Cardiovascular Research Advance Access published July 15, 2008

Cardiovascular Research
doi:10.1093/cvr/cvn178

Blood pressure variability increases connexin expression in the vascular smooth muscle of rats

Matheus L. Rocha[1]*, Alexandre H. Kihara[2], Ana P. Davel[2], Luiz R. G. Britto[2], Luciana V. Rossoni[2], and Lusiane M. Bendhack[1,3]

[1]*Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Av do café, s/n, Ribeirão Preto, Brazil;* [2]*Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil and* [3]*Department of Physics and Chemistry, Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil*

Received 15 February 2008; revised 13 May 2008; accepted 12 June 2008

Time for primary review: 49 days

KEYWORDS
Blood pressure variability;
Sinoaortic denervation;
Gap junctions;
Connexin;
Vascular smooth muscle

Aims Following sinoaortic denervation (SAD), isolated rat aortas present oscillatory contractions and demonstrate a heightened contraction for α-adrenergic agonists. Our aim was to verify the effects of SAD on connexin43 (Cx43) expression and phenylephrine-induced contraction in isolated aortas.
Methods and results Three days after surgery (SAD or sham operation), isolated aortic rings were exposed to phenylephrine and acetylcholine (0.1–10 μM) in the presence or absence of the gap junction blocker 18β-glycyrrhetinic acid (18β-GA, 100 μM). Vascular reactivity to potassium chloride (KCl, 4.7–120 mM) was also examined. The incidence of rats presenting oscillatory contractions was measured. Effects of SAD on the vascular smooth muscle expression of the Cx43 mRNA by RT–PCR and western blotting for Cx43 protein were examined. Phenylephrine-induced contraction was higher in SAD rat aortas compared with the control. In the presence of 18β-GA, the response to phenylephrine was similar in both groups. Oscillatory contractions were observed in 10/10 SAD rat aortas vs. 2/10 controls. Relaxing response to acetylcholine was similar in both groups, but in the presence of 18β-GA, the response to acetylcholine decreased significantly in the sham-operated group ($82.7 \pm 7.6\%$ reduction of relaxation), whereas a half-maximal relaxation (reduction of $46.2 \pm 5.3\%$) took place in SAD rat aortas. KCl-induced contraction was similar in both groups. Following SAD, RT–PCR revealed significantly increased levels of Cx43 mRNA (9.85 fold, $P < 0.01$). Western blot analysis revealed greater levels of Cx43 protein ($P < 0.05$).
Conclusion Blood pressure variability evoked by SAD leads to increased expression of Cx43, which could contribute to enhanced phenylephrine-induced contraction and oscillatory activity in isolated aortas.

1. Introduction

Coordination of cellular responses at vascular tissues is mediated by multiple signalling mechanisms, including intercellular communication. Direct cell-to-cell communication along the vessel wall is governed by gap junctions (GJ), which are aggregates of intercellular channels that mediate the spread of ions and second messengers (including Ca^{2+}, IP_3, $_cAMP$, $_cGMP$, and other molecules up to 1 kDa) from one cell to another,[1] enabling the production of coordinated multicellular responses.[2,3] GJ channels are formed by the apposition of two hemichannels, which are hexameric arrays formed by integral membrane proteins known as connexins (Cx). The Cx multigene family comprises around 20 members in the mammalian genome,[4] and the functional expression of several Cx has been studied in vessels. Among the studied Cx, however, none have reached the importance of Cx43, which is particularly ubiquitous in the vascular system.[5,6]

GJ expression in the vascular tissue (especially Cx43) is markedly increased in many forms of hypertension.[7–10] Segments of blood vessels isolated from hypertensive animals often contract in an oscillatory manner.[11–14] Moreover, increased vascular reactivity to contractile agonist is observed in hypertension.[8,15] Enhanced cell-to-cell communication via GJ may provide an explanation for both the occurrence of oscillatory contractions in isolated vessels from hypertensive animals and the increased response of the vascular smooth muscle from hypertensive animals to contractile agonist. Watts and Webb[8] observed that increased contraction to serotonin in aortic strips of hypertensive rats is inhibited by the blockade of the GJ. That GJ inhibition abolishes the oscillatory contractions typical of hypertension has also been demonstrated.[16]

* Corresponding author. Tel: +55 16 36024704; fax: +55 16 36024880.
E-mail address: rochaml@usp.br or matheusroch@yahoo.com.br

In spite of being elevated, blood pressure is not constant in hypertensive subjects, but it may vary spontaneously. Spontaneous variation in the blood pressure is defined as 'blood pressure variability' (BPV). In general, BPV is positively related to the severity of organ damage in hypertensive humans and rats.[17–20] A method that is widely employed for the determination of BPV is to calculate the standard deviation of the blood pressure values continuously recorded during a certain time range. With this method, investigators have found that BPV is increased in hypertensive patients[21] and in several hypertension models in rats.[17–20,22,23] Therefore, we have hypothesized that BPV might be a general phenomenon in hypertension.

Sinoaortic denervation (SAD) is a procedure that interrupts the arterial baroreceptor reflex system. The haemodynamic alterations produced by SAD have been extensively studied in many mammals, mainly in rats. There is general agreement that SAD causes a substantial increase in BPV with no changes in the mean levels of blood pressure.[24–26] Therefore, SAD rats can be used to study physiological changes caused by high BPV without sustained hypertension.

This study examines whether BPV evoked by SAD (i.e. BPV in the absence of hypertension) influences the nature of cell-to-cell communication in rat aortas. We test the hypothesis that BPV increases Cx43 expression and the formation of GJ communication, which in turn, leads to increased vascular reactivity and oscillatory contractions. The basis for this hypothesis and the experimental approach are based on the following evidence: (i) arteries that express Cx43 to a high degree, such as the rat aorta, are more sensitive to adrenergic agonists than those that express Cx43 at a low level, such as the caudal artery;[27] (ii) hypertensive rat arteries that exhibit both increased agonist-induced contraction and oscillatory contractions have higher Cx43 expressio;[8,10,16] (iii) SAD rat aortas exhibit incessant oscillatory contractions;[25] (iv) hypertension is often accompanied by BPV.[17–23] We have applied these findings to test our hypothesis that GJ communications are upregulated as a consequence of BPV, and not by hypertension.

