Flicker Light–Induced Retinal Vasodilation Is Unaffected by Inhibition of Epoxyeicosatrienoic Acids and Prostaglandins in Humans

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Submitted: June 4, 2014
Accepted: September 24, 2014

Citation: Noonan JE, Dusting GJ, Nguyen TT, et al. Flicker light–induced retinal vasodilation is unaffected by inhibition of epoxyeicosatrienoic acids and prostaglandins in humans. *Invest Ophthalmol Vis Sci*. 2014;55:7007–7013. DOI:10.1167/iovs.14-14947

Purpose. To investigate the role of epoxyeicosatrienoic acids (EETs) and prostaglandins (PGs) in retinal blood vessel calibers and vasodilation during flicker light stimulation in humans.

Methods. Twelve healthy nonsmokers participated in a balanced crossover study. Oral fluconazole 400 mg and dispersible aspirin 600 mg were used to inhibit production of EETs and PGs, respectively. Retinal imaging was performed 1 hour after drug ingestion with the Dynamic Vessel Analyzer. Resting calibers of selected vessel segments were recorded in measurement units (MU). Maximum percentage dilations during flicker stimulation were calculated from baseline calibers. We then studied six participants each after fluconazole and aspirin ingestions at 30-minute intervals for 2 hours. Within-subject differences were assessed by ANOVA and Dunnett-adjusted pairwise comparisons with significance taken at $P < 0.05$.

Results. In crossover study participants, mean (SD) arteriole and venule dilations without drug administration were 4.4% (2.0%) and 4.6% (1.7%), respectively. Neither drug affected vasodilation during flicker stimulation. Mean (SD) resting arteriole and venule calibers on no-drug visits were 119.6 (10.6) MU and 145.7 (17.0) MU, respectively. Fluconazole reduced mean (±95% CI) resting venule calibers by 5.1 (4.3) MU. In repeated measures participants, neither drug affected vasodilations, but fluconazole reduced resting venule calibers over 2 hours ($P < 0.001$).

Conclusions. Epoxyeicosatrienoic acids and prostaglandins are unlikely to be primary mediators of flicker light–induced retinal vasodilation in humans. However, EETs may play a role in the regulation of retinal vascular tone and blood flow under resting physiological conditions.

Keywords: epoxyeicosatrienoic acids, functional imaging, neurovascular coupling, prostaglandins, retinal blood flow

Retinal vasodilation, a surrogate marker of hyperemia, is a normal physiological response to flicker light stimulation. The increased blood flow provides additional oxygen and nutrients to meet the increased requirements of metabolically active cells. Retinal hyperemia in response to flicker light is thought to be a function of ganglion cell activity[1] and nitric oxide (NO) release.[2–4] However, the exact mechanism of increased retinal blood flow during flicker light stimulation in health remains unclear, which makes the significance of changes in the response during disease difficult to understand.

Experimental data from rodents challenges the view that NO is a key mediator of retinal neuronal activity-dependent hyperemia. Using ex vivo rat retinas perfused with 95% oxygen, Metea and Newman[5] found that epoxyeicosatrienoic acids (EETs) were important mediators of flicker light–induced arteriolar dilations. Although prostaglandins (PGs) did not contribute to flicker light–induced arteriolar dilations during hyperoxia, PGs did appear to mediate dilations in retinas perfused with 21% oxygen.[6] Interestingly, increasing the background NO concentration caused arterioles to constrict during flicker light stimulation.[5] Flicker light–induced arteriolar dilations were inhibited by blockade of neuron-to-glia signaling and were replicated by direct glial cell stimulation, suggesting that these vasomotor responses were mediated by glial-derived EETs and PGs.[5,6] Of the dilatory PGs, glial cells and neurons generally produce PGE_2.[7]

The in vivo production of EETs and PGs can be inhibited by fluconazole and aspirin, respectively. Fluconazole, an antifungal drug, is a potent inhibitor of CYP2C enzymes.[8] Fluconazole was previously used to demonstrate EET involve-

Table 1. Crossover Study Order of Treatments ($n = 12$)

Sequence	n	Period 1	Period 2	Period 3
A	2	Control	Fluconazole	Aspirin
B	2	Control	Aspirin	Fluconazole
C	2	Fluconazole	Control	Aspirin
D	2	Fluconazole	Aspirin	Control
E	2	Aspirin	Control	Fluconazole
F	2	Aspirin	Fluconazole	Control

ment in the control of radial artery tone and flow-mediated dilation in humans.[9,10] Aspirin, an irreversible inhibitor of cyclo-oxygenase (COX) enzymes,[11,12] has similarly been used to show that PGs can mediate cutaneous vasodilations[13,14] or vasoconstrictions[15] in response to pharmacological stimuli.

