Studies of the Effects of an Elevated Glucose Concentration on the Ultrastructure and Composite Metabolism of the Intact Rabbit Aortic Intima-Media Preparation

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SUMMARY

To assess whether the ultrastructure and composite metabolism of the arterial wall are subject to acute modification by exposure to an elevated glucose concentration, paired samples of the intact rabbit aortic intima-media preparation were incubated with 5 mM or 20 mM glucose for 1 h under conditions in which the ultrastructural and metabolic stability of samples incubated with 5 mM glucose has previously been demonstrated. Incubation with 20 mM glucose did not alter the qualitative electron-microscopic appearance of the tissue, and no significant changes were found on a quantitative morphometric evaluation of the aortic endothelium. A detectable, composite-free intracellular glucose concentration was not demonstrable in samples incubated with 5 mM or 20 mM glucose by a comparison of the ratio of glucose space:inulin space. Samples incubated with 5 mM glucose had significant sorbitol concentrations and unexpectedly high fructose concentrations but did not release fructose into the medium at detectable rates. Incubation with 20 mM glucose resulted in an increase in tissue sorbitol without an increase in tissue fructose or a detectable rate of fructose release. Tissue water content and myoinositol concentration were unaltered by incubation with 20 mM glucose, as were the rates of O_2 uptake and lactate production whether the gas phase was 5% CO_2:95% O_2 or 5% CO_2:95% air. The ultrastructure of intact aortic intima-media is not acutely altered by exposure to an elevated glucose concentration. Ambient glucose concentration is not the primary determinant of composite-free intracellular glucose concentration in arterial wall, and an increase in medium glucose concentration does not result in a significant increase in glucose utilization via the polyol pathway or induce the concomitants of increased polyol pathway activity demonstrable in a conventionally prepared aortic intima-media preparation. **DIABETES 28:720–723, August 1979.**

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In efforts to clarify the mechanisms that contribute to the increased risk of specific arterial syndromes in diabetics, the question of whether the metabolism and ultrastructure of the arterial wall are subject to acute modification by exposure to an elevated glucose concentration is important. In a previous study, paired samples of a conventional rabbit aortic intima-media preparation were incubated with 5 mM or 20 to 50 mM glucose for 2 h.[1] Increasing the glucose concentration to 20 mM resulted in increased free intracellular glucose, sorbitol, and fructose concentrations and a 3.6-fold increase in the rate of fructose released into medium. (Based on the assumption that the fructose was derived primarily from glucose utilization via the polyol pathway, the rate of fructose released into the medium was used to provide a minimal estimate of the rate of glucose utilization by way of the polyol pathway; fructose production accounted for 1.8% of the glucose uptake in samples incubated with 5 mM glucose.[1]) Increasing medium glucose concentration also resulted in an increased tissue water content, a decrease in O_2 uptake, and an increased rate of lactate production, which was associated with an increased tissue lactate: pyruvate ratio. The alterations in O_2 uptake and lactate production resulting from incubation with 20 to 50 mM glucose were prevented either by increasing the medium osmolality with mannitol or by increasing the medium O_2 tension by using 5% CO_2:95% O_2 as the gas phase instead of 5% CO_2:95% air.[1] These observations suggested that exposure to an elevated glucose concentration resulted in increased glucose utilization by way of the polyol pathway, which was associated with an increased aortic water content and impaired O_2 diffusion at physiologic O_2 tensions; the latter had frequently been suggested as a possible pathogenetic mechanism in the arterial wall.[2,3]

