Correction of Early Subnormal Superior Hemiretinal ΔPO2 Predicts Therapeutic Efficacy in Experimental Diabetic Retinopathy

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PURPOSE. To test the hypothesis that regional retinal oxygenation responses to a hyperoxic inhalation challenge are associated with reported retinopathy outcomes after different therapies in rat models of diabetic retinopathy.

METHODS. Six groups of rats were maintained for 3 months: controls (n = 8), untreated diabetic (n = 8), aminoguanidine (AMG)-treated diabetic (2.5 g/kg of diet; n = 6), untreated galactosemic (n = 7), AMG-treated galactosemic (n = 10), and WAY-509–treated (25 mg/kg body weight per day) galactosemic (n = 7). After 3 months, the change in oxygen tension was measured noninvasively from the superior to the inferior ora serrata, using a novel functional magnetic resonance imaging (fMRI) technique and a carbogen (a gas mixture of 5% carbon dioxide and 95% oxygen that has been used clinically, instead of 100% oxygen, to minimize the vasoconstrictive effects of pure O2 on retinal blood flow and oxygenation) inhalation challenge. Retinal morphometric measurements were also obtained.

RESULTS. Retinal lesions (acellular capillaries and pericyte ghosts) were not significantly (P > 0.05) present at 3 months in any experimental groups compared with the control group. Superior but not inferior hemiretinal change in partial pressure of oxygen (ΔPO2) became significantly subnormal (P < 0.05) at 3 months of diabetes or galactosemia. Aminoguanidine, which has been found to inhibit the development of retinopathy in diabetic but not galactosemic rats, inhibited the development of a subnormal ΔPO2 in diabetes but not in galactosemia. WAY-509, which has been reported to inhibit retinopathy in galactosemic rats, inhibited the ΔPO2 defect in galactosemic rats.

CONCLUSIONS. An early subnormal superior hemiretinal ΔPO2 after treatment appears to be a good predictor of the risk of development of retinopathy, as well as for assessing therapeutic efficacy in experimental diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2001;42:2964-2969)

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Retinal complications of diabetes mellitus are the principle cause of blindness and vision loss in people less than 45 years old. Good glycemic control has been shown to be an effective approach to minimizing the development of diabetic retinopathy. Nonetheless, such tight control does not prevent the development of retinopathy in all patients and is not achievable in all patients. It would be of value to identify these higher risk patients at the earliest possible time so that more intensive clinical management can be applied. In addition, the development of a test that presymptomatically predicts the risk of development of diabetic retinopathy is expected to substantially improve the testing of efficacy of new or existing treatments.1 Diabetes is a vascular disorder and thus changes in retinal hemodynamics or oxygenation will probably form the basis of such a test. Furthermore, measures of regional susceptibility to hyperglycemic insult may be important, because studies in dogs2 and humans3 have found a significantly greater incidence of diabetes-related retinal lesions in superior–temporal retina than in inferior–nasal retina.

Currently, measurements of retinal oxygenation have not been possible under clinical conditions. Instead, retinal oxygenation is usually inferred from retinal perfusion measurements. However, the retinal perfusion techniques have substantial limitations. They are not quantitative (e.g., fluorescein angiography), 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).1,4

To address these limitations, we have developed and applied a novel functional magnetic resonance imaging (fMRI) method that accurately and noninvasively measures the retinal oxygenation response to a hyperoxic inhalation challenge.5–10 Unlike standard fMRI techniques, our 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 fMRI retinal oxygenation response measurement is particularly advantageous, because it simultaneously measures regional differences in retinal oxygenation, even in the presence of cataract. Furthermore, MRI facilitates translational studies between animal models of retinopathy and human disease. Recently, we found a reduced superior oxygenation response in galactose-fed rats well before the appearance of retinal lesions (at 3.5 months on the diet) and when lesions were evident (at 15–18 months on the diet). In the present study, we attempt to extend this association by testing the hypothesis that a subnormal superior retinal oxygenation response is an early functional marker of the risk of development of diabetic retinopathy and for assessing treatment efficacy.

