Hypertension Research (2018) 41:246–252
https://doi.org/10.1038/s41440-018-0012-x

ARTICLE

ATP2B1 Gene Silencing Increases NO Production Under Basal Conditions Through the Ca^{2+}/calmodulin/eNOS Signaling Pathway in Endothelial Cells

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Received: 16 June 2017 / Revised: 19 July 2017 / Accepted: 31 July 2017 / Published online: 7 February 2018

Abstract

Emerging epidemiological and experimental evidence has shown that the ATP2B1 gene is associated with blood pressure control. Impaired eNOS activity and NO production may be among the mechanisms involved. However, little is known about how PMCA1, which is encoded by the ATP2B1 gene, regulates the activity of eNOS and NO production. In the present study, we investigated the role of the ATP2B1 gene in regulating eNOS activity and NO production under basal conditions in HUVECs and explored the mechanisms involved. Silencing ATP2B1 gene expression resulted in higher NO production and eNOS activity under basal conditions in HUVECs. Additionally, ATP2B1 gene silencing resulted in enhanced intracellular calcium concentrations compared to that in the negative siRNA-transfected HUVECs. The enhanced eNOS activity mediated by ATP2B1 gene silencing was Ca^{2+}/calmodulin dependent, as verified by the administration of the calcium chelator BAPTA-AM or the calmodulin-specific antagonist W7. Taken together, silencing ATP2B1 gene expression results in higher NO production and eNOS activity under basal conditions in HUVECs. Furthermore, the enhanced eNOS activity induced by ATP2B1 gene silencing may be mediated via higher levels of intracellular Ca^{2+}, and the effect was confirmed to be dependent on the eNOS–calmodulin interaction.

Introduction

Hypertension is an important public health challenge. The global prevalence of hypertension has been reported as ~22% in adults aged 18 years and over [1]. Raised blood pressure is a major cardiovascular risk factor that can cause myocardial infarction, cardiac failure, stroke, renal failure, etc. [2, 3]. It contributes to more than 10 million deaths per year worldwide [1, 2]. Nitric oxide, one of the important vasodilators, has been confirmed to regulate vessel tone in large human arteries and plays a key role in regulating blood pressure [4, 5]. It is well known that impaired endothelium-dependent vasodilation in the branchial, coronary and renal arteries, which is characterized by reduced nitric oxide production and bioavailability, is associated with essential hypertension [6–9].

A large-scale genome-wide association study revealed that a genomic region containing ATP2B1 showed a very strong association with systolic blood pressure, diastolic blood pressure, and hypertension in 29,136 individuals of European descent who participated in the Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) Consortium [10]. There is accumulating evidence from different ethnic groups, such as Korean [11], Japanese [12], and Chinese [13] populations, to confirm the association between SNPs relating to ATP2B1 and hypertension. Thus, the evidence suggests that ATP2B1 may play an important role in blood pressure regulation. Additionally, genetically modulated animal models, such as ATP2B1 siRNA-treated mice [14], systemic

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heterozygous ATP2B1 null mice (ATP2B1$^{+/-}$ mice) [15], and vascular smooth muscle cell-targeted ATP2B1 KO mice (VSMC ATP2B1 KO mice) [16], showed higher blood pressure than controls. An increased intracellular calcium concentration in VSMCs could augment the contractile capacity of the femoral artery and result in higher blood pressure in VSMC ATP2B1 KO mice [16]. Moreover, impaired nitric oxide production in endothelial cells may be another possible reason for hypertension in ATP2B1$^{+/-}$ mice [15]. However, overexpression of plasma membrane calcium ATPase (PMCA) proteins, which are encoded by the ATP2B1-4 genes, inhibited eNOS activity and reduced NO production in endothelial cells [17]. In view of this, the role of the PMCA1 protein in regulating eNOS activity is uncertain.

