Aerobic exercise of low to moderate intensity corrects unequal changes in BK_{Ca}-subunit expression in the mesenteric arteries of spontaneously hypertensive rats

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Short title: Exercise intensity & arterial BK_{Ca} remodeling in SHR

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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 Ca^{2+}-activated K^+ (BK_{Ca}) 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. BK_{Ca} contribution to vascular tone regulation was higher in the myocytes and arteries of SHRs compared to WKYs. SHR BK_{Ca} 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 BK_{Ca} 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 BK_{Ca} channels in myocytes of MAs from SHR.

Keywords: exercise intensity; hypertension; BK_{Ca} channel

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 Ca^{2+}-activated K^+ (BK_{Ca}) 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 Ca^{2+} concentration, inducing a hyperpolarization that opposes vasoconstriction (Brayden and Nelson 1992). Many studies have examined alterations in BK_{Ca} channels related to hypertension, though the findings have been inconsistent. Some studies have suggested increases in BK_{Ca} channels (England *et al.* 1993; Liu *et al.* 1997, 1998; Rusch and Runnells 1994), while others have reported decreases in BK_{Ca} channels (Amberg *et al.* 2003; Nieves-Cintron *et al.* 2007; Yang *et al.* 2013) with hypertension.

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 e*t 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 BK_{Ca} channels. We hypothesized that regular aerobic exercise may normalize hypertension-related alterations of BK_{Ca} 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 BK_{Ca} channel currents in mesenteric artery (MA) myocytes, the effects of aerobic exercise on hypertension-associated BK_{Ca} channel structural and functional remodeling in MA myocytes, and the relationship between exercise intensity/volume and BK_{Ca} 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: 20m/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/wk, 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/wk) than in the SHR-L (4.2 km/wk) 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 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11.2 glucose, 13.5 $NaHCO_3$, and 0.025 EDTA. Short segments of second order branches (A2) were used for contractile studies using the Multi myograph system (620M, Danish Myo Technology, Aarhus, Denmark). The non-selective

nitric oxide synthase (NOS) inhibitor, N^{ω}-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.

Patch clamp electrophysiology

Electrophysiology and solutions

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 Na_2ATP, 0.85 $CaCl_2$, 10 Glucose, 10 HEPES (pH 7.2 with KOH). The extracellular bath solution contained (mM): 134 NaCl, 6 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 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 10mV 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 at2 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 (*NP*o) was calculated from continuous gap-free data using the following equation: $NPo = \sum(t1 + t2. . .ti)$, where *t*i 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 minutes were analyzed.

Immunohistochemistry

MA sections were fixed with 4% paraformaldehyde in PBS for one hour at RT and then permeabilzed by 0.2% Triton X-100 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 *slo*β1 (Alomone Labs) antibody at a dilution of 1:200, overnight at 4 °C. The antigen was localized by 3,3'-diaminobenzedine 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 an hour. 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 1 hour. Primary Rabbit polyclonal K_{Ca}1.1 (Alomone Labs, Jerusalem, Israel; 1:200) and Rabbit polyclonal *slo*β1 (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 1 h 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-KCa1.1 (1:300) and polyclonal anti-*sloβ*1 (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 BK_{Ca} channel α (KCNMA1) (GenBank accession no. NM_031828; amplicon = 174 bp) (forward: 5′-ATAGGAAACCGCAAGAAATACG-3′ and reverse: 5′-AGGGGAGATGTTGTGAAGAAAA-3′), β1 (KCNMB1) (GenBank accession no. NM_019273; amplicon = 80 bp) (forward: 5′-ATCAAAACCAACAGTGCTCCTA-3′ and reverse: 5′-TCTGACCTTCTTCACATCAACC-3′), and the housekeeping genes β-actin (GenBank accession no. NM_031144; amplicon = 207 bp) (forward: 5′-CACCCGCGAGTACAACCTTC-3′ and reverse: 5′-CCCATACCCACCATCACACC-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 $2^{-\triangle\triangle Ct}$ 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.

