

HUVECs from newborns with a strong family history of diabetes show diminished ROS synthesis in the presence of high glucose concentrations

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Abstract

Background A family history of type 2 diabetes mellitus (DM) increases the probability to develop DM and endothelial dysfunction. The probable mechanism involves augmented reactive oxygen species (ROS) synthesis. The aim of this study was to evaluate the synthesis of ROS in human umbilical vein endothelial cells (HUVECs) obtained from healthy newborns with (experimental) and without (control) a strong family history of type 2 DM, exposed to different glucose concentrations.

Methods HUVECs were exposed to various glucose concentrations for 24 and 48 h periods, before cell proliferation, mitochondrial activity, and mitochondrial membrane potential were determined. Intracellular ROS synthesis in the presence or absence of the mitochondrial uncoupler CCCP, cytochalasin B, or diphenyleneiodonium (DPI) was also evaluated.

Results As opposed to control HUVECs, we found that experimental HUVECs exposed to 30 mmol/L glucose showed a 50% decrease in cell proliferation, a 90% reduction in mitochondrial activity, and a statistically significant inhibition of ROS synthesis in the presence of CCCP or cytochalasin B; DPI had no effect.

Conclusions Our results suggest that mitochondria and NAD(P)H-oxidase from HUVECs obtained from healthy newborns with a family history of DM have an innate deficient response to high glucose concentrations. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords endothelium; glucose; type 2 diabetes mellitus; superoxide; reactive oxygen species; oxidative stress

Introduction

A family history of type 2 diabetes mellitus (DM) is associated with an impaired vascular function in healthy offspring and adult individuals, and with the development of DM in the adult life [1–4]. The development of macro- and microvascular disease in DM is the result of continuous oxidative stress on endothelial cells [5,6]. Enhanced serum markers of oxidative stress have been demonstrated in diabetic patients [7]. Also, an increase in reactive oxygen species (ROS) synthesis has been shown in mononuclear cells isolated from type 2 diabetic patients stimulated with phorbol myristate acetate [8] and in young people with type 1 DM [9,10]. Sank *et al.* [11], and Cester *et al.* [12], have shown that human umbilical vein endothelial cells (HUVECs) isolated from newborns with type 1 diabetic mothers were 20–40% less resistant to shear stress, took up glucose more slowly, showed alterations



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in the plasma membrane, and had a more active fluid phase endocytosis and an increase in mitochondrial area, compared to control HUVECs. An increase in L-arginine transport and nitric oxide synthesis as well as an impairment in the action of insulin due to hyperglycaemia has been shown in HUVECs isolated from newborns to women with gestational diabetes [13,14]. Although we have shown the relevance of family history on endothelial susceptibility, and probably in the development of vascular damage [15], it is still unknown whether familial diabetic background augments the susceptibility of the endothelial cells to high glucose concentrations.

Since the origin of ROS in endothelial cells stimulated with supraphysiological glucose concentrations has been related to NAD(P)H-oxidase, and mitochondrial activity [16,17], and recent findings suggest that superoxide generation in the mitochondria is the first step in a vicious cycle of oxidative stress in DM [18,19], we decided to evaluate (1) the role of glucose metabolism and (2) the mitochondrial involvement in ROS synthesis. To this purpose we performed experiments in the presence or not of a mitochondrial uncoupler, an inhibitor of NAD(P)H-oxidase, and a glucose transport inhibitor, in an endothelial susceptibility model.

Materials and methods

Isolation and culture of human umbilical vein endothelial cells (HUVECs)

Umbilical cords were obtained from six newborn offspring of young non-diabetic women (18–26-year-old) with a strong family history of type 2 DM (three or more first degree relatives with the disease) (experimental HUVECs) or from 10 pregnant women (20–25 years-old) without a family history of type 2 diabetes (control HUVECs). None of the mothers in either groups had gestational diabetes at the time of delivery (Table 1). All patients signed a written informed consent.

HUVECs were isolated from each individual cord using type II collagenase (0.2 mg/mL, GIBCO/BRL). Non-pooled cells were cultured in T-75 tissue culture flasks (NUNC) in M-199 medium, whose original formula contains 5 mmol/L glucose, supplemented with 20% Fetal Bovine Serum (FBS) (HyClone), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 37.5 µg/mL of endothelial cell growth factor (ROCHE), at 37°C in a humid atmosphere containing 7% CO₂ [15]. In some experiments the M-199 medium was complemented with additional glucose in order to reach a final glucose concentration of 15 or 30 mmol/L. Cells were used within passages 4–10 and were identified as endothelial by their morphology and by uniform positive endoglin (CD105) surface antigen (BD Pharmigen) staining.

