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**ARSENIC TRIOXIDE INHIBITS THE VOLTAGE-GATED K<sup>+</sup> CHANNELS IN RAT MESENTERIC ARTERIAL SMOOTH MUSCLE CELLS**

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**Objectives** Lots of epidemiological studies have shown that excessive arsenic ingestion is strongly correlated with the development of vascular diseases. However, the underlying mechanism remains unknown. The present study was therefore designed to investigate whether the voltage-gated K<sup>+</sup> channels (K<sub>v</sub> channels), important

mediators of vascular tone, are affected by arsenic trioxide in rat mesenteric artery smooth muscle cells (SMCs) and explore the electrophysiological mechanism of arsenic-related impaired channel function.

**Methods** Reagent Arsenic trioxide was purchased from Harbin YI-DA pharmaceutical limited company. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and other cell culture reagents were obtained from Gibco (Grand Island, NY, USA). Polyclonal Kv1.5 antibody was obtained from Santa Cruz (Santa Cruz Biotech, Santa Cruz, CA), and polyclonal Kv1.2 and polyclonal Kv2.1 were obtained from Alomone (Alomone Labs, Jerusalem, Israel). Other reagents were purchased from sigma (St. Louis, Mo. USA).

The acute isolation and culture of vascular smooth muscle cells. Male Wistar rats (200–250 g) were anaesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg bodyweight). Small mesenteric arteries below the second branch of the main mesenteric arteries were dissected out and placed in ice cold isolation buffer. The isolation buffer consisted of the following (in mmol/l): NaCl 136, KCl 5.4, HEPES 10, CaCl<sub>2</sub> 1.8, NaH<sub>2</sub>PO<sub>4</sub> 0.33, MgCl<sub>2</sub> 1, Glucose 1, with pH adjusted to 7.4 with NaOH. The small arteries were cut into 4 mm-long pieces and incubated at 37°C in isolation buffer containing 1 mg/ml albumin, 1 mg/ml papain, and 1 mg/ml dithioerythritol for 30 min, and for another 30 min in the isolation buffer containing 1 mg/ml albumin, 3 mg/ml collagenase II, and 1 mg/ml hyaluronidase, single cells were released through a Pasteur pipette. SMCs were cultured in low-glucose DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin, and maintained in 95% air and 5% CO<sub>2</sub> at 37°C. SMCs were passaged regularly and subcultured to 95% confluence before experiments. In order to observe the immediate effects of arsenic trioxide on Kv currents, the freshly isolated cells were treated with a variety of drug concentrations. The effects of arsenic trioxide on Kv currents were also observed in cultured cells treated with arsenic trioxide for 48 h. All experimental procedures were approved by Animal Ethics Committee of Harbin Medical University.

#### Electrophysiological recording

The whole cell Kv currents were recorded as described previously.<sup>20</sup> The SMCs on the coverslips were mounted on the stage of an inverted phase contrast microscope (Olympus IX70). The Voltage-gated K<sup>+</sup> currents were recorded with an Axopatch 200B amplifier controlled by a Digidata 1332 interface and a Pclamp Software (V9.2, Axon Instrument Inc). Pipettes were pulled from soft microhematocrit capillary tubes with tip resistances of 2–4 MΩ when filled with the pipette solution. At the beginning of each experiment, junctional potential between the pipette solution and bath solution was adjusted to zero. The series resistance was electrically compensated to at least 70% to minimize the duration of capacitive transient. Test pulses were evoked with 10mV steps increase from -50 mV to +60 mV. The 1 mmol/l TEA was also applied to the bath solutions to minimize the activity of Ca<sup>2+</sup>-activated K<sup>+</sup> channels. The holding potential was set at -60 mV at which Kv channels are not inactivated. I–V curves were constructed using the current amplitude measured between the 300–350 ms of the test pulse when the current amplitude become sustained. The bath solution was composed of (in mmol/l): 140 NaCl, 5.4 KCl, 1.2 MgCl<sub>2</sub>·6 H<sub>2</sub>O, 10 HEPES, 1 EGTA, 10 Glucose, pH adjusted to 7.3 with NaOH. The pipette solution contained (in mmol/l): 140 KCl, 1 MgCl<sub>2</sub>·6 H<sub>2</sub>O, 10 EGTA, 10 HEPES, 5 Glucose, 2 Na<sub>2</sub>-ATP, pH adjusted to 7.3 with KOH. All experiments were conducted at room temperature (20–25°C).

#### Western Blot

The expression levels of Kv1.2, Kv1.5 and Kv2.1 protein were monitored using Western blot experiments. The vascular SMCs

were incubated at 37°C in low-glucose DMEM with and without 8 μmol/l arsenic trioxide for 48 h. The cells were then harvested from the flask (Coring) using ice-cold phosphate buffered saline (PBS) and total protein was extracted. The protein expressions of Kv channels were determined using western blot analysis and expressed as a ratio with levels of GAPDH. Denatured protein was segregated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to PVDF membrane and incubated with primary antibodies against GAPDH (Affinity Reagents), polyclonal goat anti-Kv1.5 antibody (Santa Cruz) at 1:200 dilution, polyclonal rabbit anti-Kv1.2 antibody and polyclonal rabbit anti-Kv2.1 antibody (Alomone Labs) at 1:200 dilution. Donkey anti-goat Alexa Fluor 700 (dilution 1:4000, Molecular Probes) was used as Kv1.5 secondary antibody and goat anti-rabbit Alexa Fluor 700 (dilution 1:4000, Molecular Probes) was used as Kv1.2 and Kv2.1 secondary antibody. We used the odyssey infrared fluorescent scanning system (LI-COR) to detect membrane protein. The b and densities were quantified by densitometry, using Scion Image Software.