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) ratio 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 mmHg) than in WKYs (118.0 ± 5.3 mmHg) 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 mmHg; SHR-M: 160.1 ± 5.1 mmHg). 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 (18μg/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 (*Figure 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 mmHg, respectively (*Figure 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.

Contribution of BK_{Ca} channels in vascular tone regulation

The effects of a BK_{Ca} activator on the relaxation and a BK_{Ca} inhibitor on the contraction of MA rings were examined in order to assess the BK_{Ca} 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 BK_{Ca} channel blocker (10^{-7} M) induced an increase in tension in all four groups (*Figure 1Ba and c*). 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 BK_{Ca} channel function.

The effect of NS11021 (a BK_{Ca} channel activator) was examined in MAs precontracted with 10^{-5} M NE in order to further assess the role of BK_{Ca} 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 (Figure 1Bb and d).

These data indicate that BK_{Ca} 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.

Comparison of BK_{Ca} current density

The electrophysiological properties of BK_{Ca} channels were determined in myocytes from small MAs. To compensate for differences in cell size, membrane BK_{Ca} currents are expressed relative to cell capacitance (pA/pF). As shown in Figure 2, whole-cell K^+ current densities were not significantly different among the four groups. The whole-cell K^+ currents were markedly inhibited by treatment with IbTX (100 nM) for 10 min in myocytes from all 4 groups. The IbTX-sensitive (BK_{Ca}) 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 (n = 18 cells / 6 rats, $P < 0.05$, *vs.* WKY), 13.7 ± 1.2 pA/pF in WKY (n = 18 cells / 6 rats), 22.9 ± 2.3 pA/pF in SHR-L (n = 14 cells / 6 rats, $P < 0.05$, *vs.* SHR), and 18.0 ± 2.1 pA/pF in SHR-M cells (n = 19 cells / 6 rats, $P < 0.05$, *vs.* WKY; $P < 0.05$, *vs.* SHR-L). These data indicated that the hypertension was associated with an enhancement in functional expression of BK_{Ca} channels and that exercise training attenuated this enhancement. Mean cell capacitance for WKY (17.3 ± 1.4 pF, n = 18 cells) and SHR (17.4 ± 1.8 pF, n = 18 cells) cells was similar and was not altered by exercise (SHR-L, 17.6 ± 1.7 pF, n = 14 cells; SHR-M, 17.1 ± 1.6 pF, n = 19 cells, all from 6 animals in each group; $P > 0.05$ when comparing all), indicating that current density changed because of alterations in BK_{Ca} channels.

Single channel properties of BK_{Ca} channels

An inside out single channel recording was conducted to determine whether the increase in

BK_{Ca} current amplitude in SHR myocytes was due to enhancement of BK_{Ca} channel activity. At a testing potential of +40 mV, 1 μM $[Ca^{2+}]_{free}$, the Po was significantly higher in the hypertensive group (SHR: 0.60 ± 0.06 *v.s.* WKY: 0.18±0.04). However, after exercise training, the 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 ($P < 0.05$, *Figure 3A and C*). Furthermore, the Po was significantly lower in the SHR-M compared to the SHR-L group ($P < 0.05$). There were no significant differences in BK_{Ca} channel conductance among groups (*Figure 3B and D*). BK_{Ca} channel conductance was not changed after exercise training, regardless of training volume. This suggests that the suppression of BK_{Ca} channel Po is a direct reflection of the training-induced decrease in BK_{Ca} channel activity.

BK_{Ca} channel α- and β1-subunit expression

Positive immunoreactivity for BK_{Ca} α and β1 subunits was identified in MAs from all groups (*Figure 4A*). Immunofluorescence was also performed on VSMCs isolated from MAs (*Figure 4B*). As shown in Figures 4, the positive immunoactivity of α and β1 subunit proteins was significantly increased in the SHR group and the increase in the β1 subunit was much more apparent than the increase in the α-subunit. Exercise training markedly inhibited upregulation of the α- and β1-subunit proteins and inhibition of the β1 subunit was more pronounced in SHR-M than in SHR-L group.

