Glucose Dependence of Glycolysis, Hexose Monophosphate Shunt Activity, Energy Status, and the Polyol Pathway in Retinas Isolated From Normal (Nondiabetic) Rats

Barry S. Winkler, Matthew J. Arnold, Melissa A. Brassell, and Denise R. Sliter

Purpose. To measure glucose-dependent metabolic activities and selected parameters of the polyol pathway in retinas isolated from normal rats to test the hypothesis recently proposed by Van den Enden et al that incubation of whole retinas for 2 hours with elevated concentrations of glucose results in activation of the polyol pathway, which is the cause of a redox imbalance, as measured by an increase in the retinal cytosolic lactate–pyruvate ratio and a diabetic-like state.

Methods. Retinas obtained from nondiabetic rats and separated from other ocular tissues were incubated for several hours in incubation medium containing glucose at concentrations ranging from 5 to 30 mM. Measurements were made under aerobic and anaerobic conditions of lactic acid production, retinal adenosine triphosphate (ATP), lactic acid content, the hexose monophosphate shunt pathway, aldose reductase activity, and levels of sorbitol and galactitol. Morphology was examined by light microscopy at the end of the incubations.

Results. Incubation of isolated rat retinas with 20 mM glucose increased lactic acid production by approximately 25% in comparison to the rate observed in 5 mM glucose under aerobic and anaerobic conditions. The content of ATP and lactate in the retinas after a 2-hour incubation in the presence of oxygen and 20 mM glucose was equal to the amounts found in fresh tissues, whereas these metabolites declined, respectively, by 25% and 45% when 5 mM glucose was used. The activity of the hexose monophosphate shunt pathway in isolated rat retinas was not increased significantly when the concentration of glucose was raised from 5 to 30 mM. Aldose reductase activity and polyols were below our limits of detection, 0.5 nmol/minute · mg protein and 3.5 nmol/retina, respectively, under all conditions tested. The morphologic appearance of the retina was similar in the presence of normal and high concentrations of glucose.

Conclusions. These results show that incubation of isolated rat retinas, obtained from nondiabetic rats, with elevated concentrations of glucose for 2 hours leads to increases in glycolysis and a higher tissue content of lactic acid and ATP in comparison to values obtained with 5 mM glucose. However, the magnitude of the glucose-dependent increase in the retinal level of lactate in the current study and in that of Van den Enden et al is six to seven times greater than the calculated flux of glucose through the polyol pathway. These results, therefore, do not support the hypothesis of Van den Enden et al. Rather, it is suggested that supranormal concentrations of glucose yield more lactate and ATP in a whole retina because they optimize the supply of this essential nutrient to cells throughout the tissue by overcoming diffusional limitations that result when the retina is separated from its normal choroidal and intraretinal blood supplies. Invest Ophthalmol Vis Sci. 1997;38:62–71.

In the long history of in vitro studies of the metabolic activities of isolated retinas, it is not uncommon to find that retinas were incubated for many hours in media containing supranormal concentrations of glucose, i.e., concentrations significantly greater than the 5 to 6 mM found in the plasma and ocular humor of mammals.[1] For example, Futterman and Kinoshita[2] used 33.3 mM glucose in their studies of the respiration of the cattle retina. Cohen and Noell[3] used 20 mM glucose in their classic examination of the glucose-dependent metabolic activities of the isolated rabbit retina. In a study of the ionic dependence of electroretinographic potentials, Winkler[4] used 20 mM glu-

From the Eye Research Institute, Oakland University, Rochester, Michigan.
Supported by National Institutes of Health grant EY10015 and by Core Grant for Vision Research EY05230.
Submitted for publication June 4, 1996; revised August 20, 1996; accepted August 21, 1996.
Proprietary interest category: N.
Reprint requests: Barry S. Winkler, Eye Research Institute, Oakland University, 409 Dodge Hall, Rochester, MI 48309-4401.

Investigative Ophthalmology & Visual Science, January 1997, Vol. 38, No. 1

cose in the perfusate bathing isolated rat retinas. In vitro studies of isolated frog and toad retinas also have been performed routinely under hyperglycemic conditions, i.e., 10 to 20 mM glucose.[5-10] Indeed, Winkler[11,12] has provided evidence that both electrophysiological and metabolic activities are enhanced when the glucose concentration bathing rat retinas is increased from 5 to 20 mM.

In contrast to the view that increasing the concentration of glucose in an incubation medium to 20 mM is beneficial to an isolated retina, Van den Enden et al[13] recently reported that a short-term (2-hour) incubation of an isolated rat retina obtained from a normal rat with 20 to 30 mM glucose causes a "hypoxia-like redox imbalance (pseudohypoxia) that results from increased oxidation of sorbitol to fructose." These authors suggest that this experimental condition offers a potentially new model for studying mechanisms that may contribute to diabetic retinopathy. In view of the implications of the proposal of Van den Enden et al,[13] we thought it was important to provide independent tests of their hypothesis. We have, therefore, conducted a series of experiments on the isolated rat retina similar to those performed by Van den Enden et al, and we have included new measurements of the hexose monophosphate shunt (HMPS) as a function of the glucose concentration and the activity of aldose reductase; the sum total of these experiments, we believe, has enabled us to test their hypothesis critically. The results presented in this article lead us to conclude, in contrast to the suggestion of Van den Enden et al,[13] that short-term incubation of the nondiabetic, isolated rat retina with elevated glucose concentrations neither mimics the effects of hypoxia nor represents initiating mechanisms of the complications of diabetic retinopathy. Rather, we conclude that increasing the concentration of glucose from 5 mM to 10–20 mM simply helps to overcome a diffusional limitation that is imposed when an isolated rat retina is divorced from its intraretinal and choroidal blood supplies.

