Homocysteine alters vasoreactivity of human internal mammary artery by affecting the K_{Ca} channel family

Wen-Tao Sun[1,2], Hong-Mei Xue[1,2,3], Hai-Tao Hou[1], Huan-Xin Chen[1], Jun Wang[1], Guo-Wei He[1,4,5], Qin Yang[1]

[1]Center for Basic Medical Research & Department of Cardiovascular Surgery, TEDA International Cardiovascular Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, China; [2]School of Medicine, Nankai University, Tianjin, China; [3]Department of Physiology, Hebei Medical University, Shijiazhuang, China; [4]School of Pharmacy, Wannan Medical College, Wuhu, China; [5]Department of Surgery, Oregon Health and Science University, Portland, Oregon, USA

Contributions: (I) Conception and design: Q Yang; (II) Administrative support: J Wang; (III) Provision of study materials or patients: GW He; (IV) Collection and assembly of data: WT Sun, HM Xue, HT Hou, HX Chen; (V) Data analysis and interpretation: WT Sun, Q Yang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Professor Qin Yang, MD, PhD. TEDA International Cardiovascular Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, No.61, 3rd Ave, TEDA, Tianjin 300457, China. Email: qyanghk@163.com.

Background: Hyperhomocysteinemia is an independent risk factor for atherosclerotic heart disease. We previously demonstrated that disruption of calcium-activated potassium (K_{Ca}) channel activity is involved in homocysteine-induced dilatory dysfunction of porcine coronary arteries. Recently we reported that the K_{Ca} channel family, including large-, intermediate-, and small-conductance K_{Ca} (BK_{Ca}, IK_{Ca}, and SK_{Ca}) subtypes, are abundantly expressed in human internal mammary artery (IMA). In this study, we further investigated whether homocysteine affects the expression and functionality of the K_{Ca} channel family in this commonly used graft for coronary artery bypass surgery (CABG).

Methods: Residual IMA segments obtained from patients undergoing CABG were studied in a myograph for the role of K_{Ca} subtypes in both vasorelaxation and vasoconstriction. The expression and distribution of K_{Ca} subtypes were detected by Western blot and immunohistochemistry.

Results: Both the BK_{Ca} channel activator NS1619 and the IK_{Ca}/SK_{Ca} channel activator NS309 evoked significant IMA relaxation. Homocysteine exposure suppressed NS1619-induced relaxation whereas showed no influence on NS309-induced response. Blockade of BK_{Ca} but not IK_{Ca} and SK_{Ca} subtypes significantly suppressed acetylcholine-induced relaxation and enhanced U46619-induced contraction. Homocysteine compromised the vasodilating activity of the BK_{Ca} subtype in IMA, associated with a lowered protein level of the BK_{Ca} β1-subunit. Homocysteine potentiated the role of IK_{Ca} and SK_{Ca} subtypes in mediating endothelium-dependent relaxation without affecting the expression of these channels.

Conclusions: Homocysteine reduces the expression of BK_{Ca} β1-subunit and compromises the vasodilating activity of BK_{Ca} channels in IMA. Unlike BK_{Ca}, IK_{Ca} and SK_{Ca} subtypes are unessential for IMA vasoregulation, whereas the loss of BK_{Ca} functionality in hyperhomocysteinemia enhances the role of IK_{Ca} and SK_{Ca} subtypes in mediating endothelial dilator function. Targeting BK_{Ca} channels may form a strategy to improve the postoperative graft performance in CABG patients with hyperhomocysteinemia who receive IMA grafting.

Keywords: Calcium-activated potassium channels (K_{Ca} channels); coronary artery bypass surgery (CABG); homocysteine; internal mammary artery (IMA)

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Introduction

Hyperhomocysteinemia resulting from disorders of homocysteine metabolism is an independent risk factor for atherosclerotic vascular disease and myocardial infarction (1). High concentration of homocysteine impairs vascular endothelial function by reducing nitric oxide (NO) bioavailability (2), in which downregulation of endothelial NO synthase (eNOS) expression and overproduction of reactive oxygen species play a significant role (3,4). Previous studies in hyperhomocysteinemic animal models also reported the unfavorable effect of homocysteine on endothelium-derived hyperpolarizing factor (EDHF) (5,6). EDHF-mediated vasodilatation was compromised in the renal artery of hyperhomocysteinemic rats (6) and mesenteric arteries of hyperhomocysteinemic mice (5). With electrophysiological measurements, we further demonstrated that homocysteine-induced loss of EDHF-response in porcine coronary arteries is attributed to the inactivation of intermediate- and small-conductance calcium-activated potassium (IK_{Ca} and SK_{Ca}) channels in the coronary endothelium (7). Whether homocysteine affects IK_{Ca} and SK_{Ca} channels in human vessels remains barely studied.

In addition to IK_{Ca} and SK_{Ca}, there is another K_{Ca} channel subtype called large-conductance K_{Ca} (BK_{Ca}), which is formed by a tetramer of the pore-forming α-subunit and the regulatory β-subunit (β1 isoform in vasculature) (8). BK_{Ca} channels are abundantly expressed in smooth muscle cells and play an important role in the regulation of vascular tone. Activation of BK_{Ca} channels causes membrane hyperpolarization. The consequent closure of voltage-dependent Ca^{2+} channels leads to reduced Ca^{2+} entry and subsequent relaxation of smooth muscle cells (9). BK_{Ca} channels are a target site of NO and EDHF (10-12). Electrophysiological experiments showed the inhibitory effect of homocysteine on BK_{Ca} channel activity in both animal and human vascular smooth muscle cells (13,14). Recent mechanistic studies from our group demonstrated that endoplasmic reticulum stress-mediated downregulation of BK_{Ca}β1 is involved in homocysteine-induced BK_{Ca} channel inhibition in porcine coronary arteries (15,16). There is a lack of studies regarding the effect of homocysteine on the functionality of BK_{Ca} channels in human vessels.

Internal mammary artery (IMA) is one of the most commonly used grafts for coronary artery bypass surgery (CABG). We previously reported the reduction of NO bioavailability and the impairment of endothelial dilator function of IMA caused by elevated level of homocysteine (17). Recently, we clarified the expression and distribution profile and the functionality of the K_{Ca} channel family in IMA. IMA expresses all three K_{Ca} subtypes and each subtype distributes in both endothelium and smooth muscle (18). In comparison with the BK_{Ca} subtype that significantly contributes to the dilatation of IMA, both IK_{Ca} and SK_{Ca} subtypes play a minor role in IMA dilatation under physiological conditions. Whether the function of K_{Ca} channel family alters in pathological states such as hyperhomocysteinemia needs to be further explored.

