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Nitric oxide and protein kinase G act on TRPC1 to inhibit 11,12-EET-induced vascular relaxation

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Aims Vascular endothelial cells synthesize and release vasodilators such as nitric oxide (NO) and epoxyeicosatrienoic acids (EETs). NO is known to inhibit EET-induced smooth muscle hyperpolarization and relaxation. This study investigates the underlying mechanism of this inhibition.

Methods and results Through measurements of membrane potential and arterial tension, we show that 11,12-EET induced membrane hyperpolarization and vascular relaxation in endothelium-denuded porcine coronary arteries. These responses were suppressed by *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 8-Br-cGMP, an NO donor and a membrane-permeant analogue of cGMP, respectively. The inhibitory actions of SNAP and 8-Br-cGMP on 11,12-EET-induced membrane hyperpolarization and vascular relaxation were reversed by hydroxocobalamin, an NO scavenger; ODQ, a guanylyl cyclase inhibitor; and KT5823, a protein kinase G (PKG) inhibitor. The inhibitory actions of SNAP and 8-bromo cyclic GMP (8-Br-cGMP) on the EET responses were also abrogated by shielding TRPC1-PKG phosphorylation sites with an excessive supply of exogenous PKG substrates, TAT-TRPC1^{S172} and TAT-TRPC1^{T313}. Furthermore, a phosphorylation assay demonstrated that PKG could directly phosphorylate TRPC1 at Ser^{172} and Thr^{313}. In addition, 11,12-EET failed to induce membrane hyperpolarization and vascular relaxation when TRPV4, TRPC1, or K_{Ca}1.1 was selectively inhibited. Co-immunoprecipitation studies demonstrated that TRPV4, TRPC1, and K_{Ca}1.1 physically associated with each other in smooth muscle cells.

Conclusion Our findings demonstrate a novel role of the NO-cGMP-PKG pathway in the inhibition of 11,12-EET-induced smooth muscle hyperpolarization and relaxation via PKG-mediated phosphorylation of TRPC1.

Keywords TRP channels • Nitric oxide • Epoxyeicosatrienoic acids • Vascular relaxation

1. Introduction

Nitric oxide (NO) and endothelium-derived hyperpolarizing factors (EDHFs) are two main classes of endothelium-derived vascular relaxant factors. Epoxyeicosatrienoic acids (EETs) constitute a major type of EDHFs, which are derived from arachidonic acids via the catalytic activity of cytochrome P450 (CYP) epoxygenases.[1] In addition to EETs, vascular endothelial cells can also release other types of EDHFs including K^+ ions, H_2O_2, and H_2S.[2]

Functionally, EETs elicit a prominent hyperpolarization in vascular smooth muscle cells and induce vascular relaxation through two major mechanisms.[1,2] First, EETs act on TRPV4 channels in smooth muscle cells to stimulate the activity of K_{Ca}1.1 channels.[1,3] Specifically, EETs directly activate TRPV4 via binding to a putative arachidonate recognition site on the N-terminus of TRPV4 in smooth muscle cells,[3] resulting in smooth muscle hyperpolarization and vascular relaxation.[1,4] Secondly, EETs act on TRPV4 in endothelial cells through an autocrine manner to stimulate the activity of K_{Ca}2.3 and K_{Ca}3.1 channels, causing endothelial cell hyperpolarization and a 'transfer of hyperpolarization' to the surrounding smooth muscle cells, leading to a consequential vascular relaxation.[1,5–8]

Although both EETs and NO induce vascular relaxation, thus reducing blood pressure, numerous reports have demonstrated that NO exerts an inhibitory action on EET-induced vascular relaxation.[9–11] Such an inhibitory action of NO on the EET-induced vascular relaxation is believed to have profound significance in pathological states. In pathological states

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of congestive heart failure, hypercholesterolaemia, ischaemia-reperfusion, and restenosis after coronary angioplasty, NO availability is impaired, but the action of EETs and/or other EDHFs is increased as 'the second line of defence' to compensate for suboptimal NO production.[10–12] However, despite its importance, the mechanisms related to the inhibitory effects of NO on EETs are not completely understood. One previous report suggested that NO may directly inhibit the activity of CYP epoxygenase.[13]

This study tests the hypothesis that the NO-cGMP-PKG pathway may induce TRPC1 phosphorylation, thereby causing the subsequent inhibition of EET action in coronary arterial smooth muscle cells. Furthermore, we tested the hypothesis that TRPV4, TRPC1, and $K_{Ca}1.1$ are critically involved in the EET-induced smooth muscle hyperpolarization and relaxation and that they may physically associate with each other. The results from this study demonstrate that NO and cGMP stimulate the PKG-mediated phosphorylation of TRPC1, resulting in an inhibition of EET-induced smooth muscle hyperpolarization and vascular relaxation.

2. Methods

2.1 Preparation of tissues

The animal experiments that were conducted conform the Guide for Animal Care and Use of Laboratory Animals published by the US National Institute of Health. All procedures were approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong. Fresh porcine hearts were collected from a local slaughterhouse, placed in a cold (4°C) Krebs solution, and immediately transferred to the laboratory for further experimentation. Branches of the left anterior descending artery were excised, and adherent tissue was removed. Vascular segments of ~600 μm in diameter and 2 mm in length were obtained. In all experiments, the endothelium was mechanically denuded by gently rubbing the arterial lumen with a steel wire. The successful removal of the endothelium was verified by a lack of relaxant response to 0.1 μmol/L bradykinin.