METHODS

General

Retinas were obtained from eyes of normal male Sprague–Dawley rats weighing 200 to 250 g each after euthanasia by CO_2 inhalation. Each retina (without the retinal pigment epithelium) was isolated from other ocular tissues by methods that have been described in detail.[4] 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. Typically, one retina was incubated in 5 ml of incubation medium, experiments lasted 2 hours, and metabolic activities and metabolites were measured during the experiment. To this end, samples were taken from the medium for the determination of lactate and pyruvate. After 2 hours, tissues were homogenized in appropriate solutions for determinations of retinal adenosine triphosphate (ATP), lactate, and sugar alcohol levels.

Composition of the Incubation Medium

The standard incubation medium was composed as follows, in mmol/l: NaCl, 130; KCl, 5; $NaHCO_3$, 25; D-glucose, 5; $MgSO_4$, 0.5; and $CaCl_2$, 2. The temperature was 37°C, the pH was 7.4, and the osmolarity was approximately 305 mOsm. All substitutions were made iso-osmotically with appropriate substitutes. For example, elevations in glucose concentration were made at the expense of NaCl. The oxygen tension in the medium was 95%, and the carbon dioxide tension was 5%. For the anaerobic condition, we added 1×10^{-5} M Antimycin A to the incubation medium. We have found that Antimycin A (Sigma Chemical, St. Louis, MO) rapidly (within 1 minute) and completely suppresses oxygen uptake in the isolated rat retina.[12] All chemicals were purchased from Sigma Chemical. In each case, substrates were freshly weighed and added to the medium approximately 1 hour before the start of an experiment.

Biochemical Measurements

Lactic acid production was monitored using aliquots (0.1 or 0.2 ml) of the incubation medium collected at 30-minute intervals during the experiment. Lactate was determined by the lactic acid dehydrogenase kit (826-UV; Sigma Chemical, St. Louis, MO) that couples lactate to the reduction of nicotinamide–adenine dinucleotide (NAD). The appearance of pyruvate in the medium also was monitored. In this study, we used lactic acid dehydrogenase as well in a way that differed from the estimate of lactate. A buffer reaction mixture was prepared containing 100 mM $NaPO_4$ (pH 7.4), 0.2 mM NADH, and 1 U lactic acid dehydrogenase. We added 0.8 ml of this mixture to two 1-ml cuvettes positioned in a Gilford multicompartment recording spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH). Using the offset switch and a high gain of the spectrophotometer, the recording pens were adjusted so that they exactly superimposed at the same optical density (340 nm). Then 0.2 ml of the retinal incubation medium was added to one cuvette, and 0.2 ml of an identical blank or control solution was added to the other cuvette. Immediately after these additions, both pens moved in the downward direction (less NADH) because of dilution of the NADH in the original buffer mixture. However, if pyruvate was present in the incubation medium (the "test" cuvette), the decrease in optical density in this cuvette was

greater than the decrease observed in the "blank" cuvette. Thus, the difference in the final optical density between test and blank cuvettes represented the amount of pyruvate present in the medium. The sensitivity of this method was established with an extensive set of calibration measurements using known amounts of pyruvic acid.

The content of ATP and lactate in individual rat retinas was measured in fresh retinas and in those incubated for varying periods of time. A single retina was rinsed in ice-cold saline and was transferred quickly to 0.25 ml cold 5% perchloric acid. After homogenization of the tissue, the tissue was centrifuged at 10,000*g* for 10 minutes. A 10-μl aliquot of the clear supernatant was diluted 200-fold with distilled water, and a 50-μl sample of this diluent was taken for measurement of ATP by the luciferin–luciferase method in a luminometer (Turner Systems, Mountain View, CA). Values for tissue ATP content were standardized with a series of known ATP concentrations. For tissue lactate determinations, a 150-μl aliquot of the supernatant was withdrawn and neutralized with an equal volume of 1 N KOH. The precipitate was sedimented, and 125-μl aliquots were used in the lactate assay in the same manner as for the measurement of media lactate. In these measurements, the blank consisted of a neutralized tissue supernatant added to the standard lactate assay mixture without lactic acid dehydrogenase. The value of the tissue lactate was obtained from the difference between the optical densities measured in the presence and absence of the enzyme. The assay for polyols was the same as that published previously by Reddy et al[14]; for this, a Shimadzu GC-Mini 3 Gas Chromatograph (Shimadzu, Kyoto, Japan) was used. Freshly silylated mixtures of fructose, galactose, glucose, sorbitol, and myoinositol were used as standards. In brief, one retina was homogenized in 0.5 ml $ZnSO_4$, and then 0.5 ml $Ba(OH)_2$ was added. After centrifugation, 0.5 ml of the supernatant was lyophilized. A derivatizing agent (20 μl) was added, and 1.5 μl was injected into the column. Evaluation of the measurements of standards suggest we can detect 0.1 nmol/μl. Thus, this 35-fold dilution indicates that the lower limit of detection of polyols is 3.5 nmol/retina.

