Cholesterol-induced suppression of endothelial Kir channels is a driver of impairment of arteriolar flow-induced vasodilation in humans

Sang Joon Ahn[1*], Ibra S. Fancher[1,2*], Sara T. Granados[1*], Natalia F. Do Couto[1,3], Chueh-Lung Hwang[3], Shane A. Phillips[3] and Irena Levitan[1]

[1]Department of Medicine, Division of Pulmonary, Critical Care, Allergy and Sleep, College of Medicine, University of Illinois at Chicago, [2]Department of Kinesiology and Applied Physiology, University of Delaware, [3]Department of Physical Therapy, College of Applied Health Science, University of Illinois at Chicago.

Authors contributed equally to this work.

Short title: Cholesterol and vasodilation in resistance arteries

Online Supplemental Materials

Corresponding author:

Sang Joon Ahn

Division of Pulmonary, Critical Care, Allergy and Sleep

Department of Medicine

University of Illinois at Chicago

Address: 909 S. Wolcott Ave., Room 3100, Chicago 60612 USA

Telephone: +1-312-413-1400

Fax: +1-312-996-7193

Email: sahn29@uic.edu

Irena Levitan

Division of Pulmonary, Critical Care, Allergy and Sleep

Department of Medicine

University of Illinois at Chicago

Address: 909 S. Wolcott Ave., Room 3097, Chicago 60612 USA

Telephone: +1-312-996-0441

Fax: +1-312-996-7193

Email: levitan@uic.edu

Detailed Materials and Methods

Human blood pressure measurement

After a rest for at least 10 min in a quiet room, seated blood pressure was measured with an appropriate cuff size on the right brachial artery, at a heart level. Three readings were obtained at 1-min intervals using an automatic oscillometric device (HEM-907XL, Omron Corporation, Japan) by trained research personnel. The average of the 3 readings was then calculated.

Animals

All mouse experiments were approved by The University of Illinois Animal Care Committee (ACC#19-163). Pathogen-free housing at the University of Illinois Animal Care Vivarium was used for all mice, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and fulfilled the standards of the Animal Welfare Act, the Public Health Service Policy, and the NIH Guide for the Care and Use of Laboratory Animals. All mice were humanely treated following the institutional guidelines, and performed all animal experiments in accordance with the Guide for the Care and Use of Laboratory Animals. All experiments were done to minimize the animals' pain and suffering. All animal tissues used in the experiment were extracted after euthanasia using 30% CO_2 followed by cervical dislocation.
15 weeks old ApoE$^{-/-}$ mice (8 males) were purchased from Jackson Laboratory and used in their age of 7-month-old to generate hypercholesterolemia. CRISPR modified Kir2.1^{L222I} mice were generated in C57B6/J mouse and bred with ApoE$^{-/-}$ to get homozygous Kir2.1^{L222I}/ApoE$^{-/-}$ mice. Age matched Kir2.1^{L222I}/ApoE$^{-/-}$ mice (5 males and 2 females) were used to test the effects of cholesterol insensitive mutation in Kir2.1 in FIV. Mesenteries were extracted after euthanasia, and arteries were cleaned by removing adipose tissues in HEPES buffer solution as described previously[1].

Generation of Endothelial specific Kir2.1 knock out mouse model

Floxed Kir2.1 mouse model was generated by Mouse Biology Program in University of California, Davis with the service request by Dr. Irena Levitan. A pair of male and female mice were obtained and achieved a colony. To generate conditional endothelial specific Kir2.1 knock out mouse model, floxed Kir2.1 mice were bred with Cdh5.Cre^{ERT2}ROSA^{mTmG} mouse. The colony of Cdh5.Cre^{ERT2}ROSA^{mTmG}Kir2.1$^{fl/fl}$ mouse is maintained based on the regulation of Biological Resources Laboratory in University of Illinois at Chicago. Tamoxifen was dissolved in corn oil/ethanol (10:1) and injected 2 mg/mouse for 5 consecutive days to activate Cre enzyme. Either of vehicle injected or tamoxifen injected Cdh5.Cre^{ERT2}ROSA^{mTmG} were used as control.
Genotyping primers for Kir2.1$^{fl/fl}$ mouse:
Forward: 5'-AGCTTGGCGTAATCATGGTCATAGC-3'
Reverse: 5'-GGCATGGTAAAATAGTAGGGGTGTAGC-3'