#### Statistical analysis

Data were collected from repeated experiments and were presented as mean±SE. Student's test was used for statistical analysis. A two-tail P<0.05 was taken to indicate a statistically significant difference.

**Results** We demonstrated that arsenic trioxide did not show immediately effects on Kv currents, but remarkably decreased Kv currents were observed after 48 h pretreatment with arsenic trioxide in SMCs. At +60 mV, the amplitudes of Kv currents were decreased by 33.63%, 39.21% and 75.12% with treatment of 1, 4, and 8 μmol/l arsenic trioxide for 48 h, respectively. Furthermore, the expression of Kv1.2 channel and Kv1.5 channel protein was obviously decreased after 48 h pretreatment with arsenic trioxide, but the expression of Kv2.1 did not significantly alter.

**Conclusions** The present study documented two major findings: First, arsenic trioxide did not present immediate effect on Kv currents in rat mesenteric artery SMCs. Second, after 48 h pretreatment, arsenic trioxide impaired Kv channel function mainly through suppressing Kv1.5 and Kv1.2 subunits expression.

Although lots of investigations have reported that arsenic trioxide is strongly correlated with the development of vascular diseases, they mainly focused on epidemiological studies. Recently, Lee et al found that arsenic inhibited vascular relaxation by inhibiting NO production in endothelial cells with a suppression of cGMP dependent relaxation mechanisms. On the other hand, our results revealed arsenic impaired the Kv channel function which play an important role in regulation of vascular tone. Thus, the aberrant regulation of Kv channels by arsenic trioxide may cause the imbalance of vasodilatation and vasoconstriction which may contributes to the development of vascular diseases.

The present experimental results showed that arsenic trioxide did not produce immediately inhibitory effects on the Kv currents in rat mesenteric artery SMCs, indicating that arsenic trioxide did not affect Kv channel function by regulating its activation, inactivation gate. However, after 48 h pretreatment, arsenic trioxide did result in a concentration-dependent suppression of Kv currents through inhibiting Kv subunits Kv1.5 and Kv1.2, but not Kv2.1 which all are major functional components of Kv currents in rat mesenteric artery SMCs.

Kv channels were composed of pore-forming Kvα and modulatory Kvβ subunits arranged in a complex. Among transcripts encoding the pore-forming Kvα subunits, Kv1.2, Kv1.5, and Kv2.1 mainly contributed to Kv currents and played an important role in controlling vascular tone.<sup>26–29</sup> Subunits Kv1.2, Kv1.5, and Kv2.1 are also major components of Kv currents in vascular myocytes from human pulmonary arteries and radical arteries, and rat mesenteric arteries, pulmonary arteries, cerebral vessels, etc. In human

pulmonary artery smooth muscle cells, Kv1.5 subunit was showed to contribute to functional Kv channels, and regulate cellular membrane potential, vascular tone, and vascular proliferation and apoptosis. Aberrant Kv1.5 channel expression and function takes part in vascular remodelling of idiopathic pulmonary arterial hypertension. Subunits Kv1.2 and Kv1.5 make greater contribution to Kv channels in rat coronary myocytes and regulate the resting vascular tone. Previous studies demonstrated that Kv1.2/1.5 heterotetrameric channels contribute to the resting membrane potential and diameter of rat small cerebral arteries in rat cerebral vascular SMCs.<sup>33,34</sup> The antibody for Kv1.5 subunit involves regulation of  $[Ca^{2+}]_i$  and contraction caused by hypoxia and 4-AP in pulmonary artery.<sup>34</sup> Intracellular administration of anti-Kv1.5 antibody causes more depolarisation in pulmonary arterial smooth muscle cells than anti-Kv2.1 antibody. The combination of anti-Kv1.5 and anti-Kv2.1 antibodies could produce greater depolarisation in resistance pulmonary artery than either alone. Consistently, the data from our research showed that both Kv1.2 and Kv1.5 are involved in the impairment of Kv by arsenic trioxide. The Kv1.5 expression was inhibited more potently by arsenic trioxide compared to Kv1.2 expression which implies Kv1.5 channel subunit possibly plays a major role in arsenic-associated vascular diseases.

Some studies showed that arsenic trioxide also present an inhibitory role in Kv currents in other tissues. For example, Zhou J reported that arsenic trioxide acutely decreased the Kv currents in a dose-dependent manner with a  $IC_{50}$  of 4.1  $\mu\text{mol/l}$  in human multiple myeloma cells. Nevertheless, we found that arsenic did not acutely alter Kv currents in mesenteric arterial SMCs. This disparity possibly comes from the different subunits that formed Kv channels in various tissues. In the treatment of acute promyelocytic leukaemia and other tumours, the therapeutic dosage of arsenic trioxide is ranged from 0.1  $\mu\text{mol/l}$  to 5  $\mu\text{mol/l}$ . Some epidemiological investigations also showed that arsenic concentration in plasma can reach to these levels with the environmental exposure levels of arsenic. Accordingly, the concentration of arsenic trioxide used in the present study was related to its clinical application.

Our findings indicate arsenic-induced the impairment of Kv currents by suppressing subunits Kv1.5 and Kv1.2 may be involved the development of vascular diseases.