Table 1. Characteristics of the Mexican mestizo pregnant women

	Diabetic background	Control	<i>p</i>
<i>n</i>	6	10	
Age (years)	22.8 ± 3.0	22.3 ± 1.4	N.S.
Weight (kg)	84.9 ± 7.0	64.1 ± 1.9	0.003
Height (m)	1.55 ± 0.06	1.58 ± 0.03	N.S.
BMI (kg/m ²)	35.3 ± 4.9	25.6 ± 1.0	0.02
Range BMI (kg/m ²)	(30–47)	(24–27)	
Fasting glucose (mmol/L)	4.7 ± 0.3	4.4 ± 0.2	N.S.
(Range mmol/L)	(4.1–5.5)	(4.1–4.7)	
HbA _{1c} (%)	5.5 ± 0.5	5.1 ± 0.4	N.S.
Pregnancies	1–2	1–2	
Births	1–2	1–2	
<i>Characteristics of the newborn infants</i>			
<i>n</i>	6	10	
Weight (kg)	3.15 ± 0.4	3.1 ± 0.23	
(Range kg)	(2.350–3.550)	(2.800–3.600)	
Height (m)	0.46 ± 0.02	0.44 ± 0.01	

Data represent the mean ± S.D. N.S., not statistically significant.

Cell proliferation and mitochondrial activity

HUVECs (5 × 10³ cells) were cultured for 24 h in 96-multiwell tissue culture plates with non-glucose supplemented M-199 culture medium. After this time, the culture medium was discarded and 100 µL of fresh 15 or 30 mmol/L glucose-complemented medium (without phenol red) was added to the cultures for a further 24 or 48 h. Plates were washed with Phosphate buffer saline (PBS) (PBS: 0.01 M NaH₂PO₄ • H₂O, 0.01 M NaHPO₄, 0.15 M NaCl, pH 7.2) and stained with 100 µL from 0.1% crystal violet solution (w/v) (Sigma) in 70% ethanol for 20 min, the plates were washed with deionized water, and air-dried. The bound dye was dissolved with 100 µL of a 10% acetic acid. Optical density was measured at 595 nm in a 96-multiwell-plate spectrophotometer (BIO-TEK Instruments, Inc. Highland Park, Winooski, VT, USA). Crystal violet stain allows the evaluation of cell viability as well as cellular proliferation in relation to the culture time [20,21].

Mitochondrial dehydrogenase activity was determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/mL) in sterile PBS [22]. HUVECs incubated 24 or 48 h with medium alone or supplemented with either 15 or 30 mmol/L of glucose were loaded with 20 µL of PBS-MTT for the last 4 h of the culture period. The MTT reduced end-product, formazan, was dissolved with 100 µL of 0.04 N-HCl in isopropyl alcohol, and the absorbance was recorded at 570 nm. In some experiments glucose transport was blocked with cytochalasin B (50 ng/mL Sigma) simultaneously with MTT load for the last 4 h of culture time.

Reactive oxygen species (ROS) determination

Intracellular ROS formation was determined using the fluorescent probe dichlorofluorescein diacetate bis(acetoxy-methyl) (DCFH-DA) (Molecular Probes, Eugene, OR,

USA). Endothelial cells (2×10^5 cells/well) were seeded in 6-multiwell tissue culture plates for 24 h. Culture medium was discarded and fresh 15 or 30 mmol/L glucose-supplemented culture medium (without phenol red) was added. The cultures were incubated for a further 24 or 48 h period. At the end, the cell cultures were treated with trypsin solution (0.05% trypsin/1 mmol EDTA), centrifuged at 153g for 6 min, resuspended in Hank's Balanced Salt Solution (HBSS: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.8 mmol/L, MgSO_4 0.81 mmol/L, KH_2PO_4 0.44 mmol/L, Na_2HPO_4 0.33 mmol/L, and glucose 5.5 mmol/L) and loaded with DCFH-DA 10 μM for 15 min [23]. ROS synthesis by HUVECs (1×10^4 cells) was analysed by flow cytometry (Becton Dickinson FacsCalibur instrument, San Jose, CA, USA). Positive controls consisted of HUVECs incubated with 200 μM H_2O_2 . Similar experiments were performed in the presence of the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 50 nM, Molecular Probes), the glucose transport inhibitor cytochalasin B or the NAD(P)H-oxidase inhibitor diphenyleneiodonium (DPI, 10 μM , Sigma).

Mitochondrial membrane potential

HUVECs (2×10^5 cells) were cultured on a 6-multiwell tissue culture plate with 15 or 30 mmol/L glucose-supplemented M-199 medium for 24 or 48 h; and in the end the cells were resuspended in 1 mL of HBSS, and loaded for 15 min with 50 nmol of 3,3'-dihexyloxacarbocyanine iodide (DiOC_6 (3)) (Molecular Probes), in order to determine mitochondrial membrane potential by flow cytometry in 1×10^4 cells [24].

Statistical analysis

Statistical analysis was performed with the SPSS v 11 software. Variance analysis, followed by Dunnett's test, was used. Results were shown as mean $\pm$ S.D. Statistical significance was considered as $p < 0.05$.