These observations now require reexamination, along with most of the available data on arterial metabolism derived from conventionally prepared aortic intima-media preparations.[1,4–10] For we subsequently demonstrated that the methods commonly employed to prepare aortic intima-media for incubation result in widespread irreversible

endothelial cell injury and induce persistent alterations in the composite glucose utilization and pattern of energy metabolism in this tissue.[11] Methods have been developed to obtain and incubate an intact rabbit aortic intima-media preparation that retains an unaltered electron-microscopic (EM) appearance and exhibits stable metabolic activities during a 1 h incubation with 5 mM glucose markedly different from those observed in conventional preparations.[11] This intact preparation provides a sensitive tool for in vitro studies of the effects of factors that might acutely modify the ultrastructure or composite metabolism of the arterial wall, as illustrated by its recent use to demonstrate the effects of brief periods of anoxia.[12] This preparation has been used to examine the effects of increasing medium glucose from 5 mM to 20 mM during a 1 h incubation.

METHODS

Male, white, New Zealand rabbits (1.5 to 2.5 kg), which had been fed freely on Wayne rabbit ration (Allied Mills, Chicago, IL) plus carrots and lettuce, were sedated with diazepam (2.0 mg/kg i.m.) and killed 90 min later by decapitation. The thoracic aorta was rapidly removed, and paired samples of intact aortic intima-media were prepared as previously described in detail.[11,12] Each sample, weighing about 100 mg, was transferred to a 25 ml Erlenmeyer flask containing 5.0 ml of Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, gas phase 5% CO_2:95% O_2, containing 5 mM or 20 mM glucose and 6% dialyzed bovine serum albumin (BSA) (fraction V powder, Sigma Chemical Co., St. Louis, MO) at 37 °C. The flasks were shaken at 88 cycles per minute in a Dubnoff metabolic shaker at 37 °C with continuous gassing for 5 min; the flasks were then sealed with a rubber cap and incubated for 1 h. In specific experiments, paired samples were incubated in the same medium containing 5 mM or 20 mM glucose but with 5% CO_2:95% air as the gas phase; in these experiments, 125 ml Erlenmeyer flasks containing 25 ml of medium were used for the incubation.[11] The methods for the determination of tissue water,[1] O_2 uptake,[1] tissue glucose,[13] sorbitol,[14] fructose,[14] and myoinositol concentrations,[15] and for medium glucose,[13] lactate,[16] and fructose[14] were as previously described. Separate experiments were carried out to provide samples for the determination of O_2 uptake and water content and for the determination of tissue metabolite concentrations. In those experiments in which the ratio of the glucose space:inulin space was compared, 12 μCi of (methoxy-^{3}H) inulin (specific activity 0.178 mCi/mg, New England Nuclear, Boston, MA) was added to the medium containing 5 mM or 20 mM glucose. At the end of a 1 h incubation the tissue was rapidly blotted and homogenized in 4.0 ml of 5% (w/v) $ZnSO_4$ in a glass homogenizer to which 4.0 ml of balanced 0.3 M $Ba(OH)_2$ was added, and the homogenization was repeated. After centrifugation, 1.0 ml aliquots of the supernatant were added to 15 ml of Aquasol (New England Nuclear) in a glass, counting vial and was counted in a liquid scintillation spectrometer with an internal standard (Teledyne Isotopes, Westwood, NJ); glucose was determined in aliquots of the same supernatant. $Ba(OH)_2$-$ZnSO_4$ filtrates were prepared from the incubation medium, and aliquots were counted as described above and were used for glucose determinations. Control experiments demonstrated no difference in the inulin space as a percent of wet weight in paired samples incubated for 30 min or 1 h.

The differences between the determinations made in paired samples were analyzed by the *t* test for paired comparisons.[17]

