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Research Article

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Glucose-induced Alterations of Cytosolic Free Calcium in Cultured Rat Tail Artery Vascular Smooth Muscle Cells

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Abstract

We have previously suggested that hyperglycemia per se may contribute to diabetic hypertensive and vascular disease by altering cellular ion content. To more directly investigate the potential role of glucose in this process, we measured cytosolic free calcium in primary cultures of vascular smooth muscle cells isolated from Sprague-Dawley rat tail artery before and after incubation with 5 (basal), 10, 15, and 20 mM glucose. Glucose significantly elevated cytosolic free calcium in a dose- and time-dependent manner, from 110.0±5.4 to 124.5±9.0, 192.7±20.4, and 228.4±21.9 nM at 5, 10, 15, and 20 mM glucose concentrations, respectively. This glucose-induced cytosolic free calcium elevation was also specific, no change being observed after incubation with equivalent concentrations of L-glucose or mannitol. This glucose effect was also dependent on extracellular calcium and pH, since these calcium changes were inhibited in an acidotic or a calcium-free medium, or by the competitive calcium antagonist lanthanum.

We conclude that ambient glucose concentrations within clinically observed limits may alter cellular calcium ion homeostasis in vascular smooth muscle cells. We suggest that these cellular ionic effects of hyperglycemia may underlie the predisposition to hypertension and vascular diseases among diabetic subjects and/or those with impaired glucose tolerance. (*J. Clin. Invest.* 1995. 95:763–767.) Key words: glucose • intracellular calcium • hypertension • diabetes mellitus • vascular biology

Introduction

The clinical association of hypertensive disease and non-insulin-dependent diabetes mellitus (NIDDM)[1] has long been appreciated (1, 2), but despite the clinical significance that has been attached to this association the underlying mechanisms that initiate and sustain elevated blood pressures in hypertensive diabetic subjects remain undefined. The recent findings that essential hypertension is also an insulin-resistant state, associated with hyperinsulinemia and exaggerated insulinemic responses to oral glucose loading (3, 4), has led some authors to attribute to insulin a possible pathogenetic role in the pathophysiology of essential hypertension (5) and/or in mediating the clinical coincidence of NIDDM and hypertension (2). However, circulating insulin levels may be significantly lower in NIDDM compared with weight-matched nondiabetic subjects (6), suggesting that factors other than insulin may be relevant as well.

We have recently suggested a common cellular ionic basis for the clinical association of NIDDM and hypertension (7), based in part on our observations that hyperglycemia itself, independently of insulin, may alter the ionic content of normal human erythrocytes (8). To more directly determine whether these ionic effects of glucose might be relevant to the increased peripheral vasoconstriction present in chronic diabetic states, we evaluated the effects of different concentrations of glucose on the cytosolic free calcium content (Cai) of cultured vascular smooth muscle cells (VSMC). Our results suggest that glucose, in a specific concentration- and time- dependent manner, at levels achieved clinically, and independently of insulin, may alter intracellular calcium homeostasis in VSMC.

Methods

Preparation of cultured rat tail VSMC. All studies were performed on primary cultures of VSMC isolated from Sprague-Dawley rat tail artery. Male Sprague-Dawley rats (100–200 g body wt) were anesthetized with sodium pentobarbital (65 mg/kg body wt intraperitoneally), and tail artery was dissected out and immersed in a calcium- and magnesium-free solution (Hanks' buffered saline solution [HBSS], Gibco Laboratories, Grand Island, NY) at 4°C. The blood was washed off, and the artery and the connective tissue were removed. Using a dissecting microscope the artery was cut in pieces of ~ 2 cm and placed in the HBSS solution for 30 min, at 4°C. After incubation, the arterial strips were than processed in the following solutions at 37°C: (*a*) low-calcium enzyme solution I (0.2 mM Ca^{2+}, HBSS) which was composed of collagenase/dispase (1.5 mg/ml; Boehringer Mannheim GmbH, Mannheim, Germany), elastase (0.5 mg/ml type IIa; Sigma Immunochemicals, St. Louis, MO), trypsin inhibitor (1 mg/ml, fatty acid free; Sigma Immunochemicals), and bovine serum albumin (BSA, 2 mg/ml; Sigma Immunochemicals), for 1.5 h; (*b*) the tissue was rinsed twice in calcium-free HBSS solution; and (*c*) the medium was then changed to enzyme solution II which was composed of calcium-free HBSS with collagenase (1 mg/ml, type II; Sigma Immunochemical), trypsin inhibitor (0.3 mg/ml, type I-S; Sigma Immunochemicals), BSA (2 mg/ml; Sigma Immunochemicals). Incubation in enzyme solution II lasted 1 h. All incubations in enzyme solutions I and II were carried out in a CO_2

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1. *Abbreviations used in this paper:* Cai, cytosolic free calcium content; NIDDM, non-insulin-dependent diabetes mellitus; VSMC, vascular smooth muscle cells.