2.2 Arterial tension measurement

Endothelium-denuded porcine coronary artery segments (2 mm in length) were mounted on a DMT myograph (model 610M, Danish Myo Technology, Denmark) and aerated with 95% O_2 and 5% CO_2 at 37°C. The bath solution was a modified Krebs solution containing the following (in mmol/L): 119 NaCl, 4.7 KCl, 25 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 1.2 KH_2PO_4, and 11 D-glucose. The arterial segments were stretched in a stepwise manner to obtain an optimal baseline tension of ~8 mN. This tension was equivalent to that generated when vessel diameters were 1090 ± 150 μm, which is 0.9 times the diameter of the vessel at 100 mmHg.[14,15] The segments were then allowed to equilibrate for an hour, contracted with KCl (60 mmol/L), and rinsed with Krebs solution. After equilibration, the segments were pre-contracted with U46619 or $PGF_{2\alpha}$. When appropriate, arterial segments were pre-incubated with hydroxocobalamin (200 μmol/L), ODQ (10 μmol/L), KT5823 (2 μmol/L), 14,15-EEZE (10 μmol/L), iberiotoxin (100 nmol/L), or HC067047 (1 μmol/L) at 37°C for 30 min; with SNAP (1 μmol/L) or 8-Br-cGMP (2 mmol/L) at 37°C for 40 min; with TAT-TRPC1 (TAT-$TRPC1^{S172}$ 300 nmol/L + TAT-$TRPC1^{T313}$ 300 nmol/L) or TAT-scrambled (600 nmol/L) at 37°C for 60 min; or with T1E3 (1:100) or pre-immune IgG (1:100) at 4°C overnight. In the experiments intended to reverse the action of SNAP or 8-Br-cGMP, the agents (KT5823, ODQ, hydroxocobalamin, TAT-scrambled, and TAT-TRPC1) were added at least 20 min before the addition of SNAP or 8-Br-cGMP. The administration of hydroxycobalamin, ODQ, KT5823, TAT-scrambled, and TAT-TRPC1 did not alter the pre-tone caused by U46619 (60 nmol/L) or $PGF_{2\alpha}$ (3 μmol/L). Conversely, iberiotoxin and T1E3 enhanced the pre-tone caused by U46619, whereas SNAP and 8-Br-cGMP reduced the pre-tone caused by U46619 or $PGF_{2\alpha}$. Hence, the concentration of U46619 (30–270 nmol/L) or $PGF_{2\alpha}$ (1–8 μmol/L) was adjusted to obtain a similar magnitude of pre-tone, which is ~85% of its maximal U46619 or 90% of its maximal $PGF_{2\alpha}$ contraction. After adjustment, there were no significant differences in the magnitude of the pre-tone between the compared groups. The responses of the arteries to 11,12-EET were determined after the contraction to U46619 or $PGF_{2\alpha}$ reached a plateau. Concentration-response curves were acquired through the continuous addition of 11,12-EET from 10^{-8} to 10^{-5} mol/L. All experiments were performed in a time-matched manner. The detailed U46619 concentrations are listed in *Table 1*. $PGF_{2\alpha}$ concentrations were 3 ± 1 μmol/L for control, 5 ± 1 μmol/L for SNAP, 3 ± 1 μmol/L for SNAP + KT5823, 6 ± 1 μmol/L for 8-Br-cGMP, and 4 ± 1 μmol/L for 8-Br-cGMP + KT5823. Note that, although changing the U46619 concentrations from 60 to 210 nmol/L in the absence of other agents caused a slight change in the pre-contraction level, subsequent relaxant responses to 3 μmol/L 11,12-EET were similar at 40–46%.

2.3 Membrane potential measurement

In brief, endothelium-denuded coronary arterial segments were mounted on a DMT myograph chamber filled with Krebs solution and oxygenated with 95% O_2 and 5% CO_2 at 37°C. The 2 mm arterial segments were stretched to ~8 mN.[14] In most experiments, U46619 was added to the Krebs solution to pre-constrict the vessels. Membrane hyperpolarization to 3 μmol/L 11,12-EET was then determined. When appropriate, the arterial segments were pre-incubated with various agents similar to those described in the section on 'arterial tension measurement'. Similar to the vascular tone studies, the U46619 concentration in the membrane potential experiments was adjusted, and the values are listed in *Table 1*. When the U46619 concentration varied from 60 to 210 nmol/L, the hyperpolarizing responses to 3 μmol/L 11,12-EET did not change in the range of ~16–17 mV.

Membrane potential was measured by using glass microelectrodes filled with KCl (3 mol/L) (resistance: 60–100 MΩ), as described elsewhere.[16,17] To perform the microelectrode insertion, the electrode was moved gradually towards the adventitial side. The microelectrode was inserted in the middle of the segment to reduce muscle movement problems. Successful insertion was indicated by an abrupt drop in voltage, followed by a sharp return to baseline on exit. Electrical signals were monitored continuously with an EPC9 amplifier (HEKA) and the Pulse software.

2.4 Preparation of T1E3 and pre-immune immunoglobulin G

The T1E3 antibody was raised from rabbits using the strategy developed by Xu *et al.*[18,19] In brief, a peptide (CVGIFCEQQSNDTFHSFIGT) corresponding to the E3 region near the ion permeation pore, between transmembrane region S5 and S6 of TRPC1, was synthesized. It was then conjugated to Keyhole Limpet Haemocyanin (KLH) (Alpha Diagnostic International, USA) and injected into the back of a rabbit (day 0), followed by two boost doses on Day 21 and 42. Antiserum was collected 4 weeks after the second boost. Immunoglobulin G (IgG) was purified from the T1E3 antiserum by using a HiTrap Protein G column (GE Healthcare). Pre-immune serum was purified to obtain the IgG.

2.5 TAT-mediated peptide transduction

Small peptides containing the PKG phosphorylation sites of TRPC1 (TAT-$TRPC1^{S172}$, NKKDSLRHS; TAT-$TRPC1^{T313}$, RRKPTCKKI)[18] or its corresponding scrambled peptide (TAT-scrambled, NHRLDKSKS) were conjugated to an NH_2-terminal 11-amino acid HIV Tat protein transduction domain (YGRKKRRQRRR) (Alpha Diagnostic International, USA; China-Peptides, PRC).[20] In some experiments, after the transduction process, the arterial segments were washed and cut open, and the intimal surfaces of the segments were placed upside down between two coverslips on the microscope. Images were taken at an excitation wavelength of 488 nm and an emission wavelength of 520 nm by using a FV1000 Confocal imaging system.

Table 1 Comparison of resting membrane potentials under different experimental conditions in endothelium-denuded porcine coronary artery with U46619 contractures

	U46619 (nM)	Resting membrane potential (mV)	Number of segments (animals)
Control	60 ± 10	−43 ± 3	50 (37)
SNAP (1 μM)	210 ± 30	−45 ± 2	33 (18)
Hydroxocobalamin (200 μM)	60 ± 10	−44 ± 1	7 (4)
ODQ (10 μM)	60 ± 10	−44 ± 2	9 (4)
KT5823 (2 μM)	60 ± 10	−44 ± 1	8 (4)
SNAP (1 μM) + Hydroxocobalamin (200 μM)	80 ± 20	−44 ± 2	8 (4)
SNAP (1 μM) + ODQ (10 μM)	90 ± 30	−44 ± 1	9 (5)
SNAP (1 μM) + KT5823 (2 μM)	90 ± 20	−45 ± 1	8 (4)
TAT-scrambled (600 nM)	60 ± 10	−44 ± 1	8 (4)
TAT-TRPC1(600 nM)	60 ± 10	−44 ± 1	7 (4)
SNAP (1 μM) + TAT-scrambled (600 nM)	210 ± 20	−44 ± 1	6 (4)
SNAP (1 μM) + TAT-TRPC1(600 nM)	80 ± 20	−44 ± 2	7 (4)
8-Br-cGMP (2 mM)	270 ± 20	−45 ± 2	13 (8)
8-Br-cGMP (2 mM) + KT5823 (2 μM)	100 ± 20	−44 ± 1	17 (5)
8-Br-cGMP (2 mM) + TAT-scrambled (600 nM)	270 ± 20	−45 ± 1	7 (4)
8-Br-cGMP (2 mM) + TAT-TRPC1(600 nM)	90 ± 20	−44 ± 2	8 (4)
14,15-EEZE (10 μM)	60 ± 10	−45 ± 2	7 (4)
Iberiotoxin (100 nM)	40 ± 10	−45 ± 1	10 (5)
HC067047 (1 μM)	60 ± 10	−45 ± 2	8 (4)
Pre-immune IgG (1:100)	60 ± 10	−44 ± 1	9 (4)
T1E3 (1:100)	50 ± 10	−44 ± 2	9 (4)
4-AP (5 mM)	50 ± 20	−44 ± 2	7 (4)

Values are means ± SEM. Number of animals in parentheses.

