Shear Stress Regulates ABCA1-dependent Membrane Cholesterol Content in Endothelial Cells Facilitating H_2S-dependent Vasodilation

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Abstract:

Endothelial cells (ECs) express an array of integral membrane proteins, including ion channels and transporters that contribute to blood flow regulation and cell-cell communication. Many of these membrane proteins are regulated by plasma membrane cholesterol content. The ATP-binding cassette family a1 (ABCA1) transporter is a regulator of membrane cholesterol content. We have shown increased ABCA1 mRNA expression and reduced EC membrane cholesterol in resistance mesenteric arteries compared to conduit arteries. Previous studies suggest shear stress (SS) can increase or decrease ABCA1 expression in a cell-type-dependent manner. *Hypothesis*: SS sustains lower EC membrane cholesterol concentration through ABCA1-mediated cholesterol transport, facilitating H_2S-mediated vasodilation. *Method*s: The effect of SS on ABCA1 and membrane cholesterol content was assessed in pressurized mesenteric arteries from male Sprague-Dawley rats and cultured human aortic endothelial cells. Pressure myography was used to assess the effects of ABCA1 inhibition on H_2S-mediated vasodilation. Filipin was used to assess EC membrane cholesterol content. *Results*: SS increased ABCA1 expression in the endothelium of mesenteric arteries and cultured human aortic endothelial cells and markedly reduced EC membrane cholesterol. Inhibition of ABCA1 increased EC membrane cholesterol content and abolished H_2S-induced vasodilation. *Conclusion:* SS facilitation of EC-dependent vasodilation appears to be mediated by membrane cholesterol content.

Introduction:

The endothelium comprises a monolayer of highly specialized endothelial cells (ECs) lining the luminal surface of all blood vessels and occupies a fundamental position in the cardiovascular system. The endothelium regulates complex intercellular interactions, including vascular tone, vascular permeability, and innate immune response[1,2]. The endothelium senses and integrates various stimuli to regulate vascular tone through the synthesis and release of vasoactive gas molecules, such as nitric oxide, carbon monoxide, and hydrogen sulfide (H_2S) [3].

H_2S has been reported to be an endothelial-derived hyperpolarization factor contributing to vasodilation[4] and exerting antioxidant and anti-inflammatory protective effects on the cardiovascular system[5]. The enzymatic synthesis of H_2S involves cystathionine-gamma-lyase, cystathionine-beta-synthase, and 3-mercaptopyruvate sulfurtransferase; the specific enzymatic pathways vary across vascular beds, adding complexity to the regulation of H_2S-mediated vasodilation[6,7]. Our previous work has shown that H_2S sensitivity in conduit arteries (>300 μm) is dependent on the content of membrane cholesterol in ECs, whereas resistance arteries (<150 μm) are inherently sensitive to H_2S[8].

Membrane cholesterol is a fundamental constituent of the lipid bilayer and impacts membrane fluidity, stability, and organization[9]. Interestingly, our lab has shown that native EC membrane cholesterol content is greater in EC from conduit arteries than in resistance arteries,[8] suggesting innate regional differences in membrane cholesterol content along the vascular tree. In addition, our single-cell RNAseq analysis supported by immunohistochemistry indicates that ECs from resistance arteries express greater levels

of the reverse cholesterol transport genes ATP-binding cassette protein A1 (ABCA1) and phospholipid transfer protein (PLTP) compared to ECs from conduit vessels[10]. The ATP-binding cassette (ABC) transport proteins, a family of eight genes, are responsible for transporting various hydrophobic compounds, where ABCA1 specifically facilitates membrane cholesterol efflux through a process called reverse cholesterol transport in multiple cell types[11,12]. Taken together, these findings suggest that reduced membrane cholesterol within resistance arteries enhances endothelial-mediated vasodilation, supporting the postulate that membrane cholesterol is an important mediator of EC function. However, despite the observed regional heterogeneity in ABCA1 expression within the vascular tree, it is unknown whether ABCA1 mediates innate differences in EC cholesterol content. In addition, the mechanism mediating differential ABCA1 expression between conduit and resistance arteries also remains unknown. Previous studies suggest shear stress (SS) can both increase and decrease ABCA1 expression potentially in a cell-type-dependent manner[13–15].

Vascular SS is a mechanical force resulting from friction against the endothelium generated by blood flowing along its surface. Vascular ECs sense SS through the activation of mechanosensors, translating it into biochemical signals influencing cell morphology, vascular tone, and gene expression[16,17]. As the luminal diameter of an artery decreases, SS exponentially increases[18], suggesting that differences in SS may mediate cellular heterogeneity along the vascular tree. Therefore, we hypothesized that SS sustains lower EC membrane cholesterol concentration through ABCA1-mediated cholesterol transport, facilitating H_2S-mediated vasodilation.