The present study investigated the effect of homocysteine on K_{Ca} channels in IMA. The role played by each K_{Ca} subtype in vasodilatation and vasoconstriction was studied, along with the determination of channel expression. This study for the first time revealed the impact of hyperhomocysteinemia on the K_{Ca} channel family in human vasculature.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-20-6821).

Methods

Residual IMA segments (n=76) that would otherwise be discarded were collected with the consent of the patients undergoing CABG. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study protocol was approved by the Institutional Ethics Review Board of TEDA International Cardiovascular Hospital ([2018]-0626-2).

The residual IMA segment from each patient was divided into two ring segments (3 mm length) and randomly allocated to control group and homocysteine-treated group respectively. The homocysteine-treated segment was incubated with 100 μmol/L homocysteine (Sigma Aldrich, Cat#H4628) in fresh oxygenated Krebs solution at 37 ℃ in a 5% CO_2 incubator for 24 h and the control was incubated under the same condition in Krebs containing equal volume of vehicle (distilled H_2O). After 24 h, the vessels were collected for (I) vasoreactivity studies for the functional role of K_{Ca} channels, and (II) Western blot and immunohistochemistry staining for the expression of K_{Ca} channels.

Vasoreactivity study

The IMA segments were mounted in isolated chambers of a multi wire myograph system (Model 620M, J.P.Trading, Aarhus, Denmark). The ring segments were permitted

to equilibrate for 30–60 min at 37 ℃ then normalized by setting them to an internal circumference equivalent to a 90% of the circumference at a passive transmural pressure of 100 mmHg, as in our previous studies (15-18).

Vasoconstriction

Following a 40-min equilibration period, cumulative increasing concentrations of the thromboxane A_2 (TXA_2) mimetic U46619 (–11––4.5 LogM) (Cayman Chemical, Cat#16450) were added in the myograph chamber to generate a concentration-response curve. The role played by each K_{Ca} channel subtype in the regulation of IMA constriction was further characterized by comparing a first concentration-response curve constructed in control conditions with a second concentration-response curve constructed in the same ring segment after a selective inhibition of the subtype. The BK_{Ca} subtype blocker iberiotoxin (100 nmol/L) (Alomone labs, Cat#STI-400) (group Ia), the IK_{Ca} subtype blocker TRAM-34 (1 µmol/L) (Alomone labs, Cat#T-105) (group Ib), or the SK_{Ca} subtype blocker apamin (100 nmol/L) (Alomone labs, Cat#STA-200) (group Ic) were added individually or in combination (group Id: TRAM-34 + apamin, group Ie: iberiotoxin + TRAM-34 + apamin) 30 min prior to contraction with U46619. For vessels pretreated with TRAM-34 + apamin, or iberiotoxin + TRAM-34 + apamin, the drugs were added in a randomized order.

Vasorelaxation

To assess the role of K_{Ca} channel subtypes in relaxation, the IMA ring segments were preconstricted with U46619. Cumulative dose-response curves were then established for (I) BK_{Ca} channel activator NS1619 (–9––4.5 LogM) (MedChemExpress, Cat#HY-12496), or (II) IK_{Ca}/SK_{Ca} channel activator NS309 (–9––4.5 LogM) (MedChemExpress, Cat#HY-15416), or (III) acetylcholine (–10––4.5LogM) (Sigma Aldrich, Cat#A6625) when the contraction reached a plateau.

For studies using acetylcholine, five sets of experiments (group IIa-IIe) were performed. In each set, two consecutive concentration-response curves for acetylcholine were constructed in the same ring segment: a first one in control conditions and a second one after pharmacological blockade of different K_{Ca} subtypes. Iberiotoxin (100 nmol/L) (group IIa), TRAM-34 (1 µmol/L) (group IIb), or apamin (100 nmol/L) (group IIc) were used alone or in combination to pretreat the vessels for 30 min before U46619-preconstriction. For vessels pretreated with TRAM-34 + apamin (group IId) or iberiotoxin + TRAM-34 + apamin (group IIe), the drugs were added in a randomized order.

To ensure the comparability of the relaxant response in the two consecutive relaxation studies and between control and homocysteine-treated groups, similar extent of precontraction was achieved by using varied concentration of U46619 ranging from –8.5 to –7 LogM.

Western blot analysis

The IMA segment from each patient was divided into two groups and treated without (control) or with homocysteine (100 µmol/L) for 24 h. Details of the western blot procedures were published elsewhere (7,15,16,18). In brief, whole cell protein was extracted using RIPA buffer (Solarbio, China) supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific, USA). After determination of protein concentration, the samples were aliquoted and fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (30 µg/lane) for 90 min at 100 V, followed by electro-transferred to polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, USA) for 90 min at 100 V. The PVDF membrane was then blocked with 3% BSA/TBS for 1 h at room temperature and probed overnight at 4 ℃ in 1× TBS containing 3% BSA with primary antibodies (Abcam, USA) against the protein of interest, including $BK_{Ca}\alpha$ (1:1,000, Abcam, ab3586, AB_2131256), $BK_{Ca}\beta1$ (1:200, Abcam, ab3587, AB_303932), $K_{Ca}3.1$ (IK_{Ca}) (1:500, Abcam, ab75956, AB_1952001), and $K_{Ca}2.3$ (SK_{Ca}) (1:500, GeneTex, GTX47693, AB_11173990). The membrane was washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or horse anti-mouse IgG secondary antibodies (1:3,000, Cell signaling technology, USA, 7074, AB_2099233; 7076, AB_330924) for 1h at room temperature under gentle agitation. β-actin (1:2,000, Absin, China, abs137975) was used as internal loading control. Color development was performed with electrochemiluminescence kit (Beyotime, China), followed by imaging using G:BOX gel doc system (Syngene, UK). The intensity of the bands was analyzed by Quantity One imaging system (Version 4.6.6, Bio-Rad).

Histology and immunohistochemistry staining

The IMA segment from each patient was divided into control and homocysteine-treated (100 µmol/L) groups.

