Effect of Glucose on Endothelin-1-induced Calcium Transients in Cultured Bovine Retinal Pericytes*

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Published work has shown that endothelin-1-induced contractility of bovine retinal pericytes is reduced after culture in high concentrations of glucose. The purpose of the present study was to establish the profile of endothelin-1-induced calcium transients in pericytes and to identify changes occurring after culture in high concentrations of glucose. Glucose had no effect on basal levels of cytosolic calcium or on endothelin-1-induced calcium release from intracellular stores. However, influx of calcium from the extracellular medium after endothelin-1 stimulation was reduced in pericytes that had been cultured in 25 mM D-glucose. L-type Ca2+-currents were identified by patch clamping. The L-type Ca2+-channel agonist, (-)Bay K8644, caused less influx of calcium from the extracellular medium in pericytes that had been cultured in 25 mM D-glucose than those cultured with 5 mM D-glucose. However, 3-O-methylglucose, a nonmetabolizable analogue of glucose which can cause glycation, had similar effects to those of high concentrations of glucose. The results suggest that reduced function of the L-type Ca2+-channel that occurs in bovine retinal pericytes after culture in high concentrations of D-glucose is probably due to glycation of a channel protein.

In epidemiological studies hyperglycemia is the single factor most consistently associated with diabetic microangiopathy, including retinal microangiopathy (1). Longitudinal studies in diabetes have shown that alterations in capillary function and blood flow occur in advance of the structural changes seen in diabetic retinopathy such as pericyte loss, microaneurysm formation, and neovascularization (2). Pericyte tone normally regulates retinal capillary blood flow (3). Because retinal capillaries are devoid of extrinsic innervation (4), pericyte tone is controlled to a large extent by changes in the partial pressures of blood gases (5) and endothelium-derived agonists such as endothelin-1 (ET-1) (6).

We have previously shown (7) that exposure to high ambient glucose concentrations in vitro caused a reduction in the contractile response of the bovine retinal pericyte (BRP) to ET-1 without any reduction in either ET-1 binding or generation of inositol trisphosphate (Ins(1,4,5)P3). The aim of the present study was 1) to establish the profile of ET-1-induced changes in cytosolic calcium concentrations ([Ca2+]i) in the cultured pericyte and 2) to determine whether prior exposure to raised concentrations of glucose would alter ET-1-induced calcium transients in these cells.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Bovine Retinal Pericytes—BRP were isolated as described by Gitlin and D’Amore (8). Briefly, bovine retinal homogenates were subjected to microscopically controlled enzyme digestion, and the resultant microvessel fragments were trapped on a 53-μm mesh. The microvessels were suspended in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) containing 5 mM glucose, antibiotics (200 units/ml penicillin and 200 μg/ml streptomycin, Sigma), and 20% fetal calf serum (FCS, Life Technologies, Inc.), a growth medium which promotes pericyte proliferation and discourages the growth of endothelial cells. The suspension was then seeded into 25-cm2 flasks (Falcon, Becton Dickinson Ltd., Cowley, UK) to be maintained at 37 °C in a mixture of 5% CO2 and air until confluent. The pericytes were identified by their stellate appearance and immunocytochemically by the presence of smooth muscle actin (Amersham Pharmacia Biotech); the lack of Factor VIII staining confirmed the absence of endothelial cells. All experiments were performed on BRP derived from passages 2 and 3.

Determination of [Ca2+]i by Fura-2 Fluorescence Spectroscopy—BRP from a common cell pool were seeded onto glass slips and maintained in DMEM and 20% FCS containing 5 mM glucose, 25 mM glucose, or 5 mM glucose + 20 mM 3-O-methylglucose (3-OMG) for 3, 7, or 10 days; experiments were also carried out using 5 mM glucose + 20 mM mannitol to assess the effect of osmolality. Cells were serum-starved for 24 h prior to experiments by placing them in basal DMEM containing the appropriate hexose concentration. BRP were loaded with the intracellular calcium probe, fura-2, by incubating them in basal DMEM (5 mM glucose, 25 mM glucose, or 5 mM glucose + 20 mM 3-OMG) containing 3 μM fura-2-acetoxymethyl ester and 0.02% Pluronic F127 (Molecular Probes, Eugene, OR) for 80 min at 37 °C. Cells were washed for 10 min in Hepes-buffered Krebs solution, pH 7.4, containing 0.1% bovine serum albumin, placed into the cuvette holder of a Perkin-Elmer LS-50B spectrophotometer, and stirred constantly throughout the procedure while being maintained at 37 °C. 1 mM ET-1 (Bachem UK, Saffron Walden, UK) was added to the same medium that contained either 1 mM Ca2+-free or was essentially Ca2+-free. Cells in Ca2+-free medium were previously washed in this solution and had 0.1 mM EGTA added to the cuvette 15 s prior to the addition of ET-1. In parallel experiments cells were preincubated with the calcium channel inhibitor, 0.2 mM diltiazem (Sigma), for 30 min at 37 °C prior to stimulation with ET-1 in the presence of 1 mM calcium. In a separate series of experiments cells were stimulated alone with 1 μM dihydropyridine L-type calcium channel agonist (-)Bay K8644 (Research Biochemicals Ltd., Natick, MA) (9). Fluorescence ratios were measured at 510 nM with excitation at 340 and 380 nM, and following determinations of Rmax and Rmin (the fluorescence ratios under saturating and Ca2+-free conditions, respectively), [Ca2+]i was determined by the method of Grynkiewicz et al. (10). To determine Rmax, 4 μM ionomycin was added to the cuvette at the end of the experiment, followed by 5 mM EGTA 10 s later. To determine Rmin, in alternate runs the [Ca2+]i of the medium was raised to 4 mM (3 mM in the case of Ca2+-free buffer) 10 s after the addition of ionomycin. All

