Stimulation of Retinal Capillary Pericyte Protein and Collagen Synthesis in Culture by High-Glucose Concentration

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SUMMARY

The influence of glucose concentration on cell multiplication and protein synthesis was studied in synchronized, long-term cultures of bovine retinal microvessel pericytes. The cell multiplication rate and the mitotic rate were reduced in media containing 20 mM glucose to 57% and 54%, respectively, of that obtained in media containing 5 mM glucose. Elevated glucose, however, did not change the DNA content of individual cells.

Protein and collagen synthesis were measured by the incorporation of radioactive proline and lysine, or the posttranslational production of hydroxyproline and hydroxylysine, respectively. High glucose stimulated protein and collagen synthesis per cell 2.2 ± 0.10 (SD) and 2.1 ± 0.06 times, respectively. Aspirin (0.5 mM), an inhibitor of nonenzymatic glycosylation, did not alter the effect of elevated glucose concentration on protein and collagen synthesis. DIABETES *33*:785–789, August 1984.

Loss of retinal capillary pericytes (mural cells) and thickening of microvascular basement membranes are characteristic changes of the early phase of diabetic retinal microangiopathy.[1] Hyperglycemia per se may have an etiologic role in these changes through effects on aldose reductase/sorbitol metabolism, glucosamino-proteoglycan formation, the transport of cellular metabolites, or because of increased enzymatic and/or nonenzymatic glycosylation.[2–5]

To study the potential pathogenic role of high-glucose concentrations, the growth characteristics and metabolic activities of retinal microvessel cells are being examined in tissue culture. In the present study, we show that an elevated concentration of glucose suppressed the growth curves and mitotic rate of bovine retinal capillary pericytes, but increased the total protein and collagen synthesis of individual cells.

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MATERIALS AND METHODS

Bovine eyes were obtained from a local slaughterhouse within 6 h of death. Retinal microvessels, free of neural tissue, were prepared by the technique of Meezan et al.[6] and Carlson et al.[7] with minor modifications.[8] Microvessels were incubated with bacterial collagenase (0.6%, Worthington CLS III, Freehold, New Jersey) in 5 mM $CaCl_2$, 50 mM Tris-HCl buffer, pH 7.5, at 37°C for 40 min. Cells and capillary fragments were seeded on uncoated plastic Petri dishes and incubated in Dulbecco's Modified Minimum Essential Medium containing 5 mM glucose (DMEM) and supplemented with 20% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (standard medium). After reaching confluency, the cultured cells were exposed to 0.25% trypsin (Difco, Detroit, Michigan) in Ca-Mg-free Earle's balanced salt solution (EBSS) at 37°C for 2 min, which detached pericytes but left smooth muscle cells in situ.[9] Pericyte purity after 3 passages was judged by phase-contrast microscopy, by the absence of indirect immunofluorescence for factor VIII antigen,[10] and by the lack of angiotensin-converting enzyme activity.[11]

Bovine retinal capillary pericytes (BRCP) were replated in multiwells or Petri dishes, at a density of 1.5×10^4 cells/cm^2, in standard medium. To obtain cell synchronization, the standard medium was replaced after 3 days by DMEM supplemented with 1% FCS. The serum-deficient medium was replaced 48 h later with standard medium and, after 6-h incubation, hydroxyurea (1.25 mM, final concentration) was added. The cells were washed and fresh standard medium was added 12 h later. In selected experiments, [^{14}C]-thymidine (0.5 μCi/ml, Amersham, Arlington Heights, Illinois) was added to monitor DNA synthesis and pericyte culture synchronization.

