Endothelial Dysfunction

TMEM16A Contributes to Endothelial Dysfunction by Facilitating Nox2 NADPH Oxidase–Derived Reactive Oxygen Species Generation in Hypertension

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Abstract—Ca²⁺-activated Cl⁻ channels play a crucial role in various physiological processes. However, the role of TMEM16A in vascular endothelial dysfunction during hypertension is unclear. In this study, we investigated the specific involvement of TMEM16A in regulating endothelial function and blood pressure and the underlying mechanism. Reverse transcription-polymerase chain reaction, Western blotting, communoprecipitation, confocal imaging, patch-clamp recordings, and TMEM16A endothelial-specific transgenic and knockout mice were used. We found that TMEM16A was expressed abundantly and functioned as a Ca²⁺-activated Cl⁻ channel in endothelial cells. Angiotensin II induced endothelial dysfunction with an increase in TMEM16A expression. The knockout of endothelial-specific TMEM16A significantly lowered the blood pressure and ameliorated endothelial dysfunction in angiotensin II–induced hypertension, whereas the overexpression of endothelial-specific TMEM16A resulted in the opposite effects. These results were related to the increased reactive oxygen species production, Nox2-containing NADPH oxidase activation, and Nox2 and p22phox protein expression that were facilitated by TMEM16A on angiotensin II–induced hypertensive challenge. Moreover, TMEM16A directly bound with Nox2 and reduced the degradation of Nox2 through the proteasome-dependent degradation pathway. Therefore, TMEM16A is a positive regulator of endothelial reactive oxygen species generation via Nox2-containing NADPH oxidase, which induces endothelial dysfunction and hypertension. Modification of TMEM16A may be a novel therapeutic strategy for endothelial dysfunction–associated diseases. (Hypertension. 2017;69:892-901. DOI: 10.1161/HYPERTENSIONAHA.116.08874.) • Online Data Supplement

Key Words: angiotensin II ■ endothelial cells ■ hypertension ■ NADPH oxidase ■ reactive oxygen species ■ TMEM16A

Ca²⁺-activated Cl⁻ channels (CaCCs) are expressed abundantly in many eukaryotic cell types, and they are involved in the regulation of various physiological processes, such as epithelial fluid secretion, neuronal and cardiac excitability, oocyte fertilization, and vascular tone regulation.²³ Although CaCCs have been studied at the functional level for >30 years, the molecular identity of this channel remained unclear until 2008, when 3 independent research groups found that TMEM16A (Ano1), a member of the TMEM16 family (TMEM16A–H, TMEM16J, and TMEM16K), was a component of CaCCs.³⁻⁵ Subsequent studies revealed that TMEM16A was responsible for native CaCCs in numerous cell types, including smooth muscle cells,⁶ epithelial cells,⁷ interstitial cells of Cajal,⁸ gland acinar cells,⁹ sensory neurons,¹⁰ and vascular endothelial cells.¹¹

About the cardiovascular system, previous studies have found that TMEM16A mediated cerebrovascular remodeling,⁶ ventricular hypertrophy,¹² pulmonary hypertension,¹³ and blood pressure regulation.¹⁴ Recently, it was reported that TMEM16A existed in mouse cardiac vascular endothelial cells and might play a role in the pathophysiological process of cardiac ischemia.¹¹ However, it remains unclear whether TMEM16A contributes to endothelial dysfunction and regulates blood pressure on hypertensive challenges.

It is well established that increased reactive oxygen species (ROS) formation is associated with endothelial dysfunction, which is a key feature of cardiovascular diseases, including hypertension, atherosclerosis, and diabetes mellitus.¹⁵ In recent years, it has been demonstrated that the NADPH oxidase, whose sole identified function is to generate ROS, is a predominant source of ROS in the vasculature.¹⁵ To date, 7 members (Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2) of NADPH oxidase have been
identified, among which the expression of Nox1, Nox2, Nox4, and Nox5 is largely confined to endothelial cells, and these members might be involved in the development of hypertension. Therefore, the appropriate modulation of ROS production will help to improve endothelial dysfunction in hypertension.

In this study, we generated TMEM16A endothelial-specific knockout and transgenic mice and investigated the specific involvement of TMEM16A in regulating endothelial function and blood pressure via vascular oxidative stress.

Methods
The reagents, cell isolation and culture, adenoviral infection, plasmid transfection, siRNA transfection, reverse transcription–polymerase chain reaction, Western blotting, communoprecipitation, cell fractionation, current recording, immunofluorescence, detection of ROS production, measurement of superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, NADPH oxidase activity assay, generation of endothelial-specific TMEM16A knockout and transgenic mice, animal model, assay of vasorelaxation, and statistical analyses are described in detail in the online-only Data Supplement.

All statistical analyses were performed using GraphPad Prism 5 (GraphPad software, La Jolla, CA). All data are expressed as the mean±SD. n represented the number of independent experiments on different batches of cells or different mice. A 2-tailed Student t test for independent samples was used to detect significant differences between 2 groups. One-way or 2-way ANOVA followed by Bonferroni multiple comparison test was used to compare differences when there were >2 treatment groups. The interaction in 2-way ANOVA was considered. The level for statistical significance was 0.05.

Results
TMEM16A Is a Critical Component of CaCCs in Endothelial Cells
Reverse transcription-polymerase chain reaction analysis showed that in human umbilical vein endothelial cells (HUVECs), the transcripts of the TMEM16 family, including TMEM16A, TMEM16B, TMEM16D through TMEM16F, TMEM16H, and TMEM16K, were present; others (TMEM16C, TMEM16G, and TMEM16J) were not detected (Figure 1A). The results of the Western blot analysis demonstrated that TMEM16A was expressed abundantly in

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Figure 1. TMEM16A is a critical component of Ca\(^{2+}\)-activated Cl\(^{-}\) channels (CaCCs) in human umbilical vein endothelial cells (HUVECs). A, Reverse transcription-polymerase chain reaction (RT-PCR) results for the expression of mRNAs for the TMEM16 family (TMEM16A–H, J, and K; n=4). B, Western blot showing that TMEM16A was abundantly expressed in HUVECs from 4 independent samples (n=4). C, Representative images of cells labeled with Hoechst (blue) and antibody against TMEM16A (red). Scale bars are 10 μm. D, Representative images of cells infected with RFP-labeled adenovirus (Ad)-TMEM16A (100 multiplicity of infection) for 48 h. Scale bars are 10 μm. E, Silencing of TMEM16A by Ad-TMEM16A shRNA transfection (100 multiplicity of infection) for 48 h significantly reduced Ca\(^{2+}\)-activated Cl\(^{-}\) current (\(I_{\text{Cl,Ca}}\)) in HUVECs (n=6, *P<0.05 vs con). F, Overexpression of TMEM16A by Ad-TMEM16A transfection (100 multiplicity of infection) for 48 h increased \(I_{\text{Cl,Ca}}\) in HUVECs (n=6, *P<0.05 vs con).
HUVECs (Figure 1B). Confocal image analyses revealed that TMEM16A was distributed widely in both the cell membrane and cytoplasm in HUVECs stained with anti-TMEM16A antibody (Figure 1C) or in HUVECs infected with RFP-labeled TMEM16A adenovirus (Ad; Figure 1D).

