Glucose modulation of aldose reductase mRNA expression and its activity in cultured calf pulmonary artery endothelial cells

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Summary. We examined the effect of glucose on aldose reductase mRNA expression and its activity in calf pulmonary artery endothelial cells. After the cells were exposed to 18 mmol/l glucose, aldose reductase mRNA expression began to increase at 6 h, reached a maximum (about 2.4-fold increase) at 12 h, and thereafter gradually decreased. Aldose reductase activity was found to strongly correlate with aldose reductase mRNA expression after cells were exposed to 18 mmol/l glucose. In contrast, aldose reductase mRNA expression was significantly decreased following exposure to 55 mmol/l glucose. Aldose reductase activity was also decreased at 24 h after 55 mmol/l glucose. The increase in al-

dose reductase mRNA level caused by glucose was inhibited by 1 µg/ml of actinomycin D. These phenomena appear to be glucose-specific since neither 3-O-methylglucose nor fructose affected the levels of aldose reductase mRNA. We clearly demonstrate that aldose reductase mRNA level and its activity are modulated by glucose in calf pulmonary artery endothelial cells. Our data suggest that activation of aldose reductase in endothelial cells may contribute to the development of diabetic macroangiopathy.

Key words: Diabetes mellitus, angiopathy, endothelial cells, glucose, aldose reductase.

Hyperglycaemia is a generally accepted risk factor for development of diabetic macro- and microangiopathies. According to the 'response to injury' thesis [1, 2], denuding or non-denuding injury of the endothelium may be an initial event in the pathogenesis of atherosclerosis. Unlike muscle and adipose tissues, the tissues most affected by diabetes (i.e., the lens, nerve, retina, kidney, blood vessels, and islet cells) are freely permeable to glucose and do not require insulin for glucose penetration. They are exposed to the ambient blood glucose levels, and thus accumulate glucose. In the affected cells a portion of the accumulated glucose is converted to sorbitol by the enzyme aldose reductase (AR) [3]. Because sorbitol is not freely permeable and is slowly metabolized, it accumulates within the cell [4]. In vivo experiments in rats have shown that diabetes mellitus causes an increase in vascular permeability, as measured by albumin permeation [5]. The increase in vascular permeability is accompanied by an increase in sorbitol levels [5]. These changes are corrected by treating the diabetic animals with the aldose reductase inhibitor, sorbinil [5]. Glucose uptake by the endothelial cells of large vessels also occurs independently of insulin, and the cells have been shown to accumulate sorbitol when exposed to high ambient glucose levels. Recent studies have reported on AR mRNA expression in kidney [6] and lens [7, 8]

under hypertonic conditions. In this study, we quantitated AR activity and mRNA in calf pulmonary artery endothelial cells (CPAE) under normal and high ambient glucose levels in the culture medium.

Materials and methods

Cell culture

CPAE cells were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan) and seeded in 100 mm dishes (Nunc, Copenhagen, Denmark). The growth medium was minimum essential medium (MEM) (Gibco Laboratories, Grand Island, NY, USA) containing 20% fetal calf serum (FCS). Cultures were incubated in 10 ml of medium at 37°C in a humidified 5% CO₂-95% air atmosphere. When the cells reached confluency, the concentration of FCS was decreased to 2% for 24 h, then decreased to 0.1%. After a further 48 h incubation, the cells were exposed to various concentrations of glucose. The cells were then harvested at the times indicated in the experimental conditions. The culture medium was replaced every 12 h to keep the glucose concentrations relatively constant.

Quantitative analysis of AR mRNA

We slightly modified the method of Chelly et al. [9] and Wang et al. [10] to measure mRNA levels. Total RNA was obtained using the standard method of 6 mol/l guanidine thiocyanate/CsCl centrifuga-

