Activation of canonical transient receptor potential channels preserves Ca^{2+} entry and endothelium-derived hyperpolarizing factor-mediated function in vitro in porcine coronary endothelial cells and coronary arteries under conditions of hyperkalemia

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Objectives: Although membrane depolarization by hyperkalemia is known to reduce Ca^{2+} influx in endothelial cells, the mechanism by which endothelial Ca^{2+} channel is affected by hyperkalemia remains poorly studied. We studied the effect of hyperkalemia on canonical transient receptor potential channels, in particular canonical transient receptor potential channel 3, in modulation of endothelial intracellular Ca^{2+} concentration. Endothelium-derived hyperpolarizing factor-mediated function is Ca^{2+} dependent, and hyperkalemic cardioplegia/organ preservation solutions impair endothelium-derived hyperpolarizing factor-mediated function. We explored the role of canonical transient receptor potential channel 3 in endothelium-derived hyperpolarizing factor-mediated function and investigated whether modulation of these channels preserves endothelial Ca^{2+} influx and endothelium-derived hyperpolarizing factor-mediated function under the condition of hyperkalemic/cardioplegic exposure.

Methods: Intracellular Ca^{2+} concentration was measured with fluorescent dye in primary cultured porcine coronary endothelial cells exposed to hyperkalemic/cardioplegic solutions containing mild to extreme high K^{+} concentration. Endothelium-derived hyperpolarizing factor-mediated relaxation under hyperkalemic/cardioplegic exposure was studied in small porcine coronary arteries in a myograph in the presence of cyclooxygenase and nitric oxide synthase inhibitors and nitric oxide scavenger.

Results: Canonical transient receptor potential channel 3 blocker inhibited bradykinin-induced Ca^{2+} influx and attenuated endothelium-derived hyperpolarizing factor-mediated response. Hyperkalemic exposure inhibited canonical transient receptor potential channel 3-mediated Ca^{2+} influx in a K^{+} concentration-dependent manner ($120 > 20 > 10$ mmol/L). Ca^{2+} influx decreased in porcine coronary endothelial cells exposed to histidine-tryptophan-ketoglutarate, St Thomas' Hospital, and University of Wisconsin solutions that contained mild (10 mmol/L), moderate (20 mmol/L), and extreme high (125 mmol/L) K^{+} concentration, respectively. Canonical transient receptor potential channel activator prevented the reduction of Ca^{2+} influx in porcine coronary endothelial cells exposed to solutions containing mild to moderate high $[\text{K}^{+}]_o$ and restored endothelium-derived hyperpolarizing factor-mediated response that was impaired by hyperkalemic exposure.

Conclusions: Canonical transient receptor potential channel 3 is involved in endothelium-derived hyperpolarizing factor-mediated function in coronary arteries. Hyperkalemia inhibited canonical transient receptor potential channel 3-mediated Ca^{2+} influx in endothelial cells. Canonical transient receptor potential channel activation restores Ca^{2+} influx suppressed by hyperkalemia and prevents dysfunction of endothelium-derived hyperpolarizing factor. (J Thorac Cardiovasc Surg 2014;148:1665-73)

Supplemental material is available online.

Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is a major regulator of endothelial function. Hemodynamic shear stresses or Ca^{2+} mobilizing agonists induce elevation of

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Supported by grants to Dr Yang from Research Grant Council of Hong Kong (CUHK4789/09M and 4774/12M), National Natural Science Foundation of China 81200123, CUHK Direct Grant 4054015, Tianjin Municipal Science and Technology Commission (10JCYBJC26400), Key Medical Program of Tianjin Binhai New

Area Health Bureau (2011BHKZ001 and 2012BWKZ008), and Tianjin Health Bureau (2012KZ009).

Disclosures: Authors have nothing to disclose with regard to commercial support. Drs Q.Y. and J.-H.H. contributed equally to this article.

Received for publication Sept 3, 2013; revisions received Jan 21, 2014; accepted for publication Feb 3, 2014; available ahead of print March 12, 2014.

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0022-5223/\$36.00

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<http://dx.doi.org/10.1016/j.jtcvs.2014.02.026>

Abbreviations and Acronyms

ANOVA	= analysis of variance
$[Ca^{2+}]_i$	= intracellular Ca^{2+} concentration
EDHF	= endothelium-derived hyperpolarizing factor
HTK	= histidine-tryptophan-ketoglutarate
ILH	= indomethacin, N^G -nitro-L-arginine, and oxyhemoglobin
NO	= nitric oxide
NPSS	= normal physiologic saline solution
OAG	= 1-oleoyl-2-acetyl-sn-glycerol
PCEC	= porcine coronary endothelial cell
PGI_2	= prostacyclin
Pyr3	= ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate
SD	= standard deviation
SKF96365	= 1-[2-[3-(4-Methoxyphenyl)propoxy]-2-(4-methoxyphenyl)ethyl]-1H-imidazole hydrochloride
ST	= St Thomas' Hospital
TRP	= transient receptor potential
TRPC	= canonical transient receptor potential
UW	= University of Wisconsin

intracellular Ca^{2+} in endothelial cells triggering the production of vasoactive substances, including relaxing factors such as nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF).¹⁻³

As nonexcitable cells that lack voltage-gated Ca^{2+} channels, endothelial cells mainly rely on store-operated and nonselective Ca^{2+} -permeable channels, such as transient receptor potential (TRP) channels for $[Ca^{2+}]_i$ modulation.³ Canonical TRP (TRPC) channels are a major group of members in the TRP family that have been recognized as the most important Ca^{2+} -permeable channels in vascular endothelium physiology.^{4,5}

