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ORIGINAL ARTICLE

BK_{Ca} channel activity and vascular contractility alterations with hypertension and aging via β1 subunit promoter methylation in mesenteric arteries

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Promoter methylation is a key mechanism in the epigenetic reprogramming of gene expression patterns. Here, we investigated the contribution of DNA methylation and the associated expression and function of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel in mesenteric arteries when hypertension was superimposed on aging. Male Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) at young (12 weeks), adult (36 weeks) and old (64 weeks) life stages were used. BK_{Ca} channel currents, BK_{Ca} channel activity in regulating vascular tone, and BK_{Ca} channel β1 subunit (BKβ1) function and expression were greater in mesenteric arteries from SHR than from age-matched WKY controls. Consistently, hypertension decreased CpG methylation of the BKβ1 promoter at all ages. Furthermore, aging triggered an increase in BKβ1 promoter methylation in both old WKY and SHR, with concomitant suppression of the β1 subunit and BK_{Ca} channel activity. Aging enhanced β1 gene promoter methylation and subunit expression without changing BK_{Ca} channel function between young and adult WKY animals. In contrast, aging did not alter CpG methylation but facilitated BK_{Ca} channel currents and upregulated BKβ1 expression and function in adult SHR. Taken together, our results provide compelling evidence that hypertension and aging exert opposing effects on DNA methylation at BKβ1 promoter, subsequently resulting in different β1 expression levels and functional modulation of mesenteric arterial contractility. Such information is useful in revealing the epigenetic regulation of BK_{Ca} channel function in vascular smooth muscle and in comprehensively understanding the molecular mechanisms in vascular physiology and pathophysiology.
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Keywords: aging; BK_{Ca} channel; DNA methylation; vascular smooth muscle cells

INTRODUCTION

Hypertension and aging are both recognized as major risk factors for the development of cardiovascular disease,[1–3] but their interactions are not completely understood. When hypertension is superimposed on aging, endothelial and vascular dysfunction, increased arterial stiffening, and reduced vascular reactivity can elevate blood pressure (BP).[4–6] The smooth muscle cells of small, resistance-sized arteries express abundant large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels that play an indispensable role in opposing vasoconstriction by membrane hyperpolarization.[7] The BK_{Ca} channel is a tetramer of pore-forming α subunit along with auxiliary β1 subunit (*KCNMB1*); the β1 subunit modulates BK_{Ca} channel Ca^{2+} sensitivity and gating properties.[8] Thus, β1 subunit can dynamically influence BK_{Ca} channel activity to control arterial myocyte membrane potential and vascular contractility, influencing systemic BP.[9,10] Pathological alterations in BK_{Ca} channel β1 subunit (BKβ1) expression and function are associated with cardiovascular aging and diseases, including atherosclerosis, stroke, and hypertension.[11,12]

During chronic hypertension, resistance-sized arteries undergo functional and molecular changes. Many studies have shown that the functional expression of BK_{Ca} channels or the β1 but not the α subunit in vascular smooth muscle cells (VSMCs) is upregulated in hypertension.[13–16] This is a local protective mechanism induced by hypertension to compensate for the increase in intraluminal pressure in arteries.[16,17] The molecular mechanisms underlying hypertension-mediated upregulation of BKβ1 expression in VSMCs remain undetermined. Previous studies have suggested that the BK_{Ca} channel function and expression of α and β1 subunit are both downregulated during aging.[18–20] However, in different vascular beds, this age-dependent change is not uniform. In aging coronary arteries, there is a parallel decline of α and β1 subunit, which means that the α/β1 ratio is unaltered.[19] We have recently demonstrated that aging decreases the contribution of BK_{Ca} channels to the regulation of vascular tone in mesenteric artery by asynchronous downregulation of α and β1 subunit expression.[20] Nevertheless, the pathological

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alterations of BK_{Ca} channel expression and activity in VSMCs as hypertension progresses with aging are not completely understood.

There are complex interplays between genetic and environmental factors both in the pathogenesis of hypertension and during the general aging process.[21,22] In contrast to DNA mutations, epigenetic alterations are reversible and as such are promising targets for devising preventive and therapeutic approaches for hypertension or slowing the inevitable process of aging.[21,22] DNA methylation is a chief mechanism in epigenetic modification that regulates gene transcription, and occurs at cytosine residues in the context of CpG sequences.[23] DNA hypermethylation, or increased methylation in the promoter region, generally suppresses gene transcription and directly silences the associated genes.[23] Recent studies have demonstrated that promoter methylation plays a key role in regulating ion channel expression patterns in vascular adaptation.[24–26] Little is known about the epigenetic regulation of BKβ1 gene expression patterns in vascular smooth muscle cells and its functional consequences in aging, particularly when accompanied by hypertension.

Here, we have assembled a body of evidence demonstrating that hypertension causes modulation of the BKβ1 gene via promoter demethylation and that aging adversely increases methylation sites, resulting in altered BK_{Ca} channel expression and function in mesenteric arteries. A clear understanding of how hypertension and aging influence BK_{Ca} channel function and the underlying epigenetic mechanisms, as well as whether hypertension and aging have additive or synergistic effects, are still lacking. However, this work provides important insights into vascular function, and supports the development of epigenetic studies for hypertension and aging. We performed the present study to investigate the effects of hypertension and aging on BK_{Ca} channel activity, β1 subunit function and expression, and DNA methylation in mesenteric arteries.

METHODS

Animal model

Male normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) were obtained from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) at 12 (young), 36 (adult), and 64 (old) weeks (wks) of age. Each group was composed of 24 animals. They were housed in a temperature-controlled room under a 12:12-h light-dark cycle and allowed free access to food and water. The study was approved by the ethical committee of the Beijing Sport University and performed in accordance with the animal protection laws and institutional guidelines of China.

The BP and heart rate (HR) of conscious SHR and WKY rats were measured using an indirect tail-cuff method (BP-2010A, Softron Biotechnology, Beijing).

