Diabetes Alters pH Control in Rat Retina

Andrey V. Dmitriev,[1] Desmond Henderson,[1] and Robert A. Linsenmeier[1–3]

[1]Biomedical Engineering Department, Northwestern University, Evanston, Illinois, United States
[2]Neurobiology Department, Northwestern University, Evanston, Illinois, United States
[3]Ophthalmology Department, Northwestern University, Chicago, Illinois, United States

Correspondence: Robert A. Linsenmeier, Biomedical Engineering Department, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208-3107, USA; r-linsenmeier@northwestern.edu.

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Purpose. The purpose of this study was to determine whether the ability of the rat retina to control its pH is affected by diabetes.

Methods. Double-barreled H^+-selective microelectrodes were used to measure extracellular $[H^+]$ in the dark-adapted retina of intact control and diabetic Long-Evans rats 1 to 6 months after intraperitoneal injection of vehicle or streptozotocin, respectively. Two manipulations—increasing of blood glucose and intravenous injection of the carbonic anhydrase blocker dorzolamide (DZM)—were used to examine their effects on retinal pH regulation.

Results. An increase of retinal acidity was correlated with the diabetes-related increase in blood glucose, but only between 1 and 3 months of diabetes, not earlier or later. Adding intravenous glucose had no noticeable effect on the retinal acidity of control animals. In contrast, similar injections of glucose in diabetic rats significantly increased the acidity of the retina. Again, the largest increase of retinal acidity due to artificially elevated blood glucose was observed at 1 to 3 months of diabetes. Suppression of carbonic anhydrase by DZM dramatically increased the retinal acidity in both control and diabetic retinas to a similar degree. However, in controls, the strongest effect of DZM was recorded within 10 minutes after the injection, but in diabetics, the effect tended to increase with time and after 2 hours could be two to three times larger than at the beginning.

Conclusions. During development of diabetes in rats, the control over retinal pH is partly compromised so that conditions that perturb retinal pH lead to larger and/or more sustained changes than in control animals.

Keywords: rat, retina, diabetes, pH, hydrogen ion, hyperglycemia, carbonic anhydrase

The vertebrate retina is well known for its high energy demand. It has high rates of both oxygen consumption[1] and lactic acid production,[2–4] indicating that both oxidative phosphorylation and glycolysis are employed in retinal energy metabolism. Between these two, glycolysis is a much less effective way to generate energy; 19 molecules of glucose are needed to produce the same amount of ATP that oxidative phosphorylation can yield from a single glucose molecule. Nevertheless, the seemingly irrational waste of glucose in glycolysis may not be a problem in diabetes when glucose is available in abundance. Yet, a problem can arise because glycolysis produces more lactate, so shifting toward glycolysis can potentially lead to acidosis.

We hypothesized that the retina of diabetics would be unusually acidic due to increased glycolytic metabolism. Acute hyperglycemia markedly acidifies the normal cat retina,[5] a change that was most pronounced in the inner retina. In a small number of diabetic cats that were available for study, acidosis was found in an animal with an early stage of background retinopathy.[6] Our recent work demonstrated that the retina of diabetic rats also was significantly more acidic than normal, but only temporarily between 1 and 3 months of diabetes.[7] The typical H^+-profiles recorded in control rats have the highest $[H^+]_o$ in the outer nuclear layer, where $[H^+]_o$ is typically 30 nM above arterial $[H^+]$, although in a few cases the retina was only 20 nM more acidic than blood, or as much as 50 nM more acidic. During the first month after initiation of diabetes, there were no noticeable changes in shape or amplitude of the H^+-profiles compared to age-matched controls. But later, the retinae of diabetics began to be much more acidic. In the most dramatic case, the maximum $[H^+]_o$ in the retina reached 200 nM (pH = 6.7), making it 160 nM more acidic than the blood. Additionally, H^+-profiles recorded in the same diabetic animal showed higher peak $[H^+]_o$ in some regions than in others, so the shape of the H^+-profiles in diabetics was not as uniform as in controls.[7] However, after about 3 months of diabetes, the acidity in the retina decreased, and the average retinal acidity recorded in 3- to 6-month diabetics was close to that in control animals. Diabetic rats, however, continued to demonstrate a much wider variation among their H^+-profiles than controls. At this time, some diabetic retinas still were more acidic than controls, but others were even less acidic.[7]

Increased H^+ in the retina during diabetes could imply that the retina is doing more glycolysis and producing more lactate and H^+, a Crabtree effect,[8] or it could imply that H^+ clearance mechanisms are damaged by diabetes, or both. From H^+ measurements reported to date, it is not clear which alternative is correct. It is also possible that while increased glycolysis produces more acid, this is not particularly detrimental to the retina. An increase in the amount of glucose converted to lactate may serve to limit the amount of glucose that finds its way into more damaging pathways, such as polyols or advanced glycation end products.

Manipulations superimposed on diabetes may be able to provide some information on mechanisms of diabetic pH changes in the retina. One manipulation was done here to investigate the possibility of increased H^+ production. If adding still more glucose acutely to a diabetic animal produces a further acidification, it would suggest that the diabetic retina has indeed adapted to be able to do more glycolysis and produce more acid. Such acute increases in glucose would not be expected to further damage clearance mechanisms. Another manipulation was done to attempt to interfere with one of the mechanisms important in clearance of H^+. The principal buffering system of the retina (bicarbonate/CO_2) was disturbed with dorzolamide (DZM), a carbonic anhydrase inhibitor. If clearance were already compromised by diabetes, this might be expected to have a different effect on diabetics than on control animals. The results of these manipulations will be reported and suggest that the main reason for increased acidity in diabetics was an increased utilization of glucose to produce more acid.

