

# Glucose-Specific Regulation of Aldose Reductase in Human Retinal Pigment Epithelial Cells In Vitro

Douglas N. Henry,<sup>1,2</sup> Robert N. Frank,<sup>3</sup> Seth R. Hootman,<sup>1</sup> Sandra E. Rood,<sup>1</sup> Charles W. Heilig,<sup>4</sup> and Julia V. Busik<sup>1</sup>

**PURPOSE.** To test the hypothesis that pathophysiological levels of glucose regulate aldose reductase (AR2) gene expression, protein production, and activity in human retinal pigment epithelial (RPE) cells in vitro.

**METHODS.** Primary cultures of human RPE cells were grown for up to 72 hours in media supplemented with various concentrations of glucose (5, 20, or 75 mM), or in 5 mM glucose containing media supplemented with one of the following: galactose, the transported but nonmetabolized glucose analogue 3-O-methylglucose (3-OMG), or the impermeant hexitol mannitol—so that the final hexose concentrations were equimolar to those of the various glucose concentrations used. Changes in the transcript levels for AR2 mRNA, AR2 protein content, and AR2 enzyme activity were determined. RPE glucose utilization and lactate production were determined in media containing 5 and 20 mM glucose.

**RESULTS.** Glucose utilization and lactate production increased 4.8-fold and 4.4-fold, respectively, when RPE cells were grown in media containing 20 mM versus 5 mM glucose. Glucose was more effective than any other hexose in the induction of AR2 mRNA or increased AR2 protein expression. When RPE cells were grown in media containing 20 mM mannitol, 3-OMG, or galactose they had lower levels of AR2 mRNA expression than when cells were grown in medium containing 5 mM glucose. RPE cells grown in medium supplemented with 20 or 75 mM galactose did not show a greater increase in AR2 protein expression than cells grown in medium containing 5 mM glucose. Hyperosmotic induction of AR2 mRNA was the same in medium containing 75 mM glucose or 75 mM mannitol, but was at least 50% lower when RPE cells were grown in 75 mM galactose or 3-OMG.

**CONCLUSIONS.** These data indicate that elevations in ambient glucose result in greater metabolism of glucose through glycolysis and polyol metabolism. Induction of AR2 was greatest when RPE cells were grown in pathophysiological concentrations of glucose. Hyperosmolar stress is not a necessary determinant of AR2 mRNA, AR2 protein, or AR2 protein activity in cells that form the outer blood-retinal barrier. Increased facilitative glucose transport or glucose metabolism appears to be requisite for glucose-specific and nonosmotic regulation of AR2 in the RPE cell in vitro. (*Invest Ophthalmol Vis Sci.* 2000;41:1554-1560)

**A**ldose reductase (AR2, EC1.1.1.21) has been implicated in the development of long-term complications in diabetes for more than a quarter century<sup>1,2</sup>; yet, until recently, little has been reported about AR2 regulation by

pathophysiological concentrations of glucose.<sup>3,4</sup> Much of the present understanding of AR2 gene regulation has been derived from studies examining the highly conserved adaptive response of renal medullary cells to hyperosmolarity.<sup>5-7</sup> Although these discoveries have contributed greatly to our understanding of osmotic regulation of AR2 in the renal medulla, little is known about AR2 regulation in other tissues by levels of glucose that are commonly seen in humans with diabetes.<sup>3</sup> In most diabetic humans, it is unlikely that substantial hyperosmolarity is present in most tissues or is sustained long enough to account for a hyperosmotically mediated increase in AR2 expression.

Glucose transport into the retina is a central component of the hypothesis of glucose toxicity in the pathogenesis of diabetic retinopathy.<sup>8</sup> Glucose enters the retina through two principal pathways: the inner blood-retinal barrier, formed by the retinal vascular endothelium and the outer blood-retinal barrier, formed by the retinal pigment epithelium (RPE).<sup>9</sup> The relative contributions of glucose transport into the retina from the inner and the outer blood-retinal barriers have not been precisely determined, but the majority (approximately 60%) of blood glucose entering the retina appears to be supplied by the outer blood-retinal barrier.<sup>9</sup> RPE cells are easily isolated from human eyes obtained after death, and retain well-defined phe-

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From the Departments of <sup>1</sup>Physiology and <sup>2</sup>Pediatrics and Human Development, College of Human Medicine, Michigan State University, East Lansing; <sup>3</sup>Kresge Eye Institute, Wayne State University School of Medicine, Detroit, Michigan; and <sup>4</sup>Department of Internal Medicine, Division of Nephrology, Johns Hopkins School of Medicine, Baltimore, Maryland.

