



ARTICLE

SGK1 mediates the hypotonic protective effect against H₂O₂-induced apoptosis of rat basilar artery smooth muscle cells by inhibiting the FOXO3a/Bim signaling pathway

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Serum- and glucocorticoid-inducible kinase-1 (SGK1) is a serine/threonine kinase regulated by hypotonic stimuli, which is involved in regulation of cell cycle and apoptosis. Our previous study shows that activation of volume-regulated Cl⁻ channels (VRCCs) protects rat basilar artery smooth muscle cells (BASMCS) against hydrogen peroxide (H₂O₂)-induced apoptosis. In the present study, we investigated whether SGK1 was involved in the protective effect of VRCCs in BASMCs. We showed that hypotonic challenge significantly reduced H₂O₂-induced apoptosis, and increased SGK1 phosphorylation, but did not affect SGK1 protein expression. The protective effect of hypotonic challenge against H₂O₂-induced apoptosis was mediated through inhibiting mitochondria-dependent apoptotic pathway, evidenced by increased Bcl-2/Bax ratio, stabilizing mitochondrial membrane potential (MMP), decreased cytochrome c release from the mitochondria to the cytoplasm, and inhibition of the activation of caspase-9 and caspase-3. These protective effects of hypotonic challenge against H₂O₂-induced apoptosis was diminished and enhanced, respectively, by SGK1 knockdown and overexpression. We further revealed that SGK1 activation significantly increased forkhead box O3a (FOXO3a) phosphorylation, and then inhibited the translocation of FOXO3a into nucleus and the subsequent expression of Bcl-2 interacting mediator of cell death (Bim). In conclusion, SGK1 mediates the protective effect of VRCCs against H₂O₂-induced apoptosis in BASMCs via inhibiting FOXO3a/Bim signaling pathway. Our results provide compelling evidences that SGK1 is a critical link between VRCCs and apoptosis, and shed a new light on the treatment of vascular apoptosis-associated diseases, such as vascular remodeling, angiogenesis, and atherosclerosis.

Keywords: basilar artery smooth muscle cells; volume-regulated Cl⁻ channels; hypotonic challenge; H₂O₂; apoptosis; SGK1; FOXO3a; Bim

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INTRODUCTION

Ion channel activation is essential for regulating apoptosis through the process of cell shrinkage, which is also called apoptotic volume decrease (AVD). In addition to Ca²⁺-activated K⁺ channels, the most important cation channels involved in AVD, anion channels are also required for the process of AVD [1]. Among these anion channels, volume-regulated Cl⁻ channels (VRCCs) are crucial for cell volume regulation in almost all types of cell types [2]; however, their basic components, including LRRC8a [3, 4], bestrophin-1 [5], and ClC-3 [6, 7], are still under debate. In addition to their roles in cell volume homeostasis, VRCCs have been implicated in various physiological and pathophysiological processes, such as proliferation, apoptosis, migration, angiogenesis, inflammation, neuronal excitation, and cancer [2]. Our previous work showed that the activation of VRCCs induced by hypotonic challenge protected against hydrogen peroxide (H₂O₂)-induced apoptosis in rat basilar artery smooth muscle cells (BASMCS) [8]. However, how the Cl⁻ fluxion induced by the opening of VRCCs, as Cl⁻ channels, affects the process of apoptosis remains largely unclear.

Because VRCCs have various functions, we propose that alterations in intracellular Cl⁻ concentration might activate one or more Cl⁻-sensitive kinases, subsequently resulting in a signaling cascade. The ubiquitously expressed serum- and glucocorticoid-inducible kinase-1 (SGK1) is a serine/threonine kinase that has been shown to be regulated by osmotic pressure [9]. SGK1 was first cloned as a gene activated by serum and glucocorticoids [10]. SGK1 has since been implicated in the regulation of cell growth, proliferation, survival, apoptosis, and migration, is regulated by numerous cytokines, growth factors, and cell stresses, and was identified as a cell volume-regulated gene [9]. It was reported that hypotonic challenge increased SGK1 levels in distal nephritic cells of *Xenopus leavis* [11, 12] and canine pulmonary artery smooth muscle cells [13]. In addition to being involved in the regulation of cell cycle arrest and apoptosis [14, 15], it is possible that SGK1 might serve as a mediator between VRCCs and downstream signaling and play a role in the protective effect of VRCCs against H₂O₂-induced apoptosis.

In this study, we were interested in studying the relationship between VRCCs and SGK1 in apoptosis of BASMCs and examined

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whether hypotonic challenge plays a protective role in H₂O₂-induced apoptosis via SGK1 in BASMCs. Moreover, as a downstream target of SGK1, forkhead box O3a (FOXO3a) is phosphorylated by SGK1 and subsequently inactivated and translocates from the nucleus to cytoplasm, making it unable to stimulate its proapoptotic gene targets, such as Bcl-2 interacting mediator of cell death (Bim) [16, 17]. Thus, we further explored whether the underlying mechanism of the protective effect of SGK1 on H₂O₂-induced apoptosis is related to the inhibition of the FOXO3a/Bim signaling pathway in BASMCs.

MATERIAL AND METHODS

Reagents and antibodies

Cell culture medium (Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12)), fetal calf serum, bovine serum albumin (BSA), and protease inhibitor cocktail were obtained from GIBCO/Invitrogen (Carlsbad, CA, USA). Anti-SGK1 and anti-phospho-SGK1 (Ser422) were purchased from Merck (Darmstadt, Germany). An anti-CIC-3 antibody was obtained from Alomone Labs (Jerusalem, Israel). Anti-Bcl2, anti-Bax, anti-caspase-9, anti-cleaved caspase-9, anti-caspase-3, anti-cleaved caspase-3, anti-cytochrome c (Cyt-c), anti-Bim, anti-FOXO3a, anti-phospho-FOXO3a (Ser316), anti-poly ADP-ribose polymerase (PARP), anti-cleaved PARP, anti-histone H3 and anti-Cyt-c oxidase subunit IV (COX IV) antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). Hyperfect transfection reagent was purchased from Qiagen (Valencia, CA, USA). The Cell Counting Assay Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (MD, Japan). H₂O₂ was purchased from Merck. The Annexin V-PE/7-AAD Apoptosis Detection Kit and the Mitochondrial Membrane Potential Assay Kit with JC-1 were purchased from Keygen Biotech (Nanjing, China). The Mitochondria Isolation Kit for culture cells was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Unless otherwise indicated, all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Male Sprague–Dawley rats were supplied by the Experimental Animal Center of Sun Yat-Sen University in Guangzhou, China. All procedures complied with the standards for the care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (issued by the Ministry of Science and Technology of China, Beijing) and were approved by the Ethics Committee of Zhongshan School of Medicine on Laboratory Animal Care (No. 2017-099). Rat BASMCs were cultured as described previously [18]. Briefly, rats were anesthetized with intraperitoneal phenobarbital (Merck, Darmstadt, Germany; 40 mg·kg⁻¹) and decapitated. The basilar arteries were isolated aseptically and placed in Krebs buffer. After the fat and connective tissues were removed, the artery strips were cut into 3–5 mm pieces and then plated in DMEM/F-12 containing 20% fetal calf serum (Gibco BRL, Grand Island, NY, USA) with 100 μg·mL⁻¹ streptomycin and 100 U·mL⁻¹ penicillin. BASMCs were maintained at 37 °C in a humidified incubator with a 95% O₂ plus 5% CO₂ atmosphere. The cells were identified as smooth muscle cells based on spindle morphology under a light microscope, and immunostaining was performed with a monoclonal antibody specific for smooth muscle α-actin. BASMCs from passages 8 to 12 were used when they reached 70%–90% confluence.

Solution preparation

A hypotonic solution was prepared with 75 mL H₂O and 225 mL DMEM/F-12 medium as previously described [19]. The osmolality of the solution was 225 mOsm·kg⁻¹ H₂O (measured by a freezing point depression osmometer, OSMOMAT030, Gonotec, GmbH,

Berlin, Germany). The isotonic solution contained 225 mL DMEM/F-12 medium with 75 mL mannitol solution (75 mmol·L⁻¹), and the final osmotic pressure was 300 mOsm·kg⁻¹ H₂O. BASMCs were treated with hypotonic solution for different durations, and the reactions were stopped using liquid nitrogen.

