Aerobic Exercise Upregulates DNA Methylation of *Agtr1a* and *Mas1* Genes to Improve Mesenteric Arterial Function in Spontaneously Hypertensive Rats

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Abstract

The imbalance between vasoconstrictive axis and vasodilative axis of the renin-angiotensin system (RAS) is involved in the pathogenesis of hypertension. Exercise modulates components of the RAS and influences vascular function. This study aimed to investigate the balance of RAS axes and the mechanism of DNA methylation of the *Agtr1a* ($AT_{1a}R$) and *Mas1* (MasR) genes in aerobic exercise-induced improvement of the function of mesenteric arteries (MAs) in hypertension. Spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats were subjected to exercise training or kept sedentary. Plasma RAS peptides, vascular function, and molecular properties were assessed. Aerobic exercise significantly decreased blood pressure in SHR. Plasma levels of RAS peptides increased in SHR, and the level of Ang II was much higher than Ang-(1–7), whereas exercise efficiently inhibited this inappropriate increase. In addition, Ang II-induced maximal contraction of MAs is largely through Type 1 angiotensin receptors (AT_1R), while Mas receptor (MasR) inhibits this contribution. Exercise effectively suppressed hypertension-associated mRNA and protein expression upregulation of AT_1R and MasR and increased MasR/AT_1R ratio in SHR by triggering hypermethylation of *Agtr1a* and *Mas1* genes, with increasing DNMT1 and DNMT3b protein expression and ratio of SAM/SAH. These findings suggest that aerobic exercise alleviates vascular tone by upregulating the methylation status of the *Agtr1a* and *Mas1* genes and inhibiting the imbalanced increase in the vasoconstrictive and vasodilative axes during hypertension.

Introduction

Hypertension is a large and growing public health problem worldwide and is related to approximately 1/2 of all strokes and heart failure [1]. It is one of the leading causes of morbidity and mortality globally. In clinical and animal models, hypertension is associated with vascular pathological changes, increased vasoconstriction, and arterial wall remodeling. Resistant arteries are responsible for blood pressure control and regional distribution of blood flow, which have an enhanced arterial tone in hypertension [2]. Renin-angiotensin system (RAS) has been shown to be a key factor in the development of hypertension [3]. RAS, as an endocrine system, mainly consists of a vasoconstrictor axis where circulating renin regulates cardiovascular function through angiotensin-converting enzyme (ACE), angiotensin II (Ang II), and angiotensin II receptor type 1 (AT_1R) and AT_2R on target tissues and a vasodilator axis, including ACE2, angiotensin-(1–7) [Ang-(1–7)], and its receptor, Mas (MasR). The enhanced vasoconstrictor axis-mediated vascular smooth muscle cell (VSMC) dysfunction plays a major role in hypertension [4].

The vascular and systemic RAS are involved in increased vasoconstrictor tone [5]. Exercise training has been shown to improve vasodilatory function and decrease vasoconstrictor tone in critical tissues for cardiovascular control [6, 7]. A previous study has shown that exercise training represses the mRNA and protein expression of AT_1R [5]. Ang-(1–7) participates in enhanced insulin-induced vasorelaxation through MasR after exercise [8]. Recent studies have indicated that exercise contributes to the restoration of the vasoconstrictor/vasodilator balance of the RAS axes to improve vascular function [9, 10].

Epigenetics refers to stable changes in gene function, without changes in the DNA sequence per se. Many factors such as environment can influence epigenetic modifications. DNA methylation is one of the pivotal epigenetic mechanisms in mammals and plays a crucial role in the biological processes during hypertension. Studies have suggested that DNA methylation of RAS components may be associated with hypertension. For instance, hypomethylation of the *Agt* gene promoter in rat visceral adipose tissue with a high-salt diet results in hypertension [11]. Human studies show that hypomethylation of the AT_1R gene is likely associated with the risk of hypertension [12, 13].

Studies that have focused on the RAS axes in hypertension are controversial, particularly at the level of the vasodilator axis. Here, we sought to investigate the effects of aerobic exercise on RAS axes, and the DNA methylation mechanism of exercise to characterize AT_1R and MasR gene profiles on SHRs. Our data suggest that both RAS axes are elevated in hypertension, and the vasoconstrictor axis plays a more important role in spontaneously hypertensive rats (SHRs), whereas exercise training significantly suppressed RAS axes, with more inhibition on vasoconstrictor axis than vasodilator axis. Moreover, exercise-induced hypermethylation of *Agtr1a* and *Mas1* reprograms $AT_{1a}R$ and MasR transcription and protein expression in mesenteric arteries (MAs) during hypertension. The change in gene expression is attributed to an epigenetic program with DNMTs.

Materials And Methods

Animals and exercise protocol

Twelve-week-old male normotensive Wistar-Kyoto rats (WKYs) and SHRs were randomly assigned into a sedentary (WKY-C and SHR-C) and an exercise (WKY-E and SHR-E) group ($n = 24$ in each group). After one-week habituation, rats in the exercise group were subjected to a motor treadmill (20 m/min, 0% grade, 60 min, 5 days/week, about 55–65% of maximal aerobic velocity) for 12 weeks. The maximal exercise capacity of rats was tested as described elsewhere [14]. Rats in the sedentary group were exposed to the same environments as their trained counterparts. All the rats were housed in a 12:12-h light-dark cycle environment with a temperature of 23–25°C and 40–60% humidity. The rats were provided fresh water and standard rodent chow *ad libitum*.

Body weight (BW) was measured before the rats were anesthetized, and the blood pressure of conscious WKY rats and SHRs was tested using an indirect tail-cuff method (BP-2010A, Softron Biotechnology, Beijing, China) every two weeks.

