Regulation of glucose transporters (GLUT-1 and GLUT-3) in human retinal endothelial cells

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The regulation of glucose transporters (GLUT-1 and GLUT-3), in terms of both mRNA and protein, in human retinal endothelial cells was investigated. The cells responded within 1 h of exposure to 5 mM glucose with an increase in the level of GLUT-3 mRNA that was due to an increase in the transcription of the 4.1 kb mRNA of the gene for GLUT-3. In the absence of glucose, the gene for GLUT-1 was not transcribed but the level of GLUT-3

mRNA was increased in these conditions and this was the result of an increase in the transcription of the 4.1 kb mRNA. The level of GLUT-1 and GLUT-3 mRNA was maximal when the cells were exposed to 15 mM glucose. These results are discussed in the light of the glucose regulatory potential of the retinal microvasculature and the implications that this may have for the mechanisms of diabetic retinopathy.

INTRODUCTION

Glucose is transported into cells by a sodium-dependent mechanism or by facilitative diffusion [1]. The latter process is mediated by a family of integral membrane proteins of which there are at least seven different isoforms [2,3]. Each glucose transporter has distinct kinetic and substrate specificities and their relative distributions determine the characteristic glucose regulatory potential of that particular tissue.

The breakdown of insulin-mediated glucose regulation that occurs in type 1 diabetes results in hyper- and hypoglycaemic phases that are variably controlled by exogenous insulin. The effects of poorly controlled glucose concentration have been documented in clinical studies [4], where the introduction of a tightly controlled glucose regimen significantly reduces the incidence and the severity of the microvascular complications of diabetes, namely diabetic retinopathy, nephropathy and neuropathy. We have examined the innate glucose regulatory mechanisms of the microvascular endothelium of the human retina at a range of glucose concentrations to determine the regulatory potential of the retinal vasculature at a molecular level.

GLUT-1 and GLUT-3 glucose transporter isoforms are expressed in human retinal endothelial cells (HRECs) [5–7]. In this study we describe the regulation of GLUT-1 and GLUT-3 mRNA in response to the external concentration of glucose and demonstrate that these two glucose transporters are differentially regulated in response to glucose. We also present results on the increase in the level of GLUT-1 and GLUT-3 mRNA species in response to 15 mM glucose that did not occur at higher glucose concentrations (25 mM) and discuss the potential implication of this for diabetic retinopathy.

MATERIALS AND METHODS

Cell culture

HRECs were isolated from donor eyes. The methods used have previously been described for bovine retinal endothelial cell culture [8] and subsequently modified [9]. HRECs were initially grown on a 30 mm diameter tissue-culture plate and passaged within 2–8 weeks when the cell monolayer was confluent. The

cells were grown in 75 cm³ flasks for RNA studies or in chamber slides for immunohistochemistry and were routinely used between passages 4 and 8. Glucose-free Glasgow's minimal essential medium (GMEM) (Gibco) was used and was supplemented with 2.5 % (v/v) platelet-deprived serum with various concentrations of D-glucose as specified in the text. HRECs were used when they were approx. 80 % confluent and were stepped down in glucose-free serum-free medium for a minimum of 6 h before use.

Immunohistochemistry

HRECs were fixed in chamber slides with 100 % ethanol and were incubated with 20 % (v/v) pig serum for 30 min at room temperature to block non-specific staining. Rabbit polyclonal antibody specific for GLUT-1 or GLUT-3 (East Acres Biologicals, Southbridge, MA, U.S.A.) was added to the cells for 1 h at a dilution of 1:50. The slides were washed three times with PBS and a biotinylated pig anti-rabbit antibody in 5 % (v/v) human serum was added. Specific staining was revealed with streptavidin–Texas Red and compared with negative control slides that were treated in the absence of the respective primary antibody. All of the procedures were performed at room temperature.

