11,12-Epoxyecosatrienoic Acids Mitigate Endothelial Dysfunction Associated with Estrogen Loss and Aging: Role of Membrane Depolarization

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Abstract

Endothelial dysfunction, including upregulation of inflammatory adhesion molecules and impaired vasodilatation, is a key element in cardiovascular disease. Aging and estrogen withdrawal in women are associated with endothelial inflammation, vascular stiffness and increased cardiovascular disease. Epoxyecosatrienoic acids (EETs), the products of arachidonic acid metabolism mediated by cytochrome P450 (CYP) 2J, 2C and other isoforms, are regulated by soluble epoxide hydrolase (sEH)-catalyzed conversion into less active diols. We hypothesized that 11,12-EETs would reduce the endothelial dysfunction associated with aging and estrogen loss. When stabilized by an sEH inhibitor, 11,12-EET at a physiologically low dose (0.1nM) inhibited cytokine-induced adhesion molecule upregulation on human aorta endothelial cells (HAEC) and monocyte adhesion under shear flow through marked depolarization of the HAEC when combined with TNFα. Mechanistically, neither 11,12-EETs nor 17β-estradiol (E2) at physiologic concentrations prevented activation of NFκB by TNFα. E2 at a physiological concentrations reduced sEH expression in HAEC, but did not alter CYP expression, and when combined with TNFα depolarized the cell. We also used an *in vivo* model of vascular dysfunction in adult and aged ovariectomized Norway brown rats (with and without E2 replacement) to further test the hypothesis. sEHi and 11,12-EET attenuated phenylephrine induced constriction and increased endothelial-dependent dilation of aortic rings from ovariectomized rats. There was no additional benefit when 11,12-EETs were combined with E2. Conclusions: Increasing 11,12-EETs through sEH inhibition may represent an effective strategy to preserve endothelial function and prevent atherosclerotic heart disease in postmenopausal women.

Introduction

Estrogen has many anti-inflammatory properties, which contribute to the delayed onset of cardiovascular disease in women compared to men.[1,2] Loss of estrogen leads to increased expression of inflammatory genes and proteins.[3] Similarly, epoxyecosatrienoic acids (EETs) are anti-inflammatory. Previously we identified no change in EETs levels with aging and loss of estrogen, although gender differences have been reported in other models.[4] We hypothesized that increasing EETs through inhibition of soluble epoxide hydrolase (sEH) might be an alternative approach to the use of estrogen to improve vascular function and reduce the risk of coronary disease in post-menopausal women. Increased EETs could decrease inflammation and reduce vascular disease associated with aging. 11,12-EETs have been shown to improve vascular dysfunction, but other studies have shown no effect.[5-7] To investigate this, we examined the effect of increased 11,12-EETs, which based on the literature have the most effect on the vasculature of the four EETs, and sEH inhibition on inflammatory monocyte adhesion to an endothelial layer, an early step in atherosclerosis, and on vascular function, in aged and adult rat aortas from ovariectomized (ovx) rats with and without estrogen replacement.

EETs are the products of arachidonic acid metabolism via the cytochrome P450 (CYP) pathway. The importance of arachidonic acid metabolism through the COX (cycloxygenase) and LOX (lipoxygenase) pathways, which produce prostaglandins and leukotrienes, among other mediators, is well-established in the cardiovascular system; however, the role of the CYP pathway has yet to be fully characterized.[8,9] CYP2J and CYP2C are the Cyp isoforms expressed in vascular endothelial cells, contributing to the biosynthesis of the 4 isomers, 5,6-,

8,9-, 11,12-, and 14,15-EET, which to varying degrees inhibit inflammation and promote vascular relaxation and angiogenesis.[10,11] In the heart EETs reduce ischemic injury.[12-14] EETs are primarily regulated by rapid conversion into their less active diols by sEH. It has been unclear whether DHETs (dihydroxyeicosatrienoic acids) are just less potent/inactive compared to their respective EETs, or whether DHETs actually have deleterious properties. Inhibition of sEH raises EET levels, decreases levels of the corresponding DHETs, and amplifies their biologic effects to attenuate inflammation and preserve blood pressure.[15,16] In humans, polymorphisms of genes encoding both sEH and CYPs are associated with elevated risk for cardiovascular disease.[17,18] In animal models of cardiovascular diseases pharmaceutical inhibition or genetic depletion of sEH antagonized the formation of abdominal aortic aneurysm, atherosclerotic plaque and pathological neointima.[19,20]

Endothelial dysfunction plays an important role in the pathogenesis of cardiovascular diseases. It represents a complex pathophysiological entity including upregulation of inflammatory adhesion molecules such as E-selectin, ICAM-1 and VCAM-1, impaired vasodilatation, and perturbation of the anti-coagulatory properties of the endothelial cell.[21] Importantly, studies have shown that preserving endothelial function is linked to improved clinical outcome in patients with coronary artery disease.[22, 23]

We hypothesized that 11,12-EETs would have an anti-inflammatory effect and mitigate vascular dysfunction and the endothelial inflammation associated with aging and estrogen loss. We tested this with comprehensive studies of vascular function by using isolated aortic rings from aging and ovx rats and an *ex vivo* model of TNFα-induced endothelial inflammation.

