Aerobic Exercise of Low to Moderate Intensity Corrects Unequal Changes in BKCa Subunit Expression in the Mesenteric Arteries of Spontaneously Hypertensive Rats

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Summary
Accumulating evidence indicates that hypertension is associated with "ion channel remodeling" of vascular smooth muscle cells (VSMCs). The objective of this study was to determine the effects of exercise intensity/volume on hypertension-associated changes in large-conductance Ca²⁺-activated K⁺ (BKCa) channels in mesenteric arteries (MAs) from spontaneously hypertensive rats (SHR). Male SHRs were randomly assigned to three groups: a low-intensity aerobic exercise group (SHR-L: 14 m/min), a moderate-intensity aerobic exercise group (SHR-M: 20 m/min), and a sedentary group (SHR). Age-matched Wistar-Kyoto rats (WKYs) were used as normotensive controls. Exercise groups completed an 8-week exercise program. Elevation of the α and β₁ proteins was unequal in MA myocytes from SHRs, with the β₁ subunit increasing more than the α subunit. BKCa contribution to vascular tone regulation was higher in the myocytes and arteries of SHRs compared to WKYs. SHR BKCa channel subunit protein expression, β₁/α ratio, whole cell current density and single-channel open probability was also increased compared with WKYs. Aerobic exercise lowered systemic blood pressure and normalized hypertension-associated BKCa alterations to normotensive control levels in the SHRs. These effects were more pronounced in the moderate-intensity group than in the low-intensity group. There is a dose-effect for aerobic exercise training in the range of low to moderate-intensity and accompanying volume for the correction of the pathological adaptation of BKCa channels in myocytes of MAs from SHR.

Key words
Exercise intensity • Hypertension • BKCa channel

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Introduction
Hypertension is a major risk factor for the development of strokes, coronary artery disease, heart failure, and renal disease (Schmieder 2010). In cases of chronic hypertension, small arteries and arterioles undergo extensive biological and structural adaptations in response to elevated intraluminal perfusion pressure (Joseph et al. 2013). A characteristic pathological alteration of hypertension is augmented vasoconstrictor and attenuated vasodilator responses to various physiological stimuli, resulting in elevated vascular tone in arteries and arterioles that are exposed to persistent high blood pressure (BP).

A growing body of evidence shows that hypertension is associated with “ion channel remodeling” of the vascular smooth muscle cells (VSMCs) (Harder et al. 1983). Large-conductance Ca²⁺-activated K⁺ (BKCa) channels are densely expressed in VSMCs and play a pivotal role in regulating the resting membrane potential of these cells. They are activated by membrane depolarization and increased cytosolic Ca²⁺ concentration, inducing a hyperpolarization that opposes vasoconstriction (Brayden and Nelson 1992). Many studies have examined alterations in BKCa channels related to hypertension, though the findings have been

Regular physical activity, particularly aerobic exercise, is a well-recommended non-pharmacological therapy for prevention and control of hypertension. Endurance training reduces blood pressure at rest in both normotensive and hypertensive subjects, with a more pronounced effect in hypertensive subjects (Cornelissen et al. 2005). The mechanisms underlying this effect of exercise training remain undisclosed, but are likely to be multifactorial and include vascular remodeling and/or changes in peripheral vascular function, sympathetic nervous activity, function of the nitric oxide and prostanoid system, and the renin-angiotensin system (Hellsten et al. 2015). Exercise volume depends on the intensity, duration, and frequency. Exercise intensity is a critical factor in the treatment of cardiovascular diseases and is directly linked to both the amount of improvement in exercise capacity and the risk of adverse events during exercise. Intensity ranges for aerobic exercise prescriptions are included in several guidelines and publications on the secondary prevention of cardiovascular diseases.

Over the past few years, there has been increasing evidence suggesting a link between exercise and vascular ion channels (Albarwani et al. 2010, Bowles et al. 1998, Shi et al. 2013a, Zhao and Wang 2010). However, to date, no studies have examined the effects of exercise (particularly intensity/volume) on hypertension-associated pathological molecular changes in BKCa channels. We hypothesized that regular aerobic exercise may normalize hypertension-related alterations of BKCa channels and restore vascular function in arteries via a decrease in peripheral resistance, and that is intensity/volume-dependent to a certain extent in the range of aerobic exercise.

In the current study, we investigated the molecular mechanisms of pathological alterations in BKCa channel currents in mesenteric artery (MA) myocytes, the effects of aerobic exercise on hypertension-associated BKCa channel structural and functional remodeling in MA myocytes, and the relationship between exercise intensity/volume and BKCa channels in myocytes in spontaneously hypertensive rats (SHRs).