Next, we explored the biophysical features and pharmacological profile of the Ca²⁺-activated Cl⁻ current (I_{Cl,Ca}) in HUVECs. The current recorded in the presence of a free intracellular Ca²⁺ concentration ([Ca²⁺]i) of 500 nmol/L exhibited outward rectification and time-dependent relaxation. The reversal potential of this current was −0.51±3.78 mV, which was near the equilibrium potential for Cl⁻ (0 mV) under our experimental conditions. T16Ainh-A01 (10 μmol/L), a specific inhibitor of TMEM16A,17 significantly inhibited this current (Figure S1 in the online-only Data Supplement). Moreover, Ad-TMEM16A shRNA and Ad-TMEM16A were used to silence or overexpress TMEM16A, respectively, in HUVECs (Figures S2 and S3 present infection efficiency). The results showed that silencing TMEM16A with Ad-TMEM16A shRNA (100 multiplicity of infection) for 48 hours significantly decreased the I_{Cl,Ca} (Figure 1E), whereas overexpressing TMEM16A with Ad-TMEM16A (100 multiplicity of infection) for 48 hours further increased this current (Figure 1F). These results indicate that TMEM16A is responsible for the I_{Cl,Ca} in HUVECs, whose characteristics are similar to the I_{Cl,Ca} in other cell types.3–6,11

TMEM16A Is Involved in Ang II–Induced Endothelial Dysfunction

As shown in Figure 2A and 2B, the expression of TMEM16A significantly increased in both HUVECs and aortic endothelium on angiotensin II (Ang II)–induced challenges. Moreover, Ang II significantly increased the I_{Cl,Ca} in HUVECs (Figure S4).

Figure 2. TMEM16A potentiated endothelial dysfunction during angiotensin II (Ang II)–induced hypertension. A, Western blot showing that the expression of TMEM16A was increased in human umbilical vein endothelial cells (HUVECs) treated with Ang II (100 nmol/L) for 24 h (n=6, *P<0.05 vs con). B, Representative immunofluorescence staining with TMEM16A (red) and Hoechst 33258 (blue) in sliced aorta from mice administered saline (con) or Ang II (Ang) for 4 wk. Scale bars are 200 μm (n=6). C and D, Systolic blood pressure (SBP) was measured before and after chronic Ang II infusion for 2 and 4 wk in TMEM16A endothelial-specific knockout mice (TMEKO; C), TMEM16A endothelial-specific transgenic mice (TMETg; D), and their control littermates (TMWT and TMcon for short, respectively; n=7, *P<0.05 vs TMWT and TMcon, #P<0.05 vs Ang+TMWT and Ang+TMcon). E and F, Endothelial function was measured in aortas by acetylcholine (Ach)-induced endothelium-dependent relaxation from 1×10⁻¹¹ to 1×10⁻⁵ mol/L in TMWT (E), TMETg (F), and their control littermates induced by Ang II infusion for 4 wk (n=6, *P<0.05 vs TMWT and TMWT, #P<0.05 vs Ang+TMWT and Ang+TMWT).
Because endothelial dysfunction developed during Ang II–induced hypertension, we next addressed the role of endothelial TMEM16A in cohorts of TMEM16A endothelial-specific knockout mice (TM\(^{\text{ETO}}\)) and TMEM16A endothelial-specific transgenic mice (TM\(^{\text{TG}}\)) and determined whether TMEM16A was involved in Ang II–induced endothelial dysfunction. The control cohorts were TMEM16A LoxP mice and TMEM16A transgenic mice that did not express Tie2-Cre (TM\(^{\text{TG}}\) and TM\(^{\text{ETO}}\) for short, respectively; Figures S5 and S6 present the genotyping and expression assessments). Mice at 8 weeks of age were implanted with Ang II infusion pumps for 4 weeks, and the systolic blood pressure results are shown in Figure 2C and 2D. The results indicated that blood pressure increased after Ang II infusion, which was significantly reduced in TM\(^{\text{ETO}}\) compared with TM\(^{\text{TG}}\), from 164.4±10.2 to 141.9±9.3 mm Hg, after 4 weeks of hypertension, whereas blood pressure further increased in TM\(^{\text{TG}}\) compared with TM\(^{\text{ETO}}\), from 164.4±7.6 to 178.8±8.5 mm Hg, after 4 weeks of hypertension.

Then, endothelial function was measured in TM\(^{\text{ETO}}\), TM\(^{\text{TG}}\), and their control littersates. There were no difference among all groups of mice in the basal level of endothelium-dependent relaxation responses induced by acetylcholine at concentrations from 1×10\(^{-9}\) to 3×10\(^{-5}\) mol/L in the aortas. However, during Ang II–induced hypertension, endothelial dysfunction was significantly ameliorated in the aortas from TM\(^{\text{ETO}}\) compared with those from TM\(^{\text{TG}}\) (Figure 2E), whereas Ang II–induced endothelial dysfunction was further potentiated in the aortas from TM\(^{\text{TG}}\) compared with those from TM\(^{\text{ETO}}\) (Figure 2F). In addition, almost all of the endothelium-dependent relaxation responses were blocked by L-nitro-arginine methyl ester (1 μmol/L), a nonselective NO synthase inhibitor, and there was no difference between TM\(^{\text{ETO}}\), TM\(^{\text{TG}}\), and their control littersates (Figure S7A and S7B). Moreover, there was no difference in the endothelium-independent vasorelaxation responses induced by sodium nitroprusside among all groups (Figure S7C and S7D). These findings suggest that TMEM16A is a crucial regulator of both endothelial function and blood pressure.

**TMEM16A Is Involved in Ang II–Induced ROS Generation**

Overwhelming evidence suggests that Ang II induces ROS production in the vascular wall, resulting in endothelial dysfunction.\(^{18}\) Thus, we explored whether TMEM16A could affect Ang II–induced ROS generation. Amplex Red, dihydroethidium, and 2′,7′-dichlorodihydrofluorescein diacetate were selected to examine ROS generation. The results showed that ROS production was elevated in the aortas from Ang II–induced hypertensive mice. Endothelial-specific knockdown of TMEM16A remarkably reduced Ang II–induced ROS production in the aortas (Figure 3A; Figure S8A and S8C), whereas endothelial-specific overexpression of TMEM16A showed the opposite effects (Figure 3B; Figure S8B and S8D).

To further confirm these results, ROS generation was detected in HUVECs infected with Ad-TMEM16A shRNA or Ad-TMEM16A. Although there was no difference in basal ROS production, Ang II–induced ROS generation was significantly reduced by the silencing of TMEM16A in HUVECs (Figures S9A, S10A, and S10C) and was further increased by the overexpression of TMEM16A (Figures S9B, S10B, and S10D). In addition, N-acetyl-L-cysteine, a ROS scavenger, significantly decreased Ang II–induced ROS production to the basal level, suggesting that the ROS measurements in the study were reliable (Figure S11). These results further indicate that TMEM16A mediates Ang II–induced ROS generation in endothelial cells.

Endogenous antioxidant enzymes, such as GPx and SOD, are important enzymes that scavenge intracellular ROS under physiological conditions.\(^{15}\) As shown in Figure S12, Ang II decreased the activities of GPx and SOD. The silencing of TMEM16A expression reversed the inhibitory effect of Ang II on GPx and SOD. However, the overexpression of TMEM16A enhanced the inhibitory effect of Ang II on GPx and SOD.

**TMEM16A Potentiates ROS Generation via Nox2-Containing NADPH Oxidase**

Numerous studies have shown that NADPH oxidases are a major source of ROS production in the vasculature.\(^{15,16}\) Therefore, the roles of TMEM16A in Ang II–induced NADPH oxidase activation were examined using lucigenin-based enhanced chemiluminescence. The membrane fractions were used for measurements of NADPH oxidase activity to avoid interference by other sources of ROS, such as NO synthases, mitochondria, and xanthine oxidase. Figure 3C and 3D shows that Ang II increased the NADPH oxidase activity in HUVECs, which was inhibited by the silencing of TMEM16A and further enhanced by the overexpression of TMEM16A.

