Analysis of Retinal Vasodilation after Flicker Light Stimulation in Relation to Vasospastic Propensity

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Purpose. To explore the maximum retinal vasodilation in response to repeated flicker light stimulation in relation to vasospastic propensity in healthy subjects.

Methods. Twenty-four young healthy women were grouped as vasospastic and nonvasospastic, based on their history of cold extremities and on the results of nailfold capillaroscopy. A retinal vessel analyzer was used to obtain recordings of the ocular fundus during still illumination and three flicker light stimulations. Retinal vessels were analyzed in the immediate vicinity of the optic nerve head and 2 to 3 disc diameters away from the disc. The maximum dilatory amplitudes were always the highest 1-second mean vessel diameter in response to each of the three flicker light stimuli.

Results. Maximum dilatory amplitude (in percent) was, in the proximal measurement site in the arterioles, 6.2 ± 2.6, 4.8 ± 2.1, and 6.6 ± 3.9 in the vasospastic group, and 7.9 ± 3.2, 8.6 ± 4.1, and 9.1 ± 4.7 in the nonvasospastic group in three repeated flicker stimulations. Corresponding values for distal measurement sites were 6.7 ± 2.5, 4.8 ± 3.4, and 4.7 ± 4.4 and 9.0 ± 3.7, 11.0 ± 5.2, and 12.3 ± 7.7. The maximum amplitude was significantly lower in the vasospastic group ($P = 0.001$). The maximum venule dilation was also significantly lower in the vasospastic group ($P = 0.037$). Vessel diameters failed to stabilize at the original baseline level during the 80-second recovery period, and this baseline offset had opposite signs in the arterioles in the vasospastic (remained below the original baseline) and nonvasospastic (remained above the original baseline) groups.

Conclusions. The maximum dilatory amplitude was significantly lower in vessels in the vasospastic group. An augmentation of the maximum vasodilation was observed in the nonvasospastic group after repeated flicker stimulations, a phenomenon that was missing in arterioles of vasospastic subjects. It seems that such different behavior is due to the opposite baseline offsets in interflicker periods in the two groups. (*Invest Ophthalmol Vis Sci.* 2006;47:4034–4041) DOI: 10.1167/iovs.06-0351

Due to the light sensitivity of the neural circuitry in the retina on one side, and due to direct optical accessibility of fundus blood vessels on the other, an eye offers a unique opportunity to study neurovascular coupling in vivo. Accordingly, several studies of human retinal circulation were conducted with various flicker light stimuli.[1–5] Retinal vessel response to flicker light is practically instantaneous, and these studies took advantage of the retinal vessel analyzer (Retinal Vessel Analyzer [RVA]; IMEDOS GmbH, Weimar, Germany), which offers high spatial vessel width resolution,[6] high temporal resolution,[6,7] and a high reproducibility of baseline and flicker response measurements.[3,8,9] The key role in the ocular vasodilatory response to increased neuronal activity is most likely reserved for nitric oxide.[10,11] The influence of other factors on the retinal vessel flicker response was also investigated, such as insulin-dependent diabetes,[12] hyperglycemia with insulin clamps,[13] blood pressure,[14,15] intraocular pressure (IOP),[16] lactate,[15] dopamine,[17] age, and baseline retinal vessel diameter.[14] Vasospasm is defined as inappropriate constriction or insufficient dilatation in the microcirculation, and because such vasoconstrictions are often combined with simultaneous arterial or venous dilatations in neighboring vessels or in other vascular beds, the term vascular dysregulation has been introduced.[18,19] Identifying such individuals may be clinically relevant, because systemic vascular dysregulation has been associated with several ocular diseases, including central serous chorioretinopathy,[20] glaucoma,[21] central vein thrombosis,[22] and nonarteritic anterior ischemic neuropathy.[23] Changes in retinal vessels have also been observed in patients with vascular dysregulation in other organs, such as the heart[24] or the brain.[25] However, vascular dysregulation in the eye is still poorly defined. Blood flow perturbation as a result of dysregulation is limited in time, and blood flow measurement at baseline does not necessarily show an alteration. Retinal vessel diameter at the baseline is statistically comparable between vasospastic and control subjects.[26] Therefore, a provocation test such as flicker light stimulation may be more suitable for identifying and further describing vascular dysregulation in the ocular circulation. In the present study, we analyzed maximum dilation of retinal vessels in response to flicker light stimulation with the RVA. As the proximity of the fenestrated choroidal capillaries in the optic nerve head may influence the environment for smooth muscle cells in the vessel wall,[27] in contrast to the more peripheral retina where both the inner and the outer blood–retinal barriers are in place, we explored the vasomotion in the immediate vicinity and distal to the optic disc.