Materials and Methods

Animals and exercise training protocol

After a one-week acclimation period, three-month-old male SHRs (n=54) were randomly assigned to 3 groups: a sedentary (SHR, n=18) group, a low-intensity aerobic training group (SHR-L: 14 m/min, ~40-49 % of maximal aerobic velocity, n=18), and moderate-intensity aerobic training group (SHR-M: 20 m/min, ~55-65 % of maximal aerobic velocity, n=18). Moderate intensity (approximately 60 % of maximal aerobic velocity) was set based on previous reports (Agarwal et al. 2011, Roque et al. 2013) and low intensity was considered to be less than 50 % of maximal aerobic velocity. Age-matched male Wistar-Kyoto rats (WKY, n=18) were used as normotensive controls. The training groups were subjected to low- or moderate-intensity exercise on a rodent motor-driven treadmill for 8 weeks (60 min/day continuously, 5 days/week, at 0 slope). The training duration and frequency were kept constant and the training intensity was modified between the training groups. Therefore, the training volume was higher in the SHR-M (6.0 km/week) than in the SHR-L (4.2 km/week) group. To determine the maximal exercise capacity, rats were subjected to a progressive exercise test using an incremental speed protocol (5 m/min every 3 min with no grade) until exhaustion. The treadmill exercise test was repeated after 4 weeks, in order to adjust training intensity.

All animals were housed in cages in light-dark cycle (12 h light: 12 h dark) and temperature (22±1 °C) controlled conditions and had access to food and water ad libitum. The study protocol was conformed to Beijing Sport University Guidelines for the Care and Use of Animals.

Surgical procedures and cardiovascular testing

Twenty-four animals (n=6 per group) were used for the in vivo cardiovascular study. Surgical procedures were performed 24 h after completion of 8 weeks of exercise training. Under sodium pentobarbital anesthesia (60 mg/kg i.p.), arterial and venous catheters (ID 0.01 in, OD 0.03 in), filled with heparinized saline (100 IU/ml), were implanted into the left carotid artery and right jugular vein, respectively. The free ends of the catheters were implanted, subcutaneously, in the back of the neck.

BP and heart rate (HR) were measured continuously in conscious animals two days after they recovered from the surgery. Baseline HR and BP were
recorded for ~60 min. Norepinephrine (NE, 18 μg/kg) was then administered intravenously, via the implanted catheter, and BP and HR were recorded for another 60 min. The NE dose used was based on previous studies (Shi et al. 2012, Smits et al. 1987).

**Isometric contraction studies**

Twenty-four animals (n=6 per group) were used for the isometric contraction study and the electrophysiological study. The rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and immediately euthanized by decapitation after confirming that they were fully anesthetized (e.g. no response to toe pinch). The MA and branches were then removed from each animal, and placed in cold Krebs’ solution containing (mM): 131.5 NaCl, 5 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 2.5 CaCl2, 11.2 glucose, 13.5 NaHCO3, and 0.025 EDTA. Short segments of second order branches (A2) were used for contractile studies using the Multi myograph system (620 M, Danish Myo Technology, Aarhus, Denmark). The non-selective nitric oxide synthase (NOS) inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME, 100 μM), was added 20 min prior to the contraction studies.

The contractile response of tension was evaluated by measuring the maximum peak height and expressed as a percentage of contraction to 120 mM K+ (K_{max}). The effect of the BK_{Ca} channel blocker on resting tension was examined by measuring vessel contractility after administration of 10^{-7} M IbTX (a potent BK_{Ca} channel blocker). The vasorelaxation effect of the BK_{Ca} channel activator was examined in tissues, which had been previously contracted with 10^{-5} M NE. Responses to increasing concentrations (10^{-9}-10^{-5} M) of the BK_{Ca} channel opener, NS 11021, were then tested.

Currents were sampled at 10 kHz and filtered at 2 Hz via eight-pole low-pass Bessel filter. The product of the number of channels in the patch (N) and the channel open probability (Po) was used as an index of channel steady-state activity. The BK_{Ca} channel activity (NPo) was calculated from continuous gap-free data using the following equation: \( NPo = \sum (r1 + r2 + \ldots + ri), \) where \( ri \) is the relative open time (time open/total time) for each channel level. The total number of BK_{Ca} channels in an inside-out patch was determined at a voltage of +40 mV with 100 μM free Ca^{2+} in the bath solution. Only recordings with stable Po values for a minimum 2 min were analyzed.

**Patch clamp electrophysiology**

Single VSMCs were isolated from the MAs as described previously (Shi et al. 2013a). Whole-cell K+ currents were recorded using the voltage-clamp configuration, at room temperature using an Axon700B amplifier (Axon Instruments Inc., Foster City, CA, USA). Membrane currents were low-pass filtered at 2 kHz and digitized at a sampling frequency of 10 kHz. Borosilicate glass electrodes (2-4 MΩ) were filled with pipette solution containing (mM): 110 potassium aspartate (K-Asp), 30 KCl, 1 EGTA, 3 Na2ATP, 0.85 CaCl2, 10 glucose, 10 HEPES (pH 7.2 with KOH). The extracellular bath solution contained (mM): 134 NaCl, 6 KCl, 1 MgCl2, 1.8 CaCl2, 10 glucose, and 10 HEPES (pH 7.4). Current-voltage (I-V) relationships were generated in voltage-clamp cells held at an Em of -80 mV and then stepped in 10 mV increments to +70 mV. Voltage steps were 350 ms in duration. BK_{Ca} currents were defined as the 100 nM iberiotoxin (IbTX)-sensitive component. Currents were normalized to cell capacitance to obtain the current densities.

