Analysis of glucose metabolism in diabetic rat retinas

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Ola, M. Shamsul, Deborah A. Berkich, Yuping Xu, M. Todd King, Thomas W. Gardner, Ian Simpson, and Kathryn F. LaNoue. Analysis of glucose metabolism in diabetic rat retinas. Am J Physiol Endocrinol Metab 290: E1057–E1067, 2006. First published December 27, 2005; doi:10.1152/ajpendo.00323.2005.—This study was conceived in an effort to understand cause and effect relationships between hyperglycemia and diabetic retinopathy. Numerous studies show that hyperglycemia leads to oxidative stress in the diabetic retinas, but the mechanisms that generate oxidative stress have not been resolved. Increased electron pressure on the mitochondrial electron transfer chain, increased generation of cytosolic NADH, and decreases in cellular NADPH have all been cited as possible sources of reactive oxygen species and nitrous oxide. In the present study, excised retinas from control and diabetic rats were exposed to euglycemic and hyperglycemic conditions. Using a microwave irradiation quenching technique to study retinas of diabetic rats in vivo, glucose, glucose-derived metabolites, and NADH oxidation/reduction status were measured. Studying excised retinas in vitro, glycolytic flux, lactate production, and tricarboxylic acid cycle flux were evaluated. Enzymatically assayed glucose 6-phosphate and fructose 6-phosphate were only slightly elevated by hyperglycemia and/or diabetes, but polyols were increased dramatically. Cytosolic NADH-to-NAD ratios were not elevated by hyperglycemia nor by diabetes in vivo or in vitro. Tricarboxylic acid cycle flux was not increased by the diabetic state nor by hyperglycemia. On the other hand, small increases in glycolytic flux were observed with hyperglycemia, but glycolytic flux was always lower in diabetic compared with control animals. An observed decrease in activity of glyceraldehyde-3-phosphate dehydrogenase may be partially responsible for slow glycolytic flux for retinas of diabetic rats. Therefore, it is concluded that glucose metabolism, downstream of hexokinase, is not elevated by hyperglycemia or diabetes. Metabolites upstream of glucose such as the sorbitol pathway (which decreases NADPH) and polyol synthesis are increased.

diabetic retinopathy; hyperglycemia; reactive oxygen species; mitochondria; glycolysis; polyols

DIABETIC RETINOPATHY IS A SEVERE COMPLICATION of type 1 and type 2 diabetes and is the leading cause of blindness among adults worldwide. The mechanisms of diabetes-induced damage to the retina correlate with excessive circulating levels of glucose, but it is not clear whether the toxic influence of hyperglycemia or perhaps inadequate signaling from the insulin receptor contribute more importantly to the pathogenesis (18).

Retinal glucose metabolism begins with the transport of glucose across the blood-retinal barrier. This is mediated in an insulin-independent manner (9) by the GLUT1 glucose transporter, which is highly expressed in both retinal endothelial cells and pigment epithelium (2, 35). This feature accounts for the rapid equilibration between cytosol and serum glucose under normal and pathological conditions. Large increases in glucose levels within the retina (if toxic) might damage retinal cells, particularly Müller cells. Because the $K_m$ of the transporter is 5 mM, it does not saturate with substrate except under pathological conditions (35). Therefore, interstitial levels of glucose within the retina are likely to be sensitive to increases in serum glucose. Moreover, in vivo measurements of retinal glucose transport and metabolism by NMR spectroscopy have shown that glucose transport normally exceeds metabolic rates ~16-fold (4).

Biochemical abnormalities associated with hyperglycemia and identified in diabetic retinas include activation of protein kinase C (32), nonenzymatic glycation (6), polyol formation (1), increased hexosamine synthesis (25), and activation of growth factors that promote apoptosis (40) as opposed to those that promote survival. Production of excess reactive oxygen species (ROS) may induce many of these abnormalities. There is growing evidence that oxygen-free radicals are present in excess in diabetic retinas (16, 19, 20, 30) and in vascular endothelial cells (15, 46). Oxygen-free radicals are toxic to tissue because of their high reactivity and ability to form covalent bonds nonenzymatically. They can be generated by partial reduction of oxygen with mitochondrial electron transfer complexes I and III when reducing pressure (NADH) is high and O$_2$ supply is adequate (51). Increased availability of glucose may increase the supply of NADH in the mitochondria, and the increased electron availability may cause partial reduction of oxygen to superoxide radicals in the proximal part of the electron transfer chain (36, 51). Large increases in generation of ROS are most commonly seen when downstream inhibitors of electron flow increase reduction of coenzyme Q and increase the half-life of coenzyme Q semiquinone (36, 48). Therefore, the suggestion has been made that diabetic damage to retinas is caused by mitochondrially generated superoxide radicals (23). However, clear evidence of increased cytosolic and mitochondrial NADH leading to increased electron pressure within the diabetic retina has not been demonstrated. To understand and evaluate the metabolic consequences of high glucose and diabetes on retinal metabolism, we measured glucose metabolism at 5 and 20 mM glucose in excised retinas from euglycemic and diabetic rats. Current hypotheses imply that high glucose and the diabetic state stimulate flux through the glycolytic pathway, increase cytosolic NADH, increase tissue lactate-to-pyruvate ratios, and increase tricarboxylic acid.
cycle flux (7, 15, 27, 42–44, 49, 52, 53). Surprisingly, none of these increases was observed in the studies described here, but increased polyol formation was observed along with a modest increase in glucose 6-phosphate levels and a decreased glycolytic rate with prolonged diabetes (3 mo).

