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Antioxidants prevent high-D-glucose-enhanced endothelial Ca^{2+}/cGMP response by scavenging superoxide anions

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Abstract

Very recently we proposed that hyperactivity of endothelial Ca^{2+}/cGMP signaling under hyperglycemic conditions is due to superoxide anion (O_2^-) release. The present study was designed to investigate changes in endothelial glutathione (GSH) levels in response to high D-glucose and possible prevention of the high-D-glucose-initiated changes in Ca^{2+}/cGMP signal by antioxidants. Under hyperglycemic conditions, GSH content increased by 29% within 4 h. Co-incubation with 10 mM GSH during high-D-glucose treatment normalized the Ca^{2+}/cGMP response associated with an increase in GSH content by 222%. Vitamin C (250 μM) markedly diminished the high-D-glucose-mediated hyperreactivity of endothelial Ca^{2+} entry (by 40%) and Ca^{2+} release (by 52%). Similar to GSH, co-incubation with vitamin E (α-tocopherol; 50 μg/ml) and probucol (50 μM) completely prevented the high-D-glucose-inititiated hyperreactivity of the endothelial Ca^{2+}/cGMP response. Vitamin E, probucol, GSH and vitamin C diminished the high-D-glucose-mediated O_2^- release by 78, 65, 89 and 46%, respectively. These data suggest that antioxidants prevent high-D-glucose-initiated changes in endothelial Ca^{2+}/cGMP response by scavenging the overshoot of O_2^-.

Keywords: D-Glucose oxidation; Endothelial cell; EDRF (endothelium-derived relaxing factor); Glutathione; Superoxide dismutase; Vitamin C; Vitamin E

1. Introduction

The vascular endothelium plays a crucial role in the regulation of vascular reactivity (for review see Graier et al., 1994). Thereby, endothelial cells regulate the contraction state of smooth muscle cells and prevent platelet adhesion by the production and the release of paracrine substances. Interestingly, the biosynthesis of all endothelium-derived autacoids, such as endothelin, endothelium-derived relaxing factor (EDRF; nitric oxide), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF), critically depend on increases of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (for review see Graier et al., 1994). Due to the essential role of the endothelium in vascular homeostasis, alterations of endothelial vascular function result in dysfunction of blood vessel regulation, associated with a lack of appropriate vascular responsiveness and an increased risk of atherosclerosis. Endothelium dysfunction has been demonstrated during hypercholesterolemia (Andrews et al., 1987), diabetes mellitus (Cohen, 1993) or hypertension (Lüscher, 1994). Hypercholesterolemia and hypertension result in a decrease in endothelial vascular reactivity (Lüscher et al., 1993). In contrast, diabetes mellitus initially evokes reduced peripheral resistance and increased blood flow (Houben et al., 1993; Kohner et al., 1975; Mogensen, 1971), reflecting increased endothelial activity (Williamson et al., 1993), followed by attenuation of endothelial vascular function (Cohen, 1993). Very recently, we could demonstrate enhanced Ca^{2+}/EDRF signaling under hyperglycemic conditions (Graier et al., 1993; Wascher et al., 1994a), which was shown to be due to increased superoxide anion (O_2^-) formation on high-D-glucose treatment (Graier et al., 1996). In agreement, the involvement of superoxide anions in diabetes-mellitus-induced changes in blood vessel response was also suggested by other groups who used animal models (Tesfamariam and Cohen, 1992; Williamson et al., 1993).

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The aim of the study was to investigate whether antioxidants, such as vitamin E, vitamin C or glutathione (GSH) may prevent high-D-glucose-initiated changes in endothelial Ca^{2+}/EDRF signaling, indicating a possible rationale of antioxidant therapy to avoid/delay the appearance of circulatory dysfunction during diabetes mellitus.

2. Materials and methods

2.1. Materials

Cultured media, amino acids and vitamins were purchased from Gibco-BRL Life Technologies (Vienna, Austria). Fetal calf serum was from Sebak (Suben, Austria). 2-(6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)-amino)-5-(2-(2-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acid, (acetyloxy)methylester) (Fura-2/AM) was purchased from Lambda Fluorescence Technologies (Graz, Austria). Petri dishes (10 cm diameter) were obtained from Corning (Vienna, Austria) and 6- and 24-well plates were from Falcon (Linz, Austria). Buffer salts were obtained from Merck (Darmstadt, Germany). All other materials, including superoxide dismutase, xanthine oxidase, pyrogallol and antioxidants were from Sigma (Vienna, Austria).

2.2. Cell culture

For endothelial cell isolation (Graier et al., 1993), fresh porcine aortae were incubated at 37°C with 200 U/ml collagenase (type II) in Dulbecco's minimal essential medium (DMEM) plus dilutions of (v/v) 0.02% amino acids and 0.01% vitamins and trypsin inhibitor (soybean type I; 1 mg/ml). Alternatively, 2 U/ml dispase in DMEM plus dilutions of (v/v) 0.02% amino acids and 0.01% vitamins was used. Endothelial cells were cultured up to passage 1 in Opti-DMEM containing 3% fetal calf serum. Specificity of the isolation technique was tested by the appearance of typical cobblestone morphology as well as by immunofluorescence detection of contaminating smooth muscle cells (α-actin). For experiments, only endothelial cell cultures of higher than 98% purity (normally 8 out of 10) were used.