Among TRPC proteins, TRPC3 forms channels that have significant constitutive activity.⁶ In human coronary artery endothelial cells, native TRPC3 proteins form channels that contribute to constitutive and adenosine triphosphate-dependent Ca^{2+} influx.⁷ Our recent study in porcine coronary arteries demonstrated that Ca^{2+} entry via endothelial TRPC3 channels contributes to NO release.⁸ Moreover, endothelial dysfunction during hypoxia-reoxygenation is associated with TRPC3 inhibition and the reduction of Ca^{2+} influx via TRPC3.⁸

Change of membrane potential affects the driving force for Ca^{2+} influx and therefore affects $[Ca^{2+}]_i$. The reduction of Ca^{2+} influx was observed in endothelial cells in which membrane potential is depolarized by extracellular high K^+ concentration.^{9,10} However, Ca^{2+} channels involved in

the hyperkalemia-suppressed Ca^{2+} influx remain poorly studied in endothelial cells. In this study, for the first time, we investigated whether Ca^{2+} influx via TRPC, in particular TRPC3 channels, is affected by hyperkalemia.

Hyperkalemic cardioplegia or organ preservation solutions are widely used in open surgery, including heart transplantation. K^+ is the key component, with the concentration varying in different solutions. For example, it is as high as 125 mmol/L in University of Wisconsin (UW) solution, whereas it is 20 mmol/L in St Thomas' Hospital (ST) and 10 mmol/L in histidine-tryptophan-ketoglutarate (HTK) solutions. Our previous studies have demonstrated the detrimental effect of hyperkalemia on EDHF-mediated endothelial function in both porcine and human vessels.¹¹⁻¹³ Furthermore, we reported that EDHF function is compromised in vessels exposed to cardioplegic/organ preservation solutions, including UW and ST solutions.¹⁴⁻¹⁶

In view of the prerequisite of endothelial $[Ca^{2+}]_i$ increase in EDHF function and the importance of TRPC3 channels in Ca^{2+} entry in endothelial cells,⁸ we further investigated the role of TRPC in the present study, in particular, TRPC3 channels, in EDHF-mediated relaxation and whether modulation of TRPC channels may prevent EDHF dysfunction caused by hyperkalemic or cardioplegic/organ preservation solutions.

MATERIALS AND METHODS

All experiments were in accordance with institutional guidelines. The study was approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong (reference number 12/010/GRF).

Isolation and Culture of Porcine Coronary Endothelial Cells

Fresh porcine hearts from hogs weighing approximately 30 kg, collected from a local slaughterhouse, were placed in a container filled with cold (4°C), preoxygenated (95% O_2 /5% CO_2) Krebs solution and immediately transferred to the laboratory. Small porcine coronary arteries were digested with collagenase, and freshly dispersed porcine coronary endothelial cells (PCECs) were cultured for 4 to 6 days as previously described^{8,17} (see "Supplementary Materials").

Intracellular Ca^{2+} Concentration Measurement in Porcine Coronary Endothelial Cells

$[Ca^{2+}]_i$ was measured as described previously.⁸ Briefly, primary cultured PCECs were seeded on glass coverslips 1 day before $[Ca^{2+}]_i$ measurement. Cells were loaded with 5 μ mol/L Fura-2/AM for 1 hour in the dark with 0.02% Pluronic F127 (Sigma, St Louis, Mo) for ratiometric Ca^{2+} -fluorescent measurement (see "Supplementary Materials"). The bath solution was normal physiologic saline solution (NPSS). PCECs were then transferred to Ca^{2+} -free saline solution approximately 2 minutes before bradykinin challenge, followed by reintroduction of 2.5 mmol/L $CaCl_2$ in Ca^{2+} -free saline solution. Except for the omission of $CaCl_2$ (2 mmol/L in NPSS), Ca^{2+} -free saline solution is identical to NPSS in other compositions (millimoles/liter) that include NaCl 140, KCl 5, $MgCl_2$ 1, glucose 10, and HEPES 5.

To study the effect of extracellular high K^+ or cardioplegic/organ preservation solutions on $[Ca^{2+}]_i$, saline solution (NPSS or Ca^{2+} -free saline solution) was replaced by saline solution (NPSS or Ca^{2+} -free saline

solution) containing 10, 20, or 120 mmol/L K^+ , or HTK, ST, or UW solution (see “Experimental Protocols” for details). In some experiments, cells were pretreated with the TRPC3-specific inhibitor ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate (Pyr3), shortly before bradykinin challenge. Fluorescence of $[Ca^{2+}]_i$ signals were recorded by the InCyt Basic Fluorescence Imaging System (Intracellular Imaging, Cincinnati, Ohio). $[Ca^{2+}]_i$ response was expressed as the ratio of the fluorescence signal from excitation at 340 nm to that at 380 nm (F340/F380). Changes in $[Ca^{2+}]_i$ were displayed as a ratio of fluorescence relative to the fluorescence before (baseline) the application of bradykinin.

Myograph Study of Vasorelaxation

Mounting and normalization of small porcine coronary arteries. Porcine coronary arteries with a diameter of 600 to 800 μm were cut into cylindric rings with 2 mm in length and mounted in a 4-channel Mulvany myograph (Model 610M; J. P. Trading, Aarhus, Denmark). The rings were equilibrated for 45 minutes and normalized with the internal circumference set at 90% of the circumference at a passive transmural pressure of 100 mm Hg.^{13,17-19}

Precontraction and relaxation. After equilibration, the vessels were precontracted with 9,11-dideoxy-11 α ,9 α -epoxymethanoprostaglandin $F_{2\alpha}$ (U46619, a thromboxane A_2 mimetic, 100 nmol/L) followed by relaxant response to bradykinin ($-10 \sim -6.5$ LogM). The EDHF-mediated relaxation was studied in the presence of cyclooxygenase inhibitor 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indole acetic acid (indomethacin, 7 $\mu mol/L$), NO synthase inhibitor N^G -nitro-L-arginine (300 $\mu mol/L$), and NO scavenger oxyhemoglobin (20 $\mu mol/L$).^{19,20}

Experimental Protocols

Role of canonical transient receptor potential channel 3 in bradykinin-induced intracellular Ca^{2+} concentration increase. $[Ca^{2+}]_i$ in response to bradykinin (200 nmol/L) was measured in PCECs with or without pretreatment of 1-[2-[3-(4-Methoxyphenyl)propoxy]-2-(4-methoxyphenyl)ethyl]-1H-imidazole hydrochloride (SKF96365) (nonspecific TRPC inhibitor, 10 $\mu mol/L$) or Pyr3 (TRPC3 selective inhibitor, 3 $\mu mol/L$) ($n = 3-7$ independent experiments with 15-20 cells per experiment).

