Functional lipidomics of vascular endothelial cells in response to laminar shear stress

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Abstract
Laminar shear stress generated by blood flow stimulates endothelial cells and activates signal transduction, which plays an important role in vascular homeostasis. Several lines of evidence indicate that membrane and intracellular lipids are involved in the signal transduction of biomechanical stresses. In this study, we performed global profiling of cellular lipids from human pulmonary artery endothelial cells (HPAEC) exposed to laminar shear stress. A total of 761 species of lipids were successfully annotated, with 198 of these species significantly changed in response to shear stress for 24 hours. Ether-linked lipids containing an alkyl moiety with a medium chain length (C11–C14) were uniquely upregulated, and the administration of their biosynthetic precursor 1-O-dodecyl-rac-glycerol attenuated phorbol 12-myristate 13-acetate (PMA) induced vascular cell adhesion molecule-1 (VCAM-1) expression. Given the pro-inflammatory and atherogenic roles of VCAM-1, our findings suggest that the induction of a specific group of lipids (ie, ether-linked lipids with medium length alkyl side chain) may confer atheroprotective and anti-inflammatory roles to vascular endothelial cells under flow conditions.

KEYWORDS
endothelial cells, ether lipid, lipidomics, shear stress, VCAM-1
1  |  INTRODUCTION

Endothelial cells that form the inner lining of the blood vessel wall are constantly exposed to hemodynamic forces, including hydrostatic pressure, cyclic stretch, and shear stress. Laminar shear stress is the frictional force generated by blood flow parallel to the blood vessel wall and plays a central role in maintaining normal physiologic vascular function, including thrombo-resistance, barrier function, and vascular homeostasis. In linear areas of the vasculature, steady unidirectional laminar shear stress promotes the production of vasoactive factors, including nitric oxide (NO), and atheroprotective gene expression in endothelial cells. In contrast, disturbed flow (e.g., oscillatory shear) induces several pro-inflammatory and pro-atherogenic responses, including monocyte adhesion in the region of arteries with bifurcations or curvature. In these regions, early plaque formation is prone to develop and become a cause of atherosclerosis.

The plasma membrane, which is mainly composed of a phospholipid bilayer and membrane proteins, serves as a dynamic barrier and is the major target of mechanical force. Previous studies demonstrated that shear stress affects the physical properties of the plasma membranes of endothelial cells; it increases lipid order and fluidity and decreases cholesterol content. These changes in the membrane lipid order and cholesterol content in response to shear stress are linked to downstream signaling pathways. For example, VEGFR phosphorylation is induced by laminar shear stress and attenuated when shear-induced membrane lipid order changes are prevented by cholesterol addition. Caveolae, which are characterized as unique lipid microdomains enriched in cholesterol and sphingolipids, respond to shear stress by rapidly shifting their lipid order from the liquid-ordered to liquid-disordered state. Chronic shear stress induces caveolae formation and activates downstream signaling pathways, such as AKT, nitric oxide synthase 3 (NOS3, also known as eNOS), and ERK.

Furthermore, Czarny et al. reported that vascular flow and pressure in situ rapidly and transiently induce the activity of neutral sphingomyelinase at the luminal endothelial cell surface primarily in caveolae, and ceramides generated by hydrolysis of sphingomyelin activate the MAP kinase pathway. For the intracellular lipids, shear stress activates phospholipase C (PLC), which generates lipid mediators, such as inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DG) from the membrane phospholipids. DG activates the intracellular protein kinase C (PKC) pathway, and IP3 stimulates the release of intracellular calcium. The elevated cytosolic calcium content. These changes in the membrane lipid order and cholesterol content in response to shear stress are linked to downstream signaling pathways. For example, VEGFR phosphorylation is induced by laminar shear stress and attenuated when shear-induced membrane lipid order changes are prevented by cholesterol addition. Caveolae, which are characterized as unique lipid microdomains enriched in cholesterol and sphingolipids, respond to shear stress by rapidly shifting their lipid order from the liquid-ordered to liquid-disordered state. Chronic shear stress induces caveolae formation and activates downstream signaling pathways, such as AKT, nitric oxide synthase 3 (NOS3, also known as eNOS), and ERK.

