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Exercise during pregnancy enhances vascular function via epigenetic repression of $Ca_V1.2$ channel in offspring of hypertensive rats

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ABSTRACT

Aims: Studies suggest that cardiovascular function in offspring can be epigenetically programmed by environmental changes during pregnancy. $Ca_V1.2$ channel plays a major role in the regulation of the vascular tone. This study investigated the effects and underlying mechanisms of exercise during pregnancy on $Ca_V1.2$ channel functional remodeling in hypertensive offspring.

Main methods: Exercise groups were subjected to swimming at the first day of pregnancy and on a regular schedule thereafter for 3 weeks. Their offspring (6-month-old, male) were tested for baseline blood pressure, cardiovascular response, and vascular tone of the mesenteric artery. Mesenteric artery smooth muscle cells were taken to study the whole-cell current of the $Ca_V1.2$ channel. Western blotting, RT-PCR and DNA bisulfite sequencing PCR were performed to study the protein, mRNA expression and DNA methylation of the $Ca_V1.2$ channel α1C subunit.

Key findings: Exercise during pregnancy reduced the pressor response to norepinephrine and Bay K8644, and the depressor response to nifedipine in offspring of hypertensive rats. The level of the $Ca_V1.2$ channel in norepinephrine-induced vasoconstrictions decreased, and the whole-cell current of the $Ca_V1.2$ channel declined in the SHR-EX group. Further studies found that exercise during pregnancy reduced the protein and mRNA expression of the $Ca_V1.2$ channel α1C subunit and upregulated DNA methylation of the *Cacna1c* gene promoter region in the hypertensive offspring.

Significance: These data suggest that exercise during pregnancy improves vascular functional remodeling in offspring of hypertensive rats, downregulating the $Ca_V1.2$ channel function and protein expression, a change that is most likely caused by DNA methylation.

1. Introduction

Hypertension is a major risk factor for cardiovascular diseases. As the research progressed, people gradually realized that genetic, adult environment and behavior could not fully explain the pathogenesis of hypertension. Perinatal programming studies have well confirmed the contribution of epigenetic mechanisms to the development of hypertension [1]. Epidemiological studies and clinical evidence suggest that there may be window periods of epigenetic instability during fetal development, and life events occurring during this window period are "imprinted" on the phenotype of the individual long-term and on the individual's risk of cardiovascular disease. Due to its phenotypic plasticity, the fetus is highly susceptible and produces lasting effects [2]. According to reports, negative maternal risk factors during pregnancy, such as malnutrition, smoking, and hypoxia, will increase the risk of cardiovascular diseases in adulthood. To date, most studies have focused on the effects of adverse behavior during pregnancy on the cardiovascular health in offspring [3–5].

Studies have confirmed that prenatal and the postnatal environments have an impact on the development of blood pressure in spontaneously hypertensive rat (SHR) [6,7]. There has been an increasing evidence that following a good lifestyle during pregnancy, such as regular exercising, have a beneficial effect on the physical functioning of mothers and offspring [8–15]. Studies have shown that exercise during pregnancy can reduce the incidence of gestational diabetes [16], improve the metabolic function of offspring, increase glucose tolerance

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[17], prevent the increase of leptin, and resist the increase of triglycerides [18]. In addition, many studies have shown that under normal conditions, exercise during pregnancy can affect the cardiovascular function of the offspring, reduce neonatal heart rate, and improve endothelial-dependent or independent vascular function in the offspring [19–23]. However, although many studies have linked exercise in pregnancy to the cardiovascular health of offspring, the current research on whether exercise during pregnancy has a positive effect on the offspring in terms of cardiovascular disease such as hypertension is still limited. In the future, for cardiovascular disease, if exercise during pregnancy can be shown to produce an imprinting effect on the cardiovascular function of the offspring through improving the uterine environment, and thus enhancing the cardiovascular function of the offspring in adulthood, it may become a non-medical treatment.

A hallmark of hypertension is the excessive increase in peripheral resistance vascular tone. The L-type-voltage-dependent Ca^{2+} channels ($Ca_V1.2$ channel) play a major role in the regulation of the vascular tone. Previous studies have shown that changes in the intrauterine environment can cause structural and functional remodeling of ion channels in vascular smooth muscle cells (VSMCs) [24,25]. Therefore, we hypothesized that maternal exercise during pregnancy in mothers with genetic hypertension can inhibit the function of the $Ca_V1.2$ channel of VSMCs in the offspring, thereby improving the function of the blood vessels. This change would likely be caused by DNA methylation.

2. Materials and methods

2.1. Animal studies and exercise training

Female (11 weeks old) and male (12 weeks old) SHRs and female (11 weeks old) and male (12 weeks old) Wistar-Kyoto rats (WKYs) were selected to use for breeding. The day when the vaginal plug was found was considered the day 1 of gestation. The pregnant rats were randomly divided into four groups: WKY sedentary group (WKY-SED), WKY exercise group (WKY-EX), SHR sedentary group (SHR-SED), and SHR exercise group (SHR-EX). The exercise groups were subjected to swimming at the first day of pregnancy, with 60 min per swim session for 3 weeks. The pregnant rats were placed in a bucket containing water at a depth of 40 cm. Swimming was always performed at a water temperature of 34–35 °C between 6 a.m. and 7 a.m. for 6 days a week. At the same time, the SED group was placed in a bucket with water at a depth of about 10 cm [26,27]. The caudal artery tail-cuff method (BP-2010A, Softron Biotechnology, China) was used to determine the blood pressure (BP) of male offspring (6-month-old, 6 M). Animals were purchased from Vital River Laboratory Animal Technology (Beijing, China) and kept in controlled conditions on a 12-hour light/dark cycle at an ambient temperature of 24 °C, with free access to food and water. License number: SCXK (Beijing)-2016-0006. All experiments were approved by the ethics committee of Beijing Sport University and performed in accordance with Chinese animal protection laws and institutional guidelines. The animal protocol of this study strictly complies with the National Institutes of Health guide for the care and use of Laboratory animals.

2.2. Measurement of cardiovascular responses

The animals were anaesthetized with sodium pentobarbitone (50 mg/kg *i.p.*) and underwent operation with femoral arterial and venous cannulation for the study of in vivo cardiovascular responses, as described [28]. After a 24-h recovery period, the blood pressure after *i.v.* injection of norepinephrine (NE, 18 μg/kg), $Ca_V1.2$ channel opener Bay K8644 (0.1 mg/kg), and blocker nifedipine (1 mg/kg) were monitored in vivo by PowerLab (PL3504, AD Instruments, Australia).

