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ORIGINAL ARTICLE

Exercise intensity-dependent reverse and adverse remodeling of voltage-gated Ca^{2+} channels in mesenteric arteries from spontaneously hypertensive rats

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Exercise can be regarded as a drug for treating hypertension, and the 'dosage' (intensity/volume) is therefore of great importance. L-type voltage-gated Ca^{2+} ($Ca_v1.2$) channels on the plasma membrane of vascular smooth muscle cells have a pivotal role in modulating the vascular tone, and the upregulation of $Ca_v1.2$ channels is a hallmark feature of hypertension. The present study investigated the beneficial and adverse effects of exercise at different intensities on the remodeling of the $Ca_v1.2$ channel in mesenteric arteries (MAs) of spontaneously hypertensive rats (SHRs). Moderate- (SHR-M, 18–20 m min^{-1}) and high-intensity (SHR-H, 26–28 m min^{-1}) aerobic exercise training groups were created for SHRs and lasted for 8 weeks (1 h per day, 5 d per week). Age-matched sedentary SHRs and normotensive Wistar–Kyoto rats (WKY) were used as controls. The mesenteric arterial mechanical and functional properties were evaluated. Moderate-intensity exercise training induced a lower systolic blood pressure and heart rate in these rats compared with sedentary SHRs. BayK 8644 and nifedipine induced vasoconstriction and dose-dependent vasorelaxation, respectively, in the mesenteric arterial rings. Moderate-intensity exercise significantly suppressed the increase in BayK 8644-induced vasoconstriction, tissue sensitivity to nifedipine, $Ca_v1.2$ channel current density and $Ca_v1.2$ α1C-subunit protein expression in MAs from SHRs. However, high-intensity exercise training aggravated all of these hypertension-associated functional and molecular alterations of $Ca_v1.2$ channels. These results indicate that moderate-intensity aerobic training may act as a drug and effectively reverse the remodeling of $Ca_v1.2$ channels in hypertension to restore the vascular function in MAs, but that high-intensity exercise exaggerates the adverse remodeling of $Ca_v1.2$ channels and worsens the vascular function.
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Keywords: aerobic training; calcium channel; exercise intensity; SHR

INTRODUCTION

A growing body of evidence indicates that exercise is an effective nonpharmacological therapy for preventing and controlling cardiovascular diseases such as hypertension and arterial stiffness.[1–4] The beneficial effects of regular exercise (especially aerobic exercise) on general health and in reversing hypertension have been clearly shown.[5] Therefore, it has been proposed that exercise be considered a drug. As with most drugs, the dosage is of great importance. An appropriate dosage should be chosen; otherwise, unfavorable side effects may occur. The health benefits of physical activity increase with an increasing amount of activity within a certain range; beyond a certain level, however, adverse effects will outweigh the benefits.[6] Consequently, the 'dosage' (the volume and intensity of the exercise) must be taken into account in order to achieve the best clinical outcome. Although regular physical activity has a strong positive link to cardiovascular health, the dose response and maximum safe dose of physical activity, unlike those of chemical drugs, are still largely undefined.

Hypertension is a clinical syndrome characterized by increased arterial tone.[7] In addition to the impaired endothelium-dependent vasodilatation, vascular smooth muscle cells (VSMCs) exhibit pathological alterations in response to persistent high intraluminal pressure. For instance, VSMCs are more depolarized as a consequence of the ion channel remodeling that occurs in chronic hypertension.[8] L-type voltage-gated Ca^{2+} ($Ca_v1.2$) channels are the primary Ca^{2+} entry pathway in VSMCs; they regulate smooth muscle cell contractility and control regional organ blood flow and systemic blood pressure (BP).[9–12] Hypertension is associated with an elevation in arterial smooth muscle cell $Ca_v1.2$ channel protein and current density, leading to vasoconstriction.[11,12] Thus, pharmacological $Ca_v1.2$ channel blockers are an important therapeutic option for alleviating clinical

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hypertension. The administration of potent $Ca_v1.2$ channel blockers may efficiently attenuate or prevent the development of hypertension and its effects on target organs. Because exercise training is one of the most effective interventions prescribed for hypertensive individuals, it is extremely important to understand the effects of regular physiological exercise on the pathological alterations of $Ca_v1.2$ channels caused by arteriolar myocytes during hypertension. We hypothesized that appropriate intensity of exercise can lower BP and reverse the hypertension-associated $Ca_v1.2$ channel remodeling in peripheral resistant arterioles but that high-intensity exercise might exaggerate the adverse effects on $Ca_v1.2$ channel remodeling, thereby worsening vascular function and aggravating the hypertension.

Therefore, using a genetic hypertensive model of spontaneously hypertensive rats (SHRs), we investigated the effects of moderate- and high-intensity aerobic exercise training on $Ca_v1.2$ channel remodeling associated with hypertension. The information obtained will provide a better understanding of the relationship between exercise dosing and clinical outcomes, which is important for formulating public health recommendations.

MATERIALS AND METHODS

Animal protocol

Twelve-week-old male normotensive Wistar–Kyoto rats (WKY, $n=18$) and SHRs ($n=54$) were used. Animals in the hypertension group were randomly assigned into one of the following three groups: sedentary group (SHR, $n=18$), moderate-intensity exercise-trained group (SHR-M, $n=18$) and high-intensity exercise-trained group (SHR-H, $n=18$). All rats, including sedentary animals, were habituated to treadmill exercise. Each rat walked on a motor-driven treadmill at 15 m min^{-1} (0° slope) for 5 min per day for 5 days. The animals in the SHR-M and SHR-H groups ran on a motor treadmill (0° slope) at 18–20 m min^{-1} (~55–65% of the maximal aerobic velocity) and 26–28 m min^{-1} (~75–85% of the maximal aerobic velocity), respectively. Each exercise-trained group ran 60 min per day, 5 days per week for 8 weeks. Training volume (km per week) equals exercise intensity (m min^{-1}) × duration (min per day) × frequency (days per week). Hence, because the training duration and frequency were identical among the training groups, the training volume was lower in the SHR-M group (5.4–6.0 km per week) than in the SHR-H group (7.8–8.4 km per week). To determine the maximal exercise capacity, the rats underwent a progressive exercise test using an incremental speed protocol of 5 m min^{-1} every 3 min and no grade until exhaustion. The rats were considered to be exhausted when they could no longer run at the treadmill speed.[13] The treadmill exercise test was repeated after 4 weeks to adjust the training intensity.

All experimental protocols were approved by the ethical committee of the Beijing Sport University and were performed in accordance with the Chinese animal protection laws and institutional guidelines. Every effort was made to minimize the number of animals used and their suffering.

The body weight (BW) was measured weekly. The BP and heart rate (HR) were measured using an indirect tail–cuff method (BP-2010A, Softron Biotechnology, Beijing, China).