In separate experiments (N = 8) paired samples of intact aortic intima-media were incubated for 1 h in medium containing 5 mM or 20 mM glucose and then immediately fixed and prepared for transmission EM examination as previously described.[12] Samples of freshly prepared intact aortic intima-media (N = 6) and of rabbit thoracic aortic fixed in situ (N = 6)[12] were also prepared for EM examination. Using the sampling techniques and instruments previously described[12] the EM appearance of the tissue samples was subjected to a blind qualitative evaluation. For a quantitative morphometric analysis of the EM appearance of the endothelium in paired samples of intact aortic intima-media incubated with 5 mM or 20 mM glucose for 1 h, tissue was prepared from three additional experiments. Ultrathin sections were examined from two tissue blocks randomly selected from each of the two experimental conditions employed in each paired experiment; 14 electron micrographs, seven from each section, were taken randomly at a magnification of ×7200. The electron micrographs were recorded on 70 mm film in a Zeiss EM 9 electron microscope. A carbon-grating replica with 2160 lines per millimeter was recorded on each film for calibration of the magnification, and the films were examined in a stable projector unit. The point-counting method was used to estimate the volume occupied by endothelium per unit volume of aorta, i.e., the volume density (V_v).[18] A lattice of P_T test points was placed on the negative, and the number of points enclosed within the profiles studied (P_i) was determined: V_v (volume density) = P_i/P_T.

The surface area of the endothelium per unit volume of tissue (S_v) was estimated by counting the number of intersections (I_L) of the surface profile per unit length of test lines of known length.

$$S_v = 2\ I_L$$

The average V_v and S_v observed in the seven electron micrographs from each section were determined; these values were used to calculate the mean ± SEM of the average V_v and S_v observed in the six sections from tissue incubated with 5 mM glucose and in the six sections from tissue incubated with 20 mM glucose.

RESULTS

Paired samples of intact rabbit aortic intima-media were incubated with 5 mM or 20 mM glucose for 1 h. As expected from previous studies,[11] the EM appearance of the samples incubated with 5 mM glucose was similar to that of unincubated, freshly prepared samples and to that of rabbit thoracic aorta fixed in situ. The EM appearance of the samples incubated with 20 mM glucose was qualitatively unaltered when compared with that of the paired controls incubated with 5 mM glucose. Since this qualitative evaluation might not detect minor degrees of endothelial cells' swelling or a small but significant increase in the frequency of the rare discontinuities in the endothelial surface found in samples incubated with 5 mM glucose, a quantitative morphometric analysis of the transmission EM appearance of the endothelium was carried out (see METHODS). As shown in Table 1, there were no significant differences either in the volume occupied by endothelium

TABLE 1
Morphometric evaluation of the EM appearance of the aortic endothelium after a 1 h incubation with 5 mM or 20 mM glucose

Medium glucose	Glucose 5 mM	Glucose 20 mM	P
V_v endothelium	7.38 ± 1.16	6.67 ± 1.57	NS
S_v endothelium	11.14 ± 1.67	10.91 ± 2.16	NS

Paired samples of intact rabbit aortic intima-media were fixed for EM examination immediately after a 1 h incubation with 5 mM or 20 mM glucose in KRB buffer, pH 7.4, gas phase 5% CO_2:95% O_2 containing 6% dialyzed BSA at 37°C. Tissue from three paired experiments was examined by a morphometric approach (see METHODS). V_v endothelium is the volume density of endothelium expressed as a fraction. S_v endothelium is the surface area of endothelium per unit volume of tissue expressed as a fraction. The values are the mean ± SE for the two groups of tissues.

per unit volume of aorta or in the surface area of endothelium per unit volume of aorta in paired samples incubated with 5 mM or 20 mM glucose for 1 h.

The ratio of the glucose space:inulin space in samples incubated with 5 mM glucose was consistently less than one and failed to provide evidence for a significant intracellular concentration of free glucose; the ratio was not significantly different in samples incubated with 20 mM glucose for 1 h (Table 2). Sorbitol and free fructose were present in samples incubated with 5 mM glucose for 1 h, but fructose was not released into the medium in detectable concentrations. In the paired samples incubated with 20 mM glucose, there was a significant increase in sorbitol concentration but this was not accompanied by a significant change in the tissue's fructose concentration or by a detectable release of fructose into the medium (Table 2). The water content and free myoinositol concentration in samples incubated with 20 mM glucose were not significantly different from those of the samples incubated with 5 mM glucose. The rates of O_2 uptake and of lactate production were not significantly different in samples incubated with 20 mM or 5 mM glucose (Table 2).