Methods

Animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Northwestern University's Institutional Animal Care and Use Committee. A total of 23 diabetic and 17 age-matched control adult male Long-Evans rats were used in this work. This is the same cohort of animals used by Dmitriev et al.,[7] but the present paper reports new data. Diabetes was induced with a single intraperitoneal (IP) injection of streptozotocin (STZ), and age-matched controls received an injection of 0.05 mol/L sodium citrate buffer only. Glucose measurements were initiated 2 days after the IP injection of STZ or vehicle. In diabetics, average blood glucose was 519 ± 74 mg/dL (mean ± SD) during weekly measurements prior to the H^+ recordings.

During experiments to measure intraretinal $[H^+]_o$, animals were initially anesthetized with 2.5% to 3% isoflurane/35% O_2 during preparation, and anesthesia was gradually switched to urethane (800 mg/kg IV loading followed by 75 mg-kg^{-1}-hr^{-1}) supplemented with 0.5% isoflurane. After completing surgical preparations, which took about 4 hours, the animal was paralyzed with pancuronium bromide and artificially ventilated. Recordings were then made over 5 to 8 hours, after which the animal was euthanized. Further details of the preparation have been given previously.[9] During the experiments, the blood glucose, typically measured hourly, was usually lower than in the tail blood samples of awake animals (in 22 out of 23 cases), and the average was 407 ± 80 mg/dL, which was still much higher than in controls (137 ± 41 mg/dL, $n = 17$).

Double-barreled H^+-selective microelectrodes were used to measure profiles of H^+ across the central retina of dark-adapted anesthetized rats. The microelectrodes were constructed using methods described previously for Ca^{2+}-sensitive microelectrodes.[10] The microelectrode was placed inside a metal needle inserted through the sclera and into the eye and advanced through the vitreous toward the retina as described earlier.[9] The intraretinal electroretinogram (ERG) was evaluated to determine the retinal depth of the electrode and the condition of the retina.

The original records obtained during withdrawal of H^+-selective microelectrodes were recalculated into H^+-profiles in the following three steps. First, for better comparison, the variable length of the profiles was normalized; the retinal borders with the choroid and vitreous were defined to be 100% and 0% retinal depth, respectively. The choroid/retinal boundary was considered to be the point at which $[H^+]$ began to increase. The vitreoretinal interface was identified as the location where the intraretinal ERG recorded by the microelectrode during withdrawal was the same as the vitreal ERG. Second, the voltage of the H^+-selective microelectrode (in millivolts) was recalculated into $[H^+]$ (in nanomoles/liter) based on the calibration of each electrode performed before the experiment. Absolute values of $[H^+]$ were obtained by assuming that the arterial $[H^+]$ measured just before or after each profile was the same as $[H^+]$ in the choroid. Third, data points were grouped in equal intervals, each of which was 5% of retinal depth. The values within each 5% were averaged and are presented here as the final H^+-profiles. The amplitude of a H^+-profile was determined by subtracting the value of $[H^+]$ in the choroid from the maximum $[H^+]$ in the retina. This amplitude, for one profile, is referred to as "local acidity." To obtain average retinal acidity, amplitudes of several H^+-profiles in an individual retina were averaged. Two other characteristics—average acidity of distal and proximal halves of the retina—were calculated from all profiles in a rat that were obtained under the same experimental condition by averaging all data points for the distal 50% of the retina and all data points for the proximal 50% of the retina, respectively, and subsequently subtracting the choroidal H^+ value. It should be emphasized that these are difference measurements in which the retinal pH is compared with pH of the blood, and accordingly, the term "acidity" indicates that the retina is more acidic than the blood by the given numbers of nanomoles/liter.

Two manipulations, intravenous infusions of 50% glucose to increase blood glucose or the carbonic anhydrase blocker DZM (15 mg/kg), were used to interfere with normal retinal pH regulation.

Results

The biphasic dynamics of the development of acidosis in diabetes (increasing after 1 month and then declining toward the normal level after 3 months) clearly contrasts with the dynamics of blood glucose elevation. After initiation of diabetes in rats, measurements of tail blood showed that blood glucose was about 500 mg/dL, starting with the first measurement 2 days after induction of diabetes, and it remained at this level in weekly tests. During the terminal experiments, glucose was somewhat lower, but diabetic animals were still very hyperglycemic. At 2 to 4 weeks of diabetes, blood glucose concentration was about 2.5 times higher than in controls during recording periods (diabetics: 362 ± 63 mg/dL; controls: 147 ± 14 mg/dL, here and below, mean ± SE), and the difference was larger at later times (446 ± 70 vs. 145 ± 44 mg/dL between 1 and 3 months and 393 ± 92 vs. 127 ± 49 mg/dL at more than 3 months; the exact distribution of times is shown in Fig. 3 of Dmitriev et al.[7]). But retinal acidosis was noticeable only between 1 and 3 months. During this interval, the average retinal acidity of diabetic rats was 50.6 ± 26.3 nM compared to 28.7 ± 10.4 nM for controls. This difference was highly statistically significant in spite of the large variability (for details see Dmitriev et al.[7]). Diabetics at earlier (less than 1 month) and later (more than 3 months) times during diabetes demonstrated on average the same retinal acidity as age-matched controls (28.8 ± 4.9 nM diabetic versus 32.9 ± 2.7 nM control, and 33.6 ± 20.4 nM diabetic versus 33.4 ± 7.1 nM control, respectively), but note the large variability in diabetic animals at later times.

