

Reexamining the Hyperglycemic Pseudohypoxia Hypothesis of Diabetic Oculopathy

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PURPOSE. To test the hypothesis that diabetes alters retinal NAD⁺-to-NADH ratios early in the course of the disease (e.g., the hyperglycemic pseudohypoxia hypothesis).

METHODS. In freshly excised age-matched control and diabetic rat retinas, measurements were made of the NAD⁺ and NADH content as well as a surrogate marker of NAD⁺-to-NADH ratios obtained from lactate and pyruvate levels. In addition, the effect of various hyperglycemic levels was assessed from measurements of retinal lactate and pyruvate concentrations and the rate of lactic acid production in vitro (isolated rat retinas, monolayer cultures of human retinal pigment epithelial cells, and rabbit lens epithelial cells).

RESULTS. No significant differences ($P > 0.05$) were found between control and diabetic tissues in their amount of total NAD⁺ and NADH/retina, and the ratio of NAD⁺ to NADH, or in their content of lactate, pyruvate, and adenosine triphosphate (ATP) or in the ratio of lactate to pyruvate. The content of lactate and pyruvate in retinas incubated for 2 hours in media containing 10 or 30 mM glucose was the same as found in fresh tissues, but the levels of these metabolites in retinas incubated in media containing 5 mM glucose declined in comparison to the fresh values. There were no significant differences in lactate content in cultured retinal and lens cells that were exposed to 5 or 30 mM glucose-containing media.

DISCUSSION. The present results do not support the hyperglycemic pseudohypoxia hypothesis of diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2006;47:2726–2731) DOI:10.1167/iov.06-0076

The idea that diabetic complications are linked to an increase in the activity of sorbitol dehydrogenase resulting in an increase in the intracellular concentration of NADH and an increase in the redox ratio of NADH-to-NAD⁺ was first proposed in 1993 by Williamson et al.¹ Since that first report,

several additional papers from their laboratory appear to support this redox hypothesis of diabetes, which the authors have termed “hyperglycemic pseudohypoxia.”^{2,3} Williamson et al. have long argued that the ratio of NADH to NAD⁺ in the cytoplasm of cells and tissues can be assessed on the basis of the near equilibrium between the concentration ratios of NADH/NAD⁺ and lactate/pyruvate,^{1–3} the latter measurements thus serving as surrogate measurements of the redox status in cells. However, data from other laboratories do not support the basic tenets of hyperglycemic pseudohypoxia.^{4–9} Nonetheless, Williamson et al.² have argued against these reports (see the lengthy online appendix by Nyengaard et al. published in *Diabetes* in 2004³; <http://diabetes.diabetesjournals.org>). In the specific case of work published by our laboratory,⁸ Ido and Williamson,¹⁰ Williamson and Ido,¹¹ and Nyengaard et al.³ argued that our experiments did not provide an independent test of the hyperglycemic pseudohypoxia hypothesis, because we did not measure the content of pyruvate in retinas incubated in media containing euglycemic and hyperglycemic concentrations of glucose. We had not measured pyruvate in that earlier study⁸ because we thought that measurements of lactate alone provided us with sufficient information to question the redox hypothesis proposed by Williamson et al. Recently, the role of the sorbitol pathway in diabetic complications^{12–14} has been pushed front and center in the medical literature—in part, as a result of a commentary that appeared in the “Medical News & Perspectives” section of the *Journal of the American Medical Association*.¹⁵ This article included a statement that “the new work bolstering the sorbitol pathway’s role [i.e., hyperglycemic pseudohypoxia] in diabetic complications is biochemically sound but leaves some unanswered questions.” For this reason, we believed it important to follow the suggestion of Williamson et al.^{3,10,11} and undertake additional, new experiments testing the soundness of the hypothesis of hyperglycemic pseudohypoxia by including measurements of both lactate and pyruvate in fresh retinas obtained from age-matched normal and experimental diabetic rats, to provide a more biologically relevant comparison for evaluating the hypothesis. To test the hypothesis further, we made measurements of these metabolites in retinas incubated in media containing 5, 10, or 30 mM glucose in the presence and absence of functioning mitochondria. The present results, when combined with our previous efforts,⁸ provide the strongest evidence to date against the hyperglycemic pseudohypoxia hypothesis.