Single channel currents were recorded in excised inside-out membrane patches under symmetrical K+ (145 mM) as previously described (Shi et al. 2013a). Currents were sampled at 10 kHz and filtered at 2 Hz via eight-pole low-pass Bessel filter. The product of the number of channels in the patch (N) and the channel open probability (Po) was used as an index of channel steady-state activity. The BK_{Ca} channel activity (NPo) was calculated from continuous gap-free data using the following equation: \( NPo = \sum (r1 + r2 + \ldots + ri), \) where \( ri \) is the relative open time (time open/total time) for each channel level. The total number of BK_{Ca} channels in an inside-out patch was determined at a voltage of +40 mV with 100 μM free Ca^{2+} in the bath solution. Only recordings with stable Po values for a minimum 2 min were analyzed.

**Immunohistochemistry**

MA sections were fixed with 4 % paraformaldehyde in PBS for 60 min at RT and then permeabilized by 0.2 % Triton X-100 (Sigma) for 15 min. After extensive washing with PBS, the tissue sections were blocked for 60 min with 5 % BSA for non-specific antibody binding and then incubated in primary rabbit polyclonal K_{Ca}1.1 (Alomone Labs, Jerusalem, Israel) antibody at a dilution of 1:200 or rabbit polyclonal β1 (Alomone Labs) antibody at a dilution of 1:200, overnight at 4 °C. The antigen was localized by 3,3′-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA, USA) and counterstained with hematoxylin. The negative controls were performed by substituting primary antibody with normal serum in the same dilution. Preabsorption of the primary antibody with a 10-fold excess of the blocking peptides was performed to confirm the specificity of primary antibody (Shi et al. 2013b). All immunohistochemical photographs were analyzed using Image Pro Plus (version 6.0; Media Cybernetics, Silver Spring, MD, USA).

**Immunofluorescence**

The isolated VSMCs of MA were plated on poly-L-lysine-coated coverslips and allowed settle for
For immunofluorescence staining, arterial myocytes were fixed in 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.2% Triton X-100 (Sigma) for 10 min at room temperature. After washing thrice with PBS, the VSMCs were blocked in a solution of PBS containing 5% BSA for 60 min. Primary Rabbit polyclonal K_Ca1.1 (Alomone Labs, Jerusalem, Israel; 1:200) and Rabbit polyclonal sloβ1 (Alomone Labs, Jerusalem, Israel; 1:200) antibodies were typically applied overnight at 4 °C, respectively. The next day, after washing, a second Alexa Fluor 488 Goat Anti-Rabbit IgG antibody (Molecular Probes, Grand Island, NY, USA; 1:500) was used for 60 min in the dark. Sample coverslips were placed on ProLong Gold Antifade Reagent (Molecular Probes) on the glass slides. Images were acquired using a laser-scanning confocal microscope (TCS-SP8, Leica, Wetzlar, Germany).

Western blot analysis

Membrane proteins were isolated and pooled for use in Western blots, as previously described (Shi et al. 2013a). The primary antibodies used were polyclonal anti-K_Ca1.1 (1:300) and polyclonal anti-sloβ1 (1:300) (both from Alomone Labs).

RT-PCR

MAs were placed in RNAlater (Ambion, Austin TX, USA) at 4 °C. Total RNA was isolated using the PureLink RNA Mini Kit (Ambion) and reverse transcribed into cDNA using the GoScript Reverse Transcription System (Promega, USA), according to the manufacturer’s instructions. Primers specific to BKca channel α (KCNMA1) (GenBank accession no. NM_031828; amplicon=174 bp) (forward: 5’-ATA GGAAACCGCAAGAAATACG-3’ and reverse: 5’-AGG GGAGATGTTGTGAAGAA-3’), β1 (KCNMB1) (GenBank accession no. NM_019273; amplicon=80 bp) (forward: 5’-ATCAAAACCAAACAGTGCTCTTA-3’ and reverse: 5’-TCTGACCTTCTTACCATCAC-3’), and the housekeeping genes β-actin (GenBank accession no. NM_031144; amplicon=207 bp) (forward: 5’-CAC CGCGAGTACAACCTC-3’ and reverse: 5’-CCCATA CCCACCACCATCAC-3’) were designed to identify the presence of each transcript in MA vascular smooth muscle. Real-time Quantitative PCR was performed using an ABI Prism 7500 (Applied Biosystems, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems). Amplification conditions were as follows: 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 60 s. Relative quantification to β-actin was performed using the 2^-ΔΔCt method (Livak and Schmittgen 2001).

Statistical analysis

Statistical analysis was conducted using one-way ANOVA followed by a Tukey post-hoc test for multiple comparisons. The term n represents the number of cells or animals used in each experiment. Data are expressed as mean ± SEM and P<0.05 is considered statistically significant.

Table 1. Physical characteristics of experimental rats.