We have now investigated whether EETs and PGs are important mediators of flicker light–induced retinal vasodilation or affect retinal vascular tone in humans. We hypothesized that fluconazole and aspirin would reduce the magnitude of flicker light–induced vasodilations, but have a negligible impact on resting retinal vascular calibers.

Methods

Participants

In a balanced crossover study, we studied the right eyes of 12 healthy adults (aged ≥18 years) who reported no chronic medical conditions. Exclusion criteria were smoking, eye surgery other than refractive surgery, allergies to study drugs, regular medications other than dietary supplements, pregnancy, and lactation. Additional exclusion criteria were (1) any ocular pathology detected on two-field fundus photography of the right eye centered on the macula and optic disc with a 45° digital nonmydriatic camera (CR-2; Canon, Melville, NY, USA); (2) any abnormality detected on a 12-lead electrocardiogram (ELI 150 Rx; Mortara Instrument, Milwaukee, WI, USA); and (3) any major abnormalities found during a general clinical examination by a medical professional (JEN). All participants had their age, sex, ethnicity, height and weight, heart rate, blood pressure, iris pigmentation, and IOP recorded at baseline. Abstention from alcohol and caffeine on study days was requested.

After conclusion of the crossover study, we took repeated measurements of flicker light–induced retinal vasodilation after fluconazole and aspirin ingestions using the right eyes of six participants per drug. Recruitment criteria and baseline measurements for these repeated measures studies were identical to the crossover study. Participants were eligible for any or all studies provided that they met all of the inclusion and exclusion criteria at baseline and had a washout period of at least 2 weeks between studies.

This study followed the tenets of the Declaration of Helsinki and was approved by the Royal Victorian Eye and Ear Hospital institutional review board (12/1094H). Written informed consent was obtained from all participants.

Intraocular Pressure and Hemodynamic Measurements

The right eye IOP was measured after topical oxybuprocaine hydrochloride 0.4% using fluorescein sodium with a slitlamp-mounted Goldmann applanation tonometer (Haag-Streit, Bern, Switzerland). The baseline blood pressure and heart rate were recorded in a seated position after approximately 5 minutes' rest with an automatic upper arm sphygmomanometer (HEM-7000-C1L; Omron Healthcare, Lake Forest, IL, USA).

Crossover Study

Participants were seen on 3 separate days at least 2 weeks apart. At each visit, participants received no drug (control), oral fluconazole 400 mg (Sandoz Pty. Ltd., Pyrmont Australia) or dispersible aspirin 600 mg (Reckitt Benckiser, West Ryde Australia). The drug order was randomized according to Table 1. A single 400-mg oral dose of fluconazole was expected to produce a plasma concentration of approximately 6.72 μg/mL 1 hour post ingestion,[16] similar to the plasma concentration used to implicate EETs in radial artery hemodynamics.[9,10] A single 600-mg dose of dispersible aspirin was expected to produce a plasma salicylate concentration of approximately 40 μg/mL 1 hour post ingestion.[17]

At each visit, participants had their right eye dilated (topical tropicamide, 1%), and had a 20-G cannula inserted in an antecubital fossa vein for blood sampling. Participants were seated in a quiet area for 30 minutes after cannulation. Flicker light–induced retinal vasodilation was measured at each visit and 1 hour after drug ingestions to allow time for absorption. Venous blood was collected in EDTA tubes and centrifuged at the time of the functional imaging. Plasma was stored (–80°C) until analyses.