RNA extraction and electrophoresis

HRECs were incubated for specified periods in a range of glucose concentrations (0–25 mM). The medium was drained from the cell monolayers and the cells were washed three times in PBS. Total RNA was extracted with guanidinium isothiocyanate/phenol/chloroform, with an adaptation of the Chomczynski and Sacchi method [10] as previously described [9]. The concentration of the RNA was determined by spectrophotometric analysis at 260 nm.

The RNA (10 µg per lane) was resolved by electrophoresis in a 1.2 % (w/v) agarose gel containing formaldehyde [11]. The gel was run at 60 V for 4 h and the RNA was subsequently revealed by staining with ethidium bromide, then transferred to a nylon membrane (HybondN, Amersham International, Amersham,

Abbreviations used: GLUT-1, GLUT-3, glucose transporter isoforms; GMEM, Glasgow's minimal essential medium; HRECs, human retinal endothelial cells; UTR, untranslated region.

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Bucks., U.K.) by overnight capillary blotting and immobilized by exposure to short-wave UV radiation (Spectrolinker).

Northern hybridization

The cDNA probes used for this study were as follows. The GLUT-3 probe was a 911 bp *Eco*RI fragment and the GLUT-1 probe was a 1.6 kbp *Bgl*II fragment [12]. The level of β -actin mRNA was determined with a 1.7 kb *Pst*I fragment of a genomic clone subcloned into PBR322 and was used as an internal control. The membranes were pre-hybridized at 42 °C for 6 h in buffer consisting of 50% (v/v) formamide, 10% (v/v) dextran sulphate, 0.2% polyvinylpyrrolidone, 0.2% BSA, 2% Ficoll, 0.05 M Tris/HCl, pH 7.5, 1.0 M NaCl, 0.1% sodium pyrophosphate, 1.0% SDS and denatured salmon sperm DNA (100 μ g/ml or more). The appropriate probe was radiolabelled by random priming with α -dCTP (Amersham) and the labelled fraction was purified (Appligene Nonaprimer labelling and purification kit). The specific radioactivity of the cDNA species was routinely 10^6 c.p.m./ng of DNA and was added to the existing hybridization mix. The membranes were hybridized at 42 °C for 16 h, after which the non-specific radioactivity was removed by the following washing procedures: $2 \times$ SSC (0.3 M NaCl/30 mM sodium citrate, pH 7.0) at room temperature for 5 min; $0.5 \times$ SSC/1% SDS at 65 °C (55 °C for GLUT-3) for 30 min, and finally a 30 min wash in $0.2 \times$ SSC at room temperature. Each procedure was performed in duplicate and the filters were laid down in autoradiography cassettes containing intensifying screens with Hyperfilm MP (Amersham) at -70 °C. The length of exposure varied from 1 to 7 days.

Quantification of mRNA level

The level of mRNA was determined by densitometry with Gelbase software (UVP Systems Ltd). The images were captured from autoradiography film on to floppy disks by using Imagestore and the peak absorbance value for the level of mRNA for GLUT-1, GLUT-3 and β -actin was determined for each sample ($n = 4$). The standard deviations of the respective test groups did not exceed 10%. The results are calculated relative to the level

Figure 1 Human retinal endothelial cells

The cells were grown on fibronectin-coated tissue-culture flasks showing progressive growth of the cells from isolated cells (a) to form small colonies (b, c) (top panel). As the cells continue to grow, they form a confluent monolayer (d-f) (middle panel) and on further incubation the cells align themselves with each other (g-i) (bottom panel) (magnification $\times 45$).

GLUT-1

GLUT-3

Figure 2 Immunohistochemical detection of GLUT-1 and GLUT-3 protein in HRECs

Specific staining is revealed as varying levels of intensity of fluorescence of streptavidin-Texas Red throughout the cell. GLUT-1 and GLUT-3 seem to be similarly distributed in the HRECs.

of β -actin mRNA and presented as a ratio or as a percentage change in response, as specified in the Figure legends. Student's *t* test was used to calculate the significance of the results and presented at the 99% ($P < 0.01$) or at the 99.9% ($P < 0.001$) levels of significance.

