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Soluble epoxide hydrolase expression in a porcine model of arteriovenous graft stenosis and anti-inflammatory effects of a soluble epoxide hydrolase inhibitor

William G. Sanders,1 Christophe Morisseau,2 Bruce D. Hammock,3 Alfred K. Cheung,4 and Christi M. Terry5

1Department of Pharmaceutics, University of Utah, Salt Lake City, Utah; 2Department of Entymology, University of California-Davis, Davis, California; 3Department of Entymology, University of California-Davis, Davis, California; 4Medical Service, Veterans Affairs Salt Lake City Healthcare System, Division of Nephrology and Hypertension, University of Utah, Salt Lake City, Utah; and 5Division of Nephrology and Hypertension, University of Utah, Salt Lake City, Utah

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Sanders WG, Morisseau C, Hammock BD, Cheung AK, Terry CM. Soluble epoxide hydrolase expression in a porcine model of arteriovenous graft stenosis and anti-inflammatory effects of a soluble epoxide hydrolase inhibitor. Am J Physiol Cell Physiol 303: C278–C290, 2012. First published May 30, 2012; doi:10.1152/ajpcell.00386.2011.—Synthetic arteriovenous (AV) grafts, placed between an artery and vein, are used for hemodialysis vascular access. Despite the potential for high rates of blood flow for the extracorporeal circulation, which is often achieved by the placement of a synthetic graft between an artery and a vein to create a shunt, unfortunately, such synthetic grafts fail as a result of stenosis (43, 44), and the development of hyperplasia associated with arteriovenous (AV) accesses for chronic hemodialysis (32, 48). Chronic hemodialysis requires access to high rates of blood flow for the extracorporeal circulation, which is often achieved by the placement of a synthetic graft between an artery and a vein to create a shunt. Unfortunately, ~50% of such synthetic grafts fail as a result of stenosis. Such stenosis is almost invariably due to neointimal hyperplasia formation that most frequently occurs around the graft-venous anastomosis, with the concomitant infiltration of inflammatory cells in the perivascular tissues. In the current work, we characterized inflammatory response and sEHI expression in an animal model of synthetic vascular access graft stenosis. We then tested the effects of a sEHI on the release of various inflammatory cytokines from monocytes, with the eventual

Address for reprint requests and other correspondence: C. M. Terry, Div. of Nephrology and Hypertension, Univ. of Utah, 85 N. Medical Dr. East, Salt Lake City, UT 84112 (e-mail: Christi.terry@hsc.utah.edu).
goal of exploring the potential of sEHIs as a therapeutic agent for the prevention of synthetic graft stenosis.

MATERIALS AND METHODS

Materials. sEHi cis-4-[4-(3-(4-(trifluoromethoxy)phenyl)ureido)cyclohexoxy]-benzoic acid (c-TUCB) was synthesized as previously described. Epoxyciclostaerionic acids (EETs) 8,9-EET, 11,12-EET, and 14,15-EET were purchased from Caymen Chemical (Ann Arbor, MI). Antibodies used for flow cytometry experiments were purchased from BD Biosciences (San Jose, CA). Serum-free media (SFM) was purchased from Invitrogen (Grand Island, NY). LPS (055:B5) was purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal rabbit anti-porcine-sEH antibody (38) for tissue immunostaining was generated in the laboratory of Dr. Bruce Hammock against recombinant porcine sEH as the antigen. Polyclonal rabbit anti-human sEH antibody raised against an internal region of human sEH (sEH Y-13 catalogue sc-87101; Santa Cruz, CA) were used for immunocytochemical staining and immunoblotting. Polyclonal rabbit anti-human CD3, polyclonal rabbit anti-human myeloperoxidase, and polyclonal rabbit anti-human PAX5 were from Cell Marque (Rocklin MA). Polyclonal rabbit anti-porcine MCP-1 (CCL2) was purchased from Bethyl Laboratories (Montgomery, TX). Monoclonal porcine anti-porcine phospho-inhibitory IkB (IxB), monoclonal rabbit anti-human NF-kB p65, and polyclonal rabbit anti-human phospho-stress activated protein kinase (SAPK)/COOH-terminal and anti-human phospho-JNK (Thr183/Tyr185) were purchased from Cell Signaling Technology (Danvers, MA).

Animal model. All animal work was performed using protocols approved by the Institutional Animal Care and Use Committee of the University of Utah and Veterans Affairs Salt Lake City Healthcare System and followed guidelines specified by the Guidelines for the Care and Use of Laboratory Animals. A porcine AV expanded polytetrafluoroethylene (ePTFE) graft model was utilized as described in our previous studies (62). Briefly, 3-mo-old Yorkshire crossbreed domestic swine (~30 kg) were anesthetized using a mixture of ketamine (4 mg/kg; Hospira, Lake Forest, IL), xylazine (4 mg/kg; Lloyd Laboratories, Shenandoah, IA), and tiletamine/zolazepam (4 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA). To maintain anesthesia, 1–3% isoflurane was administered via tracheal intubation. Sodium heparin (100 units/kg) was given as an intravenous bolus intraoperatively. Sterile spiral reinforced ePTFE graft (7 cm in length, 6 mm internal diameter) was sutured in place between the common carotid artery and the ipsilateral external jugular vein. After verification of graft patency and hemostasis, the wound was closed. A large animal model is required for studies on synthetic AV grafts since grafts cannot be placed in smaller animals such as mice or rabbits. Also, the large animal model provides blood flow parameters more similar to that observed in human subjects.