Real-time PCR

Endothelial Cell isolation: Age-matched littermates of Cdh5.Cre^{ERT2}ROSA^{mTmG}Kir2.1$^{fl/fl}$ mice were used (n=8). 4 mice were injected with tamoxifen, and 4 mice were injected with vehicle (corn oil/ethanol) between 9-10 weeks old. After 21 days, mesenteric arteries were taken from these mice. Each mesenteric arterial bed was separately digested in neutral protease/elastase (1 mg/ml each, Worthington) in dissociation buffer under 37 °C for 45 mins. Collagenase, Type IV (final 6 mg/ml, Worthington) in dissociation buffer was added to the tissue, and the digestion was continued for 15 mins. Purification of endothelial cells were

processed with MACS (magnetic-beads associated cell sorting) system from Miltenyi Biotech and followed company recommended procedure. Briefly, digested cells were incubated with anti-mouse CD31 antibody conjugated with magnetic beads and gone through magnetic column. Antibody captured cells were considered as endothelial cells and non-captured cells were considered as smooth muscle rich cell groups. Purity of the isolated endothelial cells were confirmed with PECAM1 and SMA expression (*S1B,C*).
RNA isolation and RT-PCR: RNA was extracted using Trizol (Invitrogen), and whole RNA were used to generate cDNA with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). All procedures were followed with company recommended protocol.
Real-time PCR: 7500 fast PCR system (Applied Biosystems) was used with 60 cycles. cDNA samples were mixed with Fast SYBR Green Master mix (Applied Biosystems) and primers for GAPDH, CD31, Smooth muscle actin, Kir2.1, respectively. All procedures followed company recommended protocol. All primers were purchased as predesigned primers from IDT Prime Time. GAPDH: Mm.PT.39a.1, CD31: Mm.PT.58.12394061, SMA: Mm.PT.58.16320644, Kir2.1: Mm.PT.58.32212507. Quantification of fold changes in gene expression was performed using the $2^{-\Delta\Delta Ct}$ method.

Cholesterol insensitive Kir2.1 mouse model

Cholesterol insensitive Kir2.1 mouse model was developed with CRISPR and homologous-directed repairment by Research Resource Center (RRC) in University of Illinois at Chicago. Briefly, the homologous fragment was developed with the leucine (CTT) of genomic Kcnj2 (gene of Kir2.1) mutated to isoleucine (ATC), and a silent mutation (TAG) was introduced in the PAM site necessary to prevent the sgRNA from cutting the mutant sequence. (Fig.2A)
sgRNA: CCTTCGAAAGAGCCACCTTG
HDR fragment: TAACCTTCGAAAGAGCCACATCGTAGAAGCT
sgRNA and HDR fragments were introduced to embryonic cells from B6 mouse by electroporation and mutant cells were selected and microinjected to FVB mother mouse. Chimeric mice were bred to get pure mutant B6 mice. A pair of male and female mice were received from the RRC and the colony was established for the mutant mice. $ApoE^{-/-}$ mice were bred together to generate cholesterol-insensitive Kir2.1 mutant with hypercholesterolemic background. Either of $Kir2.1^{L222I}$, $ApoE^{-/-}$, or WT mice were used as control to compare with $ApoE^{-/-}Kir2.1^{L222I}$ mouse. All mice were maintained based on the regulation of Biological Resources Laboratory in University of Illinois at Chicago.
Genotyping primers for $Kir2.1^{L222I}$ mouse:
Forward: 5'-GAGAGTGGGTAACCTTCGAAAGAG-3'
Reverse: 5'-CCCCTTCTGAAGTGATCCTAGATTTG-3'

Functional down regulation and recovery of Kir2.1

Functional down regulation and recovery of Kir2.1 in endothelium was performed as described previously[1, 2]. Briefly, adenoviral vector of Kir2.1 dominant negative and wild type Kir2.1 with Cdh5 promoter, and empty viral vector was purchased from Vector Biolabs. Extracted mouse mesenteric arterial beds and human subcutaneous arterioles were incubated either of Kir2.1DN, Kir2.1WT, or empty adenoviral vectors for 48 hours in DMEM (10% FBS, 1% PS) under regular cell culture condition (37 °C, 5% CO_2). We have established previously that mouse mesenteric resistance arteries maintain their reactivity to Acetylcholine and to flow, key endothelial responses, after being incubated with an empty adenoviral vector for 48 hours. This protocol was used in several in our recent studies[1-3]. In addition, we verified here that endothelium functionality of human resistance arteries remains intact after 48 hours of incubation by testing the reactivity of the vessels with flow- and acetylcholine-induced vasodilation. No difference was observed between freshly-isolated vessels and vessels incubated with the viral construct for 48 hours (*S5A,B*).