Fig. 1a, b. Time course of aldose reductase (AR) mRNA expression after addition of 18 mmol/l glucose. **a** Ethidium bromide staining of polymerase chain reaction (PCR) products separated in 2% agarose. Lanes: M, molecular weight markers; a–f, AR PCR products; g–l, β -actin PCR products; lanes a and g, 0 h; lanes b and h, 3 h; lanes c and i, 6 h; lanes d and j, 12 h; lanes e and k, 24 h; lanes f and l, 48 h. bp, base pairs. **b** The ordinate indicates AR mRNA/ β -actin mRNA ratio in arbitrary units and abscissa indicates time in h. Each point is the mean of the duplicates from a representative experiment

tion [11]. RNA was reverse-transcribed into cDNA by the following procedures as previously described [12]. Total cellular RNA (4 μ g) was heated at 70°C for 10 min, cooled slowly to 42°C, and then reverse-transcribed at 42°C for 90 min in a 25 μ l reaction mixture containing 50 mmol/l Tris-HCl (pH 8.3), 100 mmol/l KCl, 10 mmol/l MgCl₂, 10 mmol/l dithiothreitol, 0.25 mmol/l deoxynucleoside triphosphates (dNTP), 0.4 μ g oligo-dT, 400 units of RNasin (Takara Shuzo Co., Kyoto, Japan), and 11 units of Rous associated virus 2 (RAV-2) reverse transcriptase (Takara). A 20 μ l aliquot of a 1:10 diluted cDNA mixture was used as the template for amplification. Polymerase chain reaction (PCR) was performed at a final concentration of 1 $\times$ PCR buffer (50 mmol/l Tris-HCl pH 8.3, 2 mmol/l MgCl₂, 30 mmol/l KCl, 10 mmol/l dithiothreitol), 0.25 mmol/l dNTP, 1 μ g each of upstream and downstream primers, 1 $\times$ 10⁶ cpm of ³²P-end-labelled upstream primer and 2.5 units of *thermus aquaticus* DNA polymerase (taq polymerase) (Perkin-Elmer/Cetus, Norwalk, Conn., USA) in a total volume of 50 μ l. The mixture was overlaid with mineral oil and then amplified with COY Temp Cycler (Toyobo, Osaka, Japan). The amplification profile involved denaturation at 93°C for 60 s, primer annealing at 60°C for 90 s, and extension at 72°C for 90 s. At the first cycle, however, DNA was denatured for 5 min. To ensure exponential amplification, AR mRNA amplification was carried out for 25 cycles, and β -actin mRNA amplification for 22 cycles. To remove the mineral oil, the reaction mixtures were extracted once with chloroform and were then prepared for quantitation.

A 12.5 μ l aliquot of each PCR reaction mixture was electrophoresed in 2% agarose gels in Tris-acetate/EDTA buffer containing ethidium bromide. The appropriate bands were then cut from the gel and radioactivity was determined by scintillation counting. The results were expressed as the ratio between AR mRNA and β -actin mRNA. The intra-assay and inter-assay coefficients of variations were less than 5%.

Oligonucleotides used for amplification

AR primers were chosen from the bovine lens AR cDNA sequence [13]. β -Actin primers, to be used as an internal standard, were chosen from the rat β -actin cDNA sequence [14]. They were synthesized using a DNA synthesizer (Applied Biosystems, Tokyo, Japan). The 5' end of the upstream primers were labelled with [γ -³²P]ATP using T₄ polynucleotide kinase. Unincorporated nucleotides were removed using ultrafree-C3 (Japan Millipore Laboratories, Tokyo, Japan).

The AR upstream primer (5' TACCAGAATGAGAACGAGGT 3') is identical to nucleotides 87 to 106 of the cDNA. The AR downstream primer (5' TTGTAGCTCAGCAAGGTGTT 3') is complementary to nucleotides 801 to 820.

The β -actin upstream primer (5' TGTITGAGACCTTCAACACC 3') is identical to nucleotides 368 to 387 of the cDNA. The β -actin downstream primer (5' CGCTCATTGCCGATAGTGAT 3') is complementary to nucleotides 739 to 758.

Measurement of AR activity

Harvested CPAE cells were ultrasonically disrupted in 135 mmol/l Na,K-phosphate buffer pH 7.0, followed by centrifugation at 15000 rev/min for 15 min. The supernatants were assumed to be the crude aldose reductase enzyme fraction. AR activity was measured according to the method reported by Dufrane et al. [15]. Incubation was carried out in 1 ml of incubation medium (135 mmol/l Na,K-phosphate buffer pH 7.0, 0.1 mol/l LiSO₄, 0.1 mmol/l DL-glyceraldehyde, 0.03 mmol/l NADPH) at 30°C for 30 min, and was then terminated by the addition of 0.3 ml of 0.5 mol/l HCl. One ml of 6 mol/l NaOH containing 10 mmol/l imidazole was then added, fluorescent NADP was measured by fluorescence spectrophotometry at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. An excitation and an emission band pass was set at 10 nm. AR activity was corrected by the cell number.