Methods

1.1 Animal Protocol

Norway Brown rats were purchased from the National Institute of Aging and housed under standard conditions. Adult (5 months) and aged (22 months) female rats were ovariectomized and half immediately replaced with 0.36 mg 17β -estradiol (E2) 90 day slow-release pellets (Innovative Research of America, Sarasota, FL) subcutaneously, as previously described.[3] The animal protocol was approved by the University of California, Davis, Animal Research Committee (17411) in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. 9 weeks after ovariectomy, the rats were euthanized with ketamine and xylazine. Plasma and tissues were collected. The descending thoracic aorta was excised, adhering tissue removed, and cut into 3-4 mm aortic rings. The rings were treated with vehicle control, the sEHi TUPS [1-(1-methylsulfonyl piperidin-4-yl) -3-4-(4-trifluoromethoxy-phenyl)-urea (also known as 1709)][24] alone or in combination with 11,12-EET (Caymen Chemical, Ann Arbor, Michigan) for 4 hr at 37˚ in a CO_2 incubator before studies of vascular function.

1.2 Isometric tension studies

Isometric tension was measured in descending thoracic aortic rings using a standard Radnoti apparatus (Radnoti Glass Technology, Inc., Monrovia, CA) as previously described.[25,

26] A passive load of 2.0 g was applied, and the aortic segments were equilibrated for 1 h with frequent readjustment of tension until reaching a stable baseline. Two KCl (70 mM)-induced contractions were performed to train the vessels to constrict. Rings were washed and equilibrated for 40 min. The maximum contractile response to L-phenylephrine (PE, Sigma, 1 nM to 1 μM) was done as previously detailed, and 100 nM PE was used to assess vasodilation to acetylcholine (ACh, Sigma). For each rat, treatments were performed in duplicate, the results were averaged and considered as one independent experiment (n=1). All aortas were studied with intact endothelium and incubated with vehicle, sEHi alone or simultaneously with 11,12-EET for the duration of the procedure. Data were collected and analyzed using PowerLab software (AD Instruments, Colorado Springs, CO).

1.3 Flow cytometry

Low passage (passage 4-6) adult human aortic endothelial cells (HAEC) from Genlantis (San Diego, CA) were pretreated with vehicle control, sEHi alone or simultaneously with 11,12-EET or 11,12-DHET for 30 min followed by a 4 hr treatment with 0.3ng/mL TNFα (R & D Systems, Minneapolis). HAEC were detached with 0.5mM EDTA (pH 7.4), Fc blocked with normal IgG, labeled with fluorescein-conjugated antibodies against human E-selectin, ICAM-1, VCAM-1, or isotype-matched IgG control, and analyzed with FACScan flow cytometer (BD, Franklin Lakes, NJ) equipped with CellQuest software.

1.3 Monocyte adhesion assay

HAEC monolayers were pretreated with sEHi and 11,12-EET or DHET, and stimulated with TNFα as above. For consistency, monocytes in all the experiments were obtained from a single, healthy male human donor, who was not on any medication, according to Institutional Review Board-approved protocols under informed consent. Briefly, mononuclear cells (MNC) were isolated from fasting blood by sedimentation over Lymphosep density separation media (MP) and depleted of platelets by repetitive centrifugation. 10^6 MNC were resuspended in 1mL HBSS buffer with Ca^{2+}/Mg^{2+} plus 0.1% human serum albumin and perfused over preconditioned HAEC monolayers at a shear stress of 2 dyne/cm^2 for 2 min in a parallel-plate flow channel, as previously described.[27] The arrested monocytes were identified with an Alexa fluor 488-labeled antibody to the monocytic marker CD14. For each experiment, the results from 5-6 fields/channel of 1–2 channels/condition was averaged and counted as n=1.

1.4 NFκB Activation - was assessed in nuclear extracts using a DNA binding assay (Pierce), as previously described.[28]

1.5 Membrane potential measurement

The membrane potentials of HAEC were measured using bis-(1,3-Dibutylbarbituric acid)

Trimethine Oxonol (DiBAC4 (3) or bis-oxonol, (Life Technologies) and a Zeiss LSM 700 confocal microscope at room temperature. The cells were cultured on glass coverslips and mounted on a recording chamber which was continuously perfused with cell culture medium buffered with 10 mM Hepes. Cells were pre-incubated in the culture medium containing 1 μM bis-oxonol in cell culture incubator for 30 min. Treatments were added to the cell culture medium. The time lapse image acquisitions were performed with fast solution exchanges. The bis-oxonol loaded cells were excited by a 488 nm laser and the emission spectra at >530 nm wavelength were acquired. To quantify the image intensity, the cytoplasmic fluorescence signals (excluding the nucleus region) were measured and the averaged intensity was calculated after subtracting background fluorescence intensity. The signals were normalized to that measured in control medium. According to the manufacturer's information, the bis-oxonol sensitivity to membrane potential is typically ~1% per mV. Cell membrane depolarization results in more influx of the dye and an increase in fluorescence signals. Conversely, hyperpolarization induces a decrease in fluorescence. Thus membrane potential changes could be estimated by the percentage changes of the fluorescence signals. Data analysis was performed with Image J (NIH) and Origin 6.1 (OriginLab) software. A paired t-test and a one-way ANOVA were used to determine the significant differences between two groups (TNFα effects) and three groups (EETs and subsequent TNFα effects), respectively.

1.6 Western blot analysis

HAEC were treated with 0.3 or 3 ng/mL TNFα for 4 hr or E2 (1nM or 100nM, Steraloids Inc,

Newport, RI) for 72 hr and then lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail (Sigma). Westerns were performed as previously described.[25] The blots were incubated overnight at 4˚C with 1:500 anti-sEH (Cayman 10010146), 1:1000 anti-CYP2J2 and 1:1000 anti-CYP2C9 (Santa Cruz Biotech) followed by 1.5 hr incubation with an HRP-secondary antibody (Jackson). After development with chemiluminescent substrate (Pierce), signals analyzed with a ChemiDoc MP gel imaging system (Bio-Rad). The membrane was reprobed with 1:10,000 anti-GAPDH mouse antibody (Novus Biologicals) as a loading control.