**EXPERIMENTAL PROCEDURES**

**Materials**

[5-3H]glucose and [U-14C]glucose were purchased from New England Nuclear Life Science Products (Boston, MA). Hexokinase was purchased from Sigma Chemicals (St. Louis, MO), and alkaline phosphatase was from Roche Diagnostics (Indianapolis, IN).

**Animals**

Sprague-Dawley rats were used for all experiments. Rats were housed under a 12:12-h light-dark cycle and allowed free access to food and water. The experimental protocols were approved by the institutional review committees of the Pennsylvania State University College of Medicine. Animals were routinely treated in accordance with the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were made diabetic by an intraperitoneal injection of streptozotocin (65 mg/kg), and experiments were carried out 3 wk or 3 mo after streptozotocin injections. Controls were age-matched and housed with diabetic animals. Serum glucose levels of diabetic animals exceeded 17 mM.

**Isolation of the retinas.** After rats were anesthetized with ketamine-xylazine (53 mg ketamine, 5.3 mg xylazine/kg), each of the retinas was carefully dissected from excised eyes and divided into two halves as described by Lieth et al. (37). The energy status, viability, and functional integrity of retinas isolated and incubated in this way have been documented previously (37, 56). With the use of this procedure, one-half retina was used in each 1-ml incubation. Some duplicate studies of glycolytic and tricarboxylic acid cycle fluxes were done using Nembutal as an anesthetic agent (dose = 0.1 g/kg rat) instead of ketamine-xylazine. These studies demonstrated that the results obtained were independent of the anesthetic agent used.

**Incubation conditions.** Single freshly excised half-retinas were incubated at 37°C in 1 ml Krebs bicarbonate buffer containing 20 mM HEPES, pH 7.4, 118 mM NaCl, 4.7 mM KCl, 25.5 mM CaCl2, 1.2 mM KH2PO4, 1.17 mM MgSO4, 25 mM NaHCO3, and 5 or 20 mM glucose. The buffer was equilibrated with 95% O2-5% CO2. Retinas were equilibrated for 3 min in the Krebs buffer plus glucose. Experimental incubations were started with the addition of ~4 µCi [U-14C]glucose and/or ~25 µCi [5-3H]glucose. They were halted at 20, 40, or 60 min by the addition of perchloric acid (final concentration was 2%). Each of the four half-retinas from individual rats was employed in separate incubations of different incubation times. Thus half-retinas from the same individual rat were never used as duplicates for the same time point. For example, to measure the rate of oxidation of glucose, we would employ 4–5 rats. Each rat provides four half-retinas. From a single rat, one half-retina would be incubated for 20 min, another for 20 min, 40 min, and still another for 60 min. This is repeated for each rat on a rather strict time schedule. At the end of the experiment, there are four to five duplicates, each one from a different rat at a given time point. Keeping track of which half-retina came from which rat, one can calculate a rate for each animal or treat each point separately. In general, we get almost identical results independent of the method used to evaluate rates. Other experiments were used to evaluate metabolite levels after incubations for a fixed period of time. In these instances, euglycemic and diabetic rats were compared, and incubations were carried out under euglycemic (5 mM) or hyperglycemic (20 mM) conditions. In this case, two half-retinas from each rat were incubated at 5 mM and two at 20 mM. Four control rats and four diabetic rats were used in a typical experiment of this sort. Each of the four rats provided two retinas for 5 mM glucose incubations and two retinas for the 20 mM incubations. Thus metabolites in eight half-retinas from four rats were employed in a given incubation condition. Statistical analysis was carried out averaging values from the duplicate retinas and treating the average as a single data point (n = 4).

**Glycolysis.** Rates of glycolysis were measured as the formation of [3H2O] from [5-3H]glucose in incubations that included [U-14C]glucose and [5-3H]glucose. [5-3H]glucose produces 3H2O at the phosphoglycerate mutase step, where glycerate 3-phosphate is converted to glyceroate 2-phosphate. Perchloric acid extracts of retinas were sonicated, and the protein was removed by centrifugation. The supernatants were neutralized with KOH and half-milliliter aliquots of the neutral extracts were placed on Dowex-1 acetate anion exchange columns (17). The column was eluted with 5 ml of H2O, and a 0.5-ml aliquot of the eluate was counted. The eluate contained 3H2O, glucose ([14C]- and [5-3H]glucose), [14C]glutamine, and [1H]-polyols, glycosamine, and glucose. The remainder of the sample staying on the column was eluted with acetic acid to separate metabolic anions. The initial eluate was treated with hexokinase to convert glucose to glucose 6-phosphate. This was run on a second anion exchange column to remove glucose 6-phosphate, which stayed on the column. An aliquot of this second neutral fraction was taken from the eluate of the second column. It contained only 3H2O, [1H]-polyols, [1H]-glycosamine, and [14C]glutamine. Freeze-drying a portion of the second neutral fraction removed 3H2O and counting the lyophilate dissolved in 5 ml H2O via dual label counting allowed us to estimate 3H2O by difference. The disintegrations per minute of 3H2O divided by the specific activity of [5-3H]glucose provided a value for total glucose passing through the glycolytic pathway. It was also possible to determine how much of the [U-14C]glucose was converted to pyruvate without entering the tricarboxylic acid cycle. This was accomplished by isolating lactate plus pyruvate chromatographically and measuring the disintegrations per minute of carbon-14 in the lactate/pyruvate pool. The 14C disintegrations per minute were divided by the specific activity of glucose to provide a value for the amount of glucose converted to pyruvate/lactate without further metabolism.

