Drug Intervention Can Correct Subnormal Retinal Oxygenation Response in Experimental Diabetic Retinopathy

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Purpose. Early subnormal retinal oxygenation response to a hyperoxic provocation (ΔPo_2) is strongly associated with subsequent experimental diabetic retinopathy and can be reversed by drug treatments started with the induction of diabetes. It is not yet known whether drug treatment can reverse an established subnormal ΔPo_2.

Methods. Retinal ΔPo_2 was measured in two separate experimental paradigms in streptozotocin-induced diabetic rats. In a prevention study, measurements were performed in untreated diabetic rats, 3 months after the initiation of hyperglycemia (D3mo), in age-matched nondiabetic rats (C3mo), and in diabetic rats treated orally for 3 months with celecoxib, a cyclooxygenase (COX)-2-selective inhibitor, (D3mo+COX2i). In an intervention study, measurements were performed in untreated diabetic rats 4 months after the initiation of diabetes (D4mo), in age-matched nondiabetic rats (C4mo), and in diabetic rats that were untreated for 3 months and then were orally treated for an additional month with either celecoxib (D4mo+COX2i) or L-*N* (6)-(1-iminoethyl)lysine 5-tetrazole amide, a prodrug of an inhibitor of inducible nitric oxide synthase (iNOS, D4mo+ iNOSi).

Results. In the prevention arm, subnormal ($P < 0.05$) retinal ΔPo_2 was found in the D3mo group, but not in the D3mo+COX2i group ($P > 0.05$). In a previous study, it was reported that retinal ΔPo_2 also corrected in a D3mo+iNOSi group. In the intervention arm, retinal ΔPo_2 levels in the D4mo and D4mo+iNOSi, but not the D4mo+COX2i, groups were ($P < 0.05$) subnormal.

Conclusions. These results demonstrate, for the first time, that drug treatment can reverse an established subnormal ΔPo_2. Furthermore, this effect could not be predicted by a drugs' ability to prevent the development of subnormal ΔPo_2. (*Invest Ophthalmol Vis Sci.* 2005;46:2954–2960) DOI:10.1167/iovs.05-0132

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Retinal complications of diabetes mellitus are the most common cause of blindness and vision loss in people younger than 45 years. However, designing therapies that effectively prevent the development and/or progression of retinal complications in diabetes has been hampered by the use of retinal structural endpoints that develop slowly and have a variable presentation over the course of the disease. New treatments for diabetic retinopathy could be developed more effectively if other endpoints, that are either detectable earlier in the course of diabetes or progress more rapidly, were available.[1] A viable alternative is to measure changes in retinal physiology that precede the development of, but are clearly associated with, the well-described structural lesions. Because diabetic retinopathy is a vascular disorder, a technique that links changes in retinal hemodynamics or oxygenation to the development and progression of retinal structural lesions could form the basis for a more efficacious method of defining the benefits of new and existing therapies.[1]

Normally, retinal oxygen demand and supply are tightly coupled and inner retinal Po_2 is maintained through appropriate retinal vasoreactivity over a broad range of conditions.[2,3] However, if retinal oxygen demand and supply are not adequately adjusted during daily activities, then a functional oxygenation mismatch occurs. This mismatch can have long-term negative consequences for the health of the retina.[1,4] We reasoned that measuring the health of the retinovascular system's regulatory response would be useful as a clinical test that predicts the course of diabetic retinopathy and its response to treatment, while yielding important insights into the pathophysiological basis of the disease and revealing innovative targets for therapy.

Typically, retinovascular response is inferred from perfusion measurements. However, currently available retinal perfusion techniques have substantial limitations. They are either not quantitative (e.g., fluorescein angiography), are terminal (e.g., microspheres), have limited spatial resolution and sensitivity (e.g., laser Doppler velocimetry), or are limited by media opacities such as cataract (e.g., video fluorescein angiography).[1] Clearly, there is a need for an accurate and sensitive measurement of retinovascular ability to respond that can be performed in vivo in preclinical and clinical settings.

