

# $\alpha$ -Lipoic Acid Corrects Late-Phase Supernormal Retinal Oxygenation Response in Experimental Diabetic Retinopathy

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**PURPOSE.** To test the hypothesis that preventative  $\alpha$ -lipoic acid (LPA) treatment corrects an abnormal retinal oxygenation response in experimental diabetic retinopathy.

**METHODS.** Retinal oxygenation ( $\Delta\text{Po}_2$ ) was measured by MRI before (room air [ra]) and during a 4-minute carbogen inhalation challenge in five groups: control Sprague-Dawley (SD) and Lewis (LEW) rats, 3- to 4-month diabetic SD and LEW rats, and 4-month diabetic LEW rats preventatively treated with a chow LPA admix (400 mg/kg per chow). Comparisons were made between the initial 2 minutes of oxygenation change (measured using ra and first carbogen periods [ $t_1 - \text{ra}$ ]) and the next 2-minute change (assessed with first and second carbogen periods [ $t_2 - t_1$ ]) for superior and inferior hemiretinal  $\Delta\text{Po}_2$ .

**RESULTS.** In control SD rats,  $\Delta\text{Po}_2(t_1 - \text{ra}) > \Delta\text{Po}_2(t_2 - t_1)$  ( $P < 0.05$ ) was found panretinally. In diabetic SD rats, the superior, but not the inferior, hemiretina had subnormal  $\Delta\text{Po}_2(t_1 - \text{ra})$  ( $P < 0.05$ ) and supernormal  $\Delta\text{Po}_2(t_2 - t_1)$  ( $P < 0.05$ ). In control LEW rats,  $\Delta\text{Po}_2(t_1 - \text{ra})$  and  $\Delta\text{Po}_2(t_2 - t_1)$  were not significantly different ( $P > 0.05$ ). Also, control and diabetic LEW rat panretinal  $\Delta\text{Po}_2(t_1 - \text{ra})$  were lower ( $P < 0.05$ ) than in the respective SD groups. In diabetic LEW rats, a supernormal ( $P < 0.05$ ) panretinal  $\Delta\text{Po}_2(t_2 - t_1)$  was found that could be corrected with preventative LPA treatment.

**CONCLUSIONS.** These data support the hypothesis and suggest that the influence of strain differences on the interpretation of retinovascular  $\Delta\text{Po}_2$  as a surrogate of drug treatment efficacy for diabetic retinopathy may be minimized by measuring a late-phase supernormal response. The LPA data raise the possibility that oxidative stress contributes to diabetes-induced supernormal  $\Delta\text{Po}_2$ . (*Invest Ophthalmol Vis Sci.* 2006;47:4077-4082) DOI:10.1167/iovs.06-0464

The retinal oxygenation response to a hyperoxic provocation ( $\Delta\text{Po}_2$ ), measured by functional MRI, is a powerful and noninvasive biomarker of the retinovascular systems' ability to oxygenate and has been validated as a biomarker for assessing, before the appearance of retinal vascular histopathology, drug treatment efficacy in experimental diabetic retinopathy.<sup>1</sup> Recently, in a study of diabetic subjects with no clinically detectable retinopathy or mild to moderate background diabetic retinopathy, we reported *supernormal* retinal  $\Delta\text{Po}_2$  during

extended periods of a 100% oxygen provocation (i.e., late-phase).<sup>2</sup> These data appear to be in conflict with reports in diabetic rodents that  $\Delta\text{Po}_2$  measured during a shorter (2-minute, initial phase) period of carbogen (95% O<sub>2</sub>-5% CO<sub>2</sub>) provocation was significantly lower than normal (i.e., *subnormal*) in experimental diabetes before the appearance of retinal histopathology.<sup>1</sup> It is not clear whether this disparity (i.e., *supernormal* late-phase response in patients and *subnormal* initial-phase response in experimental diabetes) is due to differences in species (rodent versus human), inhalation gas (carbogen versus 100% oxygen), or duration of the hyperoxic provocation.

In our experimental studies, drug treatments that correct initial-phase subnormal retinal oxygenation response were also effective at preventing the subsequent appearance of retinal vascular structural lesions.<sup>1</sup> In addition, drugs that did not prevent diabetes-related vascular histopathology, did not correct the initial-phase subnormal  $\Delta\text{Po}_2$ .<sup>1</sup> It is not currently known whether correcting a late-phase *supernormal* response would also be linked with inhabitation of retinal vascular histopathology.