Methods

Cell Culture

Primary HAECs (Cell Applications S304-05a, P4-P6) were used for all cell culture experiments. Cells were plated on cell culture dishes coated with 0.1% Fibronectin (Sigma, FC010) and maintained at 37°C, 5% CO_2 in endothelial growth media V2 (Cell Applications). To expose cells to SS, ECs were seeded on μ-Slide 0.4 Luer ibiTreat (ibidi 80176). Unidirectional, recirculating, laminar flow was applied to confluent monolayers using a peristaltic pump [6 dyn/cm^2, 20 dyn/cm^2, or 0 ml/min for static control] with an inline wind Kessel (to dampen pulsatile oscillations) and bubble trap.

Animals

Sprague-Dawley rats (Envigo, 250–300 g, 9–10 wk. old) were used for all experiments. Rats were euthanized with a lethal concentration of pentobarbital sodium (200 mg/kg I.P.), and mesenteric arteries were collected for experiments. The Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine reviewed and approved all animal protocols. All protocols conformed to National Institutes of Health guidelines for animal use.

Quantification of Membrane Cholesterol Content

Isolated endothelial tubes were plated on glass coverslips and fixed with 4% paraformaldehyde (20 min at RT). Endothelial cell membrane cholesterol was detected by incubating cells with the fluorescent cholesterol marker filipin III (100 μg/mL) for 45 min at 37ºC under light-protected conditions, and coverslips were mounted on slides using ProlongGold Antifade (Thermo Fisher P10144). Samples were imaged using a

fluorescent confocal microscope (Zeiss LSM 800 AxioObserver.Z1/7; Gottingen, Germany) using a 353 nm laser (excitation), a 465 nm long pass filter (emission) with a Plan-Apochromat 63/1.4 numerical aperture (NA) oil immersion objective. A negative control lacking filipin was used to control for background signal. Filipin staining was quantified using ImageJ. A total of 4 tubes/artery from five animals were analyzed. The mean fluorescence intensity of each endothelial tube was measured and averaged to determine a mean fluorescence value for each animal.

HAoECs were stained as previously described above with filipin, and samples were imaged using the Zeiss-LSM800. Images contained 8-10 images with 7-10 cells per image. Regions of interest were created at approximately 1 mm from the membrane, both intra and extracellularly, to isolate the membrane from the intracellular compartments. Images were collected within one optical section showing membrane max diameter to ensure the focus was on the cell center and the mean fluorescence intensity per cell was measured and averaged to determine cholesterol content.

Quantification of ABCA1 Expression

Endothelial ABCA1 expression was quantified using immunofluorescence in *en-face* small arteries and HAECs. Small (<150 μm) were cannulated and pressurized[19]. Briefly, pressurized mesenteric arteries were fixed with 4% paraformaldehyde for 20 minutes at room temperature. Samples were blocked with 5% non-donkey serum (NDS) and treated with ABCA1 primary antibody (1:100) (Thermo Fisher, PA1-16789) overnight at 4ºC. Endothelial tubes were then blocked with 5% NDS for 1 h at 37ºC and incubated with donkey-α-rabbit 488 (1:1000) (Jackson ImmunoResearch Labs, 711-546-152) in 1% NDS for 4 h at 37ºC. Samples were mounted with Prolong Gold Antifade DAPI (Thermo

Fisher, P36935), and z-stack images were acquired using confocal microscopy (Zeiss LSM 800 AxioObserver.Z1/7) using a 488 nm laser (excitation) and 517 nm long pass filter (emission) and a Plan-Apochromat 63/1.4 NA oil immersion objective. Secondary antibody controls were used to verify the lack of non-specific staining. A total of 4 tubes/animal from five animals were analyzed. Z-stacks containing 14-16 slices each at 150nm were obtained, maximum projections were created, and the mean fluorescence intensity of each endothelial tube was measured using ImageJ and averaged to determine a mean fluorescence value for each animal. For the HAoECs, VE-Cadherin (1:50 Santa Cruz Biotech) was used to delineate the plasma membrane to create regions of interest to quantify ABCA1 fluorescence and intensity per cell. The LXR agonist, T0901317 (100 nM), is a well-established inducer of ABCA1 expression and was used to validate ABCA1 antibody specificity (Fig 6).

Application of SS to Mesenteric Arteries

Mesenteric conduit arteries (>300 μm) were cannulated and pressurized to 65mmHg using a gravity-based column system. Luminal flow was generated by adjusting the column heights [$P_{arterial}$ ~130mmHg and P_{venous} ~35mmHg (HSS)]. Arteries were continuously superfused with HPSS (5 ml/min, 37°C). Mean pressure was maintained at 65 mmHg. Vessels were exposed to flow for 6 hours, followed by a viability response (Ach-dilation) before fixation with 4% PFA for 20 minutes while continually being pressurized. Vessels were then cut along their longitudinal axis, secured *en face,* and blocked with 5% NDS for 1 hour at room temperature. Vessel segments were then incubated with ABCA1 primary antibody (1:100 – PA1-16789 Thermo) overnight at 4C in 1% NDS in phosphate-buffered saline. Secondary antibody AF-488 (1:1000 - Jackson

lab) was applied for 1 hr at room temperature in 1% NDS in phosphate-buffered saline and mounted with Prolong Gold. Five images from various parts of the artery were captured per condition. ABCA1 intensity was measured using ImageJ.

