A Study on Human Umbilical Cord Endothelial Cells: Functional Modifications Induced by Plasma From Insulin-Dependent Diabetes Mellitus Patients

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The aim of the present study was to evaluate the action of plasma from insulin-dependent diabetic (IDDM) pregnant women on nitric oxide synthase (NOS) activity in cultured human umbilical vein endothelial cells (HUVECs). We also studied the effect of the plasma on cytosolic calcium and on Na^+/K^+-adenosine triphosphatase (ATPase) activity. Dynamic fluorescence studies of membrane fluidity were contemporarily performed to detect a direct effect of plasma on the endothelial cell membrane. We observed a significant increase in NOS activity, intracellular calcium, and Na^+/K^+-ATPase activity in cultured HUVECs exposed to IDDM plasma. Our dynamic fluorescence study showed a different microenvironmental organization of the cellular membrane after incubation with plasma from IDDM pregnant women, with a marked decrease in microheterogeneity as evaluated in terms of 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) lifetime distribution width. The present investigation suggests that plasma from IDDM pregnant women can cause a generalized disturbance in the function of endothelial cells cultured from healthy subjects. Such a modification might play a central role in the pathogenesis of the vascular complications of the disease.

IT IS WELL KNOWN that vascular complications are a major cause of morbidity and mortality in diabetes mellitus and that endothelial cell damage, which might play a key role in their development, occurs in diabetic patients.[1-3] Endothelial cells produce both vasodilator substances (eg, prostacyclin and endothelium-derived relaxing factor, nitric oxide [NO]) and the vasoconstrictor endothelin-1.[4] Alterations in endothelial function have been widely described in patients with diabetes mellitus, as suggested by the presence of elevated plasma endothelin and abnormal basal NO concentrations.[5,6] Moreover, an alteration of the plasma membrane accompanied by a more active fluid-phase endocytosis has been described in human umbilical vein endothelial cells (HUVECs) obtained from pregnant women with insulin-dependent diabetes mellitus (IDDM).[7]

Normal pregnancy is characterized by a substantial vasodilatation, with attenuated vasoconstrictor responses.[8] The endothelial production of both prostacyclin and NO has been described to be enhanced in normal pregnancy, and has been hypothesized to contribute to the attenuated angiotensin II vasoconstriction.[9] On the contrary, gestational diabetes has been associated with changes in fetal endothelial cells, resulting in membrane hyperpolarization, elevated basal NO production, and decreased prostacyclin release.[10]

The aim of the present study was to evaluate the action of plasma from IDDM pregnant women on NO synthase (NOS) activity in cultured HUVECs. We also studied the effect of the plasma on cytosolic calcium, which is known to modulate endothelial NOS activity,[11] and on Na^+/K^+-adenosine triphosphatase (ATPase) activity, which has been related to endothelial basal NO release in the rabbit aorta.[12] Dynamic fluorescence studies of membrane fluidity were contemporarily performed to detect a direct effect of plasma on the endothelial cell membrane.

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SUBJECTS AND METHODS

HUVECs were purchased from Mascia Brunelli (Milano, Italy) and cultured in endothelial cell basal growth medium (ECBGM) with the addition of low serum growth supplement. At 80% confluence (~800,000 cells/mL), cultured HUVECs were incubated at 37°C with ECGBM (control samples) or 20% (vol/vol) fresh plasma (healthy nonpregnant women, healthy pregnant women, or IDDM pregnant women) filtered with Millipore (Bedford, MA) Millex-GS filter units. After incubation, the monolayers were removed by scraping and resuspended in fresh culture medium or in Tris hydrochloride 100 mmol/L, pH 7.4, for the fluorescence experiments.