Flow-induced vasodilation measurement

Flow-induced vasodilation of human subcutaneous and mouse mesenteric arteries were measured as described previously[1-3]. Briefly, human subcutaneous arteries and mouse mesenteric arteries between 100-200 μm were cannulated to the organ chamber with glass micropipettes filled with Krebs solution (pH7.4). The chamber was placed on an Olympus CKX41 microscope equipped with a camera for visualizing the artery on a monitor and artery diameters were measured using a model VIA-100 video system (Boekeler). Two reservoirs filled with Krebs buffer were connected to the both sides of the chamber and located at 60 cm height from the chamber to pressurize the cannulated vessel. The inner diameter was measured after 30 min of pre-pressurization under 60 cmH_2O. Endothelin-1 (120 pmol/L final concentration) was used to pre-constrict up to 60% of pre-pressurized inner diameter. To generate flow with pressure gradient, one side of the reservoir was gone up and the other side was gone down with equal amount to maintain the inner pressure as 60 cmH_2O. For example, to generate $\Delta 10$ cmH_2O pressure gradient, one side of the reservoir was located at 65 cm and the other side was located at 55 cm, then two reservoirs had $\Delta 10$ cmH_2O pressure difference with maintaining the average of 60 cmH_2O inner pressure[30]. Flow-induced vasodilation was determined by exposing the vessels to incremental pressure gradients of $\Delta 10$, $\Delta 20$, $\Delta 40$, $\Delta 60$, and $\Delta 100$ cmH_2O with maintaining the inner pressure at 60 cmH_2O. The change of inner diameter was measured every 30 seconds, and after 3 mins, pressure gradient was increased. The data points at 3 mins were used to determine the % changes of FIV. Inner diameter changes were measured and normalized by the following equation:
FIV(%) = (measured diameters – pre-constricted diameter)/(pre-pressurized inner diameter – pre-constricted diameters)*100.
Papaverine (100 μM) was added after the measurement of inner diameter at $\Delta 100$ cmH_2O to induce endothelial-independent maximum dilation to confirm potential dilative power of vessels. The first order mouse mesenteric arteries with 150-200 μm were cannulated to the organ chamber as described for human arteries. FIV was also determined as described for human arteries. To explore the role of Kir2.1 in FIV, all human and mouse arteries were incubated with either empty, Cdh5-promoter-driven dominant negative Kir2.1, or Cdh5-promoter-driven WT Kir2.1 expressing adenoviral vectors of about 100 MOI for 48 hours prior to measure FIV. Additionally, all vessels were exposed to $BaCl_2$ (30 μM) as well as ML133 (100 μM), well-known Kir2.1 blockers, for 30 min before determining FIV to test the role of Kir2.1 in FIV.

Cholesterol loading and Kir2.1 functional measurement in HAECs

Human aortic endothelial cells (HAECs) were cultured in EGM-2 (Lonza) media in standard cell culture incubator. HAECs were exposed to human LDL in doses of 0, 75, 150, and 250 mg/dl in 2% lipoprotein-deficient serum substituted EGM-2 media. HAECs were incubated with different concentration of LDL for 48 hours in the incubator prior to the experiments. Kir2.1 currents were recorded during patch clamp experiments described below. Cholesterol amounts were measured using Amplex Red cholesterol assay kit (Molecular Probes) according to the manufacturer's recommended guidelines and total protein amounts were measured by Bradford assay to normalize the cholesterol uptake in each condition.

Electrophysiology on freshly isolated endothelial cells

Endothelial cells from WT and Kir2.1^{L222I} C57BL/6J mice were isolated for patch clamp recording as previously described[1-3]. Briefly, mesenteric beds were cleaned and digested using an enzyme cocktail of neutral protease (0.5 mg/ml, Worthington) and elastase (0.5 mg/ml, Worthington) in HEPES buffer for an hour at 37℃. Collagenase type I (final