The relatively high O_2 requirements of the intact preparation necessitate the use of 5% CO_2:95% O_2 under the standard incubation conditions employed in the experiments reported above; this might have obviated effects of incubation with 20 mM glucose on O_2 uptake or lactate production demonstrable only at a more physiologic O_2 tension. Consequently, paired samples of the intact preparation were incubated with 5 mM or 20 mM glucose for 1 h in medium equilibrated with 5% CO_2:95% air with the fivefold increase in medium volume:tissue wt ratio required to maintain a stable rate of O_2 uptake in samples incubated with 5 mM glucose under these conditions.[11] As shown in Table 3, there were no significant differences in the rates of O_2 uptake of paired samples incubated with 5 mM or 20 mM glucose for 1 h in medium equilibrated with 5% CO_2:95% air, and there was no difference in their rates of lactate production.

DISCUSSION

Incubation with 20 mM glucose for 1 h did not noticeably alter the EM appearance of the intact rabbit aortic intima-media preparation, its water content, free myoinositol concentration, or its rate of respiration and lactate production as compared with those of paired controls incubated with a physiologic glucose concentration. These data suggest that it is unlikely that the ultrastructure and energy metabolism of the arterial wall are subject to acute modification by exposure to an elevated glucose concentration. While these data are relevant to the possible effects of a short period of hyperglycemia, the effects of chronic exposure to an elevated glucose concentration or to marked fluctuations in glucose concentration remain to be clarified.

Previous studies of the intact aortic intima-media preparation required modifications in the views of glucose and energy metabolism in the arterial wall derived from observations in conventional in vitro preparations of this tissue. In samples of the intact preparation incubated with 5 mM glucose, the rate of respiration is 55% higher, glucose uptake is 100% higher, lactate production is markedly lower, and it accounts for only a small fraction of the glucose uptake in contrast to the high rate of aerobic glycolysis previously considered characteristic of arterial wall's energy metabolism.[11] Additional modifications in these views are required as the result of the present studies. Aortic intima-media does not contain a detectable, composite-free intracellular glucose concentration when incubated with a physiologic or elevated glucose concentration, and the ambient glucose concentration is probably not the primary determinant of free intracellular glucose concentration in the vascular smooth muscle cells, which constitute the preponderant fraction of the cellular mass of the arterial wall as suggested by previous observations in conventionally

TABLE 2
Comparison of paired samples of intact rabbit aortic intima-media incubated with 5 mM or 20 mM glucose for 1 h

	Glucose (5 mM)	Glucose (20 mM)	Mean Δ + SEM	P	No.
Glucose space:inulin space	0.90 ± 0.03	0.89 ± 0.02	−0.01 ± 0.04	NS	6
Tissue sorbitol (nmol/g wet wt)	8.7 ± 1.1	22.0 ± 1.7	+13.2 ± 1.6	<0.001	8
Tissue fructose (nmol/g wet wt)	474 ± 39	414 ± 33	−60 ± 95	NS	6
Fructose production (nmol/g wet wt/h)	ND*	ND	—	—	6
Water content (g H_2O/100 g dry wt)	247 ± 4	240 ± 4	−7 ± 4	NS	6
Tissue myoinositol (μmol/g wet wt)	5.71 ± 0.43	5.49 ± 0.32	−0.22 ± 0.24	NS	6
Oxygen uptake (μl O_2/g wet wt/h)	206 ± 4	202 ± 15	−5 ± 17	NS	6
Lactate production (μmol/g wet wt/h)	2.8 ± 0.3	3.1 ± 0.1	+0.3 ± 0.2	NS	6

Paired samples were incubated at 37°C for 1 h in 5 ml of KRB buffer, pH 7.4, gas phase 5% CO_2:95% O_2 containing 6% dialyzed BSA and glucose in the concentration indicated. Values are the mean ± SEM. Significance of the difference between paired samples was assessed by the *t* test for paired comparisons.[17]

* ND stands for not detectable.