Endothelial nitric oxide synthase (eNOS), which is specifically expressed in endothelial cells and is responsible for NO generation, is also known as a Ca^{2+}/calmodulin-dependent enzyme. The enzymatic activity of eNOS has been reported to be dependent on intracellular Ca^{2+} concentrations and the interaction between eNOS and calmodulin [18–20]. PMCA1 belongs to the P_2 (subtype 2B) subfamily of P-type primary ion transport ATPase [21]. PMCA1 is ubiquitously expressed in humans and is thought to be the major high-affinity transporter for Ca^{2+} in the plasma membrane [22]. Down-regulated expression of PMCA1 results in higher intracellular calcium concentration in VSMCs from ATP2B1$^{+/-}$ mice [15] and VSMC ATP2B1 KO mice [16] than in controls. However, there is no direct evidence to show that increased intracellular Ca^{2+} levels induced by ATP2B1 gene silencing are involved in encouraging interaction between eNOS and calmodulin and subsequently in regulating eNOS activity and NO production.

In the present study, we examined the implications of ATP2B1 gene silencing on the intracellular Ca^{2+} concentration and the activity of eNOS in endothelial cells under basal conditions. Furthermore, we investigated whether high Ca^{2+} concentrations and increases in eNOS–calmodulin interactions are the mechanisms involved in increased NO production in ATP2B1-silenced HUVECs.

Methods

Cell culture

HUVECs were cultured in low-glucose DMEM supplemented with 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% FBS in a 5% CO_2, 37 °C incubator. HUVECs were transfected with negative-siRNA or PMCA1-siRNA (Santa Cruz Biotechnology, CA, USA).

NO production in HUVECs

HUVECs were seeded in 24-well culture plates and transfected with siRNA for 48 h. Following culture with ECM supplemented with 0.1% FBS for 16 h, HUVECs were lysed with cell and tissue lysis buffer for nitric oxide (Beyotime Institute of Biotechnology, Shanghai, China) on ice in 1.5 ml micro-tubes for 15 min and centrifuged for 5 min at 12,000×g at 4 °C. Supernatants were collected, and protein concentrations were measured using a Thermo Scientific Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA). The intracellular NO levels were assayed using a Total Nitric Oxide Assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions and normalized to protein content.

Measurement of the intracellular Ca^{2+} concentration

The concentration of Ca^{2+} was measured using fluo-3/AM. Briefly, after 48 h of transfection, HUVECs were harvested and washed with HANKS buffer with calcium and then suspended in serum-free low-glucose DMEM with 5 μmol/l fluo-3/AM, 0.1% BSA, 0.04% Pluronic F127, and 2 mmol/l probenecid for 30 min at 37 °C. After washing twice, the cells were suspended in assay buffer containing 2 mmol/l probenecid. The fluorescence of cells was recorded with a spectrofluorophotometer by excitation signals at 490 nm and the emission signal at 530 nm. Maximum and minimum fluorescence values (F_{max} and F_{min}) were detected by adding the calcium ionophore A23187 or A23187 plus 5 mmol/l EGTA (in Ca^{2+}-free medium), respectively. $[Ca^{2+}]i$ was calculated according to the following equation: $[Ca^{2+}]i = K_d\ (F-F_{min})/(F_{max}-F)$, where K_d is the apparent dissociation constant (400 nmol/l) of the fluorescence dye–Ca^{2+} complex.

Western blotting

Cell lysates of HUVECs were prepared in 200 μl total volume of lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) on ice in 1.5 ml micro-tubes for 15 min and centrifuged for 5 min at 12,000×g at 4 °C. The supernatant was collected, and protein concentrations were measured using the Thermo Scientific Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA). The protein samples were stored at −80 °C until further examination.

For Western blotting, cell lysates were subjected to SDS-PAGE, and immunoblotting was performed using specific antibodies against eNOS (Santa Cruz Biotechnology, Inc., TX, USA), phospho-eNOS (ser 1177) (Cell Signaling Technology, Inc., Boston, USA), PMCA1 (Santa Cruz

Biotechnology, Inc., TX, USA) and GAPDH (ZSGB-Bio, Inc., Beijing, China).

Co-immunoprecipitation

HUVECs were lysed in RIPA buffer for IP (Beyotime Institute of Biotechnology, Shanghai, China). After pre-clearing with protein A/G agarose beads (Beyotime Institute of Biotechnology, Shanghai, China), the lysates were incubated with specific antibodies against calmodulin overnight at 4 °C. Then, protein A/G agarose beads were added and incubated for 2 h. The supernatants were discarded. After washing with RIPA for IP three times, the protein A/G agarose beads were added with 2× loading buffer.

