

Altered K_{ATP} Channel Subunits Expression and Vascular Reactivity in Spontaneously Hypertensive Rats With Age

Xiaojing Liu, MS,* Peng Duan, MS,* Xingxing Hu, MS,* Ruisheng Li, PhD,† and Qinglei Zhu, PhD*

Abstract: ATP-sensitive potassium (K_{ATP}) channels link membrane excitability to metabolic state to regulate a series of biological activities including the vascular tone. However, their ability to influence hypertension is controversial. Here we aim to investigate possible alteration of K_{ATP} channel in vascular smooth muscles (VSMs) during hypertension development process. In this study, we used 16-week-old spontaneously hypertensive rats (SHRs), 49-week-old SHRs, and their age-matched Wistar-Kyoto rats to study the expression of VSM K_{ATP} subunits at the mRNA and protein level and the function of VSM K_{ATP} by observing the relaxation reactivity of isolated aorta rings to K_{ATP} modulators. We found that the expression of VSM K_{ATP} subunits Kir6.1 and sulfonylurea receptor (SUR2B) decreased during hypertension. Moreover, the expression of SUR2B and Kir6.1 in 49-week-old SHRs decreased much more than that in 16-week-old SHRs. Furthermore, the aorta rings of 49-week-old SHRs showed lower reactivity to diazoxide than 16-week-old SHRs. This study suggests that K_{ATP} channels in VSM subunits Kir6.1 and SUR2B contribute to modify the functionality of this channel in hypertension with age.

Key Words: K_{ATP} channel, hypertension, mito K_{ATP} , diazoxide, in vitro

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INTRODUCTION

Hypertension, a worldwide cardiovascular disease affecting more than 30% of the population, is defined as a complex, multifactorial, environmental, quantitative trait under a polygenic control.¹ It is known that the contraction and relaxation of vascular smooth muscles (VSMs) are related to membrane potential, which is critically determined by the activity of potassium channel.² ATP-sensitive potassium (K_{ATP}) channels exist in VSM beds and play an important

role in regulating vascular tone.^{3,4} In addition, the channel openers result in the decrease of blood pressure.^{5,6} Moreover, the channel blockers abolish the pharmacological function of channel openers to lower blood pressure.^{7,8} The K_{ATP} channel is composed of a hetero-octameric complex constituting an inward rectifier potassium channel (Kir6.1 and Kir6.2) and a sulfonylurea receptor (SUR1, SUR2A, and SUR2B), which is characterized by the inhibition of ATP and the activation of MgADP.⁹ The Kir6.x pore-forming subunit responds to the ATP sensitivity, and the SURx confers to sulfonylurea sensitivity and determines the efficacy of potassium channel openers (KCOs).¹⁰ K_{ATP} in different tissues is composed of different Kir and SUR subunits that “mix and match.” Sarcolemmal K_{ATP} (sarco K_{ATP}) channel of VSM are mainly constituted of Kir6.1/SUR2B,¹¹ which are activated by nucleoside diphosphates and are rather insensitive to ATP.

K_{ATP} channels act as important metabolic sensors and target the response of VSM to a number of pharmacological and endogenous vasodilators: adenosine, prostacyclin, α -adrenoceptor agonist, nitric oxide, neurotransmitters, calcitonin-gene-related peptide, vasoactive intestinal peptide, angiotensin II, vasopressin, noradrenaline, neuropeptide Y, endothelin, and serotonin.^{12–14} Therefore, they probably participate in the process of hypertension.

In this study, we used 16-week-old spontaneously hypertensive rats (SHRs), 49-week-old SHRs, and their age-matched Wistar-Kyoto rats (WKY) to study the expression of VSM K_{ATP} subunits at the mRNA and protein level and the function of VSM K_{ATP} by observing isolated aorta rings' relaxation reactivity to K_{ATP} modulators. We aim to clarify the alteration of the expression and the function of K_{ATP} channels in hypertension at different age.

MATERIALS AND METHODS

Animals

All experimental animals were purchased from Vital River Laboratories (Beijing, China). Male WKY and SHRs were randomly divided into 4 groups: 16-week-old WKY, 16-week-old SHRs, 49-week-old WKY, and 49-week-old SHRs. Conscious rats were instrumented with tail-cuff system (Kent Scientific) to record the blood pressure. Systolic blood pressure, diastolic blood pressure, and mean blood pressure were measured with the rat tails at 31°C as previously described.¹⁵ The investigation conforms to the Guidelines for the Care and Use of Laboratory Animals, and the procedures for care and use of animals were

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From the *Department of Cardiology, Chinese PLA General Hospital, Beijing, China; and †Research and Technology Service Center, 302 Hospital of PLA, Beijing, China.