2.3. Determination of cytosolic glutathione

Cytosolic glutathione (GSH) content was measured using the enzymatic, colorimetric recycling assay described by Tietze (1969), modified for use in microtitre plates Punchard et al. (1984). Briefly, cultured endothelial cells were grown in 24-well plates. When confluent, cells were preincubated at 37°C in DMEM containing the compound to be tested. After 24 h, the incubation medium was aspirated off and the cells were lysed by addition of 180 μl sulfosalicylic acid (1%). After 2 h at 4°C, 600 μl phosphate-buffered solution (100 mM NaH_2PO_4) containing 5 mM EDTA was added. A 100-μl aliquot of the supernatant was mixed with 100 μl of the same phosphate buffer containing 1.5 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and 0.5 mM NADPH. The reaction was started by the addition of 50 μl glutathione reductase (1 U/ml in phosphate buffer containing 5 mM EDTA). The increase in absorbance of the reaction product, 5-thio-2-nitrobenzoic acid (TNB) was monitored at 410 nm over 5 min. The slope of TNB accumulation within 5 min is proportional to the GSH concentration in the sample. A standard calibration graph was made in each assay and used for calculation of cytosolic GSH content which is expressed as nmol GSH/dish.

2.4. Ca^{2+} measurement

Intracellular free-Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined using the fura-2 technique as previously described (Graier et al., 1995a). In brief, cells preincubated for 24 h in DMEM with the compound to be tested were harvested by 1-min treatment with trypsin (0.02% in phosphate-buffered solution containing 1 mM EDTA), centrifuged and resuspended in DMEM containing 2 μM fura-2/am. After 45 min at 37°C in the dark, the cells were centrifuged, washed twice and resuspended in Hepes-buffered solution with or without 2.5 mM $CaCl_2$ containing in mM: 145 NaCl, 5 KCl, 1 $MgCl_2$, 10 Hepes-acid, pH adjusted at 7.4. Intracellular Ca^{2+} was monitored every 0.25, 0.5 or 1.0 s in monolayer or suspended cells in thermostatically controlled (37°C) dual-wavelengths spectrofluorometers (Shimadzu RF-5000PC, Hitachi F-2000PC, Hitachi F-4500 and Perkin-Elmer LS50-B/FFA) from the ratio of 340 and 380 nm excitation at 510 nm emission. A uniform experimental protocol was used throughout the study. Thereby, intracellular Ca^{2+} release was investigated in the nominal absence of free extracellular Ca^{2+} by stimulation with 100 nM bradykinin. In addition, the increase in $[Ca^{2+}]_i$ upon addition of 2.5 mM extracellular $CaCl_2$ to pre-stimulated cells was measured to demonstrate Ca^{2+} entry pathway activity.

2.5. Data acquisition

Due to the overall failure of the calibration techniques reported by many groups (Graier et al., 1995a; Sturek et al., 1991; Wang et al., 1995), intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in each experiment is expressed as the 340/380 ratio for excitation at 510 nm emission (F_{340}/F_{380}). With the instrumental setups used, there are differences in fluorescence intensity (I; $I = \quad \times I_0 \times QY \times K$) based on variations in excitation light intensity (I_0), instrumental constant (K, photomultiplier sensitivity, etc.) and the relative molar extinction coefficient of fura-2 ( ). While changes by variations on excitation intensity (I_0), the quantum yield of this compound (QY) is constant. To allow compar-

ison between different treatments of cells, a standard effect of bradykinin in untreated cells was performed on the same day with the same instrument and is shown in every figure.

2.6. Measurement of endothelial cGMP levels

Endothelial cGMP content was determined as described previously (Graier et al., 1993). Briefly, endothelial cells were subcultured in 6-well plastic plates until confluence. Prior to the experiment, the cells were washed twice with the Hepes-buffered solution described above and were preincubated for 15 min in Hepes buffer containing 2.5 mM $CaCl_2$, 1 mM 3-isobutyl-1-methylxanthine and 1 μM indomethacin. Cells were stimulated by the addition of the compound to be tested. After 4 min, the incubation medium was aspirated off and the experiment was stopped by the addition of 1.0 ml chilled HCl (0.01 M). After 1 h at 4°C, cGMP was measured using a radioimmunoassay.

2.7. Measurement of superoxide anion release

Superoxide anions were determined according to Steinbrecher (1988) as the reduction of ferricytochrome c (10 μM; horse heart type III) in the absence or presence of superoxide dismutase (476 U/ml) as previously described (Graier et al., 1996). The reduction was followed at 550 nm in a photometer. The difference in absorption between samples in the absence and the presence of superoxide dismutase gives a direct measurement of extinction due to superoxide anion-related reduction of ferricytochrome c. The amount of superoxide anion release was calculated using the molar extinction coefficient of the reduced form of ferricytochrome c (= 21.000) (Steinbrecher, 1988).

2.8. In vitro measurement of superoxide anion scavenging properties of the antioxidants

Superoxide anions (O_2^-) were generated by the reaction of xanthine oxidase (150 μU/ml) with hypoxanthine (1 mM) in Hepes-buffer containing 30 μM Ca^{2+} at 37°C (Fridovich, 1970) as described previously (Graier et al., 1995b; Graier et al., 1996). Alternatively, pyrogallol autoxidation was used in the Hepes-buffered solution containing 30 μM Ca^{2+} (Maklund and Maklund, 1974). Briefly, 40 μl of substance solution to be tested and 100 μl of Tris-HCl-EDTA buffer (in mM: 10^3 Tris-HCl, 5 EDTA, pH adjusted to 8.0 with NaOH) were added to 840 μl water and the reaction was started by the addition of 20 μl pyrogallol (10 mM in 10 mM HCl). After short stirring, the change in absorption at 320 nm was measured over 3 min. The results are given as the change in absorption per min, and superoxide anion scavenging properties are given as percentage decrease in the slope of absorption compared with control experiments without antioxidants. Superoxide dismutase, GSH and vitamin C were dissolved in buffer to the concentration indicated and probucol and vitamin E were initially dissolved in ethanol. The final ethanol concentration did not exceed 0.1%. For control experiments cells were sham-treated with 0.1% ethanol to provide proper controls. Prior the experiments, the solution was mixed with three parts of buffer and added to the mixture.

2.9. Statistics

All data points are means ± S.E.M. Experiments were performed with at least three different batches of endothelial cell preparations. The analysis of variance (ANOVA) was used for data evaluation. Significance of differences was estimated with Scheffe's *F*-test and the level of significance was defined as $P < 0.05$.