2.6 Immunoblot and immunoprecipitation

Proteins were extracted from fresh endothelium-denuded porcine coronary arteries using a detergent extraction buffer containing 1% (vol/vol) Nonidet P-40, 150 mmol/L NaCl, and 20 mmol/L Tris–HCl, pH 8.0, with the addition of protease inhibitor cocktail tablets. Extracted proteins (800 μg) were incubated with 7 μg of anti-TRPC1 (ACC-010; Alomone Laboratory), anti-TRPV4 (ACC-034; Alomone Laboratory), or anti-$K_{Ca}1.1(\alpha)$ (APC-021; Alomone Laboratory) antibody, at 4°C overnight on a rocking platform. Protein A agarose was added and incubated at 4°C for another 3 h. Immunoprecipitates were then washed thrice with saline and resolved on 8% sodium dodecyl sulphate–polyacrylamide gel electrophoresis gel. For immunoblots, the polyvinylidene difluoride membrane carrying the transferred proteins was incubated at 4°C overnight with designated primary antibodies diluted (1:200) in TBST buffer containing 0.1% Tween 20 and 5% non-fat dry milk. Immunodetection was accomplished by using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection system (GE Healthcare).[21]

2.7 Cell culture, clones, and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. The TRPC1-FLAG plasmid was obtained from Addgene (Addgene plasmid 24408).[22] TRPC1-FLAG point mutations were generated using the QuickChange Site-directed Mutagenesis Kit (Stratagene). The sequences of mutagenic oligonucleotides were GCA AAA AAC AAA AAG GAT GCC CTC CGG CAT TCC AGG for S172A and GTT TAC CGA CGC AAG CCC GCC TGT AAG AAG ATA ATG for T313A.[18] Cells were grown on 10-cm plates and transfected with Lipofectamine (Invitrogen) in accordance with the manufacturer's protocols.

2.8 TRPC1 phosphorylation assay

TRPC1 phosphorylation was measured as described elsewhere.[22,23] Briefly, the HEK293 cells were transfected with Flag-tagged wild-type TRPC1 or Flag-tagged mutant TRPC1. The cells were treated with 8-Br-cGMP (2 mmol/L) for 30 min. If needed, KT5823 (2 μmol/L) was included 20 min before the addition of 8-Br-cGMP (2 mmol/L). TAT-scrambled (600 nmol/L) or TAT-TRPC1 (600 nmol/L) were included 30 min before the addition of 8-Br-cGMP (2 mmol/L). Two milligrams of isolated proteins was incubated overnight with 7 μg of anti-FLAG (Sigma) antibody and Protein A agarose. Proteins were then electro-transferred onto polyvinylidene difluoride membranes and detected using an anti-pSer/Thr antibody (Abcam) or anti-FLAG antibody (Sigma).

2.9 Drugs

(±)11,12-EET, 14,15-EEZE, and ODQ were from Cayman chemical. Nonidet P-40, $PGF_{2\alpha}$, adenosine, bradykinin, hydroxocobalamin, 4-aminopyridine (4-AP), HC067047, and dimethyl sulfoxide were from Sigma. 8-Br-cGMP, iberiotoxin, KT5823, U46619, and SNAP were from Calbiochem. SNAP, 8-Br-cGMP, U46619, HC067047, iberiotoxin, KT5823, and ODQ were dissolved in dimethyl sulfoxide. $PGF_{2\alpha}$ was dissolved in ethanol. Hydroxocobalamin was dissolved in methanol. Others were dissolved in water.

2.10 Statistics

Data were expressed as mean ± SEM. For measurements on arterial tension and membrane potential, *n* corresponds to the number of animals used per group. For other experiments, *n* corresponds to the number of independent replications. Statistical analysis was determined using repeated measures one-way or two-way ANOVA followed by Newman–keuls or Bonferroni's

test for multiple group comparisons, respectively, using GraphPad Prism software. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1 NO-cGMP-PKG pathway inhibits 11,12-EET-induced smooth muscle hyperpolarization and vascular relaxation

Sharp microelectrodes were utilized to measure the membrane potential of smooth muscle cells in endothelium-denuded porcine coronary arterial segments. In the presence of 60 nmol/L U46619, a vasoconstrictor, the resting membrane potential of vascular smooth muscle cells was -43 ± 3 mV (*Table 1*). The application of 11,12-EET (3 μmol/L), a major type of physiological EETs,[5] elicited membrane hyperpolarization in the smooth muscle cells (*Figure 1A*). The hyperpolarizing action of 11,12-EET was inhibited by 14,15-EEZE (a selective EET antagonist, 10 μmol/L) (*Figure 1B*). SNAP (an NO donor, 1 μmol/L) and 8-Br-cGMP (a membrane-permeable activator of PKG1α, 2 mmol/L)[18,21,24] markedly reduced the 11,12-EET-induced membrane hyperpolarization (*Figure 1A, C*, and *D*). Hydroxocobalamin (an NO scavenger, 200 μmol/L), ODQ (a guanylyl cyclase inhibitor, 10 μmol/L), and KT5823 (a PKG Iα inhibitor, 2 μmol/L) attenuated the inhibitory effects of SNAP or 8-Br-cGMP on the 11,12-EET-induced membrane hyperpolarization (*Figure 1C*). In contrast, hydroxycobalamin, ODQ, or KT5823 alone had no effect on the 11,12-EET-induced membrane hyperpolarization (*Figure 1C*). The inhibitory effects of SNAP and 8-Br-cGMP on the EET actions were also observed in the absence of U46619 (data not shown). As a control experiment, SNAP (1 μmol/L) had no effect on adenosine (20 μmol/L)-induced membrane hyperpolarization (see Supplementary material online, *Figure S1*). As shown in *Table 1*, pretreatment with various agents did not significantly alter the baseline resting membrane potentials.

A wire myograph was utilized to measure the vascular tone in the endothelium-denuded porcine coronary arterial segments. The arteries were pre-constricted with 60 nmol/L U46619 and 3 μmol/L $PGF_{2\alpha}$ to 19 ± 2 and 16 ± 2 mN, respectively, which amount to 85% of the maximal U46619 contraction and 90% of the maximal $PGF_{2\alpha}$ contraction. 11,12-EET was then added to induce vascular relaxation in a dose-dependent manner (*Figure 2*). The relaxant responses to 11,12-EET were suppressed by 14,15-EEZE (10 μmol/L) (*Figure 2*). SNAP (1 μmol/L) and 8-Br-cGMP (2 mmol/L) also markedly reduced the 11,12-EET-induced vascular relaxation (*Figure 2*). The inhibitory effects of SNAP or 8-Br-cGMP on the action of 11,12-EET were reversed by hydroxocobalamin (200 μmol/L), ODQ (10 μmol/L), and KT5823 (2 μmol/L) (*Figure 2*). As controls, hydroxycobalamin, ODQ, and KT5823 alone had no effect on the 11,12-EET-induced vascular relaxation (*Figure 2C* and *D*). Furthermore, SNAP (1 μmol/L) had no effect on adenosine (20 μmol/L)-induced vascular relaxation (see Supplementary material online, *Figure S1*). Together, these data demonstrate that SNAP and 8-Br-cGMP, via their actions on PKG, inhibit the 11,12-EET-induced smooth muscle cell hyperpolarization and relaxation.