The animals were euthanized at designated time points: 1 day or 1, 2, 3, 4, 6, or 7 wk after graft placement. Briefly, after surgical exposure of the grafts in the anesthetized animal, heparin (200 units/kg) was administered intravenously, the artery was cannulated, and the lumen of the grafts and adjoining artery and vein were rinsed with saline. The animals were euthanized by the intravenous injection of pentobarbital sodium (80–100 mg/kg). The grafts and adjoining vessels were perfused in situ with 10% zinc formalin. The artery and veins were ligated under physiologic pressure to maintain lumen circumference, then explanted en bloc and further fixed in formalin overnight (27, 62). The explanted tissue was paraffin embedded, and 5-μm sections were cut perpendicular to the vein lumen (as illustrated in Fig. 1) and processed for immunostaining using standard techniques.

Neointimal hyperplasia index. The surface area of hyperplastic tissue within 5-μm cross-sections of the AV graft was manually quantified in tissue stained with Elastin von Gieson as previously described (27). The NH index was defined as the sum of the intimal hyperplasia within the lumen of the ePTFE graft (Fig. 1A) and the hyperplasia within the native vein wall divided by the sum of the medial area in the native vein wall and the ePTFE graft area in the same tissue cross-section. The denominator was used for normalization to account for the angle at which the tissue section was obtained.

Fig. 1. Foreign body giant cell (FBGC) and T-lymphocyte accumulation in arteriovenous (AV) graft hyperplasia in porcine AV graft model. A: neointimal hyperplastic tissue (outlined by the dashed line in A) in a histological cross-section obtained from the graft-vein anastomosis of an AV graft in a pig euthanized at 49 days after graft placement. B: T lymphocytes stained positive with anti-CD3 antibody appearing as rust color in the hyperplastic tissue. C: multinucleated FBGC stained positive with anti-phospho-IκB. D: CD3+ cell and FBGC accumulation at the graft-vein anastomosis of AV grafts in pigs that were euthanized at various time points after graft. FOV, field of view.
Tissue immunostaining. Formalin-fixed paraffin-embedded tissue sections from the graft-vein anastomosis region were dewaxed and rehydrated following standard protocols. Tissue sections were immunostained with anti-CD3 (1:1,000), anti-PAX-5 (undiluted), and anti-myeloperoxidase (1:1,000) antibodies to detect T lymphocytes, B lymphocytes, and neutrophils, respectively. Immunostaining with anti-MCP-1 (1:100), anti-s-EH (1:400), and anti-phospho-IκB (1:200) was performed on control ungrafted porcine external jugular veins, and graft-vein anastomotic tissues collected at 1 day and 1, 2, 3, 4, 6, or 7 wk after graft placement. For T and B lymphocyte, neutrophil and phospho-IκB detection, antigen retrieval was performed in TRIzol solution (CellMarque) by heating at 100°C for 15 min (EZ Retriever system; BioGenex Laboratories, San Ramon, CA). After nonspecific binding had been blocked with 2% goat serum for 1 h at room temperature (RT), the tissues were incubated with the respective antibody at 4°C overnight. Antibody binding was detected with horseradish peroxidase-conjugated anti-rabbit and DAB chromogen following the manufacturer’s protocol (Envision Systems; DAKO, Carpinteria, CA), and sections were counterstained with hematoxylin. For MCP-1 expression, a previously published protocol with minor modifications was followed (9). Briefly, heat-induced antigen retrieval was performed by heating the tissue sections at 95°C for 15 min in Tris-EDTA buffer, pH 9.0 (EZ-Retriever System). After nonspecific binding had been blocked with 2% goat serum for 1 h at RT, the tissues were incubated with the respective antibody at 4°C overnight. The tissue sections were then washed three times with 0.05% Tween/PBS and incubated with biotinylated anti-rabbit IgG (1:200) for 1 h at RT followed by incubation with streptavidin Alexa-fluor 546 (1:200) (Invitrogen, Carlsbad, CA) for 1 h at RT in the dark. Nuclei were stained using 4,6-diamidino-2-phenylindole (DAPI) Fluoromount (Southern Biotech, Birmingham, AL).

Monocyte isolation and cell culture. Peripheral blood samples were obtained in heparin from normal adult volunteers using protocols approved by the Institutional Review Board of the University of Utah and Veterans Affairs Salt Lake City Healthcare System. Peripheral blood mononuclear cells (PBMC) were obtained from the blood samples using Ficoll-Hypaque (GE Healthcare, Piscataway, NJ) density-gradient centrifugation following the manufacturer’s protocol. Monocytes were isolated from the PBMC layer by adherence to tissue culture plastic during a 2-h incubation in SFM culture media with 20% autologous serum. The nonadherent cells were then removed by repeat washing with 1× PBS. The remaining adherent cell population was enriched in monocytes as determined by flow cytometry assessment of immunostained cells using standard techniques. Typically, ≥70% monocytes, ≤10% CD3 T lymphocytes, ≤1% B lymphocytes, ≤8% natural killer cells, and ≤1% neutrophils were observed in each isolation procedure.