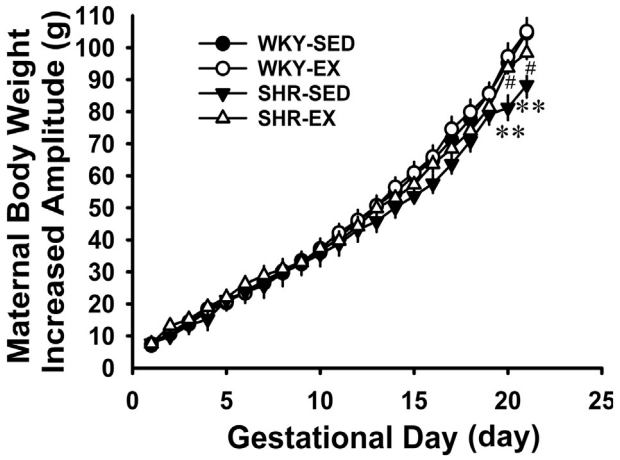

Fig. 1. Weight gain in the four groups of pregnant rats during pregnancy. The weight increased amplitude indicates the increase in body weight during pregnancy compared to the day before pregnancy. The results are expressed as mean ± SEM (n = 8). $^{**}P < 0.01$ vs. WKY-SED; $^{\#}P < 0.05$ vs. SHR-SED.

2.3. Isometric contraction study

Twenty-four animals (n = 6 per group) were used for the isometric contraction study using a Multi myograph system (620 M, Danish Myo Technology, Aarhus, Denmark). The third-order branches of the mesenteric arteries (MAs, A3) were removed and placed in cold Krebs buffer containing (in mM): 131.5 NaCl, 5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11.2 glucose, 13.5 $NaHCO_3$, and 0.025 EDTA. The tissues were then gassed with a mixture of 95% O_2 and 5% CO_2 (pH 7.4) at 37 °C. The vessel tensions were evaluated by measuring the maximum peak and expressed as percentage of maximal tension achieved in response to 60 mM K^+ (K_{max}). N^{ω}-nitro-L-arginine methyl ester (L-NAME, a non-selective nitric oxide synthase inhibitor, 100 μM) was added after K_{max} measurement. In order to investigate the role of $Ca_V1.2$ channels on vascular tone regulation, we qualified the vasorelaxation effect of the $Ca_V1.2$ channel blocker nifedipine (10^{-9}–10^{-5} M) in tissues, which had been precontracted with 10^{-5} M NE.

2.4. Cell isolation and patch clamp electrophysiology

VSMCs from MAs (A2-A3) were isolated for patch clamp electrophysiological study [29]. Whole-cell Ca^{2+} currents were measured using a conventional whole-cell patch-clamp configuration. The electrode (2–4 MΩ) solution contained (in mM): 130 CsCl, 10 HEPES, 3 Na_2ATP, 0.1 Na_2GTP, 1.5 $MgCl_2$, 10 Glucose, 10 EGTA, and 0.5 MgATP (pH 7.3 with CsOH). The extracellular bath solution comprised (in mM): 10 $BaCl_2$, 10 HEPES, 5 glucose, 1 $MgCl_2$, and 124 choline chloride (pH 7.4 with CsOH). The voltage protocol used to record whole-cell $Ca_V1.2$ channel currents consisted of a holding potential of −80 mV and a sequence of pulses for 250 ms between −70 and +70 mV in 10 mV increments. After obtaining whole-cell currents, nifedipine (100 nM) was used to verify the identity of the $Ca_V1.2$ currents. Whole-cell currents were recorded using Axon700B amplifier and pCLAMP 10.2 with Clampfit 10.2 software (Axon Instruments Inc., Foster City, CA, USA).

2.5. Western blot analysis

MAs were homogenized on ice with tissue lysis buffer and the supernatant was collected after centrifugation. Protein concentration was determined by Bradford protein assay. Proteins were separated by gel electrophoresis with SDS–10% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% nonfat milk and incubated with a primary antibody against α1C

Table 1
The body weight of the male offspring (g).

Groups	WKY-SED	WKY-EX	SHR-SED	SHR-EX
20.5D fetus	5.01 ± 0.06	4.93 ± 0.04	4.68 ± 0.08**	4.79 ± 0.05
1 M	166.21 ± 2.54	164.43 ± 1.58	159.07 ± 1.98*	161.22 ± 1.87
3 M	323.44 ± 3.39	315.05 ± 4.80	297.92 ± 4.35**	301.50 ± 4.08
6 M	417.75 ± 6.58	413.40 ± 11.48	365.20 ± 3.00**	367.60 ± 4.69

*$P < 0.05$ and **$P < 0.01$, Compared with WKY-SED. 20.5D fetus: n = 20 in each group; 1 M male offspring: n = 22 in each group; 3 M male offspring: n = 17 in each group; 6 M male offspring: n = 15 in each group.

Table 2
Blood pressure of 6 M adult offspring (mmHg).

Groups	WKY-SED	WKY-EX	SHR-SED	SHR-EX
SBP	140.9 ± 5.0	143.6 ± 3.4	219.4 ± 6.5*	213.0 ± 3.2
DBP	113.4 ± 3.9	107.8 ± 4.1	155.4 ± 4.5*	148.5 ± 5.6
MAP	122.4 ± 3.5	120.4 ± 6.3	176.8 ± 5.1*	170.3 ± 6.6

*$P < 0.01$, compared with WKY-SED; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. n = 15 in each group.

(1:500 dilution; product no. ab93892; Abcam, Inc.) and β-actin (1:4000 dilution; product no. sc-47,778; Santa Cruz Biotechnology, Inc.) overnight at 4 °C. The next day, after incubation with secondary antibody for 1 h at room temperature, the immunoreactive bands were identified with enhanced chemiluminescence and recorded with a Bio-Rad ChemiDOC XRS+ (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Quantitative real-time PCR

MAs were removed and placed in RNAlater (Ambion, Austin TX, USA) at 4 °C. The TRIzol reagent (Invitrogen, Carlsbad, CA) was used to isolate total RNA. RNA was reverse transcribed into cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher, Waltham, MA, USA), according to the manufacturer's instructions. Real-time quantitative PCR was performed using an ABI Prism 7500 (Applied Biosystems, USA) with TaqMan Fast Advanced Master Mix. The gene expression assays included the target gene *Cacna1c* ($Ca_V1.2$ channel α1C subunit gene) (Rn00709287_m1, amplicon length = 68 bp) and the normalization gene β-actin (Rn00667869_m1, amplicon length = 91 bp). All reagents were from Applied Biosystems.

2.7. DNA bisulfite sequencing PCR

Genomic DNA of MAs was isolated using a PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) and was subjected to sodium bisulfate conversion using an EZ DNA Methylation-Gold Kit (ZYMO Research, Orange, CA, USA), according to the manufacturer's recommendations. PCR amplification on the CpG island (216,566,309 to 216,566,707) of the *Cacna1c* promoter region was performed, and the products were separated using 2.0% agarose gels. The bands were excised using a TIAN gel Midi Purification Kit (Tian gen, Beijing, China).