MATERIALS AND METHODS

All rats used in these experiments were housed in rooms provided with dim cyclic white light and were cared for in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Control and Diabetic Rats

Separate groups of control (C) Sprague-Dawley rats, and 6-month-old diabetic (D) Sprague-Dawley rats were studied. Diabetes was induced

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in 200-g Sprague-Dawley rats after an overnight fast with an intraperitoneal injection of streptozotocin (55 mg/kg) within 5 minutes of its preparation in 0.01 M citrate buffer (pH 4.5). Diabetes was verified 3 days later by the presence of plasma hyperglycemia (≥ 400 mg/dL) and elevated urine volume (>60 mL/d) in nonfasted rats. Subtherapeutic levels of insulin (0–2 U of neutral protamine Hagedorn [NPH] insulin administered subcutaneously daily) were administered to maintain a level of hyperglycemia of 400 to 550 mg/dL. Only rats with this history of hyperglycemia were studied. After induction of diabetes, all animals had free access to food and water and were maintained in a 14-hour light–10-hour dark cycle. Glycated hemoglobin (GHb) levels were determined by affinity chromatography, as previously described.¹⁶ The 6-month control level of glycated hemoglobin was $4.1\% \pm 0.6\%$ ($n = 10$), and the 6-month diabetic level was $9.2\% \pm 1.8\%$ ($n = 9$).

Tissue and Cell Cultures

After the rats were killed by CO₂ inhalation or decapitation (without CO₂), each retina was isolated rapidly (within 5 seconds) from other ocular tissues by methods that have been described in detail earlier.^{17,18} Retinas were placed in a Petri dish containing ice-cold control incubation medium, and adherent vitreous humor was carefully removed. The composition of the control medium was as follows (mM): 130 NaCl, 5 KCl, 25 NaHCO₃, 0.5 MgSO₄, 5 D-glucose, and 2 CaCl₂. The pH was 7.4, and the osmolarity was approximately 310 mOsm. All substitutions were made iso-osmotically. The O₂ tension in the medium was 95%, and the CO₂ tension was 5%. Retinas were incubated in 10 mL of media in 25 mL Erlenmeyer flasks, either individually (one retina per 10 mL) or in groups of up to four retinas per flask, containing 5, 10, or 30 mM D-glucose. Incubations lasted for 2 hours at 37°C. For the anaerobic condition, media were gassed with a mixture containing 95% N₂ and 5% CO₂ before the retinas were added. Continuous gassing of the air space in the flask above the solution also continued throughout the period of incubation, thus ensuring a high partial pressure of oxygen or nitrogen.

Human retinal pigment epithelial (hRPE) cells were obtained from eyes obtained postmortem from the Michigan Eye Bank. The hRPE cell cultures were produced as described earlier.¹⁹ Cells were grown in Dulbecco's modified Eagle's Medium (DMEM cat. no. 31600; Invitrogen-Gibco, Grand Island, NY) supplemented with 15% fetal bovine serum, amphotericin B (250 μ g/mL), 250 μ g/mL sodium desoxycholate as a solubilizer (Invitrogen-Gibco), and 0.5% gentamicin.

Rabbit lens epithelial cells (LECs)²⁰ were grown in minimal essential Eagle's medium (M0643; Sigma-Aldrich, St. Louis, MO), supplemented with 8% rabbit serum and 0.5% gentamicin. Cell cultures were grown to confluence in 150-mm dishes in a humidified atmosphere of air plus 5% CO₂ at 37°C. Media were changed every 3 days. At confluence, cells (both hRPE and LECs) were split into 60-mm dishes. After 24 hours, confluent cell cultures were washed three times in serum-free minimum essential medium (MEM). Cell cultures were then incubated for 2 hours at 37°C in 4 mL of serum-free medium, containing 5 or 30 mM D-glucose. For the anaerobic condition, 0.01 mM antimycin A was added to the incubation media to block mitochondrial activity.²¹

