Similar enhancement of BK_{Ca} channel function despite different aerobic exercise frequency in aging cerebrovascular myocytes

Short title: Exercise affects BK_{Ca} in aging cerebral myocytes

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Summary

Aerobic exercise showed beneficial influence on cardiovascular systems in aging, and mechanisms underlying vascular adaption remains unclear. Large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels play critical roles in regulating cellular excitability and vascular tone. This study determined the effects of aerobic exercise on aging-associated functional changes in BK_{Ca} channels in cerebrovascular myocytes, Male Wistar rats aged 20-22 months were randomly assigned to sedentary (O-SED), low training frequency (O-EXL), and high training frequency group (O-EXH). Young rats were used as control. Compared to young rats, whole-cell BK_{Ca} current was decreased, and amplitude of spontaneous transient outward currents were reduced. The open probability and Ca^{2+}/voltage sensitivity of single BK_{Ca} channel were declined in O-SED, accompanied with a reduction of tamoxifen-induced BK_{Ca} activation; the mean open time of BK_{Ca} channels was shortened whereas close time was prolonged. Aerobic exercise training markedly alleviated the aging-associated decline independent of training frequency. Exercise three times rather than five times weekly may be a time and cost-saving training volume required to offer beneficial effects to offset the functional declines of BK_{Ca} during aging.

Key Words: Aging; BK_{Ca} channel; Aerobic exercise training; cerebral artery; biophysical properties

Introduction

The rate of age-specific mortality from heart diseases and stroke increases exponentially with age throughout later years of life (Murray *et al*. 1997; Kearney *et al*. 2005),which leads to 40%~60% of all deaths among people aged ≥65 years (Ungvari *et al*. 2010).During the process of aging, the vascular system undergoes a series of deleterious adaptations that may cause vascular diseases (Toro *et al*. 2002). Aging-related vascular changes may occur at the endothelium or smooth muscle cells (SMCs). BK_{Ca} channels are key regulators of arterial tone in many regions of the vascular tree. Because of its abundant expression and high conductance(~240 pS), BK_{Ca} activity serves as a critical hyper-polarizing and vasorelaxing force to buffer vascular constriction and maintain normal levels of vascular tone(Knot *et al*. 1998). Recent studies showed that the number of BK_{Ca} channels was reduced with aging as well as the BK_{Ca} current density was decreased along the arterial tree(Marijic and Toro 2001; Marijic J *et al*. 2001; Nishimaru *et al*. 2004; Shi *et al*. 2013). However, chronic aerobic exercise may promote peripheral vascular health by "reversing" some of the age-declined effects. e.g., restoring the nonparallel down-regulation of the molecular components of BK_{Ca} channels, therefore, enhancing vascular functions in aged mesenteric arteries (Shi *et al*. 2014). Moreover, there has been increasing evidence showing a more efficient cerebral perfusion by aerobic exercise in prevention of stroke (Aengevaern *et al*. 2013; Colcombe *et al*. 2006; Pantano *et al*. 1984). It is rational to hypothesize that aerobic exercise also may impact on BK_{Ca} channels in central arteries, which could be an important contribution against development of stroke.

An effective aerobic-training program is dependent on several critical variables, including: intensity($\%V_{max}$), duration, and frequency (Garekani *et al*. 2011). However, more than one-third of global adult population fail to meet current public health guidelines for aerobic exercise (Hallal *et al*. 2012)[i.e., ≥30 minutes of moderate-intensity exercise on 3-5

days weekly (≥150 min/week)]. Moreover, older adults are more prone to live a less active lifestyle (i.e., more sedentary behavior) (Alexander *et al*. 2010; Avlund 2010; Eldadah 2010). Therefore, to better understand exercise-related health benefits for the older, it is necessary to investigate roles of exercise parameters (intensity, frequency, mode, and duration) in optimizing health and well-being. In young rats, it was reported that exercise could up-regulate BK_{Ca} channel functions in a manner of frequency-dependent (Li *et al*. 2013). However, there has no information on effects and mechanisms of different training frequency on cerebrovascular functions in aged subjects. Such knowledge is important for health and possible prevention against stroke.

Exercise is considered to be the most accessible, effective, pluripotent, and safe intervention to improve and maintain health (Pedersen and Saltin 2006; Goldstein *et al*. 2006; Lange-asschenfeldt and Kojda 2008; Stewart *et al*. 2002).With the concept "Exercise is medicine" proposed by the American College of Sports Medicine, growing evidence implicates that exercise is as effective as drug interventions in the secondary prevention of cardiovascular and cerebrovascular mortality (Naci and Ioannidis 2013). Given the heavy societal cost of cerebrovascular diseases and the challenges of the world's changing demographics, it is critical to fully prepare for the challenge of the world's changing demographics and to create an equitable, affordable, and sustainable aging society for the future (Lucas *et al*. 2015). Thus, major efforts need to focus on prevention with emphasis on low-cost regimen such as habitual aerobic exercise. The purpose of the present study included: (1) to investigate the aging-associate functional changes in BK_{Ca} channels in middle cerebral artery (MCA) myocytes; (2) to compare the effects of 12 weeks of aerobic training with either 3 or 5 times per week on blood pressure and biophysical properties of BK_{Ca} channels in MCA myocytes in the older rats.

Materials and Methods

Animals and exercise training protocol

Male Wistar rats were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China).The rats aged 20-22 months were randomly assigned into three groups: sedentary control group (O-SED, n=10),low-frequency exercise group (O-EXL, n=10), and high-frequency exercise group (O-EXH, n=10). Young adult rats (four-month-old, n=10) rats were used as Young control. The training rats were submitted to a motor-driven treadmill training protocol (0° slope, 20 m/min, ~50-55 %VO_{2max}, 60 min/day,12 weeks) (14). The experimental protocols were approved by the ethical committee of Beijing Sport University and were performed in accordance with the Chinese animal protection laws and institutional guidelines.

