Insulin sensitivity and sorbitol production of the normal rabbit corneal epithelium in vitro

Judith Friend, Robert C. Snip, Timothy C. Kiorpes, and Richard A. Thoft

Insulin-depleted, normal rabbit corneal epithelium was incubated in vitro in tissue culture medium containing high concentrations of glucose (35 mM). These short-term incubations showed that the epithelium took up glucose equally whether or not insulin had been added to the medium, indicating that corneal epithelium is an insulin-insensitive tissue. Sorbitol accumulation showed that the normal corneal epithelium has a sorbitol pathway which can be activated in the presence of high intracellular glucose. The low level of sorbitol accumulation in these normal epithelia is probably not osmotically significant.

Key words: rabbit, insulin-sensitivity, corneal epithelium, glucose, sorbitol, osmotic cell damage

Several clinical studies have recently pointed out that the corneal epithelium may be at risk in patients with diabetes mellitus. A close correlation between diabetes and epithelial abnormalities such as superficial vascularization and defect formation has been noted. In addition, delayed epithelial healing has been measured in some diabetic experimental animals (rats), although this has not been shown for well-controlled diabetic humans or diabetic rabbits (Thoft and Friend, unpublished data).

The mechanism responsible for the epitheliopathy remains unclear. A neurotrophic cause is suggested by the observation that diabetics have decreased corneal sensitivity and by the finding of sterile ulcers in diabetic corneas. However, it is also possible that the mechanism of sorbitol pathway activation that has been proposed both for diabetic cataract formation and for the reduction of peripheral nerve conduction velocity in diabetes may be involved in corneal epithelium. It has been suggested in those systems that an excess of extracellular glucose in diabetes results in an excess of intracellular glucose with resultant activation of the sorbitol pathway. Sorbitol, produced from glucose in the presence of aldose reductase, accumulates intracellularly since the cell walls are impermeable to such sugar alcohols. This creates an osmotic imbalance. An influx of extracellular water to counteract the osmotic imbalance may then cause cataractous changes in the lens or conduction abnormalities in peripheral nerve.

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Fig. 1. Gas chromatograph of (A) sugars in a standard solution containing xylose, fructose, galactose, glucose, sorbitol, and inositol and (B) a sample of corneal epithelium after 24 hr of incubation in tissue culture medium containing 35 mM glucose. The fructose peak is at 27.2, the glucose peaks are at 33.0 and 44.2, the sorbitol peak is at 38.6, and the inositol peak is at 54.3.
Aldose reductase has been found in corneal epithelium of several animals.4,12 (2) Delayed epithelial healing in rats can be corrected by application of topical aldose reductase inhibitors.4 (3) An increased sorbitol level was found in corneal epithelium of diabetic patients when compared to normals.3

However, activation of the sorbitol pathway usually requires a high concentration of intracellular glucose. Tissues which do not require insulin for the entry of glucose into the cells, termed “insulin-insensitive,” are more apt to reflect ambient glucose levels than are “insulin-sensitive” tissues that do require insulin for entry of glucose into the cells. Thus insulin-insensitive tissues, e.g., lens and nerve, may accumulate high levels of cellular glucose when exposed to high concentrations of extracellular glucose.8–11, 13, 19 Such insulin-insensitive tissues may therefore be more subject to damage caused by high levels of intracellular glucose or products of the metabolism of that glucose, including such compounds as sorbitol. It has been observed, in general, that insulin-insensitive tissues do fare worse in diabetes than do insulin-sensitive tissues.9

The work reported here establishes that the rabbit corneal epithelium is an insulin-insensitive tissue, by measuring the effect of insulin on the uptake of glucose by insulin-depleted corneal epithelium in vitro. Furthermore, we have established that the rabbit corneal epithelium has the in vitro capability of producing sorbitol.