After incubation for 24 h, the vessels were fixed in 4% paraformaldehyde, decalcified, dehydrated, and embedded in paraffin. The embedded tissue samples were sliced into 4–5 μm sections and stained with haematoxylin and eosin after deparaffination. For immunohistochemistry staining, slides were heated at 100 ℃ in 10 mmol/L tannic acid/1 mmol/L EDTA solution (ZSGB-BIO, China) to retrieve antigens and treated with 3% H_2O_2 (ZSGB-BIO, China) to inactive endogenous peroxidase. Sections were incubated for 60 min at 37 ℃ with primary antibodies against BK_{Ca}α (1:4,000), BK_{Ca}β1 (1:500), K_{Ca}3.1 (1:800), or K_{Ca}2.3 (1:800), followed by 30-min incubation with goat anti-rabbit IgG HRP-conjugated secondary antibody at room temperature for signal detection of K_{Ca} subtypes. Afterwards, the specimens were washed in PBS and stained with 3,3'-diaminobenzidine (DAB, ZSGB-BIO, China). Counterstaining was performed with Mayer's haematoxylin (ZSGB-BIO, China). Negative controls were immunostained without the primary antibody. The immunostained images were captured using a microscope (Olympus BX43, Japan).

Immunostaining was evaluated by two independent pathologists using a blind protocol design. For each specimen, the histoscore (H-score) of each K_{Ca} channel subtype was calculated as the sum of staining intensity as previously described (18) (0–3, negative staining: 0; weak staining: 1; moderate staining: 2; and strong staining: 3) multiplied by the percentage of stained cells (0–100%).

Statistical analysis

Protein expression of targets of interest was normalized to the expression of β-actin. Relaxation was expressed as the percentage decrease in isometric force induced by U46619. The effective concentration of vasodilator/vasoconstrictor that caused 50% of maximal relaxation/constriction was defined as EC_{50}. The EC_{50} was determined from each concentration-relaxation/constriction curve by a logistic, curve-fitting equation: $E = MA^P/(A^P + K^P)$, where E is response, M is maximal relaxation/constriction, A is concentration, K is EC_{50} concentration, and P is the slope parameter. A two-sided significance level of 0.05 and a power of 80% were used to determine the sample size to show a 20% difference for comparison, based on the variance seen in our previous studies of vasoreactivity of the human IMA.

Data were expressed as mean ± SEM. Student's t-test, one-way ANOVA (Scheffe F test), and two-way repeated measures ANOVA (SPSS, version 20) were used for statistical analysis when appropriate. P<0.05 was considered statistically significant.

Results

Effect of homocysteine on the role of K_{Ca} subtypes in the regulation of IMA contractility

Blockade of the BK_{Ca} subtype with iberiotoxin significantly enhanced U46619-induced vasoconstriction (P=0.047, two-way repeated measures ANOVA) (Rmax: 71.3±12.7 *vs.* 50.1±4.9 mN in control, P=0.152; contraction at –7.5LogM: 66.3±11.9 *vs.* 37.6±5.9 mN in control, P=0.048). The sensitivity to U46619 tended to increase (EC_{50}: –9.01±0.27 *vs.* –8.34±0.25 LogM in control, P=0.096) (*Figure 1A* & *Table 1*). In comparison, inhibition of the IK_{Ca} or the SK_{Ca} subtype by TRAM-34 or apamin respectively or jointly barely affected the contractile response and sensitivity of IMA to U46619 (*Figure 1B,C,D* & *Table 1*). Interestingly, although iberiotoxin caused a marked enhancement of U46619-induced vasoconstriction, co-treatment with iberiotoxin, TRAM-34, and apamin did not significantly increase the maximal contractile response though the contraction seemed to be enhanced at low doses of U46619 (*Figure 1E*).

Homocysteine exposure augmented the contractile response of IMA to U46619 (P=0.033, two-way repeated measures ANOVA) (Rmax: 71.3±7.0 *vs.* 50.1±4.9 mN in control, P=0.032) (*Figure 1A*). The enhancement effect of iberiotoxin on the vasoconstriction was blunted after homocysteine exposure (P=0.596, homocysteine *vs.* homocysteine + iberiotoxin, two-way repeated measures ANOVA, Rmax: 66.4±16.3 *vs.* 71.3±7.0 mN, P=0.786) (*Figure 1A* & *Table 1*). Application of TRAM-34 and apamin, either individually (*Figure 1B,C*) or jointly (*Figure 1D*), showed no effect on U46619-induced constriction in homocysteine-exposed IMA. Further triple blockade of BK_{Ca}, IK_{Ca}, and SK_{Ca} subtypes also insignificantly affected the contractile response (*Figure 1E*). In addition, in homocysteine-exposed IMA, the EC_{50} values for U46619 remained unshifted after pretreatment with K_{Ca} blockers (*Table 1*).

Effect of homocysteine on the relaxant response of IMA to K_{Ca} channel activators

Both the BK_{Ca} channel activator NS1619 and the IK_{Ca}/SK_{Ca} channel activator NS309 elicited dose-dependent

