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Originals

Influence of glucose, insulin and sera from diabetic patients on the prostacyclin synthesis in vitro in cultured human endothelial cells

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Summary. The effects of glucose, insulin and sera from Type 1 (insulin-dependent) diabetic patients on the synthesis of prostacyclin in vitro were studied in confluent primary cultures of human endothelial cells. The stable metabolite, 6-keto-prostaglandin $F_{1\alpha}$, was measured in growth medium after 24 h of incubation with endothelial cells in a buffer incubated with the cells for 10 min on a rocker platform, and in a buffer solution of ruptured cells. Glucose (11, 15, 20 or 25 mmol/l) and glucose (11 mmol/l) plus insulin (10^3, 10^4 or 10^6 mU/l) in growth medium did not have any effects on the prostacyclin synthesis. The prostacyclin synthesis was significantly reduced in cell cultures incubated with medium supplemented with 10% serum from patients with Type 1 diabetes ($p < 0.01$) compared with cultures incubated with pooled serum from healthy blood donors. These data suggest that diabetic sera inhibit the prostacyclin synthesis in cultured endothelial cells unrelated to the glucose and insulin levels.

Key words: Diabetes mellitus (Type 1), endothelial cells, glucose, insulin, prostacyclin.

Microangiopathy and vaso-occlusive diseases are well recognized complications of diabetes mellitus. Vascular endothelium generate prostacyclin (PGI_2), which is a vasodilator and a potent inhibitor of platelet aggregation [1]. Blood vessels from Type 1 (insulin-dependent) diabetic patients and animals have been shown to produce less PGI_2 in vitro than vessels from normal subjects [2-4]. It has been suggested that platelet aggregation contributes to the development of macro- and microangiopathy in diabetes [5, 6].

The present study was performed in order to test the impact of the main metabolic variables in diabetes mellitus on the synthesis of PGI_2, measured as the stable metabolite 6-keto-prostaglandin $F_{1\alpha}$, (6-keto-$PGF_{1\alpha}$), in primary confluent cultures of human endothelial cells.

Materials and methods

Cell culture

Endothelial cell monolayer cultures (ECMC) were obtained by preparing cells from human umbilical veins according to the modification of Czervionke et al. [7] of a method described by Jaffe et al. [8]. The cells were cultured in plastic dishes (diameter 35 mm, Falcon Plastics, Oxnard, Calif., USA), in modified medium 199 (MM-199, Gibco Bio-Cult Ltd, Paisley, Scotland) containing glucose (11 mmol/l) and supplemented with 20% heat-inactivated fetal calf serum [8]. Confluent primary cultures of endothelial cells were obtained after 4-6 days.

Experimental conditions

Confluent ECMC were incubated in MM-199 for 24 h at 37 °C with addition of: (1) Glucose to final concentration of 15, 20 or 25 mmol/l and supplemented with 10% fetal calf serum. Control cultures were incubated with addition of urea to isomolar concentrations. (2) Insulin (Actrapid Human, Novo Industries, Copenhagen, Denmark) to final concentration of 10^3, 10^4 or 10^6 mU/l, and supplemented with 10% fetal calf serum. (3) Serum 10% from diabetic patients, and with pooled serum from six male and six female blood donors aged 20-40 years as controls. Blood samples were collected in the morning from 12 fasting patients with Type 1 diabetes, five females and seven males aged 23-64 years (mean 38.6 years) (Table 1). The sera were prepared immediately after collection by centrifugation and kept frozen at −20 °C until used in the cell cultures. The sera from diabetic patients had a mean ± SD glucose concentration of 11.8 ± 5.0 mmol/l, triglycerides 2.3 ± 1.8 mmol/l and cholesterol 6.4 ± 2.1 mmol/l. The respective values for the control sera were glucose 4.9 ± 0.4 mmol/l, triglycerides 1.3 ± 0.4 mmol/l and cholesterol 5.9 ± 0.7 mmol/l. Control cultures in medium containing 10% fetal calf serum were included in each series of experiments. ECMC were also incubated with 2 ml 100% sera from diabetic patients or controls for 2 h at 37 °C.

