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Cytochrome P450 2C8 ω3-Long-Chain Polyunsaturated Fatty Acid Metabolites Increase Mouse Retinal Pathologic Neovascularization—Brief Report

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Abstract

Objective—Regulation of angiogenesis is critical for many diseases. Specifically, pathological retinal neovascularization, a major cause of blindness, is suppressed with dietary ω3-long-chain polyunsaturated fatty acids (ω3LCPUFAs) through antiangiogenic metabolites of cyclooxygenase and lipoxygenase. Cytochrome P450 epoxygenases (CYP2C8) also metabolize LCPUFAs, producing bioactive epoxides, which are inactivated by soluble epoxide hydrolase (sEH) to transdihydrodiols. The effect of these enzymes and their metabolites on neovascularization is unknown.

Approach and Results—The mouse model of oxygen-induced retinopathy was used to investigate retinal neovascularization. We found that CYP2C (localized in wild-type monocytes/macrophages) is upregulated in oxygen-induced retinopathy, whereas sEH is suppressed, resulting in an increased retinal epoxide:diol ratio. With a ω3LCPUFA-enriched diet, retinal neovascularization increases in *Tie2*-driven human-*CYP2C8*–overexpressing mice (*Tie2-CYP2C8-Tg*), associated with increased plasma 19,20-epoxydocosapentaenoic acid and retinal epoxide:diol ratio. 19,20-Epoxydocosapentaenoic acids and the epoxide:diol ratio are decreased with

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Disclosures
None.

overexpression of *sEH* (*Tie2-sEH-Tg*). Overexpression of *CYP2C8* or *sEH* in mice does not change normal retinal vascular development compared with their wild-type littermate controls. The proangiogenic role in retina of CYP2C8 with both ω3LCPUFA and ω6LCPUFA and antiangiogenic role of sEH in ω3LCPUFA metabolism were corroborated in aortic ring assays.

Conclusions—Our results suggest that CYP2C ω3LCPUFA metabolites promote retinal pathological angiogenesis. CYP2C8 is part of a novel lipid metabolic pathway influencing retinal neovascularization.

Keywords

angiogenesis factor; cytochrome P450 CYP2C8 (human); pathologic neovascularization

Pathological neovascularization in retinopathy is a major cause of blindness.[1,2] ω3 long-chain polyunsaturated fatty acids (ω3LCPUFA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) suppress retinal neovascularization and improve visual function in animal and clinical retinopathy studies[3,4] through metabolites of cyclooxygenase and lipoxygenase.[3,5–7] Cytochrome P450s (CYPs) also metabolize both ω3LCPUFAs and ω6LCPUFAs into bioactive epoxides, which are hydrolyzed by soluble epoxide hydrolase (sEH) to form less active transdihydrodiols (diols), hence dampening the biological effects of LCPUFA epoxides (Figure 1A).[8,9] It is important to further elucidate enzymes that generate bioactive ω3LCPUFA (and ω6LCPUFA) metabolites (CYP2C) and enzymes that deactivate them (sEH) to identify pro- and antiangiogenic pathways.

CYP2C8, a dominant human epoxygenase, is induced by hypoxia,[10] which in turn is critical in retinal neovascular development. sEH is implicated in cardiovascular diseases[11] and expressed in endothelial cells (ECs)[12] and may regulate angiogenesis. ω6LCPUFA-derived epoxyeicosatrienoic acids (EETs), synthesized by CYP2C8 from arachidonic acid (AA), promote angiogenesis and EC migration.[13–17] However, the angiogenic effect of ω3LCPUFA-derived epoxy metabolites from CYP2C8: DHA-derived epoxydocosapentaenoic acids (EDPs) and EPA-derived epoxyeicosatetraenoic acids (EEQs; Figure 1A) is unknown.[18] Both EDPs and EEQs exhibit potent vasodilatory and cardioprotective effects,[19] with EDPs suggested to suppress EC migration and angiogenesis in tumors.[20]

We investigated whether CYP2C8 and its ω3LCPUFA metabolites promoted or suppressed retinal neovascularization using blood vessel system–specific *CYP2C8* and *sEH*-overexpressing mice (*Tie2-CYP2C8-Tg*, *Tie2-sEH-Tg*)[21] and germ-line knockout of *sEH* ($sEH^{-/-}$) with wild-type (WT) littermate controls with a ω3LCPUFA-enriched diet in a mouse model of oxygen-induced retinopathy (OIR). CYP2C8 and sEH metabolites from a ω6LCPUFA-enriched diet were examined similarly in OIR.