The purpose of this study was to test the hypothesis that  $\alpha$ -lipoic acid corrects an abnormal retinal oxygenation response in experimental diabetic retinopathy. To address this hypothesis, we first examined the impact of rat strain and diabetes on the temporal evolution of retinal  $\Delta\text{Po}_2$  during a carbogen challenge ( $\geq 2$  minutes). Then, we evaluated the ability of preventatively administered  $\alpha$ -lipoic acid (LPA) to correct an abnormal retinovascular oxygenation response. LPA, an antioxidant and metal chelator (among other actions), prevented early diabetes-related biochemical changes and, importantly, retinal vascular histopathology (e.g., acellular capillary formation and pericyte ghost formation).<sup>3-6</sup> The impact of LPA treatment on diabetes-induced alterations of retinal oxygenation is not yet known.

## METHODS

The animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

## Animal Groups for the MRI Experiments

Five groups were studied: control Sprague-Dawley (SD,  $n = 7$ ) and Lewis (LEW,  $n = 8$ ) rats, 3- to 4-month diabetic SD ( $n = 8$ ) and LEW ( $n = 6$ ) rats, and 4-month diabetic LEW rats treated with LPA ( $n = 6$ ). Oral treatment consisted of a diet for the diabetic rats supplemented with 400 mg LPA per kilogram of food, a dose previously shown to inhibit vascular histopathology.<sup>3</sup> Diabetic rats consume approximately 2.5 times more chow than do control animals (Kern T, Kowluru R, personal communications, 1998). This yielded an estimated dose of LPA consumed per day in each group as 25 mg/kg body weight.

Diabetes was induced with an intraperitoneal injection of streptozocin (60 mg/kg) within 5 minutes of its preparation in 0.01 M citrate buffer (pH 4.5) in rats with a body weight of approximately 200 g after an overnight fast. Diabetes was verified 3 days later by the presence of

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plasma hyperglycemia ( $\geq 300$  mg/dL) and elevated urine volume ( $>60$  mL/d) in nonfasted rats. Rat body weight and blood glucose levels were monitored weekly. Subtherapeutic levels of insulin (0–2 U of neutral protamine Hagedorn [NPH] insulin administered subcutaneously daily) were administered to maintain blood glucose levels between 450 and 550 mg/dL without urine ketones. Glycated hemoglobin (GHb) was measured after 2 months of diabetes (Glyco-Tek affinity columns, kit 5351; Helena Laboratories, Beaumont, TX).

### MRI Examination

On the day of the experiment, blood glucose levels were measured before anesthesia in each animal via a tail nick. Anesthesia was then induced by a single intraperitoneal injection of urethane (36% solution, 0.083 mL/20 g animal weight, prepared fresh daily; Aldrich, Milwaukee, WI). Then, each animal was gently positioned on an MRI-compatible homemade holder with its nose placed in a plastic nose cone. Animals were allowed to breathe spontaneously during the experiment. To maintain the core temperature, a recirculating heated water blanket was used. Rectal temperature was continuously monitored while the animal was inside the magnet, as previously described.<sup>7</sup>

MRI data were acquired in a manner similar to that previously described on a 4.7-T system using a two-turn transmit-receive surface coil (1.0-cm diameter) placed over the eye. Images were acquired with an adiabatic spin-echo imaging sequence (repetition time [TR] 1 s, echo time [TE] 22.7 ms; the shortest echo time allowed with this sequence, number of acquisitions NA 1, matrix size  $128 \times 256$ , slice thickness 1 mm, field of view  $32 \times 32$  mm<sup>2</sup>, and sweep width 25,000 Hz, with 2 minutes per image).<sup>8</sup> This resulted in an in-plane resolution of  $250 \times 125$   $\mu\text{m}^2$ . Sagittal localizer images were first collected and used to position a single 1-mm transverse slice through the center of the eye. The 1-mm slice thickness was needed to obtain adequate signal-to-noise ratio in a 2-minute image. This slice thickness resulted in some partial volume averaging so that the final image contained superior and inferior hemiretina with some relatively minor contribution from temporal and nasal hemiretina. It is important to note that steady state (room air) vitreous oxygen tension *cannot* be measured with this method, because many factors affect the preretinal vitreous water signal and its relaxation properties. In other words, simply obtaining an image of the eye during room air breathing alone cannot be used to measure retinal oxygenation.

MRI data were collected sequentially as follows: three 2-minute images while the animal breathed room air and two 2-minute images during the inhalation of carbogen. Carbogen gas exposure was started at the end of the third baseline image. Animals were returned to room air for 5 minutes, to allow recovery from the inhalation challenge and were removed from the magnet.