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

Two experimental models of diabetic retinopathy were studied in this work: the diabetic rat and the galactosemic rat. Rats were maintained in six groups for 3 months: controls (n = 8), untreated diabetic (n = 8), aminoguanidine (AMG)-treated diabetic (2.5 g/kg of diet; n = 6), untreated galactosemic (n = 7), AMG-treated galactosemic (n = 10), and WAY-509–treated (25 mg/kg body weight per day) galactosemic (n = 7). Diabetes was induced in the rat (starting weight 200–220 g) by injecting streptozotocin (55 mg/kg, 0.01 M citrate buffer, pH 4.5, intraperitoneally) after a 24-hour fast and verified 24 hours later by the presence of hyperglycemia and glucosuria in nonfasted rats. Each diabetic group started with 15 animals. Two to three animals were deemed unsuitable, due to hyperglycemia’s being too mild or to sickness during the 3-month period. An additional two to three animals did not meet our physiologic criteria during the MRI experiment, and thus were deemed unsuitable. Animals were fed normal rat chow (5001; Ralston Purina, Richmond, IN) and water ad libitum. Rat body weight, average food consumption, and blood glucose levels were monitored weekly. Subtherapeutic levels of insulin (0–4 U of neutral protamine Hagedorn [NPH] insulin administered subcutaneously up to 7 days per week, as needed) were administered to allow slow weight gain, yet maintain hyperglycemia and glucosuria. The diabetic animals produced in this study had mean plasma glucose levels of more than 400 but less than 550 mg/dl (Table 1). In the galactose-fed model rats were allowed free access to a powdered rat chow mixed with 50% galactose mixed for 3 months. Glycosylated hemoglobin was measured 1 week before the MRI examination. Final blood glucose levels were measured immediately after the MRI examination. After 3 months of hyperhexosemia, retinal lesions had not yet appeared (P > 0.05, Table 2).6

Treatments

Aminoguanidine (AMG, Sigma, St. Louis, MO) was administered at a dose of 2.5 g/kg of diet to diabetic and galactose-fed rats. This concentration of AMG was used in a recent study in which it prevented retinopathy formation at more 15 months.11 WAY-509 (kindly provided by Tom Hohman and Wyeth-Ayerst Research, Philadelphia, PA) was added to the 50% r-galactose diet so that the animals received 25 mg/kg body weight per day.12

MRI Examination

On the day of the experiment, rats (fasted for at least 12 hours) were anesthetized with urethane (1.5 g/kg, intraperitoneally, 56% solution, freshly made daily) and gently positioned prone in the MRI cradle, so that the left eye was uppermost. All rats were similarly positioned. Systemic physiologic parameters (rectal temperature, pulse, and hemoglobin oxygen saturation) were monitored and maintained during the entire experiment. A timed series of T1-weighted images were collected (repetition time, 1 second; echo time 18 msec, number of acquisitions 1, matrix size 128 × 256 pixels, slice thickness 1 mm, field of view 30 × 30 mm, 2 minutes/image) such that the animals breathed room air for six images (12 minutes) followed by one 2-minute image during carbogen breathing. An alternative experiment involving longer carbogen challenges to achieve equilibrium is possible but is associated with greater animal movement and would not be applicable to future human studies of retinal oxygenation response.13 The animals were returned to room air for 15 minutes (to allow recovery from the inhalation challenge) and removed from the magnet. Blood from the descending abdominal aorta was collected, with great care taken so that the head was not moved, during a second 2-minute carbogen challenge and analyzed for partial arterial pressure of oxygen (PaO2) and carbon dioxide (PaCO2), pH, and glucose concentration. After the MRI examination, the animals were killed.