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Corresponding author: Douglas N. Henry, Department of Physiology, Michigan State University, 108 Giltner Hall, East Lansing, MI 48824-1101. henry@psl.msu.edu

notypic characteristics for up to 30 to 40 passages in culture.<sup>10</sup> The RPE represents a homogeneous monolayer of cells that has a nutritive and supportive role for the neuroretina in vivo.<sup>11</sup> Although there is mounting evidence suggesting that functional and structural changes in the RPE occur in experimental and clinical diabetes,<sup>12,13</sup> localization of blood-retinal barrier breakdown in diabetes has been controversial, with much of the focus being on changes of the inner blood-retinal barrier. Extracellular fluid within the retina, distorting the retinal architecture, has been assumed to result from changes in the architecture and function of the retinal vasculature.<sup>13</sup> Localization of the sites of blood-retinal barrier breakdown and leakage in diabetes has been reported in the RPE.<sup>13-18</sup> Changes in the RPE outer rod-segment phagocytosis function,<sup>19</sup> plasma membrane transport and uptake,<sup>20,21</sup> cell biochemistry,<sup>16,20,22</sup> protein synthesis,<sup>23</sup> and the c-wave of the electroretinogram<sup>24</sup> have been reported. In clinical and experimental models of diabetes, the RPE is also the site of advanced glycosylation end product formation,<sup>25</sup> growth factor expression,<sup>26</sup> and accelerated apoptosis.<sup>27</sup> The RPE layer of the human eye has been shown immunohistochemically to contain large amounts of AR that are increased in diabetic retinopathy.<sup>15</sup> AR inhibitors have been reported to decrease retinal vascular endothelial growth factor production and ultrastructural change,<sup>28</sup> RPE vacuolization, and degenerative foci in the galactosemic rat.<sup>29</sup> Therefore, the physiology of glucose metabolism in the RPE cell may play a central role in glucose-mediated cytotoxicity and the pathogenesis of diabetic retinopathy. Human RPE cells are an appropriate biologic system in which to assess glucose-specific effects on AR2 expression with possible relevance to the pathogenesis of diabetic retinopathy.

Considering these questions, we investigated the effects of glucose on AR2 gene expression, glucose utilization, and lactate production in cultured human RPE cells. To test the specificity of glucose in this system, we also performed these experiments in the presence of galactose and 3-OMG, a hexose that is transported but is not metabolized. Mannitol was used as an osmotic control.<sup>3,4,30</sup>

## METHODS

### Cell Culture

Human RPE cells were isolated as previously described from eyes obtained from the Michigan Eye Bank<sup>10,30</sup> which were collected after death from a 45-year-old female donor, after the Declaration of Helsinki and approval of the institutional review board on research involving human subjects had been obtained. In brief, RPE cells were recovered with fire-polished Pasteur pipettes under direct observation with a dissecting microscope from surgically removed, sagittally bisected eyes within 24 hours after death and cultured in Ham's F-12 (Gibco, Grand Island, NY) nutrient medium containing 16% fetal bovine serum (Hyclone, Logan, UT), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 26 mM sodium bicarbonate in a 37°C, humidified 95% air and 5% CO<sub>2</sub> atmosphere. Colony-forming primary cultures were rinsed with Hanks' balanced salt solution, dispersed by incubation with 0.5 mg/ml trypsin and 0.53 mM EDTA in 0.9% saline at 37°C for 10 minutes, centrifuged for 5 minutes at 50g, resuspended in new culture medium, and replated. Confluent cultures contained a monolayer of clear polygonal cells with densely pigmented stationary cells scattered throughout. All cells appeared epithe-

lioid, with apical-basal polarity, junctional complexes, and cytoplasmic organization characteristic of RPE cells in vivo. The established primary human RPE cell lines were passaged at a density of 20,000 cells/cm<sup>2</sup> in 10-cm dishes in RPMI-1640 medium (Gibco) with 2 mM L-glutamine containing 10% calf serum and 5 mM glucose at 37°C in humidified 95% air and 5% CO<sub>2</sub>. Continuous cultures of RPE cells were easily maintained by this method. Plated cells (20,000–40,000 cells/cm<sup>2</sup>) were seeded to yield near-confluent cultures at the end of each experiment. Ten-centimeter plates were used for quantitation of AR2 mRNA, AR2 protein, AR2 activity, glucose utilization, and lactate production. Freshly plated cells were allowed to attach in standard growth medium for 24 hours and were then incubated for various times (24 hours for RNA isolation and 72 hours for AR2 activity, AR2 protein content, glucose utilization, and lactate production determinations) in RPMI-1640 medium containing 10% calf serum and 5 mM glucose supplemented with galactose, 3-OMG, or mannitol for total hexose concentrations of 20 and 75 mM. Tissue culture medium was routinely changed every day. Glucose, galactose, mannitol, 3-OMG (tissue culture grade) and all other commonly used chemicals and reagents were the highest quality available and were obtained from Sigma (St. Louis, MO). Experiments were performed on passages 5 through 25 from the RPE cell isolate.

