Hypertension Research (2017) 40, 441–449

www.nature.com/hr

ORIGINAL ARTICLE

Epigenetic regulation of L-type voltage-gated Ca^{2+} channels in mesenteric arteries of aging hypertensive rats

Jingwen Liao[1,2], Yanyan Zhang[1], Fang Ye[1], Lin Zhang[1], Yu Chen[1], Fanxing Zeng[1] and Lijun Shi[1]

Accumulating evidence has shown that epigenetic regulation is involved in hypertension and aging. L-type voltage-gated Ca^{2+} channels (LTCCs), the dominant channels in vascular myocytes, greatly contribute to arteriole contraction and blood pressure (BP) control. We investigated the dynamic changes and epigenetic regulation of LTCC in the mesenteric arteries of aging hypertensive rats. LTCC function was evaluated by using microvascular rings and whole-cell patch-clamp in the mesenteric arteries of male Wistar-Kyoto rats and spontaneously hypertensive rats at established hypertension (3 month old) and an aging stage (16 month old), respectively. The expression of the LTCC α1C subunit was determined in the rat mesenteric microcirculation. The expression of miR-328, which targets α1C mRNA, and the DNA methylation status at the promoter region of the α1C gene (CACNA1C) were also determined. *In vitro* experiments were performed to assess α1C expression after transfection of the miR-328 mimic into cultured vascular smooth muscle cells (VSMCs). The results showed that hypertension superimposed with aging aggravated BP and vascular remodeling. Both LTCC function and expression were significantly increased in hypertensive arteries and downregulated with aging. miR-328 expression was inhibited in hypertension, but increased with aging. There was no significant difference in the mean DNA methylation of CACNA1C among groups, whereas methylation was enhanced in the hypertensive group at specific sites on a CpG island located upstream of the gene promoter. Overexpression of miR-328 inhibited the α1C level of cultured VSMCs within 48 h. The results of the present study indicate that the dysfunction of LTCCs may exert an epigenetic influence at both pre- and post-transcriptional levels during hypertension pathogenesis and aging progression. miR-328 negatively regulated LTCC expression in both aging and hypertension.
Hypertension Research (2017) **40,** 441–449; doi:10.1038/hr.2016.167; published online 24 November 2016

Keywords: aging; DNA methylation; L-type voltage-gated Ca^{2+} channel; miRNA

INTRODUCTION

The elderly population has a relatively high prevalence of hypertension,[1] and aging, genetic and environmental factors are associated with the development of this condition.[2] As hypertension progresses with age, reduced reactivity in small arteries, represented by alterations of arterial stiffness, arterial wall thickening, and reduced myogenic responsiveness, typically induces total peripheral resistance and manifests as an elevation in blood pressure (BP).[3,4] Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels (LTCCs) in vascular smooth muscle cells (VSMCs) has a central role in the regulation of vascular tone by membrane potential.[5,6] Hypertension, typified by intracellular Ca^{2+} dysfunction, alters the expression of the LTCC α1C subunit in the vasculature.[7,8] In addition, aging significantly affects ion channels during arterial dysfunction.[9,10]

The mechanisms underlying hypertension are not definitively understood. However, with the surge of epigenetic studies in recent years, the pathological development of hypertension over the lifespan may be better explained, as epigenetic factors provide a unique understanding of the balance between external stimuli and heritable messages. Epigenetic regulation modifies gene expression without involving changes to the DNA sequence, and modifications can be transmitted to daughter cells.[9] Studies on DNA methylation and noncoding microRNAs (miRNA, miR) have suggested their potential contribution to hypertension during aging. Gene expression is typically negatively influenced by the level of DNA methylation of CpG islands, which refer to C-G dinucleotide-rich regions located in the promoters of mammalian genes.[10,11] Aberrant DNA methylation has important roles in the occurrence and development of complex diseases, including hypertension;[12,13] specific DNA regions show directional epigenetic changes in older individuals, suggesting the importance of these events in the aging process.[14,15] miRNAs are short noncoding RNAs (21–25 nucleotides) that negatively influence protein abundance at the post-transcriptional level through incomplete or complete complementary binding to the 3′-untranslated region of target

[1]Department of Exercise Physiology, Beijing Sport University, Beijing, China and [2]Department of Sport and Health Sciences, Guangzhou Institute of Physical Education, Guangzhou, China
Correspondence: Professor L Shi, Department of Exercise Physiology, Beijing Sport University, Beijing 100084, China.
E-mail: l_j_shi72@163.com
Received 6 May 2016; revised 18 September 2016; accepted 16 October 2016; published online 24 November 2016

mRNA;[16] the dysregulation of miRNAs could potentially result in disease development because these molecules are involved in multiple cellular functions. Several reviews have well summarized the vital roles of miRNAs in hypertension,[17–19] and as highly conserved molecules, dynamic miRNA expression also contributes to the progression of vascular aging.[20,21] Studies have demonstrated that miR-1, miR-145 and miR-328 directly target or regulate the function of LTCCs in VSMCs. Among these molecules, we focused on miR-328, which has a targeting role on the LTCC α1C subunit and has been examined in both vascular and cardiac myocytes.[22,23]

Little is known regarding how epigenetic alterations affect LTCC remodeling in both hypertension and aging arteries or whether the results of their interaction accelerate the pathological process. Thus, studies are urgently needed to establish epigenetic mechanisms in vascular electrophysiology and BP control at different life stages both during normal development and the progression of hypertension. Evidence of an association between epigenetic regulation and LTCCs during hypertension and aging is scare; therefore, this study focuses on vascular LTCC remodeling in aging hypertensive rats and investigates the potential contribution of miR-328 and DNA methylation drift on the *CACNA1C* gene, which encodes the α1C subunit of LTCCs. We hypothesized that both DNA methylation of the CACNA1C promoter and miR-328 regulate hypertension progression and that aging superimposed on pathological vasculature exaggerates LTCCs dysfunction via epigenetic factors.

METHODS

Animals and sample preparation

Male 3-month-old and 16-month-old Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) (WKY-3M, SHR-3M, WKY-16M, SHR-16M; $n=20$ in each group) were purchased from Vital River Laboratory Animal Technology (Beijing, China) and maintained in a 12:12 h light–dark cycle at a temperature of 23–25 °C and humidity level of 40–60% with free access to standard rodent chow and water. BP and heart rate were measured in warmed, restrained, conscious rats using the caudal artery tail-cuff method (BP-2010A, Softron Biotechnology, Beijing, China). The animals were killed by draining blood from the abdominal aorta under anesthesia with sodium pentobarbitone (50 mg kg^{-1}, intraperitoneal). The experimental protocols using laboratory animals were approved by the ethical committee of Beijing Sport University and performed in accordance with Chinese animal protection laws and institutional guidelines.

Histological assays

For histological assays, mesenteric arteries were carefully dissected from connective tissue, avoiding mechanical stretch, and perfused with 4% paraformaldehyde for 12 h. Subsequently, the vessels were rinsed with phosphate-buffered solution and dehydrated with ethanol. The tissues were stained with hematoxylin and eosin, and images were obtained using an inverted microscope (IX71-F22PH, OLYMPUS, Tokyo, Japan) connected to a computer.