Materials and Methods

Methods and Materials are available in the online-only Supplement.

Results

CYP2C-positive leukocytes are detected in circulating cells from WT normoxic mice (Figure 1B). The *CYP2C* mRNA level is highest in blood cells and much higher in nonperfused versus perfused retina (Figure 1C). We then identified the source of the mouse CYP2C8 homologue, CYP2C. CYP2C-positive cells are found within blood vessel lumens in normoxic retina (Figure1D and 1E) and outside vessels in P17 OIR retina (Figure 1D), when the maximal neovascularization is observed in mouse OIR.[22,23] Some F4/80-positive macrophages outside vessels express CYP2C in OIR, indicating macrophages are one of the sources of CYP2C in the OIR retina. Other sources are not excluded (Figure 1F). Pathological neovessels and neural tissue express CYP2C and sEH in OIR (Figure1G and 1H). We next examined whether OIR affects levels of CYP2C (producing active metabolites) or sEH (reducing active metabolite levels).

OIR Induces Increased CYP2C8 and Decreased sEH Retinal Levels, Resulting in an Increased Retinal DHA-Derived Epoxide:Diol Ratio

CYP2C (mRNA and protein) is induced in OIR retinas, whereas sEH is suppressed ($P<0.05$; Figure1I and 1J). In OIR versus normoxia at P14 (on normal chow) when the neovessel formation starts,[23] the DHA-derived retinal epoxide:diol ratio (19,20-EDP:19,20-dihydroxy-docosapentaenoic acid [DiHDPA]) is increased >2-fold ($P=0.017$; Figure 1K). We next determined how this increase in active metabolites influences retinal neovessel formation.

In *Tie2-CYP2C8-Tg* OIR Mice, ω3LCPUFA Feed Increases Neovascularization and Increases *VEGF-A* Expression

With ω3LCPUFA feed, *Tie2-human-CYP2C8-Tg* mice develop more OIR neovascularization than WT (*Tie2-CYP2C8-Tg:* 7.60±0.29% versus WT: 6.40±0.33% of total retinal area; $P=0.014$) at P17 (Figure 2A). Moreover, *Tie2-sEH-Tg* retinas develop less neovascularization in OIR (*Tie2-sEH-Tg*: 4.67±0.34% versus WT: 6.59±0.38%; $P=0.0027$; Figure 2B). Germ-line deletion of *sEH* (*sEH*$^{-/-}$) had no effect on neovascularization (*sEH*$^{-/-}$:7.39±0.34% versus WT: 7.35±0.32%; $P=0.95$; Figure 2C). With ω3LCPUFA feed, *Tie2-CYP2C8-Tg* OIR mice had 2.6-fold greater vascular endothelial growth factor (*VEGF*)-*A* expression than WT ($P=0.011$), whereas *Tie2-sEH-Tg* had 57% less *VEGF-A* expression ($P=0.030$). No significant difference in *VEGF-C* levels was detected (Figure2D and 2E). Significantly higher retinal interleukin-1β and interleukin-6 levels were found in *Tie2-CYP2C8-Tg* versus WT OIR mice (Figure 2D). We next examined the active and inactive metabolite levels or ratios in plasma and retina.

In OIR With ω3LCPUFA Feed, *Tie2-CYP2C8-Tg* Increase and *Tie2-sEH-Tg* Decrease Plasma Epoxide Levels and Retinal Epoxide:Diol Ratios at P17

In OIR, ω3LCPUFA-fed *Tie2-CYP2C8-Tg* mouse plasma has more 19,20-EDP (≈1.6-fold; $P=0.029$) and more 17,18-EEQ (≈1.5-fold; $P=0.030$) than WT. The DHA-derived 19,20-EDP level is 30-fold higher than EPA-derived 17,18-EEQ in both mutant and WT (Figure 2F), indicating that DHA contributes more than EPA to the effect. No significant change was found in the plasma EETs levels (Figure IIB in the online-only Data Supplement). In ω3LCPUFA-fed *Tie2-sEH-Tg* mice, 19,20-EDP and 17,18-EEQ levels were reduced by