RESULTS

HREC culture

HRECs were initially visible as isolated colonies (Figures 1a–1c), which eventually formed a confluent monolayer (Figures 1e and 1f). On further incubation the HRECs seemed to be more elongated, aligning themselves with each other (Figures 1g–1i). The endothelial cells were characterized by positive staining by immunohistochemistry with Factor VIII antibodies (results not shown). The presence of the GLUT-1 and GLUT-3 proteins was confirmed by immunohistochemical analysis of HRECs and revealed with streptavidin–Texas Red staining (Figure 2).

Figure 3 Relative levels of GLUT-1 and GLUT-3 mRNA

Top: HRECs were exposed to 5 mM glucose for 1, 3, 5 and 8 h. Total RNA was isolated from the cells and the level of GLUT-1, GLUT-3 and β -actin mRNA was determined by Northern hybridization with radiolabelled probes followed by exposure to autoradiography film at -70°C for 24 h (β -actin) or for 7 days (GLUT-1 and GLUT-3). Bottom: The mean levels of the respective mRNA species ($n = 4$) were determined by densitometry with Gelbase software (UVP Systems). The results are presented as the ratio of the mean values of GLUT-1 and GLUT-3 mRNAs to the level of β -actin mRNA. After 1 h of exposure to 5 mM glucose, the level of the 4.1 kb GLUT-3 mRNA increased significantly ($P < 0.001$) as determined by Student's *t* test. ■, GLUT-1; ▲, GLUT-3 (4.1 kb); ♦, GLUT-3 (3.2 kb).

Figure 4 The effect of actinomycin D on the levels of mRNA for GLUT-1 and GLUT-3

HRECs were exposed to glucose-free GMEM (■) or to GMEM supplemented with 5 mM glucose (▒) for 1 h in the presence or absence of 10 $\mu\text{g/ml}$ actinomycin D. Total RNA was isolated from the respective cell populations and the levels of mRNA for β -actin, GLUT-1 and GLUT-3 were determined as previously outlined. The results are presented here as (top) the autoradiographic film and (bottom) a histogram of the percentage change in the levels of the respective mRNA species in the presence of actinomycin D.

Quantification of glucose transporter mRNA in HRECs

RNA was extracted from HRECs after 0, 1, 3, 5 and 8 h exposure to 5 mM glucose.

The relative levels of GLUT-1 and GLUT-3 mRNA were determined at each time point (Figure 3). One band corresponding to a 2.8 kb mRNA was evident for GLUT-1 and two bands of 4.1 and 3.2 kb were present for GLUT-3 mRNA. The level of GLUT-1 mRNA remained relatively constant during the 8 h period whereas GLUT-3 mRNA increased after 1 h of exposure to glucose. To determine the relative changes in the two mRNA species of the gene for GLUT-3, the levels were recorded individually. The level of GLUT-3 mRNA observed after 1 h of exposure to 5 mM glucose increased significantly as determined with Student's *t* test ($P < 0.001$). The marked increase in the level of the GLUT-3 mRNA that was observed after 1 h was due to an increase in the level of the 4.1 kb mRNA species only.

Level of regulation of expression of the genes for GLUT-1 and GLUT-3 in HRECs

We examined the relative contributions of transcription and/or changes in the stability of the glucose transporter genes to the level of GLUT-1 and GLUT-3 mRNA. HRECs were incubated with actinomycin D (10 $\mu\text{g/ml}$) and the levels of mRNA for

Figure 5 The effect of glucose concentration on the levels of mRNA for GLUT-1 and GLUT-3

HRECs were exposed to 5 (solid bars), 15 (hatched bars) or 25 mM (stippled bars) glucose for 8 h. Total RNA was isolated from the cells and the levels of mRNA for β -actin, GLUT-1 and GLUT-3 were determined by Northern hybridization with radiolabelled probes as previously outlined. The level of β -actin mRNA did not change significantly in the different glucose concentrations. GLUT-1 and GLUT-3 mRNA species changed significantly in response to changes in the concentration of glucose, as indicated on the histogram. Significance levels by Student's *t* test: ** $P < 0.01$; *** $P < 0.001$.