At the end of the incubation periods (24 or 2 h) cell morphology was assessed microscopically (Diavert, Leitz, FRG). After washing the ECMC twice with 1.5 ml of a Hepes/NaCl solution (153 mmol/l, pH 7.4), the cells were incubated with 2 ml of the same buffer on a rocking platform (Bellco Glass Inc., Vineland, NY, USA) at a rate of 10 cycles/min at 22 °C for 10 min. The buffer was pipetted off and the cells were finally disrupted by freezing and thawing three times and dissolved in 1.5 ml Hepes/NaCl solution. All test samples were frozen at −80 °C without addition of cyclooxygenase inhibitors until analysis.

Table 1. Clinical data and serum levels of glucose, cholesterol and triglycerides in the diabetic patients

Patient number	Age (years)	Sex	Duration of diabetes (years)	Complications	Insulin dose (U/day)	Glucose (mmol/l)	Cholesterol (mmol/l)	Triglycerides (mmol/l)
1	23	F	11	None	32	19.9	6.1	0.8
2	36	F	24	Retinopathy	40	9.9	10.8	2.2
3	36	F	11	None	36	8.0	5.2	1.3
4	24	M	6	None	60	13.3	4.6	0.8
5	61	M	5	None	24	7.8	5.9	3.0
6	64	F	14	None	24	14.5	8.9	1.7
7	28	M	23	None	48	10.1	4.6	2.1
8	38	M	18	Retinopathy Nephropathy	32	3.2	9.1	7.4
9	32	M	10	None	68	21.0	5.4	2.6
10	45	F	1	None	24	11.3	4.9	1.3
11	46	M	6	Retinopathy	32	9.2	6.3	2.9
12	30	M	5	None	44	13.1	4.5	1.0

Table 2. The recovery of 6-keto-$PGF_{1\alpha}$ in growth medium supplemented with 10% serum from fetal calves, human controls or diabetic patients, after 24 h incubation at 37 °C ($n=3$)

Initial concentration of 6-keto-$PGF_{1\alpha}$ (ng/ml)	6-keto-$PGF_{1\alpha}$ (ng/ml) measured in:			
	Medium	Medium + fetal calf serum	Medium + control serum	Medium + serum from diabetic patients
12.5	12.7±0.4	11.1±0.1	9.4±1.0	9.8±0.4
25	29.4±1.5	28.1±1.8	29.1±1.4	27.8±0.8
50	59.2±0.8	50.8±2.4	57.1±1.1	54.8±2.1

Results are expressed as mean±SD

Table 3. The effect of glucose in the growth medium on the production of 6-keto-$PGF_{1\alpha}$ in endothelial cell cultures (ECMC) during 24 h of incubation[a,b]

Agent	No. of experiments	6-keto-$PGF_{1\alpha}$ (ng/ml) measured in:		
		Medium	Buffer incubated with ECMC	Ruptured cells
Glucose (11 mmol/l)	8	18.3±7.7	14.0±8.2	24.1±9.9
Glucose (15 mmol/l)	10	13.6±3.8	13.4±6.1	22.1±7.6
Control	10	14.0±3.8	14.3±6.0	22.0±7.2
Glucose (20 mmol/l)	4	10.9±0.4	12.9±7.6	21.8±9.4
Control	4	11.4±2.8	13.3±8.3	23.3±6.7
Glucose (25 mmol/l)	8	15.2±4.7	14.7±5.6	21.0±6.3
Control	8	15.8±8.0	14.7±6.0	20.4±6.9

[a] Results are expressed as mean±SD; [b] The control cultures were incubated with medium added urea to obtain isomolality

Table 4. Effect of insulin on the production of 6-keto-$PFG_{1\alpha}$ in endothelial cell cultures (ECMC) after 24 h of incubation with growth medium containing glucose (11 mmol/l) and 10% heat-inactivated fetal calf serum