2. Material and methods

2.1 Sinoaortic denervation

All the procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). SAD or sham-operation was performed in male Wistar rats (180–210 g) according to the method previously described by some of us.[28] Briefly, under anaesthesia with a solution of ketamine (50 mg/kg) and xylazine (5 mg/kg) i.p., the aortic and superior laryngeal nerves were isolated and dissected. The bifurcation and all the carotid branches were stripped of fibres and connective tissues. These procedures were completed bilaterally. Control rats were sham-operated (SO). After the SAD or the sham-surgery, polyethylene catheters (PE-10 connected to PE-50) were placed into the lower abdominal aorta via the left femoral artery for measurement of blood pressure and heart rate and in the left femoral vein for the subsequent administration of phenylephrine. Catheters were tunnelled subcutaneously and exteriorized at the dorsal neck region. Three days after the surgery, the blood pressure and heart rate were measured by connecting the arterial catheter to a pre-calibrated pressure transducer coupled with the amplifier recorder, and the responses were recorded using a computerized system and a Chart software 4.0 (PowerLab, ADInstruments). The total SAD was evaluated by determining the change in heart rate response to a 40 ± 10 mmHg increase in the mean blood pressure produced by the intravenous injection of phenylephrine 3–4 μg/kg. Only rats that exhibited bradycardia of less than 20 bpm were considered to be SAD rats.

2.2 Blood pressure recordings

The experiments were performed on the third day for the following reasons: (i) this period allowed for animal adaptation; (ii) the frequency of the oscillatory contractions in SAD rat aortas was higher in this period; and (iii) the blood pressure was similar in the two groups, even though the SAD group presented BPV.[26,28]

Three days after the surgery (SAD and SO), blood pressure recordings were performed in conscious rats that lasted for at least 45 min. The blood pressure and heart rate signals were digitalized by a microcomputer, and the values were determined and averaged. A widely used method for determining BPV is to calculate the standard deviation of the blood pressure recorded continuously for a certain time range.[22,23,26,28] BPV was expressed by the standard deviation of the mean value of the blood pressure.

2.3 Vascular reactivity study

Rats were killed by decapitation and the thoracic aorta was quickly removed, dissected free, and cut into 4 mm long rings. The aortic rings were placed between two stainless-steel stirrups and connected to an isometric force trans ducer (Letica Scientific Instruments) coupled with a PowerLab data acquisition unit. The responses were recorded using a computerized system and a Chart software 4.0 (PowerLab, ADInstruments), to measure tension in the vessels. The aortic rings were placed in a 10 mL organ chamber containing Krebs solution with the following composition (mM): NaCl 130, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 14.9, glucose 5.5, $CaCl_2$ 1.6. The solution was maintained at pH 7.4, and gassed with 95% O_2 and 5% CO_2 at 37°C. Rings were initially stretched to their optimum resting tension of 1 g before being allowed to equilibrate in the bathing medium. Endothelium was mechanically removed by gentle abrasion and its removal was validated by lack of relaxation to acetylcholine (1 μM) following contraction to phenylephrine (0.1 μM).

After the equilibrium period, phenylephrine (0.1 nM to 10 μM) was added cumulatively to the muscle bath. At this time, the presence of oscillatory contractions was verified. Cumulative-response curves were also carried out for KCl (4.7–120 mM) and acetylcholine (0.1 nM to 10 μM). In the latter case, the rings remained with intact endothelium and were previously contracted with phenylephrine (0.1 μM). In another series of experiments, the cumulative-response curves to phenylephrine and acetylcholine were repeated after the aorta had been incubated (45 min) with 18β-Glycyrrhetinic acid (18β-GA, 100 μM), a selective GJ blocker.[29,30]

In some experiments, low concentrations of phenylephrine (10 nM) were employed to stimulate oscillatory contractions. After the onset of agonist-induced oscillations, we added

18β-GA (30 μM) to inhibit the oscillations. Alternatively, 18β-GA (30 μM) had been pre-added to the organ bath 45 min before the oscillatory contractions have been induced by phenylephrine. In this protocol, we used a different concentration of the GJ blocker because we verified that this lower concentration was enough to inhibit the oscillations.

2.4 RNA isolation, cDNA synthesis, and real-time PCR

Aortic segments (approximately 150 mg) from SAD and SO rats were directly homogenized in 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted following the manufacturer's suggested protocol. For each 20 μL of the reverse transcription reaction, 4 μg total RNA were mixed with 1 μL oligodT primer (0.5 μg; Invitrogen), 4 μL 5× first strand buffer, 2 μL of 0.1 M DTT, 1 μL of dATP, dTTP, dCTP, and dGTP (each 10 mM), and 1 μL SuperScript III reverse transcriptase (200 U; Invitrogen).

Real-time PCR was carried out using Rotor-Gene 6000 Real-Time Rotary Analyzer (Corbett Robotics Inc., San Francisco, CA, USA) with primers purified by high-performance liquid chromatography (Invitrogen). Cx43 (forward: 5′-TTCCTCGT GCCGCAATTAC-3′, reverse: 5′-CGATTTTGCTCTGCGCTGTA-3′) and GAPDH (forward: 5′-GATGCTGGTGCTGAGTATGTCG-3′, reverse: 5′-GTGGTGCAGGATGCATTGCTGA-3′) primers resulted in 72 and 197 bp amplicons, respectively. Relative quantification of target gene expression was evaluated using the comparative CT method as previously described in detail.[31] The ΔCT value was determined by subtracting the target CT of each sample from its respective GAPDH CT value used as internal control. Calculation of ΔΔCT involved the control group mean ΔCT value as an arbitrary constant to subtract from experimental ΔCT mean value. Fold-changes in gene expression of the target gene are equivalent to $2^{-\Delta\Delta C_T}$. Values were entered into a one-way analysis of variance (ANOVA), followed by pairwise comparisons (Tukey's HSD test), with significance level set at 5%.