Biochemical Measurements

For extraction of NAD⁺, one rat retina was homogenized in 0.25 mL of 0.23 M KH₂PO₄ on ice.²² The homogenate was exposed for 1 minute in a boiling-water bath, chilled, neutralized with an equal volume of 0.2 M KOH and centrifuged at 20,000g. The supernatant was diluted 1:4 with distilled water and 0.1 mL of the diluted sample was assayed. For extraction of NADH, one rat retina was homogenized in 0.25 mL of 0.2 M KOH.²² After boiling for 1 minute and cooling, the homogenate was neutralized with 0.25 mL of 0.2 M K₂PO₄ and then centrifuged at 20,000g. The supernatant was diluted 1:2 with 0.01 M NaPO₄ (pH 7.4). Assay for NADH was performed with 0.1 mL of diluted sample. For measurement of the content of the pyridine nucleotides in the sam-

ples, the reaction mixture consisted of thiazolyl blue (MTT), bicine and phanazine ethosulfate, ethanol, and alcohol dehydrogenase. The reaction sequence includes the reduction of MTT to the corresponding formazan which absorbs maximally at 570 nm. Oxidized coenzyme is cycled back to the reduced form by the dehydrogenase and its substrate. The rate of increase in absorbance is proportional to the concentration of the coenzyme in the assay mixture. The assay was performed in a 1-mL cuvette at room temperature, using a recording spectrophotometer (Gilford model 250; Nova Biotech, El Cajon, CA). Each response was recorded as the increase in absorbance at 570 nm per 3 minutes over the blank, and rates were compared to standards for each pyridine nucleotide. For the blank, a neutralized solution of KOH-KH₂PO₄ was used in place of the cell extract.

The content of lactic acid was measured in the media at timed intervals during the incubations (to obtain an estimate of the rate of glycolysis) and in cell extracts of retinas and cultured cells. Aliquots (typically, 0.05–0.2 mL) of the incubation media were withdrawn at 1 and 2 hours during the experiments on tissues and cells. Lactate in the media was measured with a lactic acid dehydrogenase-based commercial kit (826-UV; Sigma-Aldrich) that couples lactate to the reduction of nicotinamide-adenine dinucleotide (NAD), as monitored on a spectrophotometer at 340 nm. For measurement of the content of lactate in a single isolated rat retina, a retina was removed from the incubation flask, rinsed in ice-cold saline and homogenized in 0.25 mL of 10% perchloric acid (PCA). The homogenate was centrifuged in the cold for 10 minutes at 20,000g. The PCA supernatant (0.2 mL) was diluted 1:1 with 2 M KHCO₃ and the neutralized suspension was centrifuged for 5 minutes in the cold at 20,000g to obtain a clear supernatant. The content of retinal lactate was measured using a 0.025-mL aliquot of the neutralized supernatant in a spectrofluorophotometer (RF-5000; Shimadzu, Columbia, MD) with $e_x = 340$ nm; $e_m = 460$ nm. The content of lactate in cultured hRPE and LECs was also measured on neutralized PCA extracts, as described earlier. In the case of the cells, three confluent dishes were collected together in 0.5 mL of 10% PCA, and 0.4 mL of the neutralized PCA/KHCO₃ supernatants was used in the assays. Measurements of the content of pyruvate in isolated rat retinas were performed with the spectrofluorophotometer. Because of the low content of pyruvate in a rat retina, it was necessary to homogenize four retinas together in 0.25 mL of 10% PCA. After centrifugation, the PCA supernatant (0.2 mL) was diluted 1:1 with 2 M KHCO₃, and the neutralized suspension was centrifuged for 5 minutes in the cold at 20,000g to obtain a clear supernatant. The content of retinal pyruvate was measured using a 0.25-mL aliquot. These aliquots of pooled retinas were also used for measuring lactate content, but only a 0.01-mL sample was used. In this way, data for lactate and pyruvate could be obtained from the same pooled retinas. Despite our best efforts, measurements of the content of pyruvate in pooled dishes of confluent hRPE and LECs remained below the detection limits of the fluorometer. The standard curves revealed that 1 nmol of pyruvate or lactate was the lowest amount that could be reliably detected in an individual assay (data not shown). For determination of both lactate and pyruvate, samples were incubated in the appropriate enzyme mixture containing cofactors (NAD⁺ or NADH) for 5 minutes at room temperature at which time the reactions had reached completion. Each sample was read against a lactate or pyruvate standard curve (which was run at the start of every experiment) and total nanomoles of lactate or pyruvate per retina or per 10⁶ cells were calculated. Results are expressed as nanomoles per retina or nanomoles per 10⁶ cells. To convert data on a per retina basis to a per-milligram-protein basis, we considered that averaged total homogenate protein per one rat retina was 1.1 mg and averaged cytosolic protein (equivalent to the 20,000g supernatant obtained after homogenization in phosphate buffer) was 0.33 mg. The biochemical data in the tables are presented as the mean \pm SD. Each mean value represents the number of independent measurements on single or pooled retinas. Data were analyzed by Student's paired *t*-test, and $P \leq 0.05$ was considered to be statistically significant.

