Glucose elevations alter bradykinin-stimulated intracellular calcium accumulation in cultured endothelial cells

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Abstract

Objective: Diabetes selectively injures receptor-mediated endothelium-dependent relaxation. In this study, we investigated the effect of elevated glucose concentrations on intracellular calcium (Ca^{2+}) signal transduction in response to stimulants of EDRF/nitric oxide release in cultured bovine aortic endothelial cells. Methods: [Ca^{2+}] was measured in cell suspensions using Fura-2 and fluorescence spectroscopy while nitric oxide production was evaluated using radioimmunoassay of cGMP production. Results: After 24 h exposure to 25 mM glucose in Ham’s F-12 media, the increase in endothelial cell [Ca^{2+}] in response to 100 nM bradykinin was attenuated by 40% while the response to ionomycin was unaltered. When RPMI medium was used, no reduction in response to bradykinin was observed at 25 mM glucose, but a significant reduction in [Ca^{2+}] signal was observed after exposure to 35 mM glucose for a similar time period. Defective [Ca^{2+}] signaling was also seen in cells using MEM medium. [Ca^{2+}] signal responses to ionomycin and NaF, a G-protein activator of extracellular calcium entry via calcium channels, were unaltered by elevated glucose exposure. The defect in [Ca^{2+}] signal was not mimicked by either mannose or sucrose, but was prevented by co-incubation with cytochalasin B to inhibit glucose uptake. Neither superoxide dismutase nor catalase nor the extracellular hydroxyl radical scavenger, mannitol, blocked the reduction in the bradykinin-induced increase of [Ca^{2+}] in elevated glucose-exposed cells; however, the reduction was completely blocked by the cell-permeable hydroxyl radical scavenger, dimethylthiourea. Bradykinin-stimulated but not ionomycin-stimulated cGMP production within endothelial cells or in RFL-6 detector cells was attenuated by elevated glucose exposure. Conclusions: Hyperglycemia may contribute to defective endothelium-dependent relaxation in diabetes via an attenuated increase in Ca^{2+} signal transduction for the release of nitric oxide by endothelial cells. This defect possibly arises as a consequence of hydroxyl radicals formed intracellularly.

Keywords: Diabetes; Endothelium; Calcium, intracellular concentration; Nitric oxide; Signal transduction; Bradykinin; Free radicals

1. Introduction

The endothelial cell is now known to play an important role as a paracrine organ in the regulation of vascular tone. For example, these cells are known to synthesize and release a substance originally described as an endothelium-derived relaxing factor or EDRF [1] which is now believed to be nitric oxide or a closely-related compound [2]. The release of nitric oxide from the endothelial cell activates guanylate cyclase in adjacent vascular smooth muscle cells to increase cGMP. This increase in cGMP acts on a cascade system to initiate relaxation of the blood vessel. Thus, alterations in nitric oxide production may play a key role in the regulation of vascular tone in a variety of diseases such as hypertension, atherosclerosis and diabetes mellitus.

Several investigators have shown in experimental diabetic rats and rabbits that endothelium-dependent relaxation is impaired in a variety of blood vessel types [3–8]. For details, please refer to comprehensive reviews in the literature [9,10]. This defective endothelium-dependent relaxation is not unique to experimental diabetes since it has now been confirmed in type I [11,12] and type II [13] diabetic patients. The exact mechanism of endothelial dysfunction is not yet known with certainty, but it may result from the elevation in blood glucose concentrations per se associated with diabetes. Indeed, it is known that normal blood vessels which are exposed to elevations in extracel-

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A key regulatory factor in the release of EDRF/nitric oxide from endothelial cells is the increase in intracellular calcium ($Ca^{2+}$) concentrations within the endothelial cell [18,19]. This increase is known to arise via an inositol 1,4,5-trisphosphate-mediated release from $Ca^{2+}$ stores located within the endoplasmic reticulum and influx via receptor-operated calcium channels located on the plasma membrane [19–21]. One mechanism by which endothelium-dependent relaxation could be altered in diabetes is a decrease in the $Ca^{2+}$ signal for nitric oxide release.

In this study, we investigate the role of exposure to elevated extracellular glucose concentration on alterations in $Ca^{2+}$ signal transduction in cultured bovine aortic endothelial cells. In addition, we provide a mechanism for the defective increases in bradykinin-stimulated $Ca^{2+}$ as a result of elevated glucose exposure which is related to oxygen radical formation, specifically the hydroxyl radical.