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Reprints: Qinglei Zhu, PhD, Department of Cardiology, Chinese PLA General Hospital, 28 Fuxing Rd, Beijing 100853, China (e-mail: qlzhu@yahoo.com).

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approved by the Ethics Committee of Chinese PLA General Hospital.

Vessel Contractility and Reactivity Measurement

Male rats were intraperitoneally anesthetized with 1% pelltobarbitalum natricum (Solarbo, China) 1 mL/100 g; the aorta was rapidly dissected out, placed in Krebs–Henseleit solution (KHS, in mmol/L: NaCl 115, CaCl₂ 2.5, KCl 4.6, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, NaHCO₃ 25, glucose 11.1, with pH of 7.4) at 4°C, and cleaned off the connective tissue and blood vessel endothelium. The isolated arteries were cut 3–4 mm in length and passed through the lumen of the vessel segment by 2 parallel steel triangles: one was fixed to the organ bath and the other connected to a tension transducer (TSD125B; Biopac), which was linked to AcqKnowledge software (MP150; Biopac). Vessel segments were bathed in 15 mL of KHS continuously saturated with a 95% O₂–5% CO₂ mixture at 37°C. The aortic rings were equilibrated for 90 minutes with 2.0 g of basal tension. Arterial viability was measured using KCl solution (final concentration of 60 mmol/L, the same as below), and arterial maximum contractility was determined using phenylephrine (PE, 1 μmol/L). Endothelial denudation was confirmed by acetylcholine (Ach, 10 μmol/L).¹⁶ Diazoxide (10⁻⁹ to 10⁻⁵ mol/L) or pinacidil (10⁻⁹ to 10⁻⁵ mol/L) was added to the aorta ring in the plateau phase of PE (1 μmol/L) to describe the concentration–response curves. To study the participation of the specific K_{ATP} channel on vasodilator response, the specific K_{ATP} channel blocker glibenclamide (Gli) or the mitoK_{ATP} channel blocker 5-hydroxydecanoate (5-HD) was added 30 minutes before the concentration–response curves were obtained. Gli (10 μmol/L) was used to block the vasodilator response of pinacidil, and 5-HD (10 μmol/L) was used to block the vasodilator response of diazoxide. All the changes in tension were recorded, and relaxation was expressed as a percent of the precontraction.

Quantitative Relative Real-Time Polymerase Chain Reaction

Total RNA was extracted from aorta arteries with TRIzol agent (Invitrogen, CA), and the concentration was determined by Nano Drop 2000 (Thermo Scientific). Reverse transcription–polymerase chain reaction (PCR) were performed using iScript cDNA Synthesis Kit (Bio-Rad), and quantitative relative real-time PCR was performed with the Power SYBR Green PCR Master Mix Kit (ABI) on ABI prism 7900 instrument, according to the manufacturer's instructions. Primers were based on published researches: (sequence 5' to 3') F: CAGAGGTGCTGGACATCACAGAG, R: GGCAACA GTCGGGTAGCCAATC for 36β4; F: GGAGTGCATAC TGGTCCAAACCT, R: CCCGATGCAGAGAACGAGACA CT for SUR2A; F: CATAGCTCATCGGGTTACACCA TT, R: GCATCGAGACACAGGTGCTGTTGT for SUR2B; F: AGCTGGCTGCTCTTCGCTATCA, R: CCCTCCAAA CCAATGGTCACT for Kir6.1; and F: CAACGTCGCCCA CAAGAACATC, R: CCAGCTGCACAGGAAGGACATG for Kir6.2. Quantitative relative real-time PCR consisted of 2

steps: hold at 95°C for 10 minutes; 40 cycles at 95°C for 15 seconds and at 60°C for 60 seconds. Relative expression was normalized to 36β4.

Western Blot

Total proteins were isolated from aorta tissues with RIPA buffer (Solarbo), and the concentration was measured by BCA protein assay kit (Solarbo). Protein samples (60 μg each well) were loaded on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose filter membranes. The membranes was blocked in 5% nonfat milk (BD) for 2 hours, incubated overnight at 4°C in primary antibodies, and then incubated with appropriate secondary antibodies at room temperature for 50 minutes. The primary antibody dilutions were 1:300 for Kir6.1 (ABcam, Britain), 1:500 for Kir6.2 (Alomone, Israel), 1:300 for SUR2B (Santa, Japan), and 1:30,000 for GAPDH (ABcam) antibodies. Signals were detected with the electrochemiluminescence Plus (Applygen, China). All experiments were measured in triplicate.

Statistical Analysis

Statistical analysis was performed with SPSS 17.0 and Graph Pad Prism 5.0 software. All data showed in figures were expressed as mean ± standard error of the mean. The 2 groups' unpaired data were analyzed with Student's *t*-tests. Differences in means among groups and treatments were compared using repeated-measure analysis of variance, when appropriate. Differences with 2-tailed *P* values <0.05 were considered to be statistically significant.