Adenosine triphosphate (ATP) content was measured in freshly excised retinas after homogenization of a single retina (or pooled retinas) in 0.25 mL 5% perchloric acid and centrifugation at 20,000g for 10 minutes.⁸ ATP was measured on diluted cell extracts using a luminometer (Turner Systems, Sunnyvale, CA). Sample values were compared against a standard curve for each experiment.

The activity of aldose reductase (AR) was determined in 20,000g supernatants of fresh rat retinas or cultured hRPE and LECs by observing the change in absorbance at 340 nm of NADPH at room temperature, as described previously.⁸ Substrates tested included 1 mM D,L-glyceraldehyde and 30 mM glucose. Our previous study⁸ failed to detect activity of aldose reductase when a single rat retina was homogenized in 0.5 mL of 0.1 M PO₄ buffer (pH 6.3) and 0.2 to 0.4 mL samples were tested. In the present study, two rat retinas were homogenized in 0.5 mL of phosphate buffer, and 0.4 mL of the supernatant was tested. In this way, we effectively used the equivalent of 1.6 rat retinas per assay. For measurements of aldose reductase activity in cultured cells, three dishes of confluent cells were scraped and collected into a total volume of 0.5 mL of 0.1 M PO₄. The cells were centrifuged at 20,000g for 15 minutes, and 0.4-mL samples of the supernatants were assayed for activity. Activity of lactic acid dehydrogenase (LDH) was measured in cell extracts of rat retinas and cultured cells, as previously described.¹⁹ Typically, only 5 or 10 μ L of the supernatants was needed for measurement of LDH activity.

RESULTS

Table 1 provides a summary of the data on the levels of lactate, pyruvate, and ATP content and on the lactate-pyruvate ratio in freshly excised retinas obtained from control and diabetic rats. No significant differences ($P > 0.05$) were found between control and diabetic tissues in their respective content of lactate, pyruvate, and ATP or in the ratio of lactate to pyruvate. Table 2 shows that there were no significant differences ($P > 0.05$) between the total amounts of NAD⁺ and NADH/retina, and in the ratio of NAD⁺ to NADH in control retinas and in retinas from diabetic rats.

Table 3 shows data for the aerobic content of lactate and pyruvate in freshly excised and incubated rat retinas. The averaged lactate content in a freshly excised retina obtained from rats that had been euthanized with CO₂ was 75 ± 11 nmol/retina ($n = 9$) and pyruvate content was 1.9 ± 1.1 nmol/retina ($n = 9$). The average level of freshly excised lactate content in a retina obtained from a separate group in which the rats had been decapitated without any anesthesia

TABLE 1. Lactate, Pyruvate, Lactate/Pyruvate (L/P) Ratios, and ATP Content in Fresh Retinas Obtained from Control and Diabetic Rats

| Condition | Lactate | Pyruvate | L/P | ATP |
|------------------|------------------|-------------------|-----|-------------------|
| Control retinas | 68 ± 17 (16) | 1.3 ± 0.6 (6) | 52 | 13.7 ± 3 (12) |
| Diabetic retinas | 77 ± 8 (8) | 1.2 ± 0.3 (5) | 64 | 15.1 ± 2 (7) |

Data are the mean \pm SD. Retinal levels of lactate, pyruvate, and ATP are in nmol/retina. Number of independent measurements is in parentheses. Duration of diabetes was 6 months. Lactate and ATP data were obtained in two ways: from single retinas and from four pooled retinas. Measurements of pyruvate were made using four pooled retinas. Because there were no statistically significant differences in the lactate and ATP content, respectively, between the two procedures, the data for these metabolites from single retinas and from pooled retinas were combined. Thus, the number of pyruvate measurements in control ($n = 6$) and diabetic ($n = 5$) retinas are a subset of the larger number of measurements of lactate and ATP content (e.g., $n = 16$ for lactate content in control retinas reflects that 10 measurements were performed using single retinas and six measurements came from the pooled retinas that were also used for measurements of pyruvate).