2. Methods

2.1. Materials

Fura 2-penta-acetoxymethyl ester (Fura 2-AM) was purchased from Molecular Probes, Inc. (Eugene, OR). Chemicals obtained from Sigma Chemical Co. include: gentamicin, tylosin, EGTA, digitonin, cytochalasin B, glucose, sucrose, mannose, mannotol, dimethylthiourea, bovine serum albumin, superoxide dismutase, catalase and bradykinin. Ionomycin was obtained from Calbiochem San Diego, CA. Cell culture media (i.e., Ham’s F-12, RPMI 1640 and MEM), l-glutamine, trypsin and HEPES [4-(2-hydroxyethyl)-1-piperazinethane sulfonic acid] were obtained from GIBCO (Grand Island, NY). Fetal calf serum was obtained from Hyclone (Logan, UT).

2.2. Cell culture

Bovine aortic endothelial cells (No. AG08132A, passage 2) were obtained from the N.I.A. Cell Culture Repository (Coriell Institute for Medical Research, Camden, NJ). Cells were grown in either Ham’s F-12, MEM or RPMI 1640 medium which was supplemented with 10% fetal calf serum, 1% glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamicin, 8 μg/ml tylosin and 0.25 μg/ml amphotericin in a humidified atmosphere containing 5% CO₂ and 95% air. Monolayers of endothelial cells in culture displayed the typical cobblestone morphology. Endothelial cells of passages 5 through 9 were used for these studies.

Twenty-four hours prior to $Ca^{2+}$ measurements the culture medium was removed and replaced with glucose- and serum-free medium which was supplemented with glucose to achieve a concentration of either 25 or 35 mM. Control cells containing glutamine- and serum-free normal medium were processed at the same time. Hyperosmolar control studies were performed by supplementing the medium with either mannose or sucrose instead of glucose. To ascertain a role of glucose uptake in the response of cells to elevated glucose, additional incubations were performed in the presence of 10 μM cytochalasin B. A few studies were performed in which 10 μM indomethacin was included in medium to delineate a role of prostanoit metabolism on glucose-induced changes in $Ca^{2+}$.

Endothelial cells were harvested by adding 0.03% trypsin in Puck’s medium. The supernatant containing Puck’s medium was removed following centrifugation and cells were suspended in a HEPES-buffer (pH 7.4) consisting of (in mM): 137 NaCl, 5.4 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose and 10 HEPES. In each experiment, cells were examined for viability using the trypan blue exclusion method to determine that elevated glucose concentrations did not damage the integrity of the endothelial cells which were used in the subsequent $Ca^{2+}$ measurements.

Endothelial cells were loaded with Fura-2AM (20 μM final concentration) for 30 min in the dark after which cells were diluted with 10 volumes of buffer and incubated for an additional 30 min. After loading with the calcium indicator, the cells were centrifuged, washed and resuspended to 3.2 × 10³ cells/ml using a HEPES-buffered (pH 7.4) saline solution for the determination of $Ca^{2+}$. The initial studies of $Ca^{2+}$ on cells incubated in Ham’s F-12 medium were performed on a SLM8000 fluorometer whereas all subsequent measurements were determined on a Perkin-Elmer Model LS50 spectrofluorometer in which the cuvette was thermostatically controlled at 37°C. Fluorescence was measured using excitation wavelengths of 333 and 375 nm and emission wavelength of 510 nm. Experiments were programmed to subtract for autofluorescence based upon the technique previously described [22].

Intracellular calcium concentration [$Ca^{2+}$] was calculated according to the equation:

$$[Ca^{2+}] = K_d (R - R_{min})/(R_{max} - R)/S_{max}/S_{min}$$

where the $K_d$ for the ($Ca^{2+}$:Fura-2)- complex was taken to be 224 nM at 37°C. $R_{max}$ measured after lysing cells with 50 μl of 500 μM digitonin, $R_{min}$ measured after adding 100 μl of 0.5 M EGTA. $S_{max}$ and $S_{min}$ represent the fluorescence of Fura-2 at 375 nm excitation wavelength at zero calcium and full calcium saturation, respectively.

Bradykinin or ionomycin were used to stimulate increases in $Ca^{2+}$ by receptor-dependent or receptor-independent mechanisms, respectively. Agonist-stimulated $Ca^{2+}$ was initiated using either bradykinin (100 or 200 nM) or ionomycin (100 or 190 nM) for receptor-dependent and receptor-independent stimulation, respectively. Additional studies were performed using 10 or 20 mM NaF as a receptor-independent agonist since this compound is known...
to activate Ca$_{2+}$ by extracellular calcium entry via G-protein-coupled channels [23].