2.5 Western blot analysis for Connexin43

Proteins from homogenized aorta (10 μg per lane) were subjected to 10% SDS–PAGE and then transferred electrophoretically to a nitrocellulose membrane. The membranes were incubated overnight at 4°C. Proteins from homogenized rat left ventricle (25 μG) were used as positive control. The blots were washed and probed for 1 h with a polyclonal rabbit anti-Cx43 antibody (1:1500 dilution, Zymed Laboratories, CA, USA). After washing was completed, the membrane was incubated with a 1:1000 dilution of anti-rabbit IgG antibody conjugated to horseradish peroxidase (Bio-Rad; Hercules, CA, USA). The membrane was thoroughly washed, and the immunocomplexes were detected by using an enhanced horseradish peroxidase-luminol chemiluminescence system (ECLPlus, Amersham International; Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL, Amersham International). Signals on the immunoblot were quantified with the Scion Image software. The α-actin protein expression was used to normalize Cx43 protein expression in each sample.

2.6 Statistical analysis

In the graphics, the data are presented as means ± SEM. In each set of experiments, *n* indicates the number of rats studied. The statistical analysis was performed using the GraphPad Prism version 3.0 (GraphPad Software Corp., San Diego, CA, USA). Comparisons among groups were performed using ANOVA (post-test: Newman–Keuls) and Student's *t*-test, and values of $P < 0.05$ were considered to be significant.

3. Results

3.1 Blood pressure and blood pressure variability

As shown in *Figure 1*, BPV was observed in SAD rats only. Compared with the values obtained with SO rats (104.5 ± 4.3 mmHg, $n = 9$), the mean arterial pressure remained unchanged in SAD rats (109.3 ± 6.2 mmHg, $n = 15$). The heart-rate value in SAD rats was also similar to that in SO rats (SAD: 481 ± 23 vs. SO: 459 ± 18 bpm) (data not shown). However, BPV (standard deviation of the mean blood pressure) obviously increased ($P < 0.01$) in the case of SAD rats (17.8 ± 4.1 mmHg), compared with SO rats (3.7 ± 1.9 mmHg) (*Figure 1B*).

3.2 Vascular reactivity and occurrence of oscillatory contractions in isolated aortas

Isolated SAD rat aortas either exhibited spontaneous oscillatory contractions or the contractile agonist (in low concentrations) was able to induce or intensify the oscillations. A low phenylephrine concentration (10 nM) was employed to stimulate the oscillatory contractions because the oscillations produced by contractile stimulus were steadier (no alterations in frequency and amplitude) and persisted for a long period (over 50 min). This oscillatory behaviour rarely occurred in SO rat aortas. The oscillatory contractions presented a frequency of 4.5 ± 0.50 cycles/min and an amplitude of 0.465 ± 0.05 g. *Figure 2* shows that the incidence of oscillatory contractions was higher in the aortic rings of SAD rats (100% of rats) compared with SO rats (20% of rats) ($P < 0.001$; Fisher exact test).

Oscillatory contractions in aortas isolated from SAD rats were induced by phenylephrine in control preparations (without the addition of any other drug, *Figure 3A*), and also 45 min after incubation with the GJ blocker 18β-GA (30 μM) (*Figure 3B*). Treatment with 18β-GA prevented the occurrence of oscillatory contractions. Moreover, when 18β-GA was added on the oscillatory contractions, the tone was increased and rapidly returned to baseline, while the oscillations were interrupted (*Figure 3C*).

In the experiments involving concentration-effect curves to contractile agonist, the maximal effect evoked by phenylephrine was significantly higher ($P < 0.01$) in SAD rat aortas (1.89 ± 0.12 g; $n = 10$) than in SO rat aortas (1.42 ± 0.06 g; $n = 10$) (*Figure 4A*). The phenylephrine potency, represented as pD_2 ($-\log EC_{50}$), was similar in both groups (SAD: 7.80 ± 0.09 and SO: 7.76 ± 0.08). In order to verify whether higher agonist-induced contraction could occur for other distinct contractile agonists, cumulative-response curves were also carried out for serotonin. The contractile response to serotonin was similar to that obtained in the case of phenylephrine; i.e. serotonin induced a higher contraction in SAD rat aortas compared with control rats (data not shown). The data presented in *Figure 4B* support the hypothesis that increased GJ communication plays a role in the increased vascular reactivity observed in SAD rat aortas. Aortic rings from both SAD

