aortic tissues of Sham and Unt rats (Figure 1).

Because the MAP values and contractile responses to ANG II measured in the aortic rings of the Unt and Sham rats were similar, only the Sham rats were used as controls.

Endothelium, nitric oxide (NO), AT_2R, and ANG II

To study the involvement of the endothelium and endothelium-derived NO in the decreased contractile responses of the thoracic aortic rings to ANG II after long-term coarctation, experiments were conducted using either endothelium-denuded tissues (as previously mentioned) or endothelium-intact aortic rings following incubation with L-NAME (100 μM). Figure 2A and B shows that the concentration-response curves to ANG II were left-shifted, and the maximal responses were enhanced by endothelium removal or L-NAME treatment in pressure-overloaded and control tissues, respectively. However, the contractile responses to ANG II in endothelium-denuded and L-NAME-treated pressure-overloaded aortic rings remained significantly depressed compared to control tissues (Figure 2C). On the other hand, to establish the participation of AT_2R in the depressed response to ANG II of the long-term pressure-overloaded aortic rings, experiments were conducted in endothelium-intact aortic rings following incubation with 1 μM PD123319. This selective AT_2R antagonist did not modify the contractile responses to ANG II in the endothelium-intact aortic rings of lt-Srac (Figure 2A) and Sham rats (Figure 2B). In another series of experiments, PD123319 did not influence the response to ANG II in the endothelium-denuded aortic rings of either lt-Srac or Sham rats (data not shown).

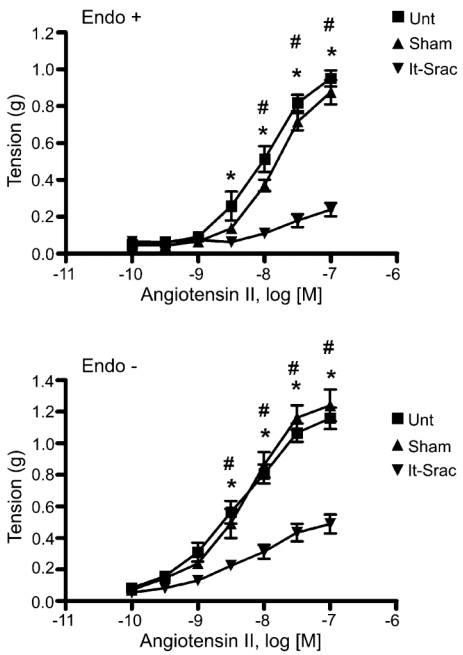

Figure 1. Contractile responses to angiotensin II in rat thoracic aortas under pressure overload. Cumulative concentration-response curves to angiotensin II were developed in endothelium-intact (Endo+) and endothelium-denuded (Endo-) thoracic aortic segments obtained from rats 8 weeks after suprarenal abdominal aortic coarctation (lt-Srac) or sham surgery (Sham). Age-matched untreated rats (Unt) were also used as controls. Data are reported as means ± SE in grams of developed force (n = 16). *P<0.05, Unt *vs* lt-Srac; #P<0.05, Sham *vs* lt-Srac (two-way ANOVA with the Bonferroni *post hoc* test).

Depression of PHE-induced contractions

To investigate the selectivity of the depression of contractile responses to ANG II in thoracic aortic rings obtained from lt-Srac rats, we conducted experiments using PHE and high K^+-depolarizing solutions. In the endothelium-denuded aortic rings, the concentration-response curves to PHE were depressed after long-term aortic coarctation compared with control tissues (Figure 3A). In contrast, high K^+ (40 and 80 mM) contractile responses in endothelium-denuded aortic preparations of lt-Srac rats remained unaltered compared with the corresponding responses in the control rings (Figure 3B).

Unchanged transient and tonic contractions

In this series of experiments, we investigated whether observed differences in contractile activity between aortic preparations from lt-Srac and Sham rats reflected changes in Ca^{2+} release from intracellular stores or Ca^{2+} entry through the plasma membrane elicited by previous activation of GPCR, but in the absence of an agonist. In Ca^{2+}-free solution, 0.1 μM ANG II caused transient contractile responses in endothelium-denuded aortic rings of lt-Srac and Sham rats; the responses did not differ significantly between the two groups (Figure 4A). Likewise, in Ca^{2+}-free media, 1 μM PHE-induced transient contractile responses in endothelium-denuded aortic tissues of lt-Srac rats were similar to those evoked in control aortic rings (Figure 4C). Also, the tonic contractions activated by adding Ca^{2+} to ANG II- or PHE-prechallenged endothelium-denuded aortic rings of lt-Srac rats did not differ from similarly evoked contractions in the aortic rings of the Sham rats (Figure 4B and D).