Isometric contraction studies

The mesenteric arteries (MAs) were removed after the rats were anesthetized with sodium pentobarbitone (60 mg kg^{-1}, intraperitoneal) and were decapitated using a guillotine. The tissues were placed in cold Krebs' solution with the following composition (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, and they were continuously gassed with 95% O_2 and 5% CO_2 (pH 7.4). Short segments of second-order branches (A2) were used for contractile studies with a Multi myograph system (620 M, DMT, Denmark).

The contractile response for tension was evaluated by measuring the maximum peak height and expressed as a percentage of contraction to 120 mM K^+ (K_{max}). To examine the effect of the $Ca_v1.2$ channel activator on resting tension, the vessel contractility was measured after the administration of 10^{-5} M BayK 8644 (a potent and effective calcium channel agonist). To examine the vasorelaxation effect of the $Ca_v1.2$ channel inhibitor, the tissues were first contracted with 10^{-5} M norepinephrine (NE). Then, the responses to the cumulative addition of nifedipine ($Ca_v1.2$ channel blocker, 10^{-9}–10^{-5} M) were tested.

Electrophysiological experiments

Cell preparation. Cells were obtained with an enzymatic isolation method. The MAs were cut into small pieces and then incubated at 37 °C in 2 mg ml^{-g} bovine serum albumin, 4 mg ml^{-1} papain, 1 mg ml^{-1} dithiothreitol and 0.6 mg ml^{-1} collagenase in physiological salt solution (in mM: NaCl 137, KCl 5.6, $MgCl_2$ 1, Glucose 10, HEPES 10, Na_2HPO_4 0.42, NaH_2PO_4 0.44, $NaHCO_3$ 4.2; pH 7.3; titrated with NaOH for 30–35 min). After enzymatic treatment, the vessel segments were washed with physiological salt solution and gently triturated to release individual smooth muscle cells with a fire-polished Pasteur pipette. Cells were cold-stored at 4 °C for up to 6 h until the experiments were performed.

Electrical recording. Whole-cell $Ca_v1.2$ currents were determined with a standard whole-cell voltage-clamp technique.[14] Micropipettes (2–4 MΩ resistance with the recording solutions) were made from capillary glass (1.2 mm OD, 0.9 mm ID, WPI, Sarasota, FL, USA) with a programmable puller (PC-10, Narashige, Japan). Experiments were performed at 25 °C using 20 mM barium chloride as the charge carrier to limit the current rundown. The composition of the pipette solution was (in mM) as follows: CsCl 130, HEPES 10, Na_2ATP 3, Na_2GTP 0.1, $MgCl_2$ 1.5, Glucose 10, EGTA 10 and MgATP 0.5; pH 7.3; titrated with CsOH. The bath solution contained the following (in mM): $BaCl_2$ 20, HEPES 10, glucose 5, $MgCl_2$ 1 and choline chloride 124; pH 7.4; titrated with CsOH. The voltage protocol used to record I_{Ba} consisted of a holding potential of −80 mV and a sequence of pulses ranging from −70 to +70 mV, for a duration of 350 ms, in 10 mV increments. The cell capacitance was measured by applying a 5-mV test pulse and correcting transients with series resistance compensation. The series resistance was 70–80%, which was compensated to give a final value below 10 MΩ. Amplified currents were filtered at 2 kHz and acquired using a Digidata 1440 (Axon Instruments, Silicon Valley, CA, USA) analog to a digital converter acquisition board at 10 kHz. Data were analyzed using the PClamp10.2 software (Axon Instruments). In a subset of cells, 0.1 μM nifedipine was used to verify the identity of the $Ca_v1.2$ channel currents, and 5 μM BayK 8644 was used to activate the $Ca_v1.2$ channel currents. The effects of drugs were determined in the same cell by comparing currents before and after drug application.

To describe the voltage dependence of the activation of $Ca_v1.2$ channels, the current amplitude relative to its maximum value was fitted with a Boltzmann function that had the form $P=1/\{1+\exp[(V_h—V)/k]\}$, where P is the current amplitude, measured as the ratio I/I_{max} and I_{max} is the maximum current amplitude of the whole-cell $Ca_v1.2$ currents. V_h is the potential for half-maximal activation, and k is a steepness factor (Boltzmann coefficient). To gain further insight into the time and voltage dependence of the inactivation of $Ca_v1.2$ channels, steady-state inactivation was determined using a standard two-step protocol. The protocol consisted of a 2000-ms conditioning prepulse to voltages from −100 to +50 mV, in steps of 10 mV, followed by a 40-ms test potential of +20 mV with a fixed 20-ms interpulse interval at the holding potential of −80 mV. Pulses were applied every 30 s. The steady-state inactivation was also measured as the ratio I/I_{max}, where I_{max} is the maximum current amplitude during the test pulse, after the most hyperpolarizing prepulse (−100 mV). The steady-state inactivation curve was drawn by fitting the data to a Boltzmann function with the form $P=1/\{1+\exp[(V-V_h)/k]\}$, where P is the relative amplitude.

Immunofluorescence

For immunofluorescence staining, the VSMCs of MA were plated on poly-L-lysine-coated coverslips and fixed in 4% paraformaldehyde in phosphate-buffered saline for 20 min. Then, VSMCs were permeabilized with 0.2% Triton X-100 for 10 min. After extensive washing with phosphate-buffered saline, the VSMCs were blocked for nonspecific antibody binding with 5% bovine serum albumin (1 h), which was followed by overnight incubation at 4 °C in primary Rabbit polyclonal anti-α1C (Alomone Labs, Jerusalem, Israel) antibody, with

1% BSA, at a dilution of 1:200. 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. The VSMCs were also stained with 4′,6-diamidino-2-phenylindole at a dilution of 1:1000 for 10 min. Sample coverslips were plated on ProLong Gold Antifade Reagent (Molecular Probes) on the glass slides, and the edges were sealed. Images were acquired using a laser-scanning confocal microscope (TCS-SP5, Leica, Wetzlar, Germany) and analyzed using the ImageJ software (NIH, Bethesda, MD, USA, version1.46r).

Western immunoblotting

Membrane proteins were isolated and pooled for use in western blots as previously described.[15] Briefly, equivalent amounts (20 μg) of total protein from the MAs were added to adjacent lanes. The antibody was polyclonal anti-α1C (1:200), which was purchased from Alomone Laboratories. After incubation with a secondary antibody (anti-rabbit IgG-HRP, 1:6000, Proteintech Group, Wuhan, Hubei, China) for 1 h, immunoreactive brands were visualized with enhanced chemiluminescence, and signals were recorded with Bio-Rad ChemiDOC XRS+ (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was detected and used to correct for the equal loading of all samples. The protein band intensities were determined using the Quantity One (Bio-Rad) software. For quantification, the protein band intensities were first normalized to GAPDH and then to the appropriate control.