The activity of aldose reductase in a cytosolic supernatant of rat retina was determined by following the change in absorbance at 340 nm of NADPH at room temperature using the method of Hayman and Kinoshita[15] with minor modifications. Substrates tested included D,L-glyceraldehyde, galactose, and glucose. Typically, one rat retina was homogenized in 0.5 ml of 0.1 M PO_4 buffer and centrifuged at 10,000*g* for 15 minutes, and an 0.2-ml sample was tested; on several occasions, a sample as large as 0.4 ml (or 80% of a single rat retina) was assayed for activity.

Activity of the HMPS was estimated by subtracting the production of $^{14}CO_2$ from (6-^{14}C)-labeled glucose (New England Nuclear, Boston, MA) contained in the incubation medium from the production of $^{14}CO_2$ from (1-^{14}C)-labeled glucose. Procedures for measuring shunt activity have been described for retinas.[16] A rat retina was cultured in capped tubes modified by the addition of a rubber stopper as a means of injecting acid into the medium at the end of the experiments. The specific activity of labeled glucose in the medium was 18,000 cpm μmol^{-1}. Appropriate blanks were subtracted from the experimental values. The total air space of the capped tubes was approximately 30 ml, and the volume of incubation fluid was 5 ml. Accordingly, both the liquid and the air space were preequilibrated thoroughly with 95% O_2–5% CO_2 for at least 30 minutes before the start of an experiment and the capping of the culture tubes. In this way, glucose oxidation experiments are conducted at this high partial pressure of oxygen, essentially yielding a maximum rate of oxygen uptake.[12]

TABLE 1. Rate of Lactate Production by Isolated Rat Retinas*

Condition	*Aerobic*	*Anaerobic*	*Pasteur*
5 mM glucose	1.25 ± 0.13 (10)	2.25 ± 0.19 (10)	1.8
10 mM glucose	1.44 ± 0.14 (3)	3.01 ± 0.22 (3)	2.1
20 mM glucose	1.55 ± 0.19 (7)	3.10 ± 0.30 (7)	2.0
30 mM glucose	1.42 ± 0.11 (5)	2.86 ± 0.28 (5)	2.0

* Results are expressed as μmol/mg dry weight/hr ± SD with number of experiments in parentheses. Measured values were obtained as described in the text. Aerobic condition is with 95% O_2/5% CO_2. Anaerobic condition is with 95% O_2/5% CO_2 plus 1×10^{-5} M antimycin A. Averaged dry weight of a single isolated rat retina was 1.25 mg (this value applies to all measured values throughout.

Histology

Retinas were fixed in a buffered 1% glutaraldehyde solution for 1 hour and were transferred to a phosphate buffer wash overnight. Tissues were dehydrated through a graded series of isopropyl alcohols and were embedded in paraffin. Microtome sections (7 μm thick) were stained with eosin and hematoxylin and were examined by light microscopy.

RESULTS

Averaged rates of aerobic and anaerobic production of lactate by the isolated rat retina were calculated from the accumulation of lactate in the incubation medium between 30 and 120 minutes (Table 1). In comparison to the aerobic rate with 5 mM glucose, lactate production was increased by 15%, 24%, and 14%, respectively, with 10, 20, and 30 mM glucose. Under the anaerobic condition, with Antimycin A added to the incubation medium, lactic acid production was increased 1.8- to 2.1-fold (the Pasteur effect)

TABLE 2. Adenosine Triphosphate Content in Fresh and Incubated Rat Retinas*

Condition	*Aerobic*	*Anaerobic*
Fresh tissue	9.44 ± 0.96 (11)	—
5 mM glucose	7.03 ± 0.96 (7)	1.92 ± 0.62 (6)
10 mM glucose	8.78 ± 0.88 (4)	4.80 ± 0.80 (4)
20 mM glucose	9.84 ± 0.90 (7)	5.26 ± 0.70 (6)
30 mM glucose	9.19 ± 1.15 (5)	5.76 ± 0.32 (4)

* Results are expressed for each condition as nmol/mg dry weight ± SD with number of experiments in parentheses. Incubations with varying concentrations of glucose in the media were carried out for 2 hours, at which time retinas were removed for determination of adenosine triphosphate as described in the text. Table 1 footnotes contain a description of aerobic and anaerobic incubations.

in the presence of the varying glucose concentrations. Relative to the rate of anaerobic lactate production in the presence of 5 mM glucose, increasing the concentration of glucose to 10, 20, and 30 mM led to increases in lactate production of 34%, 38%, and 27%, respectively. The increases in aerobic and anaerobic lactate production observed with elevated (hyperglycemic) concentrations of glucose are qualitatively similar to those found previously by Winkler[11] and Van den Enden et al.[13]

Pyruvate also accumulated in the medium bathing the isolated rat retinas, a finding in agreement with that reported by Van Enden et al.[13] In the current experiments, the averaged rate of aerobic pyruvate production was 108 nmol/hour·mg dry weight with 5 mM glucose (n = 8), and this rate of production was not significantly changed when the glucose concentration was raised to 20 mM. On a quantitative basis, the rate of pyruvic acid production was 8.6% of the rate of lactic acid production. In contrast, when the experiments were conducted in the presence of Antimycin A, pyruvate was not detected in the incubation medium, despite the fact that anaerobic lactic acid production was very high in the presence of 5 to 30 mM glucose. In the absence of glucose, neither pyruvate nor lactate accumulated in the medium to any appreciable extent aerobically or anaerobically.[11]