Measures of cardiovascular responses

24 rats (n=6 per group) were implanted with catheters in their femoral arteries as described (Shi *et al*. 2012) under anesthesia with a mixture of ketamine (75mg/kg) and xylazine (10mg/kg; i.p., Hengrui Medicine, Jiangsu, China). Two days after surgery, blood pressure (BP) were recorded continuously in conscious/unrestrained rats by using data acquisition software (BL-420S, Chengdu Technology and Market Co., China).

Cell isolation

At the end of 12-week exercise training, animals were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and sacrificed. The brain was removed immediately, and segments of the MCAs were dissected. Smooth muscle cells were isolated as reported (Maruyama *et al*. 1983). The cell suspensions were kept at 4℃ and used within 8 h.

Electrophysiological experiments and data analysis

Whole-cell recording. For measurement of whole-cell K^+ currents, conventional whole-cell configuration was conducted. The bath solution comprised (in mM): 134 NaCl, 6 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.4).The pipette (3~5 MΩ) solution contained (in mM): 110 K-Asp, 30 KCl, 1 EGTA, 3 Na_2ATP, 0.85 $CaCl_2$, 10 Glucose, and 10 HEPES (pH 7.2, with KOH). Outward K^+ currents were elicited by a series of 400 ms depolarizing voltage steps. Voltage steps were made at 10mV increments to +60mV from a holding potential of -60mV. The whole-cell patches used for analysis should be with a series resistance <20MΩ, seal resistances >2 GΩ, leakage current < 100pA. Current densities (pA/pF) were obtained for each cell by normalization of whole cell current to cell capacitance to account for differences in cell membrane surface area, using Axon700B amplifier, pCLAMP 10.2, and Clampfit 10.2 software (Axon Instruments, Foster City, CA). To assess BK_{Ca} current amplitudes, outward K^+ currents were elicited in the absence and presence of 100 nM iberiotoxin (IbTX).

Single channel recording. BK_{Ca} single channel current was recorded in excised inside-out membrane patches under symmetrical K^+ (145 mM) as previously described (Maruyama *et al*. 1983). The pipette solution contained (in mM): 145 KCl, 1 EGTA, 10 . HEPES and 5 glucose (pH 7.4 with KOH). The bath solution contained (in mM): 145 KCl, 1 EGTA, 10 HEPES, and 5 glucose (pH 7.4 with KOH). $CaCl_2$ was added to achieve the desired value of free Ca^{2+} in solution (determined using WinMAXC software; Chris Patton, Stanford University). Currents were sampled at 10 kHz and filtered at 2 Hz via eight-pole low-pass Bessel filter. The identity of BK_{Ca} currents was confirmed by the IbTX sensitivity (100 nmol/L; data not show) and by the high single-channel conductance that is typical of BK_{Ca} (Maruyama *et al*. 1983; Toro *et al*. 1998; Magleby and Pallotta 1983). The Npo was normalized to the max probability. The number of BK_{Ca} was estimated from the maximum observed current level at

relatively higher voltage and/or bath Ca^{2+} concentration. Continuous recordings of no less than 10 s were used for Po and kinetics analysis. The po-voltage relationships were fitted by Boltzmann equation to obtain the voltage for half-maximal channel activation ($V_{1/2}$), and Ca^{2+}-dependent activation was fitted with the Hill equation (Li *et al*. 2013). single exponential function was used to construct histograms showing the open/close time of BK_{Ca} channels at +40mV with 1μM Ca^{2+}. All electrophysiologic studies were performed using Axon700B amplifier, pCLAMP 10.2, and Clampfit 10.2 software (Axon Instruments Inc., Foster City, CA).

Transient BK_{Ca} currents. Spontaneous transient outward currents (STOCs) were measured using the perforated whole-cell patch clamp technique. The bath solution was the same as that for whole-cell recording descried before. Patch pipettes (3-5 MΩ) were filled with an internal solution containing (mmol/L): 110 potassium aspartate (K-Asp), 30 KCl, 1 $MgCl_2$, 10 HEPES, 0.5 EGTA, 3 Na_2ATP, and 0.2 amphotericin B (pH 7.2 with KOH). STOCs were recorded over a range of holding potentials from −50 to 0 mV. Signals were sampled at 10 kHz, filtered at 2 kHz. Data were captured on-line using a Digidata 1440 interface run under the pClamp 10.2 program (Axon Instruments, Foster City, CA). STOCs were analyzed and plotted using Mini Analysis Program (Synaptosoft, Inc., Decatur, GA).

Chemicals

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

Statistical analysis

Data are represented as mean ± SEM. When appropriate, *t* test or ANOVA were used to determine the significance of the differences among groups. A probability value of <0.05 was considered statistically significant.

Results

Basal BP and body weight

~~There was no significant difference in body weight (BW) among the O-SED, O-EXL, and O-EXH groups at the beginning of the study.~~ After exercise training for 12 weeks, BW in both exercise groups were significantly lower, however, there were no significant differences in BW between the O-EXL and O-EXH (602.8±11.67 v.s. 611.4±14.4g).As shown in Table 1, the baseline systolic BP (SBP) increased and the diastolic BP (DBP) decreased with aging. After exercise training, the SBP in O-EXL (135.8±2.9mmHg) and O-EXH (135.9±2.6 mmHg)decreased compared with that in O-SED(145.6±4.0 mmHg), although still higher than that in Young (125.6±2.8 mmHg); the DBP in both exercise groups was increased compared with that in O-SED(104.0±3.5 mmHg)and lower than that in Young. Neither SBP nor DBP was changed with an increase in training frequency (95.2±3.3 v.s. 99.5±4.0 mmHg). Pulse pressure (PP) showed the highest in O-SED (52.4 ± 4.7 mmHg) and the lowest in Young (22.0 ± 3.4 mmHg). No significant changes in (PP) were observed between training three times a week versus five times a week (38.0±1.2 v.s. 40.3±2.8 mmHg).

