Anti-inflammatory Effects of ω-3 Polyunsaturated Fatty Acids and Soluble Epoxide Hydrolase Inhibitors in Angiotensin-II–Dependent Hypertension

Arzu Ulu, MSc, Todd R. Harris, PhD,* Christophe Morisseau, PhD,* Christina Miyabe, BS,* Hiromi Inoue, DVM,† Gertrud Schuster, PhD,‡ Hua Dong, PhD,* Ana-Maria Iosif, PhD,§ Jun-Yan Liu, PhD,* Robert H. Weiss, MD,†¶ Nipavan Chiamvimonvat, MD,‖¶ John D. Imig, PhD,** and Bruce D. Hammock, PhD**

Abstract: The mechanisms underlying the anti-inflammatory and antihypertensive effects of long-chain ω-3 polyunsaturated fatty acids (ω-3 PUFAs) are still unclear. The epoxides of an ω-6 fatty acid, arachidonic acid epoxyeicosatrienoic acids also exhibit antihypertensive and anti-inflammatory effects. Thus, we hypothesized that the major ω-3 PUFAs, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), may lower the blood pressure and attenuate renal markers of inflammation through their epoxide metabolites. Here, we supplemented mice with an ω-3 rich diet for 3 weeks in a murine model of angiotensin-II–dependent hypertension. Also, because EPA and DHA epoxides are metabolized by soluble epoxide hydrolase (sEH), we tested the combination of an sEH inhibitor and the ω-3 rich diet. Our results show that ω-3 rich diet in combination with the sEH inhibitor lowered Ang-II, increased the blood pressure, further increased the renal levels of EPA and DHA epoxides, reduced renal markers of inflammation (ie, prostaglandins and MCP-1), downregulated an epithelial sodium channel, and upregulated angiotensin-converting enzyme-2 message and significantly modulated cyclooxygenase and lipoxygenase metabolic pathways. Overall, our findings suggest that epoxides of the ω-3 PUFAs contribute to lowering systolic blood pressure and attenuating inflammation in part by reduced prostaglandins and MCP-1 and by upregulation of angiotensin-converting enzyme-2 in angiotensin-II–dependent hypertension.

Key Words: ω-3 polyunsaturated fatty acids, EPA, DHA, soluble epoxide hydrolase inhibitors, angiotensin-II–dependent hypertension

(*J Cardiovasc Pharmacol*™ 2013;62:285–297)

INTRODUCTION

Cardiovascular disease (CVD) is one of the main causes of death worldwide, and thus is the subject of numerous studies to explore potential new treatment strategies. Cardiovascular risk of stroke, coronary artery disease, and hypertensive end-organ damage is increased by hypertension.[1] High blood pressure (BP) can be reduced by certain dietary modifications, such as an increased consumption of long-chain ω-3 polyunsaturated fatty acids (ω-3 PUFAs) found in fatty fish or fish oil.[2] The American Heart Association recommends that healthy adults consume a serving of fish at least 2 times a week and patients with coronary heart disease take a supplement of 1 g of eicosapentaenoic acid (EPA) (20:5) and docosahexaenoic acid (DHA), 22:6 every day.[3] In addition to hypertension, the long-chain ω-3 PUFAs have the potential to protect against many CVDs,[4,5] especially coronary heart disease and atrial fibrillation. These fatty acids have been shown to exert anti-inflammatory,[6–8] antiatherosclerotic,[9] and antiarrhythmic effects.[2,10–12] There is a general feeling that consumption of ω-3 lipids reduces cardiovascular risk; however, some of the individual studies lack power to draw solid conclusions, whereas some others have failed to show efficacy.[13–17] The mechanisms of action of ω-PUFAs are not well understood.

In the ω-6 fatty acid series, metabolites of arachidonic acid (ARA) are well known to influence inflammation and CVD.[18–20] Of particular interest, the epoxides of ARA [epoxyeicosatrienoic acids (EETs)] have anti-inflammatory and antihypertensive properties similar to the ones observed with the long-chain ω-3 PUFAs.[21–23] Moreover, in vitro DHA epoxides

Received for publication August 13, 2012; accepted April 24, 2013.

From the *Department of Entomology and UC Davis Comprehensive Cancer Center, University of California, Davis, CA; †Division of Nephrology; ‡Department of Nutrition; ‖Division of Cardiovascular Medicine, Department of Internal Medicine; §Division of Biostatistics, Department of Public Health Sciences, School of Medicine, University of California, Davis, USA; ¶US Department of Veterans' Affairs Medical Center, Sacramento, CA; and **Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, WI.

Supported by NIEHS grant R01 ES002710 and NIEHS Superfund Research Program grant P42 ES004699; NIH grants 5UO1CA86402 (Early Detection Research Network), 1R01CA135401-01A1, and 1R01DK082690-01A1; and the Medical Service of the US Department of Veterans' Affairs (all to R.H. W.). Analytical work was partially supported by the NIH and NIDDK grant U24 DK097154. Studies and analyses performed by J. D. Imig were supported by NIH grants HL59699 and DK38226. Analyses performed by A.-M. Iosif were supported by the National Center for Advancing Translational Sciences, NIH, through grant UL1 TR000002. T. R. Harris is supported by NIH T32 training grant in basic and translational cardiovascular science (T32 HL86350). B. D. Hammock is a George and Judy Marcus senior fellow of the American Asthma Foundation, and was supported by NIEHS grant R01 ES002710 and NIEHS Superfund Research Program grant P42 ES004699.

The University of California, Davis, has filed patents in the area of soluble epoxide hydrolase inhibitors for the treatment of diseases.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.jcvp.org).

Reprints: Bruce D. Hammock, PhD, One Shields Avenue, Department of Entomology, University of California, Davis CA 95616 (e-mail: bdhammock@ucdavis.edu)

[epoxydocosapentaenoic acids (EpDPEs)], similar to EETs, activate a large-conductance Ca^{2+}-activated K^+ channel[24,25] that lead to vasodilatation ex vivo.[26] Recently, regioisomeric mixtures of EPA epoxides [epoxyeicosatetraenoic acids (EpETEs)] and EpDPEs were shown to reduce inflammatory pain, in which EpDPEs were demonstrated to be more effective in reducing the nociceptive pain than the EpETEs and EETs.[27] Interestingly, the regioisomers of DHA epoxides show unequal effects on inflammatory pain. The bioactive ω-3 PUFAs compete with ARA for metabolism by cytochrome P450 enzymes (CYP), specifically CYP2C and 2J, which largely generate the EETs.[28,29] Based on these observations, we hypothesized that EPA and DHA lower arterial BP and attenuate renal markers of inflammation in vivo at least in part through their epoxide metabolites in angiotensin-II–dependent hypertension. To address this hypothesis, we conducted an experiment that included controls and Ang-II–infused animals with and without an ω-3 rich diet. We predicted that hypertensive animals treated with an ω-3 rich diet will have lower BP as compared to their Ang-II–infused counterparts. Like the EETs, the EPA and DHA epoxides are very good substrates for the soluble epoxide hydrolase (sEH).[27] Such hydrolytic metabolism has been shown to reduce the bioactivity of EETs,[30,31] and it should also reduce the bioactivity of these CYP-generated epoxy fatty acids (EpFAs) that are derived from EPA and DHA. Consequently, we tested the additional hypothesis that the presence of an sEH inhibitor (sEHI) will further increase the effectiveness of the parent compounds by increasing the tissue levels of the epoxide metabolites, EpDPEs and EpETEs. To address these additional hypotheses, we also included Ang-II–infused animals treated with the combination of ω-3 rich diet and sEHI (at low and high doses) and those treated only with the sEHI to control for the potential antihypertensive effect of the sEHI. We expected to observe lower BP in Ang-II–infused animals treated with the combination of the ω-3 rich diet and sEHI as compared to Ang-II–infused animals treated only with the ω-3 rich diet. To this end, we supplemented animals with an ω-3 rich diet in the presence of a potent sEHI in a murine model of angiotensin-II–dependent hypertension.

Metabolic profiling was used to directly quantify the metabolites of the ω-3 and ω-6 PUFAs to gain insights into the mechanism of action of the treatment with ω-3 rich diet and the combination of the ω-3 rich diet and sEHI. We observed that the ω-3 rich diet, sEHI, and the combination lower systolic blood pressure (SBP) in Ang-II–dependent hypertension. The findings of this study are consistent with our hypothesis that CYP metabolites of ω-3 PUFAs that are stabilized by sEH inhibition have a role in lowering Ang-II dependent increase in BP and in significant modulation of the COX and LOX metabolic pathways in the ARA cascade.

METHODS

Animals and Treatments

All animal studies were approved by the University of California Davis Animal Use and Care Committee and were performed in accordance with the National Institutes of Health Guide for the care and use of laboratory animals. We avoided using the C57/BL6 strain, which is poorly responsive to Ang-II compared to some other strains of mice.[32] Instead, we used Swiss Webster mice, in which the oxylipin profiles and the pharmacokinetics of the sEHIs have been well characterized,[33,34] and who do not develop renal damage induced by angiotensin-II. This allowed examining the effects of the ω-3 PUFAs solely on changes in the BP and in renal eicosanoids. Eight week old male Swiss Webster mice (Charles River Laboratories, Wilmington, MA) were acclimated to new housing conditions for 1 week and were kept under a 12 hour light–dark cycle with free access to water and food for the duration of the experiment. Baseline BPs were established for each group of mice based on the average BP taken for 3 days before treatment. Hypertension was induced by infusion of Ang-II at a constant rate (20 ng/min or 1 $mg^{-1} \cdot kg^{-1} \cdot d^{-1}$) for 14 days using subcutaneously implanted osmotic minipumps (Model 1002-Alzet, Cupertino, CA).

Mice were fed either a purified control diet (5% corn oil) or an ω-3 rich diet consisting of the 2 major long-chain ω-3 fatty acids, EPA (0.75%) and DHA (0.75%) at 90% purity (Larodan Fine Chemicals, Sweden). In the control diet, ω-3 fatty acids replaced corn oil to retain constant dietary fat. The detailed composition and preparation of the diets are given in **Supplemental Digital Content 1** (see **Table S1A**, http://links.lww.com/JCVP/A121). Based on the fatty acid analysis of each diet (see **Section A, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which describes the method for fatty acid analysis), the total percentage of the ω-3 PUFAs was 0.6% and 23% for corn oil and ω-3 rich diet, respectively (Table S1B, which presents the fatty acid composition of the ω-3 rich diet). Animals were randomly divided into 6 experimental groups: Animals in the control group underwent sham surgery and the vehicle treatment, drinking water with 1% PEG400. All groups receiving Ang-II are indicated by A in the group designation. A second group received Ang-II and the vehicle treatment (group A). The other groups receiving Ang-II were randomly assigned to receive the ω-3 rich diet (group A-ω3), sEHI (TPPU or UC1770,[35] 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea) alone (group A-TH), or the combination of the ω-3 rich diet and sEHI at a low (group A-ω3-TL) and high dose (group A-ω3-TH). All groups receiving the sEHI TPPU are indicated by T. The 2 sEHI doses were 0.2 and 0.6 mg/kg, for TL (low dose TPPU) and TH (high dose TPPU), respectively. Additionally, we included a group treated only with Ang-II (group A′) and a group treated with Ang-II and the low dose sEHI (0.2 mg/kg) (group A′-TL) to test the effect of sEHI alone on the BP in mice on a standard rodent chow diet (see **Table S1B, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which shows the fatty acid composition of the standard diet).