For each day of experiment, Ca$_{2+}$ measurements were performed in at least duplicate or triplicate for high-glucose-exposed cells and in pair-matched control cells which were processed in parallel. Occasionally, there was variability in the baseline and stimulated Ca$_{2+}$ measurements in control cells from day to day (particularly in the Ham’s F-12 study). Therefore, agonist-stimulated Ca$_{2+}$ is presented as % of the control cell response (i.e., set at 100%). For each protocol, the experiment was repeated on several days and the results averaged.

2.3. Role of reactive oxygen

Additional studies were performed to determine the mechanism of the glucose-induced defects and whether reactive oxygen was implicated in glucose-mediated defects in Ca$^{2+}$ signaling. In one set of experiments, we used a fluorescent probe and followed the acute oxidation of dichlorofluorescein in cells exposed for 20 min with 5.5 or 35 mM glucose using excitation and emission wavelengths of 500 and 525 nm, respectively [24]. In these studies, endothelial cells were loaded with 10 μg/ml dichlorofluorescein diacetate and the fluorescence analysis was performed in the fluorometer using $8 \times 10^5$ cells/ml.

To determine the potential oxidant species implicated in this defect, we performed several studies in which endothelial cells incubated with 35 mM in MEM medium were pretreated with either 100 U/ml superoxide dismutase, 100 U/ml catalase, 10 mM mannitol or 50 mM dimethylthiourea to assess the role of individual reactive oxygen species.

2.4. cGMP analysis

Additional studies were performed to measure cGMP, an index of nitric oxide production, in response to bradykinin. Endothelial cells were grown in 6-well plates in MEM medium or MEM medium which was supplemented to 35 mM glucose. Hyperosmolar controls were performed using media supplemented with sucrose. After reaching confluency, the medium was removed and replaced with a HEPES-buffered saline solution (pH 7.3) containing 5.5 mM glucose. Cells were incubated for 20 min in the presence of 100 μM 3-isobutyl-1-methylxanthine (IBMX) and 100 μM captopril to inhibit phosphodiesterase and angiotensin converting enzyme, respectively. After 20 min of incubation, cells were stimulated with 1 μM bradykinin. The reaction was terminated after 2 min stimulation with bradykinin by the addition of 1.0 ml of 6% TCA. The supernatant was washed 4 times with water-saturated ether. The pellet was dissolved in 1 M NaOH overnight and frozen for protein determination using the Bio-Rad commercial kit. The supernatant was dried under nitrogen and resuspended in buffer for radioimmunoassay of cGMP (PerSeptive Diagnostics).

Additional experiments were performed in which nitric oxide release from endothelial cells was evaluated by the action of conditioned medium from bradykinin-stimulated endothelial cells to generate cGMP in RFL-6 detector cells similar to that previously described [25]. In brief, RFL-6 cells were grown in 6-well plates. RFL-6 cells were washed twice with HEPES-buffered saline (above) and incubated for 20 min with 100 μM IBMX (in the presence or absence of 100 μM L-nitroarginine) and 5 min with 100 U/ml superoxide dismutase prior to the addition of conditioned medium from endothelial cells. Endothelial cells were incubated with 100 μM IBMX, 100 μM captopril and 100 U/ml superoxide dismutase prior to stimulation of cells with 1 μM bradykinin. In unstimulated cells or after 2 min following addition of bradykinin to endothelial cells, the medium was transferred to RFL-6 cells. After 2 min exposure of RFL-6 cells, the reaction was terminated by addition of cold TCA and processed as described above.

2.5. Statistical analysis

Data were analyzed by either paired t analysis or ANOVA for comparison between two means or multiple group means. A P-value of < 0.05 was considered to denote significance.

3. Results

Exposure of endothelial cells in Ham’s F-12 medium to 25 mM glucose for 24 h attenuated the peak increase in Ca$_{2+}$ concentration in response to bradykinin (Fig. 1, upper panel) compared to pair-matched cells processed in normal medium (i.e., calcium stimulation was 61 ± 6% of control cell response, n = 5). The increase in Ca$_{2+}$ in response to ionomycin was unaltered by elevated glucose exposure (i.e., 92 ± 10% of control cell response, n = 5). To further analyze this initial observation, we performed various experimental protocols using different culture media, hyperosmotic controls and antioxidant agents to block the effects of elevated glucose on alteration in Ca$_{2+}$ signal.