RT-PCR

MAs were placed in RNAlater (Ambion, Austin, TX, USA) at 4 °C. We isolated total RNA with a PureLink RNA Mini Kit (Ambion) and reverse-transcribed the RNA into cDNA with the GoScript Reverse Transcription System (Promega, Madison, WI, USA), according to the manufacturer's instructions. Primers specific to L-type channel α1C (Cacna1c; GenBank accession no. NM_012517; amplicon = 70 bp; forward: 5′-GAGAGCTTTCCGTGTGCTTC-3′ and reverse: 5′-GTTCAGGACCACCTGGAGAC-3′) and the housekeeping genes GAPDH (GenBank accession no. NM_017008; amplicon = 104 bp; forward: 5′-CCAGGTTGTCTCCTGTGACTTC-3′ and reverse: 5′-ACCAGGAAATGAGCTTCACAAA-3′) were designed to identify the presence of each transcript in the mesenteric arterial vascular smooth muscle. PCR was performed in a RT-PCR machine (Bio-Rad) with Taq 2X Master Mix (New England Biolabs, Beverly, MA, USA). After initial denaturation for 3 min at 94 °C, PCR cycling included 32 cycles as follows: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, which was followed by a final extension step at 72 °C for 5 min. Amplicons were verified using 1.5% agarose gel electrophoresis.

Chemicals

All chemicals were purchased from Sigma-Aldrich (China (Mainland)) unless otherwise stated.

Statistical analysis

Data were expressed as the mean ± s.e.m. The term *n* represented the number of cells or animals used in each experiment. Where appropriate, differences were evaluated for statistical significance ($P<0.05$) by a one-way analysis of variance or *t*-test.

RESULTS

Physical characteristics of the rats

Among these animals, one rat in the SHR-H group died after exercise training in the second training week and could not be used for further analysis. There were no significant differences in the BWs between the WKY and SHR groups at the beginning of the study. However, at the end of the study, the BWs in the SHR exercise groups were significantly lower than those of their sedentary counterparts (Table 1), and the BW in the high-intensity group was markedly lower than that of the moderate-intensity group. Although the heart weight (HW) in the SHRs was higher than in WKY rats, no significant differences were observed among the hypertension groups. Similarly, the HW to BW ratio (HW/BW) in the SHRs was higher than that in the normotensive WKY rats, whereas in the SHR-H group (4.38 ± 0.07), the HW/BW was higher than those of either the SHR-M group (4.19 ± 0.10) or sedentary SHR group (4.03 ± 0.07).

Table 1 Physical characteristics of rats

	WKY (n = 18)	*SHR (n = 18)*	*SHR-M (n = 18)*	*SHR-H (n = 17)*
BW (g)	349.1 ± 7.2	349.7 ± 8.5	331.4 ± 5.2[a]	319.2 ± 6.8[a,b]
HW (g)	1.15 ± 0.01	1.41 ± 0.05[c]	1.39 ± 0.05	1.40 ± 0.06
HW (mg)/BW (g)	3.29 ± 0.04	4.03 ± 0.07[c]	4.19 ± 0.10	4.38 ± 0.07[a,b]
SBP (mm Hg)	133.0 ± 6.7	196.4 ± 5.5[c]	165.3 ± 7.2[a]	208.4 ± 6.4[a,b]
HR (beats per min)	383 ± 16	433 ± 13[c]	392 ± 15[a]	425 ± 11[b]

Abbreviations: BW, body weight; HR, heart rate; HW, heart weight; SBP, systolic blood pressure; WKY, Wistar–Kyoto rats.
[a]$P<0.05$ compared with SHR.
[b]$P<0.05$ compared with SHR-M.
[c]$P<0.05$ compared with WKY.

As shown in Table 1, the systolic blood pressure (SBP) and HR in the SHRs were significantly higher than those in the WKY rats. After 8 weeks of exercise, the SBP and HR in the SHR-M group were significantly lower than those in the sedentary SHR group. However, in the SHR-H group, the SBP was higher than that in SHRs. There was no significant difference in the HR between SHR-H and SHR groups. While compared with SHR-M group, the SBP and HR in the SHR-H group were markedly higher.

Moderate-intensity exercise abrogates but high-intensity exercise aggravates the role of $Ca_v1.2$ channel in regulating vascular tone in MAs for SHRs

To determine the $Ca_v1.2$ channel contribution to the vascular tone, the effects of the $Ca_v1.2$ channel activator or inhibitor on the contraction/relaxation of MA rings were measured. In each experiment, the nonselective nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) was added 20 min before the contraction studies. The maximal response induced by KCl (120 mM) in L-NAME-treated animals was similar in the four groups. As shown in Figures 1Aa and B, BayK 8644 (10^{-5} M) elicited a tension increase in all groups that was much higher in SHRs than in WKY rats, whereas moderate-intensity exercise training (MIT, that is, ~55–65% of the maximal aerobic velocity) markedly inhibited this increase. Interestingly, high-intensity exercise training (HIT, that is, ~75–85% of the maximal aerobic velocity) significantly augmented the BayK 8644-induced tension increase.

NE (10^{-5} M) also induced a significant increase in vascular tone. The maximal NE-induced force in SHRs was 132.2 ± 6.7%*K*max, which was higher than that of the WKY (105.6 ± 5.8%*K*max, $P<0.01$) and SHR-M (114.3 ± 5.2%*K*max, $P<0.01$) groups. However, after HIT, the NE-induced maximal tension increase (SHR-H, 142.4 ± 6.5% *K*max) was significantly higher than that of SHR-M (Figures 1Ab and B). As shown in Figure 1Ab, at the plateau of NE-induced contraction, nifedipine (10^{-9}–10^{-5} M) was administered in half-log increments. A parallel leftward shift of the concentration–relaxation curve was detected in hypertensive rats. Here, the 10^{-5}-M NE-induced maximal tension increase was treated as 100% in each group. The pIC50 values (negative logarithm of the molar concentration required to block 50% of the NE-induced contraction) in the four groups were 7.14 ± 0.14 (WKY), 7.56 ± 0.07 (SHR), 7.35 ± 0.09 (SHR-M) and 7.80 ± 0.07 (SHR-H; $n=6$ each group, Figure 1C). Therefore, the ranking of the sensitivity of the MA to nifedipine was SHR-H > SHR > SHR-M > WKY (all $P<0.05$). These results indicated that the contribution