The ω-3 rich diet was supplemented 1 week before the induction of hypertension by Ang-II. The potent sEHI, TPPU was administered in the drinking water with the cosolvent 1% PEG400 (polyethylene glycol) on the same day Ang-II infusion started. Fresh water containing TPPU was readministered to the animals every 4 days. After administration of the diet for 3 weeks, the animals were euthanized and tissues were harvested and immediately frozen in liquid nitrogen to be stored at −80°C until analysis.

Measurement of BP

SBP was measured using a noninvasive tail-cuff blood pressure system (Kent Scientific Corporation, Torrington, CT), as previously described.[36–38] Animals were acclimated to the tail cuffs and the restraining procedure for 15 minutes for 3 consecutive days, and then the actual measurements were performed every day at the same time by the same qualified operator. The operator was blinded by placing cards in front of the cages to block the treatment information. The cages with those blocking cards were numbered and the data were saved with those assigned numbers, which allowed blinded data analysis as well. A standard method was followed for each animal to analyze the BP data. The first 3 values (acclimation values), highest and lowest values, and those that are associated with animal movement were discarded, and the remaining readings were averaged to determine the SBP (see **Section B, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which describes the measurement of SBP in detail).

Quantification of Oxylipins and TPPU in the Kidney

At the end of the Ang-II infusion, renal oxylipins and TPPU levels were determined using a solid phase extraction (SPE) method followed by a liquid chromatography electrospray ionization tandem mass spectrometry (LC/MS/MS) technique as previously described (see **Section C** and **Table S2, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which details oxylipin extraction and lists the analytes quantified along with their ionization conditions for LC/MS/MS analysis).[39,40]

Measurement of Proinflammatory Cytokines in the Kidney

The concentration of common proinflammatory cytokines, IL-1β, IL-6, tumor necrosis factor-α, and monocyte chemotactic protein-1 (MCP-1) were determined using a mouse selective 4-plex kit (Bio-Rad, Hercules, CA) as described before.[41] Briefly, 100 mg kidney tissue excised from the renal cortex was subjected to lysis using a Bio-Rad cell lysis kit. The assay was performed according to the instructions of the manufacturer. The obtained concentrations were normalized to the protein concentration of the lysate, and the results were reported as pg/mg protein.

Real-time Quantitative Reverse Transcription Polymerase Chain Reaction

Fold change in the mRNA expression of selected genes was determined using quantitative reverse transcription polymerase chain reaction. Relative quantification of these data was processed by ΔΔ-Ct (cycle threshold) method (see **Section D, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which details the RT-PCR method).

Statistical Analyses

All variables were summarized as mean ± SEM. The effects of Ang-II, ω-3 rich diet, and TPPU on BP over the course of the study were analyzed using mixed-effects models for longitudinal data. This approach allowed the use of all available data for each animal, whereas accounting for the correlated nature of the data because of the repeated measurements on the same individual. The model was validated with residual diagnostics.[42] In secondary analyses, we examined cumulative measures by calculating the area under the BP–time curve with respect to the control group, both for the whole period of the study and for the past 3 days of measurements (days 7, 9, and 11). The remaining data (oxylipins, cytokines, gene expression, etc) were analyzed for the effects of treatment (ω-3 rich diet, TPPU, or the combination) on dependent measures. In cases where normality was met, random-effects 1-way ANOVA (analysis of variance) was performed, followed by pairwise comparisons (corrected for multiple comparisons using a layered Bonferroni correction[43]). If the normality test failed, a Kruskal–Wallis 1-way ANOVA on the ranks was conducted, followed by Dunn's post hoc tests. A threshold of $P < 0.05$ was used in the above tests. Correlation between the variables was tested using Pearson's correlation, and the significance of the correlation was tested using a 2-tailed t test. All statistical tests were conducted in SPSS (IBM SPSS Statistics, version 19) except for analyses of residuals, which were conducted in SAS Institute, I, 2002–2010 (SAS/STAT Version 9.3, Cary, NC).

RESULTS

ω-3 PUFAs Do Not Alter Food Intake or Weight Gain in Mice

There were no significant differences detected in food intake (mice on the purified control diet consumed 4–5 g/d and mice on the ω-3 rich diet consumed 3–4 g/d) and in the body weight gain among the treatment groups during the intervention period. Weight gain in all treatment groups was as follows: Control: 2 ± 1 g, A: 1.4 ± 0.9 g, A-ω3: 1.1 + 0.5 g, A-ω3-TL: 1.6 ± 0.2 g, A-ω3-TH: 2 ± 0.5 g, and A-TH: 1.3 ± 0.4 g. Thus, our results fail to support that the ω-3 PUFAs significantly alter the food intake or weight gain in mice during the course of this study.

ω-3 PUFAs Exerted Antihypertensive Effects in Ang-II-induced Hypertensive Animals

The time course of changes in BP across all the groups is shown in Figure 1. We examined the overall effect of Ang-II and ω3/TPPU treatments on the SBP data in 2 different ways (mixed-effects models with "group" or "Ang-II, ω-3 rich diet, and TPPU" as between-subjects factor), which generated mutually supporting results. The preliminary analysis of residuals suggested that the treated groups had similar values early on day 1 followed by a sharp increase and then a plateau in the Ang-II treated group (see **Section E** and **Table S3, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which summarizes the residuals diagnostics of the mixed-effects model). Therefore, the mixed-effects model included "group" (control, A, A-ω3, A-ω3-TL, A-ω3-TH, and A-TH) as between-subjects factor and time (days 3–11) as repeated measures. This analysis revealed only a significant main effect of treatment group ($F = 15.75$, $P < 0.01$). Pairwise group comparisons using LSD (least significant difference) tests

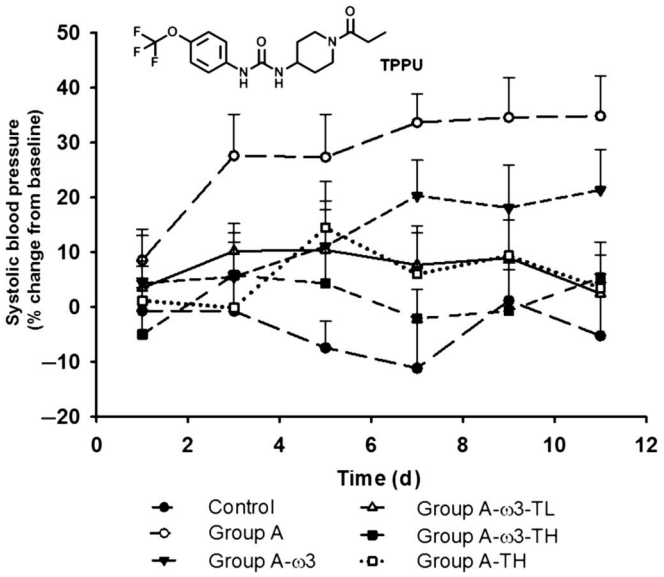

FIGURE 1. Antihypertensive effect of ω-3 PUFAs on angiotensin-II–dependent hypertension. SBP was recorded with a non-invasive tail-cuff system. The *x* axis represents the time after Ang-II delivering osmotic minipumps were implanted. Administration of the ω-3 rich diet started 1 week before the Ang-II infusion. TPPU was administered in the drinking water starting at the same time as Ang-II infusion. Results are given as percentage change from baseline taken before treatment for each group. A indicates angiotensin treatment, ω3 indicates ω3-rich diet, and TL and TH refer to TPPU treatment at low and high doses, respectively. The chemical structure of TPPU is shown. Data are mean ± SEM. Error bars were only shown unidirectional. Controls, n = 5; group A, n = 8; group A-ω3, n = 8; group A-ω3-TL, n = 7; group A-ω3-TH, n = 8; and group A-TH, n = 8.

revealed that Ang-II infusion increased SBP significantly compared to all 4 treatment groups and controls (all $P < 0.05$). Also, the SBP in group A-TH was significantly lower compared to group A-ω3 ($P = 0.03$). Furthermore, SBP was decreased in A-ω3-TH treated–animals as compared to A-ω3 ($P = 0.002$); whereas, in the A-ω3-TL, this decrease missed statistical significance ($P = 0.07$). The SBP in all treatment groups except for A-ω3-TH ($P = 0.106$) differed significantly from that of the controls ($P < 0.05$). To examine the separated effects of treatments on SBP, we analyzed the SBP data using mixed-effects model with Ang-II, ω3-rich diet and TPPU as the 3 between-subjects factors (see **Section E, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121). The results of this analysis generated qualitatively supporting results as compared to the mixed-effects model with "group" as between-subjects factor. To further explore the relationship between A-ω3 and A-ω3-TL groups (and provide further confirmation of the other group differences), we conducted supplementary ANOVAs of the SBP data expressed as area under the curve (AUC) for all days and for days 7–11 (see **Figure S1-Panel A, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121) and a focused analysis for SBP data at day 11 (when the group differences were hypothesized to be maximal; see **Section F, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121). Overall, these additional analyses generated results that were all in agreement with the results of the overall mixed-effects model: the AUC for groups A-ω3-TL and A-ω3-TH was lower than for A-ω3 in both all days and in days 7–11 (though statistical significance was reached only for the A-ω3-TH group). Similarly, at day 11, SBP was lower in A-ω3-TL and A-ω3-TH groups compared to A-ω3 (though in this case, only the former difference reached statistical significance).

We observed that SBP values after 1 week were higher than the earlier days in the experiment in group A-ω3 (Fig. 1, solid reverse triangles), suggesting that the ω-3 rich diet loses some of its effectiveness, with time in lowering BP. Therefore, we performed a post hoc analysis of these data using a paired *t* test (2-tailed), which revealed a statistically significant difference in SBP in the early versus late time points (after 1 week) ($P < 0.05$) in group A-ω3.