Ultra-performance liquid chromatography (UPLC)

Plasma for UPLC analysis was collected by centrifuging blood at 1,500*g* for 15 min (RT). Then, 500 µL of 1% TFA was added to 500 µL of the plasma sample. The samples were vortexed and centrifuged at 10,400*g* for 20 min. The pellet was discarded, and the column was prebalanced with 60% acetonitrile, 1% trifluoroacetic acid (TFA), and 39% distilled water. The column was washed with 1 mL of 1% trifluoroacetic acid (TFA), then the samples were added to the column with 1 mL 1% TFA and washed

twice. The eluent was collected in a tube and evaporated to dryness. AGT, Ang I, Ang II, and Ang-(1–7) levels were determined using the Dionex Ultimate 3000 UPLC system coupled to a TSQ Quantiva Ultra triple-quadrupole mass spectrometer (Thermo Fisher, CA, USA) and equipped with a heated electrospray ionization (HESI) probe. Samples were separated by a synergi Hydro-RP column (2.0 × 100 mm, 2.5 μm, Phenomenex, CA, USA). The mobile phase contained 10 mM tributylamine with 15 mM acetic acid in water as solvent A, and methanol as solvent B. A gradient of 10–98% of B was run for a total run time of 10 min. The gradient started with 10% B, which was changed progressively to 50% B in 1.5–2.5 min, increased in 2.5–6.5 min at 98% B, then decreased in 6.5–8 min at 10% B again, and run for the next 8.1–10 min at the same composition. Column chamber and sample tray were held at 40°C and 10°C, respectively. SAM and SAH in the plasma sample were identified according to their retention times and transitions of 399.2/250.1 and 385.2/136.2 in the negative ion mode. The resolution for precursor and fragment ion were both 0.7 FWHM. The source parameters were as follows: spray voltage, 3,000 V; ion transfer tube temperature, 350°C; vaporizer temperature, 300°C; sheath gas flow rate, 35 Arb; auxiliary gas flow rate, 12 Arb. CID gas, 1.5 mTorr. Data analysis and quantitation were performed using the software Xcalibur 3.0.63 (Thermo Fisher, CA).

Isometric contraction studies

Rats were euthanized by intraperitoneal injection with sodium pentobarbital (50 mg/kg) at the age of 25 weeks. The MAs and its branches were removed and the segments of A3 were isolated in Krebs' solution containing (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 (pH 7.4), mounted on a Multi Myograph System (620M; DMT, Aarhus, Denmark), gassing with 95% O_2 and 5% CO_2. The maximum contractile response of artery rings was elicited using 60 mM KCl (K_{max}) and arterial contractile response was evaluated by measuring the maximum peak height and expressed as a percentage of contraction to K_{max}. The non-selective NOS inhibitor N^{ω}-nitro-L-arginine methylester (L-NAME, 10^{-4} M) was added in the experiments. To assess contribution of AT_1R, AT_2R and MasR function to the vascular tone regulation, the arterial responses to NE (10^{-5} M), Ang II (10^{-5} M), losartan (AT_1R blocker, 10^{-6} M), PD123319 (AT_2R blocker, 10^{-5} M), and A779 (MasR blocker, 10^{-5} M) were examined, respectively. Each artery was used once, signals were recorded by Power-Lab system with Chart-5 software (AD Instruments, Bella Vista, Australia).

Western blotting

MAs were homogenized using RIPA buffer with protease inhibitors (complete Mini Protease Inhibitor Cocktail, Roche). Protein concentration was determined following a BCA kit (Pierce BCA protein assay, Thermo Scientific). Equal amounts of protein from MAs were separated by 3 – 8% Tris-Acetate or 4 – 12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) electrophoresis at 150 V (0.5 hour) or 200 V (1 hour) and electrotransferred to PVDF membranes by using the iBlot2 Dry Blotting System (Invitrogen) and blocked with 5% defatted milk in tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 1 hour before incubation with the primary antibodies at 4°C overnight. Antibodies and dilutions were as follows: anti-AT_1R (1:500, Abcam), anti-MasR (1:400, Alomone), anti-DNMT1 (1:500, Novus), polyclonal anti-DNMT3a

(1:500, Abcam) and anti-DNMT3b (1:1000, Abcam). Membranes were then incubated with horseradish peroxidase-linked secondary antibody in TBST. Immunoreactive brands were detected by enhanced chemiluminescence (ECL) and recorded with Bio-Rad ChemiDOC XRS+ (Bio-Rad, Hercules, CA, USA). Protein content was normalized by β-actin/GAPDH.

RT-PCR assay

To analyze the induction of the target gene *Agtr1a* ($AT_{1a}R$), *Agtr1b* ($AT_{1b}R$) and *Mas1* (MasR) transcript in MAs, total RNA was extracted with TRIzol reagent (Invitrogen). 500 ng of RNA was reverse transcribed to cDNA using Maxima First Strand cDNA Synthesis Kit after DNase treatment. Real-time PCR was performed with TaqMan Fast Advanced Master Mix and inventoried TaqMan expression assays using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The target gene used in RT-PCR assays were as follows: *Agtr1a* (Rn02758772_s1, amplicon length = 112 bp), *Agtr1b* (Rn02132799_s1, amplicon length = 150 bp), *Mas1* (Rn00562673_s1, amplicon length = 67 bp), and the normalization gene *Actb* (β-actin, Rn00667869_m1, amplicon length = 91 bp). All reagents were from Applied Biosystems. Messenger RNA abundance was calculated using the $2-\Delta\Delta CT$ method and normalized to *Actb* mRNA and expressed as a percentage of WKY-C.

DNA bisulfite sequencing PCR (BSP)

Genomic DNA from MAs were extracted using the Pure Link Genomic DNA Mini Kit (Invitrogen) and treated with sodium bisulfate with EZ DNA Methylation-GOLD Kit (Zymo Research) according to manufacturer's protocols. Unmethylated cytosine residues were converted to thymines, whereas methyl-cytosines remain unmodified. PCR amplification on the promoter and nearby regions of the *Agtr1a* (at chromosome 17 from position 35957898 to 35958307, 410 bp in length, containing 21 CpG sites) or *Mas1* (at chromosome 1 from position 48076585 to 48077039, 455 bp in length, containing 7 CpG sites) gene was performed. The products were separated with 2.0% agarose gels, and the bands were resolved using the TIAN gel Midi Purification Kit (Tiangen, Beijing, China). The purified samples were cloned into a pEASY-T1 Cloning vector (TRAN). Sample with 10 clones were sequenced. The primers used for modified BSP were 5'-ATGAGGGAGTTAGGATTAGTTGAG-3' (forward) and 5'-CACTCCRAACTCTAATCACCAC-3' (reverse) for *Agtr1a*; The primers used for modified BSP were 5'-TTTAAGAGTAGAGGGGGTTTGG-3' (forward) and 5'-TACCCTACTTAATACATAACCCCTT-3' (reverse) for *Mas1*. The percentage of methylation level of each CpG site was calculated as the ratio of methylated cytosines/total tested cytosines. The average methylation level was calculated using methylation levels of all measured CpG sites within the gene.

Chemicals and Statistical Analyses

All chemical reagents were purchased from Sigma-Aldrich (www.sigma-aldrich.com) unless otherwise stated. Data were expressed as mean ± SEM. SPSS 17.0 software were used for statistical analyses. Where appropriate, differences were evaluated for statistical significance ($P < 0.05$) by a two-way ANOVA (hypertension × exercise).