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2  |  MATERIALS AND METHODS

2.1  |  Cell culture and shear stress exposure

Human pulmonary artery endothelial cells were obtained from Clonetics (Venders, Belgium) and grown in M199 medium supplemented with 15% FBS, 2 mM L-glutamine (Gibco/Thermo Fisher Scientific, Waltham, MA, USA), 50 μg/mL of heparin, and 30 μg/mL of endothelial cell growth factor (Becton Dickinson, Franklin Lakes, NJ, USA) in 1% gelatin (Becton Dickinson, Franklin Lakes, NJ, USA) coated tissue culture flasks. The cells were used in experiments at passage 7 to 10. A parallel plate-type apparatus was used to apply laminar shear stress to the cells, as previously described. Briefly, cells were cultured on glass chambers coated with 1% of gelatin. The surfaces were held 200 μm apart with a Teflon gasket. The medium was perfused with a roller/tube pump at 15 dynes/cm², which is a physiological arterial level of shear stress, and the entire closed circuit was maintained at 37°C. The intensity of the shear stress (τ, dynes/cm²) acting on the endothelial cell layer was calculated using the formula $\tau = 6 \mu Q/a^2$, where $\mu$ is the viscosity of the perfusate (poise), $Q$ is the flow volume (mL/s), and $a$ and $b$ are the cross-sectional dimensions of the flow path (cm). HPAEC were subjected to shear stress for 6 or 24 hours for lipidomics and transcriptome analysis. To stimulate inflammation, phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA), a PKC activator, was added to the medium during shear stress exposure to a final concentration of 10 nM.

A cone-plate-type apparatus was used to apply disturbed shear stress to cells, as previously described by Garcia-Cardena et al. Briefly, the apparatus consists of a stainless steel cone driven by an electric motor and a stage that holds a 10-cm diameter culture dish with a glass plate inserted at the bottom of the dish. Rotation of the cone forces the fluid between the cone and glass plate to flow concentrically, exposing cells attached to the 1% gelatin-coated glass plate to fluid shear stress. The intensity of shear stress acting on the endothelial cells was calculated by the formula $\tau = \mu \omega / \alpha$, where $\mu$ is the viscosity of the perfusate (poise), $\omega$ is the angular velocity, and $\alpha$ is the radius of the cone.
where $\omega$ is the angular velocity of the cone and $\alpha$ is the cone angle in radians. The fluid shear stress was constant over the entire plate surface. The modified Reynolds number ($R$) was used to determine the appropriate experimental conditions to induce disturbed flow and was calculated using the formula $R \equiv r^2 \omega \alpha^2 / 12 \nu$, where $r$ is the radial distance from the apex of the cone and $\nu$ is the kinematic viscosity of the fluid.$^{23}$ With the use of this parameter, the flow was predicted to be turbulent at $R > 4$. In the present experiments, we used a $5^\circ$ cone and a rotational velocity of 120 rpm. Since $R$ is proportional to the radial dimension, turbulent flow was established at radii $\geq 2.4$ cm, which corresponded to an $R > 5$ and represented an average shear stress intensity of 1.5 dynes/cm$^2$.

Cells for the disturbed flow experiments were harvested from the outer portion of the glass plate ($\geq 2.4$ cm).