Preparation of Endothelial Tubes

The mesenteric arcade was removed and immediately placed in ice-cold HEPES buffered physiological saline solution (HPSS; in mM: 130 NaCl, 4 KCl, 1.2 $MgSO_4$, 4 $NaHCO_3$, 1.8 $CaCl_2$, 10 HEPES, 1.18 KH_2PO_4, and 6 glucose; pH adjusted to 7.4 with NaOH), containing 10 μM sodium nitroprusside and 0.1% bovine serum albumin. Veins were removed, and arteries were cleared of connective and adipose tissue. Arteries (50-150μm) were flushed of blood and pre-treated with probucol (100 μM in 2% pluronic acid) or vehicle at 37°C for 1 hour in HPSS containing 10% FBS and 0.1% bovine serum albumin. Arteries were then placed in an HPSS digestion solution (10% FBS, 0.1% bovine serum albumin, 0.62 mg/mL of papain, 1.5 mg/ mL of collagenase II, and 1 mg/mL dithiothreitol) with continued treatment with probucol or vehicle. Arteries were incubated in the digestion solution for 33 min at 37°C. Artery segments were then gently triturated to disrupt the adventitia and smooth muscle layers.

Vasodilation Studies

The mesenteric arcade was removed and immediately placed in ice-cold HPSS. After the removal of veins and adipose tissue, third- to fourth-order mesenteric arteries (100–200 μm inner diameter) were isolated and cannulated with glass micropipettes and gently flushed to remove blood from the lumen, as previously described[20,21]. The artery was stretched longitudinally to approximate in situ length and pressurized with a servo-

controlled peristaltic pump (Living Systems) to 75 mm Hg. The vessel chamber was superfused with HPSS at 5 ml/min at 37°C and allowed to equilibrate for 15 min. To determine the contribution of ABCA1 on the H_2S-mediated vasodilatory response, arteries were pretreated luminally with the specific ABCA1 antagonist, probucol (100 μM), for one hour before arteries were pre-constricted to ~30–50% with the thromboxane A_2 analog, U-46619 (Cayman Chemical). To assess H_2S-mediated vasodilation, arteries were treated with sodium hydrosulfide (NaHS) in uM; 1, 10, 30 (Cayman Chemical). Images were obtained using an Eclipse TS100 microscope (Nikon) and IonOptix CCD100M camera to measure ID using edge-detection software (IonOptix). Passive diameter was determined at the end of each experiment by superfusing the vessel in Ca^{2+}-free HPSS HPSS containing: 130 NaCl, 4 KCl, 1.2 $MgSO_4$, 3.6 EGTA, $NaHCO_3$, 10 HEPES, 1.18 KH_2PO_4, and 6 glucose; pH adjusted to 7.4 with NaOH), containing 10 μM sodium nitroprusside and 0.1% bovine serum albumin (in mM) for 30 min. For all experiments, changes in vessel diameter (plateau response) are expressed as the percent reversal of U46619-induced vasoconstriction.

Statistics and Calculations

All data are expressed as means ± SD. Statistical comparisons were made using Prism 9 (GraphPad Software). The specific statistical tests used are reported in the figure legends. A probability of <0.05 was accepted as statistically significant for all comparisons. Percent dilation was calculated as (NaHS diameter – U46619 diameter) / (Calcium Free diameter – U46619 diameter) x100%.

Results

Effect of SS on ABCA1 expression and membrane cholesterol in HAoECs

We exposed HAoECs to a SS of 6 dynes/cm^2, 20 dynes/cm^2, or static conditions for 24 hrs. Application of SS increased ABCA1 expression in HAoEC (Fig. 1). To validate that changes in ABCA1 were associated with reductions in membrane cholesterol, we employed filipin staining to demonstrate that exposing cells to SS reduces EC membrane cholesterol (Fig. 2).

Effect of SS on ABCA1 expression in mesenteric conduit arteries

To assess the effect of SS on ABCA1 expression, pressurized mesenteric arteries were exposed to 20 dynes/cm^2 for 6 hours prior to fixation. SS elicited an increase in ABCA1 expression compared to arteries under static conditions (Fig.3).

Effect of ABCA1 inhibition on membrane cholesterol and H_2S-mediated vasodilation in mesenteric resistance arteries

Inhibition of EC ABCA1 in small mesenteric arteries (<150 μm) increased EC membrane cholesterol (Fig 4). Administration of increasing concentrations of H_2S elicited a dose-dependent vasodilation in vehicle-treated arteries. Inhibition of ABCA1 attenuated H_2S-mediated vasodilation compared to control (Fig. 5).

