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# Aging decreases the contribution of MaxiK channel in regulating vascular tone in mesenteric artery by unparallel downregulation of α- and β1-subunit expression

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ABSTRACT

Vascular disease increases in incidence with age and is the commonest cause of morbidity and mortality among elderly people. Large-conductance $Ca^{2+}$-activated $K^+$(MaxiK) channel, with pore-forming α-subunit and modulatory β1-subunit, is a key regulator of vascular tone. This study explored functional and molecular evidence of MaxiK alteration with aging in the mesenteric artery(MA). Young, Middle-aged, and Old male Wistar rats were used. Selective MaxiK channel blocker (Iberiotoxin) induced a significant increase of vascular tension in MA in all three age groups. However, these effects were greatly decreased in Old animals. The amplitude and frequency of spontaneous transient outward currents were significantly decreased with aging. Single channel recording revealed that aging induced a decrease of the open probability and the mean open time, but an increase of the mean closed time of MaxiK channel. The $Ca^{2+}$/voltage sensitivity of MaxiK was also decreased. Western blotting showed that the protein expression of MaxiK β1- and α-subunit was significantly reduced with aging, and the suppression of β1 subunits was larger than that of α subunits. These data suggest that aging decreases capability of MaxiK channel in regulating vascular tone in the MA, which may be partially mediated by unparallel downregulation of α- and β1-subunit expression.



## 1. Introduction

Aging is the dominant risk factor for cardiovascular diseases (CVDs), which remains the number one cause of death in many countries (Fleg and Strait, 2012; Shepherd, 2001; Wenger, 1997). Advancing age causes remodeling of structures and changing of functions of blood vessels, leading to an increased risk of CVDs. As small vessels, mesenteric arteries (MAs) contribute importantly to the peripheral resistance. Therefore, regulation of this vascular bed may influence systemic blood pressure (BP) significantly. Accumulating evidence has shown that advancing-age leads to adaptations in mesenteric arterial myocytes to regulate vascular tone (Briones et al., 2007; del Corsso et al., 2006; Goyal et al., 2009).

One factor that influences contractile state in peripheral resistance arteries is activity of ion channels on vascular smooth muscle cells (VSMCs). In particular, large-conductance $Ca^{2+}$-activated $K^+$ (MaxiK or $BK_{Ca}$) channels play a pivotal role in the regulation of arterial tone by regulating both electrical and chemical excitability (Brayden and Nelson, 1992; Ko et al., 2008; Ledoux et al., 2006; Nelson and Quayle, 1995).

Structurally, MaxiK channels are assembled by at least two non-covalently associated subunits; the pore-forming α subunit (four α subunits form the channel pore) and a regulatory β1 subunit (Brenner et al., 2000; Lu et al., 2006; Tanaka et al., 1997). In arterial smooth muscle cells (SMCs), β1-subunit is the principle molecular and functional MaxiK channel auxiliary unit (Brenner et al., 2000; Plüger et al., 2000). Activation of MaxiK leads to membrane hyperpolarization which deactivates the voltage-dependent calcium channels, and then causes vasodilatation (Davis and Hill, 1999; Jaggar et al., 2000; Ledoux et al., 2006; Pérez et al., 2001). Although the α-subunit is responsible for the basic ion flux function of MaxiK channels, the regulatory β1 subunit can dramatically affect channel conduction by changing channel kinetics, voltage/$Ca^{2+}$ sensitivities and pharmacology (Toro et al., 1998). Thus, MaxiK channel molecular components represent excellent candidates for aging-associated changes that can contribute to altered vascular activity in the elderly.

*Abbreviations:* BP, blood pressure; BW, body weight; CVDs, cardiovascular diseases; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; DBP, diastolic blood pressure; G, conductance; HR, heart rate; HP, holding potential; HW, heart weight; IbTX, iberiotoxin; MaxiK (or $BK_{Ca}$), large-conductance $Ca^{2+}$-activated $K^+$; MA, mesenteric artery; Po, open probability; PP, pulse pressure; PSS I, physiological salt solution I; SBP, systolic blood pressure; SMCs, smooth muscle cells; STOCs, spontaneous transient outward currents; Tc, mean close time; To, mean open time; TEA, tetraethylammonium; VSMCs, vascular smooth muscle cells.