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR</th>
<th>SHR-L</th>
<th>SHR-M</th>
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</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>352.4±6.7</td>
<td>349.4±7.2</td>
<td>335.4±6.9a</td>
<td>329.2±7.1a</td>
</tr>
<tr>
<td>HW (g)</td>
<td>1.14±0.01</td>
<td>1.41±0.05*</td>
<td>1.39±0.04</td>
<td>1.38±0.06</td>
</tr>
<tr>
<td>HW (mg)/BW (g)</td>
<td>3.23±0.02</td>
<td>4.04±0.06*</td>
<td>4.14±0.07</td>
<td>4.19±0.11</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>118.0±5.3</td>
<td>185.2±6.0*</td>
<td>173.4±5.4a</td>
<td>160.1±5.1a&amp;</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>392±16</td>
<td>435±14*</td>
<td>395±16a</td>
<td>390±15a</td>
</tr>
</tbody>
</table>

*P<0.05, compared with WKY; aP<0.05, compared with SHR; &P<0.05, compared with SHR-L. HR, heart rate; HW, heart weight; BW, body weight; MAP, mean arterial pressure. n=12 in each group.

Results

Physical characteristics of experimental animals

There were no significant differences in body weight (BW) among the four groups prior to exercise. After exercise training for 8 weeks, BW in the SHR-L and SHR-M groups was significantly lower than in the SHR group, however there was no significant difference in BW when comparing the SHR-L and SHR-M groups to each other (Table 1). SHRs had a significantly higher
heart weight (HW) and HW to BW ratio (HW/BW) compared to normotensive rats (WKY). However, exercise training had no significant influence on either HW or HW/BW. Mean arterial pressure (MAP) was higher in SHRs (185.2±6.0 mm Hg) than in WKYs (118.0±5.3 mm Hg) and was markedly decreased after exercise training. Furthermore, MAP was lower in the group with the higher training intensity/volume (SHR-L: 173.4±5.4 mm Hg; SHR-M: 160.1±5.1 mm Hg). As shown in Table 1, HR in SHRs was higher than in WKYs, and HR in both the L-SHR and M-SHR groups was significantly lower than in the sedentary SHRs. However, HR was not significantly different between the SHR-L and SHR-M groups.

**Cardiovascular responses**

Intravenous injection of NE (18 μg/kg) produced significant time-dependent increases in MAP in all groups. MAP response to NE reached maximum at 0.5 min and then dropped rapidly (Fig. 1Aa). The maximal change in MAP (ΔMAP) in the WKY, SHR, SHR-L and SHR-M groups was 52.0±3.9, 76.2±4.2, 67.0±4.9 and 58.2±3.8 mm Hg, respectively (Fig. 1Ab). The NE-stimulated maximal increases in MAP were significantly higher in SHRs compared to WKYs (P<0.05). However, the NE-induced increase in MAP was markedly attenuated after exercise training and this attenuation was more pronounced in the SHR-M than in SHR-L group. The MAP returned to baseline within 6 min of the NE injection in the WKY, SHR-M, and SHR-L groups. However, MAP in the sedentary SHR group was still higher than baseline 6 min after the NE injection, indicating that exercise accelerated BP recovery time.

**Comparison of BKCa current density**

The effects of a BKCa activator on the relaxation and a BKCa inhibitor on the contraction of MA rings were examined in order to assess the BKCa channel contribution to vascular tone. In each experiment, arterial rings were preincubated with L-NAME (100 μM) for 20 min. The maximal response induced by KCl (120 mM) was similar in the WKY (14.7±1.6 mN) and SHR (15.3±1.5 mN) groups. Exercise did not modify the KCl-induced maximal response in either training group (SHR-L: 15.0±1.7 mN; SHR-M: 14.8±1.9 mN; n=6 in each group). IbTX, a specific BKCa channel blocker (10⁻⁷ M) induced an increase in tension in all four groups (Figs 1Ba and 1Bc). The increase in tension in the SHR group (28.6±3.4 %) was higher than in the WKY (11.8±2.0 %) group. However, exercise markedly inhibited the tension increase in both the SHR-L and SHR-M training groups (22.8±2.7 and 16.5±1.9 %, respectively), though the inhibition was more pronounced in the SHR-M group. This indicates that there seemed to be a possible dose-effect of exercise training intensity/volume on BKCa channel function.

The effect of NS11021 (BKCa channel activator) was examined in MAs precontracted with 10⁻⁵ M NE in order to further assess the role of BKCa channels in the maintenance of MA tension. NS11021 (10⁻⁵ M) reduced the vessel force in precontracted MAs in the WKY, SHR, SHR-L and SHR-M groups by 70.3±2.9 %, 55.6±1.2 % (P<0.05, vs. WKY), 65.3±2.6 % (P<0.05, vs. SHR) and 66.7±2.9 % (P<0.05, vs. SHR), respectively (Figs 1Bb and 1Bd). These data indicate that BKCa channel activation profoundly decreased vascular tone in the SHR group as compared to the WKY group. However, exercise appeared to effectively ameliorate this decrease in a manner that was intensity/volume-dependent.