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‡The abbreviations used are: ET-1, endothelin-1; Ins(1,4,5)P3, inositol trisphosphate; BRP, bovine retinal pericytes; [Ca2+]i, cytosolic calcium concentration; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; 3-OMG, 3-O-methylglucose.
Effect of ET-1 on \([\text{Ca}^{2+}]_i\), in pericytes grown in 5 and 25 mM glucose for 10 days. BRP cultured in 5 mM (—) and 25 mM (— —) glucose were stimulated with ET-1 (1 nM) in the presence (A) and absence (B) of 1 mM extracellular calcium. The traces shown are from a representative experiment.

Effect of Glucose on ET-1-induced Calcium Transients—Basal levels of \([\text{Ca}^{2+}]_i\), were similar in cells maintained in 5 and 25 mM glucose (Table I). Cells grown in 25 mM glucose for 10 days produced a smaller rise in \([\text{Ca}^{2+}]_i\), with ET-1 (1 nM) than those maintained in 5 mM glucose (Fig. 1A, Table II). No significant differences were seen at 3 or 7 days (results not shown). All subsequent experiments utilized cells grown for 10 days in 25 mM glucose. In \([\text{Ca}^{2+}]_i\)-free medium there was no difference in \([\text{Ca}^{2+}]_i\), in BRP was due to influx from the extracellular medium.

Characterization of \([\text{Ca}^{2+}]_i\) Channels in the Plasma Membrane of BRP—(—)-Bay K8644 (10–20 μM) generated an enhanced inward current that activated at −30 mV and peaked at +10 mV (107 ± 70 pA) (Fig. 5). 30% of those cells showing stable plateau phase \([\text{Ca}^{2+}]_i\), remained elevated (Fig. 1B). Under these conditions rises in \([\text{Ca}^{2+}]_i\), were due to ET-1-induced calcium release from the endoplasmic reticulum. If BRP were preincubated in medium containing 1 mM \([\text{Ca}^{2+}]_i\), and the calcium channel inhibitor, 0.2 mM diltiazem, for 30 min ET-1 produced an effect similar to that in \([\text{Ca}^{2+}]_i\)-free medium (Fig. 2). These observations indicate that a large portion of the ET-1-induced rise in \([\text{Ca}^{2+}]_i\), in BRP was due to influx from the extracellular medium.

Effect of Glucose on ET-1-induced Calcium Transients—Basal levels of \([\text{Ca}^{2+}]_i\), were similar in cells maintained in 5 and 25 mM glucose (Table I). Cells grown in 25 mM glucose for 10 days produced a smaller rise in \([\text{Ca}^{2+}]_i\), with ET-1 (1 nM) than those maintained in 5 mM glucose (Fig. 1A, Table II). No significant differences were seen at 3 or 7 days (results not shown). All subsequent experiments utilized cells grown for 10 days in 25 mM glucose. In \([\text{Ca}^{2+}]_i\)-free medium there was no difference in \([\text{Ca}^{2+}]_i\), response to ET-1 between the two groups of cells.
**Calcium Transients in Pericytes**

**FIG. 4.** A current voltage relationship from another cell held at -40 mV and stepped to various voltages between -25 and +40 mV and bathed in normal solution (2 mM Ca²⁺, D), 8 mM Ca²⁺ (C), and 8 mM Ca²⁺ in the presence of 1 µM nifedipine (□). The current was measured at the peak of the inward transient or at the same latency.

**TABLE I**

The basal levels of [Ca²⁺]i in pericytes cultured in 5 mM glucose, 25 mM glucose, and 5 mM glucose + 20 mM 3-OMG for 10 days

| Glucose    | n   | Basal [Ca²⁺]i |
|------------|-----|--------------|
| mM         |     |              |
| 5          | 18  | 325.4 ± 32.7 |
| 25         | 20  | 314.8 ± 20.9 |
| (+20 mM 3-OMG) | 17  | 311.8 ± 35.5 |

Values are mean ± S.E. n = the number of replicates for each condition.