Assessment of vasomotor function

The mesenteric arteries from the euthanized rats of each group were carefully excised and placed in cold Kreb's buffer containing the following (in mM): 131.5 NaCl, 5 KCl, 1.2 NaH_2PO4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11.2 glucose, 13.5 $NaHCO_3$ and 0.025 EDTA, and were continuously gassed with 95% O_2 and 5% CO_2 (pH 7.4). After equilibration, second to third (A2-A3) order mesenteric arteries were cleaned of adhering fat and connective tissue and cut into short segments. The Multi Myograph System (620 M; DMT, Aarhus, Denmark) was used for the contractile studies. The contractile response was elicited using KCl; vascular tension was evaluated by measuring the maximum peak height and expressed as a percentage of contraction to 120 mM K^+ (K_{max}). The non-selective NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) was added. The LTCC inhibitor nifedipine (10^{-9}– 10^{-5} M) was used to relax the vessels to assess the contribution of LTCC to the regulation of vascular tone after maximal contraction induced by noradrenaline (NE, 10^{-5} M).

Cell isolation and electrophysiological recording

VSMCs from A2-A3 mesenteric arteries were enzymatically isolated as previously described.[24] Whole-cell patch-clamp recordings were performed using pipette electrodes with a resistance of 2–4 MΩ in the given recording solution. Cells were perfused with a solution containing (mM): 20 $BaCl_2$, 10 HEPES, 5 glucose, 1 $MgCl_2$, and 124 choline chloride, and adjusted to pH 7.4 with CsOH. Patch electrodes were filled with a solution containing (mM): 130 CsCl, 10 HEPES, 3 Na_2ATP, 0.1 Na_2GTP, 1.5 $MgCl_2$, 10 glucose, 10 EGTA and 0.5 MgATP, and adjusted to pH 7.3 with CsOH. $BaCl_2$ was used as a charge carrier to guarantee stable currents at 25 °C. To elicit inward Ca^{2+} currents, the −30 mV potential was held and a sequence of pulses ranging from −30 to +40 mV was applied in 10 mV increments (200 ms duration). After obtaining whole-cell currents, nifedipine (0.1 μM) was used to verify the identity of the LTCC currents. The currents were amplified using a Digital 1440 (Axon Instruments, Silicon Valley, CA, USA) and analyzed using pClamp 10.2 software (Axon Instruments). VSMCs from six rats in each group were determined in the present electrophysiological study.

DNA bisulfite sequencing PCR

Genomic DNA was isolated from mesenteric artery samples using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA quality was determined based on the ratio of absorbance at 260 and 280 nm (⩾1.8 was considered qualified) and confirmed using agarose gel (1.0%) electrophoresis. Subsequently, the DNA samples were subjected to sodium bisulfate conversion using the EZ DNA Methylation-Gold Kit (ZYMO Research, Orange, CA, USA). PCR amplification of the CACNA1C promoter region on the CpG island was performed; the products were separated using 2.0% agarose gels, and the bands were resolved using the TIANgel Midi Purification Kit (Tiangen, Beijing, China). The purified bands were cloned into a pEASY-T1 Cloning vector (TRAN, Beijing, China). Subsequently, 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 sense, 5′-GAATTTATTATGTTTTT TGGAGGTGA-3′; antisense, 5′-ATCTCAACTCACTTAACTTTTACTCTAC C-3′. The results are expressed as a percentage of methylated to total cytosine (mC/tC).

Quantitative real-time PCR

A mirVana miRNA Isolation Kit (AM1560, Ambion, Waltham, MA, USA) was used to isolate total RNA from mesenteric arteries or cells according to the manufacturer's instructions. The RNA was quantified using a spectrophotometer (ND1000 NanoDrop, Watertown, MA, USA) to measure the absorbance at 260 nm (A260) and 280 nm (A280), with A260/A280 ratios above 1.8 indicating high-quality RNA. cDNA was synthesized from total RNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Subsequently, cDNA was stored at −20 °C until subsequent use in PCR reactions. The primers were designed using Primer Premier 6 and assessed using Oligo 7. The following primers were used for PCR amplification of α1C mRNA: sense, 5′-GCCGCAGTTAAGTCCAATGT-3′ and antisense, 5′-GCA CTTCTGTGAGCCAATGA-3′. The following primers were used for the PCR amplification of GAPDH mRNA: sense, 5′-CCTGCCAAGTATGATGAC-3′; antisense, 5′-GGAGTTGCTGTTGAAGTC-3′. The following primer was used for reverse transcription of miR-328: 5′-GTCGTATCCAGTGCAGGGTC CGAGGTATTCGCACTGGATACGAACGGAAGG-3′. The following primers were used for PCR amplification of miR-328: sense, 5′-ATTATATCTGG CCCTCTCTGC-3′; antisense, 5′-TCGTATCCAGTGCAGGGTC-3′. The following primers were used for reverse transcription of U6: 5′- CTCGCTTCGG CAGCACATATACT -3′. The following primers were used for PCR amplification of U6: sense, 5′-CTCGCTTCGGCAGCACATATACT-3′; antisense, 5′-ACG CTTCACGAATTTGCGTGTC-3′. The PCR reaction mixture (20 μl) contained of 10 μl of Power SYBR Green PCR Master Mix (2×) (Applied Biosystems, Bedford, MA, USA), 1 μl of forward primer, 1 μl of reverse primer, 2 μl of cDNA and 6 μl of DNase-free water. The PCR program included an initial

denaturation cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. At the end of the PCR reaction, the samples were subjected to 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s to generate the dissociation curve. Quantitative normalization was performed on U6 or GAPDH for miRNA or mRNA detection, respectively. All PCR reactions were performed in duplicate. The samples were analyzed using a Sequence Detection System (ABI 7500, Applied Biosystems). The relative expression of each gene was determined using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Mesenteric arteries or cells were homogenized on ice with lysis buffer, and the supernatant was collected after centrifugation. The protein concentration was determined by the Bradford method using bovine serum albumin as a standard. Equal amounts of proteins were separated on 10% SDS–polyacrylamide gel electrophoresis gels and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with bovine serum albumin, the membrane was washed and incubated overnight at 4 °C with primary antibody against α1C (ab84814, 1:200; Abcam, Cambridge, UK) and GAPDH (sc32233, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). On the second day, the proteins were incubated with secondary antibody, followed by autoradiography using the ChemiDoc XRS$^+$ System (Bio-Rad Laboratories, Hercules, CA, USA). The band intensities were quantified using Image Lab Software (Bio-Rad Laboratories) and are expressed as a percentage of GAPDH in each lane.

VSMCs *in vitro* transfection

Primary VSMCs were isolated and cultured from superior mesenteric arteries of WKY according to a previous study.[25] Thereafter, VSMCs were maintained in Dulbecco's modified eagle medium (DMEM, Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Paragon Biotech, Baltimore, MD, USA) and a 1% penicillin/streptomycin solution (Gibco) in an incubator at 37 °C and 5% CO_2. The transfections were performed when the cell density reached 70–90% at passage 5–7. miR-328 mimics (sense: 5′-CUGGCCCUCUCU GCCCUUCCGU-3′; antisense: 5′-GGAAGGGCAGAGAGGGCCAGUU-3′) or scramble miRNA (as negative control, NC) (sense: 5′-UUCUCCGAA CGUGUCACGUTT-3′; antisense: 5′-ACGUGACACGUUCGGAGAATT -3′) (GenePharma, Shanghai, China) were transfected into VSMCs using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. For protein quantification, cells were collected at 12, 24, 48 and 72 h after transfection, and for mRNA detection, cells were collected at 24, 48, 72 and 96 h after the transfection. Cells were washed three times with cold phosphate-buffered solution before processing for further experiments.