The electrophysiological properties of BKCa channels were determined in myocytes from small MAs. To compensate for differences in cell size, membrane BKCa currents are expressed relative to cell capacitance (pA/pF). As shown in Figure 2, whole-cell K⁺ currents were marked ly inhibited by treatment with IbTX (100 nM) for 10 min in myocytes from all 4 groups. The IbTX-sensitive (BKCa) currents were the highest in the SHR cells. For example, at +70 mV, the current density was 37.7±1.8 pA/pF in SHR-L, 22.9±2.3 pA/pF in SHR-M, 18.0±2.1 pA/pF in SHR-L and SHR-M groups (n=18 cells/6 rats, P<0.05, vs. SHR-M, 13.7±1.2 pA/pF in WKY (n=18 cells/6 rats), respectively (Figs 1Bb and 1Bd). These data indicated that BKCa channel function was inhibited in both the SHR-L and SHR-M groups  by 70.3±2.9 %, 55.6±1.2 % (P<0.05, vs. WKY), 65.3±2.6 % (P<0.05, vs. SHR) and 66.7±2.9 % (P<0.05, vs. SHR), respectively (Figs 1Bb and 1Bd). These data indicate that BKCa channel activation profoundly decreased vascular tone in the SHR group as compared to the WKY group. However, exercise appeared to effectively ameliorate this decrease in a manner that was intensity/volume-dependent.
Fig. 1. Effect of exercise on NE-induced cardiovascular responses and vascular tension in MAs. A. Effect of exercise on NE-induced cardiovascular responses. (a) NE-induced MAP response; *P<0.05, compared with the baseline level. (b) The maximal change in MAP (ΔMAP) after injection of NE. NE, norepinephrine, 18 μg/kg. B. Effects of a BK Ca channel inhibitor and activator on vascular tension in MAs. (a) Typical experimental tracings showing effect of the BK Ca blocker, IbTX (100 nM), on resting tension. (b) Effect of the BK Ca channel activator, NS11021 (10⁻⁸ to 10⁻⁵ M), on NE-induced vessel contraction. In each experiment, the arteries were pre-incubated with the non-selective NOS inhibitor, L-NAME (100 μM), for 20 min (dotted arrows). (c) Statistical diagram of IbTX effects on NE-induced vessel contraction. (d) Concentration-response curves of NS11021 effects on NE-induced contraction. NS, NS 11021. *P<0.05, compared with WKY; †P<0.05, compared with SHR; ‡P<0.05, compared with SHR-L. n=6 in each group.
Fig. 2. Whole-cell K⁺ currents in MA myocytes from WKY, SHR, SHR-L, and SHR-M. A, Typical recordings of whole-cell K⁺ currents measured during depolarizing voltage steps in the absence (left panel) or presence of IbTX (100 nM, middle panel); the mean current density versus voltage plot (right panel) in the absence (closed circles) or presence (open circles) of IbTX. B, I-V relationships of BKCa current density in VSMCs from four groups. SHR, n=18 cells from 6 rats; WKY, n=18 cells from 6 rats; SHR-L, n=14 cells from 6 rats; SHR-M, n=19 cells from 6 rats. *P<0.05, compared with WKY. †P<0.05, compared with SHR. ‡P<0.05, compared with SHR-L.
Single channel properties of \( \text{BK}_{\text{Ca}} \) channels

An inside out single channel recording was conducted to determine whether the increase in \( \text{BK}_{\text{Ca}} \) current amplitude in SHR myocytes was due to enhancement of \( \text{BK}_{\text{Ca}} \) channel activity. At a testing potential of +40 mV, 1 \( \mu \text{M} \) [\( \text{Ca}^{2+}\)]\(_{\text{free}} \), the \( \text{Po} \) was significantly higher in the hypertensive group (SHR: 0.60±0.06 vs. WKY: 0.18±0.04). However, after exercise training, the \( \text{Po} \) in the SHR-L (0.45±0.05) and SHR-M (0.31±0.07) groups was significantly lower compared with the SHR group (\( P<0.05 \), Figs 3A and 3C). Furthermore, the \( \text{Po} \) was significantly lower in the SHR-M compared to the SHR-L group (\( P<0.05 \)). There were no significant differences in \( \text{BK}_{\text{Ca}} \) channel conductance among groups (Figs 3B and 3D). \( \text{BK}_{\text{Ca}} \) channel conductance was not changed after exercise training, regardless of training volume. This suggests that the suppression of \( \text{BK}_{\text{Ca}} \) channel \( \text{Po} \) is a direct reflection of the training-induced decrease in \( \text{BK}_{\text{Ca}} \) channel activity.