The increase in partial oxygen pressure (∆PO2) in the vitreous over the room air pressure is detected as an increase in the signal intensity on a T1-weighted image.6,10 Previously, we validated that the MRI-measured ∆PO2 was similar to that determined with an oxygen electrode in normal rat retina.7 We measure the ∆PO2 in the posterior vitreous (within 200 μm from the retina, described later) as a measure of inner retinal oxygenation.14 Carbogen is a gas mixture of carbon dioxide (5% CO2) and oxygen (95% O2) that has been used clinically, instead of 100% oxygen, to minimize the vasoconstrictive effects of pure O2 on retinal blood flow and oxygenation. We, and others, have measured a roughly 50% improvement in retinal oxygenation in the rat during carbogen breathing, relative to O2 breathing.9,15 It is important to note that steady state (room air) vitreous oxygen tension cannot be measured using this method, because many factors (e.g., vitreous temperature and protein content) affect the baseline preretinal vitreous water signal and its relaxation properties. In other words, an image of the eye obtained during room air breathing alone cannot be used to measure retinal oxygenation. These factors are not likely to change on the short time scale between baseline and carbogen breathing. Thus, their contributions are expected to cancel and not contribute to the ∆PO2 measurement. The agreement between the MRI and oxygen electrode data support this interpretation.15

Data Analysis

To be included in a study, an animal must have demonstrated minimal movement (eye and head) during the examination, a regular nongasping respiratory pattern before the examination, rectal temperatures in the range of 36.5°C to 38.5°C, PaO2 higher than 350 mm Hg and PaCO2 45 to 65 mm Hg during the carbogen challenge. These quality control criteria minimized the effect of possible variations in physiological factors within and between groups that could have confounded data interpretation. A warp affine image registration was performed on a computer workstation (Sun Ultra II; Sun Microsystems, Mountain View, CA) using software written in-house. After registration, the room air images 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 on a pixel-by-pixel basis, converted to ∆PO2, and displayed as a pseudocolor parameter map as previously described.6 Data along a

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial BW (g)</th>
<th>Final BW (g)</th>
<th>Blood Glucose (mg/dl)</th>
<th>Glycosylated Hemoglobin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 8)</td>
<td>258 ± 4</td>
<td>292 ± 6†</td>
<td>161 ± 9</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>Diabetic (n = 8)</td>
<td>236 ± 4†</td>
<td>219 ± 4</td>
<td>477 ± 26†</td>
<td>12.9 ± 0.7†</td>
</tr>
<tr>
<td>Galactosemic (n = 7)</td>
<td>216 ± 1†</td>
<td>241 ± 3†</td>
<td>60 ± 6†</td>
<td>8.7 ± 0.5†</td>
</tr>
<tr>
<td>AMG-treated diabetic (n = 5)</td>
<td>235 ± 3†</td>
<td>198 ± 13†</td>
<td>462 ± 53†</td>
<td>11.2 ± 0.4†</td>
</tr>
<tr>
<td>AMG-treated galactosemic (n = 10)</td>
<td>235 ± 6†</td>
<td>251 ± 6</td>
<td>49 ± 3†</td>
<td>8.5 ± 0.4†</td>
</tr>
<tr>
<td>WAY-509–treated galactosemic (n = 6)</td>
<td>226 ± 2†</td>
<td>239 ± 5</td>
<td>69 ± 5†</td>
<td>4.9 ± 0.5†</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. BW, body weight.
* Significantly different from initial BW, P < 0.05.
† Significantly different from control group, P < 0.05.
1-pixel-thick line drawn in the preretinal vitreous space were extracted to generate a preretinal vitreous $\Delta$PO$_2$ band that was used as previously described for analysis. Note that a 1-pixel-thick line is approximately 200 $\mu$m thick, because the 30 $\times$ 30-mm$^2$ field of view was sampled by 128 $\times$ 256 data points resulting in an in-plane resolution of 234 $\times$ 117 $\mu$m. When $\Delta$PO$_2$ is measured in the posterior vitreous (within 200 $\mu$m from the retina), we refer to it as a measure of inner retinal oxygenation. Pixels in the preretinal vitreous between the optic nerve and ora serrata of the superior and inferior hemiretina were analyzed separately. Because of the 1-mm slice thickness, the superior and inferior hemiretina measurements included some contribution from temporal and nasal retina. We did not perform separate studies to deconvolute these contributions.