The Effect of Diet on Arterial Pressure

There was a large reduction in the SBP with sEHI alone (A-TH) (Fig. 1, open squares). We tested the hypothesis that some of this reduction might be caused by the trace amounts of α-linolenic acid (ALA) in the corn oil diet acting in concert with the sEHI TPPU. We switched mice to a standard diet (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, Hayward, CA) that was previously used as a control diet to accompany an ω-3 rich diet. The standard diet had higher amounts of ALA than the corn oil diet used in our study[44] (see Table S1B). We anticipated that this difference in the amount of ALA might reflect itself on changes in SBP and offer an explanation for the large reduction in SBP in animals treated with sEHI alone (group A-TH). We predicted that animals on the diet with higher amount of ALA will have lower BP as compared to those on the diet with lower amount of ALA. The mice were held on the standard diet for a week before beginning the experiment. After a 3-day acclimation period, baseline BP of these mice increased to 113 ± 2 mm Hg, which was slightly higher than the baseline BP of animals that were on either the corn oil or the ω-3 rich diet (107 ± 4 mm Hg). On the standard diet, the BP increased from 113 ± 2 mm Hg to 157 ± 3 mm Hg (excluding baseline) in group A′, a significant change of 44 mm Hg (see **Figure S2A, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which demonstrates time course of SBP in mice on a standard diet). In contrast to this increase in SBP to 157 ± 3 mm Hg on the standard diet in group A′, SBP increased only to 122 ± 4 mm Hg on the corn oil diet as in group A. The SBP resulted in an average of 140 ± 2 mm Hg in group A′-TL, indicating that TPPU lowered but did not reduce SBP back to baseline at 0.2 mg/kg dose (Figure S2A). This reduction in SBP corresponds to a percentage change from baseline of 23 ± 2% when compared to a percentage change from baseline of 7 ± 1% in group A-ω3-TL (Fig. 1, open triangles). This compares to a reduction to 107 ± 2 mm Hg on the corn oil diet in group A-TH (Fig. 1, open squares). Also, because SBP reached a plateau after day 3 in groups A′ and A′-TL, we calculated the AUCs from the SBP recorded on days 3–9 ($AUC_{t(3–9)}$). Similar to the overall results, in group A′-TL, the $AUC_{t(3–9)}$ was significantly lower than

group A′ (see **Figure S2B, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which shows the AUC $_{t\ (3–9)}$ in mice on a standard diet).

Effects of Treatments on the Plasma Levels of Ang-II and Kidney

We measured the plasma levels of Ang-II to test whether the antihypertensive effect of the ω-3 rich diet is directly associated with decreased plasma levels of Ang-II. Indeed, the plasma levels of Ang-II were reduced in group A-ω3-TL as compared to group A ($P < 0.05$) (see **Figure S3, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which shows plasma levels of Ang-II across all the groups). Inadequate amount of plasma was available from groups A-ω3 and A-TH, and thus, Ang-II levels could not be determined.

Examination of the general histology of the kidney tissue and serum kidney markers such as creatinine and urinary albumin showed no statistically significant differences across the groups as expected (see **Figure S4A and S4B, Sections G and H, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which show glomerular injury scores, renal function parameters, and results/discussion from these analyses).

Possible Mechanisms of Action of ω-3 PUFAs—Insights Gained From Metabolic Profiling

To understand the possible mechanisms by which ω-3 PUFAs affect BP and renal inflammation, we measured the levels of key oxidized metabolites of unsaturated fatty acids in the kidney. Overall, the ω-3 rich diet increased the renal levels of the EPA and DHA metabolites, specifically the epoxide metabolites (Fig. 2A and see **Tables S4 and S5, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which shows the renal levels of the epoxide and diol metabolites of ARA, linoleic acid (LA), EPA, and DHA). The epoxide, diol metabolites and their corresponding epoxide-to-diol ratios are shown in Figure 2A, Panels A–C for EPA, and Panels D–F for DHA. The EPA epoxides, EpETEs, decreased to undetectable levels (except for 8, 9-EpETE) in group A as compared to the controls. However, the renal levels of EpETE regioisomers increased in group A-ω3 (Fig. 2A, panel A). Although epoxide metabolites of DHA (EpDPEs) were more abundant than the EpETEs in the kidney (Fig. 2A, Panels A and D), supplementation of the ω-3 rich diet with TPPU resulted in a larger increase in EpETEs than the EpDPEs. Compared with group A-ω3, the renal levels of EpDPEs and EpETEs further increased in groups A-ω3-TL and A-ω3-TH ($P < 0.05$); however, this increase was not dose-dependent for the 2 doses tested. In parallel, the renal levels of each regioisomer of the DHA epoxides significantly increased in group A-ω3 ($P < 0.05$, Table S4). In contrast to ω-3 fatty acid series, the tissue levels of the epoxide metabolites of the ω-6 fatty acids decreased in group A-ω3. The epoxide, diol metabolites, and their corresponding epoxide-to-diol ratios are shown in Figure 2B, Panels A–C for ARA, and Panels D–F for LA. The availability of ARA and LA epoxides, EETs and epoxyoctadecenoic acid (EpOMEs), respectively, decreased in all groups treated with Ang-II when compared to the controls (Fig. 2B, Panels A and D). In general, the tissue levels of EETs tend to be lower in groups A-ω3, A-ω3-TL, and A-ω3-TH as compared to A-TH. As for EpOMEs, group A-ω3 showed lower tissue levels than A-TH animals. In contrast to the EETs, tissue levels of EpOMEs increased in groups A-ω3-TL and A-ω3-TH when compared to group A-TH.

In mice fed a standard diet, the renal levels of the sum of DHA and EPA epoxides were lower than those in mice fed a corn oil diet; however, EETs and EpOMEs were comparable for each diet (see **Table S6, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which shows the renal levels of the epoxide and diol metabolites of ARA, LA, EPA and DHA in mice fed a standard diet). In addition, the renal levels of EpDPEs and EpETEs but not EETs and EpOMEs were higher in group A-ω3-TL (Table S4) as compared to A′-TL (Table S6).

Considering the high abundance and their likely generation by the sEH, we examined the changes in the renal levels of the diols of EPA and DHA from EpETEs and EpDPEs, respectively [di-hydroxy-eicosatetraenoic acid (DiHETE) and di-hydroxy-docosapentaenoic acid (DiHDPE), respectively] (Fig. 2A, Panels B and E). The renal levels of DiHETE and DiHDPE decreased in group A but normalized in group A-ω3. As expected, treatment with TPPU at either dose (groups A-ω3-TL and A-ω3-TH) resulted in a significant dose-dependent decrease in the production of DiHETE and DiHDPE ($P < 0.01$). Each of the dihydroxy-fatty acid regioisomers of EPA and DHA changed similarly, and thus showed an identical pattern to the summed tissue levels of diols across all the groups. The tissue levels of the diol derivatives of the ω-6 fatty acids, dihydroxyeicosatrienoic acids (DHETs), and dihydroxyoctadecenoic acid (DiHOMEs) decreased in group A as compared to controls (Fig. 2B, Panels B and E). The tissue levels of DHETs but not DiHOMEs decreased in group A-ω3 as compared to group A. The tissue levels of both diols decreased only slightly in group A-ω3-TH in comparison with the A-ω3-TL ($P > 0.05$).

Contribution of sEH to the Antihypertensive Effects of ω-3 PUFAs

To test the hypothesis that the ω-3 PUFAs act in part through their epoxides, we further examined the epoxide-to-diol ratio of the EPA and DHA metabolites across all the groups. The trend of the change in epoxide-to-diol ratio for EPA and DHA followed an almost identical pattern to each other across all the groups (Fig. 2A, Panels C and F). Although the EpETE-to-DiHETE and EpDPE-to-DiHDPE ratios increased only slightly in group A-ω3, the ratios increased significantly in groups A-ω3-TL and A-ω3-TH with a larger increase of the EpDPE-to-DiHDPE ratio in the latter group ($P < 0.01$). In the ω-6 fatty acid series, the renal EET:DHET ratio decreased in all groups as compared to controls (Fig. 2B, panel C). As for the ω-6 linoleate, the EpOME:DiHOME ratio significantly increased in groups A-ω3-TL and A-ω3-TH as compared to groups A, A-ω3, and A-TH (Fig. 2B, panel F). To test if TPPU reached effective levels (above the IC_{50}) for inhibition of sEH, we determined the concentration of TPPU in the kidney: 7 ± 0.8 $\mu g/g_{tissue}$ and 12 ± 0.8 $\mu g/g_{tissue}$, and in the plasma: 610 ± 130 nM and 1800 ± 445 nM in groups A-ω3-TL and A-ω3-TH, respectively.

We observed that the sum of EpDPEs (includes the 10, 11-, 13, 14-, 16, 17-, and 19, 20-EpDPE) have an inverse