Results

Body weight and blood pressure

There were no significant differences in body weight (BW) between WKY rats and SHRs at baseline (12 weeks). However, BW was significantly lower in both the WKY-E and SHR-E groups when compared with their counterparts at the end of training (both $P < 0.05$, Table 1). In addition, the BW of SHR-C was significantly lower than that in the WKY-C group ($P < 0.05$). Basic systolic blood pressure (SBP), mean arterial pressure (MAP), and diastolic blood pressure (DBP) in the SHR-C were significantly higher than those in WKY-C. After 12 weeks of exercise training, SBP, DBP, and MAP significantly decreased in SHR-E compared to SHR-C (all $P < 0.05$).

Table 1
Effects of aerobic exercise on body weights and blood pressure

	WKY-C ($n = 24$)	WKY-E ($n = 24$)	SHR-C (n = 24)	SHR-E (n = 24)
BW (g)	364.5 ± 4.8	339.4 ± 4.8*	334.7 ± 6.9*	316. 4 ± 4.9#
SBP (mmHg)	139.5 ± 0.5	132.3 ± 0.6*	199.4 ± 0.5*	191.2 ± 0.7#
DBP (mmHg)	111.2 ± 1.8	110.5 ± 1.7	167.5 ± 2.3*	159.3 ± 1.5#
MAP (mmHg)	120.7 ± 1.2	117.8 ± 1.1	178.2 ± 1.5*	170.1 ± 1.0#

* $P < 0.05$ vs WKY-C; # $P < 0.05$ vs SHR-C.

Aerobic exercise decreases the plasma levels of RAS components

The plasma levels of AGT, Ang I, Ang II, and Ang-(1–7) were examined by UPLC. The plasma levels of these four peptides markedly increased in SHR-C, while decreased after exercise training in SHR-E (all $P < 0.05$, Fig. 1a–e). Figure 1f shows that the plasma Ang-(1–7)/Ang II ratio significantly decreased in SHR-C compared with WKY-C ($P < 0.05$), and exercise significantly increased the ratio in SHR-E ($P < 0.05$). These results suggest that the elevated RAS component levels from hypertension are abrogated by chronic exercise. In addition, the decrease in the amplitude of Ang II was larger than Ang-(1–7) in hypertension after exercise training.

Aerobic exercise inhibits the AT_1 R and MasR-mediated responses in contribution to vascular tone regulation in SHR MAs

To assess the function of MAs from all groups, NE was added in the presence of L-NAME. The maximal contraction in SHR-C was higher compared with that in WKY-C ($P < 0.05$), whereas MAs from SHR-E

responded with less constriction than SHR-C ($P < 0.05$, Fig. 2a). Then, Ang Ⅱ was applied to measure its effect on vasoconstriction. Figure 2b shows that in contrast to WKY-C, the increase in Ang Ⅱ-induced tension was more pronounced in the SHR-C arteries ($P < 0.05$). However, exercise training markedly inhibited this increase ($P < 0.05$). To examine the contribution of AT_1R, AT_2R, and MasR, inhibitors were used on the MAs before adding Ang Ⅱ. Losartan, an AT_1R inhibitor, almost completely suppressed the Ang Ⅱ-induced contraction (Fig. 2c). However, there were no differences in vasoconstriction between the MAs subjected to Ang Ⅱ and PD123319 (AT_2R antagonist) co-treatment when compared to the MAs treated with Ang Ⅱ alone (Fig. 2d). By contrast, suppression of MasR with A779 increased the Ang Ⅱ-induced tension in all groups, especially in hypertensive rats (Fig. 2e). Taken together, these results indicate that the tension induced by Ang Ⅱ is largely through AT_1R, not AT_2R, while MasR inhibits the contribution of Ang Ⅱ in vascular regulation and plays a pivotal role in resting tone.

Aerobic exercise suppresses increased $AT_{1a}R$ and MasR protein expression and mRNA levels in hypertension

Protein expression levels of AT_1R and MasR were measured to assess exercise-associated vasodilatation in SHR. Figure 3a and b show the results of western blot analysis, which revealed significant increases in both AT_1R and MasR protein levels of MAs in SHR-C (*vs.* WKY-C). Moreover, the expression of AT_1R and MasR both decreased in SHR after exercise, but increased in WKY under the same training conditions (all $P < 0.05$). To further determine the contribution of AT_1R and MasR in exercise, MasR/AT_1R was analyzed. The expression ratio of MasR/AT_1R in MAs was significantly downregulated in SHR-C (0.60 ± 0.04) and upregulated in SHR-E (0.69 ± 0.02, both $P < 0.05$, Fig. 3c). These findings suggest that AT_1R contributes more to the vessel tone in hypertension. However, MasR has a greater contribution in exercise-induced effects.

Then, the mRNA of $AT_{1a}R$, $AT_{1b}R$, and MasR were assessed to identify whether the differences in protein expression of AT_1R and MasR among groups were due to transcription (Fig. 3d). Compared to WKY-C, the mRNA of $AT_{1a}R$ ($P < 0.05$), not $AT_{1b}R$ ($P > 0.05$), significantly increased in SHR-C. Exercise training decreased the mRNA level of $AT_{1a}R$ in the MAs of hypertensive rats, whereas increased it in normotensive rats after exercise (both $P < 0.05$). There were no effects on the expression of $AT_{1b}R$ in either WKY or SHR after exercise training ($P > 0.05$). Similar to $AT_{1a}R$, the level of MasR in SHR-C was higher than that in WKY-C, and exercise decreased mRNA of MasR in SHR-E (both $P < 0.05$). These results on mRNA expression levels coincided with the findings of protein expression.

Aerobic exercise increases CpG methylation of the *Agtra1* and *Mas1* gene promoters in SHR MAs

To further assess the DNA methylation effect on $AT_{1a}R$ and MasR, we first evaluated DNMT functions in MAs. Figure 4a and b show that hypertension is associated with a significant decrease in the protein expression of DNMT1 ($P < 0.05$), not DNMT3b ($P > 0.05$), in SHR-C compared with WKY-C. In

normotensive rats, exercise training reduced DNMT1 and DNMT3b expression, but in hypertensive rats, DNMT1 and DNMT3b expression increased with chronic exercise ($P < 0.05$). DNMT3a levels did not change in any experimental group ($P > 0.05$). Then, the methylation capacity index (SAM/SAH ratio) was assessed. The SAM/SAH ratio was markedly reduced in SHR-C compared with WKY-C ($P < 0.05$). In contrast, the SAM/SAH ratio in plasma was significantly elevated in SHR after exercise training, but decreased in WKY-E (both $P < 0.05$, Fig. 4c). The DNA methylation status of the $AT_{1a}R$ (*Agtr1a*) and MasR (*Mas1*) genes in MAs was assessed to confirm the correlation between DNA methylation and transcription. The CpG sites detected are shown in Fig. 4d and e. In normotensive rats, there was a significant decrease in promoter methylation of the *Agtr1a* and *Mas1* genes in MAs of WKY-E compared to WKY-C. However, in hypertensive rats, exercise training significantly increased both *Agtr1a* and *Mas1* gene methylation status compared with their sedentary counterparts (all $P < 0.05$, Fig. 4f and i). These findings show that the changes in the methylation of the *Agtr1a* and *Mas1* genes may partially account for the alteration in DNMT expression in MAs observed in the hypertension and exercise training group.