### Northern Blot Analysis of mRNA and cDNA Probes

RPE cells were grown in RPMI-1640 medium with 10% calf serum supplemented with glucose (5, 20, or 75 mM) or in RPMI-1640 medium with 10% calf serum and 5 mM glucose supplemented with galactose, 3-OMG, or mannitol for total hexose concentrations of 20 and 75 mM for 24 hours. Total RNA was isolated using a modification of the acid phenol single-step extraction method.<sup>31</sup> This procedure yields approximately 100 µg of total RNA from confluent monolayers of RPE cells grown in 10-cm plates. Total RNA (10 µg) was resolved on denaturing 2.2-M formaldehyde-1% agarose gels and transferred to nylon filters (ZetaBind; Cuno, Meriden, CT) by capillary blotting. The filters were stained with methylene blue to examine the integrity of the RNA and to assess the uniformity of loading and transfer. The filters were fixed by UV cross-linking and hybridized at high stringency according to the protocol of Church and Gilbert.<sup>32</sup> Probes were labeled with <sup>32</sup>P-dCTP (α-<sup>32</sup>P dCTP; DuPont NEN, Boston, MA) using random primers to a specific activity of 10<sup>9</sup> disintegrations per minute per milligram and separated from unincorporated nucleotides by gel filtration. After 18 hours, hybridized filters were washed at high stringency. Phosphorimages and quantitation were obtained using a phosphorimager (Molecular Dynamics, Sunnyvale, CA), or autoradiograms were obtained with multiple exposures to remain within the linear range of the film and were quantitated by scanning densitometry using a high-resolution optical scanner (AGFA, Orangeburg, NY) and software (NIH Image, ver. 1.60; National Institutes of Health, Bethesda, MD). Each blot was serially hybridized with human RPE AR2 cDNA<sup>30</sup> followed by chicken β-actin cDNA<sup>33</sup> probes. Filters were stripped until free of radioactivity and were checked by rapid phosphorimaging before rehybridization with β-actin cDNA, and the abundance of transcripts was normalized to β-actin mRNA levels.

### Immunoblotting of AR2

RPE cells were grown in 10-cm plates in experimental media for 72 hours. Each plate was rinsed twice with 5 ml cold PBS, and cells were harvested and homogenized in a ground-glass homogenizer in 1.5 ml of the same. Each homogenate (0.5 ml) was combined with 1.4 ml of sodium dodecyl sulfate (SDS)-containing sample buffer and heated at 95°C for 10 minutes. After cooling, 0.1 ml of 20%  $\beta$ -mercaptoethanol was added to each sample. Samples corresponding to 10  $\mu$ g of cell protein were electrophoresed on 4% to 15% polyacrylamide minigels (Bio-Rad, Hercules, CA) along with prestained molecular weight standards. The separated proteins were electrophoretically transferred to nitrocellulose sheets as described previously.<sup>3</sup> Nitrocellulose sheets were blocked for 60 minutes at 23°C in PBS containing 10% powdered milk and 0.05% Tween-20 and then incubated for 120 minutes at 23°C in a blocking buffer containing a 1:400 dilution of a goat polyclonal antibody against human placental AR2 (kindly provided by Peter Kador, National Eye Institute<sup>3,4</sup>). After they were rinsed with PBS, the nitrocellulose sheets were incubated for 90 minutes in blocking buffer containing a 1:400 dilution of rabbit anti-goat IgG-peroxidase conjugate (Sigma) and then developed with diaminobenzidine. Abundance of AR2 protein was determined by scanning densitometry using the high-resolution optical scanner (AGFA) and software (NIH Image, ver. 1.60).

### Measurement of AR2 Activity

AR2 activity was assessed spectrophotometrically<sup>3,5</sup> at 30°C by monitoring the decrease in absorbance of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm for 10 minutes in the absence and presence of 10-mM glyceraldehyde as substrate. Enzyme activity was normalized to supernatant protein content and expressed as nanomoles of NADPH oxidized per milligram of protein per minute. Supernatant protein content was measured using the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL).

### Measurement of RPE Cell Lactate Production

RPE cells were grown for 72 hours in experimental media. Cells were rapidly washed twice in ice-cold PBS, deproteinized using 1 ml 6% perchloric acid, harvested with a rubber policeman into a 1-ml tube (Eppendorf, Fremont, CA), and vortexed. The cells were spun at 14,000 rpm in a tabletop centrifuge at 4°C for 5 minutes, and the supernatant was removed on ice for immediate lactate determinations. The pelleted protein was dissolved in 1 ml of 0.1 N NaOH, and its concentration was measured. A standard clinical lactate assay kit (Lactate Colorimetric Assay; Sigma) was used to measure lactate concentrations. Standard curves and triplicate standards and samples were run for each experiment.

