frontiers in Pharmacology

ORIGINAL RESEARCH
published: 27 March 2019
doi: 10.3389/fphar.2019.00313

Sulfur Dioxide Activates Cl^-/HCO_3^- Exchanger via Sulphenylating AE2 to Reduce Intracellular pH in Vascular Smooth Muscle Cells

*Yi Wang[1], Xiuli Wang[1], Selena Chen[2], Xiaoyu Tian[1], Lulu Zhang[1], Yaqian Huang[1], Chaoshu Tang[3,4], Junbao Du[1] and Hongfang Jin[1]**

[1] Department of Pediatrics, Peking University First Hospital, Beijing, China, [2] Division of Biological Sciences, University of California, San Diego, La Jolla, CA, United States, [3] Department of Physiology and Pathophysiology, Peking University Health Science Center, Beijing, China, [4] Key Laboratory of Molecular Cardiology, Ministry of Education, Beijing, China

OPEN ACCESS

Edited by:
Francesco Rossi,
Università degli Studi della Campania Luigi Vanvitelli, Italy

Reviewed by:
Antonella Liantonio,
University of Bari Aldo Moro, Italy
Javier Angulo,
Hospital Universitario Ramón y Cajal, Spain

***Correspondence:**
Hongfang Jin
jinhongfang51@126.com

Specialty section:
This article was submitted to Cardiovascular and Smooth Muscle Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *23 November 2018*
Accepted: *14 March 2019*
Published: *27 March 2019*

Citation:
Wang Y, Wang X, Chen S, Tian X, Zhang L, Huang Y, Tang C, Du J and Jin H (2019) Sulfur Dioxide Activates Cl^-/HCO_3^- Exchanger via Sulphenylating AE2 to Reduce Intracellular pH in Vascular Smooth Muscle Cells. Front. Pharmacol. 10:313. doi: 10.3389/fphar.2019.00313

Sulfur dioxide (SO_2) is a colorless and irritating gas. Recent studies indicate that SO_2 acts as the gas signal molecule and inhibits vascular smooth muscle cell (VSMC) proliferation. Cell proliferation depends on intracellular pH (pH_i). Transmembrane cystein mutation of Na^+- independent Cl^-/HCO_3^- exchanger (anion exchanger, AE) affects pH_i. However, whether SO_2 inhibits VSMC proliferation by reducing pH_i is still unknown. Here, we investigated whether SO_2 reduced pH_i to inhibit the proliferation of VSMCs and explore its molecular mechanisms. Within a range of 50–200 μM, SO_2 was found to lower the pH_i in VSMCs. Concurrently, NH_4Cl pre-perfusion showed that SO_2 significantly activated AE, whereas the AE inhibitor 4,4′-diisothiocyanatostilbene-2,20-disulfonic acid (DIDS) significantly attenuated the effect of SO_2 on pH_i in VSMCs. While 200 μM SO_2 sulphenylated AE2, while dithiothreitol (DTT) blocked the sulphenylation of AE2 and subsequent AE activation by SO_2, thereby restoring the pH_i in VSMCs. Furthermore, DIDS pretreatment eliminated SO_2-induced inhibition of PDGF-BB-stimulated VSMC proliferation. We report for the first time that SO_2 inhibits VSMC proliferation in part by direct activation of the AE via posttranslational sulphenylation and induction of intracellular acidification.

Keywords: sulfur dioxide, vascular smooth muscle cell, intracellular pH, Cl^-/HCO_3^- exchanger, AE2 sulphenylation

INTRODUCTION

Aberrant proliferation of vascular smooth muscle cells (VSMCs) contributes to the pathological change of vascular diseases such as hypertension, diabetic angiopathy and atherosclerosis (Owens et al., 2004; Chistiakov et al., 2015; Bennett et al., 2016). Previous studies have shown that the intracellular pH (pH_i) is an important factor involved in the regulation of cell proliferation. Additionally, cell proliferation of multiple species is dependent on the pH_i. Mitogen stimulation

promotes cell cycle progression and ultimately proliferation. DNA, RNA and protein synthesis all require an alkaline pH_i (Schreiber, 2005; Flinck et al., 2018).

To maintain pH_i homeostasis, cells utilize ionophores on the membrane to regulate the pH_i within a narrow physiological range (Cardone et al., 2005; Boron et al., 2009; Casey et al., 2010). Those ionophores include channels, pumps, exchangers and cotransporters, all of which synergistically regulate the influx and outflux of H^+/HCO_3^- ions (Concepcion et al., 2013; Chen et al., 2018). Among them, the Na^+-independent and electroneutral Cl^-/HCO_3^- exchanger (anion exchanger, AE) is encoded by the *SLC4* gene family, including *SLC4A1/AE1, SLC4A2/AE2,* and *SLC4A3/AE3*, which help regulate the pH_i, cell volume and membrane potential of various cell types (Alper, 2009; Liu et al., 2015). Genome-wide association analysis showed that *AE2* exon deletion resulted in a loss of function of AE2 in osteoclast and cell alkalization, resulting in bone resorption lacunae disorder, the genetic cause of Angoras cattle and mouse osteopetrosis (Meyers et al., 2010; Coury et al., 2013). Transforming growth factor beta 1 promotes fibroblast cell membrane AE2 expression and HCO_3^- excretion, which can neutralize tumor microenvironment H^+ ions to inhibit tumor cell invasion (Hulikova et al., 2016). Concepcion et al. (2014) found that compared with wild-type mice, the pH_i of $CD8^+$ T cells derived from *AE2* knockout mice is significantly increased, and $CD8^+$ T cell proliferation and activation levels are obviously enhanced after CD3 stimulation.

Reimold et al. (2013) constructed a mouse AE2 model devoid of transmembrane domain cysteine (Cys) residues to investigate structure-function relationships for AE2. They found that extracellular pH was alkaline-shifted by a minimum of 0.6-0.7 pH units, and the anion exchange rate was significantly decreased in the absence of transmembrane domain Cys residues (Reimold et al., 2013). Sulfur dioxide (SO_2) was found to oxidize the −SH of Cys to −SOH, which changed the protein conformation and affected protein activity levels (Svoboda et al., 2012). We confirmed that SO_2 suppressed the inflammatory response by sulphenylating NF-κB p65 at Cys^{38} in oleic acid-induced acute lung injury (Chen et al., 2017).

Recent studies have shown that aspartate aminotransferase can be catalyzed and produces SO_2 in the metabolic pathway of sulfur-containing amino acids in mammalian organisms. Furthermore, SO_2, which is considered the fourth gas signal molecule followed by NO, CO and H_2S, plays an important role in the regulation of cardiovascular physiology and pathophysiology, such as vasodilation, inhibition of vascular calcification and inflammation, anti-oxidation and protection of the myocardium (Du et al., 2008; Sun et al., 2010; Zhang et al., 2011; Huang et al., 2016; Li et al., 2016; Yu et al., 2018).

Therefore, we hypothesized that SO_2 might activate the AE in VSMCs, thereby lowering the pH_i and further inhibiting VSMC proliferation. It has been reported that AE expression varies significantly among different tissues, and only *SLC4A2/AE2* mRNA expression is detected in the VSMC cell line A7r5 cells (Brosius et al., 1997). Therefore, in this study, we aimed to determine whether SO_2 impacts the pH_i and further inhibits the proliferation of VSMCs, as well as explore underlying molecular mechanisms.

MATERIALS AND METHODS

Chemicals and Drugs

Sodium sulfite and sodium bisulfite ($Na_2SO_3/NaHSO_3$), 4,4′-diisothiocyanatostilbene -2,20-disulfonic acid (DIDS) and H89 were purchased from Sigma-Aldrich (St. Louis, MO, United States). Nigericin (N1495) and Fluo 4-AM (F14217) were purchased from Invitrogen (Eugene, OR, United States), and BCECF/AM was purchased from Thermo Scientific (Waltham, MA, United States). Dithiothreitol (DTT) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Bay K8644 was purchased from selleck (Houston, TX, United States). SO_2 donor was freshly prepared using $Na_2SO3/NaHSO_3$ dissolved in deionized water in 3:1 mole ratio. DIDS, H89, BCECF/AM and Fluo 4-AM were dissolved in DMSO. Nigericin was dissolved in ethanol.