3. Results

3.1. High-D-glucose-mediated changes in endothelial Ca^{2+}/cGMP response and GSH content

The effect of pretreatment with high D-glucose (44 mM for 24 h) on endothelial Ca^{2+} signaling is demonstrated in Fig. 1. In cells preincubated with high D-glucose, bradykinin evoked intracellular Ca^{2+} release (from 2.3 ±

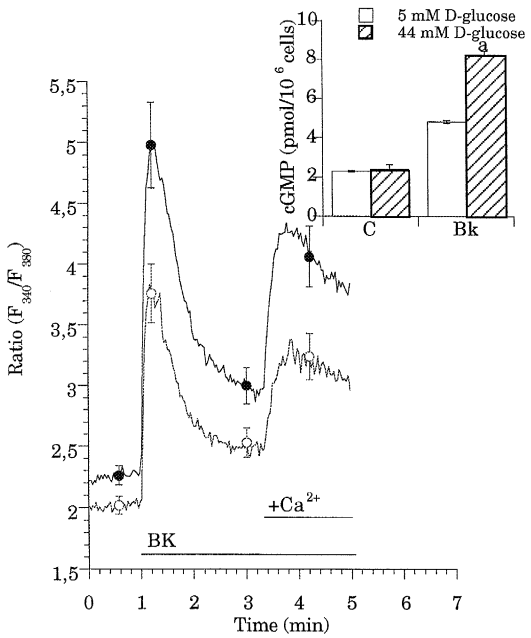

Fig. 1. Effect of high D-glucose on endothelial Ca^{2+} signaling (suspended cells) and increases in cGMP levels (inset) to bradykinin (100 nM). Endothelial cells were preincubated for 24 h in DMEM containing 5 (○) and 44 mM (●) D-glucose. Inset: effect of preincubation for 24 h in DMEM containing 5 (white column) and 44 mM D-glucose (shaded column) on control (C) and bradykinin-stimulated (1 μM; Bk) cGMP levels as biological maker for EDRF biosynthesis in the presence of 2.5 mM extracellular Ca^{2+}. [a] $P < 0.05$ vs. GSH in the presence of 5 mM D-glucose.

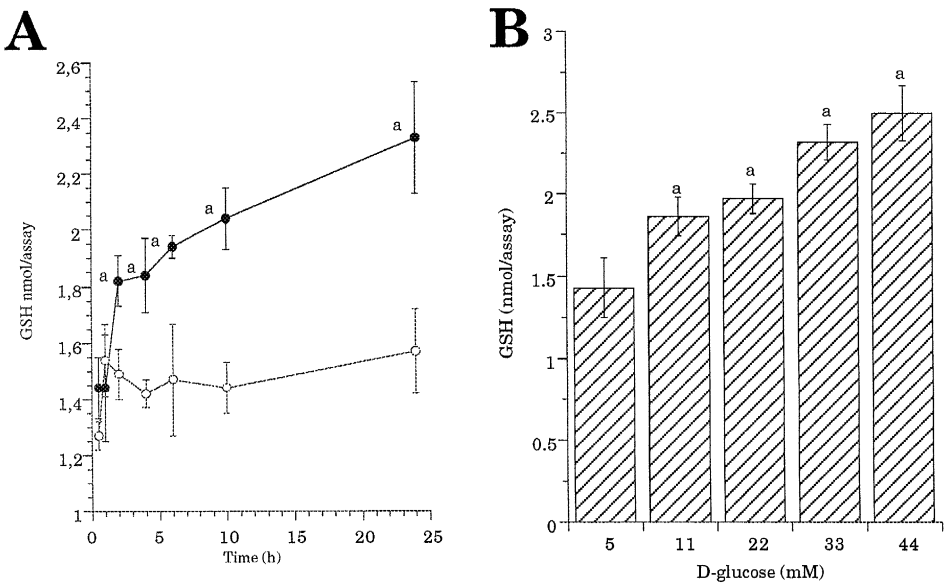

Fig. 2. Effect of acute hyperglycemia on endothelial GSH content. (A) Time course of endothelial GSH content over 24 h in DMEM containing 5 (○) and 44 mM D-glucose (●). Each point represents the mean ± S.E.M. ($n = 6$). (B) Concentration-response relationship of D-glucose on endothelial GSH content. Confluent endothelial cells were incubated for 24 h in DMEM containing D-glucose in the concentration indicated. Each column represents the mean ± S.E.M. ($n = 6$). [a] $P < 0.05$ vs. GSH in the presence of 5 mM D-glucose.

0.07 to 5.0 ± 0.35 ratio units, $n = 20$) and Ca^{2+} entry in response to addition of 2.5 mM Ca^{2+} to prestimulated cells (from 3.0 ± 0.13 to 4.4 ± 0.31 ratio units, $n = 20$). This was significantly greater than the effect of bradykinin in cells from 5 mM D-glucose-containing medium (Ca^{2+} release: from 2.1 ± 0.05 to 3.7 ± 0.24 ratio units, $n = 20$, $P < 0.05$ and Ca^{2+} entry: from 2.6 ± 0.11 to 3.2 ± 0.16 ratio units, $n = 20$, $P < 0.05$). In resting cells (i.e. no bradykinin added), addition of 2.5 mM $CaCl_2$ yielded equal increases in $[Ca^{2+}]_i$ in cells preincubated in 5 or 44 mM D-glucose-containing DMEM (0.15 ± 0.05 and 0.17 ± 0.07 ratio units, respectively, $n = 27–36$, N.S.), representing 25.0 and 12.1% of the effect of an addition of Ca^{2+} in bradykinin-prestimulated cells.

Elevation of endothelial cGMP levels in response to bradykinin was 2.2-fold higher in cells preincubated in 44 mM D-glucose-containing DMEM compared with cells preincubated in 5 mM D-glucose containing DMEM (inset to Fig. 1). In the presence of 300 μM L-N^G nitro-arginine, an inhibitor of endothelial nitric oxide synthase, bradykinin failed to increase endothelial cGMP levels under all conditions, indicating that elevation of endothelial cGMP is a useful index for EDRF formation (Graier et al., 1993).