TABLE 2. Concentrations of Pyridine Nucleotides in Fresh Retinas Obtained from Control and Diabetic Rats

| Condition | NAD ⁺ | NADH | NAD ⁺ /NADH |
|------------------|-------------------|-------------------|------------------------|
| Control retinas | 3.0 ± 0.6 (7) | 0.5 ± 0.1 (7) | 6 ± 1 (7) |
| Diabetic retinas | 2.8 ± 0.8 (3) | 0.4 ± 0.1 (3) | 7 ± 2 (3) |

Data are the mean \pm SD. Retinal levels of NAD⁺ and NADH are in nmol/retina. Number of independent measurements on individual retinas is in parentheses. Different retinas were used to measure the oxidized and reduced pyridine nucleotides. Duration of diabetes was 6 months.

was 79 ± 14 nmol/retina ($n = 12$, data not shown). No statistically significant difference ($P > 0.05$) was evident in the fresh content of lactate between CO₂-euthanized and decapitated groups of animals. Because of this similarity, Tables contain data for the content of lactate and pyruvate in incubated retinas after CO₂ narcosis. Retinas incubated aerobically for 2 hours with 5 mM glucose contained 34 nmol lactate/retina, or 55% less than the amount found in a freshly excised tissue. Pyruvate content in retinas incubated in media containing 5 mM glucose was also less than that found in fresh tissues, and this change (e.g., 1.2 vs. 1.9 nmol/retina was statistically significant; $P \leq 0.05$). In contrast, when retinas were incubated for 2 hours in media containing either 10 or 30 mM glucose, the lactate and pyruvate were maintained at levels similar to their respective content in fresh tissues (e.g., 81 and 86 nmol lactate/retina and 1.5 and 1.8 nmol pyruvate/retina; $P > 0.05$).

Table 3 also shows that the rate of aerobic lactate production (glycolysis) was 1.3μ mol/h per retina in retinas incubated in media containing 5 mM glucose. With 10 mM glucose, the rate of aerobic glycolysis increased ($P < 0.05$) to 1.8μ mol/h per retina. This rate appeared to be maximal, since it did not change ($P > 0.05$) when retinas were incubated in media containing 30 mM glucose. In an additional series of experiments, the content of lactate was measured after 2-hour incubations of isolated retinas obtained from rats killed by decapitation. The results showed that, as in the case of the CO₂-euthanized rats, incubation of retinas in oxygenated media containing 5 mM glucose led to a decline ($P < 0.05$) in the content of lactate, whereas 30 mM glucose maintained ($P > 0.05$) the level of lactate at a value similar to that found in freshly excised retinas ($n = 4$ for each glucose concentration, data not shown).

Table 4 provides information about the content of lactate and pyruvate in rat retinas incubated for 2 hours under anaerobic conditions (95% N₂ and 5% CO₂). As expected, anoxia led to increases in lactate content in retinas incubated in 5, 10, and 30 mM glucose in comparison to their respective aerobic values (compare data in Table 4, left column with data in Table 3, left column). Anaerobic lactate content was similar ($P > 0.05$) in retinas incubated with either 10 or 30 mM glucose (i.e., 138 vs. 129 nmol/retina). The anaerobic levels of pyruvate were not significantly different ($P > 0.05$) from the aerobic values for the three concentrations of glucose. Anaerobic lactate production was higher ($P < 0.05$) than aerobic lactate production for each glucose concentration tested. Relative to the rate of anaerobic lactate production in retinas incubated in media containing 5 mM glucose, increasing the concentration of glucose to 10 and 30 mM led to increases ($P < 0.05$) in lactate production of 30% to 33%, with maximum lactate production attained with 10 mM glucose.

To probe further the linkages between hyperglycemia and the redox status in ocular cells, we next examined the effects of varying the glucose concentration on the lactate content of

TABLE 3. Retinal Lactate, Pyruvate, L/P Ratios, and Lactate Production after Incubation in 5, 10, or 30 mM Glucose for 2 Hours with 95% O₂ and 5% CO₂

| Condition | Lactate Content | Pyruvate Content | Lactate/Pyruvate Ratio | Lactate Production |
|-------------------------------|-----------------|------------------|------------------------|--------------------|
| Fresh tissue (<i>n</i> = 9) | 75 ± 12 | 1.9 ± 1.1 | 39 | |
| 5 mM glucose (<i>n</i> = 8) | 34 ± 7 | 1.2 ± 1.2 | 28 | 1.3 ± 0.2 |
| 10 mM glucose (<i>n</i> = 9) | 81 ± 13 | 1.5 ± 0.5 | 54 | 1.8 ± 0.1 |
| 30 mM glucose (<i>n</i> = 6) | 86 ± 4 | 1.8 ± 1.1 | 48 | 1.7 ± 0.2 |