To address this need, we have developed and applied a novel functional magnetic resonance imaging (MRI) method that noninvasively measures retinal oxygenation responses to a hyperoxic inhalation challenge that are in agreement with those measured using oxygen electrodes (i.e., the readings are accurate).[5–8] This method measures the change in oxygen level directly through oxygen's paramagnetic relaxation of the water proton in the avascular vitreous next to the retina. The functional MRI retinal oxygenation response measurement is particularly advantageous, because it simultaneously measures regional differences in retinal oxygenation, even in the presence of cataract. MRI facilitates translational studies between animal models of retinopathy and human disease.[9]

TABLE 1. Prevention Study: Summary of Model and Physiology

Group	Blood Glucose (mg/dL)	Carbogen Challenge		
		P_aO_2 (mm Hg)	P_aCO_2 (mm Hg)	Core Temperature (°C)
C3mo (n = 9)	149 ± 15	541 ± 24	52 ± 2	35.8 ± 0.1
D3mo (n = 5)	491 ± 49*	553 ± 11	59 ± 1*	36.1 ± 0.1
D3mo + COX2i (n = 7)	521 ± 22*	598 ± 7	55 ± 2	35.6 ± 0.1

Data are expressed as the mean ± SEM.
* $P < 0.05$ compared with C.

Using this functional MRI approach in rodent models of diabetic retinopathy, we find, well before the appearance of retinal structural lesions, a lower than normal retinal oxygenation response.[5,6] Treatments that prevent the appearance of retinal structural lesions (i.e., aminoguanidine treatment of diabetic rats and ARI-509 treatment of galactose-fed rats),[5] also prevent the development of the reduced retinal oxygenation response. More recently, we found that inhibiting inducible nitric oxide synthase (iNOS) activity at the onset of diabetes using either drug treatment (L-*N*(6)-(1-iminoethyl)lysine 5-tetrazole amide [SC51], a prodrug of an inhibitor of iNOS) or iNOS knockout mice, prevents the development of reduced retinal oxygenation response in experimental diabetes.[10] It is not yet known whether initiating treatment agents after 3 months of diabetes (when subnormal retinal ΔP_{O_2} is present) can correct the subnormal retinal oxygenation response.

Retinal structural lesions (i.e., pericyte ghosts and endothelial cell dropout and the formation of acellular capillaries) that develop in rats after 12 months of diabetes appear to be irreversible.[11] The process that leads to the development of these lesions appears to become irreversible within 4 months of the onset of elevated blood hexose levels.[11] When blood hexose levels are restored to normal levels after 4 months, the severity of these lesions is not different from that in animals that remained hyperhexosemic for 20 months.[11]

In the present study, we tested the hypothesis that drug treatment can reverse an established subnormal retinal oxygenation responses (ΔP_{O_2}). In earlier studies we reported that iNOS inhibition can preserve the retinal response to carbogen inhalation in diabetic rats.[10] In this study we compared the effects of an iNOS inhibitor with those observed when the inhibitor treatment is initiated after the retinal loses its ability to respond normally to a carbogen challenge. Similarly, we compared the ability of a selective COX-2 inhibitor to preserve and restore the ability of the retina to respond to a carbogen challenge.

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 Model

In all cases, animals were randomized into three prevention or three intervention groups of 20 animals each. In the prevention study, three groups of rats were examined: untreated diabetic rats 3 months after the initiation of hyperglycemia (D3mo, n = 5), age-matched nondiabetic rats (C3mo, n = 9), and diabetic rats treated orally for 3 months with celecoxib, (D3mo+COX2i, n = 7). In the intervention study, four groups of animals were studied: untreated diabetic rats 4 months after the initiation of diabetes (D4mo, n = 6), age-matched nondiabetic rats (C4mo, n = 5), and diabetic rats that were untreated for 3 months and then orally treated for an additional month with either celecoxib (D4mo+COX2i, n = 7) or an inhibitor of iNOS (D4mo+ iNOSi, 100 ppm, $n = 6$[12]). Prevention results with the same drug, dose, and route of the iNOS inhibitor administered as were used in this study are published.[10]