Cells and Cell Culture

A7r5 VSMCs were purchased from Kunming Cell Bank of Chinese Academy of Sciences (Kunming, China). Cell culture refers to previous literature (Liu et al., 2014). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin), and cells were placed in an incubator containing 5% CO_2 at 37°C. To detect the pH_i, cells were seeded in confocal dishes and experiments were performed once the cell density reached 90%.

Measurement of pH_i in VSMCs

Measurement of pH_i was performed according to the literature (Lee et al., 2007; Galifianakis et al., 2018). Fluorescent indicator BCECF/AM was used to monitor pH_i changes. Cells were washed twice with Krebs' bicarbonate buffer, followed by the incubation with Krebs' buffer containing 0.5 μM BCECF/AM for 30 min at room temperature. Loaded cells were washed twice with fresh Krebs' buffer to remove unbound dye and left at room temperature for another 30 min to allow the dye to be fully de-esterified in the cells. The pH_i was monitored using a confocal scanning laser microscope (Leica TCS SP8 MP FLIM, Mannheim, Baden-Württember, Germany). The fluorescence intensity was measured at an excitation wavelength of 405 and 496 nm, and an emission wavelength of 535 nm was recorded. The fluorescence intensity ratio (F496/F405) was used to evaluate the pH_i. pH_i image analysis was performed using LAF-AS software (Leica). The Krebs' buffer was prepared as follows (mM): 118 NaCl, 5.4 KCl, 1.3 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 11.7 glucose, and the pH was adjusted to 7.4 with NaOH. During the measurement of pH_i, drugs were added directly to the buffer and the fluorescence intensity was recorded. A high KCl solution (130 mM KCl, 10 mM Hepes buffer, pH 6.3–9.1) was prepared, and H^+ was equilibrated with 20 μM cation ionophore nigericin to prepare a fluorescence intensity-pH_i standard curve (**Supplementary Figure S1**). The different concentrations of SO_2

in the study of pH_i were grouped into control, SO_2 (50 μM), SO_2 (100 μM), and SO_2 (200 μM). The change of pH_i was calculated as the difference in pH_i between the starting time point of SO_2 treatment and 9 min after SO_2 treatment.

Determination of AE Activity in VSMCs

AE activity was measured according to the method described in the previous literature (Simchowitz and roos, 1985; Xu and Spitzer, 1994; Lee et al., 2007). In brief, the activity of AE in A7r5 cells was evaluated by detecting the recovery rate of cells from an intracellular alkalinization. Cells were loaded with BCECF/AM as previously described. The Hepes buffer solution was prepared as follows (mM): 150 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 Hepes and 10 glucose, adjusted to pH 7.4 with NaOH. At the first min, 20 mM NH_4Cl was quickly added to the buffer. Once the cells were exposed to NH_4Cl, NH_3 rapidly diffused into the cells and combined with intracellular H^+ to form NH_4^+, leading to rapid intracellular alkalinization. The pH_i gradually decreases from the alkaline peak as HCO_3^- ions efflux during AE stimulation. The rate at which the pH_i returns within the first minute (ΔpH/min) represents AE activity. For the SO_2 and the AE activation experiment, the groups were divided as follows: control, SO_2 (100 μM) and SO_2 (200 μM), and SO_2 donor administrated 10 min before pH_i measurement. To verify that SO_2 stimulated AE via sulphenylation of AE2, we divided the groups as follows: control, SO_2, SO_2 + DTT or PDGF-BB, PDGF-BB + SO_2 and PDGF-BB + SO_2 + DTT. One hour before the pH_i measurement, cells were treated with 50 ng/ml PDGF-BB, and 50 min before pH_i measurement, cells were pretreated with 200 μM SO_2 and / or 0.4 mM DTT.

Western Blotting

Vascular smooth muscle cells were seeded in six-well plates and upon a cell density of 60–70%, were synchronized with DMEM containing 0.5% FBS for 24 h.

To confirm that AE was involved in the process of SO_2 inhibition of PDGF-BB-induced VSMC proliferation, cell were either (1) untreated; (2) treated with 50 ng/ml PDGF-BBC for 24 h; (3) pretreated with 200 μM SO_2 donor for 30 min, then 50 ng/ml PDGF-BB for 24 h; (4) treated with 200 μM SO_2 donor for 30 min; (5) treated with 50 ng/ml PDGF-BB and 30 μM DIDS for 24 h; and (6) pretreated with 200 μM SO_2 donor for 30 min, then 50 ng/ml PDGF-BB and 30 μM DIDS for 24 h.

To measure the phosphorylation level of PKA by SO_2, cells were either (1) untreated; (2) treated with 20 μM H89 for 30 min; (3) pretreated with 20 μM H89 for 30 min, and 200 μM SO_2 donor for 10 min; or (4) treated with 200 μM SO_2 donor for 10 min.

All cells were collected and lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.25% sodium deoxycholate, 1 mM PMSF, protease and phosphatase inhibitors) for 20 min at 4°C. They were then centrifuged at 12000 rpm for 10 min at 4°C, and 2x denatured protein loading buffer was added to the supernatant. The mixture was boiled at 100°C for 10 min and cooled at room temperature. Equal amounts of protein (30–60 μg) were run on an 8–10% SDS-PAGE gel. After protein separation, they were transferred to nitrocellulose membranes. The primary antibody dilutions were: 1:1000 for both PKA and p-PKA, and 1:500 for Ki67.

Measurement of VSMC Proliferation With Cell Counting Kit-8 (CCK8)

Cell proliferation was measured according to the reference (Liu et al., 2014). By using a CCK-8 kit, A7r5 cells were first seeded in 96-well plates at 2×10^3 cells / well, and divided into seven groups: (1) blank control (cell-free medium); (2) control (cell-containing medium); (3) treated with 50 ng/ml PDGF-BBC for 24 h; (4) pretreated with 200 μM SO_2 donor for 30 min, then 50 ng/ml PDGF-BB for 24 h; (5) treated with 200 μM SO_2 donor for 30 min; (6) treated with 50 ng/ml PDGF-BB and 30 μM DIDS for 24 h; and (7) pretreated with 200 μM SO_2 donor for 30 min, then 50 ng/ml PDGF-BB and 30 μM DIDS for 24 h. Ten μl of CCK8 reagent was added into a 100 μl- medium, and incubated at 37°C for 4 h. The absorbance was measured at 450 nm using a microplate reader. The mean absorbance value of the blank control group was subtracted from each group as the corrected absorbance of each group.

Measurement of AE2 Sulphenylation in VSMCs

Sulphenic acid modification of AE2 was measured as described previously (Chen et al., 2017). The cells were divided into three groups: untreated, treated with 200 μM SO_2 donor for 10 min, or simultaneously with SO_2 donor and 0.4 mM DTT for 10 min. Cells were pretreated with 50 ng/ml PDGF-BB for 1 h to induce cell proliferation. DAz-2 is a specific sulphenic acid probe used to label proteins modified by sulphenic acid. The cells were lysed with non-denaturing lysis buffer containing 5 mM DAz-2 and centrifuged at 16000 *g* for 4 min at 4°C. The supernatant was divided into two parts. 10 μl was used as total protein, which was added to denatured protein loading buffer and boiled for 10 min at 100°C. The remaining supernatant was incubated at 37°C for 2 h with gentle shaking to extract the sulphenic acid modified protein. The mixture was then incubated with 250 μM p-biotin for 2 h at 37°C with gentle shaking to label the protein with biotin. Biotinylated proteins were precipitated with UltraLinkTM Immobilized NeutrAvidinTM (Thermo Fisher Scientific, Waltham, MA, United States) and incubated for 4 h on a shaker at 4°C. The beads were washed three times with PBS, and non-denatured protein loading buffer was added and boiled at 100°C for 10 min. Total protein and sulphenic acid modified protein were subjected to WB analysis. The primary antibody for AE2 was diluted 1:500. The secondary antibody was diluted 1:2000.