Isometric contraction study

After animals were anesthetized with sodium pentobarbitone (50 $mg\,kg^{-1}$), the mesenteric arteries from each group were carefully removed and placed in ice-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 in a 95% O_2 and 5% CO_2 (pH 7.4) atmosphere. Short segments of A2-A3 were used for contractile studies using Multi Myograph System (620M; DMT, Aarhus). The contractile response for tension was evaluated by measuring the maximum peak height, and expressed as a percentage of contraction compared to 120 mM K^+ (K_{max}). In the experiments, the non-selective NOS inhibitor *N*ω-nitro-L-arginine methyl ester (L-NAME, 100 μM) was added after K_{max} measurement. To investigate the contribution of BK_{Ca} channel to vascular tone regulation, the vascular responses to iberiotoxin (IbTX, a BK_{Ca} blocker, 10^{-7} M) were examined. Each vessel was used once, and signals were recorded using the Power-Lab system with Chart-5 software (AD Instruments, Bella Vista).

Cell isolation

Cells were obtained, as described previously[27], using enzymatic isolation. The A2-A3 mesenteric arteries were cut into small pieces and then incubated at 37 °C in 2 $mg\,ml^{-1}$ bovine serum albumin, 4 $mg\,ml^{-1}$ papain, 1 $mg\,ml^{-1}$ dithiothreitol and 0.6 $mg\,ml^{-1}$ collagenase in physiological salt solution (PSS) containing (mM): 137 NaCl, 5.6 KCl, 1 $MgCl_2$, 10 Glucose, 10 HEPES, 0.42 Na_2HPO_4, 0.44 NaH_2PO_4 and 4.2 $NaHCO_3$ (adjusted to pH 7.3). After enzymatic treatment, the vessel segments were washed with PSS and gently triturated to release individual smooth muscle cells with a fire-polished Pasteur pipette. The cells were cold-stored at 4 °C for up to 6 h until the experiments were performed.

Whole-cell recording

Whole-cell K^+ currents were measured using the conventional whole-cell configuration.[16] The external solution was comprised of (mM): 134 NaCl, 6 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose and 10 HEPES (pH 7.4). The internal solution contained (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). Current–voltage (*I–V*) relationships were generated in voltage-clamp cells held at an Em of − 70 mV, and then stepped in 10 mV increments to +70 mV for 350 ms at each potential. BK_{Ca} currents were defined as the 100 nM IbTX-sensitive component.

Single-channel recording

BK_{Ca} single-channel currents were recorded in excised inside-out patches under symmetrical K^+ conditions (145 mM).[16] Cells were continuously perfused with a solution containing (mM): 45 KCl, 100 K-Asp, 1 EGTA, 10 HEPES and 5 glucose, adjusted to pH 7.4. Pipettes were filled with a solution composed of (mM) 100 KCl, 45 K-Asp, 1 EGTA, 10 HEPES and 5 glucose (pH 7.4 with KOH); the concentration of free Ca^{2+} in the bath solution was 100 nM. 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 (Po). All electrophysiologic studies were performed using Axon700B amplifier, pCLAMP 10.2 and Clampfit 10.2 software (Axon Instruments Inc., Foster City).

Western blotting

The mesenteric arteries were harvested and homogenized in ice-cold lysis buffer. These homogenates were then centrifuged at 13400*g* for 30 min (4 °C), and the supernatant was recovered. Protein concentration was determined using a BCA kit (ThermoFisher Scientific, Rockford, IL, USA). Total protein extracts were separated on 10–15% SDS polyacrylamide gels and transferred to Polyvinylidene Fluoride (PVDF) membranes. The membranes were blocked in Tris-buffered saline with Tween-20 (TBST) containing 5% BSA and incubated with primary antibody (rabbit polyclonal anti-K_{Ca}1.1 or rabbit polyclonal anti-*slo*β1 antibody from Alomone Labs) diluted in blocking buffer (1:300) at 4 °C overnight. After extensive washing in TBST, membranes were probed with goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then washed in TBST. Horseradish peroxidase bound to the immunoblot was visualized with enhanced chemiluminescence (ECL, ThermoFisher Scientific, Rockford) and signals were recorded with Bio-Rad ChemiDOC XRS^+ (Bio-Rad Laboratories, Hercules, CA, USA). β-Actin was used to normalize loading of all samples.

Immunofluorescence

Immunofluorescence labeling of dispersed myocytes was performed as described previously[28] using a polyclonal antibody specific for K_{Ca}1.1 (Alomone Labs, Jerusalem; 1:200) or *slo*β1 (Alomone Labs, Jerusalem, Israel; 1:200). The secondary antibody was an Alexa Fluor 488-conjugated goat anti-rabbit (5 $mg\,ml^{-1}$, Molecular Probes). After mounting, cells were imaged (1024 × 1024 pixel images) using a confocal system (TCS-SP8, Leica, Wetzlar) coupled with a PL APO × 63 oil immersion lens (numerical aperture 1.4). The specificity of our labeling was confirmed by performing a negative control experiment in which the primary antibodies were substituted with PBS. K_{Ca} 1.1- or *slo*β1-associated fluorescence was undetectable under these conditions (data not shown).

DNA bisulfite sequencing PCR

DNA methylation analysis was performed using a previously described method[25] with minor modifications. Briefly, genomic DNA was isolated from mesenteric arteries using a PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. DNA quality was determined based on the absorbance at 260/280 nm (≥1.8 was considered acceptable) and confirmed using 1.0% agarose gel electrophoresis. Subsequently, the DNA samples were subjected to sodium bisulfate conversion using an EZ DNA Methylation-Gold Kit (ZYMO Research, Orange). PCR amplification of the *KCNMB1* promoter region on the CpG island (at chromosome 10 from position 18911664 to 18912065, 402 bp in length, containing 7 CpG sites) was performed; the products were separated using 2.0% agarose gels, and the bands were resolved using a TIANgel Midi Purification Kit (Tiangen, Beijing, China). The purified bands were cloned into a pEASY-T1 Cloning vector (TRAN, Beijing, China). In total 10 clones from each sample were selected and sequenced; DNA bisulfite sequencing PCR (BSP) was repeated three times for each sample. The primers used for modified BSP were forward: 5′-AGAGAAAATAGAGGTTTAGAGAGGTGT-3′ and reverse: 5′-ATCAAATCT AAACACACAACTTACTCC-3′. Data are presented as the percentage of methylation at the region of interest (methylated CpG/(methylated CpG +unmethylated CpG) × 100).