The endothelial abundant Noxes include Nox1, Nox2, Nox4, and Nox5.\(^{15}\) Next, we determined which of these Noxes were responsible for TMEM16A-related NADPH oxidase activation. Figure S13 shows that the transfection of the siRNA of different Noxes, including Nox1, Nox2, Nox4, and Nox5, in all cases efficiently decreased the respective endogenous Nox protein expression in HUVECs. However, only the knockdown of Nox2 protein expression significantly reversed the effect of TMEM16A on Ang II–induced NADPH oxidase activation (Figure 3E).

**TMEM16A Increases the Protein Levels of Nox2 and p22phox**

Nox2-containing NADPH oxidase is a multisubunit complex, consisting of the membrane-bound catalytic Nox2 subunit and p22phox subunit and 3 cytosolic regulatory subunits: p47phox, p67phox, and Rac1.\(^{15}\) The expression of these subunits was examined in HUVECs. The results showed that Ang II significantly increased the expression of Nox2 and p22phox, which was attenuated by the silencing of TMEM16A and potentiated by the overexpression of TMEM16A. However, the expression of p47phox, p67phox, and Rac1 induced by Ang II was comparable after the silencing or overexpression of TMEM16A (Figure S14).

The role of TMEM16A in the expression of NADPH oxidase subunits was then investigated in TMEM16A endothelial-specific knockout and transgenic mice. As shown in Figure 4, the increased expression of Nox2 and p22phox induced by Ang II was reduced in the aortas from TM\(^{\text{ETO}}\) compared with those from TM\(^{\text{TG}}\) and increased in the aortas from TM\(^{\text{TG}}\) compared with those from TM\(^{\text{ETO}}\). In addition, the expression of p47phox, p67phox, and Rac1 was not different in the aortas from Ang II–induced hypertensive TM\(^{\text{ETO}}\) and
TMEM16A compared with those from their control littermates. To differentiate the effects between the vascular endothelium and vascular smooth muscle, cultured mouse aortic endothelial cells were used. The results in endothelial cells were consistent with those in the aortas (Figure S15). Taken together, these results from in vitro and in vivo experiments indicate that TMEM16A promotes Nox2-containing NADPH oxidase activity via increasing the expression of Nox2 and p22phox.

**TMEM16A Inhibits Nox2 Protein Degradation**

To explore how TMEM16A regulates Nox2 expression in endothelial cells, we evaluated the mRNA expression of Nox2 in HUVECs. As shown in Figure 5A and 5B, the mRNA levels of Nox2 were increased by Ang II, but were comparable between TMEM16A-silenced or TMEM16A-overexpressing cells and their control cells, suggesting that the alteration of the Nox2 protein level by TMEM16A was not because of the regulation of Nox2 protein synthesis. TMEM16A might influence Nox2 protein degradation.

Normally, Nox2 and p22phox form a heterodimer in the membrane, which is essential for the stability of these proteins. If not dimerized, Nox2 and p22phox are presumed to be quickly degraded through a proteasome-dependent endoplasmic reticulum-associated degradation pathway. Our results also showed that the degradation of Nox2 was inhibited by the proteasome inhibitor MG132 but not the lysosome blocker chloroquine (Figure S16). Moreover, MG132 normalized the difference in Nox2 protein expression between the control and TMEM16A-deficient or TMEM16A-overexpressing HUVECs (Figure 5C and 5D). After the treatment with cycloheximide (100 μg/mL) to block de novo protein synthesis, TMEM16A overexpression reduced the rate of Nox2 protein degradation, whereas TMEM16A knockdown increased the rate of Nox2 protein degradation (Figure S17). These data...
suggest that TMEM16A inhibited the degradation of Nox2 through the proteasome-dependent pathway and therefore increased Nox2 protein levels in endothelial cells.

To better understand how TMEM16A regulates the degradation of Nox2, we examined whether TMEM16A interacts with the Nox2-containing NADPH oxidase’s subunits. Interestingly, the coimmunoprecipitation of Nox2 by TMEM16A was detected in HUVECs, but not for the other subunits, including p22phox, p47phox, p67phox, and Rac1 (Figure 5E). In addition, Ang II significantly increased the protein interaction between TMEM16A and Nox2 in HUVECs (Figure S18).

Because neither the Nox2 nor the p22phox monomer was stable in primary endothelial cells and because TMEM16A only interacted with Nox2 and not with p22phox, we further studied their interaction in HEK293T cells. As shown in Figure 5F, on the ectopic overexpression of RFP-tagged TMEM16A, together with flag-tagged Nox2 and His-tagged p22phox, there was a specific interaction between TMEM16A and Nox2, but not between TMEM16A and p22phox (lanes 1, 2, and 4, top panel). On the other hand, Nox2 could form a heterodimer with either TMEM16A or p22phox (lanes 2–4, middle panel). Notably, p22phox reduced the interaction between TMEM16A and Nox2 (lanes 2 and 4, top and middle panels). Consistent with the previous results that TMEM16A facilitated Ang II–induced protein upregulation in both Nox2 and p22phox (Figure 4D; Figures S14D and S15D), the coexpression of TMEM16A with Nox2 and p22phox together also led to a substantial increase in protein expression for both Nox2 and p22phox (lanes 3 and 4, bottom panels), but the expression of TMEM16A remained unchanged in each group transfected with TMEM16A (lanes 1, 2, and 4, bottom panels). Together, these data suggested that TMEM16A protected against the degradation of Nox2 by competitively binding with Nox2 against p22phox and forming a relatively stable TMEM16A–Nox2 complex.

TMEM16A Increases the Translocation of p47phox and p67phox

The translocation of p47phox and p67phox from the cytoplasm to the cell membrane is a crucial mechanism for Nox2 activation,8 and based on our previous results that TMEM16A upregulated Nox2 protein expression, we further examined whether the translocation of p47phox and p67phox was
also increased. As shown in Figure 6A and 6B, knockdown of TMEM16A decreased the Ang II–induced translocation of both p47phox and p67phox from the cytoplasm to the cell membrane in HUVECs, whereas the overexpression of TMEM16A produced the opposite effect. Moreover, Ang II–induced interactions between Nox2 and p47phox, or p67phox, were remarkably attenuated by the knockdown of TMEM16A and further potentiated by the overexpression of TMEM16A (Figure 6C and 6D). These results indicate that TMEM16A facilitates the translocation of p47phox and p67phox from the cytoplasm to the cell membrane and the subsequent association with Nox2 in endothelial cells.

**Discussion**

In this study, we developed novel transgenic mouse models with the targeted knockout or overexpression of TMEM16A in the endothelium to investigate the specific role of endothelial TMEM16A in regulating vascular oxidative stress and hemodynamic responses. The major findings of this study are as follows: (1) TMEM16A is a crucial component of CaCCs in endothelial cells; (2) TMEM16A endothelial-specific knockout leads to a decrease in blood pressure and a protective effect on endothelial function on Ang II-induced hypertension, whereas the endothelial-specific TMEM16A transgene shows the opposite effect; (3) Ang II–induced ROS generation and
NADPH oxidase activation in endothelial cells are regulated by TMEM16A; and (4) These effects of TMEM16A on ROS generation and NADPH oxidase activation are because of the upregulation of the Nox2 protein level, which TMEM16A directly binds to and stabilizes.

These findings provide important insights into the role of endothelial TMEM16A in vascular ROS production. It is known that Ang II induces ROS production via Nox-derived ROS signaling, resulting in endothelial dysfunction.15,21 Previous studies have shown that several types of Cl− channels are related to ROS generation, such as the cystic fibrosis transmembrane regulator,22 volume-regulated Cl− channel,23 ClC-1 Cl− channel,24 and pH-activated Cl − channel.25 However, the relationship between CaCCs and oxidative stress remained unclear. The results from this study revealed that TMEM16A was responsible for CaCCs in endothelial cells and distributed in both the cell membrane and cytoplasm, suggesting that TMEM16A might play a role in endothelial functions as do other Cl− channels. Therefore, we generated novel transgenic mouse models directing TMEM16A knockout or overexpression to the endothelium under the control of the murine Tie2 promoter. With these mice, we were able to assess the importance of endothelial-specific responses to TMEM16A independent of those of the vascular smooth muscle and adventitia. As predicted, the change in TMEM16A protein expression was detected only in the vascular endothelium and not in other tissues, indicating that the transgenic modification had no effect on other tissues aside from the endothelium.