**Tricarboxylic acid cycle.** To measure oxidation of glucose to CO2, half-retinas were preincubated in 1 ml of medium containing [U-14C]glucose and [5-3H]glucose for 3 min at 37°C in glass vials. The vials were then sealed from the atmosphere. A trap containing a filter paper was suspended in the vials. Reactions were stopped at 20, 40, and 60 min by injection of 100 µl of 1 N NaOH in the traps and 100 µl of 20% perchloric acid to the medium. 14CO2 formed from glucose oxidation was allowed to diffuse out of the acidified samples in the NaOH traps. The filter paper traps were counted after shaking for several hours in liquid scintillation fluid. The disintegrations per minute of carbon-14 trapped in NaOH are divided by milligram retinal protein and by the specific activity of [U-14C]glucose to provide values for glucose oxidized to 14CO2 per minute per milligram of protein.

**Formation of glutamate.** The chromatographic procedure using Dowex-1 acetate columns separates glutamine, glutamate, aspartate, and lactate by a stepwise elution with acetic acid (17). The tricarboxylic acid cycle intermediates remaining on the Dowex columns were estimated as the difference between the total counts in the sample and the counts that were eluted. [14C]glutamate was isolated on the column and quantitated by scintillation counting of the carbon-14. The disintegrations per minute of glutamate divided by glucose specific activity allows one to estimate glutamate synthesis from glucose. Loss of the carboxyl group of pyruvate before its entry in the tricarboxylic acid cycle makes a correction necessary. The disintegrations per minute are multiplied by 3/2 and then divided by glucose specific activity as an estimate of glutamate synthesis from glucose.

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Glyceraldehyde-3-phosphate dehydrogenase activity assay. Freshly dissected retinas from control and diabetic rats were sonicated in 0.1 M Tris·HCl buffer, pH 8.3, containing 1 mM EDTA and 1 mM dithiothreitol. Both retinas from each of six rats were employed in the assay. After centrifugation, supernatant was used to measure glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity according to the Byers (8) method with little modification. Briefly, the reaction mixture (1 ml) containing 0.04 M bicine, 0.8 M sodium acetate, 0.8 mM EDTA (pH 8.5), 1 mM NAD, and 25 mM sodium arsenate was incubated at 25°C for ~5 min, and the reaction was initiated by the addition of 1 mM freshly prepared glyceraldehyde 3-phosphate. The linear increase in absorbance at 340 nm during the first 30 s after addition of substrate was used to calculate enzyme activity, which was expressed as the rate of GAPDH enzyme activity in units per milligram of protein (GAPDH unit = 1 μmol NADH oxidized/min). A Beckman model DU640 spectrophotometer was used to make the NADH measurements.

Metabolite mass assays in retina and media extracts. The mass amounts of glutamate, glutamine, lactate, glucose 6-phosphate, and fructose 6-phosphate were determined enzymatically using standard fluorometric and spectrophotometric procedures (17, 37, 54).

Measurements of fructose 6-phosphate and glucose 6-phosphate. Fructose 6-phosphate was measured fluorometrically in neutralized perchloric acid extracts of control and experimental retinas plus medium (26). The assay utilized 0.8–0.9 ml of extract from ~0.35 mg retinal protein. The fructose 6-phosphate assay buffer contained 0.2 M triethanolamine, pH 7.6, 5 mM EDTA, and 0.2 mM NADP. Because the 5 and 20 mM glucose in the samples (which included medium) interfered with the assay, the samples were pretreated with 10⁴ units of glucose oxidase and incubated at 37°C for 1 h in a nitrogen atmosphere of 100% O₂ (maintaining constant volume). To assay glucose 6-phosphate, NADPH was generated when glucose-6-phosphate dehydrogenase (5 units) was added to the reaction mixture plus sample. Generation of the NADPH was measured fluorometrically in a custom-made fluorometer (C & L Instruments, Hummelstown, PA). The NADPH generated provides a measure of glucose 6-phosphate. When the reaction was complete, phosphoglucone isomerase (10 units) was generated provides a measure of glucose 6-phosphate. When the fluorometer (C & L Instruments, Hummelstown, PA). The NADPH oxidation of the NADPH was measured fluorometrically in a custom-made fluorometer (C & L Instruments, Hummelstown, PA). The NADPH oxidation of the NADPH was measured fluorometrically in a custom-made fluorometer (C & L Instruments, Hummelstown, PA). The NADPH oxidation of the NADPH was measured fluorometrically in a custom-made fluorometer (C & L Instruments, Hummelstown, PA). The NADPH oxidation of the NADPH was measured fluorometrically in a custom-made fluorometer (C & L Instruments, Hummelstown, PA). The NADPH oxidation of the NADPH was measured fluorometrically in a custom-made fluorometer (C & L Instruments, Hummelstown, PA). The NADPH oxidation of the NADPH was measured fluorometrically in a custom-made fluorometer (C & L Instruments, Hummelstown, PA). The NADPH oxidation of the NADPH was measured fluorometrically in a custom-made fluorometer (C & L Instruments, Hummelstown, PA). The NADPH oxidation of the NADPH was measured fluorometrically in a custom-made fluorometer (C & L Instruments, Hummelstown, PA).

Protein determinations. Protein pellets obtained by sonication and centrifugation of initial perchloric acid extract of retinas were assayed and generating a second increase in the amount of NADPH equivalent to the fructose 6-phosphate in the sample.