Western blot analysis

Western blot analysis was performed as described previously [18]. BASMCs were lysed for 30 min in lysis buffer with 1% protease inhibitor cocktail. After centrifugation at 12,000 r·min⁻¹ for 15 min at 4 °C, the supernatant was obtained, and the protein concentration was measured with a BCA Kit (Beyotime, Nanjing, China). Samples containing 30 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked at room temperature for 1 h in Tris-buffered saline Tween-20 with 5% bovine serum albumin. Then, the cells were incubated with primary antibodies overnight at 4 °C and with appropriate secondary peroxidase-conjugated antibodies for 1 h at room temperature. The blots were detected by a chemiluminescence system and qualified using a computer aided 1-D gel analysis system (Bio-Red, Hercules, CA).

Measurement of [Cl⁻]_i

The intracellular Cl⁻ concentration ([Cl⁻]_i) was measured using 6-methoxy-*N*-ethylquinolinium iodide (MEQ) as described previously [20]. Briefly, MEQ was first reduced to its cell-permeable derivative 6-methoxy-*N*-ethyl-1,2-dihydroquinoline (dihydro-MEQ). MEQ reduction was carried out by the addition of 32 mol·L⁻¹ sodium borohydride to MEQ solution at room temperature under flowing nitrogen in the dark for 30 min. Dihydro-MEQ was freshly prepared, and cells were incubated with dihydro-MEQ for 15 min before the experiment. In the cytoplasm, dihydro-MEQ is quickly oxidized to MEQ, which is sensitive to the [Cl⁻]_i. Fluorescence quenching induced by Cl⁻ was monitored by MetaFluor Imaging software (Universal Imaging Systems, Chester, PA) at a 350-nm excitation wavelength and a 435-nm emission wavelength.

Transfection of adenovirus/siRNA into BASMCs

An SGK1 overexpression adenovirus was purchased from Vigene Biosciences (Rockville, USA), and the adenovirus expressed human SGK1 (NM_005627.3) with an RFP (red fluorescent protein) tag. BASMCs were transfected with the adenovirus according to a previously described method [21].

SGK1 small interfering RNA (siRNA) (5'-GGUUAUCUGCACUCCC UAA-3') was designed and synthesized by Qiagen. Scrambled RNA (5'-UUCUCCGAACGUGUCACGU-3') was used as a negative control. SGK1 siRNA and negative control siRNA were transfected into BASMCs by using HiPerfect transfection reagent according to a previously described protocol [19].

CCK-8 assay for cell viability

Cell viability was measured by the CCK-8 according to a previously described protocol [18]. At 90% confluence, BASMCs from passages 8 to 12 were growth arrested by incubation in DMEM/F-12 with 0.5% fetal calf serum for 24 h before the experiments. Then, 10 μL of CCK-8 solution was added to each well, and the cells were incubated for another 2 h. The absorbance at 450 nm was measured using a microplate reader (Bio-Tek, Winooski, VT).

Quantification of apoptosis by flow cytometry

BASMCs were cultured in 6-well plates at 70% confluence and were transfected with Ad-SGK1 or SGK1 siRNA for 24 h and then exposed to H₂O₂ in isotonic or hypotonic medium for an additional 24 h. The cells were harvested for Annexin V-PE and 7-AAD staining using the Annexin V-PE/7-AAD Apoptosis Detection Kit (Keygen Biotech) according to the manufacturer's instructions.

Briefly, BASMCs were washed with phosphate-buffered saline (PBS) and stained with Annexin V-APC and 7-AAD. After incubation for 15 min in the dark at room temperature, the stained cells were counted by flow cytometry (Coulter, Hialeah, FL) as described previously [18]. The percentages of cells in the lower and upper right corners represented the early and late apoptosis rates, respectively, and the percentages of cells in the lower and upper left corners represented the survival and necrosis rates, respectively.

MMP measurement

JC-1 exposed to BASMCs can be detected by the presence of both cytoplasmic JC-1 monomers and mitochondrial JC-1 aggregates. JC-1 forms monomers and emits green fluorescence at a low mitochondrial membrane potential (MMP), while it forms aggregates and emits red fluorescence at a high MMP. JC-1 fluorescence is usually excited by a wavelength of 488 nm by flow cytometry, and the approximate emission peaks of monomeric and aggregated JC-1 are 530 nm (green) and 590 nm (red), respectively. Thus, the fluorescence intensity ratio of monomeric JC-1 to aggregated JC-1 (green/red) represents a loss of MMP. In the experiments, cells were seeded into 6-well plates and exposed to $200 \mu\text{mol}\cdot\text{L}^{-1}$ H_2O_2 for 24 h with or without hypotonic medium. The cells were incubated with JC-1 dye for 15 min at 37°C in the dark and then analyzed by flow cytometry as previously described [18].

Isolation of mitochondria

Intact mitochondria were isolated using a Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. A pellet containing 2×10^7 cells was obtained by centrifuging the cell suspension for 2 min, and the suspension was discarded. Mitochondria isolation reagent A ($800 \mu\text{L}$) was added to the pellet, and the pellet was centrifuged at a medium speed for 5 s and incubated for 2 min on ice. Next, $10 \mu\text{L}$ mitochondria isolation reagent B was added to the tube, and the tube was centrifuged at maximum speed for 5 s and incubated on ice for 5 min. Then, $800 \mu\text{L}$ mitochondria isolation reagent C was added to the tube and centrifuged at $700 \times g$ for 10 min at 4°C . The supernatant, which was the cytosolic fraction, was transferred to a new tube and centrifuged at $12,000 \times g$ for 15 min at 4°C . The pellet, which contained isolated intact mitochondria, was washed with $500 \mu\text{L}$ reagent C and centrifuged at $12,000 \times g$ for 15 min at 4°C . The cytosolic and mitochondrial fractions were used for Western blotting. Cox IV was used as a loading control for the mitochondrial fraction.

Immunocytochemistry

BASMCs were fixed with 4% paraformaldehyde for 20 min. The cells were washed twice in PBS for 5 min at room temperature and permeabilized in PBS containing 0.2% Triton X-100 for 10 min. Nonspecific binding sites were blocked by 3% BSA in PBS for 1 h. A primary monoclonal antibody was added and incubated overnight at 4°C . Then, the cells were washed twice with PBS and incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 1 h. Finally, the cells were counterstained with Hoechst 33258 to simultaneously evaluate nuclear condensation. Cell micrographs were obtained with a CCD camera on a Zeiss LSM710 imaging microscope (Zeiss, Oberkochen, Germany).

Statistical analysis

Data analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). All data are expressed as the means \pm SEM, and the n value represents the number of independent experiments. Statistical significance was tested by one- or two-way

ANOVA followed by Bonferroni multiple comparisons test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of hypotonic solution and H_2O_2 on SGK1 activation
SGK1 expression and phosphorylation were detected by immunoblotting after BASMCs were incubated with isotonic or hypotonic solution for different durations. As shown in Fig. 1a, the SGK1 phosphorylation level was increased by treatment with hypotonic solution for 1 min and then returned to the normal level after 2 min. The SGK1 protein level was not obviously altered within 8 min under hypotonic stimulation.

H_2O_2 is one of the major reactive oxygen species (ROS) and is widely used to establish a rapid and sensitive oxidative stress-induced apoptosis model [8, 18]. Therefore, SGK1 activity was also measured after BASMCs were treated with $200 \mu\text{mol}\cdot\text{L}^{-1}$ H_2O_2 . As shown in Fig. 1b, incubation with H_2O_2 for 30 s reduced the SGK1 phosphorylation level, but the SGK1 protein level was not obviously changed.