Discussion

Endothelial function is essential for maintaining vascular homeostasis, and disruptions in endothelial signaling pathways can contribute to cardiovascular disease. This study demonstrates a novel role for ABCA1 in regulating membrane cholesterol levels in endothelial cells. To our knowledge, this is the first study to demonstrate a role for ABCA1 in SS-mediated reductions in membrane cholesterol. The findings suggest that ABCA1 regulation of membrane cholesterol impacts hydrogen sulfide (H_2S)-mediated vasodilation with potential implications for the regulation of microvascular function and disease pathophysiology. The key findings of this study are (1) SS increased ABCA1 expression in endothelial cells and (2) inhibition of ABCA1 increased membrane cholesterol levels and attenuated H_2S-mediated vasodilation.

The regulation of membrane cholesterol by ABCA1 in resistance arteries has significant physiological implications. Resistance-sized arteries are critical regulators of vascular resistance and blood pressure, and their endothelial function is highly influenced by membrane cholesterol content[22,23]. Our previous work demonstrated five up-regulated, differentially expressed genes involved in cholesterol transport (*Nfkbia*, *Apoe*, *Lrp1*, *Abca1*, *Pltp*) in ECs from small mesenteric arteries compared to ECs from large arteries. NF-kappa-B inhibitor alpha (*NFKBIA*) has been shown to enhance cholesterol transport in macrophages by inhibiting NFKB,[24,25] whereas ApoE facilitates cholesterol transport through direct binding with ABCA1[26,27]. The low-density lipoprotein receptor-related protein (*LRP1*) has been shown to regulate lipid and glucose metabolism and is a known co-activator of a transcriptional regulator of ABCA1, PPAR gamma[28,29,30]. Moreover, phospholipid transfer protein (*PLTP*) functions as an auxiliary protein

facilitating ABCA1-mediated cholesterol transport[31]. This gene ontology pathway analysis related to cholesterol transport supports the postulate that ABCA1 is an important determinant of membrane cholesterol heterogeneity.

Our previous work has shown that H_2S-mediated vasodilation in rat mesenteric arteries involves the activation of EC transient receptor potential cation subfamily V member 4 channel (TRPV4) channels and EC large conductance (BK_{Ca}) Ca^{2+}-activated potassium channels (Morin et al. 2022). TRPV4 and BK_{ca} contain cholesterol recognition/interaction amino acid consensus sequences (CRAC/CARC)[32,33]. Indeed, membrane cholesterol has been shown to negatively regulate TRPV4 and BK_{Ca} channel activity through direct protein-sterol interactions[32,34]. In addition, to channel regulation via direct protein-sterol interactions, changes in membrane cholesterol may alter signaling microdomains. We have previously demonstrated that depletion of membrane cholesterol was required to demonstrate a role for TPRV4 channels in muscarinic receptor-mediated vasodilation in rat gracilis arteries (Morin et al. 2022).

SS has been recognized as a key determinant of endothelial phenotype by influencing processes such as nitric oxide production[35], cellular polarization[36], inflammation[37,38], and permeability[39]. A previous report demonstrated a potential mechanism for enhanced ABCA1 expression in response to SS. Zhu *et al*, observed increases in key transcription factors involved in lipid homeostasis and ABCA1 regulation in human umbilical vein endothelial cells exposed to 2 dynes/cm^2 or 12 dynes/cm^2 SS. In addition, their findings suggest that SS increases mRNA levels of the CYP27A gene responsible for oxysterol production. Additionally, mRNA levels of LXRα, an oxysterol-activated transcriptional regulator of *Abca1* were increased in response to SS[13]. In

contrast, Li *et al.* demonstrated in human brain endothelial cells, SS (40 dynes/cm^2) reduced ABCA1 expression[14]. These discrepancies may be due to cell type differences, or the magnitude of the shear stress applied. Our data suggest increases in SS increase the expression of ABCA1 and that cholesterol transport mechanisms are actively modulated in response to shear stress. We demonstrated that HAoECs exposed to SS exhibit reduced membrane cholesterol levels compared to cells under static conditions. In addition, this decrease in plasma membrane cholesterol was associated with a shift to an endosomal-like localization of free cholesterol. Studies have shown that high-density lipoprotein (HDL) formation can occur by apo-A1 internalization through retro-endocytosis[40]. Indeed, ABCA1 has been shown to reside on the cell surface as well as endosomal compartments[41].