Results

Cell proliferation

Experimental HUVECs incubated with 15 or 30 mmol of glucose for 24 or 48 h showed a 40–50% decrease in cell proliferation ($n = 6$; Figure 1(A)). Contrary to experimental HUVECs, control cells showed a slight and non-statistically significant increase in cell proliferation after 24 h of culture time, which reached a 80 or 50% increase with 15 or 30 mmol/L glucose, respectively, after 48 h ($n = 10$; Figure 1(B))(* $p < 0.05$). We observed a spontaneous cluster formation in all the experimental endothelial cells (Figure 1(C)), exposed to 15 and 30 mmol/L glucose, in contrast to control HUVECs, which

showed a more homogeneous distribution (Figure 1(D)) and no cluster formation.

Mitochondrial activity

A slight decrease (20–25%) of MTT reduction was observed in experimental HUVECs after 24 h of incubation with 15 or 30 mmol glucose; interestingly, there was a 90% inhibition in MTT reduction after 48 h of culture with 15 or 30 mmol glucose ($n = 6$; Figure 2(A)). Control HUVECs ($n = 10$) cultured with supraphysiological glucose concentrations, showed a 90–100% increase in mitochondrial activity after 48 h of culture time (Figure 2(B))(* $p < 0.05$).

The excess of glucose in the diabetic state stimulates the mitochondrial activity, which in turn increases the synthesis of ROS [25]. Additionally, our results showed that formazan precipitate, which reflects mitochondrial activity, in our experimental or control endothelial cells incubated with culture medium alone was uniformly distributed around the cell nucleus, in accordance with the cellular distribution reported for endothelial cell mitochondria (data not shown) [26]. Mitochondrial activity evaluated by MTT reduction, in the presence of an inhibitor of glucose transport (cytochalasin B, 50 ng/mL), showed a 95% inhibition in the deposit of formazan precipitates in experimental and control HUVECs (data not shown). This effect was independent of the glucose concentration, time of incubation, or origin of HUVECs.

ROS determination

Figure 3(A) shows the ROS synthesis in negative (HUVECs without DCFH-DA) and positive controls (HUVECs incubated with H_2O_2 and DCFH-DA). Figure 3(B) shows the percentage of experimental HUVECs synthesizing ROS, incubated with the culture medium non-supplemented with glucose or supplemented with 15 or 30 mmol/L glucose; the percentage of ROS+ cells was not modified after 24 or 48 h of culture time. We observed that a mean of 98% of ROS+ cells ($n = 6$) were in the M1 region; a reduced percentage of ROS+ cells was found in the M2 region (<1%). An increase in the glucose concentration did not modify the percentage of ROS+ cells in either region (Figure 3(B)). In contrast, control HUVECs ($n = 10$) showed a bimodal response after incubation with the culture medium alone (M1 = $40.1 \pm 5.0\%$, M2 = $50.7 \pm 6.0\%$) (Figure 3(B)), but when the cells were exposed to 15 or 30 mmol/L glucose the percentage of cells in the M1 region diminished to $26.9 \pm 3.0\%$, and $30.9 \pm 4.0\%$, respectively, while the percentage of cells in the M2 region increased to $68.3 \pm 5.0\%$, and $62.8 \pm 7.0\%$, respectively (Figure 3(C)).