### Measurement of Glucose Utilization

Glucose utilization was determined from the formation of <sup>3</sup>H<sub>2</sub>O from [<sup>3</sup>H]glucose as described by Ashcroft and Stubbs.<sup>3,6</sup> Cells were seeded at 20,000 cells/cm<sup>2</sup> in 10-cm plates and grown for 72 hours in standard growth medium containing 5 mM glucose and in growth medium supplemented to 20 mM glucose. The medium was then replaced with 5 ml fresh medium containing these various concentrations of glucose and supplemented with 1  $\mu$ Ci [<sup>3</sup>H]glucose. Cells were then incubated at 37°C in a humidified incubator with 95% air and

5% CO<sub>2</sub>. After 30 minutes, 100- $\mu$ l aliquots of the sample media were collected from each well and centrifuged at 4°C for 5 minutes at 500g to sediment any free-floating cells and debris. Aliquots of the supernatant were acidified by addition of 20  $\mu$ l of 1 N HCl and placed in opened tubes (Eppendorf) in stoppered scintillation vials containing 0.5 ml of H<sub>2</sub>O. After equilibration overnight at 37°C, the <sup>3</sup>H<sub>2</sub>O was measured by liquid scintillation counting. <sup>3</sup>H<sub>2</sub>O production was normalized to cell protein content from each tissue culture well.

### Statistical Analysis

Results are expressed as means  $\pm$  SE of at least three experiments for mRNA, protein and activity determinations and six experiments for the measurements of lactate concentrations. Statistical significance of differences between experimental groups was determined using the nonparametric  $\chi^2$  test.  $P < 0.05$  was considered statistically significant.

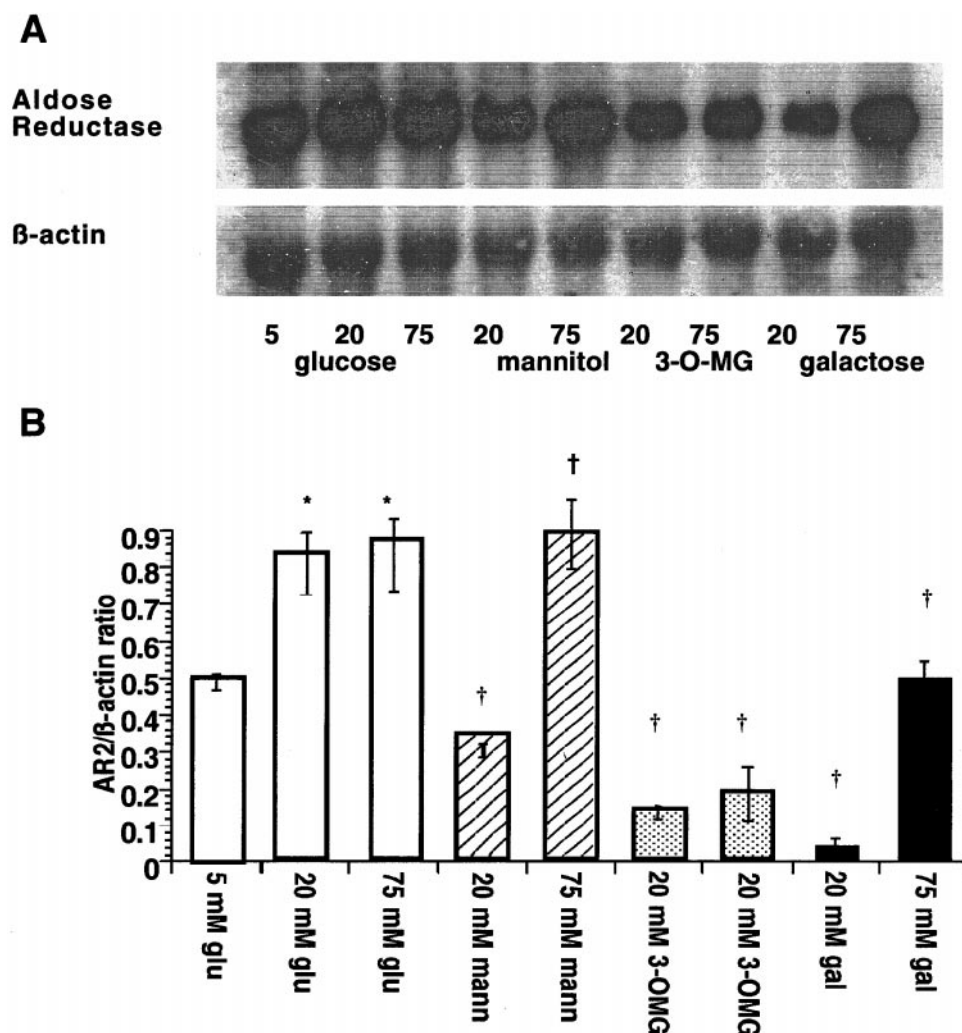
## RESULTS

### Effect of Hexose Sugars on AR2 mRNA Expression in Human RPE Cells

To determine the effects of hexose transport, metabolism, and osmotic stress on AR2 mRNA expression, RPE cells were grown in media supplemented with normal (5 mM), elevated (20 mM), or hyperosmolar (75 mM) levels of glucose or with standard growth medium supplemented with equimolar concentrations of various hexoses (3-OMG, galactose, or mannitol) for 24 hours before RNA isolation. 3-OMG and galactose are transported in a manner similar to glucose (facilitative glucose transport) but are not metabolized the same as glucose or phosphorylated by hexokinase. Galactose is metabolized by AR in an NADPH-dependent reduction to its corresponding sugar alcohol galactitol. Mannitol is considered an impermeant hexose compared with glucose. Mannitol has been used previously as an impermeant hexose in the study of osmotic regulation of AR2.<sup>3,4,30</sup>

