High-glucose incubation of human umbilical-vein endothelial cells does not alter expression and function either of G-protein α -subunits or of endothelial NO synthase

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Alterations in G-protein-controlled signalling pathways (primarily pathways controlled by G_s and G_i) have been reported to occur in animal models of diabetes mellitus. We have therefore studied the effect of a long-term exposure of human umbilical vein endothelial cells to elevated concentrations of glucose on expression and function of G-protein subunits and endothelial NO synthase. Long-term incubation in high glucose (30 mM for 15 days) did not affect the levels of $G_{i\alpha-2}$, $G_{q\alpha}$, the splice variants (long and short form) of $G_{s\alpha}$, and the G-protein β -subunits or adenylate cyclase activity: basal, as well as isoprenaline-, forskolin- and guanosine 5'-[γ -thio]triphosphate-stimulated enzyme activities were comparable in high- and low-glucose-treated cells, thus ruling out any functional changes in the stimulatory pathway. Pretreatment of endothelial cells with pertussis toxin blocked a substantial fraction (50%) of the mitogenic response to serum factor(s) which depend(s) on functional G_{i2} . The

sensitivity of cells cultured in high glucose was comparable with that of the paired controls maintained in normal glucose ($EC_{50} = 3.1 \pm 0.5$ and 3.3 ± 0.4 ng/ml respectively). Similarly, we failed to detect any differences in endothelial NO synthase expression, or intracellular distribution and basal activity of the enzyme in endothelial cells cultured in high glucose. Stimulation of NO synthase in intact cells revealed a comparable response to the calcium ionophore (A23187). In contrast, stimulation with histamine (which acts via H_1 -receptors predominantly coupled to G_q) resulted in a significantly increased response in the cells maintained in high glucose. These data are suggestive of an altered H_1 -histamine receptor- G_q -phospholipase C pathway in endothelial cells cultured in high glucose concentrations, but rule out any glucose-induced functional changes in G_s - and G_i -controlled signalling pathways.

INTRODUCTION

Endothelial dysfunction is considered an intrinsic element in the pathogenesis of diabetic angiopathy, the most common cause of mortality and morbidity in diabetic patients [1]. Among the abnormalities found in vascular functions of diabetic patients are alterations in permeability, coagulation, blood flow, basement membrane synthesis and turnover, endothelium-dependent relaxation, hormone action and growth-factor effects [2]. The long-standing elevation of blood glucose levels is considered as an important factor in the pathogenesis of diabetic angiopathy [1]; in cultured endothelial cells, high glucose levels themselves impair the fibrinolytic potential [3], increase the rate of apoptosis (S. Baumgartner-Parzer, unpublished work) and augment the expression of basal-membrane proteins [4].

G-proteins are intimately linked to the control of endothelial cell functions, which are known to be altered in diabetes mellitus. Whereas G_s -dependent increases in intracellular cAMP regulate trans-endothelial permeability [5] and exert an inhibitory effect on cell proliferation [6], signal transduction via $G_{i\alpha-2}$, the predominant endothelial $G_{i\alpha}$ subtype, stimulates endothelial cell proliferation and angiogenesis [6,7]. Several receptors, which couple to G_{i2} or to members of the G_q family, such as the receptor for bradykinin, muscarinic receptors, α_2 -adrenergic and H_1 -histamine receptors, activate eNOS [endothelial NO/EDRF (endothelium-derived relaxing factor) synthase] [8,9]. Alterations

in the levels and the function of G-proteins have been reported repeatedly in several experimental models of diabetes mellitus. In general, decreases in the mass of immunodetectable α -subunits (namely, the three subtypes $G_{i\alpha-1}$, $G_{i\alpha-2}$, $G_{i\alpha-3}$, and $G_{s\alpha}$) and loss of functional activity have been observed, and both insulin-dependent cells (hepatocytes, adipocytes) as well as insulin-independent tissues (e.g. the central nervous system) are affected [10–16]. Furthermore, the changes apparently occur irrespective of whether streptozotocin- or alloxan-treated, insulin-deficient animals or genetically obese animals with hyperinsulinaemia and insulin-resistance are examined. Decreased levels of $G_{i\alpha-2}$, as well as an altered adenylate cyclase activity, were found in platelets of patients with non-insulin-dependent diabetes mellitus [17]. Thus the elevated levels of glucose may suffice to trigger the alterations in G-proteins, and these changes may represent an important link to the pleiotropic diabetes-induced alterations found in endothelial cells.