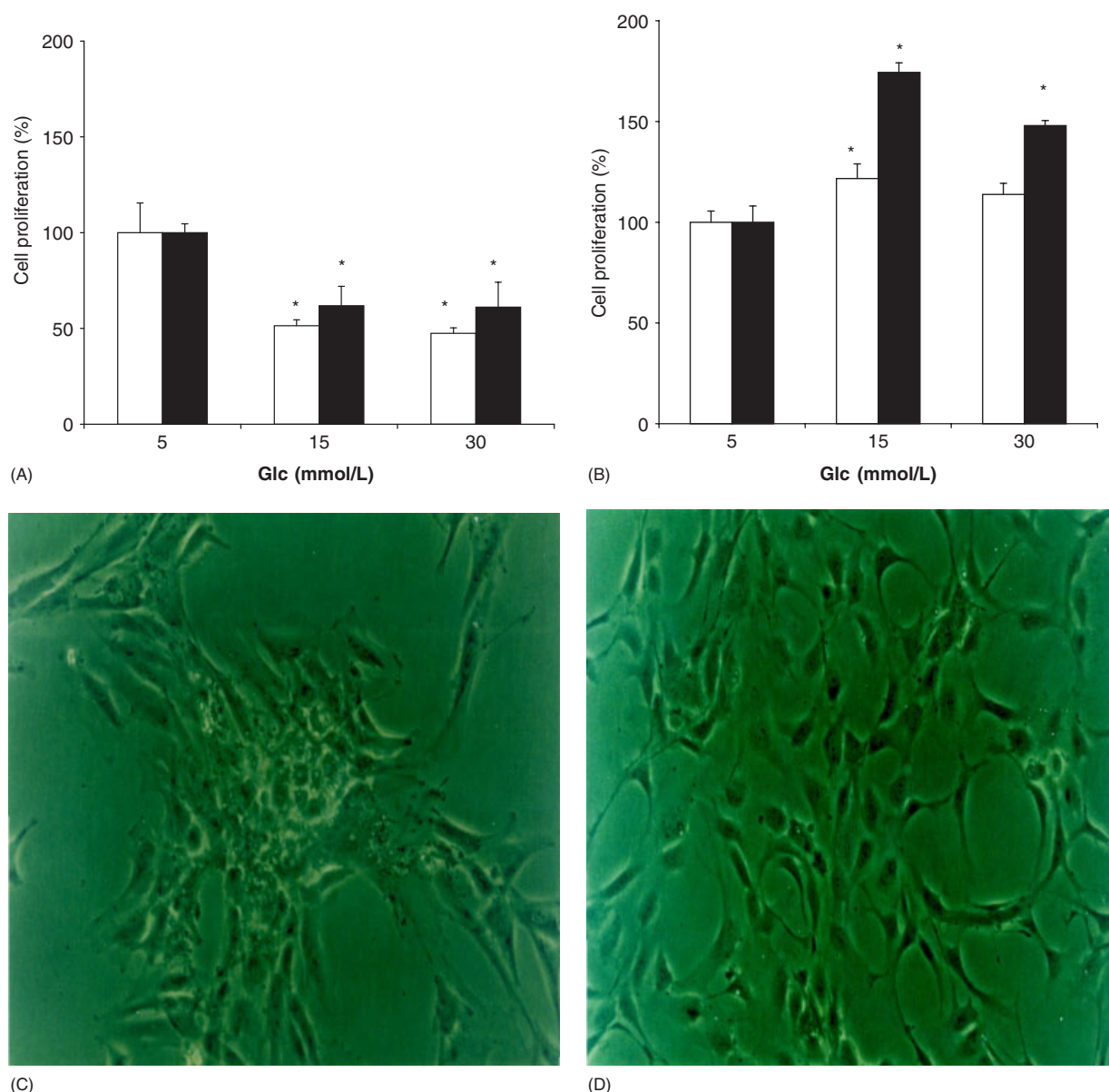


Figure 1. Cell proliferation in human umbilical vein endothelial cells (HUVECs) incubated with 5, 15 or 30 mmol/L of glucose for 24 (□) or 48 (■) h. (A) experimental and (B) control, HUVECs. (C) and (D) show the morphology of experimental and control HUVECs, respectively. Spontaneous cluster formation was observed in experimental HUVECs (C) with 15 or 30 mmol/L of glucose concentration. * $p < 0.05$ was considered statistically significant *versus* control cells

ROS synthesis in the presence of CCCP uncoupler

ROS synthesis in experimental HUVECs exposed to either glucose concentration was drastically reduced in the presence of the uncoupler (Figure 4(A)). Control HUVECs lost the bimodal pattern of ROS+ cells which we have shown in Figure 3(C), but maintained ROS synthesis, suggesting that ROS synthesis became homogeneous in all the control endothelial cells (Figure 4(B)). The synthesis of ROS in control HUVECs incubated with the uncoupler was not influenced by glucose concentration, although a minimum shift to the left was observed with 30 mmol glucose concentration (data not shown).

ROS synthesis in the presence of cytochalasin B or DPI

Cytochalasin B inhibited the synthesis of ROS in experimental HUVECs and this inhibition was not modified by glucose concentration. The mean percentage of ROS+ cells in the six experimental HUVECs which was $7.7 \pm 1.0\%$ in the M1 region of HUVECs exposed to non-glucose supplemented M-199 medium, diminished to 4.1 ± 1.5 and $4.2 \pm 1.0\%$ in cultures exposed to 15 or 30 mmol/L glucose concentrations, respectively. The percentage of ROS+ cells in the M2 region was not modified (Figure 5(A)). The bimodal response previously observed in control HUVECs was modified. The results obtained in control HUVECs ($n = 10$) showed an increase in the mean