Statistical analyses

The data are expressed as the mean ± s.e.m. Normality tests were used to determine the normal distribution of the data, and when the data were not normally distributed, log-transformation was applied. Two-way analysis of variance (ANOVA, hypertension × aging) was used to assess the interactive influence of the two factors. Selected *post hoc* analysis was performed using an independent sample *t*-test. For *in vitro* experiments, a paired *t*-test was used to compare two groups of the same observation time point. *P*-values < 0.05 were considered statistically significant. All analyses were performed using SPSS 13.0 software. The figures were obtained using SigmaPlot 11.0 (Systat Software, Chicago, IL, USA).

RESULTS

Differences in blood pressure and vascular morphology in aging and hypertensive rats

Significant differences between SHR and WKY ($P<0.01$) were observed, as hypertension significantly increased BP (diastolic BP, systolic BP and mean arterial pressure) and heart rate compared with age-matched normal group ($P<0.01$) (Table 1). ANOVA also showed that aging significantly elevated systolic BP and mean arterial pressure ($P<0.01$). In addition, the interactions between hypertension and aging for systolic BP ($P<0.05$) and mean arterial pressure ($P<0.01$) were significant.

Morphological differences among groups were determined by hematoxylin and eosin staining (Figure 1). Both hypertension and aging were significant for the arterial lumen diameter ($P<0.01$ and $P<0.01$), with significant interactions ($P<0.01$). The mesenteric arteries of WKY had a larger lumen diameter (~17.6% at 3 M and 31.1% at 16 M) than those of SHR. The arterial lumen diameters of SHR-16M and WKY-16M were reduced (~53.1% and 27.2%) compared with those of young mice in the same strain. Hypertension was significant for medial layer thickness ($P<0.01$); SHR increased higher than WKY in both young (~40.7%) and old animals (~15.0%). However, aging and the interaction with hypertension were not significant. The ratio of medial layer thickness to lumen diameter was significantly influenced by hypertension ($P<0.01$) and aging ($P<0.01$). The ratio in SHR-16M increased by ~39.8% compared with that in WKY-16M and ~32.7% compared with that in SHR-3M. The interaction between hypertension and aging was also significant ($P<0.01$).

LTCCs channel activity and gating properties in the mesenteric arteries of aging and hypertensive rats

Artery rings received KCl (120 mM) to examine the stable state and induce maximal contraction (six rats were used in each group); no significant differences were observed in the K_{max} between each group. ANOVA showed that hypertension significantly increased arterial sensitivity to NE ($P<0.01$), while aging significantly decreased arterial sensitivity ($P<0.05$), with no significant interaction. The NE-induced maximal contraction in SHR-3M was 135.1 ± 8.4% K_{max}, which was higher than that in WKY-3M (110.7 ± 6.9% K_{max}) and SHR-16M (118.8 ± 7.0% K_{max}). The contraction in WKY-16M was 95.8 ± 9.2% K_{max}, which was lower than that in both WKY-3M and SHR-16M (Figure 2a).

To determine the physiological contribution of LTCCs to the vascular tone, the effects of the LTCC inhibitor nifedipine (10^{-9}– 10^{-5} M) on the relaxation of mesenteric artery rings was measured after NE was applied to stimulate pre-contraction. At the plateau of the NE-induced contraction, nifedipine was applied in half-log increments; a parallel leftward shift of the concentration-relaxation curve was detected. The 10^{-5} M NE-induced maximal tension increase

Table 1 Blood pressure parameter of rats

	WKY-3M	*SHR-3M*	*WKY-16M*	*SHR-16M*
HR (beats min^{-1})	355 ± 10	412 ± 11**	369 ± 13	440 ± 14$^{\#,\&\&}$
SBP (mmHg)	134.9 ± 2.6	191.6 ± 2.3**	152.2 ± 5.1**	212.0 ± 6.1$^{\#\#,\&\&}$
DBP (mmHg)	93.7 ± 4.6	145.6 ± 4.2**	106.3 ± 4.31	155.6 ± 7.3$^{\&\&}$
MAP (mmHg)	107.4 ± 2.8	160.9 ± 5.2**	121.3 ± 3.3*	174.4 ± 3.9$^{\#,\&\&}$

Abbreviations: BP, blood pressure; DBP, diastolic BP; HR, heart rate; MAP, mean arterial pressure; SBP, systolic BP; SHR, spontaneously hypertensive rat.
The results are expressed as the mean ± s.e.m. ($n=20$). *$P<0.05$ and **$P<0.01$ *vs.* WKY-3M; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ *vs.* SHR-3M; $^{\&}P<0.05$ and $^{\&\&}P<0.01$ *vs.* WKY-16M.