The adherent cells were dislodged from the plastic after incubation for 15 min with calcium-free and magnesium-free PBS, then counted and seeded onto 96-well culture plates (5 × 10^3 cells/0.3 cm^2) in SFM with 20% autologous serum. Monocytes were pretreated with the sEH c-TUCB (5 μM) dissolved in DMSO, EETs (100 nM) were dissolved in ethanol, or both c-TUCB and EETs for 1 h before stimulation with LPS (10 ng/ml) for 24 h at 37°C. Pretreatment with DMSO alone or ethanol alone served as controls. The concentration of solvent was always kept below 0.1%. The media was then collected and stored at −80°C. Media containing monocytes without LPS treatment and media alone served as additional controls.

Chemokine/cytokine quantification by ELISA. Media from the tissue culture experiments was assayed for IL-6, MCP-1, macrophage inflammatory protein-1α (MIP-1α), and TNF-α using respective ELISA kits (PeproTech, Rocky Hill, NJ) in accordance with the manufacturer’s instructions. The detection range for IL-6 was 32–2,000 pg/ml, MCP-1 was 8–1,000 pg/ml, MIP-1α was 16–3,000 pg/ml, and TNF-α was 16–2,000 pg/ml.

Oxylipin quantitation. Adherent human monocytes were pretreated with the sEH c-TUCB or solvent alone and stimulated with LPS (10 ng/ml) following the same protocol described above. Monocytes without LPS treatment served as the control. After 24 h, the monocytes were collected and stored at −80°C until oxylipin analysis by liquid chromatography/tandem mass spectrometry. Cell pellets were resuspended in 1 ml cold methanol and spiked with 20 μl 50 mM internal standard I (d11–11,12-DHET and d11–14,15-EET) and 10 μl antioxidant solution (0.2 mg/ml butylated hydroxytoluene and thiamine pyrophosphate). Samples were shaken for 10 min and stored at −20°C for liquid-liquid extraction for 3 h. Samples were then thawed on ice and centrifuged for 5 min at 14,000 rpm, 4°C. The supernatants were transferred to tubes containing 6 μl trap solution (30% glycerol in methanol), and the cell pellets were re-extracted by 0.5 ml cold methanol and twice with 0.5 ml cold ethyl acetate. The supernatants from each extraction were combined and evaporated using vacuum centrifugation (Speed-Vac) until 2 μl trap solution remained in the tube. The residues were dissolved in 50 μl of methanol containing 200 nM internal standard II (1-cyclohexyl-dodecanoic acid urea, CUDIA). The samples were vortexed for 2 min, centrifuged at 14,000 rpm, and then transferred to autosampler vials for oxylipin analysis using liquid chromatography/tandem mass spectrometry as previously described (5).

Cell immunostaining. Monocytes were plated at 1.2 × 10^5/10 cm^2 in SFM with 20% autologous serum for 4-well chamber slides (Lab-Tek, Nunc, Rochester, NY). After 2 h, the nonadherent cells were removed by sequential washing with PBS. The adherent cells (the majority of which were monocytes) were then fixed in 2% paraformaldehyde (15 min at RT), washed with PBS, permeabilized using PBS + 0.1% Triton X-100 + 0.5% BSA for 30 min at RT, and incubated with anti-human-sEH antibody (1:200) alone or in the presence of a blocking peptide overnight at 4°C. Primary sEH antibody binding was detected as described above for MCP-1 detection except streptavidin Alexa-fluor 546 was performed at a 1:500 dilution. Expression and cellular location of NF-κB and phospho-JNK was detected as follows. A monocyte-enriched cell population was cultured in SFM with 20% autologous serum for 3 days that converts the cells to a macrophage phenotype before removing the serum and starving for 48 h. The cells were then pretreated with c-TUCB (5 μM) or DMSO alone for 1 h. Cells were then exposed to LPS (10 ng/ml) for 5, 15, or 45 min and fixed with 2% paraformaldehyde. Fixed cells were then stained with anti-NF-κB (1:100) or phospho-IκB (1:100) using the same antibody binding detection procedure described above for sEH. The same experiments were repeated on monocyte/macrophages that did not undergo the 3-day culturing to convert to a complete macrophage phenotype, but were adhered for 2 h, washed, pretreated with sEH antibody for 1 h, exposed to LPS, and fixed as described above. To quantitate NF-κB translocation, nuclear fluorescence intensity was measured in 15 cells in images of immunostained cells of both the LPS-treated and LPS + c-TUCB-treated groups, and the average fluorescence pixel intensity was determined. To quantitate phospho-JNK immunostaining, fluorescence intensity was measured in the cytoplasm of 15 cells in images of immunostained cells for the same treatment groups (ImageJ; National Institutes of Health, Bethesda, MD). Background fluorescence intensity was determined by measuring three cell-free areas within each image and averaging; the average background intensity was subtracted from the cell intensity values.