Exercise attenuated the aging-reduced whole-cell BK_{Ca} currents

As shown in Figure 1, outward K^+ current densities from the Young MCA myocytes were significantly higher than those from O-SED (35.43±1.55v.s.12.24±2.08 pA/pF, Young v.s. O-SED, HP=+60 mV).Exercise training attenuated the aging-associated reduction without

significant differences: 18.92±1.28 and 20.66±1.49 pA /pF between the O-EXL and O-EXH at +60mV. Following IbTX (100 nM) treatment for 10 minutes, the whole-cell K^+ currents were inhibited significantly. The residual currents were digitally subtracted from control currents. The difference between the two currents represented the magnitude of IbTX-sensitive BK_{Ca} currents. As shown in Figure 2, the peak BK_{Ca} current densities at a holding potential of +60 mV were 26.4±1.16, 4.83±0.82, 7.85±0.76,and 8.36±1.05 pA/pF in Young, O-SED, O-EXL, and O-EXH, respectively (Fig. 2A).There was no significant difference in the current density between the O-EXL and O-EXH. These data indicated that aging induced a significant decrease of functional activity of BK_{Ca} channels, which could be attenuated by exercise training. The percentage of IbTX sensitive current was the lowest in the myocytes from O-SED (Fig. 2B). Exercise partially alleviated the reduction of percentage of BK_{Ca} currents (74.50±2.91, 38±2.41, 48±2.92, 49.25±3.41% in Young, O-SED, O-EXL, and O-EXH myocytes, respectively). The residual currents of myocytes in O-SED were significantly reduced with aging (Young: 13.0±1.28; O-SED: 6.48±0.53 pA/pF; $P<0.05$), however, there was no significant difference in the residual currents among the O-SED, O-EXL, and O-EXH groups (O-SED: 6.48±0.53 pA/pF; O-EXL: 6.85±0.6 pA/pF; O-EXH: 6.31±0.53 pA/pF), indicating that BK_{Ca} currents contributed to the main changes of whole-cell K^+ currents in the MCA myocytes following aerobic exercise(Fig. 2C).

Exercise attenuated the aging-reduced transient BK_{Ca} currents

Activity of spontaneous transient outward currents (STOCs). The amplitude of STOCs was depressed by aging and significantly alleviated by the exercise. At the holding potential of −40 mV, the amplitude of STOCs was: Young (24.73±1.47pA) > O-EXL (17.09±1.91 pA) >

O-SED (9.57 ±1.06pA). It is noteworthy that a higher training frequency was equally efficient in restoring aging-associated decline of STOCs amplitude (O-EXH: 19.05±1.39 pA) compared with that in the O-EXL. There was no significant difference in the STOCs frequency among the four groups.

Kinetics of STOCs. Exercise at high frequency (O-EXH) prolonged the decay time by 43.65% compared with that of the Young group. However, Aging plays no effects on the decay time of STOCs (Figure 3F). There was no significant difference in the rise time of STOCs among the four groups.

Exercise reversed the aging-related change of BK_{Ca} channel properties

Conductance and voltage/Ca^{2+} sensitivity of BK_{Ca} channels. As show in Figure 4C, the half activation voltage ($V_{1/2}$) was rightward shifted by aging from 40.96 ± 3.49 mV (Young) to 62.97 ± 2.05 mV (O-SED) at 1 μM $[Ca^{2+}]_i$ free. Exercise at different frequency equally inhibited aging-induced reduction in voltage sensitivity ($V_{1/2}$ was 50.48±2.46 and 51.71 ± 2.37 mV for O-EXL and O-EXH). Then the Ca^{2+} sensitivity in the four groups was determined.Ca^{2+}-sensitivity can be assessed by the calcium concentration at half-maximal activation response (K_d). ThePo-Ca^{2+} curve fitted by Hill equation showed a K_d value of 0.92±0.06 in Young and 5.41±0.09 in O-SED, indicating that aging reduced the Ca^{2+} sensitivity of BK_{Ca} channels in the MCA myocytes. Exercise left-shifted the Po-Ca^{2+} curve, however, there was no significant difference in the K_d value between the O-EXL and O-EXH (2.02±0.06 v.s. 1.99± 0.05, O-EXL v.s. O-EXH). At 1 μM $[Ca^{2+}]_i$, the slope conductance of BK_{Ca} channels was (Figure 4E) 239.34 ± 9.22 pS for Young, 226.62 ± 6.25 pS for O-SED, 240.94 ± 18.14 pS for O-EXL, and 218.60±12.62 pS for O-EXH (n=12 patches from six animals each group). No significant differences in single-channel conductance were observed among the four groups.

Gating properties of BK_{Ca} channels. The open time of BK_{Ca} channels in O-SED was remarkably shorter than that of the Yong SMCs (Young: 19.22±2.08 ms; O-SED: 4.05±1.68 ms; P<0.05). The mean close time was significantly increased by aging (Young: 42.6±7.34 ms; O-SED: 219.62±17.46 ms; P<0.05). Exercise at either the high or low frequency reversed those changes (open time: 9.48±1.28 v.s. 11.28±2.16 ms, O-EXL v.s. O-EXH; close time: 71.10±7.39 v.s. 68.28±6.76 ms), indicating equally efficient for different training frequency to reverse the aging-related dysfunction of BK_{Ca} channel kenetics. Tamoxifen, a xenoestrogen, is known to activate BK_{Ca} channels only when they are associated with β1-subunit (Dick *et al*. 2001). We then examined the sensitivity of BK_{Ca} channels to tamoxifen (Figure 5). Under the condition of 100 nM $[Ca^{2+}]_{free}$, tamoxifen (1 μM) evoked 5.15-fold of increase in the Po of BK_{Ca} channels in Young patches, whereas 1.50-, 2.89-, and 3.10-fold in O-SED, O-EXL, and O-EXH patches, respectively, suggesting that the aging-reduced Ca^{2+}- and voltage sensitivity of single BK_{Ca} channel maybe resulted from a decrease in BK_{Ca} β1 functions, and this change could be removed by regular aerobic exercise without training frequency-dependent.