In addition, BK_{Ca} 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 (*Figure 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 BK_{Ca} α and β1 subunit

Finally, real-time Quantitative PCR was performed to compare BK_{Ca} 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 (*Figure 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 BK_{Ca} subunits in SHRs, with the β1subunit increasing more than the α subunit. Myocytes and arteries of SHRs display pathological adaptations to high blood pressure including higher BK_{Ca} β1/α ratios, BK_{Ca} 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 BK_{Ca} 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 BK_{Ca} 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.

BK_{Ca} 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 BK_{Ca} channels in VSMCs increases during hypertension (Liu *et al.* 1997, 1998; Rusch and Runnells 1994); K(+) channels seem to have higher activity in SHR (Pintérová *et al.* 2014); and as a compensatory activation of vasodilator, BK_{Ca} channels

enhance in all forms of hypertension, almost proportionally to BP elevation (Behuliak *et al.* 2011). In the current study, enhanced BK_{Ca} channel function was demonstrated in MAs from hypertensive animals through the use of isometric tension recordings and vascular reactivity techniques, along with a BK_{Ca} channel-specific pharmacological blocker and activator. The whole-cell K^+ current through IbTX-sensitive BK_{Ca} 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 BK_{Ca} 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 (SBP), but not diastolic blood pressure (DBP) or MAP. Exercise training also increased BK_{Ca} channel activity in MA myocytes (Shi *et al*. 2013a). Interesting, the effects of exercise on BP and tension were basically the same in SHRs in the current study, though there was a discrepancy in BK_{Ca} 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 BK_{Ca} channel expression observed in the two models. It has been demonstrated that exercise training increases BK_{Ca} channel activity by changing the gating properties of BK_{Ca} channels in various arterial smooth muscle cells (Li *et al.* 2013; Shi *et al.* 2013a; Zhao and Wang 2010), thus,

exercise itself activates BK_{Ca} channels on VSMCs. However, in hypertensive rats, BK_{Ca} channels are already upregulated in MA myocytes in response to chronically high intraluminal pressure (Joseph *et al.* 2013). Therefore BK_{Ca} channel upregulation appears to be the result, rather than a cause, of hypertension. In addition, there is evidence that K_V 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). K_V is another critical channel that plays an important role in regulating membrane potential and vascular tone. A decrease in K_V channels causes depolarization of the cell membrane, resulting in an influx Ca^{2+} from L-type calcium channels. In the current study, exercise training significantly suppressed the downregulation of K_V channels in the thoracic aorta of SHRs (Li *et al.* 2014); consequently hypertension caused by K_V 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 BK_{Ca} channel upregulation in SHRs. In addition, voltage-gated L-type Ca^{2+} ($Ca_v1.2$) channels control the Ca^{2+} influx and regulate VSMCs contractility (Joseph *et al.* 2013); exercise training, or moderate exercise can normalize SHR-associated $Ca_v1.2$ 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 contraction of aortic segments from SHRs (Rusch and Runnells 1994). *In situ* studies of cerebral arterioles from hypertensive rats also provides 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} β1 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 BK_{Ca} 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 BK_{Ca} channel remodeling, thereby suppressing the pathological adaptations of BK_{Ca} 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. BK_{Ca} 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.

Acknowledgments

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Table 1 Physical characteristics of experimental rats.

	WKY	SHR	SHR-L	SHR-M
BW (g)	352.4±6.7	349.4±7.2	335.4±6.9$^{\#}$	329.2±7.1$^{\#}$
HW (g)	1.14±0.01	1.41±0.05*	1.39±0.04	1.38±0.06
HW (mg) / BW (g)	3.23±0.02	4.04±0.06*	4.14±0.07	4.19±0.11
MAP (mmHg)	118.0±5.3	185.2±6.0*	173.4±5.4$^{\#}$	160.1±5.1$^{\#\&}$
HR (bpm)	392±16	435±14*	395±16$^{\#}$	390±15$^{\#}$

*$P < 0.05$, compared with WKY; $^{\#}P < 0.05$, 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.

Figure Legends

Figure 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^{-9} 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 20mins (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.

Figure 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 BK_{Ca} 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.