To examine correlations between retinal acidity and blood glucose, the average retinal acidity (as defined in the methods) was plotted against the average blood glucose concentration in the same animal (Fig. 1). The data are grouped into three time

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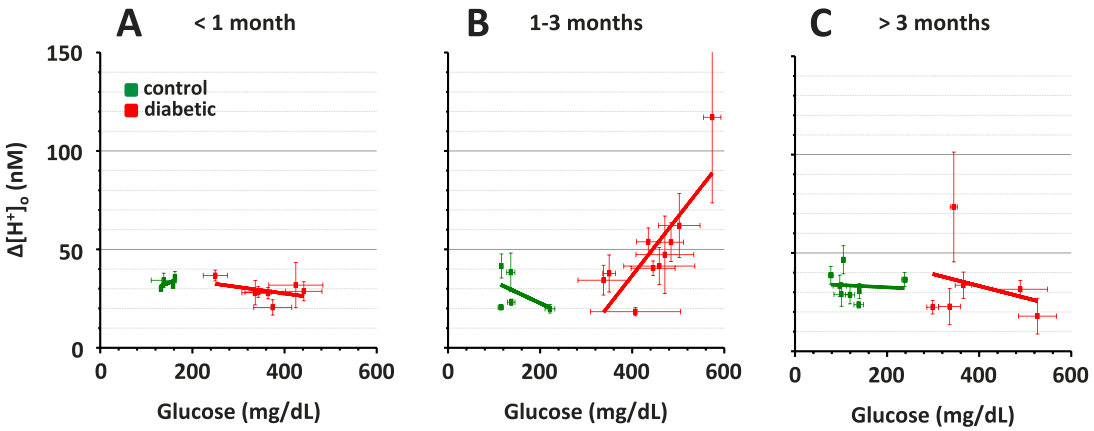

Figure 1. Retinal acidity plotted against blood glucose at three different periods of time after STZ or vehicle injections. Each point represents the mean amplitude of H^+-profiles and mean blood glucose from individual control (*green*) and diabetic (*red*) rats, with standard deviations for both. Three to 10 profiles were averaged for each point. Data are grouped for three periods of time after injection of vehicle or STZ (**A**) shorter than 1 month (four controls and seven diabetics); (**B**) 1 to 3 months (five controls and 10 diabetics); and (**C**) 3 to 6 months (eight controls and six diabetics). The lines are linear fits for corresponding data points. The ordinate is the same for all three parts (marked on the *left*). The slope of the regression is significantly different from zero only for diabetics between 1 and 3 months ($r^2 = 0.64$; $P = 0.0056$).

periods relative to injections of STZ or vehicle: less than 1 month (Fig. 1A), from 1 to 3 months (Fig. 1B), and longer than 3 months (Fig. 1C). Both average acidity and corresponding blood glucose at that time varied across animals, especially in diabetics, so the standard deviations for each parameter were marked. A strong and significant correlation between average retinal acidity and blood glucose was found in 1- to 3-month diabetics, with a positive slope of 0.30 nM of $[H^+]_o$ per 1 mM of glucose. However, no correlation was found for earlier (less than 1 month) or later diabetics (more than 3 months). There was also no correlation for earlier or later diabetics if the control and diabetic animals were included together in the regression. Interestingly, two older control rats demonstrated somewhat elevated blood glucose, but it was not accompanied by increased retinal acidity.

In a separate series of experiments, we evoked acute hyperglycemia by adding intravenous glucose. In control animals, we could increase blood glucose to as much as 600 mg/dL, but it had no noticeable effect on retinal acidity. A sample of this experiment is presented in Figure 2A. The three H^+-profiles recorded after artificial glucose elevation (light green lines) were barely distinguishable from the three H^+-profiles recorded before it (olive lines). In the case of diabetics, artificial elevation of blood glucose often (Fig. 2B), but not always (Fig. 2C), led to increasing retinal acidity.

The effect of artificial elevation of blood glucose on retinal acidity is quantitatively presented in Figure 3. In Figure 3A, local acidity (i.e., the amplitude of individual H^+-profiles) is plotted against blood glucose measured just before or after the corresponding H^+-profile. Specifically, in seven control rats, 21 H^+ profiles were recorded before artificial blood glucose elevation and 22 were recorded at least 90 minutes after glucose elevation; for diabetics, the respective numbers were 18 and 19 profiles in six rats. Diabetic animals that had moderately elevated glucose and mildly affected retinal pH before the glucose infusions were intentionally selected for these experiments to have room for both glucose and $[H^+]_o$ elevation. There was no correlation in control animals between local retinal acidity and glucose. In diabetic animals the correlation between blood glucose and the local retinal acidity was highly significant ($r^2 = 0.20$; $P = 0.0057$). The calculated slope of the H^+-glucose correlation was not large (0.08 nM of $[H^+]_o$ per 1 mM of glucose), but here the data from diabetics at various stages of the disease development (from 3 to 20 weeks) were combined. The largest correlation (0.24 nM of $[H^+]_o$ per 1 mM of glucose) was found in one approximately 3-month