Discussion

The present study investigated the effects of exercise on BK_{Ca} channel functions in the MCA myocytes of aged rats (24-month-old). The main finding showed that aging significantly decreased BK_{Ca} channel functions, whereas exercise training could reverse such kind of the decrease. Although BK_{Ca} channel conductance, the frequency, and raise time of STOCs were unchanged in the aged and exercised groups, other characterizations of BK_{Ca} channels, including whole cell currents, STOCs amplitude, voltage/Ca^{2+}sensitivity, and channel kenetics were equally benefited from exercise three time a week and five times a week.

So far as we know, only very limited data are available regarding the effects of different training frequency in elders. Even for those limited investigations, their major research objectives were resistance training (RT) and strength gaining. For example, Farinatii et al and Nelson et al reported that a higher RT frequency weekly increased muscle strength or hip flexibility to a greater extent than lower frequencies (Farinatti *et al*. 2013; Carneiro *et al*. 2015). On the contrary, DiFrancisco-Donoghue et al (2007) and Taafee et al (1999) showed a similar increase in strength between programs with high (twice) and low (once) weekly training frequencies. However, it was largely unknown regarding effects of different aerobic training frequency on aged cerebral arteries. The present study revealed the novel information that exercise-related BW loss, BP reduction, and functional remolding of BK_{Ca} channels in the MCA were equally efficient by different aerobic training frequency (three times weekly v.s. five times weekly) in aged rats, which may assist in designing rational aerobic-training prescriptions for elders.

By using *in vivo* measurement, we observed the effects of aerobic exercise on the resting BW and BP. Compared with O-SED group, the animals that performed 12-week aerobic exercise had a lower body weight. Moreover, a significant increase of baseline SBP and pulse pressure was demonstrated in the aged rats. Aging is associated with increased stiffness (reduced compliance) of large elastic arteries in healthy sedentary adults. As the large conduit arteries stiffen, aortic pulse wave velocity results in early return of reflected pressure wave, which produces significant systolic pressure augmentation and a decrease in diastolic pressure (Ungvari *et al*. 2010). The results in the present study indicated that common aging features such as arterial stiffening, following 12-week aerobic exercise, could be diminished, showing

the efficacy of the training program.Three different patterns of BK_{Ca} channel currents were observed in the determination of aging-induced cellular changes and exercise-generated adaptive responses. First, no significant increase in whole-cell BK_{Ca} currents was elicited from the group of higher training frequency. The electrochemical gradient for K^+ ions is such that opening of K^+ channels results in diffusion of this cation out of the cells and membrane hyperpolarization (Nelson and Quayle 1995; Jackson 1998). Closure of K^+ channels has the opposite effect. BK_{Ca} channel is the predominant type of K+ channel species in most arteries (Kuriyama *et al*. 1995), have a high conductance (~240 pS, in 150/150 isotonic K). Because of the large conductance of BK_{Ca} channels, the activity of relatively few channels can exert a relatively large influence on Em. Thus, activation of vascular BK_{Ca} channels may be a critical hyperpolarizing and vasorelaxing force to buffer vascular constriction and increased intravascular pressure (Knot *et al*. 1998; Anwer *et al*. 1993). Previous studies demonstrated that aerobic exercise could attenuate the aging-induced reduction of BK_{Ca} channel currents in various smooth muscle cells, including those from coronary (Albarwani *et al*. 2010), mesenteric arteries (Shi *et al*. 2013), and thoracic aorta (Shi *et al*. 2012; Zhao and Wang 2010). The present study tested MCA myocytes and revealed that the aging-declined BK_{Ca} channel currents could be partly alleviated by regular aerobic exercise in a frequency independent pattern.

STOCs oppose vasoconstriction by hyper-polarizing BK_{Ca} currents. In arterial SMCs, STOCs generate a global hyper-polarization of the cell membrane and close voltage-dependent Ca^{2+} channels, as feed-back in decreasing global $[Ca^{2+}]_i$ and arterial contraction (Nelson *et al*. 1995; Perez *et al*. 2001). Thus, the activity of STOCs is highly

associated with the auto-regulation of membrane potential by BK_{Ca} channels. Localized and transient elevations in cytosolic Ca^{2+} (Ca^{2+} sparks), caused by Ca^{2+} release from sarcoplasmic reticulum(SR), were thought to trigger opening of BK_{Ca} channels resulting in STOCs in SMCs (Pérez *et al*. 1999). In the present study using perforated patch configuration, the significant reduction in the STOCs amplitude with aging and the up-regulated effect of exercise training could conceivably reflect fundamental changes in voltage- or Ca^{2+} sensitivity. The explanation for the reduced STOCs could be a decrease in percentage of Ca^{2+} release from SR in activating a transient BK_{Ca} current. Thus, we tested Ca^{2+}/voltage sensitivity of BK_{Ca} channels using inside-out membrane patches. The data showed the characteristic features of the aging-associated single BK_{Ca} channel activity, including a decrease of voltage/Ca^{2+} sensitivity, a reduction in mean open time, and an increase in mean closed time without change in unitary conductance. However, exercise training attenuated these aging-associated changes. Previous studies reported that aerobic exercise training reversed the aging-related reduction in BK_{Ca} channel voltage/Ca^{2+} sensitivity in peripheral arteries (Shi *et al*. 2014). The present study demonstrated similar consequences occurred in cerebral arteries at cellular level as new finding. The alteration in Ca^{2+} sensitivity may be the key adaption leading to a higher STOCs activity/amplitude and whole cell BK_{Ca} currents.

Native BK_{Ca} channel is composed of four α- and four β-subunits (Knaus *et al*. 1994; Tanaka *et al*. 1997). The β1 subunit interacts with the S0 domain and the extracellular NH2 terminus of the α-subunit, thereby increasing the apparent voltage and calcium sensitivity of the channel as well as affecting gating kinetics (Nishimaru *et al*. 2004; Brenner *et al*. 2000; Tanaka *et al*. 2004). Since the aging-decreased Ca^{2+} sensitivity of BK_{Ca} channels could be efficiently prevented by exercise training, the data suggested that exercise may reverse

aging-induced down-regulation ofβ1 subunit. Tamoxifen, a pharmacological probe, could promote activity of BK_{Ca} channels by acting on β1-subunit (Dick *et al*. 2001). The present study found that tamoxifen-induced BK_{Ca} channel activation was almost the same in O-EXL and O-EXH, indicating a training frequency-independent manner in rehabilitation of functional BK_{Ca} channel in aged MCA myocytes but not in Young myocytes as reported in previous study (Li *et al*. 2013).

