Impairment of Coronary Endothelial Function by Hypoxia-Reoxygenation Involves TRPC3 Inhibition-mediated $K_{Ca}$ Channel Dysfunction: Implication in Ischemia-Reperfusion Injury

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Despite increasing knowledge of the significance of calcium-activated potassium ($K_{Ca}$) and canonical transient receptor potential (TRP) channels in endothelial physiology, no studies so far have investigated the link between these two distinct types of channels in the control of vascular tone in pathological conditions. We previously demonstrated that hypoxia-reoxygenation (H-R) inhibits endothelial $K_{Ca}$ and TRPC3 channels in porcine coronary arteries (PCAs). The present study further investigated whether modulation of TRPC3 is involved in H-R-induced $K_{Ca}$ channel inhibition and associated vasodilatory dysfunction using approaches of wire myography, whole-cell voltage-clamp, and coimmunoprecipitation. Pharmacological inhibition or siRNA silencing of TRPC3 significantly suppressed bradykinin-induced intermediate- and small-conductance $K_{Ca}$ ($IK_{Ca}$ and $SK_{Ca}$) currents in endothelial cells of PCAs (PCAECs). TRPC3 protein exists in physical association with neither $IK_{Ca}$ nor $SK_{Ca}$. In H-R-exposed PCAECs, the response of $IK_{Ca}$ and $SK_{Ca}$ to bradykinin-stimulation and to TRPC3-inhibition was markedly weakened. Activation of TRPC3 channels restored H-R-suppressed $K_{Ca}$ currents in association with an improved endothelium-derived hyperpolarizing factor (EDHF)-type vasorelaxation. We conclude that inhibition of TRPC3 channels contributes to H-R-induced suppression of $K_{Ca}$ channel activity, which serves as a mechanism underlying coronary endothelial dysfunction in ischemia-reperfusion (I-R) injury and renders TRPC3 a potential target for endothelial protection in I-R conditions.

The role of intermediate and small conductance calcium-activated K⁺ ($IK_{Ca}$ and $SK_{Ca}$) channels of endothelial cells in the control of vascular tone has been well documented. Opening of $IK_{Ca}$ and $SK_{Ca}$ channels in response to increased cytosolic Ca²⁺ is the mechanism underlying the classical endothelium-derived hyperpolarizing factor (EDHF) pathway¹. In addition, activation of these channels is also involved in the regulation of eNOS activity and NO function²,³. Disturbed function of $IK_{Ca}$/$SK_{Ca}$ channels has been reported to be associated with endothelial dysfunction in cardiovascular pathologies, e.g., hypertension and diabetes⁴.

Members of the transient receptor potential (TRP) protein superfamily form nonselective cation channels that are of great importance in [Ca²⁺], regulation in vascular endothelial cells⁵,⁶. It was reported that native canonical TRP3 (TRPC3) channels contribute to constitutive and ATP-dependent Ca²⁺ influx in human coronary artery

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both IKCa and SKCa channel inhibition in PCAECs. For experiments involving Pyr3, channel blocker Pyr3 significantly suppressed bradykinin-induced whole-cell K+ currents from 18.22 \pm 0.85 pA/pF (p < 0.01) in control, to 10.63 \pm 1.7 pA/pF (p < 0.05) (Fig. 1a and b). Further studies using gene silencing approach showed that in PCAECs transfected with TRPC3-specific siRNA, bradykinin-induced K+ currents decreased from 62.39 \pm 2.65 pA/pF to 50.71 \pm 3.49 pA/pF (p < 0.01) and both IKCa (9.46 \pm 1.12 vs. 15.67 \pm 1.29 pA/pF; p < 0.01) and SKCa (6.46 \pm 0.84 vs. 9.63 \pm 1.49 pA/pF; p < 0.05) currents in response to bradykinin were significantly inhibited (Fig. 1a and b). Successful silencing of the TRPC3 gene was confirmed by the dramatic reduction of channel expression (Supplementary Fig. S1a,b) and the significant inhibition of channel activity which is evidenced by the suppressed response to OAG stimulation (p < 0.05) in TRPC3 knockdown cells while increased to 31.04 \pm 1.61 pA/pF (p < 0.01) in control cells from 19.19 \pm 1.10 pA/pF (p < 0.05) in H-R exposed cells (Fig. 3c), suggesting that inhibition of TRPC3 by Pyr3 contributed to the inhibition of endothelial K+ activity under H-R condition.