Plasma samples were obtained from 10 healthy nonpregnant women (age, 28 ± 4 years), 10 healthy pregnant women (29 ± 5 years), and 10 pregnant women with IDDM (27 ± 5 years; disease duration, 9 ± 5 years). Gestational age was similar in the two groups (40 ± 2 weeks for healthy pregnant women and 38 ± 2 weeks for IDDM pregnant women). All subjects with IDDM were in good metabolic control at the time of delivery (hemoglobin A_{1C}, 7.4% ± 0.6% for patients and 5.6% ± 0.7% for healthy pregnant women). The plasma glucose concentration was 4.1 ± 0.4 mmol/L in healthy nonpregnant women, 3.9 ± 0.4 mmol/L in healthy pregnant women, and 6.0 ± 1.3 mmol/L in IDDM pregnant women. IDDM women were treated with multiple regular insulin injections. The last injection was given at least 12 hours before blood sampling, so the plasma insulin levels were negligible. No patient was affected by microvascular complications of diabetes.

A former set of experiments were performed by incubating cultured HUVECs for 3 hours at 37°C with ECGBM or 20% (vol/vol) fresh plasma from five healthy nonpregnant women, five healthy pregnant women, and five IDDM pregnant women. A later set of time-course studies were performed by incubating HUVECs for 12 hours with ECGBM or fresh plasma from five healthy nonpregnant women, five healthy pregnant women, and five IDDM pregnant women and measuring the biochemical parameters at the following incubation times: 5 minutes, 3 hours, 6 hours, and 12 hours.

NOS Activity

NOS activity was quantified by measurement of the nitrite levels in supernates of sonicated HUVECs as described by Chen and Mehta.[13] The nitrite level in HUVECs was measured by the Griess reaction.[14] In brief, HUVECs were suspended in NO buffer (composition in mmol/L: HEPES 25, NaCl 140, KCl 5.4, $CaCl_2$ 1, and $MgCl_2$ 1, pH 7.4)

containing 1.44 mmol/L NADPH and incubated with buffer plus L-arginine (1 mmol/L) for 1 hour at 37°C The reaction was then stopped by freeze-thawing the sample, which was then sonicated. Each sample was incubated for 1 hour at 37°C after addition of nitrate reductase (20 mU), which reduces nitrate to nitrite. After centrifugation at 10,000 rpm for 15 minutes, the supernate was allowed to react with the Griess reagent (1% sulfanilamide/0.1% naphthylenediamine dihydrochloride/2.5% H_3PO_4). The chromophore absorption was read at 543 nm. The nitrite concentration was determined with sodium nitrite in water as the standard. The assay was normalized to protein determined in the sonicated samples by the method of Lowry et al.[15]

Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} was measured in intact HUVECs using the fluorescent probe Fura 2-AM as previously described.[16] Determinations were performed in a Perkin-Elmer (Norwalk, CT) MPF-66 spectrofluorometer at 37°C according to the method of Rao.[17] Fluorescence intensity was read at a constant emission wavelength (490 nm) with changes in the excitation wavelength (340 and 380 nm).

Na^+/K^+-ATPase Activity

Na^+/K^+-activated Mg^{2+}-dependent ATPase activity was determined on HUVECs by the method of Kitao and Hattori.[18] ATPase activity was measured by incubating HUVECs after sonication at 37°C in 1 mL medium containing $MgCl_2$ (5 mmol/L), NaCl (140 mmol/L), and KCl (14 mmol/L) in 40 mmol/L Tris hydrochloride, pH 7.7. The ATPase reaction was started by the addition of 3 mmol/L Na_2ATP and stopped 20 minutes later by the addition of 1 mL 15% trichloroacetic acid. Inorganic phosphate (P_i) hydrolyzed from reaction was measured as previously described.[19] Enzyme activity was expressed as the difference in P_i released in the presence and absence of 10 mmol/L ouabain. ATPase activity assayed in the presence of ouabain was subtracted from total Mg^{2+}-dependent ATPase activity to calculate the activity of ouabain-sensitive Na^+/K^+-ATPase. The results are expressed as micromoles of P_i per milligram of membrane protein per hour. The protein concentration was determined as already described for the NOS activity assay.