Hypotonic challenge opens VRCCs, resulting in an efflux of Cl^- followed by a reduced $[\text{Cl}^-]_i$ in BASMCs. Therefore, the $[\text{Cl}^-]_i$ was measured in this study. The results showed that hypotonic stimulation remarkably decreased the $[\text{Cl}^-]_i$ from 29.96 ± 0.07 to $25.58 \pm 0.03 \text{ mmol}\cdot\text{L}^{-1}$ (Fig. 1c), whereas treatment with H_2O_2 remarkably increased the $[\text{Cl}^-]_i$ from 30.31 ± 0.19 to $38.78 \pm 0.07 \text{ mmol}\cdot\text{L}^{-1}$ (Fig. 1d). After switching to the isotonic solution, the $[\text{Cl}^-]_i$ rapidly returned to the normal level.

To further verify the relationship between the $[\text{Cl}^-]_i$ and SGK1 activation, SGK1 expression and phosphorylation were measured in BASMC lysates in a low- Cl^- environment. As shown in Fig. S1a, the phosphorylation of SGK1 was upregulated in a solution containing 20 or $10 \text{ mmol}\cdot\text{L}^{-1}$ NaCl, which represented a low- Cl^- environment, compared with that in a solution containing $30 \text{ mmol}\cdot\text{L}^{-1}$ NaCl, which represented a normal Cl^- environment. When Cl^- was substituted with gluconate, the phosphorylation level of SGK1 was not remarkably changed. Moreover, when Na^+ was substituted with K^+ , as shown in Fig. S1b, a low- Cl^- environment, but not a low-gluconate environment, remarkably increased the phosphorylation of SGK1. Taken together, these results suggest that SGK1 is activated by treatment with hypotonic solution and inhibited by treatment with H_2O_2 , which is related to the $[\text{Cl}^-]_i$.

Hypotonic challenge inhibits H_2O_2 -induced apoptosis via SGK1

Next, we performed SGK1 siRNA or adenovirus transfection to increase or decrease the corresponding protein expression level, respectively. Endogenous SGK1 protein expression was remarkably reduced to $38.7\% \pm 5.3\%$ by transfection with $40 \text{ nmol}\cdot\text{L}^{-1}$ SGK1 siRNA for 48 h (Fig. S2a) and increased $7.67\% \pm 0.47$ -fold by transfection with 200 multiplicity of infection (MOI) SGK1 adenovirus for 48 h (Fig. S2b).

To analyze whether SGK1 affects H_2O_2 -induced cell injury, the viability rate of BASMCs was evaluated by the CCK-8 assay. As shown in Fig. 2a, b, cells were cultured with $200 \mu\text{mol}\cdot\text{L}^{-1}$ H_2O_2 in isotonic or hypotonic solution for 24 h. Compared to control, H_2O_2 remarkably decreased the cell viability rate to $71.8\% \pm 1.2\%$ in isotonic solution, but the cell viability rate was reversed to $115.4\% \pm 3.2\%$ in hypotonic solution. Silencing SGK1 reduced the cell viability rate in hypotonic solution, whereas the overexpression of SGK1 increased the cell viability rate in this solution.

Next, the cell apoptosis rate was analyzed by flow cytometry. Cells were double-stained with Annexin V-FITC and propidium iodide, and the percentage in the lower right corner represented the early apoptosis rate. As shown in Fig. 2c, d, H_2O_2 remarkably increased the early apoptosis rate from $7.5\% \pm 0.5\%$ to $11.4\% \pm 0.9\%$ under isotonic conditions, and the apoptosis rate was

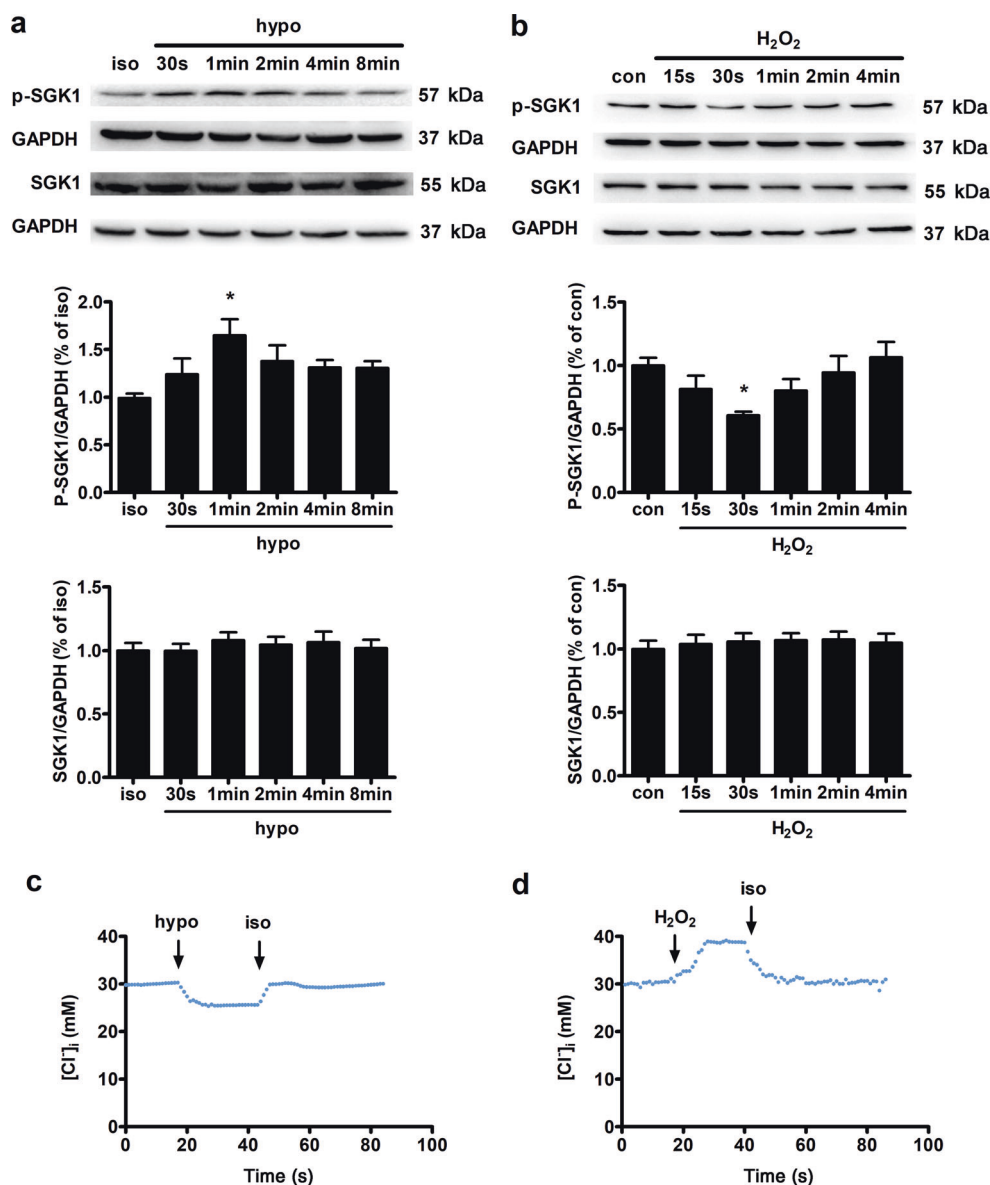


Fig. 1 The effects of hypotonic solution and hydrogen peroxide (H₂O₂) treatment on serum- and glucocorticoid-inducible kinase-1 (SGK1) phosphorylation (Ser422) and the intracellular Cl⁻ concentration ([Cl⁻]_i) in basilar artery smooth muscle cells (BASMCS). **a** The protein expression and phosphorylation of SGK1 were measured in cells incubated with isotonic (iso) or hypotonic (hypo) solution for different durations using Western blotting. Densitometric analysis of SGK1 and p-SGK1 is shown (**P* < 0.05 vs. iso, *n* = 8). **b** The protein expression and phosphorylation of SGK1 were measured in cells stimulated with H₂O₂ (200 μmol·L⁻¹) for different durations using Western blotting. Densitometric analysis of SGK1 and p-SGK1 is shown. (**P* < 0.05 vs. con, *n* = 7) **c**, **d** Cells were incubated with isotonic solution (iso, 300 mOsm·kg⁻¹ H₂O) and then incubated with hypotonic solution (hypo, 225 mOsm·kg⁻¹ H₂O) (**c**) or treated with H₂O₂ (200 μmol·L⁻¹) (**d**); after that, the solution was replaced with new isotonic solution. The [Cl⁻]_i was measured and is shown in the line charts (*n* = 5)

alleviated to 6.4% ± 0.5% by hypotonic challenge. The protective effect of hypotonic stimulation was ameliorated by SGK1 silencing and potentiated by SGK1 overexpression. These data suggest that SGK1 is a crucial signaling molecule that mediates the protective effect of VRCCs against H₂O₂-induced apoptosis in BASMCs.