A second 2-minute carbogen challenge was performed outside the magnet with care taken to not alter the spatial relationship between the animal head and nose cone. At exactly 2 minutes, arterial blood from the descending abdominal aorta was collected, as described previously.<sup>8</sup> This blood was analyzed for  $P_a\text{O}_2$ ,  $P_a\text{CO}_2$ , and pH. Note that this second inhalation challenge (outside the magnet) is needed because, in rats, it is not feasible to obtain an arterial blood sample routinely from inside the magnet ( $>40$  cm away from the magnet opening). In all cases, after the blood collection, animals were euthanized with an intracardiac potassium chloride injection.

### Data Analysis

To be included in the MRI portion of this study, the animal must have demonstrated: (1) nominal eye movement during the MRI examination. Movement artifacts (typically seen in the phase encode direction) will confound interpretation of the vitreous signal intensity changes produced during the hyperoxic challenge; (2) a nongasping respiratory pattern before and after the MRI examination. If the animal is gasping (which occurred  $<1\%$  of the time), the anesthetic has probably been inadvertently administered into an organ. This could produce a change in systemic oxygenation unrelated to the retinal changes; (3) rectal

temperatures in the range of 35.5°C and 36.5°C. Preliminary experiments (data not shown) found a strong association between core temperature and  $P_a\text{CO}_2$  and  $P_a\text{O}_2$  levels. The effect of this correlation on the precision of the measurements was minimized by using a relatively tight range of temperatures; (4)  $P_a\text{O}_2 >350$  mm Hg and  $P_a\text{CO}_2$  between 46 and 65 mm Hg during the carbogen challenge. Previously, we found that arterial oxygen levels  $>350$  mm Hg during a hyperoxic challenge are needed to produce a consistently large preretinal vitreous oxygenation response.<sup>9</sup> The range of acceptable arterial carbon dioxide levels lay within the array of values in the literature measured under carbogen breathing conditions. In addition, tight control over the acceptable blood gas value range is needed to ensure adequate quality control of each sample. Occasionally, the blood gas machine was not able to read a sample (e.g., due to a clot or excessive air in the capillary tube). In this case, the MRI data were also excluded. In general,  $\Delta\text{Po}_2$  data were collected 30 to 60 minutes after urethane injection to avoid potential errors due to variable time under anesthesia. The acceptance criteria above are needed for critical comparison of the retinal oxygenation response in these spontaneously breathing normal and sick animals while minimizing systemic differences. Based on these criteria, in this study, we excluded one control SD rat, seven control LEW rats, one diabetic SD rat, five diabetic LEW rats, and four diabetic LEW rats fed LPA.

Note that all the pixel values over a set retinal region ( $\pm 0$ –2 mm from the optic nerve was the region analyzed in the present study) were used in the comparison. For example, for the comparison of the superior hemiretina, 16 pixels per rat were used in seven rats in the control SD group and in eight rats in the diabetic SD group. Thus, 112 and 128 pixels, respectively, are being compared. Power calculations assume the acquisition of uncorrelated data. However, the pixels are spatially correlated. Thus, a power calculation is not appropriate. Instead, we took advantage of the fact that the generalized estimating equation approach fits the correlation structure between pixels. For this reason, and in our experience during the past 10 years, we find that a group size of five or more rats is adequate to determine statistical significance at the 95% confidence level.

To correct for any movement (e.g., subtle shifting of the animals' position occurred during the experiment due to settling) in the slice plane, a warp affine image coregistration was performed on each animal by using software written in-house. We insured that the selected slice was the same one used throughout the series by carefully checking for differences in the size of lens and optic nerve in each image. In addition, because the slice thickness (1 mm) is relatively large compared with the diameter of the eye ( $\sim 6$  mm), partial volumes will remain similar if the eye subtly moves out of the imaging plane, and so the data analysis results are not expected to be substantially affected. After coregistration, the MRI data were transferred to a computer (Power Mac G4; Apple Computer, Cupertino, CA) and analyzed with NIH Image (a freeware program available at <http://rsb.info.nih.gov/nih-image/> developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). Images obtained during room air breathing were averaged to improve the signal-to-noise ratio. Signal intensity changes during carbogen breathing were calculated and converted to  $\Delta\text{Po}_2$ , on a pixel-by-pixel basis, as follows.<sup>8</sup> For each pixel, the fractional signal enhancement,  $E$ , was calculated:

$$E = (S(t) - S_0)/S_0, \quad (1)$$

where  $S(t)$  is the pixel signal intensity at time  $t$  after starting the gas inhalation (with  $t_1$  representing the first 2 minutes and  $t_2$  representing the second 2 minutes of carbogen), and  $S_0$  is the control signal intensity (measured from the average of the three images obtained during room air breathing) at the same pixel spatial location.  $E$  values were converted into  $\Delta\text{Po}_2$  using theory that has been validated in the rat<sup>10</sup>:

$$\Delta\text{Po}_2 = E/(R_1 * T_k), \quad (2)$$

where  $R_1$  is the oxygen relaxivity ( $s^{-1} \cdot \text{mm Hg}^{-1}$ ), and  $T_k$  is  $T_r \cdot \exp(-T_r/T_{10})$ ,  $T_r$  is the repetition time, and  $T_{10}$  is  $T_1$  in the absence of oxygen. Using a  $T_r$  of 1 s, and assuming a vitreous  $T_{10}$  of 4 s,  $T_k = 3.52$ . This  $T_{10}$  is based on our previous measurement of the proton spin-lattice relaxation time in the rabbit vitreous (4 s), reported values in human vitreous (3.3 s), and those in cerebral spinal fluid (4.3 s), which has a high water content similar to that of vitreous.<sup>8,11-13</sup> An  $R_1$  of  $2 \times 10^{-4} s^{-1} \cdot \text{mm Hg}^{-1}$  was used. This  $R_1$  was previously measured in a saline phantom, which is assumed to be a reasonable model of vitreous (98% water).<sup>8</sup> A similar  $R_1$  was found for plasma suggesting that relatively low protein levels do not substantially contribute to the oxygen relaxivity.<sup>14</sup> Note that an  $E$  of 0.01 (i.e., a 1% signal intensity change) corresponds to a  $\Delta\text{PO}_2$  of 14 mm Hg. There did not appear to be any significant changes in vitreous  $T_{10}$  or  $R_1$  in the animals studied (data not shown).

Images were analyzed as follows. First, from enhancement images the pixel values along a 1-pixel-thick line drawn at the boundary of the retina and vitreous were set to 255 (black). We estimate that the thickness of this line, based on the in-plane resolution is approximately 100  $\mu\text{m}$ . The values in another 1-pixel-thick line drawn in the preretinal vitreous next to the black pixels were then extracted.<sup>15</sup> This procedure (1) minimized the potential for retinal/choroid pixel values' contaminating ("pixel bleed") those used in the final analysis and (2) ensured that similar preretinal vitreous space was sampled for each animal. In addition, spatial averaging over these 100- $\mu\text{m}$  regions of interest will tend to minimize the contribution from the very local preretinal oxygenation gradients next to the retinal surface.<sup>16</sup>

### Statistical Analysis

All data are presented as the mean  $\pm$  SEM, unless otherwise noted. Comparisons of these parameters were performed with a one-way ANOVA or an unpaired two-tailed  $t$ -test (unless otherwise noted).  $P \leq 0.05$  was considered significant.

Comparison of retinal  $\Delta\text{PO}_2$  between groups (i.e., control [con] SD versus diabetic [diab] SD, con SD versus con LEW, con LEW versus diab LEW, and con LEW versus D+LPA LEW) and two time points (i.e.,  $t_1 - \text{ra}$ , and  $t_2 - t_1$ ) were performed using a generalized estimating equation approach.<sup>17</sup> The generalized estimating equation method performs a general linear regression analysis using all the pixels in each subject and accounts for the within-subject correlation between adjacent pixels. Comparisons were made between the initial oxygenation change (measured using  $\text{ra}$  and first carbogen periods [ $t_1 - \text{ra}$ ]) and later change (assessed with first and second carbogen periods [ $t_2 - t_1$ ]) for superior and inferior hemiretinal  $\Delta\text{PO}_2$ .

## RESULTS

### Systemic Physiology

A summary of systemic physiology is presented in Table 1. The physiological parameters (i.e., blood gas levels, rectal temperatures, and blood glucose data) were normally distributed. As expected, compared with the respective control groups, all

diabetic animals had significantly ( $P < 0.05$ ) elevated glycated hemoglobin (GHb). Note that during carbogen breathing, only  $\text{P}_a\text{CO}_2$  was significantly ( $P < 0.05$ ) different between SD and LEW control and diabetic groups. Nonetheless, all blood gas levels were within the range expected during a carbogen challenge.<sup>8</sup>

### Retinal Oxygenation Response

**Sprague-Dawley Rats.** In control SD rats ( $n = 7$ ), superior and inferior hemiretinal regions demonstrated a similar pattern:  $\Delta\text{PO}_2(t_1 - \text{ra}) > \Delta\text{PO}_2(t_2 - t_1)$  ( $P < 0.05$ ; Fig. 1). In diabetic SD rats ( $n = 8$ ), as expected, the superior, but not the inferior, hemiretina had a subnormal  $\Delta\text{PO}_2(t_1 - \text{ra})$  ( $P < 0.05$ , one-tailed  $t$ -test; Fig. 1). Also, superior, but not inferior, hemiretinal  $\Delta\text{PO}_2(t_2 - t_1)$  was supernormal ( $P < 0.05$ ) (Fig. 1).