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The age-related changes of MaxiK channels have been reported in some vasculatures. For instance, it is demonstrated that both the number of functional channels and the expression of α-subunit protein are diminished in the coronary arteries from human and rats (Marijic et al., 2001; Nishimaru et al., 2004a). Moreover, there was a parallel reduction of its β1-subunit functional expression *via* transcript downregulatory mechanisms for both α- and β1-subunits. However, in aging cerebral myocytes, MaxiK channels remain healthy (Nishimaru et al., 2004b). These results reflect a tissue-specific alteration of MaxiK channels induced by aging. Although MaxiK channels are key regulators of arterial tone, their patterns of expression and functions in MAs during aging is largely unknown.

Therefore, the present study was designed to determine: (1) whether the contribution of MaxiK channels decreases in controlling of MA tone with aging; (2) whether MaxiK whole-cell currents in the MA myocytes decrease with aging; (3) whether single channel gating properties were changed with aging in the MA; (4) what is the molecular basis (α- and β1-subunit protein expression) for the aging-related change of MaxiK functions. Understanding the mechanisms of MaxiK alterations in the predominant peripheral resistant arteries in elderly may provide information on potential targets for molecular medicine for the well-being of the growing aging population.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats at three different ages were used [Young, 4–5 months (M); Middle-aged, 15–16 M; and Old, 22–24 M; $n = 18$ per group] (Vallée et al., 1999). The rats were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Food and water were supplied *ad libitum*. All experimental procedures and protocols conformed to the recommended guidelines on the care and use of laboratory animals issued by the Chinese Council on Animal Research. All of the procedures followed were approved by the ethical committee of Beijing Sport University.

### 2.2. Surgery and blood pressure measurement

Eighteen animals ($n = 6$ per group) were used for *in vivo* measurement of BP as described (Shi et al., 2012, 2013). The animals were anesthetized with sodium pentobarbitone (50 mg/kg I.P.), and chronic catheter (OD 0.03 in., ID 0.01 in.) was inserted into the femoral artery and advanced to the abdominal aorta. After implantation, the catheter was tunneled subcutaneously and exteriorized through a spring, sewn to the animal's back. The catheter was filled with heparin solution (50 IU/ml saline) and sealed until use. Two days after recovery from surgery, BP and heart rate (HR) were recorded continuously in conscious, unrestrained rats by using data acquisition software (BL-420S, Chengdu Technology and Market Co, Ltd, China).

### 2.3. Isometric contraction

Eighteen animals ($n = 6$ per group) were used for arterial functional evaluation and Western blotting. The MA was removed quickly and cut in 2 parts. One part was placed in cold Krebs' solution and used for isometric contraction measurement. The rest of MA was used for Western blotting to detect protein expression of MaxiK channel subunits (see below). The Krebs' solution contained (in 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, which was continuously bubbled with a mixture of 95% $O_2$–5% $CO_2$ (pH 7.4) at 37 °C. The first order branches of MA (A1) were cut into rings (4 mm) and mounted on two stainless steel wires passed through the vessel lumen. The endothelium was removed by gently rubbing the luminal surface with a stainless steel rod. Removal of the endothelium was confirmed by the lack of response to 10 μM acetylcholine. Arterial segments were first contracted with 120 mM KCl to obtain peak tension ($K_{max}$). After washing and reequilibration to baseline tone, subsequent contractions were induced by using certain potassium channel blockers. To examine the role of MaxiK channels on vascular tension, we qualified the contractility after administration of $5 \times 10^{-3}$ M tetraethylammonium (TEA, a nonselective potassium channel blocker) or $10^{-7}$ M iberiotoxin (IbTX, a potent MaxiK channel blocker). The TEA/IbTX-induced maximum contraction in Young was treated as 1, % reduction of TEA/IbTX-induced contraction in Middle-aged/Old group was calculated using the following formula:

$$\%\text{reduction} = \left(1 - \frac{\text{Tension}_{(M/O)}}{\text{Tension}_{(Y)}}\right) \times 100\%$$

### 2.4. Cell isolation and patch clamp recording

#### 2.4.1. Isolation of SMCs from rat MAs

Eighteen animals ($n = 6$ per group) were used for patch clamp recording and immunohistochemical studies. Mesenteric arteries were carefully dissected and cut in 2 parts. One part was used to measure distribution of MaxiK channels associated with aging by immunohistochemistry (see below). The other was used to isolate SMCs for electrophysiological experiments.