Discussion

In the present study, 12 weeks of aerobic exercise repressed the plasma levels of bioactive peptides including AGT, Ang I, and Ang □ and reversed the pathological compensation of Ang-(1–7) in hypertension, inhibiting both Ang □ receptor $AT_{1a}R$ and Ang-(1–7) receptor MasR-mediated vascular function in the MAs of SHRs, thereby downregulating the RAS axes. In addition, exercise-induced hypermethylation of the *Agtr1a* and *Mas1* genes via increased DNMT1 and DNMT3b expression reduced the transcription of $AT_{1a}R$ and MasR (Fig. 5). The novelty of this study is that activation of DNA methylation plays an essential role in the exercise-mediated decrease of $AT_{1a}R$ and MasR in hypertension, thereby maintaining the vasoconstriction/vasodilatation balance at the physiological level.

RAS is known as a major blood pressure regulator and is involved in the pathogenesis of hypertension. Evidence shows that in hypertension, the vasodilator axis is highly activated [15]. Reduced ACE2 and consequently decreased Ang-(1–7) production is associated with vascular injury in hypertension [16]. Therefore, an imbalance between the vasoconstrictor axis and the vasodilator axis in the RAS has emerged as a common denominator in cardiovascular disorders [17]. The present study demonstrated that levels of plasma peptides [AGT, Ang I, Ang □, Ang-(1–7)] all increased in hypertension (Fig. 1a–e). AGT is one of the candidate genes in essential hypertension. Increased plasmatic levels of AGT may induce AGT metabolism, which elevates Ang I. Next, Ang I is converted to Ang □, which is a biologically active vasopeptide that increases vascular tone. However, the level of Ang-(1–7), a vasodilator peptide, is also increased during hypertension. To determine the contribution of the vasodilator and vasoconstrictor axes, the ratio of Ang-(1–7)/Ang II was assessed (Fig. 1f). The results showed a lower ratio in hypertensive rats, which suggests that the compensatory increase of Ang-(1–7) is nevertheless insufficient in the counterbalance of Ang II during hypertension. This is accordance with research in upregulated plasma levels of both Ang-(1–7) and Ang □ in SHR conducted by Kohara [18] and Zhou [19].

The discrepancy in the results on Ang-(1–7) between Shaltout [16] and our study may be related to the use of different experimental animals (sheep *vs*. rat).

RAS regulates vascular tone and plays an important role in vascular remodeling, which is attributed to a complex interplay of alterations in vascular tone and structure. In this study, isometric contraction studies were used to examine vessel function in relation to RAS. The contractile response to Ang Ⅱ was higher in hypertensive vessels (Fig. 2b). Ang Ⅱ mediates effects via complex intracellular signaling pathways by binding to two major G protein-coupled receptors AT_1R and AT_2R. AT_1R mediates most of the pathophysiological effects of Ang Ⅱ, including vasoconstriction, inflammation growth, and fibrosis, whereas AT_2R is thought to oppose the effects of AT_1R [20]. By preincubating with an AT_1R inhibitor (losartan), Ang Ⅱ-induced vasoconstriction was almost repressed in all groups. However, the AT_2R antagonist (PD123319) did not impart any effects on Ang II-mediated vasoconstriction (Fig. 2c and d). Using a MasR blocker (A779), the vascular tension induced by Ang Ⅱ increased, suggesting the role of MasR in the regulation of vascular tone. These results are further supported at the molecular level by the demonstration of increased MasR and $AT_{1a}R$, but not $AT_{1b}R$ transcript (Fig. 3d) and protein expression (Fig. 3a and b). The MasR/AT_1R ratio also decreased in SHR-C (Fig. 3c). The ratio of the two receptors was in line with upstream Ang-(1–7)/Ang Ⅱ. Ang Ⅱ is the main active molecule of the classical RAS; upon Ang Ⅱ binding, AT_1R facilitates a variety of cytoplasmic signaling pathways that mediate VSMCs remodeling, including hypertrophy and migration. The inappropriate overactivity of the Ang Ⅱ/AT_1R interaction is involved in the genesis and progression of hypertension [21]. A sustained activation of AT_1R by Ang Ⅱ, one of the main effectors of RAS, has been proposed to contribute to increased peripheral vascular resistance in obesity-associated hypertension [22]. Tirapelli et al. [23] has observed that Ang-(1–7) is able to relax carotid rings through activation of MasR on VSMCs, as well as the aorta [24].

In the present study, DNA methylation status of the *Agtra1* and *Mas1* gene promoters was well correlated with changes in transcriptional levels of $AT_{1a}R$ and MasR, revealing a higher methylation status of the *Mas1*/*Agtra1* gene promoter in MAs in the development of hypertension. DNA hypermethylation is a hallmark of gene silencing, while DNA hypomethylation promotes active transcription. Recent studies have reported that methylation of *Mas1*/*Agtra1* plays a key role in the regulation of vascular tone [25, 26]. For instance, perinatal nicotine exposure enhances vascular contractility that is associated with decreased DNA methylation of *Agtra1* in the aorta in adult offspring [27]. In addition, hypomethylation of the *Agtra1* gene promoter is correlated with the expression of $AT_{1a}R$ in the aorta and MAs of SHRs [28]. In this study, the methylation status of the *Agtra1* gene promoter was similar to Pei in MAs from SHRs. The hypomethylation of *Mas1* was exhibited in the SHR-C as well. DNA methylation is catalyzed by DNMT. DNMT3a and DNMT3b are the *de novo* DNA methyltransferases that act on non-methylated DNA. However, DNMT1 is essential to the maintenance of methylated DNA. The present study found that the expression of DNMT1, not DNMT3a or DNMT3b, significantly decreased in hypertensive MAs (Fig. 4a and b). A pilot study on *in vitro* fertilization-embryo transfer on Ang Ⅱ-mediated vasoconstrictions in umbilical cord vein suggested that hypomethylation of *Agtr1* is caused by decreased DNMT3a expression [26]. This difference may be attributed to different vascular beds and experimental models. Recently, research

works have demonstrated that DNMT1 mediates *de novo* methylation in several cell types [29, 30]. To further confirm the contribution of DNMT1 in *de novo Agtra1* and *Mas1* methylation, the ratio of SAM/SAH was measured by UPLC, which showed a decreased ratio in SHR-C. This finding, in combination with reduced DNMT1 expression, underlies *Agtra1* and *Mas1* gene promoter hypomethylation in MAs and contributes to pathological/compensatory upregulation of $AT_{1a}R$/MasR during hypertension. In addition, it reveals that DNMT1 is not purely a maintenance methyltransferase but can also participate in *de novo* methylation.