1.7 Statistics

Data are expressed as mean ± standard error of the mean (SEM), and were analyzed with GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). Multiple groups were analyzed by ANOVA or repeated ANOVA, and differences were assessed post-hoc with a Newman-Keuls test. Two experimental groups were compared using Student's *t*-test, with pairing where appropriate. Two-tailed *P* values of ≤ 0.05 were considered statistically significant unless otherwise indicated.

Results

2.1sEH inhibitor and 11,12-EET decrease TNFα-induced CAM expression

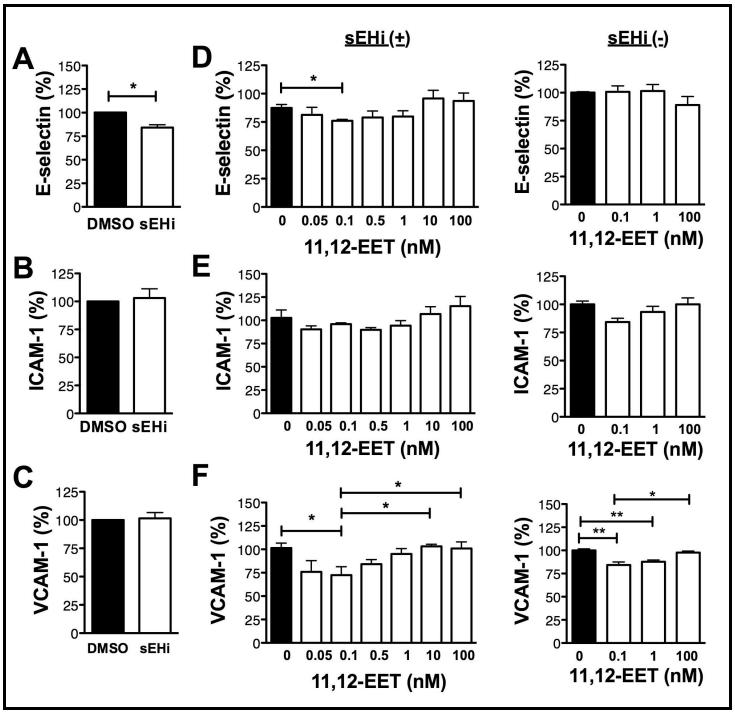

Figure 1 - 11,12-EET at physiologic concentration and inhibition of sEH decrease TNFα-induced CAM expression. HAEC monolayers were pretreated with DMSO or 125 nM sEHi in the absence or presence of different doses of 11,12-EET or DHET for 30 min, followed by another 4-hour treatment with 0.3ng/mL TNFα. Flow cytometry was applied to measure cell surface expression of E-selectin, ICAM-1 and VCAM-1. **A-C**, HAEC were treated with sEHi or vehicle control DMSO; D-F) HAEC were treated with 11,12-EET plus sEHi (left) or 11,12-EET alone (right); Significance was determined by ANOVA followed by Newman-Keuls posttest. Values are means ± SEM; $n \geq 3$. *$p \leq 0.05$; **$p \leq 0.05$.

EETs are synthesized from arachidonic acid (AA) in a reaction catalyzed by the cytochrome P450 (CYP) oxidases, and the predominant metabolism pathway for EETs is rapid hydration to DHETs by sEH. Inhibition of sEH increases the ratio of EETs to DHETs by stabilizing EETs. We examined the effect of 11,12-EET on the inflammatory response of HAEC to TNFα. We focused on 11,12-EET because previous papers have reported conflicting results for this EET in vascular disease.[5-7] Given that the physiological level of 11,12-EET remains controversial,[29-34] a dose response study was performed. HAEC monolayers were treated with 0.3ng/mL TNFα, the EC_{50} for upregulation of membrane adhesion receptors (VCAM-1, ICAM-1, or E-selectin) on HAEC.[35] Treatment with TNFα alone resulted in a 59-, 6- and 8-fold increase in E-selectin, ICAM-1 and VCAM-1 (not shown). Inhibition of 11,12-EET conversion to DHET by TUPS, a highly selective sEHi, decreased TNFα-induced E-selectin expression (12.5%) but not ICAM-1 or VCAM-1 expression (Fig. 1A-C). In the presence of sEHi, 11,12-EET at a dose as low as 0.1nM further inhibited E-selectin by 11.4% (Fig. 1D, left). The same combination significantly reduced VCAM-1 expression (22.6%,Fig. 1F, left). Experiments without the inhibitor confirmed

the inhibitory effect on VCAM-1 expression was mainly from 11,12-EET because this EET at 0.1 or 1nM decreased VCAM-1 expression at a similar level as observed in the presence of sEHi. Without sEHi, 11,12-EET had no effect on E-selectin expression, indicating this adhesion molecule is more sensitive to the inhibition of EET conversion. With or without sEHi, 11,12-EET had no effect on ICAM-1 expression (Fig. 1E). Surprisingly, with higher 11,12-EET concentrations the inhibitory effect disappeared (Fig. 1D-F). These data suggest the most effective dose of 11,12-EET in inhibiting VCAM-1 expression ranges from 0.1-1nM. In contrast, 11,12-DHET at these doses had no effect on the TNFα-induced inflammatory response in HAEC regardless of the presence of sEHi (fig. S1).