concentration 0.5 mg/ml, Worthington) was added to the cocktail for 2 to 2.5 minutes. After the digestion, the arteries were gently dissociated on ice in the enzyme solution with syringe needles (25G). Digested arteries were pipetted several times using a glass pipette to disperse ECs into a single cell suspension. Cells were allowed to adhere on 30-mm cell culture treated plate for patch clamp experiment. Soft glass pipettes (SG10 glass, Richland Glass) were pulled using a vertical pipette puller (Model PP-830) and had resistances between 2 and 4 MΩ. The perforated patch technique was performed by adding amphotericin B (250 µg/mL) to the pipette solution. Perforated patches were obtained within 2 to 5 minutes after formation of a GΩ seal, and accepted recordings for offline analysis maintained a series resistance between 10 and 30 MΩ. Currents from ECs were recorded using an EPC9/10 amplifier and accompanying acquisition and analysis software (Pulse and PulseFit, HEKA Electronik). ECs were held at −30 mV and a voltage ramp of −120 to +40 mV applied over 400 ms. Currents were low-pass filtered at 2 kHz and recordings were digitized at 10 kHz. When necessary, leak subtraction was performed offline to collect the most accurate data points at −100 mV for group analysis. To test the cholesterol insensitivity of the mutation in Kir2.1, the current density of Kir2.1 in ECs isolated from mesenteric arteries of $Kir2.1^{L222I}$ mice were compared with and without methyl-β-cyclodextrin (MβCD, $5x10^{-3}$ mol/L in serum-free EBM-2 media (Lonza)), in which ECs were incubated for 1 hour in the standard cell culture incubator. Solutions were rapidly exchanged to 60 mmol/L K^+ solution for subsequent perforated patch clamp recording.
Shear stress-induced Kir currents recording: Shear stress was applied to the freshly isolated cells using the minimally invasive flow (MIF) device[4]. Briefly, a large aliquot of cell suspension was allowed to adhere to the patch chamber before assembly. Upon whole-cell access, several currents were recorded in a static bath until a stable baseline was achieved. Application of shear stress was implemented by gravity perfusion to induce shear-activated inward K^+ currents. Shear stress (τ; 0.74 dyn/cm^2) was calculated using the equation $\tau = 6\mu Qh^2w$ where μ is the fluid viscosity (0.009 g/cm·s), Q is the flow rate (300 µl/s applied by gravity perfusion), h is the height (0.1 cm) of the MIF chamber, and w is the width (2.2 cm) of the MIF chamber.

Solutions and reagents

HEPES buffer contained in mM: NaCl 140, KCl 4, $MgCl_2$ 1, glucose 5, HEPES 10, $CaCl_2$ 2, and pH 7.4. _Krebs solution contained in mM_: NaCl 123, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 16, EDTA 0.026, glucose 11, KH_2PO_4 1.2, and pH 7.4. _Perforated patch bath solution contained in mM_: NaCl 80, KCl 60, HEPES 10, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, and pH 7.4. _Perforated patch pipette solution contained in mM_: NaCl 5, KCl 135, EGTA 5, $MgCl_2$ 1, glucose 5, HEPES 10, and pH 7.2. All the reagents were purchased from Sigma otherwise stated separately.

References

1. Ahn SJ, Fancher IS, Bian JT, Zhang CX, Schwab S, Gaffin R, Phillips SA, Levitan I. Inwardly rectifying k+ channels are major contributors to flow-induced vasodilatation in resistance arteries. *J Physiol.* 2017;595:2339-2364
2. Fancher IS, Ahn SJ, Adamos C, Osborn C, Oh MJ, Fang Y, Reardon CA, Getz GS, Phillips SA, Levitan I. Hypercholesterolemia-induced loss of flow-induced vasodilation and lesion formation in apolipoprotein e-deficient mice critically depend on inwardly rectifying k(+) channels. *J Am Heart Assoc.* 2018;7:pii: e007430. doi: 007410.001161/JAHA.007117.007430.
3. Fancher IS, Le Master E, Ahn SJ, Adamos C, Lee JC, Berdyshev E, Dull RO, Phillips SA, Levitan I. Impairment of flow-sensitive inwardly rectifying k(+)

channels via disruption of glycocalyx mediates obesity-induced endothelial dysfunction. *Arterioscler Thromb Vasc Biol*. 2020;40:e240-e255

4. Levitan I, Helmke BP, Davies PF. A chamber to permit invasive manipulation of adherent cells in laminar flow with minimal disturbance of the flow field. *Ann Biomed Eng*. 2000;28:1184-1193

Table S1

Subject characteristics. Data are mean±SE or n. BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Measures	mean±SE
Age, years	34±3
Male/female, n	9/7
Body mass index, kg/m^2	25.1±0.9
Vessel diameters, µ	148±11
Systolic BP, mmHg	120±1.9
Diastolic BP, mmHg	75±1.3
Total cholesterol, mg/dL	194±12
Triglyceride, mg/dL	94±15
LDL cholesterol, mg/dL	110±7
HDL cholesterol, mg/dL	58±3
Glucose, mg/dL	93±3
Insulin, µIU/mL	5.6±1.0

Table S2 Multiple linear regression models using enter method of factors of interest (total cholesterol, BMI, and age) to predict FIV at the pressure gradient of Δ100 cmH_2O in healthy adults (n=20). B=Unstandardized coefficients; BMI=body mass index; SE=standard error (*p <0.05).