Methods

**Tissue preparation.** Rabbits, weighing 2.5 to 3.5 kg, fasted for 24 hr to reduce endogenous insulin, were killed by an overdose of sodium pentobarbital followed by air embolism. Corneal-scleral rings and, in some cases, 1 to 5 mg (dry weight) pieces of skeletal muscle, were excised and placed in 10 ml TC-199 tissue culture medium containing 500 U/ml penicillin and 500 µg/ml streptomycin, with the glucose level adjusted to 35 mM. The osmolarity of the medium was 350 mOsm, whereas that of unaltered TC-199 was 315 mOsm. Fresh regular insulin (made from beef and pork zinc insulin crystals, Lilly) was added to some of the samples to a final concentration of 0.1 or 1.0 U/ml culture medium. The tissues were then incubated for 1 to 24 hr at 37° C in an O2-CO2 incubator, after which they were thoroughly rinsed in five changes of cold saline, and the corneal epithelium was scraped off the ocular samples with a No. 15 blade. The epithelium and muscle samples were then frozen, lyophilized, and weighed. Epithelial samples ranged in dry weight from 0.28 to 1.6 mg, with an average of 0.98 ± 0.03 (n = 87). Glycogen or glucose, sorbitol, and fructose were then measured as described below.

**Biochemical analyses.**

**Hydration.** It was not possible to obtain accurate hydration measurements on the incubated corneal epithelial samples. Therefore a normal hydration (milligram of water per milligram of dry weight) of 3.5 was assumed for the calculations converting the concentrations of glucose or sorbitol per gram dry weight to concentrations per milliliter of tissue water.

**Glycogen.** Dried tissue samples were extracted in 1 ml of 20% sodium hydroxide. Glycogen was extracted with absolute ethanol and hydrolyzed in 2N sulfuric acid for 90 min, followed by neutralization, according to the method of Reim and Cattepoel.14 Glucose was then measured by the hexokinase reaction.15 Glycogen is expressed as micromoles per gram dry weight of tissue.

**Glucose in the medium.** This was measured directly by the hexokinase reaction.15

### Table I. Muscle glucose and glycogen content after incubation in tissue culture medium with 35 mM glucose

<table>
<thead>
<tr>
<th>Hours incubated</th>
<th>Glucose (µmol/gm dry weight)</th>
<th>Glycogen (µmol/gm dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With insulin (0.1 U/ml)</td>
<td>Without insulin</td>
<td>p value</td>
</tr>
<tr>
<td>0</td>
<td>11.3 ± 0.6 (6)</td>
<td>184 ± 0 (5)</td>
</tr>
<tr>
<td>1</td>
<td>67 ± 1 (3)</td>
<td>48 ± 2 (3)</td>
</tr>
<tr>
<td>3</td>
<td>119 ± 6 (5)</td>
<td>102 ± 5 (5)</td>
</tr>
<tr>
<td>6</td>
<td>119 ± 6 (5)</td>
<td>102 ± 5 (5)</td>
</tr>
</tbody>
</table>

Values are averages ± S.E.M., with the number of determinations in parentheses. Tissues had been incubated 0, 1, 3, or 6 hr in TC-199 with 35 mM glucose with or without 0.1 U/ml insulin. The p values compare values with or without insulin.
**Fig. 2.** Corneal epithelial glucose after incubation in TC-199 with 35 mM glucose. The glucose content is plotted as averages of micromoles per gram dry weight of epithelium ± S.E.M., with the number of determinations in parentheses. There is no difference between samples incubated with 0.1 U/ml added insulin and those incubated without added insulin.

**Table II.** Corneal epithelial glucose and sorbitol levels after 4½ hr incubation in tissue culture medium with 35 mM glucose

<table>
<thead>
<tr>
<th>Insulin (U/ml of medium)</th>
<th>Glucose (µmol/gm dry weight)</th>
<th>Sorbitol (µmol/gm dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>75.1 ± 3.8 (9)</td>
<td>2.1 ± 0.2 (9)</td>
</tr>
<tr>
<td>0.1</td>
<td>71.8 ± 6.6 (4)</td>
<td>2.1 ± 0.2 (4)</td>
</tr>
<tr>
<td>1.0</td>
<td>75.3 ± 7.1 (5)</td>
<td>2.5 ± 0.6 (4)</td>
</tr>
</tbody>
</table>

Values are given ± S.E.M., with the number of determinations in parentheses. After 4½ hr of incubation, there were no significant differences in glucose or sorbitol levels between tissues incubated with no insulin or with 0.1 or 1.0 U/ml insulin.