34% (*P*=0.034) and 24% (*P*=0.016; Figure 2G). In OIR, ω3LCPUFA-fed *Tie2-CYP2C8-Tg* retinas had a 52% higher 19,20-EDP:DiHDPA ratio than WT (*P*=0.045; Figure 2H); the 17,18-EEQ:17,18-dihydroxy-eicosatetraenoic acid (DHEQ) ratio was unchanged. In ω3LCPUFA-fed *Tie2-sEH-Tg* retinas, the 19,20-EDP:DiHDPA ratio decreased by 58% (*P*=0.028; Figure 2I); the 17,18-EEQ:17,18-DHEQ ratio was unchanged. We next examined the effect of sEH and CYP2C in ex vivo transgenic mouse aortic ring sprouting to confirm our findings.

ω3LCPUFA Metabolite Promotes Aortic Vascular Sprouting From *Tie2-CYP2C8-Tg* Mice, Which Is Lost in *Tie2-sEH-Tg* Mice

The proangiogenic effect of CYP2C8 and suppressive effect of sEH with ω3LCPUFA substrate were further confirmed with aortic ring-sprouting assays. A total of 30 μmol/L DHA (versus 30 μmol/L AA as a LCPUFA DHA control) attenuated aortic sprouting in WT (*P*=0.01), which was abolished in *Tie2-CYP2C8-Tg* (*P*<0.05; Figure 2J). *Tie2-sEH-Tg* suppressed aortic sprouting versus WT with 19,20-EDP treatment (≈50%; *P*<0.01; Figure 2K), whereas there was no effect with 17,18-EEQ, confirming the relative importance of DHA versus EDP with ω3LCPUFA effects.

Discussion

Although the effects of ω3LCPUFA on human health have been reported since the beginning of the last century,[24] the knowledge of the primary mechanisms of action of these lipids is still relatively limited.[3,5,6,8] We have shown previously that the 5-lipoxygenase ω3LCPUFA metabolite 4-hydroxy-docosahexaenoic acid suppresses retinal neovascularization.[25] Here, we investigated whether CYP2C8, one of the dominant human CYP epoxygenases, also mediates angiogenic effects of ω3LCPUFA in pathological retinal neovascularization.

We found much higher levels of mouse CYP2C homologue in nonperfused retina than perfused retina, indicating that CYP2C in normal retina comes from circulating blood cells. In OIR, CYP2C is localized in some macrophages outside vessels, consistent with migration of circulating cells from increased vascular leakage in OIR.[26] Increased retinal DHA-derived epoxide:diol ratio in OIR is consistent with increased CYP2C and decreased sEH levels. Recruited *CYP2C*-expressing macrophages may contribute to the increased CYP2C in OIR retinas. The increased levels of active metabolites and decreased level of less active breakdown products in OIR, in turn, lead to an increased DHA-derived epoxide:diol ratio.

No significant difference in normal vascular development is observed in *Tie2-CYP2C8-Tg* and *Tie2-sEH-Tg* mice compared with WT littermate controls at P7 (Figure I in the online-only Data Supplement). There is no global suppression in cyclooxygenase/lipoxygenase activity in *Tie2-CYP2C8-Tg* versus WT mice on both feeds (Figure III in the online-only Data Supplement). In OIR, neovascularization is increased in *Tie2-CYP2C8-Tg* and reduced in *Tie2-sEH-Tg* with ω3LCPUFA feed, suggesting a proretinopathy role of CYP2C8 ω3LCPUFA metabolites in OIR. However, unchanged neovascularization is observed in ω3LCPUFA-fed $sEH^{-/-}$ mice, possibly because of a faster normal vascular development at P7, which is more resistant to hyperoxia (Figure I in the online-only Data Supplement), or

likely reflecting already low *sEH* expression in OIR. In OIR with ω3LCPUFA feed, *Tie2-CYP2C8-Tg* increases *VEGF-A* expression, whereas *Tie2-sEH-Tg* decreases *VEGF-A* expression, consistent with the neovascular phenotypes. We have shown that 5-lipoxygenase ω3LCPUFA metabolite 4-hydroxydocosahexaenoic acid suppresses retinal neovascularization without changing *VEGF-A* expression.[25] *VEGF-A* has been identified to be positively involved in retinal pathological angiogenesis.[27] These studies suggest an opposite effect on *VEGF-A* production of CYP2C8 ω3LCPUFA metabolites in retinal angiogenesis, possibly contributing to the increased neovessel formation.