Imaging the Intracellular Calcium in VSMCs

The intracellular calcium in VSMCs was imaged with fluorescent calcium probe, Fluo 4-AM. A7r5 cells were divided into four groups: PDGF-BB, PDGF-BB + SO_2, PDGF-BB + Bay K8644, and PDGF-BB + Bay K8644 + SO_2. Cells were incubated with 50 ng/ml PDGF-BB for 1 h, 1 μM Bay K8644, an L-type calcium channel agonist, for 30 min, and 200 μM SO_2 for 10 min. After

the treatment, the cells were washed with Krebs' buffer for twice and incubated with 5 μM Fluo 4-AM in the dark for 30 min at 37°C. The unincorporated dye was removed by washing the cells twice. Loaded cells were maintained at room temperature for another 30 min to allow Fluo 4-AM to de-esterify. Fluorescence image was obtained using a laser scanning confocal microscope (Olympus), at appropriate wavelength settings (excitation at 488 nm and emission at 520 nm).

Statistical Analysis

Data were processed using SPSS 17.0 software (SPSS Inc, Chicago, IL, United States). All data were expressed as mean ± standard error. To examine the effect of SO_2 on AE, a *t*-test was performed to compare the difference between control and DIDS. To examine the effect of different concentrations of SO_2 on AE, PKA phosphorylation and A7r5 cell proliferation, comparisons of three or more groups were analyzed by ANOVA, and the Bonferroni test or the Dunnett T3 test were used to compare the difference between two groups. $p < 0.05$ was considered statistically significant.

RESULTS

SO_2 Reduced pH_i in VSMCs

By real-time monitoring of the pH_i with the fluorescent probe, we found that the SO_2 donor at the concentrations of 50, 100, and 200 μM decreased the pH_i in VSMCs by 0.120 ± 0.012, 0.134 ± 0.011, and 0.200 ± 0.020, respectively (all $p < 0.01$, **Figures 1A,B**).

SO_2 Activated AE to Reduce pH_i in VSMCs

Anion exchanger is the main acid loader of VSMCs, which pumps out one HCO_3^- in exchange for one Cl^- into the cells, maintaining intracellular Cl^- concentration and lowering the pH_i. To further validate the effect of SO_2 on the AE, we tested the activity of the AE in VSMCs by using the widely accepted NH_4Cl perfusion method. As shown in **Figures 2A,B**, the SO_2 donor at varying concentrations of 100 and 200 μM activated the AE in VSMCs ($p < 0.05$ and $p < 0.01$, respectively).

Pretreatment with 30 μM DIDS, a Cl^-/HCO_3^- exchanger inhibitor, for 20 min significantly attenuated the pH_i reduction caused by SO_2 ($p < 0.01$, **Figures 3A,B**), suggesting that SO_2 donor reduced pH_i by activating the AE in VSMCs.

SO_2 Activated AE by Sulphenylating AE2 in VSMCs

To investigate the mechanism by which SO_2 activates AE activity in VSMCs, we tested whether SO_2 oxidizes Cys of AE2 by AE2 sulphenylation. As shown in **Figures 4A,B**, SO_2 promoted AE2 sulphenylation and AE activation ($p < 0.05$, $p < 0.01$, respectively), and reduced pH_i ($p < 0.05$). Addition of 0.4 mM DTT, a thiol reductant, reversed SO_2-induced AE2 sulphenylation and activation of the AE (both $p < 0.05$), restoring SO_2-induced pH_i reduction in VSMCs as well ($p < 0.01$, **Figure 4C**). This suggests that the thiol group is a likely target of SO_2 for activation of the AE in VSMCs.

We also aimed to understand if SO_2 affects the pH_i by activation of PKA and further activation of the exchanger in VSMCs. As shown in **Figure 4D**, SO_2 (200 μM) did not stimulate PKA within 10 min in VSMCs ($p > 0.05$). The results suggested that the PKA pathway did not mediate pH_i reduction by SO_2 in an acute phase.

SO_2 Inhibited VSMC Proliferation Depending on AE Activation

To further elucidate if SO_2 inhibit VSMC proliferation depending on AE activation, we constructed a PDGF-BB-induced VSMC proliferation cell model. In VSMCs pretreated with PDGF-BB and treated with SO_2, levels of AE2 sulphenylation and AE activity were significantly increased ($p < 0.05$, $p < 0.01$, respectively, **Figures 5A,B**) and the pH_i was significantly reduced ($p < 0.05$, **Figure 5C**).

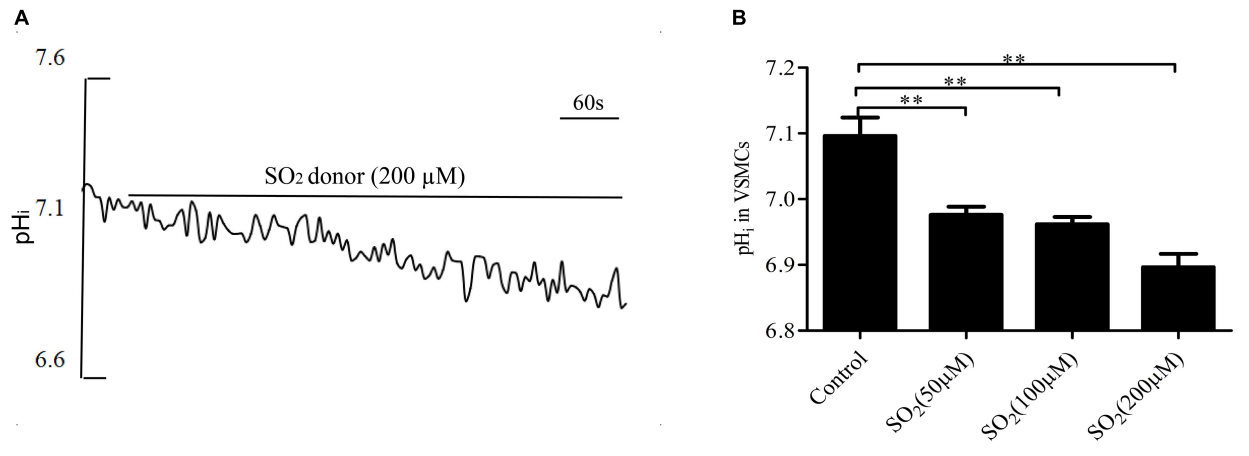

FIGURE 1 | SO_2 decreased the pH_i in VSMCs. **(A)** Representative tracing showing 200 μM SO_2 donor. **(B)** Effect of SO_2 donor on pH_i (n = 16–33). $^{**}p < 0.01$ compared with pH_i before SO_2 donor application.