Chemical reagents and statistical analysis

All chemical reagents were from Sigma-Aldrich unless otherwise stated. Values are presented as means ± s.e.m., with experimental number (n) indicating the number of arteries or cells studied unless otherwise indicated. SPSS 17.0 software was used for statistical analyses. Comparisons among the groups were determined by 2 × 2 multifactorial ANOVA, with factors defined as hypertension and age, and the interaction term determined the additive effect of these two factors. $P<0.05$ was considered significant.

RESULTS

Physical characteristics of normotensive (WKY) rats and hypertensive rats (SHR) of different ages

Significant increases in HR, SBP, DBP and MAP were observed in the SHR group compared with age-matched WKY (Figure 1). HR was unchanged with age in the WKY, whereas it continued to increase between 12 and 64 weeks in the SHR group ($P<0.05$). SBP in both the WKY and SHR groups increased significantly with age ($P<0.05$), and there was a slight rise in DBP ($P>0.05$). A marked difference in MAP was observed between 12 and 64 weeks in both the WKY and SHR groups ($P<0.05$), but not between 12 and 36 weeks or 36 and 64 weeks. Furthermore, a significant interaction between hypertension and age was found for SBP ($P<0.05$) and MAP ($P<0.05$), suggesting that hypertension and aging have an additive or synergistic effect.

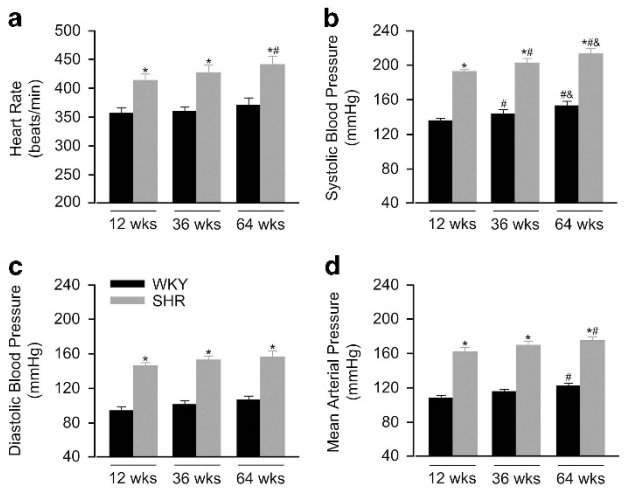

Figure 1 Blood pressure parameters in normotensive rats (WKY) and hypertensive rats (SHR). Heart rate (**a**), systolic blood pressure (**b**), diastolic blood pressure (**c**), and mean blood pressure (**d**) in WKY and SHR at different ages. Data are shown as mean ± s.e.m., with $n=24$ animals per group. Wks, weeks. $^{*}P<0.05$ compared with age-matched WKY; $^{\#}P<0.05$ compared with corresponding 12 wks; $^{\&}P<0.05$ compared with corresponding 36 wks.

Hypertension and aging altered the BK_{Ca} channel activity in arterial myocytes

To determine the functional effects of hypertension and aging on BK_{Ca} channels, patch-clamp electrophysiology was performed to measure BK_{Ca} current density in isolated arterial myocytes. In WKY-12wks myocytes, mean peak whole-cell current density was 24.3 ± 29 pA/pF (at +70 mV, $n=24$ cells). Iberiotoxin (IbTX, 100 nM), a BK_{Ca} channel-specific inhibitor, reduced mean peak current density to 9.0 ± 2.2 pA/pF, indicating that the mean iberiotoxin-sensitive current (or BK_{Ca} current) was 15.3 ± 0.7 pA/pF (at +70 mV) in arterial myocytes from WKY-12wks (Figure 2a). Hypertension dramatically increased the BK_{Ca} current to 21.3 ± 0.7 pA/pF or to 1.39 times that of WKY-12wks myocytes (at +70 mV, $n=24$ cells; $P<0.05$; Figure 2b). A similar increase in BK_{Ca} current was seen in the SHR compared with the WKY at an age of 36 weeks (WKY-36wks: 14.4 ± 0.8 pA/pF, $n=24$ cells vs SHR-36wks: 25.5 ± 1.6 pA/pF, $n=25$ cells; $P<0.05$; Figure 2c and d) and 64 weeks (WKY-64wks: 10.2 ± 0.3 pA/pF, $n=18$ cells vs SHR-64wks: 11.8 ± 0.5 pA/pF, $n=20$ cells; $P<0.05$; Figure 2e and f).

Aging reduced the mean iberiotoxin-sensitive current density in myocytes to 94.1% (36 weeks; $P>0.05$) or 73.9% (64 weeks; $P<0.05$) of that in the WKY-12wks myocytes (Figure 2c and e). Conversely, compared with SHR-12wks, there was a significant jump in BK_{Ca} current in SHR-36wks cells (1.29× higher; $P<0.05$; Figure 2d). The whole-cell BK_{Ca} current in arterial myocytes from SHR-64wks was reduced by ~55% compared with that of SHR-12wks cells ($P<0.05$; Figure 2b and f) or decreased by ~46%, when compared with SHR-36wks myocytes ($P<0.05$; Figure 2d and f). The effects of both hypertension ($P<0.05$) and aging ($P<0.05$), as well as their interaction ($P<0.05$), were significant.