3.2 NO inhibits the function of 11,12-EET through the PKG-mediated phosphorylation of TRPC1

TRPC1 contains two putative PKG phosphorylation sites,[18] located at Ser-172 and Thr-313 of TRPC1. Two membrane-permeant peptides containing the PKG phosphorylation sites of TRPC1, TAT-TRPC1^{S172}, and TAT-TRPC1^{T313} were synthesized. An abundance of these peptides should competitively inhibit PKG from binding to their active sites on endogenous TRPC1. Our results show that treatments with TAT-TRPC1 (600 nmol/L) (300 nmol/L TAT-TRPC1^{S172} + 300 nmol/L TAT-TRPC1^{T313}) reversed the inhibitory effect of SNAP and 8-Br-cGMP on the 11,12-EET-induced smooth muscle hyperpolarization and relaxation (*Figure 3*). As controls, TAT-TRPC1 and TAT-scrambled alone had no effect on the 11,12-EET-induced membrane hyperpolarization and vascular relaxation (*Figure 3B* and *D*). The successful transduction of TAT-peptides was demonstrated by the observation of FITC fluorescence in the porcine coronary arteries (see Supplementary material online, *Figure S2*).

We next determined the direct PKG phosphorylation of wild-type and mutant TRPC1 proteins. TRPC1 proteins were purified from HEK293 cells that were transfected with either wild-type TRPC1 or mutant TRPC1 (S172A-T313A), in which the two PKG phosphorylation sites on TRPC1 were mutated. The activation of PKG by 8-Br-cGMP enhanced the TRPC1 phosphorylation in HEK293 cells overexpressing wild-type TRPC1, which was not observed in the mutant TRPC1 (S172A-T313A) overexpressing cells (*Figure 4A*). KT5823 suppressed the TRPC1 phosphorylation. In addition, treatment with TAT-TRPC1 (600 nmol/L) (TAT-TRPC1^{S172} 300 nmol/L + TAT-TRPC1^{T313} 300 nmol/L) prevented the PKG phosphorylation of TRPC1 proteins in wild-type TRPC1-overexpressing HEK293 cells (*Figure 4B*). As a control, 8-Br-cGMP did not cause TRPC1 phosphorylation in non-transfected HEK293 cells (see Supplementary material online, *Figure S3*). Together, these data demonstrate that PKG could directly phosphorylate TRPC1 to inhibit the 11,12-EET-induced smooth muscle hyperpolarization and relaxation.

3.3 TRPV4, TRPC1, and K_{Ca}1.1 are critically involved in the 11,12-EET-induced smooth muscle hyperpolarization and relaxation

It has been reported that TRPC1 and TRPV4 interact with K_{Ca}1.1 to regulate vascular tone in vascular smooth muscle cells.[4,16,25] We found that the pretreatment of coronary arteries with iberiotoxin (a specific K_{Ca}1.1 blocker, 100 nmol/L), T1E3 (a TRPC1-specific blocking antibody, diluted by 1:100),[18] or HC067047 (a specific TRPV4 blocker,[26] 1 μmol/L) attenuated the 11,12-EET-induced smooth muscle hyperpolarization and relaxation (*Figure 5*). In the presence of iberiotoxin, the application of T1E3 and HC067047 had no additional inhibitory effect on the 11,12-EET-induced hyperpolarization and relaxation (*Figure 5B* and *D*). As a control, 4-AP (5 mmol/L), a blocker for delayed rectifier K^+ channel, did not affect the hyperpolarizing and relaxant responses to 11,12-EET (see Supplementary material online, *Figure S4*).

Co-immunoprecipitation experiments showed that anti-TRPV4 and anti-K_{Ca}1.1(α) antibodies could both pull down TRPC1 proteins (*Figure 6A*). Furthermore, anti-TRPV4 and anti-TRPC1 antibodies could both pull down K_{Ca}1.1 proteins (*Figure 6B*). Moreover, anti-TRPC1 and anti-K_{Ca}1.1(α) antibodies could pull down TRPV4 proteins (*Figure 6C*). For controls, the IgG purified from pre-immune serum failed to pull down TRPV4, TRPC1, or K_{Ca}1.1 proteins (*Figure 6*). The antibodies used in these experiments were previously shown to be specific to their respective targets.[18,27,28] Therefore, these results demonstrate a critical functional role of TRPV4, TRPC1, and K_{Ca}1.1 in the 11,12-EET-induced hyperpolarization and relaxation of smooth muscle cells. The data also suggest that these three proteins may be physically associated with each other.