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incubator (95% O_2 and 5% CO_2 at 37°C). The arteries were then triturated in calcium-free HBSS buffer, using a fire polished Pasteur pipette until the medium turned cloudy. The cell suspension was stored in the refrigerator at 4°C, and the calcium concentration of the suspension was increased to 2.0 mM by the stepwise addition of $CaCl_2$ over 40 min, after which the dispersed cells were plated on 35-mm Petri dishes with Dulbecco's modified Eagle's medium (DME; Gibco Laboratories) with 10% fetal bovine serum, and cultured in a humidified atmosphere of 5% CO_2, 95% O_2, at 37°C. At confluence, VSMC from second and third passage were used. 95% of the rat tail artery cells obtained were viable using the trypan blue exclusion method. Furthermore, they were determined to be smooth muscle cells based on their contractile responses to norepinephrine (9) and the localization in these cells of fluorescent antibodies to α-actin.

Measurements of intracellular Cai. For FURA-2 assays, ~ 1×10^6 cells were seeded on a microscope cover slip (25-mm circle) in DME medium. Culture medium was changed every other day until the cells were elongated and confluent. Cells were incubated for 45 min in DME containing 5 μM FURA-2 acetoxymethyl ester (AM; Molecular Probes, Inc., Eugene, OR) at 37°C, in a dark compartment. Afterwards the cells were gently washed three times with a buffer medium (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 1 mM $CaCl_2$, 0.5 mM NaH_2PO_4, 10 mM Hepes, at pH 7.4) and kept in the same buffer. After ~ 5 min, the coverslip with the cells attached was placed in a 1-ml Sykes-Moore chamber on the stage of an inverted phase-contrast microscope (Nikon, Tokyo, Japan). Fluorescence measurements were made according to the method of Grynkiewicz et al. (10), using a DM3000 spectrofluorometer (Spex Industries Inc., Edison, NJ). Intracellular calcium concentration was calculated according to the following equation:

$$\text{Cai (nM)} = K_d\,(R - R_{min}/R_{max} - R) \times b;$$

where R is the ratio of fluorescence in the sample at 340 and 380 nm. R_{max} is the fluorescence ratio obtained by adding 2 μM ionomycin. R_{min} is the fluorescence ratio subsequently obtained by 5 mM EGTA, and b is the ratio of fluorescence of FURA-2 at 380 nm at zero versus saturating calcium concentrations. K_d is the dissociation constant of calcium-bound FURA-2, taken to be 224 nM (10). Only tissue samples demonstrating consistent, reproducible elevations of cytosolic free calcium in response to KCl (30 mM, added and washed out before testing any experimental agents) were used for studies.

Experimental procedures. Confluent VSMC were exposed to the buffer media before determining intracellular calcium concentrations. Glucose was added to the buffer solution to achieve the following final concentrations: 5 (basal, no added glucose), 10, 15, and 20 mM. Cytosolic free calcium was measured before and for 60 min after the addition of each glucose concentration. To study the specificity and mechanisms of the observed glucose-induced changes, intracellular calcium measurements were also obtained after exposure of VSMC to L-glucose and mannitol, each at final concentrations of 20 mM. Lastly, intracellular calcium measurements were obtained after incubation of cells in an acidotic medium (pH = 7.1), a calcium-free medium, both before and after the addition of glucose (20 mM), as well as after the addition of lanthanum (2 mM) to the incubation medium.