Statistics

Data are expressed as the mean ± SD. Data were analyzed by one-way ANOVA. *P* values of less than 0.05 were considered statistically significant.

Result

Silencing of ATP2B1 results increased basal NO production and eNOS activity in vitro

As determined by Western blotting, the expression of PMCA1 protein was significantly decreased in the ATP2B1 siRNA-transfected HUVECs compared to the negative control siRNA-transfected cells (Fig. 1a, b). Compared to the control cells, higher intracellular NO production was found in ATP2B1-silenced HUVECs under basal conditions (Fig. 1c). Similar to the result of NO production, the higher eNOS activity and the phosphorylation levels of eNOS at site ser1177 were higher in the ATP2B1-silenced HUVECs than in the control cells under basal conditions (Fig. 1d–f). These data indicated that higher endothelial NO production is related to higher eNOS activity in ATP2B1-silenced endothelial cells.

Higher NO production obtained by silencing the ATP2B1 gene is calcium dependent in endothelial cells

To confirm whether the intracellular calcium concentration in HUVECs was altered through silencing of the ATP2B1 gene, we used the Fluo-3-acetoxymethyl ester (Fluo-3 AM) fluorescence assay. As shown in Fig. 2a, the intracellular calcium concentration was higher in the ATP2B1-silenced HUVECs at baseline than in the control cells. Furthermore, an intracellular Ca^{2+} chelator, BAPTA-AM, was found to inhibit the increased intracellular NO production and eNOS activity in the ATP2B1-silenced HUVECs under basal conditions (Fig. 2b, c).

Higher NO production obtained by silencing the ATP2B1 gene is dependent on the calmodulin signaling pathway

To test whether higher NO production induced by silencing the ATP2B1 gene is dependent on calmodulin, W7, a calmodulin antagonist, was used. As expected, intracellular NO production and eNOS activity were significantly decreased by W7 in the ATP2B1-silenced HUVECs (Fig. 3a, b). Furthermore, data from the co-immunoprecipitation experiments confirmed that higher levels of eNOS were associated with calmodulin in the ATP2B1-silenced HUVECs than in the control cells (Fig. 3c, d).

Discussion

Impaired eNOS activity and NO production have been observed in systemic heterozygous ATP2B1$^{+/-}$ mice [15]. However, overexpression of PMCAs was reported to negatively modulate eNOS activity [17]. In our present study, silencing ATP2B1 gene expression resulted in higher NO production and eNOS activity under basal conditions in HUVECs. The enhanced eNOS activity induced by silencing the ATP2B1 gene may be mediated via higher levels of intracellular Ca^{2+}, and the effect is confirmed to be dependent on the eNOS–calmodulin interaction.

Emerging epidemiological and experimental evidence shows that the ATP2B1 gene is associated with blood pressure control [10–12, 23]. As mentioned above, higher blood pressure was observed in ATP2B1 siRNA-treated mice [14], ATP2B1$^{+/-}$ mice [15], and VSMC ATP2B1 KO mice [16]. Downregulation of the ATP2B1 gene led to an increased wall:lumen ratio, myogenic response, and vascular contractility in mesenteric arteries and finally resulted in elevated blood pressure in ATP2B1 siRNA-treated mice [14]. A lack of the ATP2B1 gene in VSMCs was reported to augment vascular contractility and subsequently lead to enhanced blood pressure [16]. Additionally, impaired eNOS activity and NO production may be one of the mechanisms involved in systemic ATP2B1 gene knockdown-induced hypertension [15]. Conversely, we observed that silencing ATP2B1 gene expression results in higher NO production and eNOS activity under basal conditions in HUVECs. Consistent with our data, Angel L. Armesilla et al. revealed that overexpression of PMCA proteins in endothelial cells reduced NO production by encouraging PMCA–eNOS binding and inhibiting its activity [17]. There are several