RESULTS

Body Weight and Blood Pressure

Figure 1 shows the mean body weight and blood pressure of all groups. There was no difference in body weight between the 16-week-old SHR (314.2 ± 4.36 g) and 16-week-old WKY (307.6 ± 2.41 g) (*P* > 0.05) and also for the 49-week-old SHR (384.3 ± 10.69 g) versus 49-week-old WKY (358.5 ± 6.74 g) (*P* > 0.05) (Fig. 1A).

Blood pressure was increased in SHR groups when compared with the respective controls (*P* < 0.01). However, no difference in blood pressure level was observed between the 16-week-old (198.2 ± 3.86/152.3 ± 6.02 mm Hg) and 49-week-old SHR (184.8 ± 5.89/136.0 ± 4.08 mm Hg) (Figs. 1B, C).

mRNA Expression of Kir6.1 and SUR2B Is Reduced in Aorta SM From SHR

In aorta smooth muscle, the mRNA expression of Kir6.1 subunits was decreased by 35% in 16-week-old SHR (0.65 ± 0.11) when compared with 16-week-old controls (1.00 ± 0.16, *P* < 0.05). Moreover, it was decreased to an even lower extent of 38% in 49-week-old SHR (0.49 ± 0.11) compared with 49-week-old controls (0.79 ± 0.08, *P* < 0.05). Furthermore, compared with 16-week-old SHR, there was a reduction of about 25% in 49-week-old SHR. Similarly, VSM mRNA expression of K_{ATP} SUR2B subunits