Data are the mean ± SD. Retinal levels of lactate and pyruvate are in nmol/retina. Data for lactate and pyruvate were obtained using the same four pooled rat retinas per measurement. For example, an *n* = 8 means that a total of 32 retinas were used (four retinas/measurement × eight independent incubations). Lactate production is in μmol/hr/retina. Number of independent experiments is in parentheses.

two types of cultured ocular cells that possess AR activity. Table 5 shows that hyperglycemia (30 mM glucose) for 2 hours did not increase the lactate content in either hRPE or LECs relative to the level of lactate found in cells incubated in media containing 5 mM glucose. Lactate content in hRPE cells incubated for 2 hours in media containing 5 mM glucose was 5 nmol/10⁶ cells and in 30 mM glucose the content was 6 nmol/10⁶ cells; this small difference was not statistically significant. Similarly, in LECs there was no significant difference between the lactate content in cells incubated with 5 or 30 mM glucose. Moreover, hyperglycemia did not increase the rate of lactate production in these two cell types relative to the rate of lactate production found in the presence of 5 mM glucose. Averaged rates of lactate production per hr by hRPE or LEC were calculated from the accumulation of lactate in the incubation media in micromoles per 10⁶ cells. For both the hRPE and LECs, the averaged rate of lactate production in the presence of 5 mM glucose (0.7 μmol/h per 10⁶ cells) was the same as the rate of lactate production in cells incubated in media containing 30 mM glucose. The level of pyruvate was below the limit of detection in the fluorometer, despite the pooling of three dishes containing a total of 3 to 5 × 10⁶ cells.

Measurements of the specific activities of AR and LDH in supernatants of rat retinas showed that LDH was approximately 2500-fold higher than AR. The averaged activity of AR was 1 nmol/min per milligram protein (*n* = 5), whereas the activity of LDH was 2.5 μmol/min per milligram protein (*n* = 8). In hRPE and LECs, the activity of LDH was also much higher than AR. In hRPE, AR was 1.4 nmol/min per milligram protein (*n* = 4), and LDH was 2.2 μmol/min per milligram protein (*n* = 4). In LECs, AR was 2.5 nmol/min per milligram protein (*n* = 4), but LDH was 2.1 μmol/min per milligram protein (*n* = 4).

DISCUSSION

In the present study, we found no evidence to support the hyperglycemic pseudohypoxia hypothesis of diabetic retinopathy. We examined this hypothesis in several different ways. First, direct measures of the ratio of retinal NAD⁺ to NADH

were consistent with most previously published values in control and diabetic rat retinas^{9,22,23} and were not found to be different between control and diabetic retinas (*P* > 0.05). One exception was a reported decrease in the ratio of pyridine nucleotides in diabetic retinas obtained from rats treated for 10 weeks with alloxan.²³ However, in that study, no change was found in the rate of glycolysis in diabetic retinas, which might be expected to increase (i.e., a hypoxia-like response) if this ratio was reduced. It is possible that diabetes-induced hypoxia could be restricted to a population of sensitive cells (e.g., capillaries and pericytes), whose contribution to overall retinal metabolism is too low to be detected by our techniques. However, our measurements of lactate, pyruvate, and ATP, whose concentrations in rat retinal cells are known to be altered during a hypoxic episode,⁸ were not changed in the present study by hyperglycemia, either in the *in vitro* incubations of retinas and cells or in the *in vivo* diabetic model. Thus, although blocking mitochondrial oxygen consumption leads to an increase in the content of retinal lactate, a decrease in the concentration of retinal ATP, and a decrease in the ratio of NAD⁺-to-NADH⁸ (Winkler BS, unpublished results, 2003), none of these changes was observed under hyperglycemic conditions.