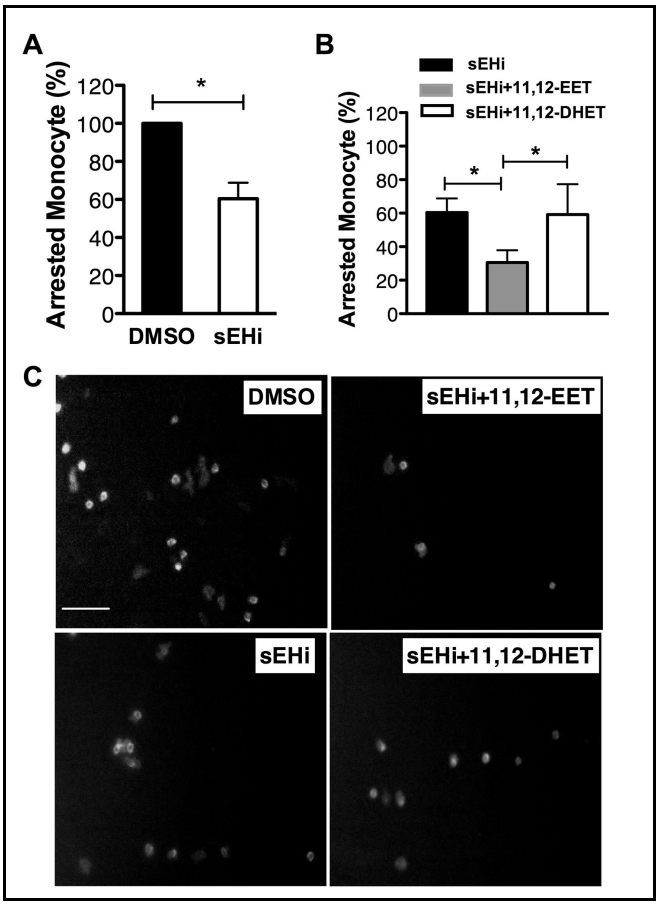

Figure 2 - sEH inhibitor and 11,12-EET, but not 11,12-DHET, decrease TNFα-induced monocyte adhesion under shear stress. HAEC monolayers were pretreated with sEHi in the absence or presence of 11,12-EET or 11,12-DHET for 30 min, followed by 4 h treatment with 0.3ng/mL TNFα. Then the monolayers were exposed to human monocytes under flow of 2 dyne/cm2 for 2 min in a parallel-plate flow channel. The arrested monocytes were identified with an Alexa fluor 488-labeled antibody to the monocytic marker CD14. A and B, Monocyte arrest was quantified and presented as percentage of DMSO control. 3 separate experiments assessed multiple monocyte arrests. *p≤ 0.05. **C**) Representative images of CD14-positive monocytes adhered to a HAEC monolayer. Scale bar = 50 m. ≤0.05.

2.2 11,12-EET inhibits monocyte recruitment to HAEC monolayer under shear stress

We used our well-established model where we pretreat HAEC monolayers and quantify cell arrest under physiologically relevant shear stress to evaluate the

effects of EETs.[35, 36] Pretreatment with sEHi significantly attenuated monocyte adhesion onto TNFα-stimulated HAEC (by 39.9%, Fig. 2A). Addition of 0.1nM 11,12-EET led to a further 29.9% reduction in monocyte recruitment (Fig. 2B). This is consistent with the fact that both E-selectin ligands and VLA-4 (α4β1 integrin) are expressed on the monocyte and their binding to endothelial E-selectin and VCAM-1 contribute to monocyte-endothelial cell interaction.[37] 11,12-DHET treatment of HAEC did not alter monocyte recruitment compared to vehicle control (Fig. 2B).

2.3 EETs, E2 and NFκB Activation by TNFα

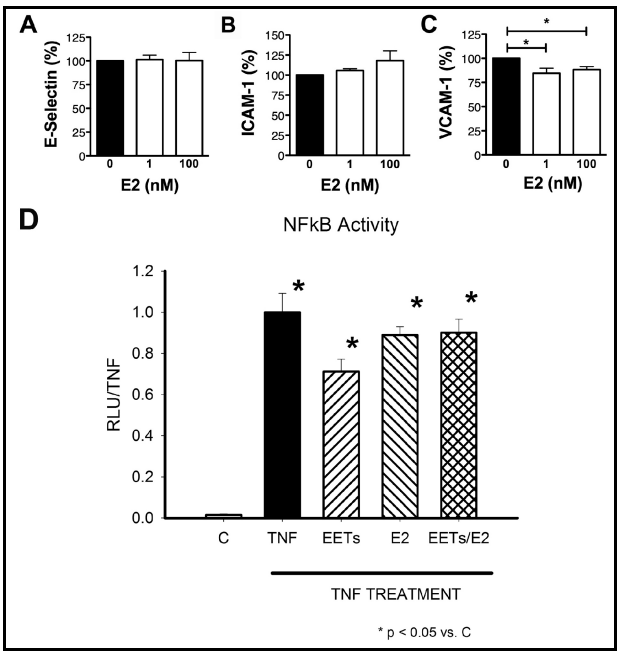

Figure 3 - A-C) Surface expression of adhesion molecules. HAEC monolayers were pretreated with DMSO (0), 1 or 100nM E2 for 72 h, followed by 4 h treatment with 0.3ng/mL TNFα. Flow cytometry was applied to measure cell surface expression of E-selectin, ICAM-1 and VCAM-1. D) NFκB activation was assessed using a DNA binding assay for P65. Nuclear extracts from cells treated with E2 or 11,12 EETs prior to treatment with TNFα. Neither 11,12-EETs nor E2 at physiologic concentrations inhibited NFκB activation. Values are means ± SEM; n = 3-15/group.*p≤0.05 vs. control (C) cells and vs. no treatment (panel C).