Bradykinin-induced intracellular Ca^{2+} concentration increase under hyperkalemic exposure and effect of canonical transient receptor potential channel activator.

The effect of hyperkalemic or cardioplegic/organ preservation solutions was studied in 2 different experimental settings to simulate clinical situations of cardiac surgery (a) and heart transplantation (b):

a. in PCECs bathed in hyperkalemic solutions

PCECs were allocated to 4 groups and bathed in Ca^{2+} -free saline solution containing different concentrations of K^+ (millimoles/liter), including 5, 10, 20, and 120. After 2 minutes of equilibration, bradykinin-induced $[Ca^{2+}]_i$ increase was determined in the absence (control) or presence of Pyr3 (3 $\mu mol/L$) or TRPC activator 1-oleoyl-2-acetyl-sn-glycerol (OAG) (100 $\mu mol/L$) ($n = 3-7$ independent experiments with 15-20 cells per experiment).

b. in PCECs preincubated in hyperkalemic solutions

PCECs were incubated for 1 hour in NPSS that contains 5 mmol/L K^+ (control) or NPSS containing 10, 20 or 120 mmol/L K^+ with or without OAG supplementation (100 $\mu mol/L$) before being transferred to Ca^{2+} -free saline solution for recording ($n = 3-6$ independent experiments with 15-20 cells per experiment).

In both a and b, to measure $[Ca^{2+}]_i$, bradykinin was applied followed by the addition of 2.5 mmol/L Ca^{2+} in Ca^{2+} -free saline solution.

For solutions containing a high concentration of K^+ , Na^+ was replaced with equivalent K^+ form. The osmolality remained unchanged. In another group, cells were bathed in Ca^{2+} -free saline solution in which Na^+ was replaced by equivalent Li^+ .

Effects of cardioplegic/organ preservation solutions and canonical transient receptor potential activator on bradykinin-induced intracellular Ca^{2+} concentration increase.

a. in PCECs bathed in cardioplegic/organ preservation solutions

Because the intrinsic concentration of Ca^{2+} differs in HTK, ST, and UW solutions, different protocols were required to study these solutions.

For those with a lack of Ca^{2+} component (UW) or a low concentration of Ca^{2+} (HTK, 0.015 mmol/L), PCECs were bathed in these solutions with $[Ca^{2+}]_i$ measured on bradykinin application followed by $CaCl_2$ introduction. Control cells were bathed in Ca^{2+} -free saline solution. For the solution containing Ca^{2+} at the level equivalent to NPSS (ST, 2.7 mmol/L), PCECs were bathed in ST solution with $[Ca^{2+}]_i$ measured on bradykinin application. Cells bathed in NPSS served as control. The effect of each solution was further studied with supplementation of OAG (100 $\mu mol/L$) ($n = 3-7$ independent experiments with 15-20 cells per experiment).

b. in PCECs preincubated in cardioplegic/organ preservation solutions

PCECs were incubated for 1 hour in NPSS (control) or HTK, ST, or UW solution with or without OAG (100 $\mu mol/L$) supplementation before being transferred to Ca^{2+} -free saline solution for $[Ca^{2+}]_i$ measurement. Bradykinin was applied, followed by Ca^{2+} addition ($n = 3-7$ independent experiments with 15-20 cells per experiment).

Role of canonical transient receptor potential channel 3 in endothelium-derived hyperpolarizing factor-mediated vasorelaxation. Bradykinin-induced EDHF-mediated relaxations were studied in vessels pretreated with or without Pyr3 (3 $\mu mol/L$).

Effect of canonical transient receptor potential channel activator on endothelium-derived hyperpolarizing factor-mediated response under hyperkalemic or cardioplegic exposure.

a. hyperkalemic exposure

Vessels were allocated ($n = 8$ in each group) and exposed for 1 hour to NPSS (control) or NPSS containing 20 mmol/L K^+ with or without OAG supplementation (100 $\mu mol/L$).

b. cardioplegic exposure

Vessels were exposed for 1 hour to NPSS (control), ST solution, or ST containing OAG ($n = 8$ in each group).

In both a and b, after washout by NPSS, bradykinin-induced EDHF-mediated vasorelaxation was studied.

Data Analysis

Relaxation was expressed as the percentage of decrease in isometric force. The effective concentration of bradykinin that caused 50% of maximal relaxation was defined as EC_{50} . The EC_{50} was determined from each concentration-relaxation curve by a logistic curve-fitting equation: $E = MA^P/(A^P + K^P)$, where E is response, M is maximal relaxation, A is concentration, K is EC_{50} concentration, and P is slope parameter.

All results were expressed as mean $\pm$ standard deviation. The statistical comparisons of the cumulative responses of relaxation under different treatments and the intracellular calcium concentration measurements were performed by analysis of variance (SPSS version 20; SPSS Inc, Chicago, Ill). The Scheffé test was used as a post hoc test to detect the individual significance. For replacement of extracellular NaCl with LiCl to exclude the role of reduction of Na^+ gradient on bradykinin-induced Ca^{2+} influx, the Student *t* test was used.