Interestingly, preincubation with high D-glucose increased cytosolic GSH content in a time-dependent (Fig. 2A) and concentration-dependent manner (Fig. 2B). In contrast to that with D-glucose, pretreatment with 22 and 44 mM D-mannitol failed to affect endothelial GSH content (data not shown). Similarly to high-D-glucose, incubation of endothelial cells with the superoxide-anion-generating mixture xanthine oxidase (150 μU/ml) and hypoxanthine (1 mM) elevated endothelial GSH levels from 1.27 ± 0.02 to 1.79 ± 0.05 nmol/assay ($n = 9$, $P < 0.05$ vs. control; assay contains 5×10^6 cells). In contrast to the preincubation with high D-glucose or xanthine oxidase/hypoxanthine, pretreatment with 300 μM hydrogen peroxide decreased cellular GSH levels by 23 ± 2.6% ($n = 12$, $P < 0.05$ vs. control).

3.2. Antioxidants prevent high-D-glucose-mediated changes in endothelial Ca^{2+}/cGMP response

3.2.1. GSH

Co-incubation of endothelial cells with 10 mM GSH during the preincubation procedure for high D-glucose blunted the effect of high-D-glucose treatment on endothe-

Table 1
Effect of co-incubation with vitamin C (250 μM) on high-D-glucose-mediated changes in endothelial Ca^{2+} signaling in response to bradykinin (100 nM)

D-Glucose (mM)	5	44	5 + Vit. C	44 + Vit. C
Basal $[Ca^{2+}]_i$	1.2 ± 0.09	1.2 ± 0.03 (100)	1.2 ± 0.03	1.2 ± 0.1 (100)
Ca^{2+} release	2.4 ± 0.19	2.8 ± 0.13 [a] (133)	2.4 ± 0.03	2.6 ± 0.12 [a,b] (116)
Ca^{2+} entry	2.7 ± 0.17	4.1 ± 0.20 [a] (193)	2.7 ± 0.09	3.6 ± 0.17 [a,b] (156)

Endothelial free Ca^{2+} concentrations are given in ratio units (ratio (F_{340}/F_{380})). Numbers in parentheses show the percentage increase compared with the effect in cells pretreated with 5 mM D-glucose. [a] $P < 0.05$ vs. the effect in cells pretreated with 5 mM D-glucose. [b] $P < 0.05$ vs. Ca^{2+} response to bradykinin in cells pretreated in 44 mM D-glucose in the absence of vitamin C.

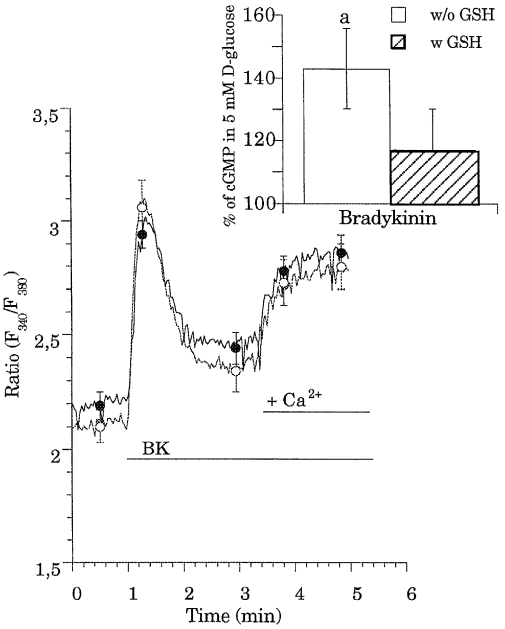

Fig. 3. Effect of GSH on high-D-glucose-initiated changes in endothelial Ca^{2+} signaling (cell monolayer) and cGMP formation as biological maker for EDRF biosynthesis (inset). In the presence of 10 mM GSH, endothelial cells were preincubated for 24 h in DMEM containing 5 (○) or 44 mM D-glucose (●) and the effect of 100 nM bradykinin on endothelial Ca^{2+} concentration was measured in the absence of GSH. Inset: effect of bradykinin (1 μM) in the presence of 2.5 mM extracellular Ca^{2+} on endothelial cGMP levels in cells pretreated in DMEM containing 44 mM D-glucose in the presence (w GSH; shaded column) and absence (w/o GSH; white column) of 10 mM GSH. Increases are expressed as percentages of bradykinin-evoked cGMP increase in cells treated in 5 mM D-glucose-containing media ($n = 12$; [a] $P < 0.05$ vs. 5 mM D-glucose).

lial Ca^{2+} signaling (Fig. 3). There were no differences in either bradykinin-initiated Ca^{2+} release and $[Ca^{2+}]_i$ increase to $CaCl_2$ addition between cells preincubated 24 h in 5 mM DMEM containing 10 mM GSH and cells preincubated in 44 mM D-glucose in the presence of 10 mM GSH.

During the incubation period, GSH increased the cytosolic GSH concentration from 0.470 ± 0.011 to 1.515 ± 0.082 nmol/10^6 cells ($n = 6$) in cells in 5 mM D-glucose, an effect which was not different from that observed in cells preincubated in 44 mM D-glucose (1.480 ± 0.023 nmol/10^6 cells; $n = 6$).

In agreement with its preventive action on the effect of high D-glucose on endothelial Ca^{2+} signaling, co-incubation with GSH during the 24 h pretreatment with 44 mM D-glucose normalized the bradykinin-induced elevation of endothelial cGMP levels ($143 \pm 12.7\%$ in 44 mM D-glucose-treated cells, $P < 0.05$ vs. 5 mM D-glucose and $117 \pm 13.3\%$ in 44 mM D-glucose plus 10 mM GSH, n.s. vs. 5 mM D-glucose in the presence of 10 mM GSH; Fig. 3 inset).