TABLE 3
Comparison of O_2 uptake and lactate production in paired samples of intact aortic intima-media incubated with 5 mM or 20 mM glucose for 1 h with 5% CO_2:95% air as the gas phase

	Glucose (5 mM)	Glucose (20 mM)	Mean Δ + SEM	P	No.
Oxygen uptake (μl O_2/g wet wt)	198 ± 7	194 ± 5	−4 ± 6	NS	6
Lactate production (μmol/g wet wt/h)	2.6 ± 0.1	2.6 ± 0.2	0.0 ± 0.1	NS	6

Paired samples were incubated at 37°C for 1 h in 25 ml of KRB buffer, pH 7.4, gas phase 5% CO_2:95% air containing 6% dialyzed BSA and glucose in the concentration indicated. Values are the mean ± SEM.

prepared aortic tissue. (The composite data do not exclude the possibility that ambient glucose concentration regulates free intracellular glucose concentration within specific cellular components.)

Alditol:NADP oxidoreductase and L-iditol:NAD oxidoreductase have been isolated from rabbit aorta,[1] and it is reasonable to assume that sorbitol and fructose in rabbit aortic intima-media preparations are derived from the reactions of the polyol pathway, although the existence of other mechanisms for the synthesis of fructose in this tissue is not excluded. There appears to be a significant enzymatic capacity for fructose synthesis in aortic intima-media, and our previous observations in a conventional preparation suggested that fructose production, presumably by way of the polyol pathway, was regulated by the ambient glucose concentration and was markedly increased when the medium glucose concentration was increased from 5 mM to 20 mM. In samples of the conventional preparation incubated with 5 mM glucose, stable sorbitol concentrations were achieved within 30 min and stable fructose concentrations within 1 h; the recovery of fructose in the medium after 30 min, 1 h, and 2 h of incubation averaged 40.9, 82.8, and 214 nmol/g, respectively.[1] The last value was sixfold greater than the steady state tissue fructose concentration and was more than 16-fold greater than the steady state sorbitol concentration. When samples of the same preparation were incubated with 20 mM glucose for 2 h, the sorbitol and fructose concentrations were both markedly higher than the steady state concentrations during incubation with 5 mM glucose, and the average amount of fructose recovered in the medium was 780 nmol/g.[1] Samples of the intact aortic intima-media preparation incubated with glucose for 1 h had a surprisingly high fructose concentration (470 nmol/g), but fructose could not be detected in the incubation medium. Samples of the intact preparation incubated with 20 mM glucose for 1 h had a significantly higher sorbitol concentration but the tissue fructose concentration was similar to that found after incubation with 5 mM glucose, and detectable quantities of fructose were not released into the medium. These observations provide no evidence that increasing the medium glucose concentration from 5 mM to 20 mM results in increased fructose production in the intact aortic intima-media preparation, although the limitations of an interpretation based on a comparison of tissue fructose concentrations must be acknowledged. It is of interest that increasing the medium glucose concentration from 5 mM to 20 mM also failed to reproduce the concomitants of increased fructose production in the conventional aortic intima-media preparation.

Conventionally prepared aortic intima-media preparations are still being used extensively (e.g., for pharmacologic studies and for studies of prostaglandin synthesis[19]), without evidence that the preparations employed are not subject to the alterations in ultrastructure and metabolism documented for our previous conventional rabbit aortic intima-media preparations. The studies reported above illustrate that conventional preparations may retain specific enzymatic capacities but they may give unreliable information about the rates at which metabolic processes occur in uninjured tissue or about the factors that regulate specific metabolic processes in the arterial wall.

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