FIGURE 1. Bradykinin-induced Ca²⁺ influx in primary cultured PCECs is inhibited by TRPC channel inhibitors (A, ****P* < .001 vs control; analysis of variance [ANOVA] and Scheffé test). SKF96365: TRPC inhibitor. Pyr3: TRPC3 selective inhibitor. Ca²⁺ influx and the TRPC3-mediated Ca²⁺ influx in response to bradykinin are suppressed by elevated concentrations of extracellular K⁺ (B-D). B, Representative traces of [Ca²⁺]_i in response to bradykinin in PCECs bathed in 5 mmol/L (5K), 10 mmol/L (10K), 20 mmol/L (20K), or 120 mmol/L (120K) K⁺. In 10K, 20K, and 120K, Na⁺ in bath solution was replaced by equivalent K⁺. C, Bradykinin-induced Ca²⁺ influx (second phase of [Ca²⁺]_i increase in B) in PCECs bathed in different [K⁺]_o in the absence or presence of TRPC3 inhibitor Pyr3. D, Pyr3-sensitive Ca²⁺ influx in PCECs bathed in different [K⁺]_o. **P* < .05. ****P* < .001 versus 5K. ANOVA and Scheffé test (C and D). Values are mean ± standard deviation (SD) of 3 to 7 independent experiments (15-20 cells per experiment). Number of independent experiments is labeled inside of the bars for each group. BK, Bradykinin; [Ca²⁺]_i, intracellular Ca²⁺ concentration; Pyr3, ethyl-1-(4-(2,3,3-trichloroacrylamide) phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate; SKF96365, 1-[2-[3-(4-Methoxyphenyl)propoxy]-2-(4-methoxyphenyl)ethyl]-1H-imidazole hydrochloride.

RESULTS

Role of Canonical Transient Receptor Potential Channels in Bradykinin-Induced Ca²⁺ Influx

In Ca²⁺-free saline solution, application of bradykinin caused a transient increase in [Ca²⁺]_i in PCECs. Subsequent introduction of Ca²⁺ in Ca²⁺-free saline solution evoked a sustained increase in [Ca²⁺]_i. The first phase of [Ca²⁺]_i increase represents Ca²⁺ release from Ca²⁺ store, and the second phase is due to Ca²⁺ influx. In the absence of bradykinin, introduction of Ca²⁺ barely caused an increase in [Ca²⁺]_i (data not shown).

TRPC inhibitor SKF96365 and TRPC3 selective blocker Pyr3 significantly inhibited bradykinin-induced Ca²⁺ influx (*P* < .001) (Figure 1, A). Pretreatment of the cells with dimethyl sulfoxide, the solvent of SKF96365 and Pyr3, at the equivalent concentration (0.2‰ in SKF96365 and 0.15‰ in Pyr3 treatment, respectively) had no effect on bradykinin-induced Ca²⁺ influx (data not shown).

Inhibition of Ca²⁺ Influx by Hyperkalemia and Restoration by Canonical Transient Receptor Potential Channel Activator

Immersion or preincubation of PCECs in hyperkalemic solutions suppressed bradykinin-induced Ca²⁺ influx (Figure 1 and Table 1). Stepwise increments of [K⁺]_o from 5 to 120 mmol/L did not affect the basal [Ca²⁺]_i and Ca²⁺ release induced by bradykinin but caused graded decreases in bradykinin-induced Ca²⁺ influx (*P* < .05, 10K vs 5K, and 20K vs 10K; *P* < .01, 120K vs 20K) (Figure 1, B and C). The Ca²⁺ influx in response to bradykinin was significantly inhibited by the TRPC3 blocker Pyr3, and the [K⁺]_o increase inhibited the Pyr3-sensitive TRPC3-mediated Ca²⁺ influx (Figure 1, C and D). OAG restored Ca²⁺ influx that was suppressed by 10 to 20 mmol/L K⁺. However, OAG was unable to normalize Ca²⁺ influx in PCECs exposed to 120 mmol/L K⁺ (*P* < .001 vs control) (Table 1). Replacement of

TABLE 1. Inhibition of endothelial Ca^{2+} influx induced by hyperkalemic solutions and the effect of supplementation of canonical transient receptor potential channel activator

Exposure to	Peak of Ca^{2+} influx ($\pm$ SD)	
	Bathed (n)	Preincubated (n)
5K	0.54 $\pm$ 0.04 (7)	0.53 $\pm$ 0.04 (6)
10K	0.46 $\pm$ 0.01* (4)	0.44 $\pm$ 0.01† (4)
10K + OAG	0.57 $\pm$ 0.08§ (3)	0.52 $\pm$ 0.03 (3)
20K	0.36 $\pm$ 0.05‡ (5)	0.39 $\pm$ 0.02‡ (3)
20K + OAG	0.48 $\pm$ 0.06§ (4)	0.50 $\pm$ 0.03 (4)
120K	0.17 $\pm$ 0.01‡ (3)	0.13 $\pm$ 0.02‡ (4)
120K + OAG	0.20 $\pm$ 0.01 (4)	0.21 $\pm$ 0.03 (4)

Values are mean $\pm$ SD. Physiologic saline solution contains 5 mmol/L K^+ (5K). Hyperkalemic solutions contain 10 mmol/L K^+ (10K), 20 mmol/L K^+ (20K), or 120 mmol/L K^+ (120K). OAG is a TRPC activator. n = the number of independent experiments (15–20 cells per experiment). K + OAG versus the K group at the same concentration without OAG (ANOVA and Scheffé test). SD, Standard deviation; OAG, 1-oleoyl-2-acetyl-sn-glycerol. * $P < .05$. † $P < .01$. ‡ $P < .001$ versus 5K. § $P < .05$. || $P < .01$.

extracellular NaCl with LiCl did not alter bradykinin-induced Ca^{2+} influx (0.54 $\pm$ 0.04 in 140 mmol/L NaCl vs 0.50 $\pm$ 0.06 in 140 mmol/L LiCl, $P = .30$, Student *t* test), suggesting that the decreased Ca^{2+} influx observed under hyperkalemic exposure was not caused by the reduction of Na^+ gradient (data not shown).