### 2.2 RNA sequencing (RNA-seq) analysis

Total RNA from HPAEC was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. Polyadenylated mRNA was selected using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, USA). Subsequently, the library was synthesized with the NEBNext Ultra RNA Library Prep Kit (New England Biolabs) using the NEB index kit, and the pooled libraries were sequenced on the Illumina NextSeq 500 or NextSeq 550 instrument using the 75-cycles High Output v2 Kit (Illumina, San Diego, CA, USA). The reads were aligned with STAR software onto the transcript reference based on hg19 (human reference).$^{24}$ The read counts for each gene were estimated using RSEM software (ver. 1.2.23).$^{25}$ The transcripts per million (TPM) for each gene were calculated as a gene expression level based on the normalized read counts between samples.$^{26}$ Differentially expressed genes (DEGs) were calculated using the EBseq package (R Bioconductor).$^{27}$ A threshold of fold-change (FC) $\geq 2$ or $\leq 0.5$ and a posterior probability of being equally expressed (PPEE) $< 0.05$ were used to identify DEGs between two groups. All raw and processed RNA-seq data have been deposited with the Genomic Expression Archive (GEA) under accession number E-GEAD-372.

### 2.3 Gene set enrichment analysis (GSEA)

To identify significantly enriched gene sets that were defined based on prior biological knowledge, GSEA (ver. 4.0.3) was performed using the RNA-seq TPM data.$^{28}$ The Molecular Signatures Database (MsigDB ver. 7.0) is a collection of annotated gene sets. These gene sets are divided into eight major collections. In this study, we used hallmark gene sets representing 50 well-defined biological states or processes, and C5 gene sets consisting of genes annotated by the same GO terms. A false discovery rate (FDR) of $q$-value $< 0.25$ is defined as significantly enriched.

### 2.4 Extraction of lipids

Lipids were extracted as a single-phase method with mixed solvent (chloroform/methanol/water, 1:2:0.2, v/v/v) as previously described.$^{29}$ Briefly, about 1 to 2 $\times 10^6$ HPAEC were suspended in 200 $\mu$L of methanol and incubated overnight at $\sim 20^\circ$C. Subsequently, chloroform (100 $\mu$L) was added to the samples, which were vortexed for 10 seconds. After a 60-min incubation at room temperature, Milli-Q water (20 $\mu$L) was added to the samples, which were vortexed and then incubated for 10 minutes. The samples were centrifuged at 2,000 $\times g$ for 10 minutes at $20^\circ$C, and a 200-$\mu$L aliquot of the supernatant was transferred to liquid chromatography-mass spectrometry (LC/MS) vials. The phosphorus content of the extracted lipids was quantified by the method of Bartlette using 20 $\mu$L of the supernatant.$^{30}$ The extracted lipids were gently dried with $N_2$ and reconstituted in MeOH/CHCl$_3$ (2:1 v/v) to a final concentration of 1 mM phosphorus.

### 2.5 Untargeted lipidomics

The lipid profile of the HPAEC was analyzed using LC-MS/MS, as described previously.$^{31}$ Briefly, a quadrupole/TOF MS (TripleTOF 6600; Sciex, Framingham, MA, USA) coupled with an ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) was used. LC separation was performed using a reverse-phase column (ACQUITY UPLC BEH peptide C18 [50 $\times$ 1.7 mm inner diameter, 2.1 $\mu$m particle size; Waters]) with a gradient elution of mobile phase A (methanol/acetonitrile/water [1:1:3, v/v/v] containing 5 mM ammonium acetate [FUJIFILM Wako Pure Chemical Industries, Osaka, Japan] and 10 nM EDTA [Dojindo, Kumamoto, Japan]) and mobile phase B (100% isopropanol containing 5 mM ammonium acetate and 10 nM EDTA). The flow rate was 300 $\mu$L/min at 45°C. MS was performed with an AB Sciex TripleTOF 6600 system. The injection volumes were 2 $\mu$L and 1 $\mu$L for the negative and positive ion modes, respectively. The data-dependent MS/MS acquisition mode was applied as previously described.$^{29}$ The parameters were set as follows: MS1 and MS2 mass ranges, $m/z$ 70-1250; MS1 accumulation time, 250 ms; MS2 accumulation time, 100 ms; collision energy, $+40/-42$ eV; collision energy spread, 15 eV; cycle time, 1300 ms; temperature, 250°C(+)/300°C(−); ion spray voltage floating, $+5.5/-4.5$ kV; declustering potential, 80 V. The other data-dependent acquisition (DDA)
parameters were: the dependent product ion scan number, 16; intensity threshold, 100 cps; exclusion time of the precursor ion, 0 seconds. The mass calibration was automatically performed using an APCI positive/negative calibration solution via a calibration delivery system (CDS).