To establish pericyte growth curves, wells containing syn-

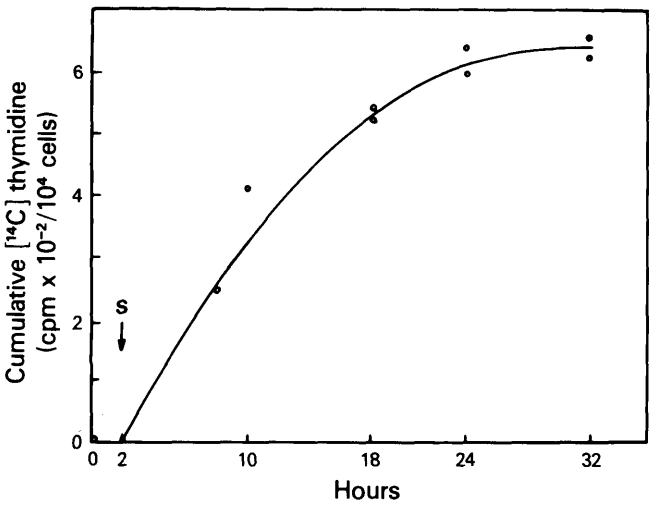

FIGURE 1. DNA synthesis (cumulative [^{14}C]-thymidine incorporation) by bovine retinal capillary pericytes (BRCP) in culture, stimulated by the addition of fresh standard culture medium (S) after serum restriction and hydroxyurea treatment for cell synchronization.

chronized cells were randomly chosen and the initial number of pericytes/well was determined after trypsinization. The remaining wells were divided into two groups. Standard medium, containing either 5 mM or 20 mM glucose, was added to odd- or even-numbered wells, respectively. At selected intervals, BRCP in subsets of wells were detached by trypsinization, counted, and cellular DNA was assayed by the method of Hinegardner[12] with minor modifications. Briefly, detached BRCP were transferred to siliconized tubes and collected by centrifugation. Cells were fixed in 10% formalin, 0.1 M Na-borate buffer, pH 8, washed with deionized H_2O (3 times), and dried at 50°C under vacuum. Diaminobenzoic acid (0.1 ml, 0.4 g/ml H_2O) was added, the tubes were placed in a 60°C water bath for 45 min, and then 1.5 ml of 1.0 N HCl was added. The mixture was vortexed and the fluorescence (excitation at 420 nm; emission at 510 nm) was measured immediately with a Ratio-Recording Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Maryland), and corrected with the appropriate blank fluorescence/light scatter measurements. Cultured feline retinal pigment epithelial cells[13] and deoxyribose (Sigma, St. Louis, Missouri) were used to construct standard curves.

To determine the effect of glucose concentration on the mitotic rate of cultured pericytes, synchronized cells were incubated for 3 days in medium containing either 5 or 20 mM glucose. Colchicine (Sigma) then was introduced (0.1 mM, final concentration) and the incubation was continued for 6 h. At 2, 4, and 6 h, 0.25% trypsin in EBSS was added, the detached cells were washed with medium, incubated with 0.075 M KCl at 37°C for 30 min, and fixed in methanol:acetic acid (3:1) at 4°C sequentially for 30, 10, and 5 min. Cell suspensions from individual dishes were air-dried on microscope slides in triplicate, stained with Giemsa (3:100) for 5 min, and mitotic figures were counted. The counting technique was modified from the method of Hooper.[14] Briefly, all stages of mitosis (i.e., prophase, metaphase, anaphase, and telophase) and all interphase nuclei of pericytes were counted by two observers in a double-blind manner. The accumulated metaphase figures were scored in at least 500 cells for each slide. These data were expressed as a percentage of the total number of nuclei scored (accumulated mitotic index), and plotted against the time of colchicine treatment. The slope of the least-squares, best-fit, linear regression lines for the data gave the cell birthrates (CBR), i.e., the new pericytes formed (as a percentage of the total number of cells) per 24 h.

Total protein synthesis 3 days after synchronization was determined in a separate experiment. BRCP, in 12 culture dishes each in either 5 mM or 20 mM glucose media, were labeled with 1 μCi/ml L-[U-^{14}C]-leucine (Amersham) and 2 mM L-leucine for 12 h. The medium was removed, the cells were washed twice with 2 mM EDTA in EBSS, and the washing and media were combined. Cells were harvested with a rubber policeman, counted, homogenized, and combined with the medium. Medium and cell proteins from individual dishes were precipitated with 10% trichloroacetic acid (TCA), dissolved in 1.0 N NaOH, 0.4 mg/ml bovine serum albumin carrier was added, the proteins were reprecipitated with 10% TCA (NaOH-TCA cycle repeated twice), washed twice with 10% TCA, and the incorporated radioactivity was measured in a scintillation counter and normalized to the cell number of the individual dishes.