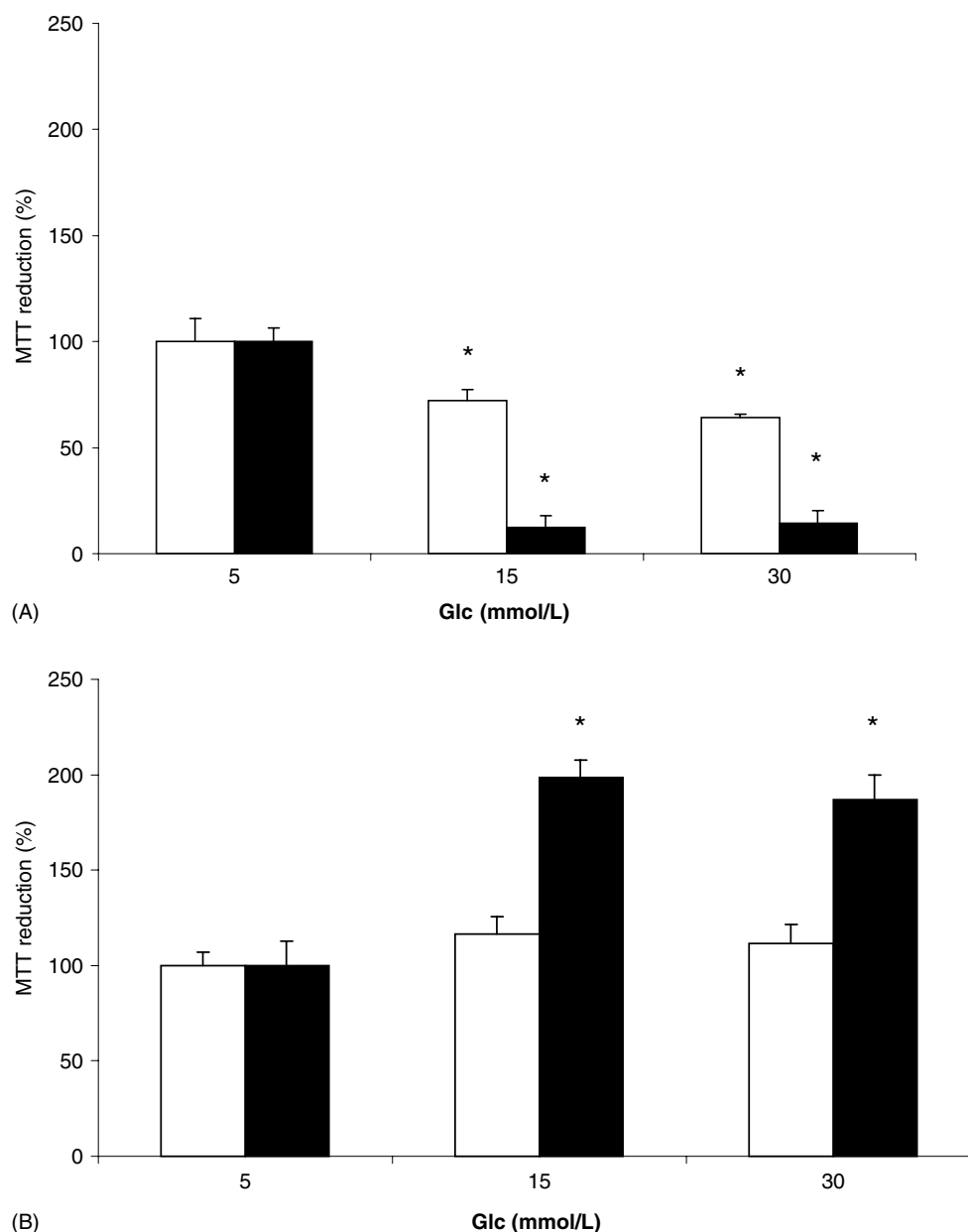


Figure 2. Mitochondrial activity determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction. HUVECs were incubated with 5, 15 or 30 mmol/L of glucose for 24 (□) or 48 (■) h. (A) Experimental, and (B) control, HUVECs. * $p < 0.05$ was considered statistically significant

percentage of cells in the M1 region and a reduction in the percentage of cells in the M2 region, independently of the glucose concentration (non-glucose supplemented, M1 = $88.1 \pm 7.0\%$, M2 = $10.1 \pm 2.0\%$; 15 mmol/L, M1 = $69.2 \pm 6.0\%$, M2 = $29.3 \pm 4.0\%$; 30 mmol/L, M1 = $69.1 \pm 4.0\%$, M2 = $28.9 \pm 3.0\%$) (Figure 5(B)).

The presence of DPI, an inhibitor of the NAD(P)H-oxidase, had a minor effect upon ROS synthesis in experimental HUVECs, independently of the glucose concentration (Figure 5(C)); these results were very similar to those obtained in the absence of DPI. In contrast, control HUVECs (Figure 5(D)) showed an increase in the mean percentage of cells in the M1 region, which was associated with a decrease in the mean percentage of cells in the M2 region, independently of the glucose

concentration (non-glucose supplemented medium, M1 = $63.5 \pm 4.0\%$, M2 = $34.9 \pm 5.0\%$; 15 mmol, M1 = $70.0 \pm 6.0\%$, M2 = $28.5 \pm 3.0\%$; 30 mmol, M1 = $65.6 \pm 6.0\%$, M2 = $32.9 \pm 3.0\%$).

Determination of mitochondrial membrane potential

Although some papers have shown that the loss of the mitochondrial membrane potential in endothelial cells incubated with high glucose is associated with apoptosis [27], we could not find significant changes in mitochondrial membrane potential under all the different experimental conditions that we used for both the experimental and control HUVECs (data not shown).