**Fig. 3.** Effects of exercise training on hypertension-associated alterations in \( \text{BK}_{\text{Ca}} \) channel activities and gating properties in MA myocytes. A. Single channel current recorded at a membrane voltage of +40 mV in WKY, SHR, SHR-L, and SHR-M ([\( \text{Ca}^{2+}\)]\(_{\text{free}} \)=1 \( \mu \text{M} \)), respectively. B. I-V relationships of \( \text{BK}_{\text{Ca}} \) current. A linear fit revealed average single channel conductances (\( G \)) of 235.3±15.1 pS (WKY, \( n=12 \) cells/6 rats), 252.4±10.56 pS (SHR, \( n=15 \) cells/6 rats), 238.6±8.6 pS (SHR-L, \( n=13 \) cells/6 rats), and 234.5±10.3 pS, \( n=10 \) cells/6 rats, respectively. C, D. Summary of \( \text{BK}_{\text{Ca}} \) channel \( \text{Po} \) (C) and conductance (D). *\( P<0.05 \), compared with WKY. #\( P<0.05 \), compared with SHR. &\( P<0.05 \), compared with SHR-L.

\( \text{BK}_{\text{Ca}} \) channel \( \alpha \) and \( \beta_1 \) subunit expression

Positive immunoreactivity for \( \text{BK}_{\text{Ca}} \) \( \alpha \) and \( \beta_1 \) subunits was identified in MAs from all groups (Fig. 4A). Immunofluorescence was also performed on VSMCs isolated from MAs (Fig. 4B). As shown in Figure 4, the positive immunoactivity of \( \alpha \) and \( \beta_1 \) subunit proteins was significantly increased in the SHR group and the increase in the \( \beta_1 \) subunit was much more apparent than the increase in the \( \alpha \) subunit. Exercise training markedly inhibited upregulation of the \( \alpha \) and \( \beta_1 \) subunit proteins and inhibition of the \( \beta_1 \) subunit was more pronounced in SHR-M than in SHR-L group.
Fig. 4. BK_{Ca} channel α and β1 subunits in MAs and VSMCs. A. Immunostaining of BK_{Ca} subunits in MAs. (a-h) Representative positive immunostaining of α subunit (a-d) and β1 subunit (e-h) in WKY, SHR, SHR-L, and SHR-M. (i) Negative control performed by substituting primary antibody with a normal serum. (j, k) Immunoreactivity was abolished when the antibody was preabsorbed with excess peptide. The black scale bar represents 100 μm. The red scale bar in the insets represents 20 μm. (l) Summary of the relative mean integrated optical density (% WKY) in four groups. B. Fluorescent microphotographs of confocal microscopy images and quantitative analysis of BK_{Ca} α (a-d) and β1 (e-h) subunits on arterial myocytes. (i, j) Bright field images of the VSMCs after staining. The inserts are representative images of VSMCs stained with α or β1 subunit antibody + blocking peptide. (k) The mean data illustrating the fluorescence intensity of the α and β1 subunit (WKY). Scale bar represents 20 μm. n=6 in each group. * P<0.05, compared with WKY. # P<0.05, compared with SHR. & P<0.05, compared with SHR-L.
In addition, BKCa channel subunit expression in MA myocytes was evaluated by Western blot. As shown in Figure 5, hypertension was associated with a significant increase in both α and β1 protein expression. Expressions of α and β1 proteins were ~1.6 and 4.4-fold higher in SHR cells than in age-matched WKY cells, respectively. The β1/α ratio was a significant ~2.5-fold higher in SHR than in WKY cells (Fig. 5A-C). These data indicate that, at both the tissue and cell level, hypertension induces a larger increase in β1 subunits with respect to α subunits. However, both the α and β1 subunit protein expressions were markedly decreased after exercise training and this decrease was greater in the SHR-M compared to the SHR-L group.

mRNA of BKCa α and β1 subunit

Finally, real-time quantitative PCR was performed to compare BKCa α and β1 message levels among the four groups. β-actin was used as the reference gene. The results showed that mean α and β1 mRNA levels were approximately 1.6 and 1.5-fold higher in arteries from the SHR compared to the WKY group, respectively (Fig. 5D), indicating that hypertension is associated with an almost equal elevation in both α and β1 subunit mRNA. Exercise training significantly reversed the elevation in α and β1 subunit mRNA and this reversal was more pronounced in the SHR-M than in the SHR-L group.

Discussion

There are two new findings in this study. First, genetic hypertension is associated with a posttranscriptional unequal upregulation of BKCa subunits in SHRs, with the β1 subunit increasing more than the α subunit. Myocytes and arteries of SHRs display pathological adaptations to high blood pressure including higher BKCa β1/α ratios, BKCa current densities, open probabilities, and BK Ca contributions to vascular tone regulation compared to myocytes and arteries of WKYs. Second, in addition to lowering BP, aerobic exercise training normalizes the hypertension-associated BKCa channel upregulation to a normotensive control level and these effects are more pronounced with exercise training of moderate-intensity compared to low-intensity. These results suggest that exercise training corrects pathological adaptations in BKCa channels in peripheral resistance-sized arteries in SHRs, in a dose-dependent
fashion. As exercise intensity, and accompanying volume, increases from low to moderate, the corrective effects also increase.