In a separate set of protein-labeling experiments, synchronized pericyte cultures in 24 Petri dishes were divided into 4 groups and cultured in DMEM containing 10% FCS and either 5 or 20 mM glucose, with or without 0.5 mM aspirin. When all cultures had reached confluency (9 days after synchronization) the medium was replaced with fresh portions of the same medium containing 1 μCi/ml L-[U-^{14}C]-proline, 1 μCi/ml L-[U-^{14}C]-lysine (Amersham), 50 μg/ml ascorbate, and 50 μg/ml β-aminopropionitrile fumarate. After 24 h, the medium was collected and the cells were harvested with a rubber policeman, counted, and homogenized. The medium and cell homogenate from each dish were combined. Vitro-

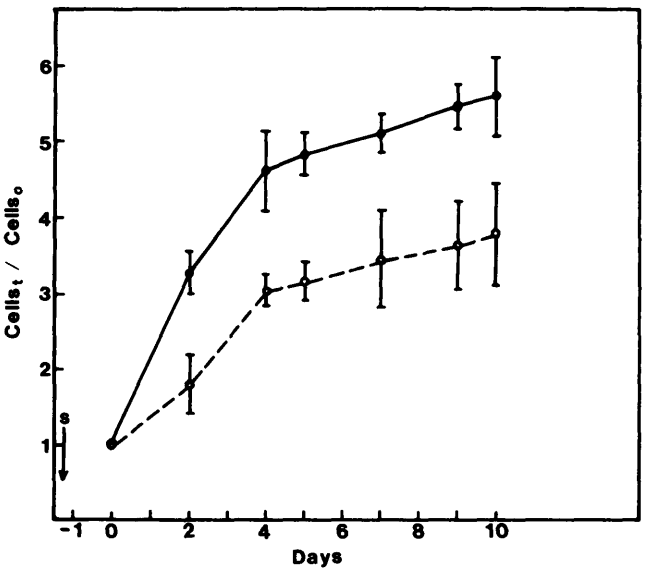

FIGURE 2. Growth curves of BRCP in cultures containing 5 mM (●——●) or 20 mM (○----○) glucose. Numbers of cells at a given time (cells$_t$) per initial cell number (cells$_o$) are plotted against days after the synchronization point (30 h after stimulation, S). Each point is the mean value (± SD) for 6 determinations.

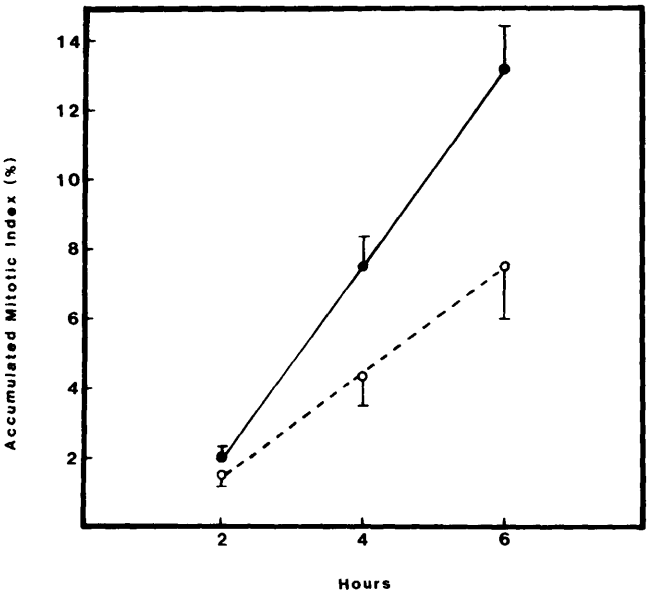

FIGURE 3. Accumulation of mitotic figures in cultured BRCP in the presence of 5 or 20 mM glucose. The mean accumulated mitotic index (cell in metaphase/total cells) is plotted against the time after the addition of colchicine to the culture. Each point is the mean value (± SD) for 9 determinations: 5 mM glucose, (●——●); 20 mM glucose, (○----○). (Lines are least-squares, best-fit, linear regression lines.) Cell birthrate was given by the slope of the accumulated mitotic index (%) versus time.

gen (20 μg/ml, type I collagen, Flow Lab, Palo Alto, California) was added as carrier, and proteins were precipitated with 10% TCA, washed repeatedly (5–7 times) until the supernate was free of radioactivity, and hydrolyzed in 6 N (constant boiling) HCl at 110°C for 24 h under vacuum. Amino acids were reacted (in glassware siliconized with dimethyldichlorosilane) with 2 mg/ml dansylchloride in acetone, and dansylated amino acid derivatives were analyzed on 7.5 × 7.5-cm, polyamide, thin-layer plates by 2-dimensional chromatography (solvents: 88% formic acid:water [2:100], benzene:glacial acetic acid [90:10]).[15] Proline, 4-hydroxyproline, lysine, and hydroxylysine were localized fluorometrically, cut out, and analyzed for radioactivity.