This study provides evidence that ABCA1 mediates cholesterol transport and membrane cholesterol levels in endothelial cells, and that reductions in membrane cholesterol content facilitate ion channel activity that contributes to H_2S signaling, promoting vasodilation. Taken together, these findings provide compelling evidence for a novel role for ABCA1 in vascular endothelial function. Our data also show that ABCA1 is a key mediator of endothelial membrane cholesterol in endothelial cells and that shear stress influences the expression of ABCA1, thereby regulating membrane cholesterol.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

J.R.A., L.V.G.B., N.L.K., and J.S.N. conceived and designed research; J.R.A. performed experiments; J.R.A. analyzed data; J.R.A., L.V.G.B., N.L.K., and J.S.N. interpreted results of experiments; J.R.A. and J.S.N. prepared figures; J.R.A. drafted manuscript; J.R.A., L.V.G.B., N.L.K., and J.S.N. edited and revised manuscript; J.R.A., L.V.G.B., N.L.K., and J.S.N. approved final version of manuscript.

Fig 1.

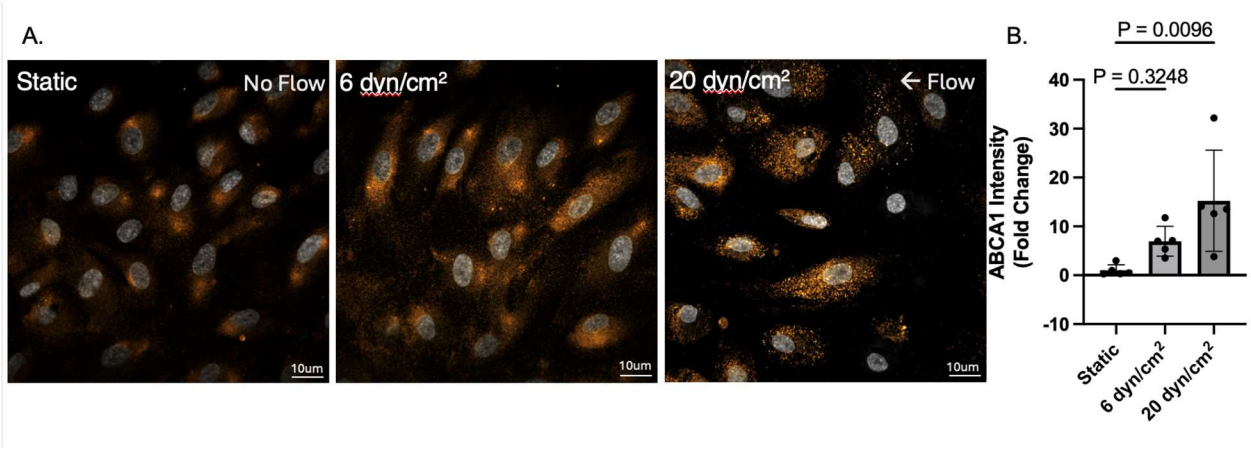
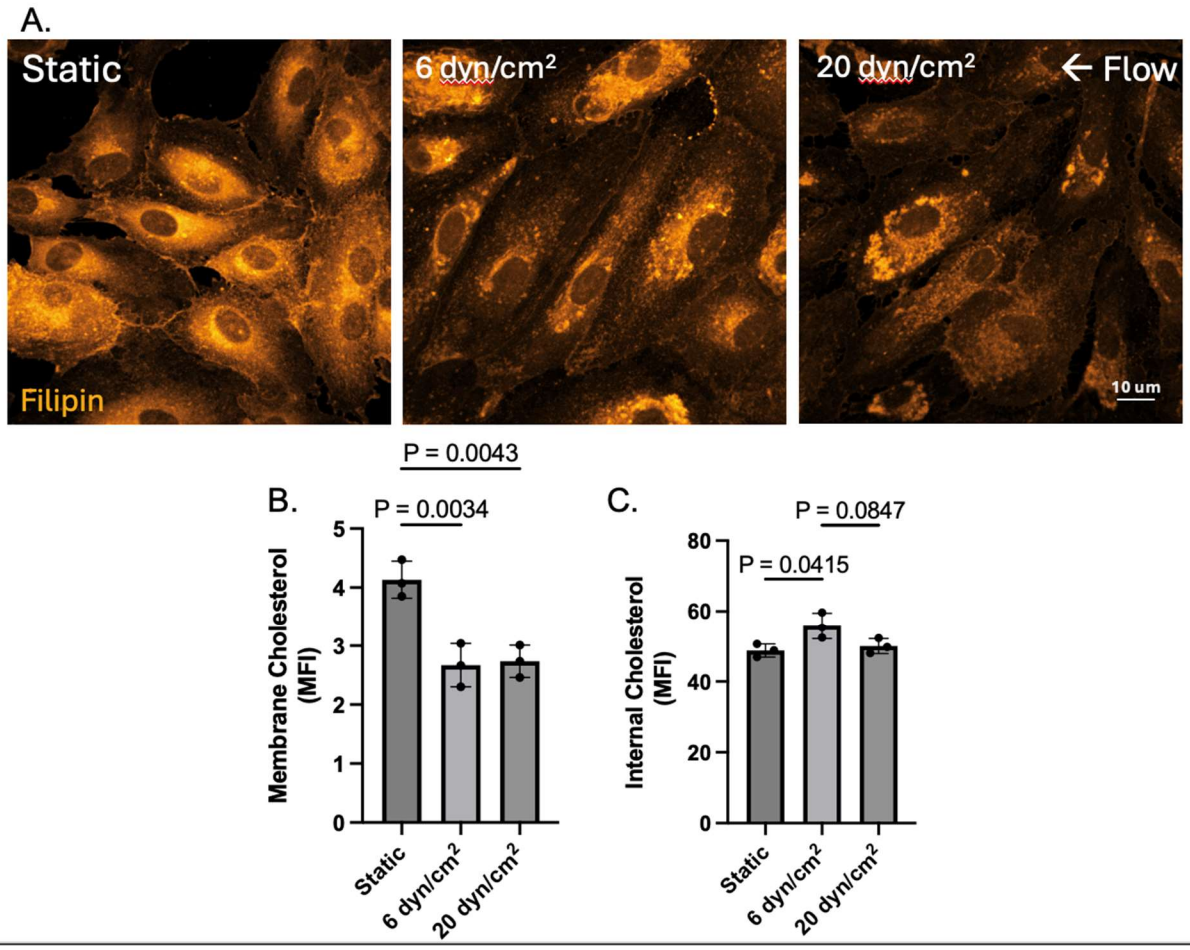

