The polyol pathway and glucose 6-phosphate in human endothelial cells cultured in high glucose concentrations

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Summary. In an attempt to identify the mechanisms underlying the ill effects of high glucose previously described in cultured human endothelial cells, we have investigated in these cells the activity of the polyol pathway and accumulation of glucose 6-phosphate, a powerful agent of non-enzymatic glycosylation. Sorbitol accumulation varied among different batches of cells (primary cultures). In presence of 5 mmol/l glucose the cellular sorbitol content ranged from 0.04 to 0.12 nmol/10⁶ cells. When cells were exposed to 20 mmol/l glucose the sorbitol content increased by 2- to 3-fold to concentrations of 0.08–0.38 nmol/10⁶ cells ($p < 0.01$). Addition to the culture medium of 100 μ mol/l Sorbinil, an inhibitor of aldose reductase, resulted in a substantial inhibition of sorbitol accumulation throughout the 14 days in culture, but the degree of inhibition varied inversely with the duration of cell exposure to high glucose (70% inhibition in cells exposed to

high glucose and Sorbinil for 1–3 days versus 14% inhibition in cells exposed for 14 days, $p < 0.01$). Sorbinil treatment failed to improve even slightly the abnormalities in cellular replication induced by high glucose. The cellular content of glucose 6-phosphate was augmented 3-fold by exposure to 20 mmol/l glucose ($p < 0.001$). In conjunction with other studies these results indicate that in this model the polyol pathway is not an osmotically or metabolically important mechanism of glucotoxicity, and that the inhibitory activity of Sorbinil on the polyol pathway of human tissues may be a function of their length of exposure to hyperglycaemia. The consequences of intracellular accumulation of glucose 6-phosphate await investigation.

Key words: Diabetes, angiopathy, endothelial cells, glucose, sorbitol, glucose 6-phosphate, aldose reductase, Sorbinil.

While it is well established that the morphology [1, 2], functional integrity [1, 3, 4] and even survival [5, 6] of vascular endothelium are jeopardized in diabetes, the mechanisms responsible for such degenerative changes are still poorly defined. A role for toxic effects of sustained hyperglycaemia is suggested by the recent demonstration that in vitro exposure of endothelial cells to high glucose levels results in accelerated death, decreased replication and signs of DNA damage [7–9].

There are at least three recognized mechanisms whereby the chronically elevated ambient glucose characteristic of diabetes may affect tissue function and structure: enhanced activity of the polyol pathway [10, 11], accelerated nonenzymatic glycosylation [12] and protracted hypertonicity [13]. Although the replicative delay induced in human endothelial cells by high glucose can to some extent be mimicked by increasing medium osmolarity with mannitol, the magnitude of the mannitol effect is much less than that observed with equimolar glucose [7]. Therefore, effects specific to the glucose molecule itself must be advocated. In

the present study we have investigated whether the polyol pathway plays a role in mediating the replicative abnormalities of human endothelial cells cultured in high glucose and whether these cells accumulate glucose 6-phosphate, which is a most potent effector of nonenzymatic glycosylation [14, 15].

Materials and methods

Cell culture

Primary cultures of endothelial cells were prepared from human umbilical veins as previously described [7, 16]. For proliferation experiments, cells were seeded at a density of 1×10^5 in 35 mm dishes (Falcon, Oxnard, Calif, USA); for metabolic measurements cells were seeded at 4×10^5 in 60 mm dishes (Falcon). The growth medium was Medium 199 (Gibco, Grand Island, NY, USA), supplemented with glutamine (2 mmol/l), Hepes buffer (17.5 mmol/l), 10 ng/ml epidermal growth factor (EGF culture grade, Collaborative Research Inc., Lexington, Mass, USA) and 14% heat-inactivated pooled human serum. Incubation volumes were 2 ml for 35 mm dishes and 4 ml for 60 mm dishes.