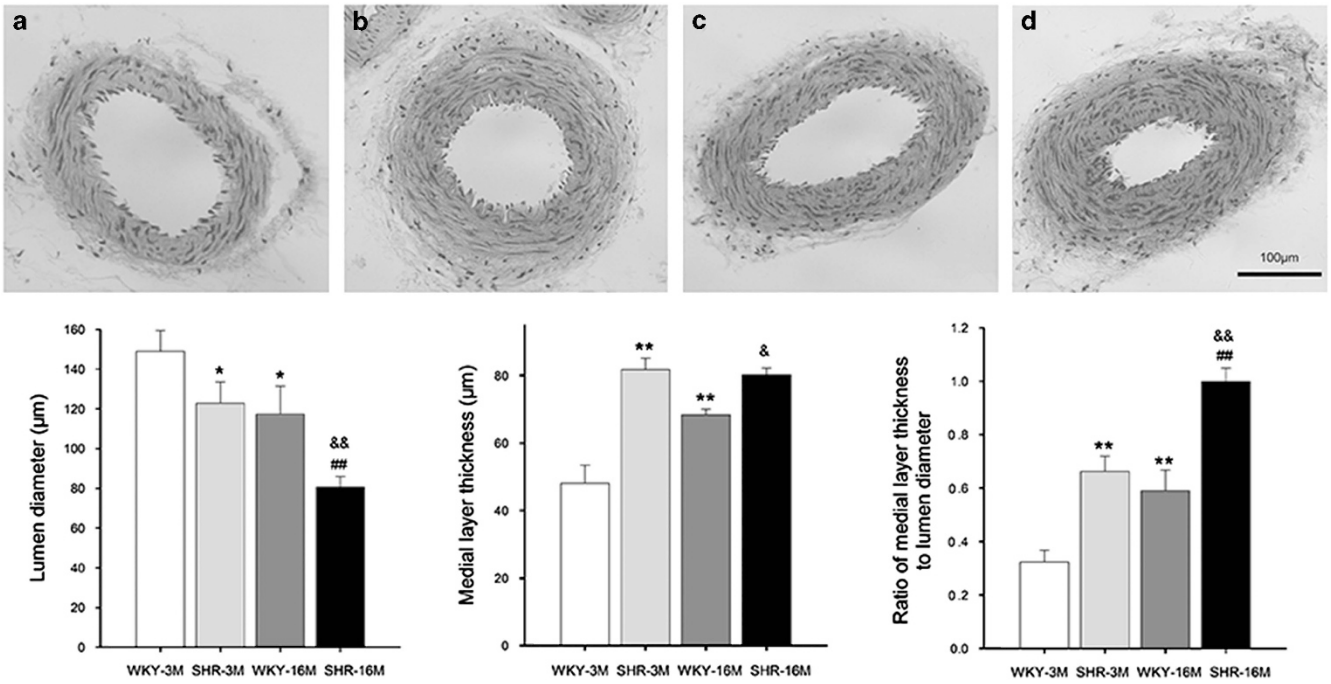

Figure 1 Histological appearance of the mesenteric arteries stained with HE in WKY-3M (**a**), SHR-3M (**b**), WKY-16M (**c**) and SHR-16M (**d**). The results are expressed as the mean ± s.e.m. ($n=6$). $^{*}P<0.05$ and $^{**}P<0.01$ *vs.* WKY-3M; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ *vs.* SHR-3M; $^{\&}P<0.05$ and $^{\&\&}P<0.01$ *vs.* WKY-16M. HE, hematoxylin and eosin; SHR, spontaneously hypertensive rat. A full color version of this figure is available at the *Hypertension Research* journal online.

was treated as 100% in each group. The values for pIC50, a negative logarithm of the 50% effective concentration, were 6.77 ± 0.05 (WKY-3M), 6.97 ± 0.08 (SHR-3M), 6.65 ± 0.06 (WKY-16M) and 6.31 ± 0.04 (SHR-16M) (Figure 2b). The sensitivity of the mesenteric artery to nifedipine was SHR-3M > WKY-3M, SHR-3M > SHR-16M and WKY-16M > SHR-16M. The effects of nifedipine on reversing contractions were higher in SHR-3M compared with the other groups. Both hypertension ($P<0.01$) and aging ($P<0.05$) and their interaction ($P<0.05$) were significant; the contribution of LTCCs to the regulation of the vascular tone were increased in hypertension and decreased with age.

The electrophysiological properties of LTCCs in VSMCs from mesenteric arteries were determined using whole-cell patch-clamp recording. We confirmed that the currents recorded were Ba^{2+} currents through LTCCs by applying nifedipine (100 nM); the inward currents were almost completely blocked compared with the control (Figure 2c). The current–voltage (*I–V*) curve of LTCC was obtained after normalizing the Ca^{2+} currents relative to the mean cell capacitance (pA/pF) to compensate for differences in cell size. The cell capacitance from the four groups was 17.2 ± 1.5 pF (WKY-3M, $n=23$ cells), 17.5 ± 2.5 pF (SHR-3M, $n=25$ cells), 18.3 ± 1.9 pF (WKY-16M, $n=20$ cells) and 18.8 ± 2.2 pF (SHR-16M, $n=24$ cells); each n represents cells from 6 rats. The mean current densities at different holding membrane potentials were obtained, and the peak LTCC current density was recorded at +20 mV in all four groups. The mean maximal LTCC current density was − 8.4 ± 1.0 pA/pF for WKY-3M ($n=20$ cells), − 12.2 ± 1.3 pA/pF for SHR-3M ($n=20$ cells), − 6.2 ± 1.1 pA/pF for WKY-16M ($n=18$ cells) and − 6.2 ± 0.8 pA/pF for SHR-16M ($n=19$ cells); each n represents cells from six rats (Figure 2d). Significant effects of hypertension ($P<0.01$) and aging ($P<0.01$) were observed for the maximal Ca^{2+} current density; the maximal current density in SHR-3M was much higher than that in WKY-3M, whereas aging markedly decreased LTCC currents in both hypertensive and normotensive rats. The interaction between hypertension and aging was also significant ($P<0.05$).

mRNA and protein expression of α1C in the mesenteric arteries of aging and hypertensive rats

The ANOVA results were significant for aging ($P<0.01$); aging inhibited α1C expression in both normotensive and hypertensive rats, as protein and mRNA expression decreased in WKY-16M and SHR-16M compared with their 3 M strains (Figure 3). However, the expression of α1C for the SHR strain was not significant, although both protein and mRNA expression increased in SHR compared with WKY in young animals. The interaction of hypertension and aging was significant ($P<0.05$).

DNA methylation status of CACNA1C in the mesenteric arteries of aging and hypertensive rats

The CpG island analyzed on the CACNA1C of rat mesenteric arteries was located near the promoter region (within 2000 bp) at chromosome 4 from position 216 566 309 to 216 566 847. This CpG island is 538 bp in length and contains 11 CpG sites. Bisulfite sequencing revealed that neither aging nor hypertension induced remarkable differences in the mean DNA methylation status. The SHR strain significantly increased the methylation of CpG 1 ($P<0.01$) and CpG 2 ($P<0.01$); the methylation status of these sites in SHR-3M and SHR-16M was elevated compared with age-matched WKY animals (Figure 4a). Although ANOVA demonstrated no significant effect for hypertension, aging and their interaction in other sites, the *post hoc* comparison showed that, in CpG 1, CpG 5, CpG 6 and CpG 8, the DNA methylation status was elevated in SHR-16M compared with SHR-3M (Figure 4b).