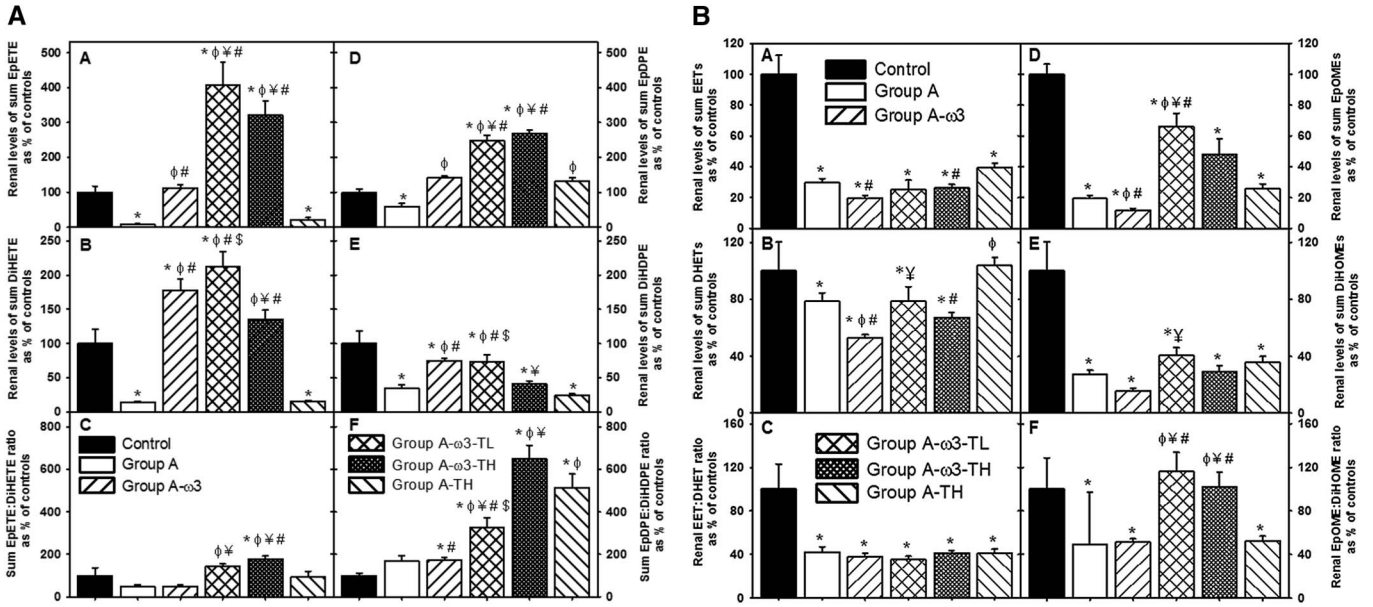

FIGURE 2. Effects of the dietary ω-3 PUFAs and TPPU on the epoxide and diol metabolites of EPA, DHA, ARA, and LA in Ang-II dependent hypertension. The changes in the abundance of the epoxides, diols, and corresponding epoxide-to-diol ratios in response to the given treatments are shown. A, Renal levels of EPA and DHA metabolites are shown in separate Panels. Panel A, sum EpETE (includes the 8, 9-, 11, 12-, 14, 15-, and 17, 18-EpETE); panel B, sum DiHETE (includes the 8, 9-, 11, 12-, 14, 15-, and 17, 18-DiHETE); panel C, sum EpETE:DiHETE ratio; panel D, sum EpDPE (includes the 10, 11-, 13, 14-, 16, 17-, and 19, 20-EpDPE); panel E, sum DiHDPE (includes the 10, 11-, 13, 14-, 16, 17-, and 19, 20-DiHDPE); and panel F, sum EpDPE:DiHDPE ratio. B, Renal levels of ARA and LA metabolites are shown in separate Panels. Panel A, sum EETs (includes the 8, 9-, 11, 12-, and 14, 15-EET); panel B, sum DHETs (includes the 8, 9-, 11, 12-, and 14, 15-DHET); panel C, sum EET:DHET ratio; panel D, sum EpOMEs (includes the 9, 10-, and 12, 13-EpOME); panel E, sum DiHOMEs (includes the 9, 10- and 12, 13-DiHOME); and panel F, sum EpOME:DiHOME ratio. Results are given as percentage of controls (n = 5). Group A, n = 8; group A-ω3, n = 8; group A-ω3-TL, n = 7; group A-ω3-TH, n = 14; and group A-TH, n = 8. Statistically significant differences were determined by 1-way ANOVA followed by pairwise comparisons. $P < 0.05$; *, compared with controls; Φ, compared with group A; ¥, compared with group A-ω3; $, compared with group A-ω3-TH; and #, compared with group A-TH. Data are mean ± SEM. For primary data see Tables S4 and S5.

correlation with SBP (R = −0.36, $P = 0.04$), whereas the sum of EpETEs (includes the 8, 9-, 11, 12-, 14, 15- and 17, 18-EpETE) have a weaker correlation (R = −0.29, $P = 0.1$), but in a similar direction as the EpDPEs. The EpDPE regioisomers, 16, 17- and 19, 20-EpDPE showed an inverse correlation with SBP (R = −0.35, $P < 0.05$). The tissue levels of sum EETs and EpOMEs did not correlate with SBP (R = −0.02 and R = −0.04, respectively).

Inhibition of sEH Enhances the Anti-inflammatory Effects of ω-3 PUFAs in the Kidney

To assess the anti-inflammatory effects of the ω-3 PUFAs, we quantified the tissue levels of common proinflammatory and anti-inflammatory metabolites produced by the COX-2 and LOX (lipoxygenase) enzymes in the ARA cascade (Fig. 3 and Table S7A and B, which show the renal levels of the COX-2 and LOX metabolites of ARA and EPA, respectively).

First, we determined the tissue levels of ARA-derived prostaglandins (PGs) in the kidney. While the tissue levels of PGE_2, PGD_2, and 6-keto-$PGF_{1\alpha}$ increased by approximately 12, 10 and 7 fold; respectively, in group A, the 3 PGs decreased in group A-ω3 ($P < 0.05$, Fig. 3, Panels A–C). In comparison with group A-ω3, PGs decreased to control levels in groups A-ω3-TL and A-ω3-TH. Also, the tissue levels of PGD_2 further decreased in group A-ω3-TL as compared to group A-ω3-TH ($P < 0.01$). Similarly, the tissue levels of thromboxane B_2 and $PGF_{2\alpha}$ increased in group A and decreased in groups A-ω3, A-ω3-TL, and A-ω-TH to control levels (Table S7A).

Second, we examined the changes in the LOX-derived ARA metabolites hydroxy-eicosatetraenoic acids (HETEs). The tissue levels of 11-, 12-, and 15-HETEs increased by 1.5- to 6-fold in group A as compared to controls (Fig. 3, Panels D–F; and Table S7A). In contrast to group A, the tissue levels of these HETEs decreased in group A-ω3, and further decreased in groups A-ω3-TL and A-ω3-TH. The tissue levels of 20-HETE slightly increased in group A ($P > 0.05$), and further increased in groups A-ω3-TL and A-ω3-TH ($P < 0.05$), but not in groups A-ω3 and A-TH (Table S7A).

We also quantified the COX-2 and LOX metabolites that are generated from EPA. Although the major LOX products of EPA, the HEPE (hydroxy-eicosapentaenoic acid) (consisting of the 5-, 8-, 12-, 15-HEPE regioisomers), decreased in group A as compared to controls, the tissue levels of these metabolites tremendously increased in group A-ω3. The presence of TPPU resulted in lower tissue levels of HEPE (except for 5- and 15-HEPE) in groups A-ω3-TL and

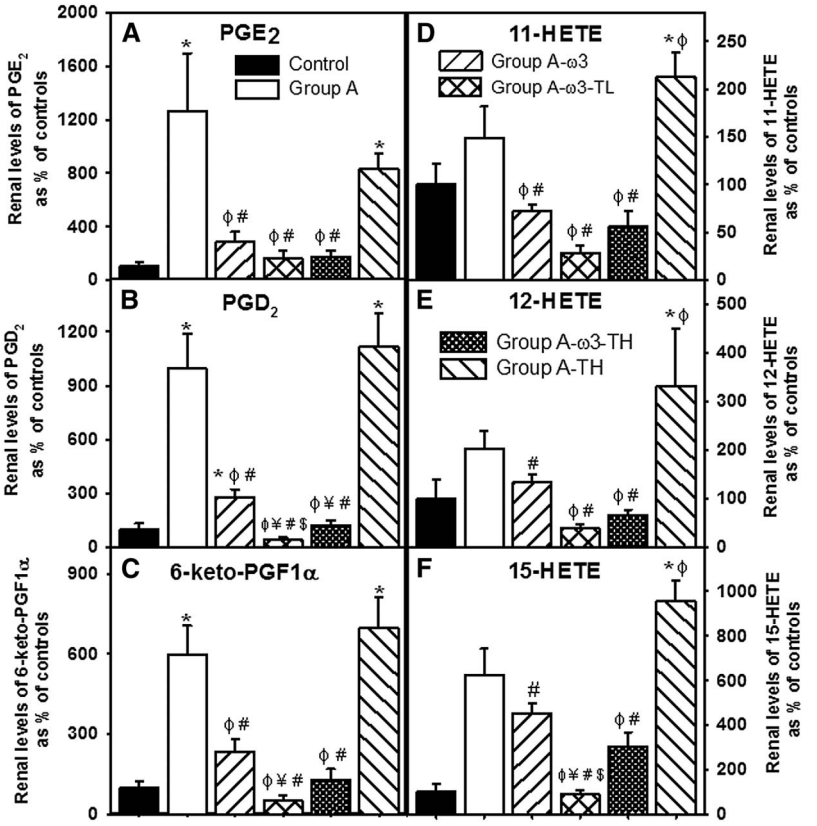

FIGURE 3. Anti-inflammatory effects of the **ω**-3 PUFAs in the kidney in Ang-II dependent hypertension. Inflammatory status in the kidney, reflected by the major PGs (panel A, PGE_2; panel B, PGD_2; and panel C, 6-keto-PGF1α) and reflected by major LOX metabolites (panel D, 11-HETE; panel E, 12-HETE; and panel F, 15-HETE) are shown. Results are given as the percentage of controls (n = 5). Group A, n = 8; group A-**ω**3, n = 8; group A-**ω**3-TL, n = 7; group A-**ω**3-TH, n = 13–14; and group A-TH, n = 7–8. Statistically significant differences were determined by 1-way ANOVA followed by pairwise comparisons. $P < 0.05$; *, compared with controls; **Φ**, compared with group A; ¥, compared with group A-**ω**3; $, compared with group A-**ω**3-TH; and #, compared with group A-TH. Data are mean ± SEM. For original data see Table S7A.

A-ω3-TH as compared to group A-ω3 (Table S7B). In addition, other metabolites of ARA and LA were reduced by the combination of ω-3 PUFAs and TPPU (see **Table S8, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which presents the renal levels of other enzymatic products of ARA and LA). We did not determine the renal levels of the COX-2 or LOX metabolites of DHA, because the analytical methods for the quantification of those metabolites are still in development.

Finally, we determined the concentrations of major proinflammatory cytokines as markers of inflammation in the kidney (Fig. 4 and see **Table S9, Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, which shows the renal levels of the major proinflammatory cytokines across all the groups). As expected, the tissue levels of MCP-1 significantly increased in group A as compared to controls ($P < 0.05$). The tissue levels of MCP-1 decreased significantly in group A-ω3 as compared to group A, and further decreased in group A-ω3-TL as compared to group A-ω3 ($P < 0.01$). The tissue levels of MCP-1 showed an inverse correlation with the renal levels of the sum EpETE and sum EpDPE ($R = -0.47$, $P < 0.01$).

The ω-3 PUFAs Modulate Renal Gene Expression of the ARA Cascade Enzymes, an Epithelial Sodium Channel and Angiotensin-Converting Enzyme-2

To obtain a better mechanistic view, we examined the renal mRNA expression profiles of the major ARA cascade enzymes, an epithelial sodium channel (ENaC) and angiotensin-converting enzyme-2 that are closely related to BP regulation (Fig. 5). First, we examined the effects of the ω-3 PUFAs on the expression of genes encoding the enzymes in the ARA cascade, *Ephx2* (epoxide hydrolase 2), *Ptgs-2* (prostaglandin endoperoxide synthase 2), and *Alox5* (arachidonate 5-lipoxygenase). The renal mRNA expression of *Ephx2* increased 10% in group A (Fig. 5, panel A). In comparison with group A-ω3, *Ephx2* was downregulated in group A-ω3-TL and A-ω3-TH ($P < 0.05$). The downregulation of *Ephx2* exhibited an inverse correlation with the tissue levels of sum EpETEs and sum EpDPEs ($R = -0.4$, $P < 0.01$). The *Ptgs-2* message decreased in the renal cortex across all the groups with a slight downregulation in groups A-ω3-TL or A-ω3-TH ($P < 0.05$) (Fig. 5, panel B). In contrast, *Alox5* message did not change across any of the groups (Fig. 5, panel C).