In the present work, we have therefore examined this hypothesis, using human venous endothelial cells maintained in medium containing normal (5 mM) or high (30 mM) levels of glucose. We have also investigated possible changes in the expression and the activity of eNOS, since this enzyme represents an important effector linked to input from G-protein-coupled receptors; NO synthase generates a diffusible messenger which controls both the function of endothelial cells as well as that of adjacent cells, thus modulating vascular tone, platelet aggre-

Abbreviations used: eNOS, endothelial NO/EDRF (endothelium-derived relaxing factor) synthase; ECL, enhanced chemiluminescence; GTP[S], guanosine 5'-[γ -thio]triphosphate; HUVECs, human umbilical-vein endothelial cells.

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gation, endothelium-leucocyte interaction and inhibiting smooth-muscle proliferation [18,19]. These cell-cell interactions are known to be affected in diabetic angiopathy [1,2]. Furthermore, in animal models, as well as in diabetic patients, functional evidence suggests that the control of NO synthase activity is altered [20,21].

MATERIALS AND METHODS

Materials

Endothelial-cell growth supplement was obtained from Laevosan (Vienna, Austria). Radioactive compounds ($[\alpha\text{-}^{32}\text{P}]\text{ATP}$, $[\text{H}^3]\text{thymidine}$) were from NEN (Boston, MA, U.S.A.). Fetal-calf serum was obtained from Life Sciences (Vienna, Austria). The materials for electrophoresis and the Coomassie Blue dye-binding kit were from Bio-Rad (Richmond, CA, U.S.A.). The ProtoBlot Alkaline Phosphatase System was from Promega (Bender Co., Vienna, Austria), the reagent kit for ECL (enhanced chemiluminescence) detection, the Megaprime DNA-labelling system and the Hybond N membrane were from Amersham. Guanosine 5'-[γ -thio]triphosphate (GTP[S]), ATP, phosphocreatine, creatine kinase and adenosine deaminase were obtained from Boehringer (Mannheim, Germany). Pertussis toxin was from Calbiochem (CA, U.S.A.). All other reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

The following antisera were used: antiserum 84, which recognizes all three subtypes of G_i [22], was kindly provided by Dr. P. Gierschik (Ulm, Germany); antiserum CS1, which is specific for G_{α} [22], and antiserum CQ, which recognizes G_{α} and $G_{11\alpha}$ [23], were kindly provided by Dr. G. Milligan (Glasgow, U.K.). Antiserum A7, which corresponds to the antiserum K521 [24] and is expected to recognize all subtypes of β -subunits, was a gift from Dr. M. Freissmuth (Vienna, Austria). Recombinant G-protein α -subunits ($G_{\alpha}\text{-S}$, $G_{\alpha}\text{-L}$, $G_{1\alpha}\text{-1}$, $G_{1\alpha}\text{-2}$) and G-protein $\beta\gamma$ -subunit complexes were purified from bacterial lysates [25,26] and bovine brain membranes respectively [27] and used to verify the specificity of the antibodies employed. Human platelet membranes, which express a high level of G_{α} and $G_{11\alpha}$, were prepared as in [28]. The antiserum N20030 against endothelial NO synthase, as well as a purified human endothelial-cell lysate (which was used as a standard), were purchased from Transduction Laboratories (Lexington, KY, U.S.A.).

Cell culture

Human umbilical-vein endothelial cells (HUVECs) were isolated by incubating the umbilical cords for 15 min with 0.2% collagenase in PBS [29]. Cells were plated in tissue-culture dishes coated with 1% gelatin and grown in medium 199 enriched with endothelial-cell growth supplement, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 units/ml penicillin, 0.25 $\mu\text{g}/\text{ml}$ amphotericin and 20% fetal-calf serum. The initial cell preparation from each individual umbilical cord was split, and the medium for one half of the cells was supplemented with glucose to a final concentration of 30 mM, versus 5 mM in the control culture. The cells were grown at 37 °C in a 5%-CO₂ humidified incubator, and the culture medium was renewed twice a week. When confluent monolayers were formed, cells were subcultured by detachment with EDTA (0.2%). Cells were used for experiments in the first passage after 13–18 days of culture. The cell population obtained consisted of > 95% endothelial cells, as verified by their cobblestone morphology and immunofluorescence staining with antibodies against Von Willebrand Factor.