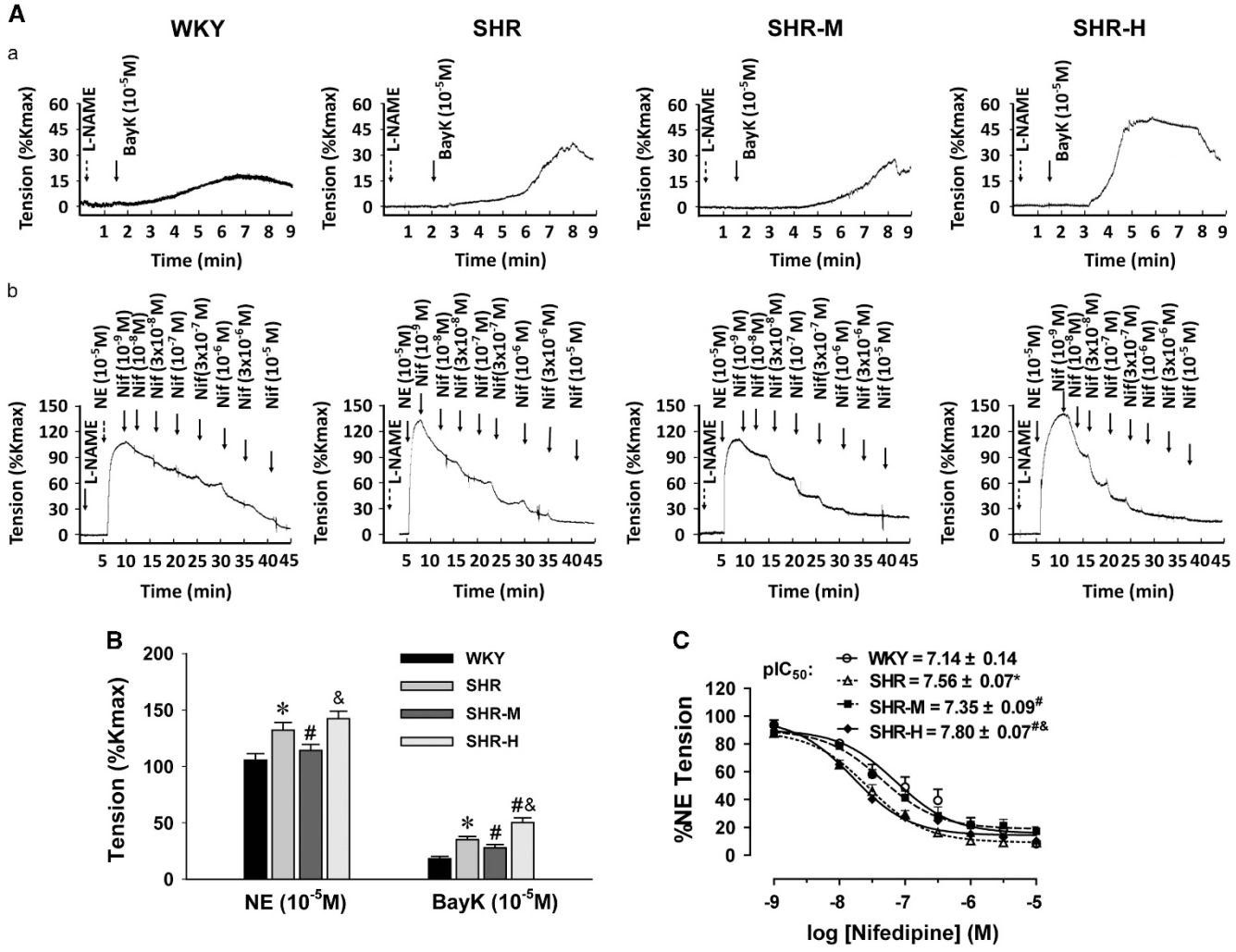

Figure 1 Effects of the $Ca_v1.2$ channel activator or inhibitor on the vascular tension in MAs from the WKY, SHR, SHR-M and SHR-H groups. (**A**) Typical experimental tracings showing the following: (a) vascular tone induced by BayK 8644 (10^{-5} M) and (b) effect of the $Ca_v1.2$ channel inhibitor nifedipine (10^{-9}–10^{-5} M) on NE-induced vessel contraction. In each experiment, the arteries were preincubated with the nonselective nitric oxide synthase inhibitor L-NAME (100 μM) for 20 min (dotted arrows). (**B**) Statistic diagram of BayK 8644-induced vessel contraction. (**C**) Concentration–response curves of nifedipine on NE (10^{-5} M)-induced contraction. $^{*}P<0.05$, *vs.* WKY; $^{\#}P<0.05$, *vs.* SHR; and $^{\&}P<0.05$, *vs.* SHR-M. $n=6$ in each group. BayK, BayK 8644 and Nif, nifedipine.

of the $Ca_v1.2$ channels in vascular tone regulation was significantly increased in SHRs but that MIT significantly ameliorated while HIT significantly augmented this change.

Moderate-intensity exercise inhibits, but high-intensity exercise enhances, the SHR-associated increase in $Ca_v1.2$ channel currents in MAs

The electrophysiological properties of $Ca_v1.2$ channels in myocytes from small MAs were determined. Figure 2A illustrates the typical records of the whole-cell Ca^{2+} currents recorded in VSMCs isolated from MAs. The peak inward current was recorded at +20 mV in all four groups. BayK 8644 (5 μM) augmented the peak inward Ba^{2+} current (I_{Ba}) and negatively shifted the *I–V* relationship (peak $Ca_v1.2$ current densities shifted to 0 mV), whereas nifedipine (0.1 μM) almost completely suppressed the inward currents. These properties suggest that the recorded inward currents were Ba^{2+} currents through $Ca_v1.2$ channels. To obtain the *I–V* curve of $Ca_v1.2$, the current densities were plotted against the corresponding command potentials (Figure 2B). The membrane Ba^{2+} currents are expressed relative to the cell capacitance to compensate for differences in the cell size (pA/pF). The mean cell capacitance for WKY (17.2 ± 1.3 pF, $n=20$ cells per six rats) and SHR (17.5 ± 1.5 pF, $n=22$ cells per six rats) cells were similar and were not significantly changed by exercise training (SHR-M, 16.8 ± 1.6 pF, $n=24$ cells per six rats; SHR-H, 17.1 ± 1.6 pF, $n=18$ cells per six rats; $P>0.05$ when comparing all). The mean peak $Ca_v1.2$ current density was −18.5 ± 2.1 pA/pF in hypertensive SHR cells compared with −9.1 ± 0.3 pA/pF in WKY cells; the former was thus approximately twofold larger (Figure 2 and Table 2). However, the $Ca_v1.2$ current densities were significantly different for different exercise-training intensities. MIT reduced the peak $Ca_v1.2$ current density in SHR cells to −11.6 ± 1.1 pA/pF (or by ~62.7%), and HIT increased it to −27.6 ± 2.7 pA/pF (or by ~149.2%).