3-OMG, mannitol, and galactose did not increase AR2 mRNA to levels greater than 5 mM or 20 mM glucose (Fig. 1). Moreover, AR2 mRNA expression was lowered when cells were grown in 20 or 75 mM galactose or 3-OMG versus equimolar concentrations of glucose. Only 75 mM mannitol-containing medium increased AR2 mRNA abundance to those levels achieved by 20 or 75 mM glucose. These findings are similar to those reported for rat AR2-luciferase reporter constructs used to transiently transfect rat aortic smooth muscle A7r5 cells grown in media supplemented with 150 mM concentrations of glucose, mannitol, 3-OMG, and galactose.<sup>4</sup>

Both pathophysiological (20 mM) and hyperosmolar (75 mM) concentrations of glucose increased AR2 mRNA expression approximately 60% higher than in medium containing 5 mM (Fig. 1), a finding similar to the glucose-mediated changes in AR2 mRNA in CAPAN-1 human pancreatic duct epithelial cells previously reported by our laboratory.<sup>3</sup> Although there were small differences in loading of total RNA for Northern blot analysis, we found no systematic effect of these hexoses on  $\beta$ -actin mRNA levels in the RPE cells. The absence of effect of these hexoses on  $\beta$ -actin mRNA levels was consistent with our previous report in pancreatic duct epithelia<sup>3</sup> and findings reported recently by Aida et al.<sup>4</sup>



**FIGURE 1.** Glucose-specific induction of AR mRNA in RPE cells. **(A)** Autoradiogram of Northern blot analysis of AR mRNA expression in RPE cells exposed to normal (5 mM), pathophysiological (20 mM), or hyperosmolar (75 mM) concentrations of glucose or equimolar concentrations of mannitol, 3-OMG, or galactose. **(B)** Quantitation of AR mRNA expression in RPE cells. Glucose (glu, *open bars*), mannitol (mann, *hatched bars*), 3-OMG (*stippled bars*), or galactose (gal, *solid bars*). \* $P < 0.05$  in 5 mM glucose versus 20 or 75 mM glucose. † $P < 0.05$  in 5 mM glucose versus 20 or 75 mM hexoses. Results represent means  $\pm$  SE of three experiments.

### Effect of Glucose on AR2 Protein Expression

To determine whether the increases in AR2 mRNA were paralleled by an increase in AR2 protein content, RPE cells were cultured in media containing 5, 20, and 75 mM glucose for 72 hours and then analyzed by immunoblot (Fig. 2). After development with diaminobenzidine, AR2 protein was identified by size (approximately 38 kDa) using molecular weight markers (Fig. 2A). Immunologically detectable AR2 protein levels increased 50% after exposure to 20 mM glucose for 72 hours and 100% after exposure to 75 mM glucose ( $P < 0.05$ , Fig. 2B). The increase in AR2 protein content paralleled the increases in AR2 mRNA when cells were grown in pathophysiological and normal concentrations of glucose-containing media (Fig. 1).

### Substrate Specificity of Hexoses for AR2 Activity and Protein Expression

RPE cells were grown in the various concentrations of glucose or in medium containing high concentrations of galactose for 72 hours, and AR2 activity was determined. At 20 mM, galactose increased AR2 activity (statistically nonsignificant) but not to as great an extent as did medium containing 20 mM glucose (Fig. 3,  $P < 0.05$ ). RPE cells were grown in medium containing 5 mM glucose or in medium supplemented with equimolar concentrations of galactose for 72 hours and then analyzed by immunoblot for AR2 protein content (Fig. 4). Galactose-sup-