Figure 3. Effects of exercise training on hypertension-associated alterations in BK_{Ca} channel activities and gating properties in MA myocytes. A: Single channel current recorded at a membrane voltage of +40mV in WKY, SHR, SHR-L, and SHR-M ($[Ca^{2+}]_{free}$ = 1μM), respectively. B: I–V relationships of BK_{Ca} current. A linear fit revealed average single channel conductances of (G) 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 BK_{Ca} channel Po (C) and conductance (D). $^{*}P < 0.05$, compared with WKY. $^{\#}P < 0.05$, compared with SHR. $^{\&}P < 0.05$, compared with SHR-L.

Figure 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 20μm. 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. (a-h). (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.

Figure 5. Protein and mRNA in BK_{Ca} α and β1 subunits in mesenteric smooth muscle cells. A: Representative Western blot illustrating BK_{Ca} α-subunit, β1-subunit, and β-actin. Specificity of the antibody was tested in mesenteric arterial smooth muscle lysate in Wistar rats. The immunoreactive band in the arterial tissue was abolished by preincubating with the corresponding control antigen, indicating specificity of the antibody for its intended epitope. B, C: Mean values of BK_{Ca} α-subunit and β1-subunit proteins (B), and the β1/α ratio (C). D: Mean quantitative PCR data for BK_{Ca} α and β1 subunit mRNA in MAs normalized to β-actin and then to age-matched WKY controls. $n = 6$ in each group. *$P < 0.01$, compared with WKY; $^{\#}P < 0.01$, compared with SHR. $^{\&}P < 0.01$, compared with SHR-L.