Inhibition of Ca^{2+} Influx by Cardioplegic/Organ Preservation Solutions and Restoration by Canonical Transient Receptor Potential Channel Activator

Bradykinin-induced Ca^{2+} influx was suppressed in PCECs bathed in cardioplegic/organ preservation solutions. The sustained Ca^{2+} influx in response to bradykinin was attenuated by ST solution ($P < .01$), manifested by the suppressed plateau phase of $[\text{Ca}^{2+}]_i$ increase (Figure 2, A and B). Likewise, HTK and UW solutions inhibited bradykinin-induced Ca^{2+} influx (Figure 2, C and D). The inhibition caused by UW was more potent than that of HTK. In PCECs bathed in OAG-containing ST and HTK solutions, the suppressed $[\text{Ca}^{2+}]_i$ response was restored (Figure 2, A, B, and D).

The inhibitory effect of hyperkalemia or cardioplegic/organ preservation solutions on Ca^{2+} influx was even observed after washout (Table 1 and Figure 3). The potency of inhibition is UW > ST > HTK. The inhibitory effect of HTK and ST on Ca^{2+} influx was abolished by OAG supplementation in these solutions. Although OAG may partially restore Ca^{2+} influx in PCECs preincubated in 120 mmol/L K^+ and UW solution (Table 1 and Figure 3, B), it failed to prevent the reduction of Ca^{2+} influx in the cells bathed in these solutions (Table 1 and Figure 2, D).

Role of Canonical Transient Receptor Potential 3 Channels in Endothelium-Derived Hyperpolarizing Factor-Mediated Vasorelaxation

Bradykinin induced significant vasorelaxation (95.6% $\pm$ 4.8%; EC_{50} : -7.83 ± 0.31 LogM). The EDHF response

(66.3% $\pm$ 10.8%) was inhibited by TRPC3 blocker Pyr3 (44.6% $\pm$ 10.5%, $P < .01$), whereas EC_{50} remained unchanged (-7.29 ± 0.65 LogM vs -7.62 ± 0.42 LogM) (Figure 4, A).

Protection of Endothelium-Derived Hyperpolarizing Factor-Mediated Response by Canonical Transient Receptor Potential Channel Activator Under Hyperkalemic/Cardioplegic Exposure

Exposure to 20 mmol/L K^+ for 1 hour significantly decreased bradykinin-induced, EDHF-mediated relaxation (52.5% $\pm$ 11.3% vs 67.2% $\pm$ 12.2%, $P < .05$), which was prevented by supplementation of OAG in the hyperkalemic solution (61.9% $\pm$ 9.9%) (Figure 4, B). No differences in EC_{50} s were observed (-7.53 ± 0.42 in control vs -7.12 ± 0.40 in 20 mmol/L K^+ vs -7.54 ± 0.48 LogM in 20 mmol/L K^+ containing OAG). Likewise, EDHF response was inhibited by 1-hour exposure to ST solution (52.2% $\pm$ 13.6% vs 67.5% $\pm$ 11.3%, $P < .05$), which was prevented by OAG supplementation (65.0% $\pm$ 13.5%) (Figure 4, C).

DISCUSSION

Potassium cardioplegia is widely used in cardiac surgery. The deleterious effects of high potassium in cardioplegic solutions on endothelial function have been recognized recently.^{11–13} In this study, for the first time, we demonstrated that (1) Ca^{2+} influx via TRPC3 channels is reduced by hyperkalemia in endothelial cells; (2) the reduction of endothelial Ca^{2+} influx caused by hyperkalemia or cardioplegic/organ preservation solutions could be restored by TRPC activator; and (3) TRPC3 channels are involved in EDHF function and TRPC activation prevents EDHF dysfunction caused by hyperkalemic or cardioplegic/organ preservation solutions.

Previous studies have demonstrated that TRPC channels participate in vasoactive agonist-induced Ca^{2+} entry in vascular endothelial cells.^{21,22} In our recent study, with direct measurement of NO, we demonstrated that bradykinin-induced Ca^{2+} influx via TRPC3 contributes to endothelial NO release from porcine coronary endothelium.⁸ In the present study, we further observed that the vasorelaxation resistant to NO synthase and cyclooxygenase inhibition was reduced in porcine coronary arteries pretreated with TRPC3 blocker, suggesting that TRPC3 is involved in the EDHF-mediated response in this vasculature. Ca^{2+} influx via TRPC3 underlies the role of this channel in EDHF function. Further studies are warranted to elucidate the signaling pathways involved in the functional association of TRPC3 and EDHF.