A number of studies have reported inhibition of NFκB by E2, but used high levels of E2. 11,12-EETs, as well as 8,9- and 14,15-EETs have been reported to inhibit NFκB by preventing nuclear translocation of p65.[5, 38, 39, 40] However, these studies used 100 nM[4] to 1 μM EETs concentrations, which greatly exceeds *in vivo* concentrations. In another approach, the sEH inhibitor,1-adamantan -3-(5-(2-(2-ethylethoxy)ethoxy)pentyl)urea (AEPU), blocked activation of NFκB in a TAC model of hypertrophy.[16] However, it was not established that activation of NFκB is inhibited with physiologic levels of EETs.

We investigated treatment with 0.5 nM E2 (136 pg/ml) as more reflective of mean estrogen level in mature females. Neither E2 nor E2 plus 11,12-EETs at the concentration, which inhibited monocyte adhesion (0.1 nM), significantly reduced NFκB activation by TNFα, as assessed by a DNA binding assay using nuclear fractions (fig.3D). Thus, NFκB inhibition was not the mechanism reducing VCAM-1 and E-selectin on the endothelial cell membrane after TNFα stimulation. We hypothesized that changes in membrane potentials could be a mechanism by which TNFα induced upregulation of VCAM-1 and E-selectin was inhibited. HAEC were cultured on glass coverslips and loaded with bis-oxanol, a cell permeable fluorescent dye, which increases fluorescence intensity as membrane potentials become depolarized. We pretreated cells with E2 or vehicle (DMSO) and then added 0.1 nM 11,12-EETS with TUPS, as in previous experiments. 0.3 ng/ml of TNFα was then added. A special setup on the microscope stage allowed rapid solution changes. We monitored fluorescence using confocal microscopy throughout the experiments. As shown in fig.4A-C, in control DMSO treated cells, the addition of TNFα caused mild membrane depolarization. In contrast, pretreatment with EETs led to significant depolarization when TNFα was added. Cells were

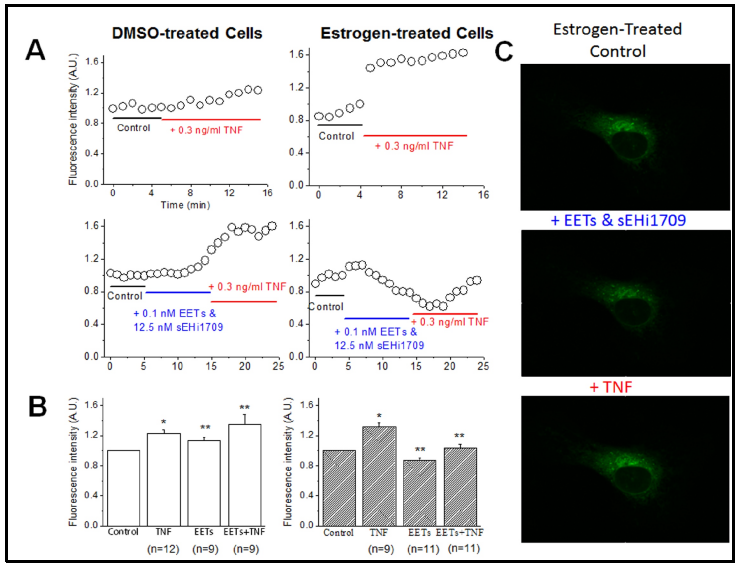

Figure 4 - EETs hyperpolarizes the membrane potential of the E2-treated HAECs. A) Representative traces of the bis-oxonol fluorescence signals from HAECs treated with either DMSO or E2. TNFα only, or EETs & sEHi TUPS (1709) and subsequent TNFα were applied to test their effects on the fluorescence signal, an indicator of the membrane potential. B) Summary of the effects of TNF and EETs & sEHi TUPS on membrane potentials (*P<0.05, compared to control group by paired t-Test; ** P<0.05, compared to control by one-way ANOVA). C) Representative images of the bis-oxonol loaded cell with estrogen treatment at different conditions. Data is sum of 3 experiments studying 9-12 cells/group.

grown in E2 for 4 days to model chronic E2 effects rather than the well-described rapid, protective response to E2, and then studied. Addition of TNFα caused marked depolarization (fig. 4A, upper right panel), similar to that seen with 11,12-EETs treated cells in DMSO control group (fig. 4A, lower left panel). If 11,12-EETs alone were added to E2 treated cells, this caused membrane hyperpolarization (fig.4A, lower right panel). These results suggest that TNFα added to 11,12-EETs or E2 treated cells results in marked depolarization of the cell membrane, which could interfere trafficking of intracellular proteins to the cell membrane. Interestingly, the combination of 11,12-EETs and E2 together with TNFα caused less depolarization(fig. 4A, lower right panel).