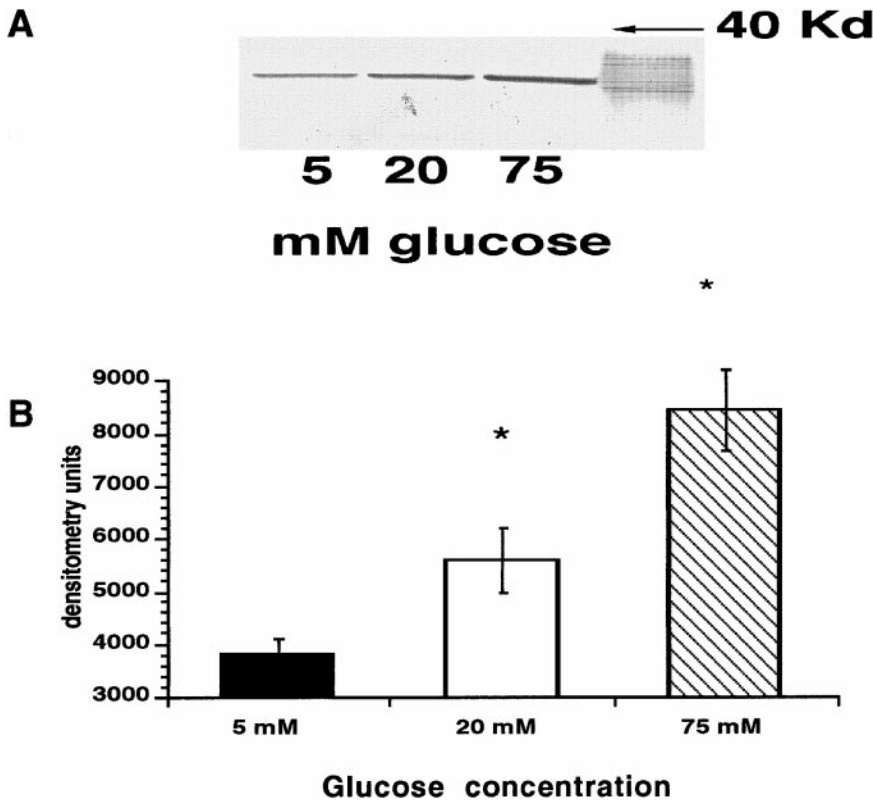
plemented medium decreased AR2 protein content more than did 5 mM glucose-containing medium (Fig. 4B,  $P < 0.05$ ). Thus, AR2 protein content and activity paralleled the changes in AR2 mRNA content induced by elevated glucose and galactose.

### Glucose Changes in RPE Cell Glucose Utilization and Lactate Production

Induction of AR2 by pathophysiological concentrations of glucose suggests that high concentrations of glucose enter the RPE cells and increase the metabolism of glucose by polyol metabolism. To determine whether glucose metabolism increased through glycolysis, glucose utilization and lactate production were measured in RPE cells grown in high glucose and control media for 72 hours. The media were changed every day. Glucose utilization (Fig. 5) and lactate production (Fig. 6) were significantly greater (4.8-fold and 4.4-fold,  $P < 0.05$ , respectively) in RPE cells cultured in medium containing 20 mM glucose than in that containing 5 mM glucose. Lactate production was lower in media containing mannitol, 3-OMG, and galactose (Fig. 6).

### DISCUSSION

In the present experiments a human RPE cell line was used as an in vitro model to study the effects of elevated hexose levels

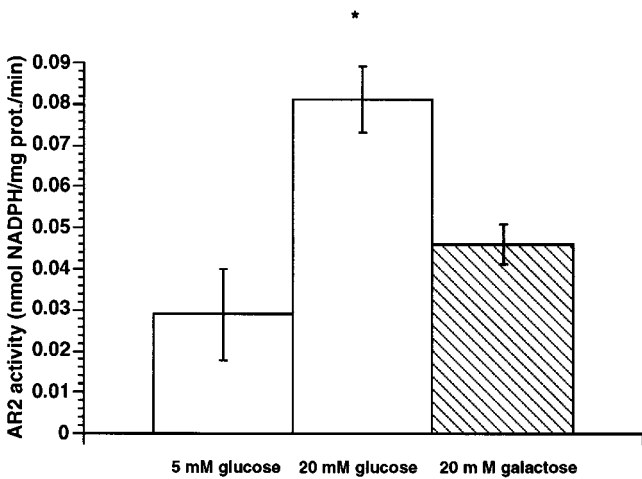


**FIGURE 2.** Effect of glucose on AR protein expression in RPE cells. **(A)** Immunoblots of AR protein from RPE cells cultured in 5, 20, or 75 mM glucose for 72 hours. AR protein appeared as a single band of approximately 38 kDa. **(B)** Quantitation of AR protein expression from immunoblots. Results represent means  $\pm$  SE of three experiments.

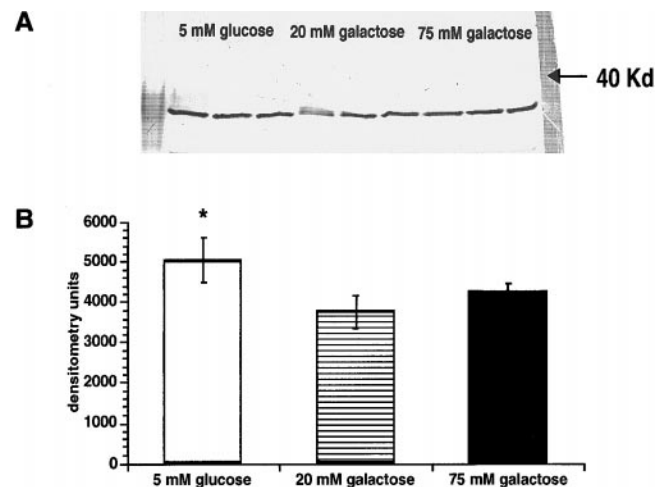
on the expression of AR2. An increase in the glucose concentration in the medium to 20 mM, similar to plasma levels observed in diabetic patients, increased the expression of AR2. Other hexoses did not reproduce the effects of glucose on AR2 mRNA content, AR2 protein levels, or AR2 activity. These findings suggest that hyperosmolar concentrations of glucose (~75 mM) are not necessary to regulate AR2 expression in RPE cells as they are in the renal medulla<sup>6,7</sup> where AR2 is osmoregulated and sorbitol acts as a nonperturbing osmolyte. These findings serve as an important confirmation of similar findings