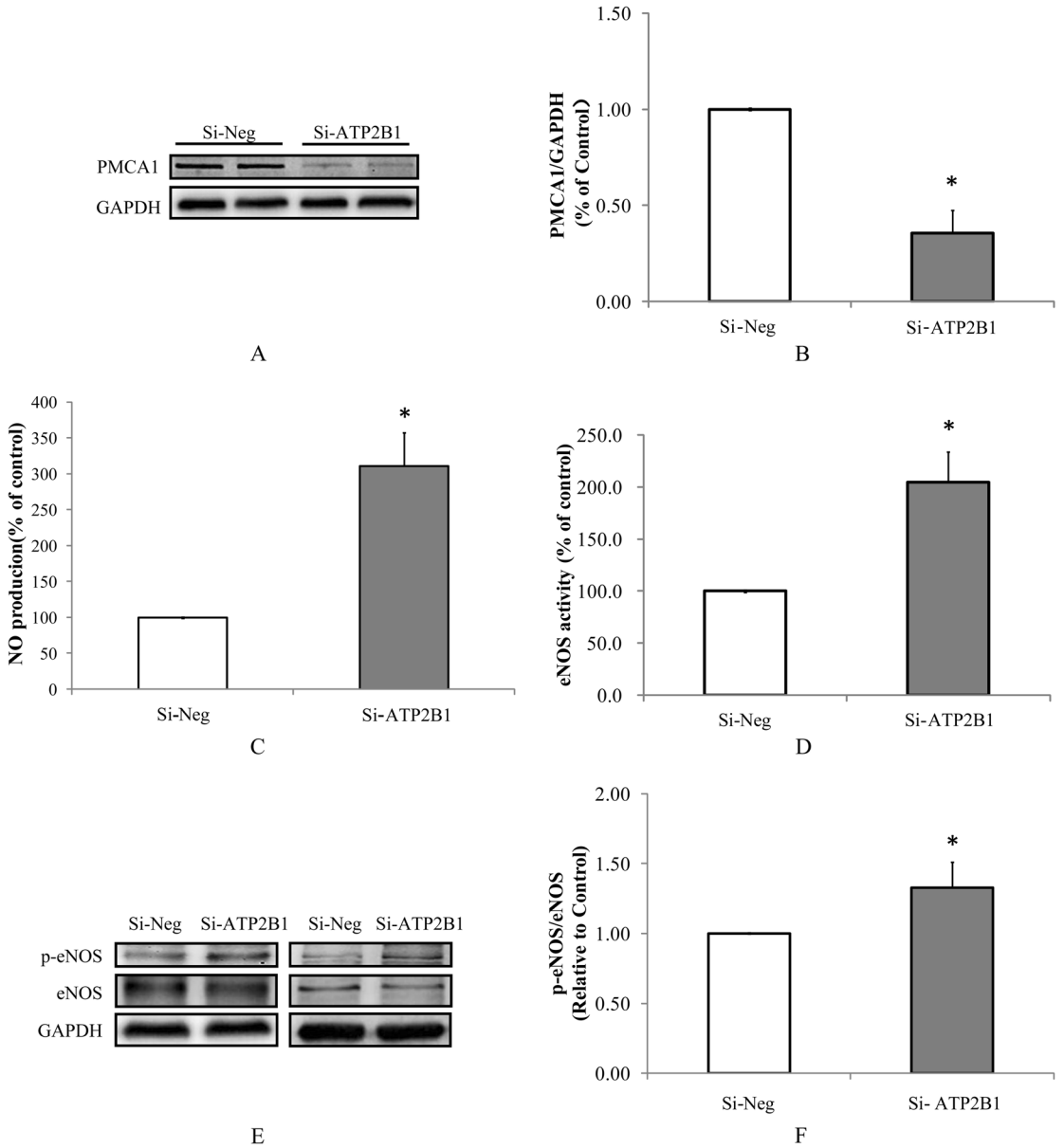

Fig. 1 ATP2B1 gene silencing results in increased basal NO production and eNOS activity in HUVECs. **a** Representative Western blotting results of PMCA1 protein and GAPDH. **b** The fold changes in PMCA1 protein relative to that in the control cells were quantified by densitometry ($n = 4$). Forty-eight hours after transfection, NO production (**c**) and eNOS activity (**d**) were detected in HUVECs. **e** Representative Western blotting results of p-eNOS, eNOS and GAPDH ($n = 3$). **f** Fold changes in phosphor-eNOS vs. total eNOS relative to its basal levels in control cells. $^*P < 0.05$