Next, we examined whether the ω-3 PUFAs may reduce arterial BP by altering the gene expression of an ENaC, and thus regulate sodium balance in the kidney. We determined the mRNA levels of the α-subunit of ENaC gene, namely *Scnn1a* (Fig. 5, panel D). In group A, *Scnn1a* mRNA expression increased by ~ 50% compared with controls. The *Scnn1a* message was not altered in group A-ω3 as compared to group A; however, it was significantly downregulated in group A-ω3-TH ($P < 0.05$) and further downregulated in group A-ω3-TL ($P < 0.01$). The expression profile of the *Scnn1a* message showed a significant inverse correlation with the tissue levels of sum EpETE and sum EpDPE ($R = -0.4$, $P < 0.01$).

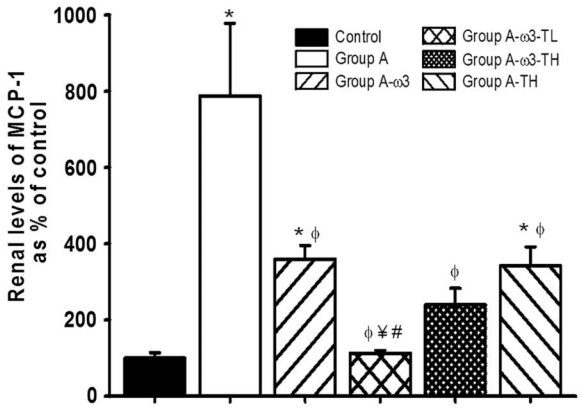

FIGURE 4. Anti-inflammatory effects of the ω-3 PUFAs on MCP-1 in the kidney. The renal levels of a proinflammatory cytokine, MCP-1, were determined in the kidney at the end of the experiment. Results are given as percentage of the controls (n = 5). Group A, n = 8; group A-ω3, n = 8; group A-ω3-TL, n = 7; group A-ω3-TH, n = 13–14; and group A-TH, n = 7–8. Statistically significant differences were determined by 1-way ANOVA followed by pairwise comparisons. $P < 0.05$; *, compared with controls; Φ, compared with group A; ¥, compared with group A-ω3; $, compared with group A-ω3-TH; and #, compared with group A-TH. Data are mean ± SEM. See Table S9 for original data and other cytokines.

Last, we tested the hypothesis that upregulation of the *Ace-2* (angiotensin-I-converting enzyme-2) might be involved in the mechanism of action of the ω-3 PUFAs and sEH inhibition. The *Ace-2* message was upregulated by 3-fold in A-ω3-TL and A-ω3-TH as compared to the controls ($P < 0.05$, Fig. 5, panel E). The *Ace-2* message inversely correlated with last day SBP (R = −0.3, $P = 0.1$), plasma levels of Ang-II (R = −0.5, $P = 0.09$), and the tissue levels of sum EpDPEs and sum EpETEs (R = 0.7, $P < 0.01$). Among the DHA epoxides, the tissue levels of 19, 20-EpDPE and 16, 17-EpDPE showed a strong correlation (R = 0.6, $P < 0.01$), whereas 13, 14-EpDPE showed a moderate correlation (R = 0.4, $P < 0.01$) with the upregulation of *Ace-2*.

DISCUSSION

Our major goal was to elucidate the biology and mechanism of action of the ω-3 PUFAs in a murine model of Ang-II dependent hypertension. We found that the ω-3 PUFAs in combination with an sEH inhibitor contributes effectively to the reduction of Ang-II dependent increase in BP and to the attenuation of the renal markers of inflammation. Metabolic profiling methods that directly quantify the oxylipins that are derivatives of ω-3 and ω-6 PUFAs suggest that the epoxides of the ω-3 PUFAs are in part responsible for the antihypertensive and anti-inflammatory effects of these compounds. In addition, our results suggest that sEH has a significant role in the metabolism and function of the epoxides of the ω-3 PUFAs. Dietary ω-3 PUFAs, when sEH was inhibited, not only increased the bioactive epoxides, but also modulated both the COX and the LOX pathways in the ARA cascade in Ang-II–infused mice. The increase in EpDPEs on supplementation with an ω-3 rich diet and sEH inhibitor and their inverse correlation with the SBP suggest that these

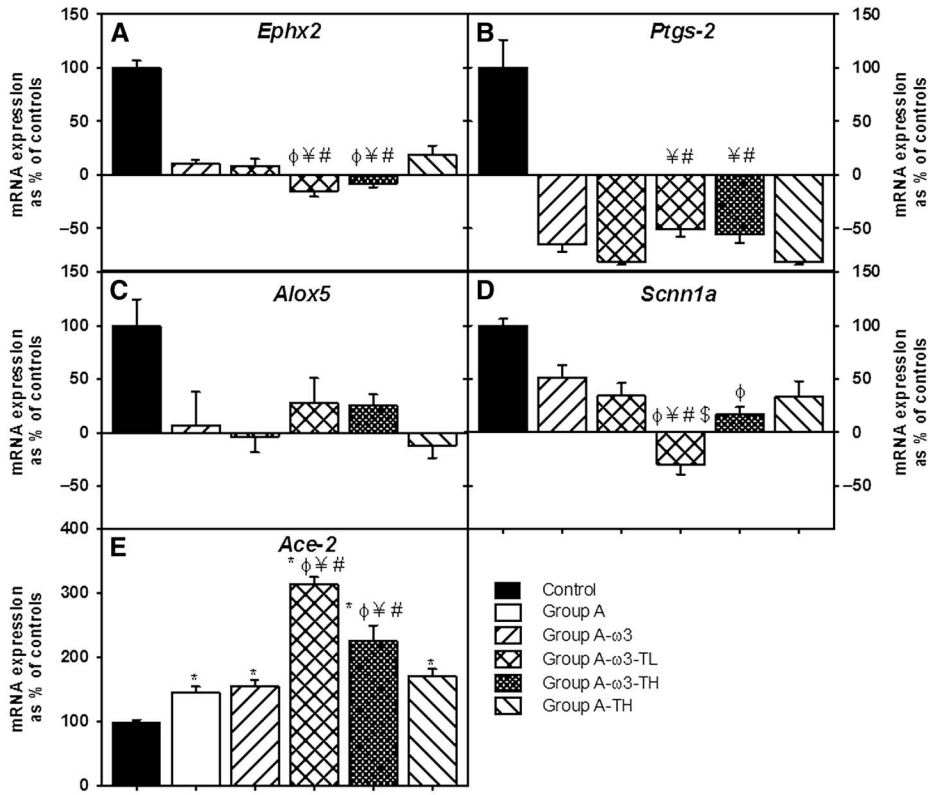

FIGURE 5. Changes in the mRNA expression of genes encoding the major ARA cascade enzymes (*Ephx2*, *Ptgs-2*, and *Alox5* genes) and genes encoding an ENaC, α-ENaC (*Scnn1a* gene), and an angiotensin I-converting enzyme-2 (*Ace-2*) in the kidney. Panel A, *Ephx2*; panel B, *Ptgs-2*; panel C, *Alox5*; panel D, *Scnn1a*; and panel E, *Ace-2*. Positive mRNA expression values represent upregulation, whereas negative values represent downregulation of the gene of interest. Results are given as percent of the controls (n = 5). Group A, n = 8; group A-**ω**3, n = 8; group A-**ω**3-TL, n = 7; group A-**ω**3-TH, n = 14; and group A-TH, n = 8. Statistically significant differences were determined by 1-way ANOVA followed by pairwise comparisons. $P < 0.05$; *, compared with controls; **Φ**, compared with group A; ¥, compared with group A-**ω**3; $, compared with group A-**ω**3-TH; #, compared with group A-TH. Data are mean ± SEM.

epoxides contribute to the reduction of SBP and attenuation of renal inflammation in Ang-II–dependent hypertension. Furthermore, it seems that upregulation of *Ace-2* and *Scnn-1a* might contribute to the mechanism of action of the ω-3 PUFAs and sEH inhibition.

Antihypertensive Effects of the ω-3 PUFAs

Animals receiving Ang-II had significantly higher SBP compared to the rest of the groups suggesting that treatment with the ω-3 rich diet, TPPU or combination of both were effective in lowering BP in this model. The antihypertensive effects of the ω-3 PUFAs have been demonstrated in spontaneously hypertensive rats and in humans.[12,45,46] Consistent with these previous reports, the ω-3 PUFAs reduced SBP in Ang-II dependent hypertension. The ω-3 PUFAs were as effective as TPPU at 0.6 mg/kg in reducing Ang-II induced hypertension for the first several days of exposure (Fig. 1); however, this positive effect decreased with time to where the 2 doses of sEHI with or without the ω-3 rich diet were better than the ω-3 rich diet alone. We do not have a full explanation for the decreased antihypertensive effect observed with the ω-3 rich diet on days 7, 9, and 11 of the study (Figure S1). However, coadministration of the ω-3 rich diet with an sEHI resulted in a return to near normotensive BP. The results of the mixed-effects model with Ang-II, ω-3 rich diet and TPPU as between-subjects factor supported these findings that main effects of each of these factors are statistically significant, and there is an interaction between the ω-3 rich diet and TPPU in reducing SBP after day 1 in Ang-II–dependent hypertension.

Furthermore, our hypothesis that by the end of the treatment period, the combination of the ω-3 rich diet and TPPU would have a stronger effect on SBP compared to the ω-3 rich diet alone was supported by the results of the ANOVA of SBP at day 11, which showed significantly lower SBP with A-ω3-TL, and a trend for lower SBP with A-ω3-TH ($P = 0.08$). However, it is likely that the SBP decrease in A-ω3-TH also represents a real effect rather than an artifact, because the mixed-effects model for the SBP data and AUC analysis for days 7 through 11 (Figure S1, panel A) all showed significantly lower SBP with A-ω3-TH compared to the ω-3 rich diet alone. Because there were no detectable differences in the SBP between groups A-ω3-TL and A-ω3-TH (see Figure S1, panel B), the 2 doses of TPPU studied here did not result in a dose-dependent effect on the BP. Furthermore, the sEHI in combination with the ω-3 rich diet clearly reduced biochemical indicators of inflammation in the kidney better than either treatment alone.