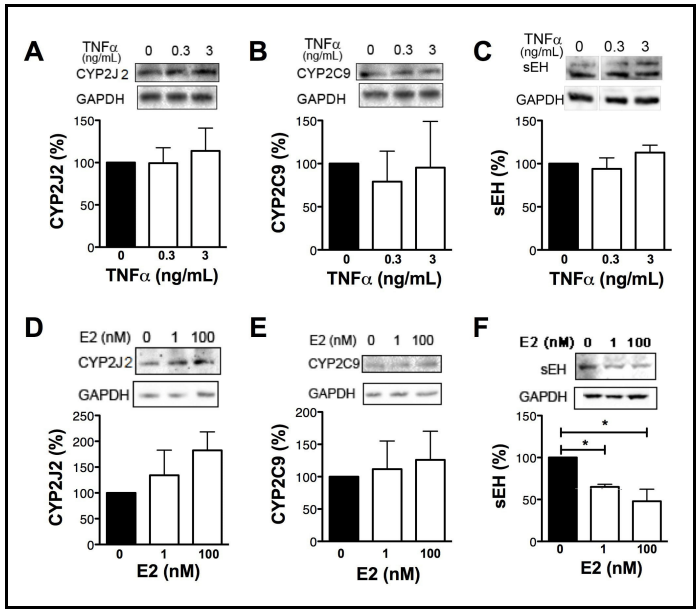

Figure 5 - 17β-estradiol, TNFα and CYP/sEH expression in HAEC - HAEC monolayers were pretreated with 0.3 or 3ng/mL TNFα(A-C) for 4 hour, or 1 or 100nM E2 (D-F) for 72 hr. Then the cells were lysed and analyzed by Western Blot for CYPs and sEH expression. Panel A-C and D-F images were from the same blot. Densitometry was used for quantification. Values are means ± SEM; $n \geq 3$.*$p \leq 0.05$.

2.4 TNFα, 17β-estradiol and the expression of CYPs and sEH, enzymes responsible for 11,12-EET production and hydration to 11,12-DHET

CYPs are widely expressed, and metabolize arachidonic acids to EETs. Different CYPs have distinct catalytic efficiency. The CYP2C and CYP2J oxidase enzymes have been implicated in olefin oxidation of arachidonic acid to its four EET isomers (5,6-, 8,9-, 11,12-, 14,15-EET) in the cardiovascular system.[8, 9] Previous work focused on

human umbilical vein EC (HUVECs), cell lines and nonhuman EC. We examined CYP2C9 and CYP2J2 expression in HAEC lysates following 4 hours of stimulation with TNFα. To investigate whether E2 increases CYP expression cells were treated with E2 for 72 hr., but there was no significant difference compared with control cells (Fig. 5A, B, D, E). TNFα stimulation did not alter sEH expression (Fig. 5C). However, sEH expression was significantly decreased by 72-hour-treatment with E2 either at a physiological (1nM) or a pharmacologic (100nM) concentration (Fig. 5F). Furthermore, E2 treatment resulted in a significant decrease in TNFα-induced VCAM-1 expression (Fig. 3 A-C).

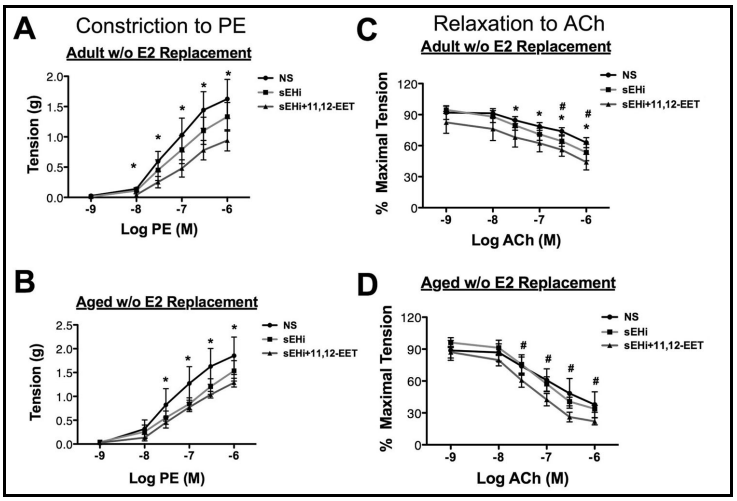

Figure 6 - Effect of 11,12-EETs on the aortic function in adult and aged rats with estrogen withdrawal. Aged (22 months) and Adult (5 months) Norwegian brown rats were ovariectomized with or without E2 slow-release pellets implanted sc and studied 9 weeks later. 11,12-EETs had no added effect in E2 replaced group (Supplemental figure 2). A) Isometric tension developed in a dose response to PE. B) Dose-response curves to ACh were obtained in 100nM PE pre-contracted aortic rings. Each group contains 5-6 rats (n=5-6). *$p \leq 0.05$, NS vs. sEHi+EET. # $p \leq 0.05$, sEHi vs. sEHi+EET.

2.5 sEHi and 11,12-EET attenuated vascular stiffness caused by E2-withdrawal or aging

EETs have vasodilatory properties. Previously we reported that aging is associated with increased constriction in response to PE, and that aging combined with estrogen loss leads to impaired relaxation.[25] We hypothesized that with TUPS or TUPS plus 11,12-EETs would improve vascular function. Aortic rings were prepared from ovx aged

and adult Norway Brown rat with and without E2 replacement, as previously described.[25] After confirming the previously reported increase in constriction with PE in aged rats without E2 replacement (data not shown), we examined whether TUPS or TUPs plus 11,12-EET would mitigate increased constriction. Developed tension was significantly decreased by the combination of TUPS and 11,12-EETs in both groups without E2 (fig.6 A,B); however TUPS and 11,12-EETs had no effect in E2 groups (fig.S2). Similarly, TUPS plus 11,12-EETs improved vascular relaxation to acetyl choline only in groups without E2 (fig. 6C,D, fig.S2). TUPS plus 11,12-EETs was superior to TUPS alone (fig. 6C,D). These data suggest deregulation of EETs is, at least partly, responsible for vascular dysfunction that is associated with estrogen withdrawal during aging.