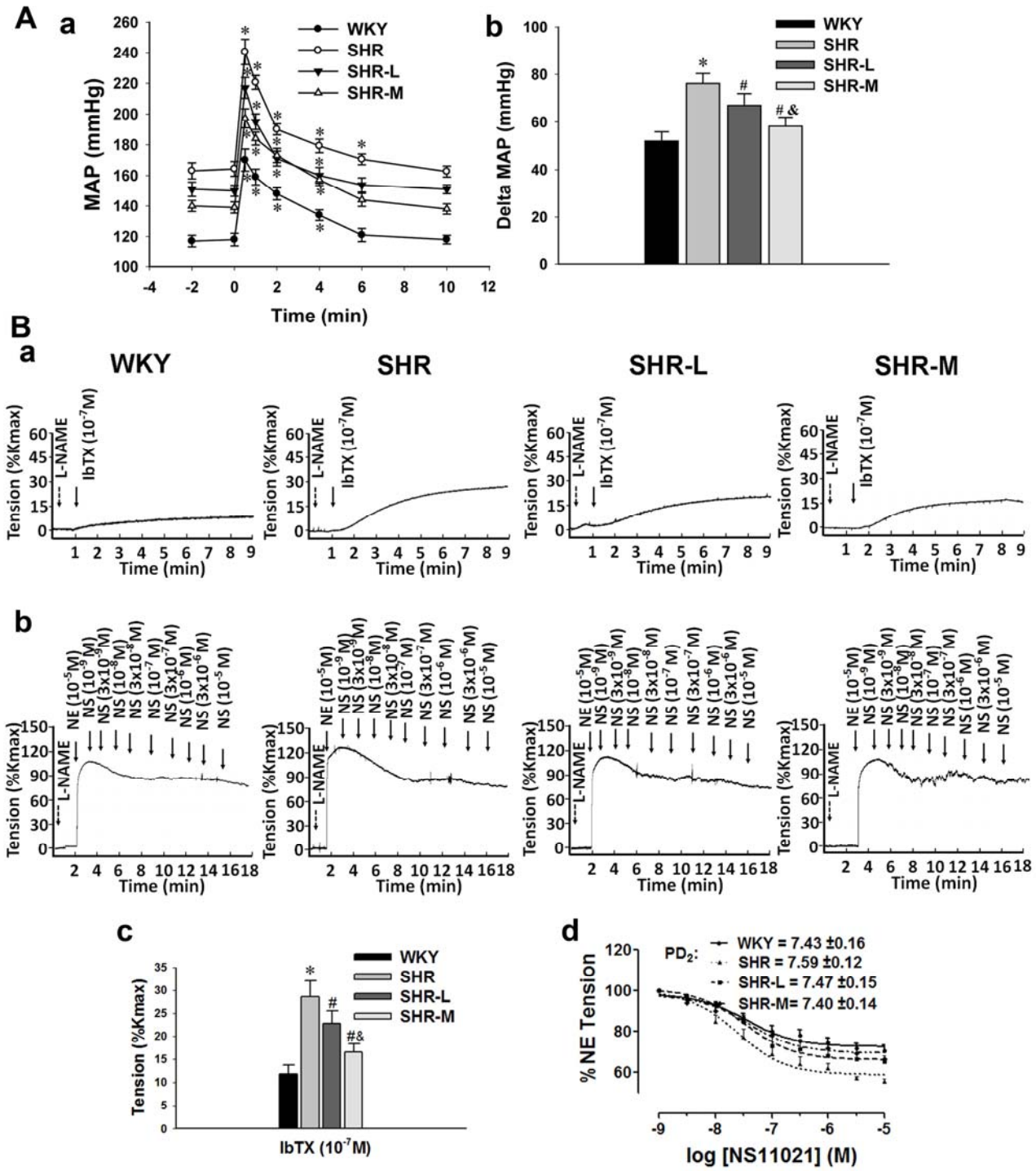


Figure 1. Effect of exercise on NE-induced cardiovascular responses and vascular tension in MAs.