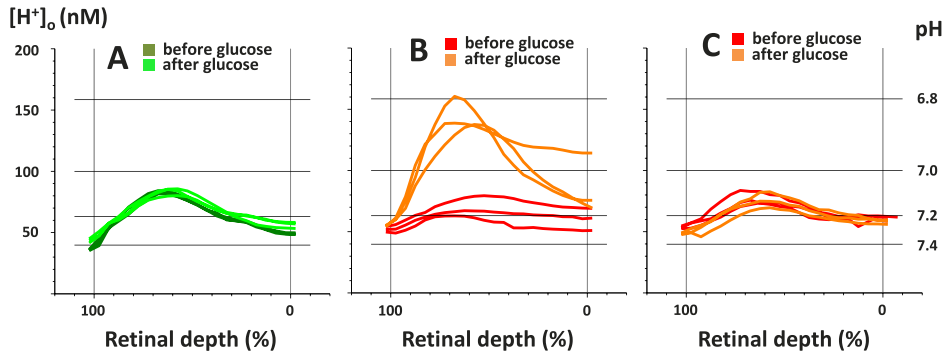

Figure 2. Samples of H^+-profiles obtained before and after artificial elevation of blood glucose in three individual rats. The panels are for a control rat 6 weeks after vehicle injection (**A**) and diabetics 14 weeks (**B**) and 20 weeks (**C**) after STZ injection. In panel **A**, three profiles were recorded before elevation of glucose (*olive lines*) and three were recorded at least 90 minutes after glucose elevation (*light green lines*). Panels **B** and **C** are similar, with *red* lines being profiles prior to glucose elevation and *orange lines* representing profiles after glucose elevation. The scales are the same in all three parts; $[H^+]_o$ concentration is marked in nanomoles/liter near the vertical axis in part **A** and the corresponding pH is marked on the *right* of the horizontal grid lines in part **C**.

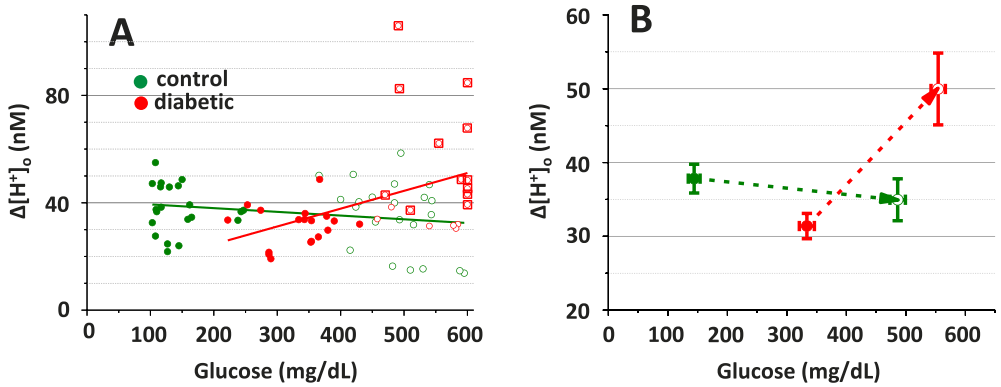

Figure 3. Effect of artificial elevation of blood glucose on retinal acidity in diabetic and control rats. (**A**) Each point represents the amplitude of H^+-profiles plotted against blood glucose at the time of the measurement; control rats (*green*) and diabetic rats (*red*). *Filled symbols* indicate before addition of glucose directly in the blood of the rat; *open symbols* indicate 90 or more minutes after the glucose addition. The lines (*green* for controls, *red* for diabetics) are linear fits. *Squares* around *open circles* mark data obtained from 7- to 14-week diabetics (others obtained at 3, 4, and 20 weeks). (**B**) The mean amplitude of H^+-profiles plotted against mean blood glucose with corresponding standard errors for control (*green*) and diabetic (*red*) rats before blood glucose elevation (*closed symbols*) and after it (*open symbols*). *Arrows* point to the changed mean amplitude of H^+-profiles and blood glucose after the manipulation.

diabetic rat. Two diabetic rats showed no positive correlation at all, and those were the animals at the earliest (2 weeks) and the latest (27 weeks) stages of diabetes tested. Viewed in terms of averages rather than regressions (Fig. 3B), adding intravenous glucose in control animals elevated plasma glucose from 144.2 ± 10.0 to 486.2 ± 13.0 mg/dL, but this had an insignificant effect (with a slightly negative trend) on the amplitude of the H^+-profiles (37.8 ± 1.9 and 35.0 ± 2.8 nM, respectively). In contrast, similar injections of glucose in diabetic rats significantly increased both blood glucose (from 333.6 ± 12.6 to 554.3 ± 12.1 mg/dL) and the amplitude of the H^+-profiles (from 31.4 ± 1.7 to 49.9 ± 4.9 nM; $P = 0.0012$). Besides this increase in retinal acidity in diabetics (but not in controls), artificial hyperglycemia evoked a slight (a few nanomoles/liter) acidification of the blood in both controls and diabetics, which was a bit larger in controls (controls: 7.40 ± 0.03 before and 7.31 ± 0.04 during hyperglycemia [nine rats]; diabetics: 7.37 ± 0.04 before and 7.32 ± 0.05 during hyperglycemia, [eight rats]).