$[\text{H}^3]\text{Thymidine}$ incorporation

HUVECs were allowed to grow to confluency and were subsequently synchronized by serum starvation for 48 h in medium 199 containing 1% BSA. Thereafter, the cells were removed from culture dishes by brief trypsin treatment and seeded at 10⁴ cells/well in flat-bottomed 96-well tissue-culture plates in medium 199 containing 5% fetal-calf serum and the respective glucose concentrations. After 2 h, which allows the cells to adhere, pertussis toxin was added, to the final concentrations indicated in the Figure legends. $[\text{H}^3]\text{Thymidine}$ was added 48 h thereafter, to a final concentration of 1 $\mu\text{Ci}/\text{ml}$. After a further 4 h, cells were lysed by a freeze-thaw cycle; the radioactivity incorporated into DNA was trapped on glass-fibre filters by using a Skatron cell harvester and counted by liquid scintillation.

Immunoblots

For the quantification of G-protein levels, cells were prepared in buffer containing 50 mM Tris/HCl (pH 7.4), 1 mM EDTA and 20 μM aprotinin. After a rapid freeze-thaw cycle in liquid nitrogen, cells were homogenized by using an Ultra-Turrax and centrifuged for 10 min at 39 000 *g*. The resulting membrane pellet was resuspended in HME buffer (20 mM Hepes/NaOH, pH 8.0, 2 mM MgSO₄, 1 mM EDTA). For immunoblots, 30–50 μg of membrane protein was dissolved in Laemmli sample buffer containing 2% SDS and 40 mM dithiothreitol and resolved by electrophoresis on SDS/polyacrylamide gels. Proteins were transferred on to nitrocellulose membranes. The nitrocellulose membranes were stained with Ponceau S to verify that equal amounts of protein had been applied. After a blocking step (1% BSA in 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20), immunoreactive bands were revealed by consecutive incubation with the primary antiserum and the second antibody, either conjugated to alkaline phosphatase (Protoblot system) or coupled to horseradish peroxidase (ECL system).

eNOS levels were determined as follows. Whole-cell lysates were obtained by sonication for 4 $\times$ 15 s in buffer (50 mM triethanolamine, 1 mM EDTA, 10 mM mercaptoethanol, 20 μM aprotinin). Then 20 μg of protein of each sample was subjected to electrophoresis and blotted as described above, and detection was carried out with the ECL system. To determine the intracellular distribution of eNOS, whole-cell lysates were centrifuged for 180 min at 45 000 *g* at 4 °C. The supernatant represents the cytosol, and the pellet the membrane fraction. Corresponding amounts of cytosol and membranes were subjected to electrophoresis and blotted as described above. For quantitative analysis of immunoblots, X-ray films (ECL system) or nitrocellulose blots (alkaline phosphatase staining) were subjected to laser-scan densitometry. The absorbance in the samples derived from the cultures maintained in 5 mM glucose (control) was set at 100% for the purpose of normalization. The absorbance of the corresponding samples derived from the paired cultures maintained in 30 mM glucose was expressed as percentage of the 100% control value. Statistical analysis was carried out with Student's *t* test for paired determinations.

Determination of adenylate cyclase activity

Adenylate cyclase activity was determined in a volume of 100 μl containing 10–12 μg of endothelial membrane protein, 40 mM Hepes/NaOH (pH 8.0), 1 mM EDTA, 4 mM MgCl₂, 0.5 mM $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, 5 mM phosphocreatine, 5 units of creatine kinase and 0.1 mM phosphodiesterase inhibitor rolipram (RO 20-1724;

Research Biochemicals International, Natick, MA, U.S.A.). Activating ligands were added at the concentrations indicated in the respective Figure legend. After 25 min at 25 °C, the reaction was quenched and the [32 P]cAMP formed was separated by sequential chromatography on Dowex 50W-X8 and neutral alumina [30].