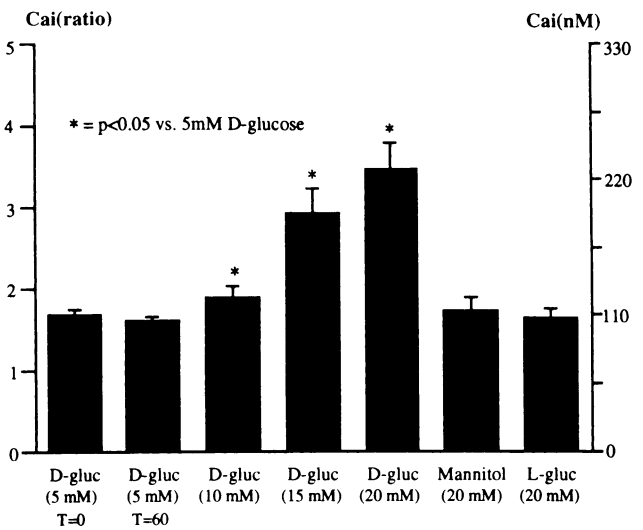

Figure 1. Effects of D-glucose (*D-gluc*) at 5 mM (before, T = 0, and after 1 h of incubation, T = 60) and at 10, 15, and 20 mM; L-glucose (*L-gluc* at 20 mM); and mannitol (at 20 mM) on intracellular cytosolic free calcium concentrations (Cai, nM) in primary cultures of VSMC.

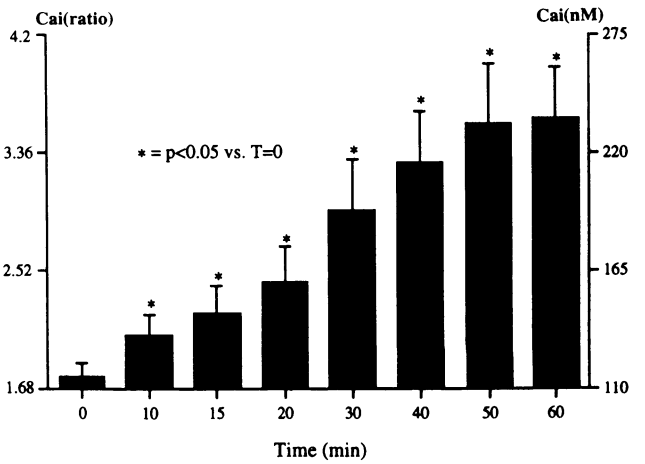

Figure 2. Time-dependent effects of D-glucose (20 mM) on cytosolic free calcium concentrations (Cai, nM) in primary cultures of vascular smooth muscle.

Data analysis. All data values are reported as the mean±SEM. Paired *t* tests were used for comparison between mean values of the free calcium before and after incubation with glucose at a given concentration, for a given group of cells. Repeated-measures analysis of variance and subsequent Neuman-Keul's multiple test were used to assess the effects of glucose over time and over the range of concentrations tested, for different groups of cells. A P value < 0.05 was considered statistically significant.

Results

Effect of glucose on intracellular free calcium concentrations. The basal cytosolic free calcium concentration in this cell preparation (5 mM glucose) was 110±5.438 nM (fluorescence ratio = 1.679±0.083, n = 12). In the absence of further additions of glucose, this value was stable over the incubation periods used in this study (106.2±3.9 nM after 1 h). Incremental doses of glucose above 5 mM induced consistent and dramatic elevations in cytosolic free calcium which appeared to be dose-dependent over the range of concentrations tested (10 mM, 124.5±9.0 nM; 15 mM, 192.7±20.4 nM; 20 mM, 228.4±21.9 nM, sig = 0.05 vs 5 mM glucose at all tested concentrations) (Fig. 1). The glucose-induced changes in Cai also evolved over time, calcium progressively accumulating over the course of 1 h, as shown in Fig. 2 and 3.

The effect of glucose in raising Cai also appeared to be specific, since no significant intracellular free calcium increase (108.3±6.8 nM, P = NS vs basal) was observed when VSMC were incubated with 20 mM L-glucose. Washout of hyperglycemic media with basal, 5 mM glucose-containing media did not