Foreign-body giant cell detection. A number of antibodies reported to be specific for macrophages were tested for cross-reactivity against porcine macrophages. No antibody tested was found to be sufficiently specific for reliable quantification of macrophages in the paraffin-embedded porcine tissue sections. Foreign-body giant cells (FBGC), which are formed by the fusion of multiple macrophages in response to frustrated phagocytosis, were thus used as a measure of macrophage recruitment to the graft-vein anastomotic region. Phosphorylation of IκB releases NF-κB for translocation to the nucleus and subsequent transcription of genes involved in inflammation. To determine the activation state of the FBGCs, tissue sections were stained (10 ng/ml) following the same protocol described above.
with anti-phospho-IkB, FBGC accumulation in the AV graft anastomotic region was assessed by visual inspection of the phospho-IkB-stained tissue sections with light microscopy. A cell was counted as a FBGC if it contained two or more nuclei regardless if it stained positive for phospho-IkB. Four fields of view at 40× magnification were counted in each tissue section, and the number of FBGCs in the four fields of view were averaged.

**Immunoblot analysis for sEH protein.** The monocye-enriched cell population was isolated using Ficoll-Hypaque as described above. Adherent cells were lysed with RIPA buffer containing a protease inhibitor cocktail (Complete mini; Roche Diagnostic, Mannheim, Germany) and sonicated. The soluble lysate was collected by centrifugation, and protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Lysate (20 µg) was resolved on 4–12% NuPAGE Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA). Membranes were blocked in 5% nonfat dry milk for 1 h at RT and then incubated with anti-human-sEH antibody (1:2,000) alone or in the presence of blocking peptide overnight at 4°C. The antibody binding was detected by incubation with anti-rabbit IgG–horseradish peroxidase (Santa Cruz Biotechnology) and chemiluminescence substrate following the manufacturer’s instructions (Supersignal West Dura Extended Duration Substrate kit; Thermo Scientific, Rockford, IL). The bands on the immunoblot were quantified using densitometry analysis (ImageJ).

**Cell harvest and culture from wild-type and sEH-knockout mice.** The procedures and animal care related to these mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of Utah. Wild-type (WT) C57BL/6 mice (Jackson Laboratories, Sacramento, CA) and Ephx2 gene-deleted mice (Ephx2−/−) [sEH knockout (KO)] were utilized for bone marrow cell harvest. Bone marrow cells (BMCs) were harvested from femurs and tibias of both WT and sEH KO mice and differentiated into bone marrow macrophages (BMMΦ) as described previously (15). Briefly, isolated BMCs were plated in DMEM (Invitrogen, Carlsbad, CA) and Ephx2 gene-deleted mice (Ephx2−/−) [sEH knockout (KO)] were utilized for bone marrow cell harvest. Bone marrow cells (BMCs) were harvested from femurs and tibias of both WT and sEH KO mice and differentiated into bone marrow macrophages (BMMΦ) as described previously (15). Briefly, isolated BMCs were plated in DMEM (Invitrogen) containing 10% L929 conditioned media, 1% fetal calf serum, 1% non-essential amino acids, 1% sodium pyruvate, 1% HEPES, and antibiotics/antimycotics for 10 days, allowing the cells to mature to a macrophage phenotype. Cells were seeded at 5 × 10^4 cells/0.3 cm^2 in 96 well plates in 20% fetal calf serum. BMMΦs were pretreated with the sEH c-TUCB, EETs, or both, or solvent alone and stimulated with LPS (10 ng/ml) following the same protocol mentioned above for human monocytes. After 24 h, the media was collected from each well and stored at −80°C until MCP-1 quantification by ELISA. BMMΦs without LPS treatment or media alone served as additional controls.

**Statistical analysis.** Data presented for inflammatory cell [foreign-body giant cell detection (FBGC)] accumulation was expressed as means ± SD. For cytokine-release studies, the cytokine released from cells treated with LPS alone was set as 100% and results from other treatments (LPS plus c-TUCB, or LPS plus c-TUCB plus EETs) were calculated as percentage of LPS alone. All statistical analyses were performed on the log scale, and results were expressed as the ratio of geometric means. Mixed-effects analysis of variance, treating the individual blood donor or mouse as a random effect and treatment as a fixed effect, were performed, and significance was determined using the Holm–Bonferroni procedure for each cytokine/chemokine tested in humans and MCP-1 in mice. For the human and mouse data, LPS plus c-TUCB versus LPS alone and LPS plus c-TUCB plus EETs versus LPS alone were the two primary comparisons analyzed for each cytokine/chemokine. For the mouse data, cytokine release from LPS alone in WT mice versus LPS alone in the KO mice and basal cytokine release from untreated WT mice versus untreated KO mice were also compared by two-sample unpaired t-tests.

**RESULTS**

**Inflammatory cell infiltrate at graft-vein anastomosis in porcine AV graft model.** Both CD3+ cells (T lymphocytes; Fig. 1B) and FBGC (macrophages; Fig. 1C) accumulated at the graft-vein anastomotic region preceding or concomitant with the increase of neointimal hyperplasia as indicated in Fig. 1D. The majority of FBGCs examined in multiple tissue sections stained positive for phospho-IkB expression (data not shown), suggesting the activation of NF-κB.

Neutrophil infiltration was observed around the graft sutures within the first week after graft placement, but their numbers declined rapidly thereafter (data not shown). Sporadic clusters of B lymphocytes were occasionally observed in the hyperplastic tissue at various time points (data not shown).

sEH expression is upregulated in the graft-vein anastomosis. Figure 2 shows sEH expression was low in ungrafted porcine external jugular vein. In contrast, sEH expression was intense in the neointimal hyperplastic tissue at the graft-vein anastomosis and appeared to increase over time up to 4 wk. The majority of FBGC (white arrows at right) stained positive for sEH but some FBGC showed low sEH expression (middle arrow).