Under most conditions in the present study, aerobic exercise performed three times weekly improved intrinsic characteristics of BK_{Ca} channels as the same as those of the five times a week in the aged MCA myocytes. However, when exercise five times a week, the decay time of STOCs was prolonged greatly, even longer than that in Young group. In MCA, BK_{Ca} channels are activated by micro-molar intracellular Ca^{2+} delivered by local calcium release from the sarcoplasmic reticulum(Ca^{2+} sparks) (Nelson *et al.* 1995). Our results indicate that the density of BK_{Ca} currents in SMCs of MCA was obviously reduced with aging, thus, it is unlikely that the augmented decay time of STOCs was attributable to an increase in functional BK_{Ca} channels. Since the kinetic characteristics is highly related to that of Ca^{2+} sparks, it is tempting to speculate that the altered kinetics in the group O-EXH may reflect a prolonged decay time of Ca^{2+} sparks in the MCA myocytes. Whether and how exercise could impact on kinetics of Ca^{2+} sparks requires further studies with approach such as confocal scanning laser microscopy.

It is known that increased arterial stiffness is a clinically important phenotype associated with vascular aging in humans (Ungvari *et al*. 2010). Recent studies reported that peripheral arterial stiffness was strongly relevant to perceived fatigability during physical activity due to its relationship with muscle perfusion and energy expenditure during exercise (Kizu *et al*. 2003; Ferreira *et al*. 2002). Thus, compared with young adults, the perceived fatigability during exercise may augment in older adults because of the aging-induced arterial stiffness.

Therefore, it is possible that older adults more prone to feel tired when trained at a larger volume, and may reduce their daily physical activity level at the rest time of the training (Alexander *et al*. 2010; Eldadahba 2010). The loss of frequency dependent of channel functional remolding may attributed to a fatigability-related physiological changes.

Increasing evidence demonstrated that regular aerobic exercise promotes brain health/cognitive functions in the aging (Colcombe *et al*. 2006; Shay and Roth 1992; Buchman *et al*. 2007; Erickson *et al*. 2011). Little is known about whether and how the functional adaption by exercise was promoted via the improvement of cerebral vascular re-activity. Recently, Chapman et al investigated the effect of 12-week aerobic training (50-75% $V_{O2\,max}$) on the brain, cognition, and cardiovascular fitness in the aging (Chapman *et al*. 2013). They proposed that exercise training increased the cerebral blood flow (CBF) and lead to memory performance improvement. However, the mechanism underlying the adjustment of CBF by aerobic exercise remains unknown. Previous studies demonstrated that BK_{Ca} channels can be significantly activated by high pressure leading to forced dilation of cerebral arteries(Nelson *et al*. 1995; Brayden and Nelson 1992). Clinical studies suggest that aging impairs auto-regulatory protection to high blood pressure in the human brain (Castellani *et al*. 2006). It is interesting to consider that a loss of the regulatory β1 subunit of BK_{Ca} channels in aged cerebral arteries may contribute to dysfunction of vasodilation that mediate the impairment of vascular autoregulation protection. Aerobic exercise partly restored the degeneration by increasing Ca^{2+}/voltage sensitivity of BK_{Ca} channels, which is substantially important in maintaining CBF on pressurized cerebral arteries. After all, cognitive functions, particularly in the domains of executive functions, processing speed, and episodic memory has been reported to be declined by aging, and could be exacerbated by cardiovascular diseases and cardiovascular risk factors. Multiple recent studies indicate that aerobic exercise programs may slow the progression of age-related neural changes and reduce the risk for cognitive

impairment as well as dementia (Lucas *et al*. 2015; Chapman *et al*. 2013; Zheng *et al.* 2015; Thomas *et al*. 2013). Whether and how exercise affects cognition via improvement of cardiovascular functions is worth of further investigation.

We considered limitations in assessing the data in the present study. Although the functional β1 subunit of BK_{Ca} channels was detected according to it's sensitivity to tamoxifen, there may need further evidence to determine whether molecular alteration of BK_{Ca} channels might lead to a blunted coupling between α and β1 subunit. In addition, this work focused on the electrical remodeling of BK_{Ca} channels at cell level, determination of vascular re-activity at tissue level could be helpful to verify BK_{Ca}-mediated dysfunction in the MCA. Those limitations also offer new opportunities for future investigation. Our research data showed the moderate improvement by aerobic exercise performed three times a week as the same as five time a week in older rats, which indicates that chronic moderate aerobic training at a frequency of three times a week could possibly be a more appropriate training volume required to have beneficial effects to offset the declines in BW, BP, and BK_{Ca} channels in the MCA in aged adults. Based on the preliminary data from animals, human trial is warranted for further study, the results will be helpful to design aerobic-training programs that are more time and cost efficient.

Conclusion

Regular aerobic exercise may improve BK_{Ca} channel activity in aged MCA myocytes by affecting its biophysical properties in a manner of training frequency independent, which partly underlies the beneficial effect of exercise on restoring aging-associated reduction of vasodilatory properties in the MCA. Given the key role of BK_{Ca} channels in regulation of BP and vessel dilation, exercise is a non-pharmacological intervention recommended for the prevention of vascular diseases such as stroke.

Acknowledgments

This work was supported by Beijing Natural Science Foundation (5132017), National Natural Science Foundation of China (31371201), the Chinese Universities Scientific Fund (2015ZD008), 2016 Research project of General Administration of Sport of China.

Li N and Liu B contributed equally to this work. First Hospital of Soochow University and Beijing Sport University contributed equally to this work.