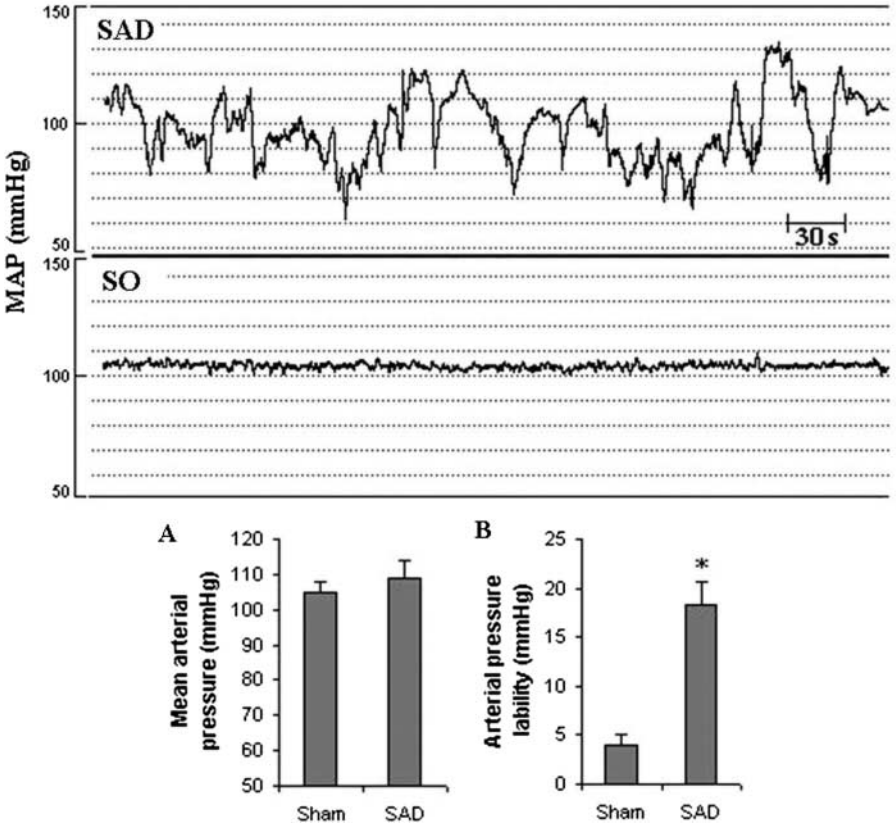

Figure 1 Representative tracings of the mean arterial pressure (MAP) measured 3 days after SAD ($n = 10$) or Sham-operation (SO, $n = 10$). (*A*) Mean arterial pressure. (*B*) Measurement of the arterial pressure variability. *Significant differences ($P < 0.01$) between the two groups.

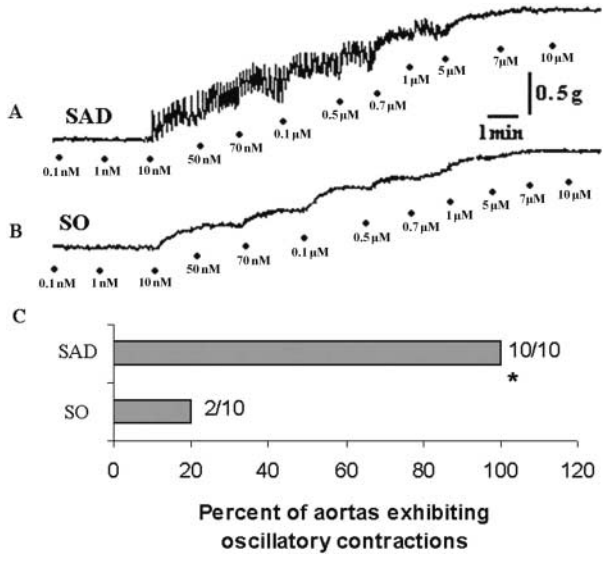

Figure 2 Simple representative tracings of oscillatory contractions induced by phenylephrine (from 0.1 nM to 10 μM, points in the traces) in SAD rat aortas (*A*; $n = 10$), but not in Sham-operated rat aortas (*B*; $n = 10$). C). Incidence of oscillatory contractions in SAD and Sham-operated rat aortic rings. Oscillatory contractions were observed in 10/10 SAD vs. 2/10 Sham-operated rats. *Significant differences (Fisher's exact test, $P < 0.05$) between the two groups.

and SO rats were treated with the GJ inhibitor 18β-GA (100 μM) and concentration-response curves to phenylephrine were then examined. The blockade of the GJ communications normalized the concentration-response curves to phenylephrine in SAD rat aortas. The maximum effect was statistically similar (1.47 ± 0.10 g, $n = 13$) to that response obtained in SO aortas in the presence of 18β-GA (100 μM) (1.38 ± 0.09, $n = 11$) ($P > 0.05$). These data suggest that GJ activity is probably enhanced in the SAD rat aorta because a GJ inhibitor normalizes the SAD aorta response to contraction at least in the case of phenylephrine.

Figure 5 shows the concentration-dependent contraction induced by KCl. Note that the maximum KCl effect was similar in SAD (1.35 ± 0.17 g, $n = 8$) and SO rat aortas (1.21 ± 0.11 g, $n = 7$). In the same way, the potency of KCl was similar in both groups.

Acetylcholine induced a similar concentration-dependent relaxation of phenylephrine-pre-contracted SAD and SO rat aortas, without significant difference in the potency and maximum effect (*Figure 6*). However, when aortas from both SAD and SO rats were treated with the GJ inhibitor, there was a significant difference in the acetylcholine response. 18β-GA had larger inhibitory effect ($P < 0.001$) on SO rat aortas (reduction of $82.7 \pm 7.6\%$ in relaxation; $n = 9$) than on SAD ones (reduction of $46.2 \pm 5.3\%$; $n = 8$).

3.3 Cx43 gene expression is upregulated after sinoaortic denervation

In order to evaluate Cx43 gene expression following SAD, we used quantitative real-time PCR, a method that provides more precision than end-point methods.[32] By using primers specifically designed for rat Cx43, we generated amplification plots from cDNA serial dilutions. Linear regression analysis of the amplification plots revealed a high correlation

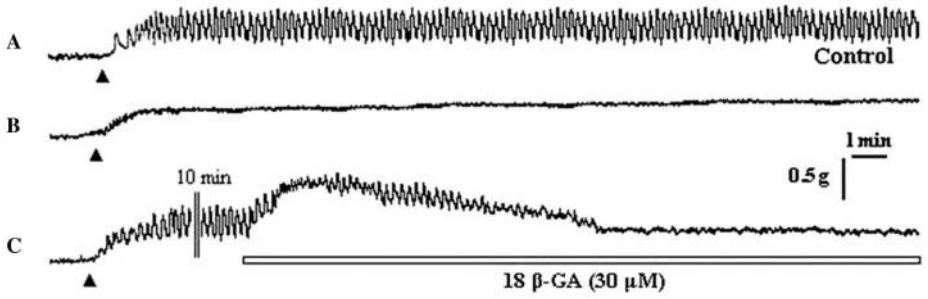

Figure 3 Effect of gap junction inhibitor (18β-GA) on oscillatory contractions induced by phenylephrine (‡, 10 nM) in SAD rat aortas. (*A*) Control preparations showing typical oscillatory contractions ($n = 6$). (*B*) Incubation with 18β-GA (30 μM, 30 min) inhibited the appearance of oscillatory contractions ($n = 5$). (*C*) A representative trace illustrating the effect of 18β-GA (30 μM) on the oscillatory contractions ($n = 5$). 18β-GA was added after stabilization of the oscillations induced by phenylephrine. Calibration bars of the force and time apply to all traces.