Variables	Model 1		Model 2		Model 3	
	B	SE	B	SE	B	SE
Total cholesterol	-0.32*	0.07	-0.29*	0.08	-0.24*	0.07
BMI			-0.70	0.79	-0.17	0.74
Age					-0.61	0.26

Table S3 Multiple linear regression models using enter method of factors of interest (total cholesterol, BMI, and age) to predict Kir2.1-dependent maximal FIV in healthy adults (n=20). B=Unstandardized coefficients; BMI=body mass index; SE=standard error (*p <0.05).

Variables	Model 1		Model 2		Model 3	
	B	SE	B	SE	B	SE
Total cholesterol	-0.43*	0.08	-0.37*	0.09	-0.37*	0.10
BMI			-0.49	0.74	-0.18	0.72
Age					-0.47	0.27

Figure S1

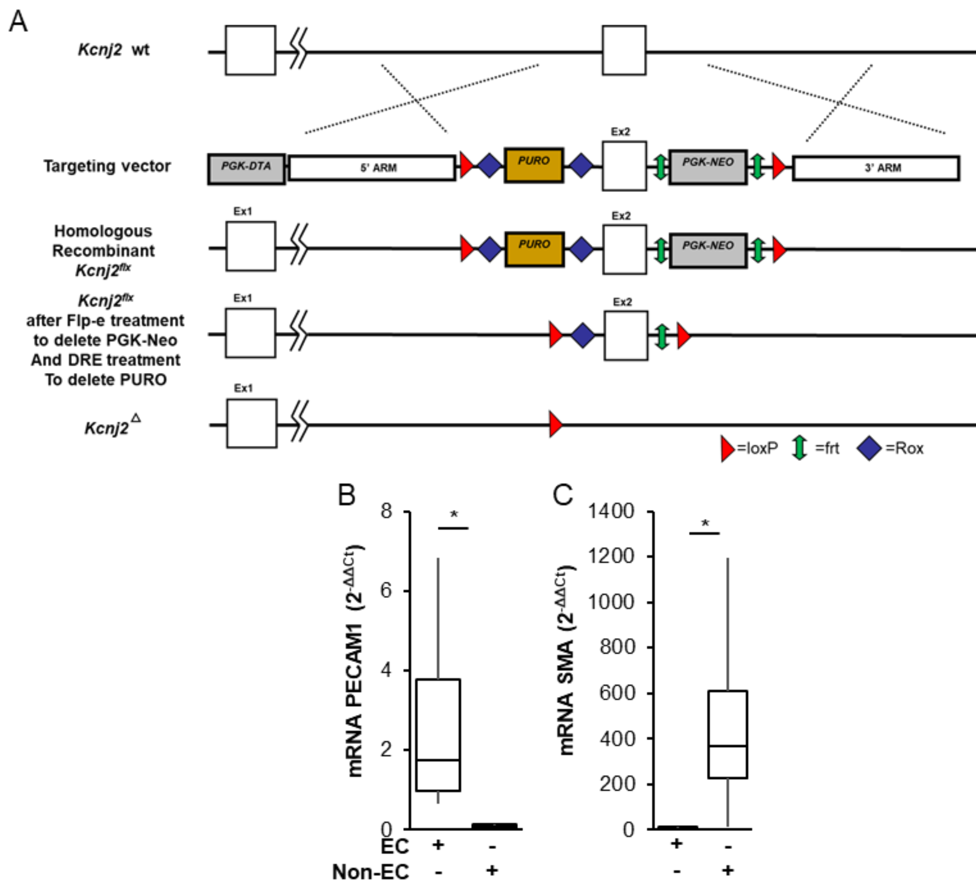

Figure S1. A. Schematic design of Kir2.1 flox mice. Deletion target is located in Exon 2 and generate the homozygous mouse pairs with multiple steps. Puromycine and neomycine resistance sites were removed in the middle of the steps. B,C. Confirmation of endothelial cell purity in EC-portions with real-time PCR (n=4 pairs, *p<0.05).