Control and untreated diabetic rats in both arms of the study were fed normal rat chow (5001; Ralston Purina, Richmond, IN) and water ad libitum. The COX-2 inhibitor celecoxib was admixed in the chow at a concentration of 200 ppm to yield a daily dose of approximately 20 mg/kg body weight (D+COX2i). The iNOS inhibitor was admixed in the chow at a concentration of 100 ppm to yield an approximate daily dose of 10 mg/kg.

Diabetes was induced with an intraperitoneal injection of streptozotocin (55 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 plasma hyperglycemia (≥200 mg/dL) and elevated urine volume (>60 mL/d) in nonfasted rats. Rat body weight, average food consumption, 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 an appropriate level of hyperglycemia (Tables 1, 2). For example, 1 U insulin given for 2 days and skipped for 2 days is sufficient to maintain blood glucose under 600 mg/dL, negative urine ketone levels and body weight loss to less than 15%. Glycated hemoglobin (GHb) was measured after 2 months of diabetes with affinity columns (Glyco-Tek kit 5351; Helena Laboratories, Beaumont, TX).

MRI Examination

On the day of the experiment, anesthesia was induced by a single intraperitoneal injection of urethane (36% solution, 0.083 mL/20 g animal weight, prepared fresh daily; Aldrich, Milwaukee, WI). 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.[5]

MRI data were acquired on a 4.7-T system using a two-turn transmit–receive surface coil (1.5 cm in diameter) placed over the eye. Images were acquired with an adiabatic spin-echo imaging sequence (repetition time [TR], 1 second; echo time [TE], 22.7 ms—the shortest echo time allowed with this sequence): number of acquisitions [NA], 1; matrix size, 128 × 256; slice thickness, 1 mm; field of view, 32 mm^2; sweep width, 25,000 Hz; 2 minutes/image).[7] This resulted in an in-plane resolution of 250 × 125 μm. Examining the initial retinal oxygenation response to a hyperoxic challenge requires careful attention to experimental timing and makes high demands on the animals' physiology, compared with steady state measurements. We reasoned that the retinal circulation is often necessary to maintain an adequate oxygenation level under different conditions and the hyperoxic challenge acts as an acute "stress" test of the retinal circulation's ability to oxygenate. Other stress tests (e.g., response to hypoxia and/or reduced

TABLE 2. Intervention Study: Summary of Animal Model and Physiology

Group	Blood Glucose (mg/dL)	GHb (%)	Carbogen Challenge		
			P_aO_2 (mm Hg)	P_aCO_2 (mm Hg)	Core Temperature (°C)
C4mo ($n = 5$)	93 ± 9	4.4 ± 0.1	534 ± 21	55 ± 2	36.8 ± 0.3
D4mo ($n = 6$)	390 ± 46*	14.8 ± 0.9*	558 ± 21	55 ± 1	36.7 ± 0.1
D4mo + COX2i ($n = 7$)	507 ± 46*	13.7 ± 0.8*	585 ± 9	56 ± 2	36.8 ± 0.2
D4mo + iNOSi ($n = 6$)	483 ± 60*	14.8 ± 0.5*	540 ± 24	57 ± 1	36.6 ± 0.2

Data are expressed as the mean ± SEM.
* $P < 0.05$ compared with C.

blood pressure) can also be envisioned. However, relatively smaller signal intensity changes are expected from these challenges compared with that found during carbogen breathing. This would probably make analysis of the data difficult to detect or interpret. In the present study, we chose to use a carbogen challenge because large signal intensity changes are produced in the rat allowing for the detection of subtler pathophysiology.