The purified bands were cloned into a pEASY-T1 Cloning vector (TRAN, Beijing, China). Ten clones from each sample were sequenced. The forward primer used for bisulfate-modified PCR was 5′-GAATTTA TTATGTTTTTTGGAGGTGA-3′, and the reverse primer was 5′-ATCTCA ACTCACTTAACTTTTACTCTACC-3′. Data are presented as the percentage of methylation at the region of interest (methylated CpG/(methylated CpG + unmethylated CpG) × 100).

2.8. Statistical analysis

Statistical analysis was conducted using two-way analysis of variance (ANOVA, hypertension × exercise). Selected post hoc analysis was performed using an independent sample *t*-test. Data are expressed as a mean ± SEM. All analyses were performed using SPSS 19.0 software, and $P < 0.05$ was considered statistically significant. Concentration–response curves were analyzed by computer-assisted non-linear regression to fit the data using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) to obtain -logEC50 (pIC50) and the maximal response. Electrophysiological data analysis and curve fitting were performed offline using Clampfit 10.2.

3. Results

3.1. Maternal and litter characteristics

The weight of the four groups of pregnant rats showed a gradual upward trend throughout pregnancy. Compared with WKY pregnant rats, the weight gain of SHR decreased significantly during the last two days of pregnancy (n = 8, $P < 0.01$), and exercise during pregnancy significantly inhibited this decline (n = 8, $P < 0.05$ vs. SHR-SED) (Fig. 1). The litter size in the four groups was 10.31 ± 0.33 (WKY-SED, $n = 8$ pregnant rats), 10.1 ± 0.55 (WKY-EX, $n = 8$ pregnant rats), 9.6 ± 0.67 (SHR-SED, $n = 8$ pregnant rats), and 10 ± 0.46 (SHR-EX, $n = 8$ pregnant rats). There was no significant difference in each group.

From fetus of 20.5 days to 6-month-old, the body weight was lower in the male offspring of the SHR-SED group than WKY-SED group ($P < 0.05$). Exercise during pregnancy has no significant effect on male offspring weight (Table 1). Compared with the WKY-SED group, the systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) were higher in the 6 M offspring of the SHR-SED group (n = 15, $P < 0.01$); no significant difference was observed in the SBP, DBP, and MAP between the SED and Ex groups. However, compared with the SHR-SED group, the SBP, DBP and MAP of the SHR-EX group have downward trends, but these trends did not reach statistical significance (Table 2).

3.2. Cardiovascular reactivity in the offspring of hypertensive rats which exercised during pregnancy

NE (18 μg/kg, *i.v.*) caused a significant increase in the BP of the offspring of all four groups (Fig. 2A). When compared with the WKY group (52.0 ± 6.1 mmHg, $n = 6$), the male adult offspring of the SHR group (99.0 ± 7.9 mmHg, n = 6, $P < 0.01$) had a significant increase in the pressor response, but exercise during pregnancy significantly inhibited this change (75.1 ± 4.9 mmHg, $n = 6$, $P < 0.05$ vs. SHR-SED) (Fig. 2D). The interaction of exercise during pregnancy and hypertension was significant ($P < 0.05$). Bay K8644 (0.1 mg/kg, *i.v.*) produced a rapid increase in arterial BP in all groups (Fig. 2B). The maximal increases of the MAP induced by *i.v.* injection of Bay K8644 were larger in the 6 M offspring of the SHR-SED group (38.5 ± 3.7 mmHg, $n = 6$, $P < 0.05$) compared with the WKY-SED group (29.0 ± 2.6 mmHg, n = 6). Exercise during pregnancy markedly decreased the pressor responses in the 6 M offspring of the SHR-EX group (29.5 ± 2.7 mmHg, $n = 6$, $P < 0.05$ vs. SHR-SED) (Fig. 2E). Nifedipine (1 mg/kg, *i.v.*) elicited a rapid decrease in arterial BP in all groups (Fig. 2C). Compared with the 6 M offspring of the WKY-SED group (33.5 ± 2.0 mmHg, $n = 6$), the depressor responses induced by *i.v.* injection of nifedipine were increased in the 6 M offspring of the