Insulin (mU/l)	No. of experiments	6-keto-$PGF_{1\alpha}$ (ng/ml) measured in:		
		Medium	Buffer incubated with ECMC	Ruptured cells
0	10	15.9±8.0	14.5±7.8	23.9±13.4
10^3	10	12.8±4.8	14.7±6.1	25.3±9.0
10^4	4	20.1±8.1	17.0±9.8	34.9±5.0
10^6	12	11.1±5.1	15.6±6.9	26.0±12.9

Results are expressed as mean±SD

Table 5. Influence of 10% human sera from controls and diabetic patients on the production of 6-keto-$PGF_{1\alpha}$ in endothelial cell cultures (ECMC) during 24 h of incubation

Serum from	No. of experiments	6-keto-$PGF_{1\alpha}$ (ng/ml) measured in:		
		Medium	Buffer incubated with ECMC	Ruptured cells
Controls	8	19.3±7.6	12.2±5.3	15.8±4.3
Diabetic patients	12	12.7±4.1[a]	11.9±3.8	14.4±3.4

Results are expressed as mean±SD. Significance of differences compared to control human sera; [a] $p<0.001$

Analyses

6-keto-$PGF_{1\alpha}$ was determined by radioimmunoassay as described by Salmon [9] with one modification: the incubation time with dextran-coated charcoal was 10 min. Antibody against 6-keto-$PGF_{1\alpha}$ and the 6-keto-$PGF_{1\alpha}$ standards were kindly donated by Dr. J.A. Salmon, Wellcome Research Laboratories, Beckenham, Kent, UK. ^{3}H-6-keto-$PGF_{1\alpha}$ was purchased from New England Nuclear, Boston, Mass., USA. The specificity and sensitivity (detection limit 50–100 pg/ml) of the method has recently been described [10]. The PGI_2 synthesis from ECMC was estimated by measuring 6-keto-$PGF_{1\alpha}$ in the growth medium, the stimulated cellular release after 10 min of rocking and in the buffer solution of disrupted cells. 6-keto-$PGF_{1\alpha}$ was also measured in the serum from diabetic patients and controls. In preliminary experiments, 6-keto-$PGF_{1\alpha}$ (Sigma Chemical Co., St. Louis, USA) was incubated in final concentrations of 12.5, 25 and 50 ng/ml in MM-199 without and with supplement of 10% fetal calf serum, 10% control human serum or 10% serum from diabetic patients for 24 h at 37 °C. At the end of the incubation period no significant differences in the recovery between the different incubation media were observed (Table 2).

Serum glucose was measured by the hexokinase method ("Gluco Quant", Boehringer Mannheim, Mannheim, FRG). The coefficient of variation was 3.3%. Serum cholesterol was measured according to the method of Trinder [11]. The coefficient of variation was 1.4%. Serum triglycerides were measured by the fully enzymatic kit from Boehringer Mannheim (combined lipase and glycerolkinase reaction). The coefficient of variation was 6.4%.

Statistics

Two-tailed Student's t-test for paired and unpaired data was used for the statistical evaluation of the results. The results were expressed as mean ± SD.

Results

The in vitro synthesis of 6-keto-$PGF_{1\alpha}$ in ECMC was not influenced by varying the glucose concentrations in medium (MM-199) from 11 to 25 mmol/l or by increasing osmolality in the medium by addition of urea (Table 3). Incubations of ECMC with medium containing glucose (11 mmol/l) and insulin in final concentrations of 10^3, 10^4 or 10^6 mU/l did not have any significant effects on the synthesis of 6-keto-$PGF_{1\alpha}$ (Table 4). Incubation of ECMC with 100% serum from patients or controls for 2 h or with medium supplemented with 10% serum from diabetic patients for 24 h did not change the cell morphology or density.

The 6-keto-$PGF_{1\alpha}$ production from ECMC after 24 h of incubation with growth medium containing 10% serum from each of the blood donors was similar whether the serum was prepared from males or females. The sera were therefore pooled and used as controls. Incubation of the cells for 24 h with medium containing 10% serum from diabetic patients gave a significantly lower medium concentration of 6-keto-$PGF_{1\alpha}$ than incubations with pooled control serum ($p < 0.01$) (Table 5). Detectable amounts of 6-keto-$PGF_{1\alpha}$ (> 0.05 ng/ml) were not found in the sera from diabetic patients or in the control sera before incubation with endothelial cells.