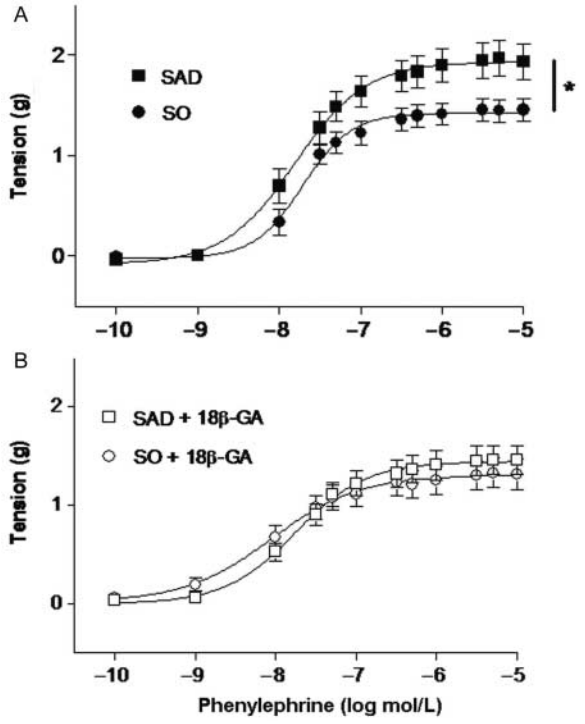

Figure 4 Concentration-response curves for phenylephrine were determined in SAD ($n = 10$) and SO ($n = 10$) aortic rings after (*A*) and before (*B*) treatment with the gap junction inhibitor 18β-GA (100 μM). Force values are expressed as mean ± SEM. Significant difference *$P < 0.01$.

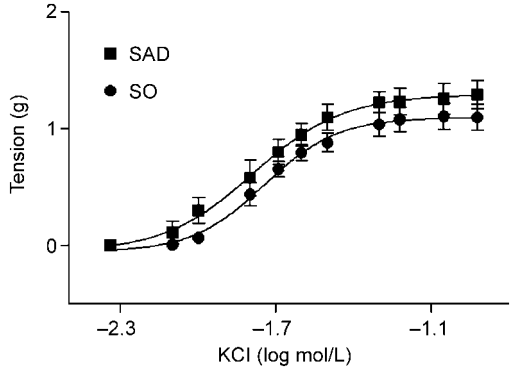

Figure 5 Concentration-response curves for KCl were determined in SAD ($n = 8$) and SO ($n = 7$) aortic rings. Force values are expressed as mean ± SEM.

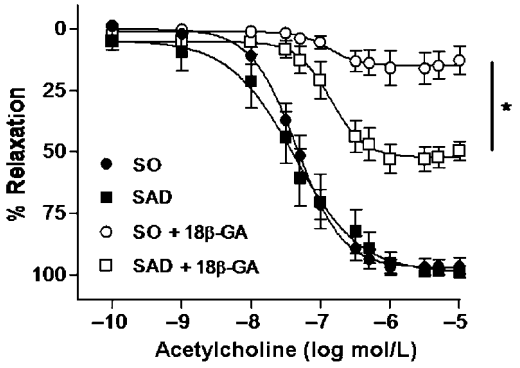

Figure 6 Concentration-response curves for acetylcholine were determined in SAD ($n = 8$) and SO ($n = 9$) aortic rings after (black symbols) and before (white symbols) treatment with gap junction inhibitor 18β-GA (100 μM). Values are expressed as mean ± SEM of relaxation in relation to pre-contraction induced by phenylephrine. Significant difference *$P < 0.001$.

($R^2 > 0.98$), confirming the amplification linearity (*Figure 7A*). Dissociation curves of these PCR products were obtained by heating samples from 60 to 95°C. The single peak observed matched to theoretical melting temperature previously calculated, indicates specificity of the primers (*Figure 7B*). As shown in *Figure 7C*, Cx43 transcripts were massively upregulated after SAD (9.85 fold, $P < 0.01$). In these experiments, GAPDH gene expression was used as internal control (*Figure 7D*).

3.4 Cx43 protein expression increases following sinoaortic denervation

The data in *Figure 8* provide molecular evidence for increased vascular GJ communication in SAD rats. Western blot analysis was employed to measure changes in Cx43 expression in the aortas after SAD. Cx43 protein expression was detected in arteries from both groups (SAD and SO) and it was higher ($P < 0.05$) in the aortas from SAD rats (*Figure 8*). As expected, the Cx43 protein was found in abundance in the left ventricle extracts. The left ventricle serves as positive control for the detection of the Cx43 protein due to its abundance in the heart tissue. Results are expressed after normalization to α-actin.

4. Discussion

Several studies have shown that GJ expression is upregulated in the vascular smooth muscle of hypertensive animals, resulting in the occurrence of oscillatory contraction and increased