The activation and steady-state inactivation curves of mesenteric $Ca_v1.2$ channels and the mean values of the Boltzmann fit parameters are summarized in Figure 2C and Table 2. The voltage dependence of the half-maximal $Ca_v1.2$ current activation (V_h) and slope (k) were similar in the WKY, SHR, SHR-M and SHR-H myocytes. The voltage dependence of the half-maximal inactivation and slope were also

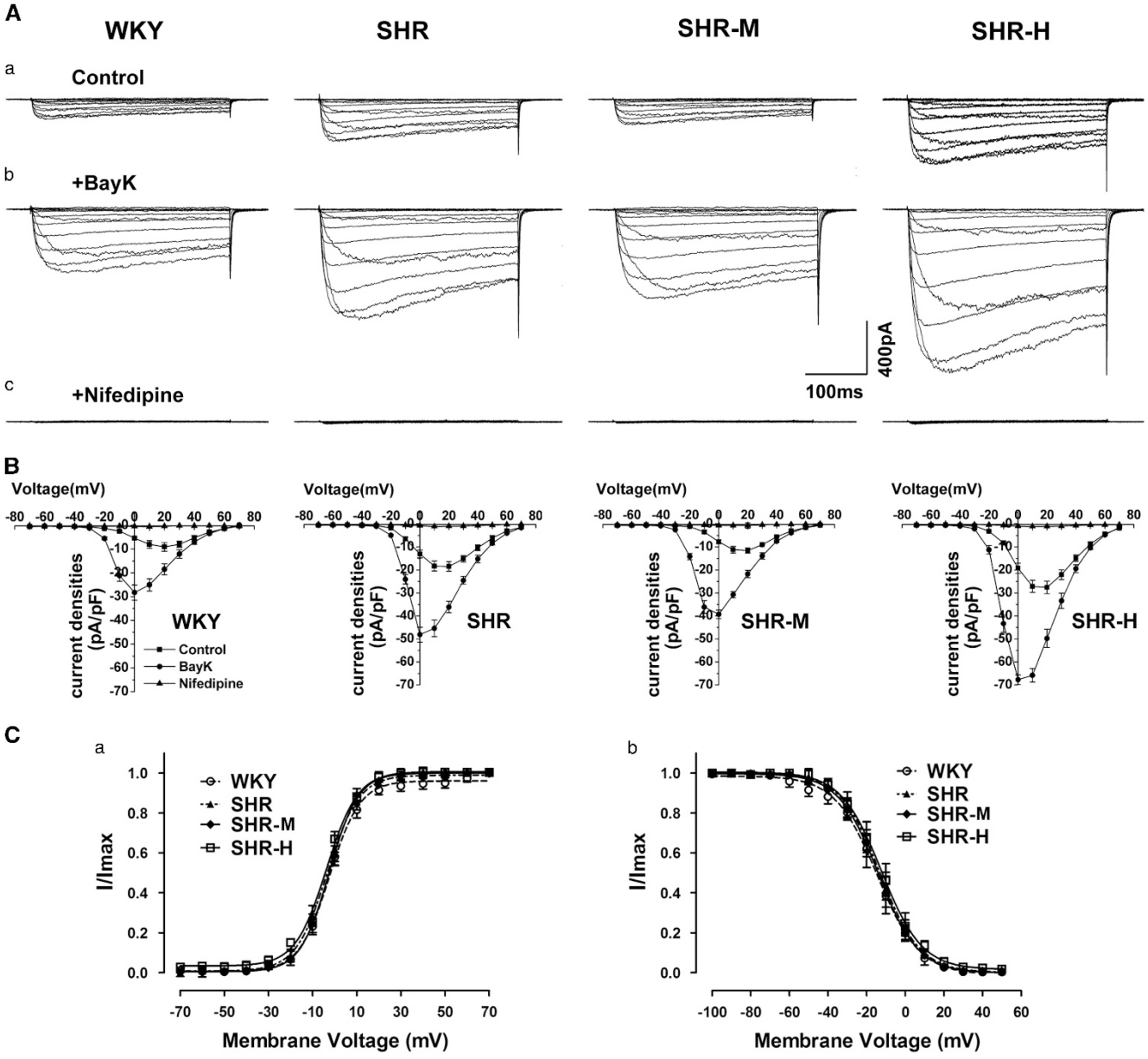

Figure 2 Whole-cell $Ca_v1.2$ currents recorded in the myocytes of the MAs from the WKY, SHR, SHR-M and SHR-H groups. Arterial myocytes were freshly isolated from the MAs. (**A**) Traces of Ca^{2+} channel currents evoked by command potentials (−70 to +70 mV in a 10 mV step) in the absence (a) or presence of BayK 8644 (b, 5 μM) or nifedipine (c, 0.1 μM). (**B**) Current–voltage relationships of the $Ca_v1.2$ currents in the VSMCs. The current amplitudes at various command potentials were normalized by the cell capacitance and plotted. (**C**) Steady-state inactivation and activation curves of the Ba^{2+} currents in mesenteric myocytes from the WKY, SHR, SHR-M and SHR-H myocytes.

similar in the four groups. All of these data indicated that hypertension was associated with an enhancement of the functional expression of $Ca_v1.2$ channels. MIT attenuated these changes, whereas HIT exaggerated the adverse functional remodeling of $Ca_v1.2$ channels in MA myocytes from SHRs.

Moderate-intensity exercise suppresses, but high-intensity exercise augments, the upregulation of $Ca_v1.2$ channel α1C protein expression in the MAs from SHRs

To investigate the molecular mechanism underlying the $Ca_v1.2$ current modifications, we examined the $Ca_v1.2$ channel α1C subunit protein expression by immunohistochemical staining and western blotting.

Mesenteric myocytes were freshly isolated for immunocytochemical analysis, and the fluorescence intensities of a total of 32 cells were analyzed individually. As shown in Figures 3A and B, the fluorescence signals from the SHR cells labeled with the anti-α1C antibody were higher than those in the WKY cells when exposed for equivalent times, showing a significantly increased average intensity of ~2.9-fold in the SHRs. Similarly, MIT suppressed this alteration (~1.5-fold of WKY), but HIT reinforced it (~3.1-fold of WKY).

Subsequently, western blot analysis was used to compare the expression of the α1C subunit protein in the four groups (Figures 4a and b). The α1C subunit was overexpressed in the mesenteric myocytes of SHRs (3.51 ± 0.25, $n = 6$) compared with

Table 2 Properties of $Ca_v1.2$ currents from mesenteric arterial myocytes

	WKY (n = 6)	*SHR (n = 6)*	*SHR-M (n = 6)*	*SHR-H (n = 6)*
I–V relationship				
Peak current density (pA/pF)	−9.1 ± 0.3 (15)	−18.5 ± 2.1 (18)[a]	−11.6 ± 1.1 (16)[b]	−27.6 ± 2.7 (15)[b, c]
Voltage-dependent activation				
$V_{h\text{-}act}$ (mV)	−2.33 ± 0.55 (12)	−3.35 ± 0.65 (12)	−2.49 ± 0.52 (12)	−3.69 ± 0.72 (12)
Slope (*k*)	6.87 ± 0.48 (12)	7.11 ± 0.53 (12)	6.53 ± 0.45 (12)	7.07 ± 0.54 (12)
Voltage-dependent inactivation				
$V_{h\text{-}inact}$ (mV)	−14.50 ± 1.04 (10)	−13.20 ± 0.97 (9)	−13.99 ± 0.94 (10)	−12.37 ± 1.09 (8)
Slope (*k*)	−10.92 ± 0.93 (10)	−10.38 ± 0.86 (9)	−10.34 ± 0.83 (10)	−10.47 ± 0.97 (8)

Abbreviations: WKY, Wistar–Kyoto rats.
Numbers in parenthesis indicate experimental number of cells from six animals in each group.
[a]$P<0.05$ compared with WKY.
[b]$P<0.05$ compared with SHR.
[c]$P<0.05$ compared with SHR-M.