Repeated Measures Studies

Similar to the main crossover study, participants received oral fluconazole 400 mg or dispersible aspirin 600 mg, and the right eye was dilated with topical tropicamide 1%. However, participants in these repeated measures studies were not cannulated. Flicker light–induced retinal vasodilation was measured at baseline and at 30-minute intervals for 2 hours after drug ingestion. We have previously shown that 30 minutes between tests is sufficient to allow retinal flicker response recovery.[18]

Flicker Light–Induced Retinal Vasodilation

Flicker light–induced retinal vasodilation was measured with the Dynamic Vessel Analyzer (DVA; IMEDOS Systems UGI, Jena, Germany) as described previously.[18] Briefly, participants seated in a dimly lit room were instructed to fixate on the tip of a fixation bar inside the camera while the fundus was examined under green light (1 = 530–600 nm) with the mydriatic camera (FF450plus; Carl Zeiss AG, Jena, Germany) set to a viewing angle of 30°. The light source was set to an average luminance of 130 cd/m^2 for all tests, measured with an ILT1700 Research Radiometer (International Light Technologies, Peabody, MA, USA). A unique fundus location was chosen as a tracking target for the DVA software (IMEDOS Systems UGI). Next, we selected a straight temporal arteriole and venule segment 0.5 to 2.0-disc calibers from the optic disc margin and at least one vessel caliber from any bifurcation or neighboring vessel.

Superior vessels were chosen where possible to reduce upper eyelid interference.

Vessel diameters were automatically and continuously measured in real-time for 350 seconds. This consisted of 50 seconds of constant light, followed by three cycles of 20 seconds of diffuse luminance flicker at 12.5 Hz, and 80 seconds of constant light. Repetition mode was used for all follow-up tests to ensure that the same vessel segments were studied. If repetition mode was unable to automatically re-identify the vessel segments, the same segments were manually re-selected using an image of the previous examination location. Baseline vessel calibers were reported in MU, where 1 MU is equivalent to 1 μm for the Gullstrand eye.[19] Maximum vessel dilation was calculated from the mean of the three measurement cycles as the maximum percentage increase in vessel caliber relative to baseline during 20 seconds of flicker stimulation.

Extraction of Plasma ($n = 12$) for Fluconazole Quantitation

Milli-Q Water (70 μL; EMD Millipore, Billerica, MA, USA) was added to plasma (50 μL) in a 2-mL Eppendorf tube. Ethyl acetate (100%) (600 μL) was added and the sample vortexed (1 minute) and centrifuged (2,200*g*, 5 minutes, room temperature [RT]). A 550-μL aliquot (upper polar phase) was transferred into a glass insert, dried in vacuo then re-dissolved in 40 μL ethyl acetate for subsequent fluconazole quantitation by gas chromatography–mass spectrometry (GC-MS).

Extraction of Plasma ($n = 12$) for Salicylic Acid Quantitation

Plasma (50 μL) was transferred into a 1.5-mL Eppendorf tube. Cold (4°C) methanol (150 μL) was added, vortexed (1 minute), placed on ice (10 minutes), then centrifuged (2,200*g*, 10 minutes, RT) to precipitate protein. A 75-μL aliquot was transferred into a glass insert, dried in vacuo, and placed in a snaplock bag with silica gel before derivatization by GC-MS. For derivatization, plasma samples were treated for 30 minutes with *N,O-bis*-(trimethylsilyl)trifluoroacetamide (40 μL) with mixing at 500 rpm at 37°C and allowed to rest for 60 minutes before injection into the GC-MS.

Gas Chromatography–Mass Spectrometry

Samples (1 μL) were injected into a GC-MS composed of a Gerstel 2.5.2 Autosampler (Gerstel GmbH, Mülheim, Germany), a 7890A Agilent gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), and a 5975C Agilent quadrupole MS (Agilent Technologies, Santa Clara, CA, USA). The MS was adjusted according to the manufacturer's recommendations using *tris*-(perfluorobutyl)-amine (CF43). Gas chromatography was performed on a 30-m VF-5MS column with 0.2 μm film thickness and a 10-m Integra guard column (Agilent Technologies).