There are several sources of ROS in the vasculature, but the only enzymes whose sole identified function is to generate ROS are NADPH oxidases.15 Of the 7 members (Nox1–5, Duox1, and 2) of NADPH oxidases that are expressed in blood vessels, Nox2-containing NADPH oxidase predominantly exists in endothelial cells and is a major source of the excessive ROS production during hypertension.16 Nox2-containing NADPH oxidase is a multisubunit flavoprotein complex in endothelial cells, consisting of the membrane-bound catalytic Nox2 subunit and p22phox subunit and 3 cytosolic regulatory subunits: p47phox, p67phox, and Rac1.15 In this study, we found that TMEM16A enhanced Nox2-containing NADPH oxidase activity and increased the protein levels of Nox2 and p22phox induced by Ang II, whereas the expression of p47phox, p67phox, and Rac1 was not changed. These results from cell culture experiments were consistent with those from experiments with TMEM16A endothelial-specific knockout and transgenic mice, indicating that TMEM16A facilitated

![Figure 6. TMEM16A facilitated the translocation of p47phox and p67phox from the cytoplasm to the membrane and the subsequent interaction with Nox2. A and B. The expression of p47phox and p67phox were measured in human umbilical vein endothelial cells (HUVECs) infected with adenovirus (Ad)-TMEM16A shRNA (A), Ad-TMEM16A (B), and their corresponding controls on stimulation with angiotensin II (Ang II; 100 nmol/L) for 24 h. Cell components were separated into cytoplasm and membrane fractions and then subjected to immunoblotting (n=5, *P<0.05 vs con, †P<0.05 vs Ang). C and D, HUVECs were infected with Ad-TMEM16A shRNA (C), Ad-TMEM16A (D), and their corresponding controls on stimulation with Ang II (100 nmol/L) for 24 h. Lysates were immunoprecipitated with anti-Nox2 and immunoblotted with anti-p47phox or anti-p67phox (n=5, *P<0.05 vs con).](http://hyper.ahajournals.org/)

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**Figure 6.** TMEM16A facilitated the translocation of p47phox and p67phox from the cytoplasm to the membrane and the subsequent interaction with Nox2. A and B. The expression of p47phox and p67phox were measured in human umbilical vein endothelial cells (HUVECs) infected with adenovirus (Ad)-TMEM16A shRNA (A), Ad-TMEM16A (B), and their corresponding controls on stimulation with angiotensin II (Ang II; 100 nmol/L) for 24 h. Cell components were separated into cytoplasm and membrane fractions and then subjected to immunoblotting (n=5, *P<0.05 vs con, †P<0.05 vs Ang). C and D, HUVECs were infected with Ad-TMEM16A shRNA (C), Ad-TMEM16A (D), and their corresponding controls on stimulation with Ang II (100 nmol/L) for 24 h. Lysates were immunoprecipitated with anti-Nox2 and immunoblotted with anti-p47phox or anti-p67phox (n=5, *P<0.05 vs con).
ROS generation via increasing Nox2-containing NADPH oxidase activity in the vascular endothelium.

It was well established that the protein expression of Nox2 and p22phox is upregulated in response to Ang II in the cardiovascular system, including the aorta, heart, and resistance arteries.\(^\text{15,26,27}\) Nox2 and p22phox form a heterodimer in the membrane, which is essential to the stability of these proteins.\(^\text{19,20}\) If not dimerized, Nox2 and p22phox are presumed to be quickly degraded through a proteasome-dependent endoplasmic reticulum-associated degradation pathway.\(^\text{28}\) Consistent with these findings, our results revealed that the expression of both Nox2 and p22phox was upregulated on Ang II–induced hypertensive challenges. Moreover, the silencing of TMEM16A was associated with the marked upregulation of Nox2 protein degradation via the proteasome-dependent endoplasmic reticulum-associated degradation pathway, whereas the overexpression of TMEM16A had the opposite effect, indicating that TMEM16A played a role in stabilizing Nox2 protein levels. Interestingly, TMEM16A had a direct interaction with Nox2, but not p22phox, suggesting that TMEM16A might compete with p22phox to form a more stable TMEM16A–Nox2 complex to avoid Nox2 degradation. A recent study also reported that a protein named negative regulator of ROS limited ROS generation by directly interacting with the Nox2 monomer, but not p22phox, which facilitated the degradation of Nox2 through the endoplasmic reticulum-associated degradation pathway.\(^\text{29}\) Therefore, we speculated that TMEM16A might combine with Nox2 against p22phox and protect against Nox2 degradation.

It is well known that the renin-angiotensin system plays an important role in the control of arterial blood pressure.\(^\text{30,31}\) Ang II, a major member in this system, is involved in the pathogenesis of hypertension, which is in part mediated by vascular NADPH oxidase–derived ROS.\(^\text{15,18}\) For example, basal blood pressure is reduced in Nox2-deficient mice.\(^\text{32}\) A suppressor dose of Ang II was shown to have a smaller effect in Nox2 knockout mice compared with wild-type mice.\(^\text{33}\) A previous study reported that transgenically overexpressed Nox2 in the endothelium of mice further supported a significant pressor response induced by Ang II compared with that in wild-type mice, although there was no change in the basal blood pressure.\(^\text{34}\) In this study, it was noteworthy that the aortic relaxation responses were partially restored in TMEM16A endothelial-specific knockout mice but further deteriorated in TMEM16A endothelial-specific transgenic mice during Ang II–induced hypertension, in combination with the corresponding pressor responses. Moreover, these effects were related to the change in Nox2 protein expression. Actually, TMEM16A-mediated CaCCs have been reported to potentiate membrane depolarization in response to vasoconstrictors and enhance pulmonary vasoreactivity in pulmonary hypertension.\(^\text{13,35}\) Another study suggested that T16A-inh-A01, a specific TMEM16A inhibitor,\(^\text{36}\) blocked CaCCs in vascular smooth muscle cells (VSMCs) and directly relaxed blood vessels.\(^\text{30}\) Recently, a study showed that mice lacking vascular TMEM16A had a lower systemic blood pressure and reduced hypertensive response after administration of vasoconstrictors.\(^\text{34}\) The results of all of these studies are consistent with those of this study, and we further found that the upregulation of endothelial TMEM16A aggravated Ang II–induced endothelial dysfunction and hypertension by activating Nox2-containing NADPH oxidases.

Our findings have important implications for understanding how endothelial TMEM16A contributes to ROS–dependent signaling in the vascular wall. Vascular NADPH oxidases in general, and oxidases in VSMCs in particular, are known to play key roles, especially Nox1-containing NADPH oxidase that predominates in VSMCs.\(^\text{15}\) Moreover, the targeted disruption of TMEM16A in VSMCs was reported to result in lower basal blood pressure,\(^\text{14}\) but it was not established whether this response was related to Nox1. We now demonstrate that Nox2, but not Nox1, can reverse TMEM16A-related Ang II–induced NADPH oxidase activation in the vascular endothelium and that the modulation of endothelial TMEM16A is sufficient to alter total vascular ROS generation and the hemodynamic response to Ang II. Additional studies need to determine how endothelial and VSMC TMEM16A together contribute to vascular ROS generation in the pathogenesis of vascular disease states.