**Glucose, fructose, and sorbitol in tissue samples.** These sugars were measured in single epithelial or muscle samples by gas chromatography. Prior to tissue preparation, inositol (10 nmol) was added to each sample as an internal standard. Inositol has not been previously reported and was not detected in normal corneal epithelial samples by using our method. Therefore, even if it is present, it is there in such small amounts in epithelium that it would not interfere with our use of inositol as an internal standard. Samples were extracted by sonification of the sample in 0.2 ml of water followed by removal of the protein with chloroform-methanol (2:1). The aqueous phase, containing the sugars, was lyophilized, and the dry residue was then stored in a vacuum desiccator over phosphorous pentoxide for at least 24 hr or until derivatization. Tissues were derivatized for 90 min in 200 µl of a trimethylsilyl reagent with pyridine (Sigma Sil A), followed by redrying and resuspension in hexane to a final volume of 25 µl. Injection volume was 5 to 10 µl.

The gas chromatograph used was an FID Hewlett-Packard 5830A. The column was ¼ inch by 6 ft, packed with 100/200 mesh Gaschrom Q coated with 3% silicone GE SE-30. The detector temperature was 290° C, injection temperature was 250° C, oven temperature was 140° to 200° C at 1°/min (except 0.01°/min from 34 to 44 min), and carrier gas (nitrogen) flow rate was 30 ml/min. Approximate retention times were fructose, 27.2 min; α-glucose, 33.0 min; β-glucose, 44.2 min; sorbitol, 38.5 min; and inositol, 54.2 min. Inositol recovery was 84% ± 7 in 20 consecutive samples. Levels as low as 0.1 µmol/gm dry weight of tissue were detectable.

**Results**

Samples of skeletal muscle, an insulin-sensitive tissue, were run as controls for the methods used. Table I shows that it was possible to differentiate samples incubated with insulin from those incubated without, on the basis of the glucose and glycogen content. Muscle incubated with insulin had higher levels of glucose and glycogen than muscle incubated without insulin, although the tissues were rapidly losing glycogen during the incubation.

Glucose accumulated linearly ($r^2 = 0.93$) in the incubated rabbit corneal epithelium for the first 6 hr (Fig. 2 and Table II). During that time, the glucose levels at any given time were the same whether the tissue had been incubated with or without added insulin in the high-glucose medium. By 6 hr, assuming a normal hydration of 3.5, the glucose concentration in the tissue was in equilibrium with that in the medium and remained so up to 18 hr. By 24 hr, however, the glucose levels in the tissue had decreased.

Sorbitol could not be detected in the unincubated samples or in samples incubated only a short time. After 3 hr, sorbitol could be detected in the corneal epithelial cells.
The sorbitol levels were the same whether insulin had been added to the medium or not (Fig. 3 and Table II).

Fructose was found in none of the samples incubated for short terms and only in some of the 18 and 24 hr samples, in any of which it was no higher than 2.1 μmol/gm dry weight. Fructose could not be detected (i.e., was less than 0.1 μmol/gm dry weight) in three of the six 18 hr samples and in six of 10 of the 24 hr samples. Again, no influence of insulin was found.

Glycogen levels were unrelated to the presence or absence of insulin. They were normal for the first few hours of incubation but by 24 hr were only about 60% of normal (Table III).

Discussion

The incubated corneal epithelial samples took up glucose and produced sorbitol for at least 18 hr. By 24 hr, there was a loss of glucose from the samples, which may reflect a washout when the tissues were rinsed during preparation, an indication that the cells were deteriorating and becoming leaky by that time. Further evidence that by 24 hr the tissues were no longer healthy is the decreased glycogen (to 60% of normal) and the failure to produce (or retain) more sorbitol. However, the epithelium may be assumed to be functioning normally for the first few hours of incubation, as has been demonstrated for this system in the past and confirmed here by the relative retention of glycogen (up to 76% of normal) and by the continued production of sorbitol for the first 6 hr.

If, on that basis, we further assume a normal epithelial hydration of 3.5 for the first few hours, we can calculate that the tissue glucose was in equilibrium with the medium—about 34 mM at 6 and 18 hr. However, the incubation medium is hyperosmotic, and it is possible the cells would dehydrate to match the osmolarity of the medium. If a 15% reduction in cell hydration occurred (to match the 15% increase in tissue culture medium osmolarity vs. normal), the glucose concentration at 6 and 18 hr would be 39 mM, a level which is not significantly different from the surrounding medium, indicating no active concentration of glucose by the epithelial cells.