**Lewis Rats.** In control and diabetic LEW rats ( $n = 8$  and 6, respectively), superior and inferior hemiretinal  $\Delta\text{PO}_2(t_1 - \text{ra})$  were both less ( $P \leq 0.05$ ) than that in similar regions in SD rats, although  $\Delta\text{PO}_2(t_2 - t_1)$  was not ( $P > 0.05$ ) different between the strains. Within the LEW rat group, no differences ( $P > 0.05$ ) were found in superior or inferior hemiretinal  $\Delta\text{PO}_2(t_1 - \text{ra})$  between the control and diabetic groups, and in  $\Delta\text{PO}_2(t_2 - t_1)$  in the control group (Fig. 2). In diabetic LEW rats, supernormal ( $P < 0.05$ ) superior and inferior  $\Delta\text{PO}_2(t_2 - t_1)$  were found (Fig. 2). Supernormal response in both superior and inferior hemiretinas were corrected ( $P > 0.05$ ) in diabetic rats with LPA treatment ( $n = 6$ ; Fig. 3).

## DISCUSSION

The major findings in this study are (1) that, in response to a similar carbogen provocation, control and diabetic SD and LEW rats demonstrated substantial differences in initial magnitude and subsequent evolution of superior and inferior hemiretinal oxygenation, with the following exception: (2) diabetic SD and LEW exhibited a similar late-phase supernormal retinal  $\Delta\text{PO}_2$ , and (3) that pathways sensitive to LPA treatment contributed to the development of diabetes-induced supernormal  $\Delta\text{PO}_2$ .

To the best of our knowledge, retinal vascular structure and neuronal anatomy are grossly similar in SD and LEW rats. However, it has been reported that in response to different provocations, such as cyclic hyperoxia exposure,<sup>18</sup> extended increases in intraocular pressure,<sup>19</sup> or experimental autoimmune encephalomyelitis,<sup>19</sup> the SD and LEW strains demonstrate distinct retinal vascular and/or neuronal outcomes. The results from the present study add to the literature by demonstrating significant differences in retinovascular oxygenation response to hyperoxic provocation, not only with diabetes, but also in the absence of an insult (i.e., control conditions; Figs. 1, 2). These considerations support our previous findings that MRI  $\Delta\text{PO}_2$  is a sensitive metric for evaluating diabetic retinopathy.<sup>1</sup>

TABLE 1. Summary of the Animal Model and Physiology during a 2-Minute Carbogen Challenge

	Weight (g)	GHb (%)	$\text{P}_a\text{CO}_2$ (mm Hg)	$\text{P}_a\text{O}_2$ (mm Hg)	pH	Temp. ( $^{\circ}\text{C}$ )
SD C ( $n = 7$ )	288.7 $\pm$ 14.2	4.0 $\pm$ 0.1	51 $\pm$ 1	566 $\pm$ 22	7.31 $\pm$ 0.02	36.3 $\pm$ 0.2
SD D ( $n = 8$ )	267.6 $\pm$ 5.2	12.5 $\pm$ 0.9*	55 $\pm$ 1	495 $\pm$ 32	7.30 $\pm$ 0.02	36.8 $\pm$ 0.2
LEW C ( $n = 8$ )	239.1 $\pm$ 5.6	4.2 $\pm$ 0.0	60 $\pm$ 1†	574 $\pm$ 9	7.30 $\pm$ 0.01	36.4 $\pm$ 0.3
LEW D ( $n = 6$ )	241.1 $\pm$ 3.9	10.6 $\pm$ 0.5*	63 $\pm$ 1†	513 $\pm$ 32	7.28 $\pm$ 0.01	36.2 $\pm$ 0.2
LEW D + LPA ( $n = 6$ )	242.8 $\pm$ 2.7	10.5 $\pm$ 0.5*	57 $\pm$ 1	506 $\pm$ 42	7.29 $\pm$ 0.03	37.1 $\pm$ 0.2

Data are the mean  $\pm$  SEM.

\*  $P < 0.05$ , compared with the respective control group.