Methods

Subjects

Forty healthy nonsmoking women were screened for the study. The protocol adhered to the tenets of the Declaration of Helsinki. After approval by the ethics committee, we obtained the informed consent of the subjects. A notification in the University Eye Clinic of Basel informed potential volunteers (collaborators, students, parents and friends of patients) of the opportunity to participate in a scientific research project. Subjects were screened for ocular and systemic diseases. A detailed medical and ophthalmic history was recorded, and all subjects completed an ophthalmic examination. Included were individuals with no history of ocular or systemic disease, no history of

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chronic or current systemic or topical medication (except for contraceptive pills, subjects were entirely drug free for at least 1 week before examination, including over-the-counter drugs), and no history of drug or alcohol abuse. Further inclusion criteria were normal systolic (100–140 mm Hg) and diastolic (60–90 mm Hg) blood pressure, best corrected visual acuity >20/25 in both eyes, ametropia within −3.0 to +3.0 D of spherical equivalent and <1 D astigmatism in each eye, an IOP lower than 20 mm Hg in each eye by (Goldmann) applanation tonometry, and no pathologic findings after slit lamp examination and indirect funduscopy. Subjects were classified as having vasospasm if they related a clear history of frequently cold hands (answering yes to the questions: "Do you always have cold hands, even during the summer?" and "do other people tell you that you have cold hands?") and as healthy subjects if they reported no such history. Vasospastic propensity or the absence of it had to be confirmed by nailfold capillaroscopy, with the examiner unaware of the history of cold hands. Subjects with contradictory findings from nailfold capillaroscopy and history or describing sometimes having cold hands were excluded from the present analysis. A positive history of contraceptive pills use was not an exclusion criterion. As hormonal status may influence ocular circulation,[28] subjects not taking contraceptive pills all had to be in the postovulation phase of the cycle, which was verified by a subsequent phone interview ascertaining that menstrual bleeding had occurred less than 2 weeks after the study examination day.

Nailfold Capillaroscopy

Nailfold capillaroscopy was performed in a room with a constant temperature of approximately 23°C (range, 21–25°C). Before the examination, the hands of the subjects were warmed in a water bath of 40°C. The skin of the nailfold was made transparent by a drop of oil, rendering the capillaries running parallel to the skin surface and the flow of cellular elements visible under a light microscope. A microscope coupled to a television monitor, which was coupled to a video recorder, was used, allowing the observed blood flow to be videotaped and to be analyzed off-line. After the baseline flow recording, the nailfold area was cooled down to 14°C to 15°C for 60 seconds by rapidly decompressing carbon dioxide (gas stream temperature, −15°C), and the occurrence and duration of blood flow standstill was recorded. A closure of one or more visible capillaries with an average stop-time longer than 12 seconds was defined as a vasospastic reaction.[29]

Retinal Vascular Diameter Measurements

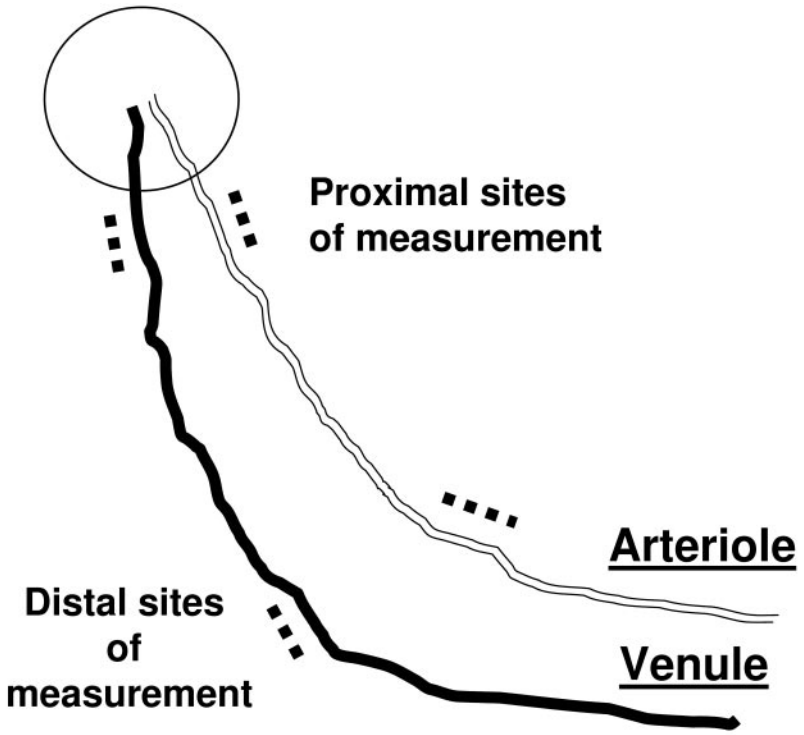
Retinal vessel diameter was measured with the RVA (IMEDOS GmbH).[6,7] An essential part of the RVA device is the fundus camera (FF450; Carl Zeiss Meditec, Jena, Germany), which allows the examination and recordings of the ocular fundus. It incorporates the illumination and the observation optical pathway. After being reflected from the retina, the light is delivered through the observation pathway to the observation ocular and to the charge-coupled device (CCD) chip of the video camera simultaneously. The standard video signal from the CCD then goes to the RVA control computer and to the SVHS recorder, which enables subsequent offline measurements of the recorded session later on. The measuring principle of the RVA is as follows: Inside the walls of the retinal blood vessels, there is a column of red blood cells, separated from the walls by the plasma edge stream. Red blood cells absorb one part of the light. The RVA measures the diameter of the column of the red blood cells. For measurements, the fundus camera is adjusted to the dilated pupil, and a clear fundus image with good contrast and no reflections is obtained on the monitor. For the current experiment, temporal resolution was set at 40 ms, which translated to 25 captured video frames per second. The RVA produces one vessel width measurement, expressed in units of measurement (UM), for each segment length of 12.5 UM. In an emmetropic person, 1 UM = 1 μm. An optoelectronic shutter, inserted in the camera, interrupts the observation light (530–600 nm, irradiance at the fundus $\sim 1.96 \times 10^{-4}$ W/cm^2) over the entire 30° visual field of the retinal camera and produces a bright-to-dark contrast ratio of at least 25:1. The chosen frequency of 12.5 Hz of rectangular light interruption provided a sequence of one normal illuminated and one dark single frame at a video frequency of 25 Hz. This frequency lies in the range of the maximally exciting flicker frequency.[5] The optoelectronic shutter is controlled by a special program running on the RVA computer. It produces practically no sound, but subjects are visually aware of the flickering light. However, these conditions affected both groups equally. We used the protocol described by Nagel et al.[3,4]: After the baseline recording of 50 seconds, three flicker periods of 20 seconds each were applied, interrupted with 80 seconds of still illumination.