Using RPMI 1640 medium rather than Ham’s F-12 medium, basal Ca$_{2+}$ concentration was unaltered by 24 h of exposure to 25 mM glucose (normal medium 107 ± 12 nM vs. high glucose medium 122 ± 12 nM, n = 26 determinations each). Furthermore, exposure to 25 mM glucose in RPMI 1640 medium did not alter the peak Ca$_{2+}$ signal in response to either ionomycin (not shown) or bradykinin (Fig. 1, middle panel). If the concentration of glucose in RPMI 1640 medium was increased to 35 mM, the bradykinin-elicited Ca$_{2+}$ signal was significantly diminished (Fig. 1, lower panel) while the ionomycin-elicited
Fig. 1. Upper panel: Reduction in the increase in peak bradykinin-stimulated Ca\(^{2+}\) but not ionomycin-stimulated Ca\(^{2+}\) following exposure of endothelial cells in Ham’s F-12 medium to 25 mM glucose for 24 h. Final concentration of bradykinin BK and ionomycin equals 100 nM. Middle panel: Effect of exposure of endothelial cells in RPMI 1640 medium for 24 h to 25 mM glucose (upper panel) or 35 mM glucose (lower panel) on alterations in bradykinin-stimulated and ionomycin-stimulated Ca\(^{2+}\). The increase in Ca\(^{2+}\) signal is calculated as a ratio of the increase observed in high-glucose-exposed cells divided by the increase observed in paired-control medium cells which were processed at the same time. Each bar graph represents the mean ± s.e.m. of \(n = 5\) (Ham’s F-12 studies) or \(n = 7–9\) (RPMI 1640 studies). Cell viability was > 95% for each group. * \(P < 0.05\) indicates significant difference from control cell response.

Fig. 2. Failure of mannose or sucrose supplementation for 24 h in RPMI 1640 medium to alter peak bradykinin (BK, 200 nM)-stimulated Ca\(^{2+}\) in endothelial cells. Each bar represents the mean ± s.e.m. of \(n = 4–6\) determinations each. * \(P < 0.05\) indicates significant difference from individual basal values; (\(\#\)) \(P < 0.01\) indicates significant difference from peak value compared to control, sucrose and mannose groups.

In addition to diminished peak Ca\(^{2+}\) response to bradykinin (which reflects intracellular calcium release), the Ca\(^{2+}\) at the plateau after bradykinin (which reflects extracellular calcium entry) was also diminished in cells exposed to 35 mM glucose relative to cells exposed to control medium (see tracings in Fig. 3). This effect was reproduced in studies using both RPMI and MEM culture media (summarized in Fig. 4). The Ca\(^{2+}\) concentration at the peak in cells exposed to high glucose was similar to the concentration present at the plateau phase of control cells. Both peak and plateau phase Ca\(^{2+}\) concentration was decreased in high-glucose-exposed cells compared to pair-matched control cells using either RPMI or MEM culture medium. In the presence of indomethacin, Ca\(^{2+}\) accumulation was also reduced in high-glucose-exposed cells versus control cells (Fig. 5).

In contrast to bradykinin responses, the Ca\(^{2+}\) signal response to ionomycin was not phasic but increased rapidly and remained elevated. The peak Ca\(^{2+}\) concentration in
response to ionomycin was not altered by high glucose concentration compared to pair-matched control cells incubated in either RPMI or MEM medium (Fig. 6).

To further assess extracellular calcium entry, additional experiments were performed using NaF as the agonist. In this case, exposure to high glucose did not alter Ca^{2+} signal in response to NaF (Fig. 7).

To assess a role of glucose uptake in the alterations in signal transduction by elevated glucose exposure, additional studies were performed in which cells were incubated with cytochalasin B during the 24 h glucose exposure period. In contrast to the previous observations, there was no difference in bradykinin-stimulated Ca^{2+} response in cells which had been co-incubated with 10 μM cytochalasin B during the 24 h exposure period (Fig. 8).

The addition of superoxide dismutase to endothelial cells which were exposed to elevated glucose did not
Fig. 8. Effects of co-incubation with 10 μM cytochalasin B during 24 h incubation of endothelial cells with 35 mM glucose on Ca\(^{2+}\) accumulation in response to 200 nM bradykinin BK compared to pair-matched control cells \((n = 10\) determinations each). There was no difference between experimental groups at baseline, peak and plateau phases.

To verify that oxygen radical production is augmented by the addition of elevated glucose concentration, we performed additional paired-analysis of oxidation of dichlorofluorescein. The rate of oxidation was increased from 2.1 ± 0.2 to 3.1 ± 0.3 arbitrary fluorescence units per minute in cells exposed to 5.5 and 35 mM glucose, respectively \((n = 6\) paired tests, \(P < 0.01\)).