A.
Static
No Flow
6 dyn/cm²
20 dyn/cm²
← Flow
10um
10um
10um
B.
P = 0.0096
P = 0.3248
ABCA1 Intensity
(Fold Change)
40
30
20
10
0
-10
Static
6 dyn/cm²
20 dyn/cm²

Fig 2.


A.
Static
6 dyn/cm²
20 dyn/cm²
← Flow
Filipin
10 um
P = 0.0043
B.
P = 0.0034
Membrane Cholesterol (MFI)
0
1
2
3
4
5
Static
6 dyn/cm²
20 dyn/cm²
C.
P = 0.0847
P = 0.0415
Internal Cholesterol (MFI)
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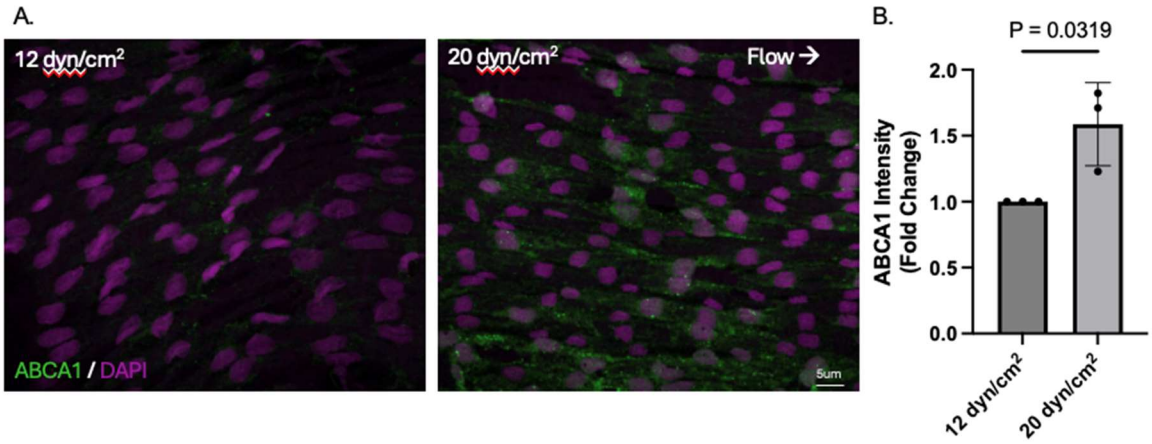
20
40
60
80
Static
6 dyn/cm²
20 dyn/cm²