### 2.6 Lipidomics data processing

Data processing was performed using MS-DIAL (version 3.70), as previously described. Briefly, the raw MS files (WIFF format file) were converted to ABF (analysis base file format) using the freely available Reifys file converter (http://www.reifys.com/AbfConverter/) and imported into MS-DIAL. The MS/MS spectra of the identified lipids were confirmed and manually corrected, as necessary. In the negative ion mode, ceramide (Cer), fatty acid (FA), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI), lysophosphatidylserine (LPS), phosphatidylcholine (PC), ether-phosphatidylethanolamine (ePC), phosphatidyl ethanolamine (PE), ether-phosphatidylethanolamine (ePE), phosphatidyl-inositol (PI), ether-phosphatidylinositol (ePI), phosphatidylserine (PS), ether-phosphatidylserine (ePS), and sphingomyelin (SM) with anionic species (e.g., −H−, +CH₃COO−) were quantified. Because of the limited data in the negative ion mode, specific ions were selected for analysis. In the positive ion mode, acylcarnitine (CAR), fatty acid (FA), glycerophosphocholine (GPC), lysophosphatidylcholine (LPC), lysophosphatidyl ethanolamine (LPE), lysophosphatidylinositol (LPI), lysophosphatidylserine (LPS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), ether phosphatidylethanolamine (ePE), phosphatidyl inositol (PI), ether phosphatidylinositol (ePI), phosphatidylserine (PS), ether phosphatidylserine (ePS), and sphingomyelin (SM) with cationic species (e.g., +H⁺, +NH₄⁺) were quantified. Because TG and eTG species with equivalent total chain lengths are co-eluted, the best possible structure predicted from the MS/MS spectrum is shown at the molecular level. For example, TG 52:3 contained a mixture of TG 16:0_18:1_18:2 and TG 16:1_18:1_18:1.

### 2.7 Alkylglycerol supplementation and PMA treatment

Human pulmonary artery endothelial cells were maintained in HuMedia-EB2 medium supplemented with 2% FBS, 10 ng/mL of hEGF, 1.34 μg/mL of hydrocortisone, 5 ng/mL of hFGF, 10 μg/mL of heparin, 50 μg/mL of gentamicin, and 50 ng/mL of amphotericin, according to the supplier’s instructions (Kurabo, Osaka, Japan). HPAEC were grown on collagen-I coated dishes and incubated with 1-O-dodecyl-rac-glycerol (12AG, Santa Cruz, Dallas, TX, USA), 1-O-hexadecyl-rac-glycerol (16AG, Santa Cruz), 1-O-octadecyl-rac-glycerol (18AG, Sigma-Aldrich), or 1-lauroyl-rac-glycerol (12MG, Tokyo Chemical Industry, Tokyo, Japan), followed by treatment with 10 nM PMA for 4 hours.