Experimental Procedures

After evaluation of inclusion criteria and the nailfold capillaroscopy, an RVA measurement was scheduled. The investigator performing RVA measurement was masked to the history and nailfold capillaroscopy results. Retinal vessel diameters were measured in the morning in one randomly chosen eye, after the subject had fasted for 12 hours overnight. Participants were instructed to abstain from a large meal, alcohol consumption (including alcohol-containing products and drugs), and physical exercise for 24 hours before the measurements. On the day of the experiments (in the morning hours between 8 and 9 AM), the subjects were seated for 30 minutes in the laboratory, and local tropicamide was applied in one randomly selected eye three times every 5 minutes for pupil dilation. Blood pressure was measured every 10 minutes for at least 30 minutes. After stabilization of blood pressure, retinal vessel diameter was assessed. The fellow eye was covered to improve fixation during imaging with the fundus camera. The image of the retina was adjusted on the screen of the real-time monitor, and a recording was taken of the inferior temporal quadrant. This quadrant's vessels were chosen because they are among the largest in the retina and are readily accessible for the recording performed in the present study. Blood pressure (BP) and pulse-rate readings were taken during the measurement. The recordings were stored on a videotape, and evaluated off-line, outlining a region of interest on the desired site of measurement along the major temporal inferior branch arteriole and venule, as described in the following text. After retinal vessel assessment, IOP was measured by Goldmann applanation tonometry.

Analysis of Maximum Vessel Dilation

We tested the hypothesis that the maximum vasomotion in response to flicker light stimulation is different between the vasospastic and nonvasospastic groups and that the proximity to the optic disc influences this response. In each subject, two segments of the major temporal inferior branch of the arteriole and venule entered the analysis. Each chosen segment length was at least 500 UM long (corresponding in an emmetropic eye to 500 μm). One (proximal) segment was in the immediate vicinity of the optic disc, the other (distal) was 2 to 3 optic disc diameters away from the disc (Fig. 1). The whole recording lasted 350 seconds, as detailed earlier. Subjects stabilized their fixation and positioning during the first 30 seconds of the recording, and the last 20 seconds before the start of the first flicker vessel diameter was averaged and taken as the first baseline value. The point of maximum dilation was determined for each flicker stimulation as the highest 1-second mean vessel diameter during the period of 50 seconds after the beginning of the corresponding flicker stimulus. This period was chosen, as the study from Nagel et al.[4] has shown that after the flicker stimulus of 20 seconds, it takes ~30 seconds for the vessel diameter to stabilize again. Thus, three points of maximum dilation were obtained in all measuring sites (proximal and distal in arterioles and venules) and all were expressed as a percentage of the corresponding first baseline value. These maxima entered a three-way analysis of variance (ANOVA) model, where one factor was the difference between the vasospastic and nonvasospastic group), difference between sites of measurement (proximal/distal) was the second factor, and the third factor was the



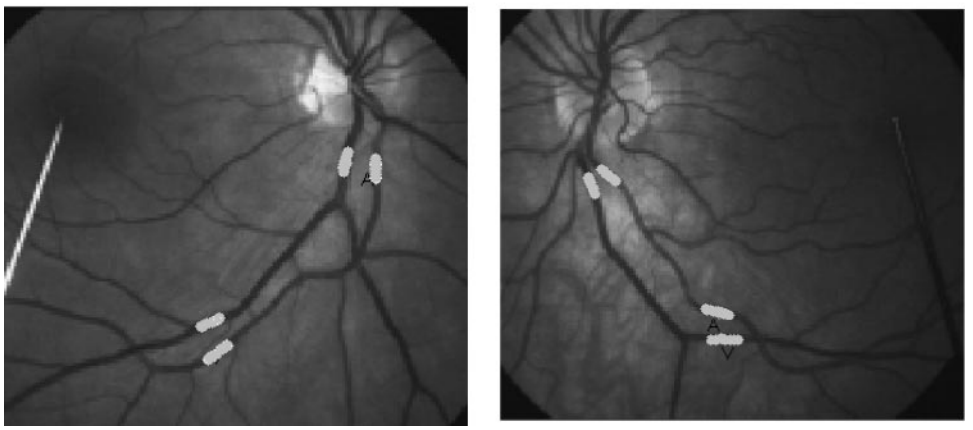


FIGURE 1. Schematic representations of the sites of measurement in the present study. Two fundus photographs show examples of the chosen measurement sites (the RVA enabled simultaneous paired measurement; depicted are two pairs in each picture that were measured offline from the band).

difference in maximum dilation between three-flicker stimulation. The analysis was performed separately for arterioles and venules.