Hypertension and aging modified the BK_{Ca} channel contribution to vascular tone regulation in mesenteric arteries

To investigate functional BK_{Ca} channel activity in regulating mesenteric arterial contractility, IbTX (100 nM) was used to stimulate vasoconstriction. As depicted in Figure 3a and b, the iberiotoxin-induced increase in tension in the SHR group was higher than in age-matched WKY controls ($n=6$ in each group, all $P<0.05$). The IbTX-induced vasoconstriction (myogenic tone) in WKY-36wks was slightly enhanced, yet in SHR-36wks was markedly increased ($P<0.05$) when compared with WKY-12wks and SHR-12wks, respectively (Figure 2b). Furthermore, the increase in myogenic tone in SHR-36wks was much more pronounced than that in either SHR-12wks or WKY-36wks ($P<0.05$; Figure 2a and b). In contrast, aging inhibited the IbTX-induced tension increase in both WKY-64wks and SHR-64wks (~30% and ~29%, respectively; both $P<0.05$) compared with 36-week WKY and SHR. The effects of both hypertension and aging were individually significant ($P<0.05$ and $P<0.05$), but there was no significant interaction between them.

These data suggest that compared with age-matched WKY, hypertension increases BK_{Ca} channel activity in vascular tone. Interestingly, the contribution of BK_{Ca} channels in vascular tone regulation increases

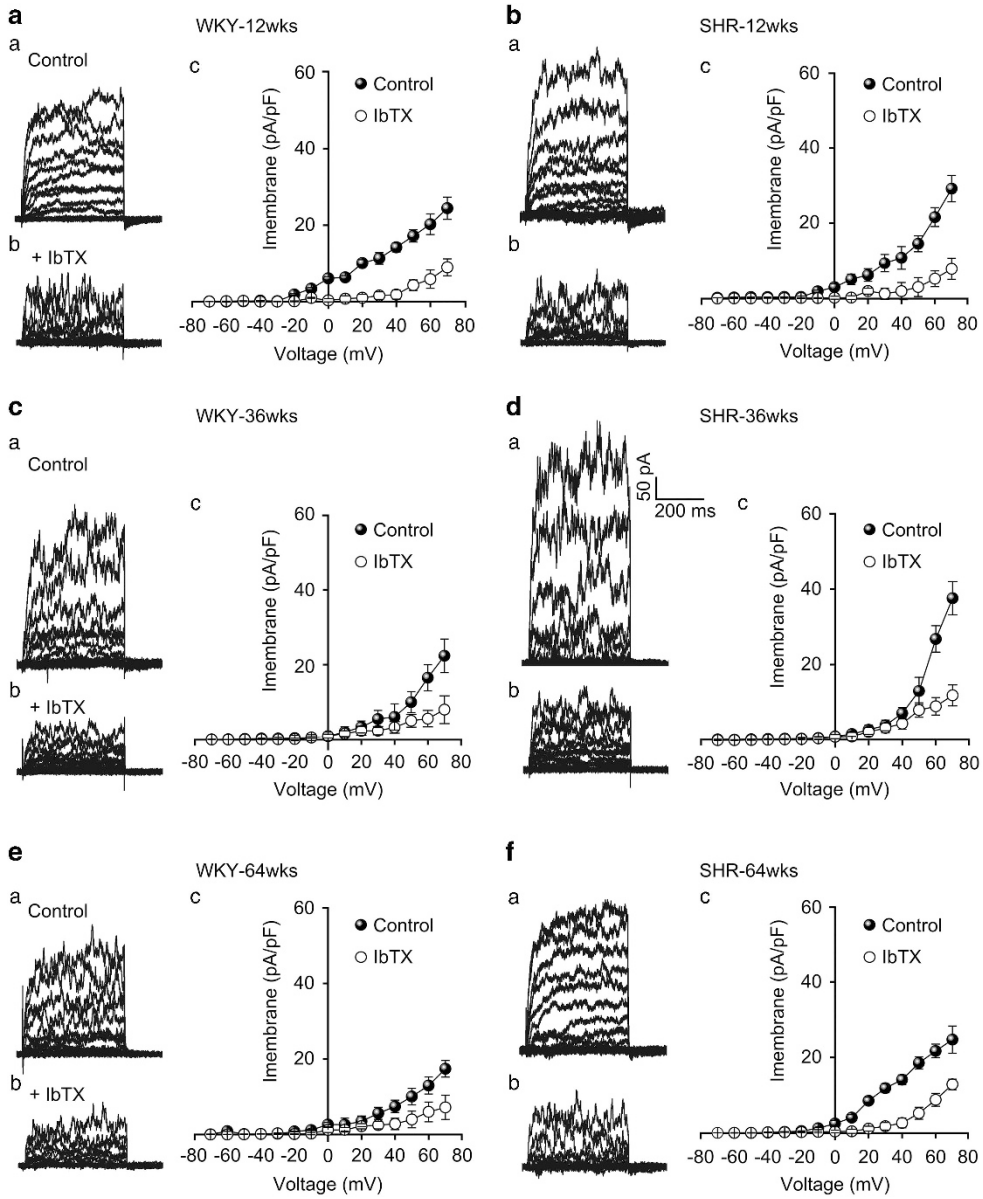

Figure 2 Whole-cell K^+ currents recorded using conventional patch-clamp methods in mesenteric artery smooth muscle cells. (**a**) WKY-12wks; (**b**) SHR-12wks; (**c**) WKY-36wks; (**d**) SHR-36wks; (**e**) WKY-64wks; (**f**) SHR-64wks. (a) Representative whole-cell K^+ currents measured during depolarizing voltage steps; (b) Example of whole-cell K^+ current blockade by IbTX (100 nM) for 10 min; (c) Current–voltage relationships showing the effect of IbTX on peak macroscopic K^+ current in myocytes from different WKY and SHR age groups. The current inhibited by IbTX was identified as BK_{Ca} current (that is, IbTX-sensitive current). IbTX, iberiotoxin.

before 36 weeks; aging subsequently diminishes this change in both WKY and SHR.