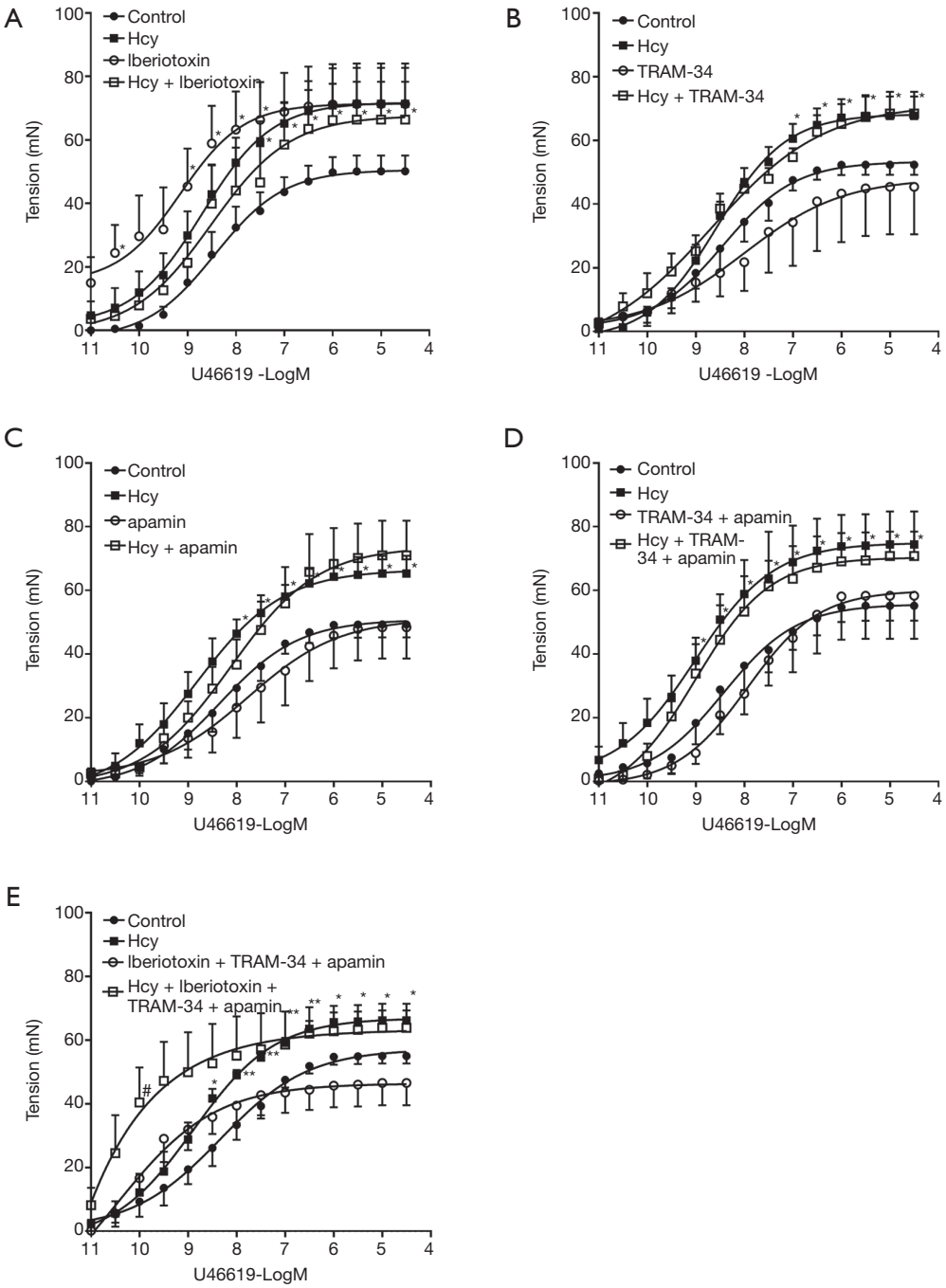

Figure 1 Effect of homocysteine (Hcy) on the role of K_{Ca} subtypes in the regulation of internal mammary artery (IMA) contractility. Hcy enhances the contractile response of IMA to U46619 and compromises the activity of the BK_{Ca} channel subtype in opposing vasoconstriction (A). Neither IK_{Ca} nor SK_{Ca} channel subtype is involved in U46619-induced contraction in IMAs exposed or not exposed to homocysteine (B,C,D). The K_{Ca} channel family is barely involved in the regulation of IMA contractility in hyperhomocysteinemic condition (E). n=6. *P<0.05, **P<0.01 *vs.* control; #P<0.05 *vs.* Hcy. Iberiotoxin: BK_{Ca} channel blocker; TRAM-34: IK_{Ca} channel blocker; apamin: SK_{Ca} channel blocker.

Table 1 Effect of K_{Ca} channel blockers on EC_{50} values for U46619 in internal mammary artery (IMA) subjected or not subjected to homocysteine exposure (n=6 in each group)

Group	Without homocysteine exposure		With homocysteine exposure		P value
	EC_{50} (LogM)	P value (*vs*. control)	EC_{50} (LogM)	P value (*vs*. control)	
Ia					
Control	−8.34±0.25		−9.05±0.42		0.176
Iberiotoxin	−9.01±0.27	0.096	−8.90±0.41	0.804	0.830
Ib					
Control	−8.43±0.28		−8.59±0.15		0.614
TRAM-34	−7.71±0.43	0.186	−8.61±0.48	0.966	0.189
Ic					
Control	−8.51±0.45		−8.71±0.17		0.681
Apamin	−7.61±0.39	0.165	−7.95±0.36	0.097	0.538
Id					
Control	−8.39±0.25		−9.03±0.42		0.223
TRAM-34 + apamin	−7.95±0.24	0.234	−8.48±0.44	0.390	0.338
Ie					
Control	−8.58±0.44		−8.77±0.08		0.684
Iberiotoxin + TRAM-34 + apamin	−9.49±0.28	0.114	−9.92±0.68	0.125	0.571

K_{Ca} channel, calcium-activated potassium channel; EC_{50}, concentration for 50% of maximal effect.

relaxation in IMA. Homocysteine exposure significantly suppressed NS1619-induced relaxation (Rmax: 31.8%±1.7% *vs.* 51.0%±3.1% in control, P<0.001) (*Figure 2A*) whereas showed no influence on NS309-induced relaxation (Rmax: 81.5%±5.2% *vs.* 79.0%±6.7% in control, P=0.760) (*Figure 2B*).

Effect of homocysteine on the role of K_{Ca} subtypes in endothelium-dependent relaxation in IMA

Blockade of BK_{Ca} channels with iberiotoxin significantly inhibited acetylcholine-induced relaxation (P<0.0001, two-way repeated measures ANOVA; Rmax: 41.9%±5.5% *vs.* 72.6%±3.4%, P<0.001) (*Figure 3A*). Application of the IK_{Ca} channel blocker TRAM-34 and the SK_{Ca} channel blocker apamin, individually (*Figure 3B*,*C*) or jointly (*Figure 3D*), barely suppressed the relaxant response of IMA to acetylcholine. Combination of TRAM-34, apamin, and iberiotoxin showed similar inhibition on acetylcholine-induced relaxation (36.9%±6.1% *vs.* 79.9%±4.7%, P<0.001) as compared to iberiotoxin alone, which further suggests the dominant contribution of the BK_{Ca} subtype to IMA dilatation (*Figure 3E*).