Perspectives
Our present study provides evidence that TMEM16A plays a specific and critical role in mediating the hemodynamic response to Ang II through promoting Nox2-containing NADPH oxide–derived ROS generation. These results suggest that TMEM16A is a promising target for the treatment of endothelial dysfunction–associated cardiovascular diseases, such as hypertension, stroke, atherosclerosis, and diabetes mellitus.

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Disclosures
None.

References
Novelty and Significance

What Is New?

- **TMEM16A facilitates endothelial dysfunction and regulates blood pressure during hypertension.**
- **TMEM16A protects against Nox2 degradation and increases Nox2-containing NADPH oxidase activity, resulting in increased reactive oxygen species generation.**

What Is Relevant?

- **TMEM16A is involved in the regulation of endothelial function and blood pressure, which contributes to the development of cardiovascular diseases.**

Summary

Our findings demonstrate that TMEM16A, a Ca2+-activated Cl- channel, is a regulator of endothelial function and blood pressure on hypertensive challenges, which underlies the effects caused by the activation of Nox2-derived reactive oxygen species generation. The data suggest that the modification of TMEM16A may be a novel therapeutic approach for endothelial dysfunction—associated cardiovascular diseases, such as hypertension, stroke, atherosclerosis, and diabetes mellitus.
TMEM16A Contributes to Endothelial Dysfunction by Facilitating Nox2 NADPH Oxidase–Derived Reactive Oxygen Species Generation in Hypertension
Ming-Ming Ma, Min Gao, Kai-Min Guo, Mi Wang, Xiang-Yu Li, Xue-Lin Zeng, Lu Sun, Xiao-Fei Lv, Yan-Hua Du, Guan-Lei Wang, Jia-Guo Zhou and Yong-Yuan Guan

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TMEM16A contributes to endothelial dysfunction by facilitating Nox2 NADPH oxidase-derived ROS generation in hypertension

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Supplemental material

Materials and methods

Reagents
Antibody to TMEM16A, Nox1, Nox4, and Nox5 were from Abcam (Cambridge, MA). Antibodies to p47phox was from Millipore (Billerica, MA). Antibodies to Nox2, p67phox, p22phox, Na⁺-K⁺-ATPase, Protein G beads and IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to Rac1 were from Signalway Antibody (College Park, MD). Antibodies to β-actin and all secondary antibodies were from Cell Signaling (Boston, MA). M199 medium, DMEM medium, fetal calf serum, and L-glutamine were obtained from Gibco (Life Technologies, Grand Island, NY). Recombinant human endothelial growth factor β (β-ECGF) was purchased from BD Biosciences (San Jose, CA). Angiotensin II (Ang II), lucigenin, NADPH, 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), dihydroethidium (DHE), N-acetyl-L-cysteine (NAC), T16Ainh-A01, MG132, cycloheximide, chloroquine, acetylcholine (Ach), L-nitro-arginine methylester (L-NAME), sodium nitroprusside (SNP), and phenylephrine (PE) were obtained from Sigma-Aldrich (St. Louis, MO). Synthesis of specific primers was performed by BGI Tech (Shenzhen, CN). Other chemicals, if not indicated, were all from Sigma-Aldrich (St. Louis, MO).

Cell isolation and culture
Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described previously. The experiments were approved by the medical research ethics committee of Sun Yat-Sen University and conducted according to the principles expressed in the Declaration of Helsinki. Informed consents were obtained from all subjects. In brief, HUVECs were harvested from the umbilical vein digested by 0.125% trypsin with 0.01% EGTA. Then the cells were cultured in M199 medium containing 20% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, 25 U/ml heparin, 2 mmol/L L-glutamine, and 5 ng/ml β-ECGF at 37°C, 5% CO₂ humidified atmosphere. Cells were used between passages 4 and 8 in this study.

Mouse aortic endothelial cells (ECs) and aortic smooth muscle cells (SMCs) were isolated and cultured as described previously. Briefly, male mouse was anesthetized with 60 mg/ml pentobarbital sodium intraperitoneally and the aorta was dissected and immersed in ice-cold Kreb’s solution. For isolation of ECs, after connective tissues were carefully removed, the vessel was cut into small pieces about 1-2 mm² and placed in a fibronectin-coated culture dish with the intima side faced downwardly. About 3-5 days, the ECs began to migrate from the aortic segments. The ECs were cultured in M199 medium and passaged in 0.125% trypsin as that of HUVECs. For isolation of SMCs, the thoracic aorta was subjected to digestion in DMEM medium supplemented with 200 units/ml collagenase type III, 0.1 mg/ml elastase and 0.5 mg/ml soybean trypsin inhibitor at 37°C for 30 min and then in medium containing 130 units/ml collagenase type III, 0.1 mg/ml elastase and 0.5 mg/ml soybean trypsin inhibitor at 37°C for 45 min. After that, the medial tissue was transferred to fresh medium with 130 units/ml collagenase type III, 0.1 mg/ml elastase and 0.5 mg/ml soybean trypsin inhibitor, minced with scissors and further
digested at 37°C for 60 min. Cells were centrifuged at 200 g for 3 min and then seeded for primary culture in DMEM with 20% FBS, 100 U/ml penicillin and 100 U/ml streptomycin.

**Adenoviral infection**
Ad-TMEM16A shRNA and Ad-TMEM16A were designed and produced by Sunbio Medical Biotechnology (Shanghai, CN). The sequence of Ad-TMEM16A (NM_018043) shRNA is 5'-CCGGAGCACGATTGTCTAT-3'. The cDNA of TMEM16A plasmid was a kind gift from Dr. Jan LY (University of California, San Francisco, USA). Adenovirus was infected using the protocol previously described. Briefly, cultured HUVECs at 50% confluence were infected with adenovirus encoding TMEM16A shRNA and TMEM16A for 4 h, and then cells were washed and incubated in fresh medium before experimentation.

**Plasmid transfection**
The cDNA of Nox2 and p22phox plasmids were kind gifts from Dr. Brandes RP (Klinikum der J. W. Goethe-Universität, Germany). Plasmids were transfected with lipofectamine reagent (Invitrogen, Life Technologies, Grand Island, NY) as previously described. Briefly, plasmids were mixed with lipofectamine in OPTI-MEM medium (Invitrogen, Life Technologies, Grand Island, NY) and incubated with cells according to the manufacturer’s instruction. 6 h later, cells were rinsed with PBS and cultured in the previous medium for 42 h before cell lysates collection.

**siRNA transfection**
The sequences of siRNA against human Nox1, Nox2, Nox4, and Nox5 were synthesized by Invitrogen (Life Technologies, Grand Island, NY). The sequences are as follows: Nox1, 5'-GAAAUCCAUCUGGUACAAA TT-3'; Nox2, 5'-CCUCUCAUGACUUGGAAUTT-3'; Nox4, 5'-CAGAACAUUCCAUAUACTT-3'; Nox5: 5'- TTCTATCGGAATCTAGGGTT-3'. A scrambled RNA was used as negative control. HUVECs were transfected with siRNA and negative control as previously described. Briefly, siRNA was diluted in 100 μl culture medium and mixed with 12 μl HiPerFect reagent (Qiagen, Valencia, CA) for 20 min at room temperature. And then the complex was incubated with cells for 3 h in culture medium without serum (the final siRNA concentration was 40 nmol/L). Finally, cells were incubated in normal culture medium for another 45 h.

**Reverse-transcription polymerase chain reaction (RT-PCR)**
RT-PCR was performed as previously described. Briefly, total RNA was isolated using Trizol reagent (Invitrogen, Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. 1 pg of total RNA was reverse-transcribed in a total volume of 50 μl on a Thermal Cycler (Thermo Scientific, Waltham, MA) according to the instructions of One-step RT-PCR Kit (Qiagen, Valencia, CA). Reactants underwent a reverse transcription (50°C, 30 min), an initial denaturation (95°C, 15
min), 35 amplification cycles (94°C, 45 s; 55°C, 45 s; 72°C, 60 s), and a final extension (72°C, 10 min). PCR products were detected in 1.5% agarose gel containing 0.1% ethidium bromide. The specific primers were performed in Table S1 and Table S2.