Figure S2

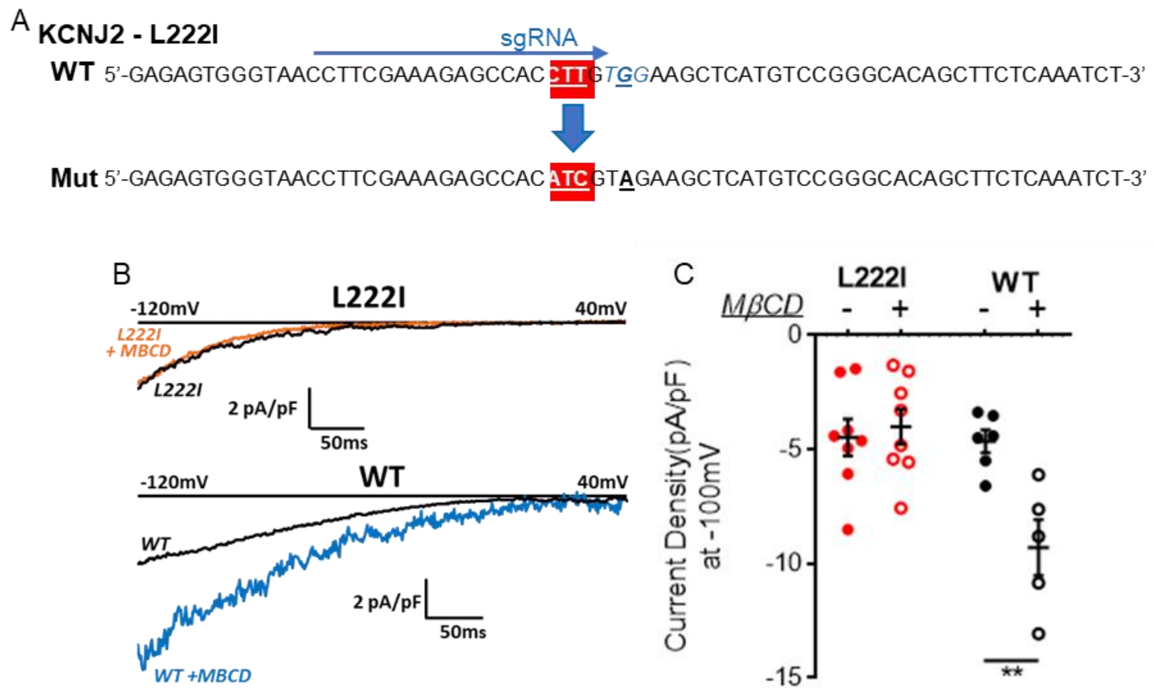

Figure S2. A. Schematic design of generating L222I mice using CRISPR. sgRNA was designed with the sequence under blue arrow. Leucine at 222 (CTT) was mutated to Isoleucine (ATC) and the pam sequence of TGG was also mutated as TAG to prevent the cleavage after homologous recombination. B. Representative trace of electrophysiological recording of Kir2.1 in mesenteric arterial ECs from Kir2.1^{L222I} and WT mice with and without MβCD (5 mmol/L, 1 hour). C. Average Kir current densities from CRISPR L222I (n=9-10) and wild type mice (n=5-6, **$p < 0.01$)

Figure S3

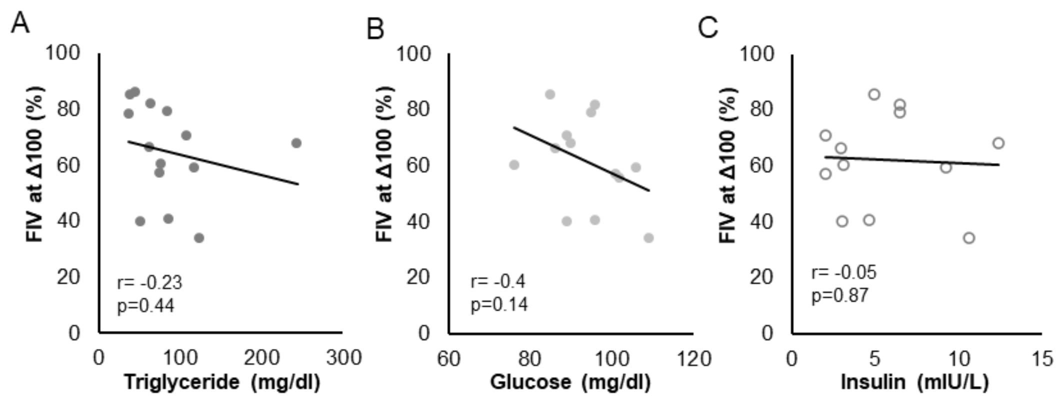

Figure S3. A, B, C. Correlation between FIV at Δ100 cmH_2O and triglycerides, glucose, and insulin of human participants (n=16).