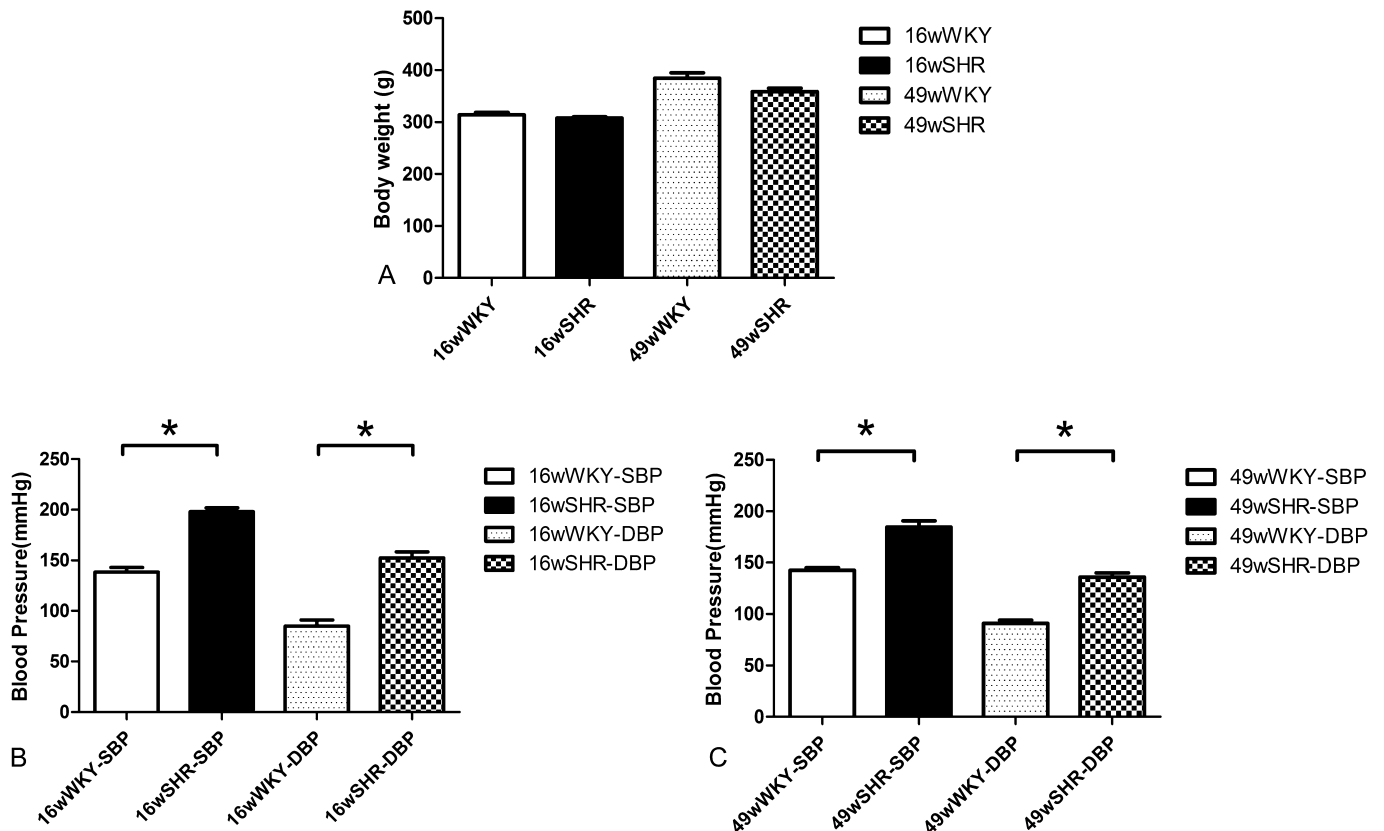


FIGURE 1. Body weight and blood pressure of rats. A, Body weight, (B) systolic blood pressure (SBP), and (C) diastolic blood pressure (DBP). Data shown are the means \pm standard error of the mean of 6 separate experiments. * $P < 0.05$ for the SHR group compared with the respective WKY controls.

showed a 26% reduction in 16-week-old SHRs (0.74 ± 0.06) when compared with 16-week-old controls (1.00 ± 0.11 , $P < 0.05$), and a further reduction of 61% in 49-week-old SHRs (0.36 ± 0.14) compared with 49-week-old controls (0.92 ± 0.18 , $P < 0.05$). Moreover, SUR2B subunits were decreased by 51% in 49-week-old SHRs when compared with 16-week-old SHRs ($P < 0.05$). However, there were no differences in the Kir6.2 and SUR2A of the mRNA expression between 16-week-old and 49-week-old SHRs when compared with their respective controls (Figs. 2A–D).

Protein Expression of Kir6.1 and SUR2B Is Reduced in Aorta SM From SHRs

To examine whether the K_{ATP} expression is altered in the SHRs, we used Western blotting to measure the protein expression. Consistent with the mRNA data above, in both 16-week-old SHRs and 49-week-old SHRs, the protein expression of Kir6.1 and SUR2B was decreased than the respective controls. For Kir6.1, there was an almost 22% reduction in 16-week-old SHRs (0.78 ± 0.02 , $P < 0.05$) and about 54% reduction in 49-week-old SHRs (0.45 ± 0.05 , $P < 0.01$). For SUR2B, 20% reduction in 16-week-old SHRs (0.80 ± 0.02) and 34% reduction in 49-week-old SHRs (0.66 ± 0.06 , $P < 0.01$) was observed. Moreover, there was a significant difference in Kir6.1 and SUR2B between

16-week-old and 49-week-old SHRs. Compared with 16-week-old SHRs, Kir6.1 decreased 44% and SUR2B reduced 25% in 49-week-old SHRs ($P < 0.01$) (Figs. 3A–C).

Vascular Reactivity to Diazoxide Is Reduced in Aorta SHRs

To evaluate K_{ATP} channel functional condition, tissue bath myography was determined in this study. Both diazoxide and pinacidil evoked a concentration-dependent relaxation response in PE-precontracted aortic segments isolated from either WKY or SHR groups. The relaxation response of diazoxide was apparently lower than that of pinacidil. No difference was observed in 16-week-old SHR aorta response to diazoxide compared with 16-week-old controls. However, diazoxide caused a marked lower relaxation in 49-week-old SHRs compared with 49-week-old WKY at concentrations of 10^{-6} mol/L (12.06 ± 0.61 vs. 20.87 ± 2.92 , $P < 0.05$) and 10^{-5} mol/L (16.82 ± 0.26 vs. 48.72 ± 4.31 , $P < 0.001$). The area under curve (AUC) of diazoxide response of 49-week-old SHRs decreased when compared with 49-week-old WKY ($P < 0.05$). Furthermore, the AUC of diazoxide response of 49-week-old SHRs also showed a greater decrease than 16-week-old SHRs ($P < 0.05$). MitoK_{ATP} channel inhibitor 5-HD blocked diazoxide-induced relaxation to a similar extent in SHRs and normotensive rats. Thus, the delta area under