Fluorescence Studies

Fluorescence lifetime measurements were performed with a multifrequency phase fluorometer using 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) as a probe as previously described.[20,21] The instrument was equipped with an ISS (Urbana, IL) ADC interface for data collection and analysis; the excitation wavelength was set at 360 nm. Fluorescence was measured through a Corion (Santa Clara, CA) LG 370 S filter. The range of modulation frequencies used for TMA-DPH was 5 to 190 MHz. Data were accumulated at each modulation frequency until the standard deviations of the phase and modulation values were below 0.1° and 0.004°, respectively. The lifetime measurements were obtained using the 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP) lifetime value (1.35 ns) as a reference. The experimental data were analyzed by a model that assumes a continuous distribution of the lifetime values characterized by lorentzian shape centered at a time c and having a width w. For this analysis, the program minimizes the reduced chi-square defined by an equation reported elsewhere.[22,23] The program was provided by ISS. The temperature of the samples was maintained at 37°C with an external bath circulator (Haake F3, Berlin, Germany).

Statistical Analysis

Results are expressed as the mean ± SD. Statistical analyses were performed using ANOVA. To reduce the probability of significant differences arising by chance, Bonferroni correction was applied to the data following ANOVA. Differences were considered significant at a P level less than .05. Linear regression analysis was used to study within-group (healthy nonpregnant women, healthy pregnant women, and IDDM pregnant women) relations between the plasma glucose level and the change in endothelial cell properties after 3 hours of incubation.

Table 1. Effect of Incubation With Plasma From Healthy Nonpregnant Women, Healthy Pregnant Women, and Pregnant Women With IDDM on HUVEC NOS Activity (nmol/min/mg protein, mean ± SD)

	Incubation Time			
Group	5 min (n = 5)	3 h (n = 10)	6 h (n = 5)	12 h (n = 5)
Control	2.62 ± 0.33	2.50 ± 0.35	2.44 ± 0.39	2.43 ± 0.27
Nonpregnant	2.59 ± 0.35	2.74 ± 0.38	2.56 ± 0.31	2.60 ± 0.37
Pregnant	3.13 ± 0.29*	3.19 ± 0.28*	3.20 ± 0.31*	3.21 ± 0.29*
IDDM	4.25 ± 0.23*	4.37 ± 0.38*	4.28 ± 0.31*	4.34 ± 0.42*

*$P < .05$ *v* control.

RESULTS

Incubation with plasma from healthy nonpregnant women did not change NOS activity, reflected in nitrite formation in HUVEC supernates, at any incubation time. Plasma from healthy pregnant women caused a slight but significant increase in activity ($P < .05$). Incubation of HUVECs with plasma from IDDM pregnant women greatly enhanced NOS activity not only after 3, 6, and 12 hours but also after 5 minutes ($P < .05$) (Table 1).

In a similar way, intracellular calcium concentrations did not show any change after incubation with plasma from nonpregnant controls, whereas they significantly increased after incubation with plasma from healthy pregnant women and increased to an even greater extent with plasma from IDDM women ($P < .05$). Such an effect was detectable after 5 minutes' incubation and remained constant with time (Table 2).

Incubation with plasma from either nonpregnant controls or healthy pregnant women significantly increased Na^+/K^+-ATPase activity ($P < .05$). A more marked increase in activity was observed after the addition of IDDM plasma at every incubation time ($P < .05$) (Table 3).

The analysis of dynamic fluorescence showed a significant increase in the distribution width (w_1) of TMA-DPH fluorescence after incubation with plasma from healthy nonpregnant women compared with culture medium alone, whereas a significant decrease in w_1 was observed after incubation with

Table 2. Effect of Incubation With Plasma From Healthy Nonpregnant Women, Healthy Pregnant Women, and Pregnant Women With IDDM on HUVEC Intracellular Calcium Concentrations (nmol/L, mean ± SD)

	Incubation Time			
Group	5 min (n = 5)	3 h (n = 10)	6 h (n = 5)	12 h (n = 5)
Control	75 ± 9	78 ± 11	80 ± 12	81 ± 10
Nonpregnant	77 ± 11	80 ± 12	78 ± 8	82 ± 11
Pregnant	95 ± 9*	98 ± 10*	99 ± 10*	101 ± 11*
IDDM	107 ± 12*	112 ± 14*	116 ± 15*	120 ± 13*

*$P < .05$ *v* control.