Fig. 9. Effect of co-incubation with 10 mM mannitol or dimethylthiourea (DMTU) on glucose-induced reductions in bradykinin BK, 200 nM-stimulated Ca\(^{2+}\) in endothelial cells incubated in RPMI 1640 medium containing 35 mM glucose (high glucose, HG) for 24 h. Each bar graph represents the mean ± s.e.m. of \(n = 11–15\) determinations. Cell viability was 94 ± 2% (each normal medium plus mannitol or DMTU), 95 ± 1% (high glucose medium) 93 ± 1% (high glucose medium plus mannitol) and 94 ± 1% (high glucose medium plus DMTU). * \(P < 0.05\) indicates significant difference from control cell response.

To prevent the increase in the response of the stimulus, we used an inhibitor of hydrogen peroxide release, catalase, to avoid damage to the endothelial cell. Catalase administration did not prevent the rise in the bradykinin-stimulated Ca\(^{2+}\) signal (i.e., 112 ± 4% of the response seen in pair-matched, glucose-exposed cells without superoxide; \(n = 14\)). Net increases in bradykinin-stimulated Ca\(^{2+}\) were 122 ± 34 nM (control cells), 54 ± 7 nM (glucose-exposed cells) and 58 ± 8 nM (superoxide dismutase-treated, glucose-exposed cells).

Additional experiments were performed to assess the role of hydroxyl radicals using the classical hydroxyl radical scavengers, mannitol and dimethylthiourea. Dimethylthiourea had no effect on the bradykinin-stimulated Ca\(^{2+}\) signal. The increase in Ca\(^{2+}\) was 89 ± 24% \((n = 7\) determinations) of the response seen in pair-matched, glucose-exposed cells without catalase treatment.

To verify that oxygen radical production is augmented by the addition of elevated glucose concentration, we performed additional paired-analysis of oxidation of dichlorofluorescein. The rate of oxidation was increased from 2.1 ± 0.2 to 3.1 ± 0.3 arbitrary fluorescence units per minute in cells exposed to 5.5 and 35 mM glucose, respectively \((n = 6\) paired tests, \(P < 0.01)\).

Bradykinin caused a significant increase in endothelial cell cGMP content over baseline cGMP in cells exposed to normal glucose conditions (Fig. 10). In contrast, the extent of increase in bradykinin-stimulated cGMP concentration from basal cGMP in unstimulated cells was blunted as a consequence of exposure to 35 mM glucose for 24 h.

These experiments were repeated using another protocol in which conditioned medium from bradykinin-stimulated endothelial cells was transferred to RFL-6 detector cells. Similar to the results shown for endothelial cell cGMP production, cGMP was increased in RFL-6 cells from bradykinin-stimulated medium (Fig. 11, upper panel) and this increase was blunted if the cells had been exposed to 35 mM glucose for 24 h prior to bradykinin stimulation. The increase in bradykinin-stimulated cGMP production in RFL-6 cells was nearly completely blocked by prior incubation of endothelial cells with 100 μM L-nitroarginine to inhibit endothelial cell nitric oxide synthase. Bradykinin-stimulated cGMP production in RFL-6 cells was not altered in cells exposed to equimolar concentration of sucrose (i.e., normal cells 3.3 ± 0.3 pmol/mg protein; sucrose cells 2.9 ± 0.2 pmol/mg protein; \(n = 5\) paired tests).

In contrast to results with bradykinin, high glucose exposure did not alter cGMP production by cells stimulated with ionomycin (Fig. 11, upper panel). Incubation of endothelial cells with DMTU during the 24 h high-glucose exposure, restored bradykinin-stimulated cGMP generation in RFL-6 detector cells (e.g., DMTU-treated control =...
Fig. 11. cGMP generation in RFL-6 detector cells from bradykinin-stimulated endothelial cells (upper panel, \( n = 9-10 \)) or ionomycin-stimulated endothelial cells (lower panel, \( n = 7 \)) previously incubated for 24 h with 5.5 or 35 mM glucose. Concentration of bradykinin and ionomycin equals 1 \( \mu M \) each. \( P < 0.01 \) vs. corresponding baseline.

3.5 ± 0.6 pmol/mg protein; DMTU-treated high glucose = 3.5 ± 0.7 pmol/mg protein, \( n = 6 \) each.