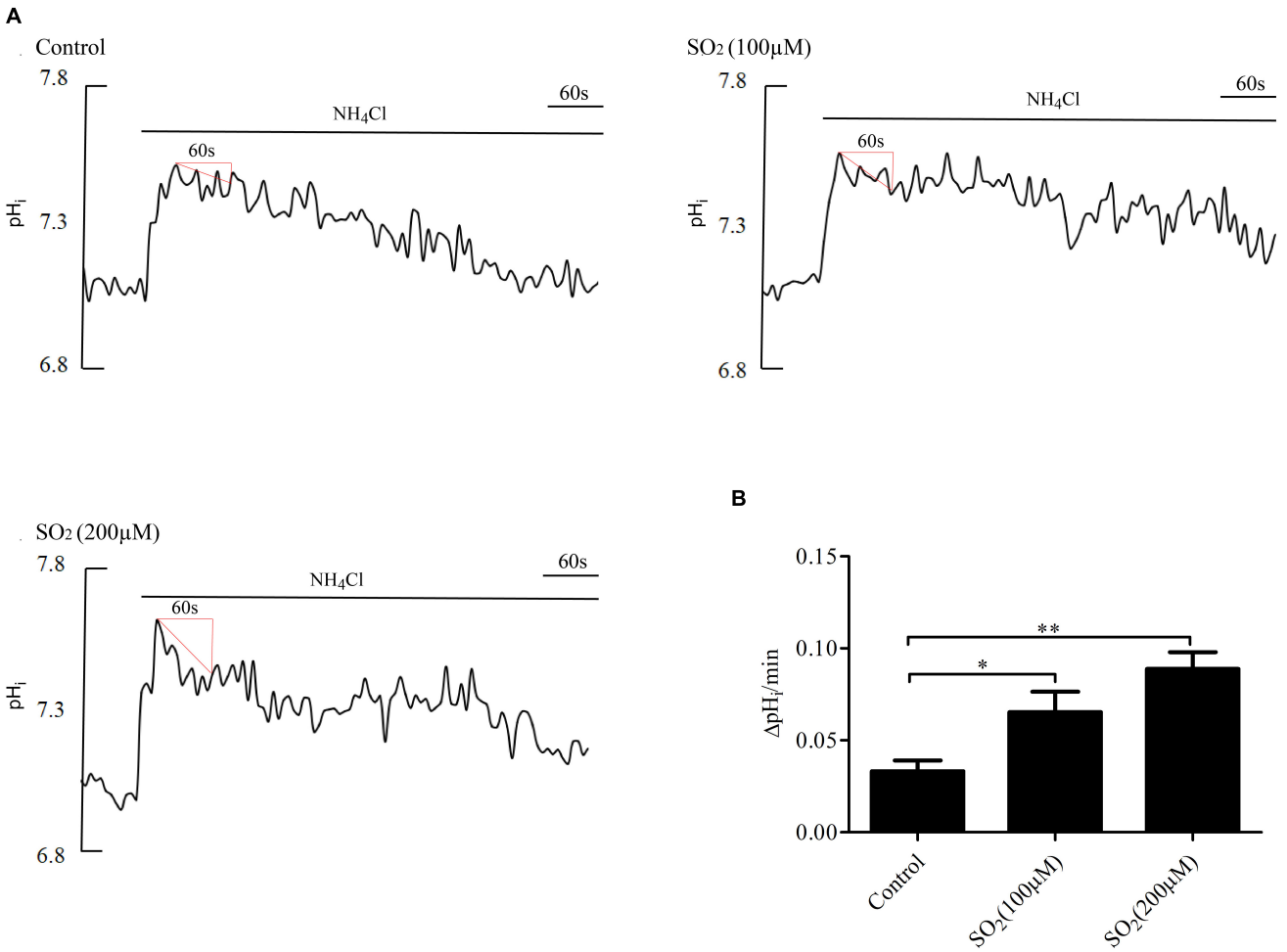

FIGURE 2 | Effect of SO_2 on AE activity. **(A)** Representative tracings of the rapid alkalinization of 20 mM NH_4Cl and recovery from higher pH_i at different concentrations. SO_2 was given 10 min before measurement. **(B)** Results were shown in mean ± SEM (n = 25–39). *$p < 0.05$, **$p < 0.01$ compared with control.

VSMC proliferation evaluated by Ki67 protein expression and CCK8 activity was significantly inhibited by SO_2 ($p < 0.05$, $p < 0.01$, respectively, **Figures 5D,E**), as compared with those in VSMCs pretreated with PDGF-BB only. However, SO_2 failed to inhibit PDGF-BB-induced VSMCs proliferation once AE was inhibited by DIDS (both $p > 0.05$, **Figures 5D,E**).

SO_2 Reduced the Intracellular Calcium by Inhibiting L-Type Calcium Channel in VSMCs

To investigate the effects of SO_2 on intracellular calcium in VSMCs, we used Fluo 4-AM to image the intracellular calcium in VSMCs. The results showed that SO_2 reduced PDGF-stimulated intracellular calcium in VSMCs. While, Bay K8644, an L-type calcium channel agonist, blocked SO_2-reduced intracellular calcium content in PDGF-stimulated VSMCs (**Figure 6**). The results indicated that SO_2 might decrease the calcium level by inhibiting L-type calcium channel in VSMCs.

DISCUSSION

As well known, pH_i is precisely controlled and needs to be maintained within physiological range. The imbalance of pH_i is an important pathological basis for the abnormal cell metabolism and life activities. Therefore, pH_i is a vital target for clinical treatment of diseases. In the present study, we firstly reported that SO_2 donor decreased the pH_i of VSMCs by enhancing AE2 sulphenylation to activate the AE, which might partially mediate the inhibitory effect of SO_2 on VSMC proliferation.

At first, by monitoring pH_i in real time, we found that the treatment of 50 ∼ 200 μM SO_2 donor for 9 min decreased pH_i in VSMCs by 1.7–2.8%, respectively. The mechanisms by which SO_2 reduced pH_i in VSMCs, however, have yet been unclear. AE is the main acid loader in VSMCs and plays an important role in the regulation of pH_i (Vigne et al., 1988). Previous studies reported that pH_i was increased significantly when NH_4Cl loaded cells, and consequently AE was activated to extrude HCO_3^-, which eventually restored the pH_i to normal levels (Xu and Spitzer, 1994; Lee et al., 2007). Therefore, we

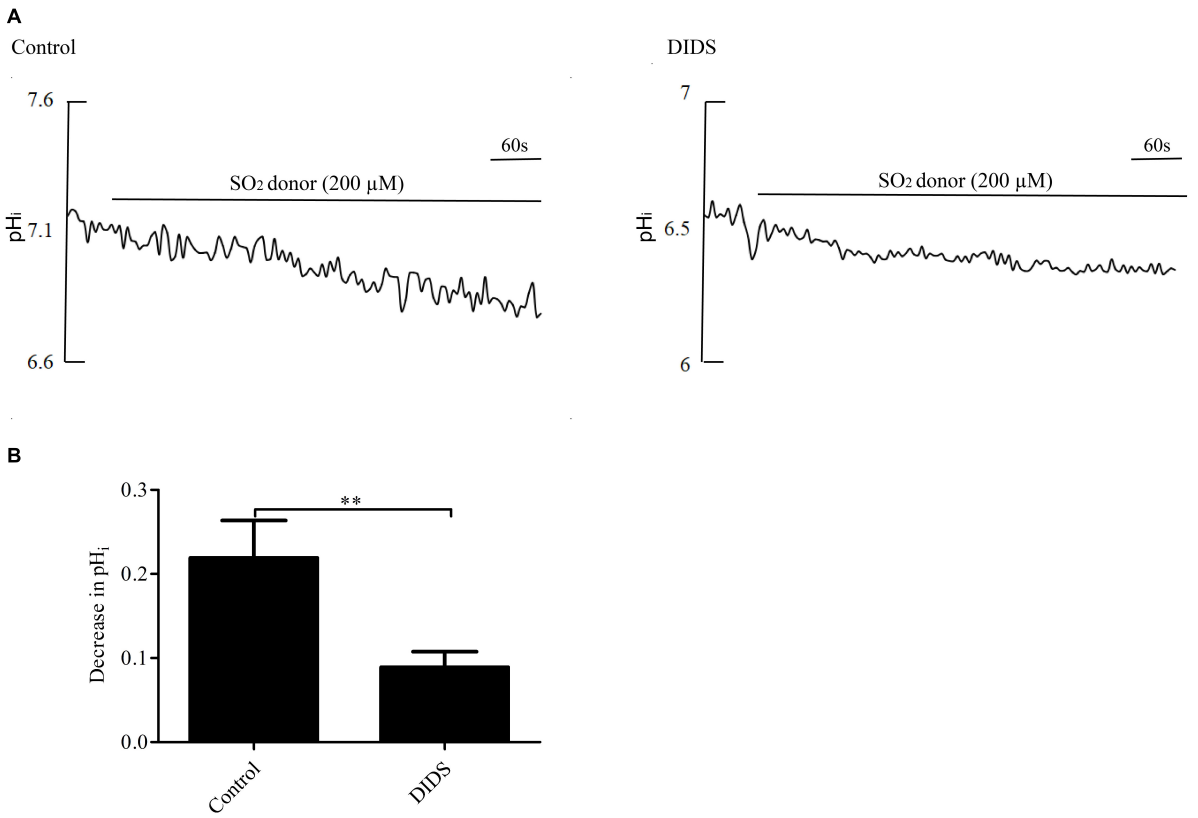

FIGURE 3 | Effect of SO_2 on pH_i in the presence or absence of DIDS in VSMCs. **(A)** Representative tracings of 200 μM SO_2 donor alone (n = 19) and in the presence of 30 μM DIDS (n = 19). **(B)** Results were shown in mean ± SEM. $^{**}p < 0.01$ compared with control.