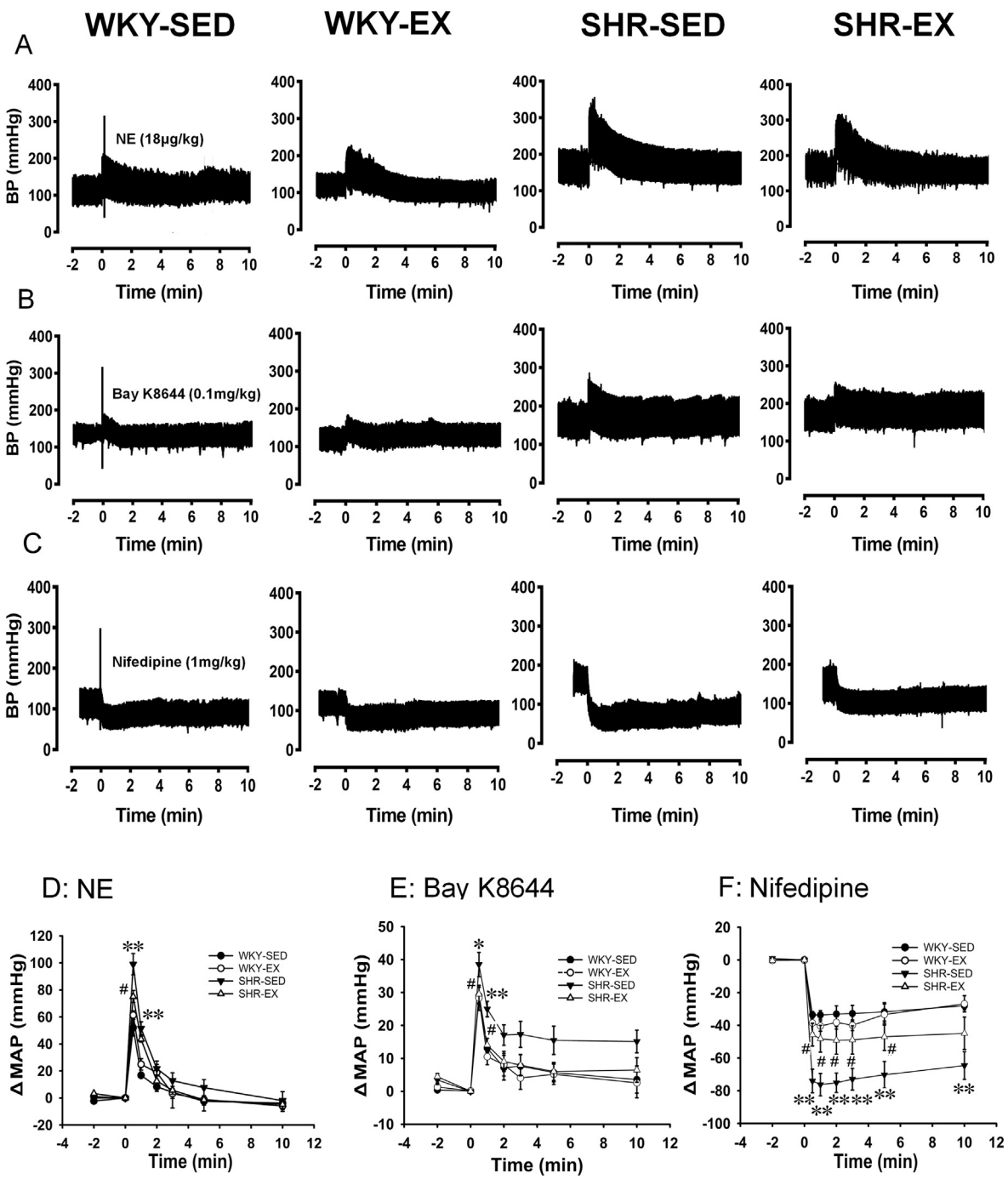

Fig. 2. Drug-induced blood pressure response. A-C, the blood pressure response caused by intravenous injection of NE (18 μg/kg; A), Bay K8644 (0.1 mg/kg; B) and Nifedipine (1 mg/kg; C); D, the mean arterial pressure increase induced by noradrenaline; E, the mean arterial pressure increase induced by Bay K8644; F, the mean arterial pressure decrease induced by nifedipine. The time point 0 min indicates the time of the intravenous injection of drug. The results are expressed as the mean ± SEM (n = 6). *$P < 0.05$ and **$P < 0.01$ vs. WKY-SED; $^{\#}P < 0.05$ vs. SHR-SED. NE: norepinephrine.

SHR-SED group (74.0 ± 7.2 mmHg, n = 6, $P < 0.01$). Exercise during pregnancy markedly attenuated the depressor responses in the 6 M offspring of the SHR-EX group (45.7 ± 7.0 mmHg, n = 6, $P < 0.05$ vs. SHR-SED) (Fig. 2F). This indicated that the function of the $Ca_V1.2$ channels was enhanced in SHRs, and exercise during pregnancy ameliorated this change. The interaction of exercise during pregnancy and hypertension was significant ($P < 0.05$).

3.3. Exercise during pregnancy inhibited the role of the $Ca_V1.2$ channel in regulating the vascular tone in MAs of hypertensive offspring

The maximal contraction elicited by KCl (60 mM) was similar in the offspring of all groups. The amplitude of vasoconstriction caused by 10^{-5} M NE in the four groups was 122.8 ± 2.2% K_{max} (WKY-SED, $n = 6$), 119.4 ± 7.2% K_{max} (WKY-EX, $n = 6$, $P > 0.05$ vs. WKY-SED), 166.1 ± 10.6% K_{max} (SHR-SED, $n = 6$, $P < 0.01$ vs. WKY-SED), and 135.9 ± 1.3% K_{max} (SHR-EX, $n = 6$, $P < 0.01$ vs. SHR-SED). To investigate the effect of $Ca_V1.2$ channels in regulating the tone of MAs, nifedipine (10^{-9}–10^{-5} M) was measured in MAs precontracted with

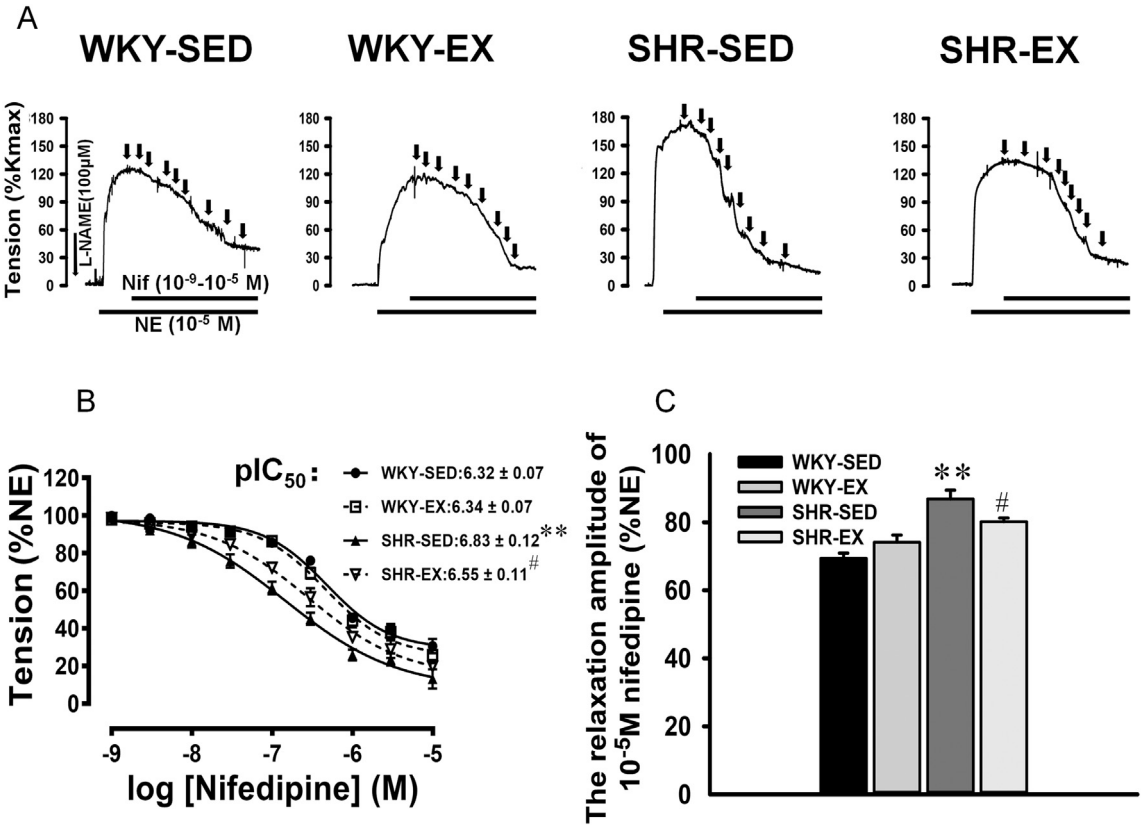

Fig. 3. Effects of nifedipine on the vascular tension in MAs. A, The effect of the nifedipine (10^{-9}–10^{-5} M) on noradrenaline-induced vessel contraction. B, Concentration–response curves for the effects of nifedipine on the NE-induced concentration (NE: 10^{-5} M). C, The relaxation amplitude of nifedipine (10^{-5} M) on noradrenaline -induced vessel contraction. The results are expressed as the mean ± SEM (n = 6). $^{**}P < 0.01$ vs. WKY-SED; $^{\#}P < 0.05$ vs. SHR-SED. NE: norepinephrine.

