Hypercapnia Impairs Vasoreactivity To Changes In Blood Pressure And Intraocular Pressure In Rat Retina

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Abstract

Significance: The balance between oxygen and carbon dioxide balance sets the resting tone (or diameter) of retinal blood vessels. Eyes that are hypercapnic use up their "vasodilatory reserve" and therefore, fail to respond adequately to changes in intraocular or blood pressure.

Purpose: Retinal vessels are regulated by both myogenic and metabolic mechanisms. We considered whether alteration of metabolic status would modify the vascular response to ocular perfusion pressure (OPP) lowering in rat retina.

Methods: In pentobarbital anesthetized adult Brown Norway rats, normocapnia or hypercapnia was achieved by artificially ventilating animals with air or 5% carbon dioxide in ~30% oxygen, respectively. OPP was gradually reduced to ~20 mmHg by either lowering blood pressure (slowly drawing blood from a femoral artery/vein) or manometrically increasing intraocular pressure (IOP) under normocapnic or hypercapnic conditions. In all four groups (n = 7 eyes for each), a confocal scanning laser ophthalmoscope was used to acquire image sequences centered on the optic nerve throughout pressure modification. The diameter of arterioles and venules at various OPP levels was measured and expressed as percentage relative to their own baseline. The response of arterioles and venules to OPP lowering was compared between normocapnic and hypercapnic groups.

Results: Average arterial carbon dioxide partial pressure was 36.9 ± 2.6 mmHg in normocapnic and 64.1 ± 5.9 mmHg in hypercapnic ($P < .001$) animals. In the normocapnic groups, blood pressure lowering and IOP elevation resulted in significant vasodilatation of both arterioles and venules ($P < .0001$). In the hypercapnic groups, OPP lowering induced vasodilation was significantly attenuated compared to the corresponding normocapnic groups ($P < .0001$ for both, 2-way ANOVA).

Conclusions: Hypercapnia significantly modified myogenic vascular autoregulation in response to OPP reduction.

Introduction

The retina is one of the most metabolically active tissues in the body. To sustain this demand in the absence of stored energy, blood supply is tightly autoregulated by local myogenic and metabolic mechanisms.[1, 2] Myogenic regulation refers to the adjustment of vascular resistance in order to maintain stable blood flow in face of fluctuating ocular perfusion pressure (OPP). Myogenic regulation is triggered by activation of stretch sensitive channels on the vascular endothelium[3] and vascular smooth muscle cells,[4, 5] which stimulate the releases of vasoactive peptides to either constrict or relax smooth muscle cells.[4, 5] Metabolic regulation refers to the capacity for the retinal vascular bed to adjust blood supply in response to changes in local metabolic status, including oxygen (O_2) and carbon dioxide (CO_2) tensions, pH and the local concentrations of a range of other metabolic products.[1, 2, 6, 7]

As CO_2 is a major metabolic product, its local tension is known to impact local vessel tone, with hypercapnia reducing (relative vasodilatation) and hypocapnia increasing (relative vasoconstriction) vessel tone.[8-11] Changes in local CO_2 tension modify the balance of a range of vasoactive substances including nitric oxide,[12, 13] prostaglandin E_2 (PgE_2)[14] and adenosine.[15] Given that local CO_2 tension impacts vessel tone, it is likely that the capacity for the vasculature to cope with OPP lowering will be affected during hypercapnia.

Studies in the cerebral circulation show that metabolic perturbation affects vasoreactivity to perfusion pressure changes. *Ex vivo* experiments of isolated rat diaphragmatic arterioles show that alteration of CO_2 levels in the perfusate significantly modify myogenic responses to changes in intraluminal

pressure.[16] *In vivo*, results show that hypercapnia shortens the range of the cerebral autoregulatory plateau in response to changes in blood pressure.[17] Furthermore, in human middle cerebral arteries, hypercapnia slows their responses to a rapid change in blood pressure.[18] How retinal myogenic autoregulation is modified by metabolic status has yet to be investigated.

In the present study, we hypothesized that hypercapnia would impair the vasoreactivity of retinal blood vessels to OPP lowering induced by blood pressure (BP) and IOP. We tested this hypothesis in rats, where a robust myogenic autoregulatory response has been documented[19] and a species increasingly used in studies of vascular physiology and ocular diseases.