In separate parallel experiments, confluent cells grown in standard medium with 5 mM glucose were transferred to serum-free medium (HB-101, Hana Biologics, Berkeley, California) supplemented with 2% FCS, 4 mM L-proline, 50 μg/ml ascorbate, and 10 μCi/ml L-[2,3,4,5-^{3}H]-proline. Proteins precipitated from medium-cell homogenates by 10% TCA were determined according to Bradford.[16] Proteins were digested with bacterial collagenase (Advance Biofactures Corp., Lynbrook, New York) at a ratio of 3 U collagenase/100 μg protein. The collagen fraction of the total protein was calculated according to Diegelmann et al.[17]

RESULTS

Homogeneous, angulated pericytes were seen on phase-contrast microscopy after 3 passages. Retinal capillary endothelial cells did not proliferate under the present culture conditions, because they require special anchoring surfaces and tumor-conditioned medium for growth. The cultured pericytes were not reactive immunofluorescently for factor VIII antigen, and lacked angiotensin-converting enzyme activity, both of which were demonstrated in cultured bovine retinal capillary endothelial cells (unpublished observations). Cell viability (trypan-blue exclusion) was 97–98% and was not affected by the different glucose concentrations in the culture media.

The cumulative [^{14}C]-thymidine-labeling index reached a plateau 30 h after the addition of fresh standard medium to pericyte cultures that had been exposed to serum-deficient medium and hydroxyurea (Figure 1), and 30 h was selected as the synchronization point for replacement with experimental media. The growth curves for synchronized pericytes, cultured in medium containing either 5 mM or 20 mM glucose, showed that the cells had passed the lag period and had entered the growth phase at this point (Figure 2). A difference in growth rate was observed by day 2 (Figure 2). The doubling times, calculated from the 48-h data, were 40.6 ± 3.2 (SD) and 73.4 ± 9.5 h for pericytes grown in medium containing 5 mM and 20 mM glucose, respectively. In the first 4 days, the cells in both groups increased linearly with time (Figure 2); during this period, the rate of multiplication of pericytes grown in medium containing 20 mM glucose was only 57% of the rate in medium containing 5 mM glucose. After 9 days, when the cells in both groups entered a stationary phase, the cell numbers were 5.5 or 3.6 times the initial cell number for pericytes cultured with 5 mM or 20 mM glucose, respectively. The total DNA content/cell did not differ significantly between the 20 mM and 5 mM glucose cultures at any given point on the growth curve.

The accumulation of mitotic figures of pericytes after colchicine treatment was determined when the growth rates in both culture media were linear (3 days). The slopes for both groups differed significantly ($P < 0.01$) from zero, indicating a significant accumulation of mitotic figures (Figure 3). Elevated glucose (20 mM) suppressed the accumulation of mi-

TABLE 1
[^{14}C]-proline, hydroxy-[^{14}C]-proline, [^{14}C]-lysine, and hydroxy-[^{14}C]-lysine in proteins synthesized by cultured pericytes

Glucose (mM)	Aspirin (mM)	[^{14}C]-proline*	HO-[^{14}C]-proline*	[^{14}C]-proline/ HO-[^{14}C]-proline*	[^{14}C]-lysine*	HO-[^{14}C]-lysine*	[^{14}C]-lysine/ HO-[^{14}C]-lysine
5	—	14788 ± 1175	2240 ± 475	6.6	2976 ± 152	304 ± 16	9.8
5	0.5	17160 ± 504	2640 ± 445	6.5	3196 ± 287	311 ± 26	10.2
20	—	34038 ± 1624†	4661 ± 357†	7.3	6397 ± 253†	615 ± 37†	10.4
20	0.5	37230 ± 2880†	4983 ± 249†	7.5	6467 ± 452†	643 ± 38†	10.1

*Radioactive amino acids analyzed from each individual Petri dish. Numbers (dpm/10^5 cells) are the mean values from 6 dishes, (N = 6) ± SD.
†Differed significantly from 5 mM glucose culture results ($P < 0.01$, Student's one-tailed *t* test).

totic figures significantly ($0.005 < P < 0.01$); the cell birthrates were 36.0 ± 9.6%/24 h and 67.2 ± 9.2%/24 h when the culture glucose concentration was 20 mM and 5 mM, respectively. Total protein synthesis, measured 3 days after synchronization, was increased 61.5 ± 4.9%/cell when the glucose concentration was increased from 5 to 20 mM.