Depolarizing cardioplegia is widely used for protecting the heart against ischemia during open surgery. The major depolarizing agent in cardioplegic solutions is K^+ , usually 10 to 20 mmol/L.²³ For example, HTK solution contains

FIGURE 2. Effect of bathing in HTK, ST, and UW solutions and supplementation of TRPC activator on bradykinin-induced $[Ca^{2+}]_i$ response in primary cultured PCECs. The protocol used for studying $[Ca^{2+}]_i$ response in PCECs bathed in Ca^{2+} -containing ST solution (2.7 mmol/L Ca^{2+}) is different from those bathed in UW (no Ca^{2+}) or HTK (very low concentration of Ca^{2+} , 0.015 mmol/L) (see representative traces in A and C, and related experimental protocols in “Materials and Methods”). B, $[Ca^{2+}]_i$ increase at 2 minutes of bradykinin challenge in PCECs bathed in NPSS (control), ST, or ST containing the TRPC activator OAG. D, Bradykinin-induced Ca^{2+} influx in PCECs bathed in Ca^{2+} -free saline solution (control), HTK, or UW solution with or without OAG supplementation. Values are the mean $\pm$ SD of 3 to 7 independent experiments (15–20 cells per experiment). Number of independent experiments is labeled inside of the bars for each group. ** $P < .01$. *** $P < .001$ versus control. ANOVA and Scheffé test. BK, Bradykinin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HTK, histidine-tryptophan-ketoglutarate; OAG, 1-oleoyl-2-acetyl-sn-glycerol; ST, St Thomas’s Hospital; UW, University of Wisconsin.

10 mmol/L K^+ and ST solution contains 20 mmol/L K^+ . Both solutions are currently used for heart preservation, as is the UW solution. Regardless of protection of cardiomyocytes, because of the differences between cardiac myocytes and vascular cells, depolarizing cardioplegic/organ preservation solutions have been reported to compromise endothelial function.^{24–27} Studies from our laboratory have provided extensive evidence of the unfavorable effect of hyperkalemic or depolarizing cardioplegic/organ preservation solutions on EDHF-mediated vasorelaxation and hyperpolarization.^{11,15,16,18} The compromised EDHF function was again demonstrated in the present study after exposure to 20 mmol/L K^+ or ST solution.

Previous investigations have demonstrated that extracellular high K^+ concentrations lead to reduction of Ca^{2+} influx in endothelial cells by depolarizing membrane potential and therefore decreasing the driving force for Ca^{2+} entry.^{9,10} However, the Ca^{2+} channels involved have been barely studied. The current study demonstrated, for the first time, that the decreased endothelial $[Ca^{2+}]_i$ caused by hyperkalemia is associated with the inhibition

of Ca^{2+} entry via TRPC3. Together with the finding that TRPC3 participates in EDHF-mediated vasorelaxation, our results suggested that TRPC3 inhibition by high concentrations of K^+ may be a mechanism underlying the impairment of EDHF function in coronary arteries exposed to hyperkalemic or depolarizing cardioplegic/organ preservation solutions.

The suppressed Ca^{2+} influx was observed not only in endothelial cells bathed in hyperkalemic or depolarizing cardioplegic/organ preservation solutions but also in cells preincubated in these solutions. The inhibition of Ca^{2+} influx in endothelial cells preincubated in hyperkalemic/cardioplegic solutions suggested that the depolarizing effect of these solutions on endothelial membrane potential may exist even after washout for a certain period. In fact, previous studies from our laboratory have demonstrated that after washout, EDHF responses remain compromised in coronary and pulmonary arteries preincubated in cardioplegic/organ preservation solutions.^{15,28}

Another important finding of the present study is that the reduced Ca^{2+} influx in endothelial cells caused by

FIGURE 3. Effect of 1-hour preincubation in HTK, ST, and UW solutions with or without supplementation of TRPC activator on bradykinin-induced $[Ca^{2+}]_i$ response in primary cultured PCECs. A, Representative traces for $[Ca^{2+}]_i$ in response to bradykinin in PCECs preincubated in NPSS (control), HTK, ST, or UW solution. B, Bradykinin-induced Ca^{2+} influx in PCECs preincubated in NPSS (control), HTK, ST, or UW with or without supplementation of the TRPC activator OAG. Values are the mean $\pm$ SD of 3 to 6 independent experiments (15-20 cells per experiment). Number of independent experiments is labeled inside of the bars for each group. * $P < .05$. ** $P < .01$. *** $P < .001$ versus control. ANOVA and Scheffé test. BK, Bradykinin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HTK, histidine-tryptophan-ketoglutarate; OAG, 1-oleoyl-2-acetyl-sn-glycerol; ST, St Thomas' Hospital; UW, University of Wisconsin.

hyperkalemic or depolarizing cardioplegic/organ preservation solutions could be restored by OAG, an activator of TRPC3/6/7. The beneficial effect of OAG on Ca^{2+} influx is prominent in solutions containing a mild or moderate high concentration of K^+ , such as NPSS containing 10 to 20 mmol/L K^+ , HTK, and ST. However, in solutions containing an extremely high concentration of K^+ , such as NPSS containing 120 mmol/L K^+ or UW that contains 125 mmol/L K^+ , OAG failed to restore the reduction of Ca^{2+} influx. This might be explained by the complete loss of K^+ gradient and therefore the driving force for Ca^{2+} across the cell membrane.

Study Limitations

The present study was performed in vitro. Although this is an ideal way to investigate cellular mechanisms, caution needs to be exercised in extrapolating the in vitro findings to clinical settings. Fresh porcine hearts collected from a local slaughterhouse were used to harvest coronary arteries for this study. Although multiple measures were taken to minimize the impact of ischemia/hypoxia, including immediate harvest and placement of the heart in cold (4°C), preoxygenated (95% O_2 /5% CO_2) Krebs solution as soon as the pig was slaughtered and immediate transfer of the heart to the laboratory to perform vessel dissection in ice-cold Krebs aerated with 95% O_2 /5% CO_2 , a short period of warm ischemia (<10 minutes) was inevitable during the heart harvest. Every experiment had a control group; therefore, the impact of warm ischemia on the study and data interpretation was minimized. Whether this short-term warm ischemia had an effect on TRPC- Ca^{2+} signaling in coronary endothelial cells remains to be explored.