In Ca^{2+}-free KBS, addition of 10 mM caffeine induced transient contractions in the endothelium-denuded aortic rings of lt-Srac and Sham rats. Phasic contractions

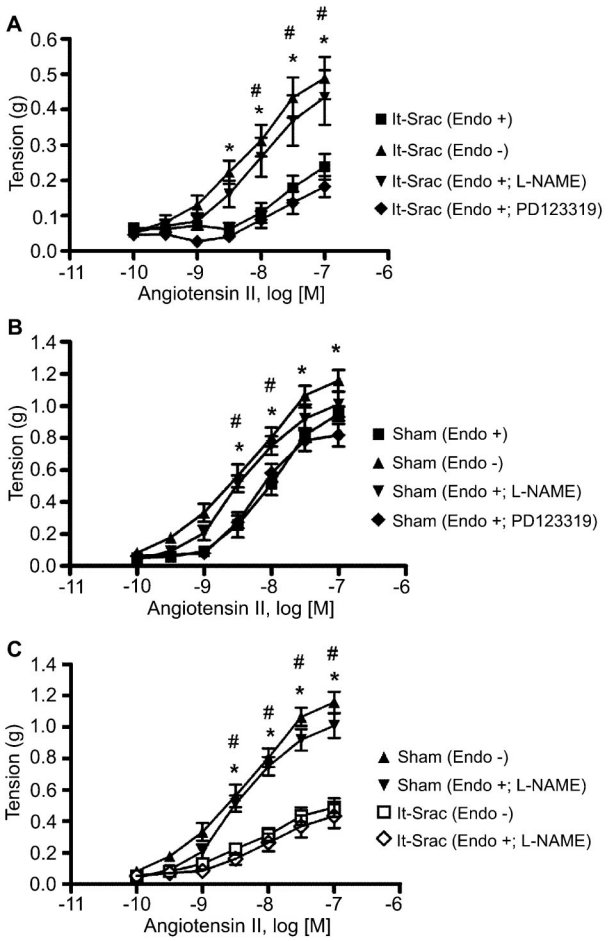

Figure 2. Influences on the contractile responses to angiotensin II in rat thoracic aortas under pressure overload. Concentration-response curves for angiotensin II were elicited in the aortic rings under the presence (Endo+) or absence (Endo-) of endothelium, the presence of endothelium and 100 μM N^G-nitro-L-arginine methyl ester (Endo+; L-NAME), and the presence of endothelium and 1 μM PD123319 (Endo+; PD123319). The thoracic aortas were dissected from rats 8 weeks after suprarenal abdominal aortic coarctation (lt-Srac) and sham surgery (Sham). *A*, Comparison of the cumulative concentration-response curves to angiotensin II in the aortic rings of lt-Srac rats. *B*, Comparison of the cumulative concentration-response curves to angiotensin II in aortic rings of Sham rats. *C*, Selective comparison of the cumulative angiotensin II concentration-response curves for the aortic rings of lt-Srac and Sham rats. Data are reported means ± SE in grams of developed force (n = 14-16). *A* and *B*, *P<0.05, (Endo+) *vs* (Endo-); #P<0.05, (Endo+) *vs* (Endo+; L-NAME); P not significant, (Endo+) *vs* (Endo+; PD123319). *C*, *P<0.05, Sham (Endo-) *vs* lt-Srac (Endo-); #P<0.05, Sham (Endo+; L-NAME) *vs* lt-Srac (Endo+; L-NAME) (two-way ANOVA with the Bonferroni *post hoc* test).

induced by caffeine in the endothelium-denuded aortic rings were comparable for the lt-Srac (0.16 ± 0.01 g, n = 14) and Sham rats (0.15 ± 0.02 g, n = 15; P>0.5).

In the endothelium-denuded aortic rings of lt-Srac and Sham rats, the transient contractile responses induced by PHE in Ca^{2+}-free KBS were depressed by 1 μM thapsigargin relative to their respective controls in the absence of Ca^{2+}-ATPase inhibitor (Figure 5). In the presence of thapsigargin, however, there was no difference between PHE-induced transient contractile responses in the endothelium-denuded aortic rings of lt-Srac and Sham rats (Figure 5).