### 2.8 Western blot analysis

Human pulmonary artery endothelial cells were quickly rinsed with ice-cold PBS then lysed with lysis buffer (75 mM NaCl, 3% SDS, Complete Mini EDTA-free protease inhibitors [Roche, Basel, Switzerland], and Halt phosphatase inhibitor cocktail [Thermo Scientific, Waltham, MA, USA] in 50 mM HEPES, pH 8.5). Lysed samples were boiled for 5 min. The cell extracts were sonicated for 30 seconds using the Handy Sonic UR-20P (TOMY, Tokyo, Japan) and centrifuged at 12,000 × g for 10 minutes. The supernatants were mixed with 2× Laemmli Sample Buffer (BIO-RAD, Hercules, CA, USA) and boiled at 95°C. Protein concentrations were determined by the Micro BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein were separated on 4-20% Mini-PROTEAN TGX gels (BIO-RAD) and transferred onto PVDF membranes using the Trans-Blot Turbo Transfer System (BIO-RAD). The membranes were blocked with StartingBlock T20 (TBS) Blocking Buffer (Thermo Scientific) for 30 minutes, and then incubated at room temperature for 1 hours with VCAM-1 (E1E8X, Cell Signaling Technology, Danvers, MA, USA) or β-Actin (ACTB) (13E5, Cell Signaling Technology) rabbit monoclonal antibody in Can Get Signal Solution1 (TOYOBO, Osaka, Japan). After washing with TBST buffer (10 mM Tris base pH 8.0, 150 mM NaCl, 0.2% Tween-20), the membranes were incubated for 1 hours at room temperature with donkey anti-rabbit HRP-linked IgG whole Ab (1:10,000, GE Healthcare, Chicago, IL, USA) in Can Get Signal Solution2. Signals were enhanced by Immobilon Forte Western HRP substrate (Merck Millipore, Burlington, MA, USA) and detected using the ImageQuant LAS 4000 (GE Healthcare) in chemiluminescence mode.

### 2.9 Quantitative polymerase chain reaction (qPCR)

For the generation of cDNA, 50 ng total RNA was reverse-transcribed using SuperScript VILO Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in a final volume of 20 μL. The TaqMan probes (Thermo Fisher Scientific) for VCAM-1 (Hs01003372_m1), which was labeled with FAM, and ACTB (Hs01060665_g1), which was labeled with VIC, were used for duplex qPCR. The reaction mixture contained 2 μL of cDNA, 0.5 μL of each TaqMan probe, 5 μL of TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific), and 2 μL of nuclease-free water. Each real-time PCR reaction was performed in four qPCR technical replicates using the
QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific), following the manufacturer’s instructions. The relative VCAM-1 gene expression values were normalized to ACTB levels using the following formula: $2^{(ACTB \text{ Ct} - \text{VCAM-1 Ct})}$. Statistical significance and standard error were determined from the relative VCAM-1 gene expression values.

### 2.10 Statistics

Lipid abundance for each sample was calculated based on the peak height. Statistical analysis of normalized quantile data was performed using the student’s $t$ test with post hoc correction according to the method of Benjamini and Hochberg (adjusted $P$-value). Heatmaps, PCA, and volcano plots were created using the R programming language. The statistical significance between groups for the qPCR gene expression analysis was determined using the Welch’s $t$ test. $P < .05$ indicated statistical significance. The results are presented as the mean ± standard deviation (SD). Microsoft Excel 2013 and R software (version 3.5.2) were used for the statistical analysis.

### 3 RESULTS

#### 3.1 Shear stress induces anti-inflammatory effects on HPAEC

In this study, we used a system in which HPAEC in separated chambers were cultured on the gelatin-coated glass plate under stress generated by a continuous laminar flow (Figure 1A). This system allowed more than $1 \times 10^6$ HPAEC to be cultured under laminar shear stress, which is a high enough number of cells for transcriptome analysis.