Cultures were incubated at 37°C in a humidified 5% CO₂-95% air atmosphere and all media were filtered and supplemented with penicillin (50 U/ml), streptomycin (50 µg/ml) and fungizone (0.25 µg/ml) purchased from Irvine Scientific, Santa Ana, Calif, USA. Media were changed every third day. At regular intervals, the endothelial cell monolayers were tested with FITC-conjugated goat anti-factor VIII related antigen antibodies (Atlantic Antibodies, Scarborough, Me, USA) and always found to be brightly and uniformly stained. The pH of all incubated cultures varied between 7.2 and 7.35.

To study the effects of high glucose concentrations the growth medium was supplemented with D-glucose (Mallinckrodt, Inc., Paris, Ky, USA) to achieve a final concentration of 20 mmol/l. Exposure to high glucose was started the day after plating or at the indicated times before cell harvest. Glucose concentration in control medium was 5 mmol/l.

In experiments addressing the effect of aldose-reductase inhibition on polyol accumulation and endothelial cell proliferation, Sorbinil (kindly provided by Pfizer Research, Groton, Conn, USA) was added to the growth medium in concentrations of 2, 4, 10 and 100 µmol/l. Additions were performed either the day after plating and renewed at every medium change, or at the indicated times prior to cell harvest. Sorbinil was suspended in normal saline and solubilized by sonication after titrating to pH 9.5 with 0.1 N NaOH. The solution was then back titrated to pH 7.4 with 0.1 N HCl. The low concentrations of Sorbinil (2–10 µmol/l) were chosen on the basis of the IC₅₀ of this compound on human placenta aldose reductase [17]; the 100 µmol/l concentration was employed after witnessing the ineffectiveness of the lower concentrations. The stability of Sorbinil in the tissue culture medium was checked by testing the effect of 100 µmol/l Sorbinil, incubated with endothelial cells for 3 days in presence of 20 mmol/l glucose, on the accumulation of sorbitol by human erythrocytes.

Measurements

In proliferation experiments addressing whether treatment with Sorbinil would prevent or reduce the proliferative delay induced by 20 mmol/l glucose, cell counts were performed every other day from day 4 to day 12–14 in culture. The procedures employed have been previously described [7].

Metabolic intermediates were measured in neutralized perchloric acid extracts of endothelial cells and, in selected experiments, of human erythrocytes. For preparation of extracts, cell culture dishes were cooled on ice, the monolayers were washed twice with phosphate-buffered saline (PBS, 0.15 mol/l, pH 7.4), the wash fluid was carefully and completely aspirated and 100 µl of chilled 6% perchloric acid was added to each dish in order to precipitate cellular material, which was harvested with a rubber policeman. On the basis of cell counts obtained from companion dishes, the appropriate number of dishes from each treatment were pooled to yield the desired cell number (at least 4×10^6 endothelial cells for each polyol assay and 20×10^6 cells for each glucose 6-phosphate assay). The harvested material was sonicated and centrifuged. The supernatant was neutralized with K₂CO₃ (2 mol/l), the excess salt removed by centrifugation and the supernatant filtered through a 0.45 µm membrane. When subjected to high-performance liquid chromatography (HPLC) or gas-liquid chromatography (GLC) the cellular extracts were further purified by application to a column of mixed acidic cation and basic anion exchange resin (Dowex MR-3, Sigma Chemical Co., St. Louis, Mo, USA) and then evaporated under vacuum. Before application to the HPLC column the sample was resuspended in 30 µl of HPLC grade water (Fisher Scientific Co., Fair Lawn, NJ, USA). For GLC analysis the sample was resuspended in 200 µl of distilled water, dried under nitrogen and derivatized with 50 µl of trimethylsilyl reagent (Sigma). When erythrocytes were employed, these were obtained from heparinized blood collected from normal volunteers, washed three times with PBS and incubated in 3 volumes of the same culture medium employed for endothelial cells and containing 5 or 20 mmol/l glucose. After 4 h of incubation at 37°C,

erythrocytes were sedimented by centrifugation, washed twice with PBS and aliquots precipitated with 3 volumes of chilled 6% perchloric acid. The remainder of the extraction procedure was as described above for endothelial cells.