Protein and collagen synthesis were studied when the cells in both culture media had entered the stationary phase (10 days). The radioactivity of [^{14}C]-proline or [^{14}C]-lysine incorporated into newly synthesized proteins, and of hydroxy-[^{14}C]-proline or hydroxy-[^{14}C]-lysine formed by posttranslational modification of newly synthesized collagen, were used as indicators of total protein or collagen synthesis, respectively (Table 1). Pericytes incubated with 20 mM glucose synthesized 2.2 ± 0.10 times as much total protein and 2.1 ± 0.06 times as much collagen/cell as did those incubated with 5 mM glucose, in the presence or absence of 0.5 mM aspirin (Table 1). The ratios of [^{14}C]-proline/hydroxy-[^{14}C]-proline and [^{14}C]-lysine/hydroxy-[^{14}C]-lysine of newly synthesized proteins were not significantly ($P > 0.05$) altered by elevated glucose or aspirin in the culture media (Table 1). Collagenase digestion solubilized 4.8 ± 0.5% (N = 12) of the total radioactivity precipitated by TCA from pericyte culture medium-cell homogenates.

DISCUSSION

In the present study, when the growth curve, mitotic rate, DNA content, and protein/collagen synthesis per individual cell were determined, only the single variable, the glucose concentration in the medium, was changed in the synchronized cell cultures. The metabolic behavior of cells may be dramatically different during different phases of the cell cycle. Even though cells are located at the same phase (e.g., mitosis) a rather wide variation in cell mass and possibly in DNA content in cultured cells may be observed.[18] However, the large size differences between daughter cells disappears by the start of the S-phase.[18,19] The synchronization technique used in the present study is capable of collecting cells at G_1 and S-boundary, and, therefore, the synchronized cells in the present study were not only located at the same phase but also would have similar mass.[20] The growth curves and DNA content/cell were studied here up to the time that the steady state was obtained. The mitotic rate and protein/collagen synthesis were studied at selected times during this period. Synchronization of cultured cells offers the advantage that age-matched cells may be analyzed during the growth period.

The cell birthrate (CBR) of bovine retinal capillary pericytes (BRCP) in the presence of a physiologic concentration (5 mM) of glucose was 67%/24 h. This is a substantially higher CBR than that of low-reentry-rate cells such as fetal pancreatic β-cells.[21] The restoration of DNA synthesis after the serum-deficient medium was replaced by the standard medium may be taken as evidence that BRCP growth was controlled by an R-point mechanism.[18,22] The high cell birthrate may be due to the fact that the quiescent BRCP have a relatively high transition probability of passing the restriction (R) point occurring in the late G_1-phase, and enter the cell division cycle with relative ease. In the present study, the mitotic rate decreased significantly when the BRCP were cultured in a higher glucose concentration (20 mM), indicating that the elevated glucose may have blocked BRCP reentry. The higher glucose concentration stimulated protein synthesis at the time when the mitotic rate was decreased. When the general rate of protein synthesis is stimulated, the amount of U-protein, a special trigger protein required to overcome the R-point threshold,[24] might have been expected to increase as well, and the higher glucose concentration then should have facilitated the R-point passage. This paradox implied that BRCP may be under the influence of other control mechanisms superimposed on the R point mechanism.[22–26] In the present study, the average DNA content/cell was unchanged by increased glucose. Since most proteins are synthesized continuously throughout interphase, unlike DNA, which is replicated only once during the S-phase,[19,27] it is not necessarily surprising that DNA synthesis did not parallel protein synthesis.

The present findings that an elevated glucose concentration suppressed pericyte growth curves and increased protein and collagen synthesis in culture indicate that glucose per se is a crucial factor in the alterations of the growth characteristic and biosynthetic behavior of pericytes in culture. The increased protein and collagen synthesis induced by high levels of glucose in pericyte cultures suggests that hyperglycemia itself may have an etiologic role in the early microvascular basement membrane thickening of diabetic retinopathy.

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