WKY rats (1.00 ± 0.00, $n=6$), which was ameliorated by MIT (SHR-M, 2.51 ± 0.31, $n=6$). However, HIT significantly increased, rather than inhibited, the α1C protein expression (6.51 ± 0.50, $n=5$) compared with its sedentary counterparts (all $P<0.05$).

Moderate-intensity exercise reverses, but high-intensity exercise aggravates, the upregulation of the $Ca_v1.2$ channel α1C mRNA in the MAs from SHRs

We also examined the $Ca_v1.2$ channel α1C expression at the transcriptional level using conventional RT-PCR (Figures 4c and d). GAPDH was used as the reference gene. The results showed that the mean α1C mRNA levels were ~2.5-fold (2.53 ± 0.18, $n=6$) higher in SHRs compared with the arteries from WKY rats (1.00 ± 0.00, $n=6$). However, MIT significantly reversed (1.08 ± 0.13, $n=6$), but HIT markedly aggravated (3.14 ± 0.17, $n=6$) this elevation.

DISCUSSION

In our recent study, we demonstrated that chronic aerobic exercise can normalize the changes in the $Ca_v1.2$ and Kca1.1 channels in the MAs from SHRs.[16] The present study focuses primarily on the effect of the exercise intensity/volume on $Ca_v1.2$ channel remodeling in arterioles from SHRs. Consistent with our hypothesis, this study provides the initial functional and molecular evidence that high-intensity exercise aggravates hypertension and exaggerates the adverse remodeling of $Ca_v1.2$ channels in mesenteric arterial smooth muscle cells from SHRs, whereas moderate-intensity exercise reduces the BP and reverses the $Ca_v1.2$ channel remodeling on arteriole myocytes.

SHRs exhibited a higher HR, pressure and pulse pressure compared with WKY rats. Although it was recently proposed that exercise can be considered a drug, it is not easy to issue a public recommendation for physical activity in patients with hypertension.[2] The dosage (volume and intensity of the exercise), frequency of administration (sessions per week), type (aerobic *vs.* resistance exercise) and side effects of exercise must be taken into account to achieve the best clinical outcome.

In the present study, at the end of the 8 weeks of moderate-intensity aerobic exercise training, rats in the SHR-M group had a decreased SBP and HR, suggesting that an appropriate intensity of exercise training may be an effective therapy for hypertension. This decrease in the BP may be attributed to the attenuation of the sympathetic activity to the heart, which can lead to bradycardia and, consequently, a reduction in the cardiac output.[17,18] This lower sympathetic activity induced by exercise training could also be attributed to an improvement of the arterial baroreflex and chemosensitive cardiopulmonary baroreflex sensitivity in SHRs,[18–21] which modulates the peripheral autonomic nervous system. In addition, other peripheral mechanisms are very important. The small arteries and arterioles undergo extensive biological and structural adaptations in response to the elevated intraluminal perfusion pressure that occurs during chronic hypertension. The underlying pathophysiological processes appear to be complex, and they likely involve vascular remodeling, endothelial dysfunction, smooth muscle cell hypertrophy and changes in the extracellular matrix composition and function.[22,23] The net effect of these adaptive changes is augmented vasoconstriction and impaired vascular relaxation to various physiological stimuli, which result in elevated vascular tone in the arteries and arterioles.[24,25] Azevedo *et al.*[26] demonstrated that the observed BP decrease in moderate-intensity treadmill-trained SHRs was due to the attenuation in the total peripheral vascular resistance.[26] The probable mechanisms of this reduction include an improvement in endothelial function that occurs after increasing the shear stress and stimulating an effect on the production of nitric oxide by the endothelium.[27] In addition, the pathological vascular remodeling in the arteries and arterioles during hypertension is markedly suppressed.

The benefits of exercise on cardiovascular health are almost certain; however, the negative effects of exercise overdose on cardiovascular function are largely unknown. One previous study showed that high-intensity aerobic exercise may accelerate hypertensive heart disease and improve fibrosis.[28] In our study, one animal in the SHR-H group died after one session of high intensity of exercise training. Moreover, the BP and HR in the SHR-H group did not decrease; instead, they increased significantly compared with the sedentary SHR group. These results are in line with the results obtained by da Costa Rebelo *et al.*,[28] who suggested that high-intensity aerobic exercise must be considered an important risk factor rather than a therapeutic intervention.

There are considerable data in the literature suggesting that augmented Ca^{2+} influx through the $Ca_v1.2$ channels contributes to the augmented peripheral resistance and contractile responses of the vascular smooth muscle in hypertension. Using vascular isometric contraction recording along with $Ca_v1.2$ channel-specific pharmacological activators (BayK 8644) and blockers (nifedipine), we demonstrated the enhanced function of the $Ca_v1.2$ channels in the arterioles of SHRs, which was in line with previous reports.[29,30] However, at the end of MIT, both the NE-induced vasoconstriction and the sensitivity