Hypotonic challenge increases the Bcl-2/Bax ratio via SGK1
 The anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax regulate the mitochondrial pathway of apoptosis. An increased or decreased Bcl-2/Bax ratio appears to determine the survival or death of cells under apoptotic stimulation. As shown in Fig. 3, H₂O₂ decreased Bcl-2 protein expression and

increased Bax protein expression, resulting in a decline in the Bcl-2/Bax ratio. Hypotonic solution reversed the effects of H₂O₂ and induced an upregulation in the Bcl-2/Bax ratio, which was alleviated and strengthened by silencing and overexpressing SGK1, respectively.

Hypotonic challenge prevents the loss of MMP and the release of Cyt-c via SGK1
 H₂O₂-induced apoptosis is regulated by the loss of MMP. To test whether hypotonic challenge protected H₂O₂-induced apoptosis through the SGK1-mediated mitochondrial pathway, the MMP level was measured using JC-1 fluorescent dye. This dye usually accumulates in the mitochondria as aggregates and emits red

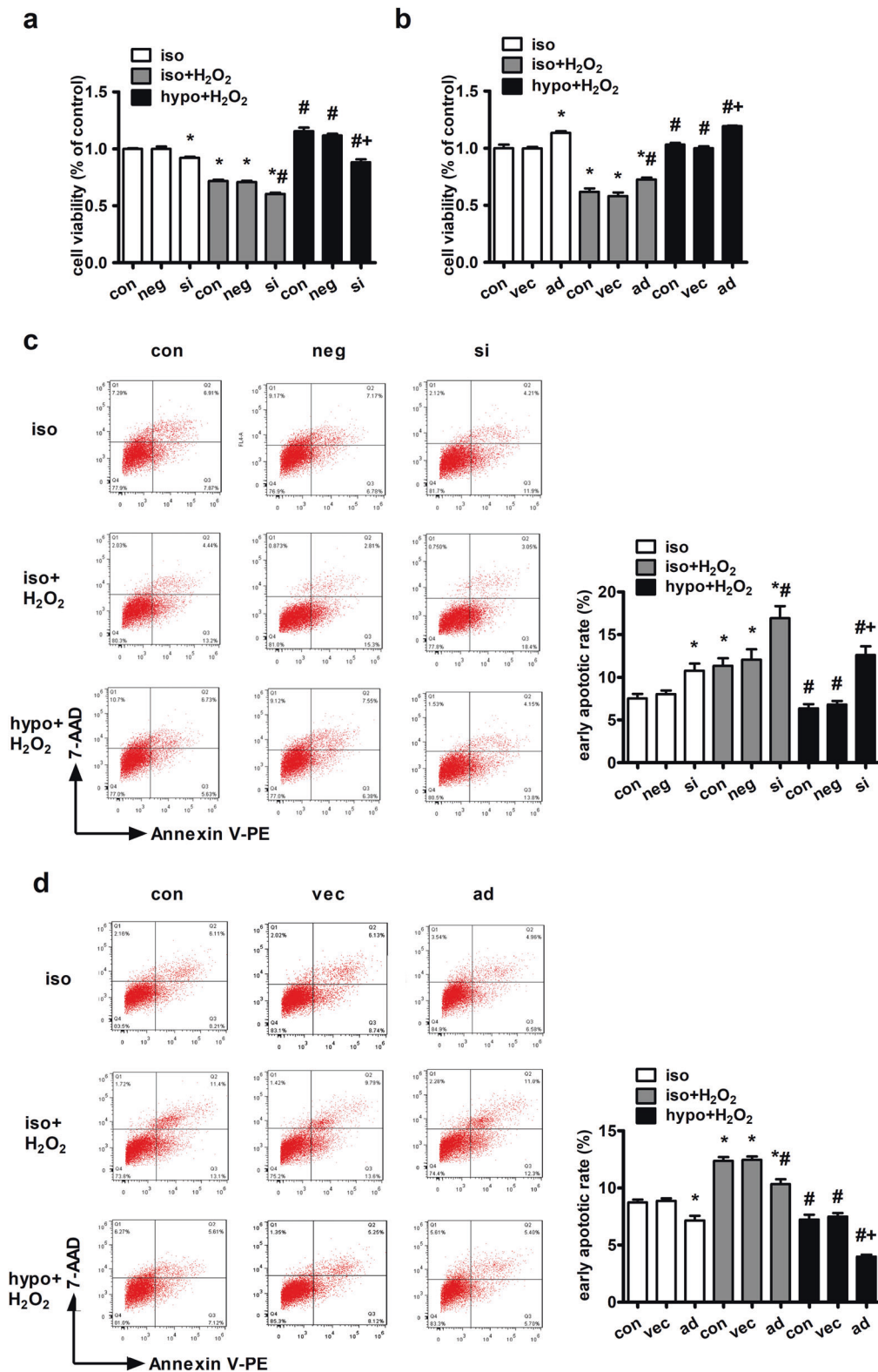


Fig. 2 Hypotonic challenge protected against H₂O₂-induced apoptosis via SGK1 in BASMCs. Cells were transfected with SGK1 siRNA (si, 40 nmol·L⁻¹), SGK1-expressing adenovirus (ad, 200 MOI), or the respective negative control (neg and vec, respectively) for 24 h and then treated with H₂O₂ (200 μmol·L⁻¹) in isotonic (iso) or hypotonic (hypo) solution for another 24 h. **a, b** SGK1 mediated the protective effect of hypotonic challenge on H₂O₂-induced cell injury. The effects of hypotonic challenge plus SGK1 silencing (**a**) or overexpression (**b**) on H₂O₂-induced apoptosis were evaluated by detecting cell viability, which was measured by the CCK-8 assay and is shown in the bar graphs (**P* < 0.05 vs. con in iso, #*P* < 0.05 vs. con in iso + H₂O₂, +*P* < 0.05 vs. si or ad in iso + H₂O₂, *n* = 6). **c, d** SGK1 mediated the protective effect of hypotonic challenge against H₂O₂-induced apoptosis. The effects of hypotonic challenge plus SGK1 silencing (**c**) or overexpression (**d**) on H₂O₂-induced apoptosis were measured by flow cytometry. The percentage of early apoptotic cells (lower right corner) was quantitatively analyzed (**P* < 0.05 vs. con in iso, #*P* < 0.05 vs. con in iso + H₂O₂, +*P* < 0.05 vs. si or ad in iso + H₂O₂, *n* = 6)