by our laboratory in human pancreatic duct epithelia<sup>3</sup> and more recently by Aida et al.<sup>4</sup> in rat aortic smooth muscle cells.

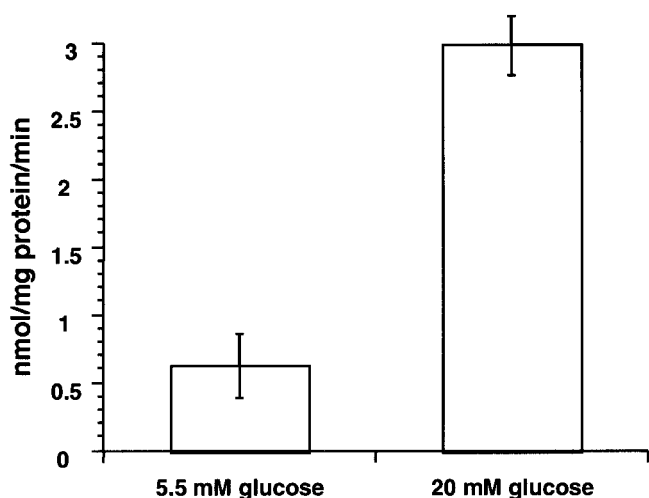
Increased glucose metabolism occurred when RPE cells were grown in 20 mM versus 5 mM glucose-containing medium as evidenced by the 4.8-fold increase in glucose utilization, 4.4-fold increase in lactate production, and the 2.6-fold greater AR2 activity. Some cultured cells (renal proximal tubule cells) tend to function far more anaerobically than their



**FIGURE 3.** Effect of elevated glucose and galactose on AR enzyme activity in RPE cells. AR enzyme activity was measured in RPE cells exposed to 5 mM glucose or 20 mM galactose for 72 hours. \* $P < 0.05$  in 20 mM glucose versus 5 mM glucose or 75 mM galactose. Results represent means  $\pm$  SE of three experiments.



**FIGURE 4.** Effect of galactose on AR protein expression in RPE cells. **(A)** Immunoblots of AR protein from RPE cells cultured in 5 mM glucose or 20 and 75 mM galactose for 72 hours. RPE cell protein (10  $\mu$ g) was run in each lane from each condition in triplicate experiments. **(B)** Quantitation of AR protein expression from the immunoblot shown in Figure 3A. \* $P < 0.05$  in 5 mM glucose versus 20 or 75 mM galactose. Results represent means  $\pm$  SE of three experiments.



**FIGURE 5.** Effect of elevated glucose on RPE cell glucose utilization. Rate of glucose utilization was determined in RPE cells exposed to 5 or 20 mM glucose for 72 hours. \* $P < 0.05$  in 20 mM versus 5 mM glucose. Results represent means  $\pm$  SE of three experiments.

counterparts in vivo.<sup>37</sup> Nonetheless, RPE cells in vitro produced more lactate when cultured in 20 mM than in 5 mM glucose-containing medium. The hexitol mannitol, the transported but not metabolized glucose analogue 3-OMG, and the transported and polyol pathway-metabolized hexose galactose did not reproduce the effects of glucose on AR2 expression at pathophysiologically equivalent concentrations of these sugars. Furthermore, AR2 mRNA content was significantly lower in medium containing galactose or 3-OMG than in medium containing 5 mM glucose. Limiting glucose transport or lowered levels of glycolytic intermediates possibly necessary for nonosmotic regulation of AR2 may have accounted for the lower levels of AR2 mRNA when RPE cells were exposed to equimolar concentrations of 3-OMG or galactose. Direct competition of these various sugars for glucose transport was not determined. However, mannitol is considered to be an impermeable hexitol that is often used as an osmotic control. We determined that <sup>14</sup>C-mannitol entered the RPE cell after 48 hours' exposure (data not shown). Lower levels of AR2 expression with mannitol versus glucose may have been the result of mannitol's entering the cell, which resulted in a lower osmotic gradient than glucose alone.