Results

TRPC3 inhibition contributes to the inhibition of endothelial K+ activity under H-R condition. We examined the role of TRPC3 channels in endothelial K+ channel activation with using pharmacological tools and siRNA knockdown technique. Pretreatment of PCAECs for 30 min with the specific TRPC3 channel blocker Pyr3 significantly suppressed bradykinin-induced whole-cell K+ currents (45.69 \pm 2.77 vs. 65.45 \pm 3.01 pA/pF; p < 0.01) with the IKCa channel current decreasing from 16.89 \pm 0.93 pA/pF to 6.71 \pm 0.85 pA/pF (p < 0.001) and the SKCa channel current from 10.63 \pm 1.69 pA/pF to 5.51 \pm 1.17 pA/pF (p < 0.05) (Fig. 1a and b). Further studies using gene silencing approach showed that in PCAECs transfected with TRPC3 specific siRNA, bradykinin-induced K+ currents decreased from 62.39 \pm 2.65 pA/pF to 50.71 \pm 3.49 pA/pF (p < 0.01) and both IKCa (9.46 \pm 1.12 vs. 15.67 \pm 1.29 pA/pF; p < 0.01) and SKCa (6.46 \pm 0.84 vs. 9.63 \pm 1.49 pA/pF; p < 0.05) currents in response to bradykinin were significantly inhibited (Fig. 1a and b). Successful silencing of the TRPC3 gene was confirmed by the dramatic reduction of channel expression (Supplementary Fig. S1a,b) and the significant inhibition of channel activity which is evidenced by the suppressed response to OAG stimulation (p < 0.05) in TRPC3 silRNA-transfected cells while increased to 31.04 \pm 1.43 pA/pF (p < 0.01) in control cells from 19.19 \pm 1.84 pA/pF (Supplementary Fig. S1c). With a subsequent application of Pyr3, the TRPC3 channel current was able to be differentiated and the data further confirmed the loss of TRPC3 channel activity in the gene knockdown cells (4.69 \pm 1.08 vs. 8.50 \pm 0.35 pA/pF in control, p < 0.05) (Supplementary Fig. S1d).

TRPC3 channels do not physically associate with K+ channels in PCAECs. The results of co-immunoprecipitation experiments showed that in PCAECs, TRPC3 protein co-precipitates with neither KCa.3.1 nor KCa.2.3 protein, suggesting that TRPC3 channels do not physically associate with KCa channels in PCAECs (Fig. 2).
period. After H-R exposure, the cells were moved to patch clamp recording chamber and the recording was usually completed within 1 hour.

**Activation of TRPC3 channels protects endothelial K\textsubscript{Ca} channels from H-R-induced inhibition.** Incubation of PCAECs with OAG during H-R exposure enhanced IK\textsubscript{Ca} and SK\textsubscript{Ca} channel activity. OAG was added to the bath solution just before the onset of hypoxia and the subsequent current recording

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**Figure 1.** In PCAECs pre-treated with the TRPC3 selective blocker Pyr3 or transfected with TRPC3 specific siRNA, both IK\textsubscript{Ca} and SK\textsubscript{Ca} currents in response to bradykinin (BK) were significantly inhibited. (a) Representative traces and current-voltage relationship of whole-cell K\textsuperscript{+} current in PCAECs from different treatment groups before and after BK stimulation with further application of the IK\textsubscript{Ca} channel blocker TRAM-34 and the SK\textsubscript{Ca} channel blocker apamin. *p < 0.05, **p < 0.01, BK vs. basal; #p < 0.05, ##p < 0.01, BK + TRAM-34 vs. BK; ^p < 0.05, ^^p < 0.01, BK + TRAM-34 + Aapamin vs. BK + TRAM-34. (b) Summarized data of BK-induced IK\textsubscript{Ca} and SK\textsubscript{Ca} currents from 5 independent experiments, each obtained from cell isolates of different heart. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA and Scheffe post-hoc test.
was performed by moving the cells to patch clamp recording chamber after H-R exposure. Compared with the H-R-exposed cells, cells exposed to H-R and co-treated with OAG exhibited significantly larger current density of IKCa (15.08 ± 0.99 vs. 8.89 ± 0.86 pA/pF in H-R, p < 0.01) and SKCa (9.62 ± 0.89 vs. 4.92 ± 0.53 pA/pF in H-R, p < 0.05) upon bradykinin stimulation, leading to increased whole-cell K+ current (56.67 ± 1.79 vs. 39.79 ± 0.82 pA/pF in H-R, p < 0.01). Further experiments revealed that TRPC3 channel activation mediates OAG-induced protection against H-R on KCa channel activity, which is suggested by the findings of loss of enhancement of bradykinin-induced K+ (38.93 ± 1.22 pA/pF, p < 0.01) and IKCa (7.94 ± 0.32 pA/pF, p < 0.01) and SKCa (4.15 ± 0.27 pA/pF, p < 0.01) channel currents in OAG-incubated cells in which current recording was performed in the presence of Pyr3 (Fig. 4a and b). Under H-R condition, activation of TRPC3 also preserves the constitutive activity of IKCa and SKCa channels, suggested by the finding of an increase of basal K+ currents in OAG-treated cells (28.90 ± 1.42 vs. 23.59 ± 1.75 pA/pF in H-R, p < 0.05) that is diminished by Pyr3 (22.03 ± 2.32 pA/pF) (Fig. 4a).