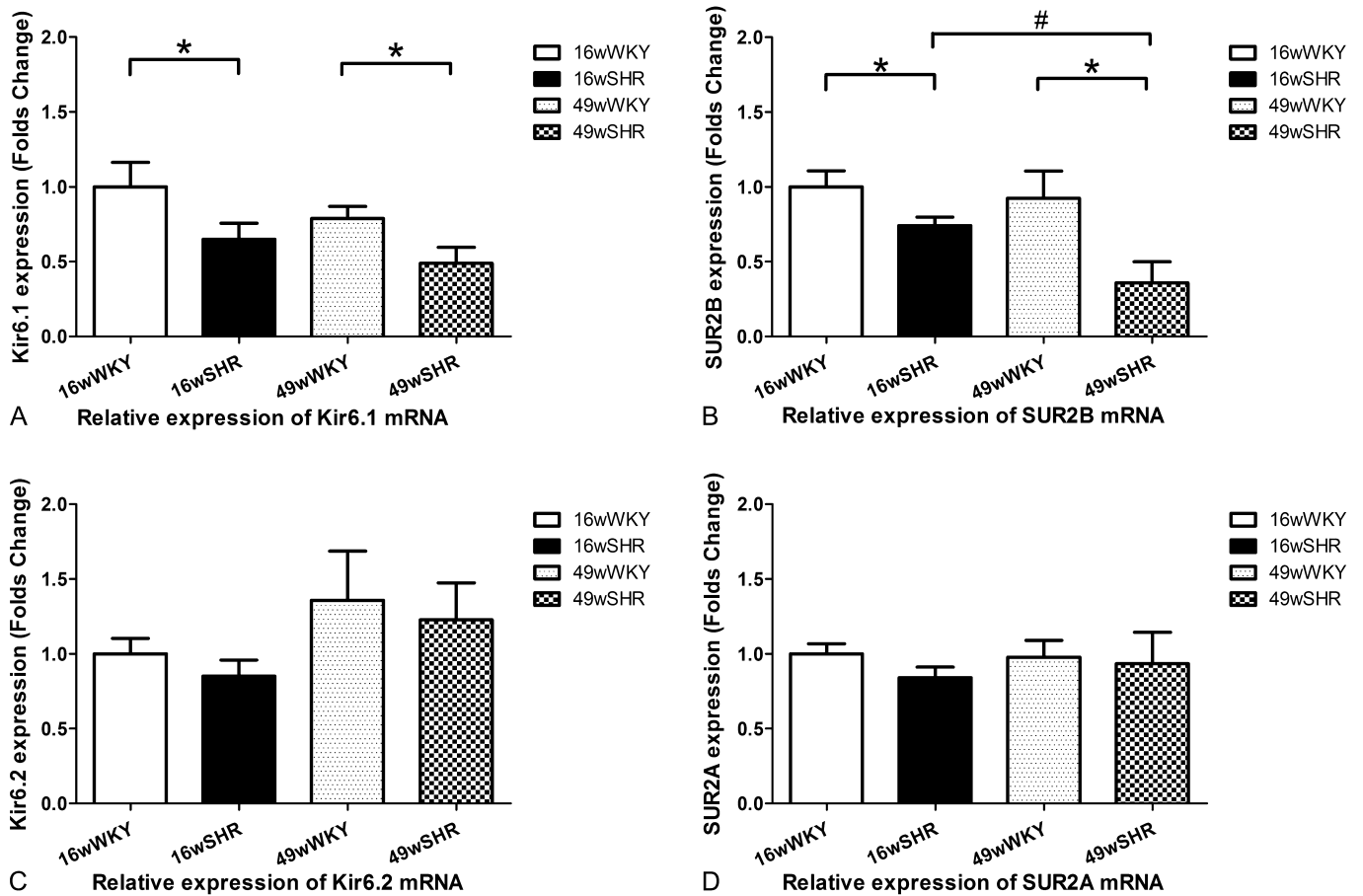


FIGURE 2. Relative expression of Kir6.1, Kir6.2, SUR2A, and SUR2B mRNA. A, Kir6.1, (B) SUR2B, (C) Kir6.2, and (D) SUR2A. Data shown are the means \pm standard error of the mean of 6 separate experiments. * $P < 0.05$ for the SHR group compared with the respective WKY controls. # $P < 0.05$ for the 49-week-old SHR group compared with the 16-week-old SHR group.

curve (dAUC) of 5-HD ranged from 13.14% to 16.50%, and there was no statistical differences in both strains (Figs. 4E, G).

Pinacidil elicited similar concentration-dependent relaxation curves in denuded aorta segments from SHRs and normotensive rats (both 16-week-old and 49-week-old SHRs).

The AUC of pinacidil showed no difference in both strains. Furthermore, the pinacidil-induced relaxation was abolished by the K_{ATP} blocker Gli (10 $\mu\text{mol/L}$) to a similar extent. Thus, the dAUC of Gli kept unchanged in both strains about 86.65%–98.11% (Figs. 4F, H).