The levels of ATP and lactate in freshly excised and incubated rat retinas were measured as a function of the concentration of glucose in the incubation medium (Tables 2, 3). The duration of the retinal incubations was 2 hours, and the values measured for ATP and lactate at the end of the incubations can be compared readily with the same measurements carried out on the fresh tissues. Fresh retinas were isolated from the other ocular tissues, placed in ice-cold saline, stripped of the adherent vitreous, and placed in 5% perchloric acid (see Methods); elapsed time for this procedure averaged 30 seconds per retina. At the low temperature of the saline solution, the production and use of ATP and lactate by isolated retinas was essentially zero (Winkler BS, unpublished observations, 1983), and leakage of lactate from the retinal cells was minimal over this short period of time in the ice-cold saline. The importance of removing the vitreous humor was evident from the known high concentration (12 mM) of lactic acid in this ocular medium.[1] Similarly, the incubated retinas (also stripped free of adherent vitreous at the start of the experiment) were removed from the incubation flasks by capture on a lens loop, rinsed in ice-cold saline, and immersed in 5% perchloric acid.

It can be seen in Table 2 that the amount of ATP in retinas incubated aerobically for 2 hours with 5 mM glucose was approximately 25% less than the value in fresh tissues (7.03 versus 9.44 nmol/mg dry weight). Under aerobic conditions, the ATP content was maintained in retinas incubated with 10 to 30 mM glucose at values similar to the ATP content found in the fresh tissue. Under the anaerobic condition, it can be seen that the ATP content of retinas incubated with 5 mM glucose fell to less than 30% of that found under the aerobic condition (1.92 versus 7.03 nmol/mg dry weight. However, in retinas incubated with 10 to 30 mM glucose, the decline in ATP was much smaller, to 53% of the control in the case of 20 mM glucose, (5.26 versus 9.84 nmol/mg dry weight).

The averaged lactate content in a freshly excised rat retina was 80.2 nmol/mg dry weight (Table 3). Retinas incubated aerobically for 2 hours with 5 mM glucose contained approximately half as much lactate (43.8 versus 80.2 nmol/mg dry weight). In contrast, with 20 mM glucose, the lactate content was maintained aerobically at essentially the same value as that found in the fresh tissue. Inhibition of retinas with the mitochondria poison Antimycin A (anaerobic condition) led to increases in the lactate content in retinas incubated with 5 and 20 mM glucose. With 5 mM glucose, the net increase in lactate content over the 2-hour period amounted to 28 nmol (71.8 versus 43.8 nmol); with 20 mM glucose, the net increase was 40.6 nmol (125.6 versus 87 nmol).

The activity of the HMPS was measured in retinas during a 2-hour incubation with varying concentra-

TABLE 3. Lactic Acid Content in Fresh and Incubated Rat Retinas*

Condition	*Aerobic*	*Anaerobic*
Fresh tissue	80.2 ± 10 (6)	—
5 mM glucose	43.8 ± 11 (6)	71.8 ± 8 (3)
20 mM glucose	87.0 ± 13 (6)	125.6 ± 12 (3)

* Results are expressed for each condition as nmol/mg dry weight ± SD with number of experiments in parentheses. Incubations with varying concentrations of glucose in the media were carried out for 2 hours, at which time retinas were removed for determination of lactate as described in the text. Table 1 footnotes contain a description of aerobic and anaerobic incubations.

TABLE 4. Effects of Varying Glucose Concentration on the Activity of the Hexose Monophosphate Shunt in Rat Retinas*

Condition	C-1	C-6	[C-1] − [C-6]
5 mM glucose	140 ± 23 (10)	128 ± 19 (7)	12
20 mM glucose	154 ± 22 (12)	134 ± 31 (6)	20
30 mM glucose	137 ± 24 (8)	122 ± 6 (4)	15

* Results are expressed as nmol CO_2/retina per 2 hours and are mean ± SD with number of experiments in parentheses. The shunt activity is taken as the difference between CO_2 production from C-1 labeled glucose and CO_2 production from C-6 labeled glucose, i.e., [C-1] − [C-6].

tions of glucose. Overall, the results presented in Table 4 show that shunt activity was low when retinas were incubated with 5 to 30 mM glucose, as shown by the value of 1.1 to 1.2 for the ratio of glucose-1-[^{14}C] oxidized to glucose-6-[^{14}C] oxidized. Indeed, the actual amounts of CO_2 produced by the shunt pathway were only approximately 10% to 15% of the amount of CO_2 produced from mitochondrial oxidation. Moreover, HMPS activity showed only a small, statistically insignificant increase with increasing concentrations of glucose. Similarly, the mitochondrial oxidation of glucose essentially was unchanged after an increase in glucose concentration from 5 to 30 mM.

Because cytosolic NADP-dependent malic enzyme and NADP-dependent isocitrate dehydrogenase may generate NADPH in the retina, [17] retinas were incubated in medium containing 1×10^{-5} M Antimycin A and were equilibrated with nitrogen to isolate the contribution of the HMPS pathway to NADPH generation. Under this condition of mitochondrial blockade, the activities of malic enzyme and isocitrate dehydrogenase were inhibited because the appropriate substrates ceased to be produced. The amounts of $^{14}CO_2$ produced anaerobically from the oxidation of ^{14}C-1 glucose were low after 2 hours, amounting to 4 ± 1 nmol/retina (n = 4) with 5 mM glucose and 6.5 ± 1 nmol/retina (n = 4) with 30 mM glucose; the difference was 2.5 nmol $^{14}CO_2$ produced after an increase in glucose concentration in the medium.