Polyols. To estimate the formation of ³H-polyols from [5-³H]glucose, the ³H-polyols were separated from other tritiated products, and disintegrations per minute in the radiolabeled material were measured. Small amounts of tritiated impurities in the commercial [5-³H]glucose interfered with accurate assessment of polyol formation. Therefore, commercial [5-³H]glucose was treated with hexokinase for 1 h, and the reaction mixture was applied to the Dowex acetate ion exchange column. The [5-³H]glucose 6-phosphate binds to the ion exchange resin, and impurities were washed away with excess distilled water and discarded. Finally, the [5-³H]glucose 6-phosphate on the column was treated with alkaline phosphatase. The enzyme converted the [5-³H]glucose 6-phosphate to [5-³H]glucose, which was eluted from the column with H₂O. This purified glucose was used for experiments in which polyols were measured. Retinas from rats that had been diabetic for either 3 wk or 3 mo were incubated for 40 min in 1 ml of the Krebs bicarbonate buffer substituting [5-³H]- for [U-¹⁴C]glucose. Incubations were stopped with the addition of perchloric acid, and extracts were sonicated, centrifuged, and neutralized with KOH. Aliquots (0.5 ml) were applied to the Dowex acetate columns, which removed [5-³H]glucose 6-phosphate and other anions. The columns were eluted with 5 ml H₂O. An aliquot of the 5-ml neutral fraction was counted, and the remainder was treated with hexokinase to phosphorylate [5-³H]glucose. The hexokinase-treated reaction mixture was applied to another anion exchange column. H₂O and ³H-polyols remain in the eluted neutral fraction, and an aliquot of that fraction was counted. After removal of H₂O by lyophilization, the lyophilate was reconstituted in 5 ml H₂O, and a 0.5-ml aliquot was counted. The ¹⁴H remaining was assumed to be mainly polyols. Blanks were run that had been incubated without retinas, and ¹⁴H disintegrations per minute remaining in the blank samples were subtracted from the experimental samples. A flow chart showing the methods for separation and analysis of metabolites is provided in Fig. 1. The production of H₂O by [5-³H]glucose was used to estimate total glycolysis, independent of lactate consumption by the mitochondria.

Flowchart for Analyses

![Flowchart for Analyses](image)

Fig. 1. Flow Chart for analyzing products of retinal incubations.
to obtain protein values for each incubation. Incubations employing half-retinas included ~0.4 mg retinal protein. NaOH (0.5 ml of 1 M) was added to the precipitated protein, and the solubilized protein was assayed immediately using the Bio-Rad reagent.

**Microwave fixation and metabolite extraction.** The microwave fixation technique for tissue extraction of the brain is far better than freezing the entire brain in liquid nitrogen because it overcomes the technical difficulty of achieving rapid access to brain tissue (14, 45). The rapidity of denaturation eliminates rapid postmortem changes in lactate and NADH.

Anaesthetized euglycemic and diabetic rats were placed in a holder and heads positioned within the SGF Venostat microwave chamber (Cober Electronics, Norwalk, CT). An irradiation time of 1 s at 4 kW was used, and this fixed and denatured the tissues. After irradiation, the retinas and a section of forebrain were dissected and placed (separately) in tared 0.5-ml Eppendorf centrifuge tubes containing 100–200 µl of ice-cold 0.55 M (3.6%) perchloric acid. The tissue was homogenized using a Minibead beater (Biotips Spec, Barleesville, OK) for 20 s at 4,200 beats/min, after which the homogenate was placed on ice for 15 min. Next, the sample was centrifuged for 2 min at 10,000 g in an Eppendorf microfuge. An aliquot of the acidic supernatant was transferred to another tube and neutralized using KHCO$_3$. Metabolites in neutralized extracts were analyzed enzymatically.

**Statistical Analysis.**

Values shown are means ± SE. These data were analyzed for significance by Standard Student’s $t$-tests and where appropriate, ANOVA, with $P < 0.05$ considered to be significant.

**RESULTS**

Studies were carried out using retinas from euglycemic control rats or rats that had been diabetic for either 3 wk or 3 mo. The initial incubations with both [U-14C]glucose and [5-3H]glucose were conducted using retinas from 3-wk diabetic rats. Subsequent studies of 3-mo diabetic rats were conducted with [5-3H]glucose in the media but not [U-14C]glucose. This made estimation of 3H-polyols more accurate, since the necessity for double-label counting was eliminated.

**Glucose Oxidation**

The production of 14CO$_2$ from [U-14C]glucose was measured in 3-wk diabetic and euglycemic retinas incubated with 5 or 20 mM glucose (Fig. 2, A and B, respectively). 14CO$_2$ production was linear between 20 and 60 min in retinas from euglycemic and diabetic rats but was significantly ($P < 0.01$) lower in retinas from diabetic animals exposed to 5 mM glucose. The difference disappeared when the level of glucose was increased to 20 mM. As shown in Fig. 2, C and D, [14C]glutamate increased in a nonlinear way but reflected differences seen in the 14CO$_2$ production. The [14CO$_2$] and [14C]glutamate data are related, since both are products of tricarboxylic acid cycle flux. Over the same time period, the mass amount of glutamate declined even in the presence of 20 mM glucose (Fig. 2, E and F). Thus, despite having excess glucose in the retina, diabetic animals oxidized less glucose to CO$_2$ than did euglycemic controls.