Discussion

Endothelial inflammation and impaired vasodilation play critical roles in atherosclerotic heart disease. Endothelial inflammation is manifest as upregulated expression of CAMs leading to monocyte recruitment and infiltration into the arterial wall. Although the vasodilatory effect of 11,12-EETs has been recognized, aspects of the anti-inflammatory properties remain controversial. 11,12-EETs at a concentration as high as 100nM blocked monocyte recruitment to inflamed endothelium through down regulation of adhesion molecule expression, including E-selectin, ICAM-1 and VCAM-1 by inhibiting NFκB activity.[5] However, the physiologic levels of 11,12 EETs used here did not inhibit NFκB. Others found that 14,15-EET increased U937 (monocyte cell line) cell adhesion to HUVECs.[41] Minimally oxidized LDL induced EETs and promoted adhesion.[6,7] 11,12-EET or 14,15-EET given at the time of reperfusion mitigated the

increase in endothelial permeability with reperfusion in ischemia induced lung injury.[42] In a model of arteriovenous graft stenosis pre-treatment of monocytes with an sEHi reduced the release of MCP-1 and TNFα, but not MCP-1 or IL-6 in response to LPS.[43] Most interestingly, Kundu et al. have reported that DHETs are essential for monocyte recruitment by MCP-1.[44] In other studies, pretreatment with 11,12-EET or 14,15 had no effect.[43] In the current study, we found that physiologic concentrations of 11,12-EETs and E2 led to mild hyperpolarization of the cell membrane. Both EETs and E2 are known to activate the large conductance Ca-activated K^+ channel (BK channels) in both vascular smooth muscle cells and EC, which may contribute to membrane polarization changes.[45, 46] When TNFα was added to EETs or E2, the membrane was markedly depolarized. This change in membrane polarization could interfere with trafficking of adhesion molecules to the membrane.

Endothelium-dependent vasodilatation in premenopausal women correlates with circulating estrogen levels, and blood pressure is lower in premenopausal women than in age-matched men.[47] Accordingly, pre-menopausal women have lower cardiovascular risk than men. The difference disappears after menopause when cardiovascular disease becomes the predominant cause of death in women.[48] Induction of vasodilation is also one of the most important cardioprotective effects of EETs. Studies have primarily focused on models in young animals, while increased vasconstriction is a problem of aging. EETs relax preconstricted mesenteric arteries, renal arteries, cerebral arteries, and coronary arteries.[49, 50] Endothelial overexpression of CYPs lowers blood pressure and attenuates hypertension-induced renal injury in mice.[51] 11,12-EET has been reported to increase cAMP levels and activate protein phosphatase 2A (PP2A), and these signaling pathways contribute to the activation of

the BKCa channel and vasodilation in mesenteric resistance arteries and renal microvessels.[45] In porcine coronary vasculature, 11,12-EET activates both smooth muscle cell BKCa channel and endothelial cell small (SKCa) and intermediate (IKCa) conductance calcium-activated K+ channels.[45] Thus a number of studies support that 11,12-EETs can activate the BKCa channel. Furthermore, previous work,[52] as well as the current study, supports that E2 increases EETs through inhibition of sEH expression and also potentiates the effects of EETs. Combining with these previous results, our data imply that both estrogen itself and its effects on 11,12-EET metabolism contribute to the beneficial effect of estrogen replacement on vascular function via different mechanisms in postmenopausal females.

11,12-EET and Monocyte Adhesion - Applying a state of the art atherosclerosis-relevant *ex vivo* model using HAEC primed with low dose of TNFα over a large dose range of 11,12-EET, we observed differential changes in the expression of the vascular adhesion molecules, as well as a biphasic response. sEH inhibition alone or with 11,12-EET had no effect on ICAM-1 expression. While sEH inhibition alone is sufficient to inhibit E-selectin expression, 11,12- EET plus sEHi is required to inhibit VCAM-1 expression. This is consistent with the distinct transcriptional regulation of different adhesion molecules on the endothelial cell surface. Moreover, in the presence of sEH inhibition, 11,12-EET at a dose of 0.1nM was most effective in inhibiting expression of adhesion molecules including E-selectin and VCAM-1. The disparity between our results and the previous studies may be due to the different conditions applied for the experiments.[5] For example, 30-fold less TNFα (i.e., 0.3ng/mL vs. 10ng/mL) was used in the current study. Flow cytometry, rather than ELISA, was employed providing assessment of changes in membrane expressed adhesion molecules. To prevent the conversion of 11,12-EET to 11,12-DHET, we used an sEH inhibitor. Our data support that 11,12-DHET has little

anti-inflammatory, but is not detrimental.

The precise concentration of EETs in the blood remains controversial because of the use of different models and different measurement techniques. Furthermore, plasma/tissue concentrations may not reflect localized tissue concentrations. In published reports, 14,15-EET ranges from 3.9 ng/mL (~13.0nM) to 0.101ng/mL (~0.3nM) in human plasma; and 11,12-EET from 2 ng/mL (6.7nM) to less than 0.1ng/mL (<0.3nM).[29-32,34] Based on recent studies with HLPC–MS/MS, the plasma total EETs in healthy humans is ~0.19ng/mL(~0.6nM) and 14,15-EET is 0.1ng/mL (~0.3nM) while 11,12-EET is below the lower limit of detection in both healthy individuals and patients with coronary artery disease.[33] Although the effective dose of 11,12-EET found in our study is far lower than that by other groups, it is closer to physiological concentrations in human as well as in experimental animals blood, as discussed. The availability of sEH inhibitors makes it possible to increase EETs levels. SEH inhibitors are already being investigated in clinical trials of hypertension treatment.[53]