Hypertension and aging remodel BK_{Ca} single-channel activity via β1 subunit in arterial myocytes

We have previously shown that BK_{Ca} channel β1 subunit is upregulated during hypertension, whereas aging reduces BKβ1 expression and function.[16,20] To study the involvement of the β1 subunit in BK_{Ca} channel activity during hypertension and aging, the effects of tamoxifen (1 mM, 100 nM $[Ca^{2+}]_{free}$) on BK_{Ca} channels were examined. Tamoxifen, a (xeno) estrogen and β1 subunit-specific BK_{Ca} channel activator, is known to activate BK_{Ca} channels only when associated with the β1 subunit.[29] As illustrated in Figure 3c and d, the increases in the Po of BK_{Ca} channels that tamoxifen evoked in WKY rats were significantly lower than those of age-matched SHR (all $P<0.05$).

In normotensive rats, tamoxifen increased BK_{Ca} channel mean Po in 12-week myocytes ($n=16$ cells) nearly 3.8 times, while in 36-week ($n=20$ cells) and 64-week ($n=15$ cells) patches, tamoxifen evoked a 4-fold and 3.2-fold increase, respectively. Although aging enhanced the tamoxifen-evoked increase by ~5% in WKY-36wks myocytes ($P>0.05$), it inhibited this increase in WKY-64wks ($P<0.05$; Figure 3d). Importantly, there was a dramatic elevation of tamoxifen-induced increase in arterial myocytes from SHR-36wks (9.2-fold increase, $n=20$ cells) compared with that in SHR-12wks (6.2-fold increase, $n=18$ cells; $P<0.05$). In contrast, aging decreased the tamoxifen-evoked effects in SHR-64wks (6.8-fold increase, $n=12$ cells) when compared with SHR-36wks myocytes ($P<0.05$).

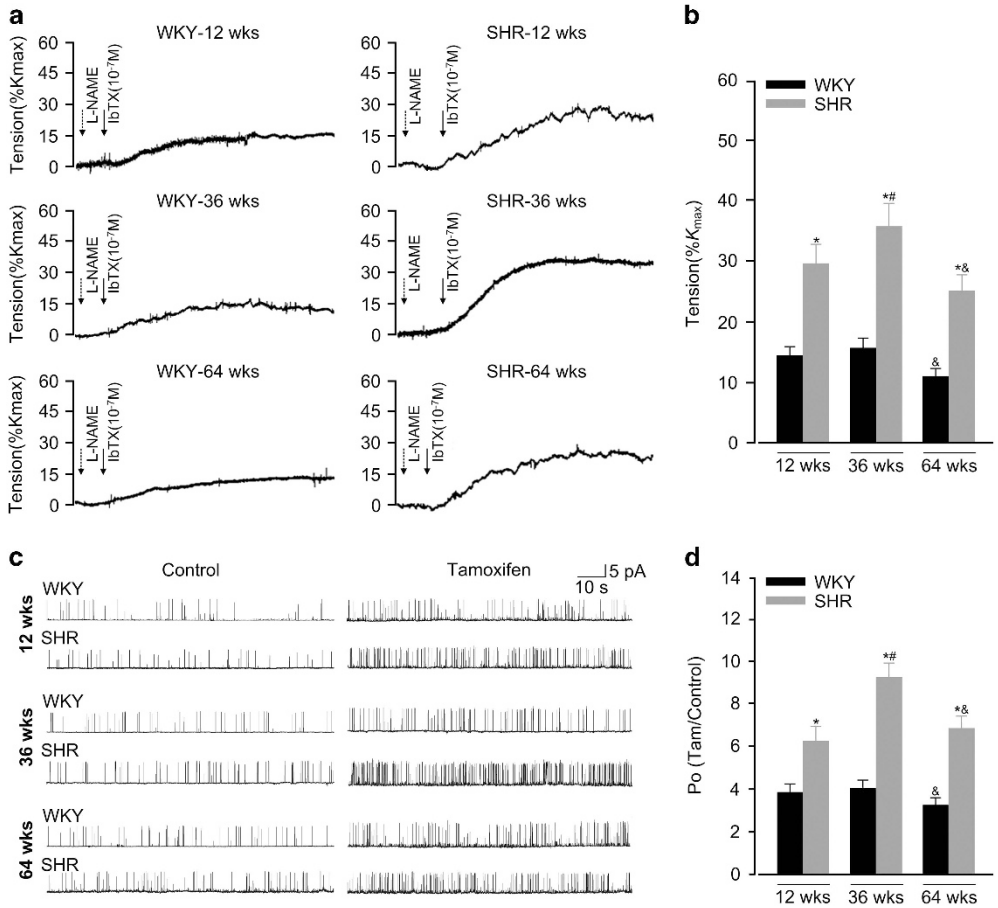

Figure 3 Effects of BK_{Ca} channel modulators on vascular tension and single-channel activity in mesenteric arteries. (**a**) Representative experimental tracings showing the effect of the BK_{Ca} blocker iberiotoxin (IbTX, 100 nM) on the vascular tension in mesenteric arteries. (**b**) Statistical diagram of the effect of IbTX on vascular tone ($n=6$ for each). (**c**) Effects of tamoxifen (Tam, 1 μM) on BK_{Ca} single-channel activity. $[Ca^{2+}]_{free}$ in the bath solution: 100 nM; HP = +40 mV. (**d**) Bar plot summarizing the mean ± s.e.m. fold change in the Po of BK_{Ca} channels after the application of Tam ($n=6$ for each). $^{*}P<0.05$ compared with age-matched WKY; $^{\#}P<0.05$ compared with corresponding 12 wks; $^{\&}P<0.05$ compared with corresponding 36 wks.

These data indicate that compared with age-matched WKY, hypertension increases β1 function in mesenteric arterial myocytes. Furthermore, β1 function in WKY is not altered during aging. However, with hypertension, aging elevates BK_{Ca} channel activation via a β1 ligand, which has negative effects on β1 function in old SHR. The effects of both hypertension and aging were significant ($P<0.05$ and $P<0.05$), but had no significant interaction.