possible reasons for the adverse results in these studies. First, the research conducted by Akira Fujiwara et al. was performed in vivo, while we and Angel L. Armesilla conducted studies in endothelial cells cultured in vitro. Second, the decreased eNOS activity was revealed in mice with noncell-specific but not endothelial cell-specific ATP2B1 gene deletion. Therefore, other mechanisms that are involved in impaired eNOS activity in ATP2B1$^{+/-}$ mice cannot be ruled out. Further investigations are warranted in endothelial cell-specific ATP2B1 gene knockout mice. Third, we detected eNOS activity at 48–72 h after the transient transfection of siRNA in the present study. However, the decrease in eNOS activity was examined in ATP2B1$^{+/-}$ mice at 3 months of age. The implication of the ATP2B1 gene on the regulation of eNOS activity deserves further investigation.

Impaired endothelium-dependent vasodilation, which is characterized by reduced NO production in endothelial cells, has been demonstrated to be one of the most important mechanisms involved in the progression of hypertension, atherosclerosis, and cardiovascular disease. eNOS, the predominant NOS isoform in vascular endothelial cells, is

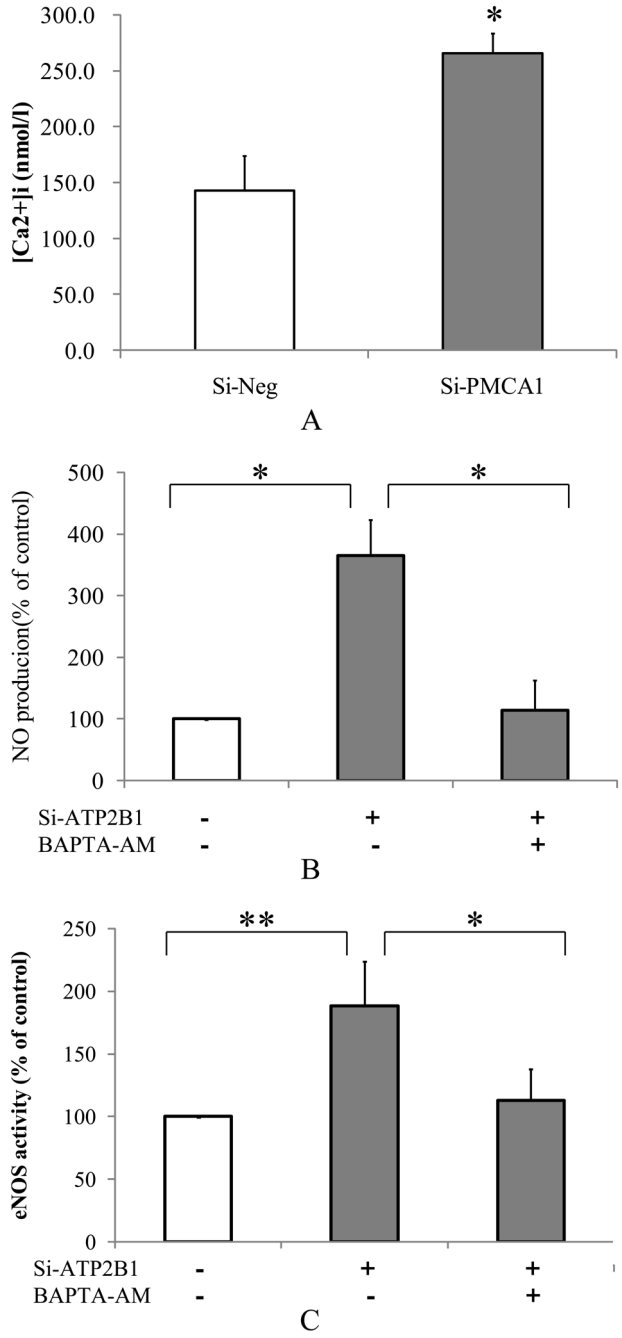

Fig. 2 Higher NO production by silencing the ATP2B1 gene is calcium dependent in endothelial cells. **a** The intracellular concentration of Ca^{2+} was measured using fluo-3/AM in HUVECs 48 h after transfection ($n = 6$). At 2 h after BAPTA administration, NO production (**b**) and eNOS activity (**c**) were determined in HUVECs 48 h after transfection. *$P < 0.05$, **$0.01 > P > 0.001$