3.2.2. Vitamin E

Similar to GSH, vitamin E prevented the high-D-glucose-initiated enhancement of the bradykinin-induced Ca^{2+} signal (Fig. 4) and cGMP elevation (inset Fig. 4). In contrast to incubation with GSH, vitamin E failed to increase endothelial GSH content. Moreover, increases in GSH by preincubation with 44 mM D-glucose were measured even in the presence of vitamin E (Fig. 5). In the absence of vitamin E, preincubation of endothelial cells with 44 mM D-glucose increased the GSH content from 0.260 ± 0.010 nmol/10^6 cells (5 mM D-glucose) to 0.363 ± 0.013 nmol/10^6 cells ($n = 6$; $P < 0.05$ vs. cells in 5 mM D-glucose). In the presence of 0.05 mg/ml vitamin E, the GSH content was similarly elevated by the increase of D-glucose from 5 to 44 mM (from 0.280 ± 0.090 to 0.397 ± 0.018 nmol/10^6 cells; $n = 6$, $P < 0.05$; Fig. 5).

3.2.3. Probucol

In agreement with our data about the inhibitory action of co-incubation with vitamin E, probucol also prevented high-D-glucose-mediated changes in endothelial Ca^{2+} re-

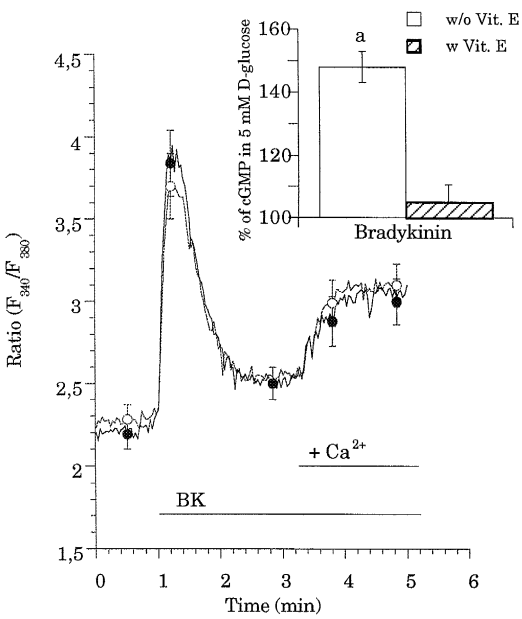

Fig. 4. Effect of vitamin E (α-tocopherol) on high-D-glucose-initiated changes in endothelial Ca^{2+} signaling (supended cells) and cGMP formation as biological maker for EDRF biosynthesis (inset). In the presence of vitamin E (0.1% final ethanol concentration) cells were preincubated for 24 h in DMEM containing 5 (○) or 44 mM D-glucose (●) and the effect of 100 nM bradykinin on endothelial Ca^{2+} concentration was measured in the absence of GSH. Inset: effect of bradykinin (1 μM) in the presence of 2.5 mM extracellular Ca^{2+} on endothelial cGMP levels in cells pretreated in DMEM containing 44 mM D-glucose in the presence (w Vit. E; shaded column; 0.1% ethanol) and absence (w/o Vit. E; white column; sham treated with 0.1% ethanol) of 50 μg/ml vitamin E. Increases are expressed as percentages of bradykinin-evoked cGMP increase in cells treated in 5 mM D-glucose containing media ($n = 12$; [a] $P < 0.05$ vs. 5 mM D-glucose).

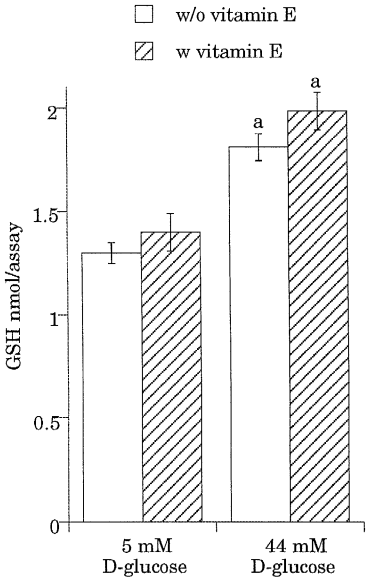

Fig. 5. Co-incubation with vitamin E while 44 mM D-glucose was present did not prevent increases in the intracellular GSH level, due to the high sugar concentration. Cultured endothelial cells were incubated for 24 h in DMEM containing 5 or 44 mM D-glucose in the absence (white columns) or presence (shaded columns) of 50 μg/ml vitamin E (α-tocopherol). Each column represents the mean ± S.E.M. ($n = 12$). [a] $P < 0.05$ vs. cells in 5 mM D-glucose containing DMEM.

sponse and cGMP elevation. In cells pretreated with 50 μM probucol in 5 and 44 mM D-glucose-containing DMEM, the addition of 100 nM bradykinin in nominally Ca^{2+}-free solution increased $[Ca^{2+}]_i$ from 1.8 ± 0.05 to 3.3 ± 0.07 ($n = 12$) and 1.7 ± 0.04 to 3.2 ± 0.05 ($n = 17$), respectively (experiments performed with cell monolayer). Similarly to the bradykinin-initiated Ca^{2+} release, elevation of $[Ca^{2+}]_i$ in response to addition of 2.5 mM extracellular $CaCl_2$ to bradykinin pre-stimulated cell was identical in cells pretreated with 50 μM probucol in 5 and 44 mM D-glucose-containing media. As expected, normalization of the bradykinin-initiated Ca^{2+} response by probucol co-incubation prevented the increase in cGMP formation in response to bradykinin in high-D-glucose-pretreated cells also (data not shown).