Extraction and analysis of RNA

Total cellular RNA was purified by homogenization in guanidium isothiocyanate and phenol/chloroform/3-methylbutan-1-ol extraction. A 20 μ g portion of each RNA sample was electrophoresed on a 1%-agarose gel containing formaldehyde, transferred on to Hybond N nylon membranes by standard capillary-blotting techniques and hybridized to the 32 P-labelled probe, which was generated as follows. The 1.4 kDa *Bam*HI-*Eco*RI restriction fragment (nucleotides 2732–4097) of cDNA coding for eNOS, kindly given by Dr. Kenneth D. Bloch, Harvard Medical School, Massachusetts General Hospital, Boston, MA, U.S.A. [31], was labelled by employing the Megaprime DNA labelling system. Hybridization was performed at 65 °C overnight in a solution containing 6 $\times$ SSC (SSC = 0.15 M NaCl/0.015 M sodium citrate), 5 $\times$ Denhardt's solution and 100 mg/ml salmon sperm DNA and the labelled cDNA probe. The blots were washed with 2 $\times$ SSC and twice with 2 $\times$ SSC/0.1% SDS and twice with 0.1 $\times$ SSC for 15 min each at 65 °C. The 28 S rRNA oligonucleotide probe (Clontech Laboratories, Palo Alto, CA, U.S.A.) used as a control was labelled by the kinase reaction, and hybridization was carried out at 41 °C in 900 mM NaCl/180 mM Tris/HCl/12 mM EDTA/1 $\times$ Denhardt's solution containing 20 mg/l salmon sperm DNA, overnight, followed by five washing steps (20 min each) at room temperature with 2 $\times$ SSC/0.1% SDS. The membranes were exposed to Kodak XAR5-Omat films at -80 °C.

Measurement of NO synthase activity in endothelial-cell extracts

NO synthase activity was measured as described by Mayer et al. [32]. Cell extracts from HUVECs were prepared by sonication for 4 $\times$ 15 s in homogenization buffer containing 50 mM triethanolamine/HCl (pH 7.3), 0.5 mM EDTA, 10 mM 2-mercaptoethanol and 0.3 mM PMSF. Incubation was carried out in 50 mM triethanolamine/HCl containing 10 μ M L-arginine, 5 μ M FAD, 5 μ M FMN, 0.5 mM NADPH, 10 μ M (6R)-tetrahydrobiopterin, 10 μ g/ml calmodulin, 0.5 mM CaCl₂ and 60000–80000 c.p.m. of purified L-[2,3,4,5- 3 H]arginine.

The reaction was stopped by addition of 800 μ l of 20 mM sodium acetate, pH 5.0, containing 200 μ M EDTA and 1 mM L-citrulline. [3 H]Citrulline was quantified after separation from [3 H]arginine by the cation exchanger Dowex 50W. NO synthase activity is given as pmol of [3 H]citrulline formed/min per mg of protein.

Determination of intracellular cGMP levels

The method for determination of NO-triggered intracellular cGMP was modified from Schmidt et al. [33]. Briefly, paired confluent monolayers of HUVECs, grown in 12-well plates, were washed with 10 mM Hepes buffer, pH 7.4, containing 2.5 mM CaCl₂, 1 mM MgCl₂, 5 mM KCl and 145 mM NaCl. Monolayers were then incubated for 15 min with 1 mM 3-isobutyl-1-methylxanthine at 37 °C and then treated with 0.1 μ M A23187, 1 μ M bradykinin or 10 μ M histamine, or with vehicle, in a final volume of 800 μ l for 4 min. The reaction was stopped by removing the supernatant and adding 1 ml of 0.01 M HCl. Intracellular cGMP was extracted for 1 h at 4 °C and then quantified by

radioimmunoassay, with acetylation of the sample for maximal sensitivity. The detection limit was 20 pmol/l.

Data are reported as means $\pm$ S.D.

RESULTS AND DISCUSSION

Previous reports have indicated that *in vivo* diabetes mellitus is associated with an impaired function of both G_i and G_s [10–17]. We have used a well-defined model *in vitro*, in which a major cellular target for diabetes-induced alterations, namely endothelial cells, are continuously exposed to high levels of glucose.