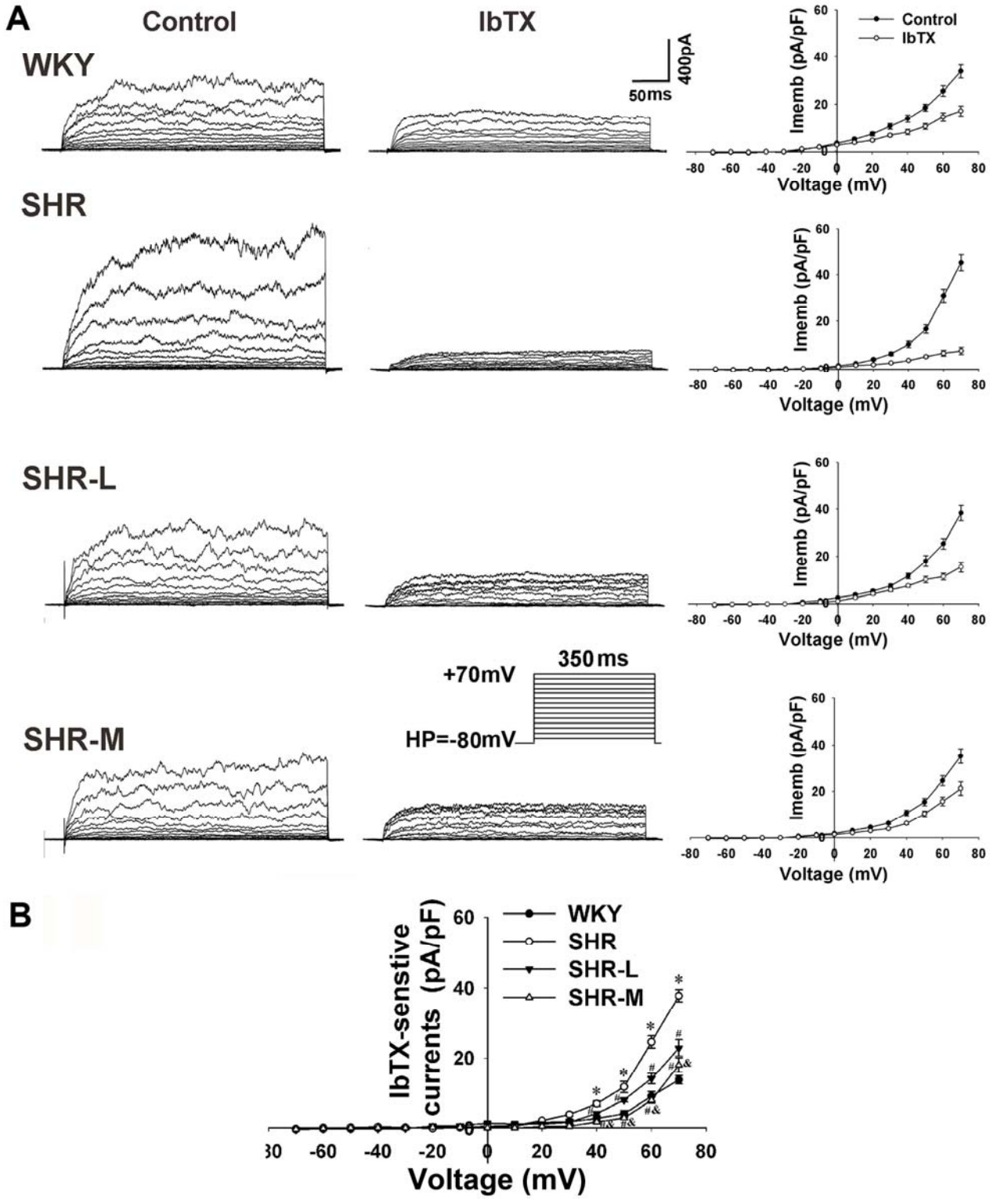


Figure 2. Whole-cell K^+ currents in MA myocytes from WKY, SHR, SHR-L, and SHR-M.