Temperatures were set at 250°C for the injection inlet, 280°C for the MS transfer line, 250°C for the ion source, and 150°C for the quadrupole. The carrier gas (helium) flow rate was 0.8 mL/min (fluconazole) or 1.0 mL/min (salicylic acid). Sample analysis was under the following temperature program: start injection at 50°C, hold for 1 minute, ramp temperature by 15°C/min (fluconazole) or 25°C/min (salicylic acid) to 325°C and heat for 2 minutes (fluconazole) or 3 minutes (salicylic acid).

Retention times and mass spectra (unique qualifier ions) were identified and compared directly using commercially available standards of fluconazole (≥98% [HPLC], F8929-100MG; Sigma-Aldrich, Sydney, Australia) and salicylic acid (≥99% [BioXtra], SF922-100G; Sigma-Aldrich). Drug concentrations were quantified from prepared calibration curves in the linear range: 10, 25, 50, 75, 100, 125, and 150 μM for fluconazole, and 10, 25, 50, 75, and 100 μM for salicylic acid.

Mass spectra were recorded at two scans per second with an *m/z* 50 to 600 scanning range. Both chromatograms and mass spectra were evaluated using the Agilent MassHunter Workstation Software, Quantitative Analysis, Version B.05.00/ Build 5.0.291.0 for GC-MS. All matching mass spectra were additionally verified by determination of the retention time with authentic fluconazole and salicylic acid standards.

Plasma PGE_2 Metabolite Concentration

Plasma PGE_2 metabolite concentrations before and after flicker light–induced retinal vasodilation on no-drug visits were quantified on previously unthawed samples using the Prostaglandin E Metabolite EIA Kit (Cayman Chemical, Ann Arbor, MI, USA) as per manufacturer's instructions. Briefly, proteins were precipitated with acetone and samples derivatized (37°C) overnight then further purified after acidification by ethyl acetate extraction. The standard curve (in duplicate) was obtained from eight 1:1 serial dilutions of standard from 50 pg/ mL to 0.39 pg/mL. Samples were assayed undiluted in triplicate. The plate was developed by addition of Ellman's reagent, incubated for 90 minutes, and the absorbance of light at a wavelength of 412 nm was measured. The intra-assay coefficient of variation was 7.5%.

Statistical Analyses

The mean maximum relative dilations during flicker stimulation and the resting arteriole and venule calibers were compared within each study. Group means were compared using ANOVA for continuous variables or Fisher's exact test for categorical variables. Pairwise comparisons with control or baseline groups used Student's *t*-tests with Dunnett's adjustments for multiple comparisons. Data were analyzed in STATA (version 12.1; StataCorp LP, College Station, TX, USA); $P < 0.05$ was considered significant. Based on our previous data,[18] a crossover study with 12 participants would have 94% power to detect a 0.8% absolute (approximately 20% relative) reduction in arteriolar dilations 1 hour after test-drug, assuming a within-subject SD of 0.5% and two-sided significance level of 0.05.

Results

The participants' characteristics are described in Table 2. In total, 20 unique participants were recruited for the crossover and repeated measures studies. One participant completed all three studies, one completed the crossover and fluconazole repeated measures studies, and another completed the fluconazole and aspirin repeated measures studies. Different studies were separated by at least 4 weeks in participants who completed two or more studies.

In general, participants were young, mostly male and Caucasian, with normal blood pressure, body mass index, and IOP. The proportion of Caucasians and blood pressure levels were lower in the crossover study compared with the fluconazole and aspirin repeated measures studies.

Crossover Study

The maximum relative dilations of arteriole and venule segments are shown in Figure 1A. Drug treatments had no significant effect on arteriolar or venular dilations. No