The spatial heterogeneity of the H^+ distribution in diabetic retinas that was described earlier[7] was enhanced by artificial elevation of blood glucose. Figure 4A shows a 3D reconstruction of the H^+ distribution in a diabetic retina 14 weeks after STZ injection. The data for this 3D reconstruction were obtained when seven adjacent H^+-profiles were recorded in one plane with the electrode rotated horizontally after each profile by about 2° (eccentricity axis on Fig. 4) around the point where it penetrated the sclera. This rotation caused a lateral separation between H^+-profiles of 160 to 170 µm. The 3D reconstruction shows that the variability in H^+-profiles recorded in diabetics was not random but rather was spatially organized. In this example, the retinal acidity was greater at higher eccentricity (on the right). The average blood glucose level during these H^+ measurements was 335 mg/dL. When blood glucose was elevated to an average of 537 mg/dL by intravenous glucose, and the electrode was used to record a new set of profiles parallel to those in Figure 4A, the acidity dramatically increased in those regions of the retina where it already was high, but did not change much in the area where it was lower (Fig. 4B).

In order to further investigate disturbances of pH regulation, we tested the effects of the nonspecific carbonic anhydrase blocker DZM. Blocking of carbonic anhydrase disrupts the principal pH buffering system in the retina, the CO_2-bicarbonate buffer, which results in a significant increase of retinal $[H^+]_o$ concentration, as shown previously in other

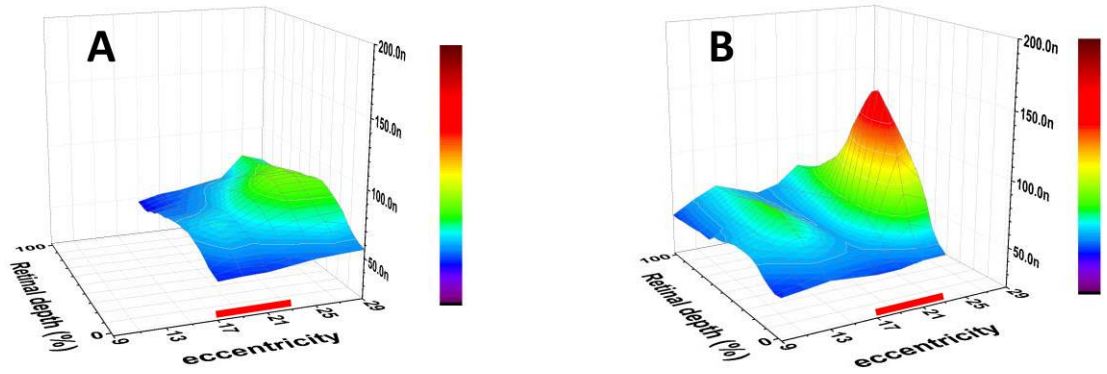

Figure 4. Effect of artificial blood glucose elevation on retinal acidity in diabetic retinas is strongly dependent on location. (**A**) Three-dimensional presentation of $[H^+]$ distribution in one diabetic retina (14 weeks) before the glucose injection (average glucose = 335 mg/dL). The X-Y plane shows position, and the Z direction is $[H^+]$. (**B**) $[H^+]$ distribution in the same retina after the glucose injection (average glucose level = 537 mg/dL). Profiles used to generate the smooth surface in (**A**) were recorded along a nasal-caudal line with the distance between profiles of about 170 µm (1° of eccentricity is equal to approximately 85 µm). The profiles used for part **B** were recorded along a line that was parallel to and about 170 µm away from the profiles in part **A**. The areas of spatial overlap in the lateral direction are marked by *red lines* on the axis.

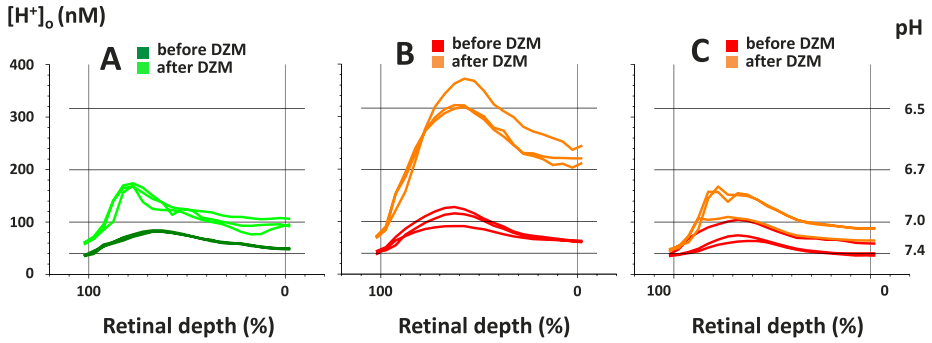

Figure 5. Samples of H^+-profiles obtained before and after pharmacologic suppression of carbonic anhydrase in three individual rats. The panels are for a control rat 6 weeks after vehicle injection (**A**) and diabetics 6 weeks (**B**) and 11 weeks (**C**) after STZ injection. Three individual profiles in each animal were recorded before application of carbonic anhydrase blocker DZM (*olive lines* for control and *red lines* for diabetics) and three profiles after DZM (*light green lines* for control and *orange lines* for diabetics). The scales are the same in all three parts; $[H^+]_o$ concentration is marked in nanomoles/liter near the vertical axis in panel **A**, and the corresponding pH is marked on the right of horizontal grid lines in panel **C**.

animals[11,12] (Wangsa-Wirawan N, et al. *IOVS* 2001;42:ARVO E-Abstract 5367). In rat retina, application of DZM also led to a large acidification. For example, the amplitude of H^+-profiles for the control animal presented in Figure 5A increased by about 90 nM, and the minimum pH dropped below 6.8. When tested on diabetics, the effects of an equivalent dose of DZM on retinal acidity were inconsistent; in some cases the effect was larger than in controls (Fig. 5B), but in other cases it was weaker (Fig. 5C). Figure 6 shows that, on average, suppression of carbonic anhydrase by DZM dramatically, but almost equally, increased the acidity in control retinas (from 36.5 ± 1.6 to 126.9 ± 9.4 nM, $n = 17$ and 13) and in diabetic retinas (from 44.3 ± 3.3 to 129.4 ± 13.3 nM, $n = 30$ and 24). As in other situations, diabetics demonstrated wider variability (Fig. 6A, 6C).