Homocysteine exposure suppressed the relaxant response of IMA to acetylcholine (P=0.028, two-way repeated measures ANOVA) with the maximal relaxation decreasing from 72.6%±3.4% to 56.5%±5.3% (P=0.029) (*Figure 3A*). The EC_{50} remained unchanged after homocysteine exposure (*Table 2*). Iberiotoxin showed no inhibition on acetylcholine-induced relaxation in homocysteine-exposed IMA (56.2%±7.7%) (*Figure 3A*). The diminished inhibitory effect of iberiotoxin suggested that the role of BK_{Ca} subtype in IMA dilatation was compromised by homocysteine. In contrast, after homocysteine exposure, acetylcholine-induced relaxation was significantly inhibited by TRAM-34 (P<0.0001, two-way repeated measures ANOVA; Rmax: 38.3%±10.6% *vs.* 65.9%±4.9%, P=0.056; relaxation at −5.5LogM: 34.6%±10.6% *vs.* 63.3%±4.3%, P=0.046) (*Figure 3B*) and apamin (P=0.018, two-way repeated measures ANOVA; Rmax: 28.9%±8.1% *vs.* 58.2±3.7%, P=0.013) (*Figure 3C*). Combined application of TRAM-34 and apamin (Rmax: 36.3%±5.5% *vs.* 54.4%±7.0%), even in conjunction with iberiotoxin (Rmax: 42.5%±7.6%

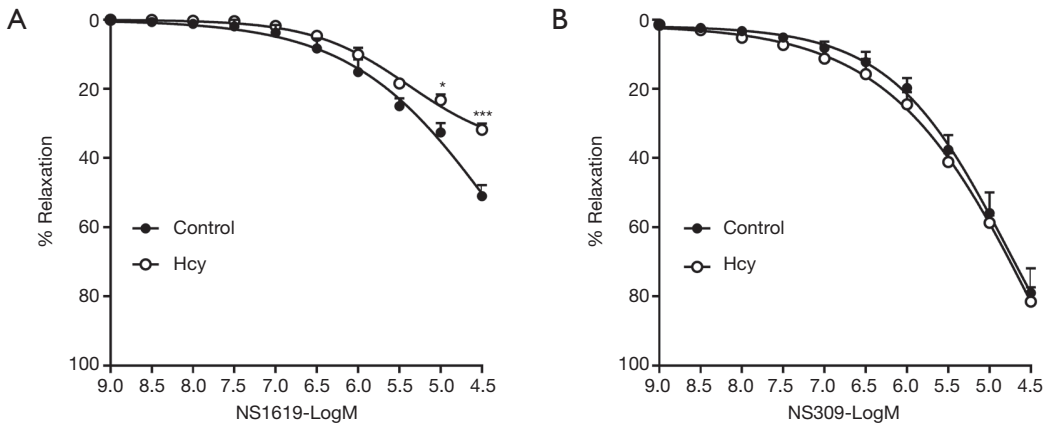

Figure 2 Effect of homocysteine (Hcy) on the relaxant response of internal mammary artery (IMA) to K_{Ca} channel activators. Hcy exposure significantly suppressed the BK_{Ca} channel activator NS1619-induced relaxation (A) whereas showed no influence on the IK_{Ca}/SK_{Ca} channel activator NS309-induced response (B). n=6. *P<0.05, ***P<0.001 *vs.* control.

vs. 56.1%±6.2%), did not show further inhibition on the relaxation in homocysteine-exposed IMA (*Figure 3D*,*E*), as compared to TRAM-34 or apamin alone. The significant inhibitory effect of TRAM-34 and apamin suggested that contribution of IK_{Ca} and SK_{Ca} subtypes to IMA dilatation increases under hyperhomocysteinemic conditions.

Application of different K_{Ca} blockers did not shift the EC_{50} of acetylcholine. Only in IMA treated with blockers of all three K_{Ca} subtypes, the EC_{50} was right-shifted, which however was reverted by homocysteine exposure (*Table 2*).

Table 3 showed the resting force and U46619-induced preconstriction in each set of the experiments. No significant differences were observed in the resting force and precontraction between the groups incubated with or without homocysteine or between pre- and post-treatment with K_{Ca} blockers (P>0.05 in all sets of experiments). This ensured the reliability and accuracy of the comparison between control and homocysteine-treated IMAs concerning the role of K_{Ca} channels in relaxation.

Effect of homocysteine on the expression of K_{Ca} subtypes in IMA

Western blot results showed that homocysteine exposure significantly lowered the protein level of $BK_{Ca}\beta1$ whereas without influencing the level of $BK_{Ca}\alpha$ in IMA. The protein level of IK_{Ca} and SK_{Ca} subtypes was unaltered by homocysteine exposure (*Figure 4A*,*B*). Downregulation of the BK_{Ca} β1-subunit by homocysteine was further shown by immunohistochemistry staining (*Figure 4C*,*D*). H-score calculated from seven independent experiments suggested that the BK_{Ca} β1-subunit was significantly less in homocysteine-exposed IMA as compared to the vessels without homocysteine exposure (P=0.038). The H-score of IK_{Ca} or SK_{Ca} subtypes did not differ between control and homocysteine-exposed IMAs, which was consistent with the western blot results showing the unaltered protein level of IK_{Ca} and SK_{Ca} subtypes after homocysteine exposure.

Discussion

IMA subjected to homocysteine exposure showed significantly suppressed response to acetylcholine. Such suppression could be attributed, at least partially, to the inhibition of BK_{Ca} channels, which was suggested by the diminished efficacy of iberiotoxin in inhibiting acetylcholine-induced relaxation in homocysteine-exposed IMA. The blunted relaxant response of IMA to the BK_{Ca} channel activator NS1619 after homocysteine exposure also supported the notion that homocysteine inhibits the vasodilatory activity of the BK_{Ca} subtype in IMA. Previously we reported that decrease of eNOS-NO bioavailability is involved in homocysteine-induced endothelial dysfunction of IMA (17). Taking into account that BK_{Ca} channels are an effector of NO (11,19), we suggested that compromised BK_{Ca} channel activity may also be partially responsible for the impaired NO-mediated relaxation in IMA in hyperhomocysteinemic states.

The significant vasodilatory role of the BK_{Ca} subtype in IMA and the inhibition by homocysteine was further