Change of Vessel Diameter between Flicker Stimulations

This part of the analysis was result driven. The hypothesis that in the present protocol with 80 seconds recovery time before next flicker stimulus, the vessel diameter returns to baseline, hence there is no offset to the first baseline, was postulated and tested. It was tested with a three-way ANOVA model, analyzing in original units the first baseline (as described earlier, mean vessel diameter during 20 seconds before the first flicker stimulus), second (20 second before the second flicker), and third baseline (20 seconds before the third flicker). Factor 1 was the difference between the vasospastic and nonvasospastic groups, factor 2 was the difference between the proximal and distal measuring sites, and factor 3 was the differences between three baselines.

Corrected Maximum Vessel Dilation

If an offset to the first baseline is indeed present and taken into account, does the maximum vessel dilation differ between the vasospastic and nonvasospastic group? To answer this question, the point of maximum dilation (as described earlier) was expressed as a the percentage of the immediately preceding baseline and tested with the same three-way ANOVA model, as described earlier.

RESULTS

Forty healthy nonsmoking women were screened for the study. Sixteen subjects were excluded due to a mismatch between the nailfold capillaroscopy and the history of cold hands and feet, because they described sometimes having cold hands and feet, due to a poor RVA recording (unstable fixation during the 350 seconds of RVA measurements), or because the

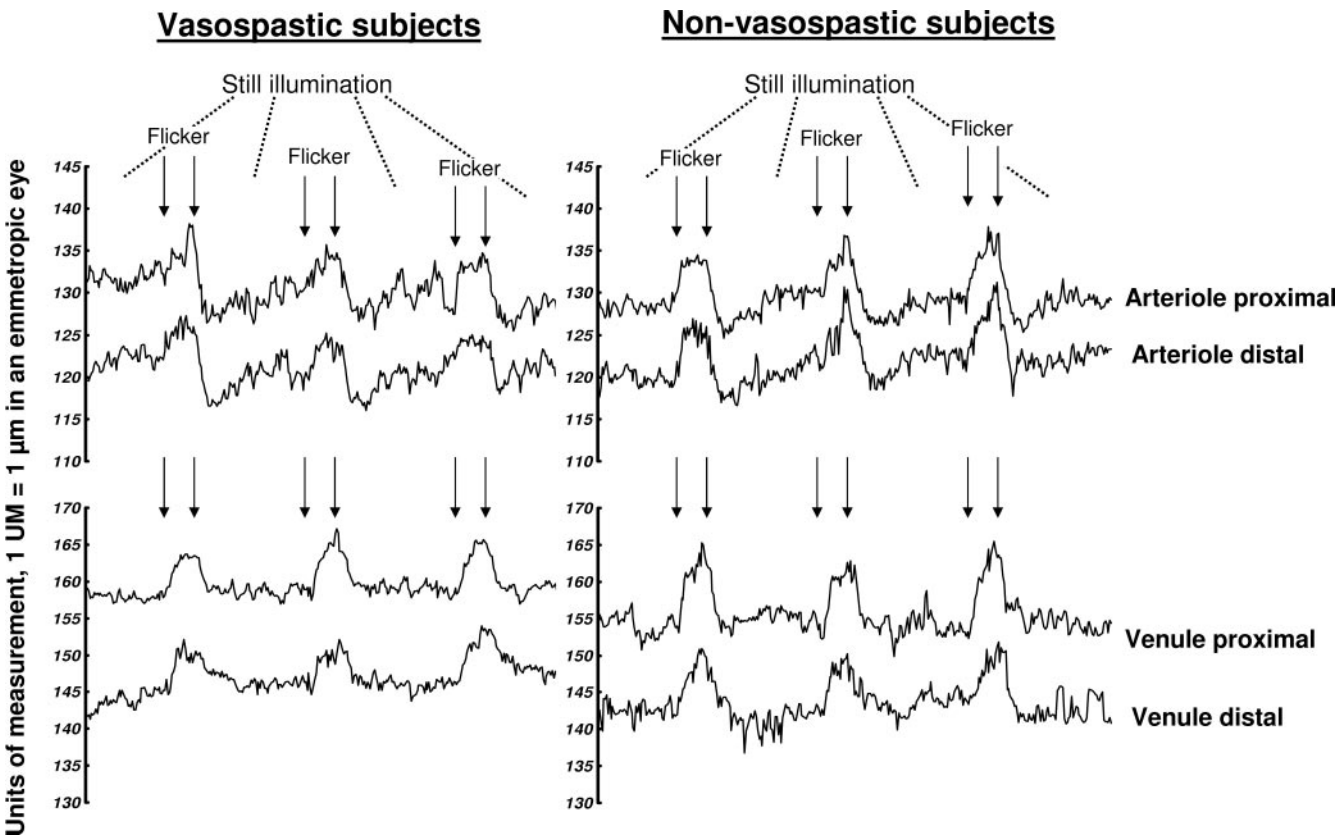


FIGURE 2. Trace recordings of the vessel diameter of the whole 350 seconds in original units of measurements (1 UM = 1 μm in an emmetropic eye). Data were averaged to 1-second data, and then averaged within the groups of vasospastic and normal control subjects. The first sequence of still illumination lasting 50 seconds was followed by three cycles of flicker light stimulation (20 seconds) and still illumination (80 seconds).