The MRI data were collected as follows. 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 an 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 the superior and inferior hemiretina with some relatively minor contribution from the temporal and nasal hemiretina. A capillary tube (1.5 mm inner diameter) filled with distilled water was used as the external standard. It is important to note that steady state (room air) vitreous oxygen tension *cannot* be measured using 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 cannot be used alone to measure retinal oxygenation.

Data were collected as follows: three images while the animal breathed room air and one image during the inhalation of carbogen. Carbogen inhalation was started at the end of the third 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 inhalation 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.[7] This blood was analyzed for glucose, P_aO_2, P_aCO_2, and pH. Note that this second inhalation challenge (outside the magnet) is needed because it is not feasible to obtain an arterial blood sample routinely from inside the magnet (>40 cm away from the magnet opening) from rats. In all cases, after the blood collection, animals were euthanatized with an intracardiac potassium chloride injection.

Data Analysis

To be included in this study, the animal must have demonstrated: (1) minimal eye movement during the MRI examination. Movement artifacts (typically, in the phase encode direction) 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 was probably improperly administrated (e.g., not intraperitoneal). This error could produce a change in systemic oxygenation unrelated to the retinal changes. (3) Rectal temperatures in the range of 35.5°C to 36.5°C. Preliminary experiments (data not shown) found a strong association between core temperature and P_aCO_2 and P_aO_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_aO_2 higher than 350 mm Hg and P_aCO_2 between 46 and 65 mm Hg during the carbogen challenge. Previously, we found that arterial oxygen levels above 350 mm Hg during a hyperoxic challenge were needed to produce a consistently large preretinal vitreous oxygenation response.[13] 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, ΔPo_2 data were collected approximately 60 minutes after urethane injection to avoid potential errors due to variable time under anesthesia. The acceptance criteria are necessary for critically comparing the retinal oxygenation response in these spontaneously breathing normal and sick animals while minimizing systemic differences. Because such tight criteria were used, only approximately 50% of the animals that started the study were used in the final analysis. Based on our previous experience in rats, $n \geq 5$ is sufficient to draw statistically valid conclusions.

To correct for any movement in the slice plane, a warp affine image coregistration was performed on each animal with software written in-house. This procedure was performed for all the animals used in the final analysis but was only necessary in approximately half of them, regardless of group (e.g., subtle shifting of the animals' position occurred during the experiment because of settling on the gauze packing). Because the slice thickness (1 mm) was relatively large compared with the diameter of the eye (approximately 3 mm), partial volumes would be similar if the eye subtly moved out of the imaging plane, and so the data analysis results were 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 using NIH Image (a freeware program available at http://rsb.info.nih.gov/nih-image/available by ftp at zippy.nimh.nih.gov/; 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. All pixel signal intensities in the average room air image and the 2-minute carbogen image were then normalized to the external standard intensity. Signal intensity changes during carbogen breathing were calculated and converted to ΔPo_2, on a pixel-by-pixel basis, as follows.[7] 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, and S_0 is the control signal intensity (measured from the average of the three control images) at the same pixel spatial location. E is converted into ΔPo_2 according to a theory that has been validated in the rat[14]:

$$\Delta PO_2 = E/(R_1 \cdot T_k), \quad (2)$$

where R_1 is the oxygen relaxivity (seconds per mm Hg), and $T_k = T_r \cdot \exp(-T_r/T_{10})$, T_r is the repetition time, and T_{10} is the T_1 in the absence of oxygen. Using a T_r of 1 second and assuming a vitreous T_{10} of 4 seconds, $T_k = 3.52$. This T_{10} is based on our previous measurement of the proton spin-lattice TR in the rabbit vitreous (4 seconds) and reported values in human vitreous (3.3 seconds) and cerebral spinal fluid (4.3 seconds), which has a high water content similar to vitreous.[7,15–17] An R_1 of 2×10^{-4} s^{-1}/mm Hg^{-1} was used. This R_1 had been measured in a saline phantom, which is assumed to be a reasonable model of vitreous (98% water).[7] A similar R_1 was found for plasma, suggesting that relatively low protein levels do not substantially contribute to the oxygen relaxivity.[18] Note that an E of 0.01 (i.e., a 1% signal-intensity change) corresponds to a ΔPo_2 of 14 mm Hg.