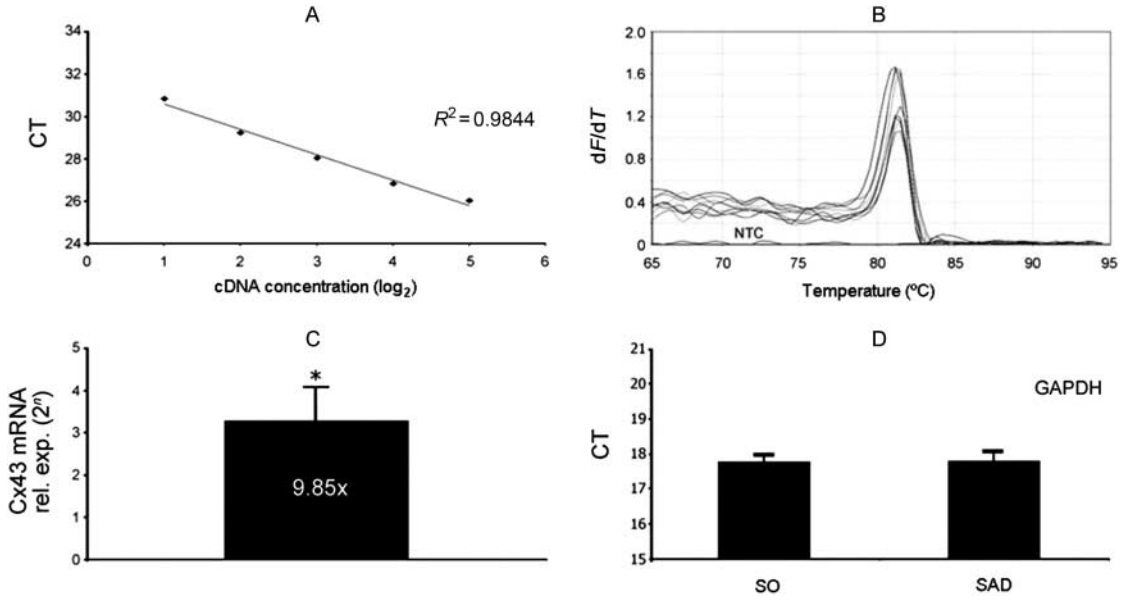

Figure 7 Vascular smooth muscle connexin43 mRNA expression via real-time PCR. (*A*) Linear regression analysis using data from different concentrations of cDNA. $R^2 = 0.9844$ indicates a strong correlation between logarithmic estimated cDNA concentration (2, 4, 8, 16, and 32 nG) and threshold cycle (CT). (*B*) Graph of the first derivative plot from normalized fluorescence against temperature. The melting temperature of PCR products (indicated by the peak of the lines) matches exactly the theoretical Tm expected for the amplicon, previously calculated. The no template control (NTC) curve shows no amplification of specific PCR product (NTC line). (*C*) Relative Cx43 mRNA expression after sinoaortic denervation. (*D*) GAPDH gene expression (internal control). $^*P < 0.01$.

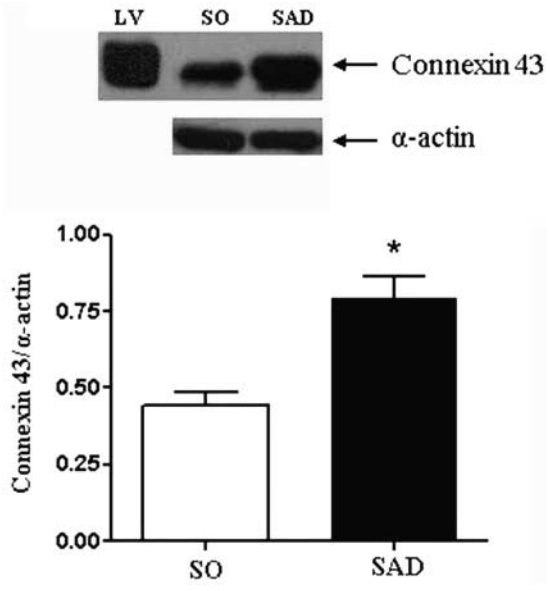

Figure 8 Representative western blot (Top) and quantitative analysis (Bottom) for connexin43 protein expression in thoracic aortas of control (SO, $n = 5$) and sinoaortic denervated rats (SAD, $n = 5$). Left ventricle (LV) served as a positive control for Cx43 detection. Results (means $\pm$ SEM) are expressed as the ratio between signal for the connexin43 protein and signal for α-actin in the corresponding aorta. Student's *t*-test, $^*P < 0.05$ vs. SO.

agonist-induced contraction in isolated arteries.[7–16] Associated with this information, we found in the literature that hypertension is frequently accompanied by high BPV.[17–23] In the present study, we provide evidence that the changes in the vascular smooth muscle caused by increased GJ communication in arteries from hypertensive animals could be, at least to some degree, a consequence of BPV, and not of hypertension '*per se*'. The premise for this information is that arteries from SAD rats, which present high BPV with no hypertension, also presented the same changes found under hypertensive conditions.

Intercellular communications are important for agonist-induced contraction[2,33] and relaxation,[34,35] indicating that GJ can regulate the vascular tone. In addition, it has been reported that GJ activity is crucial to the appearance of oscillatory contractions.[8,16] In the present study, we found that BPV induces elevated contractile effect and increases oscillatory contractions in response to an α-adrenergic agonist. The increased contractile responses are normalized and the oscillatory contractions are abolished by the inhibition of GJ.

Oscillations in the tone observed for SAD rat aortas are completely inhibited by addition of the GJ inhibitor, indicating that the functional GJ communication is necessary for the generation and/or maintenance of the oscillations. To evaluate the role of intercellular communications, we employed a structurally distinct inhibitor of GJ. Several 'relatively selective' GJ uncoupling agents have been characterized to date, e.g. octanol, heptanol, sucrose, halothane, etc., but among these the glycyrrhetinic acid derivatives, such as 18β-GA, are the most commonly employed.[29,30] Although peptide inhibitors of intercellular communications have more recently been employed, they have their apparent interpretational limitations as well.[36]

Coordination of responses among vascular wall cells is decisive for modulation of vasomotor tone and maintenance of circulatory homeostasis. There are evidences indicating that neither release of transmitter from perivascular innervation nor regenerative electrical events, such as the propagated action potentials, are sufficient to guarantee syncytial activation of the vascular smooth muscle in many blood vessels.[33,37,38] Moreover, although diffusion through the media may take place in some vessels, it has also been argued that diffusion distances, neurotransmitter instability, tissue tortuosity factors, and neuronal and non-neuronal uptake processes make it unlikely that neurotransmitter

and agonist concentrations would be sufficient to activate all the muscle cells, not being able to reach the deepest layers of cells in the vascular wall.[2,33,37,39]

In this work, we found that aortas from rats with BPV exhibit superior contraction to phenylephrine. This result can be attributed to the larger GJ expression in these vessels. Since GJ mediates the spread of ions, such as Ca^{2+}, and second messengers, such as IP_3, from one cell to another,[1] the increase in GJ should enhance the diffusion potential, leading to direct activation of a greater fraction of smooth muscle cells in the preparation.[2,40] The increased maximal responses could result from the recruitment of additional cells subsequent to the enhanced activation of a similar proportion of cells, resulting in a greater driving force for the passive diffusion of relevant second messenger molecules/ions.[2,33] Supplying a support for this information, we found that increased agonist-induced contraction in SAD rat aorta can be abolished by GJ inhibition, making the SAD and SO aortic contractions equivalent. These findings support the strong association between oscillatory contractions, responsiveness to a contractile agonist, and increased GJ communication. Our results resemble data observed in hypertensive rat arteries.[8,16]

In fact, we have found more direct evidence supporting our hypothesis that BPV evoked by SAD leads to increased vascular GJ. Despite the large amount of the recently identified connexin protein, Cx43 appears to be a predominant GJ protein present between most vascular smooth muscle cells.[5,33] In the PCR assessment, we observed that Cx43 gene expression (mRNA) is upregulated after SAD. Through complementary data obtained by western blotting technique, we have demonstrated that Cx43 protein level increase following SAD. This increase in the protein levels may well lead to increased GJ communication; the presence of a large amount of protein could lead to the additional formation of hemichannels.