The spatial distribution and expression of BK_{Ca} channel α and β1 subunits

Using immunofluorescence and confocal imaging, we examined the cellular distribution of BK_{Ca} channel α and β1 subunits in myocytes of small mesenteric arteries. The resulting data illustrated that a large fraction of BK_{Ca} α subunits were plasma membrane-localized in arterial myocytes; no positive fluorescence was observed intracellularly. In contrast, β1 subunits were not only present at the myocyte surface, but a fraction of the β1 subunits were located intracellularly in arterial myocytes (Figure 4a). As shown in representative cells (Figure 4a), BK_{Ca} channel β1 subunits localized at the sarcolemma, and the intracellular level was higher in mesenteric artery myocytes from SHR than in normotensive myocytes. Furthermore, aging increased the β1 subunit distribution in both adult WKY and SHR myocytes but inhibited this increase in the corresponding old groups.

We next studied the molecular mechanisms underlying the functional alteration of BK_{Ca} in mesenteric arteries, focusing on BK_{Ca} channel α and β1 subunit protein expression. Representative immunoblots, which are depicted using bar graphs (Figure 4b), showed that no significant changes in α subunit expression were observed during hypertension and aging (Figure 4b and c). In contrast, compared with age-matched WKY, the protein expression level of the β1 subunit observed in SHR was always significantly increased ($n=6$ in each group, all $P<0.05$). There was a significant increase in β1 subunit expression between WKY-12wks (1.00 ± 0.14) and WKY-36wks (1.57 ± 0.12); aging subsequently reduced β1 subunit expression in WKY-64wks mesenteric arteries to 0.71 ± 0.10, ~45.2% of the level in WKY-36wks ($P<0.05$). Consistent with these findings in the WKY group, in the hypertension groups, aging induced a significant increase in β1 subunit expression in SHR-36wks (3.02 ± 0.15) compared with SHR-12wks (2.17 ± 0.13). However, this increase drastically faded as aging continuously progressed (SHR-64wks: 1.30 ± 0.18; $P<0.05$). ANOVA analysis revealed significant differences between SHR and WKY ($P<0.05$), and within the age groups ($P<0.05$). The comparisons showed a significant interaction between hypertension and age ($P<0.05$).

Taken together, these findings suggest that the expression of β1 subunit is upregulated not only during hypertension, but also at the age of 36 weeks in both WKY and SHR.

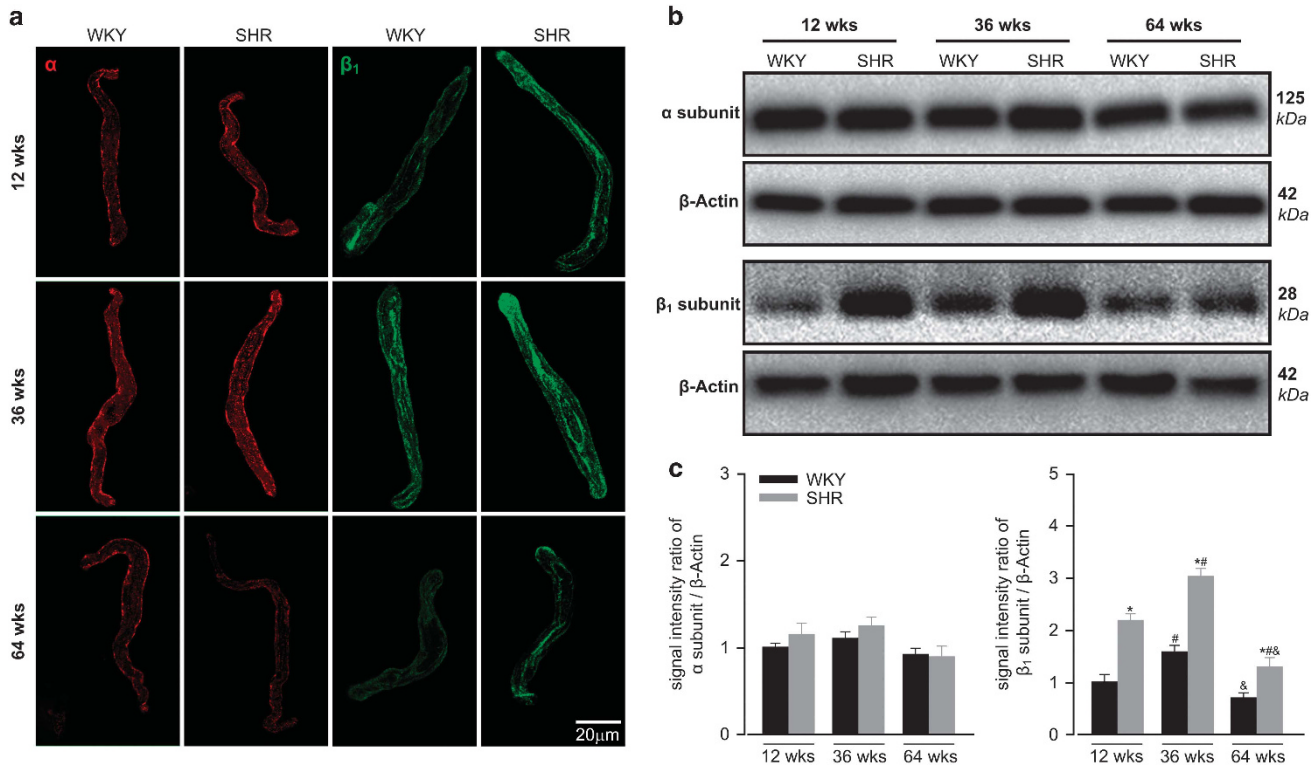

Figure 4 Protein expression levels of BK_{Ca} channel (α and β1) subunits in mesenteric artery smooth muscle cells. (**a**) Representative confocal images of arterial myocytes labeled with a BK_{Ca} α-specific antibody (red) or β1-specific antibody (green) from WKY and SHR at different ages. (**b**) Representative Western blots of BK_{Ca} channel (α and β1) subunits in mesenteric smooth muscle cells; immunoreactive bands corresponding to the BK_{Ca} α subunit, β1 subunit, and β-Actin. (**c**) Mean data for α subunit and β1 subunit protein levels expressed as a ratio to β-Actin ($n=6$ for each). $^{*}P<0.05$ compared with age-matched WKY; $^{\#}P<0.05$ compared with corresponding 12 wks; $^{\&}P<0.05$ compared with corresponding 36 wks.