Contribution of Diet to Arterial Pressure

Mice are known to concentrate ω-3 PUFAs in a very short time even on a low-fat diet,[47] and ALA was shown to prevent ω-3 PUFA deficiency–induced hypertension in the offspring that are on a 10% ALA rich diet during the prenatal period and 24 weeks until after the weaning.[48] In our study, we used a control diet supplemented with corn oil compared to a diet rich in ω-3 PUFAs. The sEHI alone resulted in a significant reduction of SBP in animals on the corn oil diet (Fig. 1). This could be in part due to the sEHI stabilizing trace levels of epoxides from ω-3 PUFAs derived from ALA present in the corn oil (Table S1B). Of course, sEHIs are well known to stabilize epoxides of ω-6 PUFAs, notably the antihypertensive EETs. Therefore, we hypothesized that the ω-3 PUFAs in the corn oil diet that are comprised mostly of ALA (Table S1B) might have contributed to the normalized BP in group A-TH. To this end, we used a second control diet from Harlan that was previously used as a standard diet to compare with an ω-3 rich diet. When we put mice on the standard diet for 7 days, Ang-II infusion increased BP as expected (Figure S2A). A low dose of sEHI of 0.2 mg/kg still reduced BP, which is consistent with previous studies.[37,49,50] Our data lacked the power to determine whether this reduction was different from the high dose of sEHI alone on our standard diet without ω-3 lipids.

Even though the amount of ALA was lower in the corn oil diet as compared to the standard diet, we observed higher renal levels of the DHA and EPA epoxides in mice fed a corn oil diet as compared to mice fed a standard diet. In contrast to the epoxides of ω-3 PUFAs, the ARA and LA epoxides did not differ in mice on these 2 different diets. The differences in the SBP and renal DHA and EPA epoxides emphasize the contribution of the diet to the changes in BP. We do not have a full explanation of why the amount of ALA did not reflect itself in tissue levels in our experiment. Of course, ALA conversion into DHA is low, and small changes in levels of DHA epoxides likely are overshadowed by endogenous EETs, which have strong antihypertensive effects. However, the higher tissue levels of EPA and DHA epoxides in animals fed a corn oil diet are consistent with the lower SBP observed in this group. We do not know whether there are dietary sources other than the ALA that might explain the increase in tissue levels of DHA and EPA in mice fed the corn oil diet as compared to mice fed the standard diet (see **Supplemental Digital Content 1**, http://links.lww.com/JCVP/A121, footnote in Table S1B explaining the differences in formulation and analysis of the fatty acid composition between the corn oil and standard diet).

Based on these results, it seems that mainly the increase in the EPA and DHA epoxides and not the ALA content of the diet are major contributors to the normalized BP in group A-TH. However, because of the significant increase in the tissue levels of the epoxides of ω-3 PUFAs and lower SBP in group A-ω3 as compared to group A, we cannot attribute the observed antihypertensive effects to sEH inhibition alone in groups that received the combined treatment. Moreover, the data showing that BP is only normalized in A-ω3-TH and not in A-TH suggest that ω-3 PUFAs (in addition to TPPU) also contribute to the reduction in BP in Ang-II dependent hypertension. Also, because it is hard to remove all ω-3 lipids from the diet, we cannot say that the action of the sEHI occurred in the absence of ω-3 lipids (ie, in group A-TH). However, we can say that the sEH inhibitor was effective even at the low dose in reducing BP to a certain extent even when the amount of ω-3 lipids in the diet is low.

Overall, the decrease in the effectiveness of the ω-3 PUFAs at days 7 through 11 (Fig. 1), and the data showing that TPPU alone does not reduce BP back to control levels at the low dose (Figure S2A) suggest that both the ω-3 rich diet and TPPU were needed to maintain a stable reduction in BP as in A-ω3-TL and A-ω3-TH throughout the experiment. In support of this argument, *ACE-2* message was downregulated

and *Scnn1a* message was upregulated in groups A-ω3-TL and A-ω3-TH as compared to A-ω3 and A-TH (Fig. 5). Also, the anti-inflammatory effects (PGD_2, 6-keto-PGF1α, 15-HETE, and MCP-1) observed with group A-ω3-TL as compared to groups A-ω3 and A-TH are consistent with the combined effects of the ω3-PUFAs and TPPU.

This observation is further supported by the mixed-effects model with 3 between-subjects factor. As summarized in Table S3, both the ω-3 rich diet and the sEHI treatments contribute to reduced BP as indicated by the arbitrary units derived from the model. This model additionally indicates that sEHI treatment contributes more to reduced BP alone or in combination with the ω-3 PUFAs than the ω-3 alone ($P = 0.004$).

Potential Role of Epoxide Metabolites in the Action of ω-3 PUFAs

The overall increase in the EPA and DHA metabolites suggests that 1 week pretreatment and the following 2-week intervention with the ω-3 rich diet allowed enough time for EPA and DHA to be incorporated into the membranes and to be accumulated in the tissues. The inverse correlation of EpDPEs with BP is consistent with the hypothesis that EpDPEs contribute to lower BP in mice on the ω-3 rich diet. Moreover, this correlation was stronger for EpDPEs than for EpETEs. Given the high abundance of EpDPEs in the kidney, these results suggest that DHA epoxides contribute to the reduction of SBP more than the EpETEs. Collectively, these results support the notion that DHA has antihypertensive effects as has been previously shown in spontaneously hypertensive rats.[46,51] DHA has further been shown to activate large-conductance Ca^{2+}-activated K^+ channels on vascular smooth muscle cells[25] and cause vasodilatation.[24] These antihypertensive effects of DHA have been shown to be dependent on CYP epoxygenase activity. Although our results suggest that DHA is more important than EPA in the kidney in Ang-II–dependent hypertension, recent recommendations in the literature propose consumption of both EPA and DHA for maximum benefit from the ω-3 PUFAs in cardiovascular diseases.[3] In addition, information on the total fatty acid composition of the renal tissue would be helpful to understand the contribution of the major parent fatty acids (ARA, ALA, DHA, and EPA) to the biosynthesis of the EPA and DHA epoxide metabolites.

We provide evidence that sEH inhibition increases the epoxide-to-diol ratio of the EPA and DHA epoxides in vivo. Assessment of the epoxide-to-diol ratio also allowed us to elucidate the contribution of the sEH to the metabolism of epoxides that are derived from EPA and DHA. We show that TPPU increases the epoxides of both EPA and DHA and reduces their corresponding diols, which results in an increased epoxide-to-diol ratio. These observations are in line with previous reports suggesting a key role for sEH in the metabolism and function of DHA and EPA epoxides, which are excellent substrates for sEH.[27,28] However, the effects of TPPU on the tissue levels of DHA and EPA epoxides were not dose-dependent at the doses selected in our study. Our findings agree with studies demonstrating that EpDPEs are more abundant than the EpETEs.[27,29] The ratio of EpDPEs to DiHDPEs provides a better indication of sEH inhibition (target engagement) than the corresponding EET:DHET ratio. Accordingly, tissue levels of TPPU were at least 1000 times above its IC_{50} (2 nM or 2.50 10^{-4} $\mu g/g_{tissue}$) determined against the recombinant mouse sEH using fluorescent α-cyanocarbonate as substrate. This suggests near-complete inhibition of sEH by TPPU in the kidneys. There are possible explanations for the unchanged EET:DHET ratio. The higher abundance of EPA and DHA, which competes with ARA metabolism, might have affected the availability of the precursor fatty acid reservoir. Of course, further metabolism and excretion of the diols and alternate routes of epoxide metabolism including relative incorporation into phospholipids could have an impact.[52] These results are consistent with our hypothesis that EpDPE regioisomers have a role in the mechanism of action of DHA in reducing Ang-II induced hypertension. Among the DHA epoxides, we found 19, 20-EpDPE to be the most abundant DHA epoxide in the kidney. However, it is currently not possible to make assumptions based on its high abundance whether this would be the most effective regioisomeric DHA epoxide in reducing BP and inflammation. The 3 regioisomeric epoxides of DHA, 13, 14-, 16, 17-, and 19, 20-EpDPE have been shown to have excellent antihyperalgesic activities in a rat inflammatory pain model.[27] Among these 3 regioisomeric DHA epoxides, 19, 20-EpDPE has been shown to be the most abundant regioisomer in the central nervous system of these rats; however, a less abundant regioisomeric epoxide, 13, 14-EpDPE is found to be more efficacious than the 19, 20-EpDPE in this model. While studies examining the vascular reactivity and channel activity in response to DHA epoxides support the vasodilator effects of DHA epoxides,[24,26,53] the bioactivity and efficacy of different regioisomeric epoxides of DHA in hypertension needs to be explored in future studies.

Inhibition of sEH Increases the Efficacy of the Anti-inflammatory Effects of the ω-3 PUFAs in the Kidney

We examined the changes in renal eicosanoid levels across the treatment groups, because these metabolites are known to effectively contribute to the long-term regulation of BP in the kidney. As expected, we found that the ω-3 PUFAs were more effective in attenuating renal markers of inflammation when sEH was inhibited at either dose than when administered alone. This increased effectiveness might be because of an increase in the epoxides when sEH was inhibited. Of the 2 doses, the anti-inflammatory effects of the ω-3 PUFAs were most evident in the presence of the lower dose TPPU. This is an advantageous effect, because intake of drugs or drug-like compounds at high doses is usually associated with a higher risk of adverse effects in comparison with intake at low doses. Even the higher dose of TPPU (0.6 mg/kg) used in this study, can still be considered a low dose when compared to many drugs on the market. For example, NSAIDs are usually taken at high doses, which would be equivalent to over 1 or more mg/kg dose in mice. In group A-ω3-TL, we observed enhanced tissue levels of the epoxides (ie, EpETEs and EpDPEs), reduced renal

PGs (primarily PGE_2 and PGD_2), reduced LOX products (mainly HETEs), and reduced MCP-1 levels in comparison with group A-ω3 (Figs. 2–4). Among the LOX products, the proinflammatory and the powerful vasoconstrictor, 20-HETE, followed a different pattern than the other HETE regioisomers across the groups, which might be because this metabolite is produced by a CYP rather than an LOX enzyme.