Activation of TRPC3 channels preserves vasodilatory function of endothelial KCa channels under H-R condition. Porcine small coronary arteries pretreated with Pyr3 showed attenuated EDHF-type relaxation to bradykinin (Rmax: 43.3 ± 4.7% vs. 69.5 ± 3.8%, p < 0.01) (Fig. 5a). Exposure to H-R compromised bradykinin-induced EDHF-mediated relaxation (Rmax: 40.4 ± 5.1% vs. 70.1 ± 2.7% in normoxic control, p < 0.01). Treatment with OAG throughout the H-R exposure period prevented the inhibition of EDHF-mediated relaxant response (61.3 ± 3.5%, p < 0.05 vs. H-R), indicating that activation of TRPC3 channels preserves KCa channel-related endothelial function under H-R condition (Fig. 5b). No differences were observed among different treatment groups with regard to resting force and the U46619-induced pre-contraction (Supplementary Table S1).

Discussion
The present study demonstrated that in porcine coronary arteries (1) TRPC3 channels play a fundamental role in the regulation of KCa channel activity in endothelial cells; (2) the functional association between TRPC3 and KCa channels does not involve physical interaction; (3) reduction of membrane TRPC3 contributes to H-R-induced suppression of IKCa and SKCa channel activity; (4) activation of TRPC3 protects IKCa and SKCa channels from H-R-induced inhibition thereby preserving EDHF-type vasorelaxation.

Previous investigations have shown the significance of TRPC3 channels in arterial tone and blood flow regulation in both animal and human vasculatures. In our prior studies in porcine coronary arteries, we reported that TRPC3 channels regulate vascular tone through promoting NO production and EDHF-type relaxation. The present study further demonstrated that TRPC3 channels participate in EDHF-type relaxation through mediating activation of endothelial IKCa and SKCa channels, which is in agreement with the findings in rodent vascular beds. By using sharp electrode to measure membrane potential, Senadheera and colleagues observed that in the presence of cyclooxygenase and eNOS inhibitors, inhibition of TRPC3 channels with Pyr3 significantly reduced the amplitude of ACh-induced hyperpolarization in rat mesenteric artery endothelial cells, which was the first published electrophysiological evidence suggesting the functional role of TRPC3 in KCa-dependent EDHF mechanism. A subsequent study by the same group on mouse cerebral arteries yielded similar results and further linked TRPC3 channel activation and SKCa channel activation in the EDHF-response with support of channel current recording data. In the present study, we observed that in PCAECs, pharmacological inhibition of TRPC3 channels and gene silencing of TRPC3 significantly suppressed TRAM-34-sensitive and apamin-sensitive whole-cell K+ currents in response to bradykinin, suggesting the requirement of TRPC3 channels in the activation of both IKCa and SKCa channels in porcine coronary endothelium.

Figure 2. TRPC3 channels do not physically interact with IKCa and SKCa channels in PCAECs. The cell lysates were incubated with anti-TRPC3 antibody, and the immunoprecipitates were subjected to immunoblot by anti-KCa3.1 and anti-KCa2.3 antibodies respectively (a and b). The immunoprecipitation and immunoblot experiments were further performed in reversed order (c and d). Lysate supernatant (20 μg of protein) was used as input control. Mouse IgG was used as a negative control. Full-length western blot images are presented in Supplementary Figure S2. Experiments in each order were performed for four times with using cell isolates from different hearts.
Figure 3. Inhibition of TRPC3 channels contributes to H-R-induced suppression of IKCa and SKCa channel activity in PCAECs. Representative traces and current-voltage relationship of whole-cell K+ current in PCAECs from different treatment groups before and after bradykinin (BK) stimulation with further application of the IKCa channel blocker TRAM-34 and the SKCa blocker apamin (a). In H-R group, cells were exposed to 60-min hypoxia and 30-min reoxyegenaion before patch-clamp recording. *p < 0.05, **p < 0.01, BK vs. basal; #p < 0.05, ##p < 0.01, BK + TRAM-34 vs. BK; ^p < 0.05, ^^p < 0.01, BK + TRAM-34 + Apamin vs. BK + TRAM-34. Pretreatment of PCAECs with the selective TRPC3 channel blocker Pyr3 significantly inhibited BK-induced IKCa and SKCa channel currents under both normoxic and H-R conditions (b). Pyr3 exhibited less inhibition on both components of IKCa and SKCa induced by BK when compared with the cells without H-R exposure (c). *p < 0.05, **p < 0.01, one-way ANOVA and Scheffe post-hoc test (a and b) and unpaired t test (c). n = 5 in each group. N indicates the number of independent experiments, each obtained from cell isolates of different hearts.
Given the knowledge that TRP channels may associate with other proteins to form a signaling complex, for example, colocalization of TRPC1 or TRPC3 with BKCa was found to underlie the role of these TRP members in the activation of BKCa channels. We investigated whether the functional link between TRPC3 and