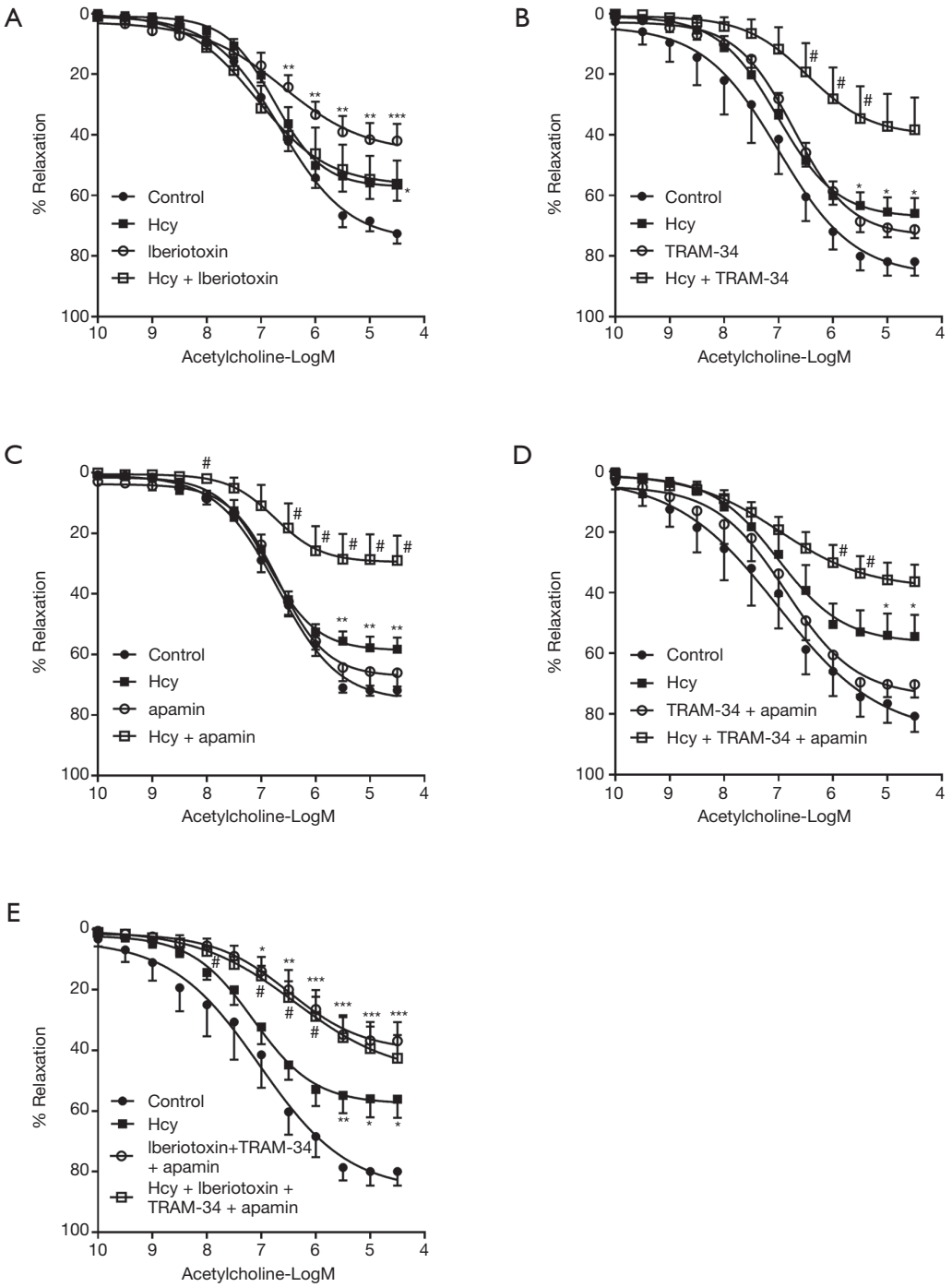

Figure 3 Effect of homocysteine (Hcy) on the role of K_{Ca} subtypes in endothelium-dependent relaxation in internal mammary artery (IMA). Hcy impairs acetylcholine-induced relaxation in internal mammary artery (IMA) with a significant suppression on BK_{Ca}-mediated response, suggested by the diminished inhibitory effect of the BK_{Ca} channel blocker iberiotoxin (A). Both IK_{Ca} and SK_{Ca} subtypes play a minor role in IMA dilatation whereas their contribution increases after homocysteine exposure, shown by the increased efficacy of IK_{Ca} and SK_{Ca} channel blockers TRAM-34 and apamin in inhibiting acetylcholine-induced relaxation (B,C,D). In homocysteine-exposed IMA, triple blockade of K_{Ca} channels shows no further inhibition on the relaxation as compared to double blockade of IK_{Ca} and SK_{Ca} channels, further suggesting the loss of BK_{Ca}-mediation caused by homocysteine (E). n=6. *P<0.05, **P<0.01, ***P<0.001 *vs.* control; #P<0.05 *vs.* Hcy.

Table 2 Effect of K_{Ca} channel blockers on EC_{50} values for acetylcholine in internal mammary artery (IMA) subjected or not subjected to homocysteine exposure (n=6 in each group)

Group	Without homocysteine exposure		With homocysteine exposure		P value
	EC_{50} (LogM)	P value (*vs*. control)	EC_{50} (LogM)	P value (*vs*. control)	
IIa					
Control	−6.59±0.25		−6.87±0.12		0.349
Iberiotoxin	−7.13±0.35	0.256	−6.94±0.29	0.841	0.687
IIb					
Control	−7.46±0.48		−7.03±0.16		0.471
TRAM-34	−6.70±0.08	0.097	−6.65±0.27	0.273	0.849
IIc					
Control	−6.71±0.09		−6.89±0.10		0.208
Apamin	−6.68±0.08	0.818	−6.57±0.16	0.156	0.587
IId					
Control	−7.32±0.57		−7.04±0.19		0.648
TRAM-34 + apamin	−7.11±0.30	0.733	−6.87±0.25	0.627	0.551
IIe					
Control	−7.43±0.43		−7.13±0.14		0.511
Iberiotoxin + TRAM-34 + apamin	−6.48±0.21	0.048*	−6.71±0.28	0.259	0.509

*, P<0.05. K_{Ca} channel, calcium-activated potassium channel; EC_{50}, concentration for 50% of maximal effect.

Table 3 Resting force and U46619-precontraction of internal mammary artery (IMA) in the study of acetylcholine-induced relaxation (n=6 in each group)

Group	Resting force (mN)		U46619-precontraction (mN)	
	Without homocysteine exposure	With homocysteine exposure	Without homocysteine exposure	With homocysteine exposure
IIa				
Control	16.3±0.8	18.1±1.1	51.8±3.6	56.3±2.6
Iberiotoxin	12.6±2.2	15.9±2.1	57.7±10.6	63.5±1.1
IIb				
Control	14.5±2.4	15.3±2.1	42.1±6.2	55.5±3.1
TRAM-34	16.2±3.8	18.4±1.5	42.5±6.5	52.1±8.2
IIc				
Control	17.2±3.2	12.4±1.7	42.2±10.8	59.1±6.3
Apamin	16.2±3.2	16.9±2.4	50.6±7.6	49.8±3.3
IId				
Control	17.2±2.2	11.9±1.7	48.3±9.1	59.5±7.6
TRAM-34 + apamin	12.7±1.9	14.1±1.7	44.8±10.0	63.2±13.5
IIe				
Control	17.7±1.9	14.0±1.5	48.8±7.8	52.8±3.7
Iberiotoxin + TRAM-34 + apamin	16.9±2.5	18.0±1.4	46.9±9.5	59.2±4.8