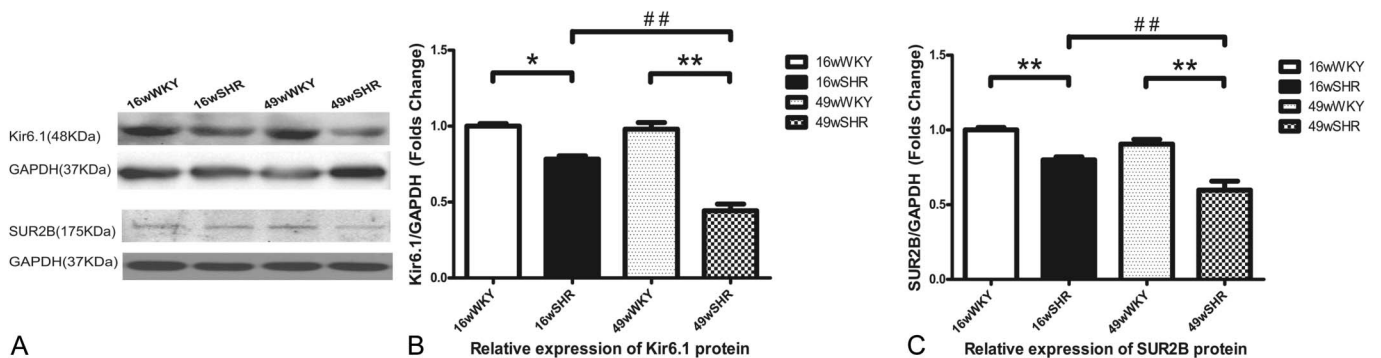


FIGURE 3. Representative Western blots for Kir6.1 and SUR2B expression. A, Western blot of Kir6.1, SUR2B, and GAPDH. B, Analysis of relative gray intensity of Kir6.1. C, Analysis of relative gray intensity of SUR2B. Results are expressed as the ratio between the signal for the Kir6.1/SUR2B expression and the signal for GAPDH. Data shown are the means \pm standard error of the mean of 3 separate experiments. ** $P < 0.01$ for the SHR group compared with the respective WKY controls. ## $P < 0.01$ for the 49-week-old SHR group compared with the 16-week-old SHR group.

