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Glucose and insulin independently reduce the fibrinolytic potential of human vascular smooth muscle cells in culture

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Summary Hyperglycaemia and hyperinsulinaemia have both been related to accelerated atherosclerosis in non-insulin-dependent diabetes mellitus (NIDDM). Plasma fibrinolytic potential is reduced in NIDDM and it is known that glucose and insulin can modulate plasminogen activator inhibitor (PAI-1) and tissue-plasminogen activator (t-PA) secretion and can therefore regulate local fibrinolysis. Vascular smooth muscle cells (vSMC) play an important role in the development of atherosclerotic lesions; however, the role of insulin and glucose in regulating PAI-1 and t-PA production in vSMC is presently not known. Therefore, we cultured arterial vSMC explanted from human umbilical cords and exposed them to increasing concentrations of glucose (5, 12, 20, 27, 35 mmol/l) or insulin (0.1, 0.5, 1, 10 nmol/l) in a serum free medium. After 24 h, PAI-1 and t-PA antigens and activity were evaluated in the culture medium; in cells exposed to 20 mmol/l glucose and to 0.5 nmol/l insulin PAI-1 gene expression was also evaluated. An increase in PAI-1 antigen was observed at each glucose concentration (by 138, 169, 251 and 357 % as compared to 5 mmol/l glucose) which was paralleled by an increase in PAI-1 activity. t-PA concentration was also increased by glucose but its activity was sharply reduced. An increase in PAI-1 antigen was detected at each insulin level (by 121, 128, 156 and 300 % as compared to no insulin). PAI-1 activity was slightly increased at the lowest insulin concentrations but markedly increased by 10 nmol/l insulin. t-PA antigen was also increased by insulin; however, its activity was markedly reduced at each concentration. As compared to control cells, PAI-1 mRNA was increased by 2.5 and 2.0 fold by 20 mmol/l glucose and 0.5 nmol/l insulin, respectively. We conclude that in human vSMC both glucose and insulin can affect the fibrinolytic balance so as to reduce fibrinolytic potential. This might contribute to decreased local fibrinolysis and thereby might accelerate the atherothrombotic process in NIDDM subjects. [Diabetologia (1996) 39: 1425–1431]

Keywords PAI-1, t-PA, fibrinolysis, vascular smooth muscle cells, glucose, insulin.

Diabetes mellitus is associated with an increased risk for atherosclerosis [1, 2]. The mechanism(s) responsible for this increased risk have not been completely elucidated. It is known, however, that diabetes is associated with attenuated endogenous fibrinolytic activity which might predispose to thrombosis [3]. The balance between tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) is one of the main factors regulating fibrinolytic potential [4]: increased circulating levels of PAI-1 [5, 6] and decreased overall plasma fibrinolytic activity [7] have been demonstrated in subjects with diabetes. Increased PAI-1 activity in plasma has also been correlated with prevailing insulin levels [5, 8], which are increased in insulin-

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Abbreviations: NIDDM, Non-insulin-dependent diabetes mellitus; PAI-1, plasminogen activator inhibitor-1; t-PA, tissue-plasminogen activator; vSMC, vascular smooth muscle cells; PBS, phosphate buffer saline; FCS, fetal calf serum; CM, conditioned medium.

resistant non-insulin-dependent diabetic (NIDDM) subjects.

Although circulating levels of t-PA and PAI-1 can undoubtedly affect thrombosis and fibrinolysis, synthesis and local release of these factors by the cells of the arterial wall could be at least as important in determining the development of atherosclerotic lesions [9, 10]. NIDDM is characterized by hyperglycaemia and, often, by elevated peripheral insulin levels [11] and both these factors have been implicated in the pathogenesis of accelerated atherosclerosis in this disease [12, 13]. In this regard, several studies have shown that both glucose [14–16] and insulin [17, 18] can modulate t-PA and PAI-1 synthesis and release in cultured endothelial cells so as to reduce fibrinolytic potential in this system. However, no data are presently available about the effect of insulin and glucose on fibrinolytic potential in vascular smooth muscle cells (vSMC). Information about this would be of outmost importance since vSMC are the main component of the atherosclerotic plaque and it has been demonstrated that these cells can actively produce PAI-1. Therefore, in the present study, we investigated the effect of insulin and glucose on the regulation of fibrinolytic potential in human vSMC in culture.