Arteries were enzymatically dissociated to obtain individual SMC as previously described (Shi et al., 2013). Briefly, MAs (A1–A3) were placed in $Ca^{2+}$-free ice-cold physiological salt solution I (PSS I) containing (mM): 137 NaCl, 5.6 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose, and 0.03 sodium nitroprusside (pH 7.4 with NaOH). They were cut into pieces (0.5–1.0 mm in both length and width), and incubated for 30 min at 37 °C with papain (0.3 mg/ml, Worthington Biochemical, Lakewood, NJ, USA), dithiothreitol (1 mg/ml, Sigma–Aldrich), and BSA (1 mg/ml, Bovogen Biologicals, Australia). Then the tissue was incubated for 20 min (37 °C) with another digestion buffer supplemented with collagenases Type F (1.5 mg/ml, Sigma–Aldrich), Type 1-S (1 mg/ml, Sigma–Aldrich), and BSA (1 mg/ml, Bovogen Biologicals, Australia). After that, tissues were washed 3–4 times with PSS II containing (mM): 137 NaCl, 5.6 KCl, 1 $MgCl_2$, 10 Hepes, 10 glucose, 0.42 $Na_2HPO_4$, and 0.44 $NaH_2PO_4$ (99.9% $O_2$ for 40 min, pH 7.3–7.4 with NaOH), and triturated gently using a wide bore pipette to release single cells. Separated MA myocytes were used within 6–8 h after isolation.

#### 2.4.2. Perforated whole cell recording

Spontaneous transient outward currents (STOCs) were measured using the perforated whole-cell patch-clamp technique. The composition of the HEPES-PSS bath solution was (in mM): 134 NaCl, 6 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.4). Patch pipettes (8–10 MΩ) were filled with an internal solution that contained (in mM) 110 potassium aspartate (K-Asp), 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES, 0.05 EGTA, and 0.2 amphotericin B (pH 7.2). Holding potential was −60 mV. Depolarizing test potentials were gradually increased in 10 mV increments from −50 to 0 mV.

#### 2.4.3. Single channel recording

MaxiK single channel currents were recorded from inside-out patches under symmetrical $K^+$ (145 mM) at RT as described (Shi et al., 2013). The pipette solution consisted of (in mM): 100 KCl, 45 K-Asp, 1 EGTA, 10 HEPES, and 5 glucose, adjusted to pH 7.4 with KOH. The bath solution contained: 45 KCl, 100 K-Asp, 1 EGTA, 10 HEPES, and 5 glucose, adjusted to pH 7.4 with KOH. Free $Ca^{2+}$ in solution was adjusted to the desired value by adding $CaCl_2$ (determined using WinMax C software, Stanford University, Stanford, CA, USA; http://www.maxchelator.stanford.edu/).

Currents were sampled at 10 kHz and filtered at 2 kHz with a Bessel filter (8 pole). As an index of channel steady-state activity, we used the product of the number of channels in the patch ($N$) and the channel open probability ($P_o$).

Voltage dependent behavior of the channel $P_o$ was modeled with the Boltzmann function of the form (Zhao and Wang, 2010):

$$P_o = \frac{1}{1 + \exp[-ZF/RT(V - V_{1/2})]} \quad (1)$$

where $V_{1/2}$ is the membrane potential for the halfmaximal channel activation.

The $Ca^{2+}$-dependent activation was fitted with the Hill equation:

$$P_o = \frac{[Ca^{2+}]_i \eta^H}{K_d \eta^H + [Ca^{2+}]_i \eta^H} \quad (2)$$

where $\eta^H$ is the Hill coefficient, and $K_d$ the dissociation constant.

#### 2.4.4. Conventional whole-cell recording

Whole-cell $K^+$ currents were measured using the conventional whole-cell configuration of the patch-clamp technique. The composition of bath solution was the same as the 6 mM $K^+$ HEPES-PSS described above. The internal 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). From a holding potential of −60 mV, outward $K^+$ currents were elicited by a series of 400 ms depolarizing voltage steps. Voltage steps were made at 10 mV increments to +80 mV from a holding potential of −60 mV. All electrophysiologic studies were performed using Axon700B amplifier, pCLAMP 10.2 and Clampfit 10.2 software (Axon Instruments Inc., Foster City, CA, USA).

### 2.5. Immunohistochemistry

The MA tissue was incubated with specific primary antibody against MaxiK α-subunit [Anti-$K_{Ca}$1.1 (1097–1196)] or MaxiK β1-subunit [Anti-slo beta 1 (KCNMB1)] (1:200; Alomone Labs, Jerusalem, Israel) at 4 °C overnight (Adebiyi et al., 2007; Bolognesi et al., 2007; Xie et al., 2010). The sections were then incubated for 1 h with horseradish peroxidase-labeled goat anti-rabbit IgG (1:200 dilution; Proteintech Group Inc, Chicago, Illinois). The antigen was localized by