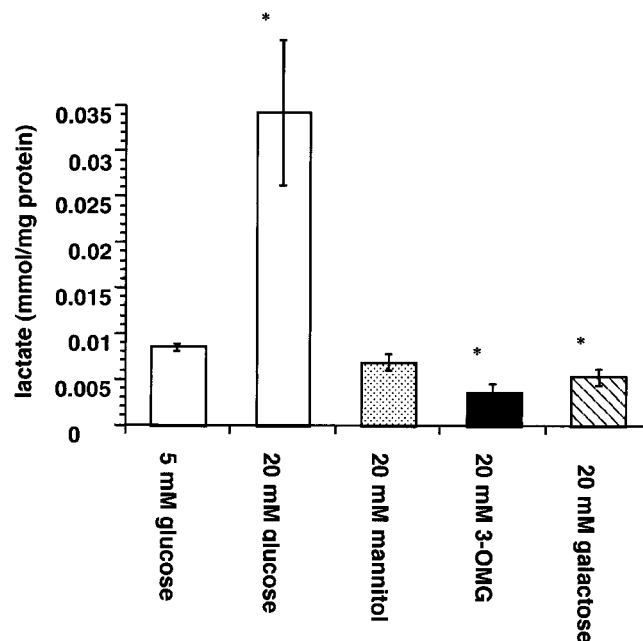
These findings suggest in part that 3-OMG and galactose competes with glucose for the facilitative glucose transporter I (GLUT1), the predominant glucose transporter of the blood-retinal barrier.<sup>38</sup> Putative pathogenetic mechanisms that may determine glucose-specific and nonosmotic regulation of AR2 warrant further investigations using glucose response element (GIRE<sup>24</sup>) reporter constructs or determining if GIRE are present in the AR2 gene.

Only 75 mM mannitol was effective as a hyperosmolar inducer of AR2 relative to glucose. Although we determined that mannitol enters the cell over 48 hours, hyperosmolar (75 mM) concentrations of mannitol were necessary to produce osmotic induction of AR2. These data suggest that in the RPE cell, AR2 is regulated by glucose (or its metabolism) at nonhyperosmolar concentrations of glucose (20 mM) and by hyperosmolarity at high concentrations of glucose or mannitol (75 mM). We did not measure intracellular mannitol concentrations in the RPE cells, but mannitol probably enters the cell

very slowly relative to the other hexoses used and therefore was a more effective osmotic stressor.

The mechanisms of glucose or galactose toxicity may be relevant to diabetic retinopathy, because at least in dogs and potentially in rats and mice, galactose causes retinopathy that is morphologically similar to, yet not identical with, that caused by diabetes.<sup>39</sup> The mechanisms by which glucose and galactose produce retinopathy are likely to be similar (glucose and galactose toxicity, respectively) but quantitatively and qualitatively distinct, because the galactose-induced effects that we have observed in RPE cells are different from those induced by glucose. The observed galactose or glucose toxicity in the RPE does not necessarily constitute *prima facie* evidence for its role in the development of diabetic retinopathy. Rather, we conclude that glucose-specific transport and/or metabolism may have specific effects on RPE AR2 expression not recapitulated by other hexoses. Furthermore, we have demonstrated that AR2 is nonosmotically regulated by glucose in the human RPE in vitro. Slight differences in AR2 mRNA, AR2 protein expression, and AR2 protein activity are noted. The cause for these differences are not known in the RPE, but may be attributable to changes in AR2 mRNA and AR2 protein half-life under hyperosmolar conditions, as previously reported by Smardo et al.,<sup>7</sup> or to differences in the activity of the AR2 protein when different hexoses serve as substrates.

Further evidence is accumulating that glucose or glucose transport specifically regulates gene expression related to diabetic nephropathy and perhaps to other long-term complications of diabetes.<sup>40-43</sup> These glucose-specific effects may be determined by upregulation of facilitated glucose transporters and/or by increased glucose transport. We have recently reported that GLUT1 regulates AR2, protein kinase C- $\alpha$ , and native GLUT1 expression in renal mesangial cells in vitro.<sup>40</sup> In



**FIGURE 6.** Effect of elevated glucose on RPE cell lactate production. Lactate content was determined in RPE cells exposed to 5 and 20 mM glucose and media with high concentrations of galactose, mannitol, or 3-OMG for 72 hours. \* $P < 0.05$  in media with high concentrations of glucose, galactose, or 3-OMG versus 5 mM glucose or high concentrations of galactose. Results represent means  $\pm$  SE of six experiments.

that report, we constitutively expressed the GLUT1 transporter in rat renal mesangial cells, which resulted in high constitutive AR2 expression in medium containing only 8 mM glucose.<sup>40</sup> Osmotic induction of AR2 was not necessary for the activation of AR2.<sup>40</sup> GLUT1 expression, accelerated glucose transporter exchange,<sup>44</sup> increased glucose entry, or glucose metabolism may play important proximate roles in the activation of AR2 and other putative pathways of glucose-mediated cell toxicity in the development of diabetic retinopathy.

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