In contrast with the results obtained with the phenylephrine-induced contraction, we verified that contraction induced by KCl is similar in SAD and SO rat aortas, which can be explained by the discoveries of Christ *et al.*[33] These authors found that phenylephrine-induced contraction is GJ dependent, as discussed before. However, the KCl, due to its low molecular weight and higher concentration (i.e. 120 000-fold greater than the concentration of phenylephrine), is able to penetrate the tissue in sufficient amounts to contract all cells, independently of junction communications, by opening voltage-dependent Ca^{2+} channels on all of the smooth muscle cells simultaneously, and thus eliciting a syncytial tissue contraction.

The intercellular communications are also essential to endothelial-induced relaxation.[34,35] The present study provides evidence that endothelial NO-induced relaxation in the vascular smooth muscle relies on the intercellular communications provided by GJ. We have shown that preparations treated with a GJ inhibitor (18β-GA) are significantly less responsive to acetylcholine. These results corroborate with previous findings, which demonstrated that the blockade of GJ impairs the vasodilatation induced by endothelial NO.[34,41] Curiously, 18β-GA exerted larger inhibitory effects on the SO rat aortas than in the SAD ones, which can be due to a larger GJ expression in the SAD rat aortas.

An alternative explanation for the effects of 18β-GA on acetylcholine-induced relaxation is based on the dependence of the NO-cGMP pathway on the GJ that exists at a step in the pathway prior to the NO activation of guanylate–cyclase.[34] However, it is doubtful that NO itself travels through GJ, since its free-radical structure makes it highly diffusible through the cell membrane.[42] Christ *et al.*[43] have shown that NO may have a small effective diffusion radius, so diffusion cannot fully account for the relaxation caused by NO. These authors proposed that NO-induced vasodilation in peripheral tissues is dependent on the cell-to-cell diffusion of cGMP through the GJ. Although we think these hypotheses are reasonable, we cannot state here the real mechanism by which GJ inhibition reduces relaxation to acetylcholine. We consider important to point out that our experiments showed that in SAD rat aortas there is less inhibition compared with SO rat aortas, possibly due to the largest GJ expression in these vessels.

In conclusion, BPV evoked by SAD causes increased Cx43 expression in rat aortas. The functional experiments revealed oscillatory contractions and greater isometric force generation to agonist-induced contraction in isolated SAD rat aortas, when compared with control rats. These responses are normalized by a selective gap junction inhibitor.

Acknowledgements

The authors would like to thank Juliana A. Vercesi and Miriam C.C. Melo for technical assistance.

Conflict of interest: none declared.

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Proc. no. 04/09739-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

1. Kumar NM, Gilula NB. The gap junction communication channel. *Cell* 1996;**84**:381–388.
2. Christ GJ. Modulation of α1-adrenergic contractility in isolated vascular tissue by heptanol: a functional demonstration of the potential importance of intercellular communication to vascular response generation. *Life Sci* 1995;**56**:709–721.
3. Evans WH, Martin PE. Gap junctions: structure and function. *Mol Membr Biol* 2002;**19**:121–136.
4. Sohl G, Willecke K. Gap junctions and the connexin protein family. *Cardiovasc Res* 2004;**62**:228–232.
5. Bennett MVL, Barrio LC, Bargiello T, Spray DC, Hertzberg E, Saez JC. Gap junctions: new tools, new answers, new questions. *Neuron* 1991;**6**: 305–320.
6. Beyer EC. Gap junctions. *Int Rev Cytol* 1993;**137C**:1–37.
7. Berry LB, Sosa-Melgarejo JA. Nexus junctions between vascular smooth muscle cells in the media of the thoracic aorta in normal and hypertensive rats: a freeze-fracture study. *J Hypertens* 1989;**7**:507–513.
8. Watts SW, Webb RC. Vascular gap junctional communication is increased in mineralocorticoid-salt hypertension. *Hypertension* 1996;**28**:888–893.
9. Haefliger J-A, Castillo E, Waeber G, Bergonzelli GE, Aubert J-F, Sutter E *et al*. Hypertension increases connexin43 in a tissue-specific manner. *Circulation* 1997;**95**:1007–1014.
10. Haefliger JA, Meda P. Chronic hypertension alters the expression of Cx43 in cardiovascular muscle cells. *Braz J Med Res* 2000;**33**:431–438.
11. Holloway ET, Bohr DF. Reactivity of vascular smooth muscle in hypertensive rats. *Circ Res* 1973;**33**:678–685.
12. Myers JH, Lamb FS, Webb C. Norepinephrine-induced phasic activity in tail arteries from genetically hypertensive rats. *Am J Physiol Heart Circ Physiol* 1985;**248**:H419–H423.