Depression of extracellular Ca^{2+}-dependent TPA-induced contractions

TPA was employed to provoke muscle contractions in both Ca^{2+}-free KBS and standard KBS to examine the possibility that long-term suprarenal aortic coarctation could influence aortic rings through an extracellular Ca^{2+}-dependent PKC-mediated mechanism. In the endothelium-denuded aortic rings of Sham rats, the steady-state contractions induced by 1 μM TPA in Ca^{2+}-free medium were significantly smaller than those obtained in standard KBS (Figure 7). Remarkably, contractions evoked by TPA in endothelium-denuded aortic rings of lt-Srac rats were depressed compared with those evoked in the tissues of Sham rats when extracellular Ca^{2+} was present (Figure 6). In the absence of Ca^{2+}, however, the data showed that TPA sensitivity of the aortic rings of lt-Srac and Sham rats was similar (Figure 6). Therefore, in endothelium-denuded aortic rings of lt-Srac rats, there was no difference between contractions evoked by TPA in KBS and Ca^{2+}-free KBS (Figure 6).

ANG I and II levels remained unaltered

One aim of the present study was to investigate the possibility that ANG I and II were present at higher concentrations in the aortas of lt-Srac than Sham rats. However, ANG I and II levels (fmol/g tissue) did not differ significantly (unpaired Student *t*-test) between the aortas of lt-Srac and Sham rats (n = 8 each). The values were as follows: ANG I Sham 9.20 ± 4.12 *vs* ANG I lt-Srac 11.67 ± 5.20; and ANG II Sham 18.18 ± 5.72 *vs* ANG II lt-Srac 19.55 ± 5.82.

Unchanged expression of AT_2R

AT_2R protein expression in the thoracic aortas was compared between lt-Srac and Sham rats. Single bands of approximately 44 kDa were observed in Western blots of the thoracic aortas of both lt-Srac and Sham rats (Figure 7). The approximate molecular mass of AT_2R was consistent with previously reported values (16). Densitometric analysis of the 44-kDa band demonstrated that band intensity was similar between the aortas of lt-Srac and Sham rats (Figure 7). The AT_2R signal for lt-Srac and Sham rat aortas was low (Figure 7).

Discussion

This study was designed to examine the contractile

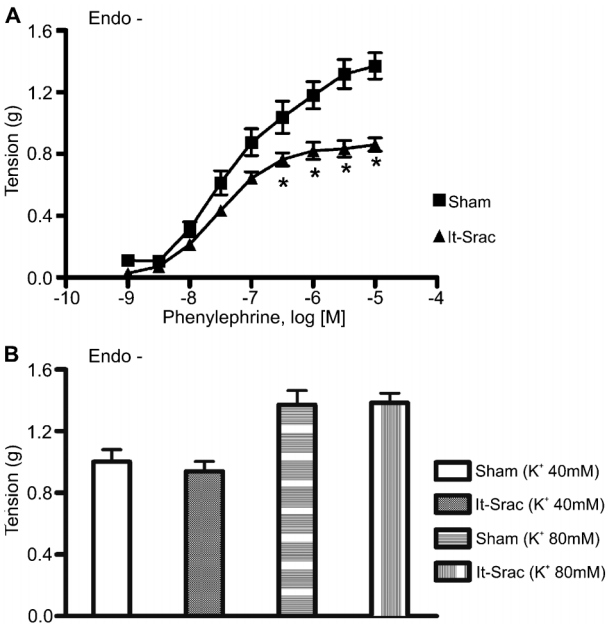

Figure 3. Contractile responses to phenylephrine and high K^+ in pressure-overloaded rat thoracic aortas. Contractile responses to phenylephrine (*A*) and high K^+ (*B*) were constructed in endothelium-denuded (Endo-) aortic rings obtained from rats 8 weeks after suprarenal abdominal aortic coarctation (It-Srac) or sham surgery (Sham). Data are reported as means ± SE in grams of developed force (n = 13-16). *A*, *P<0.05, It-Srac *vs* Sham (two-way ANOVA with the Bonferroni *post hoc* test). *B*, P not significant, Sham (K^+ 40 mM) *vs* It-Srac (K^+ 40 mM), and Sham (K^+ 80 mM) *vs* It-Srac (K^+ 80 mM) (unpaired Student *t*-test).