![FIGURE 1 Shear stress system and anti-inflammatory effect of shear stress. A, Schematic model of the shear stress system. B and C, GSEA analysis using Hallmark gene sets identified the inflammatory response gene set as an enriched pathway in cells treated with PMA for 4 hours compared to the control cells (static) (B) and cells treated with PMA for the last 4 hours of the 24-h exposure to shear stress (C). These results indicated the anti-inflammatory effect of shear stress. NES represents the normalized enrichment score that reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list, normalized for differences in gene set size, and correlated with the expression dataset. The upregulated pathways were defined by an NES > 0; downregulated pathways were defined by an NES < 0. FDR represents the false discovery rate that estimates the probability that a gene set with a given NES represents a false positive finding.](image-url)
and untargeted lipidomics. HPAEC under static conditions were also cultured on the same gelatin-coated glass plate, since extracellular matrix plays pivotal roles on the phenotype of endothelial cells in vitro. Gene expression analysis revealed that 790 and 2,144 transcripts were significantly changed 6 or 24 hours after exposure of HPAEC to shear stress, respectively (GEA accession number E-GEAD-372, experiment 1). As expected, GSEA analysis using the C5 collection showed that the “GO RESPONSE TO LAMINAR FLUID SHEAR STRESS” gene set was upregulated significantly in both the 6-h (FDR q-value = 0.046) and 24-h (FDR q-value = 0.17) shear stress groups compared to the static condition group. This gene set included flow-sensitive transcription factors, such as Kruppel-like factor 2 (KLF2), KLF4, and nuclear factor erythroid 2-like 2 (NFE2L2, also known as NRF2), which were induced by shear stress. A series of vasoprotective, antioxidant (e.g., heme oxygenase 1 [HMOX1, also known as HO-1], NAD(P)H quinone dehydrogenase 1 [NQO1], glutamate-cysteine ligase modifier subunit [GCLM], and ferritin heavy chain 1 [FTH1]), antithrombotic (e.g., eNOS and thrombomodulin [THBD]), and vasodilatory (e.g., argininosuccinate synthase 1 [ASS1], natriuretic peptide C [NPPC], and COX-2) genes were significantly upregulated in response to laminar shear stress (Table S1A).

To investigate the anti-inflammatory effects of shear stress on vascular endothelial cells, HPAEC were stimulated by PMA with or without laminar shear stress (E-GEAD-372, experiment 2). GSEA using the hallmark gene sets demonstrated that the “HALLMARK INFLAMMATORY RESPONSE” gene set was upregulated by PMA treatment, and PMA-induced inflammatory gene expression was significantly attenuated by shear stress (Figure 1B). For example, expression of cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1),

FIGURE 2 Shear stress altered lipid profiles significantly. A and B, Shear stress treatment for 6 h versus static condition in negative ion mode (A) and positive ion mode (B). C and D, Shear stress treatment for 24 hours versus static condition in negative ion mode (C) and positive ion mode (D). The x-axis of the volcano plot indicates the log-transformed fold-change (threshold of 2 or 0.5), and the y-axis signifies the log-transformed adjusted P-value (threshold of .05). Some Cer, FA, ePC, ePE, TG, and eTG species were increased predominantly, whereas some TG and eTG species were decreased by exposure to shear stress for 24 hours.
and E-selectin (SELE), were dramatically upregulated (> 100-fold) by PMA. However, their upregulation was suppressed under shear stress conditions (Table S1B). These results demonstrated that anti-inflammatory and atheroprotective changes in gene expression patterns occurred when HPAEC were exposed to laminar shear stress.

3.2 Untargeted lipidomics revealed lipid profiles of HPAEC under laminar shear stress

To obtain the comprehensive lipid profiles, we applied LC-QTOF-MS-based untargeted lipidomics to HPAEC exposed to laminar shear stress for 6 or 24 hours. As shown in Table S2, a total of 761 species of lipids were successfully annotated. The volcano plots showed that 110 lipids (34 in the negative ion mode; 76 in the positive ion mode) were altered significantly (adjusted P-value < .05; FC ≥ 2 or ≤ 0.5) by the shear stress administered for 6 h, while 198 lipids (59 in the negative ion mode; 139 in the positive ion mode) changed after 24 hours of treatment (Figure 2). Ether-containing lipids (e.g., ePC, ePE, eDG, and eTG) were among the significantly changed lipids. Of interest, the ether-containing lipids with a medium length alkyl side chain, such as the C12 alkyl moiety, were significantly upregulated by shear stress, while eTG with a longer alkyl chain length (≥C18) were downregulated (Figures 3 and 4). The abundance of ether-containing lipids with medium length alkyl chain was low under static conditions but dramatically increased by shear stress. Time-course differences were observed between the different species of ether lipids. In particular, ePC and ePE gradually increased up to 24 hours, whereas eDG and eTG were rapidly increased at 6 hours (Figure 5). When compared with disturbed flow

**Figure 3** Time-course profiles of altered ether lipids. The normalized intensities of the significantly altered ether lipids (ePC, ePE, eDG, and eTG) were used for heatmap analysis. The ether lipid species were ordered by the alkyl chain length. The ether lipid species with a medium length alkyl chain moiety were increased by laminar shear stress, whereas eTG species with a longer alkyl chain moiety were decreased.
condition in addition to static condition, ether-containing lipids with a medium length alkyl side chain were significantly increased only under laminar flow condition (Figure S1).