It is known that distal and proximal halves of the retina are different in the way they process glucose in energy metabolism; the distal retina relies considerably on glycolysis, but the proximal retina uses almost exclusively oxidative phosphorylation.[3,13] Accordingly, metabolism of glucose will result in accumulation of both CO_2 and lactic acid in distal retina, but mostly CO_2 in proximal retina. Due to this difference in metabolic waste, we investigated the effects of the carbonic anhydrase blocker separately for the distal and proximal halves of the retina. Thus, we compared average acidity in distal and proximal retina (which were averages of the first 10 and the second 10 points of the H^+-profiles, respectively; see Methods) before and after application of DZM for both control (Fig. 6B) and diabetic (Fig. 6C) rats. Data from each animal are presented as a pair of floating columns (left for distal retina and right for proximal retina). The bottom of the columns show the average acidity before application of DZM, and the top shows the average acidity after DZM. Here three to six H^+-profiles were averaged for each data point. For control rats, the results of carbonic anhydrase blocking by DZM were consistent and reproducible (Fig. 6B). The acidity increased more in distal retina than in proximal retina, both in absolute amount (increase of 42.4 to 54.6 nM in distal and 19.5 to 26.2 nM in proximal retina) and relative measures (increase of 245% to 336% of the pre-DZM value in the distal retina and 203% to 253% in the proximal retina). The larger effect on the distal retina was expected because it produces more lactic acid than proximal retina, and with reduced action of the CO_2-bicarbonate buffer system under carbonic anhydrase blocker, freshly produced H^+ cannot be easily converted into highly diffusible CO_2 for evacuation from the retina.

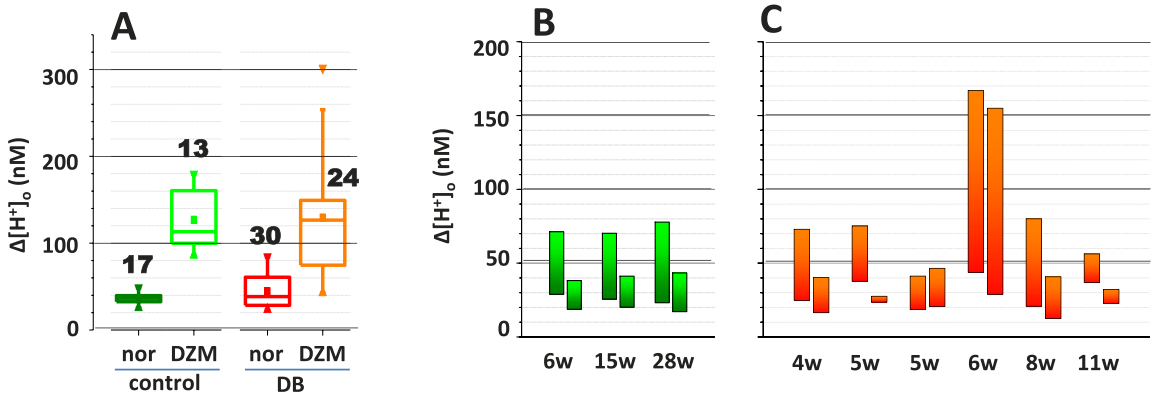

Figure 6. Effect of a carbonic anhydrase blocker on retinal acidity in diabetics and controls. (**A**) The descriptive statistics of retinal acidity for control (*olive* before DZM application; *light green* after DZM) and diabetic rats (*red* before DZM application; *orange* after DZM): ■, the mean of H^+-profile amplitudes; ▲, minimum; ▼, maximum; *boxes* cover from 25% to 75% of the values in each group; *solid lines* in *boxes* show the median. Numbers of profiles in each group are marked near corresponding boxes. (**B**) The floating columns represent the average acidity in the distal (*left* in each pair) and proximal (*right* in each pair) halves of the retina of control rats evoked by DZM application. The *bottom* of the floating columns is the average acidity before DZM application, and the *top* of the floating columns is the average acidity after DZM application. Three pairs of data corresponding to three individual rats; the time after injection of vehicle (in weeks) is marked under each group. (**C**) The same as part **B** for diabetics. Six pairs of data corresponding to six individual rats; the time after initiation of diabetes (in weeks) is marked under each group. Scale for the ordinate is the same as on part **B** (marked on the *left*).