**Human monocytes express sEH.** Adherent human monocytes stained strongly for sEH protein in the cytoplasm and to a lesser extent in the nuclei (Fig. 3, A and B), similar to that recently reported in rat neutrophils, macrophages, and brain cortical astrocytes (8, 46). Immunoblotting of the monocyte lysate confirmed sEH expression in human monocytes (Fig. 3C). Incubation with an sEH blocking peptide decreased the sEH expression in both confocal microscopy and immunoblotting (by ~60% determined by densitometry), indicating that
the staining was specific for sEH. Results shown are representative of two experiments.

Upregulation of chemokine/cytokine expression in porcine graft-vein anastomotic tissue. TNF-α expression, as evaluated by visual assessment of immunohistochemical staining, increased from a low level at day 1, similar to that seen in the control vein, to widespread intense expression in the hyperplastic tissues at 7 wk after graft placement (data not shown). Expression of TNF-α was often more intense in FBGCs than in the surrounding tissues (Fig. 4). MCP-1 staining in the control vein was minimal, whereas the vein-graft anastomosis tissue had elevated expression of MCP-1 by 1 wk (Fig. 5) that remained elevated at 6 wk, which was the latest time point tested (data not shown).

Attenuation of chemokine/cytokine release from human monocytes by sEH. LPS was used to stimulate monocytes seeded on tissue culture polystyrene instead of on PTFE as others have reported similar induction of cytokines by LPS when cells were seeded on either material (50). c-TUCB is a potent and selective inhibitor of sEH, with a 50% inhibition (IC₅₀) against recombinant sEH activity occurring at ~0.6 nM (19). LPS-stimulated MCP-1 and TNF-α release from adherent human monocytes was significantly inhibited by pretreatment with c-TUCB in the presence or absence of 14,15-EETs,

Fig. 3. sEH expression in human monocytes. Confocal images (60×) of immunocytofluorescence of adherent human monocytes immunostained with anti-sEH (green) alone (A) or in the presence of sEH antibody blocking peptide (B) merged with the fluorescence of the nuclear marker, 4,6-diamidino-2-phenylindole (DAPI; blue), are shown. C: immunoblot analysis of lysate from adherent human monocytes. The blocking peptide decreased sEH staining in both confocal images and immunoblotting, indicating the staining was specific for sEH.

Fig. 4. TNF-α expression in hyperplastic tissues at the porcine graft-vein anastomosis. A: tissues obtained at 2 wk after graft placement showed moderate expression of TNF-α (rust color) but expression in FBGCs (white arrow) was typically more intense. B: tissues obtained at 7 wk after graft placement showed high expression of TNF-α with even greater expression in FBGCs (white arrow).
compared with LPS alone ($P < 0.05$) (Fig. 6, A and B). There was no appreciable effect on LPS-induced MCP-1 and TNF-$\alpha$ release by the addition of 14,15-EETs to the c-TUCB–treated cells, or by the addition of 14,15-EETs alone (data not shown). The effects of 8,9-EETs and 11,12-EETs were also tested but neither inhibited MCP-1 or TNF-$\alpha$ release when added alone or in conjunction with c-TUCB (data not shown). The secretion of IL-6 (Fig. 6C) or MIP-1$\alpha$ (Fig. 6D) after LPS exposure was not inhibited by pretreatment with c-TUCB alone or c-TUCB with EETs. EETs alone had no discernible effect (compared with LPS alone) on the release of any of the chemokine/cytokines tested (data not shown).

**Effect of sEH inhibitors on LPS-stimulated human monocyte EET-to-DHET ratios.** Enzyme activity analysis required a large amount of blood since monocytes are a small percentage of the total blood cell population. Adherent monocytes were collected from the blood of three donors and pretreated with c-TUCB, then exposed to LPS for 24 h. Levels of EETs and the respective diols within the cells and in the tissue culture media were then measured. Although statistical significance was not reached in the sample size analyzed, the ratio of EET to DHET for combined intracellular and extracellular 11,12 oxylipins was lessened in cells treated with LPS alone, compared with cells treated with LPS in the presence of c-TUCB ($7.81 \pm 1.74$ vs $10.16 \pm 2.9$, respectively; $P = 0.08$, not significant). A trend toward decreased total 11,12-EET levels was also observed in the cells treated with LPS alone compared with those treated with LPS and c-TUCB but the difference also did not reach significance (data not shown). Levels of 8,9-EET were below detection, and levels of 14,15-EET were largely unchanged.

**Response of BMM$\Phi$ from WT and sEH knockout mice to sEHI.** LPS-stimulated release of MCP-1 from WT murine BMM$\Phi$ was significantly inhibited by preincubation with the sEHI c-TUCB ($P < 0.01$; Fig. 7), similar to that seen in the human monocyte experiments (Fig. 6). However, no inhibition was observed when BMM$\Phi$ from sEH KO animals were exposed to LPS after preincubation with c-TUCB (Fig. 7). There were no discernible effects on LPS-stimulated MCP-1 release by the addition of EETs to c-TUCB–treated cells from either WT or KO animals (data not shown). However, untreated as well as LPS-treated BMM$\Phi$ from sEH KO animals had elevated levels of MCP-1 release compared with BMM$\Phi$ from WT animals (untreated: 2,163 ± 599 pg/ml vs. 1,416 ± 1,011 pg/ml; LPS-treated: 6,531 ± 5,537 pg/ml vs. 2,256 ± 1,424 pg/ml; $P < 0.009$).