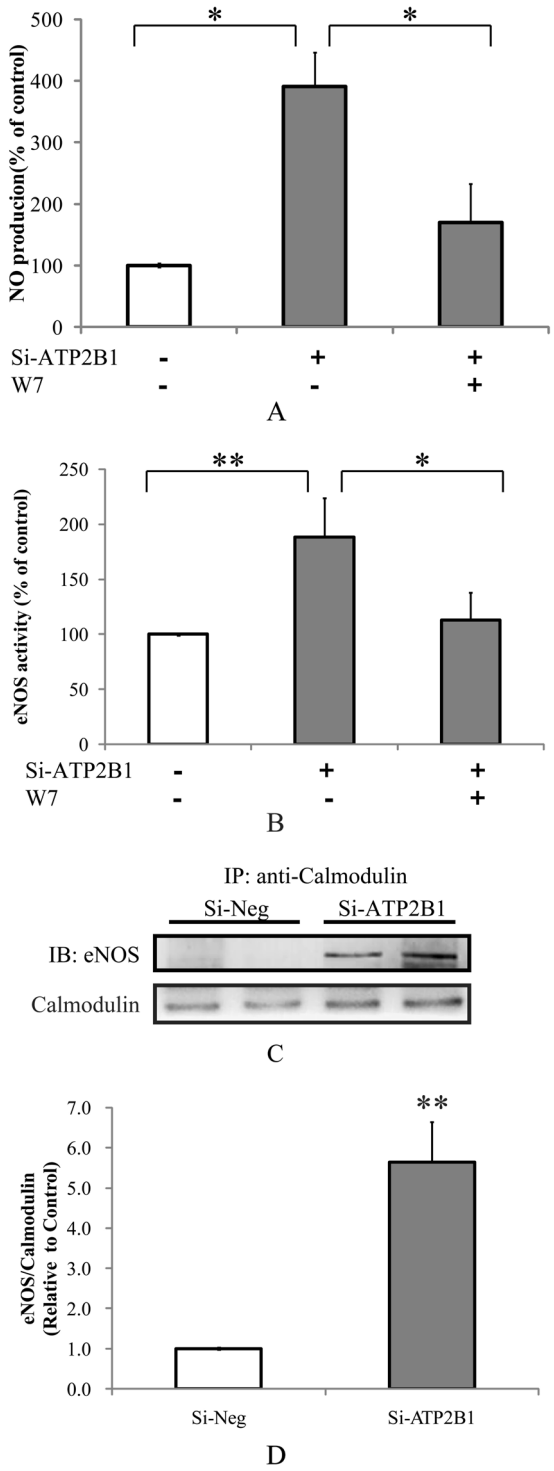

Fig. 3 Higher NO production by silencing the ATP2B1 gene is dependent on the calmodulin signaling pathway. At 2 h after W7 administration, NO production (**a**) and eNOS activity (**b**) were detected in HUVECs 48 h after transfection. Furthermore, co-immunoprecipitation was performed 48 h after transfection. **c** Representative Western blotting results of eNOS that was co-immunoprecipitated with calmodulin ($n = 3$). In addition, the fold changes in calmodulin-associated eNOS relative to basal levels in control cells were quantified by densitometry. *$P < 0.05$, **$0.01 > P > 0.001$

responsible for most NO production and plays a key role in vessel tone regulation, anti-coagulation, and anti-thrombogenesis [13]. The activity of eNOS is regulated by post-translational modification (such as multi-site phosphorylation, acylation, and S-nitrosylation), calcium–calmodulin binding and interaction with protein