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Table 1. Effects of Exercise Training on BW and Basal Arterial BP of Aged Rats

	BW (g)	SBP(mmHg)	DBP (mmHg)	PP (mmHg)
Young	573.3 ±13.5	125.6 ±2.8	104.0 ±3.5	22.0 ±3.4
O-SED	702.3 ±20.2*	145.6 ±4.0*	84.8 ±1.5*	52.4 ±4.7*
O-EXL	602.8 ±11.7$^{\#}$	135.8 ±2.9$^{\#}$	95.2 ±3.3$^{\#}$	38.0 ±1.2$^{\#}$
O-EXH	621.4 ±15.4$^{\#}$	135.9 ±2.6$^{\#}$	99.5 ±4.0$^{\#}$	40.3 ±2.8$^{\#}$

Notes: BP = blood pressure; BW = body weight; DBP = diastolic blood pressure; SBP = systolic blood pressure; PP = pulse pressure.

*$p < .05$, compared with Young; $^{\#}p < .05$, compared with O-SED group. Values are means ± SEM.

Figure legend

Figure 1. Whole-cell K^+ currents in myocytes of middle cerebral artery (MCA) measured using the conventional whole-cell configuration of the patch clamp technique. (A) Young; (B)O-SED; (C) O-EXL; (D) O-EXH. (a) Representative recordings of whole-cell K^+ currents measured during depolarizing voltage steps. (b) Example of whole-cell K^+ current blockade by iberiotoxin (IbTX, 10^{-7} M) for 10 min. (c) The mean current density versus voltage plot, in the absence or presence of IbTX in myocytes from four groups. Group data are shown as mean ± SEM; *P<0.05, IBTX vs without IBTX. n=18-20 cells from 6 rats in each group.

Figure 2. Histograms showing differential blockade of middle cerebral artery (MCA) myocytes current densities by iberiotoxin (IbTX) at a holding potential of +60 mV. A, BK_{Ca} current densities obtained by digital subtraction of residual currents in the presence of 100 nm IbTX from control currents. B, The BK_{Ca} current as a percentage of the total current in the MCA myocytes of the four groups. C, The residual currents after full blockade of BK_{Ca} currents in the four groups. Bars represent the mean ± SEM. *, P < 0.05, compared with Young; $^{\#}$, P < 0.05, compared with O-SED. n= 20~30 cells from 6 rats in each group.

Figure 3. Measurement of spontaneous transient outward current (STOC) activity in freshly isolated middle cerebral artery (MCA) myocytes from Young, O-SED, O-EXL and O-EXH rats. A, Representative STOCs recorded at test potentials ranging from -40 to -10 mV in young animals. B, Expanded time scale showing STOCs in each group. HP=-40 mV. C-F: Bar graphs of STOCs amplitude (C), frequency (D), raise (E) and decay time (F)in the four groups. *p < 0.05, compared with Young; $^{\#}$p < 0.05, compared with O-SED; $^{\&}$p<0.05, compared with O-EXL. n= 18~20 cells from 6 rats in each group.

Figure 4. Effect of exercise training on Ca^{2+}/voltage sensitivity and gating properties of

BK_{Ca} channel in MCA myocytes from Young, O-SED, O-EXL and O-EXH groups. A,B, Representative recordings of single-channel currents in inside-out patches(HP=-40 mV) from Young, O-SED, O-EXL and O-EXH VSMCs exposed to different [Ca^{2+}]. C, Voltage dependence of BK_{Ca} channel at 1 μM [Ca^{2+}]. Curves were fitted with the Boltzmann equation. D, Effect of exercise training on Ca^{2+} dependence of BK_{Ca} channel. The data points were fitted with the Hill equation to obtain the calcium concentration necessary to open half of the channels (K_d) and the Hill coefficient(η^H). E and F, Summary of BK_{Ca} channel conductance (E) and mean open/closed time (F). *$p < 0.05$, compared with Young; $^{\#}p < 0.05$, compared with O-SED. n= 20~30 cells from 6 rats in each group.

Figure 5. Effect of tamoxifen (1 μM, Tam) on BK_{Ca} channel activity in the four groups. A, representative recording showing that tamoxifen increased the activity of BK_{Ca} channels. B, Bar plot summarizes the mean ± SEM fold change in the normalized Npo of BK_{Ca} channels after the application of Tamoxifen (Tam). HP= + 40 mV; 0.1μM [Ca^{2+}] free in the bath solution; **$p < 0.01$, compared with Young group; $^{\#}p < 0.05$, compared with O-SED group. n = 16~20 cells form 6 rats in each group.

Figure 1

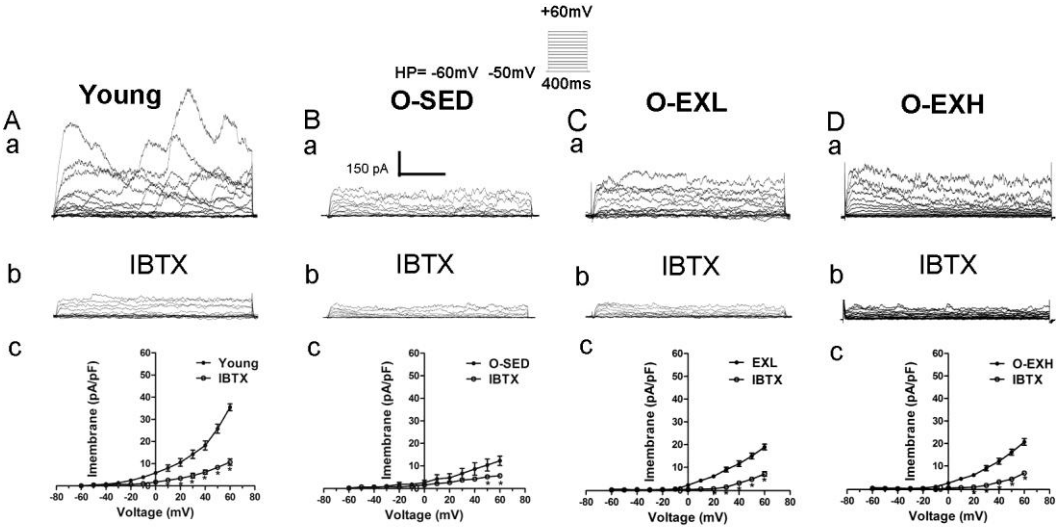

+60mV
HP= -60mV -50mV
400ms
A
a
Young
B
a
O-SED
150 pA
C
a
O-EXL
D
a
O-EXH
b
IBTX
b
IBTX
b
IBTX
b
IBTX
c
Imembrane (pA/pF)
Young
IBTX
Voltage (mV)
c
Imembrane (pA/pF)
O-SED
IBTX
Voltage (mV)
c
Imembrane (pA/pF)
EXL
IBTX
Voltage (mV)
c
Imembrane (pA/pF)
O-EXH
IBTX
Voltage (mV)