Statistical Analysis
The physiological data (blood gas, rectal temperature, and blood glucose) were normally distributed. Comparisons between groups were performed using an ANOVA. The hemiretinal MRI oxygenation responses and morphometric data were not normally distributed, and comparisons were made by using a Kruskal-Wallis test. In all cases, $P < 0.05$ was considered significant.

Trypsin Digest
In randomly chosen animals from each group that were examined by MRI, one half retina of one eye per animal was fixed in 10% buffered formalin. Trypsin digests of retinas were performed as previously described. In a masked fashion, the digests were analyzed for acellular capillaries and pericyte ghosts in multiple fields across the entire sample and were expressed as the median frequencies of acellular capillaries per square millimeter of retinal area and pericyte ghosts per 1000 capillary cells, respectively. Pericyte ghosts were defined as out-pouching of basement membrane on capillaries possessing at least one endothelial or pericyte nucleus. Acellular capillaries and pericyte ghosts are among the earliest markers of histopathology accepted as characteristic of diabetic retinopathy.

RESULTS

Model Characteristics
The two models' characteristics are presented in Table 1. The final body weights of the untreated and treated diabetic rats at 3 months were not greater ($P > 0.05$) than the respective initial body weights of these groups. There was a difference between initial and final body weights in the untreated galactose-fed rats ($P < 0.05$), but not in the two treated galactose-fed groups ($P > 0.05$). As expected, compared with control animals, the blood glucose level was significantly elevated ($P < 0.05$) in treated and untreated diabetic groups, but not elevated ($P > 0.05$) in any of the galactose-fed groups. Blood galactose levels were not measured. Glycosylated hemoglobin levels were statistically higher than control levels in all groups except the WAY-509–treated galactose-fed rats. Note that there was no significant difference ($P > 0.05$) between the glycosylated hemoglobin levels of the treated and untreated diabetic rats.

Morphometric Analysis
As expected in the short duration of the study, there was no morphometric evidence of retinal lesions in any of the groups (Table 2). No statistical differences ($P > 0.05$) in the number of acellular capillary and pericyte ghosts were found between any of the groups.

Magnetic Resonance Imaging
As shown in Figure 1, in the untreated 3-month diabetic and galactosemic groups the superior hemiretinal $\Delta$PO$_2$, but not that of the inferior hemiretina, was significantly ($P < 0.05$) subnormal. AMG treatment prevented the decrease of the superior hemiretinal $\Delta$PO$_2$ in diabetic but not in galactosemic rats. In addition, the superior hemiretinal $\Delta$PO$_2$ was also normal in the WAY-509–treated galactose-fed rats (Fig. 1). Furthermore, no significant differences ($P > 0.05$) in arterial blood gas, pH, and core temperatures during the carbogen challenge were found between any of the groups (Table 3).

DISCUSSION
This study demonstrates a novel oxygenation measurement that appears to provide an early marker of treatment efficacy in experimental diabetic retinopathy. The results of this work confirm our previous finding of an early subnormal retinal $\Delta$PO$_2$ in galactose-fed rats, extend this finding to diabetic rats, and suggest that this approach may be useful in the evaluation of treatment efficacy. We studied the diabetic and galactose-fed rat models because they produce similar retinal lesions after 15 months in 100% of the animals. This histopathology appears similar to that found in the early stages of human diabetic retinopathy. There are differences between these two models, however, that were useful for evaluating the extent to which the response of retinal $\Delta$PO$_2$ to therapy predicted the ability of therapy to inhibit retinal histopathology. The exact mechanism of this AMG-effect is not known, but because different groups have published similar results, the differential morphometric outcome to AMG in diabetic and galactosemic rats appears reproducible. AMG significantly inhibited development of the decrease in $\Delta$PO$_2$ in superior hemiretina in diabetics (where the drug also has been reported to prevent development of retinal histopathology), and did not correct the defect in $\Delta$PO$_2$ in galactosemic animals where the drug has been reported to have no effect on the development of retinal histopathology. As an additional test, we examined galactose-fed rats given the drug (WAY-509). We chose this drug because two separate laboratories have reported that WAY-509 prevents long-term retinal lesion development in galactosemic rats. The effect of WAY-509 treatment on retinal lesion development in diabetic rats has not been reported and so was
not studied here. Nonetheless, these results confirm and extend our previous findings in galactose-fed rats.\textsuperscript{6} The present results strongly support our hypothesis that a subnormal superior hemiretinal oxygenation response is an early functional marker of the risk of development of diabetic retinopathy and for assessing treatment efficacy.