Figure 4. Activation of TRPC3 channels protects IKCa and SKCa channels from H-R-induced inhibition in PCAECs. (a) Representative traces and current-voltage relationship of whole-cell K+ current in PCAECs from different treatment groups before and after bradykinin (BK) stimulation with further application of the IKCa channel blocker TRAM-34 and the SKCa channel blocker apamin. Under H-R condition, PCAECs treated with OAG shows enhanced basal and BK-induced K+ currents compared with the cells without OAG-treatment and such enhancement is diminished by Pyr3. \( * p < 0.05, * * p < 0.01, \) BK vs. basal; \( ^p < 0.05, *^p < 0.01, \) BK + TRAM-34 vs. BK; \( \ddagger p < 0.05, \ddagger^p < 0.01, \) BK + TRAM-34 + Apamin vs. BK + TRAM-34. (b) Summarized data of BK-induced IKCa and SKCa currents from 5 independent experiments, each obtained from cell isolates of different hearts. PCAECs were subjected to H-R exposure in the absence or presence of the TRPC3/6/7 activator OAG before patch-clamp recording. For cells subjected to H-R exposure and OAG treatment, the current recording was performed under the condition without (H-R + OAG-treated) or with Pyr3 application (H-R + OAG-treated + Pyr3). \( * p < 0.05, *^p < 0.01; \) one-way ANOVA and Scheffe post-hoc test.
TRPC3 channels play an important role in EDHF-mediated relaxation in porcine small coronary arteries (a, n = 8 in each group). H-R exposure impairs the EDHF-mediated vasorelaxation and activation of TRPC3 preserves the EDHF-type response under H-R condition (b, n = 8 in each group). The EDHF-response was induced by bradykinin in the presence of indomethacin, N\textsuperscript{G}-nitro-L-arginine, and oxyhemoglobin.

\textbf{Figure 5.} TRPC3 channels play an important role in EDHF-mediated relaxation in porcine small coronary arteries. (a) Bradykinin-induced IK\textsubscript{Ca} and SK\textsubscript{Ca} currents in control and H-R-exposed PCAECs, TRPC3 channel blocker Pyr3 inhibits bradykinin-induced IK\textsubscript{Ca} and SK\textsubscript{Ca} channel currents, however, the extent of inhibition is significantly less in H-R-exposed cells in comparison with cells without H-R exposure (Fig. 3). Further recordings showed that activation of TRPC3 channels effectively prevents H-R-induced suppression of endothelial IK\textsubscript{Ca} channel activity (Fig. 4). Both basal K\textsuperscript{+} currents and IK\textsubscript{Ca} and SK\textsubscript{Ca} currents in response to bradykinin were enhanced in cells exposed to H-R and co-treated with OAG, in which blocking TRPC3 channels with Pyr3 diminished the protective effect conferred by OAG. In our previous study, we demonstrated that in preparation of PCAECs, the basal K\textsuperscript{+} current is predominantly composed of IK\textsubscript{Ca} and SK\textsubscript{Ca} currents\textsuperscript{26}. Taken together, these results suggest that loss of TRPC3 channel activity contributes to H-R-induced IK\textsubscript{Ca} and SK\textsubscript{Ca} channel inhibition in coronary endothelium. In conjunction with the finding of decreased cell surface expression of IK\textsubscript{Ca} (K\textsubscript{Ca}3.1) and insignificant change in cell surface expression of SK\textsubscript{Ca} (K\textsubscript{Ca}2.3) following H-R exposure, our results suggest that the inhibition of IK\textsubscript{Ca} current induced by H-R is a combined effect of less IK\textsubscript{Ca} in the membrane and less Ca\textsuperscript{2+} signals for K\textsubscript{Ca} channel activation in PCAECs. Further studies of how Ca\textsuperscript{2+} signals bridge TRPC3 and K\textsubscript{Ca} channels, e.g., initiation and location of the signal, and/or cascades involved in Ca\textsuperscript{2+} signaling, need to be undertaken in order to fully understand the functional role of TRPC3 in K\textsubscript{Ca} channel activation.

IK\textsubscript{Ca}/SK\textsubscript{Ca} channels involves direct physical association of the channel proteins. Our co-immunoprecipitation experiments showed that in PCAECs, TRPC3 protein neither binds with K\textsubscript{Ca}2.1 nor K\textsubscript{Ca}2.3 protein, suggesting that TRPC3 does not physically interact with IK\textsubscript{Ca}/SK\textsubscript{Ca} channels to regulate the K\textsubscript{Ca} channel activity in porcine coronary endothelium. As to the study of SK\textsubscript{Ca} channel proteins, among the three subtypes SK1 (K\textsubscript{Ca}2.1), SK2 (K\textsubscript{Ca}2.2) and SK3 (K\textsubscript{Ca}2.3), we focused on SK3 (K\textsubscript{Ca}2.3), which is because (1) previous investigation demonstrated that no mRNA encoding SK1 is detected in porcine coronary endothelium and the apamin-sensitive component of the EDHF-response is mediated by SK3 while not by SK2\textsuperscript{24}, and (2) our latest study added a further piece of evidence concerning the significance of K\textsubscript{Ca}2.3 subtype in the regulation of endothelial function in this vasculature\textsuperscript{25}.