We had chosen to perform sorbitol measurements with the enzymatic method based on the activity of sorbitol dehydrogenase [18]. The specificity of sorbitol dehydrogenase (L-iditol:NAD 5-oxidoreductase EC 1.1.1.14 from sheep liver; Sigma) was tested against glucose, galactitol, mannitol, fructose, myoinositol and xylitol (all purchased from Sigma). Reactivity of the enzyme with glycerol is known to be very low [19] and was not checked. As expected [19], all the compounds tested yielded only background readings even at 20 µg/ml concentration, with the exception of xylitol, which reacted in the assay exactly as sorbitol. We thus tested endothelial cells for the presence of measurable amounts of xylitol by enzymatic and chromatographic methods. The enzymatic measurement of xylitol was carried out as previously described [20] employing xylitol:NADP 4-oxidoreductase L-xylulose forming (EC 1.1.1.10 from pigeon liver, Sigma). GLC separation was performed in a Varian Model 3700 Gas Chromatograph on a 3% SE30 column (Supelco Inc., Bellefonte, Pa, USA) at a flow rate of 50 ml/min and temperature increments of 2°C/min (140–215°C). HPLC separation was performed in a Spectra-Physics 8700 instrument equipped with a U.V. detector, employing a 300 × 7.8 mm Aminex HPX-87H organic acid column (Biorad, Richmond, Calif, USA) which was maintained at 40°C, equilibrated in 30% buffer A (10 mmol/l H₂SO₄) and 70% buffer B (purified HPLC water) and eluted in the isocratic mode at a flow rate of 0.5 ml/min. The HPLC effluent was monitored at 195 nm [21].

The glucose 6-phosphate (G6P) content of cellular extracts was measured spectrophotometrically at 320 nm by following the reduction of NADP to NADPH in the presence of 50 mmol/l Tris, pH 7.35 and 1 U of glucose 6-phosphate dehydrogenase (EC 1.1.1.49, Sigma) in a final volume of 0.2 ml [22]. An enzyme blank, all components except the sample, and a sample blank, all components except the enzyme, were included and had an absorbance change that was less than 5% of the absorbance change of the full mixture.

Statistical analysis

Data are presented as mean ± SD in the indicated number of experiments. In each experiment determinations were obtained in duplicate-triplicate. Statistical analysis was performed by analysis of variance and by linear or polynomial regression (time-course of inhibition of sorbitol accumulation by Sorbinil).

Results

Since sorbitol dehydrogenase, the enzyme employed to measure cellular sorbitol content, showed substrate cross-reactivity with xylitol, we tested human endothelial cells for the presence of measurable amounts of xylitol. The enzymatic assay based on xylitol:NADP 4-oxidoreductase yielded concentrations of xylitol of 0.20 ± 0.06 nmol/10⁶ cells. When, however, the same cellular extracts were tested by GLC or HPLC no xylitol peak was observed. A graphic demonstration of the absence of a xylitol peak in HPLC tracings clearly showing a sorbitol peak is provided in Figure 1. Since the same pattern was evident in GLC separation, we concluded that the enzymatic assay for xylitol was measuring some compound other than xylitol and that in fact, under our culture conditions, human endothelial cells do not accumulate detectable amounts of xylitol. On the basis of the additional specificity studies re-

Fig. 1. HPLC tracings of (a) standards for glucose, sorbitol and xylitol; (b) extracts of cells cultured in 5 mmol/l glucose; (c) extracts of cells cultured in 5 mmol/l glucose + Sorbinil; (d) extracts of cells cultured in 20 mmol/l glucose; (e) extracts of cells cultured in 20 mmol/l glucose + Sorbinil. Duration of all treatments was 14 days. Abscissa: time; ordinate: intensity of signal detected at 195 nm; arrows: Sorbitol peak (12.5–12.7 min)

ported in the Methods section, it could thus be assumed that the readings obtained with sorbitol dehydrogenase quantitatively represented true sorbitol.