In OIR with ω3LCPUFA feed, *Tie2-CYP2C8-Tg* increase and *Tie2-sEH-Tg* decrease plasma epoxide levels and retinal epoxide:diol ratios at P17. Higher levels of DHA-derived 19,20-EDP in both mutant and WT suggest 19,20-EDP as the main active CYP2C ω3LCPUFA metabolite versus EPA-derived 17,18-EEQ mediating a proretinopathy effect in OIR. These results suggest that with ω3LCPUFA feed, CYP2C8 may potentiate neovascularization primarily by increasing plasma 19,20-EDP and the retinal 19,20-EDP:DiHDPA ratio, which reflects both the activity of *CYP2C8*, which produces bioactive 19,20-EDP and the activity of sEH, which attenuates bioactivity through conversion to less active diol. The effects of CYP2C and sEH in transgenic mouse aortic ring sprouting confirm that *Tie2-CYP2C8-Tg* promotes angiogenesis with ω3LCPUFA (primarily DHA) and suggests that decreased neovascularization in *Tie2-sEH-Tg* may be directly attributable to accelerated degradation of 19,20-EDP by overexpressed *sEH*.

Finding new approaches to prevent or treat neovascularization are important. Here, we find a novel role of CYP2C8 (and sEH) ω3LCPUFA metabolites in retinopathy. CYP2C8 overexpression potentiates retinal neovascularization with ω3LCPUFA feed by increasing plasma DHA-derived 19,20-EDP (and increasing the retinal 19,20-EDP:DiHDPA ratio), reflecting both production and elimination of 19,20- EDP. A recent study shows that EDPs inhibit ECs migration in tumor angiogenesis by suppressing *VEGF-C*, but not *VEGF-A*, in human umbilical vein endothelial cells in vitro.[20] VEGF-C is a critical mediator of lymphangiogenesis (as well as angiogenesis)[28] and considered as an important therapeutic target for cancer. In current studies, no change in *VEGF-C* expression is observed in *Tie2-CYP2C8-Tg* and *Tie2-sEH-Tg* retina. The different expression pattern of *VEGF-A* and *VEGF-C* may contribute to the opposite angiogenic function of CYP2C8 metabolite 19,20-EDP observed in the retina and in tumor, indicating a tissue-specific role of CYP2C8. Previously, cardiomyocytes expressing CYP2C8 were shown to increase recovery after cardiac ischemia/reperfusion, whereas ECs expressing CYP2C8 reduced the recovery.[12]

OIR neovascularization is potentiated by the CYP2C ω6LCPUFA metabolite (14,15-EET; Figures IV and V in the online-only Data Supplement) and the CYP2C ω3LCPUFA metabolite 19,20-EDP, suggesting that inhibition of CYP2C8 might reduce retinal neovascularization by suppressing ω3LCPUFA and ω6LCPUFA diet-induced proretinopathy lipid metabolites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

AA	arachidonic acid
CYP	cytochrome P450 epoxygenase
DHA	docosahexaenoic acid
DHEQ	dihydroxy-eicosatetraenoic acid
DiHDPA	dihydroxy-docosapentaenoic acid
EC	endothelial cell
EDP	epoxydocosapentaenoic acid
EEQ	epoxyeicosatetraenoic acid
EET	epoxyeicosatrienoic acid
EPA	eicosapentaenoic acid
LCPUFAs	long-chain polyunsaturated fatty acids
OIR	oxygen-induced retinopathy
sEH	soluble epoxide hydrolase
VEGF	vascular endothelial growth factor
WT	wild-type

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Significance

Cytochrome P450 epoxygenase induces retinal neovascularization; inhibition of cytochrome P450 epoxygenases presents a new target for retinopathy treatment. Retinal CYP2C from circulating blood cells increase levels of the proangiogenic CYP ω3-long-chain polyunsaturated fatty acid metabolite 19,20-epoxydocosapentaenoic acid (and ω6-long-chain polyunsaturated fatty acid metabolite 14,15-epoxyeicosatrienoic acid).