3.2.4. Vitamin C

In addition to the lipophilic antioxidants, vitamin E and probucol, the hydrophilic antioxidant vitamin C diminished high-D-glucose-mediated alterations of the endothelial Ca^{2+}/cGMP response (Fig. 6). In cells preincubated for 4 h in 44 mM D-glucose-containing DMEM, the bradykinin-evoked intracellular Ca^{2+} release and Ca^{2+} entry were much greater than those observed in cells pretreated in 5 mM D-glucose-containingg DMEM (133 and 193%, respectively). Co-incubation with 250 μM vitamin C together with high D-glucose, slightly diminished the effect of high D-glucose on bradykinin-initiated Ca^{2+} release and Ca^{2+} entry (116 and 156%, respectively; Table 1).

Like vitamin E and probucol, vitamin C failed to normalize the high-D-glucose-mediated elevation of cytosolic

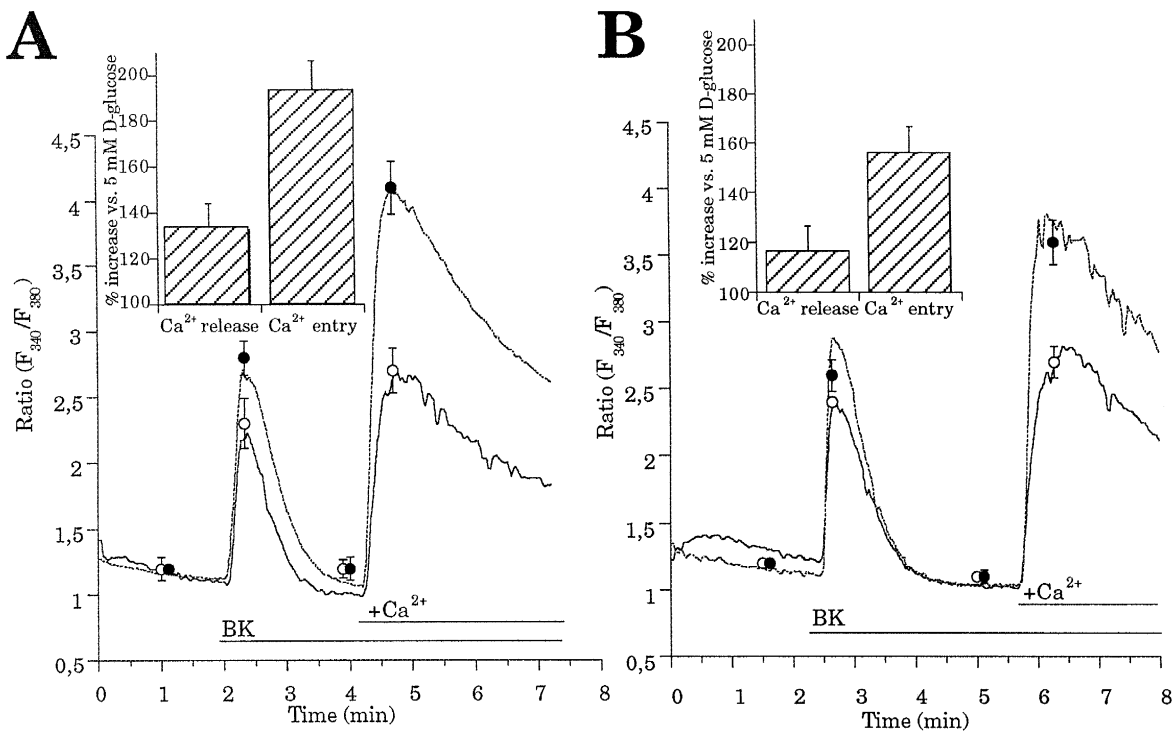

Fig. 6. Effect of co-incubation with vitamin C on high-D-glucose-initiated increase in endothelial Ca^{2+} signaling stimulated by bradykinin in cell suspension. Cultured endothelial cells were preincubated for 4 h in DMEM containing 5 (○) or 44 (●) mM D-glucose in the absence (A) or presence (B) of 250 μM vitamin C. Afterfura-2/am loading procedure, intracellular Ca^{2+} signaling in response to stimulation with 100 nM bradykinin in the nominal absence of free extracellular Ca^{2+} and the readdition of 2.5 mM Ca^{2+} was measured. Each point represents the mean ± S.E.M. ($n = 9$–12).

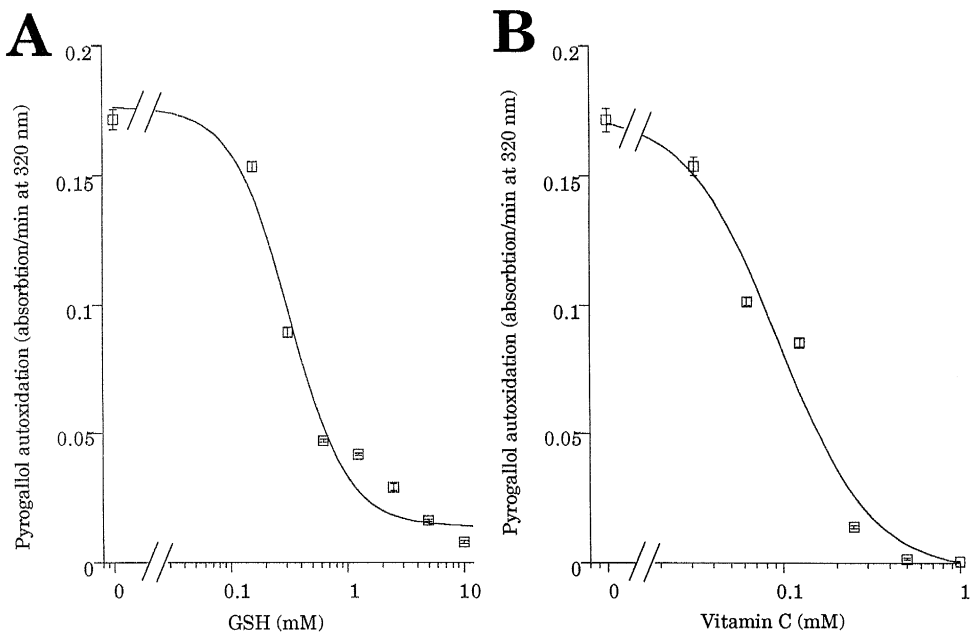

Fig. 7. Superoxide anion scavenging properties of GSH (A) and vitamin C (B). Superoxide anions were generated by pyrogallol autoxidation which yields an increase in absorption at 320 nm. Scavenging potency of the compounds tested reflects decreased changes in absorption of oxidized pyrogallol. Each point represents the mean ± S.E.M. ($n = 7$).