Fig 3


A.
12 dyn/cm2
ABCA1 / DAPI
20 dyn/cm2
Flow →
5um
B.
P = 0.0319
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(Fold Change)
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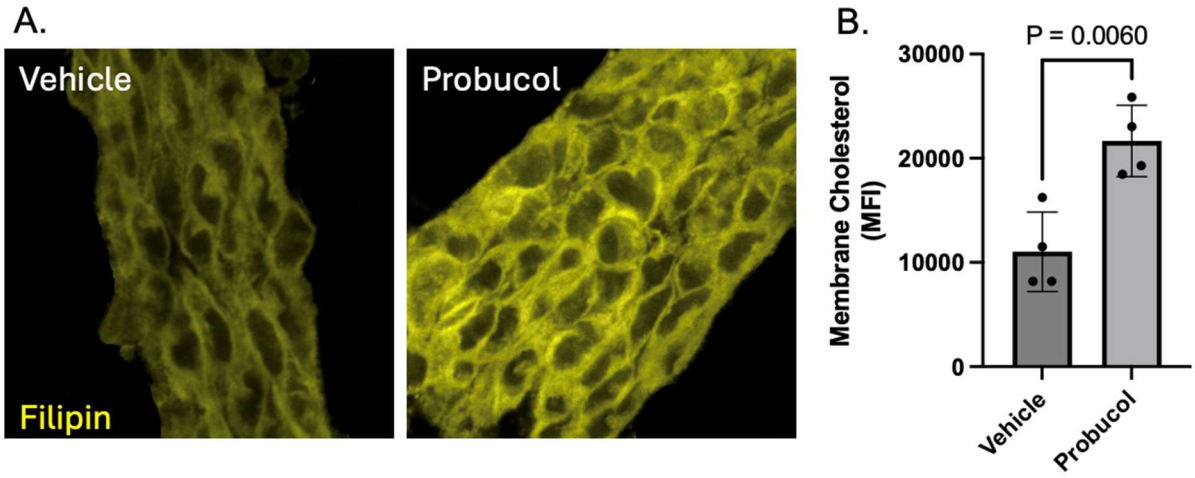
1.5
1.0
0.5
0.0
12 dyn/cm2
20 dyn/cm2

Fig 4.


A.
Vehicle
Filipin
Probucol
B.
P = 0.0060
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20000
10000
0
Vehicle
Probucol

Fig 5.

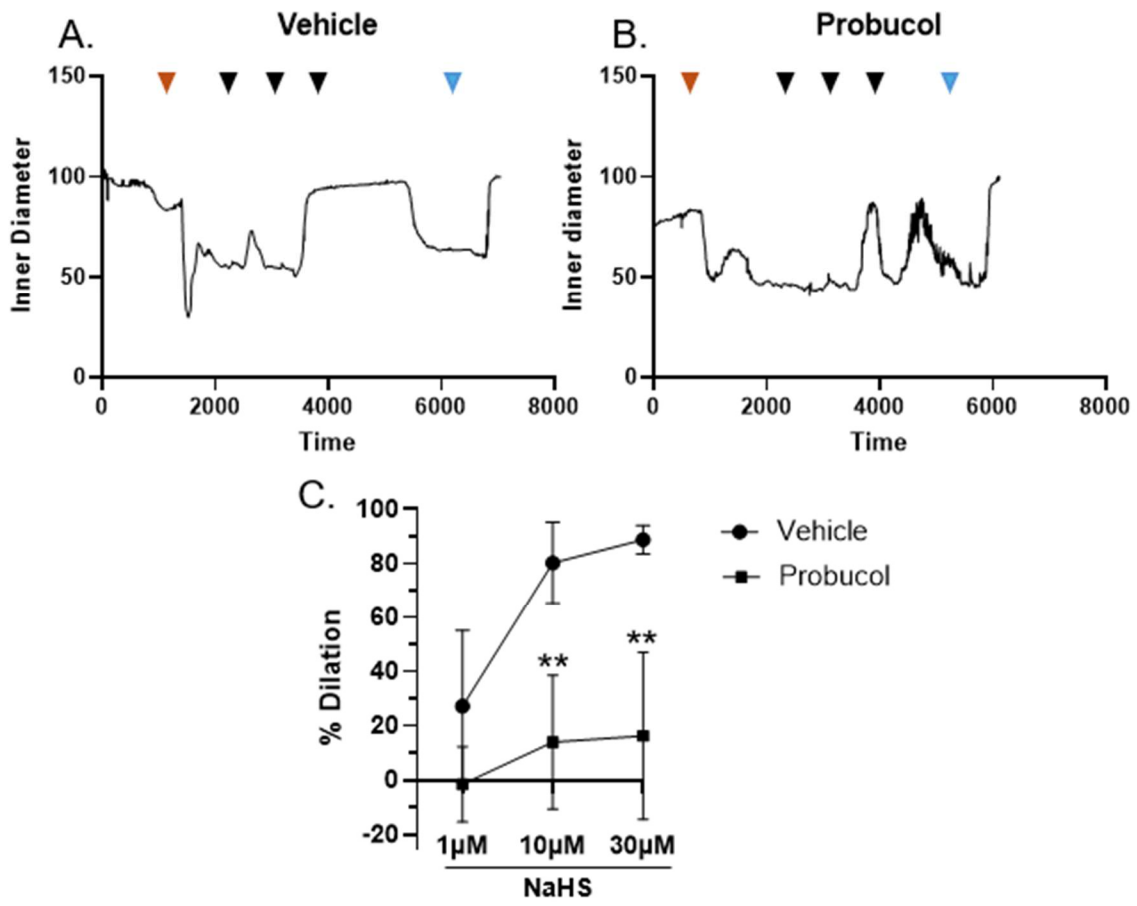
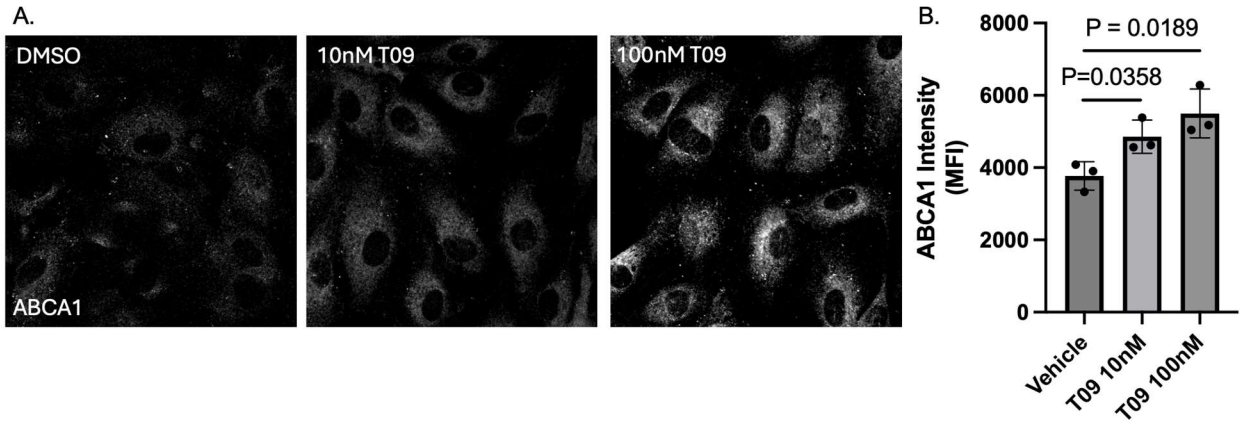