Aerobic exercise is often considered the cornerstone of nonpharmacological treatment of hypertension. However, the exact mechanism by which exercise improves vascular dysfunction in hypertension remains unclear. In this study, we have proven a disproportional reduction in RAS peptides in hypertension, especially the major bioactive content of Ang Ⅱ and Ang-(1–7) after 12 weeks of exercise. In addition, the increase in the Ang-(1–7)/Ang Ⅱ ratio is a more important contributor to enhancing the function of hypertensive MAs after exercise, indicating that exercise restores the abnormal balance of vascular RAS and adjusts this to a lower level. The transcript and protein expression of receptors (AT_1R, MasR) suppressed in SHR-E group. Studies suggest that epigenetics is influenced by the environment, and exercise is associated with DNA methylation [31]. In support of this notion, DNA-modifying enzymes in MAs and intermediate metabolites (SAM, SAH) were assessed. Exercise upregulated the expression of DNMT1 and DNMT3b in MAs and increased SAM/SAH, thus influencing *Agtra1* and *Mas1* gene promoter methylation, which led to the downregulation of $AT_{1a}R$ and MasR. By contrast, in normotensive rats, exercise-mediated enhancement of $AT_{1a}R$ and MasR expression was correlated with decreased methylation status of *Agtra1* and *Mas1* gene promoters, which were attributed to reduced DNMT1 and DNMT3b expression and decreased SAM/SAH ratio. The difference in the results between physiological and pathological conditions may be multifactorial. We hypothesize that exercise training mainly restores the inappropriate overactivity of RAS to a lower level in hypertension, whereas exercise enhances vasoconstriction by increasing $AT_{1a}R$ and MasR expression at the physiological level.

Conclusions

In summary, the present set of data shows that exercise reduces RAS and its counter-regulatory axes to improve vascular function in hypertensive rats. Most important, our results demonstrate that aerobic exercise decrease $AT_{1a}R$ and MasR via hypermethylation of *Agtra1* and *Mas1* gene, counterbalancing the pathological increase. These results provide mechanistic evidence that unlike currently available pharmacological anti-hypertensive therapies, aerobic exercise plays a favorably role in treatment for hypertension. Our finding reveals crucial insight into the epigenetic mechanism by which exercise exerts beneficial effects in hypertension.

Declarations

Author Contributions Conceptualization,Y.C. and L.J.S; formal analysis, Y.Y.Z; investigation, Y.C, S.S.L, H.R.Z; methodology, Z.X.X; writing-review and editing, Y.C and L.J.S; funding acquisition, L.J.S.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Conflicts of Interest The authors declare no conflict of interest.

Ethical approval All experiments were approved by the ethical committee of Beijing Sport University and were performed in accordance with the Chinese animal protection laws and institutional guidelines.