†  $P < 0.05$ , compared with the respective SD group.

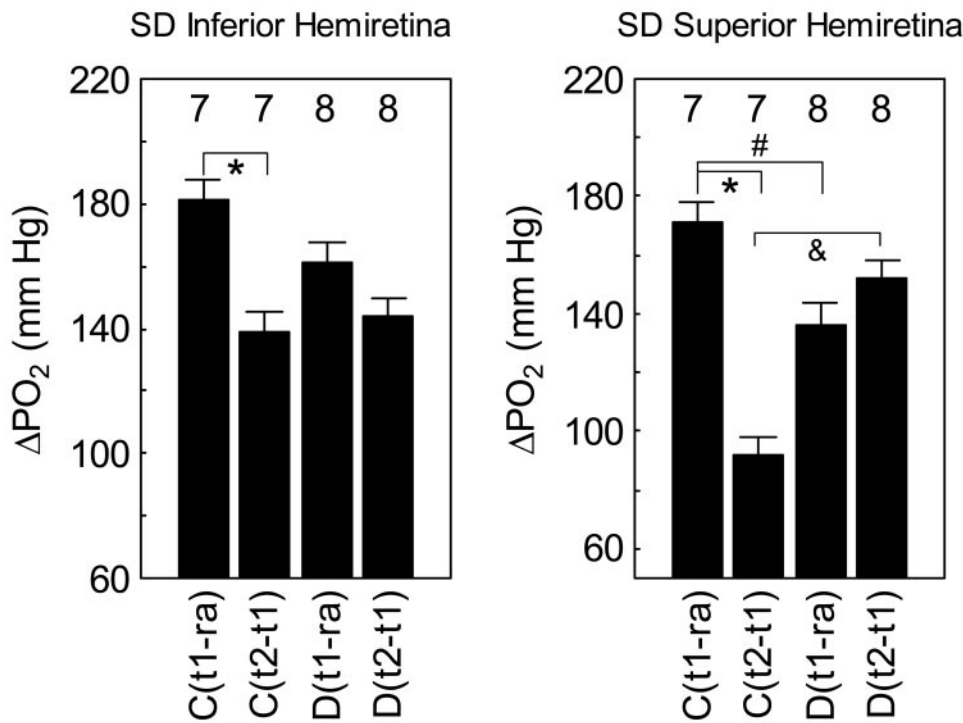


FIGURE 1. Summary of preretinal  $\Delta\text{PO}_2$  temporal evolution in SD control (C), and diabetic (D) rats. In the D group, superior hemiretina  $\Delta\text{PO}_2$  was subnormal during the first 2 minutes of carbogen breathing (i.e.,  $t_1 - \text{ra}$ ) and supernormal during the next 2 minutes (i.e.,  $t_2 - t_1$ ). The number of animals used to generate the data is listed *above* each bar. Error bars, SEM; \*,#,& $P < 0.05$ .

Preretinal vitreous oxygenation responses represent the difference between changes in supply and consumption. Our current hypothesis is that the regulation of retinovascular oxygen supply is the major factor in determining the magnitude of preretinal vitreous  $\Delta\text{PO}_2$ . Previous data collected in rodents indirectly supported this hypothesis. For example, the combination of hyperoxia and hypercapnia (e.g., the gas mixture carbogen, 95% O<sub>2</sub>-5% CO<sub>2</sub>) produce a significantly larger oxygenation response than 100% oxygen breathing.<sup>1</sup> Also, intravitreal injection of the potent vasoconstrictor endothelin-1 produces a subnormal  $\Delta\text{PO}_2$  response in control rats.<sup>1</sup> In addition,

a range of vasoactive drugs including aminoguanidine, aldose reductase inhibitors, and iNOS and COX-2 inhibition corrected subnormal superior retinal  $\Delta\text{PO}_2$  in experimental diabetic retinopathy.<sup>1,20</sup>

Using multiple strains and time points in the present study, we find two notable examples that also support our retinovascular regulation hypothesis. First, as expected, in the diabetic SD group superior hemiretinal  $\Delta\text{PO}_2(t_1 - \text{ra})$  was subnormal<sup>7,21-23</sup>. Results in the literature are consistent with this finding of an initial-phase subnormal superior hemiretinal  $\Delta\text{PO}_2$  in the SD diabetic group, supporting the lack of spurious