Materials and methods

Cell cultures. Umbilical cords were obtained from healthy women after delivery. Permission to harvest the cords was obtained from the subjects and from the Atri Municipal Hospital ethical committee. vSMC were isolated by the explant technique [19] from pieces of human umbilical artery after delivery. Briefly, tissue specimens were washed with phosphate buffered saline (PBS) and the tunica media was isolated by microdissection and cut into 1–2 mm pieces. These were placed in a petri dish (100 mm; Costar, Cambridge, Mass., USA) and covered with M-199 (Sigma Chemicals, St. Louis, Mo., USA) containing 20 % fetal calf serum (FCS) (HyClone, Logan, Utah, USA). The explants were then incubated in 5 % CO_2-95 % air at 37 °C.

After 15–20 days, vSMC grown from the explants reached confluency and were subcultured using the split ratio of 1:3. Cells were confirmed as smooth muscle cells by their typical "hill and valley" morphology and by positive immunofluorescent staining with a monoclonal antibody against alpha-actin (Sigma) [20]. Of the cells tested 95–98 % showed a positive staining for alpha-actin. Contamination by endothelial cells was ruled out by negative immunofluorescent staining with an anti-von Willebrand factor VIII antibody. All vSMC used in this study were between passages 1 and 5.

Glucose and insulin treatment. vSMC were plated at a density of 75000 cells/well in 12-well dishes (Costar). At subconfluency cells were exposed to M-199 plus 0.1 % FCS to allow PAI-1 and t-PA to decline to basal levels. After 48 h cells were confluent and quiescent and were incubated with 0.5 ml/well serum free medium (5 mmol/l glucose) with or without addition of insulin (Novo Industries, Copenhagen, Denmark) or excess of D-glucose (Sigma) for 24 h.

Insulin was used at concentrations of 0.1, 0.5, 1.0 and 10 nmol/l and D-glucose was added to the medium to reach concentrations of 5 mmol/l (no extra glucose added), 12, 20, 27 and 35 mmol/l. Glucose concentration in the conditioned media, spectrophotometrically measured (SVR Glucose Test; Behring Diagnostics, Somerville, N.J., USA) at the end of the 24 h of incubation was 1.8 ± 0.3 mmol/l when the initial glucose level was 5 mmol/l and 17.3 ± 1.9 mmol/l when the initial level was 20 mmol/l, similar to that reported by Kaiser et al. [21].

At the end of experiments, conditioned media (CM) were collected on ice, centrifuged at 2000 rev/min for 10 min at 4 °C and supernatants were aliquoted and stored at –80 °C. In each series of experiments, one set of cells was counted.

In separate experiments, fixed doses of insulin (10 nmol/l) or glucose (20 mmol/l) were used to stimulate vSMC for different time periods (3, 6, 18, 24 h). At the time points indicated CM were collected and stored as described above.

In additional experiments cells exposed to 20 mmol/l glucose or to 0.5 nmol/l insulin for 24 h were harvested, washed with PBS and guanidine hydrocloride was added for RNA extraction.

SDS-PAGE fibrin autography and reverse fibrin autography. CM samples (15 μl) were applied to SDS-PAGE and electrophoresed at 200 V for 40 min at room temperature with 10 % resolving and 4 % stacking acrylamide gels [22]. Marker proteins with known molecular weight, applied in each SDS-PAGE run, were stained with 0.1 % Coomassie blue. The portion of gel containing the samples was soaked in 2.5 % Triton and placed on a fibrin agar indicator film to localize plasminogen activator activity. The gels were incubated at 37 °C in a moist chamber and photographed after development of the lysis areas. Reverse fibrin autography was performed after discontinuous SDS-PAGE on 4 % stacking and 10 % separating gels. The gels were loaded with 15 μl CM samples. Low molecular weight standard proteins were applied to each gel and stained separately after the run [22].