Materials and methods

Animals

Experiments were conducted in young adult Brown-Norway rats (131 ± 21 days). Rats were maintained in 22℃, 12-h light/12-h dark environment. Normal rat chow and water were available *ad libitum*. Experiments were undertaken between 8:00AM to 12:00AM in a dedicated quiet animal surgery suite maintained at 22^0C with adequate ventilation. All experimental methods and animal care procedures adhered to Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Institutional Animal Care and Use Committee (Legacy Health System, Portland, Oregon).

Anesthesia and surgical preparation

General anesthesia was induced by inhalation of 2% isoflurane followed by maintenance at 1 - 2% isoflurane. The femoral arteries and veins were cannulated with polyethylene tubing (PE10 or 50, BD Intramedic, Franklin Lakes, NJ). One femoral artery was connected to a pressure transducer (BLPR2; World Precision Instruments, Sarasota, FL) and a four-channel amplifier system (Lab-78 Trax-4/24T; World Precision Instruments) for continuous BP monitoring. A femoral vein was used for administration of drugs. The other artery or vein was used for blood drawing to induce systemic hypotension as detailed below. An orotracheal intubation was performed and the anesthesia was switched to continuous intravenous infusion of sodium pentobarbital (2-5 mg/kg/hr, Nembutal, Oak Pharmaceuticals, Lake Forest, IL) via an infusion pump (Aladdin, World Precision Instruments). The reservoir was set initially at a height to maintain a baseline level of 10 mmHg. Animals were ventilated with a respirator (RSP 1002, Kent Scientific Co., Torrington, CT) with 30% oxygen at a rate between 60 and 90 breaths/min. The arterial oxygen partial pressure (PaO_2) was maintained at average of 110.6 ± 14.0 mmHg and a pH of 7.44 ± 0.10, as measured using a blood gas analyzer (i-STAT©, Abbott Inc. Princeton, NJ). A heating pad, set at 37º C, was used to maintain body temperature throughout the experiments. Both eyes were dilated with tropicamide (0.5%; Alcon Laboratories, Inc., Fort Worth, TX), and topical anesthetized with 0.5% proparacaine hydrochloride (Bausch & Lomb, Inc., Bridgewater, NJ).

Monitoring of $EtCO_2$ and $PaCO_2$

In order to avoid the need for repeated blood sampling, which makes stable continuous imaging difficult, arterial carbon dioxide partial pressure ($PaCO_2$) was calibrated to the end tidal carbon dioxide

level ($EtCO_2$). In 12 rats, $PaCO_2$ ranging from 30 mmHg to 60 mmHg was measured (n = 27 samples) using the blood gas analyzer. The corresponding $EtCO_2$ was recorded simultaneously using a capnometer (RSP-300, Kent Scientific Co., Torrington, CT). The relationship between $EtCO_2$ and $PaCO_2$ was determined using linear regression ($EtCO_2$ = 0.76 mmHg · $PaCO_2$ - 3.5309, R^2 = 0.79). This function was used to convert $EtCO_2$ to $PaCO_2$.

In the current study, we set $PaCO_2$ in normocapnic groups between 30 to 40 mmHg (or $EtCO_2$ between 20 and 27 mmHg), which was maintained by adjusting the respiratory rate (between 60 and 90 breaths/min) and the inspiration/expiration ratio (between 15% and 45%). The $PaCO_2$ in the hypercapnic groups was set between 60 and 70 mmHg (or $EtCO_2$ between 45 and 50 mmHg) and achieved by adding 5% CO_2 to the inspired air.

Gradual OPP reduction by BP lowering and IOP elevation

OPP reduction was induced by BP lowering and IOP elevation. For BP lowering, a syringe pump was used to slowly draw blood from either a femoral artery or a vein at a rate of 0.84 ± 0.24 ml/min. Drawing started after the mean BP had stabilized between 95 and 100 mmHg. The blood draw ended when BP fell to between 20 and 25 mmHg. This rate of blood draw resulted in an average rate of blood pressure lowering of 10.7 ± 3.1 mmHg/min and the average blood volume drawn was 6.0 ± 1.8 ml.