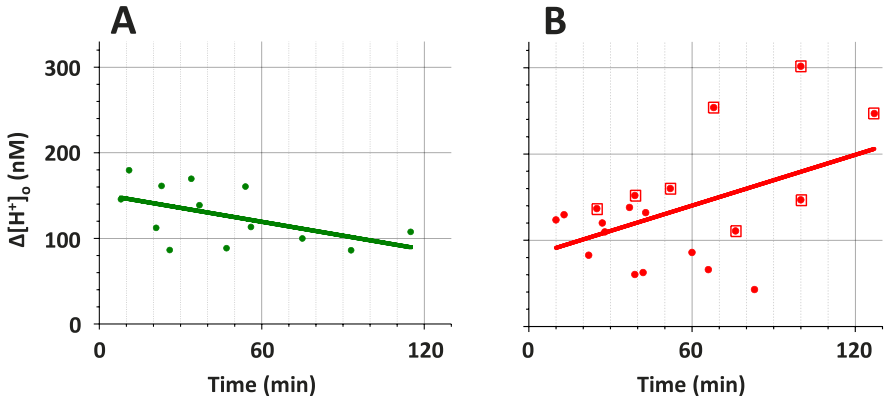

Figure 7. Development of the effect of DZM on retinal acidity in time. (**A**) Amplitudes of H^+-profiles in retinas of control rats after application of carbonic anhydrase blocker DZM plotted against time after DZM; three rats. The slope of the regression line was not significant ($P = 0.07$). (**B**) The same for diabetics; *squares* around *circles* mark data obtained from 6- to 8-week diabetics (others obtained on 4-, 5-, and 11-week animals); six rats. The slope of the regression line was significantly different from zero ($P = 0.041$).

In contrast, blocking of carbonic anhydrase in retinae of diabetic rats produced widely variable results (Fig. 6C). In distal retina, the DZM-induced increase of acidity varied from 19.3 to 123.3 nM, and the range was even larger in proximal retina (from 4.0 to 126.1 nM). Accordingly, relative changes ranged from only 117% of predrug amplitude to 538% (both for proximal retina).

While the average effects of DZM were similar in control and diabetic animals, one difference was clear. Changes in retinal acidity had opposite time courses after DZM application in control and diabetic retinas (Fig. 7). In controls, the strongest effect was recorded within 10 minutes after the injection, and then the effect slightly decreased (Fig. 7A). This partial recovery toward normal acidity was the main source of variability for controls. In diabetics, the effect of DZM tended to increase with time, and after 2 hours could be two to three times larger than at the beginning (Fig. 7B). Again, this increase in acidity with time did not happen in all diabetics, and the best representation of the trend was found in rats 6 and 8 weeks after STZ injection. This difference in time course of the acidosis was not due to a difference in the magnitude or time course of the systemic effect of DZM. DZM led to a decrease in blood pH of 0.13 pH units in controls (from 7.34 ± 0.05 to 7.21 ± 0.04) and 0.12 pH units in diabetics (from 7.34 ± 0.06 to 7.22 ± 0.06).

Discussion

The retina produces acid as an inevitable waste product of glucose utilization during energy metabolism. In diabetes, retinal $[H^+]_o$ increases, and we showed previously that this did not happen until after the first month of diabetes and was maintained to about 3 months.[7] We had not previously analyzed the dependence of this retinal acidosis on glucose level, but we showed here that in that same time frame, acidity was greater in diabetics when blood glucose was spontaneously higher during experiments (Fig. 1) and when glucose was intentionally elevated (Fig. 3). We could not measure retinal glucose, but there is evidence that it follows blood glucose,[14] so it is highly probable that these effects were due to alterations in retinal glucose. The variability of blood glucose in control animals was small and did not allow us to determine whether blood glucose also influenced retinal acidity in those animals, but with infusions of glucose, there was no sign of increased retinal acidity with increased glucose. Thus, there is some relatively slow adaptation that occurs in diabetics regarding retinal pH. This is reminiscent of an adaptation with a similar time course that is seen in the ERG a- and b-waves of Zucker Diabetic fatty (ZDF) type II rats, where a change in glucose from euglycemic to hyperglycemic affects the ERG in diabetics after about a month, but does not affect the ERG of congenic controls.[15] We do not wish to imply that pH changes are causal for the ERG changes, but only point out the slow adaptation of both to diabetes. (Our own ERG work shows a similar effect and will be reported separately in a larger study of the ERG b- and c-waves [Dmitriev and Linsenmeier, manuscript in preparation, 2019]).

The acidity of the diabetic retina could increase either because H^+ production increases or H^+ clearance decreases. Increased H^+ production could theoretically be from either oxidative or glycolytic metabolism. CO_2 production from oxidative metabolism could increase acidity as CO_2 is converted to H^+ and bicarbonate, but this is probably a very small contributor. Increased CO_2 production would be accompanied by increased photoreceptor O_2 consumption, which did not occur in diabetics.[16] In addition, CO_2 diffusion is about 20 times faster than O_2 diffusion, so most of the CO_2 probably leaves the retina by diffusion. And finally, the peak H^+ concentration, in diabetics as well as controls, is in the outer nuclear layer, where there are no mitochondria to produce CO_2. An increase in glycolytic H^+ production in response to high glucose, caused by a modification of the retina's glucose-utilizing machinery, seems more likely, and at least some relevant enzymes of glycolysis have been shown to change in diabetic rats. At 3 weeks, Salceda et al.[17] showed that retinal lactate dehydrogenase activity was about 80% higher in diabetic Long-Evans rats than in controls, and Ola et al.[18] found higher retinal activity of GAPDH, an important glycolytic enzyme, in diabetic Sprague-Dawley rats at 3 months compared to those at 3 weeks. Once the change to the new state occurs, the diabetic retina can increase $[H^+]_o$ further when challenged with larger concentrations of glucose. A decrease in H^+ clearance as the cause of greater $[H^+]_o$ cannot be completely ruled out but seems less likely. We did not detect mRNA changes in the principal enzymes and transporters in the retina that handle H^+ at either 1 or 6 months of diabetes, compared to age-matched controls, although we did find changes in these genes in severe metabolic acidosis.[19]

An increased capacity of the retina for glycolysis/lactate production, or a shift from oxidative metabolism to glycolysis, in the presence of high glucose could be called a Crabtree