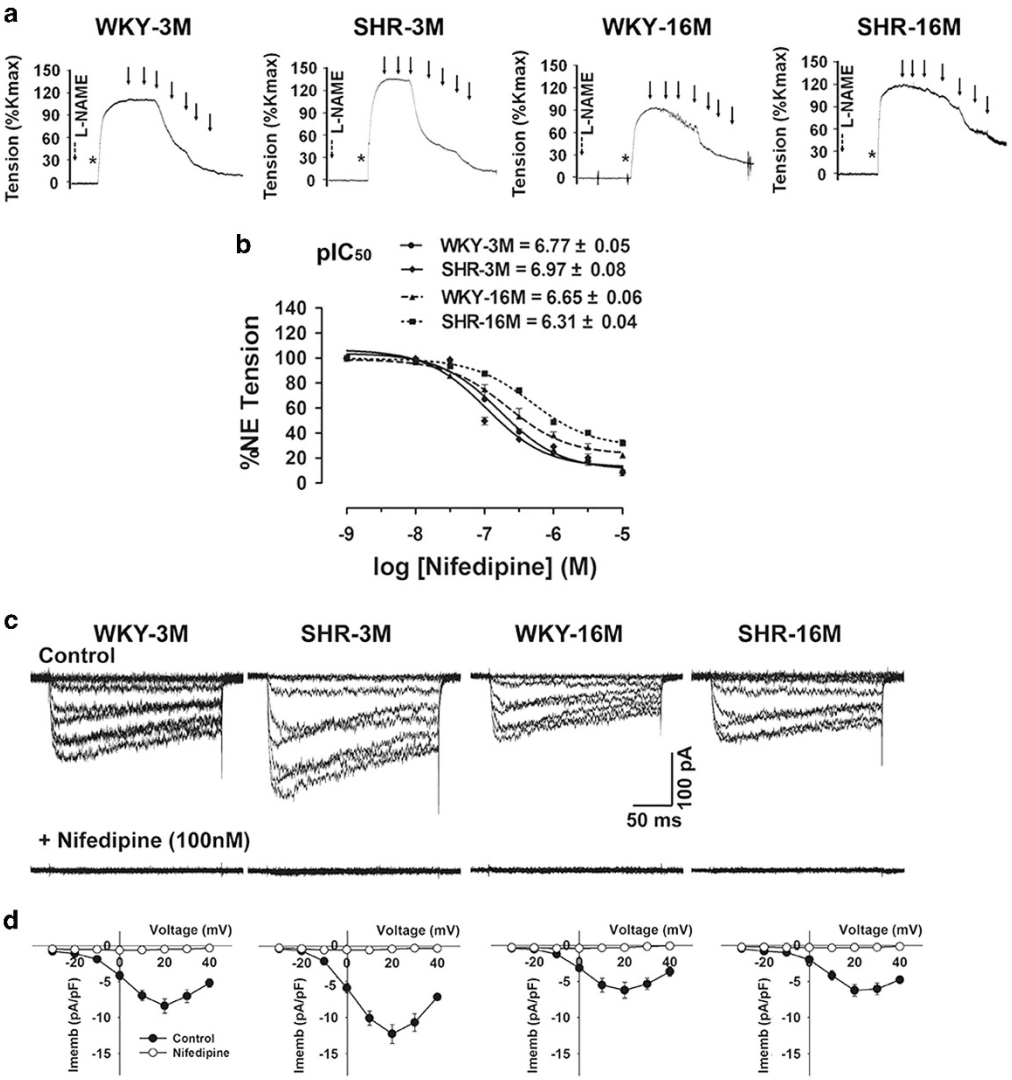

Figure 2 LTCC channel activity and gating properties in rat mesenteric arteries. (**a**): The effect of the LTCC inhibitor nifedipine (10^{-9}– 10^{-5} M) on NE-induced vessel contraction. (**b**) Concentration–response curves for the effects of nifedipine on the NE-induced concentration (NE: 10^{-5} M) ($n=6$). (**c**) Traces of Ca^{2+} currents evoked by command potentials (–30 to +40 mV in a 10 mV step) in the absence (upper) or presence of nifedipine (100 nM) (lower). (**d**) Relative current–voltage relations of LTCC currents. The current amplitudes at various command potentials were normalized to the cell capacitance to correct for differences in the membrane surface area ($n=6$). LTCC, L-type voltage-gated Ca^{2+} channels; NE, noradrenaline.

miR-328 expression in the mesenteric arteries of aging and hypertensive rats

The expression of miR-328 was significantly inhibited by hypertension ($P<0.01$) and enhanced by aging ($P<0.05$), with a significant interaction between them ($P<0.01$). As shown in Figure 4c, miR-328 expression in hypertensive mesenteric arteries was decreased, with a 61.9% reduction in SHR-3M and a 40.1% reduction in SHR-16M for each age-matched group. However, aging enhanced miR-328 levels in both normotensive and hypertensive mesenteric arteries.

The expression level of α1C mRNA and protein in VSMCs after *in vitro* transfection

The targeting role of miR-328 on α1C in VSMCs was further investigated after transfecting exogenous miR-328 mimics into cells. The results showed that transient transfection of the miR-328 mimic significantly decreased mRNA expression of α1C at 12 and 24 h compared with each NC ($P<0.01$) (Figure 5a). In addition, protein expression of α1C was also significantly inhibited at 24 and 48 h after transfection ($P<0.01$) (Figures 5b and c). There was an obvious silencing effect of miR-328 on α1C in cultured VSMCs.

DISCUSSION

In the present study, we investigated whether hypertension and aging altered the functions of LTCCs within the microcirculation and identified potential epigenetic factors during vascular remodeling. LTCC dysfunction in aging and hypertension could be regulated by epigenetic mechanisms. The results of the present study demonstrated that LTCC function, which is well correlated with the α1C subunit protein and mRNA expression, increased in hypertensive mesenteric arteries, but decreased with aging. miR-328 is responsible for the differential LTCC expression at various stages in both normotensive and hypertensive vessels, and this finding been further confirmed in experiments using cultured VSMCs. However, the level of DNA

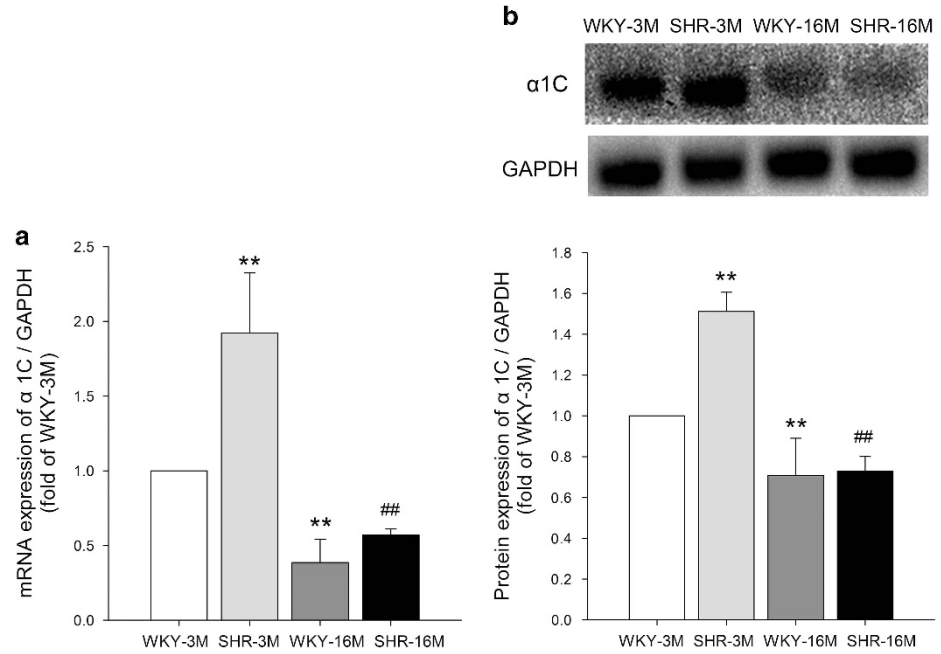

Figure 3 The mRNA (**a**) and protein (**b**) expression of the LTCC α1C subunit in rat mesenteric arteries. The results are expressed as the mean ± s.e.m. ($n=6$) normalized to WKY-3M. $^{*}P<0.05$ and $^{**}P<0.01$ *vs.* WKY-3M; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ *vs.* SHR-3M. LTCC, L-type voltage-gated Ca^{2+} channels; SHR, spontaneously hypertensive rat.