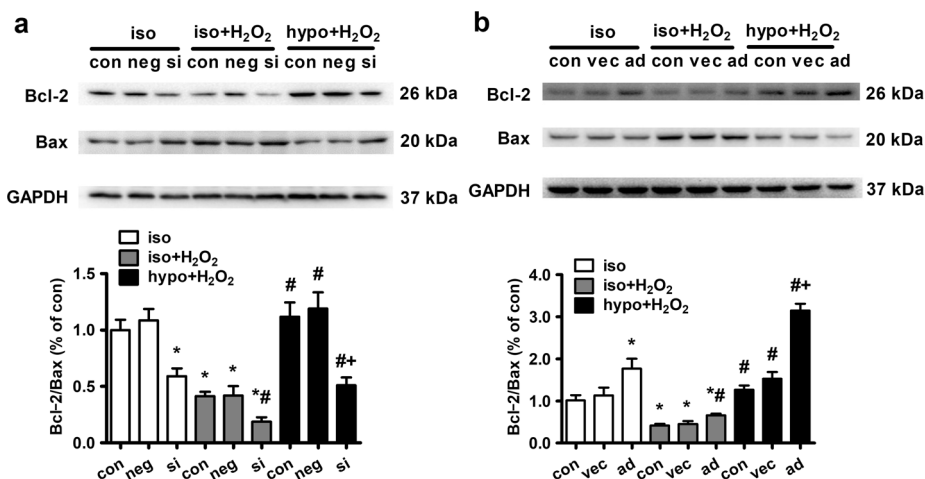


Fig. 3 Hypotonic challenge increased the Bcl-2/Bax ratio via SGK1 during H₂O₂-induced apoptosis in BASMCs. **a, b** Cells were transfected with SGK1 siRNA (si, 40 nmol·L⁻¹), SGK1-expressing adenovirus (ad, 200 MOI), or the respective negative control (neg and vec, respectively) for 24 h and then treated with H₂O₂ (200 μmol·L⁻¹) in isotonic (iso) solution or hypotonic (hypos) solution for another 24 h. The effects of hypotonic challenge plus SGK1 silencing (**a**) or overexpression (**b**) on H₂O₂-induced apoptosis were evaluated by detecting the expression of Bcl-2 and Bax, which was measured by Western blotting, in different treatment conditions. Densitometric analysis of the Bcl-2/Bax ratio is shown in the bar graphs (**P* < 0.05 vs. con in iso, #*P* < 0.05 vs. con in iso + H₂O₂, +*P* < 0.05 vs. si or ad in iso + H₂O₂, *n* = 6)

fluorescence in healthy cells. However, it flows out into the cytoplasm as monomers and emits green fluorescence in apoptotic cells when MMP decreases. Thus, a decrease in the red/green fluorescence ratio represents a loss of MMP. As shown in Fig. 4a, b, the *x*- and *y*-axis in the flow cytometry plot represent the green and red fluorescence intensity, respectively. H₂O₂ decreased the red/green fluorescence ratio, indicating a promoting effect of MMP loss, which was protected in cells treated with hypotonic solution. The protective effect of hypotonic solution was reversed and enhanced by transfection with SGK1 siRNA and SGK1 adenovirus, respectively.

MMP loss induces Cyt-*c* release from the mitochondria into the cytosol. Therefore, Cyt-*c* expression in both the cytosol and mitochondria was determined by Western blotting. As shown in Fig. 4c, d, treatment with H₂O₂ enhanced the release of Cyt-*c* from the mitochondria into the cytosol, resulting in an increased Cyt-*c* level in the cytosol and a reduced Cyt-*c* level in the mitochondria. These effects were prevented by hypotonic challenge. Silencing SGK1 attenuated the effect of hypotonic solution on Cyt-*c* release, whereas overexpressing SGK1 further potentiated this effect.

Hypotonic challenge reduces caspase activation via SGK1
An increase in the cytosolic Cyt-*c* level leads to the activation of caspases, such as caspase-9 and its downstream caspase-3, which are crucial proteases that cleave cellular proteins and, as a consequence, induce mitochondria-dependent apoptosis. As shown in Fig. 5, the cleavage of caspase-9 and caspase-3 was promoted by treatment with H₂O₂ and was restored to a normal level by hypotonic challenge. The effects of hypotonic stimulation were remarkably attenuated and strengthened by silencing and overexpressing SGK1, respectively. Accordingly, the cleavage of PARP, one of the downstream targets of caspase-3, induced the same changes as those in cleaved caspase-3. Taken together, these results demonstrate that SGK1 mediates the protective effect of hypotonic challenge on H₂O₂-induced mitochondrial pathway-dependent apoptosis.

Hypotonic challenge inhibits FOXO3a activation via SGK1
FOXO3a, a downstream target of SGK1, is phosphorylated by SGK1. This process inhibits the translocation of FOXO3a from the

cytosol to the nucleus and results in the disruption of FOXO3a-dependent transcription, cell cycle arrest, and apoptosis. In this study, hypotonic challenge induced an increase in FOXO3a phosphorylation within 1 min, and the protein expression of FOXO3a was not affected (Fig. S3). The phosphorylation of FOXO3a was upregulated by hypotonic stimulation, which was inhibited by silencing SGK1 and enhanced by overexpressing SGK1 (Fig. 6a, b), indicating that the hypotonic-induced upregulation of FOXO3a phosphorylation was mediated by SGK1.

Next, H₂O₂-induced FOXO3a translocation was evaluated using Hoechst staining to label cell nuclei and an anti-FOXO3a antibody with a fluorescent secondary antibody to determine the location of FOXO3a. As shown in Fig. 6c, d, the results showed that H₂O₂ increased the localization of FOXO3a in the cell nucleus, suggesting increased transcription of apoptosis-related proteins, and this was reversed by hypotonic challenge. This effect of hypotonic solution was alleviated and promoted by silencing and overexpressing SGK1, respectively. A similar result was also obtained using Western blotting (Fig. S4), which showed that H₂O₂ increased the translocation of FOXO3a from the cytosol to the cell nucleus and that this effect was reversed by hypotonic challenge. Silencing and overexpressing SGK1 increased and decreased this translocation of FOXO3a, respectively.

Hypotonic challenge downregulates Bim expression via SGK1
Bim is a FOXO3a target that is related to mitochondria-dependent apoptosis. In this study, we found that the expression of Bim was upregulated under treatment with H₂O₂. Hypotonic challenge reduced Bim expression, which was attenuated in cells transfected with SGK1 siRNA and enhanced in cells transfected with SGK1 adenovirus (Fig. 7). These results demonstrate that the activation of VRCCs protects against H₂O₂-induced apoptosis via SGK1, the inhibition of downstream FOXO3a translocation into the nucleus and Bim transcription.

DISCUSSION

In the present study, we investigated whether the protective effect of VRCCs against H₂O₂-induced apoptosis is mediated by SGK1 in BASMCs. The results show that the activation of VRCCs