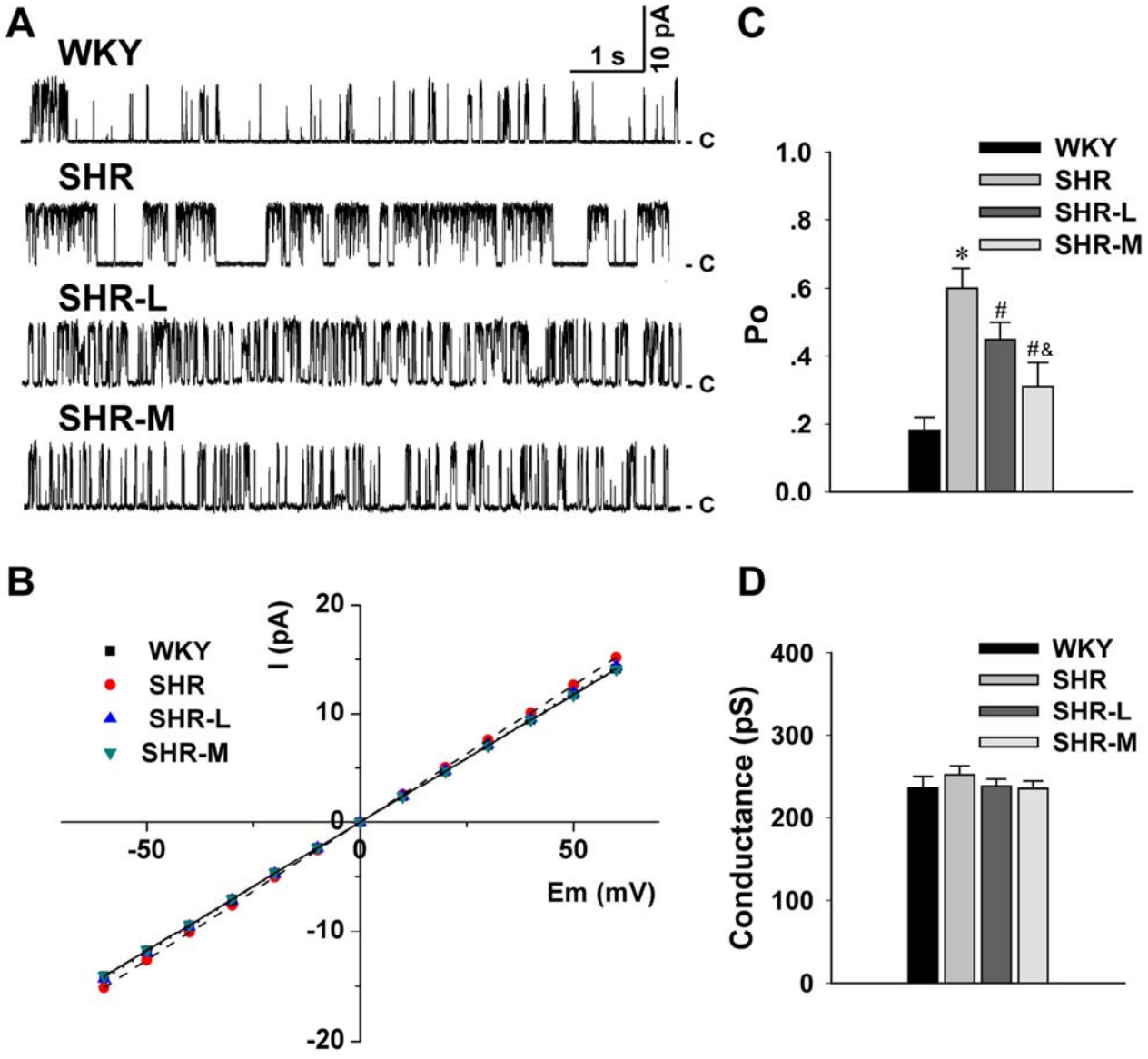


Figure 3. Effects of exercise training on hypertension-associated alterations in BK_{Ca} channel activities and gating properties in MA myocytes.