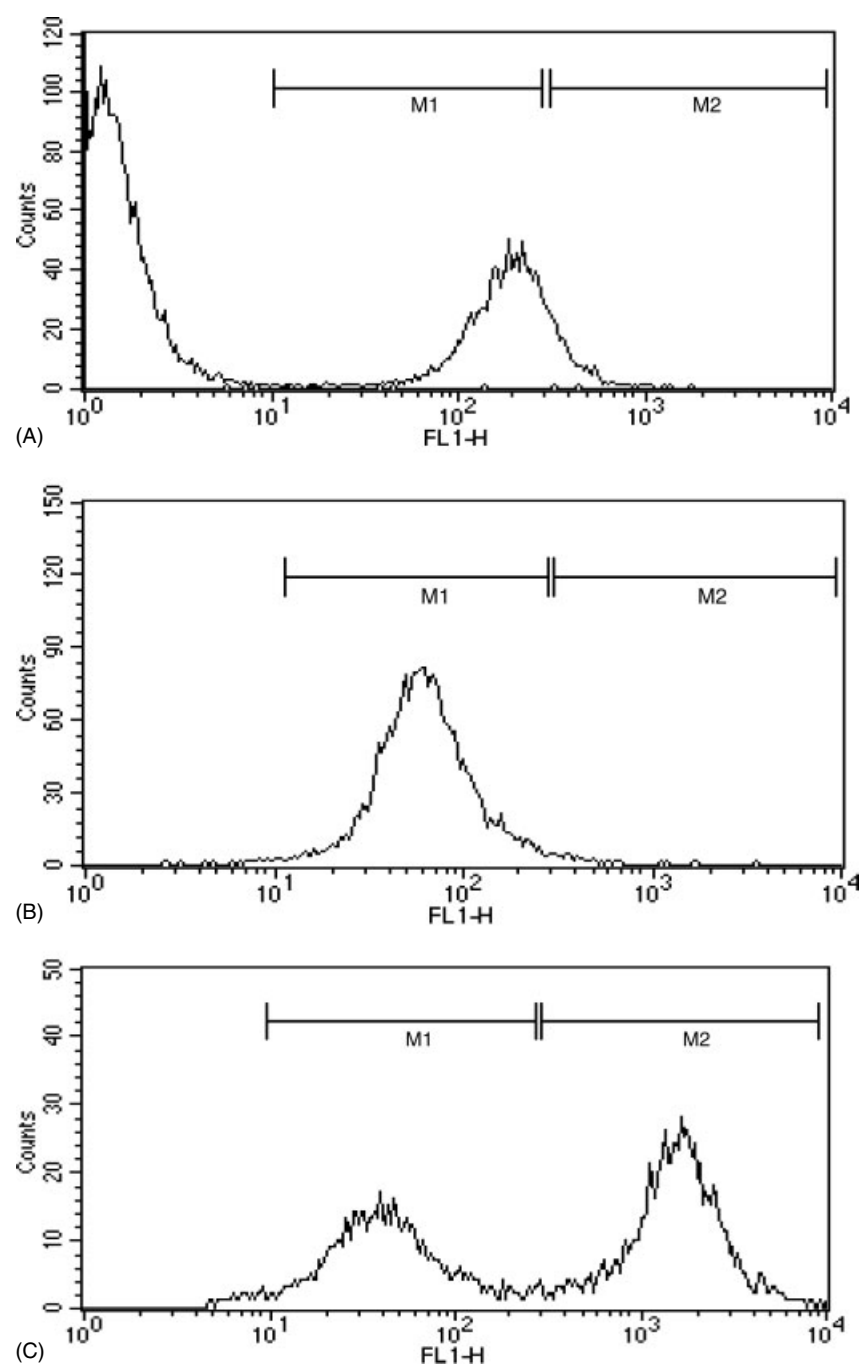


Figure 3. Reactive oxygen species (ROS) synthesis detected by DCFH-DA ($10 \mu\text{M}$). Experimental controls are shown in Figure (A) which illustrates HUVECs without and with DCFH-DA and $200 \mu\text{M}$ H_2O_2 . Experimental cells (B) showed a single cell population (M1 region) that produced ROS versus two cell populations (M1 and M2 regions) in control HUVECs (C). Although ROS synthesis was uniform in experimental HUVECs, in control HUVECs, there was a decrease in the percentage of ROS+ cells in the M1 region and an increase in the M2 region as the glucose concentration increased. The figure shows the results of a 48 h representative experiment

Discussion

A family history of type 2 DM has been recognized as an important risk factor for vascular dysfunction [18,28,29]. Impaired vascular function in healthy offspring of mothers with type 2 DM, has also been described [2,3]. The vascular abnormalities in DM range from endothelial dysfunction to low-grade or sub-clinical inflammation [30].