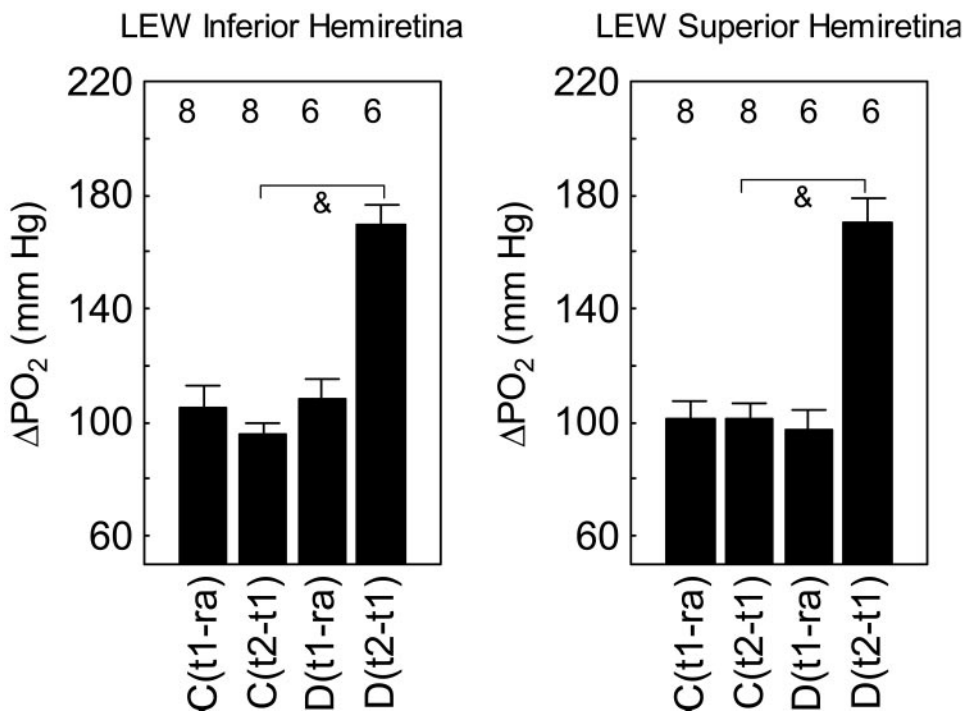
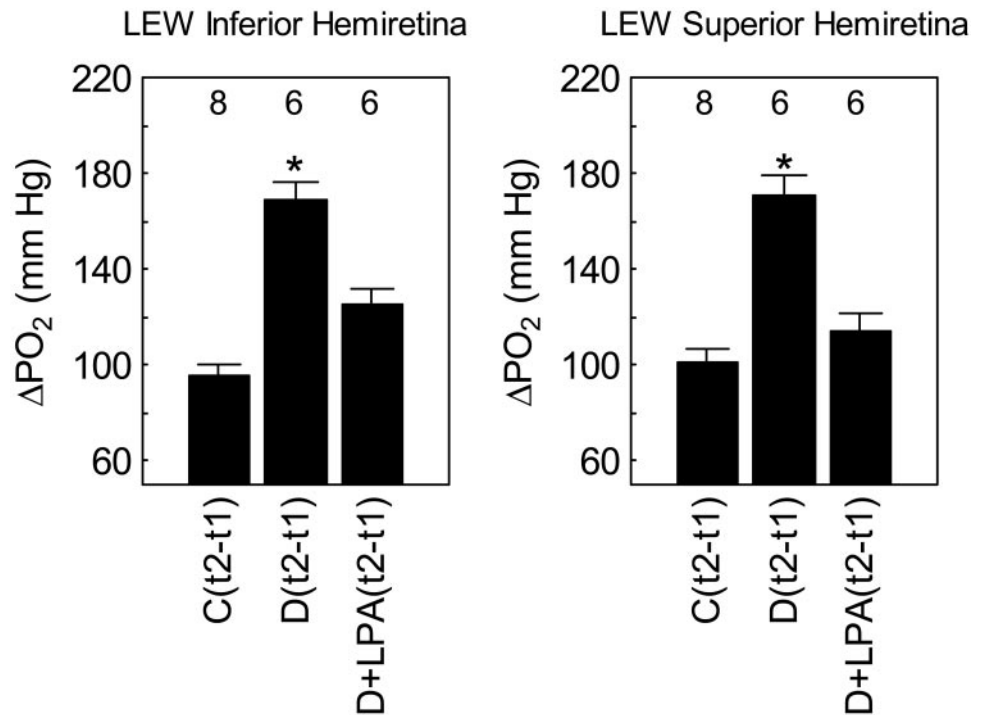


FIGURE 2. Summary of preretinal  $\Delta\text{PO}_2$  temporal evolution in LEW control (C), and diabetic (D) rats. In the D group, inferior and superior hemiretina  $\Delta\text{PO}_2$  were supernormal during the last 2 minutes of carbogen breathing (i.e.,  $t_2 - t_1$ ). The number of animals used to generate the data is listed *above* each bar. Error bars, SEM; & $P < 0.05$ .