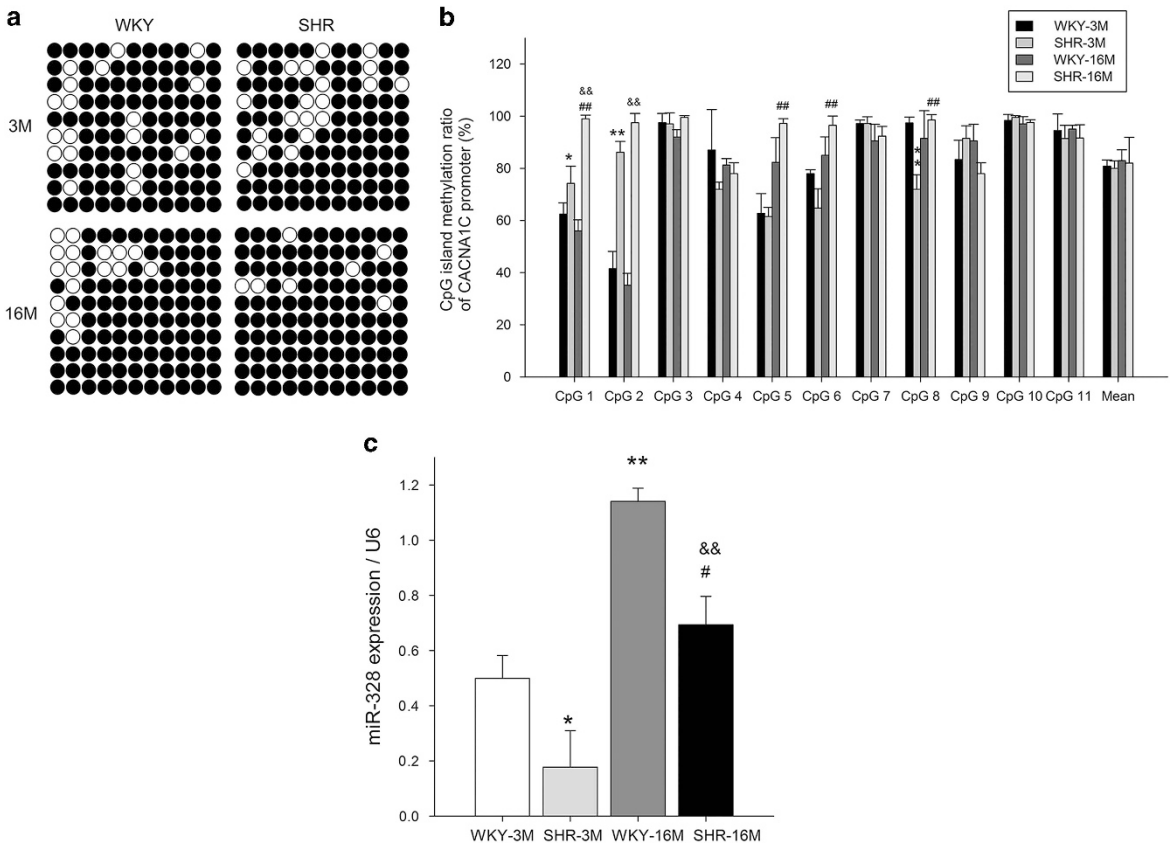

Figure 4 (**a**) Comparison of the DNA methylation status of the α1C gene (CACNA1C) promoter in rat mesenteric arteries. The black and white circles indicate methylated and unmethylated cytosines, respectively. A total of 11 out of 43 CpG sites in the CpG island were subjected to sequencing. (**b**) Methylation percentages of the 11 CpG sites within the CACNA1C promoter. (**c**) Expression of miR-328 normalized to U6 in rat mesenteric arteries. The results are expressed as the mean ± s.e.m. ($n=6$). $^{*}P<0.05$ and $^{**}P<0.01$ *vs.* WKY-3M; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ *vs.* SHR-3M; $^{\&}P<0.05$ and $^{\&\&}P<0.01$ *vs.* WKY-16M. SHR, spontaneously hypertensive rat.

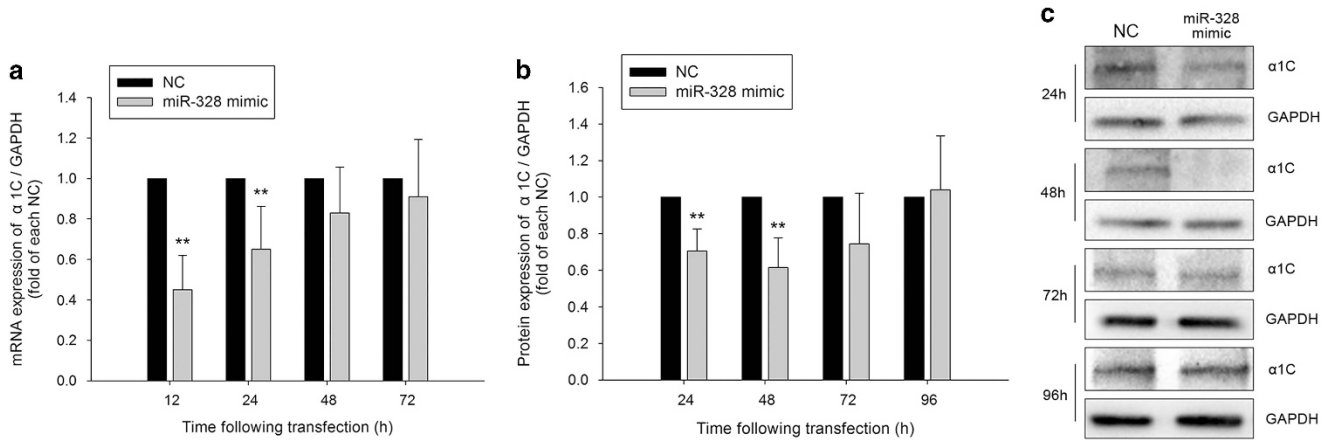

Figure 5 Expression of mRNA (**a**) and protein (**b** and **c**) of LTCC α1C subunit after transfection of the miR-328 mimic in VSMCs. The results are expressed as the mean ± s.e.m. ($n=9$) normalized to each NC. $^{*}P<0.05$ and $^{**}P<0.01$ *vs.* NC. LTCC, L-type voltage-gated Ca^{2+} channels; NC, negative control; VSMCs, vascular smooth muscle cells.

methylation in the LTCC gene promoter was also changed in hypertension progression, regardless of the overall stable methylation status.

Hypertension and aging influence blood pressure and arteries

Both hypertension and aging increased BP in rats and induced obvious remodeling in mesenteric arteries, as evaluated by hematoxylin and eosin staining. These results are consistent with data from both animal and human studies,[3,14] suggesting a superimposed effect of aging and hypertension on BP and vessel morphology. The morbidity from hypertension is much higher in elderly people, and persistent high BP accelerates age-related vascular pathology.[26] The continuous high peripheral vascular resistance during hypertension primarily manifests as an increase in the medial layer and arterial responsiveness in the arterioles, resulting from pathological or physiological alterations to the VSMCs. Sehgel *et al.*[3] reported that the increased stiffness and adhesion of VSMCs contributed to the vascular changes observed during hypertension superimposed on aging. The contractile regulatory mechanisms in VSMCs are damaged in both hypertension and aging.

LTCCs dysfunction in hypertension and aging arteries

The dominant role in vascular contraction is primarily played by Ca^{2+} regulation; elevated Ca^{2+} influx through LTCCs contributes to peripheral resistance and the contractile responses of VSMCs in hypertension.[19] Studies are beginning to reveal the underlying causes of some of the characteristic changes that occur in both hypertension and aging. In the present study, we used mesenteric artery rings and whole-cell patch-clamp recording to demonstrate the enhanced LTCC function in hypertension arterioles, consistent with data from a previous study.[27] NE-induced vasoconstriction and arterial sensitivity to nifedipine were enhanced in SHRs in both young and older groups. The peak Ca^{2+} current density of young hypertensive arteries was much higher than that in the normal control; however, this difference in the Ca^{2+} current of aged arteries was not observed. The diversity of the Ca^{2+} current density between SHRs and WKYs might reflect the number of Ca^{2+} channels instead of the channel activities, as Ohya *et al.*[28] showed that the voltage-dependent kinetics of channel activation and inactivation did not differ between the two rat strains. The upregulated protein and mRNA expression of the LTCCs α1C subunit in the present study confirmed this point, and the expression of both molecules was strongly correlated with channel electrophysiology. Overexpression of LTCCs in vascular myocytes has been demonstrated in several hypertensive models.[29] In addition, antagonists of LTCCs could relieve the symptoms of hypertension[8,20] but not in normotensive subjects,[23,24] suggesting a minimal contribution to normal aged vascular activities.