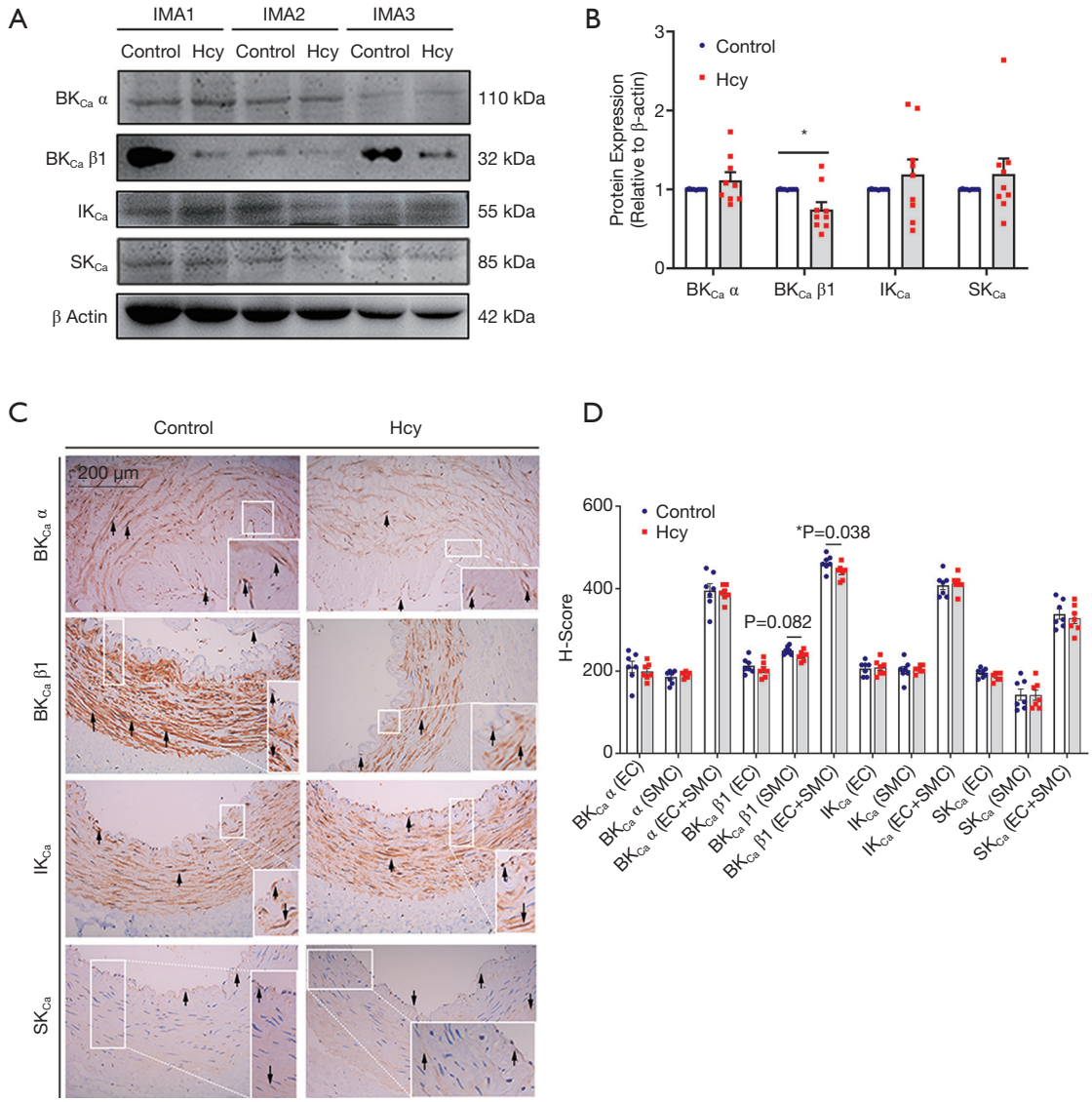

Figure 4 Effect of homocysteine (Hcy) on the expression of K_{Ca} channels in internal mammary artery (IMA). Immunoblotting of cell lysate prepared from IMA samples using the indicated antibodies (A). Protein levels of K_{Ca} subtypes in homocysteine-exposed IMAs compared with those without homocysteine exposure (B, n=9). Immunohistochemistry images (magnification 400×) showing the distribution and expression of K_{Ca} subtypes in IMA. The IMA sections were incubated with the indicated antibodies before stained with 3,3'-diaminobenzidine and counterstained with Mayer's haematoxylin (C). Comparison of the histoscore (H-score) of each K_{Ca} subtype in the endothelial cell (EC) and the smooth muscle cell (SMC) layer respectively as well as the overall H-score of each K_{Ca} subtype in both cell layers (EC + SMC) (D, n=7) between IMAs with or without homocysteine exposure. *P<0.05, Hcy *vs.* control.

demonstrated in the study of vasoconstriction. Previous studies in hyperhomocysteinemic animal models revealed simultaneously increased TXA_2 activity both in vessels and platelets (20). We therefore used U46619, a TXA_2 analog, as the vasoconstrictor to study the contractility changes of IMA and the regulatory role of K_{Ca} channels under hyperhomocysteinemic condition. Pretreatment of IMA with iberiotoxin significantly enhanced U46619-induced constriction. Homocysteine exposure enhanced the response of IMA to U46619 whereas the enhancement effect of iberiotoxin on the vasoconstriction became ignorable, suggesting that homocysteine inhibited the activity of BK_{Ca} channels in opposing vasoconstriction in IMA. Previous studies in human coronary arterial myocytes found that TXA_2 prostanoid receptor (TP) physically associates with BK_{Ca} α-subunit, resulting in channel inhibition after agonist stimulation. Such TP-induced trans-inhibition of BK_{Ca} channel activity promotes vasoconstriction induced by U46619 (21). Further study demonstrated that the β1-subunit of BK_{Ca} can by itself interact with TP meanwhile can assemble in a tripartite complex with BK_{Ca}α and TP. β1 is able to reduce TP agonist TXA_2-induced BK_{Ca}α inhibition and thus vasoconstriction. It was observed that U46619-induced contractile response of aortic rings was increased in $\beta1^{-/-}$ mice compared with those from $\beta1^{+/+}$ animals (22). In our study, we found that homocysteine decreased the expression of BK_{Ca} β1-subunit in IMA, which may explain the enhancement of U46619-induced IMA constriction after homocysteine exposure. Previously we demonstrated that homocysteine induces ER stress in porcine coronary arteries and PERK-dependent activation of forkhead box O3a (FoxO3a) mediates BK_{Ca} β1 degradation via upregulating the E3 ubiquitin ligase atrogin-1. This mechanism might also be involved in homocysteine-induced loss of BK_{Ca} β1 in IMA, though further studies are warranted to validate this.