**TABLE II**

Effect of ET-1 (1 nM) on [Ca²⁺]i in BRP grown in 5 mM glucose, 25 mM glucose, or 5 mM glucose + 20 mM 3-OMG for 10 days

| Glucose     | n  | [Ca²⁺]i,max | [Ca²⁺]i,plateau |
|-------------|----|-------------|-----------------|
| mM [Ca²⁺]i |    |             |                 |
| 1 mM        |    |             |                 |
| 5           | 34 | 349 ± 64    | 158 ± 16        |
| 25          | 28 | 191 ± 25    | 110 ± 9         |
| (+20 mM 3-OMG) | 30  | 173 ± 21³   | 102 ± 9⁴        |
| 0 mM [Ca²⁺]i |    |             |                 |
| 5           | 14 | 78 ± 16     |                 |
| 25          | 15 | 73 ± 13     |                 |
| (+20 mM 3-OMG) | 13  | 88 ± 13     |                 |

* Results are shown as increases in [Ca²⁺]i, mean ± S.E.; plateau phase results are taken at 210 s poststimulation with ET-1.
  * p < 0.005; 5 mM glucose versus 25 mM glucose or 5 mM glucose + 3-OMG.
  * p < 0.05; 5 mM glucose versus 25 mM glucose or 5 mM glucose + 3-OMG.
  ³ p < 0.001; 5 mM glucose versus 25 mM glucose or 5 mM glucose + 3-OMG.
  ⁴ [Ca²⁺], calcium concentration of the medium.

**TABLE III**

Effect of (-)-Bay K8644 (1 µM) on [Ca²⁺]i in cultured BRP grown in 5 mM glucose, 25 mM glucose, or 5 mM glucose 3-OMG for 10 days

| Glucose     |      |       |
|-------------|------|-------|
| 5 mM        | 152 ± 36 | 101 ± 11³ |
| 25 mM + 20  |      |       |
| 5 mM        | 147 ± 43 | 117 ± 32b |
| 5 mM + 20 mM 3-OMG |      |       |

³ p < 0.03.
  b p < 0.04.

**DISCUSSION**

ET-1 produced a biphasic rise in [Ca²⁺]i, that consisted of an initial peak followed by a sustained plateau phase. The plateau phase was abolished in Ca²⁺-free medium, showing that this phase arose from influx through Ca²⁺ channels. The probability that these were L-type Ca²⁺ channels was suggested by identification of a nifedipine-sensitive inward current and by (-)-Bay K8644-induced increases in [Ca²⁺]i. The plateau phase was depressed in high concentrations of glucose; the implication of this is that culture in high concentrations of D-glucose alters the properties of L-type Ca²⁺ channels in BRP.

The initial phase of the ET-1-induced Ca²⁺ transient is composed of Ins(1,4,5)P₃-induced Ca²⁺ release from the endoplasmic reticulum, and this is superimposed on the earliest part of influx from the extracellular medium. In the absence of extracellular Ca²⁺, the Ins(1,4,5)P₃-induced increase in [Ca²⁺]i, was
similar in BRP grown in 5 or 25 mM glucose. This is consistent with previous results that showed that ET-1 binding and \( \text{Ins(1,4,5)P}_3 \) production by BRP were unaffected by glucose under similar culture conditions (7). Thus, the present results show that continued exposure to high concentrations of glucose attenuates the \([\text{Ca}^{2+}]_i\) rise produced by ET-1 by an action on L-type \( \text{Ca}^{2+} \) channels but has no effect on that mediated by \( \text{Ins(1,4,5)P}_3 \). The results from studies with 3-OMG strongly suggest that glucose affects the \( \text{Ca}^{2+} \) channel by a mechanism involving glycation. We have not been able to detect any evidence of free radical attack, at least on lipids, either in this study where F\(_2\)-isoprostanes were measured or in a previous study (12) where malondialdehyde was measured after 10 days of exposure to 25 mM glucose; we have detected increased malondialdehyde after 18 days only. The attenuated increase in \([\text{Ca}^{2+}]_i\), may explain, at least in part, the previously observed reduced contractile response to ET-1 of BRP grown in high concentrations of glucose despite the fact that ET-1 binding and \( \text{Ins(1,4,5)P}_3 \) production were unchanged (7).

In conclusion, culture of BRP in high concentrations of glucose for extended periods leads to reduced function of L-type \( \text{Ca}^{2+} \) channels; the observed effects may be due to glycation of a channel protein. These results suggest that glycation of \( \text{Ca}^{2+} \) channel proteins may affect the quality of retinal pericyte response to ET-1 and, hence, control of retinal capillary blood flow in the presence of sustained hyperglycemia.

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