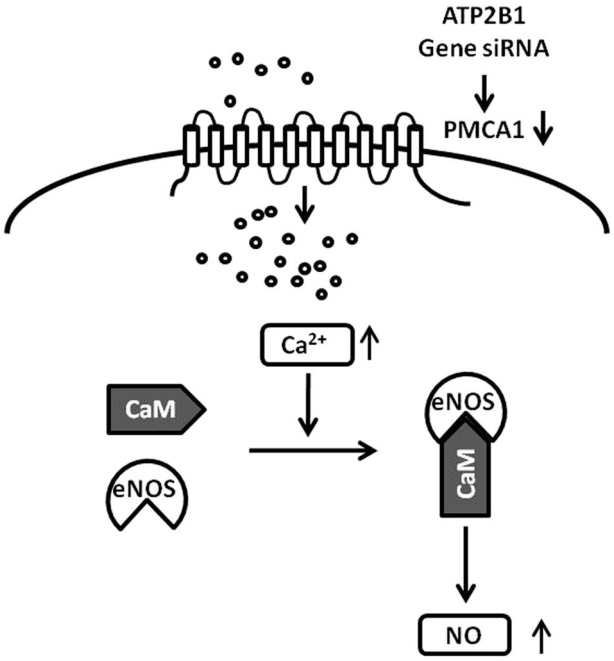

Fig. 4 Proposed model of the role of the PMCA1 protein in regulating eNOS activity and NO production under basal conditions in endothelial cells. ATP2B1 gene silencing, which results in decreased expression of the PMCA1 protein, results in elevated intracellular Ca^{2+} levels in endothelial cells. Increased intracellular Ca^{2+} promotes interaction between calmodulin and eNOS and leads to enhanced eNOS activity and NO production under basal conditions. ATP2B1 ATPase plasma membrane Ca^{2+} transporting 1, PMCA1 plasma membrane calcium ATPase 1, CaM calmodulin, Akt protein kinase B, eNOS endothelial nitric oxide synthase, NO nitric oxide

partners (such as caveolin, Akt, hsp90, and cytoskeleton actin, among others). Several studies have reported that PMCAs are involved in regulating eNOS activity. An inhibitory interaction between PMCA and eNOS may be one of the mechanisms involved in negatively regulating eNOS activity [17]. The activity of calcineurin, a Ca^{2+}–calmodulin-dependent protein, was also reported to be inhibited by binding with PMCA. Furthermore, decreased eNOS activity induced by PMCA may be due to the calcineurin–PMCA interaction, which leads to a decrease in the calcineurin-mediated dephosphorylation of Thr-495 of eNOS and subsequent inhibition of eNOS activity [17]. In our present study, the elevated eNOS activity and NO production in ATP2B1-silenced endothelial cells may be due to the increase in the intracellular Ca^{2+} concentration and eNOS–calmodulin interaction.

In summary, silencing ATP2B1 gene expression results in higher NO production and eNOS activity under basal conditions in HUVECs. ATP2B1 gene silencing directly resulted in a higher intracellular Ca^{2+} concentration. Furthermore, the Ca^{2+}–calmodulin signaling pathway is one of the mechanisms involved in enhanced eNOS activity and NO production in ATP2B1-silenced HUVECs under basal conditions. In an attempt to show how the PMCA1 protein regulates eNOS activity and NO production under basal conditions in endothelial cells, a figure of the proposed model is provided (Fig. 4). However, there are some limitations to the current study. First, this research was conducted on HUVECs cultured in vitro, and in vivo animal experiments were not performed. Therefore, we do not know whether silencing the ATP2B1 gene increases eNOS activity and NO production in endothelial cells in vivo. Second, we reported that the enhanced eNOS activity and NO production in ATP2B1-silenced HUVECs under basal conditions are due to an increased intracellular Ca^{2+} concentration and subsequent calmodulin–eNOS interaction in this article. However, it is not the only mechanism involved. Some studies have shown that PMCA1 directly binds to eNOS and inhibits its activity [17]. Others have indicated that Ca^{2+}–calmodulin-dependent proteins, such as calcineurin [17] and Akt [24], are also involved in regulating eNOS activity. Additionally, Akt, which is regulated by phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) [25], directly phosphorylates eNOS at serine 1177 and activates eNOS [24]. Further studies are needed to confirm these underlying mechanisms in silenced ATP2B1 gene-mediated higher NO production.

Acknowledgements This work was supported by the Joint Project of People' Government of Luzhou and Sichuan Medical University under Grant no. 2015SX-0032 and by the Scientific Research Fund of SiChuan Provincial Education Department under Grant no. 17ZA0450.

Funding This work was supported by the Joint Project of the People' Government of Luzhou and Sichuan Medical University under Grant no. 2015SX-0032 and by the Scientific Research Fund of SiChuan Provincial Education Department under Grant no. 17ZA0450.

Compilance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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