Table 2. Baseline Characteristics of Participants

Parameter	All Studies, $n = 20$	Crossover, $n = 12$	Repeated Measures		P
			Fluconazole, $n = 6$	Aspirin, $n = 6$	
Age, y	25.6 (4.6)	25.7 (4.6)	27.8 (4.0)	25.2 (5.2)	0.561
Ratio of men to women	13:7	9:3	4:2	4:2	1.000
Ratio of Caucasians to other ethnicities	15:5	7:5	6:0	6:0	0.046
Ratio of brown to other iris pigmentations	9:11	7:5	2:4	3:3	0.863
Systolic blood pressure, mm Hg	113.9 (11.3)	108.2 (7.6)	122.3 (8.4)	124.7 (12.1)	0.002
Diastolic blood pressure, mm Hg	74.4 (8.6)	70.2 (5.6)	79.2 (7.0)	79.5 (8.8)	0.013
Heart rate, beats/min	63.6 (11.2)	61.6 (9.5)	63.7 (15.7)	63.5 (12.6)	0.920
Body mass index, kg/m^2	24.9 (3.2)	24.6 (2.9)	25.0 (2.8)	25.0 (4.4)	0.952
IOP, mm Hg	13.8 (3.8)	12.5 (3.7)	15.2 (4.4)	13.5 (2.7)	0.371

Data are expressed as mean (SD) unless otherwise indicated. Participant characteristics were compared between studies by ANOVA for continuous variables or Fisher's exact test for categorical variables.

significant sequence, period, or carryover effects were identified for the crossover study ($P > 0.05$ for all).

The resting calibers of arteriole and venule segments before flicker stimulation are shown in Figure 1B. Only venule calibers were significantly different between drug treatments ($P = 0.034$ by ANOVA), being driven by fluconazole, which reduced the mean (±95% confidence interval [CI]) resting venule calibers by 5.1 (4.3) MU ($P = 0.020$ after Dunnett's adjustment). Similar to dilations, no significant effect on calibers was identified for test periods or the sequence of drugs and no significant carryover effects were found ($P > 0.05$ for all).

Mean (SD) plasma fluconazole and salicylate levels 1 hour after ingestion of 400 mg oral fluconazole and 600 mg dispersible aspirin were 6.75 (2.62) μg/mL and 13.7 (11.9) μg/mL, respectively. At control visits, mean (SD) plasma prostaglandin E_2 metabolite levels were 15.10 (17.08) pg/mL and 16.03 (19.33) pg/mL immediately before and after DVA tests, respectively. This corresponded to a mean (SD) difference in concentrations of 0.93 (3.37) pg/mL ($P > 0.05$).

Changes in retinal vasodilations during flicker stimulation and resting vessel calibers between control and drug visits are presented in Table 3, stratified by tertiles of plasma drug concentrations. No significant differences between tertiles of plasma drug concentrations were identified for arteriole or venule dilations during flicker stimulation or resting calibers ($P > 0.05$ for all).

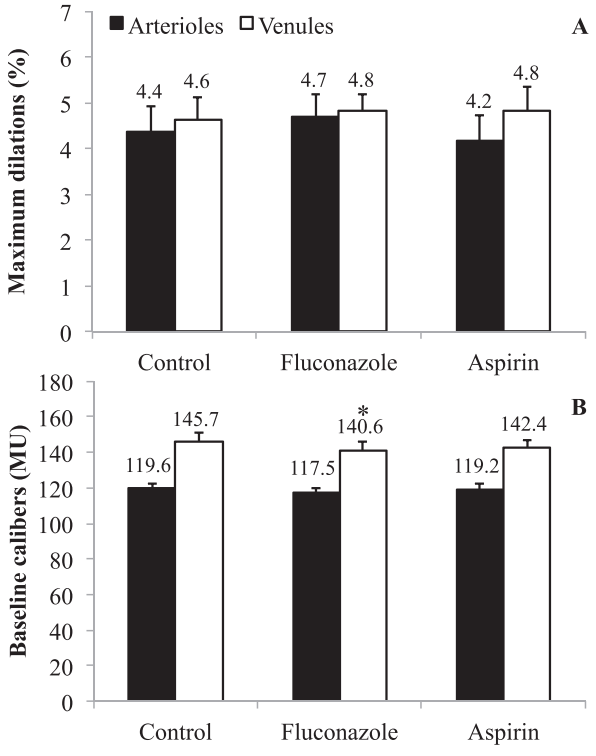

Figure 1. Crossover study. (**A**) Maximum retinal arteriole and venule dilations during flicker light stimulation. (**B**) Preflicker retinal arteriole and venule calibers. Data are expressed as means (SEM). *$P < 0.05$ versus control.