The present study focused on endothelial TRPC3. Because TRPC3 is also expressed in cardiomyocytes and hyperkalemic cardioplegia was designed and is still widely used for myocardial protection, further studies on this channel in cardiomyocytes are warranted to fully understand the effect of targeting TRPC during cardioplegic arrest on postoperative cardiac performance.

Clinical Implications

Exposure of coronary endothelium to cardioplegic/organ preservation solutions is inevitable in cardiac surgery and heart transplantation. The widely used depolarizing (hyperkalemic) solutions are detrimental to coronary endothelial function, especially the function mediated by EDHF. Although our previous studies suggested that the impairment of EDHF-mediated endothelial function caused by hyperkalemic exposure may tend to be reversible after a prolonged washout period, the unfavorable effect of these solutions on endothelial function lasts for at least 1 hour during the reperfusion period and may be longer.^{15,28} Considering that the early reperfusion period after cardiac arrest is critical for the recovery of myocardial function that can be hampered by coronary dysfunction, to achieve better postoperative cardiac performance, satisfactory protection of endothelial function during the operation and the early phase of reperfusion is of great importance. The present study demonstrated that supplementation of TRPC activator in hyperkalemic/hyperkalemic cardioplegic solutions prevents endothelial $[Ca^{2+}]_i$ disturbance and preserves EDHF responses both during solution exposure and after a certain period of washout, which therefore may provide new insight for the future development of cardioplegic/organ preservation solutions for better protection of endothelial function.

FIGURE 4. Role of TRPC3 channels in EDHF-mediated relaxation in small porcine coronary arteries (A, n = 8 in each group). The EDHF response was induced by bradykinin in the presence of indomethacin, N^G-nitro-L-arginine, and oxyhemoglobin (ILH). *P < .001 versus control. #P < .01. ##P < .001 versus ILH. ANOVA and Scheffé test. Preincubation in 20 mmol/L K⁺ (B, n = 8 in each group) or ST solution (C, n = 8 in each group) for 1 hour decreased the EDHF-mediated relaxant response to bradykinin in the presence of ILH, which was prevented by supplementation of the TRPC activator OAG. Values are mean ± SD. *P < .05. **P < .01. ***P < .001 versus control. ANOVA and Scheffé test. Pyr3: TRPC3 selective blocker. EDHF, Endothelium-derived hyperpolarizing factor; ILH, indomethacin, N^G-nitro-L-arginine, oxyhemoglobin; OAG, 1-oleoyl-2-acetyl-sn-glycerol; Pyr3, ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate; ST, St Thomas' Hospital.

CONCLUSIONS

The present study demonstrated that TRPC3 participates in EDHF-mediated endothelial function in porcine coronary arteries. The reduction of Ca²⁺ influx via TRPC3 is involved in the decreased Ca²⁺ entry caused by hyperkalemia in endothelial cells. Activation of TRPC channels restores Ca²⁺ influx suppressed by hyperkalemic or depolarizing cardioplegic/organ preservation solutions and prevents the impairment of EDHF function. This study may provide new insight for the improvement of cardioplegic/organ preservation solutions for better protection of endothelial function.

References

- Govers R, Rabelink TJ. Cellular regulation of endothelial nitric oxide synthase. *Am J Physiol Renal Physiol*. 2001;280:F193-206.
- Millanvoeye-Van Brussel E, David-Duflilio M, Pham TD, Iouzalet L, Aude Devynck M. Regulation of arachidonic acid release by calcium influx in human endothelial cells. *J Vasc Res*. 1999;36:235-44.
- Nilius B, Droogmans G. Ion channels and their role in vascular endothelium. *Physiol Rev*. 2001;81:1415-59.
- Nilius B, Droogmans G, Wondergem R. Transient receptor potential channels in endothelium: solving the calcium entry puzzle? *Endothelium*. 2003;10:5-15.
- Yao X, Garland CJ. Recent developments in vascular endothelial cell transient receptor potential channels. *Circ Res*. 2005;97:853-63.
- Trebak M, Vazquez G, Bird GS, Putney JW Jr. The TRPC3/6/7 subfamily of cation channels. *Cell Calcium*. 2003;33:451-61.
- Smedlund K, Vazquez G. Involvement of native TRPC3 proteins in ATP-dependent expression of VCAM-1 and monocyte adherence in coronary artery endothelial cells. *Arterioscler Thromb Vasc Biol*. 2008;28:2049-55.
- Huang JH, He GW, Xue HM, Yao XQ, Liu XC, Underwood MJ, et al. TRPC3 channel contributes to nitric oxide release: significance during normoxia and hypoxia-reoxygenation. *Cardiovasc Res*. 2011;91:472-82.
- Laskey RE, Adams DJ, Johns A, Rubanyi GM, van Breemen C. Membrane potential and Na(+)-K+ pump activity modulate resting and bradykinin-stimulated changes in cytosolic free calcium in cultured endothelial cells from bovine aorta. *J Biol Chem*. 1990;265:2613-9.
- Schilling WP, Rajan L, Strobl-Jager E. Characterization of the bradykinin-stimulated calcium influx pathway of cultured vascular endothelial cells. Saturability, selectivity, and kinetics. *J Biol Chem*. 1989;264:12838-48.
- He GW, Yang CQ, Graier WF, Yang JA. Hyperkalemia alters EDHF-mediated hyperpolarization and relaxation in coronary arteries. *Am J Physiol*. 1996;271:H760-7.
- He GW. Hyperkalemia exposure impairs EDHF-mediated endothelial function in the human coronary artery. *Ann Thorac Surg*. 1997;63:84-7.
- Yang Q, Liu YC, Zou W, Yim AP, He GW. Protective effect of magnesium on the endothelial function mediated by endothelium-derived hyperpolarizing factor in coronary arteries during cardioplegic arrest in a porcine model. *J Thorac Cardiovasc Surg*. 2002;124:361-70.
- He GW, Yang CQ. Impaired endothelium-derived hyperpolarizing factor-mediated relaxation in coronary arteries by cold storage with University of Wisconsin solution. *J Thorac Cardiovasc Surg*. 1998;116:122-30.
- Zhang RZ, Yang Q, Yim AP, He GW. Alteration of cellular electrophysiologic properties in porcine pulmonary microcirculation after preservation with University of Wisconsin and Euro-Collins solutions. *Ann Thorac Surg*. 2004;77:1944-50.
- Ge ZD, He GW. Altered endothelium-derived hyperpolarizing factor-mediated endothelial function in coronary microarteries by St Thomas' Hospital solution. *J Thorac Cardiovasc Surg*. 1999;118:173-80.
- Yang Q, Huang JH, Man YB, Yao XQ, He GW. Use of Intermediate/small conductance calcium-activated potassium channel activator for endothelial protection. *J Thorac Cardiovasc Surg*. 2011;141:501-10. 510.e1.
- Yang Q, Zhang RZ, Yim AP, He GW. Effect of 11,12-epoxyeicosatrienoic acid as an additive to St. Thomas' cardioplegia and University of Wisconsin solutions on