**Glycolysis**

To estimate glycolytic rates in retinas from age-matched euglycemic and 3-wk diabetic rats, 3H$_2$O, [14C]lactate, and lactate mass were quantified after 20-, 40-, and 60-min incubations in Krebs bicarbonate buffer containing either 5 or 20 mM glucose. 3H$_2$O, [14C]lactate, and lactate mass increased linearly between 20 and 60 min in all incubations. Total glycolysis ([3H]O) generated by euglycemic and diabetic retinas was the same, but hyperglycemia increased glycolytic flux by 16 and 25%, respectively, as shown in Table 1. Similarly, there was no significant difference ($P > 0.05$) between the amount of [14C]lactate produced by retinas from euglycemic rats compared with the amount produced by retinas from diabetic rats. However, an increase in glycolytic flux was observed in the presence of 20 mM compared with 5 mM glucose. The rates of production of lactate mass were similar under all conditions. To determine the effect of duration of diabetes, these experiments were repeated using 3-mo diabetic rats and their age-matched controls. All experiments with the older animals and some experiments with the younger rats were carried out with [5-3H]glucose but no [U-14C]glucose in the media. In those cases, samples were taken at time ($t$) = 0 and $t = 40$ min only. Linearity was assumed, and rates only for all data are shown in Table 1.

Table 1 summarizes all the in vitro data related to glycolysis using retinas from 3-wk and 3-mo diabetic and euglycemic rats. Values shown are rates in glucose equivalents. Thus, 3H$_2$O and [14C]lactate are divided by specific activity of glucose, and lactate mass is divided by two, since 1 mol glucose produces 2 mol lactate. The [14C]lactate production was linear and passed through the origin of the graph. Lactate mass also increased linearly, but the curve did not pass through the origin. The values of lactate at $t = 0$ are subtracted from the lactate assayed at 20, 40, and 60 min and then divided by two to obtain the rate of glucose converted to lactate. The difference between total glycolysis (3H$_2$O appearance) and [14C]lactate appearance is because of further metabolism of lactate and pyruvate. The difference between total glycolysis (3H$_2$O appearance) and [14C]lactate generation is slightly greater, under some conditions, than the formation of 14CO$_2$, especially in the younger rats. Some [14C]lactate synthesized may be used for synthesis of intermediates other than 14CO$_2$ (e.g., glutamate, glutamine, tricarboxylic acid cycle intermediates, etc.). It is clear that glycolytic flux declines as diabetes progresses from 3 wk to 3 mo, and, although raising medium glucose concentration to 20 mM normalizes glycolytic rates in retinas from 3-wk diabetic rats, it is unable to do so in retinas from rats that have been diabetic for 3 mo. This may be secondary to a time-dependent increase in oxidative damage to one or more glycolytic enzymes (23).

**Lactate-to-pyruvate ratio.** The cytosolic ratio of NADH/NAD reflects the availability of reducing equivalents generated by glycolysis in the cytosol, and it can be monitored as the ratio of lactate/pyruvate since lactate dehydrogenase catalyzes equilibration of the products and reactants of the lactate dehydrogenase reaction. The measurements of this parameter were carried out in neutralized perchloric acid extracts of control retinas and retinas from 3-wk and 3-mo diabetic rats incubated in vitro (Fig. 3). It is apparent that as diabetes progresses the cytosolic oxidation-reduction potential of NADH/NAD becomes more oxidized, not more reduced. This may be a result of the decrease in production of cytosolic NADH that occurs when glycolysis decreases.

Because retinal mitochondria may experience a lack of O$_2$ in vivo but not in vitro, we also measured lactate-to-pyruvate...
Table 1. Differences between glycolytic fluxes, rates of lactate appearance, and tricarboxylic acid cycle flux

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<th>Condition</th>
<th>Glucose, mM</th>
<th>Δ[3H]2O</th>
<th>Δ[14C]Lactate</th>
<th>ΔLactate mass</th>
<th>Δ[14CO2]</th>
<th>3H2O-[14C]Lact</th>
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<td>22.85±1.15</td>
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<tr>
<td>3 Month euglycemic</td>
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Values shown represent means ± SE; n = 8 determinations from 2 independent experiments. Δ[3H]2O, rate of generation of tritiated water from [5-3H]glucose; Δ[14C]Lactate, rate of generation of [14C]lactate from [U-14C]glucose; Δ[14CO2], rate of generation of [14CO2] from [U-14C]glucose; 3H2O-[14C]Lact, difference between total glycolysis (Δ[3H]2O) and the rate of generation of [14C]lactate (the glucose carbon generates products other than lactate); 3H2O-Lact Mass, difference between total glycolysis (Δ[3H]2O) and the rate of generation of unlabeled lactate (Lactate mass). These differences should equal Δ[14CO2], P < 0.05, significant difference between diabetic and its age- and glucose concentration-matched euglycemic control (•) and significant difference because of glucose concentration (†).
support the hypothesis that diabetic retinas are relatively hypoxic.

Glucose 6-Phosphate

The first step in glucose metabolism after its transport in cells is phosphorylation to glucose 6-phosphate. The in vitro levels of glucose 6-phosphate in excised retinas, incubated with 5 or 20 mM glucose, were determined in retinas from 3-wk or 3-mo diabetic rats and from their age-matched controls, as described in Methods. Measurements were made in neutralized perchloric acid extracts of retinas incubated for 40 min. The concentration of glucose in the incubation medium had no significant influence on glucose 6-phosphate levels in retinas from euglycemic or diabetic rats (Fig. 4). Retinal glucose 6-phosphate levels were not increased by incubation of retinas in 20 mM glucose as opposed to 5 mM glucose. However, retinas from rats that had been diabetic for 3 mo and were incubated in 20 mM glucose exhibited slightly higher glucose 6-phosphate levels than retinas of rats diabetic for only 3 wk and the age-matched euglycemic control rats that were incubated in 20 mM glucose. The glucose 6-phosphate levels were also measured in retinas in vivo. Levels of retinal glucose 6-phosphate are lower in vivo than in vitro and lower in the in vivo retinas than the in vivo brain (Table 2). The in vivo glucose 6-phosphate levels were slightly, but significantly, higher in the diabetic animals. This is true even though tissue glucose levels were much higher in the retinas of diabetic compared with euglycemic rats. This demonstrates important control of glycolytic flux at hexokinase, rather than transport.