In a randomly chosen eye, gradual IOP elevation was achieved by cannulating the anterior chamber with a 33-G needle connected to a reservoir filled with balanced salt solution (Baxter, Deerfield, IL). To induce a gradual increase in IOP at a rate that matched the BP lowering, the reservoir was raised from 10

to 70 mmHg at a rate of 9.6 ± 0.3 mmHg/min using a modified peristaltic pump (Longer Precision Pump Co., Ltd, Hebei, China). The final target IOP (70 mmHg) was confirmed using a rebound tonometer (TONOLAB, Vantaa, Finland). The OPP was calculated as the difference between mean arterial BP and IOP. With mean arterial BP providing an estimate of ophthalmic arterial pressure, which does not require correction for hydrostatic pressure differences in rats, whereas IOP provides an estimate of retinal venous pressure.

Changes in vessel diameter in response to two methods of gradual OPP reduction (BP lowering and IOP elevation) were assessed in both normocapnia (control) and hypercapnia. Animals were randomly allocated to 4 groups (n = 7 eyes, each): normocapnia with BP lowering; normocapnia with IOP elevation; hypercapnia with BP lowering; hypercapnia with IOP elevation. A minimum sample size of 6 was needed as determined for 2 groups with 8 repeated measures (ANOVA) assuming an effect size of 0.5, alpha value 0.5, power = 0.8 (G*Power, http://www.gpower.hhu.de/) [20]. Two experiments were excluded, either for excessive eye movements that impacted image quality or poor baseline blood pressure that could not be stabilized between 95-100 mmHg.

Vessel responses to BP lowering and IOP elevation were also compared with two groups of spontaneously hypercapnic rats (n = 7 eyes for BP group and n = 5 eyes for IOP group), in which animals were anesthetized in the same way but breathed normal room air, rather than being intubated and artificially ventilated (i.e., spontaneous hypercapnia versus controlled hypercapnia). In these spontaneously hypercapnic animals average $PaCO_2$ was 63.4 ± 11.6 mmHg, PaO_2 was 117.5 ± 32.58 mmHg

and pH was 7.33 ± 0.07.

Vessel diameter imaging and quantification

Following general preparation, rats were placed on a stage in front of a spectral-domain optical coherence tomography/confocal scanning laser ophthalmoscope (SD-OCT/cSLO, Spectralis; Heidelberg Engineering, Heidelberg, Germany). Customized rigid gas-permeable contact lenses (3.5-mm posterior radius of curvature, 5.0-mm optical zone diameter, and +5.0-diopter back vertex power) were placed on both eyes. Changes in retinal vessel diameter across a 30^{o} field centered on the optic nerve head were recorded in infrared reflection mode (cSLO-IR) at baseline and throughout the course of OPP lowering (BP lowering or IOP elevation). More specifically, baseline vessel diameter was recorded over the first 20-seconds, after which OPP modification was initiated and continuous recording ensued until BP was decreased to 20-25 mmHg or IOP was increased to 70 mmHg.

Changes in vessel diameter during OPP lowering were quantified using Fiji (ImageJ v2.0.0; National Institutes of Health, Bethesda, MD) as described previously.[21] Image sequences were combined into a single stack and registered with custom-software to allow the same regions to be analyzed across time. In each eye, 3 to 6 arterioles and 3 to 6 venules were assessed. ImageJ was used to extract the light intensity profile perpendicularly across the vessel at one-disc diameter from the optic disc margin. Using custom written software, vessel diameter was quantified as the distance between the two intensity troughs, which correspond to the two edges of the vessel. Changes in diameter for each vessel were expressed as a percentage relative to its own baseline. Group data were averaged into bins spanning 10 mmHg of OPP.

Data analysis and statistics

The percentage change in vessel diameter with OPP lowering (binned every 10 mmHg) in each experimental group was analyzed using one-way repeated measures ANOVA with Dunnett's post hoc test. Differences in vessel reactivity under normocapnia and hypercapnia were compared using 2-way ANOVA (OPP and $PaCO_2$) for BP lowering and IOP elevation challenges separately. The OPP at which vessel diameter began to increase or decrease with OPP lowering was determined by segmental linear regression analysis using individual diameter measurements at corresponding OPP.

The critical probability (P) to reject our null hypothesis was set at 5%. Data are presented as mean ± SD (standard deviation) in the figures and within the text. All analyses were performed using Prism 7 (GraphPad Software, Inc., La Jolla, CA).

Results

Table 1 shows baseline parameters (prior to OPP manipulation) for the four experimental groups. The values including $PaCO_2$ and vessel diameters in controlled hypercapnic groups were significantly higher than the normocapnic rats ($P < 0.05$), while baseline OPP in each group was similar.