study measurement took place more than 2 weeks before menstrual bleeding. Ultimately, 24 subjects entered the analysis, 12 with a positive history of cold hands and feet and a positive nailfold capillaroscopy result, and 12 with negative history and negative capillaroscopy result. The average age was 22.75 ± 2.8 and 23.4 ± 3.6 years (*t*-test for independent samples, $P = 0.61$), IOP in the experimental eye was 12.9 ± 1.8 and 12.2 ± 2.5 mm Hg ($P = 0.38$), systolic blood pressure 112.1 ± 10.0 and 114.3 ± 13.3 mm Hg ($P = 0.67$), diastolic blood pressure 72.6 ± 9.2 and 73.3 ± 9.1 mm Hg ($P = 0.87$), pulse rate 69.2 ± 8.6 and 70.8 ± 10.5 beats per minute ($P = 0.70$), and ocular perfusion pressure 44.3 ± 5.6 and 45.8 ± 5.6 mm Hg ($P = 0.53$). Spherical equivalent in the experimental eye was −2.25 ± 1.5 (0 to −3.0) and −1.0 ± 1.2 D (+0.5 to −3.0; $P = 0.11$) in the nonvasospastic and vasospastic groups, respectively. Both groups were also comparable in contraceptive pills use (9 positive/3 negative versus 10 positive/2 negative in the nonvasospastic and vasospastic groups, respectively; χ^2 $P = 0.62$).

Before any flicker stimulation, first baseline vessel diameters (mean ± SD, given in units of measurement, 1 UM = 1 μm in an emmetropic eye) at the proximal and distal arteriolar measuring sites were 132.1 ± 14.0 and 122.4 ± 15.0 in the vasospastic group, and 128.4 ± 23.7 and 119.6 ± 23.2 in the nonvasospastic group, respectively (independent *t*-test for difference between the vasospastic and nonvasospastic groups, for proximal arterioles $P = 0.65$, distal arterioles $P = 0.72$). For venules, baseline diameters were at proximal and distal measuring sites 157.7 ± 16.8 and 144.4 ± 13.0 in the vasospastic group and 153.1 ± 26.6 and 142.8 ± 15.8 in the nonvasospastic group (independent *t*-test, for proximal venules $P = 0.62$, distal venules $P = 0.79$).

Figure 2 shows original trace recordings of vessel diameters averaged within groups on a 1-second basis.

Maximum Vessel Dilation

Maximum dilation amplitudes as percentages of the corresponding first baseline are reported in Table 1 for arterioles

TABLE 1. Maximum Dilation Amplitude of Arterioles and Venules

Maximum Dilation Response	Nonvasospastic Subjects	Vasospastic Subjects
Proximal arterioles		
First flicker stimulation	7.9 ± 3.2	6.2 ± 2.6
Second flicker stimulation	8.6 ± 4.1	4.8 ± 2.1
Third flicker stimulation	9.1 ± 4.7	6.6 ± 3.9
Distal arterioles		
First flicker stimulation	9.0 ± 3.7	6.7 ± 2.5
Second flicker stimulation	11.0 ± 5.2	4.8 ± 3.4
Third flicker stimulation	12.3 ± 7.7	4.7 ± 4.4
Proximal venules		
First flicker stimulation	10.7 ± 5.1	5.5 ± 2.6
Second flicker stimulation	10.8 ± 4.6	6.8 ± 4.0
Third flicker stimulation	10.9 ± 5.6	6.6 ± 3.3
Distal venules		
First flicker stimulation	6.3 ± 5.5	6.7 ± 2.2
Second flicker stimulation	8.7 ± 4.7	7.7 ± 4.1
Third flicker stimulation	10.1 ± 6.6	7.9 ± 2.5

Maximum dilation response is the highest 1-second mean vessel diameter during the period of 50 seconds after the beginning of each flicker stimulation, expressed as a percentage of the corresponding first baseline. Data are expressed as mean percentages ± SD.

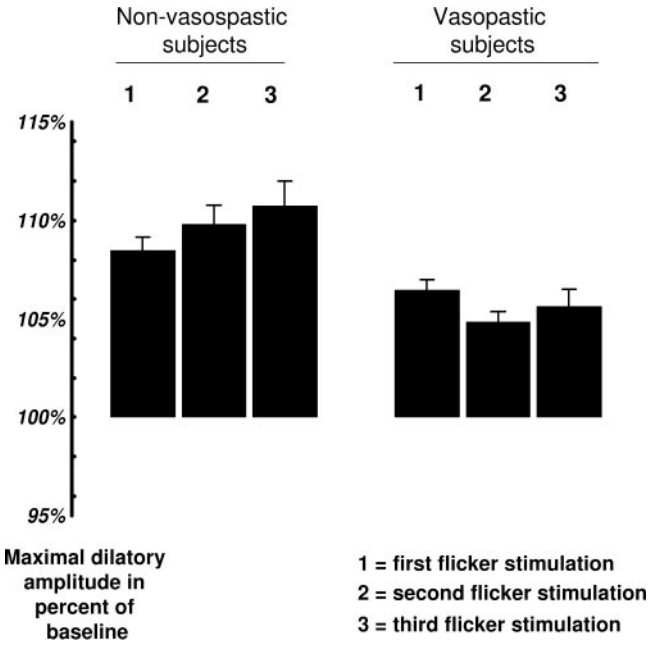


FIGURE 3. Maximum dilatation amplitude of arterioles (the highest 1-second mean vessel diameter value during the period of 50 seconds after the beginning of each flicker stimulation expressed in percents of the corresponding first baseline). Data from distal and proximal site of measurement in arterioles are pooled together in this graph to graphically depict the different time course of parameter in vasospastic and control subjects (date are expressed as mean percentages ± SE).

and venules. Results of the 3-way ANOVA (factor 1: the difference between vasospastic and nonvasospastic subjects; factor 2: the difference between sites of measurement proximal versus distal; factor 3: the difference between responses to three repeated flicker stimulations) for arterioles were: factor 1, $P = 0.001$; factor 2, $P = 0.26$; and factor 3, $P = 0.37$. The only significant interaction was between factors 1 and 3, $P = 0.033$; other interactions were $0.11 \leq P \leq 0.91$. The results demonstrated lower maximum dilation of arterioles in vasospastic subjects (factor 1) and a different time course between groups in three repeated flicker stimulations (interaction factors 1–3), graphically depicted in Figure 3.