10^{-5} M NE. 10^{-5} M NE-induced maximal tension increase was treated as 100% in each group. The pIC50 values, a negative logarithm of the 50% effective concentration, were 6.32 ± 0.07 (WKY-SED, $n = 6$), 6.34 ± 0.07 (WKY-EX, n = 6, $P > 0.05$ vs. WKY-SED), 6.83 ± 0.12 (SHR-SED, n = 6, $P < 0.01$ vs. WKY-SED), and 6.55 ± 0.11 (SHR-EX, n = 6, $P < 0.05$ vs. SHR-SED) (Fig. 3B). When compared with the 6 M offspring of the WKY-SED group (69.39 ± 1.53, n = 6), the amplitude of vasodilation caused by 10^{-5} M nifedipine in the SHR-SED group (86.85 ± 2.57, n = 6, $P < 0.01$) was significantly increased, while exercise during pregnancy (80.16 ± 1.09, n = 6, $P < 0.05$ vs. SHR-SED) significantly inhibited the increase in the amplitude of vasodilation caused by hypertension (Fig. 3C). These data reveal that the activation of the $Ca_V1.2$ channel increased vascular tone much more in the 6 M offspring of the SHR group than in the WKY group. However, exercise during pregnancy appeared to ameliorate this change significantly.

3.4. Exercise during pregnancy reduced the currents through the $Ca_V1.2$ channel in MAs in the offspring of SHR

To determine the effects of hypertension and exercise during pregnancy on the function of $Ca_V1.2$ channels, patch-clamp electrophysiology was used to determine $Ca_V1.2$ channel currents in the VSMCs of MAs. Fig. 4A shows whole-cell $Ca_V1.2$ currents recorded in the VSMCs of MAs from the adult offspring of the four groups. The peak density of the inward current was recorded at +10 mV in the offspring of all four groups. Bay K8644 (5 μM) strengthened the peak of inward Ba^{2+} current and negatively shifted the I–V relationship. That is, the peak of the $Ca_V1.2$ current densities shifted from +10 to 0 mV. Nifedipine (100 nM) almost completely blocked the inward current. These observations showed that the inward current recorded was the Ba^{2+} current through the $Ca_V1.2$ channel. The membrane Ba^{2+} currents are expressed relative to the cell capacitance (pA/pF) in order to avoid the influence of different cell surface areas on the $Ca_V1.2$ channel current. There were no significant differences between the capacitance of VSMCs from the offspring of the four groups. The maximal calcium current density in the 6 M offspring of the SHR-SED group (−19.6 ± 3.1 pA/pF, $n = 20$ cells from 6 animals, $P < 0.01$) was significantly higher than in the WKY-SED (−11.5 ± 1.6 pA/pF, $n = 22$ cells from 6 animals). Exercise during pregnancy significantly reduced the maximal calcium current density in hypertensive rats (−15.1 ± 1.7 pA/pF, n = 22 cells from 6 animals, $P < 0.05$ vs. SHR-SED), but had no significant effect on WKY rats (−14.7 ± 0.6 pA/pF, n = 24 cells from 6 animals, $P > 0.05$ vs. WKY-SED).

The activation and inactivation curves of $Ca_V1.2$ channels (Fig. 4B) in VSMCs show that there were no significant differences in the half-maximal voltages (V_h) and slopes (k) in the offspring of the four groups of rats. We suggest that the effect of hypertension and exercise during pregnancy on the function of the $Ca_V1.2$ channel largely depended on the changes in channel expression and had nothing to do with the characteristics of the channel itself.

3.5. Exercise during pregnancy downregulated the protein and mRNA expression of the $Ca_V1.2$ channel α1C subunit in MAs in hypertensive offspring

$Ca_V1.2$ channel α1C subunit protein expression in MA myocytes was measured by Western blotting. As shown in Fig. 5B, compared with the 6 M offspring of the WKY-SED group (1.00 ± 0.00, $n = 6$), the protein expression of the $Ca_V1.2$ channel α1C subunit was significantly increased in the SHR-SED group (1.59 ± 0.07, n = 6, $P < 0.01$). Exercise during pregnancy markedly inhibited the expression of the