**Western blot**
Western blot was performed as previously described. Briefly, cells or tissues were lysed with RIPA lysis buffer containing protease inhibitor cocktail (Merk, Germany). Protein was separated with 8% SDS-PAGE and transformed to PVDF membrane. After blocked with 5% milk at room temperature for 1 h, the membrane was incubated with primary antibody at 4°C overnight, and then incubated for 1 h with secondary antibody at room temperature. Bands were detected with Pierce ECL western blotting substrate and quantified with the computer-aided 1-D gel imaging system (Bio-Red, Hercules, CA).

**Co-immunoprecipitation**
Co-immunoprecipitation was performed according to the protocol previously described. Briefly, cell lysates were incubated with pre-coupled antibodies bound to protein G beads overnight at 4°C. The beads were centrifuged and washed, and then boiled in protein SDS sample buffer. Samples were resolved on 8% SDS-PAGE gels and transferred to PVDF membranes. The bound proteins were determined by immunoblotting with the indicated antibodies.

**Cell fractionation**
Subcellular fractions were performed using the Qproteome Cell Compartment Kit (Qiagen, Valencia, CA), according to the manufacturer’s protocol. Briefly, cell suspension was rinsed by PBS, and then lysed by ice-cold Extraction Buffer CE1 for cytoplasm and ice-cold Extraction Buffer CE2 for membrane.

**Current recording**
Whole-cell patch experiments were performed using an Axopatch 200B patch clamp amplifier (Axon instrument, Foster City, CA) as described previously. Patch pipettes were made from borosilicate capillary tubes with a Sutter P-97 horizontal puller (Sutter Instrument, Novato, CA). The resistance of the pipettes were 3-6 MΩ after filling with pipette solution. In experiments, 70-90% series resistance was compensated. The currents were elicited with voltage steps from -100 mV to +100 mV in +20 mV increment for 250ms, and with an interval of 5s from a holding potential of -50 mV. Currents were filtered at 2 kHz and sampled at 5 kHz using pCLAMP8.0 software (Axon Instruments).

The extracellular solution contained (mmol/L): NMDG-Cl 125, KCl 5, CaCl₂ 1.5, MgSO₄ 1, HEPES 10, Glucose 10, pH was adjusted to 7.4 with NMDG. The pipette solution contained (mmol/L): CsCl 130, Mg·ATP 1, MgCl₂ 1.2, HEPES 10, EGTA 2, CaCl₂ 1.639, pH was adjusted to 7.4 with CsOH. The intracellular Ca²⁺ concentration was 500 nmol/L.
**Immunofluorescence**
Hoechst 33258/TMEM16A fluorescent double staining was preceded as described previously. Samples were fixed in 4% paraformaldehyde for 15 min and blocked with 5% BSA for 30 min, and then incubated with anti-TMEM16A (1:100) overnight at 4°C. After that, samples were rinsed and incubated with secondary antibody Alex555 Red for 1 h. Then samples were rinsed and incubated with Hoechst 33258 (1 μg/ml) for 5 min to locate nuclei. All the images were captured by Confocal Laser Scanning Microscope (Olympus, Tokyo, JP).

**Detection of ROS production**
ROS levels were measured using DCF-DA and DHE probe. HUVECs were rinsed with Hank’s buffer, and then incubated with DCF-DA (10 μmol/L) or DHE (10 μmol/L) at 37°C for 30 min. To measure ROS production in isolated aortas in situ, frozen descending thoracic aorta section were stained with DCF-DA (10 μmol/L) or DHE (10 μmol/L) at 37°C for 30 min. The fluorescent signal was detected by Confocal Laser Scanning Microscope (Olympus, Tokyo, JP) at 504-nm excitation and 524-nm emission for DCF or at 535-nm excitation and 610-nm emission for DHE.

H₂O₂ levels were determined using the Amplex Red H₂O₂ assay kit (Invitrogen™, Life Technologies, Grand Island, NY) according to the manufacturer's instruction. Briefly, aortic arteries were dissected, cleaned, and cut into pieces as 4 mm in length before transferred to a 96-well plate. The pieces were incubated with Amplex Red (50 μmol/L) and horseradish peroxidase (0.1 μmol/L) at 37°C for 1 h. Fluorescence was measured in a luminometer (MGM Instruments, Hamden, CT) using an excitation filter of 530 nm and an emission filter of 590 nm. To measure H₂O₂ production in HUVECs, cells were also incubated with Amplex Red (50 μmol/L) and horseradish peroxidase (0.1 μmol/L) at 37°C for 1 h, and recorded the fluorescence as described in tissues.

**Measurement of superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities**
SOD and GPx activities were measured by SOD assay kit and GPx assay kit (Beyotime, Nanjing, CN) according to previously described. The final results were corrected for protein content.

**NADPH oxidase activity assay**
The NADPH oxidase activity of cell membrane was measured by lucigenin-enhanced chemiluminescence as described previously. Briefly, for cultured HUVECs, cell suspension was created by detachment with phosphate buffer (50 mmol/L) containing EGTA (1 mmol/L) and protease inhibitor cocktail (pH 7.0). And then, cells were sonicated for 10 s on ice and centrifuged at 28000 × g for 15 min at 4°C. The membrane pallet was resuspended and incubated with lucigenin (5 μmol/L) and NADPH (100 μmol/L) at 37°C for 15 min. The light emission was recorded every 15 s for 20 min in a luminometer (MGM Instruments, Hamden, CT). Background counts
determined in cell-free preparations were subtracted from the total count. The NADPH oxidase activity was calculated from the ratio of mean light units to total protein level and expressed as arbitrary units.

Endothelial-specific TMEM16A knockout and transgenic mice
The TMEM16A LoxP and transgenic mice were designed and produced by Cyagen (Suzhou, CN). For TMEM16A LoxP mice, the F1 genotyping strategy was shown in Figure S5A. Two LoxP sites were introduced into the 5’ and 3’ of exon 12 separately as previously indicated, which could remove 53 amino acids of the second transmembrane domain and the extracellular loop between the first and second transmembrane domains. A flanked neomycin selection cassette was cloned into the 3’ of exon 12 in front of the second LoxP site. This targeting vector was linearized and electroporated into R1 embryonic stem cells, which were screened for homologous recombination by PCR and Southern blot analysis. A clone with successful homologous recombination was injected into C57BL/6 blastocysts and transferred into foster mice. The floxed line was backcrossed with a line expressing a Cre transgene driven by Tie2 kinase promoter/enhancer (B6.Cg-Tg(Tek-cre)12Flv/J, The Jackson Laboratory, Sacramento, CA).

For TMEM16A transgenic mice, the transgene construct consisted of the following components: pRP.ExBi-CMV-LoxP-Stop-LoxP-TMEM16A, and was microinjected into fertilized C57BL/6 mouse eggs to generate transgenic mice. Due to “stop codon” exist, TMEM16A was not overexpressed in this strain. To obtain mice harboring endothelial-specific overexpressing TMEM16A, transgenic founders were bred with mice expressing a Tie2-Cre transgene.

Genotypes of TMEM16A LoxP, transgene, and Tie2-Cre offspring were determined by PCR on tail DNA as previously described using the primers shown in Table S3. Specific expression of TMEM16A in endothelium was confirmed by Western blotting.

Animal model
C57BL/6J mice were supplied by the Experimental Animal Center of Sun Yat-Sen University in Guangzhou, China. All animals were maintained in pathogen-free facilities with a 12-hour light/dark cycle. All experimental procedures were performed in accordance with the policies of the Sun Yat-Sen University Animal Care and Use Committee and conformed to the “Guide for the Care and Use of Laboratory Animals” of the National Institute of Health in China. The number used followed the principle of minimal requirement and showed in the figure legends.