directly detected the activity of AE using NH_4Cl stimulation as previously reported (Simchowitz and roos, 1985). The data showed that SO_2 donor markedly steepened the recovery slope of pH_i trace following the pH_i peak due to NH_4^+-induced alkalinization, suggesting that SO_2 activated AE in VSMCs as we expected. Subsequently, the fact that an inhibitor of Cl^-/HCO_3^- exchanger, DIDS blunted the SO_2-induced decrease in pH_i in VSMCs supported the speculation that the activation of AE was involved in the effect of SO_2 on the pH_i in VSMCs.

However, up to now, the mechanisms by which SO_2 activated the AE in VSMCs remain unclear. Cysteine thiol group (−SH) can be oxidized to sulphenic acids (−SOH) and the process is termed as protein sulphenylation, which is an important mechanism for regulating protein function. Hourihan et al. (2016) found that sulphenylation of inositol-requiring enzyme 1 could inhibit endoplasmic reticulum stress and activate antioxidant response. The sulphenylation of NF-κB p65 by SO_2 resulted in an inactivation of NF-κB pathway (Chen et al., 2017). While Cys residues mutation within transmembrane domain of AE2 could affect the activity of AE2 (Reimold et al., 2013). Interestingly, our data showed that SO_2 sulphenylated AE2 in VSMCs in association with the enhancement of the activity of AE. While, a thiol reductant DTT blocked the effect of SO_2 on sulphenylation and the activity of AE2. Moreover, PKA pathway was also reported to participate the activation of AE (Puceat, 1999) and SO_2 treatment for 30 min activated cAMP/PKA pathway (Liu et al., 2014). But, in the present study, SO_2 incubation for 9 min could not enhance the phosphorylation of PKA, which excluded the possibility that SO_2 indirectly promoted the activation of AE via PKA pathway. Those above results suggested that SO_2 might directly activate the AE by inducing the sulphenylation of AE2 at the posttranslational level.

To investigate the significance of SO_2-induced reduction of pH_i in VSMCs in its inhibitory effect on VSMC proliferation, DIDS was used to block the SO_2-induced reduction of pH_i in VSMCs, and then Ki67 expression and CCK8 activity were analyzed as the markers of VSMC proliferation. We found that SO_2 inhibited PDGF-BB-induced VSMC proliferation, while DIDS abolished the inhibitory effect of SO_2 on PDGF-BB-induced VSMC proliferation, which suggested that SO_2 inhibited VSMC proliferation at least partly through decreasing pH_i in VSMCs.

However, we also found an interesting detached phenomenon that SO_2 donor alone did not inhibit the proliferation of VSMC but inhibited PDGF-BB-induced VSMC proliferation. In fact, under physiological condition, VSMC-derived endogenous SO_2 was sufficient enough to inhibit cell proliferation. Therefore, the supplement of additional exogenous SO_2 donor to the VSMC on the basis of sufficient endogenous SO_2 level would not further exert the anti-proliferative effect (Liu et al., 2014). However,

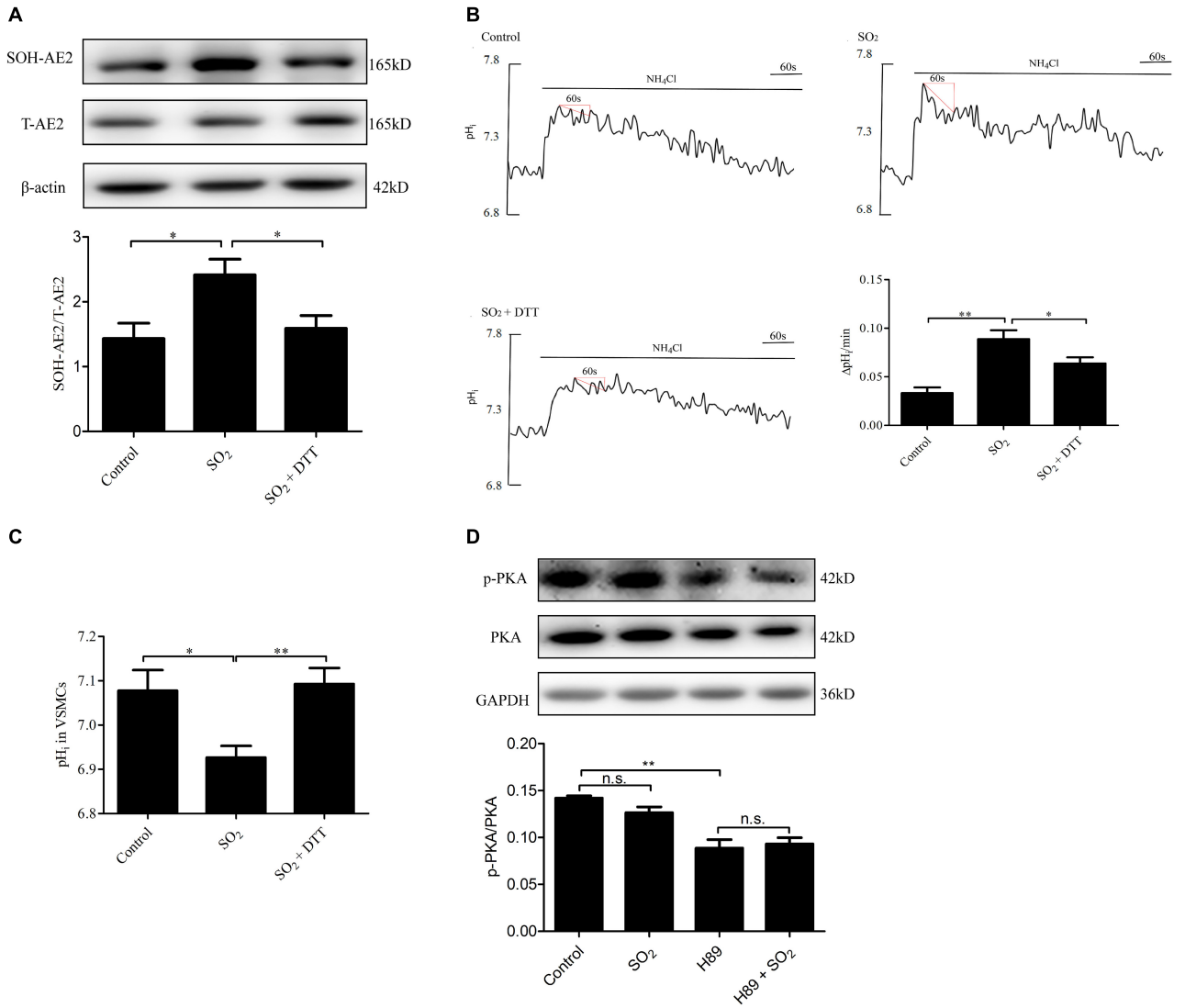

FIGURE 4 | Mechanisms by which SO_2 donor activated AE. **(A)** Sulphenylation of AE2 by 200 μM SO_2, and 0.4 mM DTT reversed the process. SO_2 and DTT were given 10 min before collecting cells (n = 6). **(B)** Representative tracings of AE activity in the absence or presence of 200 μM SO_2 donor and 0.4 mM DTT. Results were shown as mean ± SEM (n = 25–39). **(C)** The change of pHi in VSMCs in each group. **(D)** Effect of SO_2 donor on PKA phosphorylation. Cells were pretreated with H89 30 min, followed by SO_2 donor in 10 min (n = 6). *$p < 0.05$, **$p < 0.01$, n.s.$p > 0.05$.