Systemic differences (blood glucose, glycosylated hemoglobin) between diabetic and galactosemic groups do not seem able to explain responses of $\Delta$PO\textsubscript{2} to therapy. For example, the superior hemiretinal $\Delta$PO\textsubscript{2} in two groups reported not to show development of retinal lesions at more than 15 months (AMG-treated diabetic rats and WAY-509–treated galactosemic rats) were not subnormal even though these two groups had significantly different blood glucose and glycosylated hemoglobin levels ($P > 0.05$, 462 vs. 69 mg/dl and 11.2 vs. 4.9%, respectively). In addition, no differences in morphology or response to the carbogen challenge were found between any of the groups (Tables 2, 3). The blood glucose levels in all the galactosemic groups were significantly lower ($P < 0.05$) than those in the other groups in this study and in our previous work with galactose-fed rats.\textsuperscript{6} The reason for the lower blood glucose levels in this study is not known. The AMG-treated galactose-fed group had the lowest blood glucose level (49 mg/dl, not significantly different from untreated galactose blood glucose levels ($P > 0.05$)).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{\(\Delta\text{PO}_2\) (mean $\pm$ SEM) of superior (right) and inferior (left) hemiretina in control (C, $n = 8$), untreated diabetic (D, $n = 8$), AMG-treated diabetic (D+AMG, $n = 6$), untreated galactosemic (G, $n = 7$), AMG treated galactosemic (G+AMG, $n = 10$), or WAY-509–treated galactosemic (G+WAY, $n = 6$) rats. *$P < 0.05$, compared with control rats.}
\end{figure}
level, \( P > 0.05 \) and the lowest oxygenation response (Fig. 1). It is possible that lower blood glucose contributes to the development of subnormal \( \Delta \text{PO}_2 \). Nonetheless, for most of the groups studied in the present work, the fMRI data appear to reflect local changes in retinal physiology and not differences in systemic physiology.

Determining the effect of therapeutic intervention in experimental diabetic retinopathy requires an accurate and precise measurement. Previously, we have noted that the preretinal \( \Delta \text{PO}_2 \) pixels in any given animal can vary between 2 and more than 400 mm Hg. However, the 99% confidence levels of the mean and median for all groups is 15 to 20 mm Hg. In other words, 99% of the data are within 15 to 20 mm Hg of the mean or median. Only a few points are outside this confidence interval and so are unlikely to substantially alter the statistical comparisons. Indeed, as discussed in the introduction and elsewhere, the MRI measurement accurately reports the preretinal \( \Delta \text{PO}_2 \) relative to oxygen electrode measurements.

To address the issue of the precision (or reproducibility) of the MRI measurement, we compared the preretinal \( \Delta \text{PO}_2 \) from control and galactose-fed animals in the present work to those published more than 3 years earlier. Previously, we measured median preretinal \( \Delta \text{PO}_2 \) in the control and 3.5 month galactose-fed rats of 141 and 84 mm Hg, respectively. In the present study, we measured median preretinal \( \Delta \text{PO}_2 \) in the control and 3-month galactose-fed rats of 122 and 99 mm Hg. Neither control nor galactose levels are statistically different (\( P > 0.05 \)) between these two time points. Taken together, these considerations underscore the accuracy and reproducibility of the MRI method.