Figure 2

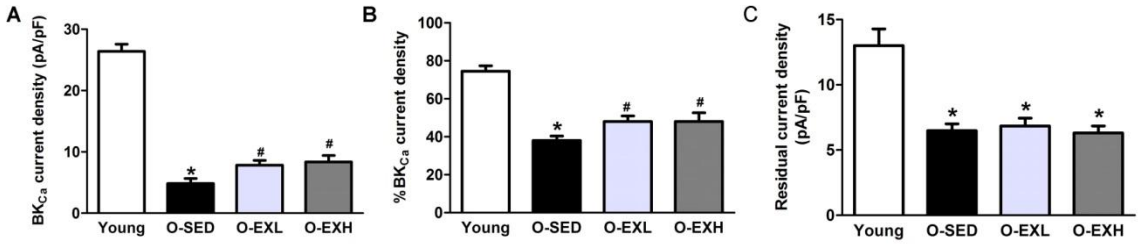

A
BK_{Ca} current density (pA/pF)
30
20
10
0
*
#
#
Young
O-SED
O-EXL
O-EXH
B
%BK_{Ca} current density
100
80
60
40
20
0
*
#
#
Young
O-SED
O-EXL
O-EXH
C
Residual current density (pA/pF)
15
10
5
0
*
*
*
Young
O-SED
O-EXL
O-EXH

Figure 3

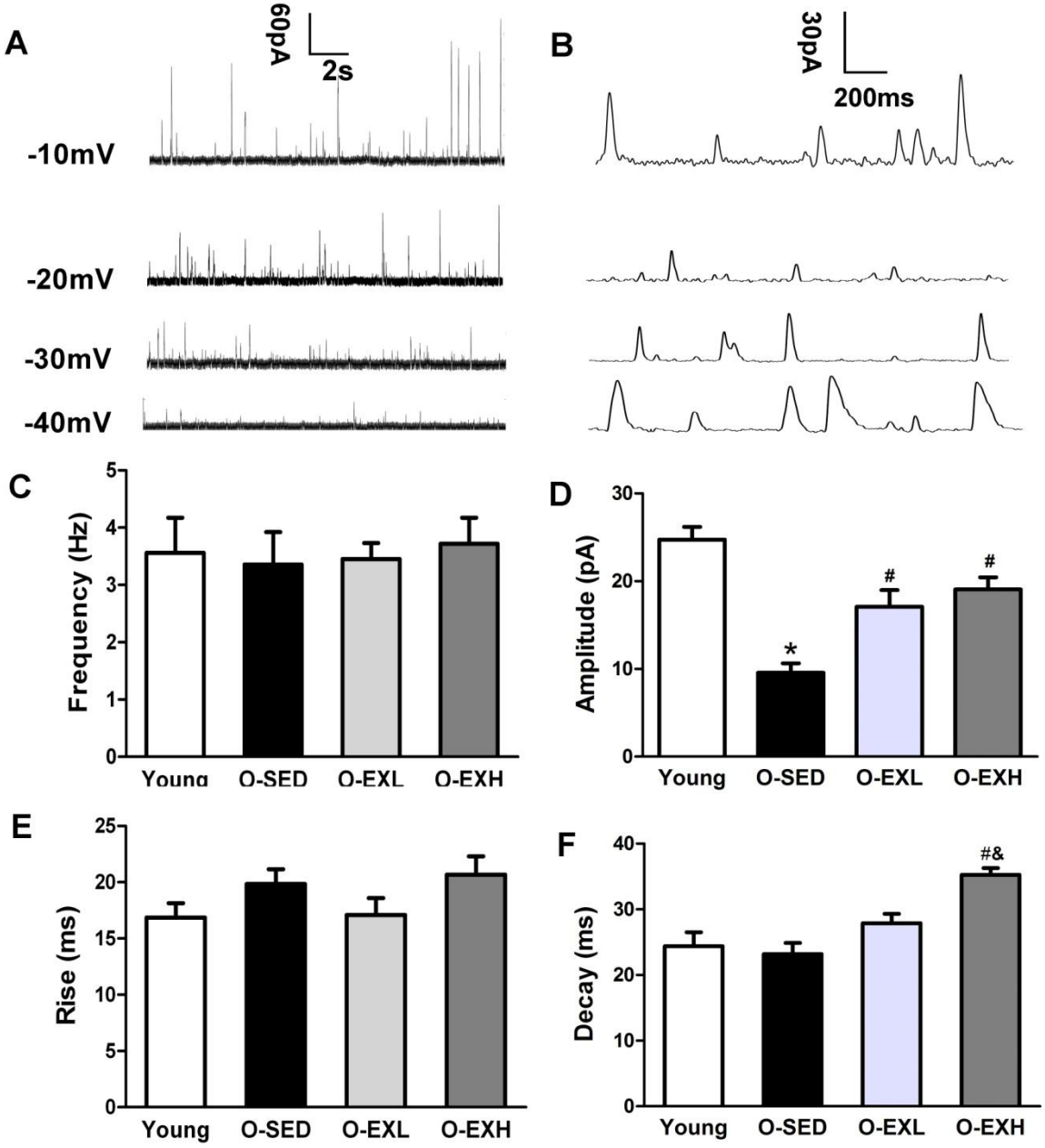

A
60pA
2s
-10mV
-20mV
-30mV
-40mV
B
30pA
200ms
C
Frequency (Hz)
Young
O-SED
O-EXL
O-EXH
D
Amplitude (pA)
*
#
#
E
Rise (ms)
F
Decay (ms)
#&