3.3 | Supplementation of alkylglycerols induces ether-containing lipids in endothelial cells

Ether-containing lipids are one of the major components of the cell membrane and play physiological roles. To investigate the effect of ether-containing lipids with different alkyl chain lengths, a series of alkylglycerols was added to the HPAEC as biosynthetic precursors (Figure 6A). To facilitate the biosynthesis of C12, C16, and C18 alkyl moiety containing lipids in endothelial cells, HPAEC were incubated with 12AG, 16AG, or 18AG for 72 hours. Untargeted lipidomics successfully annotated 911, 835, and 826 lipid species, respectively (Table S3-S5). Of these lipid species, 314, 281, and 233 lipids, respectively, were significantly changed (adjusted P-value < .05; FC ≥ 2 or ≤ 0.5) in response to the supplementation with 12AG, 16AG, and 18AG. With the 12AG supplementation, medium length (C11-14) alkyl chain containing lipids (ie, ePC, ePE, eDG, and eTG) were significantly increased in HPAEC (Figure 6B-E).

3.4 | Ether-containing lipids reduce VCAM-1 expression induced by PMA

To investigate the effects of ether-containing lipids on endothelial cell function, we conducted transcriptome analysis on HPAEC supplemented with 12AG. When compared to the shear stress conditions, 159 DEGs (23 upregulated and 136 downregulated) overlapped between 12AG + PMA versus PMA (E-GEAD-372, experiment 2) and shear + PMA versus PMA (E-GEAD-372, experiment 3). Among these DEGs, VCAM-1, a vascular inflammatory gene, was downregulated (Figure S2A). Immunoblot analysis confirmed that both shear stress and 12AG-treatment attenuated the VCAM-1
induction in response to PMA (Figure S2B-D). This effect was dose- and time-dependent. Of interest, VCAM-1 inhibition was not observed in HPAEC treated with 16AG, 18AG, or 12MG, suggesting a 12AG structure-specific activity in the attenuation of the inflammatory response in HPAEC (Figure 7).

4 | DISCUSSION

VCAM-1 serves as a receptor for very late antigen (VLA)-4, which is involved in monocyte recruitment. Its expression is upregulated by cytokines (eg, TNF-α) and other inflammatory mediators in early atherosclerotic lesions. Previous reports demonstrated a variety of mechanisms that regulate VCAM-1 expression in vascular endothelial cells by exposure to shear stress. Here, we demonstrated for the first time that lipid metabolism in endothelial cells plays an important role in regulating VCAM-1 expression, suggesting a novel mechanism by which laminar shear stress could confer anti-inflammatory and atheroprotective roles by modulating intracellular lipid metabolism in vascular endothelial cells.