In comparison with the BK_{Ca} subtype that is of importance in mediating relaxation of IMA, IK_{Ca} and SK_{Ca} subtypes play a minor role in the tone regulation of IMA under physiological condition despite their expression abundance. Blockade of the IK_{Ca} and SK_{Ca} subtypes individually or jointly barely suppressed the relaxant response of IMA to acetylcholine, which was consistent with the finding in a previous study from our group (18). Moreover, inhibition of IK_{Ca} and SK_{Ca} subtypes had no impact on U46619-induced vasoconstriction. However, after homocysteine exposure, IK_{Ca} and SK_{Ca} subtypes became involved in endothelium-dependent relaxation, evidenced by the significant inhibitory effect of TRAM-34 and apamin on the relaxation. The "unmasking" of the role of IK_{Ca} and SK_{Ca} channels in mediating endothelium-dependent relaxation may be explained by the inhibition of NO pathway under hyperhomocysteinemic condition. The loss of NO-mediated relaxation may augment the vasodilating activity of EDHF whose action is mediated by endothelial IK_{Ca} and SK_{Ca} channels (23). The augmentation of IK_{Ca} and SK_{Ca} channel involvement may be a mechanism to compensate for homocysteine-induced loss of BK_{Ca} channel function. It is unclear how homocysteine augmented the role of IK_{Ca} and SK_{Ca} channels in IMA relaxation. Both western blot and immunohistochemistry staining experiments showed unaltered protein levels of IK_{Ca} and SK_{Ca}. It might be possible that although homocysteine does not alter the overall expression of these channels, it may affect cell surface density of IK_{Ca} and SK_{Ca} channels and/or modulate the activity of single channels in the endothelial cells of IMA. Further experiments including cell surface protein analysis and patch clamp recording will be required to test this speculation. In addition, homocysteine did not affect the "no involvement" status of IK_{Ca} and SK_{Ca} subtypes in the constriction of IMA, indicating that although IK_{Ca} and SK_{Ca} channels are abundantly expressed in the smooth muscle of IMA, the function of smooth muscle IK_{Ca} and SK_{Ca} remains elusive, at least they are unessential to smooth muscle tone regulation in IMA. Whether these subtypes are involved in inflammatory responses of endothelial and smooth muscle cells and take part in the process of atherosclerosis in IMA in hyperhomocysteinemia is a topic worthy of investigation.

Study limitations

The present study investigated the effect of hyperhomocysteinemia on K_{Ca} channels in IMA by using an *in vitro* tissue culture model. The IMA samples were collected from 76 CABG patients who were free from diabetes. Hypertension (68.42%) and dyslipidemia (32.89%) are common comorbidities in these patients and current smokers account for 35.53%. Preoperative medications including anti-hypertensive, lipid-lowering, anti-thrombotic, and coronary dilating drugs were quite common (Table S1). Considering that these cardiovascular risk factors and drug therapies may affect vascular reactivity, we used "paired sample" design in this study to avoid misinterpretation of the results. In each experiment, the IMA sample taken from each patient was divided and treated with or without homocysteine. In this

way, although we could not rule out the influence posed by the risk factors and pharmacotherapies on the vascular reactivity, we were able to clarify the effect of homocysteine on IMA vasoreactivity and K_{Ca} channels. Future studies with stratification shall help further clarify the influence of the risk factors on the effect of homocysteine. Besides, there are several limitations of this study that need to be addressed. First, the concentration of homocysteine was 100 μmol/L, which is higher than the plasma homocysteine level in most hyperhomocysteinemic patients (20–30 μmol/L). Although it is not unusual to use high dose of exposure for *in vitro* studies and 100 μmol/L even millimolar concentrations were often used in *in vitro* investigation of the effect of homocysteine (7,18,24,25), studies using homocysteine at concentrations more commonly seen in hyperhomocysteinemic patients for prolonged exposure period may be further considered.

Second, it has to be mentioned that the plasma homocysteine concentration of these IMA donors was unknown since CABG patients are not routinely checked for homocysteine level in our hospital. Considering the incidence of hyperhomocysteinemia in patients with coronary artery disease, it is possible that some of the IMAs had already been subjected to high concentration of homocysteine *in vivo* before the *in vitro* exposure. However, in this study, the IMA used in every single experiment was divided from a same patient. In comparison to random assignment of IMAs from different individuals to control and treatment groups, this design helped rule out the influence of clinical characteristics of different patients on the K_{Ca} channels and at the same time minimized the impact of plasma homocysteine status on the data interpretation. Nevertheless, further strengthening the study with comparison of K_{Ca} channels in IMAs between hyperhomocysteinemic and non-hyperhomocysteinemic patients will be the direction for future studies.

Another limitation is that although we demonstrated that homocysteine inhibits the BK_{Ca} channel activity and augments the activity of IK_{Ca} and SK_{Ca} channels in IMA dilatation, direct electrophysiological measurements of the channel activities were not performed despite our experience in the patch-clamp recording of K_{Ca} channels in vascular cells (7,15,16,26). Due to the limited amount of residual IMA graft obtained from each patient, we were unable to enzymatically disassociate enough endothelial and smooth muscle cells for satisfactory primary culture for the subsequent patch-clamp recording. Optimization of tissue preparation procedures and cell disassociation and culture conditions etc. needs to be implemented to help realize electrophysiological measurements in future studies.

Conclusions

Homocysteine compromises the vasodilating activity of BK_{Ca} channels in IMA, which is associated with a loss of the BK_{Ca} β1-subunit. Unlike BK_{Ca}, IK_{Ca} and SK_{Ca} channels are unessential for the regulation of vasoreactivity in IMA, while the loss of BK_{Ca} channel functionality in hyperhomocysteinemia enhances the role of IK_{Ca} and SK_{Ca} channels in mediating endothelial dilator function. These findings provided new mechanistic insights into the dysregulation of vascular tone in patients with hyperhomocysteinemia. Moreover, we suggest that targeting BK_{Ca} channels may form a strategy to improve the postoperative graft performance in CABG patients with hyperhomocysteinemia who receive IMA grafting.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Ethics Review Board of TEDA International Cardiovascular Hospital ([2018]-0626-2). Written informed consent was obtained from all patients.

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