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Figures

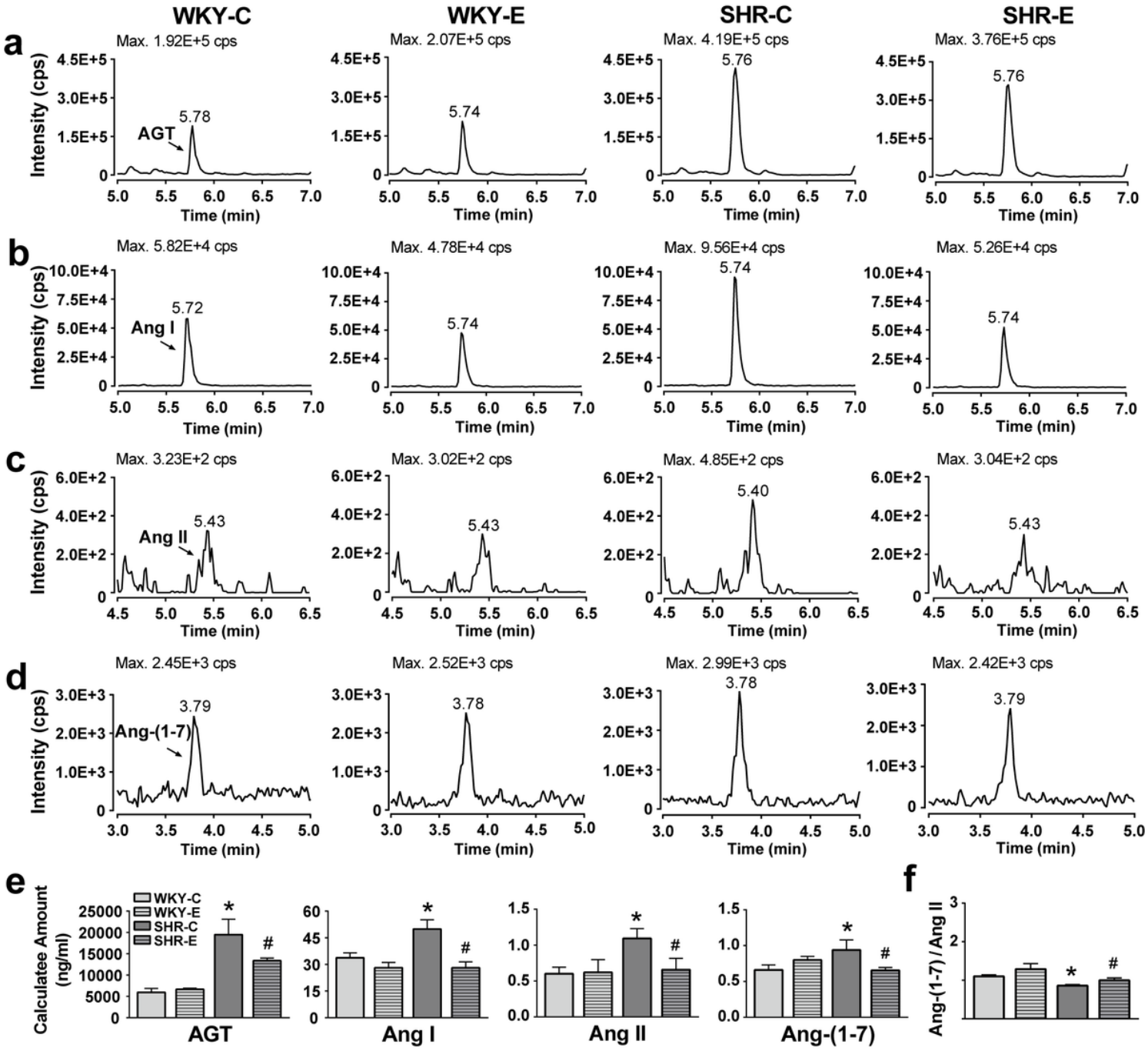

Figure 1

The levels of RAS components in plasma. Plasma of AGT, Ang I, Ang II, Ang-(1-7) levels, respectively (a–d), comparison of plasma levels of AGT, Ang I, Ang II and Ang-(1-7) (e), Ang-(1-7)/ Ang II in WKY-C, WKY-E, SHR-C, SHR-E plasma samples (f). n = 5-6. * $P < 0.05$ vs WKY-C; # $P < 0.05$ vs SHR-C.

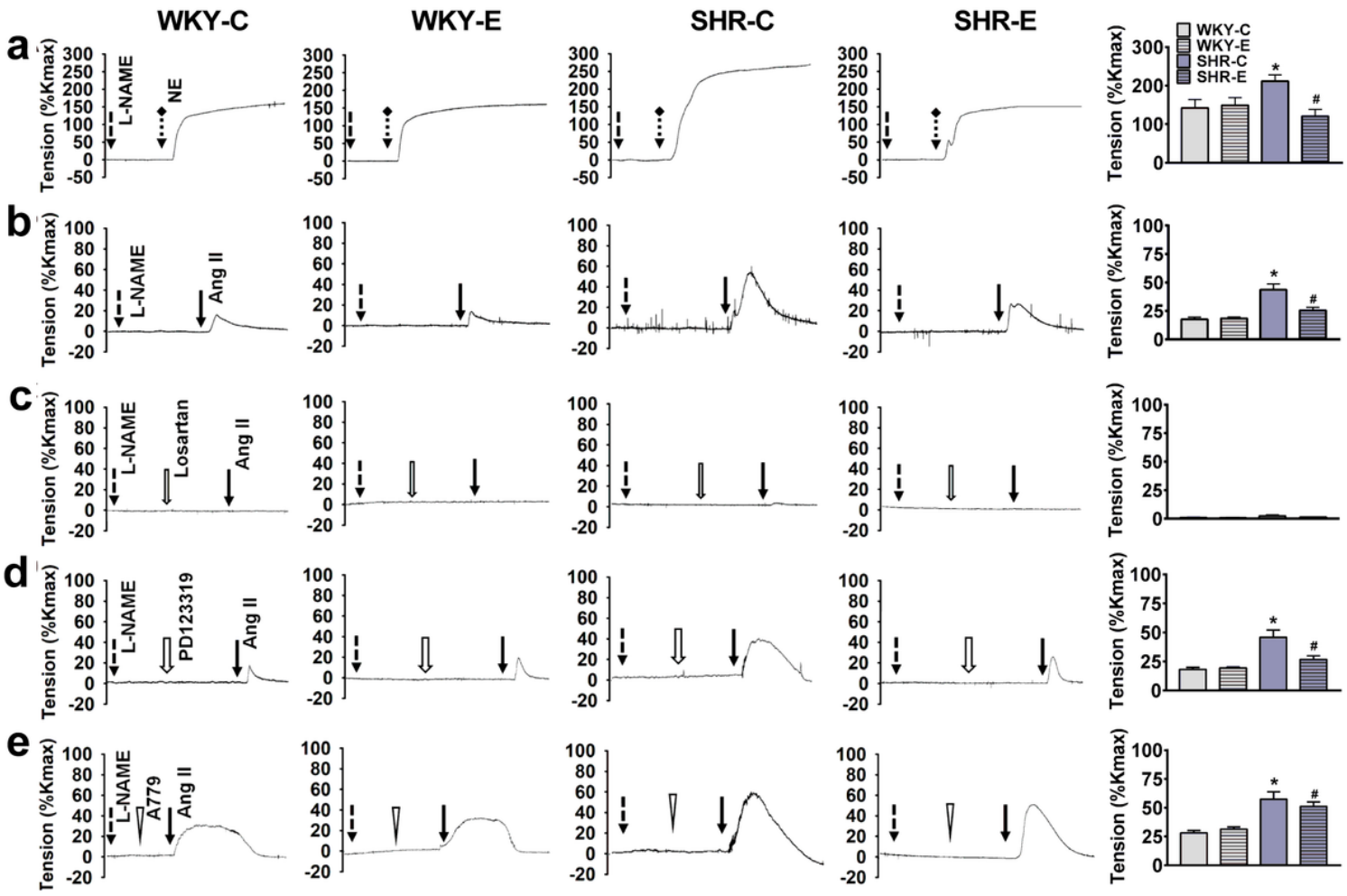

Figure 2

Effects of RAS components on the vascular tension in MAs. Effects of NE on the vascular tension in MAs (a), effect of Ang Ⅱ (10−5 M) on the vasoconstriction (b), effect of AT1R blockers losartan (10−6 M), AT2R inhibitor PD123319 (10−5 M), MasR antagonist A779 (10−5 M) on Ang Ⅱ-induced artery contraction (c–e). In each experiment, the arteries were preincubated with nonselective nitric oxide synthase inhibitor Nω-nitro-l-arginine methyl ester (L-NAME, 100 μM) for 20 min (dotted arrows). n = 6 in each group. * P < 0.05 vs WKY-C; # P < 0.05 vs SHR-C.