It has been reported that in vascular endothelial cells IK\textsubscript{Ca} channels predominantly reside in myoendothelial projections and SK\textsubscript{Ca} channels preferentially locate at the sites of homocellular endothelial gap junctions and caveolin-rich domains\textsuperscript{36}. In rat mesenteric arteries, based on the findings of strong expression of TRPC3 in myoendothelial contact regions, Senadheera and colleagues proposed that a close proximity of TRPC3 and IK\textsubscript{Ca} channels facilitates IK\textsubscript{Ca} activation via TRPC3-dependent Ca\textsuperscript{2+} signaling that may involve Ca\textsuperscript{2+} influx and/or refilling of the IP\textsubscript{3}-mediated endoplasmic reticulum Ca\textsuperscript{2+} stores\textsuperscript{23}. The exact localizations of TRPC3 and IK\textsubscript{Ca} channels were not characterized in the present study, however, with the findings of no binding of TRPC3 with K\textsubscript{Ca} proteins whereas significant role of TRPC3 in K\textsubscript{Ca} channel activity, the present study favors the theory of TRPC3-mediated Ca\textsuperscript{2+} signals for K\textsubscript{Ca} channel activation in PCAECs. Further studies of how Ca\textsuperscript{2+} signals bridge TRPC3 and K\textsubscript{Ca} channels, e.g., initiation and location of the signal, and/or cascades involved in Ca\textsuperscript{2+} signaling, need to be undertaken in order to fully understand the functional role of TRPC3 in K\textsubscript{Ca} channel activation.

The most important and novel finding of the present study is that inhibition of TRPC3 channels contributes to H-R-induced suppression of channel activity of IK\textsubscript{Ca} and SK\textsubscript{Ca}. We previously reported that H-R suppresses K\textsubscript{Ca} and TRPC3 channel activity in porcine coronary arterial endothelial cells\textsuperscript{8,26}. In the present study, given our demonstration that TRPC3 is integral to endothelial IK\textsubscript{Ca} and SK\textsubscript{Ca} channel activation in porcine coronary artery, we further explored the relationship between TRPC3 channel inhibition and K\textsubscript{Ca} channel inhibition and its significance in endothelial dysfunction related to I-R injury. Patch-clamp data showed that in both control and H-R-exposed PCAECs, TRPC3 channel blocker Pyr3 inhibits bradykinin-induced IK\textsubscript{Ca} and SK\textsubscript{Ca} channel currents, however, the extent of inhibition is significantly less in H-R-exposed cells in comparison with cells without H-R exposure (Fig. 3). Further recordings showed that activation of TRPC3 channels effectively prevents H-R-induced suppression of endothelial K\textsubscript{Ca} channel activity (Fig. 4). Both basal K\textsuperscript{+} currents and IK\textsubscript{Ca} and SK\textsubscript{Ca} currents in response to bradykinin were enhanced in cells exposed to H-R and co-treated with OAG, in which blocking TRPC3 channels with Pyr3 diminished the protective effect conferred by OAG. In our previous study, we demonstrated that in preparation of PCAECs, the basal K\textsuperscript{+} current is predominantly composed of IK\textsubscript{Ca} and SK\textsubscript{Ca} currents\textsuperscript{26}. Taken together, these results suggest that loss of TRPC3 channel activity contributes to H-R-induced IK\textsubscript{Ca} and SK\textsubscript{Ca} channel inhibition in coronary endothelium. In conjunction with the finding of decreased cell surface expression of IK\textsubscript{Ca} (K\textsubscript{Ca}3.1) and insignificant change in cell surface expression of SK\textsubscript{Ca} (K\textsubscript{Ca}2.3) following H-R exposure, our results suggest that the inhibition of IK\textsubscript{Ca} current induced by H-R is a combined effect of less IK\textsubscript{Ca} in the membrane and less Ca\textsuperscript{2+} signals for K\textsubscript{Ca} channel activation in PCAECs. Further studies of how Ca\textsuperscript{2+} signals bridge TRPC3 and K\textsubscript{Ca} channels, e.g., initiation and location of the signal, and/or cascades involved in Ca\textsuperscript{2+} signaling, need to be undertaken in order to fully understand the functional role of TRPC3 in K\textsubscript{Ca} channel activation.
Recent evidence suggests that trafficking of TRPC3 to the plasma membrane facilitates SKCa channel activation in mouse cerebral endothelium. In a prior study, we demonstrated that in H-R-exposed PCAECs, decreases of TRPC3 channel currents and TRPC3-mediated Ca2+ entry are attributable to the inhibition of TRPC3 trafficking that results in reduction of TRPC3 channels presented at the endothelial cell surface. In light of these findings, we therefore propose that inhibition of TRPC3 trafficking is the likely mechanism underlying the pathological significance of TRPC3 in endothelial Kc channel dysfunction associated with H-R injury.