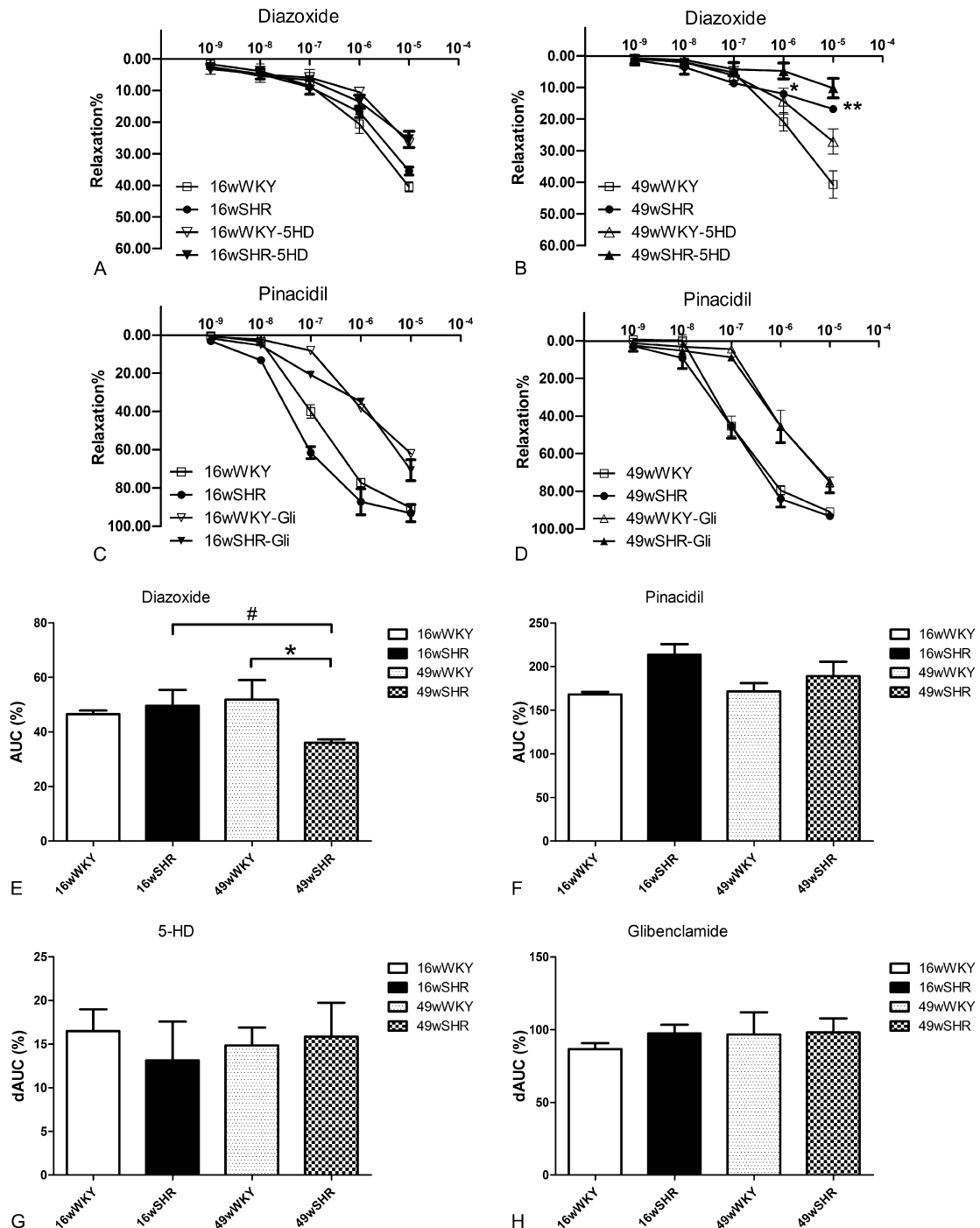


FIGURE 4. Vascular reactivity to K_{ATP} channel openers and blocker. Aorta rings were saturated in KHS and diazoxide or pinacidil were accumulatively added (10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ mol/L). A, Concentration–relaxation curves of diazoxide in the presence or absence of 5-HD in the 16-week-old WKY and 16-week-old SHR group. B, Concentration–relaxation curves of diazoxide in the presence or absence of 5-HD in 49-week-old WKY and 49-week-old SHR group. C, Concentration–relaxation curves of pinacidil in the presence or absence of Gli in 16-week-old WKY and 16-week-old SHR group. D, Concentration–relaxation curves of pinacidil in the presence or absence of Gli in 49-week-old WKY and 49-week-old SHR group. E, AUC for diazoxide. F, AUC for pinacidil. G, dAUC is expressed as the difference between AUC for diazoxide and corresponding AUC for aortic segments in the presence of 5-HD; H, dAUC is expressed as the difference between AUC to pinacidil and the corresponding AUC for aortic segments in the presence of Gli. Results are expressed as percent of previous contraction with PE. Data shown are the means ± standard error of the mean of 4–8 separate experiments. *P < 0.05, **P < 0.01 for the SHR group compared with the respective WKY controls; #P < 0.05 for the 49-week-old SHR group compared with the 16-week-old SHR group.

DISCUSSION

In this study, we present the following findings: (1) At the mRNA and protein level, expression of Kir6.1 and SUR2B is reduced in the aorta from 16-week-old and 49-week-old SHR. (2) Moreover, the expression of Kir6.1 and SUR2B in 49-week-old SHR decreases much more than that in 16-week-old SHR. (3) The vasodilator response to diazoxide decreases in 49-week-old SHR, although this trend of decline is not observed in 16-week-old SHR. (4) The vasodilator response to pinacidil remains unchanged, and its inhibition by Gli is also similar in both strains of WKY and SHR.

K_{ATP} channel is a tetrameric complex of Kir and SUR. In rat aorta, K_{ATP} channel has been demonstrated to be Kir6.1 and SUR2B.^{11,17} In this study, we found that at the mRNA and protein level, expression of Kir6.1 and SUR2B is reduced in the aorta for 16-week-old and 49-week-old SHR, which is consistent with previous studies.^{18,19} However, in this study, we found that the protein expression of Kir6.1 and SUR2B in 49-week-old SHR showed a much larger decrease than that in 16-week-old SHR, although, the blood pressure remained unchanged between 49-week-old and 16-week-old SHR. This is the first time we reported that the expression of Kir6.1 and SUR2B was reduced with age in SHR. Although this different expression of VMS K_{ATP} channel subunits did not directly relate to the regulating of blood pressure, it probably affected other functions, such as the channel subunits affinity and the channel gating-dynamic, etc.

K_{ATP} channels act as targets of many vasoactive molecules and are widely distributed in various tissues and cells with different subunits and participate in both physiological and pathophysiological conditions including insulin resistance, hyperglycemia, hypoglycemia, hypoxia, and ischemia.²⁰ Activation of K_{ATP} channel in VSM leads to a diminution of intracellular Ca^{2+} levels and an increase in vascular diameter, and thus lowers the vascular resistance.³ In this study, we evaluated the vasorelaxant response to the KCOs pinacidil and diazoxide in thoracic aorta from 16-week-old and 49-week-old SHR. We found that pinacidil elicited similar concentration-dependent relaxation curve in both SHR and WKY, and the relaxation was abolished by the K_{ATP} blocker Gli to a similar extent. However, the relaxant response to diazoxide was evidently declined in 49-week-old SHR. 5-HD abolished the diazoxide-induced relaxation to a similar extent. These results indicate that the response to pinacidil, Gli, and 5-HD seems to be preserved, whereas the response to diazoxide diminishes in 49-week-old SHR.