Under the electrophoretic conditions employed, G_i α -2 migrates as a distinct band, whereas G_i α -1 and G_i α -3 co-migrate [22,34]. All subtypes are recognized by antiserum 84. A single immunoreactive band was detected in endothelial cell membranes, irrespective of whether cells had been cultured in the presence of 5 or 30 mM glucose. This band co-migrated with the

Figure 1 Effect of high glucose on G-protein expression

HUVEC membrane preparations (50 μ g) were separated by SDS/PAGE on 10% gels, blotted and probed with antibodies against G-protein α and β subunits. Key: 5, 5 mM glucose; 30, 30 mM glucose. (A) Antibody 84 recognizes all three subtypes of G_i α : G_i α -1, 30 ng of recombinant G_i α -1; G_i α -2, 30 ng of recombinant G_i α -2. (B, C) Antibody CS1 recognizes both splice variants of G_s α : G_s α S+L, 30 ng of recombinant G_s α S and G_s α L; G_i α -2, 30 ng of recombinant G_i α -2. The blot depicted in (B) was additionally probed with antibody 84; the extra band in (C) is a non-specific band resulting from staining with the ECL system. (D) Antibody CQ recognizes G_q α and G₁₁ α ; 50 μ g of human platelet membranes expressing high amounts of G_q protein was used as a standard. (E) Antibody A7 recognizes the β -subunit; β , 30 ng of recombinant β -subunit.

Figure 2 Effect of high glucose on adenylate cyclase activity

Adenylate cyclase (AC) activity in membranes prepared from HUVECs cultured in 5 mM (white symbols) or 30 mM glucose (black symbols). Each symbol pair represents one batch of HUVECs. Key: basal, AC activity in the absence of any stimulating agent; GTP γ S, AC activity in the presence of 10 nM GTP[S]; GTP γ S + ISO, AC activity in the presence of 10 nM GTP[S] and 50 μ M isoprenaline; Forsk., AC activity stimulated by 20 μ M forskolin.

purified G α -2 standard. Although modest decreases occurred in some cell batches maintained in the presence of 30 mM glucose (see Figure 1A), these were not consistently seen (e.g. G α -2 in Figure 1B), such that, in the six paired cultures, the levels of G α -2 after high-glucose incubation amounted to $98 \pm 4\%$ of those in control incubations, as assessed by densitometric analysis of the immunoblots. The levels of G β were also determined by using the two detection systems. The top band depicted in Figure 1(C) is a non-specific band resulting from staining with the ECL system (not seen in Figure 1B). The amounts of the long and short splice variants, G β -L and G β -S, were found to be comparable by densitometry of the paired endothelial membrane preparations [30 mM glucose samples containing $110 \pm 15\%$ and $101 \pm 11\%$ ($n = 6$) respectively of the amount in the corresponding 5 mM glucose control]. Similarly, the levels of G α and G α -1 were not altered by long-term incubation with high glucose levels [Figure 1D; 30 mM glucose samples containing $91 \pm 2\%$ ($n = 6$) of the amount in the corresponding 5 mM glucose control]. Upon activation, the heterotrimeric G-protein dissociates, and both the α -subunit as well as the $\beta\gamma$ -dimer may modulate their effector molecules. Screening for β -subunit expression was carried out with antiserum 7, which is directed against a sequence shared by all β -subtypes. As shown in Figure 1(E), the amount of β -subunit was virtually identical in both groups [30 mM glucose samples containing $97 \pm 5\%$ ($n = 6$) of the amount in the corresponding 5 mM glucose control].

The functional integrity of G β was tested by measuring the ability of G β to stimulate adenylate cyclase. Analysis of basal activity and activity stimulated by either the direct activator forskolin or the hydrolysis-resistant guanine nucleotide analogue GTP[S] was determined in paired membrane preparations from HUVECs derived from three batches of cells cultured in normal and high glucose (Figure 2). Although the absolute activities varied widely between the individual cell batches, we failed to detect any significant changes (basal, forskolin-stimulated and GTP[S]-stimulated activities of 30 mM-glucose-treated cells being $123 \pm 22\%$, $98 \pm 8\%$ and $116 \pm 29\%$ of the 5 mM glucose