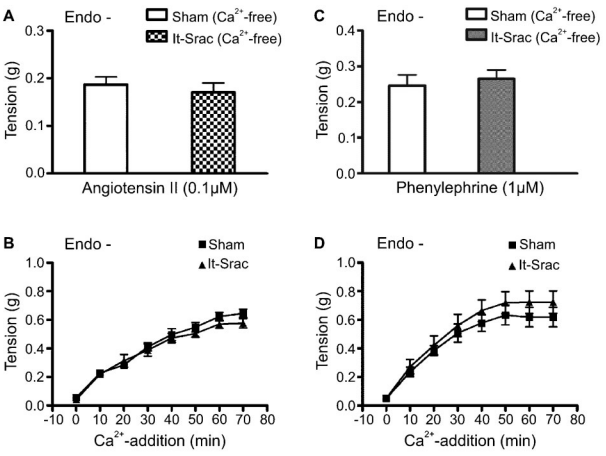

Figure 4. Transient and tonic contractions induced by angiotensin II and phenylephrine in rat thoracic aortas under pressure overload. Transient contractile responses to angiotensin II (*A*) and phenylephrine (*C*) were elicited in Ca^{2+}-free medium, and the subsequent tonic contractions were evoked by the addition of normal Ca^{2+} in the absence of angiotensin II (*B*) or phenylephrine (*D*) in endothelium-denuded aortic rings prepared from rats 8 weeks after suprarenal abdominal aortic coarctation (It-Srac) or sham surgery (Sham). The increase in contractile tone induced by Ca^{2+} restoration is represented as a function of time. Data are reported as means ± SE in grams of developed force. (n = 14-16). *A* and *C*, P not significant (unpaired Student *t*-test). *B* and *D*, P not significant (two-way ANOVA).

responsiveness of rat thoracic aortas under pressure overload after long-term (8 weeks) suprarenal abdominal aortic coarctation. In particular, we examined whether AT_2R-mediated contractile depression of rat aortas under long-term pressure overload could be part of a compensatory response to avoid mechanical stress. Endothelium-dependent depression of contractile responses to ANG II mediated by AT_2R mRNA upregulation has already been reported in pressure-overloaded thoracic aortas of rats (1) and mice (2-4). In endothelium-intact aortic rings from rats subjected to short-term abdominal aortic coarctation, the depressed contractile responses to ANG II were restored to control values with either 1 μM of the AT_2R antagonist PD123319, endothelium denudation, or NOS inhibition by 100 μM L-NAME (1). Our results show that long-term suprarenal aortic coarctation decreased contractile responses to ANG II in endothelium-intact rings of rat thoracic aortas. However, 1 μM PD123319 did not alter the responses to ANG II in these endothelium-intact tissues, and a decreased contractile response to ANG II was also observed in endothelium-denuded rings of long-term pressure-overloaded aortas. Furthermore, AT_2R protein expression was not upregulated in the thoracic aortas of It-Srac rats (compared with Sham rats). Thus, the observed effect of long-term suprarenal aortic coarctation on rat thoracic aortas was found to be endothelium independent and AT_2R independent.

In this study, endothelium denudation and NO synthesis inhibition with 100 μM L-NAME resulted in a left shift of ANG II-related concentration-contraction curves of aortic rings of It-Srac rats; however, the same phenomenon was observed in the aortic tissues of Sham rats. Moreover, although endothelium denudation and L-NAME treatment increased ANG II-induced contractile responses in the aortas of It-Srac rats, the responses remained significantly depressed compared to control tissues. Studies have shown that, in rat aortas, NO plays a fundamental role in endothelium-dependent depression of vasoconstrictor responses (17-20). Hence, the data indicate that the endothelium – principally, the basal or induced release of NO – depressed ANG II-evoked contractile responses evoked in aortic rings of both It-Srac and Sham rats. If depression of contractile responses to ANG II in long-term pressure-overloaded aortic rings was fundamentally endothelium dependent, major endothelial mechanical or chemical disruption would reverse the depressed contractile responses to values comparable to those obtained in control tissues, as has been reported for short-term pressure-overloaded aortas of rats and mice (1-4). Obviously, this was not the case.