Fructose 6-Phosphate

Fructose 6-phosphate is a branch point in glycolysis and acts as the precursor for both fructose 1,6-bisphosphate and for UDP-N-acetylglucosamine and then glucosamine. The glucosamine pathway mediates many of the adverse effects of glucotoxicity, and enhanced flux through this pathway has been proposed to lead to diabetic complications. The levels of fructose 6-phosphate in retinas from rats that had been diabetic for 3 wk or 3 mo were assayed after 40 min incubation with 5 or 20 mM glucose (Fig. 5). After 3 wk of diabetes, fructose 6-phosphate was not increased significantly in retinas of diabetic compared with euglycemic rats. After 3 mo of diabetes, the levels of fructose 6-phosphate in the retina were significantly higher compared with retinas of rats that had been diabetic for only 3 wk. However, they were not higher than retinas from their own age-matched euglycemic controls. The fructose 6-phosphate levels were also measured in vivo in 3-wk diabetic rats, and, although the in vivo values were lower than in vitro, the values measured in vivo in diabetic rats were not significantly higher than those measured in vivo in euglycemic rats (Table 2). Because fructose 6-phosphate is a precursor of glucosamine, the data argue against a role for the glucosamine pathway as a mediator of hyperglycemia-induced toxicity.

Polyl Formation

The enzyme aldose reductase normally converts certain toxic aldehydes to nontoxic alcohols. However, under hyperglycemic conditions, it converts glucose to sorbitol. In doing so, it has been proposed that the enzyme consumes NADPH, which may leave cells vulnerable to oxidative stress. To measure polyl content as an indicator of pathway activation, we purified commercially available [5-3H]glucose as outlined in Methods. Retinas from diabetic and euglycemic rats were incubated for 40 min in Krebs bicarbonate buffer containing 25 μCi purified [5-3H]glucose plus either 5 or 20 mM glucose.

Table 2. In vivo metabolic levels in euglycemic and diabetic retinas

<table>
<thead>
<tr>
<th>Retina</th>
<th>[Glucose], nmol/mg protein</th>
<th>[G-6-P], nmol/mg protein</th>
<th>[F-6-P], nmol/mg protein</th>
<th>[Pyr], nmol/mg protein</th>
<th>[Lact], nmol/mg protein</th>
<th>L/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euglycemic</td>
<td>59.0±7.5</td>
<td>0.68±0.04</td>
<td>0.20±0.02</td>
<td>2.40±0.34</td>
<td>40.5±5.0</td>
<td>17.1±0.9</td>
</tr>
<tr>
<td>Diabetic</td>
<td>232.3±22.4*</td>
<td>0.98±0.09*</td>
<td>0.27±0.05</td>
<td>2.47±0.02</td>
<td>39.8±1.1</td>
<td>16.2±0.6</td>
</tr>
<tr>
<td>Brain</td>
<td>47.1±4.3</td>
<td>1.88±0.09</td>
<td>0.39±0.04</td>
<td>0.36±0.02</td>
<td>8.2±0.5</td>
<td>22.7±1.1</td>
</tr>
<tr>
<td>Diabetic</td>
<td>110.8±7.1*</td>
<td>2.22±0.14</td>
<td>0.52±0.05</td>
<td>0.44±0.01</td>
<td>8.9±1.0</td>
<td>18.1±2.4</td>
</tr>
</tbody>
</table>

Values shown represent means ± SE, n = 6 experiments. Three-week diabetic rats were anesthetized and subjected to microwave irradiation, as described in Methods. Metabolism was stopped within 100 ms by the irradiation. Neutralized perchloric acid extracts of these retinas and forebrains were assayed for glucose, glucose 6-phosphate (G-6-P), fructose 6-phosphate (F-6-P), pyruvate (Pyr), and lactate (Lact). Brackets denote concentration. The lactate/pyruvate ratio (L/P) is also shown. *P < 0.05, diabetic different from euglycemic.

AJP-Endocrinol Metab • VOL 290 • JUNE 2006 • www.ajpendo.org
The tritium-labeled polyol fraction was isolated, and disintegrations per minute of polyols were compared with blanks incubated without retinas. The disintegrations per minute of 3H-polyols minus the blank were divided by specific activity of glucose to obtain values for nanomoles glucose converted to polyol (sorbitol). The data are summarized in Fig. 6. As expected from the kinetic parameters and specificity of aldose reductase, polyols in the retinal extracts were much higher when retinas were incubated with 20 mM glucose as opposed to 5 mM. It is also apparent from Fig. 6 that retinas from diabetic rats produced more polyols than retinas from euglycemic rats. Also, rats that had been diabetic for 3 mo produced more polyols than rats diabetic for 3 wk. The same age-dependent trend is apparent in euglycemic rats but to a lesser extent. These data are consistent with a role for polyols in rodent diabetic retinopathy.

GAPDH Activity

GAPDH activity is sensitive to oxidative damage, and the observation that its activity is low in endothelial cells exposed to high glucose has been used as evidence for ROS damage secondary to exposure of endothelial cells to excess glucose (15). However, measurements of the GAPDH activity in whole retinas of diabetic rats have not been reported. To assess possible oxidative damage in diabetic retinas in situ, we measured the GAPDH activity in freshly dissected rat retinas from 3-mo diabetic rats and their age-matched controls. Interestingly, in diabetic retinas, a significant decrease (22.3%) in GAPDH activity was observed compared with control retinas, as shown in Fig. 7.