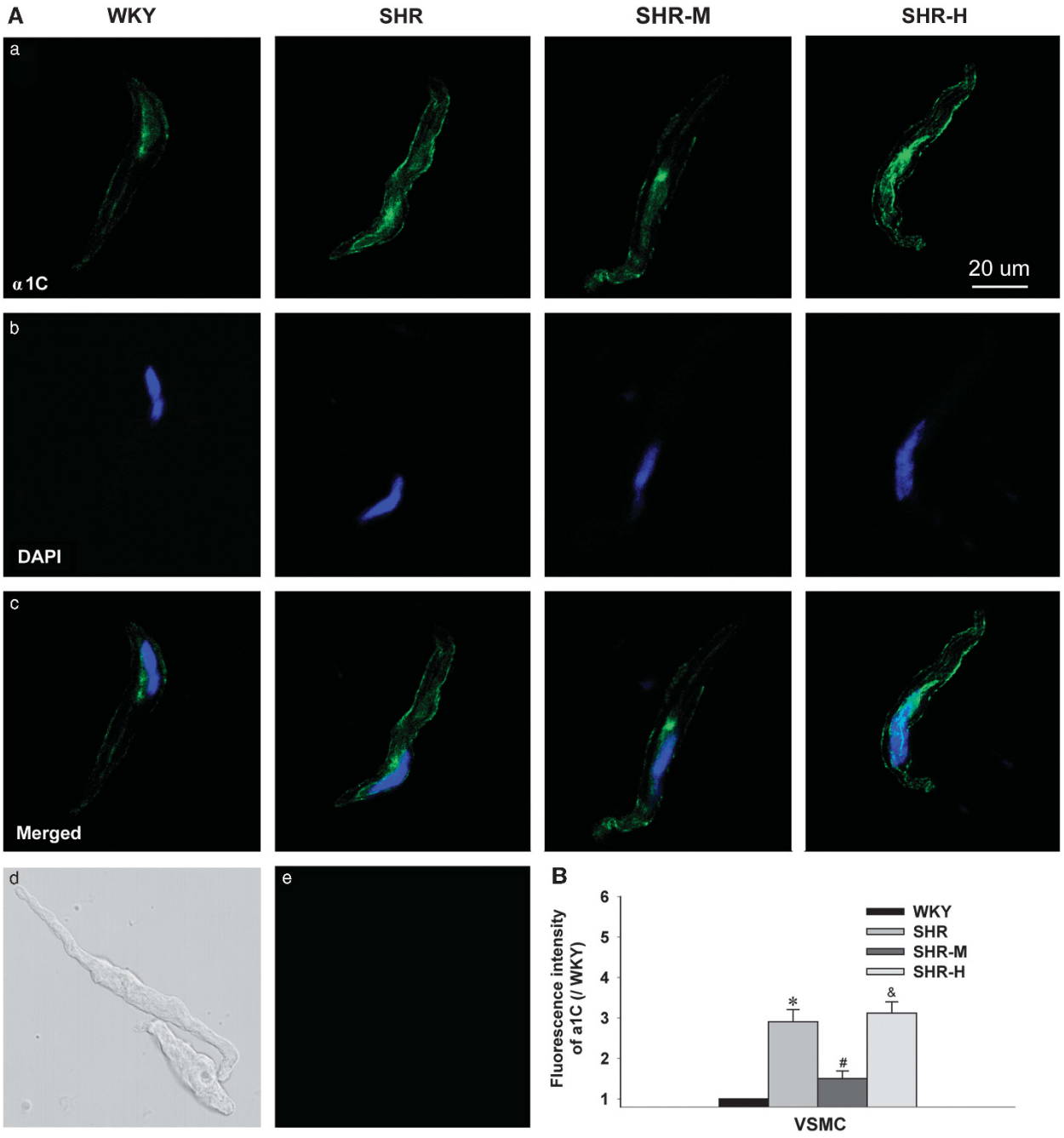

Figure 3 Fluorescent microphotographs of confocal microscopy images and quantitative analysis of $Ca_v1.2$ α1C subunit arterial myocytes. (**A**a) Representative positive immunostaining of the α1C subunit in isolated VSMCs from the WKY, SHR, SHR-M and SHR-H groups. (b) Blue indicates 4′,6-diamidino-2-phenylindole (DAPI) staining of the nuclei. (c) Overlay images with the α1C subunit (green) and DAPI (blue). (d) Bright field image of the VSMCs after staining. (e) Representative image of VSMCs stained with α1C antibody + blocking peptide. (**B**) The mean data illustrating the fluorescence intensity of the α1C subunit (WKY). $^{*}P<0.05$ *vs.* WKY; $^{\#}P<0.05$ *vs.* SHR; and $^{\&}P<0.05$ *vs.* SHR-M.

of the MA to nifedipine were decreased compared with untreated SHRs, thus providing strong support that appropriate exercise may correct the upregulation of the $Ca_v1.2$ channel function in maintaining vascular tone in SHRs. Interestingly, HIT induced the opposite effects. NE-induced vasoconstriction increased even more in the SHR-H group than in sedentary SHRs, which indicated that the hypertension-associated increase in the vasoconstriction in response to vasoconstrictors became even heavier after HIT. Both BayK 8644-induced vasoconstriction and the sensitivity of MA to nifedipine were higher in the SHR-H group than in SHRs, indicating that the role of the $Ca_v1.2$ channel in vascular tension regulation was elevated even more for an overdose of exercise training.

Previous electrophysiological studies have demonstrated elevated Ca^{2+} currents in freshly isolated VSMCs from cerebral, mesenteric, renal, skeletal and pulmonary arteries in various hypertensive animal models.[29–31] In addition, the enhanced amplitude of the whole-cell

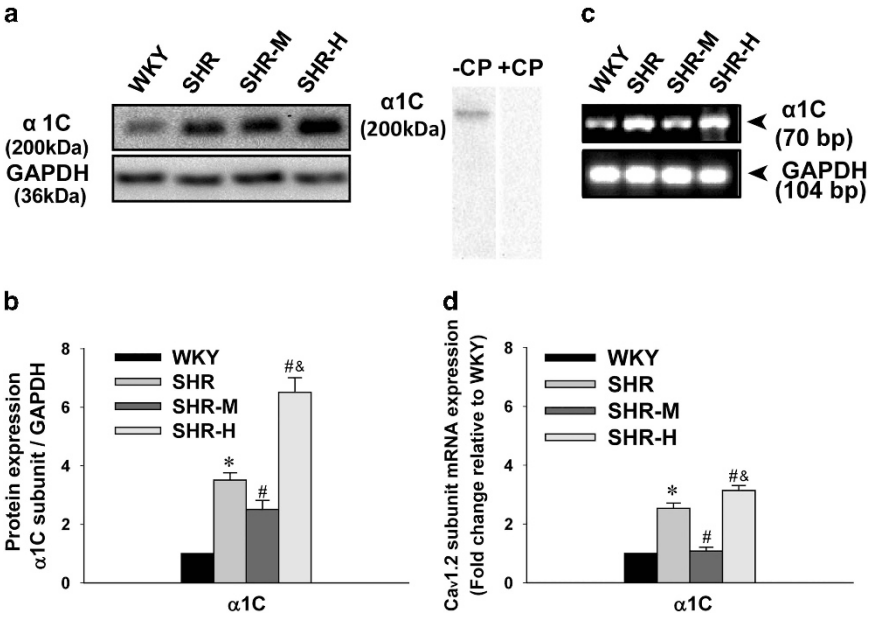

Figure 4 Protein expression of the $Ca_v1.2$ channel (α1C−) subunit in the mesenteric smooth muscle cells. (**a**) Immunoreactive bands corresponding to the α1C− subunit and GAPDH detected by western blot analysis (left panel). Right panel, the α1C− subunit was recognized in the left lane as a 200-kDa band in the MAs of WKY rats (−CP), but it was absent in the right lane after competing peptide inhibition (+CP). (**b**) Summarized data of the α1C− subunit protein levels expressed as a ratio to the GAPDH levels ($n=6$ for the WKY, SHR and SHR-M groups; $n=5$ for SHR-H). (**c**) Representative agarose gel showing the $Ca_v1.2$ channel α1C− subunit and GAPDH (control) mRNA amplicons from the arterial myocytes in the four groups. (**d**) The mean PCR data for the $Ca_v1.2$ channel α1C mRNA in the MAs normalized to GAPDH and then to age-matched WKY controls. $n=6$ in each group. $^{*}P<0.01$ compared with WKY rats; $^{\#}P<0.01$ compared with SHRs; and $^{\&}P<0.05$ *vs.* SHR-M.