The morphology of isolated rat retinas was examined after incubation of the tissues for 3 hours in media containing 5 to 30 mM glucose (Fig. 1). As can be seen in these light microscopic sections, retinal morphology was well preserved during the incubations. There appeared to be no significant structural differences between fresh tissues and retinas incubated with 5 mM glucose or with elevated concentrations of glucose (20 to 30 mM).

The activity of aldose reductase was examined in cytosolic fractions of freshly excised and incubated rat retinas. Under the assay conditions used, activity of this enzyme was not detected in supernatants prepared from freshly excised retinas (n = 12)—that is, activity was below the limit of detection in the assay (approximately 0.5 nmol/minute · mg protein). Thus, the slopes of NADPH oxidation in the presence of 0.5 mM D,L-glyceraldehyde, 25 mM galactose, and 25 mM glucose were the same in the presence and the absence of the retinal supernatants. Aldose reductase activity was not detected in supernatant fractions of retinas incubated for 2 hours in medium containing 5 mM (n = 4) or 20 mM (n = 6) glucose. In contrast, when the same assay condition was used with a cytosolic fraction of a freshly excised rabbit lens, aldose reductase activity was detected readily with 0.5 mM D,L-glyceraldehyde as the substrate—that is, 65 nmol/minute per whole lens (n = 4).

In the final series of experiments, the levels of sugar alcohols (sorbitol and galactitol) were measured in fresh and incubated retinas. In retinas isolated from normal rats, polyols were not measurable in the assay, whose detection limit was estimated to be 3.5 nmol/retina. The content of polyols in retinas incubated for 2 hours with either 5 mM glucose (n = 3), 30 mM glucose (n = 3), or 5 mM glucose and 30 mM galactose (n = 2) was also below detection. The latter substrate condition was included because it offered the best opportunity to see an increase in polyol content. Galactitol, if produced, is poorly metabolized by sorbitol dehydrogenase.

DISCUSSION

The focus of the current experiments was on whether short-term incubation of freshly excised, normal rat retinas with increasing concentrations of glucose leads to a stimulation of the polyol pathway and a diabetes-like status.[13] The polyol pathway consists of a set of two reactions: The first is catalyzed by the NADPH-dependent aldose reductase, in which glucose is converted to sorbitol, and the second is catalyzed by the NAD-dependent sorbitol dehydrogenase, in which sorbitol is converted to fructose. Interest in the activity of the polyol pathway has centered on the pathologic potential of a glucose-dependent increase in this pathway, resulting in the intracellular accumulation of sugar alcohol, which leads to an influx of water, intracellular volume imbalance, and deleterious changes in sensitive tissues. In ocular tissues, the lens, retinal vasculature, and retinal pigment epithelium represent the most sensitive cellular targets of the pathologic effects of hyperglycemia. Nevertheless, the role of the polyol pathway in the pathogenesis of diabetes in ocular and other tissues is still controversial.[18] Because our experiments did not involve use of in vivo animal models of diabetes,[19–26] we have compared our results with those of Van den Enden et al.[13]

Data in Table 1 confirm and extend previous results from our laboratory,[11] and they agree qualita-