DISCUSSION

Purpose of the Study

Major objectives of this study were to evaluate changes in metabolic pathways that occur as a result of the diabetic state and identify changes likely to cause long-term damage to retinas.

An excess of ROS (e.g., superoxide anions and H2O2) has been detected in tissues of experimental animals with diabetes, and in cultured retinal Müller cells and cultured vascular endothelial cells (16, 31, 41). The potential role of ROS in the pathology of diabetes is well documented, but the origin of the excess oxygen free radicals is not well established (3, 7, 21,
and in complex I (36), can form free radicals and initiate electron transport chain, for example in complex III the mitochondria is high (51). Coenzyme Q in the mitochondria is a single step (12), thus precluding ROS formation. However, this NADH can reduce O2 to H2O (51). Cytochrome oxidase is the final member in the electron transport chain. Its heme group, like Hb, binds molecular oxygen at very low oxygen pressures. The protein's structure and its copper- and iron-binding sites are such that transfer of four electrons to oxygen along with protons reduces O2 to H2O in what is almost a single step (12), thus precluding ROS formation. However, this protective mechanism can fail if delivery of electrons to the mitochondria is too rapid or if the oxygen tension surrounding the mitochondria is high (51). Coenzyme Q in the mitochondrial electron transport chain, for example in complex III (10, 16) and in complex I (36), can form free radicals and produce ROS when O2 tension is high. The half-life of quinone-free radicals can increase when NADH delivery is excessive (36, 51).

Evaluation of Glucose Metabolism in Relation to ROS Production

The proposal has been made that high serum glucose levels, common in diabetic patients and experimental animals with diabetes, can increase intracellular glucose levels, and this induces an increase in the rate of glycolysis. If this were true, increased substrate delivery to the mitochondria could increase accumulation of ROS (7, 23, 42).

Our in vivo data show that diabetes causes a profound increase in retinal intracellular glucose, much more so than in the brain. However, the in vitro glycolytic flux data and the in vivo measurements of glucose 6-phosphate, fructose 6-phosphate, and pyruvate show that the rate of glycolysis is not proportionally increased by the increases in glucose. Moreover, when the condition is prolonged from 3 wk to 3 mo, the rate of glycolysis decreases. Because the measured decline in glycolytic flux in the diabetic compared with the euglycemic state is accompanied by a decrease in the tissue levels of lactate and pyruvate and by an increase in hexose phosphates, one could conclude that an enzyme or enzymes between phosphofructokinase and pyruvate kinase are impaired or downregulated. Our measurements of GAPDH activity in euglycemic and diabetic retinas confirm this possibility, and by implication it confirms oxidative damage to diabetic retinas.

Measurements of glucose oxidation to CO2 and glutamate indicate that the flow of electrons through the electron transport chain is not increased. This is perhaps attributable to the lower levels of pyruvate in the tissue rather than to hypoxia or pseudohypoxia (26). The unchanged ratios of tissue lactate-to-pyruvate both in vivo and in vitro rule out those possibilities. The measurements of lactate-to-pyruvate ratios and the rate of glucose oxidation to CO2 do not support the concept that the mitochondria in diabetic retinas produce excessive amounts of ROS.

Another potentially important source of ROS is provided by oxidation of cytosolic NADH by NADH oxidase (38, 50). Moreover, increases in flux through the polyol pathway may deplete NADPH, since the first step in the polyol pathway is reduction of glucose to sorbitol by NAPDH. The depletion of NADPH, which maintains the level of glutathione in the reduced state, may allow H2O2 levels to rise (11, 28, 34, 47). Aldose reductase is the first and rate-limiting step in the polyol pathway. The activation of the polyol pathway and the depletion of NADPH may make the retina vulnerable to oxidative stress, thereby accelerating retinopathy.

Another source of free radicals are the microglia, which in neural and retinal tissue subserves the function of macrophages as the primary immune cell. When activated, microglia export toxins and cytokines to kill bacteria. Our own data in fact show that, in diabetes, activation of these macrophage-like cells can cause neuronal apoptosis (33, 40). To test another possibility, we evaluated the formation of tritiated polyols from tritiated glucose. Excess formation of polyols (sorbitol) via aldose reductase may lower cytosolic NADPH, possibly leading to depletion in glutathione and accumulation of H2O2. Our estimates of polyols provide evidence for increased flux through this pathway in diabetic rats. Retinas incubated with 20 mM glucose had much higher polyol levels than those incubated with 5 mM glucose. Moreover, retinas from diabetic rats had higher polyol levels than those from euglycemic rats. Rats that had been diabetic for 3 mo had higher polyol levels than those that had been diabetic for only 3 wk.

The amounts of polyols and the influence of medium glucose on those amounts are very similar to those observed recently by Dagher and coworkers (13) in euglycemic human retinas in organ culture with 5 or 30 mM glucose. Indeed, Dagher et al.’s study revealed similar rates of high-glucose-induced polyol formation in rat and human retinas. Our studies and those of Dagher et al. suggest that there is an increase in the polyol synthetic pathway in the diabetic retina, which can deplete NADPH levels. However, clinical studies of the effect of aldose reductase inhibitors over the past few years have been negative and argue against a role for aldose reductase in the pathology of diabetic retinopathy (22).