Repeated Measures Studies

Fluconazole. The effects of fluconazole on flicker light–induced retinal vasodilation and resting arteriole and venule calibers are shown in Figure 2. Maximum vessel dilations were unchanged from baseline by fluconazole at any time point ($P > 0.05$ for all). Fluconazole did not significantly affect arteriole calibers over 2 hours, although the mean (±95% CI) of resting arteriole calibers was reduced by 3.0 (3.3) MU at 120 minutes after ingestion. In contrast, fluconazole significantly affected venule calibers over 2 hours ($P < 0.001$). Fluconazole reduced the mean (±95% CI) resting venule calibers by 2.8 (2.3), 5.1 (2.3), and 5.5 (2.3) MU at 60, 90, and 120 minutes after fluconazole, respectively.

Aspirin. The effects of aspirin on flicker light–induced retinal vasodilation and resting arteriole and venule calibers are shown in Figure 3. Aspirin had no significant effect on the maximum dilations or resting calibers of arterioles or venules ($P > 0.05$ for all).

Discussion

Neither fluconazole nor aspirin reduced flicker light–induced retinal vasodilation in humans in vivo. This was a consistent finding both in our crossover and repeated measures studies. Indeed, our repeated measures studies appeared to suggest that fluconazole may actually increase, rather than decrease, dilations during flicker stimulation. However, in further analyses (not shown), fluconazole had no effect on maximum absolute vessel calibers and the apparent increase in relative dilations appeared to be a result of smaller calibers before flicker stimulation. Given that fluconazole and aspirin inhibit the enzymes CYP2C and COX, respectively, our findings suggest that these EETs and PGs are not major contributors to retinal hyperemia during flicker light stimulation in humans.

Table 3. Changes in Retinal Vasodilations and Resting Calibers Between Control and Drug Visits by Tertiles of Plasma Drug Concentrations

			Change vs. Control			
n	Tertile	Range, µg/mL	Arteriole Dilation, %	Venule Dilation, %	Arteriole Caliber, MU	Venule Caliber, MU
Fluconazole						
4	1	<5.00	0.0 (2.6)	−0.2 (2.0)	−1.1 (7.9)	−5.6 (3.0)
4	2	5.00 to <7.80	−0.3 (1.6)	1.1 (0.5)	−2.1 (5.8)	−4.7 (5.3)
4	3	≥7.80	1.2 (1.6)	−0.2 (1.6)	−3.3 (1.3)	−5.0 (5.2)
		P	0.572	0.438	0.871	0.963
Salicylic acid						
4	1	<8.00	0.5 (1.8)	0.1 (0.8)	0.4 (2.6)	−6.7 (10.6)
4	2	8.00 to <11.00	0.4 (0.1)	−0.3 (0.6)	−1.5 (2.9)	−2.7 (3.8)
4	3	≥11.00	−1.6 (1.7)	0.8 (2.2)	−0.3 (2.7)	−0.6 (1.4)
		P	0.119	0.533	0.622	0.439

Data are expressed as mean (SD). Results between tertiles of plasma drug concentrations were compared by ANOVA.

Our results contrast with recent rodent data that indicate these responses are a result of glial-derived EETs and PGs.[5,6] This discrepancy may be due in part to the experimental models. These rodent studies used ex vivo retinas that had been surgically removed and perfused by oxygen bubbled through saline, not blood. Furthermore, the arterioles were preconstricted with a thromboxane analog. In vivo studies with cats[2,3] and humans[4] have consistently implicated NO as a major contributor to retinal hyperemia during flicker light stimulation. Our results suggest that EETs and PGs are not major contributors to retinal hyperemia in humans, and are consistent with the paradigm that NO is the primary mediator of these responses.[1]

We did not find a significant effect of fluconazole on resting retinal arteriole calibers in either study. Arterioles were probably slightly constricted at 120 minutes after fluconazole ingestion, but the change was small and our study was not powered to detect differences in this outcome. This is consistent with the previous finding of radial artery constriction with intra-arterial fluconazole and L-NMMA, but not with either drug alone.[10] In contrast, fluconazole consistently and significantly induced venule constriction in both our crossover and repeated measures studies. The greatest change was seen at 120 minutes after ingestion, with a constriction of approximately 4% from baseline. This suggests that EETs might play a role in the regulation of retinal blood flow under physiological conditions via a venous dilatory effect. Aspirin ingestion had no effect on either retinal arteriole or venule calibers.