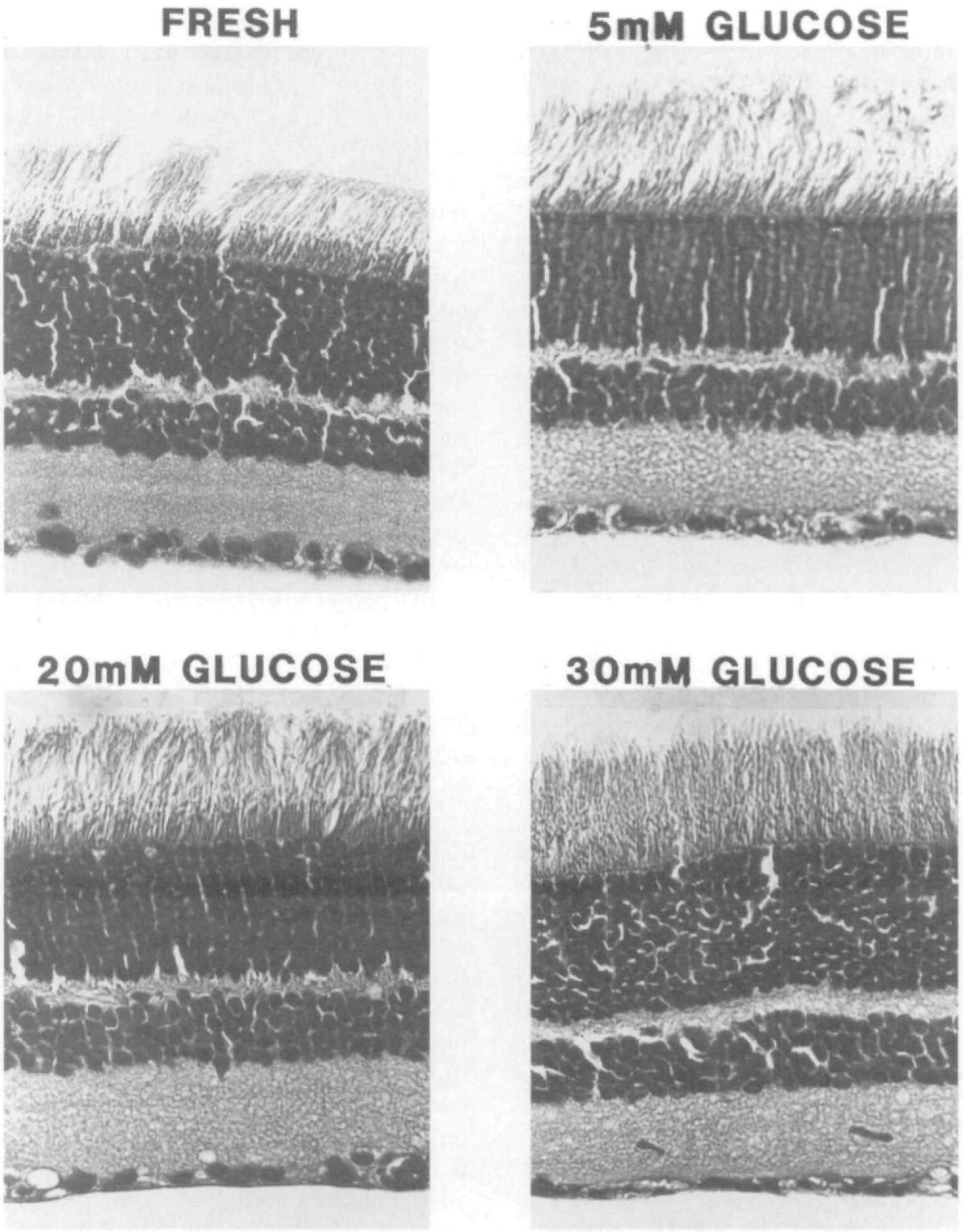

FIGURE 1. Light micrographs of 7-μm-thick sections of isolated rat retinas, stained with hematoxylin and eosin, incubated for 3 hours in medium containing varying concentrations of glucose. (*top left*) Freshly excised tissue; (*top right*) 5 mM glucose; (*bottom left*) 20 mM glucose; (*bottom right*) 30 mM glucose.

tively with the results of Van den Enden et al[13] concerning the dependence of aerobic glycolysis on glucose concentration. Thus, we found that aerobic glycolysis was increased by 24% and anaerobic glycolysis was increased by 33% between 5 and 20 mM glucose. However, there are significant quantitative differences between our results and those of Van den Enden et al when the data are compared on a common basis (e.g., per retina). Van den Enden et al[13] express their data on a per microgram DNA basis but fail to indicate the microgram DNA content in a whole rat retina. Organisciak et al[27] have reported the DNA content in the visual cells of a rat retina to be approximately 175 μg, and, if we assume that visual cell DNA accounts for 75% of total retinal DNA, we can calculate lactate production on a per retina basis by taking the published values of Van den Enden et al and multiplying by 250 (the approximate number of micrograms of DNA in a whole rat retina). For the data in the current study, which are expressed on a milligram dry weight basis, we must multiply our numbers by 1.25 (the dry weight [in mg] of a whole rat retina). Thus, our rate of aerobic lactic acid production (see Table 1) becomes 1.56 μmol/hour per retina in 5 mM glucose and 1.94 μmol/hour per retina in 20 mM glucose, amounting to an increase of 0.38 μmol/hour

per retina. In the article by Van den Enden et al[13] (Fig. 1), the aerobic lactic acid production, with 5 mM glucose, is 3.25 μmol/hour per retina. This rate is approximately two times higher than in the current study and is considerably higher than other published values for the rate of aerobic glycolysis in a mammalian retina.[28]

The freshly excised rat retina contains 9.44 nmol ATP/mg dry weight and 80.2 nmol lactate/mg dry weight. Data in Tables 2 and 3 clearly show that incubation of isolated rat retinas with 5 mM glucose and oxygen for 2 hours leads to declines in the tissue levels of both ATP (−25%) and lactate (−46%). However, with 10 to 20 mM glucose in the bathing medium, ATP and lactate are maintained at values essentially identical to the values measured in fresh tissue. On this basis, it seems reasonable to conclude that optimal incubation conditions for isolated rat retinas are achieved using an oxygenated incubation medium containing at least 10 mM glucose.

The use of high glucose concentrations also leads to a significantly greater preservation of ATP content under the anaerobic (mitochondrial inhibited) condition, as previously reported.[11,12,28] For example, with 10 to 30 mM glucose, anaerobic ATP content is equal to, on average, 56% of the amount in the fresh tissue, whereas with 5 mM glucose, anaerobic ATP content after 2 hours was only 20% of the level in the freshly excised tissue. The significantly improved energy (ATP) status of the hyperglycemic retina under the anaerobic condition is most probably the result of a greater compensatory increase in glycolysis (enhanced Pasteur effect), as shown in Table 1, and probably accounts as well for the reduced sensitivity of electroretinographic potentials in diabetic rats to hypoxemia.[29]