Aging could act as an independent factor to induce vascular problems and influence BP, as Fukuda *et al.*[30] showed that anti-hypertensive drugs that relax vascular contraction by blocking LTCCs diminish therapeutic potentials in aging normotensive and hypertensive rats. To determine the precise effects of aging on LTCCs, particularly in small arteries that control BP, we used an older animal model (16 M) and quantified LTCC function and expression in the mesenteric arteries. The results demonstrated that aging weakened the relaxation effects of nifedipine in both hypertensive and normotensive rats. However, LTCC expression (protein and mRNA) and the peak Ca^{2+} current density through this channel were suppressed by aging in the mesenteric arteries of both WKY and SHR animals, underscoring the decreased number of LTCCs in aging vascular myocytes. Consistent with previous studies,[10,26] we provided an explanation for the limited effectiveness of anti-hypertensive drugs (LTCCs blocker) on BP control in old age. However, the selection of animals yielded discrepant results. For example, del Corsso *et al.*[31] reported dysfunction in Ca^{2+} signaling, but the LTCC current densities were preserved in 30-month-old C57B1/6 mice, indicating that channel expression remained unchanged with aging. Although hypertension and aging synergistically elevated BP, the changed vasomotor response from aged arteries might not reflect LTCC dysfunction.

The role of DNA methylation on LTCC expression in hypertension and aging arteries

Among the epigenetic mechanisms, DNA methylation may be the most dominant, particularly in age-related disease.[15] Thus, we determined whether the effects of aging and hypertension on the methylation status would modify CACNA1C expression. Although the results showed that, on average, methylation of the CACNA1C promoter showed relatively stable levels in the mesenteric arteries, despite aging and hypertension, specific CpG sites (CpG 1 and CpG 2) were elevated by hypertension. The high-level methylation status in hypertensive arteries indicated a potential silencing effect on gene expression, but we observed overexpression of LTCCs. These results

may reflect the lack of contribution of these CpG sites to the CACNA1C transcription process; another potential explanation is that the pre-transcriptional modulation of methylation on LTCCs, if any, may be offset by other factors during the development of hypertension. In addition, in hypertensive rats, aging increased the methylation status of CpG 1, CpG 5, CpG 6 and CpG 8 and thus inhibited CACNA1C expression. The upregulation of methylation status was inversely correlated with LTCC expression in SHR-16M animals. DNA methylation of the CACNA1C promoter (CpG 1, CpG 5, CpG 6 and CpG 8) was likely associated with damaged LTCC in the arteries of aging hypertensive rats.

The role of miR-328 on LTCCs expression in hypertension and aging arteries

miRNAs are also functionally responsible for the acceleration of vascular remodeling in aging and hypertension.[17,32] The targeting effects of miR-328 on the LTCC α1C subunit have been established in several studies. Guo *et al.* showed that miR-328 overexpression ameliorated the symptoms through inhibition of α1C expression in hypoxia-induced pulmonary hypertension.[33] Choutry *et al.*[34] reported that miR-328 is involved in atrial electric remodeling in atrial fibrillation by targeting CACNA1C. In the present study, we showed that the expression of miR-328 in SHR arteries was downregulated by hypertension, while aging had an opposite effect. Until recently, miR-328 has not been analyzed in relation to hypertension in aged arterioles. Here, we provided the first evidence that both hypertension and aging could independently influence miR-328 expression and thus modify LTCCs. Next, we performed *in vitro* experiments to confirm the targeting effect of miR-328 on the LTCC subunit α1C. Overexpression of miR-328 through transient transfection into cultured VSMCs inhibited α1C mRNA and protein expression. These results highlight the important regulatory role of miR-328 in controlling the specific gene, which significantly contributes to BP.

Alterations of epigenetic patterns in adulthood are generally considered to be age-associated deleterious changes rather than necessary and programmed variations during early biological development.[34] The changes in the DNA methylation of CACNA1C and miR-328 might be involved in decreasing LTCC expression during aging, while DNA methylation contributed less to LTCC dysfunction in hypertension development. Consistently, previous studies have shown that post-transcriptional mechanisms, instead of elevated α1C transcripts, accounted for increases in arterial LTCC in hypertension.[20,35,36] In an attempt to determine whether differences in miRNAs are involved in the etiology of vascular remodeling, investigators are encouraged to perform and delineate the global dynamic profiles of miRNAs in hypertension arteries during aging.

Perspectives and conclusion

The present study on the epigenetic alterations in aging and hypertensive arterioles demonstrated the complexity of LTCC regulation when hypertension is superimposed with aging. Characterizing the epigenetic mechanisms may guide us toward potential therapeutic targets that directly control vascular contraction in the hypertensive population during aging. Furthermore, with the side effects of pharmacological channel blockers, which target LTCCs,[37,38] it may be advantageous to ameliorate anomalous Ca^{2+} influx by preventing the dysregulation of LTCC expression as a desirable therapeutic method.

In summary, the findings of the present study showed that hypertension and aging accelerates vascular remodeling and dysfunction in the mesenteric arteries of rats. LTCCs in VSMCs are upregulated in hypertension and downregulated with aging. The epigenetic factor, miR-328, may be responsible for the disruption of LTCCs in both hypertension pathogenesis and the aging progress.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was financially supported by grants from the National Natural Science Foundation of China (31371201); the Chinese Universities Scientific Fund (2015ZD008, 2016RC001); Research project of the General Administration of Sport of China (2015B035); and the Beijing Natural Science Foundation (to LS).