control). The EC $_{50}$ values for GTP[S]-mediated activation of adenylate cyclase were comparable, being 23.7 ± 10 nM (5 mM glucose) and 49 ± 5 nM (30 mM glucose), (mean $\pm$ S.E.M., $n = 3$). Endothelial cells express β_2 -adrenergic receptors [35]. We failed to obtain any functional evidence for altered coupling between the receptor and G β , as the isoprenaline-mediated increment in adenylate cyclase activity was similar in membranes prepared from cells exposed to high glucose concentrations ($90 \pm 2\%$; Figure 2). Similarly, the EC $_{50}$ for isoprenaline was unchanged (40 ± 8 and 50 ± 10 nM for cells maintained in normal and high glucose, respectively).

In order to investigate whether functional changes in G β protein are induced by high glucose levels, the G β -dependent growth of endothelial cells was evaluated. Pertussis toxin ADP-ribosylates G β *in vivo* and progressively inhibits the proliferative response of endothelial cells to serum factors [6]. In endothelial cells subjected to long-term incubation in 30 mM glucose, as well as in control cells, pertussis toxin caused the same concentration-dependent inhibition of [3 H]thymidine incorporation, with a comparable EC $_{50}$ of ~ 3 ng/ml and a maximum decrease by 50% (Figure 3).

Taken together, these results clearly rule out that glucose itself is the systemic factor which induces alterations in the G-proteins G β and G β and the cellular signalling pathways which are controlled by these proteins in diabetes mellitus. No changes in the level of G-protein subunits (G α -2, G β -S, G β -L and G β), nor evidence for any functional impairment in the ability of G β to transduce signals from receptor to effector, was found, since the regulation of adenylate cyclase was unaffected by glucose pretreatment. The same holds true if the G β -dependent mitogenic response to serum factors is examined. We believe that this proliferation assay ought to be of particular functional relevance and sensitivity, since it determines a long-term effect of G β -dependent signalling. The inhibitory effect on cell proliferation of cholera toxin, which constitutively activates G β , and forskolin was also comparable in both groups (results not shown). Thus, although diabetes mellitus, as well as incubation of endothelial cells in high glucose, affects functional responses which are also under the control of G β and G β [1,2], we can rule out the possibility that these alterations can be linked to abnormalities in these G-proteins. The only G-protein subunit the expression of which was not investigated in our study is the γ -subunit. However, alterations of γ -subunits seem very unlikely, since β - and γ -subunits form stable, tightly associated dimers, and free γ -subunits do not exist. Hence, the levels of expression of the β - and the γ -subunit are expected to be co-regulated [36]. Furthermore, only very little, if any, difference has been found in the ability of the various $\beta\gamma$ combinations to activate adenylate cyclase, phospholipase C or K $^+$ channels [37–39].

In endothelial cells, receptor-activated EDRF/NO release represents an important effector system controlled by G β [8,9]. EDRF/NO not only relaxes the underlying smooth-muscle cell, but also protects the vascular intima from platelet aggregates [19]. Previous evidence suggests additional protective functions of NO in the vasculature, such as inhibition of leucocyte adherence and inhibition of smooth-muscle proliferation [18]. Thus EDRF/NO modulates cell–cell interactions, which are altered in diabetic angiopathy [1,2]. Most studies that have examined the role of NO in vascular responsiveness of diabetes mellitus have focused on the vasodilatory effect of NO; a decreased function of eNOS has been demonstrated in several diabetic animal models [40,41], as well as in diabetic patients [21]. Hence, it has been postulated that this decreased EDRF/NO-dependent relaxation is an important factor in the development of diabetic angiopathy [20]. However, the role of NO in the

Figure 3 Effect of high glucose on pertussis-toxin-mediated inhibition of cell proliferation

Concentration-dependent inhibition of DNA synthesis on serum-stimulated [3 H]thymidine incorporation by pertussis toxin in HUVECs cultured in 5 mM (white symbols) or 30 mM (black symbols) glucose. [3 H]thymidine incorporation in the presence of 5% fetal-calf serum was set at 100% for the purpose of normalization. Data are given as means $\pm$ S.E.M. ($n = 4$).

pathogenesis of diabetic angiopathy remains controversial, since in some diabetes models no alteration, or an increased release of NO, was observed [42,43].