Inhibition of mitochondrial activity leads to substantial increases in the tissue content of lactic acid in the presence of 5 and 20 mM glucose. This change is consistent with an anaerobic-induced increase in the NADH–NAD ratio. This increased ratio enables pyruvate to be reduced at a higher rate anaerobically, limiting its availability for efflux into the medium. Under the aerobic condition, when reduction of pyruvate is slower, its availability for export may be increased. We offer this as a tentative explanation for our finding of pyruvate in the incubation medium only when retinas were incubated aerobically. An alternative possibility is that transport of pyruvate out of retinal cells is inhibited after mitochondrial blockade. Based on our results and those of Van den Enden et al,[13] it seems that the normal concentration of pyruvate (0.3 to 0.7 mM) in vitreous humor[1] may result from diffusion from retinal cells as well as from the lens, another ocular tissue with a high glycolytic capacity.[30]

At the outset, we indicated that the impetus for undertaking the current experiments was the suggestion put forth by Van den Enden et al[13] that the glucose-dependent increase in the lactate–pyruvate ratio in an isolated rat retina is caused by stimulation of the polyol pathway. In support of this suggestion, these authors found that elevated glucose levels increased retinal levels of sorbitol and triose phosphates and that an inhibitor of aldose reductase prevented these increases. These authors[13,31] use the phrase pseudohypoxia to describe the hypoxia-like redox changes, an increase in the lactate–pyruvate ratio they think results from the glucose-dependent stimulation of aldose reductase. To test this hypothesis, we measured specific components of the polyol pathway in isolated rat retinas and compared quantitatively changes in this pathway with changes in retinal lactate content. Our results and calculations fail to support the interpretation of Van den Enden et al[13] or their use of pseudohypoxia as the reason for the increase in the retinal lactate–pyruvate ratio after an increase in glucose concentration.

Using cytosolic fractions of whole rat retinas, we have not been able to detect aldose reductase activity. We tested the ability of D,L-glyceraldehyde (0.1 to 1.0 mM), glucose (up to 25 mM), and galactose (up to 25 mM) to serve as substrates for aldose reductase; none of these substrates was capable of eliciting enzymatic activity. In addition, no activity was found in the presence of $LiSO_4$, which has been reported to increase substantially the activity of aldose reductase in whole lens[15] and in Y-79 cells.[32] The sensitivity of the spectrophotometric system enables us to detect activities as low as 0.5 nmol/minute · mg protein. Kennedy, Frank, and Varma[33] found that the specific activity of aldose reductase (assayed with 0.1 mM D,L-glyceraldehyde) was 0.4 and 0.7 nmol/minute · mg protein, respectively, in bovine retina and bovine retinal pericytes; these values are at our limit of detection. In addition, in a number of studies, immunohistochemical procedures have been used to evaluate the presence of aldose reductase in nondiabetic eyes. In whole eye sections of rats[34] and dogs,[35] staining for aldose reductase, although prominent in corneal endothelium and epithelium and in lens epithelium and cortex, was much less consistent in the retinas. In a recent study on the expression of aldose reductase in normal and diabetic human retina and retinal pigment epithelium, Vinores et al[36] reported that in nondiabetic eyes, these tissues were "completely devoid of staining for AR" whereas "eyes from long-term-diabetic patients manifested positivity." However, immunohistochemical expression of aldose reductase has been found in mural cells from retinal capillaries in digest preparations of vessels from nondiabetic human[37] and dog[38] retinas, in cultured pericytes from normal rhesus monkeys[39] and humans,[40] and in cultured human retinal pigment epithelial cells.[41] It seems reasonable to conclude that differences exist in the extent of ex-

pression of aldose reductase in nondiabetic retinas and that enhanced expression of this enzyme is seen in diabetic retinas.

In the current study, the levels of polyols in fresh retinas and in those incubated for 2 hours with either 5 to 30 mM glucose or with 30 mM galactose were below our limits of detection (3.5 nmol/retina). Using a more sensitive assay system, Van den Enden et al[13] found that retinas incubated for 2 hours with 5 mM glucose contained 0.7 nmol sorbitol/retina, whereas retinas incubated with 30 mM glucose had 4.1 nmol/retina. Thus, the glucose-dependent increase in polyol content amounted to 3.4 nmol/retina in the study of Van den Enden et al. In chronic diabetic animals, there are much greater increases in sugar alcohol levels in retinas freshly isolated from these animals. For example, Poulsom et al[42] induced diabetes in rats by the injection of streptozotocin and reported a 10-fold increase in sorbitol relative to the level found in retinas from nondiabetic littermates—that is, sorbitol increased from 0.61 nmol/retina to 6.6 nmol/retina based on 10 mg as the approximate wet weight of a rat retina. In galactosemic dogs, the concentration of galactitol rose more than 40 times in the retina, and this increase was blocked 90% to 96% by sorbinil, an inhibitor of aldose reductase.[43] In alloxan-treated rabbits, sorbitol was elevated in all retinal layers in comparison to the low levels found in nondiabetic rabbits.[19]