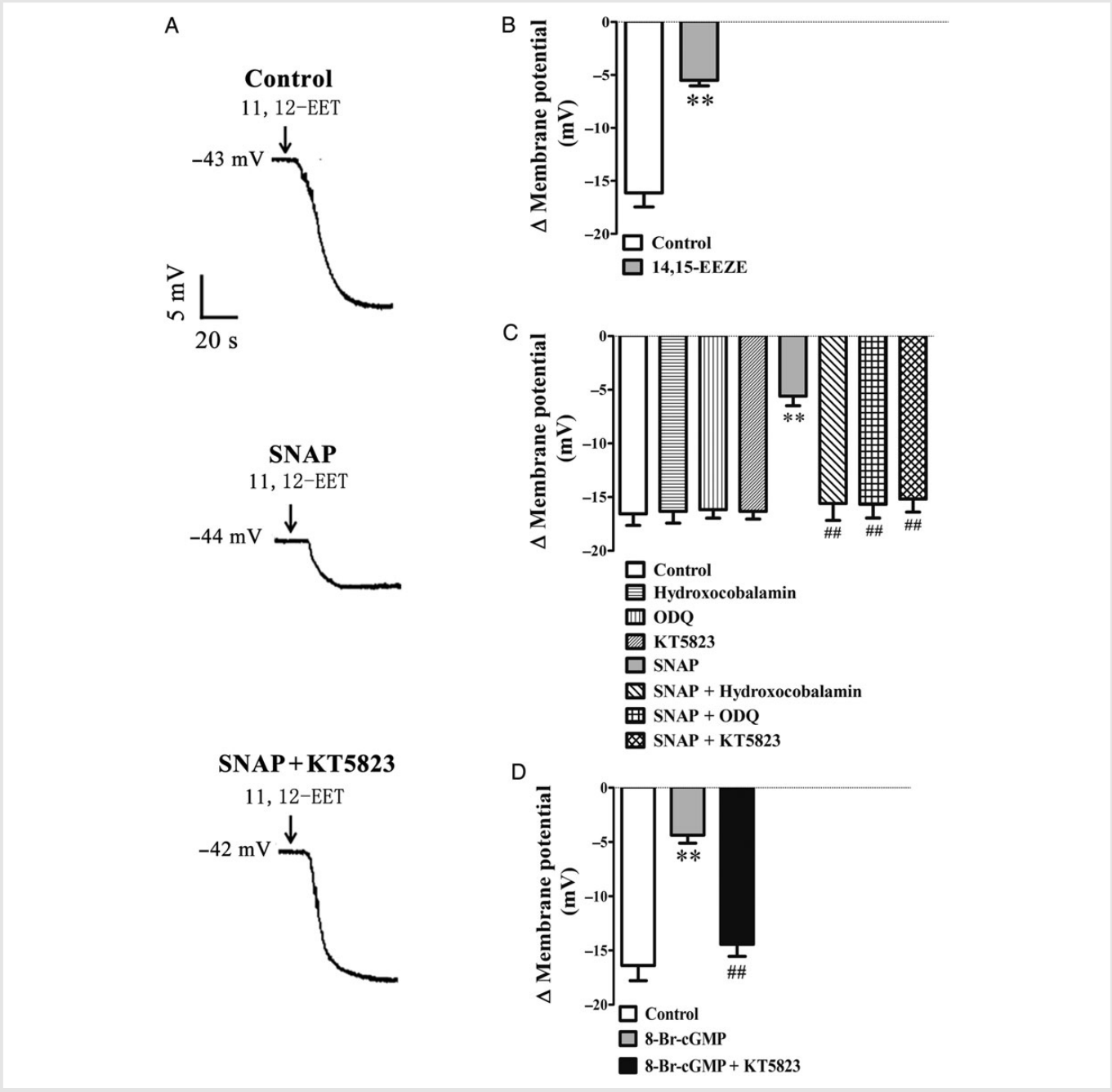

Figure 1 The NO-cGMP-PKG pathway prevents 11,12-EET-induced smooth muscle hyperpolarization in endothelium-denuded porcine coronary arterial segments. (*A*) Representative traces showing 11,12-EET (0.01–10 μmol/L)-induced changes in membrane potentials and the effects of different agents on the hyperpolarization of smooth muscle cells in the presence of U46619. (*B–D*) Maximal changes in membrane potential in response to the addition of 11,12-EET (3 μmol/L) and the effect of various treatments. Agent concentrations: 14,15-EEZE (10 μmol/L); SNAP (1 μmol/L); hydroxocobalamin (200 μmol/L); ODQ (10 μmol/L); KT5823 (2 μmol/L); and8-Br-cGMP (2 mmol/L). Mean ± SEM ($n = 4–6$). $^{**}P < 0.01$ compared with control, $^{\#\#}P < 0.01$ compared with SNAP or 8-Br-cGMP.

4. Discussion

There are several major findings in this study. First of all, the NO-cGMP-PKG pathway inhibits the smooth muscle hyperpolarization and relaxation induced by 11,12-EET in endothelium-denuded porcine coronary arteries. Secondly, TRPC1 is a critical component in 11,12-EET-induced smooth muscle hyperpolarization and relaxation. The inhibition of TRPC1 attenuates the 11,12-EET-induced vascular responses. Finally, TRPC1 is the phosphorylation target of PKG. From these data, we conclude that the NO-cGMP-PKG pathway results in PKG-mediated phosphorylation of TRPC1, thus reducing the 11,12-EET-induced smooth muscle hyperpolarization and relaxation. These results provide a novel mechanistic explanation for the negative modulation by NO of EET actions.

NO and EETs are two important vasodilators in the cardiovascular system. NO can inhibit the EET-induced vascular relaxation.[10,11,29]