under certain pathophysiological conditions, when VSMCs were insulted by the exogenous injury stimuli, endogenous SO_2 production was decreased and the anti-proliferative effect was weakened, resulting to the excessive cell proliferation. In such a case, the supplement of SO_2 donor, on the basis of the deficient endogenous SO_2 level, would exert a markedly anti-proliferative effect on the proliferating VSMC (Sun et al., 2010; Liu et al., 2014; Wu et al., 2016; Yu et al., 2016). In brief, this discrepancy effect of SO_2 donor on the VSMC proliferation on the different conditions might provide a novel idea for the treatment of vascular remodeling in vascular-injury diseases.

In addition to pH_i, Ca^{2+} mobilization is another important stimulus for cell migration and proliferation (Yamamura, 2014; Luo et al., 2018). Endogenous SO_2 and its derivates could inhibit L-type calcium channel, which might help explain the mechanism of vasorelaxant function (Du et al., 2008). Therefore, we observed the effect of SO_2 on the L-type calcium channel. The data showed that 200 μM SO_2 could inhibit the PDGF-BB-stimulated increase in the cytosolic Ca^{2+} concentration in VSMCs. However, pretreatment of Bay K8644, a specific activator of L-type calcium channel, could block the inhibitory effect of SO_2 on the cytosolic Ca^{2+} concentration, suggesting that L-type calcium channel inhibition occurs under experimental conditions in the presence of SO_2 donor.

However, our study still has some limitation. For example, we studied the effect of SO_2 on pH_i only in cultured VSMCs, but whether the phenomenon occurs in the complex vascular wall

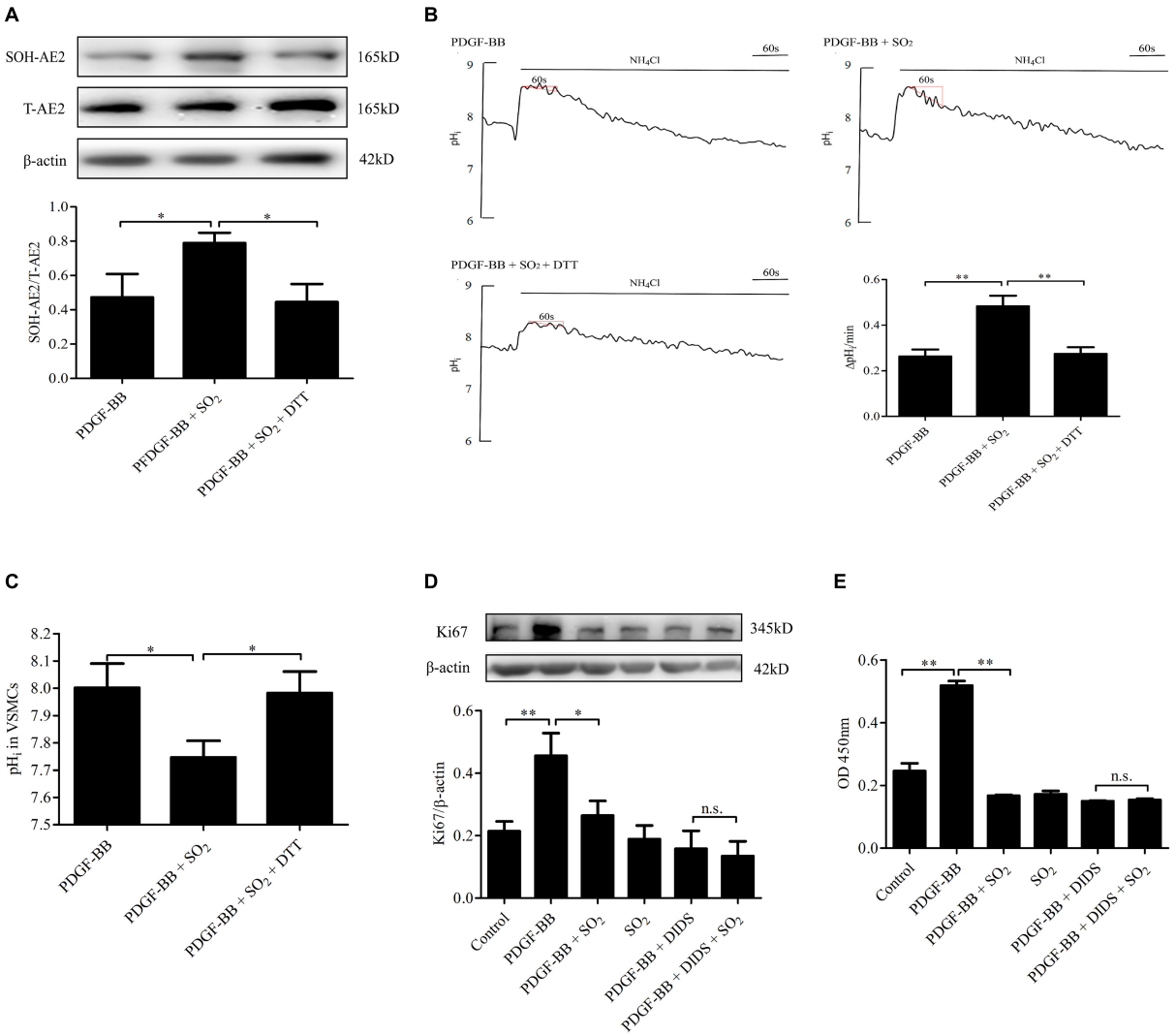

FIGURE 5 | Activation of AE was responsible for SO_2 to inhibit VSMC proliferation. **(A)** Sulphenylation of AE2 by SO_2 in the presence of PDGF-BB. Cells were pretreated with 50 ng/ml PDGF-BB 1 h, followed by 200 μM SO_2 and 0.4 mM DTT 10 min (n = 6). **(B)** Representative tracings of AE activity in the absence or presence of 200 μM SO_2 donor and 0.4 mM DTT when cells were pretreated with 50 ng/ml PDGF-BB 1 h (n = 31–39). **(C)** pH_i change in each group. **(D,E)** WB and CCK8 were used to measure the proliferation of VSMCs. 50 ng/ml PDGF-BB, 200 μM SO_2 and 30 μM DIDS were administered for 24 h (n = 6). *$p < 0.05$, **$p < 0.01$, n.s.$p > 0.05$.