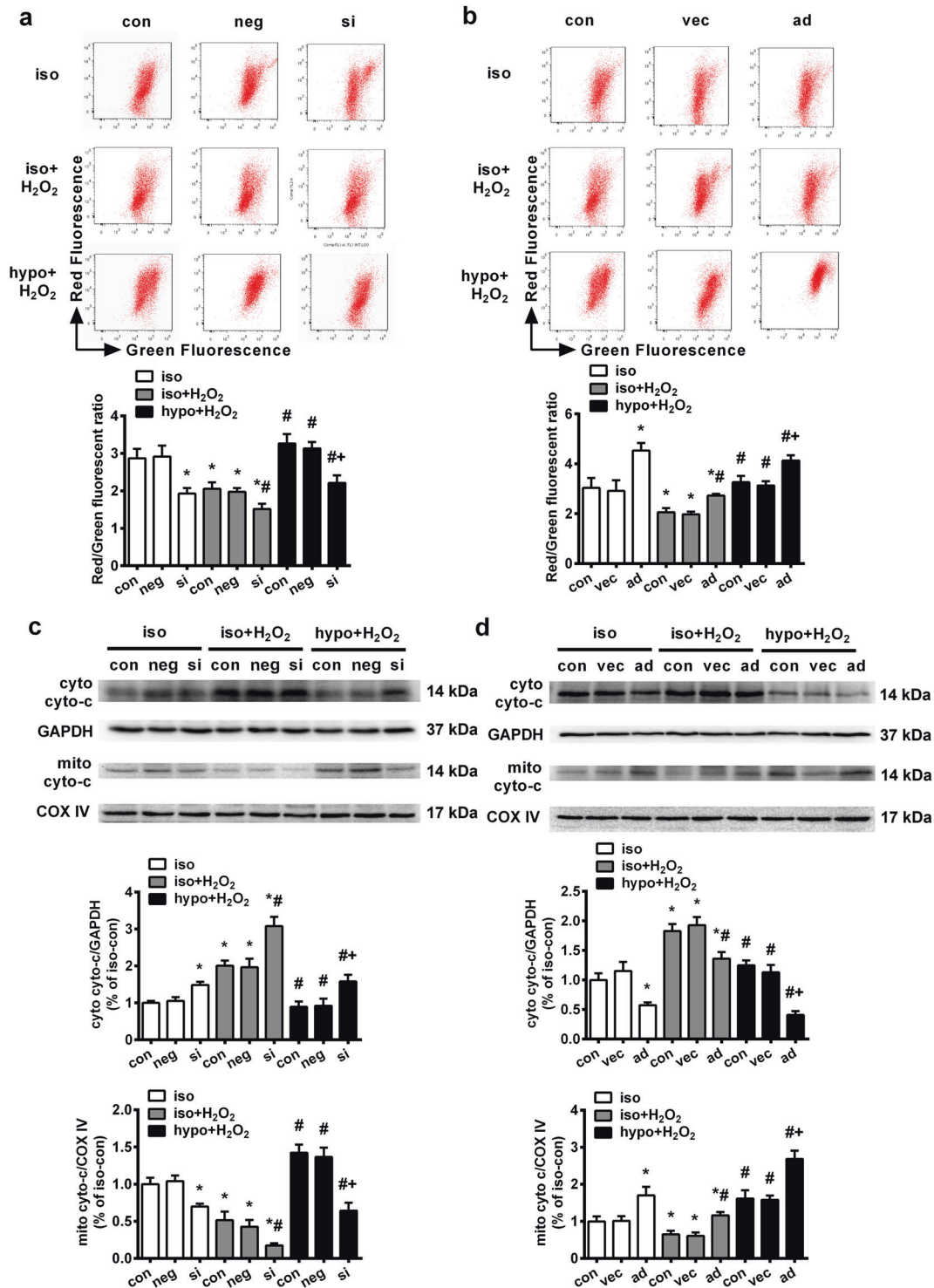


Fig. 4 Hypotonic challenge prevented mitochondrial membrane potential (MMP) loss and cytochrome c (Cyt-c) release via SGK1 during H₂O₂-induced apoptosis in BASMCs. Cells were transfected with SGK1 siRNA (si, 40 nmol·L⁻¹), SGK1-expressing adenovirus (ad, 200 MOI), or the respective negative control (neg and vec, respectively) for 24 h and then treated with H₂O₂ (200 μmol·L⁻¹) in isotonic (iso) solution or hypotonic (hypo) solution for another 24 h. **a, b** SGK1 mediated the protective effect of hypotonic challenge against H₂O₂-induced MMP loss. The effects of hypotonic challenge plus SGK1 silencing (**a**) or overexpression (**b**) on H₂O₂-induced apoptosis were evaluated by detecting MMP loss, which was measured by JC-1 staining. A reduced ratio of red/green fluorescence represented the loss of MMP. Densitometric analysis of the ratio of red/green fluorescence intensity is shown in the bar graphs (**P* < 0.05 vs. con in iso, #*P* < 0.05 vs. con in iso + H₂O₂, +*P* < 0.05 vs. si or ad in iso + H₂O₂, *n* = 6). **c, d** SGK1 mediated the protective effect of hypotonic challenge against H₂O₂-induced Cyt-c release from the mitochondria (mito) into the cytosol (cyto). The effects of hypotonic challenge plus SGK1 silencing (**c**) or overexpression (**d**) on H₂O₂-induced apoptosis were evaluated by detecting the expression of Cyt-c in the cyto and mito, which was measured by Western blotting. Densitometric analysis of Cyto-c in the cyto and mito is shown in the bar graphs (**P* < 0.05 vs. con in iso, #*P* < 0.05 vs. con in iso + H₂O₂, +*P* < 0.05 vs. si or ad in iso + H₂O₂, *n* = 6)

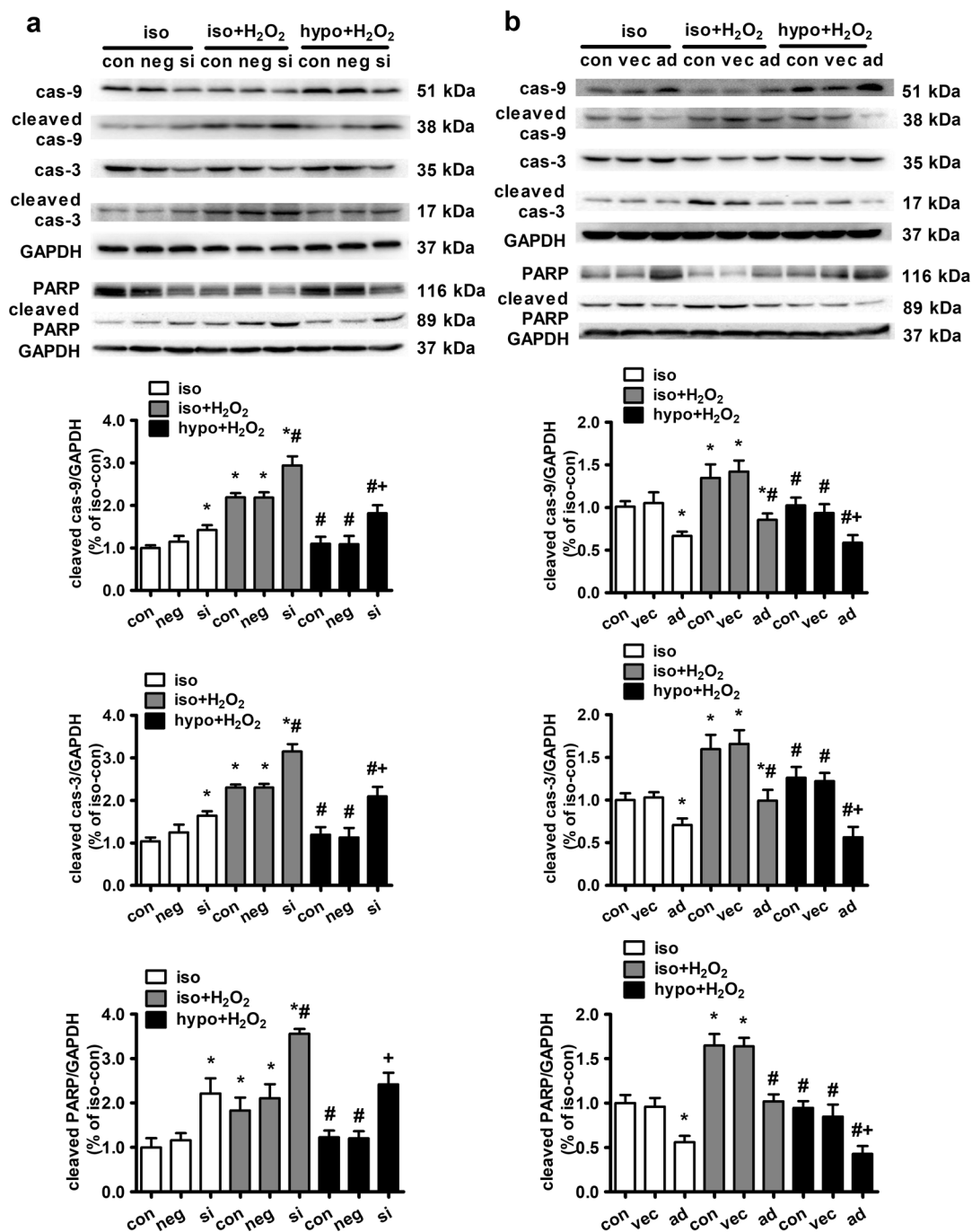


Fig. 5 Hypotonic challenge inhibited caspase and PARP activation via SGK1 during H₂O₂-induced apoptosis in BASMCs. Cells were transfected with SGK1 siRNA (si, 40 nmol·L⁻¹), SGK1-expressing adenovirus (ad, 200 MOI) or the respective negative control (neg and vec, respectively) for 24 h and then treated with H₂O₂ (200 μmol·L⁻¹) in isotonic (iso) solution or hypotonic (hypo) solution for another 24 h. **a, b** The effects of hypotonic challenge plus SGK1 silencing (**a**) or overexpression (**b**) on H₂O₂-induced apoptosis were evaluated by detecting the expression and activation of caspase-9 (cas-9), caspase-3 (cas-3), and PARP, which were measured by Western blotting, in different treatment conditions. Densitometric analysis of the expression of cleaved cas-9 and cleaved cas-3 is shown in the bar graphs (**P* < 0.05 vs. con in iso, #*P* < 0.05 vs. con in iso + H₂O₂, +*P* < 0.05 vs. si or ad in iso + H₂O₂, *n* = 6)

protects against H₂O₂-induced mitochondria-dependent apoptosis via SGK1, which inhibits FOXO3a translocation from the cytosol into the nucleus and subsequent Bim transcription.