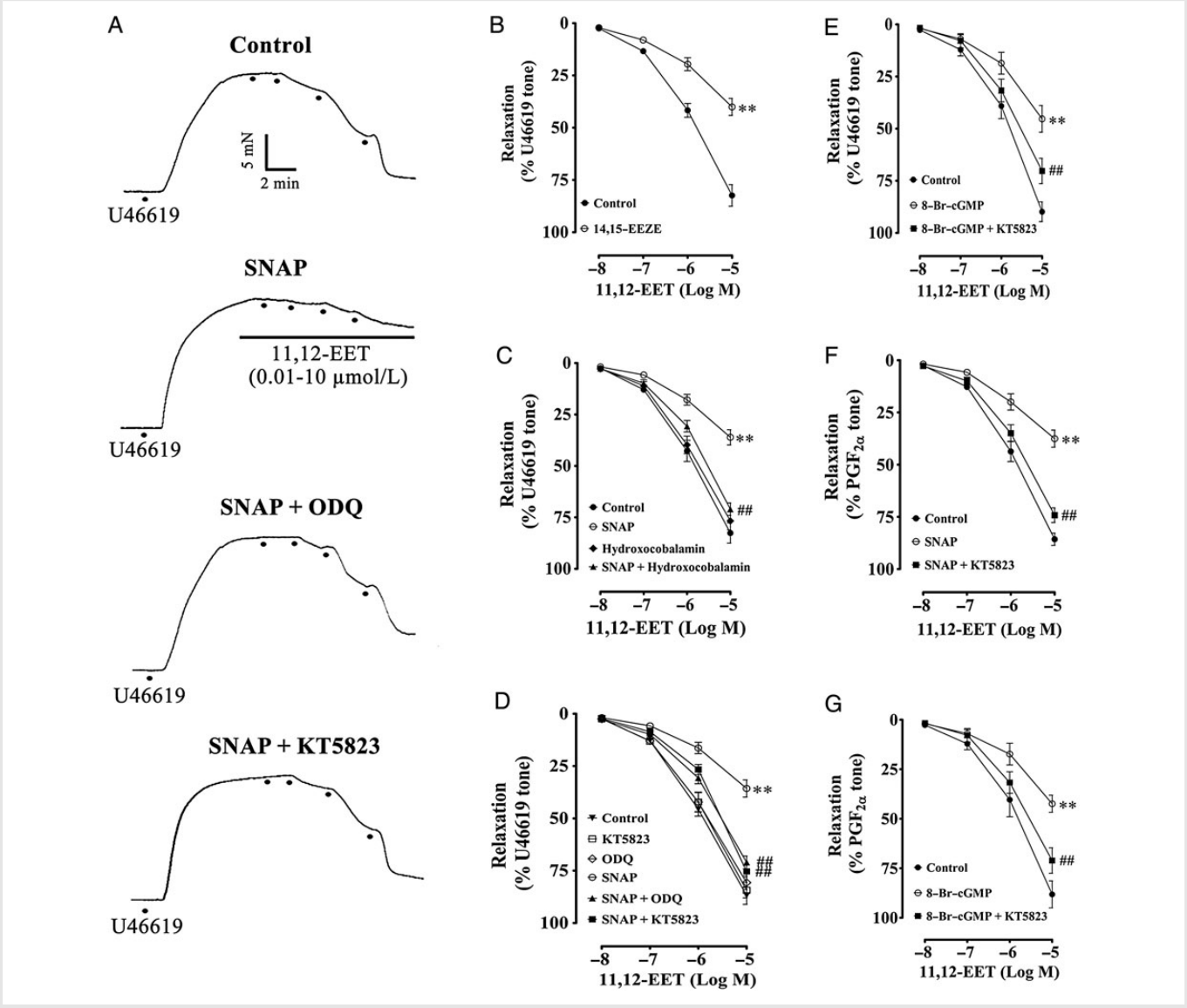

Figure 2 The NO-cGMP-PKG pathway prevents 11,12-EET-induced vascular relaxation in endothelium-denuded porcine coronary arterial segments. (*A*) Representative traces of dose-dependent vascular relaxation to 11,12-EET (0.01−10 μmol/L) and the effects of different agents on the relaxant responses. (*B*−*E*) 11,12-EET-induced vascular relaxation with U46619 contractures and the effect of various treatments. (*F*−*G*) 11,12-EET-induced vascular relaxation with $PGF_{2\alpha}$ contractures and the effect of various treatments. Agent concentrations: 14,15-EEZE (10 μmol/L); SNAP (1 μmol/L); hydroxocobalamin (200 μmol/L); ODQ (10 μmol/L); KT5823 (2 μmol/L); and 8-Br-cGMP (2 mmol/L). Mean ± SEM ($n = 4$−6). $^{**}P < 0.01$ compared with control, $^{\#\#}P < 0.01$ compared with SNAP or 8-Br-cGMP.

During pathological situations, EETs serve as back-up vasodilators when NO production is compromised.[2,12] A previous report indicated that NO could directly inhibit the activity of CYP epoxygenases,[13] which are the enzymes catalyzing the production of EETs. However, the molecular mechanism of NO inhibition on the EET actions has not been fully elucidated.[29,30] In this study, we utilized porcine coronary arteries to study the physiological interactions of NO and EETs in vascular tone control. We selected coronary arteries, because both NO and EETs are known to be important vasodilators in this artery type.[9] In all experiments, the endothelium layer was removed from the arteries to eradicate the autocrine actions of EETs on endothelial cells, which may interfere with the interpretation of experimental results. Therefore, our method allowed a direct demonstration of the mechanism of EET action on vascular smooth muscle cells without interference from endothelium.

We demonstrated that in porcine coronary arteries, SNAP and 8-Br-cGMP prevented 11,12-EET-induced smooth muscle hyperpolarization and relaxation. This inhibition was reversed by KT5823, thereby suggesting an involvement of PKG. Furthermore, the inhibitory action of 8-Br-cGMP and SNAP on the function of EETs was abrogated by shielding TRPC1-PKG phosphorylation sites with an excessive supply of exogenous PKG substrates, TAT-$TRPC1^{S172}$, and TAT-$TRPC1^{T313}$. Moreover, phosphorylation assays demonstrated that PKG could phosphorylate wild-type TRPC1, but not mutated TRPC1 (S172A/T313A), in which two PKG phosphorylation sites on TRPC1 were removed. As a result, we established that the NO-cGMP-PKG pathway could enable

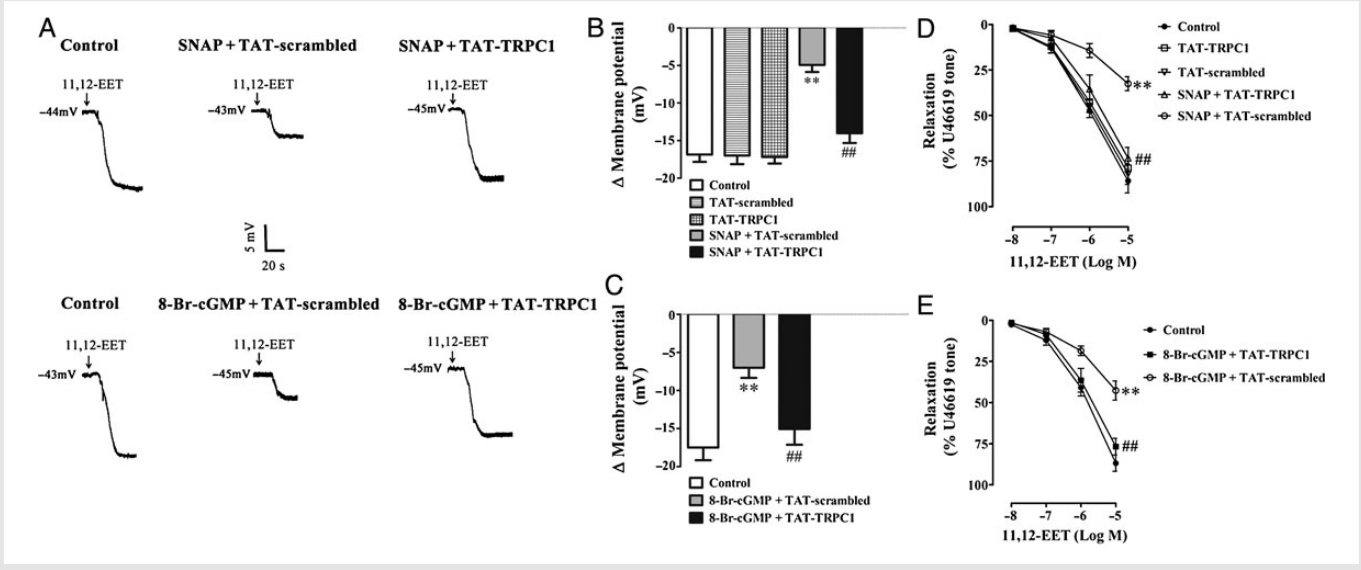

Figure 3 Effect of TAT-peptides on smooth muscle hyperpolarization and relaxation induced by 11,12-EET in endothelium-denuded porcine coronary arterial segments. (*A*) Representative traces showing 11,12-EET (3 μmol/L)-induced changes in membrane potentials and the effects of different treatments on the hyperpolarization of smooth muscle cells in the presence of U46619. (*B*–*C*) Maximal changes in membrane potentials in response to 11,12-EET (3 μmol/L) with U46619 contractures and the effect of various treatments. (*D*–*E*) Effects of different treatments on 11,12-EET-induced vascular relaxation with U46619 contractures. Agent concentrations: SNAP (1 μmol/L); 8-Br-cGMP (2 mmol/L); TAT-scrambled (600 nmol/L); and TAT-TRPC1 (300 nmol/L TAT-TRPC1^{S172} + 300 nmol/L TAT-TRPC1^{T313}). Mean ± SEM ($n = 4$–6). $^{**}P < 0.01$ compared with control, $^{\#\#}P < 0.01$ compared with SNAP or 8-Br-cGMP.