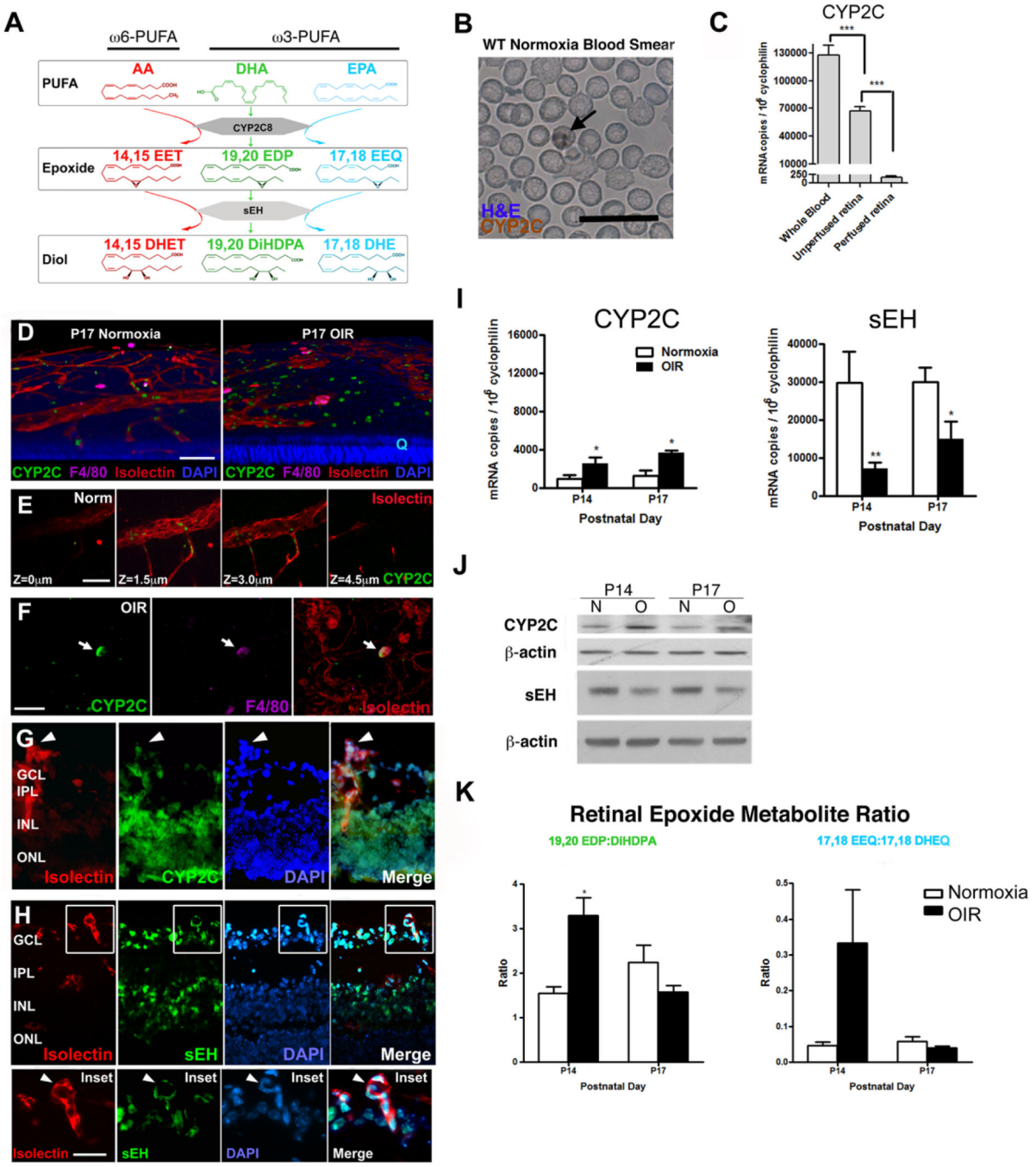

Figure 1.

Retinal expression of cytochrome P450 epoxygenases (CYP2C8) homologue, soluble epoxide hydrolase (sEH), and their products ratio in normoxia vs oxygen-induced retinopathy oxygen-induced retinopathy (OIR). **A**, Schematic diagram of CYP2C8 and sEH metabolism of arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA). **B**, Wild-type (WT) blood smear indicates CYP2C-positive leukocytes (arrows). Scale bar, 20 μm. **C**, mRNA level of *CYP2C* in blood and retina with or without perfusion. **D**, Three-dimensional (3D) reconstruction of confocal images of postnatal day (P) 17 WT normoxia and oxygen-induced retinopathy (OIR) retinal flat-mount stained with CYP2C (green), F4/80 (purple), isolectin (red), and 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bar, 100 μm. **E**, Layer-by-layer confocal image across a vein of