The release of 6-keto-$PGF_{1\alpha}$ from ECMC was not influenced by incubation for 2 h with 100% serum from diabetic patients (6.9 ± 6.0 ng/ml) compared with control sera (5.0 ± 4.3 ng/ml).

There was no correlation between the reduction in 6-keto-$PGF_{1\alpha}$ synthesis in endothelial cells incubated with serum from diabetic patients and the degree of hyperglycaemia or the concentration of serum total cholesterol and triglycerides. Furthermore, no correlation could be established between the reduction in 6-keto-$PGF_{1\alpha}$ production and the duration of diabetes, the occurrence of complications or the dosage of insulin used in the individual patient.

Discussion

The present study demonstrated that serum from diabetic patients contained one or more substances which inhibited the synthesis of PGI_2 (measured as the stable metabolite 6-keto-$PGF_{1\alpha}$) in cultured human endothelial cells. This is quite in accordance with a previous report by Paton et al. [12] about diminished synthesis of PGI_2 by endothelial cells cultured in medium containing serum from diabetic patients with proliferative retinopathy. We did not, however, find any effect on the in vitro prostacyclin synthesis from ECMC either by increasing the glucose or the insulin concentrations in the growth medium. It should be stressed that we only used glucose concentrations in the hyperglycaemic range (11–25 mmol/l). A recent study [13] has, however, shown that a glucose concentration ranging from 5.6 to 22.4 mmol/l did not modify PGI_2 production in cultured human endothelial cells. Our results seem to be in conflict with the study of Jeremy et al. [14], which demonstrated a stimulatory effect on the synthesis of PGI_2 in vitro from rat aortic rings incubated with glucose at concentrations between 10 and 35 mmol/l, while insulin inhibited the PGI_2 synthesis at concentrations 10 and 50 mU/l by 10% and 25%, respectively. Species differences, shorter incubation period and use of much lower concentrations of insulin without glucose supplement to the medium may explain the differences. When they tested the insulin effect in the presence of glucose (10 mmol/l) a stimulatory effect of insulin (10 mU/l) was found. An inhibitory effect of insulin in concentrations of 2.5, 0.5 and 0.25 mU/l on the synthesis of PGI_2 from rat aortic ring after 1 h of incubation in buffer containing glucose (2.8 mmol/l) has recently been reported [15].

Our study suggests that the reduction in synthesis of PGI_2 in vitro from ECMC exposed to sera from diabetic patients is confined to one or more unidentified serum factors, independent of glucose and insulin. Soluble immune complexes from diabetic patients have been found to enhance platelet aggregation [16, 17], and a pathogenetic role for soluble immune complexes in the development of diabetic angiopathy has been postulated [18].

Lipid and lipoprotein abnormalities are integrated parts of the metabolic derangements in diabetes [19–21]. Decreased synthesis of PGI_2 from ECMC by high concentrations of LDL in the growth medium has been found previously [22]. However, the total cholesterol levels in all except 3 sera from the diabetic patients were within normal limits, while 6 of 12 patients had elevated triglyceride levels. Furthermore, no correlation could be established between the serum cholesterol or triglyceride concentrations and the inhibitory effects on PGI_2 production in vitro. Increased levels of plasma FFA have been reported in poorly controlled diabetic patients [23]. The concentration and composition of FFA were not determined in the present study. As, however, no inhibitory effects were observed when the cell cultures were incubated with 100% sera from diabetic patients for 2 h, this possibility also seems unlikely.

In a previous study, Arbogast et al. [24] found a toxic effect of sera from diabetic rats on cultured porcine aortic endothelial cells. We were unable by morphologic criteria to demonstrate a similar effect in our cell cultures.

In conclusion, we have shown that sera from diabetic patients inhibit the prostacyclin synthesis in cultured human endothelial cells. This effect seems unrelated to the glucose and insulin levels. If a similar reduced synthesis of prostacyclin in the vascular wall also occurs in vivo, it may contribute to the development of vascular disease in diabetes.

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