For venules, results of the 3-way ANOVA (factors as detailed earlier) were: factor 1, $P = 0.037$; factor 2, $P = 0.54$; and factor 3, $P = 0.023$. Interactions were not significant. The results demonstrated lower maximum dilation of venules in vasospastic subjects (factor 1) and increasing dilation amplitude in the repeated flicker stimulations in both groups (factor 3).

Vessel Diameter Change between Flicker Stimulations

Results of the first, second, and third baseline tests are presented in Table 2. For arterioles, there was no difference in overall baseline diameters between groups ($P = 0.85$). The second and third baselines behaved differently in two groups: In nonvasospastic subjects there was a positive offset, with arterioles failing to reach the original baseline within the 80 seconds of interflicker recovery; in vasospastic subjects, the offset was negative, with undershooting below the original baseline in the interflicker periods. This difference in behavior was statistically significant ($P = 0.013$). For venules, there was also no difference in overall baseline diameters between groups ($P = 0.69$). the first, second, and third baselines differed among themselves ($P = 0.016$), with second and third baselines higher than the first, but this variation was comparable between the vasospastic and nonvasospastic group ($P = 0.61$).

Corrected Maximum Vessel Dilation

Because the analysis of baseline vessel diameters between flicker stimulation revealed a significant offset to first baseline, we tested whether the corrected maximum dilation amplitudes (expressed in percent of the immediately preceding baseline rather than of first baseline) are different between vasospastic and nonvasospastic subjects, in proximal and distal measurement locations. The results are shown in Table 3. Corrected maximum responses were indeed higher in the nonvasospastic group in both arterioles ($P = 0.028$) and venules ($P = 0.029$); however, there was no significant variability of these responses in repeated flicker stimulations (factor 3 was alone or the interactions were statistically nonsignificant). Of note, the proximal and distal measuring sites demonstrated somewhat

TABLE 2. First, Second, and Third Baseline Measurements in Arterioles and Venules

Baseline Vessel Diameters	Nonvasospastic Subjects	Vasospastic Subjects	ANOVA Results
Proximal arterioles			
First baseline	128.4 ± 23.7	132.1 ± 14.0	Factor 1 $P = 0.85$
Second baseline	130.5 ± 23.0	131.0 ± 14.5	Factor 2 $P = 0.015$
Third baseline	129.8 ± 24.5	131.6 ± 14.8	Factor 3 $P = 0.75$
Distal arterioles			Factors 1 & 2 $P = 0.87$
First baseline	119.6 ± 23.2	122.4 ± 15.0	Factors 1 & 3 $P = 0.013$
Second baseline	121.1 ± 22.4	121.2 ± 15.0	Factors 2 & 3 $P = 0.93$
Third baseline	121.5 ± 24.5	121.1 ± 15.1	Factors 1, 2, & 3 $P = 0.65$
Proximal venules			
First baseline	153.1 ± 26.6	157.7 ± 16.8	Factor 1 $P = 0.67$
Second baseline	156.0 ± 28.6	158.4 ± 17.6	Factor 2 $P = 0.0054$
Third baseline	154.6 ± 24.8	158.8 ± 16.4	Factor 3 $P = 0.016$
Distal venules			Factors 1 & 2 $P = 0.80$
First baseline	142.8 ± 15.8	144.4 ± 13.0	Factors 1 & 3 $P = 0.61$
Second baseline	142.6 ± 15.5	146.2 ± 12.8	Factors 2 & 3 $P = 0.55$
Third baseline	145.3 ± 17.1	145.3 ± 13.7	Factors 1, 2, & 3 $P = 0.09$

Baseline measurements were obtained at the last 20 seconds before the next flicker stimulation. Units of measurement (1 UM = 1 μm in an emmetropic eye, mean ± SD). Results of three-way ANOVA: factor 1, the difference between vasospastic and nonvasospastic subjects; factor 2, the difference between the proximal and distal measuring sites; and factor 3, the difference between the three baselines.