Given that the reported upregulation of AT_2R mRNA in

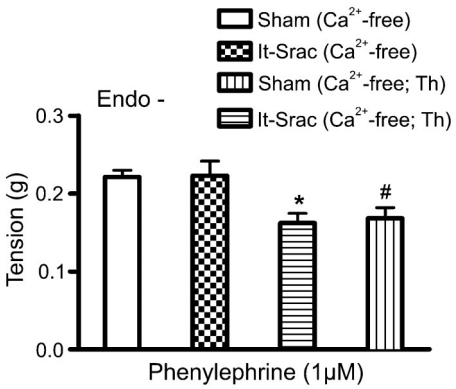

Figure 5. Transient contractile responses to phenylephrine in Ca^{2+}-free medium after treatment with the sarco-/endoplasmic ATPase inhibitor thapsigargin (1 µM for 10 min). Transient contractile responses to phenylephrine (1 µM) were elicited in Ca^{2+}-free medium in the presence or absence of thapsigargin (Th) in endothelium-denuded (Endo-) aortic rings prepared from rats 8 weeks after suprarenal abdominal aortic coarctation (It-Srac) or sham surgery (Sham). Data are reported as means ± SE grams of developed force (n = 13-15). *P<0.05, Sham (Ca^{2+}-free) *vs* Sham (Ca^{2+}-free; Th); #P<0.5, It-Srac (Ca^{2+}-free) *vs* It-Srac (Ca^{2+}-free; Th); P not significant, Sham (Ca^{2+}-free) *vs* It-Srac (Ca^{2+}-free), and Sham (Ca^{2+}-free; Th) *vs* It-Srac (Ca^{2+}-free; Th) (one-way ANOVA with the Tukey *post hoc* test).

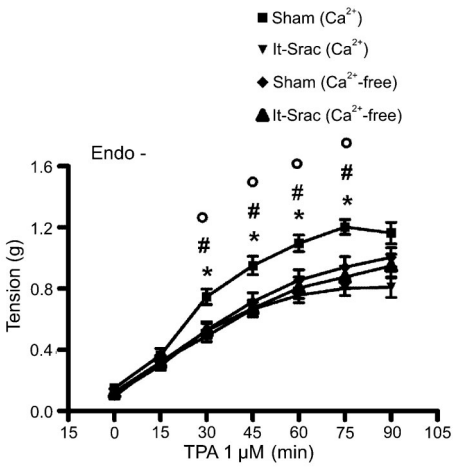

Figure 6. Contractile responses to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in rat thoracic aortas under pressure overload. TPA-induced contractions were developed either in a Ca^{2+}-free solution (Ca^{2+}-free) or a standard Ca^{2+}-containing medium (Ca^{2+}) in endothelium-denuded (Endo-) aortic rings obtained from rats 8 weeks after suprarenal abdominal aortic coarctation (It-Srac) or sham surgery (Sham). Data are reported as means ± SE in grams of developed force as a function of time (min) (n = 13-16). *P<0.05, Sham (Ca^{2+}) *vs* It-Srac (Ca^{2+}); #P<0.05, Sham (Ca^{2+}) *vs* It-Srac (Ca^{2+}-free); °P<0.05, Sham (Ca^{2+}) *vs* Sham (Ca^{2+}-free) (two one-way ANOVA with the Bonferroni *post hoc* test).

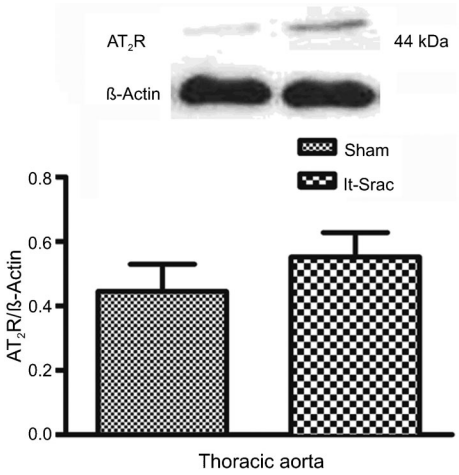

Figure 7. Western blot analyses for AT_2R protein in long-term pressure-overloaded rat aortas. Thoracic aortas were obtained from rats 8 weeks after suprarenal abdominal aortic coarctation (It-Srac) and sham surgery (Sham). *Upper*, A representative Western blot is depicted. *Lower*, The intensity of the bands was quantified using densitometric analysis and normalized with the corresponding β-actin. Data are reported as means ± SE of 12 rats by group. Data were not significantly different (unpaired Student *t*-test).

aortas of rats with suprarenal aortic coarctation was not correlated with plasma renin activity but was dependent on previous activation of AT_1R by endogenous ANG II (1), we investigated the possibility that ANG I and II were present at higher concentrations in the aortas of It-Srac rats than those of Sham rats. Total ANG I and II values in aortic tissues of It-Srac and Sham rats were similar. Most likely, therefore, ANG I and II derived from circulation or produced in situ were not increased in aortas of It-Srac rats compared with Sham rats.