We also tested the pseudohypoxia hyperglycemia hypothesis by examining the levels of lactate, pyruvate, and ATP in control and diabetic retinas and found no differences. Previous studies of the lactate and pyruvate content in control and diabetic retinas have yielded conflicting results. Heath et al.²⁴ reported no difference in the pyruvate content between control and diabetic retinas, whereas retinal lactate content increased in starch-fed but not in sucrose-fed diabetic rats. Salceda et al.²⁵ reported early increases (7–14 days) in lactate and pyruvate content in retinas after administration of streptozotocin to rats. However, by 45 days into the diabetic state, there was no significant difference in lactate content between control and diabetic retinas, whereas the pyruvate content in diabetic retinas declined to a value below the control level. Obrosova et al.²⁶ found that retinal lactate levels were not increased in 3- to 6-week diabetic rats in comparison to control animals, but pyruvate levels were lower than in the control.

TABLE 4. Retinal Lactate, Pyruvate, L/P Ratios, and Lactate Production after Incubation in 5, 10, or 30 mM Glucose for 2 Hours with 95% N₂ and 5% CO₂

| Condition | Lactate Content | Pyruvate Content | Lactate/Pyruvate Ratio | Lactate Production |
|-------------------------------|-----------------|------------------|------------------------|--------------------|
| 5 mM glucose (<i>n</i> = 4) | 52 ± 14 | 1.0 ± 0.2 | 52 | 2.4 ± 0.1 |
| 10 mM glucose (<i>n</i> = 4) | 138 ± 23 | 1.4 ± 0.1 | 99 | 3.2 ± 0.2 |
| 30 mM glucose (<i>n</i> = 4) | 129 ± 31 | 1.6 ± 0.3 | 81 | 3.1 ± 0.1 |

Data are the means ± SD. Number of experiments is in parentheses. Retinal content of lactate and pyruvate are in nmol/retina. Data for lactate and pyruvate content were obtained using the same four pooled rat retinas per measurement. For example, an *n* = 4 means that a total of 16 retinas were used (four retinas/measurement × four independent incubations). Lactate production is in μmol/hr/retina.

TABLE 5. Lactate Content and Rate of Lactate Production by Human Retinal Pigment Epithelial Cells and Lens Epithelial Cells after Incubation in 5 or 30 mM Glucose for 2 Hours

| Cell Type | Condition | Lactate Content | Lactate Production |
|----------------------------|---------------|-----------------|--------------------|
| hRPE cells (<i>n</i> = 6) | 5 mM glucose | 5 ± 2 | 0.7 ± 0.2 |
| hRPE cells (<i>n</i> = 6) | 30 mM glucose | 6 ± 2 | 0.7 ± 0.2 |
| LEC (<i>n</i> = 6) | 5 mM glucose | 6 ± 2 | 0.7 ± 0.1 |
| LEC (<i>n</i> = 6) | 30 mM glucose | 6 ± 1 | 0.7 ± 0.1 |

Data are the mean ± SD. Levels of lactate content are in nmol/10⁶ cells. Lactate production is in μmol/h per 10⁶ cells. Number of experiments is in parentheses.

More recently, Ola et al.⁹ reported no differences in the content of lactate and pyruvate between control rat retinas and 3-week/3-month diabetic rats, a result similar to that found in the longer-term diabetic rat retinas in the present study (see Table 1). These considerations support our data showing that the lactate-to-pyruvate ratio in rat retinas is not elevated by diabetes.

The experimental evidence motivating the present work and that led to the hypothesis that an elevation of glucose concentration causes a hypoxia-like redox imbalance, termed “hyperglycemic pseudohypoxia,” was originally obtained after short-term incubations (2 hours) of isolated rat retinas (obtained from nondiabetic animals) in media containing either 5 or 30 mM glucose.^{2,3} The fundamental finding at the core of this hypothesis is that incubation of retinas in media containing 30 mM glucose leads to an increase in the lactate-to-pyruvate ratio relative to the ratio found in retinas incubated with 5 mM glucose. On a quantitative basis, the increase in lactate in retinas incubated with 30 mM glucose amounted to approximately 100 nmol/retina, whereas the increase in pyruvate amounted to only 1 to 2 nmol/retina. That hyperglycemia and hypoxia each cause an increase in the lactate-to-pyruvate ratio led Williamson et al.² to link these effects into a common hypothesis that diabetic complications are related to a hypoxia-like redox imbalance caused by increased oxidation of sorbitol to fructose by the NAD⁺-dependent sorbitol dehydrogenase.