current is attributable to the increased number of the $Ca_v1.2$ channel openings instead of the change in the single channel conductance, open-time distribution or voltage sensitivity.[32] In the present study, we found that the peak $Ca_v1.2$ currents were about twice as high in the mesenteric arterial myocytes of SHRs than in those of WKY rats, which was in agreement with previous reports.[29–31,33,34] Some previous studies have also investigated the effects of exercise on the $Ca_v1.2$ channels in specific arteries from normal animals. Bowles *et al.*[35] demonstrated that the smooth muscle L-type Ca^{2+} current density is increased within the coronary arterial bed by endurance exercise training in miniature swines. This increased voltage-gated calcium channel density may provide an important mechanistic link between functional and cellular adaptations in the coronary circulation in response to exercise training. However, in hypertensive animals, the Ca^{2+} currents in myocytes from various arteries are significantly increased.[29–31] Will exercise training continue to increase the Ca^{2+} currents or suppress it instead? No answer can be found in previous reports. In the present study, we demonstrated that after a period of MIT, this increase in the $Ca_v1.2$ channel functional expression was effectively ameliorated. However, after HIT, the discrepancy in the $Ca_v1.2$ current density between WKY rats and SHRs increased, thus providing further evidence that HIT accelerates the adverse remodeling of the $Ca_v1.2$ channels in MA myocytes from hypertension. No changes were observed in the voltage dependence activation and inactivation kinetics among the four groups.

Immunostaining in single myocytes and the western blot assay showed that the molecular expression of the pore-forming α1C subunit of the $Ca_v1.2$ channel was significantly elevated in the arteries of hypertensive animals compared with age-matched normotensive animals. This finding indicates that the profound upregulation in the vascular $Ca_v1.2$ channel function observed in hypertensive animals is largely because of the increased channel expression. With respect to the relationship between the exercise dose and effects, we found, for the first time, that MIT significantly inhibited the protein expression of the α1C subunit protein, which was associated with hypertension. Conversely, HIT exaggerated this pathological remodeling of the $Ca_v1.2$ channels in MA myocytes. All of these immunostaining and western blotting results strongly supported the functional study.

At the mRNA level, we also found that the $Ca_v1.2$ α1C mRNA levels in the MAs were higher in SHRs than in WKY rats. Obviously, the increase in α1C (~2.5-fold) mRNA cannot fully account for the elevation in its protein (~3.5-fold) during hypertension. These data indicate that both transcriptional and post-translational mechanisms elevate α1C proteins in the MA myocytes during hypertension. In other words, in addition to transcriptional activity, post-transcriptional mechanisms, such as increased translation efficiency, increased trafficking of channel proteins to the plasma membrane and increased stability of the channel protein complex, may contribute to the upregulation of α1C protein in the vasculature during hypertension.[36] After MIT, α1C decreased to ~1.1-fold of WKY rats at the mRNA level; however, at the protein level, α1C decreased to ~2.5-fold of WKY rats. By contrast, after HIT, α1C increased to ~3.1-fold of WKY rats at the mRNA level, whereas it increased to ~6.5-fold of WKY rats. These results suggest that exercise training affects the post-transcriptional processes much more than the transcriptional activity of $Ca_v1.2$ α1C in the MAs from SHRs.

Data presented in the literature show that there is a strong positive correlation between the BP and number of functional $Ca_v1.2$ channels in the VSMCs *in vivo*.[27] For instance, the SBP was found to be linearly correlated to the membrane densities of the $Ca_v1.2$ channel currents in VSMCs from the small MAs of SHRs and WKY rats.[31] This may be an explanation for the results of the present study, that is, a reduction in the SBP by treatment with moderate-intensity aerobic exercise training resulted in a concomitant decrease in the $Ca_v1.2$ channel current

densities in the VSMCs of SHRs. In contrast, an aggravated high BP resulted in a concomitant exaggerated increase in the $Ca_v1.2$ channel current densities. However, how is this process initiated? What comes first—BP-lowering or a reduction in the number of calcium channels? The answer to this question is not clear. The results of these experiments alone cannot answer this question, and more studies are needed to clarify it. However, in the present study, we provide solid evidence that exercise training may regulate the $Ca_v1.2$ channel function and pore-forming α1C subunit molecular expression, which is closely related to the exercise-training intensity.

There are several notable limitations in the present study. First, we only examined the pore-forming α1C subunit molecular expression at the protein and mRNA levels. $Ca_v1.2$ channels consist of pore-forming α1 and auxiliary α2δ and β subunits.[37] It is still unclear whether these auxiliary subunits are changed during hypertension and whether MIT and HIT have different effects on their subunit expression. Recently, it has been reported that genetic hypertension is associated with an elevation in the α2δ-1 expression, which promotes the surface trafficking of the $Ca_v1.2$ channels in cerebral artery myocytes.[11] Recently, $\alpha 2\delta$-1 was identified as critical for the functional trafficking of $Ca_v1.2$ α1 subunits to the plasma membrane (surface) in arterial myocytes.[38] In addition, it has been reported that the β3 subunit is a critical regulatory protein that is required to upregulate arterial $Ca_v1.2$ channels and fully develop Ang II-dependent hypertension in C57BL/6 mice.[39] Obviously, post-translational alterations of the α1C subunit by regulatory β and α2σ subunits may also alter the expression of $Ca_v1.2$ by regulating its formation or its targeting and stability in the plasma membrane.[40,41] At the moment, it is unclear to what extent the α2σ and β subunits are expressed and functionally important for the changes in the function of VSMCs as well as which factors affect their interaction with the α1C subunit. Therefore, further studies will be needed to determine the exact molecular basis of this abnormality. Another limitation that should be mentioned is that in the present study we did not investigate the effects of the exercise intensity on the $Ca_v1.2$ function in normotensive (WKY) animals. According to the present data, it is difficult to interpret the cause–effect relationship between changes in the calcium channel function/expression and exercise intensity with concurrent changes in the BP. To answer this question, future studies should be conducted by repeating the same protocol in normotensive control animals.

CONCLUSION

In summary, using a combination of mechanical, electrophysiological and biochemical approaches, the present study provided functional and molecular evidence that moderate-intensity aerobic exercise training reverses pathological $Ca_v1.2$ channel remodeling, inducing vessel dilation in hypertension, whereas high-intensity aerobic exercise training aggravates the adverse remodeling of $Ca_v1.2$ channels in association with hypertension. These data indicate that for the intervention treatment of hypertension an appropriate training protocol should be chosen. The study may contribute to optimize the treatment regimens of hypertensive patients who are performing aerobic exercise. Further studies on the regulation of $Ca_v1.2$ channel transcription, translation and trafficking should provide more insights into the molecular mechanisms.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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