GLUT-1, GLUT-3 and β -actin were determined after 1 h of exposure to glucose-free GMEM or to GMEM supplemented with 5 mM glucose as described above. The presence of actinomycin D significantly decreased the levels of mRNA for GLUT-1 and GLUT-3 in cells exposed to 5 mM glucose (Figure 4). In glucose-free medium a decrease in the level of GLUT-3 mRNA was observed although of a smaller magnitude. No major change was evident for the GLUT-1 mRNA level in the absence of glucose.

The role of increased glucose concentration in the regulation of glucose transporter mRNA

The levels of mRNA for GLUT-1 and GLUT-3 were determined after exposure to 5, 15 or 25 mM glucose for 8 h (Figure 5). The levels of the mRNA for GLUT-1 and GLUT-3 that was evident after 8 h of exposure to 5 mM glucose (see Figure 3) was increased in 15 mM glucose ($P < 0.001$). The levels of glucose transporter mRNA species in 25 mM glucose were similar to those evident in HRECs exposed to 5 mM glucose.

DISCUSSION

In considering the potential impact that glucose concentration has on the retinal endothelial cells in diabetes, it is important to use a human model system to eliminate any potential species-specific effects. We have previously described changes in the level of GLUT-3 mRNA in bovine retinal endothelial cells in response to glucose concentration [9]. However, the gene for GLUT-3 in non-primate species expresses only one mRNA species of 4.1 kb, whereas a second smaller mRNA (3.2 kb) is present in primates [7,13], generated by alternative polyadenylation within the 3' untranslated region (UTR) [14]. We have used HRECs to investigate the potential regulatory role of the 3.2 kb mRNA and

to examine specifically the differential regulatory responses of the GLUT-3 mRNAs and the expression of GLUT-1 by the same cells.

In the present study we found that GLUT-3 mRNA increased significantly after 1 h of exposure to 5 mM glucose. The increase was due to an increase in the transcription of the 4.1 kb mRNA of the gene for human GLUT-3. We have also shown that the level of the GLUT-1 and the two GLUT-3 mRNA species decreased in the presence of actinomycin D in these conditions but there was no evidence for the transcription of the gene for GLUT-1 in the absence of glucose. In the presence of 15 mM glucose, the levels of mRNA for GLUT-1 and GLUT-3 increased from those observed in 5 mM glucose, although the levels observed in 25 mM glucose were similar to those in 5 mM glucose.

The HRECs used in this study were grown in a glucose concentration approximating a euglycaemic concentration (5 mM) and were depleted of intracellular stores of glucose by removing both glucose and serum for a minimum of 6 h before analysis. The distinct pattern of response that was observed in the levels of the mRNA of the two glucose transporter isoforms is likely to reflect the distinct kinetic properties of these transport proteins [12] and in the transcription of the respective genes and/or the inherent stability of the mRNAs. In the presence of 5 mM glucose both GLUT-1 and the 4.1 kb mRNA of the gene for GLUT-3 were transcribed, although the failure to demonstrate a net increase in the level of the GLUT-1 mRNA demonstrates a fundamental difference in the stability and/or degradation of the two mRNA species. GLUT-3 has a very low K_m to maximize glucose uptake in tissues that are highly metabolically active, e.g. retina and brain, to overcome potential limitations in nutrient availability. This might in part explain the rapid increase in the GLUT-3 mRNA level.