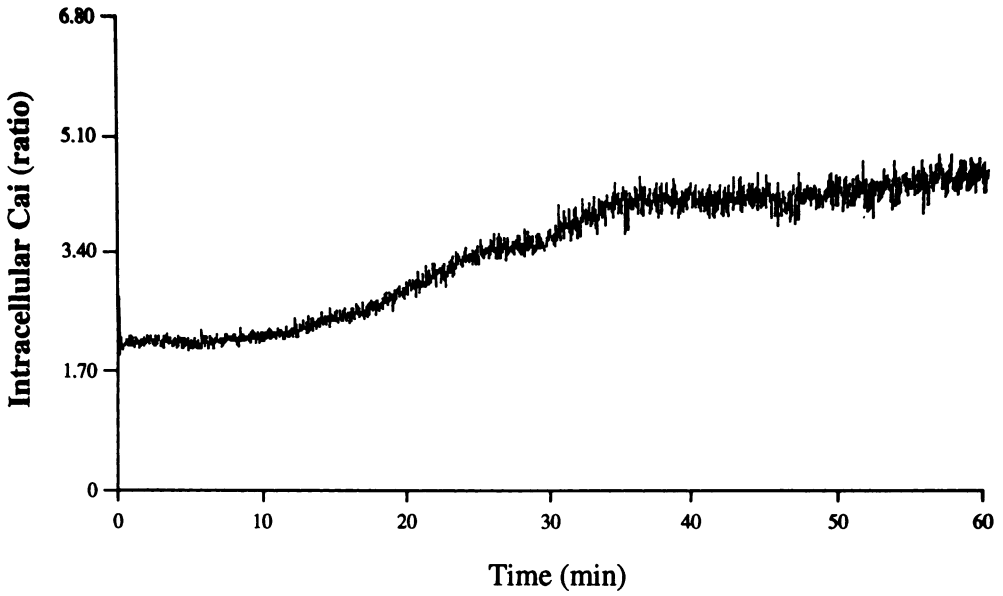

Figure 3. Continuous tracing of intracellular cytosolic free calcium in isolated VSMC exposed to 20 mM D-glucose over the course of 60 min.

change the elevated Cai levels over the course of an additional 1 h of incubation. Further evidence that this effect did not merely reflect an altered osmotic environment was also indicated by the lack of effect of 20 mM mannitol to alter Cai in these cells (113.6±10.8, P = NS vs basal) (Fig. 1).

Mechanistically, preliminary data suggest that this glucose effect is dependent on the ambient extracellular pH, as well as on the availability of extracellular calcium. Thus, when the medium pH was lowered from 7.4 to 7.1, the addition of 20 mM glucose did not produce any change in cytosolic free calcium (97.9±6.6 nM, P = NS vs basal) (Fig. 4, *left*). When a pH of 7.4 was restored to these same cells, the subsequent addition of 20 mM glucose once again elevated Cai (to 155±14.9 nM, $P < 0.05$ vs basal glucose). At a pH of 7.4, the glucose-induced free calcium increase was prevented by a calcium-free medium (104.6±6.6 nM, P = NS vs basal) (Fig. 4, *right*). The addition of the competitive calcium antagonist, lanthanum (2 mM), to the incubation medium had no effect on basal Cai but blunted the rise in Cai induced by 20 mM glucose by 57% (96.3±3.0 to 140.6±10.8 nM, n = 14, P = 0.0037 vs 20 mM glucose alone).

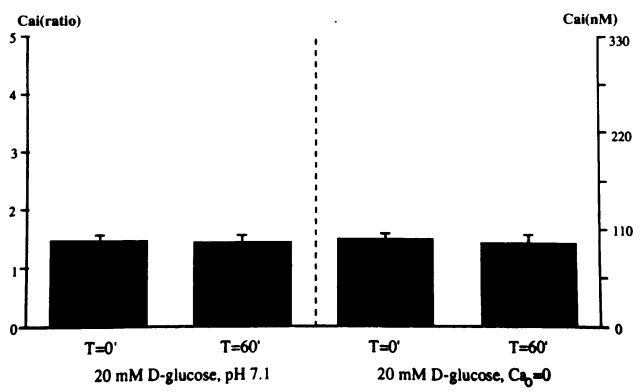

Figure 4. The inhibition by extracellular acidosis (pH = 7.1) and a calcium-free medium (Ca_o = 0) of the effect of D-glucose on intracellular cytosolic free calcium in primary cultures of VSMC.

Discussion

The clinical association of hypertension and accelerated atherosclerotic vascular disease with abnormalities of glucose and insulin metabolism such as obesity and NIDDM is well known (1–5). Hyperglycemia is the characteristic metabolic abnormality of diabetes mellitus, and early epidemiologic studies involving diabetic and nondiabetic subjects have also shown that both systolic and diastolic blood pressures are positively related to plasma glucose levels (1, 11). Consistent with this epidemiologic observation is the reduction of systolic and diastolic blood pressures clinically observed with improved metabolic control of diabetes (12). Experimentally, even in the absence of clinical diabetes, excess sucrose ingestion may elevate blood pressure in different rat strains (13).

Our group has attempted to understand the relationship between diabetes and hypertension from a cellular point of view, proposing that hypertension, peripheral insulin resistance, and hyperinsulinemia may be different clinical manifestations of a common underlying cellular defect of mineral ion metabolism, represented at least in part by elevated cytosolic free calcium and suppressed intracellular free magnesium levels (7, 14, 15). Indeed, elevated cytosolic free calcium levels and/or increased cellular calcium influx have been observed in erythrocytes (15), VSMC (2, 16), and platelets (3, 17) of patients with essential hypertension and in erythrocytes of diabetic and obese subjects (15).