The ΔPo_2 parameter image was analyzed as follows. First, the pixel values along a 1-pixel-thick line drawn at the boundary of the retina–choroid and vitreous were set to 255 (black). We estimate that the thickness of this line, based on the in-plane resolution is approximately 100 μm. The values in another 1-pixel-thick line drawn in the preretinal vitreous next to the black pixels were then extracted.[19] This procedure minimized retinal/choroid pixel values from potentially contaminating ("pixel bleed") those used in the final analysis and insured that similar preretinal vitreous space was sampled for each animal. In addition, spatial averaging over these 100-μm regions of interest tends to minimize the contribution from the very local preretinal oxygenation gradients next to the retinal surface.[20] An average ΔPo_2 band was constructed based on the within-group mean for each pixel.

Statistical Analysis

The physiological parameters measured after the MRI experiment (i.e., blood gas levels, rectal temperatures, and blood glucose data) were normally distributed and are presented as the mean ± SEM. Comparisons were performed with an unpaired two-tailed *t*-test and $P \leq 0.05$ was considered significant.

Comparison of retinal ΔPo_2 between control and experimental groups were performed with a generalized estimating equation approach.[21] This method performs a general linear regression analysis using all the pixels in each subject and accounts for the within-subject correlation between adjacent pixels. In all cases, we tested to determine whether the difference between the control ΔPo_2 and that of the experimental group is positive, based on our previous experience (i.e., a one-tailed test); $P \leq 0.05$ was considered significant. Our results were not corrected for the number of group comparisons, and so it is possible that significant findings were due to random chance, although chance significance is most likely to occur when many comparisons are performed. Bonferroni corrections are considered overly conservative and there is no clear consensus on how one should control for multiple comparisons.[22] In this work, actual, uncorrected probabilities are reported. Results from the literature are consistent with our present finding of a subnormal retinal ΔPo_2 in the diabetic groups, supporting the lack of spurious findings of significance.

Results

Systemic Physiology

Summaries of systemic physiology are presented in Tables 1 and 2. As expected, compared with control rats, all diabetic animals had either significantly ($P < 0.05$) elevated glucose levels (prevention study) or glycated hemoglobin (GHb; intervention study). GHb levels in the rats in the prevention arm were not measured, because the kits were unavailable from the vendors at the time. A summary of the blood parameters measured during a 2-minute hyperoxic challenge and core temperature during the experiment are also presented in the tables. No significant differences ($P > 0.05$) were observed between any of the groups, except for the D3mo arterial carbon dioxide level (Table 1). Note that the P_aCO_2 in all groups are within the range expected during carbogen provocation.[7]