Table 3. Effect of Incubation With Plasma From Healthy Nonpregnant Women, Healthy Pregnant Women, and Pregnant Women With IDDM on HUVEC Na^+/K^+-ATPase Activity (μmol P_i/mg protein/h, mean ± SD)

	Incubation Time			
Group	5 min (n = 5)	3 h (n = 10)	6 h (n = 5)	12 h (n = 5)
Control	2.52 ± 0.23	2.43 ± 0.31	2.31 ± 0.28	2.43 ± 0.34
Nonpregnant	2.91 ± 0.21*	2.93 ± 0.22*	2.94 ± 0.25*	2.94 ± 0.25*
Pregnant	3.02 ± 0.28*	3.09 ± 0.21*	3.11 ± 0.33*	3.05 ± 0.34*
IDDM	3.83 ± 0.43*	3.73 ± 0.35*	3.56 ± 0.45*	3.43 ± 0.34*

*$P < .05$ *v* control.

plasma from IDDM pregnant women ($P < .05$). Both effects were detectable after 5 minutes' incubation and remained constant with time (Table 4).

No difference was observed in the lifetime values after incubation with plasma from healthy nonpregnant, healthy pregnant, or IDDM pregnant women (Table 4).

The linear regression analysis did not find any significant correlation between plasma glucose levels and HUVEC NOS activity, intracellular calcium, Na^+/K^+-ATPase activity, w_1, and lifetime values of TMA-DPH after 3 hours' incubation of HUVECs with plasma from healthy nonpregnant, healthy pregnant, or IDDM pregnant women (data not shown).

DISCUSSION

Endothelial cells regulate hemostasis, platelet function, and vascular tone.[24] A disturbance in endothelial function has been described in diabetes mellitus and has been related to the increased frequency of cardiovascular disease.[25] In particular, recent studies described a modification in NO synthesis by endothelial cells in diabetes mellitus[26] related this alteration to a compromised endothelium-dependent relaxation in vascular segments from diabetic patients.[25] Previous studies reported that a 3-hour incubation with glucose caused modifications both in Na^+/K^+-ATPase activity and in basal NO synthesis in rabbit aortic rings.[12] In the present study, we observed that plasma from IDDM pregnant women significantly modified HUVEC plasma membrane organization, Na^+/K^+-ATPase activity, NOS activity, and intracellular calcium concentrations after just 5 minutes' incubation, and this effect was maintained for as long as 12 hours. Plasma membrane alterations can be caused by short periods of cell incubation with different substances,[27,28] whereas the short time is not sufficient to cause transcription-related changes in the cells. On the basis of these time-course studies, it might therefore be supposed that the modifications observed in the present experimental conditions were determined by an action of plasma on the cellular membrane.

Intracellular calcium concentrations were significantly increased in HUVECs after just 5 minutes' incubation with IDDM plasma. Such an increase might secondarily affect NOS activity, as the endothelial cell constitutive NOS is dependent on cytosolic calcium levels.[11] The increase in cytosolic calcium might be caused by a modified transport across the plasma membrane or by a higher release from intracellular stores.[29] The present data support the former hypothesis, ie, the action of diabetic plasma on intracellular calcium is mediated by a modification of the endothelial cell plasma membrane, with a secondary alteration in transmembrane ion transport. In fact, our dynamic fluorescence study showed a different microenvironmental organization of the cellular membrane after incubation with plasma from IDDM pregnant women, with a marked decrease in microheterogeneity evaluated in terms of TMA-DPH lifetime distribution width. This sustained alteration in membrane organization is consistent with a modified conformation of plasma membrane calcium channels, as the membrane modification showed the same time-course pattern as the alterations in cytosolic calcium levels.