The conversion of glucose to sorbitol by aldose reductase requires NADPH, which must be regenerated for this reaction to continue. In rabbit and rat lenses, oxidation of glucose by the HMPS pathway was shown to be linked closely to aldose reductase: An increase in the concentration of glucose (or galactose) caused a linear increase in the production of $^{14}CO_2$ from ^{14}C-1 labeled glucose and an increase in the concentration of sorbitol (or galactitol).[30] A similar set of results has been found in human erythrocytes.[44] In addition, Yokoyama et al[45] reported that HMPS activity in dog lens epithelial cells was stimulated 2.5 times by 30 mM galactose. For these reasons, we evaluated the effects of varying the extracellular concentration of glucose on the HMPS pathway in incubated retinas; indeed, it has long been known that the HMPS is a source of NADPH in the retina.[3,16,46,47] The current results, obtained with freshly excised rat retinas incubated over a 2-hour period, failed to find an increase in HMPS activity at glucose concentrations between 5 and 30 mM. For this range of glucose concentrations, the total activity of the shunt pathway is low in the isolated rat retina (see Table 4), as it is in the isolated rabbit retina incubated with 20 mM glucose for 1 hour under similar conditions.[3]

When retinas were incubated under conditions of mitochondrial blockade to eliminate NADPH production from malic enzyme and isocitrate dehydrogenase[17] and to isolate the contribution of the HMPS pathway, we found only a small glucose-dependent increase in CO_2 production from ^{14}C-1 glucose, amounting to 2.5 nmol $^{14}CO_2$ produced per retina per 2 hours. Under the assumption that this entire increase in CO_2 production is caused by an increase in the polyol pathway, the maximum production of NADPH is 5 nmol, which can convert 5 nmol of glucose into sorbitol. Thus, it is possible that a short-term incubation of a normal isolated rat retina with elevated glucose concentrations may lead to a small increase in HMPS activity, which could reflect a low rate of flux of metabolites through the polyol pathway.

Van den Enden et al[13] hypothesize that an increase in the oxidation of sorbitol to fructose causes an increase in the lactate–pyruvate ratio. They reported that "increased retinal lactate-pyruvate ratios in 30 versus 5 mM glucose were accounted for by an approximately two fold increase in lactate content versus only an approximately 30% increase in pyruvate" (p. 1678). Retinal lactate content in their experiments was 98 nmol/retina in 5 mM glucose; a 2-fold increase brings this value to 196 nmol/retina. Similarly, pyruvate content was 4.3 nmol/retina in 5 mM glucose and 5.5 nmol/retina in 30 mM glucose. Thus, increasing the glucose concentration from 5 to 30 mM increased lactate content by 98 nmol, whereas pyruvate content was increased by only 1.2 nmol. Clearly, for Van den Enden et al's hypothesis to be valid, the rate of oxidation of sorbitol to fructose must account quantitatively for the measured increase (+98 nmol) in lactate content. Yet, Van den Enden et al found that the level of sorbitol increased by 3.4 nmol/retina, and the production of fructose increased by 4 nmol. Adding these two quantities provides an estimate of the glucose-dependent flux through the polyol pathway (7.4 nmol/retina every 2 hours). Glucose equivalents of the increased lactate content are 49 nmol (2 nmol lactate/nmol glucose). Thus, using the data from Van den Enden et al,[13] it can be calculated that the magnitude of the increase in lactate content is 6.6-fold greater than the estimated flux through the polyol pathway. The current experiments also show that the glucose-dependent increase in retinal lactate content is not caused by a stimulation of the polyol pathway. For example, under the anaerobic condition in which measurements of HMPS activity in 5 and 30 mM glucose revealed an upper limit for the conversion of glucose to sorbitol to be 5 nmol/retina per 2 hours, the anaerobic retinal lactate content increased by 67.3 nmol (per retina basis, see Table 3) or 33.6 glucose equivalents. Thus, in the current experiments, the increase in retinal lactate exceeds the putative formation of sorbitol by at least 6.7-fold. Nevertheless, it is important to consider that whole retinal measurements of the content of lactate and pyruvate represent the summed amounts in all the different classes of retinal

cells. The fact that the retina contains many different cell types with different levels of glycolytic intermediates and different levels of polyol pathway activity precludes interpreting measurements (i.e., lactate–pyruvate ratios) as if they have been made on a single, homogeneous cell. Clearly, studies designed to evaluate potential mechanisms of diabetic retinopathy should be focused on retinal capillary cells, which make up a small fraction of the retina.

In summary, our experiments do not support the recent suggestion by Van den Enden et al[13] that an hyperglycemia-induced increase in the retinal lactate–pyruvate ratio in isolated rat retinas results directly from increased oxidation of sorbitol to fructose. We conclude that raising the glucose concentration in the bathing medium surrounding normal isolated rat retinas enhances its rate of diffusion from the choroidal and vitreal surfaces to the center of the 200-μm thick tissue, thereby increasing its availability to retinal neurons and glia and enabling these cells to metabolize glucose at a higher rate, which accounts for the observed increases in the rate of glycolysis and in retinal lactate content. Support for this view comes from recent work[12] showing that a reduction in the partial pressure of oxygen from 95% to 20% (ambient) in the medium bathing isolated rat retinas led to an approximately 50% decline in the rates of oxygen consumption and mitochondrial glucose oxidation, an effect most probably caused by a diffusional limitation in the entry of oxygen into tissue, which is overcome by using an elevated oxygen tension.

Key Words

aldose reductase, glycolysis, hexose monophosphate shunt pathway, polyols, retinal glucose metabolism

Acknowledgments

The authors thank Dr. L.-R. Lin and Ms. Jane Tsui, who performed measurements of retinal polyols. They also thank Alice Carleton for expert typographical assistance.

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