More importantly, however, at no time was there a difference in glucose, sorbitol, or glycogen levels between the epithelial samples incubated with and those incubated without added insulin (Figs. 2 and 3, Tables...
Table IV. Sorbitol levels in corneal epithelium

<table>
<thead>
<tr>
<th>Hours incubated</th>
<th>μmol/gm dry weight</th>
<th>mmol/L of tissue water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Not detected (4)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Not detected (6)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.8 ± 0.2 (7)</td>
<td>0.23</td>
</tr>
<tr>
<td>4½</td>
<td>2.0 ± 0.1 (8)</td>
<td>0.58</td>
</tr>
<tr>
<td>6</td>
<td>2.5 ± 0.3 (11)</td>
<td>0.71</td>
</tr>
<tr>
<td>18</td>
<td>4.4 ± 0.7 (6)</td>
<td>1.26</td>
</tr>
<tr>
<td>24</td>
<td>3.7 ± 0.5 (10)</td>
<td>1.06</td>
</tr>
</tbody>
</table>

Sorbitol levels in corneal epithelium after incubation in TC-199 with 35 mM glucose. Values are expressed as μmol/gm of dry weight, averages ± S.E.M. with the number of determinations in parentheses or as mmol/L calculated from μmol/gm of dry weight assuming a corneal epithelial hydration of 3.5. Since there was no difference between samples incubated with or without insulin (0.1 U/ml), those values are pooled.

*After incubation in tissue culture medium containing 35 mM glucose.
†Calculated from μmol/gm dry weight assuming a corneal epithelial hydration of 3.5.

II and III). This indicates that unlike muscle (in which it is possible to differentiate between tissue samples incubated in insulin and those incubated without insulin [Table I]), the corneal epithelium is an insulin-insensitive tissue not requiring insulin for the entry of glucose into the cells. As such, it is similar to lens and nerve, both of which also seem to be insulin-insensitive.8-10, 13, 19 This lack of insulin sensitivity makes it likely that the corneal epithelium will readily reflect ambient glucose levels. Indeed, it did equilibrate its glucose levels with those in the medium in about 6 hr (Fig. 2). Thus it is possible that high levels of extracellular glucose may result in high levels of intracellular glucose, which in turn might lead to an accumulation of glucose metabolites, including sorbitol and fructose, products of the sorbitol pathway.

In lens, increased levels of sorbitol and fructose have been found in diabetic humans and animals and can be induced by incubation of the tissue in medium containing high concentrations of glucose (35 mM). The in vitro intracellular sorbitol accumulation in the lens, up to 15 mM in 1 day and 40 mM in 4 days, has been correlated to increased wet weight and to cataractous changes.8, 10, 13

Corneal epithelium in the short-term incubations reported here, however, showed far less dramatic increases in sorbitol after 24 hr than were seen in the lens studies, and virtually no accumulation of fructose was measured. Sorbitol levels were increased during incubation approximately 5.5-fold from 3 to 18 hr, but the levels are still very low and much lower than was observed in lenses at comparable times (Table IV).8, 10 The osmotic effect of the amount of sorbitol measured can be judged only by determining the concentration of the molecule in the tissue water. If we assume that the sorbitol is uniformly distributed in the epithelium and that the epithelial hydration is normal (3.5), the sorbitol concentration at its peak is only about 1.3 mM, or 1.3 milliosmolar (Table III, 18 hr). This seems unlikely to be osmotically significant. Other studies also found little evidence for a highly active sorbitol pathway.

Measurement of sugar levels in normal whole rat cornea after incubation in high glucose medium (35 mM) showed fructose accumulation but little sorbitol accumulation.20 It is possible, however, that future studies will demonstrate enhanced sorbitol accumulation in diabetic corneal epithelium. Analysis of nonincubated diabetic lenses showed higher sorbitol levels than are found in normal lenses. This may be the result of either a chronically elevated blood sugar or an increased ability of diabetic tissues to accumulate polyol in the presence of high levels of extracellular sugar.21-23

However, unless either the total intracellular sorbitol accumulation reported here is confined to only a portion of the cells or the small increase interferes directly with cell function (e.g., by altering pyridine nucleotide ratios), it is unlikely that activation of the sorbitol pathway causes profound osmotic alteration of the normal rabbit corneal epithelial cells in the presence of high concentrations of external glucose.

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REFERENCES