4. Discussion

Little information exists regarding the mechanism by which diabetes or diabetes-related factors alter intracellular signal transduction pathways for EDRF/nitric oxide release from the vascular endothelium to elicit the phenomenon known as ‘endothelium-dependent relaxation’. In this study, we provide new evidence that exposure of cultured endothelial cells to elevated glucose concentrations produces a defect in both the increase in \( \text{Ca}^{2+} \) and cGMP production in response to the nitric oxide releasing agent, bradykinin. In addition, our studies provide new information regarding the role of hydroxyl radicals as a mechanism responsible for production of this defect resulting from exposure to elevated glucose concentrations. This reduction in the increase in \( \text{Ca}^{2+} \) signal could provide a potential mechanism to explain the decrease in endothelium-dependent relaxation shown previously in our laboratory [17] and elsewhere [14–16,26] after exposure of normal blood vessels both in vitro and in vivo to elevated glucose concentrations.

Interestingly, the defect in \( \text{Ca}^{2+} \) signal which we observed in endothelial cells following elevated glucose exposure was shown to be selective for receptor-mediated \( \text{Ca}^{2+} \) pathways since responses to bradykinin were altered while responses to ionomycin and NaF were unaltered. Our observations provide an important link in explaining the selective impairment in receptor-mediated, endothelium-dependent relaxation observed after acute exposure of normal blood vessels to elevated glucose concentrations both in vivo [17] and in vitro [14] or in intact blood vessels taken from diabetic animals as observed in our laboratory [3,27] and elsewhere [8]. Collectively, these studies suggest that elevated glucose concentrations which exist under diabetic conditions appear to target receptor-coupled pathways which mediate release of nitric oxide from endothelial cells to produce endothelium-dependent relaxation.

Our studies also provide valuable insight into the location of the defect in \( \text{Ca}^{2+} \) signaling. In our study, we observed that both peak and plateau levels of \( \text{Ca}^{2+} \) in response to bradykinin were diminished in cells exposed to elevated glucose concentrations. This suggests that both intracellular calcium release and extracellular calcium entry are altered following high glucose conditions. Since extracellular calcium entry is coupled to intracellular calcium release, in the case of bradykinin, we extended our studies to measure calcium entry using NaF, an agonist which is known to couple calcium entry via G-protein-coupled activation of plasma membrane calcium channels [23]. Accordingly, we observed that NaF-stimulated \( \text{Ca}^{2+} \) signal to be unaltered. This suggests that the defect in bradykinin-induced \( \text{Ca}^{2+} \) signal might be due to a defect in bradykinin-coupled pathways for release of calcium from intracellular calcium stores and/or a defect in coupling of intracellular calcium release with subsequent extracellular calcium entry.

In addition to defective \( \text{Ca}^{2+} \) signaling, we have also shown a blunted response to bradykinin-stimulated cGMP generation, an indirect measure of nitric oxide production, in cells exposed to elevated glucose. Our observation of attenuation of cGMP production is consistent with the study by Weisbrod et al. [28] using porcine aortic endothelial cells in which they observed a decrease in the bradykinin-stimulated endothelial cell cGMP formation after 6 h exposure to 44 mM glucose. These authors concluded that although glucose increased eicosanoid production there was a dissociation between glucose-induced effects on eicosanoid production and NO release as assessed by their indomethacin experiments. Our additional studies using indomethacin also suggest that eicosanoid production is not a significant pathway in the development of glucose-induced defects in \( \text{Ca}^{2+} \) signaling. Furthermore, since basal and stimulated cGMP production were
performed in the presence of IBMX [28, and the present study], differences in phosphodiesterase activity within endothelial cells cannot account for the blunted agonist-stimulated cGMP formation.

To further strengthen this observation and to suggest that glucose-induced decreases in intracellular calcium signaling could alter nitric oxide production by endothelial cell nitric oxide synthase, we performed additional studies using cGMP generation in RFL-6 cells. Bradykinin stimulation of endothelial cells produced an increase in cGMP generation in RFL-6 detector cells which was eliminated by prior incubation of endothelial cells with the nitric oxide synthase inhibitor, L-nitroarginine. This suggests that the cGMP generation is specific for nitric oxide in both control endothelial cells and endothelial cells exposed to high glucose concentration. The observation that cGMP generation was blunted following exposure of endothelial cells to high glucose suggests that nitric oxide production is also decreased following this intervention. Furthermore, our additional studies showing that elevated sucrose levels did not modify agonist-stimulated cGMP production suggest that the defect in cGMP generation is likely not due to hyperosmolarity.