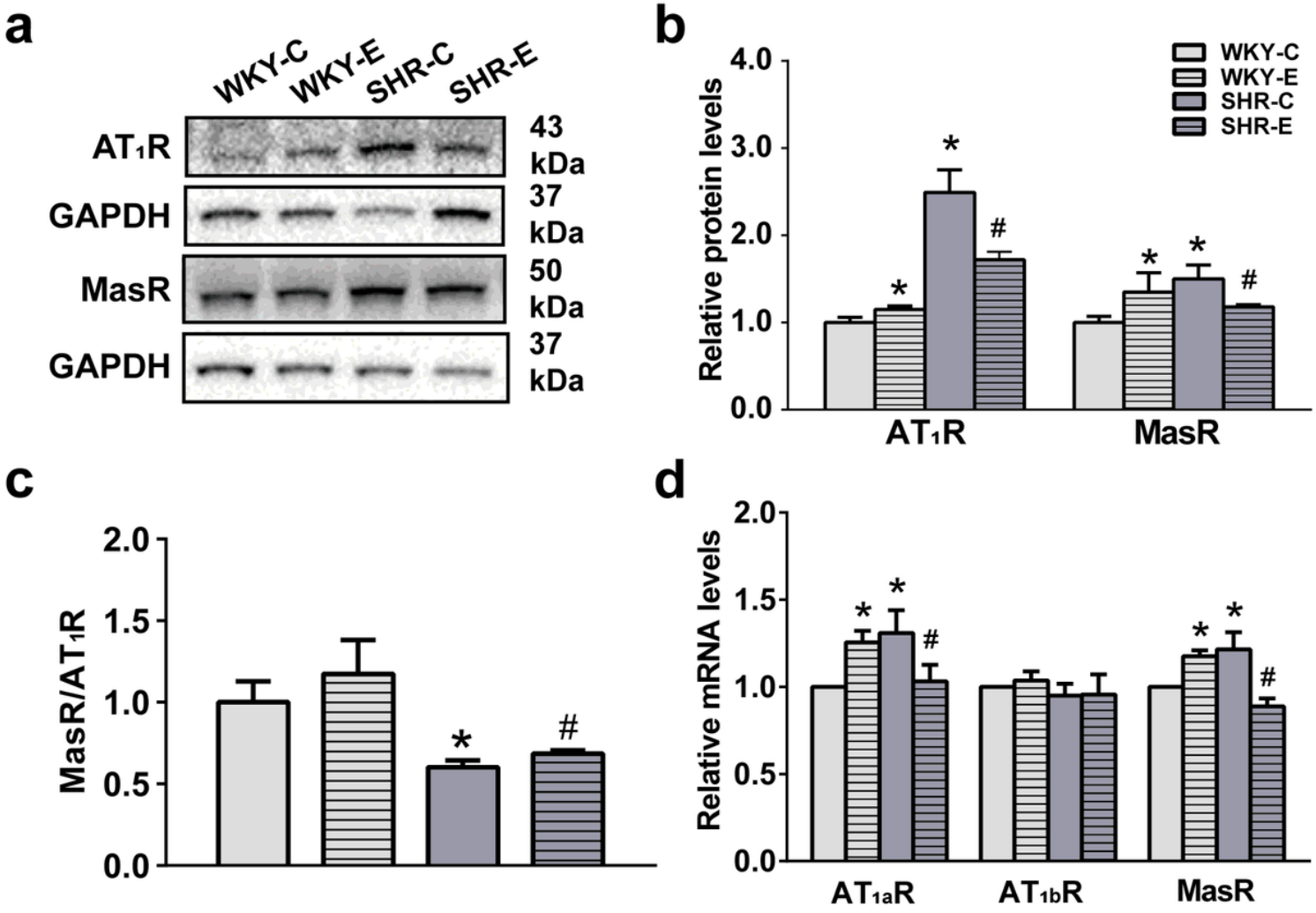

Figure 3

Protein expression and transcript level of AT1R and MasR in MAs. Protein expression of AT1R and MasR (a), corresponding densitometry summary data (b), ratio of MasR/AT1R protein levels in WKY-C, WKY-E, SHR-C, SHR-E (c), bar plot summarizing quantitative real-time PCR data for AT1aR, AT1bR and MasR transcript (d). n = 6 in each group. * $P < 0.05$ vs WKY-C; # $P < 0.05$ vs SHR-C.