Taken together, our data do not contradict those obtained for pressure-overloaded thoracic mouse aortas, because the authors of the studies using mice (2-4) clearly established that endothelium-dependent depression of aortic contractions in response to ANG II requires upregulation of AT_2R, which was a short-lived event that lasted approximately 4-6 weeks. In contrast, AT_2R mRNA expression in rat aortas remained significantly elevated 4 weeks after coarctation (1). However, AT_2R mRNA or protein expression values after that period were not quantified. Thus, we intended to provide additional information about long-term regulation of AT_2R in pressure-overloaded aortas. Notably, AT_2R protein expression was not significantly increased in the thoracic aortas of It-Srac rats compared to Sham rats. We may infer that increased molecular and functional expression of AT_2R may have developed but then weakened several weeks after coarctation. Thus, our results could reflect a transition from short-term to long-term pressure-overload-induced aortic responses.

An additional important finding was that the contractile

responses to PHE in endothelium-denuded thoracic aortas under long-term pressure overload were also attenuated. Accordingly, the contractile responses to ANG II were not selectively depressed in the aortic tissues of It-Srac rats. ANG II and PHE are GPCR agonists that trigger varied and complex cellular signaling pathways (21,22). However, both agonists, acting on different receptors, mainly use the $Gq/_{11}$-phospholipase C signaling pathway to stimulate vascular smooth muscle constriction via release of intracellularly stored Ca^{2+} and influx of extracellular Ca^{2+} (9,11,23-25). We investigated whether Ca^{2+} released from intracellular stores or Ca^{2+} entering through the plasma membrane after α_1-AR or AT_1R activation, respectively, is altered in thoracic aortas of It-Srac rats. Initially, endothelium-denuded aortic rings were challenged with ANG II or PHE in Ca^{2+}-free KBS to obtain transient contractions (which are attributed to IP_3-mediated Ca^{2+} release from intracellular stores), and then Ca^{2+} was added (in the absence of agonists) to induce tonic contractions through store-operated channels (SOCs), which are activated by the emptying of intracellular Ca^{2+} stores (6-11). We found that the transient contractions induced by either ANG II or PHE in Ca^{2+}-free medium did not differ between the aortic tissues of It-Srac and Sham rats. Otherwise, caffeine, which stimulates the Ca^{2+}-induced Ca^{2+} release mechanism (25,26), caused transient contractions in Ca^{2+}-free KBS that were also similar in magnitude in the aortic segments from both groups of rats. Thus, the observed difference in vascular reactivity between aortic rings of the It-Srac and Sham rats did not appear to depend on either the IP_3-induced Ca^{2+} release mechanism or the Ca^{2+}-induced Ca^{2+} release mechanism. Similarly, long-term suprarenal aortic coarctation did not modify the vasoconstriction induced by Ca^{2+} supplementation in ANG II- or PHE-prechallenged endothelium-denuded aortic rings, suggesting that long-term pressure overload cannot influence vasoconstriction induced by GPCR-agonist-activated extracellular Ca^{2+} entry through SOCs. In addition, we found that the contractile responses to high K^+ solutions in endothelium-denuded thoracic aortas of It-Srac and Sham rats were very similar. It is well known that extracellular Ca^{2+} entry through VDCC is the major cause of high K^+-induced contractions in rat aortas (27). It appears, therefore, that long-term pressure overload did not directly affect VDCC function in endothelium-denuded rat thoracic aortas.

In selective experiments with endothelium-denuded aortic rings from It-Srac and Sham rats, PHE-induced transient contractions in Ca^{2+}-free medium were analyzed in the presence of thapsigargin. Thapsigargin is a useful pharmacological tool for blocking the refilling of Ca^{2+} stores because it inhibits SERCA activity (8,11). Our results indicate that, when thapsigargin was added, a partial inhibition of the response to PHE was obtained in aortic tissues of both It-Srac and Sham rats. Nonetheless, there was no difference between the PHE-induced phasic responses of aortic tissues of It-Srac and Sham rats with or without thapsigargin treatment. This suggests that the intracellular Ca^{2+} store mobilized by PHE in rat aortas is at least partly dependent on SERCA activity, but it is not altered by long-term suprarenal aortic coarctation.