Our study which contains new information regarding Ca$_{2+}$ signaling provides a missing link to explain the reduction in bradykinin-stimulated increase in cGMP formation following elevated glucose exposure. Collectively, the present study and the report by Weisbrod et al. [28] strongly suggest that elevated glucose may limit the endothelial cell production of nitric oxide via defects in the Ca$_{2+}$ signal transduction pathway. Thus, defects in endothelial cell Ca$_{2+}$ signal transduction may be an important intracellular mechanism which contributes to impaired endothelium-dependent relaxation in hyperglycemic states such as diabetes mellitus.

Our observations of defective Ca$_{2+}$ signal transduction and cGMP generation are contrasted by recent studies by another group using porcine aortic endothelial cells [29,30]. These investigators observed that exposure of these endothelial cells to 44 mM glucose for 24 h increased rather than decreased Ca$_{2+}$ signal and cGMP generation in response to bradykinin.

The precise reason for the discrepancy in the results by these investigators compared with the experiments conducted by Weisbrod et al. [28] and in our study is unclear. Differences in culturing conditions, differences in media and medium composition, the species of cells used, or cell-passage number are all factors which could potentially account for these differences. It is difficult to reconcile the type of endothelial cells and passage number can explain these divergent results. While we used bovine endothelial cells at passage #5-9, our Ca$_{2+}$ and cGMP data are consistent with the cGMP measurements performed by Weisbrod et al. [28] using passage #2 porcine endothelial cells. Interestingly, the use of porcine cells and early passage are experimental conditions similar to those used by investigators who reported enhanced bradykinin-stimulated Ca$_{2+}$ signals and cGMP in response to elevated glucose [29,30]. Thus, other unknown factors including the buffer or medium used during the glucose incubation period could potentially account for these divergent findings.

The fact that we verified defective bradykinin-stimulated Ca$_{2+}$ signaling in endothelial cells using three separate media indicates that this glucose-induced toxicity is independent of the medium used. Interestingly, endothelial cells grown in Ham’s F-12 medium appeared to be more susceptible to elevated glucose concentrations than cells grown in RPMI 1640 medium. The exact reason for this enhanced susceptibility is uncertain, but would be consistent with the observation that oxidation of low-density lipoproteins is augmented in Ham’s medium compared to other media [31].

While it is not feasible to identify which particular constituent in individual media contributes to either enhanced susceptibility or enhanced protection to glucose-mediated cellular toxicity, the presence of copper and iron salts in Ham’s F-12 medium may be a factor. Both copper and iron are well-known to facilitate metal-ion-catalyzed hydroxyl radical formation. Indeed, it has been suggested that metal ions can catalyze the production of hydroxyl radicals from either superoxide anion radicals or hydrogen peroxide which can be generated under conditions of elevated glucose concentrations [32].

Further evidence for this hypothesis derives from studies in which 25 mM glucose (but not 5 mM glucose) potentiates the autoxidative potential of CuSO$_4$ [33]. Thus, it is reasonable to suspect that enhanced metal-catalyzed hydroxyl radical formation could explain the greater susceptibility of endothelial cells grown in Ham’s F-12 versus RPMI 1640 medium to alterations in Ca$_{2+}$ signal after elevated glucose exposure. Furthermore, the presence of glutathione in RPMI 1640 versus Ham’s F-12 medium could be important. Glutathione is known to protect endothelial cells which are exposed to elevated glucose concentrations from an increased susceptibility to hydrogen-peroxide-induced cytotoxicity [34].

Alternative to hydroxyl radical formation via auto-oxidation of glucose in the presence of metal ions, a significant portion of glucose is known to be metabolized by the sorbitol pathway [35]. Activation of this pathway by high glucose concentrations could lead to intracellular generation of oxygen radicals as well [36,37]. Other possible sources of oxygen radical generation include: the cyclo-oxygenase pathway [15,16], protein kinase C pathway [37], cytochrome P450 [38], mitochondria [39], and xanthine oxidase [40]. Because of the multiple potential sites of oxygen radical production, the present studies do not differentiate the source of potential glucose-induced oxygen radical production for eliciting these calcium signaling defects.