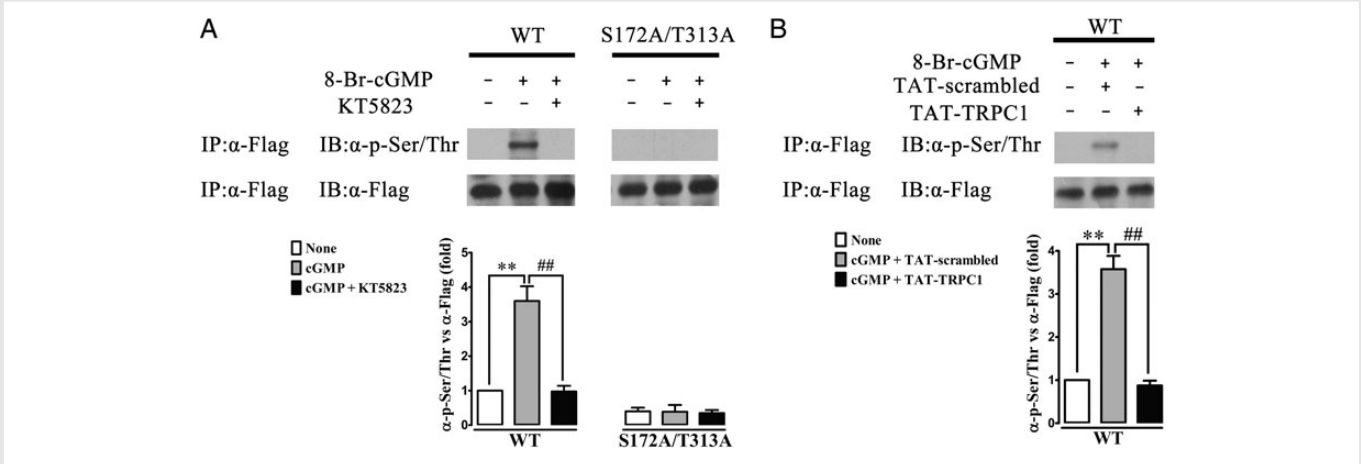

Figure 4 Specific recognition of TRPC1 phosphorylation at Ser^{172} and Thr^{313} by a pSer/Thr antibody. (*A*) PKG-dependent phosphorylation of TRPC1 proteins at Ser^{172} and Thr^{313} in TRPC1 (WT)- and TRPC1 (S172A/T313A)-expressing HEK293 cells. HEK293 cells were treated with KT5823 (2 μmol/L) for 20 min before the addition of 8-Br-cGMP (2 mmol/L) for 30 min. (*B*) TRPC1 (WT)-expressing HEK293 cells were treated with TAT-scrambled (600 nmol/L) or TAT-TRPC1 (TAT-TRPC1^{S172} 300 nmol/L + TAT-TRPC1^{T313} 300 nmol/L) for 30 min before the addition of 8-Br-cGMP (2 mmol/L) for 30 min. Shown are representative images (upper panels) and data summary (lower panels). IP and IB are abbreviations designating immunoprecipitation and immunoblotting, respectively. Mean ± SEM ($n = 5$). $^{**}P < 0.01$ compared with no cGMP or TAT, $^{\#\#}P < 0.01$ compared with cGMP but no KT/(TAT-TRPC1).

the phosphorylation of TRPC1, thereby causing the inhibition of 11,12-EET-induced smooth muscle cell hyperpolarization and relaxation.

We next determined the TRPC1 signalling pathway in EET-induced smooth muscle hyperpolarization and relaxation. Our previous publication demonstrated that TRPC1 allows a Ca^{2+} influx to stimulate $K_{Ca}1.1$, resulting in smooth muscle cell hyperpolarization and relaxation.[16] This notion was recently confirmed by Marrelli's group using TRPC1 knockout mice.[31] Furthermore, another publication of our group demonstrated that heteromeric TRPV4-TRPC1 channels were present in vascular endothelial cells.[18] Other studies have shown that EETs could directly target TRPV4.[1,3] In this study, we found that TRPV4, TRPC1, and $K_{Ca}1.1$ were endogenously present in porcine coronary arterial

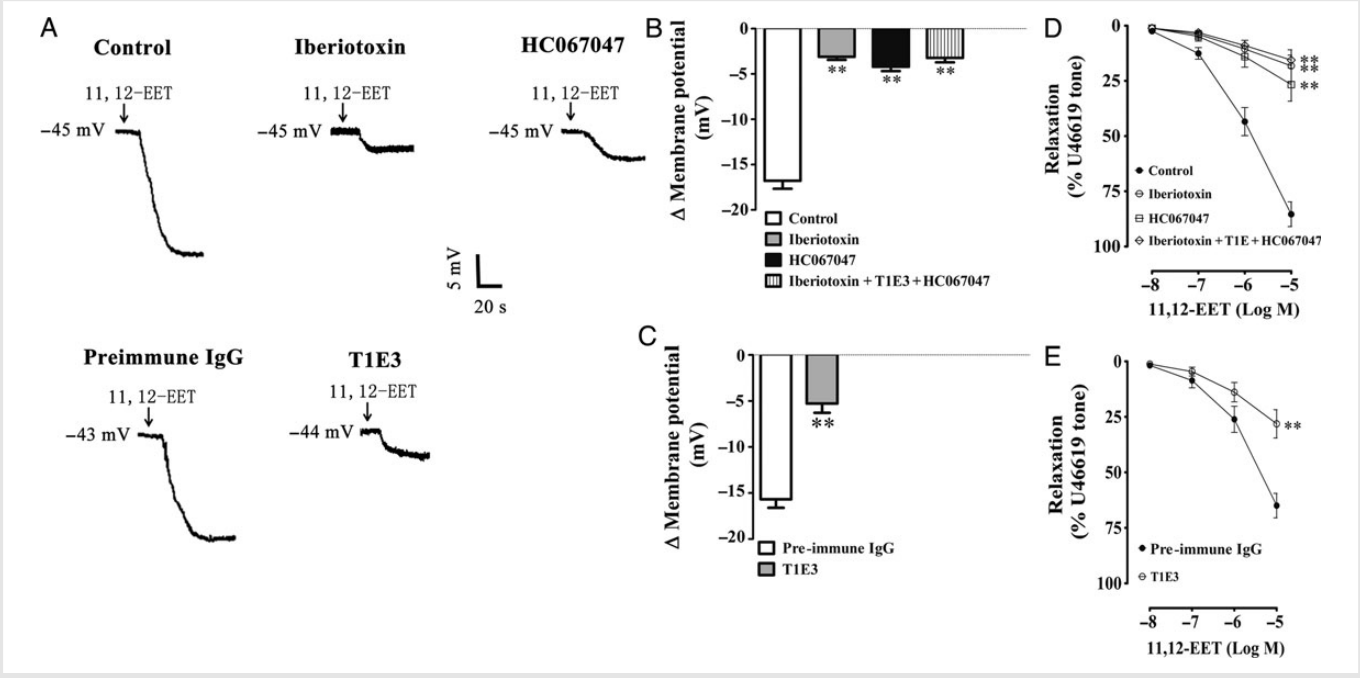

Figure 5 The effect of T1E3, iberiotoxin, and HC067047 on 11,12-EET-induced hyperpolarization and relaxation in endothelium-denuded porcine coronary arterial segments. (*A*) Representative traces showing 11,12-EET (3 μmol/L)-induced changes in membrane potentials and the effects of different treatments on the hyperpolarization of smooth muscle cells in the presence of U46619. (*B*–*C*) Maximal changes in membrane potentials in response to 11,12-EET (3 μmol/L) with U46619 contractures and the effects of various treatments. (*D*–*E*) Effects of different treatments on 11,12-EET-induced vascular relaxation with U46619 contractures. Agent concentrations: iberiotoxin (100 nmol/L); HC067047 (1 μmol/L); and T1E3 (1:100). Mean ± SEM ($n = 4$–6). $^{**}P < 0.01$ compared with control or pre-immune IgG.