Previously, in the galactose-fed rat model, we measured a subnormal superior hemiretinal \( \Delta \text{PO}_2 \) before (at 3.5 months) and during (at 15–18 months) the appearance of retinal lesions. The results of the present study extend this initial observation to diabetic rats and underscore the superior hemiretina as a potentially important early site of retinal pathophysiology. This regional specificity may at first seem surprising, given the gross symmetry of the rat retinal circulation and expected uniform insult of systemic hyperglycemia in diabetes. It should be noted that an early subnormal superior hemiretinal \( \Delta \text{PO}_2 \) does not imply that the later forming retinal lesions will necessarily be distributed unequally across the retina. Pathobiologic mechanisms that may be involved in the regional oxygenation response within the retina are beyond the scope of this study, but may include regional differences in the density of ganglion cells and catecholamine-containing amacrine cells. In any event, the present MRI data underscore the possibility of regional biochemical and physiological changes early in diabetes, and these may be useful as predictors of later risk of development of diabetic retinopathy.

The exact biochemical changes that contribute to the development of subnormal retinal \( \Delta \text{PO}_2 \) are not known. Both AMG and WAY-509 treatments have relatively broad activity. For example, in experimental diabetes AMG has been considered an inhibitor of inducible nitric oxide synthase activity, PKC activation, oxidative stress, and advance glycation end product formation. WAY-509 is reported to be an aldose reductase inhibitor, an inhibitor of prostaglandin metabolism, an antioxidant, and an inhibitor of PKC activation. The lower glycohemoglobin levels in the WAY-509–treated galactosemic rats has been reported, which, if caused by lesser elevation of blood galactose, may contribute to the lower rate of development of retinopathy in those animals. Recently, we presented proof-of-concept data in normal subjects that human retinal \( \Delta \text{PO}_2 \) measurements are possible. We speculate that measuring the retinal \( \Delta \text{PO}_2 \) in patients with diabetes may be advantageous in the clinical management of patients that either do not respond to tight glycemic control or in whom good control is not achievable, as well as in the response of diabetic retinopathy to therapeutic interventions.

Acknowledgments

The authors thank Wei Zhang and Hongmei Luan for help in collecting some of the MRI data.

References


Table 3. Summary of Arterial Blood Parameters Measured during a 2-Minute Carbogen Challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>PaO\text{2} (mm Hg)</th>
<th>PaCO\text{2} (mm Hg)</th>
<th>pH</th>
<th>Core Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (( n = 8 ))</td>
<td>570 ± 15</td>
<td>52 ± 2</td>
<td>7.34 ± 0.02</td>
<td>37.0 ± 0.1</td>
</tr>
<tr>
<td>Diabetic (( n = 8 ))</td>
<td>529 ± 21</td>
<td>55 ± 2</td>
<td>7.31 ± 0.05</td>
<td>37.2 ± 0.2</td>
</tr>
<tr>
<td>Galactosemic (( n = 8 ))</td>
<td>560 ± 21</td>
<td>47 ± 0</td>
<td>7.35 ± 0.01</td>
<td>37.0 ± 0.1</td>
</tr>
<tr>
<td>AMG-treated diabetic (( n = 5 ))</td>
<td>522 ± 33</td>
<td>57 ± 2</td>
<td>7.34 ± 0.04</td>
<td>37.1 ± 0.1</td>
</tr>
<tr>
<td>AMG-treated galactosemic (( n = 10 ))</td>
<td>587 ± 9</td>
<td>54 ± 1</td>
<td>7.29 ± 0.01</td>
<td>37.1 ± 0.1</td>
</tr>
<tr>
<td>WAY-509–treated galactosemic (( n = 6 ))</td>
<td>517 ± 27</td>
<td>51 ± 2</td>
<td>7.29 ± 0.02</td>
<td>37.2 ± 0.2</td>
</tr>
</tbody>
</table>

Data are mean ± SEM.