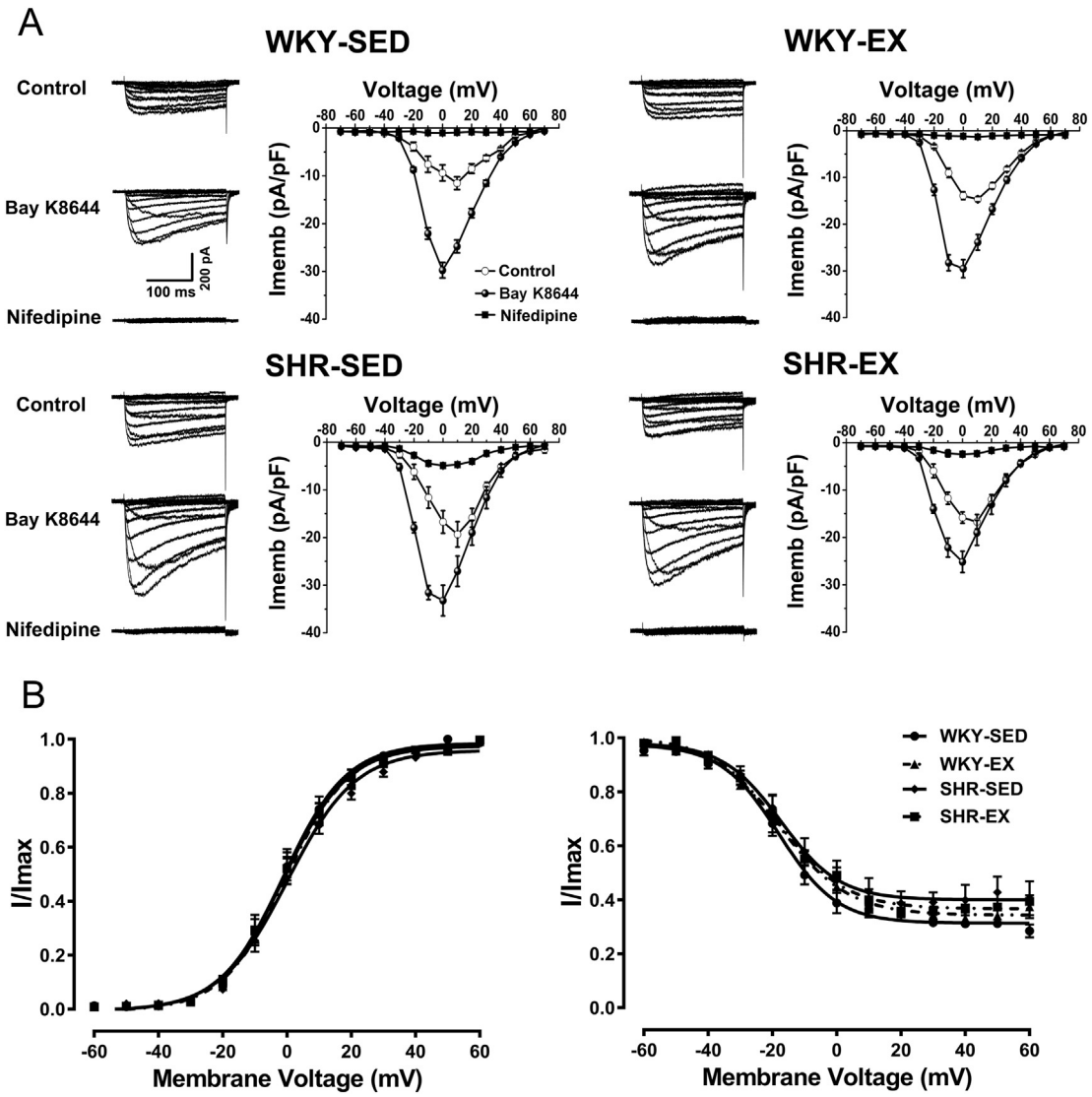

Fig. 4. Whole-cell $Ca_V1.2$ channel currents recorded in the VSMCs of MAs. A, Traces of Ca^{2+} channel currents evoked by command potentials (−70 to +70 mV in a 10 mV step) in the absence (upper panel) or presence of Bay K8644 (5 μM) (center panel) or nifedipine (100 nM) (lower panel) on the left. Mean current density is the voltage of $Ca_V1.2$ currents in VSMCs on the right (n = 6). B, Steady-state activation (left) and inactivation (right) curves of the Ba^{2+} currents in MAs (n = 6).

$Ca_V1.2$ channel α1C subunit in the 6 M offspring of the SHR-EX group (1.20 ± 0.12, n = 6, *P < 0.05* vs. SHR-SED).

RT- PCR was performed to compare the mRNA expression of α1C among the 6 M offspring of four groups. The results showed that the mean mRNA levels of α1C were approximately 1.2fold higher in MAs from the 6 M offspring of the SHR-SED group (1.18 ± 0.05, n = 6, *P < 0.01*) compared to the WKY-SED group (1.00 ± 0.00, n = 6). Exercise during pregnancy significantly reversed the elevation of α1C mRNA in the 6 M offspring of the SHR-EX group (1.03 ± 0.03, n = 6, *P < 0.01* vs. SHR-SED) (Fig. 5C).

3.6. The role of DNA methylation on the downregulation of the expression of the $Ca_V1.2$ channel α1C subunit

Bisulfite sequencing was used to study the DNA methylation of the *Cacna1c* promoter. The CpG island is located near the *Cacna1c* promoter from 216,566,309 to 216,566,707. Its length is 399 bp, and it contains 9 CpG sites (The second CpG site is a SNP and is not counted in the statistics.). As shown in Fig. 6, there was no significant difference in the DNA methylation status of the SHR-SED group compared with the 6 M offspring of the WKY-SED group (n = 6, P > 0.05), but the exercise during pregnancy was significantly upregulated (n = 6, P < 0.05 vs. SHR-SED) DNA methylation status of the *Cacna1c* gene promoter in 6 M offspring of hypertensive rats.

4. Discussion

The new findings are as follows: (1) exercise during pregnancy improves the cardiovascular response of adult male offspring of SHR to NE, Bay K8644, and nifedipine. (2) Exercise during pregnancy reduced the current density and the expression of the $Ca_V1.2$ channel protein and mRNA in the VSMCs of hypertensive adult male offspring. (3) Exercise during pregnancy significantly upregulated the DNA methylation of *Cacna1c* gene in the MAs of hypertensive adult male offspring. These novel results suggest that downregulation of $Ca_V1.2$ channels induced by exercise during pregnancy may be an important cause of the cardiovascular response improvement in adult male offspring of SHR, and DNA methylation may be one of the important mechanisms of this.

Hypertension during pregnancy causes maternal vascular dysfunction, reduces placental volume and leads to decreased placental perfusion. These injuries cause fetal undernutrition, resulting in lower birth weight than normal. Low birth weight is associated with an

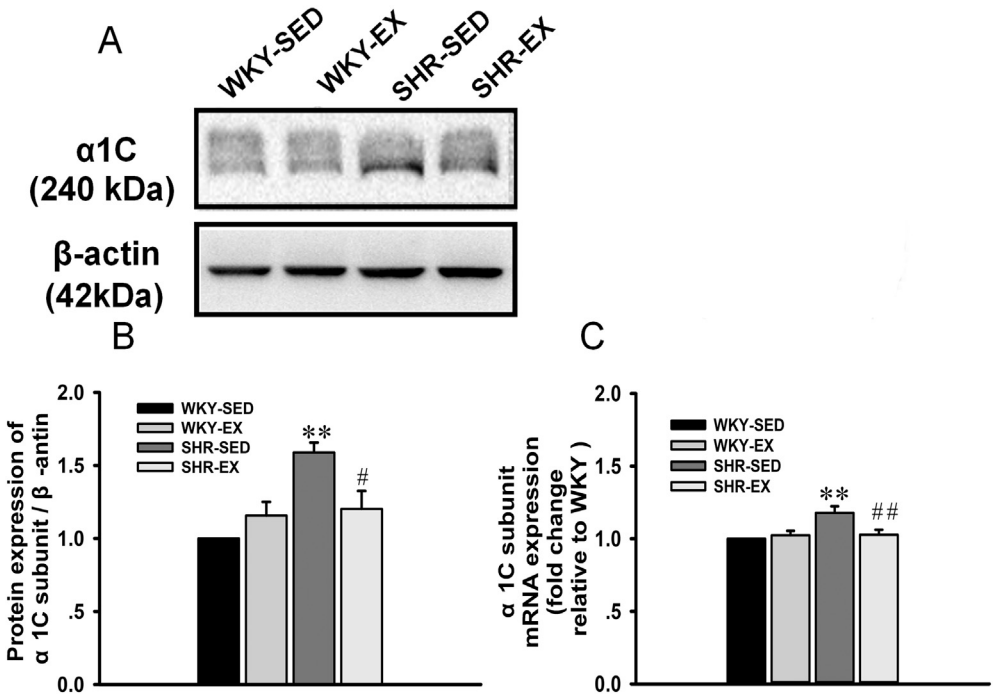

Fig. 5. The protein and mRNA expression of the $Ca_V1.2$ channel α1C subunit in MAs. A, Immunoreactive bands corresponding to the $Ca_V1.2$ channel α1C subunit and β-actin. B, Summarized data of α1C subunit protein levels in the MAs expressed as a ratio to β-actin. C, Summarized PCR data for the $Ca_V1.2$ channel α1C mRNA in the MAs normalized to β-actin. The results are expressed as the mean ± SEM (n = 6). $^{**}P < 0.01$ vs. WKY-SED; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. SHR-SED.