Sorbitol accumulation by human endothelial cells in primary culture was quite variable among different batches of cells. In presence of physiologic glucose levels cellular sorbitol content ranged from 0.04 to 0.12 nmol/ 10^6 cells. When cells were exposed to glucose levels mimicking diabetic hyperglycaemia (20 nmol/l) the sorbitol content increased by 2- to 3-fold to concentrations of 0.08–0.38 nmol/ 10^6 cells ($p < 0.01$).

Addition to the culture medium of 100 μ mol/l Sorbinil, an inhibitor of aldose reductase, resulted in a substantial inhibition of sorbitol accumulation by endothelial cells. It was, however, noted (Table 1) that the degree of inhibition achieved varied inversely with the duration of cell exposure to high glucose ($r = -0.957$, $p = 0.01$). While in cells exposed to high glucose and Sorbinil for 24 h sorbitol accumulation was inhibited by 71%, in cells exposed for 14 days inhibition was only 14% ($p < 0.01$). That the declining efficacy of Sorbinil was a function of length of cellular exposure to high glucose and not of loss of inhibitory activity of

Table 1. Sorbitol accumulation in human endothelial cells exposed to high glucose and effect of an aldose reductase inhibitor (Sorbitol 100 $\mu\text{mol/l}$)

Glucose in medium/ duration	Sorbitol/ duration	Sorbitol ^a nmol/ 10^6 cells mean $\pm$ SD	Inhibition by Sorbinil ^b
5 mmol/l 14 days	—	0.053 $\pm$ 0.006	89%
5 mmol/l 14 days	+ 24 h	0.006 $\pm$ 0.001	
20 mmol/l 24 h	—	0.100 $\pm$ 0.006	71%
20 mmol/l 24 h	+ 24 h	0.029 $\pm$ 0.006	
20 mmol/l 3 days	—	0.090 $\pm$ 0.02	70%
20 mmol/l 3 days	+ 3 days	0.027 $\pm$ 0.01	
20 mmol/l 8 days	—	0.086 $\pm$ 0.01	59%
20 mmol/l 8 days	+ 8 days	0.036 $\pm$ 0.008	
20 mmol/l 14 days	—	0.082 $\pm$ 0.008	14%
20 mmol/l 14 days	+ 14 days	0.071 $\pm$ 0.002	

Cells were studied for sorbitol content after 12–14 days in culture and after the indicated duration of treatments. ^aValues represent mean $\pm$ SD of determinations obtained in three experiments. ^bThe correlation coefficient between duration of exposure to high glucose (days) and percent inhibition of sorbitol accumulation by Sorbinil was -0.957 ($p=0.01$)

Table 2. Intracellular accumulation of glucose 6-phosphate in human endothelial cells cultured in high glucose

Culture conditions	Glucose 6-phosphate (nmol/ 10^6 cells)	
5 mmol/l glucose	0.23 $\pm$ 0.11 ^a	$p < 0.001$
20 mmol/l glucose	0.70 $\pm$ 0.08 ^a	
20 mmol/l glucose switched to 5 mmol/l for 5 h	0.21 $\pm$ 0.09 ^b	

^aValues represent the mean of 6 experiments $\pm$ 1 SD. ^bValues represent the mean of 2 experiments $\pm$ 1 SD

Fig. 2. Sorbinil treatment (2–10 $\mu\text{mol/l}$ left panel, 100 $\mu\text{mol/l}$ right panel) failed to prevent the proliferative delay observed in human endothelial cells cultured in high glucose ($\circ$ glucose 5 mmol/l; $\bullet$ glucose 5 mmol/l + Sorbinil; $\square$ glucose 20 mmol/l; $\blacksquare$ glucose 20 mmol/l + Sorbinil). Each point represents the mean $\pm$ SD of the determinations obtained in five experiments. The proliferation curves of cells exposed to 20 mmol/l glucose in presence or absence of Sorbinil are significantly different ($p < 0.005$ by ANOVA) from those of cells exposed to 5 mmol/l glucose