This study has several limitations that must be acknowledged. The negative results of our co-immunoprecipitation experiments suggest that TRPC3 and Kc channels are not physically associated with each other in PCAECs. In the present study, the protein-protein interactions of TRPC3 and either IKCa and SKCa were only studied in endothelial cells under unstimulated condition and no further efforts were made to explore whether these may change when cells are upon stimulation, e.g., by agonists such as bradykinin, or by pathological factors such as I-R/H-R. Considering the fact that internal and external stimuli may dynamically regulate ion channels through mechanisms including, but not limited to, forward trafficking and endocytosis of channels, further studies of protein-protein interaction of TRPC3 and Kc in stimulated condition is therefore necessary to derive a complete and dynamic understanding of the relationship of these two channels in the control of coronary arterial tone. In addition, although we demonstrated that TRPC3 physically interacts with neither IKCa nor SKCa channels in PCAECs, current results could not answer the question whether TRPC3 and Kc channels are colocalized or located in close proximity in these cells. Further studies with double immunofluorescence staining and fluorescence resonance energy transfer measurement shall reveal the spatial proximity between these channels thereby deepen our understanding of how TRPC3 functions to regulate Kc channel activity under physiological condition and how H-R disrupts the functional interaction between TRPC3 and Kc.

In conclusion, the present study demonstrated that TRPC3 channels play an important role in IKCa and SKCa channel activation in coronary arterial endothelial cells. The functional association between TRPC3 and Kc channels does not involve physical interaction. Inhibition of TRPC3 channels contributes to H-R-induced suppression of IKCa and SKCa channel activity, which serves as a mechanism underlying endothelial dysfunction under H-R condition and renders TRPC3 channels a potential target for endothelial protection in ischemic status.

Materials and Methods
Fresh hearts of young adult pigs (~35 kg) were collected from a local slaughterhouse. Once excised, the hearts were placed in a container filled with cold (4 °C), pre-oxygenated (95%O2/5%CO2) Krebs solution and immediately transferred to the laboratory for dissection of small coronary arteries as in our previous studies. All experiments were in accordance with institutional guidelines and approved by the Animal Experimentation Ethics Committee, and Safety Office of the Chinese University of Hong Kong (Ref. No. 14/010/GRF).

Isolation and primary culture of porcine coronary arterial endothelial cells (PCAECS). PCAECs were isolated by enzymatic digestion from porcine small coronary arteries and cultured as previously described. Briefly, small porcine coronary arteries taken from the branches of left anterior descending artery (LAD) were dissected into tiny strips and treated with 0.2% collagenase (type I, Sigma) at 37 °C for 15 min. The suspension was centrifuged at 1600 rpm for 5 min and cells were resuspended in 5 mL culture medium containing 90% RPMI and 10% FBS with 100 μg/ml penicillin and 100 μg/ml streptomycin. After 2-hour incubation at 37 °C, the medium was replaced once to remove unattached cells. Attached endothelial cells were cultured in medium containing antibiotics in a humidified incubator with 5% CO2 at 37 °C for ~6 days. For maintaining electrophysiological properties of isolated coronary endothelial cells, only primary cells were used for experiments. The endothelial nature of the cultured cells was identified by immunostaining for von Willebrand Factor (vWF) as previously described. Briefly, cultured cells seeded on glass coverslips were fixed with 4% formaldehyde for 15 min at room temperature and blocked in PBS containing 2% BSA for 60 min, then incubated overnight at 4 °C with rabbit anti-vWF antibody (1:200), followed by 1 hour incubation with Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Abcam, 1:500) at room temperature in the dark. Immunostaining for alpha-smooth muscle actin (α-SMA) was used to evaluate the contamination by smooth muscle cells, which was performed by incubation of the cells with anti-α-SMA antibody (1:200) followed by Alexa Fluor® 555-conjugated donkey anti-rabbit antibody (Abcam, 1:500). The coverslip was placed on a microscope slide and covered with a drop of Prolong Gold antifade reagent with DAPI (Invitrogen). After rinsing with PBS, the cells were observed and photographed using a fluorescent microscope (Leica DM 6000 FS, Germany).