Mini-Osmotic pumps (Model 1002) were obtained from Alzet Durect Corp (Cupertino, CA). Ang II-induced hypertensive mice were operated according to manufacturer’s instruction. Briefly, Ang II (1.5 mg/kg/d) or saline was infused into Alzet osmotic pumps. Before the operation, mice (18-22 g, male) were anesthetized with 60 mg/ml pentobarbital sodium intraperitoneally. Then the already infused pump was implanted subcutaneously between the scapulae as the instruction indicated. All of the mice implanted with Ang II-infused pump would develop hypertension within 4 weeks. Systolic blood pressure (SBP) was measured in conscious mice by tail-cuff
plethysmography (BP-98A, Softron, Japan). The blood pressure measurements were taken 3 times consecutively for each mouse. The averaged data represented the blood pressure at that time point.

**Assay of vasorelaxation**

Vasorelaxation was assayed by organ chamber (DMT 620M, Winnipeg, CA). Assay of vasorelaxation was performed according to the manufacturer’s protocol. Briefly, before sacrifice, mice were anesthetized with pentobarbital sodium (60 mg/kg) intraperitoneally and weighted. Aortas were removed and immediately separated carefully.

Contractile responses of aortic rings were evoked using PE (1 μmol/L) to elicit reproducible responses. At the plateau of contraction, Ach (1×10^{-9} to 3×10^{-5}) or SNP (1×10^{-11} to 1×10^{-5}) was progressively added to the organ bath to induce endothelium-dependent relaxation and endothelium-independent relaxation, respectively. L-NAME (1 μmol/L) was preincubated for 20 min before adding Ach in the corresponding group.

**Statistics**

All statistical analyses were performed using GraphPad Prism 5 (GraphPad software, La Jolla, CA). All data are expressed as the mean±SD. N represented the number of independent experiments on different batches of cells or different mice. A 2-tailed Student’s t test for independent samples was used to detect significant differences between two groups. One-way or two-way ANOVA followed by Bonferroni multiple comparison test was used to compare differences when there were more than two treatment groups. The interaction in two-way ANOVA was considered. The level for statistical significance was 0.05.
References
### Supplemental Tables

**Table S1.** Sequences of the primers for members of TMEM16 family in HUVECs

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<th>gene</th>
<th>Primer pair (5'-3')</th>
<th>Size</th>
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<td>TMEM16A</td>
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<td></td>
<td>Rw: GCCGTATTACCGCCATCAT</td>
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<tr>
<td>TMEM16B</td>
<td>Fw: CATCATTTCCTCATCCATCTTT</td>
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<td></td>
<td>Rw: GGAGGCAAAGTTCATCAGGTA</td>
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<tr>
<td>TMEM16C</td>
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<td></td>
<td>Rw: TCCCAGCCAAGCAAGTATA</td>
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<td>Rw: GGGGCAGTGTCAGTCAGTATA</td>
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<td>TMEM16E</td>
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<td>Rw: AGGGCAACAAGCTCATG</td>
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<td>Rw: CTTTGAGGCAGTGACAGTAG</td>
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<td>Rw: GCCGTGGCTTACGATG</td>
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Fw, forward primer; Rw, reverse primer
**Table S2.** Sequences of the primers for determining Nox2 mRNA levels in HUVECs

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer pair (5'-3')</th>
<th>Size</th>
</tr>
</thead>
</table>
| Nox2        | Fw: GTCACACCCTTCGCATCCATTCTCAAGTCAGT  
Rw: CTGAGACTCATCCAGCCAGCGAGGTAG  | 225bp |
| GAPDH       | Fw: GCCAAAAGGGTCATCATCTC  
Rw: TTTGGGCAGGTTTTTCTAGACG  | 417bp |

Fw, forward primer; Rw, reverse primer
Table S3. Sequences of the primers for TMEM16A LoxP and transgenic mice genotyping

<table>
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<th>Primer name</th>
<th>Primer pair (5’-3’)</th>
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<tbody>
<tr>
<td>LoxP-1</td>
<td>Fw: GGTATCACCACCAAGGTAACCATCCA</td>
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<tr>
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<td>Rw: CAACCCTCTCTATCCCTGTACATG</td>
<td>WT: 264bp</td>
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<td>LoxP-2</td>
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<tr>
<td>Tg-TMEM16A</td>
<td>Fw: TCATGTCTGGATCCCCATCAAGC</td>
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<tr>
<td></td>
<td>Rw: GAGTACTTCTCGGGGACCCTCA</td>
<td></td>
</tr>
<tr>
<td>Tie2-Cre</td>
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<td>~100bp</td>
</tr>
<tr>
<td></td>
<td>Rw: GTGAAACAGCATTGCTGTCCTTT</td>
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</table>

Fw, forward primer; Rw, reverse primer
Figure S1. T16A_{inh}-A01 (10 μmol/L) significantly blocked calcium-activated Cl⁻ current (I_{Cl, Ca}) in HUVECs. Representative traces of I_{Cl, Ca} and I-V curve of current densities from the experiments were shown (n=6, *P<0.05 vs. 0 nmol/L [Ca²⁺]_{i}, #P<0.05 vs. 500 nmol/L [Ca²⁺]_{i}).
Figure S2

**A.** Cells were infected with Ad-TMEM16A shRNA in different MOI for 48 h. Western blot showed that the dose of 100 MOI was suitable for silencing TMEM16A (n=5, *P<0.05 vs. con).

**B.** Cells were infected with Ad-TMEM16A shRNA in 100 MOI for different time course. Western blot showed that the time course of 48 h was suitable for silencing of TMEM16A (n=5, *P<0.05 vs. con).

**Figure S2.** Effect of Ad-TMEM16A shRNA infection on the expression of TMEM16A in HUVECs. **A.** Cells were infected with Ad-TMEM16A shRNA in different MOI for 48 h. Western blot showed that the dose of 100 MOI was suitable for silencing TMEM16A (n=5, *P<0.05 vs. con). **B.** Cells were infected with Ad-TMEM16A shRNA in 100 MOI for different time course. Western blot showed that the time course of 48 h was suitable for silencing of TMEM16A (n=5, *P<0.05 vs. con).
Figure S3. Effect of Ad-TMEM16A infection on the expression of TMEM16A in HUVECs. **A.** Cells were infected with Ad-TMEM16A in different MOI for 48 h. Western blot showed that the dose of 100 MOI was suitable for overexpressing TMEM16A (n=5, *P<0.05 vs. con). **B.** Cells were infected with Ad-TMEM16A in 100 MOI for different time course. Western blot showed that the time course of 48 h was suitable for overexpressing TMEM16A (n=5, *P<0.05 vs. con).
**Figure S4.** $I_{Cl,Ca}$ was increased in HUVECs treated with Ang II (100 nmol/L) for 24 h. Representative traces of $I_{Cl,Ca}$ and I-V curve of current densities from the experiments were shown ($n$=6, *P*<0.05 vs. con).
Figure S5. Identification of TMEM16A endothelial-specific knockout mice (TM\textsuperscript{EKO}). A. F1 genotyping strategy of TM\textsuperscript{EKO}. B. Mice were identified by PCR amplified from mouse genomic DNA using primers specific for LoxP-1, LoxP-2, and Tie2-Cre respectively. Mice containing all of LoxP-1, LoxP-2, and Tie2-Cre were considered as TM\textsuperscript{EKO}. Mice containing LoxP-1 and LoxP-2, but not Tie2-Cre were considered as TM\textsuperscript{WT}. C. The protein expression of TMEM16A was successfully knockdown in cultured aortic endothelial cells of TM\textsuperscript{EKO} compared with TM\textsuperscript{WT}, but not in cultured...
aortic smooth muscle cells (n=4, *P<0.05 vs. TM\textsuperscript{WT}). D. The protein expression of TMEM16A was comparable in other tissues, including heart, liver, and kidney of TM\textsuperscript{EKO} and TM\textsuperscript{WT} (n=4). E. I\textsubscript{Cl, Ca} was fully abolished in cultured aortic endothelial cells from TM\textsuperscript{EKO} compared with those from TM\textsuperscript{WT}. Representative traces of I\textsubscript{Cl, Ca} and I-V curve of current densities from the experiments were shown (n=5, *P<0.05 vs. TM\textsuperscript{WT}).
Figure S6. Identification of TMEM16A endothelial-specific transgenic mice (TMETg). A. Mice were identified by PCR amplified from mouse genomic DNA using primers specific for transgene-TMEM16A and Tie2-Cre, respectively. Mice containing transgene-TMEM16A and Tie2-Cre were considered as TMETg. Mice containing transgene-TMEM16A, but not Tie2-Cre were considered as TMcon. B. The protein expression of TMEM16A was successfully upregulated in cultured aortic endothelial cells of TMETg compared with TMcon, but not in cultured aortic smooth muscle cells (n=4, *P<0.05 vs. TMcon). C. The protein expression of TMEM16A was comparable in other tissues, including heart, liver, and kidney of TMETg and TMcon (n=4). D. ICa was further potentiated in cultured aortic endothelial cells from TMETg compared with those from TMcon. Representative traces of ICa and I-V curve from the experiments were shown (n=5, *P<0.05 vs. TMcon).
Figure S7