GSH concentration. In cells preincubated for 24 h in DMEM containing 44 mM D-glucose, the GSH level was 1.1520 ± 0.031 nmol/well, which represents an increase of 0.2833 nmol/well compared with GSH levels in cells incubated in 5 mM D-glucose-containing DMEM (0.8687 ± 0.0317 nmol/well). In the presence of vitamin C, 44 mM D-glucose treatment increased the GSH content within 24 h from 1.0312 ± 0.0290 (5 mM D-glucose) to 1.3083 ± 0.0488 nmol/well (i.e., an increase in GSH content by 0.2771 nmol/well).

3.3. Properties of antioxidants used to scavenge superoxide anions

All antioxidants used in this study were tested for their superoxide anion-scavenging properties at the concentration used (Fig. 7). The IC_{50} values to prevent the autoxidation of 20 μM pyrogallol for GSH (Fig. 7A) and vitamin C (Fig. 7B) were 325.9 (121.85–871.52) and 95.18 (50.30–180.12) μM, respectively. The anti-oxidative potency of the liphophilic compounds, vitamin E and probucol, was demonstrated by Arudi et al. (1983).

3.4. Antioxidants prevent high-D-glucose-mediated elevation of endothelial superoxide anion release

Very recently, we have demonstrated that the high-D-glucose-mediated changes in endothelial Ca^{2+}/EDRF signaling are due to generation of superoxide anions (Graier et al., 1996). Thus, the hypothesis was tested that the normalization of the endothelial Ca^{2+}/cGMP response in high-D-glucose-containing medium by antioxidants correlates with the normalization of superoxide anion release in high-D-glucose-treated cells. As shown in Fig. 8, all antioxidants able to prevent the effect of high D-glucose on

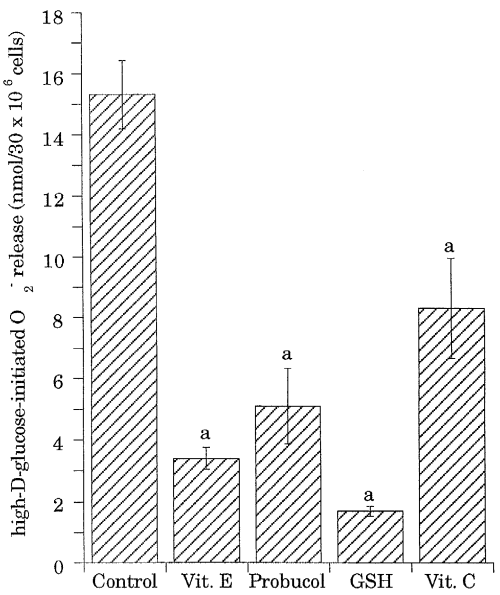

Fig. 8. Effect of co-incubation with antioxidants during high D-glucose treatment. Cultured endothelial cells were incubated for 4 (vitamin C) or 24 h (all other antioxidants) in DMEM containing 5 or 44 mM D-glucose in the absence (control) or presence of vitamin E (Vit. E; 50 μg/ml), probucol (50 μM), GSH (10 mM) and vitamin C (Vit. C; 250 μM). Columns represent the difference in superoxide anion release from cells incubated in 5 and 44 mM D-glucose under control conditions (no antioxidants added) or in the presence of vitamin E, probucol and GSH. Each column represents the mean ± S.E.M. [a] $P < 0.05$ vs. control ($n = 3$–6).

endothelial Ca^{2+}/cGMP response significantly decreased superoxide anion release in cells pretreated in high D-glucose with the antioxidants to be tested.

4. Discussion

Despite the increasing number of publications showing the protective effect of antioxidants against diabetes-mediated blood vessel dysfunction, the cellular responses to antioxidants are unknown. This study showed for the first time, that antioxidants prevent the high-D-glucose-mediated overshoot of superoxide anion release and normalize endothelial Ca^{2+}/cGMP responses in high-D-glucose-pretreated endothelial cells.

Increased vascular release of superoxide anions is not only a diabetic-related phenomenon (Cohen, 1993; Graier et al., 1996; Mazière et al., 1995; Tesfamariam et al., 1990; Tesfamariam and Cohen, 1992), but is also observed in hypercholesterolemia (Keaney et al., 1995), septic shock (Goligorsky, 1989), hypoxia and ischemia reperfusion injury (Lüscher, 1994). Significantly, all these pathological phenomena are linked to a blood vessel dysfunction which is manifested as attenuated endothelium-dependent relaxation. Very recently, we have shown that preincubation of endothelial cells with high D-glucose increases superoxide anion release which augments endothelial Ca^{2+}/EDRF signaling (Graier et al., 1996) and vascular cell proliferation (Graier et al., 1995b). In fact, extracellular superoxide anions might be of particular importance for vascular wall homeostasis. As long as superoxide anions do not dramatically scavenge nitric oxide, the initial effect of superoxide anion formation in endothelial cells is beneficial for nitric oxide formation and might play a crucial role in reduced peripheral resistance and increased blood flow in the early stages of diabetes mellitus. However, depending on the ratio of superoxide anions and nitric oxide and the capacity of the vasculature to prevent an overshoot of superoxide anions and, perhaps, hydroxyl free radicals by superoxide dismutase and catalase, nitric-oxide-mediated relaxation may eventually be diminished (Wascher et al., 1994b; Williamson et al., 1993). Besides decreased amounts of nitric oxide beeing able to activate smooth muscle soluble guanylyl cyclase, superoxide anions might inhibit prostacyclin synthase, knocking out the second vasorelaxing pathway derived from the endothelial cells (Cohen, 1993), resulting in the release of vasocontracting prostanoids (Tesfamariam et al., 1990; Tesfamariam et al., 1995) under hyperglycemic conditions. Thus, even if an increased endothelial Ca^{2+} signal might be beneficial to compensate nitric oxide scavenging by superoxide anions in early stages of diabetes mellitus, it turns out to be a massive drawback through its strong activation of prostanoid synthase, finally resulting in prostaglandin H_2, thromboxane A_2 and 15-hydroxyeicosatetraenoic acid production.