Ether-containing lipids are characterized by an ether linkage of an alkyl chain moiety at the sn-1 position of the glycerol backbone. We found that four classes of ether lipids (ePC, ePE, eDG, and eTG) with a medium length alkyl chain were increased by laminar shear stress, but not by disturbed shear stress. Ether-phospholipids (ePC and ePE) are the major component of cell membranes, including alkyl-acyl-phospholipids and alkenyl-acyl-phospholipids. Platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; PAF) is an alkyl-acyl-phospholipid with a 1-O-alkyl ether linkage, whereas plasmalogen is an alkenyl-acyl-phospholipid with a vinyl-ether linkage. Shear stress increased 1-O-alkyl-2-acyl-phosphatidylcholine with a longer acyl chain at the sn-2 position (C14–C22), but PAF was not detected. Plasmalogens are the most abundant subclass of the ether phospholipids. They are not only structural components of cell membranes but also have roles in facilitating membrane fusion, ion transport, cholesterol efflux, decreasing membrane fluidity, and protecting membrane lipids from oxidation at its vinyl-ether moiety. The major ePC species increased by shear stress were alkyl-acyl-phosphatidylcholine, whereas most of the ePE species were alkenyl-acyl-phosphatidylethanolamine (Figure 3). There are few studies describing the function of neutral ether lipids, such as eDG and eTG. Higher levels of eDGs are found in the cerebral cortex, fat tissue, and liver of aged mice compared to the equivalent tissues in young mice. In vascular smooth muscle cells, eDG reduces...
cell growth by inhibiting platelet-derived growth factor (PDGF)-stimulated ERK activation in a PKCe-dependent manner. A previous study showed that up to 20% of neutral lipids in lipid droplets are eTG, suggesting that cellular droplets play a central role in ether lipid metabolism and intracellular lipid traffic. Furthermore, eTG is abnormally accumulated in Wolman’s disease, which is defined by a deficiency in lysosomal acid lipase (LIPA). Alkylglycerol is abundant in shark liver oil. Its beneficial effects include lowering radiotherapy-induced injury,
reducing tumor growth, and improving vaccination efficacy and are part of traditional medicine in Norway and Sweden.\textsuperscript{57} Alkylglycerol is also present in mammalian tissues, such as bone marrow and the spleen.\textsuperscript{58} Because alkylglycerol enters the plasmalogen biosynthetic pathway downstream of the peroxisomal step, it is expected to show pharmacological effects on peroxisomal disorders that have impaired plasmalogen biosynthesis.\textsuperscript{57,59} Indeed, the administration of alkylglycerol can restore plasmalogen levels in vitro and in vivo.\textsuperscript{60,61} Furthermore, alkylglycerol modulates endothelial permeability by altering lipid signaling.\textsuperscript{62} In this study, we investigated, for the first time, the global lipid profiles of HPAEC upon treatment with alkylglycerols (12AG, 16AG, or 18AG). As expected, the alkyl chain of the ether-containing lipids induced in HPAEC was correlated with that of the alkylglycerols added to the cells as metabolic precursors.

Of particular interest, a previous study demonstrated that 12AG inhibited PMA-induced arachidonic acid release more potently than other alkylglycerols, which is consistent with our observation that 12AG attenuated PMA-induced VCAM-1 expression.\textsuperscript{63} PMA binds to PKC and enhances its translocation from the cytosol to the plasma membrane, where PKC phosphorylates and activates a number of substrate proteins.\textsuperscript{64-67} In Madin-Darby canine kidney cells, 12AG promoted PKC displacement from the membrane fraction and inhibited PKC translocation after PMA treatment.\textsuperscript{68} Suppression of VCAM-1 by 12AG was time- and concentration-dependent, suggesting active metabolism of 12AG may play a role in the inhibition of PMA-induced VCAM-1 expression. Laminar shear stress may elicit anti-inflammatory and atheroprotective effects in vascular endothelial cells by controlling the endogenous metabolism of ether-containing lipids with a medium length alkyl chain. Furthermore, 12AG elicits antibacterial and antifungal activities with greater potency than other alkylglycerols.\textsuperscript{69,70} Future in vivo studies may clarify the role of 12AG in maintaining vascular homeostasis and its therapeutic potential.

In summary, untargeted lipidomics revealed for the first time that laminar shear stress could induce a series of ether-containing lipids with a medium length alkyl moiety in vascular endothelial cells. Our findings provided new insights into the causal relationship between endothelial lipid metabolism and vascular homeostasis and demonstrated the potential benefit of 12AG supplementation for reducing cardiovascular risk.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

M. Arita designed the research. T. Hirata performed the experiments. Both M. Arita and T. Hirata wrote the paper. K. Yamamoto supervised the shear stress experiments. K. Ikeda supported the lipidomics experiments. All authors contributed to the analysis of data.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.