Figure 4

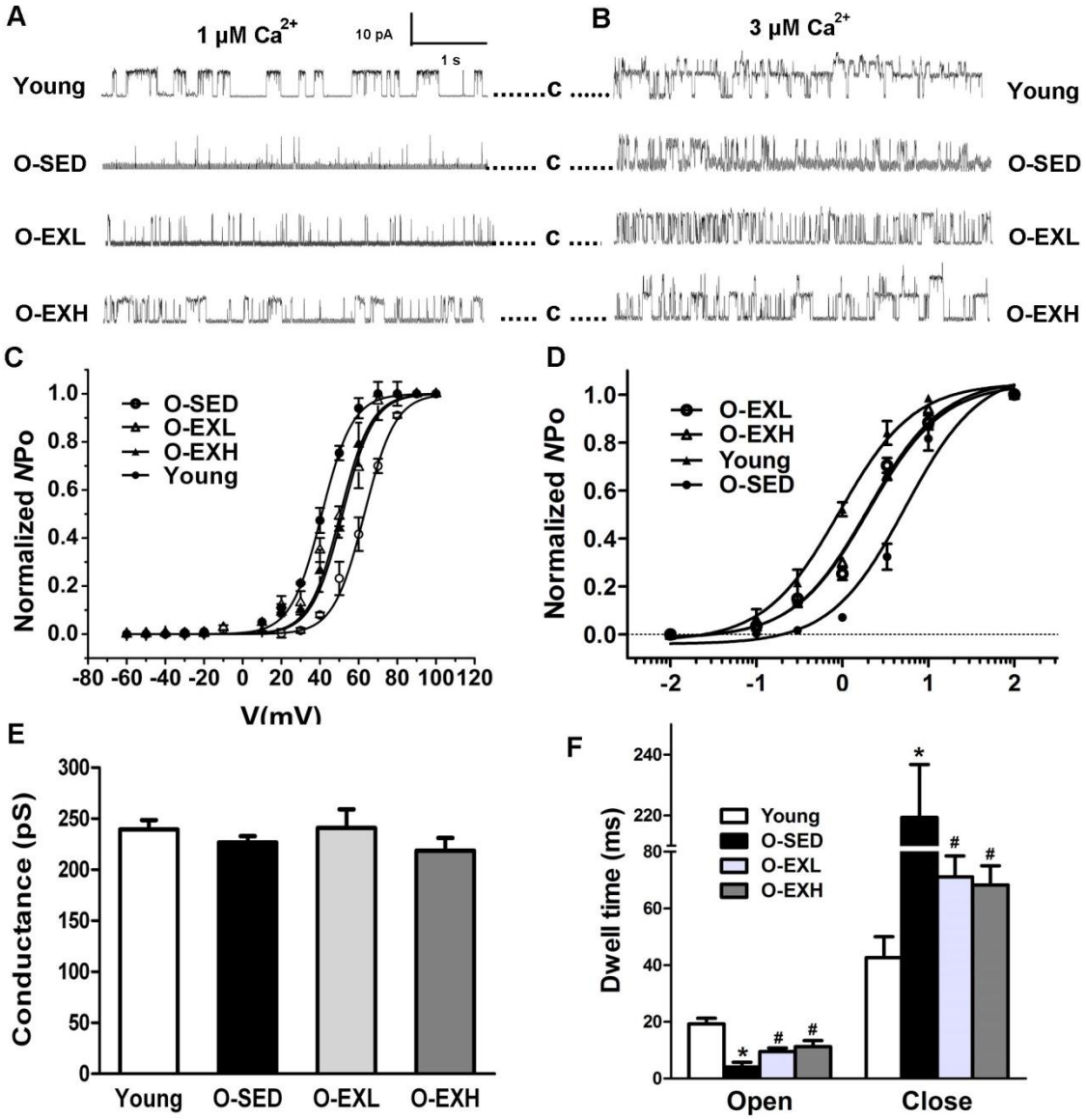
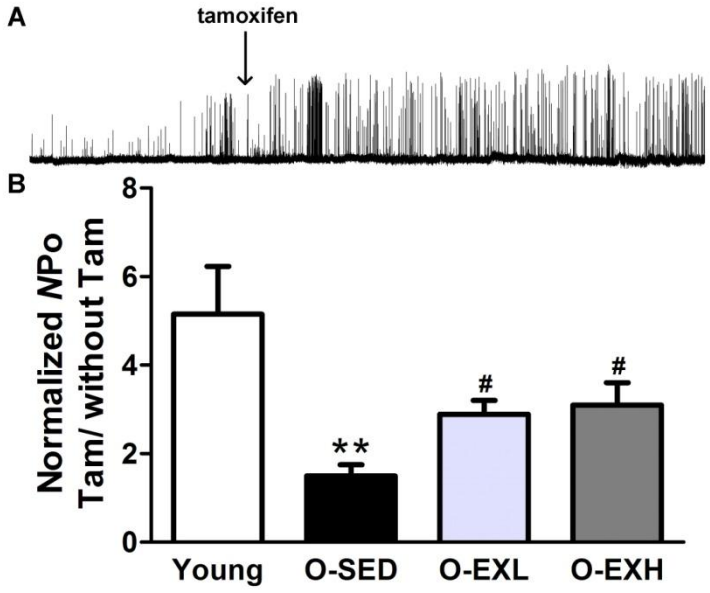

A
1 μM Ca2+
10 pA
1 s
Young
O-SED
O-EXL
O-EXH
B
3 μM Ca2+
C
Normalized NPo
O-SED
O-EXL
O-EXH
Young
V(mV)
D
Normalized NPo
O-EXL
O-EXH
Young
O-SED
E
Conductance (pS)
Young
O-SED
O-EXL
O-EXH
F
Dwell time (ms)
Young
O-SED
O-EXL
O-EXH
Open
Close

Figure 5


A
tamoxifen
B
Normalized NPo
Tam/ without Tam
8
6
4
2
0
**
#
#
Young
O-SED
O-EXL
O-EXH