Since regulation of mRNA levels has been found to be a control point in the regulation of eNOS [19], and hyperglycaemia has been shown to modify gene expression in endothelial cells [44], we determined whether eNOS mRNA levels were altered by maintaining cells in 30 mM glucose. Total RNA from ten paired HUVEC cultures was analysed by Northern blotting (Figure 4). No significant changes were detected by densitometric analysis of the RNA blots [30 mM glucose samples containing $112 \pm 8\%$ ($n = 10$, $P > 0.5$) of that in the corresponding 5 mM glucose control].

Figure 4 Effect of high-glucose incubation on eNOS mRNA expression

Total RNA was purified from ten paired cultures of HUVECs incubated in normal (5 mM) or high (30 mM) glucose for 14–20 days. A 20 μ g portion of total RNA was separated on a 1% agarose gel, blotted and probed with the 1.4 kDa *Bam*HI–*Eco*RI restriction fragment of eNOS cDNA. Northern blots from six paired HUVEC cultures are shown. Key: 5, 5 mM glucose; 30, 30 mM glucose.

Figure 5 Effect of high-glucose incubation on eNOS protein expression

(A) Whole cell lysates of confluent HUVECs after a 14–20 day period in normal or high glucose were separated by SDS/PAGE on a 7% gel, blotted and analysed with an antibody directed against a 20.4 kDa protein corresponding to amino acids 1030–1209 of human eNOS. One Western blot with three from the six paired HUVEC cultures is shown. (B) eNOS distribution in cytoplasmic and membrane fractions derived from HUVECs cultured in normal or high glucose. The distribution of one representative paired culture is shown. Key: St, standard; Cp, cytoplasmic fraction; M, membrane fraction; 5, 5 mM glucose; 30, 30 mM glucose.

Similarly, the level of eNOS protein in whole cell preparations was virtually identical in six paired cultures (Figure 5A), and only minor variations were found between the different cell batches [30 mM glucose samples containing $113 \pm 15\%$ ($n = 6$) of the amount in the corresponding 5 mM glucose control]. Co- and post-translational modifications are important determinants for the regulation of eNOS activity. Myristoylation is thought to promote the membrane association of eNOS. Under resting conditions, eNOS is almost entirely membrane-bound [45]; persistent receptor-induced activation leads to phosphorylation of the enzyme, which results in translocation of NO synthase from the membrane to the cytosol [46,47]. To address the question whether long-term incubation in high glucose affects the state of eNOS activation, and thus the subcellular distribution, we have compared the levels of the protein in membrane and cytosolic fractions of cells cultured in 30 mM versus 5 mM glucose. The majority of eNOS ($> 95\%$) was found in the membrane fraction, irrespective of the incubation conditions; in some paired cultures essentially no reactivity was detected in the cytoplasmic fraction, whereas in others a weak signal was seen. However, the pattern of distribution was identical in paired cultures (Figure 5B).

Similarly, long-term incubation in high glucose did not affect the basal activity of eNOS (8.5 ± 0.5 and 9.5 ± 1.0 pmol/min per mg in control and high-glucose-treated cells, respectively; $n = 6$; $P > 0.5$). Thus no glucose-induced changes in endothelial NO synthase were observed with respect to the amount of protein expression, the subcellular distribution and the activity of the enzyme.

NO synthase activity is stimulated by shear stress and in response to a variety of receptor ligands; the receptors for bradykinin, histamine and acetylcholine stimulate NO synthase via G_i and G_q proteins [8,9]. The NO generated activates the soluble form of guanylate cyclase in the vascular smooth-muscle cell, as well as in the endothelial cell itself. The rise in endothelial cGMP was measured, after phosphodiesterase inhibition with 3-isobutyl-1-methylxanthine, after stimulating HUVECs with bradykinin (acting via G_i and G_q), histamine (acting via G_q),

Table 1 Effect of high-glucose incubation on eNOS activity

Receptor- and A23187-stimulated accumulation of cGMP. Confluent HUVECs were stimulated with 0.1 μ M A23187, 1 μ M bradykinin, 10 μ M histamine or vehicle for 4 min. cGMP levels varied in the individual cell batches from 200 to 1000 fmol/sample (1×10^5 cells) and were set to 1 for the purpose of normalization. *Significantly different from 5 mM glucose [*t* test for unpaired data, means $\pm$ S.E.M. ($n = 4$), $P < 0.05$].