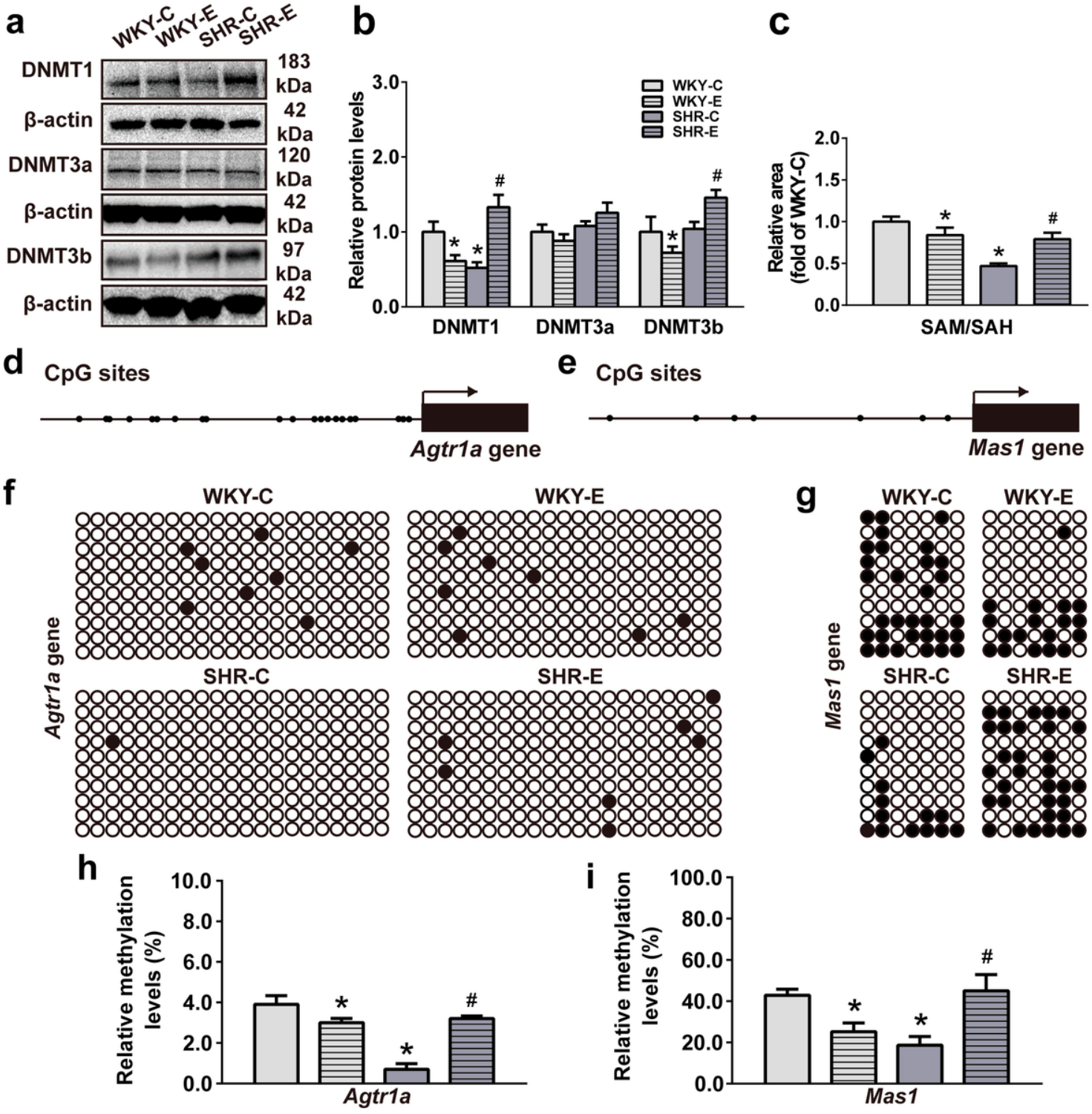

Figure 4

DNA methylation status of Agtr1a and Mas1 gene in MAs. Immunoreactive bands corresponding to the DNMT1, DNMT3a, DNMT3b and β-actin in MAs (a), corresponding densitometry summary data (b), a summary of SAM/SAH averaged from each group (c), CpG sites in regions of Agtr1a (35957898 to 35958307, 410 bp) and Mas1 (48076585 to 48077039, 455 bp) gene, respectively (d and e), representative DNA methylation status of the CpG sites of Agtr1a and Mas1 gene; the solid circles represent methylated cytosines, and hollow circles denote unmethylated ones; a total of 21 CpG sites of

the Agtr1a gene, 7 CpG sites of the Mas1 gene and 10 clones were subjected to sequencing (f and g), the average total percentage of methylation levels of all measured CpG sites within Agtr1a and Mas1 gene in MAs (h and i). n = 6 in each group. * P < 0.05 vs WKY-C; # P < 0.05 vs SHR-C.

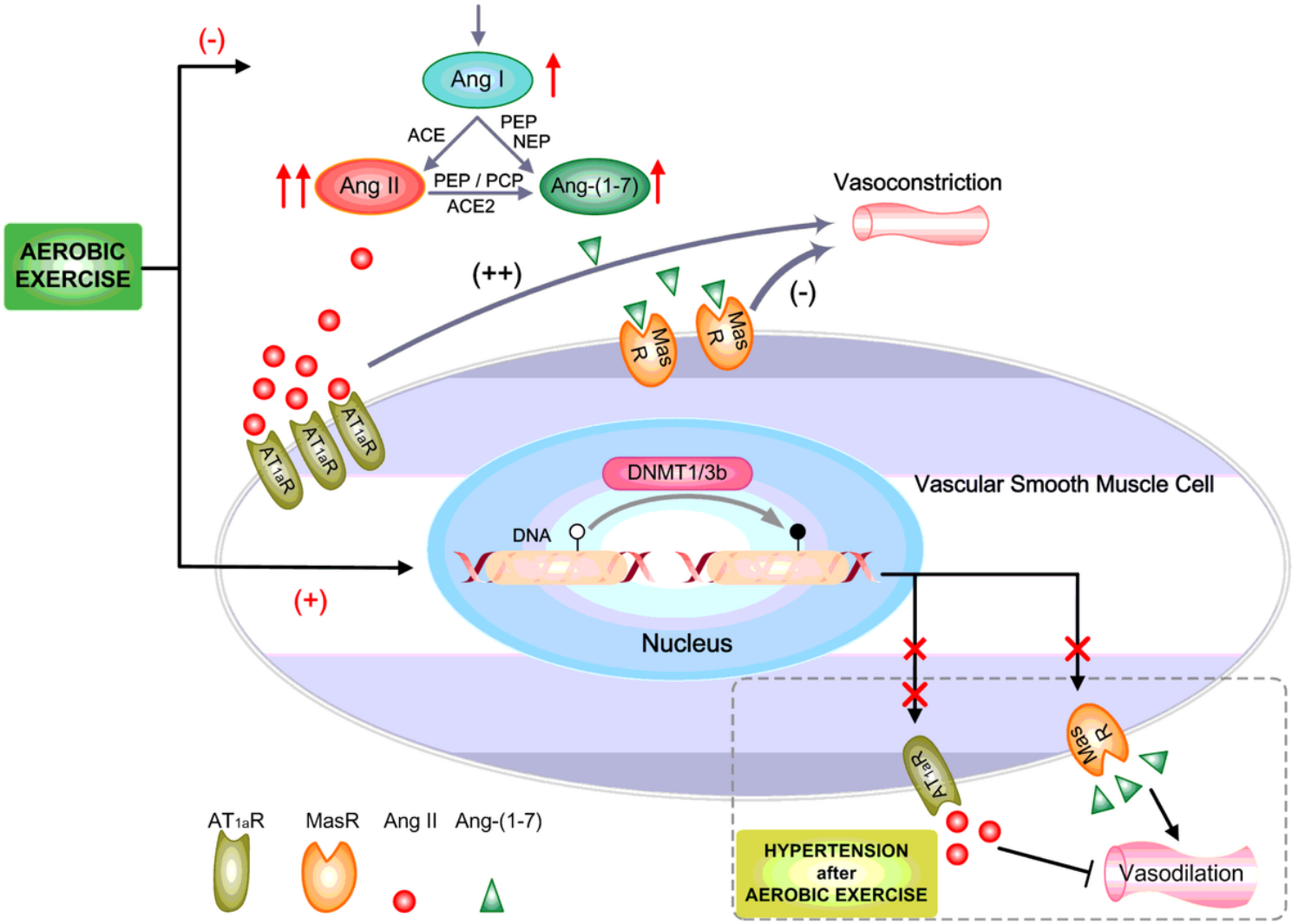

Figure 5

Proposed model for exercise-mediated repression of AT1aR and MasR via hypermethylation of Agtr1a and Mas1 gene in hypertension. Under hypertensive conditions, unbalanced increase with RAS axes lead to vasoconstriction. Aerobic exercise suppresses AT1aR and MasR in hypertension contributing to vasodilation via hypermethylation of Agtr1a and Mas1 gene and increases DNMT1, DNMT3b and SAM/SAH. PEP, prolyl endopeptidase; NEP, neutral endopeptidase; PCP, proline carboxypeptidase.