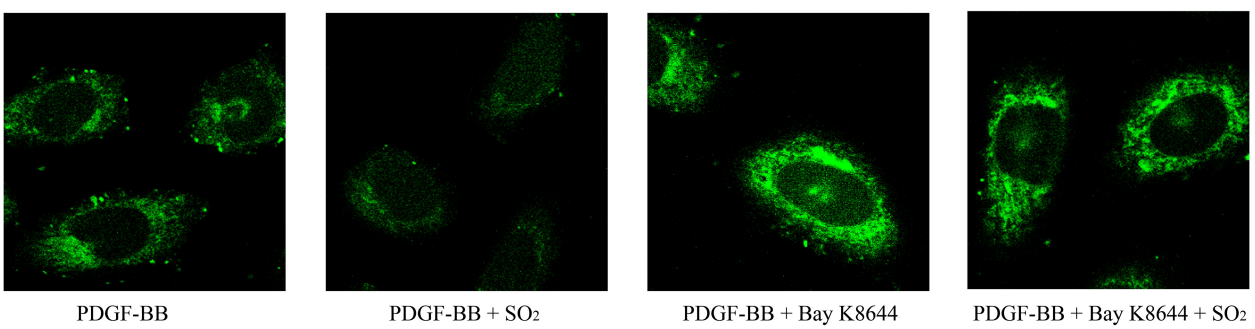

FIGURE 6 | SO_2 decreased the intracellular calcium level by inhibiting L-type calcium channel in VSMCs. Cells were incubated with 50 ng/ml PDGF-BB for 1 h, 1 μM Bay K8644 for 30 min and 200 μM SO_2 for 10 min. Fluorescence intensity represents calcium levels.

is still unknown. As we all know, vascular wall is composed of complex multicellular tissue. It needs to respond to various stimuli such as mechanical stress and neurological and humoral factors in a coordinate manner. The intercellular communication among the constituent cells of vessel wall plays an important role in the regulation of vascular structure and activity and is indispensable for the synchronous response by the wall of vessels. The gap junction was found to exert the abovementioned function of intercellular communication (Haefliger et al., 2004; Sorensen and Holstein-Rathlou, 2012; Yang et al., 2017). The gap junction is composed of a kind of transmembrane proteins termed connexin and form a direct conduit for the exchanges of intercellular signals such as ions and bioactive metabolites, which allows vessel cells to sense the functional and metabolic state of neighbor cells and rapidly modulate the activity by themselves, and therefore synchronously respond to the stimuli (Haefliger et al., 2004; Sorensen and Holstein-Rathlou, 2012; Yang et al., 2017). In addition to the gap junction, gasotransmitters also participate the intercellular communication. Since 1980s, endothelial nitric oxide as the first gasotransmitter was found to regulate the VSMC relaxation via a paracrine pathway, which is partly due to its unique properties including small molecular weight, rapid transmembrane diffusion and extensive action (Wang, 2014; Huang et al., 2016; Kimura, 2016; Nagpure and Bian, 2016). In the previous study, pulmonary artery smooth muscle cell-derived SO_2 was found to inhibit the collagen accumulation in the pulmonary artery fibroblasts (Yu et al., 2016). Therefore, we speculated that SO_2 might act as an intercellular signal molecule to transduce the massagers among the constitute cells in the vessels, which participated in the synchronicity of vascular function. However, more experiments are needed to extend the effect of SO_2 on single channel activity to complex vascular wall.

CONCLUSION

We discovered the effect of SO_2 on pH_i of VSMCs and clarified the mechanism by which SO_2 decreased pH_i of VSMCs. Most importantly, we demonstrated that SO_2-induced decrease in pH_i of VSMCs might participate the inhibitory effect of SO_2 donor on the VSMC proliferation stimulated by mitogen such as PDGF-BB. We expect that those interesting results maybe provide a new idea for the potential clinical prevention and treatment of vascular remodeling in vascular injury diseases such as hypertension.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

YW, JD, and HJ designed the study. YW performed the experiments, analyzed the data, and wrote the manuscript. HJ, JD, and SC revised the manuscript. CT and YH provided useful suggestion and comments to the design of the research. XW, XT, and LZ were involved in the experiments. All the authors read the manuscript and approved the final version.

FUNDING

This study was supported by National Natural Science Foundation of China (Nos. 31130030 and 81622004).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00313/full#supplementary-material

FIGURE S1 | The fluorescence intensity-pH_i standard curve obtained with nigericin.

REFERENCES

Alper, S. L. (2009). Molecular physiology and genetics of Na^+-independent SLC4 anion exchangers. *J. Exp. Biol.* 212, 1672–1683. doi: 10.1242/jeb.029454

Bennett, M. R., Sinha, S., and Owens, G. K. (2016). Vascular smooth muscle cells in atherosclerosis. *Circ. Res.* 118, 692–702. doi: 10.1161/CIRCRESAHA.115.306361

Boron, W. F., Chen, L., and Parker, M. D. (2009). Modular structure of sodium-coupled bicarbonate transporters. *J. Exp. Biol.* 212, 1697–1706. doi: 10.1242/jeb.028563

Brosius, F. C. III, Pisoni, R. L., Cao, X., Deshmukh, G., Yannoukakos, D., Stauart-tilley, A. K., et al. (1997). AE anion exchanger mRNA and protein expression in vascular smooth muscle cells, aorta, and renal microvessels. *Am. J. Physiol.* 273, F1039–F1047. doi: 10.1152/ajprenal.1997.273.6.F1039

Cardone, R. A., Casavola, V., and Reshkin, S. J. (2005). The role of disturbed pH dynamics and the Na^+/H^+ exchanger in metastasis. *Nat. Rev. Cancer.* 5, 786–795. doi: doi.org/10.1038/nrc1713

Casey, J. R., Grinstein, S., and Orlowski, J. (2010). Sensors and regulators of intracellular pH. *Nat. Rev. Mol. Cell. Biol.* 11, 50–61. doi: 10.1038/nrm2820

Chen, G. S., Lee, S. P., Huang, S. F., Chao, S. C., Chang, C. Y., Wu, G. J., et al. (2018). Functional and molecular characterization of transmembrane intracellular pH regulators in human dental pulp stem cells. *Arch. Oral. Biol.* 90, 19–26. doi: 10.1016/j.archoralbio.2018.02.018

Chen, S., Huang, Y., Liu, Z., Yu, W., Zhang, H., Li, K., et al. (2017). Sulphur dioxide suppresses inflammatory response by sulphenylating NF-κB p65 at Cys38 in a rat model of acute lung injury. *Clin. Sci.* 131, 2655–2670. doi: 10.1042/CS20170274

Chistiakov, D. A., Orekhov, A. N., and Bobryshev, Y. V. (2015). Vascular smooth muscle cell in atherosclerosis. *Acta Physiol.* 214, 33–50. doi: 10.1111/apha.12466

Concepcion, A. R., Lopez, M., Ardura-Fabregat, A., and Medina, J. F. (2013). Role of AE2 for pHi regulation in biliary epithelial cells. *Front. Physiol.* 4:413. doi: 10.3389/fphys.2013.00413

Concepcion, A. R., Salas, J. T., Sarvide, S., Saez, E., Ferrer, A., Lopez, M., et al. (2014). Anion exchanger 2 is critical for $CD8^+$ T cells to maintain pHi homeostasis and modulate immune responses. *Eur. J. Immunol.* 44, 1341–1351. doi: 10.1002/eji.201344218

Coury, F., Zenger, S., Stewart, A. K., Stephens, S., Neff, L., Tsang, K., et al. (2013). SLC4A2-mediated Cl^-/HCO_3^- exchange activity is essential for calpain-dependent regulation of the actin cytoskeleton in osteoclasts. *Proc. Natl. Acad. Sci. U.S.A.* 110, 2163–2168. doi: 10.1073/pnas.1206392110

Du, S. X., Jin, H. F., Bu, D. F., Zhao, X., Geng, B., Tang, C. S., et al. (2008). Endogenously generated sulfur dioxide and its vasorelaxant effect in rats. *Acta Pharmacol. Sin.* 29, 923–930. doi: 10.1111/j.1745-7254.2008.00845.x

Flinck, M., Kramer, S. H., and Pedersen, S. F. (2018). Roles of pH in control of cell proliferation. *Acta Physiol.* 223:e13068. doi: 10.1111/apha.13068

Galifianakis, N. V., Placantonakis, D. G., and Chesler, M. (2018). Intracellular pH measurements in glioblastoma cells using the pH-sensitive dye BCECF. *Methods Mol. Biol.* 1741, 103–109. doi: 10.1007/978-1-4939-7659-1_8

Haefliger, J. A., Nicod, P., and Meda, P. (2004). Contribution of connexins to the function of the vascular wall. *Cardiovasc. Res.* 62, 345–356. doi: 10.1016/j.cardiores.2003.11.015

Hourihan, J. M., Moronetti Mazzeo, L. E., Fernández-Cárdenas, L. P., and Blackwell, T. K. (2016). Cysteine sulfenylation directs IRE-1 to activate the SKN-1/Nrf2 antioxidant response. *Mol. Cell.* 63, 553–566. doi: 10.1016/j.molcel.2016.07.019

Huang, Y., Tang, C., Du, J., and Jin, H. (2016). Endogenous sulfur dioxide: a new member of gasotransmitter family in the cardiovascular system. *Oxid. Med. Cell. Longev.* 2016:8961951. doi: 10.1155/2016/8961951

Hulikova, A., Black, N., Hsia, L. T., Wilding, J., Bodmer, W. F., and Swietach, P. (2016). Stromal uptake and transmission of acid is a pathway for venting cancer cell-generated acid. *Proc. Natl. Acad. Sci. U.S.A.* 113, 5344–5353. doi: 10.1073/pnas.1610954113

Kimura, H. (2016). Hydrogen polysulfide (H2Sn) signaling along with hydrogen sulfide (H_2S) and nitric oxide (NO). *J. Neural Transm.* 123, 1235–1245.