Our present findings suggest that glucose itself may alter cellular calcium homeostasis in VSMC. Specifically, we found that elevated extracellular glucose concentrations in vitro raise cytosolic free calcium concentrations in primary cultures of VSMC. This effect was both time- and dose-dependent, as well as being specific for D-glucose, no effect being observed with equiosmolar concentrations of L-glucose or mannitol.

These data support our previous work and are also consistent with other reports as well. We have reported a rise in Cai following increasing concentrations of glucose in normal human erythrocytes (7). Glucose also elevates Cai in pancreatic beta cells (4, 18) and in insulinoma cells (5, 19). In isolated fat cells,

glucose potentiates the effect of insulin to elevate cytosolic free calcium but had no effect in the absence of insulin (20). On the other hand, preincubating cultured aortic VSMC for 48 h with different glucose concentrations caused a reduction in cellular calcium uptake and efflux (21). These latter data suggest that longer-term exposure to hyperglycemia may produce secondary, compensatory changes, or may rather represent the effects of longer-term tissue culture conditions per se. Thus, regulation of glucose transporters in VSMC has been shown to be inversely and reversibly regulated by glucose itself (22). Furthermore, alterations in intrinsic glucose transporter activity have been demonstrated previously in association with altered metabolic states (23), fasting (24), as well as incubation of cells with various agents (25). Indeed, an alteration in the glucose transporter protein with multiple passages in tissue culture may also help to explain why we failed to detect any effect of glucose on Cai in cultured VSMC when we used cells after the third or fourth passage or from established cell lines (A10) (data not shown).

The mechanism(s) by which glucose modulates intracellular ion content remains unclear. Osmotic or other nonspecific effects seem ruled out by the failure of equiosmolar amounts of L-glucose or mannitol to reproduce the D-glucose effect (see Results, Fig. 1). More relevant, perhaps, are the observations of Davis and colleagues (26), who demonstrated a direct inhibitory effect of hyperglycemia on plasma membrane CaATPase, which could promote calcium accumulation from the extracellular space. Deziel et al. (27) have more extensively documented this inhibitory action on plasma membrane CaATPase, which appears to be mediated by enzymatic glycosylation. This mechanism is consistent with our observations that the glucose effect on Cai is extracellular calcium dependent, based on the lack of glucose effect on Cai when incubated in a calcium-free medium, and by the inhibition of this effect by the addition of lanthanum, a competitive antagonist of ambient extracellular calcium (see Results and Fig. 4). This mechanism is also consistent with the failure of glucose washout over the course of an hour to significantly lower glucose-induced elevated Cai levels. A second potential mechanism for the observed glucose effects involves glucose-induced stimulation of diacylglycerol formation and protein kinase C activity (28–30), potentially recruiting calcium from intracellular storage sites via IP_3 formation. The fact that glucose has similar calcium-stimulating effects in the red cell, devoid of intracellular organelles, makes this mechanism less likely. A third mechanism involves the activation of glycolysis by increased cellular glucose uptake, which would increase organic acid formation and thus lower intracellular pH, as we recently observed in red blood cells (7). Intracellular pH is known to affect cytosolic free calcium (6, 17, 31, 32), and a decrease in intracellular pH is associated with an increase in Cai (33). We did not measure intracellular pH in the present study and thus cannot comment on the cellular mechanism of its possible involvement. Nevertheless, the importance of pH as a factor contributing to our results is strongly suggested by the failure of glucose to stimulate Cai in cells exposed to an acidotic environment (see Results and Fig. 4). Regardless of the mechanisms involved, however, this preliminary report suggests that future investigations must consider the direct contribution of extracellular glucose to cellular ion homeostasis.

Lastly, since Cai is a critical element regulating peripheral vascular tone (34), we believe our results may be clinically significant. First, glucose-induced stimulation of Cai occurs in vitro at circulating glucose concentrations (> 10 mM) observed in vivo in subjects with impaired glucose tolerance as well as frank diabetes mellitus, consistent with the ability of hyperglycemia to constrict coronary arteries (35), and with the increased incidence of hypertension in diabetes (1, 11). Also of interest, our results support and may represent one mechanism underlying the recent observations of improved clinical vascular outcomes with stricter metabolic control of hyperglycemia in diabetes (36). These possibilities, previously put forward as part of an overall ionic hypothesis of cardiovascular and metabolic disease (7), need to be the focus of future research in this area.

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