Statistical analysis

Data are means $\pm$ SEM. Statistical analysis was carried out with Student's *t*-test.

Results

In this study we used PCR to examine whether or not AR mRNA is expressed in CPAE cells. The amplified AR cDNA and β -actin cDNA were found as a single band after agarose gel electrophoresis and migrated at a predicted size of 734 and 391 base pairs, respectively (Fig. 1a). Direct sequencing of the amplified DNAs using the asymmetric PCR technique [16] demonstrated over 95% homology to bovine lens cDNA and rat β -actin cDNA (data not shown).

Time course of AR mRNA expression

We studied the ability of glucose to influence AR mRNA expression. The time course of AR mRNA expression after exposure of quiescent CPAE cells to 18 mmol/l glucose was determined using PCR quantitative analysis. As shown in Figure 1 (a and b), AR mRNA levels began to increase at 6 h after glucose exposure, reached a maximum at 12 h (about 2.4-fold increase), and then gradually decreased. Figure 1b shows the AR mRNA/ β -actin mRNA ratio.

Fig. 2. Time course of aldose reductase (AR) activity after addition of 18 mmol/l glucose. The ordinate indicates AR activity, and the abscissa indicates time in h. The open and closed circles indicate 5.5 mmol/l and 18 mmol/l glucose, respectively. Values are means $\pm$ SEM for three independent experiments. * $p < 0.05$ vs control culture

Time course of AR activity

A time course evaluation of AR activity was performed following the addition of 18 mmol/l glucose. AR activity was found to correlate with AR mRNA levels. AR activity reached a maximum (about 1.3-fold) at 24 h, and thereafter gradually decreased (Fig. 2). In contrast, at a glucose concentration of 55 mmol/l, AR activity gradually decreased until 24 h after glucose exposure, followed by a plateau (about 0.4-fold, data not shown).

Concentration dependency of AR mRNA expression

Figure 3a shows the concentration dependency of AR mRNA expression at 12 h after glucose exposure. The expression of AR mRNA was found to follow a biphasic pattern. The AR mRNA/ β -actin mRNA ratio increased up to 18 mmol/l glucose; however, at higher concentrations of glucose, the AR mRNA/ β -actin mRNA ratio gradually decreased.

Concentration dependency of AR activity

The concentration dependency of AR activity is shown in Figure 3b. AR activity was found to have a strong correlation with AR mRNA level. After exposure to glucose, AR activity reached a maximum at 18 mmol/l glucose, but decreased when the glucose concentration was raised over 30 mmol/l.

The effect of actinomycin D on AR mRNA expression

We also studied whether the stimulatory effects of glucose on AR mRNA levels occurred via a transcriptional or non-transcriptional mechanism. One microgram per millilitre of actinomycin D was added 30 min before the addition of glucose and CPAE cells were exposed to glucose for 12 h. As shown in Figure 4, AR mRNA expression by glucose was completely suppressed.

The effect of other sugars on AR mRNA expression

By testing other sugars and osmolytes, we also studied whether the induction of AR mRNA expression is specific for glucose. As shown in Figure 5, glucose, fructose or 3-O-methylglucose at 18 mmol/l were each added to the medium. There was an increase in AR mRNA expression only after the addition of 18 mmol/l glucose.

Discussion

Diabetic patients have an increased incidence and severity of atherosclerosis. Several theories have been proposed to explain why diabetic patients are at an increased risk for such vascular disorders. In uncontrolled diabetes, excess glucose causes a glycation of various proteins [17], a decrease in the level of oxygen dissociation in erythrocytes, an increase in platelet aggregation [18, 19], an increase in the circulatory level of very low density lipoproteins, and a decrease in high density lipoproteins [20]. In addition, Yamauchi et al. [21] recently reported that elevated glucose levels enhance the secretion of endothelin-1 in cultured bovine aortic endothelial cells.