A.
Vehicle
Inner Diameter
150
100
50
0
0
2000
4000
6000
8000
Time
B.
Probucol
Inner diameter
150
100
50
0
0
2000
4000
6000
8000
Time
C.
% Dilation
100
80
60
40
20
0
-20
**
**
1μM
10μM
30μM
NaHS
Vehicle
Probucol

Fig 6.


A.
DMSO
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100nM T09
ABCA1
B.
P = 0.0189
P=0.0358
ABCA1 Intensity (MFI)
8000
6000
4000
2000
0
Vehicle
T09 10nM
T09 100nM

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Figure Legends

Figure 1. Shear stress increases ABCA1 expression. Representative images of ABCA1 expression (yellow) in response to static, 6 dynes/cm^2, or 20 dynes/cm^2 in human aortic endothelial cells (A) Quantification of mean fluorescence intensity for ABCA1 expression (B). 8-10 images were acquired per condition (n=5/group) with 6 – 7 cells per image. One-way ANOVA was used for statistical analysis.

Figure 2. Shear stress decreases membrane cholesterol. Representative images of membrane cholesterol expression in response to static, 6 dynes/cm^2, or 20 dynes/cm^2 in human aortic endothelial cells (A). Quantification of mean fluorescence intensity for filipin III staining (orange) for the plasma membrane (B) and internal cholesterol content (C). 8-10 images per condition (n=3/group) with 6 – 7 cells per image were acquired. One-way ANOVA was used for statistical analysis.

Figure 3. Increasing shear stress increases ABCA1 expression. Representative images of ABCA1 expression in response to increasing shear stress in intact large mesenteric arteries (A). Quantification of mean fluorescence intensity expressed as fold change for ABCA1 expression (B). Five images per artery were acquired per condition (n=3/group). Nuclear stain DAPI (magenta), ABCA1 (green). Student's t-test was used for statistical analysis.

Figure 4. Inhibition of ABCA1 increases membrane cholesterol. Representative images of membrane cholesterol in response to vehicle (A) or probucol (B) in endothelial tubes isolated from small mesenteric arteries (< 150 μm). Quantification of mean fluorescence intensity for filipin III staining (yellow). 4 tubes/artery from 5 animals. Student's t-test was used for statistical analysis.

Figure 5. Inhibition of ABCA1 attenuates H_2S-mediated vasodilation. A. representative trace of vehicle treated artery, The red arrow indicates pre-constrictor U46619, black arrows indicate H_2S dosage, and the blue arrow indicates Ca^{2+} free PSS. B. representative trace of probucol treated artery. C. Summary responses of NaHS-dependent dilation in arteries treated with vehicle (n = 5) or the ABCa1 inhibitor probucol (100 μM, n = 5). Unpaired students t test was used for statistical analysis.

Figure 6. The liver X receptor agonist (T0901317) increases ABCA1 expression in HAoECs. Representative images of ABCA1 expression (cyan) in response to vehicle (DMSO) or T09 (10 μM and 100 μM) (A). Quantification of mean fluorescence intensity for ABCA1 expression (B). VE-cadherin staining was used to outline endothelial cells. 8-10 images per condition (n=3) with 6 – 7 cells per image. One-way ANOVA was used for statistical analysis.