**Effects of sEHI on NF-$\kappa$B translocation in human monocytes.** EETs have previously been reported to inhibit IxB kinase (41, 67), which phosphorylates IxB. Phosphorylation of IxB results in its dissociation from the transcription factor.
NF-κB, allowing NF-κB to translocate to the nucleus and participate in transcription of genes involved in inflammation. Using a different sEHI than c-TUCB, others have reported the inhibition of NF-κB translocation in mouse cardiomyocytes (67). We tested the effects of c-TUCB on NF-κB translocation in adherent human monocytes. NF-κB was present in the cytoplasm and nucleus of control monocytes as determined by immunofluorescent staining (Fig. 8). Exposure of cells to LPS (10 ng/ml) was associated with NF-κB translocation to the nucleus, but preincubation with c-TUCB had no appreciable inhibitory effect on that translocation (pixel intensity of anti-NF-κB immunostaining in nucleus: LPS alone, 29.6 ± 8.9 vs. LPS + TUCB, 28.3 ± 6.2; P < 0.05). No inhibition of NF-κB translocation was observed at any time point examined or when cells were adhered for either 2 h or 3 days before experimentation (data not shown).

**Effect of c-TUCB on JNK in adherent human monocytes.** The activation of JNK, a member of the MAPK pathway, has been reported to be involved in LPS-induced expression of MCP-1 in microglia (71) and TNF-induced expression of MCP-1 in astrocytes (14). When adherent human monocytes were exposed to LPS, phosphorylation of JNK was enhanced (Fig. 9). However, when cells were exposed to LPS after pretreatment with c-TUCB, phospho-JNK levels were markedly decreased, as assessed by immunofluorescent staining (Fig. 9) (pixel intensity of anti-phospho-JNK immunostaining in cytoplasm: LPS alone, 13.5 ± 2.2 vs. LPS + c-TUCB, 4.1 ± 0.7; P < 0.05).

**DISCUSSION**

Others have investigated cell phenotypes in human AV graft and native AV fistula vein samples collected during surgical revision of stenosed accesses and reported the presence of numerous macrophages (48, 56). However, the percentage of grafts that had undergone percutaneous transluminal angioplasty before the surgical revision was unknown, which complicates interpretations of the data since angioplasty itself initiates inflammation. The porcine model is a well-accepted animal model of AV graft stenosis that recapitulates the neointimal hyperplasia development that occurs in human AV grafts but in a shorter time frame (24, 48). One caveat to this model is the absence of uremia, which may affect inflammation. Studies in uremic animal models and human cells/tissue from patients with end-stage renal disease are desirable to confirm the findings presented here.
T lymphocytes and macrophages accumulated in large numbers in our porcine model after AV graft placement. T lymphocytes were particularly notable for their early appearance, and this is the first report to our knowledge on their presence in AV graft tissues. A previous study reported the accumulation of T lymphocytes in clotted native AV fistulas likely due to thrombosis (4). T lymphocytes may influence the macrophage response to the PTFE graft material as T cells have been shown in vitro to enhance the adherence of macrophages, and the formation of FBGC, on certain synthetic biomaterials (2).

In another study, rat aortic smooth muscle cells exposed to conditioned media from human PBMC exposed to PTFE had increased proliferation rates compared with smooth muscle cells exposed to conditioned media from unexposed PBMC (32). In vivo studies reported that T cell–deficient mice had lower numbers of macrophages adhered to polyether urethane implants, compared with WT mice (47).

We, and others, have reported the expression of growth factors, cytokines, and chemokines within the anastomotic tissues of the AV grafts (27, 34, 65). Inflammatory cells are likely sources of these factors. Because macrophages and T lymphocytes are early and prevalent inhabitants of the AV graft tissue and release cytokines and chemokines that induce cellular migration and proliferation, they are attractive targets for pharmacotherapy against AV graft stenosis.

The possibility that sEH may play a role in the marked inflammation observed in AV grafts has not previously been investigated. This hypothesis is plausible because the sEH enzyme catabolizes anti-inflammatory EETs to the less biologically active diols (Fig. 10). Indeed, sEH has been shown to play a role in a number of other inflammatory conditions. For example, in mice rendered hypertensive by DOCA-salt treatment, urinary MCP-1 levels were significantly decreased in sEH KO mice compared with WT mice (31). MCP-1 protein and NF-κB activity were decreased in lung homogenates from sEH KO mice compared with WT mice after in vivo LPS exposure (7) and intraperitoneal administration of a dual EET agonist/sEH inhibitor significantly attenuated plasma levels of TNF-α and MCP-1 in heme oxygenase-2 KO mice (54).