Interestingly, TPA-induced contractions in long-term pressure-overloaded thoracic aortic rings were reduced in the presence but not in the absence of extracellular Ca^{2+} compared to control tissues. A fraction of the ANG II and PHE contractions that occur in rat aortas result from PKC stimulation (12). PKC activity mediates the sensitization to Ca^{2+} of the vascular smooth muscle's contractile apparatus and contributes to the excitability of smooth muscle by activating depolarizing cation currents (12,14). In Ca^{2+}-free KBS, the contractile responses elicited by the PKC activator TPA were equivalent in endothelium-denuded aortic rings of It-Srac and Sham rats; under this experimental condition, TPA-induced aortic smooth muscle constriction has been mainly correlated with the extent of PKC-mediated Ca^{2+} sensitization (13). In the presence of extracellular Ca^{2+}, however, TPA-induced increases in the tone in aortic tissues of Sham rats, but not It-Srac rats, were higher than those obtained in Ca^{2+}-free medium. Hence, direct or indirect PKC-dependent increases in Ca^{2+} entry may contribute to TPA-elicited aortic smooth muscle constriction in endothelium-denuded aortic tissues of control rats. However, this hypothetical mechanism appears to be inhibited in endothelium-denuded aortic rings of It-Srac rats. In sum, these findings indicate that the depression of contractile activity observed in long-term pressure-overloaded rat aortas may result from inhibition of PKC-dependent stimulation of extracellular Ca^{2+} currents without the participation of PKC-mediated Ca^{2+} sensitization. We surmised that impairment of PKC-activated Ca^{2+}-permeable nonselective cation channels (14,28) may have caused contractile depression of rat thoracic aortas after long-term suprarenal aortic coarctation. However, the literature regarding mechanistic insights into how PKC contributes to cation currents in rat aorta remains to be defined. In addition, it is necessary to elucidate whether endothelium-independent depression of rat aorta contractile responses under long-term pressure overload is the result of a compensatory mechanism to avoid mechanical stress or a consequence of the mechanical damage itself.

Acknowledgments

Research supported by Secretaría de Investigación y Posgrado del Instituto Politécnico Nacional (SIP-IPN), Comisión de Operación y Fomento de Actividades Académicas del IPN (COFAA), and Consejo Nacional de Ciencia y Tecnología (CONACYT).