The modification of the membrane physicochemical properties might cause an alteration not only in calcium transport but also in the active sodium transport, as Na^+/K^+-ATPase is greatly dependent on its lipid microenvironment.[30] The activation of Na^+/K^+-ATPase activity of the HUVEC plasma membrane caused by IDDM plasma confirms previous data on erythrocyte membranes incubated with plasma from IDDM patients.[31]

The increase in NOS activity observed in the present study in endothelial cells exposed to IDDM plasma is in agreement with previous observations by Sobrevia et al[10] of an enhanced rate of L-arginine transport accompanied by an increased production of NO in HUVECs obtained from women with gestational diabetes. NO can be rapidly inactivated via quenching by oxygen-derived free radicals, producing the more cytotoxic peroxynitrite.[32] This phenomenon might be enhanced during diabetes mellitus, as hyperglycemia causes an increased generation of oxygen radicals.[33] Peroxynitrite is a strong oxidant that might initiate lipid peroxidation, playing a role in endothelial dysfunction in diabetes mellitus.[34]

NO has been recently identified as a relevant factor in the regulation of Na^+/K^+-ATPase activity in the rabbit aorta, playing a role in its activation by agonists.[12] It might therefore be suggested that the activation of the sodium pump during incubation with IDDM plasma is dependent not only on the modification of the membrane microenvironment but also on increased NO formation.

The stimulatory effect of plasma from healthy pregnant women on endothelial NOS activity might be involved in

Table 4. Lorentzian Distribution Analysis of TMA-DPH Fluorescence Decay in Cultured HUVEC Plasma Membranes After Incubation With Plasma From Healthy Nonpregnant Women, Healthy Pregnant Women, and Pregnant Women With IDDM

	5 min (n = 5)		3 h (n = 10)		6 h (n = 5)		12 h (n = 5)	
Group	c_1	w_1	c_1	w_1	c_1	w_1	c_1	w_1
Control	4.31 ± 0.24	0.58 ± 0.08	4.21 ± 0.23	0.61 ± 0.10	4.25 ± 0.19	0.62 ± 0.09	4.26 ± 0.18	0.64 ± 0.10
Nonpregnant	4.39 ± 0.31	0.80 ± 0.07*	4.41 ± 0.30	0.90 ± 0.11*	4.32 ± 0.28	0.91 ± 0.08*	4.38 ± 0.17	0.93 ± 0.09*
Pregnant	4.46 ± 0.33	0.65 ± 0.10	4.30 ± 0.28	0.66 ± 0.12	4.39 ± 0.25	0.66 ± 0.09	4.41 ± 0.22	0.68 ± 0.09
IDDM	4.03 ± 0.20	0.21 ± 0.09*	3.99 ± 0.20	0.11 ± 0.04*	4.11 ± 0.18	0.09 ± 0.03*	4.12 ± 0.20	0.10 ± 0.03*

NOTE. c_1 = center and w_1 = width of Lorentzian distribution analysis, in nanoseconds (mean ± SD).
*$P < .05$ *v* control.

pregnancy-associated vascular refractoriness. As already suggested for IDDM plasma, a relation might exist between intracellular calcium concentrations and NOS activity, as the higher intracellular calcium might secondarily stimulate the enzymatic activity.

Multiple mechanisms might be the basis of the modifications in HUVEC properties caused by IDDM pregnant plasma: hyperglycemia, quantitative and/or qualitative modifications in plasma lipids, and hormonal changes related to diabetic pregnancy. The lack of statistical correlation between the plasma glucose concentration in IDDM women and the modifications induced in HUVECs suggests that the acute exposure to high glycemic levels might not be the direct cause of such modifications. However, further studies are necessary to clarify the molecular mechanisms involved in this action of IDDM pregnant plasma, and to verify whether it is specifically related to the pregnant state or is present also in IDDM nonpregnant patients.

In conclusion, the present study suggests that plasma from IDDM pregnant women can cause a generalized disturbance in the function of endothelial cells cultured from healthy subjects. Such a modification might play a central role in the pathogenesis of vascular complications of the disease.

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