Hypertension decreased, whereas aging increased, CpG methylation at *KCNMB1* gene promoter

We performed a further investigation of the DNA methylation status at β1 (*KCNMB1*) gene promoter to gain more insight into the molecular mechanisms underlying the functional alteration of BK_{Ca} in mesenteric arteries during hypertension and aging.

As shown in Figure 5, CpG dinucleotides at *KCNMB1* gene promoter were highly methylated in normotensive rats. Hypertension decreased CpG methylation at *KCNMB1* gene promoter (all $P<0.05$). This hypertension-mediated demethylation was not seen in mesenteric arteries from WKY. In contrast, the average total methylation rate of *KCNMB1* gene promoter in WKY tended to rapidly increase as aging progressed (Figure 5, all $P<0.05$). Contrary to expectations for the hypertensive rats, the methylation levels of the CpG island in mesenteric arteries from SHR-36wks increased slightly with age, albeit not significantly. Interestingly, in SHR-64wks, aging further induced significant increases in CpG methylation at *KCNMB1* gene promoter compared with SHR-12wks and SHR-36wks (Figure 5, both $P<0.05$). Furthermore, both hypertension ($P<0.05$) and aging ($P<0.05$) significantly affected CpG methylation at *KCNMB1* gene promoter, and their interaction was also significant ($P<0.05$).

DISCUSSION

The present study provides evidence that hypertension and aging have opposing effects on DNA methylation at β1 gene promoter, resulting in differential BKβ1 expression and functional modulation of arterial contractility. In addition, hypertension-induced demethylation is a major contributor to β1 upregulation in young and adult rats, and as aging progresses, aging-mediated hypermethylation is a stronger regulator of BKβ1 than hypertension. The opposite effects of hypertension and aging suggest distinct epigenetic regulatory mechanisms, as well as the significance of BK_{Ca} channel alterations in pathological and declined physiological situations.

Old spontaneously hypertensive rats are the most commonly studied model of aging and hypertension, with several reports[5,6] of increased SBP, accelerated changes in vascular dysfunction, increased arterial stiffening, and decreased vascular reactivity in the aging SHR model. Morbidity from hypertension is much higher in elderly individuals, and persistently elevated BP exacerbates age-related vascular changes.[30] This study has shown that hypertension increased both SBP and MAP, and these increases were both augmented by aging. This synergistic phenomenon in BP may involve various mechanisms.[4,5,31,32] For example, the interplay of VSMC stiffness and adhesion contributes to the interaction of hypertension and aging in accelerating vascular stiffness, thereby elevating aortic pressures.[5] In addition, hypertension and aging additively cause the enhancement of sympathetic activity, the impairment of autonomic function, BP variability, and vascular senescence and dysfunction.[32]

During hypertension, ion channels in the plasma membrane of VSMCs undergo aberrant expression and function in response to the abnormally elevated arterial tone.[33] Our and other previous reports[13,14,16,27,33] have shown that hypertension is associated with an increase in BK_{Ca} channel expression and function, including enhanced BK_{Ca} currents, higher α subunit expression, or increased expression of β1, but not the α subunit. Questions about these diverse intrinsic mechanisms remain unanswered, but are of great interest and

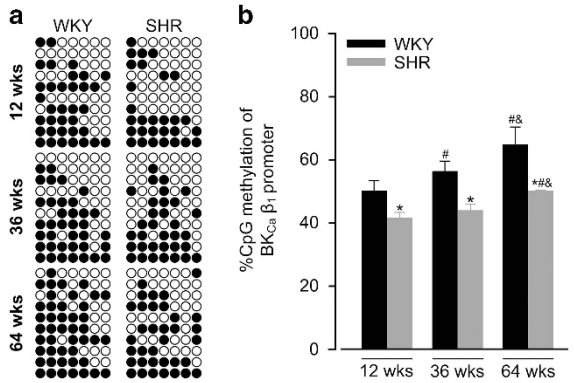

Figure 5 Comparison of DNA methylation status of the BKβ1 gene (*KCNMB1*) promoter in the mesenteric arteries from WKY and SHR. (**a**) Representative DNA methylation status of the CpG island in the promoter region of *KCNMB1* gene; the black and white circles denote methylated and unmethylated cytosines, respectively. A total of 7 CpG sites in the CpG island and 10 clones were subjected to sequencing. (**b**) The average total percentage of methylated CpG in *KCNMB1* promoter in mesenteric arteries of WKY or SHR ($n=6$ for each). $^{*}P<0.05$ compared with age-matched WKY; $^{\#}P<0.05$ compared with corresponding 12 wks; $^{\&}P<0.05$ compared with corresponding 36 wks.