In addition to our demonstration of decreased
bradykinin-stimulated Ca\textsuperscript{2+} as a consequence of exposure to elevated glucose concentrations, we have provided a potential mechanism to explain this endothelial cell defect which might be related to oxygen radical production. Furthermore, we provide additional direct evidence of oxidant production in endothelial cells during incubation with elevated glucose concentration using fluorescence spectroscopic measurements of the oxidation of dichlorofluorescein which is consistent with the hypothesis concluded using oxygen radical scavengers. That reactive oxygen species per se can alter calcium signal transduction has been well documented. While the immediate response to exogenously-generated reactive oxygen is usually an increase in resting cytosolic Ca\textsuperscript{2+} [41–44], this increase is usually transient. Furthermore, following exposure and removal of the oxygen-radical-generating system, agonist-stimulated increases in cytosolic Ca\textsuperscript{2+} are attenuated [42–44], which is consistent with our results following elevated glucose exposure.

Our studies also provide new information regarding the potential oxygen radical species which might mediate glucose-induced defects in Ca\textsuperscript{2+} signal formation in endothelial cells. Accordingly, our results suggest the possibility that hydroxyl radicals might be involved since the defect in Ca\textsuperscript{2+} signal was completely blocked by DMTU.

It was previously shown that other oxygen radical scavengers such as the enzymes superoxide dismutase or catalase individually prevented the impaired endothelium-dependent relaxation observed after exposure of normal rabbit and rat arteries to elevated glucose concentrations in vitro [15] and in vivo [16]. These studies suggest that either superoxide anion radical and/or hydrogen peroxide, or perhaps a radical species produced distally to these steps was the oxygen radical species which mediates glucose-induced reductions in endothelium-dependent relaxation. Nevertheless, since these studies were conducted in intact blood vessels (under high oxygen tensions) rather than in purified endothelial cell suspensions, it is not possible to rule out the possibility that superoxide dismutase may have acted on other non-endothelial cell types such as the vascular smooth muscle cell or on modulating the biological activity of nitric oxide after its release from the endothelial cell.

In contrast to the studies cited above, our results do not appear to indicate a direct role of superoxide anion radicals or hydrogen peroxide (at least extracellularly-derived) in glucose-induced changes in Ca\textsuperscript{2+} signal transduction since superoxide dismutase and catalase both failed to prevent the change in Ca\textsuperscript{2+} signal transduction. It is possible that the actions of superoxide dismutase and catalase were ineffective because both are large-molecular-weight substances which are likely not to act at intracellular sites of oxygen radical production. Therefore, it is not possible to totally exclude intracellular generation of both superoxide anion and hydrogen peroxide (known precursors of hydroxyl radical formation) as contributing to this defect.

In support of our findings, Weisbrod et al. [28] observed that superoxide dismutase failed to prevent the decrease in bradykinin-stimulated cGMP production in porcine endothelial cells which are exposed to elevated glucose concentrations. Thus, it is unlikely that superoxide anion radicals directly mediate the reduction in bradykinin-stimulated Ca\textsuperscript{2+} signal transduction following exposure of endothelial cells to elevated glucose concentrations.

In contrast to superoxide anion radicals, our studies suggest the possibility that the hydroxyl radical is the potential oxygen radical species which might mediate glucose-induced alterations in Ca\textsuperscript{2+} signal transduction. In this regard, we demonstrated that DMTU, a cell-permeable hydroxyl radical scavenger, completely prevented the defective Ca\textsuperscript{2+} signal transduction in bradykinin-stimulated cells. The lack of efficacy of the hydroxyl radical scavenger, mannitol, could be explained by its action to scavenge extracellular hydroxyl radicals only. A direct effect of DMTU on Ca\textsuperscript{2+} signal transduction was excluded since DMTU did not augment bradykinin-stimulated Ca\textsuperscript{2+} signals in control cells or alter ionomycin-stimulated Ca\textsuperscript{2+} signals in glucose-exposed cells. That intracellular actions or metabolism of high glucose are responsible for this impairment in Ca\textsuperscript{2+} signal transduction is also supported by the absence of the defect in bradykinin-stimulated Ca\textsuperscript{2+} signal transduction using cytochalasin B to inhibit GLUT 1 transport of glucose.

In summary, our studies provide new information which indicates that exposure of endothelial cells to elevated concentrations of glucose causes a reduction in bradykinin-stimulated Ca\textsuperscript{2+} signal transduction. Our studies suggest the possibility that the mechanism for this defect potentially arises from an oxygen-radical-mediated pathway which is distal to superoxide anion radical or hydrogen peroxide generation. The studies presented suggest that intracellular generation of the hydroxyl radical might be responsible. Thus, our study suggests that defects in the Ca\textsuperscript{2+} signal transduction pathway for the release of nitric oxide may be an important mechanism to explain the reductions in endothelium-dependent relaxation observed in hyperglycemic states and in diabetes mellitus.

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