The presence of two mRNA species of the gene for GLUT-3 in primates provides an ideal system for examining the differential regulation of mRNA species and thereby the potential role of the 3' UTR sequence present in the 4.1 kb mRNA [14]. The increase in the level of the GLUT-3 mRNA is mainly the result of an increase in the level of the 4.1 kb mRNA, which is largely due to an increase in the specific transcription of the gene for GLUT-3 generating the larger mRNA rather than an increase in the stability of the mRNA in the experimental conditions examined. The differential responsiveness of the two GLUT-3 mRNA species might reflect a specific regulatory potential of the 3' UTR sequence. The 3' polyadenylated sequence and any associated protein complex has a protective role for mRNAs that might be modified in response to hormonal and nutritional changes [15]. This might be mediated at a variety of different levels, including interactions of specific sequences with transcriptional regulatory proteins. Parallel experiments indicated that the decrease in the levels of the two glucose transporter mRNA species was not uniform in the presence of actinomycin D, indicating that there is a fundamental difference in the abilities of the two GLUT-3 mRNA species to respond to the presence of glucose. Differential regulation of the two mRNA species arising from the gene for GLUT-3 is also evident in glucose-free conditions as the presence of actinomycin D does not uniformly decrease the level of the GLUT-3 mRNA. Thus transcriptional changes are not solely due to sequences in the 5' UTR: other factors are clearly important.

This did not seem to be an exclusive effect of glucose because even in the absence of glucose a similar pattern of regulation of GLUT-3 mRNA was observed, although there was a net decrease in the mRNA levels that were examined. This observation contrasts with the glucose-dependent transcription of the gene

for GLUT-1, but is consistent with results from other studies [16]. It seems likely that this effect is mediated by a mechanism of regulation that includes the presence of a glucose-specific regulatory element in the 5' UTR of the gene for GLUT-1 that is able to interact with an intracellular metabolite of glycolysis and/or by the level of GLUT-1 protein occupancy that changes in the presence of glucose [17]. The former mechanism seems more likely given the down-regulation of the GLUT-1 mRNA in HRECs in the presence of high glucose concentrations (25 mM). This has been observed in other systems. For example, the insulin gene is regulated by glucose concentration via regulatory proteins that interact with specific sequence elements in the 5' UTR [18,19]. The ability of the β -cell to respond to glucose concentrations is mediated via a complex series of events [20] that act in concert to maintain glucose homeostasis. An increase in the level of genes that are involved in transcriptional regulation, e.g. *c-fos* and *c-jun*, has been demonstrated after exposure to high glucose concentrations [21] and the resulting AP-1 complex is known to be involved in the regulation of gene expression. Despite the evidence of glucose-dependent transcription of GLUT-1 during 1 h of exposure to 5 mM glucose there was no net increase in the level of mRNA at that time and so transcription of GLUT-1 mRNA must be balanced with the translation and/or degradation of the GLUT-1 mRNA.

The presence of glucose transporters with low K_m values ensures the efficient uptake of glucose by the retinal endothelial cells. The marked increase in the level of glucose transporter mRNA that was observed in 15 mM glucose illustrates a potential mechanism by which retinal endothelial cells might maximally utilize glucose from the circulation. However, it is perhaps this innate regulatory mechanism of the retinal endothelial cells that predisposes this tissue to glucose-mediated damage and subsequent disease. In the brain, GLUT-3 has been reported to be present predominantly in the neuronal tissue although a relatively minor portion is present in the microvessels [22]; however, the present study shows that GLUT-3 is a major functional glucose transporter of the retinal endothelial cells. Thus diabetic retinopathy might be the culmination of the damage mediated by the high glucose concentrations that circulate throughout the retinal microvasculature. In more excessive glucose concentrations (25 mM), down-regulation of the glucose transporters did occur although in these

extreme conditions it might be insufficient to serve as a protective mechanism. It would be of great interest to study further the mechanism(s) by which down-regulation occurs in order to develop specific intervention therapies for this debilitating disease and to extend our knowledge of the role of 3' UTRs in glucose-mediated regulation by using HRECs.

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