Relationship of the Present Data to Those of Other Workers

Recent studies by Brownlee and coworkers (7, 15, 42) have used bovine aortic vascular endothelial cells from euglycemic animals to study toxicity resulting from hyperglycemia. These
workers provide compelling data that show that high levels of glucose (30 mM) in cultured aortic vascular cells produce increased levels of ROS, increased flux through the aldose reductase pathway, increased glycolysis, and increased oxidation of glucose to CO$_2$. These results should be interpreted with some caution since microvessels may alter their capacity to transport and metabolize glucose when placed in culture (5). Nevertheless, the results may be relevant to glucose toxicity in vascular endothelial cells of the retina.

Later studies by the same group (23) emphasize the role of glyceraldehyde phosphate dehydrogenase in the glucose-induced toxicity imposed on endothelial cells. The excess amounts of ROS generated by hyperglycemia damaged glyceraldehyde phosphate dehydrogenase and produced a blockade of carbon flow in the glycolytic pathway. This caused increases in fructose 6-phosphate and glyceraldehyde 3-phosphate in the vascular cells. By adding a thiamine analog (benzotiamine) to the incubation mixture, they converted excess fructose 6-phosphate (via thiamine-dependent transketolase) to pentose phosphate. This decreased the damage to the cells by shunting carbon away from synthesis of glucosamine and methyl glyoxal (a precursor for intracellular glycation). The data are reminiscent of our in vitro studies of the decrease in glycolysis after 3 mo of diabetes and the concomitant rise in glucose 6-phosphate. Our data are consistent with an inhibition or decrease in activity of glyceraldehyde phosphate dehydrogenase in the whole retina after 3 mo of diabetes.

Therefore, conclusions drawn from our present studies do not contradict those of Hammes and coworkers (23) since their data were generated from vascular endothelial cells. The vascular cells of the intact retina most likely experience oxidative stress, and, since glucose entry in and out of vascular endothelial cells is so high, these cells may experience higher cytosolic glucose levels than other retinal cells. However, isolated endothelia lack the presence of cells that utilize glucose, so the whole retina studies may be more relevant to human disease.

Kern and coworkers (16, 30, 31) have studied intact excised retinas, cultured retinal vascular endothelial cells, and Müller cells in an effort to evaluate the extent of ROS production and their pathological impact. These studies all report the presence of oxidative damage in the retina. The most recent study reports ROS production measured luminometrically in isolated cultured vascular cells, Müller cells, and intact retinas from control and diabetic rats. Hyperglycemia increased the rate of ROS production in the cultured cells. However, the incubation media of intact retinas did not include any added glucose or other substrate. ROS production was faster in retinas from diabetic rats than from control retinas. Moreover, an inhibitor of mitochondrial complex II decreased ROS production more in the diabetic cells than control cells. This was interpreted to mean that the mitochondria were the source of the ROS. An alternate interpretation might be that the diabetic cells retained excess glucose fueling the increased electron flow through the mitochondria of the diabetic retinas and therefore increased ROS production.

Our data conflict with several laboratories who report elevated ratios of lactate to pyruvate in retinas taken rapidly from diabetic rats compared with those taken from control rats. The ratios they measure are higher than we measure in vitro or in vivo in diabetic rats (24, 44, 49). Although these workers intended to measure in vivo values, the procedures used involved removal of retinas from experimental animals followed by rapid freezing or treatment with perchloric acid. The surgical procedures used were probably not fast enough to prevent anoxia from developing before metabolism was stopped. As we demonstrate here, when very rapid methods are used to quench metabolism in <1 s, no evidence of high lactate-to-pyruvate ratios is found in retinas from diabetic rats. The microwave irradiation method we employed to measure volatile metabolites in situ is thought to be the most reliable for compounds that have rapid metabolic turnover times (14, 45). In vitro retinas recover metabolically from removal from the animals during the 3-min preincubation period (37, 56). Subsequent metabolic quenching by addition of perchloric acid to the in vitro medium is almost instantaneous.

Van den Enden et al (52) report a high ratio of lactate/pyruvate in euglycemic retinas incubated with high glucose and refer to this as a pseudohypoxic state. Other workers have questioned their conclusions and in particular the methodology used to generate the high lactate-to-pyruvate ratios (55).

In summary, our data showed that, despite high glucose levels and duration of diabetes, glycolysis did not increase. Tricarboxylic acid cycle flux was also unaffected by high glucose in both euglycemic and diabetic retinas, suggesting that excess ROS are not generated by mitochondria. Our data do not support the pseudohypoxia hypothesis of Nyengaard et al. (43) and Williamson et al. (53) because neither hyperglycemia nor diabetes caused an increase in the lactate-to-pyruvate ratio. The duration of diabetes and exposure to high glucose had little influence on glucose 6-phosphate and fructose 6-phosphate or the glucosamine pathway in the excised retina, suggesting that these pathways are not major sources of oxidative stress. Measurements of polyols gave evidence of increased flux through the polyol pathway as the duration of diabetes increased and as hyperglycemia developed. Thus the polyol pathway may deplete NADPH and contribute significantly to oxidative stress in diabetic retinopathy of the rat. However, clinical studies of aldose reductase inhibition in human subjects suffering from diabetes argue against this possibility in humans (22). The slow glycolysis seen in rats after 3 mo of streptozotocin-induced diabetes may be because of oxidative damage to glyceraldehyde phosphate dehydrogenase and a subsequent decrease in its activity.

Thus we provide little support for major pathways of glucose toxicity except for the production of excess polyols and oxidative stress. ROS may be generated by microglia and their half-lives prolonged by low NADPH levels while other processes such as glutamate excitotoxicity (37) may tip the scale toward neuronal apoptosis (40).

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REFERENCES