Effects of controlled hypercapnia on baseline vessel diameters (prior to OPP manipulation)

Representative baseline images in Figure 1 shows that compared with a normocapnic animal (Fig. 1a), vessels were more dilated in a hypercapnic animal (Fig. 1b). The average baseline arteriolar diameter was larger in the two hypercapnic groups (41.4 ± 5.2 μm) than the two normocapnic groups (35.8 ± 4.5 μm).

This represents a 19.6% hypercapnia induced increase in basal arteriolar diameter ($P < .001$). Similarly, hypercapnia induced a 27.1% increase in venular diameter (normocapnia: 39.5 ± 4.2 µm vs hypercapnia: 49.8 ± 5.7 µm , $P < .0001$). These are summarized in Table 1.

Effects of hypercapnia on vascular responses to BP lowering

The baseline OPP prior to blood drawing was 82.2 ± 1.4 and 83.0 ± 4.4 mmHg in the normocapnic and controlled hypercapnic groups ($P = .07$), respectively. Blood drawing induced similar BP reductions of 70.2 ± 3.4 mmHg (from 94.1 ± 2.8 to 23.9 ± 2.3 mmHg) in normocapnic and 69.2 ± 5.5 mmHg (from 94.6 ± 3.0 to 22.8 ± 4.1 mmHg) in hypercapnic groups, respectively ($P = .48$). Under normocapnic conditions, retinal arteriolar and venular diameters remained stable with moderate OPP lowering. As OPP fell to 52.2 ± 1.2 mmHg for arterioles and 67.7 ± 1.7 mmHg for venules, vessel diameters started to increase steadily and reached peaks of 15.4% and 12.1% above baseline, respectively ($P < .0001$, 1-way ANOVA for both, Fig. 2).

Under hypercapnic conditions, arteriolar and venular responses were significantly attenuated compared with normocapnia ($P < .001$, 2-way ANOVA for both, Fig. 2). Specifically, arteriolar dilation only reached a maximum of 5.2% compared to the 15.4% seen in the normocapnic group (Fig. 2a). During hypercapnia, arteriolar diameter started to decline when OPP dropped to very low levels at 23.9 ± 0.9 mmHg, this is in contrast to the dilation seen in the normocapnic group at similar OPPs (Fig. 2a). For venules, hypercapnia completely abolished the vasodilation seen in the normocapnic group (Fig. 2b). At a very low OPP of 23.3 ± 1.5 mmHg retinal venules showed significant constriction ($P < .01$, Fig. 2b).

Normocapnic and controlled hypercapnic animals were also compared against those that were

naturally ventilated (spontaneously hypercapnic groups). Figure 2a and 2b show that vessel responses to BP lowering in spontaneously hypercapnic groups were not significantly different to those under controlled hypercapnia (P = .47 and P = .08 for arterioles and venules, respectively).

Effects of hypercapnia on vascular response to IOP elevation

The baseline OPP prior to IOP elevation was similar in normocapnic (86.3 ± 2.2 mmHg) and controlled hypercapnic groups (85.6 ± 3.8 mmHg, P = .13). In the normocapnic group, gradual IOP elevation from 10 to 70 mmHg induced vasodilation in both arterioles and venules. For arterioles and venules, significant dilation started when OPP was lowered to 59.6 ± 2.1 mmHg and 74.6 ± 2.0 mmHg, respectively. Peak vasodilatations of 11.9% and 4.5% was reached by arterioles and venules, respectively (P < .0001, Fig. 3).

Hypercapnia completely blunted both arteriolar and venular vasodilatation in response to IOP elevation (P = .97 and P = .12, respectively, Fig. 3). Compared to the normocapnic groups, both arteriolar and venular responses were significantly attenuated in controlled hypercapnia groups (P < .0001, 2-way ANOVA for both, Fig. 3). At very low OPPs (24.3 mmHg, Fig. 3b), venules were significantly constricted (P = .0002), whereas arterioles did not (P = .26). These hypercapnia data were adequately described using a single line regression model, rather than the two-line regression model needed to describe data from normocapnic groups. Vessel response to IOP elevation in the spontaneously hypercapnic group was blunted compared with normocapnia; an effect that was similar to the controlled hypercapnic group (Fig. 3).

Discussion

We evaluated the impact of altered metabolic status induced by hypercapnia on myogenic regulation in the rat retinal circulation. We found that hypercapnia significantly increased basal vessel diameter and blunted the capacity for vessels to dilate to cope with OPP lowering.