Conclusions - In the current study we found that both 11,12-EETs and E2 at physiologic concentrations led to marked endothelial cell depolarization when TNFα was added. This depolarization was associated with a decrease in the translocation of specific CAMs to the cell membrane in response to TNFα. E2 treatment for 72 h decreased VCAM-1 movement to the cell membrane after TNFα. Inhibition of metabolism of EETs to DHETs with TUPS, a selective sEH inhibitor, reduced E-selectin movement to the plasma membrane. The combination of TUPS and 11,12-EETs treatment not only decreased E-selectin, but also VCAM-1 presentation on the cell membrane. Collectively, these findings suggest that CYP-mediated arachidonic

acid metabolism is deregulated in endothelial cells during aging and estrogen loss. Modulation of 11,12-EET metabolism may represent an effective strategy to preserve endothelial function and prevent atherosclerotic heart disease in postmenopausal women.

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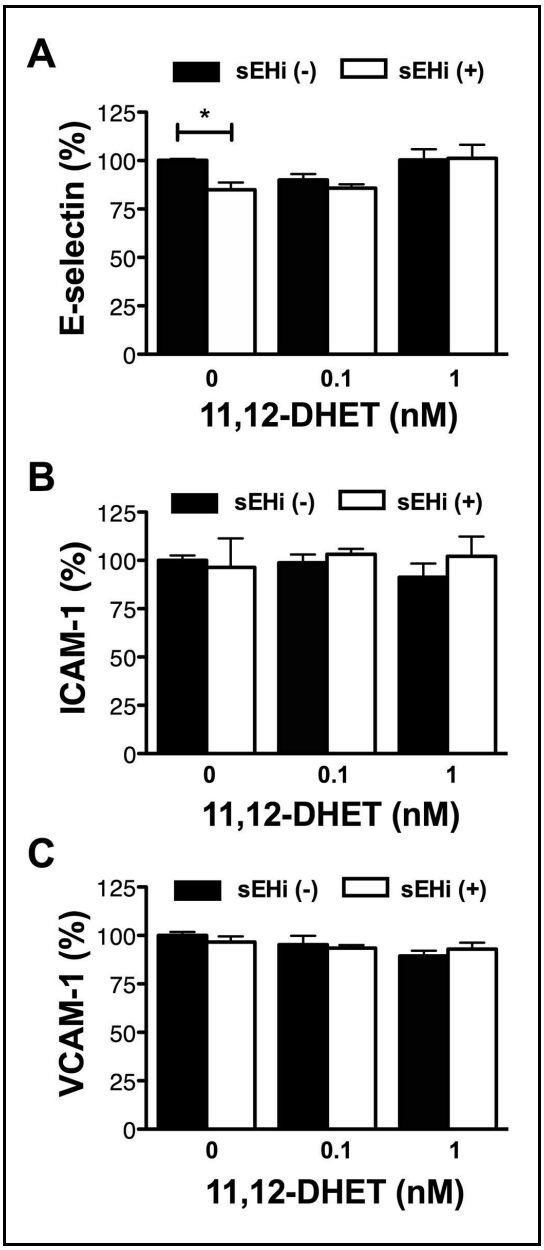
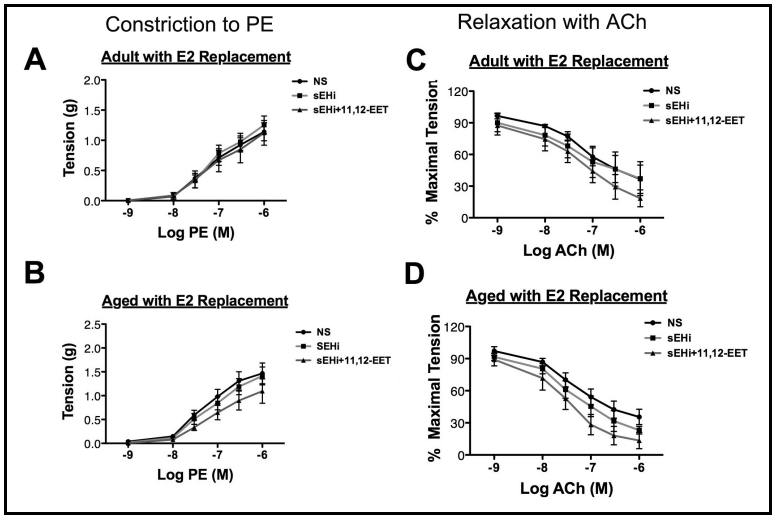


Supplemental Figure 1 - 11,12-DHET does not affect TNFα-induced CAM expression. HAEC were treated with 11,12-DHET alone (solid) or 11,12-DHET in combination with sEHi (clear) for 30 min, followed by another 4hour treatment with 0.3ng/mL TNFα. Flow cytometry was applied to measure cell surface expression of E-selectin, ICAM-1 and VCAM-1. Significance was determined by ANOVA followed by Newman-Keuls post-test. Values are means ± SEM; $n \geq 3$. *$p \leq 0.05$.



Supplemental Figure 2 - Effect of 11,12-EETs on the aortic function in adult and aged rats with estrogen replacement. Aged (22 months) and Adult (5 months) Norwegian brown rats were ovariectomized with or without E2 slow-release pellets implanted sc and studied 9 weeks later. As shown here, 11,12-EETs had no added benefit in E2 replaced group. A) Isometric tension developed in a dose response to PE. B) Concentration-response curves to ACh were obtained in 100nM PE pre-contracted aortic rings. Each group contains 5-6 rats (n=5-6).