SUR2B subunit is mainly responsible for channel affinity with K_{ATP} channel openers and blockers.^{21,22} The SUR2B possesses 3 transmembrane domains (TMD0, TMD1, and TMD2), 2 cytoplasmic nucleotide binding domains (NBD1 and NBD2), and the Walker A, Walker B, and Linker L consensus sequences.²³ It functions as a regulatory subunit to mediate gating of the Kir6.1 pore by sulfonylurea drugs, such as Gli. Uhde et al identified 2 regions, KCOI and KCOII, in rat SUR2B. Region KCOI was located from Thr1059 to Leu1087 and region KCOII was located from Arg1218 to Asn1320.²⁴ The simultaneous presence of 2

regions was necessary for high-affinity binding of SUR2B, suggesting that the opener site could be made up of association of both regions. The downregulation of SUR2B expression in hypertension could directly affect the channel open state, gating regulation, and the affinity with modulators.

KCOs exhibit an extreme chemical diversity and comprise a number of different structural classes such as the benzopyrans (cromakalim), cyanoguanidines (pinacidil), benzothiadiazines (diazoxide), and nicotinamides (nicorandil).^{25,26} Diazoxide and pinacidil have been recognized as the first-generation K_{ATP} channel modulators, and both can act on Kir6.1/SUR2B. Basically, diazoxide activates mito K_{ATP} channel, and pinacidil nonselectively activates both mito K_{ATP} and sarc K_{ATP} channels. The potency of pinacidil is much stronger than diazoxide. Diazoxide activates the channel in a concentration-dependent manner with a half-maximal effective concentration of 30–60 μmol , but pinacidil has a half-maximal effective concentration of 2 μmol .²⁷ Notably, diazoxide possesses a high affinity with SUR2B in the cardiovascular system, SUR2B (dissociation constant, $K_d = 18 \mu\text{mol}$) > SUR2A ($K_d = 76 \mu\text{mol}$).²⁸ Researchers have also found that the transmembrane helix 16 and 17 in TMD2 and the section of the cytosolic loop linking helix 13 and 14 of SUR2B are required for the action of pinacidil, levocromakalim, and P1075, rather than diazoxide.^{24,29} Furthermore, the present regions such as TMD1, TMD2, and NBD1 cannot totally explain the binding site of diazoxide. Although the detailed binding mechanism remains to be studied, it is clear that diazoxide has a binding site distinct from all other openers.^{23,30}

Taking the above discussion into account, the reason that the response to diazoxide diminished whereas the response to pinacidil was preserved may be explained as follows. First, diazoxide shows higher potency and efficacy to open mito K_{ATP} than pinacidil.^{31–33} The expression of Kir6.1, which is also a subunit of mito K_{ATP} in 49-week-old SHR decreases much more than that in 16-week-old SHR; hence, the response to diazoxide diminished in 49-week-old SHR. Second, both diazoxide and pinacidil are able to bind to SUR2B and activate K_{ATP} channel. Pinacidil is reported to bind to TMD2 domains of SUR2B. Different from pinacidil, diazoxide is potent and has a more complex binding site in which SUR2B C-terminal domains are involved.³⁴ Furthermore, diazoxide has a 4-fold higher affinity for SUR2B than SUR2A.^{35,36} Therefore, the much more decreased expression of SUR2B in 49-week-old SHR may be another reason. Third, opening sarc K_{ATP} channel plays a much more important role in the vasodilation than mito K_{ATP} .^{37,38} Compared with diazoxide, pinacidil activates sarc K_{ATP} channel in addition. The function of mito K_{ATP} channel is mainly based on the modulation of mitochondrial function, including changes in mitochondrial matrix volume, mitochondrial potential, and oxygen consumption.²⁷ In this study, we also found that pinacidil induced a stronger relaxation than diazoxide. Although the mito K_{ATP} decreased in 49-week-old SHR, the response of pinacidil to sarc K_{ATP} channel seemed to be preserved in 49 weeks.

In brief, this study found that (1) the expression of Kir6.1 and SUR2B reduced in SHR, and the decrease in

49-week-old SHR was much more than that in 16-week-old SHR. (2) The response to diazoxide diminished, whereas the response to pinacidil, G_i, and 5-HD seems to be preserved in 49-week-old SHR. Although the detailed mechanisms still need to be clarified, this study demonstrated that the decreased expression of the VSM K_{ATP} subunits Kir6.1 or SUR2B correlate with the functional changes of K_{ATP} channel in hypertension with age.

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