The relationship between physical activity and health benefits has been well established (Foulds et al. 2014, Wen et al. 2011). The dose-response nature of the relationship between physical activity and improved health has also been demonstrated with studies of morbidity, overall mortality, and numerous chronic conditions (Kesaniemi et al. 2001, Kohl 2001, Wen et al. 2011). Exercise training is now considered to be nonpharmacological therapeutic strategy substitute for hypertensive patients and is recommended by a number of organizations and agencies. In the present study, the HR, BW, and MAP of hypertensive rats were significantly reduced after eight weeks of treadmill training. These positive effects demonstrate the efficacy of aerobic exercise training. Interestingly, the improvement in hypertension was exercise intensity/volume dependent.

BKCa channels are highly expressed in VSMCs and play a crucial role in vascular relaxation via an endogenous compensatory mechanism to buffer vasoconstriction, particularly in the intense myogenic constriction of resistance vessels exposed to high intraluminal pressure (Hill et al. 2010, Kim et al. 2003). Most previous studies have reported that the functional expression of BKCa channels in VSMCs increases during hypertension (Liu et al. 1997, Liu et al. 1998, Rusch and Runnells 1994); K+ channels seem to have higher activity in SHR (Píntrová et al. 2014); and as a compensatory activation of vasodilator, BKCa channels enhance in all forms of hypertension, almost proportionally to BP elevation (Behuliak et al. 2011). In the current study, enhanced BKCa channel function was demonstrated in MAs from hypertensive animals through the use of isometric tension recordings and vascular reactivity techniques, along with a BKCa channel-specific pharmacological blocker and activator. The whole-cell K+ current through IbtX-sensitive BKCa channels was also found to be higher in VSMCs from MAs of SHRs when compared with WKYs. This finding is in line with previous studies of the aorta and MA in different models of hypertension (England et al. 1993, Liu et al. 1997, Rusch et al. 1992, Xu et al. 2005). Single channel recordings revealed that the channel activity (Po) was significantly enhanced in SHRs vs. WKYs. Interestingly, exercise training diminished these differences. Moreover, moderate-intensity exercise reversed the hypertension-associated BKCa channel alterations more effectively than did low-intensity exercise.

In a recent study of normotensive rats, we found that exercise training significantly reduced systolic blood pressure, but not diastolic blood pressure or MAP. Exercise training also increased BKCa channel activity in MA myocytes (Shi et al. 2013a). Interestingly, the effects of exercise on BP and tension were basically the same in SHRs in the current study, though there was a discrepancy in BKCa channel expression and activity between hypertensive rats in our study and normotensive animals in the previous study. There are some possible explanations for the contradictory effect of exercise on BKCa channel expression observed in the two models. It has been demonstrated that exercise training increases BKCa channel activity by changing the gating properties of BKCa channels in various arterial smooth muscle cells (Li et al. 2013, Shi et al. 2013a, Zhao and Wang 2010), thus, exercise itself activates BKCa channels on VSMCs. However, in hypertensive rats, BKCa channels are already upregulated in MA myocytes in response to chronically high intraluminal pressure (Joseph et al. 2013). Therefore BKCa channel upregulation appears to be the result, rather than a cause, of hypertension. In addition, there is evidence that Kv channels are significantly reduced in hypertensive rats compared to normotensive rats, which may be one reason for the high vascular tone (Zhang et al. 2005). Kv is another critical channel that plays an important role in regulating membrane potential and vascular tone. A decrease in Kv channels causes depolarization of the cell membrane, resulting in an influx Ca2+ from L-type calcium channels. In the current study, exercise training significantly suppressed the downregulation of Kv channels in the thoracic aorta of SHRs (Li et al. 2014); consequently hypertension caused by Kv channel downregulation was eliminated, at least partially. Previous studies demonstrated that a greater intracellular free calcium concentration and net potassium efflux in lymphocytes from spontaneously hypertensive stroke-prone rats (Furspan and Bohr 1986, Furspan and Bohr 1990). This resulted in a BP decrease, subsequently abolishing BKCa channel upregulation in SHRs. In addition, voltage-gated L-type Ca2+ (Ca1.2) channels control the Ca2+ influx and regulate VSMCs contractility (Joseph et al. 2013); exercise training, or moderate exercise can normalize SHR-associated Ca1.2 up-regulation to restore mesenteric arterial function (Chen et al. 2015, Shi et al. 2015). However, from the present study it is not possible to ascertain whether the...
observed exercise-induced BK$_{Ca}$ changes might be a consequence of lowered BP and/or attenuated calcium influx. Exercise appears to directly activate BK$_{Ca}$ channels, but further studies are needed to determine whether the net effect of exercise corrects the pathological remodeling of BK$_{Ca}$ channels in hypertensive animals.

In fact, the level of whole-cell K$^+$ current through BK$_{Ca}$ channels in VSMCs is positively correlated to BP in hypertensive animals (Rusch and Runnells 1994). Existing research suggests that the decrease in BP produced by exercise is a critical factor affecting in BK$_{Ca}$ expression and function. For example, the angiotensin-converting enzyme inhibitor, ramipril, lowered BP in SHRs by normalizing elevated BK$_{Ca}$ current densities. In addition, ramipril abolished TEA-induced constriction of aortic segments from SHRs (Rusch and Runnells 1994).