ROS which stimulate endothelial cell proliferation [31,32] are synthesized by the mitochondrial respiratory chain and vascular NAD(P)H-oxidases, among others [31], and depend on glucose uptake and metabolism [33]. We found an inhibition in cell proliferation and repressed ROS synthesis in experimental HUVECs exposed to high glucose concentrations. The actual status of the mitochondria in experimental HUVECs is unknown, but it has been shown that the defective energy homeostasis

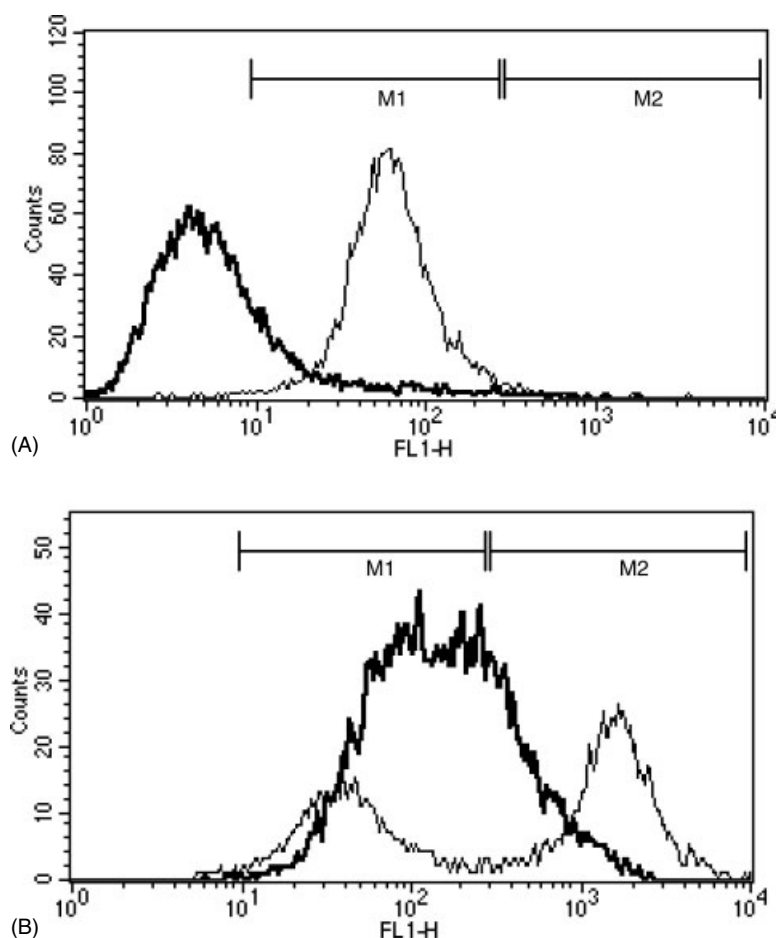


Figure 4. ROS synthesis in experimental (A) and control (B) HUVECs incubated with CCCP (50 nM) in the presence of 5, 15, and 30 mmol/L glucose. CCCP inhibited ROS synthesis in experimental HUVECs, whereas in control cells CCCP was associated with a slight inhibition of ROS+ cells. Bold line shows HUVECs with CCCP, whereas the thin line shows HUVECs without the uncoupler. The figure shows the results of a 48 h representative experiment

by mitochondria alone induces type 2 DM [34] and that high glucose concentrations affect the shape and Ca_2^+ homeostasis of mitochondria in endothelial cells [35]. Interestingly, endothelial cells with type 1 DM background have a deficient glucose uptake [11] and probably a low ROS production. In contrast to our results other authors mention an overproduction of ROS together with an inhibition in cell proliferation in endothelial cells without diabetic background exposed to high glucose concentrations for periods of time above 48 h that we evaluated [36,37]. The latter suggests that the inhibition in cell proliferation by non-diabetic background endothelial cells is not ROS-dependent, as opposed to our experimental HUVECs.

We found an inhibition in ROS synthesis in the presence of CCCP independently of the HUVECs background, but interestingly the inhibition was more severe in experimental HUVECs. CCCP stimulates oxygen consumption [38], but in hepatic mitochondrias from diabetic rats it decreases respiration [39]. Our results suggest that mitochondrias from HUVEC from newborns with DM background have a diminished cellular respiratory capacity thus corroborating the results of Nishikawa *et al.* and Peterside *et al.* results [39,40].

Contrary to the results by Recchioni *et al.* and Ido *et al.* [27,41] we did not find alterations in the mitochondrial membrane potential probably because we used shorter experimental times and HUVECs with a diabetic background (data not shown). NAD(P)H-oxidase activity has been linked with endothelial cell damage in DM [42]. We found, contrary to control HUVECs, that there was no inhibition of ROS synthesis in experimental HUVECs incubated with DPI (an inhibitor of NAD(P)H-oxidase), thus suggesting that the activity of NAD(P)H-oxidase is abolished or has low activity, as a consequence of an altered utilization of glucose.