increased risk of cardiovascular disease in adulthood [30]. This study found that exercise during pregnancy can improve the slow weight gain of hypertensive rats in late pregnancy. The weight of newborns in SHR-EX group tends to increase. This may be beneficial for the cardiovascular function of the offspring.

Most previous studies have focused on the health outcomes of cardiovascular function in offspring that are associated with adverse uterine environments during pregnancy [31–34]. However, rare studies have explored whether healthy behaviors during pregnancy improve the cardiovascular function of the offspring [35]. Studies have shown that exercise during pregnancy can reduce the blood pressure of spontaneously hypertensive pregnant rats, improve the function of the placenta, and promote the formation of fetal neovascularization [36,37]. In this study, although the effect of exercise during pregnancy was not significant on blood pressure in the offspring of hypertension, the downward trends of SBP, DBP and MAP were observed. In addition, the study found that exercise during pregnancy reduced the blood pressure response of hypertensive offspring to the sympathetic hormones. Studies have reported [25] that there is no significant change in the resting blood pressure of offspring in hypoxic during pregnancy, and the offspring showed a significant increase in blood pressure after giving a high-salt diet from 4 to 12 week. There are also research reports [24,38] that prenatal nicotine or caffeine exposure can increase the response of the offspring to vasoconstrictor drugs. This suggests that although the effects of different interventions during pregnancy on the blood pressure of the offspring are not significant, the potential effects already exist.

In order to find potential mechanisms for these effects, we conducted a series of experiments. Given the potential link between hypertension and vascular tone, a possible mechanism which can explain the effect of exercise in pregnancy on the cardiovascular function of hypertensive offspring is an improvement in abnormal peripheral vascular resistance. Our study found that exercise during pregnancy significantly reduced NE-induced vasoconstriction of the resistance vessels in offspring of hypertension. L-NAME inhibits the effects of eNOS, highlighting the improvement of exercise during pregnancy on vascular smooth muscle cell function.

The programming effect of the intervention during pregnancy on the cardiovascular function of the offspring is achieved to some extent by affecting the ion channel. Previous studies reported that fetal exposure to caffeine or hypoxia altered vascular functions by the downregulation of the $Ca_V1.2$, K_V, and BK_{Ca} channels in adult offspring [24,25]. A hallmark feature of hypertension is the exaggerated contraction of small arteries and arterioles mediated by Ca^{2+} influx through the $Ca_V1.2$ channel [39,40]. Moosmang et al. [41] found that the blood pressure of the smooth muscle $Ca_V1.2$ gene-specific inactivated mice were reduced by 30–40 mmHg. This suggested that the $Ca_V1.2$ channel is a key factor in regulating blood pressure and vascular myogenic tension. Therefore, $Ca_V1.2$ channel dysfunction may lead to abnormal myogenic tension and blood pressure changes. We confirmed that exercise in pregnancy indeed reduced the role of $Ca_V1.2$ channels in adult offspring of SHR through intravenous injection of Bay K8644 and nifedipine. The pathogenesis of essential hypertension is very complicated, involving endocrine factors, neuromodulation, vascular abnormalities and other factors. The function of $Ca_V1.2$ channels is downregulated. However, there may be damage caused by other regulatory factors. For example, there may be damage to the diastolic function of the vascular endothelium. This may not be significantly improved, or other compensatory mechanisms may be generated, resulting an insignificant change in the blood pressure in the offspring of SHR-EX group. In addition, the downregulation of $Ca_V1.2$ channels may not reach a certain threshold, so that it reduced the SBP, DBP and MAP only to a certain extent and did not achieve the level of significant changes in basic blood pressure.

Microvascular tension test shows that as a benign stimulation of the uterine environment, exercise during pregnancy acts on the peripheral vasculature of the offspring, reduces the role of the $Ca_V1.2$ channel in NE-induced vascular contraction, and improves the function of the $Ca_V1.2$ channel in VSMCs of the MAs. Whole-cell patch-clamp experiments further confirmed that exercise during pregnancy reduced $Ca_V1.2$ channel current of MA VSMCs in hypertensive adult offspring. An increase in $Ca_V1.2$ channel current has been detected early in the development of hypertension and does not seem to correspond to an altered voltage sensitivity of the channel [42,43]. In contrast, single channel recording has detected an increased number of $Ca_V1.2$ channel openings in SHR, while single channel conductance or open time distribution did not change [44]. All of these findings are consistent with the hypothesis that an increase in the number of functional $Ca_V1.2$