In the present study, we showed that, after AV graft placement, the expression of sEH was markedly upregulated in the vessel wall, initially in the medial layer, then in the adventitia and eventually in the neointima at the graft-vein anastomosis. Expression of sEH has been shown to be altered in endothelial cells by shear stress (29). Disordered blood flow is characteristic at the venous anastomosis, thus may contribute to sEH upregulation in this setting. Also, the sEH gene promoter region contains recognition sites for a number of transcription factors that respond to oxidative stress, growth factors, and inflammation (60), conditions that are prevalent in the AV graft tissue (65). Immunostaining with anti-sEH antibody showed that FBGC expressed sEH. Adherent human monocytes also expressed sEH, as demonstrated by both immunoblotting and immunocytochemistry. We postulated that the elevated sEH
expression in both the vascular tissues and infiltrating inflammatory cells contribute to the inflammation and consequently stenosis in the AV graft, by decreasing anti-inflammatory EET levels. Subsequently, studies were undertaken to investigate the effect of a pharmacological inhibitor of sEH (c-TUCB) on monocyte/macrophages.

LPS induction of MCP-1 and TNF-α was significantly inhibited by c-TUCB exposure. A trend of increased EET-to-DHET ratio for 11,12 oxylipins was observed in LPS-stimulated monocytes pretreated with c-TUCB compared with monocytes exposed to LPS alone. These results support that the inhibition of MCP-1 and TNF-α that was observed with c-TUCB exposure may occur through increasing 11,12-EETs. It is also possible that levels of other epoxides such as 5,6-EET are altered by the c-TUCB treatment, or that there are changes in the ratio of the different regioisomers of 11,12- or 14,15-EET that could be involved in c-TUCB effects on cytokine release. However, 5,6-EET is unstable in aqueous solutions and the regioisomers are very difficult to differentiate using HPLC MS/MS.

P450 epoxygenases convert arachidonic acid to EETs. Recently, an inhibitor of epoxygenase activity was shown to induce TNF-α mRNA expression in human macrophages (3). In that study, TNF-α protein from the human macrophages could not be reliably measured due to variability and low cell numbers, and EET levels in the human macrophages were not reported. However, the addition of 11,12- or 8,9-EET significantly inhibited basal TNF-α release in a human monocytic cell line (THP-1). Exogenously applied EETs have been shown to be anti-inflammatory in other settings as well (7, 41). For example, exogenous EETs inhibited cell adhesion molecule expression in endothelial cells (41). Also, overexpression of P450 epoxygenase 2J2 in endothelial cells decreased LPS induction of MCP-1, but coincubation of these cells with a P450 inhibitor elicited an increase in MCP-1 (7). These studies further support that EETs participate in decreasing MCP-1 and TNF expression. In the present study, the administration of EETs did not significantly augment the inhibitory effects of c-TUCB on cytokine release, suggesting the concomitant endogenous increase in EETs was maximally effective. However, the addition of EETs alone did not inhibit LPS-induced cytokine release, even though a trend in decreased 11,12-EET was observed with LPS exposure. Possible explanations for this include 1) EETs may be metabolized to DHETs too rapidly if sEH is not inhibited (11), 2) albumin, supplemented in the cell culture media in the form of fetal calf serum, may reduce the cellular uptake of EETs (1), or 3) exogenous EETs may be

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**Fig. 9.** Effect of the sEHI c-TUCB on LPS-stimulated phosphorylation (phospho) of JNK in adherent human monocytes. Shown are confocal images of cells immunostained with anti-phospho-JNK (phospho-JNK) (red) (top), then merged with images of DAPI nuclear stain (blue) (bottom). Low levels of cytoplasmic phospho-JNK (arrowhead) were observed in untreated cells (first column), whereas increased levels (arrowhead) were observed 15 min after LPS treatment (middle column). Pre-exposure to c-TUCB markedly reduced phospho-JNK levels (red) (last column). All images were obtained at 60×.
depleted via peroxisomal β-oxidation (10) and phospholipid bilayer esterification pathways (12).

Because sEH is not expressed in sEH KO mice and their EET levels have been reported to be elevated (30), one might expect a decreased basal expression of MCP-1 in BMMΦ collected from these animals. However, basal MCP-1 release was paradoxically elevated from untreated as well as LPS-stimulated BMMΦ from sEH KO mice. Similarly, Luria et al. (30) showed that although sEH KO mice had elevated plasma levels of 14,15-EET and other cytochrome P-450 epoxygenase metabolites, they did not have decreased basal blood pressure compared with WT mice, which was unexpected since EETs are potent vasodilators (22, 57). However, there were also concomitant fourfold increases in production of the potent vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE) in the sEH KO mouse kidneys and urine (30). It was postulated that the elevated 20-HETE levels counteracted the hypotensive actions of the elevated levels of EETs. Of note, sEH KO mice had markedly less hypotension after an LPS infusion compared with WT mice, and their blood pressure recovered more quickly afterward. It is possible that other pathways are also upregulated in response to sEH inhibition in monocyte/macrophage cells. Other lipoxygenase metabolites (12-HETE and 15-HETE) are known to induce MCP-1 in murine peritoneal macrophages (66), and derangements in their levels may occur in response to sEH deletion, resulting in elevated MCP-1 levels. Further studies are needed to investigate such possibilities.

A previous study has shown that human peripheral blood monocytes isolated by column elution had no detectable sEH activity against trans-stilbene oxide (51). In contrast, our present study demonstrated the expression of sEH in monocyte/macrophages. This finding is supported by four observations in the present study. First, a blocking peptide specific for sEH markedly decreased anti-sEH antibody binding in immunoblots of the human monocyte lysate. Second, the release of MCP-1 and TNF-α from normal human monocytes was inhibited by a selective pharmacological sEHI, c-TUCB. Third, treatment with c-TUCB elevated the EET-to-DHET ratio of 11,12 oxylipins in LPS-stimulated monocytes compared with cells treated with LPS alone. Finally, the sEHI inhibited MCP-1 release from BMMΦ from WT mice, but not in sEH KO mice.