normoxia retina. **F**, Colocalization of CYP2C and F4/80 (arrow) in OIR retinal flat-mount. **G**, Retinal cross-sectional staining with isolectin (red), CYP2C (green), and DAPI (blue) shows CYP2C is expression in neovascular tufts (arrowhead), as well as in the neurons of the ganglion cell layer (GCL), inner nuclear layers (INL), and outer nuclear layer (ONL). **H**, Retinal cross-sectional staining with isolectin (red), sEH (green), and DAPI (blue) shows sEH is expressed in neovascular tufts (arrow-head), as well as in neurons of GCL and INL. Scale bar, 10 μm. **I**, *CYP2C* and *sEH* mRNA expression in retina during OIR (n=6). **J**, CYP2C and sEH protein expression in normoxia (N) versus OIR (O) retina. **K**, The ratio of corresponding DHA and EPA epoxides to diols by liquid chromatography/mass spectrometry/mass spectrometry oxylipid analysis (n=4–6 per group; 2-way ANOVA with Bonferroni post test; **P*<0.05). Three-dimensional reconstruction was made using Volocity 3D Image Analysis Software. All confocal images were taken by Leica SP2 confocal microscope with ×40 objective lens. Blood smear pictures was taken by Zeiss AxioObserver microscope under ×20 objective lens. DHET indicates dihydroxyeicosatrienoic acid; DiHDPA, dihydroxy-docosapentaenoic acid; EDP, epoxydocosapentaenoic acid; EET, epoxyeicosatrienoic acid; EEQ, epoxyeicosatetraenoic acid; and PUFA, polyunsaturated fatty acid.