Previous studies have shown that increasing $PaCO_2$ increases basal arteriolar diameter. The magnitude of such increases have been found to range between 1.4% [22] to 8.5% [14] in brain arterioles and from 3% [23] to 14% [24] in retinal arterioles. In the current study, arteriolar diameter was increased by 19.6% under hypercapnic conditions. A likely reason for the difference between ours and previous reports may lie in differences in induced magnitude of $PaCO_2$ changes.[25-27] In previous studies on human subjects, $PaCO_2$ levels are most often mildly elevated by between 12.0% to 25.4%.[23, 24] In our study, direct delivery of a 5% CO_2 mixture by artificial ventilation raised $PaCO_2$ by ~70%, which would account for the larger basal dilation observed in our study.

Dissolved carbon dioxide in blood reacts with water to form carbonic acid, which is further disassociated into HCO^{3-} and H^+ [28]. The latter is believed to be a major mediator leading to change in vascular tone[29] by stimulating release of vasoactive substances from vascular and/or glial cells.[30, 31] In particular nitric oxide, which is synthesized by astrocytes,[32] Müller cells [33] and endothelial cells,[34] relaxes SMCs via a cyclic guanosine monophosphate signaling pathway.[35] Hypercapnia may also enhance the synthesis of prostaglandin E_2 through cyclooxygenase-1 and glutathione pathways,[36] as well it reduces calcium levels within and thus relaxes SMC.[37-39] Inhibition of prostaglandin E_2 reverses hypercapnia-induced vasodilation.[14]

189 Whilst relaxation of SMC is relevant to increased arteriolar diameter, it cannot explain our observation
190 that venules dilated by 27.1% under hypercapnia as rat venules have virtually no SMC. Since the inner
191 retinal circulation is a closed system, venular dilation could arise from passive distention by increased
192 blood volume in upstream arterioles. However, we found that retinal venules dilated significantly more
193 than arterioles (27.1% vs. 19.6%, *P* = .003). Differences in the way that arterioles and venules respond to
194 stimuli have been reported in previous studies. For example, while flickering light induced dilation of
195 retinal arteries, veins showed little change.[40] In the cerebral circulation, arterioles have been shown to
196 dilate (10%) more than venules (2%) in response to xenon gas inhalation.[41] In response to hypercapnia in
197 awake mice, cerebral arterioles dilated by 15.8%, whereas venules dilated by only 2.3%.[27] These studies
198 suggest that changes in venular diameter do not passively follow arterial changes.[21]

199 Myogenic mechanisms maintain stable blood flow over a wide range of OPPs; often referred to as the
200 autoregulation plateau along the pressure-flow curve.[6] With extremely high or low OPPs (< 30 mmHg), the
201 pressure difference between tissue and intraluminal pressure results in forceful dilation and constriction,
202 respectively. In the cerebral circulation, hypercapnia impairs vascular responses to change in perfusion
203 pressure,[42, 43] as evidenced by narrowing of the autoregulation plateau.[17] Dineen et al.,[18] showed that
204 hypercapnia delays the initial transient hemodynamic responses to a rapid change in perfusion pressure
205 in human middle cerebral arteries. Consistent with these studies, we showed for the first time that
206 hypercapnia blunts the capacity of the retinal vasculature to compensate for OPP lowering (Figs. 2 and 3).
207 These data support the idea that metabolic perturbation is detrimental to pressure-induced myogenic
208 autoregulation in the retina.

Since basal vessel diameter was already increased by hypercapnia (Table 1, Fig. 1), further vasodilation in response to OPP lowering might be limited by an already low myogenic tone; or a reduced "vasodilatory reserve".[44] A previous study in isolated arterioles revealed that severe hypercapnia inhibit myogenic response by suppression of SMC and activation of endothelium dependent mechanisms.[16] Hypercapnia induced reductions in pH are also known to significantly attenuate vasodilation.[45] In addition to local CO_2 levels, other mechanical factors are also likely to influence the retinal vasodilatory reserve.[46]

The results from this study highlights the importance of maintain normal $PaCO_2$ levels when studying vascular physiology. As indicated by Moult et al.,[47] depending on the type of anesthetic used, the respiratory system of experimental animals can be depressed to different degrees. Respiratory depression will result in accumulation of CO_2. We show here that in pentobarbital anesthetized rats spontaneously breathing room air, $PaCO_2$ was as high as those rats breathing 5% CO_2 (63.4 vs. 64.2 mmHg). Thus, without knowing or controlling $PaCO_2$, studies employing spontaneous room air breath will underestimate vasoreactivity.