The expression of MCP-1 was elevated in the porcine model. MCP-1 is a potent chemoattractant that recruits peripheral monocytes and memory T cells into sites of inflammation (40). Besides monocytes/macrophages, many other cell types, including T cells, endothelial cells, smooth muscle cells, and fibroblasts, also secrete MCP-1 (33). Increased MCP-1 expression occurs in other experimental models of neointimal hyperplasia. For instance, upregulation of MCP-1 appeared early in arteries after balloon injury in hypercholesterolemic rabbits (35) and femoral artery interpositional venous bypass grafting in rats (55). Treatment using either a neutralizing antibody for MCP-1 or a plasmid coding for NH2-terminal deletion mutant MCP-1 also was effective in inhibiting macrophage infiltration and neointimal hyperplasia in a rat carotid injury (13) and a mouse or monkey periarterial injury model (39). Recently, in-stent restenosis in porcine coronary arteries has been reported to be inhibited by oral treatment with bindarit, a selective small-molecule inhibitor of MCP chemokines (20). Thus the ability of sEHI to inhibit MCP-1 and TNF-α release from monocytes/macrophages makes it a potential therapeutic agent for the treatment of neointimal hyperplasia.

Schmelzer et al. (49) reported that AUDA (another sEHI) decreased plasma levels of IL-6, TNF-α, and MCP-5 in LPS-
challenged mice and concluded that this effect was derived from the blocking of NF-κB nuclear translocation. Olearczyk et al. (42) reported that oral sEH diminished urinary MCP-1, inhibited kidney NF-κB activity, and attenuated macrophage infiltration into the kidney in hypertensive rats (42). Others reported that sEH KO mice rendered hypertensive with DOCA-salt had decreased NF-κB activity, decreased urinary MCP-1, and fewer macrophages in the kidney compared with hypertensive WT mice (31). Herein, LPS induced the nuclear translocation of NF-κB in adherent human monocytes, yet pretreatment with c-TUCB did not inhibit the NF-κB translocation under the conditions tested. This inability of c-TUCB to inhibit NF-κB translocation is consistent with our data showing c-TUCB did not significantly alter IL-6 or MIP-1α release from monocytes, since NF-κB is also a key inflammatory transcription factor involved in the production of these chemokine/cytokines (18, 70). These contradictory findings indicate the role of sEH and EETs in the NF-κB pathway is complex and likely cell specific.

Enzymes in the MAPK pathway (ERK1/2, p38, and JNK) (17) are also activated by LPS exposure (16, 63). Each of these kinases has been reported to participate in MCP-1 and TNF-α secretion in a variety of scenarios, and inhibition of these kinases is associated with decreased MCP-1 and TNF-α release (6, 64, 68). The glucocorticoid dexamethasone inhibits JNK and suppressed MCP-1 release in microglia (71) and TNF-α release in murine macrophages (59). Of note, overexpressing CYP2C9 (an epoxygenase) in endothelial cells resulted in increased intracellular EET levels and a concomitant dephosphorylation (inactivation) of JNK, suggesting that EETs inhibit JNK activity (45). EETs have also been reported to inhibit the phosphorylation (activation) of JNK in hepatocytes (52). In the present study, we showed that the pretreatment with sEH almost completely abolished the phosphorylation of JNK in adherent human monocytes in response to LPS stimulation. Thus, in human monocytes, sEH inhibition likely attenuates MCP-1 and TNF-α release via an effect on the JNK pathway and not the NF-κB pathway. Since NF-κB and other pathways have been shown to mediate the expression of MCP-1 and TNF-α, the inability by sEH to affect the NF-κB pathway in monocytes may explain the lack of complete knockdown of both MCP-1 and TNF-α release by c-TUCB.

This study is the first to report that sEH can inhibit MCP-1 and TNF-α release from monocyte/macrophages. Furthermore, this inhibition appears to be mediated by its effect on the JNK pathway instead of the NF-κB pathway (Fig. 10). The biological activities of MCP-1 and TNF-α, their role in the pathogenesis of neointima in other models, and their high expression level in our porcine AV graft anastomotic tissues suggest that these chemokine/cytokines may play a role in hyperplasia formation in AV graft stenosis. Because inflammation is a likely contributor to stenosis development, sEH expression is increased in the AV graft tissue and prevalent macrophages, and an inhibitor of sEH has shown efficacy against MCP-1 and TNF release in vitro, pharmacological sEH may represent a novel therapeutic approach for anti-stenotic therapy. This work sets the stage for in vivo efficacy studies using sEH in the porcine AV graft stenosis model.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: W.G.S., B.D.H., A.K.C., and C.M.T. concept and design of research; W.G.S. performed experiments; W.G.S. and C.M.T. analyzed data; W.G.S. and C.M.T. interpreted results of experiments; W.G.S. and C.M.T. prepared figures; W.G.S. drafted manuscript; W.G.S., B.D.H., A.K.C., and C.M.T. edited and revised manuscript; W.G.S., A.K.C., and C.M.T. approved final version of manuscript.

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