Lee, S. W., Cheng, Y., Moore, P. K., and Bian, J. S. (2007). Hydrogen sulphide regulates intracellular pH in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 358, 1142–1147. doi: 10.1016/j.bbrc.2007.05.063

Li, Z., Huang, Y., Du, J., Liu, A. D., Tang, C., Qi, Y., et al. (2016). Endogenous sulfur dioxide inhibits vascular calcification in association with the TGF-beta/smad signaling pathway. *Int. J. Mol. Sci.* 17:266. doi: 10.3390/ijms17030266

Liu, D., Huang, Y., Bu, D., Liu, A. D., Holmberg, L., Jia, Y., et al. (2014). Sulfur dioxide inhibits vascular smooth muscle cell proliferation via suppressing the Erk/MAP kinase pathway mediated by cAMP/PKA signaling. *Cell. Death Dis.* 5:e1251. doi: 10.1038/cddis.2014.229

Liu, Y., Yang, J., and Chen, L. M. (2015). Structure and function of slc4 family transporters. *Front. Physiol.* 6:355. doi: 10.3389/fphys.2015.00355

Luo, L. M., Hong, X. Y., Diao, B., Chen, S. Y., and Hei, M. Y. (2018). Sulfur dioxide attenuates hypoxia-induced pulmonary arteriolar remodeling via Dkk1/Wnt signaling pathway. *Biomed. Pharmacother.* 106, 692–698. doi: 10.1016/j.biopha.2018.07.017

Meyers, S. N., McDaneld, T. G., Swist, S. L., Marron, B. M., Steffen, D. J., O'Toole, D., et al. (2010). A deletion mutation in bovine SLC4A2 is associated with osteopetrosis in red angus cattle. *BMC. Genomics* 11:337. doi: 10.1186/1471-2164-11-337

Nagpure, B. V., and Bian, J. S. (2016). Interaction of hydrogen sulfide with nitric oxide in the cardiovascular system. *Oxid. Med. Cell. Longev.* 2016:6904327. doi: 10.1155/2016/6904327

Owens, G. K., Kumar, M. S., and Wamhoff, B. R. (2004). Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol. Rev.* 84, 767–801. doi: 10.1152/physrev.00041.2003

Puceat, M. (1999). pHi regulatory ion transporters: an update on structure, regulation and cell function. *Cell. Mol. Life Sci.* 55, 1216–1229. doi: 10.1007/s000180050368

Reimold, F. R., Stewart, A. K., Stolpe, K., Heneghan, J. F., Shmukler, B. E., and Alper, S. L. (2013). Substitution of transmembrane domain cys residues alters pHo-sensitive anion transport by AE2/SLC4A2 anion exchanger. *Pflugers Arch.* 465, 839–851. doi: 10.1007/s00424-012-1196-6

Schreiber, R. (2005). Ca^{2+} signaling, intracellular pH and cell volume in cell proliferation. *J. Membr. Biol.* 205, 129–137. doi: 10.1007/s00232-005-0778-z

Simchowitz, L., and roos, A. (1985). Regulation of intracellular pH in human neutrophils. *J. Gen. Physiol.* 85, 443–470. doi: 10.1085/jgp.85.3.443

Sorensen, C. M., and Holstein-Rathlou, N. H. (2012). Cell-cell communication in the kidney microcirculation. *Microcirculation* 19, 451–460. doi: 10.1111/j.1549-8719.2011.00149.x

Sun, Y., Tian, Y., Prabha, M., Liu, D., Chen, S., Zhang, R., et al. (2010). Effects of sulfur dioxide on hypoxic pulmonary vascular structural remodeling. *Lab. Invest.* 90, 68–82. doi: 10.1038/labinvest.2009.102

Svoboda, L. K., Reddie, K. G., Zhang, L., Vesely, E. D., Williams, E. S., Schumacher, S. M., et al. (2012). Redox-sensitive sulfenic acid modification regulates surface expression of the cardiovascular voltage-gated potassium channel Kv 1.5. *Circ. Res.* 111, 842–853. doi: 10.1161/CIRCRESAHA.111.263525

Vigne, P., Breittmayer, J. P., Frelin, C., and Lazdunski, M. (1988). Dual control of the intracellular pH in aortic smooth muscle cells by a cAMP-sensitive HCO_3^-/Cl^- antiporter and a protein kinase C-sensitive Na^+/H^+ antiporter. *J. Biol. Chem.* 263, 18023–18029.

Wang, R. (2014). Gasotransmitters: growing pains and joys. *Trends Biochem. Sci.* 39, 227–232. doi: 10.1016/j.tibs.2014.03.003

Wu, H. J., Huang, Y. Q., Chen, Q. H., Tian, X. Y., Liu, J., Tang, C. S., et al. (2016). Sulfur dioxide inhibits extracellular signal-regulated kinase signaling to attenuate vascular smooth muscle cell proliferation in angiotensin II- induced hypertensive mice. *Chin. Med. J.* 129, 2226–2232. doi: 10.4103/0366-6999.189927

Xu, P., and Spitzer, K. W. (1994). Na-independent $Cl^-HCO_3^-$exchange mediates recovery of pHi from alkalosis in guinea pig ventricular myocytes. *Am. J. Physiol.* 267, H85–H91. doi: 10.1152/ajpheart.1994.267.1.H85

Yamamura, A. (2014). Pathological function of Ca^{2+}-sensing receptor in pulmonary arterial hypertension. *J. Smooth Muscle Res.* 50, 8–17. doi: 10.1540/jsmr.50.8

Yang, G. M., Peng, X. Y., Wu, Y., Li, T., and Liu, L. M. (2017). Involvement of connexin 43 phosphorylation and gap junctional communication between smooth muscle cells in vasopressin-induced ROCK-dependent vasoconstriction after hemorrhagic shock. *Am. J. Physiol. Cell Physiol.* 313, C362–C370. doi: 10.1152/ajpcell.00258.2016

Yu, W., Jin, H. F., Tang, C. S., Du, J. B., and Zhang, Z. R. (2018). Sulfur-containing gaseous signal molecules, ion channels and cardiovascular diseases. *Br. J. Pharmacol.* 175, 1114–1125. doi: 10.1111/bph.13829

Yu, W., Liu, D., Liang, C., Ochs, T., Chen, S., Du, S., et al. (2016). Sulfur dioxide protects against collagen accumulation in pulmonary artery in association with downregulation of the transforming growth factor β1/smad pathway in pulmonary hypertensive rats. *J. Am. Heart Assoc.* 5:e003910. doi: 10.1161/JAHA.116.003910

Zhang, R. Y., Du, J. B., Sun, Y., Chen, S., Tsai, H. J., Yuan, L., et al. (2011). Sulfur dioxide derivatives depress L-type calcium channel in rat cardiomyocytes. *Clin. Exp. Pharmacol. Physiol.* 38, 416–422. doi: 10.1111/j.1440-1681.2011.05528.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.