**FIGURE 3.** Summary of preretinal  $\Delta PO_2$  during the last 2 minutes of carbogen breathing (i.e.,  $\Delta PO_2(t_2 - t_1)$ ) in LEW control (C), diabetic (D), and diabetic rats treated orally with LPA for 3 months (D+LPA). The number of animals used to generate the data is listed *above* each bar. Error bars, SEM; \* $P < 0.05$ .

significant findings.<sup>1</sup> It is possible that a subnormal response implies an increased retinal demand for  $O_2$ . If retinal oxygen demand was increased by diabetes in both strains, one might expect a subnormal response in both strains and at both time points: ( $t_1 - ra$ ) and ( $t_2 - t_1$ ). However, this initial-phase subnormal response was not observed in the diabetic LEW group or during later periods of carbogen breathing in any group. Thus, the data do not support a role for increased retinal oxygen consumption leading to an initial phase subnormal response. Second, both strains demonstrated a supernormal superior hemiretinal  $\Delta PO_2(t_2 - t_1)$ . It is possible that this supernormal response represents a decreased retinal demand for  $O_2$ , due perhaps to a suppression of activity or loss of neural elements. In this case, the fraction of oxygen from the choroidal circulation that contributes to the preretinal  $\Delta PO_2$ , which is negligible in control rats,<sup>24,25</sup> may become greater than normal. In the present study, if decreased demand for  $O_2$  had occurred in the diabetic retina of both strains, one might expect to see a supernormal response at both time points: ( $t_1 - ra$ ) and ( $t_2 - t_1$ ). This was not observed in any group. Considered together, these considerations suggest that the various magnitudes and temporal evolution patterns observed in this study cannot be coherently explained by changes in retinal oxygen demand. More work is needed to understand the mechanisms underlying the strain-related  $\Delta PO_2$  magnitudes and evolution.

Retinal vessel autoregulation is known to be impaired in diabetes.<sup>26</sup> A late-phase supernormal  $\Delta PO_2$  could develop in both strains of diabetic rats if the retinal circulation were unable to autoregulate in response to the inhalation challenge. In this case, the magnitude of the supernormal effect would increase at longer durations of a hyperoxic provocation due to a greater cumulative buildup of oxygen in the preretinal vitreous space (a tissue with negligible oxygen consumption) over time, compared with the control. Thus, the present data appear consistent with a key role for impaired retinovascular reactivity differences in the late-phase supernormal response in diabetic rats. We speculate that during the initial phase of carbogen breathing (i.e., [ $t_1 - ra$ ]), the subnormal superior hemiretinal oxygenation response found in SD but not LEW

rats reflects a genetic influence on retinovascular regulation in response to carbogen. In any event, the data in this study highlight the duration of the inhalation challenge as a likely explanation for the apparent conflict between previously reported subnormal  $\Delta PO_2(t_1 - ra)$  in diabetic rats and supernormal oxygenation response in patients with type I diabetes, even before the appearance of retinopathy.<sup>2</sup>

In diabetic rat retinas, preventative oral LPA treatment reduced oxidative stress, normalized NF $\kappa$ B activation and angiotensin-2 expression, and decreased nitrotyrosine and vascular endothelial growth factor levels.<sup>3,5,27</sup> Of note, two groups have shown that LPA inhibited the appearance of acellular capillaries and pericyte ghosts in the rat STZ model.<sup>3,5</sup> Because diabetic LEW rats exhibited strong panretinal hemiretinal-symmetric late-phase supernormal responses, relative to the SD group, we chose to evaluate the effect on LPA treatment only in the LEW group. More studies are now needed, perhaps in genetically modified models, to understand better the possible role of antioxidants in the development of a supernormal  $\Delta PO_2$ .<sup>28,29</sup>

Regardless of mechanism, LPA was found to correct the late-phase supernormal response. Because LPA also inhibits the development of retinal vascular histopathology, it appears that supernormal retinal  $\Delta PO_2(t_2 - t_1)$  may also be useful as an early predictor of whether a pharmacological agent will minimize later development of retinal vascular lesions.<sup>1</sup> Based on these observations, we speculate that correction of a late-phase supernormal oxygenation response in patients with type I diabetes before the appearance of retinopathy, will allow more rapid evaluation of drug efficacy and prognosis.<sup>2</sup>

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