HUVECs with diabetic background showed a spontaneous cluster formation in the presence of 15 or 30 mmol/L of glucose, whereas control HUVECs had no changes. Rellier *et al.* [43], have shown that the carbohydrate composition of the glycoconjugates that constitute the glycocalyx of microvascular cells, are modified in the presence of high glucose concentrations, affecting cell–cell interactions. Likewise, high glucose concentrations are known to induce changes in the morphology of endothelial cells, via the glucose-mediated reorganization of F-actin [44] and it also has been shown

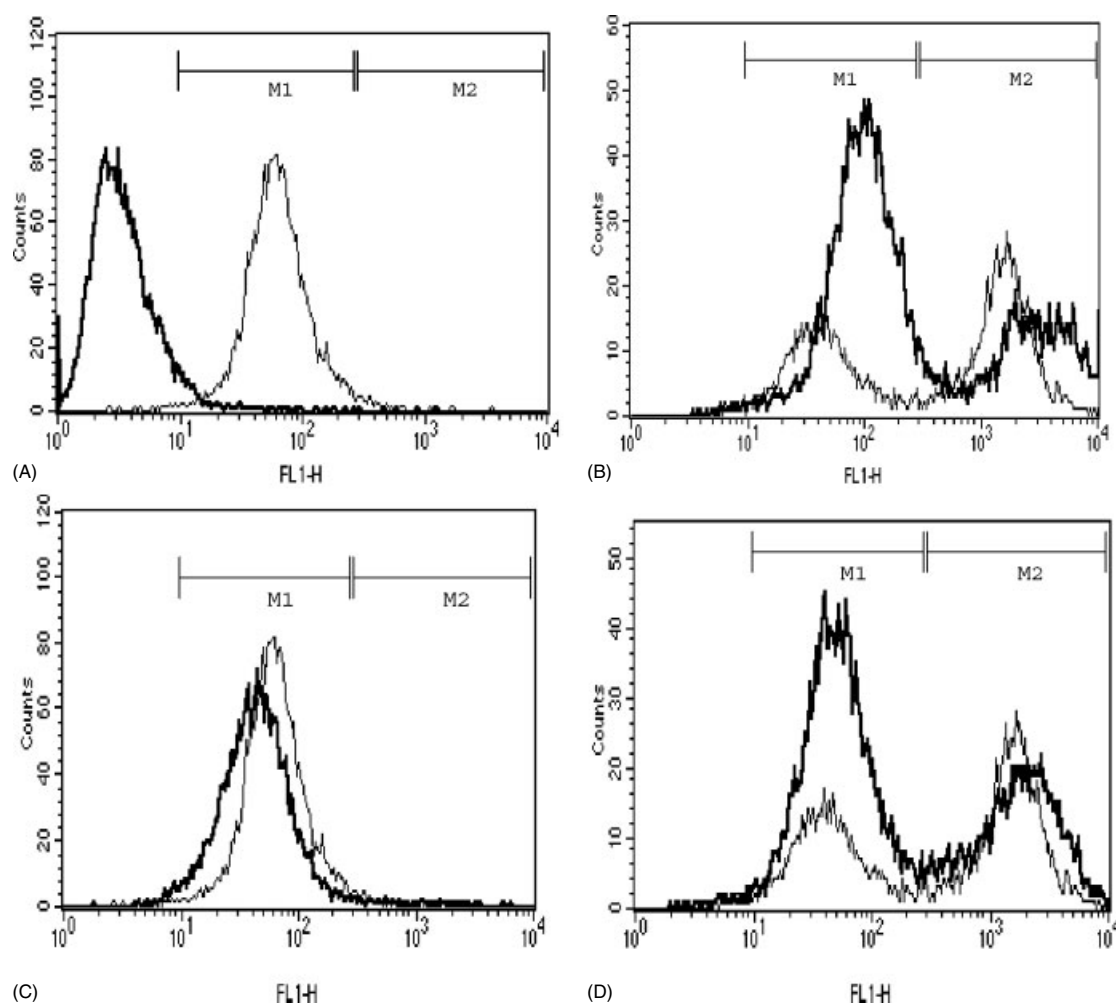


Figure 5. ROS synthesis in HUVECs incubated with cytochalasin (B) or DPI. Experimental HUVECs showed an inhibition in ROS synthesis (A), whereas the percentage of ROS+ cells in the M2 region of control HUVECs (B) showed a decrease in the presence of cytochalasin (B). In the presence of DPI, the percentage of ROS+ cells was slightly modified in the experimental HUVECs (C), whereas in control HUVECs (D) there was a diminution in the percentage of ROS+ cells in the M2 region and an increase in the M1 region. Bold line shows HUVECs with cytochalasin (B) or DPI, and the thin line shows HUVECs without cytochalasin (B) or DPI. The figure shows the results of a 48 h representative experiment

that the endothelial cell function may be modified by glycosylation pattern differences in the extracellular matrix of endothelial cells with type 1 DM background [11]. This endothelial damage might also be related to the greater retraction of fibrin clots, which promote the exposure of sub-endothelial layers and play a major role in thrombogenesis in HUVECs from infants with poorly controlled insulin dependent diabetic mothers [45].

We must emphasize that our results derive from individualized non-pooled HUVECs; the use of pooled HUVECs are known to produce important variations in some assays [46].

Endothelial damage in DM is mediated by ROS [36,47] although its source is controversial (activated leukocytes vs endothelial cells) [48]. Our results seem to confirm the endothelial cell as the most important ROS source and show that there is an increase in the susceptibility to develop metabolic alterations, in the presence of high glucose concentrations, in endothelial cells with a strong family diabetes background.

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