Figure S4

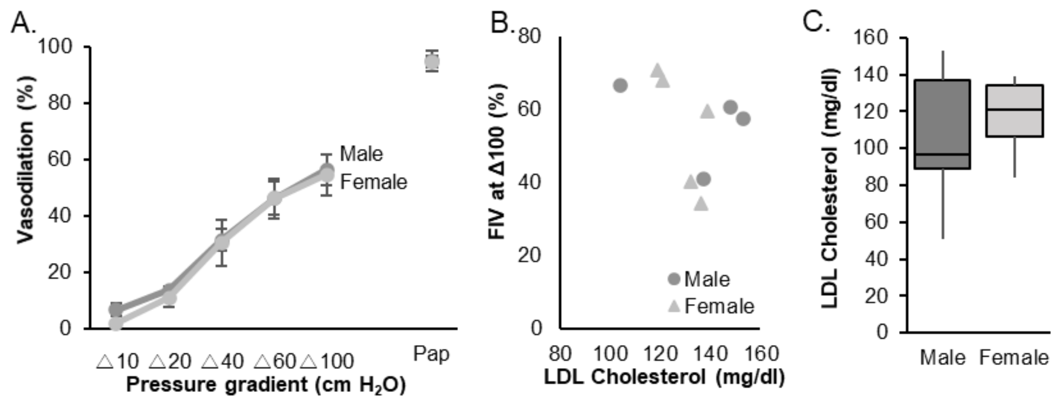

Figure S4. A. FIV of male and female participants (n=9 and 7, respectively). B. Comparison of FIV at Δ100 cmH_2O and LDL cholesterol above 100 mg/dl (n=4 and 5, respectively). C. The level of LDL cholesterol in male and female participants (n=9 and 7, respectively).