Regulatory volume decrease is the mechanism by which cells maintains a constant volume when hypotonic challenge favors the influx of free water, which is related to VRCC activation [22]. It has been proven that the opening of VRCCs protects against H₂O₂-

induced apoptosis via a mitochondria-dependent pathway in vascular smooth muscle cells (VSMCs) [8, 23] and by inhibiting oxidative stress in PC12 cells [24]. Ischemia/reperfusion-induced apoptosis is also inhibited by the activation of VRCCs in the myocardium [25]. Moreover, VRCCs act as dual sensors of both hypoosmolarity and low p*H*_o and reduce neuronal injury during ischemia stimulation and *N*-methyl-*D*-aspartate-induced apoptosis

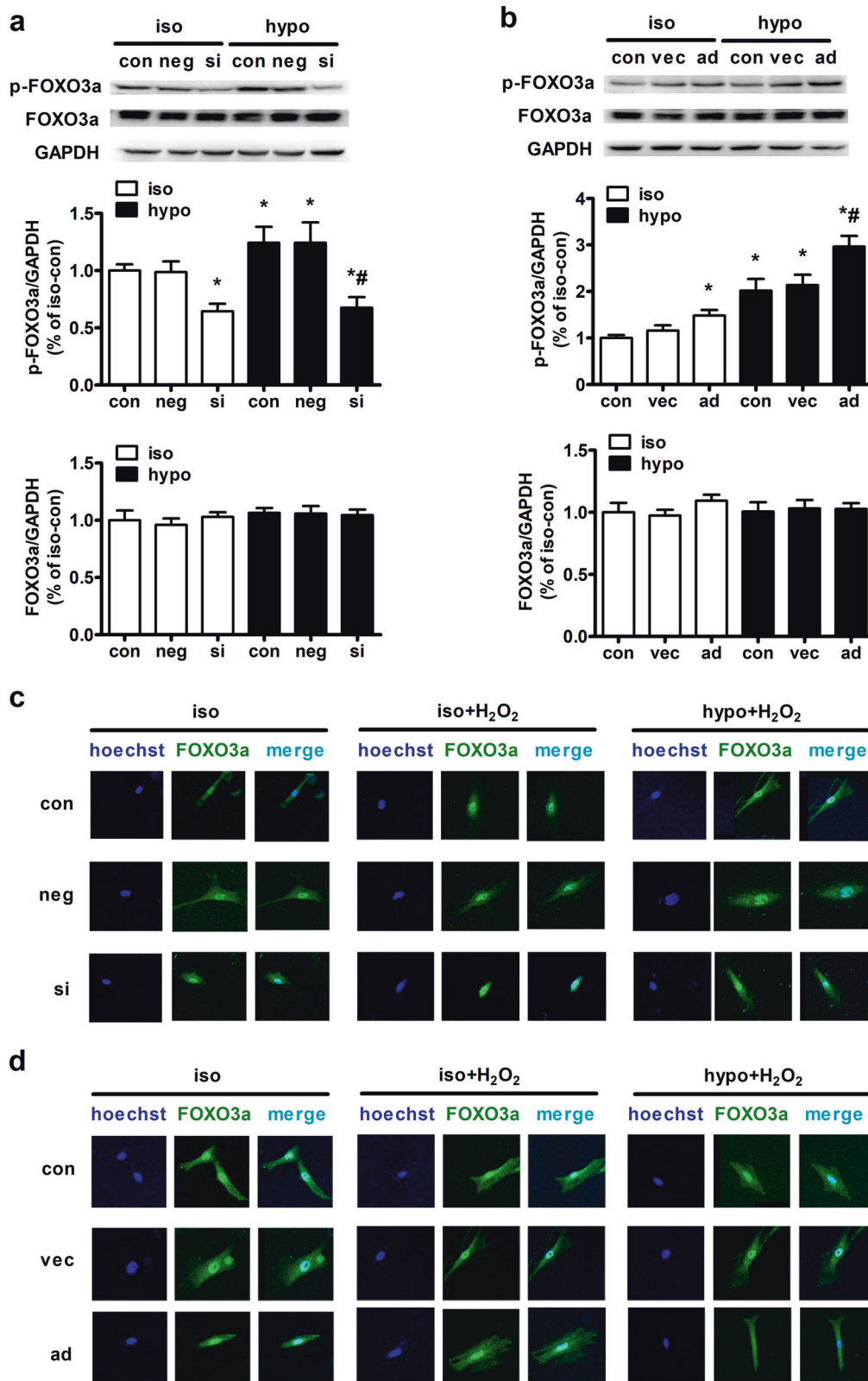


Fig. 6 Hypotonic challenge inhibited forkhead box O3a (FOXO3a) activation via SGK1 during H₂O₂-induced apoptosis in BASMCs. **a, b** Cells were transfected with SGK1 siRNA (si, 40 nmol·L⁻¹), SGK1-expressing adenovirus (ad, 200 MOI), or the respective negative control (neg and vec, respectively) for 24 h and then incubated in isotonic (iso) solution or hypotonic (hypo) solution for another 24 h. The effects of hypotonic challenge plus SGK1 silencing (**a**) or overexpression (**b**) on the expression and phosphorylation of FOXO3a in different treatments were measured by Western blotting. Densitometric analysis of FOXO3a phosphorylation is shown in the bar graphs (**P* < 0.05 vs. con in iso, ***P* < 0.05 vs. con in hypo, *n* = 6). **c, d** Cells were transfected with SGK1 siRNA (si, 40 nmol·L⁻¹), SGK1-expressing adenovirus (ad, 200 MOI), or the respective negative control (neg and vec, respectively) for 24 h and then treated with H₂O₂ (200 μmol·L⁻¹) in isotonic (iso) solution or hypotonic (hypo) solution for another 24 h. The effects of hypotonic challenge plus SGK1 silencing (**c**) or overexpression (**d**) on H₂O₂-induced FOXO3a translocation were evaluated by fluorescence microscopy. Cell nuclei were stained with Hoechst (blue), and FOXO3a was stained with a FITC-labeled antibody (green) (*n* = 5)