In this study, the antioxidants, GSH and vitamin C were shown to have strong scavenging properties for superoxide anions, which was also shown for vitamin E and probucol (Arudi et al., 1983). The liphophilic agents tested, vitamin E and probucol, as well as GSH, completely normalized the high-D-glucose-initiated hyperreactivity of endothelial Ca^{2+} signaling, while the hydrophilic antioxidant, vitamin C, was less active. The reason for this difference is unclear, but may be related to different distribution of these compounds within the cell (i.e. cell membrane or cytosol).

Since autacoid-initiated formation of nitric oxide, and cGMP as biological marker for nitric oxide (Martin et al., 1988) depends strictly on elevation of intracellular Ca^{2+} concentration (Busse et al., 1993; Graier et al., 1994), the normalization of bradykinin-initiated cGMP formation in high-D-glucose-pretreated cells by antioxidants might be due to their normalizing properties on Ca^{2+} signaling.

Pretreatment with high D-glucose stimulated anti-oxidative pathways of endothelial cells, as indicated by the increase in GSH in cells preincubated in high-D-glucose-containing media. This is consistent with the observation of Ceriello et al. (1996) who demonstrated that pretreatment with high D-glucose results in the induction of antioxidant enzymes in cultured endothelial cells, indicating a high anti-oxidative capacity of endothelial cells to respond to increased oxidative stress under hyperglycemic conditions. In contrast to high D-glucose, high D-mannitol as hyperosmotic control failed to affect endothelial GSH content, which is in agreement with our previous report that D-mannitol had no effect on the endothelial Ca^{2+}/cGMP response (Graier et al., 1993) and cell proliferation (Graier et al., 1995b). Interestingly, the time course of high-D-glucose-induced changes in cellular GSH content shown here mirrored the time course of high-D-glucose-mediated changes in endothelial Ca^{2+} signaling demonstrated previously (Graier et al., 1993). Moreover, preincubation with the superoxide anion-generating mixture, xanthine oxidase/hypoxanthine, mimicked the effect of high D-glucose on cellular GSH levels while hydrogen peroxide yielded the opposite effect. These results further support our hypothesis that superoxide rather than peroxides is the candidate for the reactive species involved in blood vessel modulation in initial hyperglycemia.

Neither vitamin E, probucol nor vitamin C could prevent the high-D-glucose-mediated increases in cytosolic GSH levels. One might speculate that vitamin C does not enter the cytosol within our incubation time, while the hydrophobic vitamin E and probucol might accumulate within the plasma membrane. Thus, all compounds will be able to interfere with superoxide anions leaking out of the cells, while they fail to have a significant impact on the anti-oxidative capacity within the cells.

In view of the potential role of endothelium-derived superoxide anions in the processes of atherogenesis, one might expect antioxidants, which restored superoxide anion release, to be effective against increased lipoprotein

oxidation (Hiramatsu et al., 1987; Hunt et al., 1994; Mazière et al., 1995) and vessel dysfunction in diabetes mellitus (Cohen, 1993). While the inducing agent for increased superoxide anion release, D-glucose, is clear, the generating pathways for D-glucose, resulting in superoxide anion formation are unknown. While we could exclude aldose reductase as a key enzyme for D-glucose-initiated superoxide anion formation in endothelial cells (Graier et al., 1996), there is evidence for the involvement of D-glucose autoxidation (Hunt et al., 1994; Graier et al., 1996) and NADPH oxidase in high-D-glucose-mediated superoxide anion release (Pagano et al., 1995; Graier et al., 1996).

Consistent with the present results, antioxidants have been demonstrated to prevent hypertension in diabetic individuals (Ceriello et al., 1991), diabetes-mellitus-initiated protein kinase C activation (Kunisaki et al., 1994), peripheral nerve dysfunction in streptozotocin-diabetic rats (Cameron et al., 1993) and dysfunction of endothelium-dependent blood vessel relaxation (Cameron and Cotter, 1992; Tesfamariam and Cohen, 1992). While these studies were focused on diabetes mellitus-related phenomena which occur in later stages of diabetes mellitus, there is evidence that even in the early stages of diabetes mellitus, where increased blood flow and reduced peripheral resistance can be observed, superoxide anion scavenging agents prevent vascular and neural dysfunction (Williamson et al., 1993) and increased retinal blood flow (Kunisaki et al., 1995).

Thus, at all stages of diabetes mellitus, increased superoxide anion formation may play a crucial role in the clinical manifestation of diabetes mellitus in the vasculature. The mechanisms of interactions between this type of radical with endothelial vascular function are multiple, including increased Ca^{2+}/EDRF signaling (Graier et al., 1993, 1996), enhanced prostanoid formation (Tesfamariam et al., 1990), the release of contracting arachidonic acid metabolites (Tesfamariam et al., 1995) and scavenging of nitric oxide (Mian and Martin, 1995). This study, for the first time, showed clearly that high-D-glucose-initiated changes in some endothelial vascular function (Ca^{2+} increase and endothelial NO synthase-mediated cGMP elevation) can be normalized by antioxidants, due to their properties to diminish high-D-glucose-stimulated release of superoxide anions. The results, together with with results from the literature, strongly point to the potential use of antioxidants to prevent or to delay the appearance of diabetes-accompanied vascular complications.

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