must be studied further. The BK_{Ca} channel is primarily involved in a mechanism inhibiting further increases in VSMC tone, and this appears to be part of a compensatory mechanism for protection in pathological conditions.[33] The present study examined BK_{Ca} channel currents in arterial myocytes, BK_{Ca} channel contributions to vascular tone regulation, and α and β1 subunit expression of mesenteric arteries from WKY and SHR of different ages. The results revealed that hypertension significantly upregulated BK_{Ca} channel signaling mechanisms, including BK_{Ca} channel activity (Figures 2 and 3a and b) and β1, but not α, subunit expression (Figure 4). Hypertension also facilitated tamoxifen-induced BK_{Ca} channel activation in arterial myocytes (Figure 3c and d), which represented an increase in BKβ1 function. These data are consistent with the results of Western blot analysis showing that β1 function was augmented in hypertensive mesenteric arteries. More importantly, our study has provided novel evidence of an effect of hypertension on BKβ1 promoter demethylation that plays a role in elevated β1 gene expression, and hence upregulated β1 subunit expression, increased BK_{Ca} channel activity in arterial myocytes, and myogenic tone in genetic hypertension at all ages. To our knowledge, this is the first study to show an epigenetic modification of β1 promoter demethylation in hypertension-induced reprogramming of BK_{Ca} channel β1 subunit expression and function in mesenteric arteries. Transcriptional regulation by DNA methylation is commonly observed in CpG islands located around promoter regions, and CpG island methylation of transcriptional start sites is associated with long-term silencing.[23,24] Here, we analyzed the CpG island located near the *KCNMB1* gene promoter region (within 2000 bp) at chromosome 10 from position 18911664 to 18912065, which is 402 bp in length and contains 7 CpG sites. At all ages, the average methylation of the 7 CpG sites *KCNMB1* gene promoter in WKY was invariably higher than in age-matched SHR (Figure 5), suggesting hypertension-induced β1 gene promoter demethylation, which is consistent with the upregulation of β1 subunit expression and function. Thus, promoter methylation level plays a crucial role in regulating BK_{Ca} channel and vascular contractility in hypertension. However, there are two limitations in this approach. Previous studies in sheep demonstrated that the specificity protein-1 (Sp-1) binding site at BKβ1 promoter was highly methylated in the uterine arteries of nonpregnant sheep, and that methylation inhibited transcription factor binding and BKβ1 promoter activity.[24] We did not determine whether hypertension decreased CpG methylation at specific transcription factor binding sites, as increased DNA methylation at the promoter is generally associated with gene silencing by preventing binding of transcription factors.[24,34] The other limitation is that we did not provide evidence of a cause-and-effect relationship with DNA methylation inhibition. 5-Aza-2′-deoxycytidine, a non-selective DNA methyltransferase (DNMT) inhibitor, ablated hypoxia-induced methylation of the $Sp\text{-}1_{-520}$ and USF_{-15} binding sites at the estrogen receptor-α (ER-α) promoter, restored the binding of Sp-1 and upstream stimulatory factor (USF) to the promoter, and reinstated the expression of ER-α in uterine arteries.[26] Further investigations are required to better understand the underlying mechanisms of hypertension-mediated *KCNMB1* promoter demethylation and upregulation of BKβ1 expression to buffer pressure-induced vasoconstriction in mesenteric arteries.

Epigenetic changes constitute an important component of the aging process, among which DNA methylation has become a dominant hallmark of aging.[22,35] DNA methylation increased with age (Figure 5) in both WKY and SHR, and the increase in WKY was rapid. Unlike WKY-36wks, although there appeared to be a trend of increased methylation in SHR-36wks, aging had no significant effect on BKβ1 promoter methylation, suggesting a minor role in regulating β1 gene expression in mesenteric arteries of adult hypertensive rats. However, CpG methylation was significantly increased in SHR-64wks, an older age for assessing longer-term vascular remodeling events, suggesting that aging plays a major role in suppressing β1 gene expression in older hypertensive animals. On the basis of DNA methylation data, in theory, the expression and function of the β1 subunit should decline with increasing age. As expected, in both old WKY and SHR groups, with an elevation of DNA methylation status there was a concomitant reduction in BK_{Ca} channel activity and β1 subunit function and expression. Consistent with previous studies,[18–20] the contribution of the BK_{Ca} channel to the regulation of vascular tone in arteries was downregulated with advancing age. Between young and adult normotensive rats, aging not only enhanced CpG methylation at *KCNMB1* gene promoter but also upregulated β1 subunit expression. These adverse effects observed in adult normotensive rats are most likely attributable to a compensatory mechanism in response to slightly elevated intraluminal perfusion pressure in mesenteric arteries, suggesting that a post-transcriptional modulation of the β1 subunit exists. In addition, the lack of detectable changes in BK_{Ca} channel activity and β1 function may be due to physiological modulation; physiological stimuli can regulate subunit trafficking to control functional BK_{Ca} channel activity in arterial myocytes and vascular contractility.[11,36] Unexpectedly, in the adult SHR, aging did not alter CpG methylation but significantly facilitated BK_{Ca} channel currents and upregulated β1 subunit expression and function. These rather surprising results may be attributable not only to compensation from hypertension-induced *KCNMB1* demethylation but also to the post-transcriptional modulation of β1 subunit expression. As mentioned above, aging plays a minor role in regulating β1 gene expression in the mesenteric arteries of adult hypertensive rats; instead, hypertension-mediated demethylation is predominantly involved in β1 gene expression regulation as a pathological compensatory mechanism during this period. An additional implication is that the SHR model can develop and adapt to hypertension gradually, which may affect the temporal nature of its remodeling process.[5] Thus, with further advancement to an older age when longer-term vascular remodeling

events can be assessed, aging surpasses hypertension itself in making major contributions to the downregulation of BKβ1. Nevertheless, it is worth highlighting that we cannot exclude the pre-transcriptional modulation of methylation on the *KCNMB1* gene.

The epigenetic mechanisms of ion channel regulation in vascular smooth muscle cells and their adaptation to hypertension and aging remain poorly understood, despite the identification of several microRNAs directly targeting or regulating the function of ion channels in VSMCs, including miRNA-328, miRNA-145 and miRNA-153.[25,37,38] In addition, there is evidence for another epigenetic mechanism in which promoter demethylation at sequence-specific transcription factor binding sites dysregulates BK_{Ca} channel expression and activity in uterine vascular adaptations to pregnancy.[24] However, little is known about the methylation levels of enhancers, insulators and gene bodies, which have been largely overlooked yet closely influence the binding and function of regulatory proteins.[23] More investigations are clearly required to identify the methylation status of *KCNMB1* gene enhancers and other regulatory elements, particularly to elucidate the mechanisms that mediate BKβ1 expression in vascular smooth muscle during hypertension and aging. Furthermore, it is also important to determine the mechanisms of BK_{Ca} subunit trafficking to control channel activity and the functions of arterial myocytes in hypertension-related pathology or aging-mediated physiologic decline.

In summary, we have shown that hypertension and aging can divergently alter CpG methylation at *KCNMB1* promoter, subsequently resulting in differential BKβ1 expression and functional modulation of mesenteric arterial contractility. These findings are useful for comprehensively understanding the molecular mechanisms regulating BK_{Ca} channel activity and vascular contractility during hypertension and aging.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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