the drug was verified in two ways. First, we compared the effect of freshly prepared Sorbinil versus Sorbinil incubated with endothelial cell cultures for three days (the maximum interval between medium changes) on the accumulation of sorbitol by human erythrocytes (RBC). Sorbitol content of RBC incubated for 3 h at 37°C in presence of 5 mmol/l glucose was 7.7 ± 2.6 nmol/ml RBC and increased to 14.6 ± 0.9 nmol/ml RBC in presence of 20 mmol/l glucose. The addition of Sorbinil reduced this amount to 8.8 ± 0.8 nmol/ml RBC (fresh Sorbinil) and to 8.4 ± 0.3 nmol/ml RBC (incubated Sorbinil). Second, we contrasted the effects of chronic Sorbinil treatment (14 days) on sorbitol accumulation by cultures exposed to 5 or 20 mmol/l glucose for 14 days (Fig. 1). While the HPLC tracing of cells cultured in 5 mmol/l glucose showed, in the presence of Sorbinil, obvious disappearance of the sorbitol peak, the latter was practically unmodified in cells cultured in 20 mmol/l glucose in the presence of Sorbinil. No attempts at quantitation of sorbitol were made in these experiments, in which the amount of cellular extracts derived from the 5 mmol/l glucose cultures was maximized in order to ensure a detectable sorbitol signal.

We had previously documented [7] that in endothelial cell cultures exposed to 20 mmol/l glucose the achievement of saturation density is substantially delayed, with cell counts throughout the growth curve being 70–80% of control. We thus investigated whether the reduction in sorbitol content achieved with Sorbinil would modify the proliferative delay induced by exposure to high glucose. As illustrated in Figure 2, none of the concentrations of Sorbinil tested (2–100 $\mu\text{mol/l}$) succeeded in modifying the hindering effects of high glucose on endothelial cell proliferation. Both in the presence or absence of Sorbinil the growth curve of the cultures exposed to high glucose differed significantly from control ($p < 0.005$).

The intracellular accumulation of glucose 6-phosphate (Table 2) was enhanced 3-fold in endothelial cells cultured in high glucose: 0.70 ± 0.08 nmol/ 10^6 cells versus 0.23 ± 0.11 in controls ($p < 0.001$). In order to exclude that the increased G6P found in high glucose cultures might be contributed by G6P released from cell membranes during the preparation of extracts, we measured G6P in extracts of endothelial cells that – after having been chronically exposed to 20 mmol/l glucose – were switched to culture medium containing 5 mmol/l glucose for 5 h. The content of G6P in these cells (Table 2) was the same as in cells chronically exposed to 5 mmol/l glucose, thus excluding that G6P detected in extracts of cells cultured in high glucose could derive from dissociation of the sugar from adducts possibly formed with cellular constituents. When reported to cellular protein content (≈ 200 $\mu\text{g}/10^6$ cells) the amount of G6P found in endothelial cells was substantially higher than that reported in the literature for human erythrocytes (≈ 30 nmol/ml

RBC, 1 ml RBC = 371 mg protein). We thus tested the accuracy of our assay procedure by measuring G6P content in human RBC. The G6P content of RBC incubated for 3 h in 5 mmol/l glucose was 39 ± 5 nmol/ml RBC, and is in good agreement with published data [23, 24].

Discussion

Abnormalities of function and structure of vascular endothelium are likely to represent a central pathogenetic event in the development of diabetic angiopathy [1–6], and clarification of the mechanisms of endothelial cell injury should assist in developing rational strategies for clinical intervention. Although the tissue culture environment is quite remote from the complex and dynamic *in vivo* milieu, it offers the opportunity to study vascular endothelial cells of human origin not otherwise readily accessible.

We have previously shown [7, 8] that human endothelial cells in primary culture are susceptible to “glucose toxicity” as manifested by the occurrence of accelerated death, decreased replication and DNA damage upon exposure to high glucose concentrations. The combined results obtained in this and other studies indicate that the polyol pathway is not of major importance in mediating the ill effects of high glucose observed in this model. The amount of intracellular sorbitol accumulated in the presence of high glucose – in good agreement with previous observations [25] – was two orders of magnitude below that expected to possibly increase intracellular osmotic pressure [10]. It could be argued that measurements of intracellular sorbitol content might grossly underestimate sorbitol production if leakage of the metabolite to the medium is substantial. From studies in other tissues it is, however, apparent that, when production of sorbitol is high, this continues to be mirrored in the intracellular sorbitol content in spite of obvious signs of cellular leakage [26].