Whole-cell recording using the perforated patch-clamp technique for IKCa, SKCa, and TRPC3 channel currents in PCAECs. Whole-cell K+ currents of PCAECs were recorded by using the whole-cell perforated patch-clamp technique (EPC10, HEKA, Lambrecht, Germany) at room temperature (20–24 °C) with further differentiation of the IKCa and SKCa components. Briefly, patch pipettes with resistance of 3–5 MΩ were filled with a solution containing (mmol/L): NaCl 20, KCl 40, K-aspartate 80, MgCl2 1, Mg-ATP 3, EGTA 10, and K-HEPES 5 (pH adjusted to 7.2 using KOH). The pipette solution contains amphotericin B (500 μg/mL) as the perforating agent. The bath solution contains (mmol/L): NaCl 140, KCl 5.4, MgCl2 1, CaCl2 1.8 and Na-HEPES 10 (pH 7.4 adjusted with NaOH). Whole-cell currents were evoked by 500-ms voltage steps from −100 mV to +100 mV in 20 mV increments from a holding potential of −60 mV. K+ currents induced by bradykinin (100 nmol/L) were recorded with further application of the specific IKCa channel blocker TRAM-34 (1 μmol/L) followed by SKCa blocker apamin (100 nmol/L). Currents were recorded after achievement of the full perforation of the membrane patch that usually took 3–5 min, as judged from the development of repetitive currents in response to step depolarizations. Liquid junction potentials, series resistance, and cell capacitance were compensated. For recording of TRPC3 channel currents, current patches were filled with a solution containing (mmol/L): Cs-methanesulphonate 120, CsCl 20, HEPES 10, MgCl2 1, and EGTA 1 (pH 7.2 with KOH). The
bath solution contains (mmol/L): NaCl 137, KCl 5.4, HEPES 10, glucose 10, MgCl₂ 1, and CaCl₂ 2 (pH 7.4 with NaOH). Membrane currents were recorded in whole-cell mode using a voltage-clamp protocol (voltage ramps from −100 to +100 mV, 0.6 V/s, 0.2 Hz, holding potential −70 mV). Current in response to the TRPC3/6/7 activator 1-oleyl-2-acetyl-sn-glycerol (OAG) (100 µmol/L) were recorded with further application of the TRPC3 selective blocker Pyr3 (10 µmol/L) to differentiate the TRPC3 component. Data were analyzed with PulseFit software (HEKA). The current was normalized by cell capacitance into current densities (pA/pF). Only recordings with seal resistance >500 MΩ, and series resistance <10 MΩ were included in the study and experiments were only analyzed if changes in series resistance were no more than 2 MΩ in the entire recording period.

siRNA knockdown of endogenous TRPC3 channels. Primary cultured PCAECs were transfected with the TRPC3-specific siRNA (sense: 5′GCUCGAGGAUCAUGUCUATT3′, antisense: 5′UAGGCAUUGAUCUCGAGCTT3′) by using LipofectamineTM 2000 (Invitrogen), according to manufacturer’s instructions. Knockdown efficiency of TRPC3 was determined at both mRNA level by RT-PCR and protein level by western blotting.

RT-PCR analysis of mRNA expression of TRPC3. Total RNA from PCAECs was extracted using RNAiso reagent (Takara) and mRNA was converted to cDNA using PrimeScriptTM RT Master Mix (Takara), according to manufacturer’s instructions. PCR amplification was performed using GoTaq® G2 Flexi DNA Polymerase (Promega). For TRPC3 amplification, PCR parameters were as follows: 95 °C activation (30 s) followed by 95 °C dissociation (30 s), 46.6 °C anneal (30 s), and 72 °C elongation (1 min) for 30 cycles. Primers used for TRPC3 amplification were: forward: 5′GCAAACAGGCACAGCAGTA3′, reverse: 5′TTGAGACACAGCAGAGATCA3′. As the internal loading control, GAPDH was amplified for 30 cycles with the forward primer 5′GGTGCGAGTGAAAGGGATT3′ and the reverse primer 5′ATTGGTGTGTTGGCGGATT3′ under the following condition: activation (95 °C, 30 s) - dissociation (94 °C, 30 s) - anneal (43.3 °C, 30 s) - elongation (72 °C, 1 min). Products were identified by electrophoresis and digitally imaged for analysis (Geliance 600 Imaging System, PerKinElmer, UK).

Western blot analysis of protein expression of TRPC3. Whole cell protein was extracted from primary cultured PCAECs with ice-cold RIPA lysis buffer, composed of 30 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 3.75 mmol/L KCl, 1% NP-40, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate. Protease (1 tablet in 50 ml) and phosphatase (1 tablet in 10 ml) inhibitor cocktail tablets were freshly added to the buffer before use (Roche Diagnostics). The protein extraction was centrifuged at 4 °C for 20 min at 12000 rpm and the supernatant was then mixed with loading buffer, heated up to 100 °C for 10 min, then fractionated by denaturing 8% sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (25 µg per lane) for 90 min at 120 V and electro-transferred to polyvinylidene difluoride membrane (Thermo scientific) for 90 min at 100 V. The membrane was blocked with 5% BSA/TBST for 1 hour at room temperature and incubated with the primary antibody against TRPC3 (1:1000 dilution, Abcam) or β-tubulin (1:2500, Abcam) in 5% BSA/TBST overnight at 4 °C. The membrane was then washed in TBST followed by incubation with secondary IRDye800®-infrared fluorescent dye-conjugated goat anti-rabbit antibody (1:10000, Rockland) in TBST for 1 hour at room temperature. Imaging was performed by using Odyssey gel imaging scanner (Li-Cor Biosciences) at a wavelength of 800 nm and band intensities were analyzed by Quantity One imaging software (Version 4.6.6, Bio-Rad). β-tubulin served as internal loading control.