Overall, these results are consistent with our hypotheses that the epoxide metabolites of ω-3 PUFAs can be stabilized by sEH inhibition and EpDPEs and EpETEs contribute to the observed anti-inflammatory effects of their corresponding parent fatty acids in Ang-II–dependent hypertension. Moreover, the inverse correlation between the tissue levels of MCP-1 and that of epoxides offers a promising clue to the mechanism of action for the anti-inflammatory effects of the ω-3 PUFAs in the kidney in Ang-II dependent hypertension. Based on the data on the largely inflammatory cytokines MCP-1, IL-1β, and IL-6, the lower dose of TPPU at 0.2 mg/kg with the ω-3 PUFAs has an additive effect in reducing these indicators of renal inflammation (Fig. 4). This is possibly because TPPU alone significantly reduced the tissue levels of the proinflammatory cytokines MCP-1, tumor necrosis factor-α, and IL-1β ($P < 0.05$) as compared to group A (Table S9). Not only the tissue levels of MCP-1 but also the diol metabolites of EPA, DHA, and ARA decreased after TPPU treatment in our study (Fig. 2A and B, Panels B and E). This finding is consistent with a recent study reporting that the diol metabolites that are derived from ARA, the DHETs, promote MCP-1 mediated–monocyte chemotaxis, and suggesting that sEH inhibitors act in part by inhibiting MCP-1 induced–monocyte chemotaxis through a decrease in DHET levels.[54] It was also intriguing that the ω-3 rich diet showed a further decrease in the tissue levels of the LOX metabolites when sEH was inhibited. The metabolites in the LOX pathway altered by ω-3 PUFAs are largely proinflammatory,[55] and thus the observed decrease is indeed a favorable anti-inflammatory effect.

DHA has been shown to alter the major PGs and LOX metabolites by competing with ARA.[29] While we observed that the tissue levels of EETs and EpOMEs (Fig. 2B) decreased, the tissue levels of EpETEs and EpDPEs (Fig. 2A) increased in groups treated with the ω-3 rich diet as compared to the controls. Such differences in the relative abundance of epoxides of the ω-3 and ω-6 fatty acid series are suggestive of selective formation of epoxides of ω-3 PUFAs among all 4 parent fatty acids (ARA, LA, and EPA/DHA). Thus, our results on the P450 branch of the ARA cascade are in good agreement with previous studies[7,28,29] suggesting that ω-3 PUFAs exert their anti-inflammatory effects, in part, by competitively inhibiting the conversion of ARA to its respective metabolites in the COX and LOX branches.

Effects of the ω-3 PUFAs on mRNA Expression of *Ephx2*, *Ptgs-2*, *Alox5*, *Scnn1a*, and *Ace-2*

The gene expression data provided additional insights into the biological effects of the ω-3 PUFAs. Overall, the effects of the ω-3 PUFAs on gene expression were reversed by TPPU, which may be interpreted as a control of homeostasis and vascular tone in this model. Although the gene expression data correlated generally with changes in oxylipin expression for *Ephx2*, *Ptgs-2*, and *Scnn1a*, the mRNA expression of *Alox5* was unaltered despite the reduced LOX metabolites. In groups A-ω3-TL and group A-ω3-TH, the downregulation of *Ephx2* and *Ptgs-2* was consistent with the reduced levels of diols from EPA/DHA and reduced levels of PGs, respectively. Moreover, assuming that changes in the *Ephx2* message are closely associated with the activity of sEH, reduced sEH expression should result in reduced metabolism and thus increased levels of EpETE and EpDPE. Indeed, we determined higher levels of EpFAs in both groups A-ω3-TL and A-ω3-TH. Interestingly, *Ephx2* was downregulated by the ω-3 PUFAs only in the presence of TPPU, although TPPU alone did not alter the mRNA expression of the *Ephx2* message. These data suggest that the ω-3 epoxides are involved in the downregulation of *Ephx2* message. A previous report has demonstrated a decrease in *Ephx2* expression in the liver on dietary supplementation with ω-3 PUFAs.[9] However, our study has examined the expression of *Ephx2* in the kidney not in the liver, and in a different disease model. The downregulation of the *Ephx2* message by the ω-3 PUFAs in the presence of an sEHI is likely a desirable effect, because sEH has been shown to be involved in the development and maintenance of hypertension.[40]

The gene expression profile of the *Scnn1a* provides another potential mechanism underlying the ω-3 PUFAs action in BP regulation. ENaC is a constitutively active membrane-bound sodium channel, located at the apical membrane of the epithelial cells in the kidney. ENaC is a major contributor to the regulation of BP, because it constitutes the ultimate step of renal sodium handling.[56] The EETs inhibit ENaC and elicit natriuresis by regulating tubular sodium transport and inhibit sodium reabsorption by altering the pressure–natriuresis mechanism in the kidney.[57–59] Because the changes in the transcription or translation of ENaC are closely associated with the number of channels and aldosterone-induced ENaC expression is specific to the α-subunit of the ENaC,[60] we looked at the α-subunit of ENaC, *Scnn1a* mRNA expression in the kidney. We found that the ω-3 PUFAs downregulated the *Scnn1a* message in the presence of TPPU, and this downregulation showed an inverse correlation with the renal EpETE and EpDPE levels. These results suggest that the epoxides of the ω-3 PUFAs, very much like the EETs, contribute to sodium handling in the kidney, which may be responsible, in part, for their antihypertensive effects.

The upregulation of *Ace-2* and its inverse correlation with SBP suggest that the combination of the ω-3 rich diet and TPPU modulates the renin–angiotensin–aldosterone system (RAAS). ACE-2 is considered as the counter regulatory component of the RAAS, because ACE-2 inactivates both Ang-I and Ang-II by its exopeptidase action, which results in the formation of Ang 1, 7. The Ang 1, 7 opposes the vasoconstrictor actions of Ang-II and induces vasorelaxation.[61] The ω-3 PUFAs reduce ACE-2 activity[62–64]; and ACE-2 mRNA has been reported to be downregulated in the aorta of the spontaneously hypertensive rats fed a diet rich in ALA.[65] Last, the strong correlation between the upregulation of *Ace-2* and the tissue levels of the EpDPEs and EpETEs and plasma levels of Ang-II suggests that these epoxides might modulate *Ace-2* and thus contribute to the regulation of BP.

CONCLUSION

We provided evidence that the long-chain ω-3 PUFAs, sEH inhibition, and coadministration of the ω-3 PUFAs with sEHIs lower arterial BP and attenuate markers of renal inflammation in a murine model of angiotensin-II dependent hypertension. Our results also suggest that the ω-3 PUFAs, in combination with an sEHI exhibit improved anti-inflammatory effects and modulate the ARA cascade by blocking both the COX and the LOX pathways. Results on the anti-inflammatory and antihypertensive effects of the ω-3 PUFAs and sEH inhibition are consistent with decreased tissue levels of PGs, LOX products, and MCP-1, increased tissue levels of epoxides derived from EPA and DHA, the downregulation of the *Scnn1a* and upregulation of *Ace-2* message in the kidney. Even though the ω-3 rich diet and sEHI lead to changes in the tissue levels of the EPA and DHA metabolites which is reflected in the inflammatory state of the kidney, we were unable to show the clear reflection of these changes on SBP.

Our study forms a basis for understanding the biology and underlying mechanisms of the long-chain ω-3 PUFAs in Ang-II dependent hypertension. We provide evidence for the anti-inflammatory effects of ω-3 PUFAs and their modulation of the COX and LOX pathways in the ARA cascade. Furthermore, our data suggest that the epoxide metabolites of ω-3 PUFAs are among the mediators of the antihypertensive and anti-inflammatory effects of ω-3 PUFAs in Ang-II dependent hypertension. The combination of ω-3 supplementation and TPPU treatment presents an exciting alternative to current anti-inflammatory and antihypertensive drugs on the market. The modulation of key enzymes and metabolites in the COX, LOX, and P450 pathways and the accompanying reduction in SBP and proinflammatory cytokines are exciting and have implications for not only hypertensive renal injury but also atherosclerosis and vascular remodeling. Future studies are warranted to identify mediators of the anti-inflammatory and antihypertensive effects of EPA and DHA independently from each other in angiotensin-II–dependent hypertension.

ACKNOWLEDGMENTS

The authors thank Dr Alan Buckpitt and Dr Kent Pinkerton for their valuable assistance with renal metabolic profiling and survival surgeries.

REFERENCES

1. Izzo JL Jr, Sica DA, Black HR. *Hypertension Primer*. 4th ed. Dallas, Texas: American Heart Association, Lipincott Williams & Wilkins; 2008.
2. Mozaffarian D, Wu JH. (n-3) fatty acids and cardiovascular health: are effects of EPA and DHA shared or complementary? *J Nutr.* 2012;142: 614S–625S.
3. Kris-Etherton PM, Harris WS, Appel LJ. Omega-3 fatty acids and cardiovascular disease: new recommendations from the American Heart Association. *Arterioscler Thromb Vasc Biol.* 2003;23:151–152.
4. Harris WS, Kris-Etherton PM, Harris KA. Intakes of long-chain omega-3 fatty acid associated with reduced risk for death from coronary heart disease in healthy adults. *Curr Atheroscler Rep.* 2008;10:503–509.
5. Cabo J, Alonso R, Mata P. Omega-3 fatty acids and blood pressure. *Br J Nutr.* 2012;107(suppl 2):S195–S200.
6. Mozaffarian D, Wu JH. Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. *J Am Coll Cardiol.* 2011;58:2047–2067.
7. Seki H, Tani Y, Arita M. Omega-3 PUFA derived anti-inflammatory lipid mediator resolvin E1. *Prostaglandins Other Lipid Mediat.* 2009; 89:126–130.
8. Serhan CN, Hong S, Gronert K, et al. Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J Exp Med.* 2002;196: 1025–1037.
9. Mavrommatis Y, Ross K, Rucklidge G, et al. Intervention with fish oil, but not with docosahexaenoic acid, results in lower levels of hepatic soluble epoxide hydrolase with time in apoE knockout mice. *Br J Nutr.* 2010;103:16–24.
10. Wu JH, Lemaitre RN, King IB, et al. Association of plasma phospholipid long-chain omega-3 fatty acids with incident atrial fibrillation in older adults: the cardiovascular health study. *Circulation*. 2012;125: 1084–1093.
11. Kris-Etherton PM, Harris WS, Appel LJ. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Arterioscler Thromb Vasc Biol.* 2003;23:e20–30.
12. Levinson PD, Iosiphidis AH, Saritelli AL, et al. Effects of n-3 fatty acids in essential hypertension. *Am J Hypertens.* 1990;3:754–760.
13. Rizos EC, Ntzani EE, Bika E, et al. Association between omega-3 fatty acid supplementation and risk of major cardiovascular disease events: a systematic review and meta-analysis. *JAMA*. 2012;308: 1024–1033.
14. Rauch B, Schiele R, Schneider S, et al. *OMEGA*. A randomized, placebo-controlled trial to test the effect of highly purified omega-3 fatty acids on top of modern guideline-adjusted therapy after myocardial infarction. *Circulation*. 2010;122:2152–2159.
15. Tavazzi L, Maggioni AP, Marchioli R, et al. Effect of n-3 polyunsaturated fatty acids in patients with chronic heart failure (the GISSI-HF trial): a randomised, double-blind, placebo-controlled trial. *Lancet*. 2008;372:1223–1230.
16. von Schacky C, Angerer P, Kothny W, et al. The effect of dietary omega-3 fatty acids on coronary atherosclerosis. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med.* 1999;130:554–562.
17. Noreen EE, Brandauer J. The effects of supplemental fish oil on blood pressure and morning cortisol in normotensive adults: a pilot study. *J Complement Integr Med.* 2012;9.
18. Imig JD. Epoxides and soluble epoxide hydrolase in cardiovascular physiology. *Physiol Rev.* 2012;92:101–130.
19. Deng Y, Theken KN, Lee CR. Cytochrome P450 epoxygenases, soluble epoxide hydrolase, and the regulation of cardiovascular inflammation. *J Mol Cell Cardiol.* 2010;48:331–341.
20. Campbell WB, Falck JR. Arachidonic acid metabolites as endothelium-derived hyperpolarizing factors. *Hypertension*. 2007;49:590–596.
21. Fleming I. Cytochrome P450 epoxygenases as EDHF synthase(s). *Pharmacol Res.* 2004;49:525–533.
22. Imig JD, Hammock BD. Soluble epoxide hydrolase as a therapeutic target for cardiovascular diseases. *Nat Rev Drug Discov.* 2009;8:794–805.
23. Konkel A, Schunck WH. Role of cytochrome P450 enzymes in the bioactivation of polyunsaturated fatty acids. *Biochim Biophys Acta.* 2011;1814:210–222.
24. Wang RX, Chai Q, Lu T, et al. Activation of vascular BK channels by docosahexaenoic acid is dependent on cytochrome P450 epoxygenase activity. *Cardiovasc Res.* 2011;90:344–352.
25. Lai LH, Wang RX, Jiang WP, et al. Effects of docosahexaenoic acid on large-conductance Ca2+-activated K+ channels and voltage-dependent K+ channels in rat coronary artery smooth muscle cells. *Acta Pharmacol Sin.* 2009;30:314–320.
26. Ye D, Zhang D, Oltman C, et al. Cytochrome p-450 epoxygenase metabolites of docosahexaenoate potently dilate coronary arterioles by activating large-conductance calcium-activated potassium channels. *J Pharmacol Exp Ther.* 2002;303:768–776.
27. Morisseau C, Inceoglu B, Schmelzer K, et al. Naturally occurring monoepoxides of eicosapentaenoic acid and docosahexaenoic acid are bioactive antihyperalgesic lipids. *J Lipid Res.* 2010;51:3481–3490.
28. Arnold C, Markovic M, Blossey K, et al. Arachidonic acid-metabolizing cytochrome P450 enzymes are targets of {omega}-3 fatty acids. *J Biol Chem.* 2010;285:32720–32733.