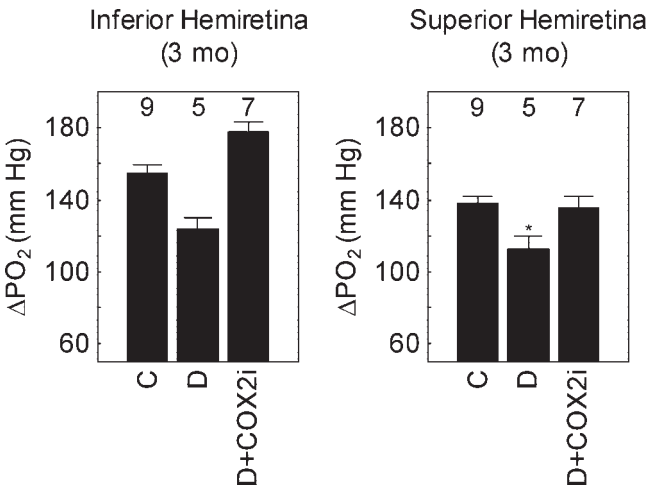

Figure 1. Summary of hemiretinal ΔPo_2 in the 3-month prevention study of control (C), diabetic (D), and diabetic rats treated orally for 3 months with celecoxib, a COX-2-selective inhibitor (D+COX2i). A subnormal (*$P < 0.05$) superior hemiretinal ΔPo_2 was found only in the D group. The number of animals used to generate the data are listed above each bar. Error bars, SEM. Only superior hemiretinal oxygenation responses have been found to be a useful early surrogate marker of treatment efficacy.[5,6,10,23]

Functional MRI

Figure 1 represents a summary of the functional MRI results in the prevention study. Inferior hemiretina ΔPo_2s were not different ($P > 0.05$) between any groups. Superior hemiretina ΔPo_2s were subnormal ($P = 0.045$) in the D3mo rats but normal ($P = 0.38$) in the D3mo+COX2i group. In the intervention study, inferior hemiretina ΔPo_2s were not different ($P > 0.05$) between any groups. Superior hemiretinal ΔPo_2 for the D and D+iNOSi groups were significantly subnormal ($P = 0.035$ and 0.014, respectively, right panel, Fig. 2), but the differences between the nondiabetic animals and the D+COX-2 group were not significant ($P > 0.05$).

Discussion

The major findings in this study are (1) that the early subnormal oxygenation response associated with diabetes is reversible with drug treatment, (2) that intervention with a COX-2 activity inhibitor corrects the early oxygenation response defect but an iNOS activity inhibitor does not, and (3) that the ability of a drug to prevent the development of subnormal ΔPo_2 does not predict its success in correcting a subnormal response when used interventionally.[5,6,10,23] We also found that the severity of the superior oxygenation defect between groups of rats after 3 or 4 months of diabetes was similar (25% and 21%, respectively) suggesting that the vascular functional lesion does not worsen after 3 to 4 months of diabetes. In agreement with several previous reports, we confirmed a lack of significant difference in the inferior hemiretina ΔPo_2 measured before the appearance of histopathology between control and diabetic animals (Figs. 1, 2).[5,6,10,23]

Understanding the pathophysiology underlying the regional asymmetry between superior and inferior hemiretinal responses in diabetic rodents is currently an active area of research in our laboratory. At first, this finding may seem remark-

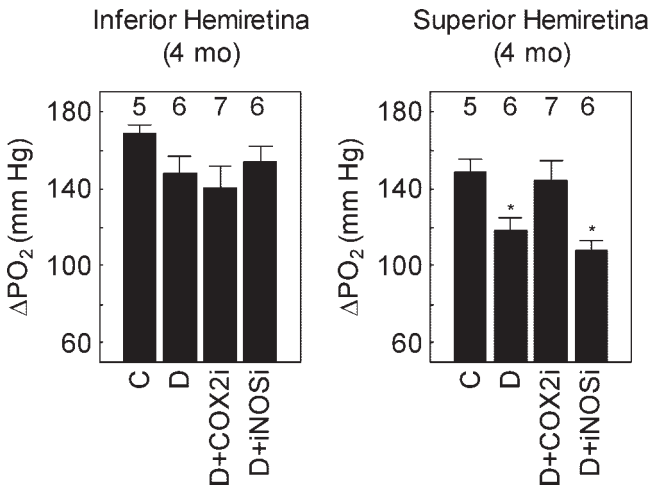

FIGURE 2. Summary of hemiretinal ΔP_{O_2} in the 4-month intervention study of control (C), diabetic (D), and diabetic rats that were untreated for 3 months and then orally treated for an additional month with either celecoxib (D+COX2i) or SC51, a prodrug of an inhibitor of inducible nitric oxide synthase (iNOS, D+iNOSi). Only in the D and D+iNOSi groups were subnormal ($^*P < 0.05$ for both) superior hemiretinal ΔP_{O_2} levels found. The numbers of animals used to generate the data are listed above each bar. Error bars, SEM.