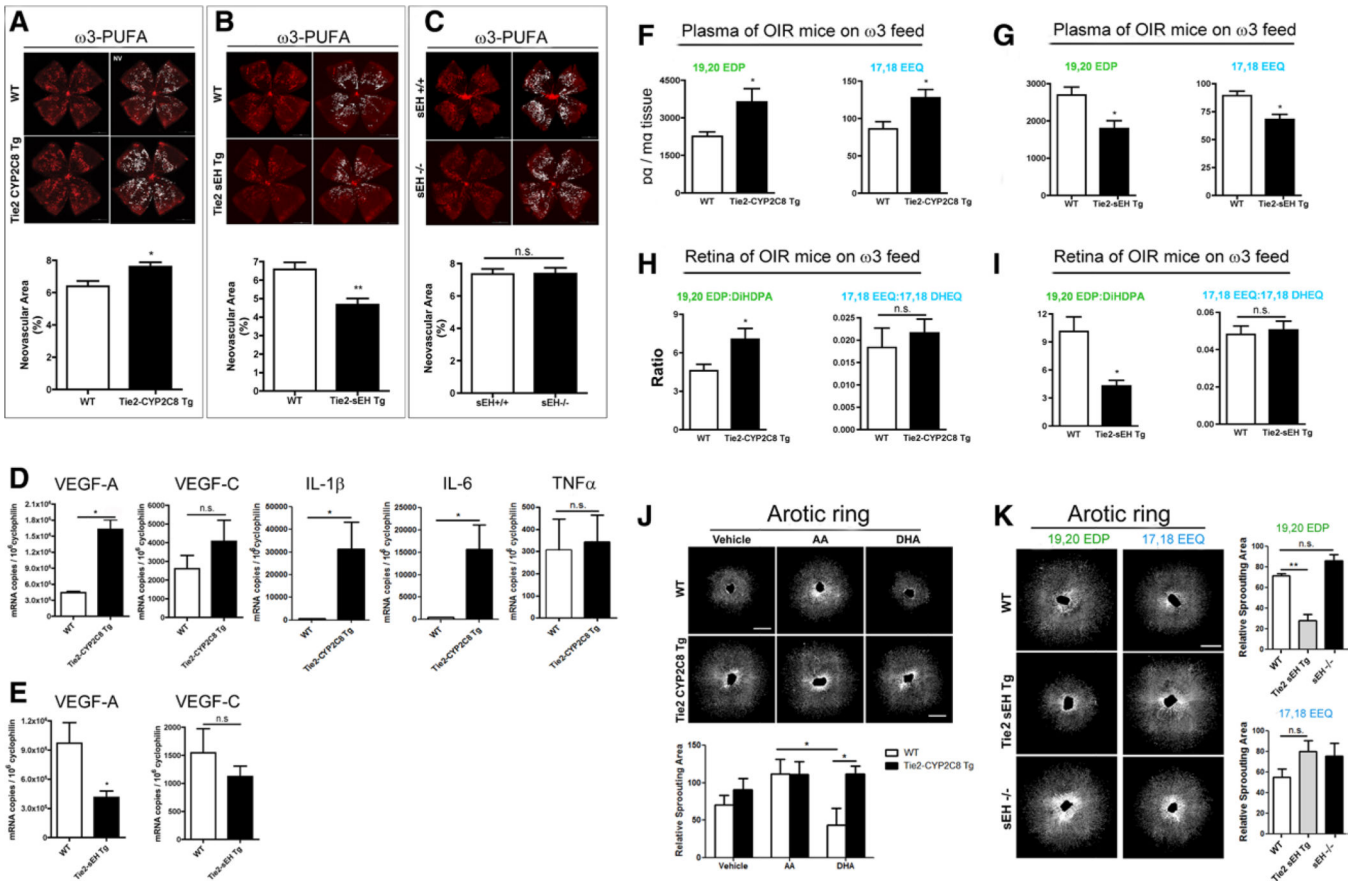

Figure 2.

With ω3-long-chain polyunsaturated fatty acid (ω3LCPUFA) feed, oxygen-induced retinopathy (OIR) neovascularization and corresponding epoxides level are modified in *Tie2-CYP2C8-Tg* and *Tie2-sEH-Tg* mice; the alternation of angiogenesis was also shown in aortic ring sprouting using *Tie2-CYP2C8-Tg* and *Tie2-sEH-Tg* treated with docosahexaenoic acid (DHA) and eicosapentaenoic acid (AA) or epoxide metabolites. **A**, Neovascular area of *Tie2-CYP2C8-Tg* mice exposed to OIR comparing with wild-type (WT) littermate control (n=11–13 per group). Scale bar, 500 μm. **B**, Neovascular area in OIR of *Tie2-sEH-Tg* comparing with WT (n=14–19 per group). **C**, Neovascular area in OIR of systemic soluble epoxide hydrolase (*sEH*) knockout (*sEH*−/−; n=8–15 per group). **D** and **E**, Reverse transcription polymerase chain reaction of *vascular endothelial growth factor (VEGF)-A*, *VEGF-C*, *interleukin (IL)-1β*, *IL-6*, and *tumor necrosis factor (TNF)*-α in OIR *Tie2-CYP2C8-Tg* (**D**) and *VEGF-A*, *VEGF-C* in OIR *Tie2-sEH-Tg* (E) compared with WT (*t* test; **P*<0.05 and ***P*<0.01). Retinal whole mount pictures were taken by Zeiss AxioObserver microscope under ×5 objective lens. **F**, Plasma levels of 19,20-epoxydocosapentaenoic acid (EDP) and 17,18-epoxyeicosatetraenoic acid (EEQ) in *Tie2-CYP2C8-Tg* mice (n=4–6 per group). **G**, Plasma levels of 19,20-EDP and 17,18-EEQ in *Tie2-sEH-Tg* mice (n=4–6 per group). **H**, Retinal 19,20-EDP:dihydroxy-docosapentaenoic acid (DiHDPA) and 17,18-EEQ:17,18-dihydroxy-eicosatetraenoic acid (DHEQ) ratio of *Tie2-CYP2C8-Tg* vs WT (n=4–6 per group). **I**, Retinal 19,20-EDP:DiHDPA and 17,18-EEQ:17,18-DHEQ ratio of *Tie2-sEH-Tg* vs WT (n=4–6 per group; *t* test; **P*<0.05 and ***P*<0.01). **J**, Vehicle control, AA (30 μmol/L) or DHA (30 μmol/L) induced aortic ring sprouting of WT and *Tie2-CYP2C8-Tg* mice (n=3–7 per group). **K**, Aortic sprouting from *Tie2-sEH-Tg* and *sEH*−/− treated with 17,18-EDP and 19,20-EEQ (n=4–8 per group). Scale bars, 50 μm (*t* test; **P*<0.05 and ***P*<0.01). Aortic ring pictures were taken by Zeiss AxioObserver microscope under ×10 objective lens. n.s. indicates not significant.