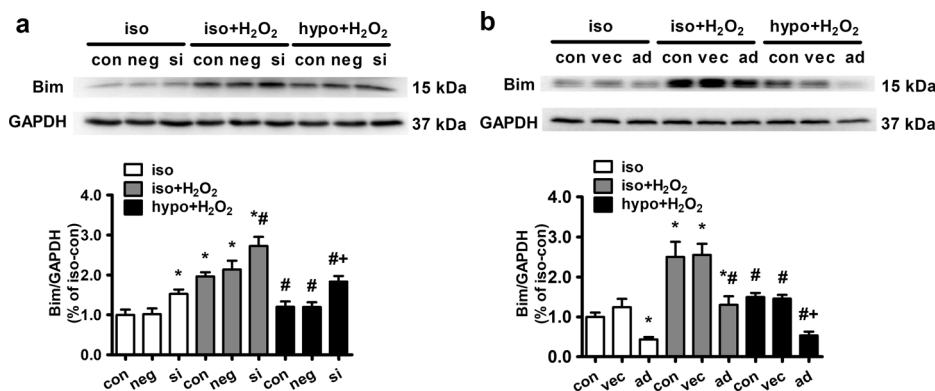


Fig. 7 Hypotonic challenge reduced Bim expression via SGK1 during H₂O₂-induced apoptosis in BASMCs. Cells were transfected with SGK1 siRNA (si, 40 nmol·L⁻¹), SGK1-expressing adenovirus (ad, 200 MOI) or the respective negative control (neg and vec, respectively) for 24 h and then treated with H₂O₂ (200 μmol·L⁻¹) in isotonic (iso) solution or hypotonic (hypo) solution for another 24 h. **a**, **b** The effects of hypotonic challenge plus SGK1 silencing (**a**) or overexpression (**b**) on H₂O₂-induced apoptosis were evaluated by detecting the expression of Bim, which was measured by Western blotting, in different treatment conditions. Densitometric analysis of the expression of Bim is shown in the bar graphs (**P* < 0.05 vs. con in iso, #*P* < 0.05 vs. con in iso + H₂O₂, +*P* < 0.05 vs. si or ad in iso + H₂O₂, *n* = 6)

[26]. However, the mechanism by which VRCCs regulate apoptosis needs further exploration.

Apoptosis relies on an intracellular proteolytic cascade, which includes two types of caspases, namely, initiator caspases (such as caspase-9) and executioner caspases (such as caspase-3). Apoptosis is essentially mediated by two important pathways, the extrinsic pathway and the intrinsic (or mitochondrial) pathway [27]. Notably, the mitochondrial pathway can be activated following oxidative damage via ROS, which induce outer mitochondrial membrane permeabilization followed by Cyt-c release from the mitochondria. The translocation of Cyt-c into the cytosol initiates the cleavage of caspase-9 and subsequently caspase-3, resulting in the cleavage of downstream targets, such as PARP, and induces apoptotic cell death [28]. It is well established that SGK1 activation is critical to cell survival and is related to the onset and progression of several pathophysiological disorders [9]. Extensive studies have demonstrated that the inhibition of SGK1 remarkably triggers apoptosis in breast cancer cells [29], colon cancer cells [30], prostate cancer cells [31], intestinal epithelial cells [32], and human umbilical vein endothelial cells [33]. In SGK1-null animals, the absence of SGK1 results in embryonic and extraembryonic angiogenic defects and increased apoptosis of endothelial cells and VSMCs [15]. In the present study, we observed that the silencing of SGK1 increased apoptosis, whereas the overexpression of SGK1 inhibited it, through the mitochondria-dependent intrinsic pathway in BASMCs. These results are consistent with the results of previous studies.

FOXO3a, also called forkhead transcription factor FKHL1, is an intracellular target of SGK1 [17]. FOXO3a is phosphorylated by SGK1, resulting in its translocation from the nucleus to the cytoplasm and its inability to stimulate its pro-apoptotic gene targets, such as p27 and Bim [16, 17]. It has been reported that Wnt signaling inhibits FOXO3a-induced apoptosis by increasing SGK1 synthesis [30]. However, the inhibition of SGK1 promotes autophagy-dependent apoptosis via the mTOR-FOXO3a pathway [31]. The present results revealed that the activation of SGK1 by hypotonic challenge increases FOXO3a phosphorylation levels, resulting in the inhibition of FOXO3a translocation into the cell nucleus and the suppression of Bim expression. This result provides compelling evidence that SGK1 is an important regulator of mitochondria-dependent apoptosis via the regulation of FOXO3a phosphorylation.

SGK1 activity is regulated by extracellular osmotic pressure. Previously, the human isoform of SGK1 was identified as a cell

volume-regulated gene [34]. This observation was also proven by other research groups, which showed that hypotonic conditions increase SGK1 levels in distal nephritic cells of *Xenopus leavis* [11, 12] and canine pulmonary artery smooth muscle cells [13]. However, other studies have demonstrated that hypertonic conditions also increase SGK1 levels in some other cells, such as hepatocytes [35], pancreatic acinar cells [36], mammary tumor cells [37], and renal collecting duct cells [38]. These results indicate that SGK1 is involved in osmotic cell adaptation via the dual regulation of cell volume in a cell type-dependent manner. Our results demonstrated that hypotonic challenge remarkably increased SGK1 phosphorylation and that the phosphorylation of SGK1 in BASMCs recovered to normal levels within several minutes. Therefore, these data only provide evidence that acute alterations in the intracellular Cl⁻ concentration affects the phosphorylation of SGK1 and this effect is transient. However, we cannot exclude the possibility that downstream signaling, such as FOXO3a signaling, is not already stimulated. A similar result showing that the phosphorylation of SGK1 is increased after 5 min of incubation with hypotonic culture medium and returns to the normal level within 20 min in pulmonary artery smooth muscle cells has been reported [13]. We speculate that the difference in time course between this study and our study was due to the different cell types. To achieve long-term regulation of SGK1 phosphorylation, we used SGK1 siRNA and adenovirus to alter the expression of SGK1 in subsequent experiments. The results showed that increased SGK1 prevented H₂O₂-induced apoptosis in BASMCs. Therefore, it appears that the opening of VRCCs induces the activation of SGK1, resulting in a subsequent inhibitory effect on the apoptosis signaling cascade.

Regarding the mechanism of VRCC-induced SGK1 phosphorylation, we speculate that there are at least two possible mechanisms. The first is the phosphoinositide-3-kinase (PI3K) signaling pathway. It has been demonstrated that SGK1 is highly homologous to the Akt kinase family, sharing similar upstream activators, such as PI3K [9, 14]. Previous works have reported that silencing candidate basic components of VRCCs, such as LRRC8a [3, 4] and CIC-3 [6, 7], remarkably reduces the activation of PI3K/Akt signaling pathway [13, 39, 40], indicating that VRCCs might regulate SGK1 activity via PI3K. The second pathway is the WNK1 signaling pathway. WNK1 is a serine-threonine protein kinase with an atypically placed catalytic lysine, and the WNK family is sensitive to the [Cl⁻]_i [41]. WNK1 has been found to activate SGK1 directly to regulate

the action of epithelial sodium channels [42]. In addition, hypotonic challenge can evoke WNK phosphorylation [19]. Therefore, WNK1 might be another link between VRCCs and SGK1. Moreover, WNK1 activates SGK1 in a PI3K-dependent manner, indicating an effect of crosstalk between PI3K and WNK1 on SGK1 activity [43]. However, these speculations need further exploration in the future.

As of now, the functional significance of SGK1 is still far from understood. Functional analysis of gene-targeted mice lacking SGK1 has provided insights into the SGK1-dependent regulation of physiological functions. Although SGK1 knockout does not lead to a severe phenotype, the multiple physiological deficits it induces, such as a decreased ability to retain salt, an enhanced ability to excrete K^+ [44, 45], resistance to hypertension [46], blunted intestinal glucose uptake and decreased uptake of glucose into peripheral tissues [47], point to the broad functional role of SGK1 [9]. In this regard, targeting SGK1 might present a novel therapeutic strategy to benefit SGK1-related diseases.

In summary, our findings show that SGK1 mediates the protective effect of VRCCs against H_2O_2 -induced mitochondria-dependent apoptosis in BASMCs. This work highlights the role of SGK1, which is downstream of VRCCs, in H_2O_2 -induced apoptosis in BASMCs and sheds new light on the treatment of apoptosis-associated cardiovascular diseases, such as vascular remodeling, angiogenesis, and atherosclerosis.

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AUTHOR CONTRIBUTIONS

YYG designed the study; MMM and BYC wrote the manuscript; BYC, CCH, XFL, HQZ, YJZ, and LS performed the experiments; BYC, CCH, XFL, and MMM analyzed the data; YYG and GLW revised the manuscript; YYG and MMM are the guarantors of this work and, as such, had full access to all the data in this study and take responsibility for the integrity of the data and the accuracy of the data analysis.

ADDITIONAL INFORMATION

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