TABLE 3. Corrected Maximum Dilation Amplitude of Arterioles and Venules

Corrected Maximal Dilation Response	Nonvasospastic Subjects	Vasospastic Subjects	ANOVA Results
Proximal arterioles			
First flicker stimulation	7.9 ± 3.2	6.2 ± 2.6	Factor 1 $P = 0.028$
Second flicker stimulation	6.6 ± 2.4	5.7 ± 1.5	Factor 2 $P = 0.074$
Third flicker stimulation	8.1 ± 4.2	7.1 ± 4.9	Factor 3 $P = 0.40$
Distal arterioles			Factors 1 & 2 $P = 0.042$
First flicker stimulation	9.0 ± 3.7	6.7 ± 2.5	Factors 1 & 3 $P = 0.82$
Second flicker stimulation	9.5 ± 5.6	5.9 ± 1.9	Factors 2 & 3 $P = 0.77$
Third flicker stimulation	10.7 ± 7.5	5.9 ± 3.4	Factors 1, 2, & 3 $P = 0.43$
Proximal venules			
First flicker stimulation	10.7 ± 5.1	5.5 ± 2.6	Factor 1 $P = 0.029$
Second flicker stimulation	8.9 ± 3.9	6.3 ± 4.0	Factor 2 $P = 0.45$
Third flicker stimulation	9.6 ± 4.4	5.8 ± 3.0	Factor 3 $P = 0.081$
Distal venules			Factors 1 & 2 $P = 0.038$
First flicker stimulation	6.3 ± 5.6	6.7 ± 2.2	Factors 1 & 3 $P = 0.99$
Second flicker stimulation	8.8 ± 3.8	6.3 ± 2.6	Factors 2 & 3 $P = 0.36$
Third flicker stimulation	8.3 ± 5.4	7.3 ± 3.9	Factors 1, 2, & 3 $P = 0.10$

Maximum dilation amplitude is the highest 1-second mean vessel diameter during the 50 seconds after the beginning of each flicker stimulation, expressed as a percentage of the immediately preceding baseline. Data are expressed as the mean percentage ± SD. Results of three-way ANOVA: factor 1, the difference between vasospastic and nonvasospastic groups; factor 2, the difference between the proximal and distal measuring sites; and factor 3, the difference between the corrected maximum responses in three repeated flicker stimulations.

different behavior: In arterioles, the magnitude of difference between the vasospastic and nonvasospastic groups was higher in the distal than in the proximal measuring sites (value for this different behavior: $P = 0.042$), and in venules, the difference in response was higher in the proximal than in the distal sites ($P = 0.038$).

Taking contraceptive pills had no influence on the parameters of interest (data not shown).

DISCUSSION

In the present study, we analyzed retinal vasomotion in response to flicker light stimulation in vasospastic and nonvasospastic subjects. Vasospastic subjects demonstrated a significantly weaker maximum dilatory response in both arterioles and venules. In addition, an augmentation of the maximal dilation in arterioles was observed in nonvasospastic subjects in repeated flicker stimulations, whereas arterioles of vasospastic subjects demonstrated an exhaustion of the maximum dilatory amplitude after the first flicker stimulus. Additional analysis revealed that this inverse behavior is mostly due to the offset to original first baseline after the flicker stimulus. After correction for offset, vasospastic subjects still demonstrated on the average smaller maximum dilatory amplitudes in both arterioles and venules.

Women have an overwhelming tendency toward vasospasm.[30–33] To eliminate an effect of gender, we recruited only female subjects. One negative aspect of such an approach is that the relevance of the study findings for men is unclear. Attempting optimal separation between the vasospastic and nonvasospastic groups, only subjects who had a clear positive history of cold hands confirmed by a positive nailfold capillaroscopy result were included in the vasospastic group and vice versa. On the other hand, groups were balanced in age, blood pressure, IOP, refraction, contraceptive pills history and menstrual cycle.

An important difference between this and most of the studies dealing with the neurovascular coupling in the retina is that we were not primarily interested in the hemodynamic effect of vascular flicker response. In the present study the parameter of interest was the short-term vasomotion, aiming to further characterize vascular dysregulation in the eye. This is why a relatively short moment of maximum dilation was chosen rather than the average over longer time periods. Possible phase differences in response to flicker stimulation in two groups were not an object of the present analysis. However, as the point of maximum dilation was defined as the highest 1-second mean vessel diameter at any point during the period of 50 seconds after the beginning of the corresponding flicker stimulus, it was not fixed on the time axis and thus could not be influenced by the possible response time lag between the groups.

That we took a brief moment of maximum dilation, rather than averaging on a larger scale, is a reason that our results are higher than those usually reported in the literature. It is also in part a possible explanation of why an augmentation of the flicker response almost uniformly escaped attention in the previous studies. As a matter of fact, a response augmentation to repeated flicker stimulation has already been observed[2]; however, no further analysis was attempted. In addition, other studies did not differentiate between vasospastic and nonvasospastic subjects, obtaining in fact an averaged response in the general healthy population. The pattern of altered behavior of ocular blood vessels when vasospastic subjects, defined as in the present study, are analyzed as a separate group has already been observed in several studies dealing with ocular blood flow, with[31,33] or without provocation tests.[30,32] The maximum dilatory response was reduced in the vasospastic group, even when corrected for the baseline offset after the flicker stimulus. The baseline offset accounts for the observed response augmentation in nonvasospastic subjects and a lack thereof in vasospastic subjects. A physiological role of the response augmentation observed in nonvasospastic subjects remains at present unclear. It obviously takes longer than 80 seconds for the retinal circulation to return to the original equilibrium.

Measurements were obtained in retinal arterioles and venules in the immediate vicinity of and distal to the optic nerve head. The environment for smooth muscle cells in the vessel walls may well be different in these two locations. The source of interstitial fluid in the optic nerve head comes in part from the fenestrated choroidal capillaries,[27] whereas more

peripherally, both the inner and the outer blood–retinal barriers are in place. In the study by Polak et al.,[5] which did not differentiate between healthy vasospastic and nonvasospastic subjects, no influence of the site of measurement on vessel response was found. On average, this was also the case in the present study. However, a magnitude of difference in corrected maximum dilatory amplitude the vasospastic and nonvasospastic groups was dependent on the measuring site.