1 Kaplan NM. Hypertension in the elderly. *Annu Rev Med* 1995; **46**: 27–35.
2 Binder A. A review of the genetics of essential hypertension. *Curr Opin Cardiol* 2007; **22**: 176–184.
3 Sehgel NL, Sun Z, Hong Z, Hunter WC, Hill MA, Vatner DE, Vatner SF, Meininger GA. Augmented vascular smooth muscle cell stiffness and adhesion when hypertension is superimposed on aging. *Hypertension* 2015; **65**: 370–377.
4 Sun Z. Aging, arterial stiffness, and hypertension. *Hypertension* 2015; **65**: 252–256.
5 Hill MA, Meininger GA, Davis MJ, Laher I. Therapeutic potential of pharmacologically targeting arteriolar myogenic tone. *Trends Pharmacol Sci* 2009; **30**: 363–374.
6 Chen Y, Zhang H, Zhang Y, Lu N, Zhang L, Shi L. Exercise intensity-dependent reverse and adverse remodeling of voltage-gated Ca^{2+} channels in mesenteric arteries from spontaneously hypertensive rats. *Hypertens Res* 2015; **38**: 656–665.
7 Gollasch M, Nelson MT. Voltage-dependent Ca^{2+} channels in arterial smooth muscle cells. *Kidney Blood Press Res* 1997; **20**: 355–371.
8 Pesic A, Madden JA, Pesic M, Rusch NJ. High blood pressure upregulates arterial L-type Ca^{2+} channels: is membrane depolarization the signal? *Circ Res* 2004; **94**: e97–104.
9 Saco TV, Parthasarathy PT, Cho Y, Lockey RF, Kolliputi N. Role of epigenetics in pulmonary hypertension. *Am J Physiol Cell Physiol* 2014; **306**: C1101–C1105.
10 Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, Schubeler D. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet* 2007; **39**: 457–466.
11 Lam LL, Emberly E, Fraser HB, Neumann SM, Chen E, Miller GE, Kobor MS. Factors underlying variable DNA methylation in a human community cohort. *Proc Natl Acad Sci USA* 2012; **109**: 17253–17260.
12 Pei F, Wang X, Yue R, Chen C, Huang J, Li X, Zeng C. Differential expression and DNA methylation of angiotensin type 1A receptors in vascular tissues during genetic hypertension development. *Mol Cell Biochem* 2015; **402**: 1–8.
13 Glier MB, Green TJ, Devlin AM. Methyl nutrients, DNA methylation, and cardiovascular disease. *Mol Nutr Food Res* 2014; **58**: 172–182.
14 Jones MJ, Goodman SJ, Kobor MS. DNA methylation and healthy human aging. *Aging Cell* 2015; **14**: 924–932.
15 Jung M, Pfeifer GP. Aging and DNA methylation. *BMC Biol* 2015; **13**: 7–15.
16 Fiedler J, Gupta SK, Thum T. MicroRNA-based therapeutic approaches in the cardiovascular system. *Cardiovasc Ther* 2012; **30**: e9–e15.
17 Shi L, Liao J, Liu B, Zeng F, Zhang L. Mechanisms and therapeutic potential of microRNAs in hypertension. *Drug Discov Today* 2015; **20**: 1188–1204.
18 Batkai S, Thum T. MicroRNAs in hypertension: mechanisms and therapeutic targets. *Curr Hypertens Rep* 2012; **14**: 79–87.
19 Albinsson S, Sward K. Targeting smooth muscle microRNAs for therapeutic benefit in vascular disease. *Pharmacol Res* 2013; **75**: 28–36.
20 Menghini R, Stohr R, Federici M. MicroRNAs in vascular aging and atherosclerosis. *Ageing Res Rev* 2014; **17**: 68–78.
21 Jansen F, Yang X, Nickenig G, Werner N, Vasa-Nicotera M. Role, function and therapeutic potential of microRNAs in vascular aging. *Curr Vasc Pharmacol* 2015; **13**: 324–330.
22 Guo L, Qiu Z, Wei L, Yu X, Gao X, Jiang S, Tian H, Jiang C, Zhu D. The microRNA-328 regulates hypoxic pulmonary hypertension by targeting at insulin growth factor 1 receptor and L-type calcium channel-alpha1C. *Hypertension* 2012; **59**: 1006–1013.
23 Lu Y, Zhang Y, Wang N, Pan Z, Gao X, Zhang F, Shan H, Luo X, Bai Y, Sun L, Song W, Xu C, Wang Z, Yang B. MicroRNA-328 contributes to adverse electrical remodeling in atrial fibrillation. *Circulation* 2010; **122**: 2378–2387.
24 Shi L, Zhang H, Chen Y, Liu Y, Lu N, Zhao T, Zhang L. Chronic exercise normalizes changes in Cav 1.2 and KCa 1.1 channels in mesenteric arteries from spontaneously hypertensive rats. *Br J Pharmacol* 2015; **172**: 1846–1858.
25 Golovina VA, Blaustein MP. Preparation of primary cultured mesenteric artery smooth muscle cells for fluorescent imaging and physiological studies. *Nat Protoc* 2006; **1**: 2681–2687.
26 Garcia-Palmieri M. Hypertension in old age. *P R Health Sci J* 1995; **14**: 217–221.
27 Pratt PF, Bonnet S, Ludwig LM, Bonnet P, Rusch NJ. Upregulation of L-type Ca^{2+} channels in mesenteric and skeletal arteries of SHR. *Hypertension* 2002; **40**: 214–219.

28 Ohya Y, Abe I, Fujii K, Takata Y, Fujishima M. Voltage-dependent Ca^{2+} channels in resistance arteries from spontaneously hypertensive rats. *Circ Res* 1993; **73**: 1090–1099.
29 Sonkusare S, Fraer M, Marsh JD, Rusch NJ. Disrupting calcium channel expression to lower blood pressure: new targeting of a well-known channel. *Mol Interv* 2006; **6**: 304–310.
30 Fukuda T, Kuroda T, Kono M, Miyamoto T, Tanaka M, Matsui T. Attenuation of L-type Ca^{2+} channel expression and vasomotor response in the aorta with age in both Wistar-Kyoto and spontaneously hypertensive rats. *PLoS ONE* 2014; **9**: e88975.
31 del Corsso C, Ostrovskaya O, McAllister CE, Murray K, Hatton WJ, Gurney AM, Spencer NJ, Wilson SM. Effects of aging on Ca^{2+} signaling in murine mesenteric arterial smooth muscle cells. *Mech Ageing Dev* 2006; **127**: 315–323.
32 Zhang H, Yang H, Zhang C, Jing Y, Wang C, Liu C, Zhang R, Wang J, Zhang J, Zen K, Li D. Investigation of microRNA expression in human serum during the aging process. *J Gerontol A Biol Sci Med Sci* 2015; **70**: 102–109.
33 Li N, Bates DJ, An J, Terry DA, Wang E. Up-regulation of key microRNAs, and inverse down-regulation of their predicted oxidative phosphorylation target genes, during aging in mouse brain. *Neurobiol Aging* 2011; **32**: 944–955.
34 Choudry FA, Frontini M. Epigenetic control of haematopoietic stem cell aging and its clinical implications. *Stem Cells Int* 2016; **2016**: 5797521.
35 Barana A, Matamoros M, Dolz-Gaiton P, Perez-Hernandez M, Amoros I, Nunez M, Sacristan S, Pedraz A, Pinto A, Fernandez-Aviles F, Tamargo J, Delpon E, Caballero R. Chronic atrial fibrillation increases microRNA-21 in human atrial myocytes decreasing L-type calcium current. *Circ Arrhythm Electrophysiol* 2014; **7**: 861–868.
36 Rau F, Freyermuth F, Fugier C, Villemin JP, Fischer MC, Jost B, Dembele D, Gourdon G, Nicole A, Duboc D, Wahbi K, Day JW, Fujimura H, Takahashi MP, Auboeuf D, Dreumont N, Furling D, Charlet-Berguerand N. Misregulation of miR-1 processing is associated with heart defects in myotonic dystrophy. *Nat Struct Mol Biol* 2011; **18**: 840–845.
37 Grossman E, Messerli FH. Calcium antagonists. *Prog Cardiovasc Dis* 2004; **47**: 34–57.
38 Meka N, Katragadda S, Cherian B, Arora RR. Combination therapy in hypertension: a focus on angiotensin receptor blockers and calcium channel blockers. *Am J Ther* 2010; **17**: 61–67.