Fig. 3. **a** Concentration dependency of aldose reductase (AR) mRNA at 12 h after glucose exposure. The ordinate indicates AR mRNA/ β -actin mRNA ratio in arbitrary units, and abscissa indicates glucose concentration. Each point is the mean of the duplicates from a representative experiment. **b** Concentration dependency of AR activity at 24 h after glucose exposure. The ordinate indicates AR activity, and the abscissa indicates glucose concentration. Values are means $\pm$ SEM for three independent experiments. * $p < 0.05$ vs control culture

Fig. 4. The effect of actinomycin D on aldose reductase (AR) mRNA expression. The ordinate indicates AR mRNA/ β -actin mRNA ratio in arbitrary units. The open and closed circles indicate 5.5 mmol/l and 18 mmol/l glucose, respectively. Actinomycin D was added 30 min before glucose exposure. The cells were incubated for 12 h. The results are the means of the duplicates from representative experiments

Fig. 5. The effect of other sugars on aldose reductase (AR) mRNA expression. Glucose, fructose and 3-O-methylglucose were added to the medium to study the effect of other sugars on AR mRNA expression. Each medium was prepared at 18 mOsm/kg except for control medium (5.5 mOsm/kg). The ordinate indicates AR mRNA/ β -actin mRNA ratio in arbitrary units. The results are the means of duplicates from representative experiments

The above-listed conditions are considered to cause alterations in the function of endothelial cells, and are positive risk factors for the development of atherosclerosis.

Abnormal glucose metabolism and the subsequent accumulation of intracellular sorbitol caused by the activa-

tion of AR in addition to the decrease in myo-inositol levels are reported to be one of the factors responsible for diabetic complications such as cataract, retinopathy, neuropathy and macroangiopathy [22–25].

In this study we have examined the modulation of AR mRNA expression and its activity by glucose in cultured CPAE cells. An important finding in this study is that AR mRNA expression and its activity increased when exposed to high glucose concentrations, commonly encountered in diabetic patients. This phenomenon is likely due to the induction of AR gene expression by glucose but not by other sugars or other osmolytes.

Our previous study demonstrated that diabetic patients with complications had an increased erythrocyte sorbitol/blood glucose ratio [26]. These findings may be explained by the induction of AR gene expression by glucose, although further studies are needed in human endothelial cells.

Interestingly, AR mRNA expression and its activity are inhibited at higher glucose concentrations (55 mmol/l). At present the precise mechanism is unknown, but two possibilities should be considered one being a feed-back inhibition in response to intracellular sorbitol accumulation. Bondy et al. [27] have reported that galactose feeding in the rat resulted in an increase in the renal medullary galactitol and profound decrease in renal medullary AR mRNA levels via a down regulation in response to AR end-product accumulation. Another possibility is a toxic effect under conditions of high ambient glucose. A toxic effect of sustained hyperglycaemia is suggested by the recent demonstration that in vivo exposure of endothelial cells to high glucose levels results in accelerated death, decreased replication and signs of DNA damage [28–30]. In fact, a toxic effect of glucose was observed in the cultures at the higher glucose concentrations (e.g. decrease in cell number and in adherence to dish) (data not shown).

Recent studies have reported that the products of the polyol pathway are stronger glycating agents than glucose [31, 32]. A logical extension of this hypothesis is that induction of AR gene expression by glucose, although it may be transient, may result in cell dysfunction not only because of the increase in sorbitol level or the decrease in myoinositol level, but also because of the increase in glycation of intracellular proteins.

The present study is to our knowledge the first report demonstrating that glucose at a concentration observed in diabetic patients increases the levels of AR mRNA expression and AR activity in endothelium. Data from our study suggest that high blood glucose levels in endothelial cells increase AR mRNA expression with a subsequent increase in sorbitol and decrease in myo-inositol and possibly an increased glycation of proteins. This could result in damage to the endothelial cells and accelerate the development of vascular complications in diabetic patients. Investigations are now underway to determine whether AR mRNA expression is also induced by glucose in diabetic patients in vivo.

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