In situ studies of cerebral arterioles from hypertensive rats also provide evidence for upregulation of vascular BK$_{Ca}$ channel function (Liu et al. 1998). When IbTX (100 nM) was topically applied to pial arterioles via a cranial window preparation, arterioles from SHRs constricted more compared to arterioles from normotensive WKY rats. Thus, the upregulation of vascular BK$_{Ca}$ channel function seems to act as an adaptive mechanism to diminish elevated vascular excitability and vasospastic episodes in microcirculatory beds during hypertension (Joseph et al. 2013). Exercise training efficiently lowers MAP, in a dose-dependent manner, in those with hypertension (Foulds et al. 2014). This may explain the differing effects on BK$_{Ca}$ channel alterations in SHRs produced by different intensity/volumes of exercise in our study.

The BK$_{Ca}$ β1 subunit confers Ca$^{2+}$ sensitivity to BK$_{Ca}$ channels and mediates the coupling of Ca$^{2+}$ sparks to BK$_{Ca}$ channel activation (Brenner et al. 2000). Changes in the stoichiometry of α and β1 subunits have been implicated in a variety of physiological and pathophysiological conditions (Amberg and Santana 2003, Zhao et al. 2007). Previous studies demonstrated that the pore-forming α subunit of the BK$_{Ca}$ channel is increased in both aorta and microcirculatory beds (Cox and Rusch 2002, Liu et al. 1997) in hypertension. In the current study, the protein expressions of both the α and β1 subunits were significantly higher in MA myocytes from SHRs compared to WKY rats. Furthermore, hypertension induced a larger increase in β1 subunits with respect to α subunits in SHRs. The increased β1/α subunit stoichiometry in MAs of SHRs enhanced BK$_{Ca}$ channel activation, leading to membrane hyperpolarization and decreased vascular tone.

According to previous studies, the mRNA level in the α subunit of BK$_{Ca}$ is the same in the aortas of SHRs and WKY rats. During hypertension, the expression pattern of the ancillary BK$_{Ca}$ β subunit differs from that of the pore-forming α subunit in the vasculature. For example, mRNA expression of the β1 subunit is reduced in cerebral and MAs from SHRs and in angiotensin II-infused hypertensive mice, however the expression of the BK$_{Ca}$ channel pore-forming α subunit mRNA was unchanged in these animals (Amberg et al. 2003, Nieves-Cintron et al. 2007, Amberg and Santana 2003). In the present study, we found that both α and β1 mRNA levels were higher in MAs from SHRs compared to WKY rats. Inconsistent with the protein level, there was a similar increase in β1 and α mRNA in hypertension. In this study, the increases in β1 (~1.6-fold) and α (~1.5-fold) mRNA cannot fully account for the elevation in β1 (~4.4-fold) and α (~2.5-fold) proteins in hypertension. These data indicate that both transcriptional and posttranslational mechanisms elevate β1 and α proteins in MA myocytes during hypertension. The β1/α protein level is elevated more than the β1/α mRNA level, suggesting that hypertension-associated changes in posttranslational events also contribute to increased expression of BK$_{Ca}$ β1 subunits in hypertension. Thus, posttranscriptional modifications and the protein stability of the pore-forming α subunit of the BK$_{Ca}$ channel, and/or its association with auxiliary regulatory proteins, may play an important role in the increased functional expression of vascular BK$_{Ca}$ channels during hypertension. The mRNA levels of both the α and β1 subunits decreased a similar amount after exercise training. In addition, protein levels, α and β1 subunit protein expression, and the β1/α ratio were markedly decreased compared to SHRs. This result suggests that exercise training can ameliorate the pathological changes in BK$_{Ca}$ channels that result from hypertension and that this effect is dependent on the intensity/volume of aerobic exercise, at least in the range of low to moderate.

**Conclusions**

Many studies have demonstrated an increase in BK$_{Ca}$ channel function and molecular expression in arterial myocytes to compensate the increased BP in hypertension. This study provides further evidence that the development of genetic hypertension is associated
with a posttranscriptional unequal upregulation of BKCa subunits, with the β1 increasing more than α subunit, in MA myocytes. In addition to lowering BP, aerobic exercise training may correct the hypertension-associated BKCa channel remodeling, thereby suppressing the pathological adaptations of BKCa channels that result from high blood pressure.

Limitations

There were some limitations in the current study. For example, we just focused on the endothelium-independent regulation of the MA (i.e. BKCa channels in VSMCs). It has been demonstrated that exercise training may improve NO bioavailability in the endothelium in small MAs from SHRs that have undergone exercise training compared to sedentary SHRs (Roque et al. 2013). Therefore, other mechanisms such as reduced oxidative stress and/or greater NO bioavailability associated with increased endothelial NOS (eNOS) expression/activation may be also involved in the exercise-induced effects. This need to be further studied.

Conflict of Interest

There is no conflict of interest.

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