References

1. Yayama K, Horii M, Hiyoshi H, Takano M, Okamoto H, Kagota S, et al. Up-regulation of angiotensin II type 2 receptor in rat thoracic aorta by pressure-overload. *J Pharmacol Exp Ther* 2004; 308: 736-743, doi: 10.1124/jpet.103.058420.
2. Hiyoshi H, Yayama K, Takano M, Okamoto H. Stimulation of cyclic GMP production via AT2 and B2 receptors in the pressure-overloaded aorta after banding. *Hypertension* 2004; 43: 1258-1263, doi: 10.1161/01.HYP.0000128022.24598.4f.
3. Yayama K, Hiyoshi H, Imazu D, Okamoto H. Angiotensin II stimulates endothelial NO synthase phosphorylation in thoracic aorta of mice with abdominal aortic banding via type 2 receptor. *Hypertension* 2006; 48: 958-964, doi: 10.1161/01.HYP.0000244108.30909.27.
4. Hiyoshi H, Yayama K, Takano M, Okamoto H. Angiotensin type 2 receptor-mediated phosphorylation of eNOS in the aortas of mice with 2-kidney, 1-clip hypertension. *Hypertension* 2005; 45: 967-973, doi: 10.1161/01.HYP.0000164571.77710.19.
5. Yayama K, Okamoto H. Angiotensin II-induced vasodilation via type 2 receptor: role of bradykinin and nitric oxide. *Int Immunopharmacol* 2008; 8: 312-318, doi: 10.1016/j.intimp.2007.06.012.
6. Noguera MA, Calatayud S, D'Ocon MP. Actions of alpha-adrenoceptor blocking agents on two types of intracellular calcium stores mobilized by noradrenaline in rat aorta. *Naunyn Schmiedebergs Arch Pharmacol* 1993; 348: 472-477, doi: 10.1007/BF00173205.
7. Noguera MA, Ivorra MD, Chulia S, D'Ocon P. Capacitative Ca^{2+} entry associated with alpha1-adrenoceptors in rat aorta. *Naunyn Schmiedebergs Arch Pharmacol* 1997; 356: 83-89, doi: 10.1007/PL00005033.
8. Noguera MA, Madrero Y, Ivorra MD, D'Ocon P. Characterization of two different Ca^{2+} entry pathways dependent on depletion of internal Ca^{2+} pools in rat aorta. *Naunyn Schmiedebergs Arch Pharmacol* 1998; 357: 92-99, doi: 10.1007/PL00005156.
9. Guibert C, Ducret T, Savineau JP. Voltage-independent calcium influx in smooth muscle. *Prog Biophys Mol Biol* 2008; 98: 10-23, doi: 10.1016/j.pbiomolbio.2008.05.001.
10. Parekh AB, Penner R. Store depletion and calcium influx. *Physiol Rev* 1997; 77: 901-930.
11. Leung FP, Yung LM, Yao X, Laher I, Huang Y. Store-operated calcium entry in vascular smooth muscle. *Br J Pharmacol* 2008; 153: 846-857, doi: 10.1038/sj.bjp.0707455.
12. Morgan KG, Leinweber BD. PKC-dependent signalling mechanisms in differentiated smooth muscle. *Acta Physiol Scand* 1998; 164: 495-505, doi: 10.1046/j.1365-201X.1998.00445.x.
13. Sato K, Hori M, Ozaki H, Takano-Ohmuro H, Tsuchiya T, Sugi H, et al. Myosin phosphorylation-independent contraction induced by phorbol ester in vascular smooth muscle. *J Pharmacol Exp Ther* 1992; 261: 497-505.
14. Earley S, Straub SV, Brayden JE. Protein kinase C regulates vascular myogenic tone through activation of TRPM4. *Am J Physiol Heart Circ Physiol* 2007; 292: H2613-H2622, doi: 10.1152/ajpheart.01286.2006.
15. de Lannoy LM, Danser AH, Bouhuizen AM, Saxena PR, Schalekamp MA. Localization and production of angiotensin II in the isolated perfused rat heart. *Hypertension* 1998; 31: 1111-1117, doi: 10.1161/01.HYP.31.5.1111.
16. Karpe PA, Gupta J, Marthong RF, Ramarao P, Tikoo K. Insulin resistance induces a segmental difference in thoracic and abdominal aorta: differential expression of AT1 and AT2 receptors. *J Hypertens* 2012; 30: 132-146, doi: 10.1097/HJH.0b013e32834d9efe.
17. Kaneko K, Sunano S. Involvement of alpha-adrenoceptors in the endothelium-dependent depression of noradrenaline-induced contraction in rat aorta. *Eur J Pharmacol* 1993; 240: 195-200, doi: 10.1016/0014-2999(93)90898-R.
18. Martin W, Furchgott RF, Villani GM, Jothianandan D. Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. *J Pharmacol Exp Ther* 1986; 237: 529-538.
19. Matsuda K, Sekiguchi F, Tojo M, Shimamura K, Sunano S. Effects of N^G-nitro-L-arginine on alpha-agonists-induced contraction of aortae from Wistar Kyoto rats and stroke-prone spontaneously hypertensive rats. *J Smooth Muscle Res* 1995; 31: 51-60, doi: 10.1540/jsmr.31.51.
20. Vinet R, Brieva C, Pinardi G, Penna M. Modulation of alpha-adrenergic-induced contractions by endothelium-derived relaxing factor in rat aorta. *Gen Pharmacol* 1991; 22: 137-142, doi: 10.1016/0306-3623(91)90324-Y.
21. Hunyady L, Catt KJ. Pleiotropic AT_1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. *Mol Endocrinol* 2006; 20: 953-970, doi: 10.1210/me.2004-0536.
22. Guimaraes S, Moura D. Vascular adrenoceptors: an update. *Pharmacol Rev* 2001; 53: 319-356.
23. Maguire JJ, Davenport AP. Regulation of vascular reactivity by established and emerging GPCRs. *Trends Pharmacol Sci* 2005; 26: 448-454.
24. Allen BG, Walsh MP. The biochemical basis of the regulation of smooth-muscle contraction. *Trends Biochem Sci* 1994; 19: 362-368, doi: 10.1016/0968-0004(94)90112-0.
25. Collier ML, Ji G, Wang Y, Kotlikoff MI. Calcium-induced calcium release in smooth muscle: loose coupling between the action potential and calcium release. *J Gen Physiol* 2000; 115: 653-662, doi: 10.1085/jgp.115.5.653.
26. Sato K, Ozaki H, Karaki H. Multiple effects of caffeine on contraction and cytosolic free Ca^{2+} levels in vascular smooth muscle of rat aorta. *Naunyn Schmiedebergs Arch Pharmacol* 1988; 338: 443-448, doi: 10.1007/BF00172125.
27. Nelson MT, Quayle JM. Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol* 1995; 268: C799-C822.
28. Earley S, Brayden JE. Transient receptor potential channels and vascular function. *Clin Sci* 2010; 119: 19-36, doi: 10.1042/CS20090641.