We measured plasma fluconazole and salicylate concentrations to confirm the effectiveness of our oral drug administration. Plasma fluconazole levels were almost identical to those

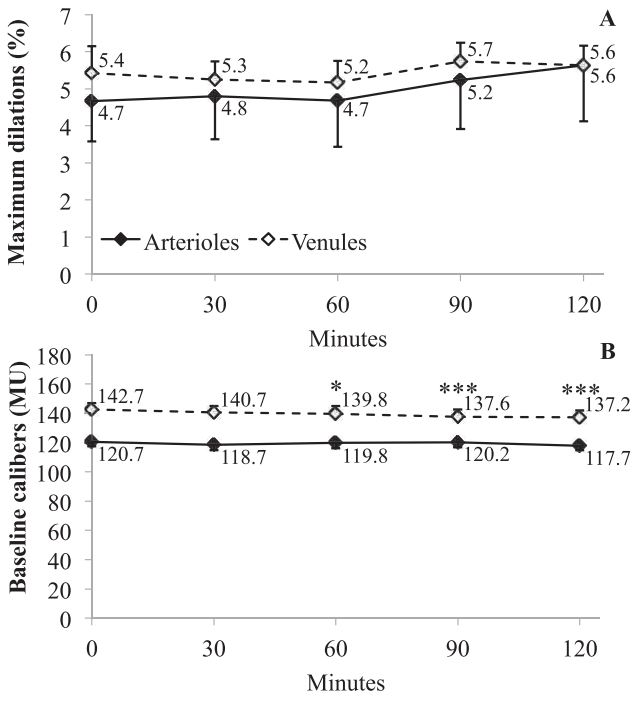

Figure 2. The effects of fluconazole over 2 hours on (**A**) maximum flicker light–induced retinal vasodilations, and (**B**) baseline retinal vessel calibers. Data are expressed as means (SEM). *$P < 0.05$; ***$P < 0.001$ versus baseline.

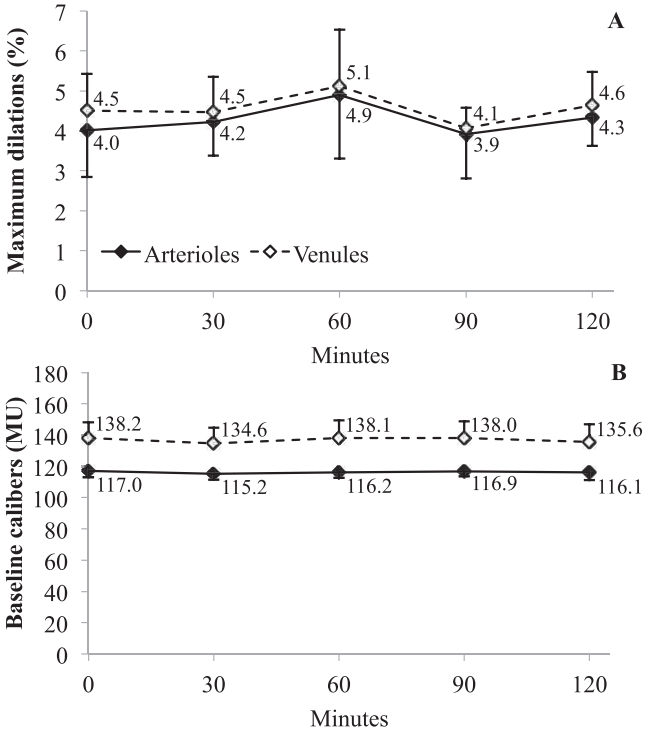

Figure 3. The effects of aspirin over 2 hours on (**A**) maximum flicker light–induced retinal vasodilations, and (**B**) baseline retinal vessel calibers. Data are expressed as means (SEM).