A number of limitations of our study are worth noting. First in the current study we elected to use changes in retinal vessel diameter as an index of vascular responses to OPP challenge. However, the actual blood flow resulting from changes in diameter is not known. Second, although $PaCO_2$ was constantly adjusted to maintain levels within our target range by monitoring $EtCO_2$, PaO_2 was only sampled once. Although PaO_2 variation can influence vasoreactivity,[10] by ventilating animals with 30% O_2 we believe that small variations in PaO_2 would not have substantially influenced our results. Finally, as IOP was

continuously changing during the experiment, it was challenging to maintain a consistent focus during imaging. As a result, the image quality in experiments with IOP elevation was more variable than BP lowering manipulations.

Our current study provides evidence that alteration of metabolic status induced by hypercapnia profoundly impacts pressure-initiated myogenic autoregulation in rat retinal vessels. These findings have implication for understanding how the retinal vasculature copes with OPP fluctuation in conditions such as glaucoma. Tissues that already have low oxygen or high carbon dioxide tension (which can also occur with low pH arising from increased anaerobic glycolysis) would tend to have lower vessel tone, and thus reduced "vasodilatory reserve". In those whose vasculature is already impaired (e.g. diabetes induced vascular damage, arteriosclerosis with long term systemic hypertension), a narrower autoregulatory plateau means that only a small reduction in OPP would exceed the lower limit of an already compromised "vasodilatory reserve".

Conflict of Interest: The authors declare that they have no conflict of interest.

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Author Contributions:

Study design: l.W., B.V.B. and G.C; Resources: l.W., B.V.B; Writing the original manuscript: l.W. and G.D.L; Figures: G.D.L. and l.W; Project administration: G.D.L. and G.C; Data collection: G.C. and G.D.L; Analysis: G.D.L. and l.W; Review & Editing: B.V.B., l.W., G.C. and G.D.L.

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Figure legends

Fig.1 Representative baseline cSLO images from one eye in normocapnic group (a) and another in hypercapnic group (b). Vessel diameters were measured at 1-optic disc (dotted circle) distance from the optic disc margin (solid circle). a (red): arterioles; v (white): venules.

Fig. 2 Effect of hypercapnia on retinal arteriolar and venular diameters (percentage change, %) in response to BP modification. Mean (± SD) diameter change (%) summarized into 10 mmHg OPP bins for arterioles (a) and venules (b) under normocapnic (blue, n = 7), controlled hypercapnic (red, n = 7) and spontaneously hypercapnic (black, n = 7) conditions. Unfilled circles indicate significant difference from baseline (first symbols). Asterisks (*) indicate significant difference between normocapnic and controlled hypercapnic groups. Baseline arteriole diameter was 35.5 ± 3.7 µm, 39.3 ± 2.7 µm and 40.9 4.8 ± 5.2 µm for normocapnia, controlled hypercapnia and spontaneously hypercapnia groups, respectively. Baseline venule diameter was 39.4 ± 2.9 µm, 47.3 ± 3.0 µm and 48.7 ± 5.2 µm for normocapnia, controlled hypercapnia and spontaneously hypercapnia groups, respectively.

Fig. 3 Effect of hypercapnia on retinal arteriolar and venular diameters in response to IOP elevation. Mean (± SD) diameter change (%) summarized into 10 mmHg OPP bins for arterioles (a) and venules (b) under normocapnic (blue, n = 7), controlled hypercapnic (red, n = 7) and spontaneously hypercapnic (black, n = 5) conditions. Unfilled circles indicate significant difference from baseline (first symbols). Asterisks (*) indicate significant difference between normocapnic and controlled hypercapnic groups. Baseline arteriole diameter was 40.5 ± 5.2 µm, 43.6 ± 7.1 µm and 42.8 ± 5.3 µm for normocapnia, controlled hypercapnia

356 and spontaneously hypercapnia groups, respectively. Baseline venule diameter was 39.4 ± 2.9 μm, 47.3 ±

357 3.0 μm and 54.3 ± 2.8 μm for normocapnia, controlled hypercapnia and spontaneously hypercapnia groups,

358 respectively.