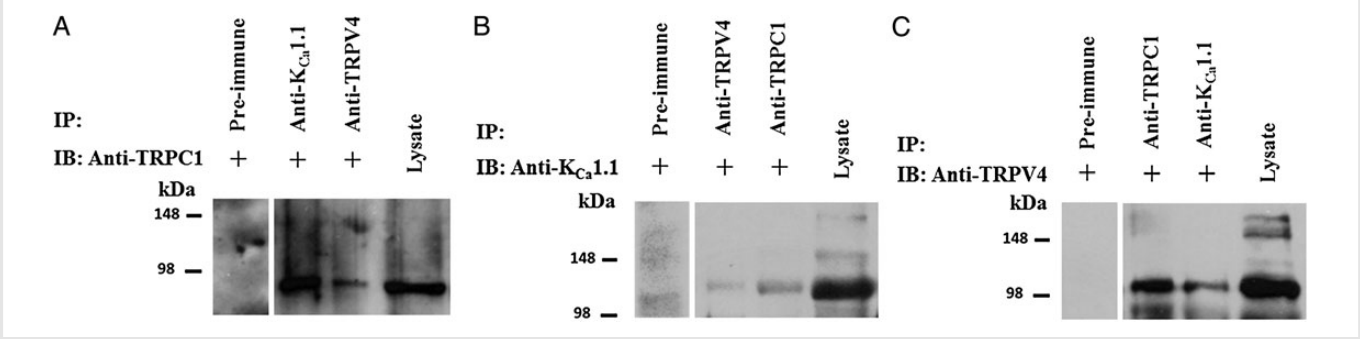

Figure 6 Co-immunoprecipitation of TRPV4, TRPC1, and $K_{Ca}1.1$ in the lysate prepared from endothelium-denuded porcine coronary arteries. (*A*) TRPC1 pull-down by anti-$K_{Ca}1.1(\alpha)$ and anti-TRPV4 antibodies. (*B*) $K_{Ca}1.1$ pull-down by anti-TRPC1 and anti-TRPV4 antibodies. (*C*) TRPV4 pull-down by anti-TRPC1 and anti-$K_{Ca}1.1(\alpha)$ antibodies. IP and IB are abbreviations designating immunoprecipitation and immunoblotting, respectively; $n = 5$.

smooth muscle cells. By inhibiting TRPV4, TRPC1, and $K_{Ca}1.1$ with their respective blockers (HC067047, T1E3, and iberiotoxin), the 11,12-EET-induced smooth muscle cell hyperpolarization and relaxation were suppressed. Our co-immunoprecipitation study also demonstrated the molecular association between TRPV4, TRPC1, and $K_{Ca}1.1$ in the smooth muscle cells. Based on these observations, a likely scenario is that EETs first act on TRPV4. TRPC1 may be downstream of TRPV4 or may heteromerize with TRPV4. The activity of TRPV4 and TRPC1 may then enhance Ca^{2+} influx to stimulate $K_{Ca}1.1$, resulting in smooth muscle cell hyperpolarization and vascular relaxation. However, note that our present data are still insufficient to prove the functional interaction of TRPV4, TRPC1, and $K_{Ca}1.1$. Future investigation is needed to further clarify the issue. For instance, the presence of functional heteromeric TRPV4-TRPC1 channels needs to be verified by patch-clamp electrophysiological recording.

It has already been well established that an increased level of NO and cGMP could activate PKG, which in turn directly phosphorylates $K_{Ca}1.1$ to cause vascular relaxation.[32] Our study provides novel evidence that NO-cGMP-PKG can act on TRPC1 to counter the EET activation on $K_{Ca}1.1$. Thus, our present data complements the generally accepted scheme that PKG activates $K_{Ca}1.1$. In other words, NO itself causes vascular relaxation, but it also inhibits EET-mediated vascular relaxation

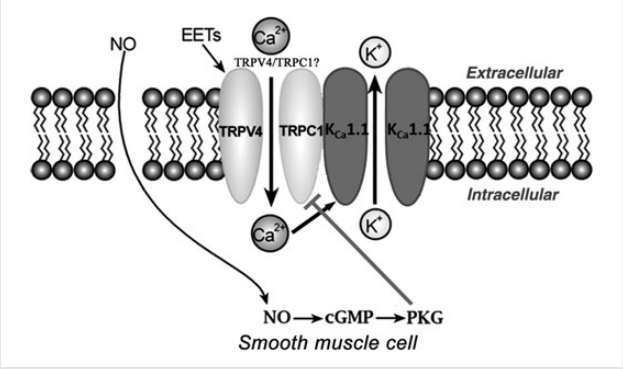

Figure 7 Schematic presentation of the postulated mechanism by which EETs interact with the NO-cGMP-PKG pathway in regulating vascular tone. EETs may act on the TRPV4-TRPC1-K_{Ca}1.1 complex to induce hyperpolarization in smooth muscle cells and vascular relaxation. The NO-cGMP-PKG pathway negatively regulates TRPC1 to counteract the effects of EETs on smooth muscle hyperpolarization and vascular relaxation.

(*Figure 7*). Such a mechanism of NO inhibition on EETs could allow EETs to serve as a back-up system for 'the second line of defence' when NO production is compromised in pathological conditions.[12] It should, however, be noted that NO and EETs may also interact through alternative pathways to influence vascular tone. In addition to the well-documented NO inhibition on EET synthesis,[13] another report also showed that EETs may stimulate NO production.[33] It is still unclear whether these different mechanisms may co-exist in the same arteries or whether they may function separately in different arteries.

In conclusion, our results provide a novel signalling pathway by which NO can elicit inhibition of the EET-induced hyperpolarization and relaxation of vascular smooth muscle. We found that NO inhibition on the EET-induced vascular responses was due to PKG-mediated phosphorylation of TRPC1. Furthermore, TRPV4, TRPC1, and K_{Ca}1.1 are important components in facilitating the EET-induced vascular smooth muscle cell hyperpolarization and vascular relaxation.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflicts of interest: none declared.

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