	cGMP (fold)	$\pm$ S.E.M.	<i>P</i>
Bradykinin, 5 mM glucose	1.40	0.07	
Bradykinin, 30 mM glucose	1.90	0.40	
A23187, 5 mM glucose	5.00	1.00	
A23187, 30 mM glucose	6.20	0.70	
Histamine, 5 mM glucose	6.20	0.70	
Histamine, 30 mM glucose	12.60	3.50	*

and A23187, which stimulates eNOS receptor-independently by increasing intracellular calcium concentrations. Table 1 summarizes the results obtained; the basal levels of cGMP varied between 200 and 1000 fmol/well (1×10^5 cells) in the different cell batches, but no significant differences were detected between cells maintained in normal and high glucose. For the purpose of normalization, all values were expressed as fold stimulation over the basal level. No significant differences were seen after stimulation with A23187. The stimulatory effect of bradykinin, a predominantly G_i -dependent response, was low, resulting in a 1.4- and 1.9-fold increase in cGMP levels in normal and high-glucose cultures respectively. In contrast, addition of histamine caused a marked elevation of intracellular cGMP, and this response was significantly increased in cells which had been subjected to high-glucose incubations (Table 1).

To summarize, our observations rule out the possibility that long-term exposure of endothelial cells alters the levels of NO synthase with respect to both the amount of protein as well as the basal enzyme activity. Similarly, glucose does not affect the subcellular distribution of NO synthase. We have also evaluated the regulation of NO production in intact cells by determining the increase in cGMP. The finding that the calcium ionophore A23187 leads to comparable increases in cGMP levels is in line with the observation that a long-term incubation in glucose does not alter endothelial NO synthase activity. It is a drawback of our work that the G_i -linked regulation of endothelial NO-production by bradykinin was barely measurable. We have also tested the cGMP response to α_2 -adrenergic receptors, which are exclusively coupled via G_i to eNOS [8]; however, the expression of α_2 -adrenergic receptors is lost if human umbilical cells are maintained in long-term cultures, as was observed for the bradykinin receptor (K. Schmidt, unpublished work). While we have no evidence for any effect of glucose on the G_i -dependent regulation of endothelial NO production, it is therefore difficult to draw any firm conclusions based solely on the modest bradykinin-induced increases in cGMP accumulation. Given the fact that the G_i -dependent proliferative response and the levels of G_i were unaltered by glucose, it is highly improbable that a high glucose concentration is associated with a selective impairment of the G_i -stimulated endothelial NO production. In contrast, we note that cGMP accumulation in response to histamine was significantly increased after high-glucose incubation. Histamine activates NO synthase via binding to H_1 -histamine receptors, which couple via G-proteins of the G_q group to the phospholipase C pathway [48]. This enzyme generates two second messengers, i.e. inositol trisphosphate, which elevates Ca^{2+} and thereby stimulates NO synthesis, and diacylglycerol,

which activates the typical and the novel isoforms of protein kinase C [49]. An increased Ca^{2+} -sensitivity of eNOS can be ruled out, since the responsiveness to the calcium ionophore A23187 was unaltered. Thus the glucose-induced alterations must occur either at the level of the interaction between H_1 -histamine receptor and G_q proteins or at the level of phospholipase C regulation. Our results clearly rule out the possibility that the alteration occurs at the level of $G_q\alpha/G_{11}\alpha$ expression, since comparable levels were present in paired membranes of endothelial cells maintained in 5 and 30 mM glucose (see Figure 1D).

Our results show that long-term incubation of endothelial cells in elevated glucose concentrations does not affect the expression of G-proteins in human endothelial cells. Any alterations in receptor-controlled signalling pathways must therefore occur upstream at the level of the receptors involved and/or at the level of downstream effectors. Examples include an increased receptor-mediated formation of inositol trisphosphate, which was described in endothelial cells after a 24 h incubation in high glucose [50], and, furthermore, hyperglycaemia results in increased protein kinase C activity in capillary endothelial cells [51].

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