A and B. Endothelial function was measured in aortas by Ach-induced endothelium-dependent relaxation from $1 \times 10^{-11}$ to $1 \times 10^{-5}$ mol/L after the use of L-NAME (1 μmol/L) for 20 min in TMEKO (A), TMETg (B), and their control littermates induced by Ang II for 4 weeks (n=6).

C and D. SNP-induced endothelium-independent relaxation was also measured in TMEKO (C), TMETg (D), and their control littermates induced by Ang II for 4 weeks (n=6).
Figure S8. TMEM16A facilitated endothelial ROS generation during Ang II-induced hypertension. A to D. Ang II-induced ROS generation was reduced in aortas of TM\textsuperscript{EKO}, and further raised in aortas of TM\textsuperscript{ETg}. Representative pictures of the sliced aortas stained with DHE (A and B) and DCF-DA (C and D), whose fluorescence intensity represented for the amount of ROS generation, in TM\textsuperscript{EKO} (A and C), TM\textsuperscript{ETg} (B and D), and their control littermates upon Ang II-induced hypertension. Scale bars are 500 μm.
Figure S9. Ang II-induced H$_2$O$_2$ generation was restrained by silence of TMEM16A, and accelerated by overexpression of TMEM16A in HUVECs. **A** and **B**. Bar charts showed the amount of H$_2$O$_2$ generation in HUVECs infected with Ad-TMEM16A shRNA (**A**), Ad-TMEM16A (**B**), and their corresponding control upon the stimulation of Ang II (100 nmol/L) for 24 h (n=5, *P*<0.05 vs. con, #P<0.05 vs. Ang).
Figure S10. TMEM16A facilitated Ang II-induced ROS generation in HUVECs. A to D. Ang II-induced ROS generation was restrained by silence of TMEM16A, and accelerated by overexpression of TMEM16A. Representative pictures of HUVECs stained with DHE (A and B) and DCF-DA (C and D) in Ad-TMEM16A shRNA (A and C), Ad-TMEM16A (B and D), and their corresponding control infected group. Ang II (100 nmol/L) was added 24 h before staining. Scale bars are 50 μm.
Figure S11. N-acetyl-L-cysteine (NAC), a ROS scavenger, significantly decreased Ang II-induced ROS production in HUVECs. **A to C.** Cells was treated with Ang II (100 nmol/L) for 24 h, NAC (1 mmol/L) was added 30 min before the incubation of Ang II. ROS production was determined by H$_2$O$_2$ generation (**A**), DHE dying (**B**), and DCF-DA dying (**C**). Scale bars are 50 μm (n=5, *P<0.05 vs. con, #P<0.05 vs. Ang).
Figure S12

Figure S12. TMEM16A further reduced the activity of GPx and SOD upon Ang II-induced challenges. A to D. Ang II-induced decrease in GPx and SOD activity was restored by silence of TMEM16A, and further reduced by overexpression of TMEM16A in HUVECs. Bar charts showed the corresponding activity of GPx and SOD in HUVECs infected with Ad-TMEM16A shRNA (A and B), Ad-TMEM16A (C and D), and their corresponding control upon the stimulation of Ang II (100 nmol/L) for 24 h (n=6, *P<0.05 vs. con, #P<0.05 vs. Ang).
Figure S13. The protein expression of Noxes was significantly reduced via siRNA transfection. **A** to **D.** HUVECs were transfected with negative control (neg) or different Noxes siRNA (40 nmol/L) for 48 h, including Nox1 (**A**), Nox2 (**B**), Nox4 (**C**), and Nox5 (**D**), and then the protein expression was examined by western blotting (n=4, *P<0.05 vs. con).
Figure S14. TMEM16A further increased the protein expression of Nox2 and p22phox induced by Ang II in HUVECs. A to F. Silence of TMEM16A blunted the upregulation of Nox2 and p22phox induced by Ang II, whereas, overexpression of TMEM16A showed the opposite effects. The expression of p47phox, p67phox, and Rac1 was not changed by the alteration of endothelial TMEM16A. The expression of NADPH oxidase subunits were measured in HUVECs infected with Ad-TMEM16A shRNA (A to C), Ad-TMEM16A (D to F), and their corresponding control upon the stimulation of Ang II (100 nmol/L) for 24 h (n=5, *P<0.05 vs. con, #P<0.05 vs. Ang).
Figure S15. TMEM16A further increased the protein expression of Nox2 and p22phox induced by Ang II in mouse aortic endothelial cells. A to F. Silence of TMEM16A blunted the upregulation of Nox2 and p22phox induced by Ang II (100 nmol/L) for 24 h, whereas, overexpression of TMEM16A showed the opposite effects. The expression of p47phox, p67phox, and Rac1 was not changed by the alteration of endothelial TMEM16A. The expression of NADPH oxidase subunits were measured in aortic endothelial cells of TM^{EKO} (A to C), TM^{ETg} (D to F), and their control littermates (n=4, *P<0.05 vs. TM^{WT} and TM^{con}, #P<0.05 vs. Ang+TM^{WT} and Ang+TM^{con}).
The degradation of Nox2 was inhibited by MG132, the proteasome inhibitor, but not chloroquine (ClQ), the lysosome blocker. The expression of Nox2 was examined using western blotting in HUVECs pretreated with MG132 (10 μmol/L) or chloroquine (10 μmol/L) for 6 h (n=5, *P<0.05 vs. con).
Figure S17. TMEM16A stably the protein levels of Nox2. A to D. The degradation of Nox2 protein was restrained in TMEM16A overexpressed cells, and accelerated in TMEM16A knockdown cells. After knockdown (A and C) or overexpression (B and D) of TMEM16A and incubated with Ang II (100 nmol/L) for 24 h in HUVECs, the expression of Nox2 was examined using western blotting in cells pretreated with cycloheximide (100 μg/ml) for different time course (n=4, *P<0.05 vs. con).
Figure S18. Ang II increased the interaction between Nox2 and TMEM16A. HUVECs were incubated with Ang II (100 nmol/L) for 24 h, and then cell lysates were immunoprecipitated with anti-Nox2 and immunoblotted with anti-TMEM16A (n=6, *P<0.05 vs. con).