previously reported at 1 hour after ingestion of 400 mg.[16] Surprisingly, plasma salicylate levels were only 13.7 μg/mL at 1 hour after ingestion of 600 mg dispersible aspirin, roughly one-third of what was expected.[17] This may have been due in part to the participants' nonfasting status or samples taken too early to capture peak salicylate concentrations.[20] However, as an irreversible inhibitor, the duration of aspirin is dependent on the rate of COX turnover, which is roughly 6 hours in humans.[21] Similar doses of aspirin have potentiated,[15,22] reduced,[13,14,23] or had no effect[24,25] on cutaneous hyperemia during iontophoresis, but no study has examined salicylate concentrations with functional responses, as we have. We also compared changes between control and drug visits in retinal vessel dilations during flicker stimulation and resting calibers by tertiles of measured drug concentrations. We did not find any differences between tertiles of drug concentrations, but with only four participants per tertile, our crossover study may have had limited power to detect such differences.

Given that PGE_2 is the main vasodilator prostaglandin of glia,[7] we hypothesized that plasma PGE_2 metabolite levels may increase immediately after retinal flicker stimulation. Plasma PGE_2 metabolite concentrations were highly variable between participants, but remained stable before and after our tests. Our results did not implicate PGE_2 as a mediator of retinal functional hyperemia, although this was not unexpected given that our samples were very diluted from the source and obtained far from the eye at the cubital fossa.

Strengths of our study included our use of pharmacological inhibitors to investigate directly the contribution of EETs and PGs to flicker light–induced retinal vasodilation in humans and our quantification of drug levels. Second, we approached our research question using two study designs. The crossover study minimized bias and the effects of potential confounders, whereas our repeated measures studies characterized acute drug effects over 2 hours. Both approaches were statistically efficient and enabled testing for within-person effects without requiring large participant numbers. Finally, our DVA system allowed us to obtain accurate real-time measurements of retinal vessel calibers. Although vasodilation is considered an indirect measure of hyperemia, this technique is currently more reliable than other techniques, such as laser Doppler velocimetry.[18,26]

A major limitation of in vivo functional studies in humans with systemic drugs is that we are restricted to drugs approved for human use. Unfortunately, many of the drugs used by Newman and colleagues[5,6] are not safe for human research. In addition, we cannot determine how completely fluconazole and aspirin inhibited their respective enzymes at these doses. Finally, we did not measure visual acuity or refractive errors in our participants. However, only four of our participants had myopia and we did not previously find any relationship between refractive error and retinal flicker responses in young people.[18] We did not consider our sample sizes to be a major limitation, as our studies were designed to detect large within-person effects, which should have been evident if EETs or PGs were major mediators.

Given our results, additional in vivo animal studies are required to confirm the importance of EETs, PGs, and NO in retinal functional hyperemia. In particular, studies are needed to identify the cellular source of flicker light-stimulated NO production. Recent epidemiological studies indicate that diabetic retinopathy progression is associated with wider retinal vessels.[27] Considering that EETs appear to regulate retinal venule calibers, the relationship between wider retinal vessels and EETs or PGs merits study in diabetes.

Our data do not support a major role for EETs or PGs in flicker light–induced retinal vasodilation. Given previous findings,[2–4] NO is probably the principal mediator of this response. EETs appear to regulate retinal venular caliber to a small degree in vivo and might therefore play a minor role in the physiological regulation of retinal blood flow at rest.

Acknowledgments

We thank Jing Xie for expert statistical advice and Andrzej Januszewski (National Health and Medical Research Council Clinical Trials Centre, Sydney, Australia) for the PGE_2 metabolite assay.

Supported by the Royal Victorian Eye and Ear Hospital. The Centre for Eye Research Australia receives Operational Infrastructure Support from the Victorian government. Also supported by the Australian National Health and Medical Research Council Postgraduate Medical Scholarship (ID1038701) (JEN) and Australian National Health and Medical Research Council Research Fellowships (ID1045280 and 1003113) (ELL and GJD).

Disclosure: **J.E. Noonan**, None; **G.J. Dusting**, None; **T.T. Nguyen**, None; **A.J. Jenkins**, None; **R.E.K. Man**, None; **W.J. Best**, None; **D.A. Dias**, None; **N.S. Jayasinghe**, None; **U. Roessner**, None; **E.L. Lamoureux**, None

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