A more important consideration is that intracellular sorbitol content does not in itself reflect other possible ill effects of enhanced polyol pathway activity, the best recognized of which is compromised energy balance secondary to myoinositol depletion [11]. In various tissues [11, 26, 27] such ill effects have uniformly been prevented by Sorbinil treatment. In human endothelial cells, however, treatment with Sorbinil failed to improve even slightly the proliferative delay induced by high glucose, despite reducing cellular sorbitol content to concentrations below control levels throughout a good portion of the growth curve. Moreover, a major role for myoinositol depletion in the replicative delay of human endothelial cells exposed to high glucose has been specifically excluded by studies involving supplementation of the culture medium with supraphysiologic concentrations of myoinositol [28].

It cannot be determined from these studies whether the minimal extent of polyol pathway activation by high glucose is a function of tissue culture conditions, type of endothelial cells studied or human origin of the cells. The latter characteristic is, however, likely to be important, since vascular endothelial cells of the human retina do not stain for aldose reductase [29], and aldose reductase from other human tissues (lens) shows a higher *K_m* for glucose than the enzyme present in other species [30].

Exposure of endothelial cells to Sorbinil uncovered an aspect of Sorbinil action of potential relevance to its clinical applications. This was the progressively lesser inhibition of sorbitol formation achieved by Sorbinil with increasing time of exposure of endothelial cells to high glucose. Since the phenomenon could not be ascribed to diminished intrinsic potency of the drug, it may represent a consequence of the “activation” of human aldose reductase. Das and Srivastava have shown [31] that, in human tissues chronically exposed to high glucose, aldose reductase changes its kinetic properties and becomes less sensitive than in its native form to inhibition by various drugs, including Sorbinil. Together with the low activity of the polyol pathway in human tissues this phenomenon may contribute to the difficulties in establishing a clear-cut effectiveness of aldose reductase inhibitors in the treatment of diabetic complications in humans.

In spite of the saturation kinetics of hexokinase, accumulation of glucose 6-phosphate (G6P) appears to be a common occurrence in tissues obtained from diabetic humans or animals; the phenomenon can be ascribed to the relief by high ambient glucose of the G6P inhibition of hexokinase [24]. The amount of G6P found in endothelial cells cultured in physiologic glucose was almost an order of magnitude above that reported for erythrocytes [23], brain [13] and kidney [32], but superimposable to that found in rat myocardium [33]. The high G6P content of endothelial cells could not be attributed to inaccuracy in the assay nor to release of G6P from possible adducts; it is unclear at this time whether it represents a tissue-related characteristic (as in myocardium) or a consequence of the *in vitro* incubation conditions. Nevertheless, the almost 3-fold increase in G6P observed upon exposure to high glucose indicates that endothelial cells respond akin other tissues to ambient glucose levels. Owing to its open ring structure G6P is a powerful “glycosylator”, ten times as potent as glucose on a molar basis [14, 15]. Additionally, reducing sugars have been shown to undergo auto-oxidation in aqueous milieu with consequent generation of free radicals and intermediates of dioxygen reduction, and, albeit less potent than other monosaccharides, G6P is also in this respect more potent than glucose [34]. Thus, either through non enzymatic glycosylation or through free radicals production, accumulation of G6P might damage intracellular constituents and, among them, nucleic acids [35, 36]. We are particularly interested in exploring

this possibility to explain the replicative delay [7, 9] and DNA damage [8] observed in vascular endothelial cells maintained in a high glucose milieu, since these abnormalities could conceivably be prodromes of the decreased endothelial cell density [2] or accelerated disappearance [5, 6] described in the diabetic state.

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