Figure S5

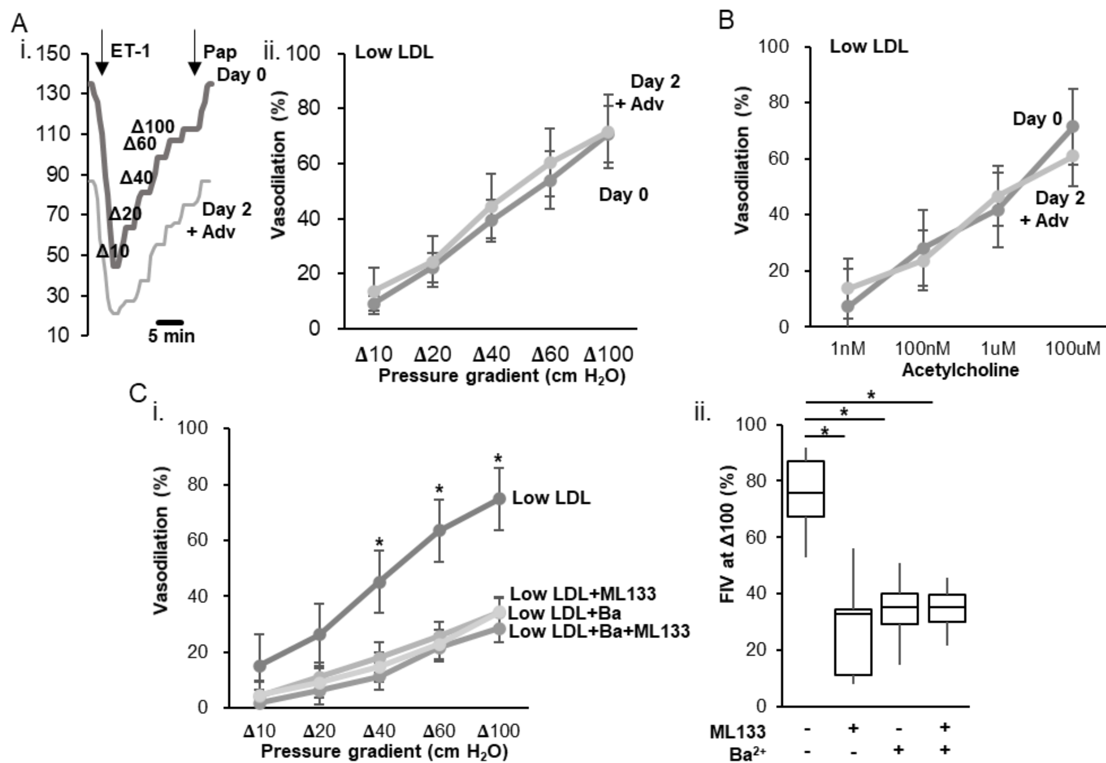

Figure S5. A. i. Representative trace of FIV. ii. FIV on the day of isolation and the day after 48-hour adenoviral vector incubation in human gluteal fat arteries from human subjects with LDL<100 mg/dl. (n=5 vessels from 4 subjects) B. Acetylcholine-induced vasodilation on the day of isolation and the day after 48-hour adenoviral vector incubation in human gluteal fat arteries from human subjects with LDL<100 mg/dl. (n=5 vessels from 4 subjects) C. i. FIV of human gluteal fat arteries from human subjects with LDL<100 mg/dl with Ba^{2+} (30 μM, Ba) and/or ML133 (100 μM), a Kir2.1-specific inhibitor. (n=5 vessels from 4 subjects, *p<0.05) ii. Comparison of FIV at Δ100 cmH_2O. (n=5 vessels from 4 subjects, *p<0.05)

Figure S6

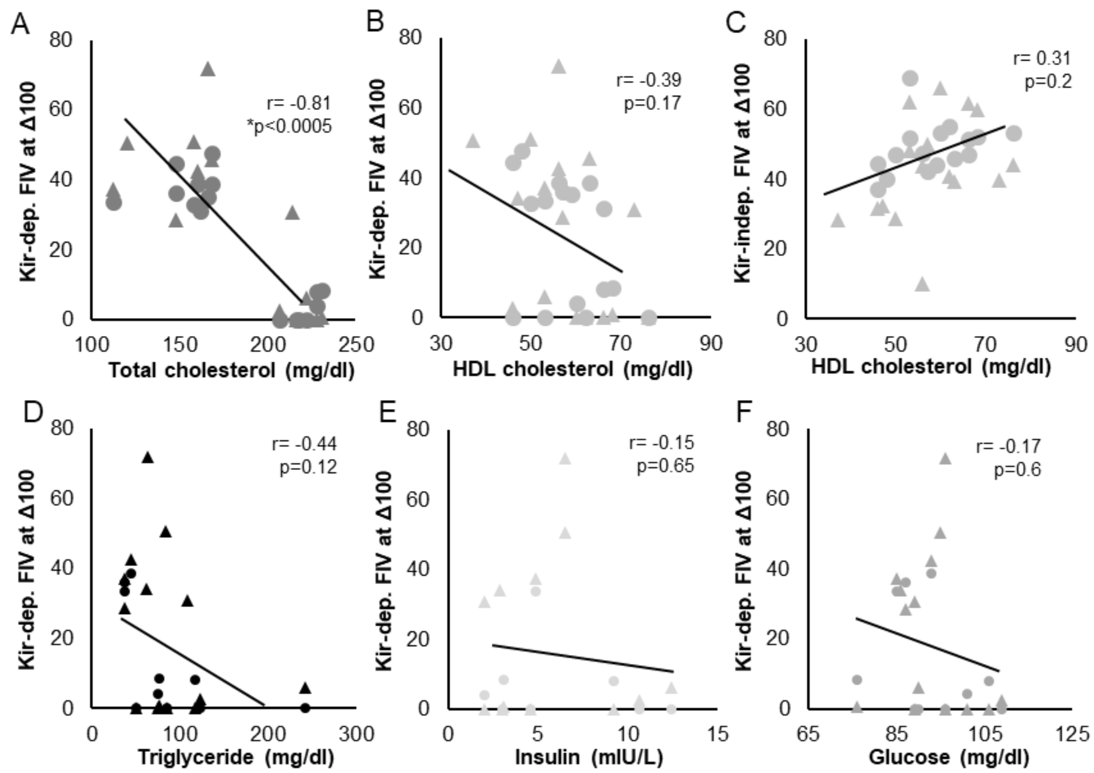

Figure S6. A. Correlation between Kir2.1 dependent FIV at Δ100 cmH_2O and HDL in human participants (n=20, *p<0.05). B. Correlation between Kir2.1 dependent FIV at Δ100 cmH_2O and HDL in human participants (n=20). C. Correlation between Kir2.1 independent FIV at Δ100 cmH_2O and HDL in human participants (n=20). C, D, E. Correlation between Kir2.1 dependent FIV at Δ100 cmH_2O and triglyceride, insulin, and glucose (n=16). Kir2.1 dependency were determined either by Ba^{2+} (triangle) or Cdh5-dnKir2.1 (circle).