13. Sunano S, Sasaki F, Osugi S, Shimamura K. Comparison of endothelium-dependent and -independent tension oscillation in aorta of stroke-prone spontaneously hypertensive rats and Wistar Kyoto rats. *J Smooth Muscle Res* 1994;**30**:135–145.
14. Shimamura K, Sekiguchi F, Sunano S. Tension oscillation in arteries and its abnormality in hypertensive animals. *Clin Exp Pharmacol Physiol* 1999; **26**:275–284.
15. Linder AE, Weber DS, Whitesall SE, D'Alecy LG, Webb RC. Altered vascular reactivity in mice made hypertensive by nitric oxide synthase inhibition. *J Cardiovasc Pharmacol* 2005;**46**:438–444.
16. Tsai ML, Watts SW, Loch-Caruso R, Webb RC. The role of gap junctional comunication in contractile oscillations in arteries from normotensive and hypertensive rats. *J Hypertension* 1995;**13**:1123–1133.
17. Parati G, Pomidossi G, Albini F, Malaspina D, Mancia G. Relationship of 24 h blood pressure mean and variability to severity of target-organ damage in hypertension. *J Hypertens* 1987;**5**:93–98.
18. Su DF, Miao CY. Reduction of blood pressure variability: a new strategy for treatment of arterial hypertension. *Trends Pharmacol Sci* 2005;**26**: 388–390.
19. Zhang LM, Cheng Y, Liu JH. Blood pressure variability and target-organ damage in aged rats with spontaneously hypertension. *Chin J Hypertens* 1996;**4**:7–10.
20. Liu JG, Gong K, Cheng Y. Effects of changing blood pressure variability on target organs damage in spontaneously hypertensive rats. *Acad J Sec Mil Med Univ* 1998;**9**:515–518.
21. Mancia G, Ferrari A, Gregorini L, Parati G, Pomidossi G, Bertinieri G *et al*. Blood pressure and heart rate variabilities in normotensive and hypertensive human beings. *Circ Res* 1983;**53**:96–104.
22. Blanc J, Ponchon P, Laude D, Elghozi JL, Jover B. Blood pressure variability in established L-NAME hypertension in rats. *J Hypertens* 1999;**17**: 1527–1534.
23. Miao CY, Shen FM, Su DF. Blood pressure variability is increased in genetic hypertension and L-NAME-induced hypertension. *Acta Pharmacol Sin* 2001;**22**:137–140.
24. Norman RA, Coleman TG, Dent AC. Continuous monitoring of arterial pressure indicates sinoaortic denervation rats are not hypertensive. *Hypertension* 1981;**3**:119–125.
25. Rocha ML, Bendhack LM. Endothelial nitric oxide has inhibitory effect on the rhythmic contractions in aortas of sinoaortic deafferented rats. *J Cardiovasc Pharmacol* 2007;**50**:510–518.
26. Eto M, Toba K, Akishita M, Kozaki K, Watanabe T, Kim S *et al*. Reduced endothelial vasomotor function and enhanced neointimal formation after vascular injury in a rat model of blood pressure lability. *Hypertens Res* 2003;**26**:991–998.
27. Rummery NM, Hickey H, McGurk G, Hill CE. Connexin37 is the major connexin expressed in the media of caudal artery. *Arterioscler Thromb Vasc Biol* 2002;**22**:1427–1432.
28. Rocha ML, Bendhack LM. Spontaneous oscillatory contractions in aortas of rats with arterial pressure lability caused by sinoaortic denervation. *Clin Exp Pharmacol Physiol* 2007;**34**:708–713.
29. Davidson JS, Baumgarten IM. Glycyrrhetinic acid derivatives: a novel class of inhibitors of gap junction intercellular communication. Structure–activity relationships. *J Pharmacol Exp Ther* 1988;**246**:1104–1107.
30. Yamamoto Y, Fukuta H, Nakahira Y, Suzuki H. Blockade by 18β-glycyrrhetinic acid of intercellular coupling in the guinea-pig arterioles. *J Physiol* 1998;**511**:501–508.
31. Medhurst AD, Harrison DC, Read SJ, Campbell CA, Robbins MJ, Pangalos MN. The use of TaqMan RT–PCR assays for semiquantitative analysis of gene expression in CNS tissues and disease models. *J Neurosci Methods* 2000;**98**:9–20.
32. Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ, Reed MW. Quantitative reverse transcription–polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal Biochem* 2000;**285**:194–204.
33. Christ GJ, Brink PR, Zhao W, Moss J, Gondré CM, Roy C *et al*. Gap junctions modulate tissue contractility and α1-adrenergic agonist efficiency in isolated rat aorta. *J Pharmacol Exp Ther* 1993;**266**:1054–1065.
34. Javid PJ, Watts SW, Webb RC. Inhibition of nitric oxide-induced vasodilation by gap junction inhibitiors: a potential role for a cGMP-independent nitric oxide pathway. *J Vasc Res* 1996;**33**:395–404.
35. Fukuta H, Koshita K, Yamamoto Y, Suzuki H. Inhibition of the endothelium-dependent relaxation by 18β-glycyrrhetinic acid in the guinea-pig aorta. *Jpn J Physiol* 1999;**49**:267–274.
36. Berthoud VM, Beyer EC, Seul KS. Peptides inhibitors of intercellular communication. *Am J Physiol Lung Cell Mol Physiol* 2000;**279**:L619–L622.
37. Burnstock G, Gannon B, Iwayama T. Sympathetic innervation of vascular smooth muscle in normal and hypertensive animals. *Circ Res* 1970;**27**: 5–23.
38. Gibbins IL, Morris JL, Furness JB, Costa M. *Nonadrenergic Innervation of Blood Vessels*. Boca Raton, FL: CRC Press, Inc; 1988.
39. Bevan JA, Torok J. Movement of norepinephrine through the media of rabbit aorta. *Circ Res* 1970;**27**:325–331.
40. Kenakin TP. One the importance of agonist concentration-gradients within isolated tissues. Increased maximal responses of rat vasa deferentia to noradrenaline after blockade of neuronal uptake. *J Pharm Pharmacol* 1980;**32**:833–838.
41. Watts SW, Tsai ML, Loch-Caruso R, Webb RC. Gap junctional communication and vascular smooth muscle reactivity : use of tetraethylammonium chloride. *J Vasc Res* 1994;**31**:307–313.
42. Ignarro LJ. Signal transduction mechanisms involving nitric oxide. *Biochem Pharmacol* 1991;**41**:485–490.
43. Christ GJ, Brink PR, Ramanan SV. Dynamic gap junctional communication: a delimiting model for tissue responses. *Biophys J* 1994;**67**:1335–1344.