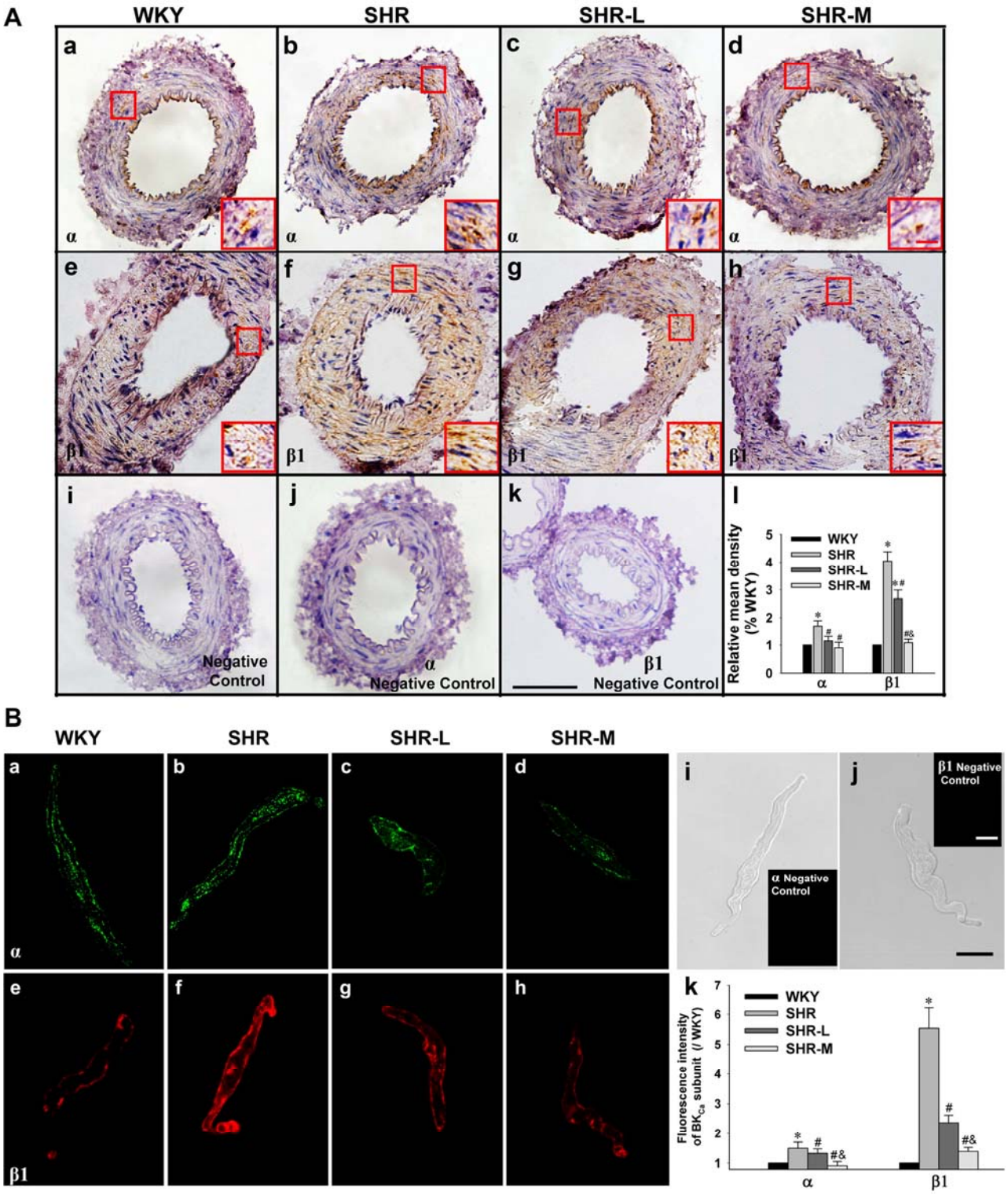
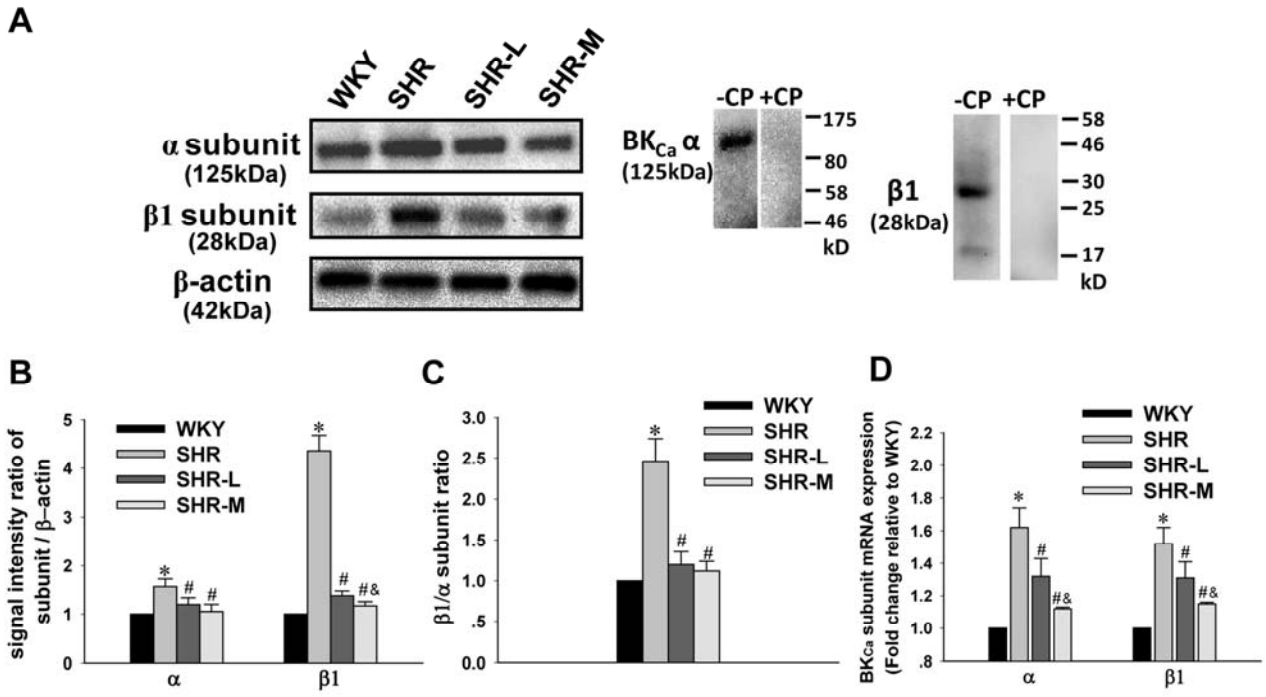


Figure 4. BK_{Ca} channel α and β1 subunits in MAs and VSMCs.



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