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effect, which was first demonstrated in yeast. We argue that such a change may occur in diabetics and may explain the increase in $[H^+]_o$ that we found here. However, the previous literature is complex because methods and rat strains have differed, so there is uncertainty about whether an increase in glycolysis in the retina occurs with acute or chronic hyperglycemia[17,20,21] or not.[5,18,22,23] Some in vitro experiments where increased glucose led to greater lactate production[20] have been challenged on the grounds that the initial level of glucose may not have been sufficient for normal metabolism,[22] even though bath levels of glucose were similar to those normally found in the blood. The diffusion limitation caused by unstirred layers in in vitro preparations frequently causes substrate concentrations at the tissue to be lower than in the bath, so the "control" condition may have been partial starvation and the "high" glucose condition was not really high. Other work, in which concentrations of lactate rather than rates of lactate production were measured, has been criticized because the isolation of the retina may have been slow enough that hypoxia, rather than a Crabtree effect, caused the increased glycolysis. Ola et al.,[18] who found no dependence of lactate production or retinal lactate concentration on glucose in control or diabetic Sprague-Dawley rats, argued that hypoxia may have distorted the data of Heath et al.[21] and was responsible for the higher retinal lactate-to-pyruvate concentration ratio (L/P) that Heath et al.[21] found in normal animals (21 ± 2.9 in Heath et al.[21] and 17.1 ± 0.9 in Ola et al.[18]). But if the control and diabetic rats were treated similarly during experiments, then some component of any increase in the L/P ratio could have been due to hypoxia, but a difference between control and diabetic groups would have been due to diabetes. This difference between groups was substantial. Heath et al.[21] found that after 3 weeks on a starch diet, diabetic Wistar rats had 40% higher retinal lactate and 52% higher retinal L/P than control rats. The results of Salceda et al.[17] in diabetic Long-Evans rats were similar at a similar time point.[17] A further complication is that lactate concentrations and L/P ratios do not necessarily reflect metabolic rates, and it is possible that higher L/P in diabetics resulted from a reduced ability of the retina to clear lactate. Significantly, plasma lactate increased by 100% in the diabetics in the study by Heath et al.,[21] so the energetics of moving lactate from the retina to the blood would have been less favorable and lactate transport could have been reduced. We did not measure blood lactate; however, the blood acidified equally during hyperglycemia in our control and diabetic rats, so we do not believe that blood lactate in our diabetic animals could have been much higher than in controls. We conclude that there is evidence for a Crabtree effect that takes time to develop, but that it may be small and therefore difficult to measure directly, and it may vary among rat strains. With pH recordings, we can see that most of the increased $[H^+]_o$ comes from the outer retina, whereas all the other studies referred to could not differentiate inner and outer retina. All these things may contribute to the divergence in results.

After 3 to 4 months of diabetes, $[H^+]_o$ does not depend strongly on blood glucose, and average acidity in the retina is not different from that in controls, although the larger variability at this time indicated that some animals were still producing more H^+. The change at these later times possibly occurs partly because unknown compensatory mechanisms become active to stabilize $[H^+]_o$ and partly because there is progressive damage to retinal cells, particularly photoreceptors,[24] which decreases glycolytic capacity. Ola et al.[18] showed that at 3 months, lactate production was lower in diabetics than it was at 3 weeks, and both Ola et al.[18] and Salceda et al.[17] showed that the L/P ratio was lower at this time. Ola et al.[18] also found that polyol production increased with diabetic duration, so some of the glucose was taking a new metabolic pathway.

The tendency is to assume that more acidity is bad and is another sign of diabetic damage. It is also possible that increased glucose utilization, which happens to be associated with acidosis, is not very damaging, at least up to some level of acidity. It may instead primarily reflect a useful adaptation to hyperglycemia and limit the amount of glucose that takes more deleterious pathways.

DZM presumably blocked both the intracellular CA isoform (CA II)[25–27] and the extracellular isoform (CA-XIV),[28] which are the only two CA isoforms that have been demonstrated in the retina. It was already known that blocking carbonic anhydrase with intravenous blockers would acidify the retina (Wangsa-Wirawan N, et al. *IOVS* 2001;42:ARVO E-Abstract S367)[11] but we were interested in knowing here whether there would be a differential effect in diabetics and controls. If the activity of CA in diabetics was greater than in controls, then blocking it should have led to a larger increase in $[H^+]_o$ in diabetics. On the other hand, if CA were initially less active in diabetics, then blocking it might have had a smaller effect in diabetics. The results are complicated by the large variability that we are now accustomed to seeing in diabetics, but on average the effect of DZM was similar in both diabetics and controls, suggesting that no important changes in CA activity accompanied diabetes. We can offer two tentative possibilities for the puzzling result that the effect of CA blockade grew with time in diabetics and decreased with time in controls. First, CA blockade may have been more damaging in normal retina, preventing the generation of $[H^+]_o$, whereas the diabetic retina was more accustomed to high $[H^+]_o$, so CA blockade was less damaging to H^+ production, allowing H^+ to accumulate. Second, it is possible that the normal retina has a more effective blood-retinal barrier (BRB) and was able to limit DZM entry, whereas a damaged BRB in diabetics gradually allowed the concentration of blocker within the retina to increase, so it became more effective in blocking buffering with time.

In summary, we found that not only is retinal $[H^+]_o$ higher than in control animals, but it is also less stable when blood glucose fluctuates, as it normally would in diabetics. It is also more variable in response to both glucose and CA manipulations.

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