able because the rat retinal circulation is grossly symmetric around the optic nerve head. To explain this asymmetric oxygenation response, we considered the impact on ΔP_{O_2} of several systemic variables, such as baseline arterial P_{O_2} and P_{CO_2}, hematocrit, and ocular perfusion pressure that could have been different in diabetic rats compared with controls. Previously, we reported baseline P_{O_2} and P_{CO_2} of control rats on urethane and have collected blood samples periodically since that report to confirm that baseline blood gas levels fall within similar levels in urethane anesthetized control and diabetic rats (P_{O_2} 80–100 mm Hg and P_{CO_2} 35–45 mm Hg; data not shown).[7] With regard to possible hematologic differences between control and diabetic rats, Alder et al.[24] reported that control and 6-week diabetic rats have similar hematologic parameters (except for expected elevations of blood glucose, glycated hemoglobin, and 2,3 diphosphoglycerate in the diabetic group compared with controls), including similar P_{50} (the P_{O_2} at which the oxygen-carrying capacity of blood is 50% of maximum). With regard to differences in intraocular pressure, rats made diabetic for 2 months do not demonstrate different baseline intraocular pressure than do control animals.[25] In summary, there is no evidence in the literature of differences in baseline measures of arterial P_{O_2} and P_{CO_2}, hematocrit, and ocular perfusion pressure between control and diabetic rats. Consequently, these parameters appear unlikely to have contributed to the group differences reported in this and previous studies. We speculate that systemic factors that could influence retinal ΔP_{O_2} would affect both the superior and the inferior hemiretina equally. Based on this reasoning, our working assumption is that the lack of differences in inferior hemiretinal responses between groups in this study and in previous works implies a minimum impact of systemic differences on ΔP_{O_2}. These considerations suggest that the regional ΔP_{O_2} asymmetry is more likely to be due to local physiological retinal differences between control and diabetic rats.

Our current hypothesis is that, at least in rats, the regulation of retinovascular oxygen supply is the major factor in determining the magnitude of preretinal vitreous ΔP_{O_2}. In support of this hypothesis, we reported that, in rats, the combination of hyperoxia and hypercapnia (e.g., the gas mixture carbogen, 95% O_2-5% CO_2), produces a significantly larger oxygenation response than 100% oxygen breathing.[7,26] Other investigators have strongly confirmed these results in rat,[26,27] cat (Chung E, et al. *IOVS* 2003;44:ARVO E-Abstract 4975), and pig.[28] In contrast, in humans, the relative response of the retinal circulation to breathing carbogen remains somewhat controversial. Some studies report vasoconstriction during both oxygen and carbogen.[29] In contrast, Hickam and Frayser[30] reported that carbogen-like gas inhalation prevents the large degree of vasoconstriction typically associated with 100% oxygen breathing. Other studies have reported that carbogen can correct the vasoconstriction associated with 100% oxygen breathing.[31,32] These data are consistent with the notion that, at least in rats, cats, and pigs, carbogen minimizes the retinal vasoconstriction associated with 100% oxygen breathing. In humans, more studies are needed for clarification.

Regulation of the retinovascular oxygen supply is known to be impaired in diabetes.[1] For example, retinal vessels in diabetic subjects do not vasoconstrict to the same extent during a 100% oxygen inhalation provocation as in control subjects.[1] Consistent with these data, we found a greater than normal retinal ΔP_{O_2} in diabetic patients during 100% oxygen provocation compared with age-matched control subjects (Trick GL, et al. *IOVS* 2004;45:ARVO E-Abstract 2997). Impaired dilatation of cerebral vessels during hypercapnia has been associated with increased severity of diabetes.[33] In addition, we have reported that subnormal superior retinal ΔP_{O_2} in experimental diabetic retinopathy can be corrected by preventative vasoactive drugs including aminoguanidine, aldose reductase inhibitors, iNOS inhibition, and protein kinase C knockout mice.[5,10,23] Further work is ongoing to clarify exactly which aspect of retinal oxygen supply and demands are perturbed by diabetes and why diabetes produces an asymmetric retinal oxygenation response. Based on these considerations, diabetes appears to produce a more rigid pipelike condition in retinal blood vessels, which can be studied using functional MRI and an inhalation provocation.