The diameter of the vessel depends on the transmural pressure gradient and the compliance of the vessel wall. Neurovascular coupling is a highly active process, most likely involving the retina as in the brain gliovascular units and mediation through glutamate, NMDA receptors, and nitric oxide synthase.[34–36] The following is the possible sequence of events after initiation of the flicker: the first event is likely to be the relaxation of the wall in small arterioles (these vessels are out of measuring range of RVA), producing a local imbalance in factors mentioned earlier, and resulting in local dilation. Thus, locally, a new equilibrium is being reached, but this implies a lower intraluminal pressure in this vessel segment. Because of the new pressure gradient inside the vessel lumen, blood starts moving faster, increasing the shear stress in longer arteriolar segments, including those reliably measured by the RVA (>80 μm). An intact endothelium senses shear stress and induces changes in the luminal diameter to keep shear stress constant at a predetermined level.[37–39] Further dilation ensues, again probably through the release of nitric oxide,[40] since blocking its release blunts the retinal vascular flicker response.[11] The resistance in the local vascular tree decreases as further volumetric flow increases. Ultimately, shear stress increases also in venules, and they dilate. A delay in flicker response between retinal arterioles and venules has been documented.[4] Venules experience half of the wall shear stress present in retinal arterioles, and shear stress seems to be constant in first- and second-order venule branches, whereas in arterioles it decreases toward smaller calibers.[41] The different behavior of arterioles and venules in the present study could be a consequence of shear stress distribution in retinal vessels. Of interesting, blood viscosity and rheologic properties are altered in persons with Raynaud phenomenon.[42–44] This may have a bearing, not only on the validity of RVA measurements, as the device measures the diameter of the red blood cell column in a vessel and not a vessel lumen or a vessel wall itself, but also on the exerted level of shear stress. However, women with Raynaud phenomenon seem to have an intact endothelium response to shear stress.[45] Moreover, none of our subjects reported classic symptoms of Raynaud (tricolor phenomenon). An explanation for the observed differences between the vasospastic subjects and their counterparts should therefore consider other factors as well. A suggested underlying cause of vascular dysregulation is an endotheliopathy,[18,19] and the observed different vasomotion may be an indicator of endotheliopathy. In diseases that involve vasospasm and affect the eye[46,47] or heart,[48,49] nitric-oxide–mediated dilation is impaired, not only in the affected organs, but also systemically. Subjects in the present study were healthy. Otherwise healthy vasospastic subjects have increased serum endothelin-1 levels[50]; an increased propensity to vasoconstriction may explain lower dilatory amplitudes in our vasospastic group.

Based on the present data, it is not possible to pinpoint the culprit in the observed group differences. Retinal vessels in vasospastic subjects are inherently neither constricted nor dilated at baseline: we did not observe any differences in original (first) baselines compared with nonvasospastic subjects, confirming previous results.[26] Nevertheless, immediately after responding to a challenge, in this case a flicker stimulation, the vessels tended to become more constricted. A regression to the mean is unlikely, as the baseline conditions were strictly standardized in all participants. In addition, it is by no means explanatory of the observed differences in corrected maximum dilatory response. At present, we merely describe the observed phenomena, which we are not yet able to understand completely, including also an increased variability or instability of the arteriole's diameter in vasospastic subjects during still illumination periods, as depicted in Figure 2.

Present data lend further support to previous observations that there is a strong association between peripheral and ocular circulation. Vascular dysregulation in the eye itself has been elusive and insufficiently characterized—a likely reason for the low awareness in this regard in the ophthalmology community. Several ocular diseases have been associated with systemic vascular dysregulation.[20–23] In a recent study, an altered response to reduction of IOP was observed in patients with vasospastic glaucoma and in those with ocular hypertension, with possible influences on the decision to treat and on setting the target pressure.[51] Having so many prevalent diseases linked to this type of vascular dysregulation makes understanding what is happening in an otherwise healthy eye with vascular dysregulation relevant. Without longitudinal studies, we cannot tell whether our subjects with vascular dysregulation have a higher propensity for ocular diseases; however, using different methods (retinal oxygenation response) others have shown that impaired retinovascular responses are indeed predictive of onset and progression of retinopathy.[52] We need to clarify and define the differences between normal and abnormal vascular regulation in otherwise healthy eyes and then move to the diseased state and look for similar vascular changes in, for example, patients with glaucoma or venous occlusion. A proper definition of vascular dysregulation in the eye would, in the clinical setting, translate to the possibility of its objective assessment and treatment. The results of the present study describe one of the manifestations of vascular dysregulation in retinal vessels.

No correction of for magnification effect was used in the present study. Two groups were balanced in terms of refractive errors. Parameters of interest were expressed either as a percentage of baseline, like the maximum dilatory amplitude, or the relevant analysis was based on intraindividual comparisons, like the time course of baseline diameters between flicker stimulations. As mentioned before, the original baseline vessel diameters were not significantly different between the groups. Of note, baseline vessel diameter does not influence relative magnitude of the flicker response.[5,14]

In conclusion, vasospastic subjects demonstrated different retinal vasomotion in response to flicker stimulation than did nonvasospastic subjects. The maximum dilatory response in both arterioles and venules was lower in vasospastic subjects. The time course of the dilatory response to repeated flicker stimulation was altered in arterioles of vasospastic subjects, showing an exhaustion of response rather than an augmentation, as was observed in the nonvasospastic group.

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