In this study, our *in vitro* results (e.g., lactate and pyruvate content, and the lactate-to-pyruvate ratio in Table 3) are quite similar to those of Williamson et al. in their 1995² and 2004³ papers; but, when these data are compared with those obtained in freshly excised retinas, a different picture emerges. Although hyperglycemia increased the levels of lactate and pyruvate in incubated rat retinas, the maximum effect was observed with 10 mM glucose. A similar pattern of changes in the content of lactate and pyruvate was observed in retinas incubated in nitrogenated media. Anoxia increased the levels of lactate and pyruvate and the lactate-to-pyruvate ratio, and the effects were maximal in the presence of 10 mM glucose (see Table 4).

One concern is whether the lactate levels measured in fresh tissues after CO₂ narcosis are indicative of a chronic hypoxic state, and thus the higher fresh values measured relative to those found after a 2-hour incubation in media containing 5 mM glucose may simply reflect a distortion of the “true” *in vivo* level. If this were the case, then incubations of retinas in media containing the higher glucose concentrations (10 and 30 mM) would simulate hypoxia, even in the presence of a high oxygen tension. Our retinal lactate data in rats killed by decapitation without any anesthesia, and the data from other laboratories,^{3,26,27} do not support this possibility. In addition, in our surgical technique for isolation of a rat retina, only approxi-

mately 5 seconds elapsed between the time of decapitation or CO₂ narcosis and the removal and placement of the tissue into ice-cold media. Given the similarity of the starting content of lactate in retinas isolated under these two different conditions of death and the short time needed for the surgical isolation of a rat retina, it seems unlikely that the fresh retinas are hypoxic. That our averaged value for ATP content in freshly excised rat retinas is similar to that reported for rat retinas dissected from frozen *in situ* tissue using a funnel freezing procedure²⁸ lends support to our contention that our fresh retinas are not hypoxic.

It appears then that the use of 10 mM glucose, but not 5 mM glucose, is sufficient to preserve the levels of lactate and pyruvate in incubated rat retinas. Although this result may appear surprising, it should be noted that the plasma glucose concentration measured under normoglycemic conditions in rat blood is 11 to 12 mM.^{28,29} This result supports an earlier suggestion from our laboratory⁸ that “raising the glucose concentration in the bathing medium (from 5 to 10 mM) surrounding normal isolated rat retinas enhances its rate of diffusion from the choroidal and vitreal surfaces to the center of the 200 micron thick tissue.” This suggestion is further supported by the present findings showing that the rates of aerobic and anaerobic lactic acid production are also maximal with 10 mM glucose. An important point from the present *in vitro* incubations is to include measurements of metabolites in freshly excised tissues to establish the *t* = 0 baseline (i.e., a value as close as possible to what is present *in vivo* that can be compared with values obtained during the ongoing incubations). Although 5 mM glucose is commonly used in solutions used in *in vitro* studies of tissues and cultured cells, it is not always the case that this concentration is sufficient to support optimal metabolic activities. Our findings that incubation of retinas in media containing 5 mM glucose lowers the levels of lactate and pyruvate, but that 10 mM glucose maintains their levels in comparison to their respective values in freshly excised retinas reinforce this point. A similar situation is seen with oxygen, because equilibration of incubation media bathing an isolated rat retina with 20% oxygen (a partial pressure slightly greater plasma P_{O₂}) is not sufficient to sustain a maximal rate of respiration in this tissue.⁸

To provide an additional test of the role of diffusion as an explanation for the differences in results obtained in rat retinas incubated in media containing 5 or 10 mM glucose, we examined the effects of varying the concentration of glucose in media bathing monolayer cultures of hRPE and LEC, two cell types that possess AR activity, though this activity was several orders of magnitude lower than the activity of LDH (see the Results section). We reasoned that the use of monolayer cultures would eliminate to a large degree diffusion limitations for the availability of glucose in these cell models. The results (see Table 5) show unequivocally that there are no significant differences in lactate content and lactate production in cultured cells incubated with either 5 or 30 mM glucose.

In summary, this study raises the possibility that the original *in vitro* data² suggesting the hyperglycemic pseudohypoxia hypothesis in retina were based on a glucose concentration in the incubation media that was too low to support metabolism. After repeating the *in vitro* experiments with a more appropriate level of glucose in the incubation media and examining *in vivo* data from normoglycemic and diabetic rat retinas, we⁸ and other investigators^{4-7,9} do not find support for this hypothesis as contributing to the development of diabetic abnormalities in the retina.

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