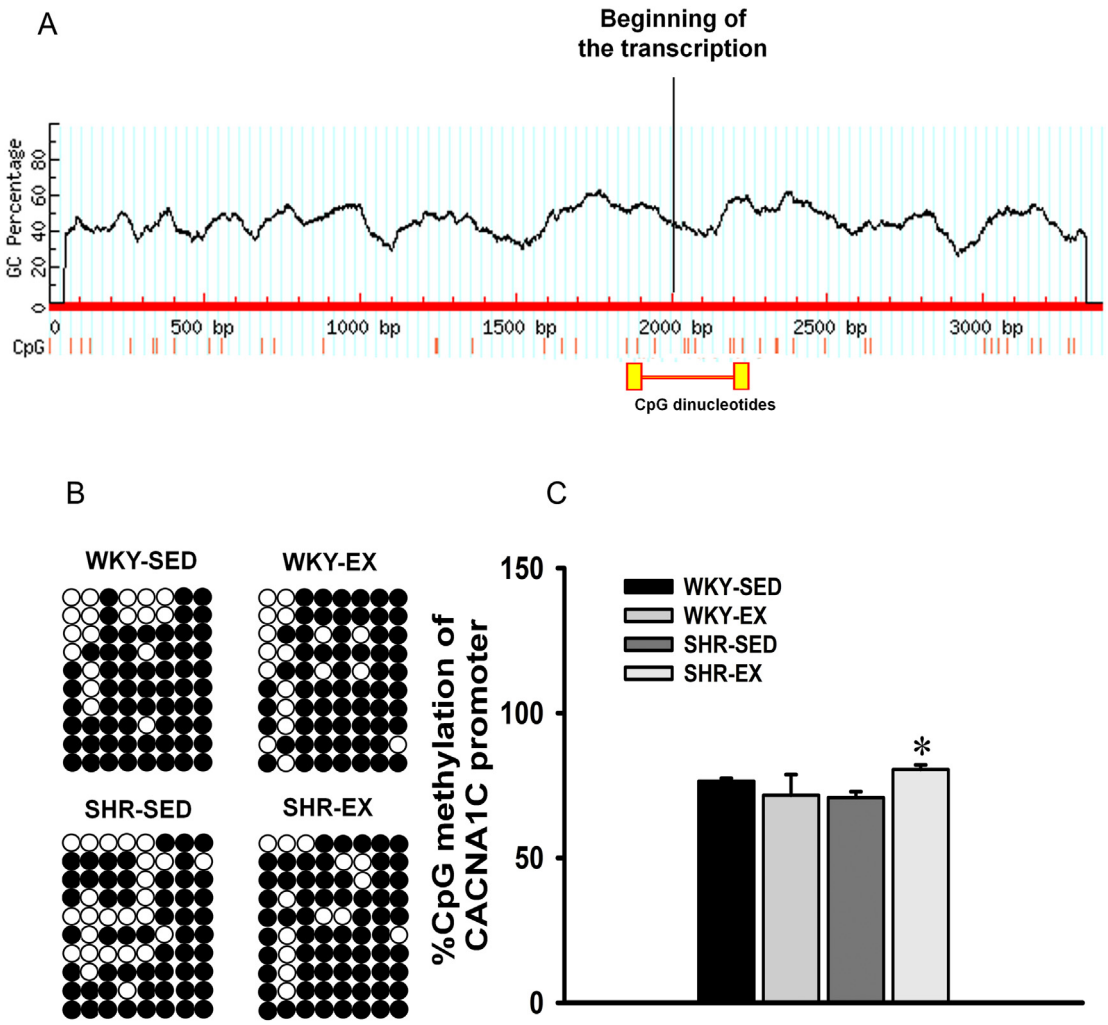

Fig. 6. The DNA methylation of *Cacna1c* promoter in MAs. A, The CpG island in the promoter region of the *Cacna1c* gene. B, Representative DNA methylation level of the CpG island in the promoter region of the *Cacna1c* gene; the black and white circles indicate methylated and unmethylated cytosines, respectively. A total of 8 CpG sites in the CpG island and 10 clones were subjected to sequencing. C, Methylation percentages of the 8 CpG sites in the *Cacna1c* promoter. The results are expressed as the mean ± SEM (n = 6). $^{*}P < 0.05$ vs. SHR-SED.

channel proteins, rather than altered channel characteristics, may be responsible for the increase in the $Ca_V1.2$ channel current in VSMCs of SHR.

The $Ca_V1.2$ channel has a pore-forming protein, α1C, and three other auxiliary regulatory subunits (β, α2δ, and γ) [45,46]. Gene knockout experiments were initially performed to understand the effects of α1C and β on the regulation of vascular tone and blood pressure. Depolarization-induced vasoconstriction and myogenic tension in smooth muscle-specific α1C gene knockout mice were abolished, blood pressure was significantly reduced, and conventional knockout of the β subunit showed no significant changes in blood pressure. There is no doubt that the α1C subunit plays a crucial role in regulating vascular reactivity [47]. Western blot analysis confirmed that the inhibitory effect of exercise in pregnancy on the function of the $Ca_V1.2$ channel in hypertensive rats is mainly achieved by downregulating the protein expression of the $Ca_V1.2$ channel α1C. Decreased the protein expression of α1C subunit can cause downregulation of $Ca_V1.2$ channel function, which may be one of the key factors leading to the improvement of vascular function.

Numerous studies have confirmed that the health of the offspring can be programmed by epigenetics [48–50]. Studies have found that exercise during pregnancy can reduce the blood pressure [51], improve the placental function [52], and affect the cardiovascular development of the offspring in hypertensive mother [53]. It is not clear whether this process is regulated by epigenetics. However, studies have found that long-term exercise intervention can alter the DNA methylation of various tissues, including skeletal muscle and adipose tissue [54,55]. Therefore, we envisage that exercise during pregnancy can regulate the $Ca_V1.2$ channel function of offspring through epigenetics. As an important component of epigenetics, DNA methylation plays a direct role in regulating transcription levels. Therefore, we first verified whether the protein expression of the $Ca_V1.2$ channel α1C is regulated by transcriptional levels. The results showed that exercise during pregnancy significantly downregulated the mRNA expression of the $Ca_V1.2$ channel α1C subunit in adult offspring of hypertensive rats. This result reflected the observation that exercise during pregnancy regulated the expression of α1C subunits at the transcriptional level in the adult offspring of mothers with hypertension. The results of the DNA methylation assay also validated this inference. We performed a DNA bisulfite sequencing PCR test on the promoter region of the *Cacna1c* gene (about 399 bp). It was found that hypertension didn't caused a significant change of DNA methylation in the *Cacna1c* promoter region, but exercise during pregnancy significantly upregulated DNA methylation status in the *Cacna1c* promoter region. Therefore, genetic factors may be the key to up-regulation of the $Ca_V1.2$ channel α1C protein and mRNA expression in SHR, and DNA methylation may not play a significant role. Liao et al. also found that there was no significant difference in the DNA methylation level of the *Cacna1c* promoter region in

SHR rats compared with WKY rats [56]. However, Zhang et al. found that hypertension can significantly reduce the level of DNA methylation of BKβ1 gene (KCNMB1) [57]. Although hypertension does not affect the DNA methylation of *Cacna1c* promoter, exercise during pregnancy can improve the vascular function by up-regulating the DNA methylation status in the *Cacna1c* promoter region and down-regulating the $Ca_V1.2$ channel α1C protein expression.

5. Conclusion

This study provides evidence that exercise during pregnancy may reduce the mRNA and protein expression of the $Ca_V1.2$ channel α1C subunit by regulating the DNA methylation pattern in the offspring of hypertensive rats, thereby inhibiting the upregulation of $Ca_V1.2$ channel function during hypertension. Exercise during pregnancy improves cardiovascular function in the adult offspring of SHR. Due to the particular circumstances of pregnancy, drug treatment can have certain side effects and limitations, and, therefore, a non-pharmacological treatment may be an important step forward.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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