Co-immunoprecipitation. Experiments were performed using methods described elsewhere with slight modifications. Proteins were extracted from PCAECs 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 and phosphatase inhibitor cocktail (Roche Diagnostics). Extracted protein (1000 µg) was incubated overnight with 10 µg of antibody against TRPC3 (Alomone Labs), Kcα 2.3 (Alomone Labs), or Kcα 3.1 (Alomone Labs) at 4 °C on a rocking platform. Protein A agarose was added and incubated at 4 °C for another 4 hours. Immunoprecipitates were then washed twice with saline and resolved on 8% sodium dodecyl sulphate-polyacrylamide electrophoresis gel. For immunoblots, the polyvinylidene difluoride membrane carrying the transferred proteins was incubated with the TRPC3-specific siRNA (sense: 5′GCUCGAGGAUCAUGUCUATT3′, antisense: 5′UAGGCAUUGAUCUCGAGCTT3′) by using LipofectamineTM 2000 (Invitrogen), according to manufacturer’s instructions. Knockdown efficiency of TRPC3 was determined at both mRNA level by RT-PCR and protein level by western blotting.

Isometric force study. Porcine small coronary arteries with the diameter of 300~500 µm were dissected from the branches of LAD and cut into cylindrical rings with 2-mm in length. The rings were mounted, normalized, and equilibrated in a four-channel Mulvany myograph (Model 610 M, J.P. Trading, Aarhus, Denmark) as previously described with 1% (vol/vol) Nonidet P-40, 150 mmol/L NaCl, and 20 mmol/L Tris-HCl pH 8.0, with the addition of protease and phosphatase inhibitor cocktail (Roche Diagnostics). Extracted protein (1000 µg) was incubated overnight with 10 µg of antibody against TRPC3 (Alomone Labs), Kcα 2.3 (Alomone Labs), or Kcα 3.1 (Alomone Labs) at 4 °C on a rocking platform. Protein A agarose was added and incubated at 4 °C for another 4 hours. Immunoprecipitates were then washed twice with saline and resolved on 8% sodium dodecyl sulphate-polyacrylamide 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% BSA. Immunodetection was accomplished by 1-hour incubation with secondary IRDye800®-infrared fluorescent dye-conjugated goat anti-rabbit antibody (1:10000, Rockland) in TBST at room temperature. Imaging was performed at a wavelength of 800 nm with using Odyssey gel imaging scanner (Li-Cor Biosciences). To complete the protein-protein interaction analysis of TRPC3 and Kcα channels, immunoprecipitate obtained with anti-TRPC3 antibody was subjected to immunoblot analysis using anti-Kcα 3.1 or anti-Kcα 2.3 antibodies. The immunoprecipitation and immunoblot experiments were then performed in reversed order, i.e., immunoprecipitation with anti-Kcα 3.1 or anti-Kcα 2.3 antibody, followed by immunoblotting with anti-TRPC3 antibody.

Induction of H-R. As described in our previous studies, small coronary arteries or primary cultured PCAECs (seeded on glass coverslips) were placed in a plastic cover-sealed chamber filled with bath solution
that was continuously bubbled with 95%N₂-5%CO₂ at 37.0 ± 0.1°C to induce hypoxia exposure (PO₂ < 5 mmHg, monitored by Oxygen Meter Model 781, Strathkelvin Instrument, Glasgow, Scotland, UK) for 60 min, followed by 30 min of reoxygenation before isometric force study and voltage-clamp recording in relevant protocols. For studies using coronary arteries, the bath solution was Krebs solution (pH 7.4) that contains (in mmol/L): Na⁺ 143.4, K⁺ 2.5, Ca²⁺ 1.2, Cl⁻ 128.7, HCO₃⁻ 25, SO₄²⁻ 1.2, H₂PO₄⁻ 1.2, and glucose 11.1. While in studies with PCEAECs, the bath solution was RPMI-1640 medium.

Data analysis. Data were presented as mean ± s.e.m. Statistical analyses were performed using unpaired t test or one-way ANOVA followed by Scheffe test (SPSS, version 20) when appropriate. Differences were considered significant when p < 0.05.

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**Author Contributions**

X.-C.W., W.-T. S., J.F., and J.-H.H. performed experiments; C.-M.Y., M.-J.U., and G.-W.H., contributed to data analysis, involved in scientific discussion and paper writing; Q.Y. designed the study, supervised experiments, and wrote the paper.

**Additional Information**

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