Regardless of the mechanism, early changes in retinal ΔP_{O_2} have been shown to be a useful early predictor of whether a pharmacologic agent will minimize later development of retinal vascular lesions.[5] Hyperglycemia causes a variety of biochemical changes including upregulation of COX-2, increased production of retinal nitric oxide, and increased retinal oxidative stress.[34–36] Because each of these pathways can regulate the others, it has not yet been possible to unravel the contributions and regulation of each pathways.[6,37] We, and others, have published evidence to show that nitric oxide is significantly increased in the rat retina 2 to 3 months after the initiation of diabetes.[10,35] In our previous experiments, we confirmed that a relatively selective inhibitor of iNOS activity (SC-51; prodrug L-*N*(6)-(1-iminoethyl)lysine 5-tetrazole amide) prevents the diabetes-induced accumulation of retinal NO and corrects the early subnormal retinal oxygenation response.[10] In addition, we have shown that treatment with aminoguanidine, a relatively selective inhibitor of iNOS activity, also preserves the ability of the retina to respond to a carbogen challenge.[5] Consistent with the latter observations, the retinal oxygenation response remained normal after 4 months of diabetes in mice in which the iNOS gene was deleted (iNOS knockout mice).[10] Note that we cannot rule out the possibility that nNOS and eNOS also contribute to or compensate for the beneficial effects of inhibiting or knocking out iNOS. In addition, we cannot rule out the possibility that the COX-2 and the iNOS inhibitors used in this study affected ΔP_{O_2} through a systemic mechanism instead of a local (retinal) mechanism. However, this possibility is considered unlikely, because no

change in inferior hemiretinal ΔPo_2 was found between groups. Nonetheless, in the present study, 3 months of celecoxib treatment in diabetic rats prevented the oxygenation defect (Fig. 1).

The goal of the present study was to test whether interventional inhibition of COX-2 or iNOS treatment, initiated after the appearance of a subnormal retinal oxygenation response, would restore the oxygenation response to normal. We observed that intervention with a COX-2 inhibitor, but not with an iNOS inhibitor could correct the response to the carbogen challenge. These results were somewhat surprising, because both the COX-2 inhibitor and the iNOS inhibitor, tested at the same doses used in the intervention study, preserved ΔPo_2 when inhibitor treatment was initiated on the same day that diabetes was induced.[10] We speculate that retinal COX-2 and iNOS, which appear to be have central roles in the pathogenesis of diabetes, are initially upregulated at a similar time during induction of diabetes, but during a later "chronic" period of diabetes, regulation of COX-2 and iNOS activity dissociate.[37,38] For example, by 3 months of diabetes, COX-2 activity may be elevated, so that a COX-2 inhibitor would be effective, whereas iNOS activity may have normalized, so that an iNOS inhibitor would not be effective.

The results of the present study clearly demonstrate that early subnormal ΔPo_2 is reversible with COX-2 inhibitor treatment. These data strongly suggest the availability of a temporal therapeutic window of opportunity for drug intervention to correct pathophysiology associated with diabetic retinopathy.[39] In addition, functional MRI is expected to be useful in examining biochemical changes involved in the reversible and irreversible phases of diabetic retinopathy. Future studies are needed to define better what other biochemical interventions can reverse the oxygenation lesion and when, during the course of diabetes, ΔPo_2 damage becomes irreversible. These data highlight the application of functional MRI measures of oxygenation as a powerful approach to monitoring drug treatment regimens.

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