- endothelium-derived hyperpolarizing factor-mediated function in coronary microarteries: influence of temperature and time. *Ann Thorac Surg.* 2003;76:1623-30.
19. Ge ZD, Zhang XH, Fung PC, He GW. Endothelium-dependent hyperpolarization and relaxation resistance to N(G)-nitro-L-arginine and indomethacin in coronary circulation. *Cardiovasc Res.* 2000;46:547-56.
 20. Yang Q, Ge ZD, Yang CQ, Huang Y, He GW. Bioassay of endothelium-derived hyperpolarizing factor with abolishment of nitric oxide and the role of gap junctions in the porcine coronary circulation. *Drug Dev Res.* 2003;58:99-110.
 21. Liu CL, Huang Y, Ngai CY, Leung YK, Yao XQ. TRPC3 is involved in flow- and bradykinin-induced vasodilation in rat small mesenteric arteries. *Acta Pharmacol Sin.* 2006;27:981-90.
 22. Freichel M, Suh SH, Pfeifer A, Schweig U, Trost C, Weissgerber P, et al. Lack of an endothelial store-operated Ca^{2+} current impairs agonist-dependent vasorelaxation in TRP4-/- mice. *Nat Cell Biol.* 2001;3:121-7.
 23. Hearse DJ, Braimbridge MV, Jynge P. *Protection of the Ischemic Myocardium: Cardioplegia.* New York: Raven Press; 1981.
 24. Mankad PS, Chester AH, Yacoub MH. Role of potassium concentration in cardioplegic solutions in mediating endothelial damage. *Ann Thorac Surg.* 1991;51:89-93.
 25. Jeng LB, Lin PJ, Yao PC, Chen MF, Tsai KT, Chang CH. Impaired endothelium-dependent relaxation of human hepatic arteries after preservation with the University of Wisconsin solution. *Arch Surg.* 1997;132:7-12.
 26. Yang Q, He GW. Effect of cardioplegic and organ preservation solutions and their components on coronary endothelium-derived relaxing factors. *Ann Thorac Surg.* 2005;80:757-67.
 27. Pearl JM, Laks H, Drinkwater DC, Sorensen TJ, Chang P, Aharon AS, et al. Loss of endothelium-dependent vasodilatation and nitric oxide release after myocardial protection with University of Wisconsin solution. *J Thorac Cardiovasc Surg.* 1994;107:257-64.
 28. Wu M, Dong YY, Yang Q, Yim AP, He GW. Cellular electrophysiological and mechanical effects of celsior solution on endothelial function in resistance coronary arteries. *Transplantation.* 2005;80:1765-72.

SUPPLEMENTARY MATERIALS

Methods

Isolation and culture of porcine coronary endothelial cells. Fresh porcine hearts from hogs (either sex) weighing approximately 30 kg, collected from a local slaughterhouse, were placed in a container filled with cold (4°C) Krebs solution and immediately transferred to the laboratory. Small porcine coronary arteries were dissected into approximately 1 × 2-mm (width × length) strips and treated with 0.2% collagenase (type I; Sigma, St Louis, Mo) in phosphate-buffered saline at 37°C for 25 minutes. After the enzyme digestion, the suspension was centrifuged at 1600 rpm for 5 minutes. The cells were resuspended in 5 mL of culture medium containing 90% Roswell Park Memorial Institute medium and 10% fetal bovine serum with 100 U/mL penicillin and 100 µg/mL streptomycin. After 1-hour incubation at 37°C, the medium was replaced once to remove unattached cells. Attached endothelial cells were

primary cultured in medium containing antibiotics in a humidified incubator with 5% CO₂ at 37°C for 4 to 6 days.

Intracellular Ca²⁺ concentration measurement in porcine coronary endothelial cells.

Primary cultured PCECs were seeded on glass coverslips 1 day before [Ca²⁺]_i measurement. Cells were loaded with 5 µmol/L Fura-2/AM for 1 hour in the dark with 0.02% Pluronic F127 at 37°C. The cells were bathed in NPSS oxygenated with a gas mixture of 95% O₂ and 5% CO₂. NPSS contained in millimoles/liter: 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 5 HEPES (pH adjusted to 7.4). Fura-2/AM fluorescence signals were measured using dual excitation wavelengths at 340 and 380 nm with a digital InCyt Basic Fluorescence Imaging System (Intracellular Imaging, Cincinnati, Ohio). Fura-2/AM ratio changes (340/380) were then converted to [Ca²⁺]_i on the basis of the calibration using a calibration kit (Invitrogen, Carlsbad, Calif). All experiments were performed at room temperature.