29. Westphal C, Konkel A, Schunck WH. CYP-eicosanoids—a new link between omega-3 fatty acids and cardiac disease? *Prostaglandins Other Lipid Mediat.* 2011;96:99–108.
30. Theken KN, Deng Y, Kannon MA, et al. Activation of the acute inflammatory response alters cytochrome P450 expression and eicosanoid metabolism. *Drug Metab Dispos.* 2011;39:22–29.
31. Chiamvimonvat N, Ho CM, Tsai HJ, et al. The soluble epoxide hydrolase as a pharmaceutical target for hypertension. *J Cardiovasc Pharmacol.* 2007;50:225–237.
32. Hartner A, Cordasic N, Klanke B, et al. Strain differences in the development of hypertension and glomerular lesions induced by deoxycorticosterone acetate salt in mice. *Nephrol Dial Transplant.* 2003;18:1999–2004.
33. Liu JY, Yang J, Inceoglu B, et al. Inhibition of soluble epoxide hydrolase enhances the anti-inflammatory effects of aspirin and 5-lipoxygenase activation protein inhibitor in a murine model. *Biochem Pharmacol.* 2010;79:880–887.
34. Liu JY, Tsai HJ, Hwang SH, et al. Pharmacokinetic optimization of four soluble epoxide hydrolase inhibitors for use in a murine model of inflammation. *Br J Pharmacol.* 2009;156:284–296.
35. Rose TE, Morisseau C, Liu JY, et al. 1-Aryl-3-(1-acylpiperidin-4-yl)urea inhibitors of human and murine soluble epoxide hydrolase: structure-activity relationships, pharmacokinetics, and reduction of inflammatory pain. *J Med Chem.* 2010;53:7067–7075.
36. Daugherty A, Rateri D, Hong L, et al. Measuring blood pressure in mice using volume pressure recording, a tail-cuff method. *J Vis Exp.* 2009;27.
37. Elmarakby AA, Williams JM, Imig JD, et al. Synergistic actions of enalapril and tempol during chronic angiotensin II-induced hypertension. *Vasc Pharmacol.* 2007;46:144–151.
38. Feng M, Whitesall S, Zhang Y, et al. Validation of volume-pressure recording tail-cuff blood pressure measurements. *Am J Hypertens.* 2008;21:1288–1291.
39. Yang J, Schmelzer K, Georgi K, et al. Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry. *Anal Chem.* 2009;81:8085–8093.
40. Koeners MP, Wesseling S, Ulu A, et al. Soluble epoxide hydrolase in the generation and maintenance of high blood pressure in spontaneously hypertensive rats. *Am J Physiol Endocrinol Metab.* 2011;300:E691–E698.
41. Burne-Taney MJ, Yokota-Ikeda N, Rabb H. Effects of combined T- and B-cell deficiency on murine ischemia reperfusion injury. *Am J Transplant.* 2005;5:1186–1193.
42. Laird NM, Ware JH. Random-effects models for longitudinal data. *Biometrics.* 1982;38:963–974.
43. Holm S. A simple sequentially rejective multiple test procedure. *Scand J Stat.* 1979;6:65–70.
44. Arendash GW, Jensen MT, Salem N Jr, et al. A diet high in omega-3 fatty acids does not improve or protect cognitive performance in Alzheimer's transgenic mice. *Neuroscience.* 2007;149:286–302.
45. Liu JC, Conklin SM, Manuck SB, et al. Long-chain omega-3 fatty acids and blood pressure. *Am J Hypertens.* 2011;24:1121–1126.
46. Engler MM, Engler MB, Pierson DM, et al. Effects of docosahexaenoic acid on vascular pathology and reactivity in hypertension. *Exp Biol Med (Maywood).* 2003;228:299–307.
47. Tang X, Li ZJ, Xu J, et al. Short term effects of different omega-3 fatty acid formulation on lipid metabolism in mice fed high or low fat diet. *Lipids Health Dis.* 2012;11:70.
48. Begg DP, Sinclair AJ, Stahl LA, et al. Hypertension induced by omega-3 polyunsaturated fatty acid deficiency is alleviated by alpha-linolenic acid regardless of dietary source. *Hypertens Res.* 2010;33:808–813.
49. Jung O, Brandes RP, Kim IH, et al. Soluble epoxide hydrolase is a main effector of angiotensin II-induced hypertension. *Hypertension.* 2005;45:759–765.
50. Imig JD, Walsh KA, Hye Khan MA, et al. Soluble epoxide hydrolase inhibition and peroxisome proliferator activated receptor gamma agonist improve vascular function and decrease renal injury in hypertensive obese rats. *Exp Biol Med (Maywood).* 2012;237:1402–1412.
51. Rousseau-Ralliard D, Moreau D, Guilland JC, et al. Docosahexaenoic acid, but not eicosapentaenoic acid, lowers ambulatory blood pressure and shortens interval QT in spontaneously hypertensive rats in vivo. *Prostaglandins Leukot Essent Fatty Acids.* 2009;80:269–277.
52. Spector AA, Norris AW. Action of epoxyeicosatrienoic acids on cellular function. *Am J Physiol Cell Physiol.* 2007;292:C996–C1012.
53. Agbor LN, Walsh MT, Boberg JR, et al. Elevated blood pressure in cytochrome P4501A1 knockout mice is associated with reduced vasodilation to omega-3 polyunsaturated fatty acids. *Toxicol Appl Pharmacol.* 2012;264:351–360.
54. Kundu S, Roome T, Bhattacharjee A, et al. Metabolic products of soluble epoxide hydrolase are essential for monocyte chemotaxis to MCP-1 in vitro and in vivo. *J Lipid Res.* 2013;54:436–447.
55. Schmitz G, Ecker J. The opposing effects of n-3 and n-6 fatty acids. *Prog Lipid Res.* 2008;47:147–155.
56. Sun Y, Zhang JN, Zhao D, et al. Role of the epithelial sodium channel in salt-sensitive hypertension. *Acta Pharmacol Sin.* 2011;32:789–797.
57. Imig JD. Epoxygenase metabolites. Epithelial and vascular actions. *Mol Biotechnol.* 2000;16:233–251.
58. Sun P, Lin DH, Yue P, et al. High potassium intake enhances the inhibitory effect of 11,12-EET on ENaC. *J Am Soc Nephrol.* 2010;21:1667–1677.
59. Honetschlagerova Z, Sporkova A, Kopkan L, et al. Inhibition of soluble epoxide hydrolase improves the impaired pressure-natriuresis relationship and attenuates the development of hypertension and hypertension-associated end-organ damage in Cyp1a1-Ren-2 transgenic rats. *J Hypertens.* 2011;29:1590–1601.
60. Loffing J, Pietri L, Aregger F, et al. Differential subcellular localization of ENaC subunits in mouse kidney in response to high- and low-Na diets. *Am J Physiol Renal Physiol.* 2000;279:F252–F258.
61. Moon JY. ACE2 and angiotensin-(1-7) in hypertensive renal disease. *Electrolyte Blood Press.* 2011;9:41–44.
62. Das UN. Essential fatty acids and their metabolites could function as endogenous HMG-CoA reductase and ACE enzyme inhibitors, anti-arrhythmic, anti-hypertensive, anti-atherosclerotic, anti-inflammatory, cyto-protective, and cardioprotective molecules. *Lipids Health Dis.* 2008;7:37.
63. Hamden K, Keskes H, Belhaj S, et al. Inhibitory potential of omega-3 fatty and fenugreek essential oil on key enzymes of carbohydrate-digestion and hypertension in diabetes rats. *Lipids Health Dis.* 2011;10:226.
64. Cicero AF, Ertek S, Borghi C. Omega-3 polyunsaturated fatty acids: their potential role in blood pressure prevention and management. *Curr Vasc Pharmacol.* 2009;7:330–337.
65. Ogawa A, Suzuki Y, Aoyama T, et al. Dietary alpha-linolenic acid inhibits angiotensin-converting enzyme activity and mRNA expression levels in the aorta of spontaneously hypertensive rats. *J Oleo Sci.* 2009;58:355–360.