PAI-1 and t-PA assays. The concentration of PAI-1 antigen in CM samples (active and latent) was measured by means of a double monoclonal antibody enzyme-linked immunosorbent assay (Imulyse PAI-1; Biopool, Umea, Sweden). PAI-1 complexed to t-PA and u-PA is poorly detected by this method, therefore only changes in free PAI-1 concentration in CM were measured in our experiments. The concentration of t-PA antigen in CM samples (free t-PA, t-PA/alpha2-antiplasmin complexes and t-PA/PAI-1 complexes) was measured by means of a double monoclonal antibody enzyme-linked immunosorbent assay (Imubind-5 t-PA; American Diagnostica inc., Greenwich, Conn., USA).

RNA extraction and hybridization. Total cellular RNA was isolated by single extraction by a modification of the guanidine hydrochloride method [23]. Total RNA was quantified spectrophotometrically at 260 nm. Total RNA, 10 μg/lane, was fractioned on a 1 % formaldehyde agarose containing 0.66 mol/l formaldehyde. Electrophoresis was carried out at 100 V for 2 h in 0.2 mol/l 4-morpholinepropane-sulphuric acid (Fluka Buchs CH), gels were transferred to nylon membranes (Hybond N; Amersham, Amersham, UK) and cross-linked by ultraviolet irradiation (120 mJ, Stratalinker, Stratagene, La Jolla CA). The membranes were prehybridized and hybridized at 42 °C in a hybridization oven (Hybridization oven; Appligene, Rome, Italy) following standard procedures. Gel-purified fragments of DNA random primed with ^{32}P (2×10^8 counts · min^{-1} · μg^{-1}) were used for hybridization. The probe used was human PAI-1, 1250 bp ECO RI cDNA fragment excised from PGM3 plasmid (a generous gift from Prof. DJ Loskutoff, La Jolla CA). The size of the transcripts relative to 18S and 28S rRNA

were 3.4 and 2.3 kb. For normalization purposes, the autoradiogram was scanned by a Ultrascan XL densitometer (LKB-Pharmacia, Uppsala, Sweden) and peak area intensity was measured: to compare PAI-1 mRNA signals their intensity was expressed relative to the signals obtained by a cDNA probe for glyceraldehyde dehydrogenase.

Statistical analysis. Results are expressed as mean ± SEM of three different experiments. Differences between group means were assessed by Student's *t*-tests or analysis of variance followed by modified Bonferroni post hoc test when approriate. Significance was defined as p less than 0.05.

Results

Effect of elevated glucose concentration on t-PA and PAI-1 antigen levels and activity in CM of vSMC (Fig. 1) Glucose concentrations were selected in a range between physiologic blood glucose levels (5 mmol/l) and severe hyperglycaemia (35 mmol/l). Since glucose-free medium could non-specifically compromise t-PA and PAI-1 synthesis by deprivation of substrate needed for cell metabolism, our analysis of the potential response of t-PA and PAI-1 synthesis to increments in glucose concentrations focused on the changes elicited by concentrations over 5 mmol/l. Regardless of glucose concentration, cell counts at the end of incubation were between 195000 and 210000 cells per well.

t-PA protein concentration in CM significantly increased at each glucose level and this effect was more pronounced at the maximal glucose concentration used (35 mmol/l) (Fig. 1A). Control fibrin autography analysis (Fig. 1A, inset) exhibited two distinct lysis areas at 100 kDa and 66 kDa, corresponding to t-PA/PAI-1 complex and free t-PA, respectively. Treatment of cells for 24 h with increasing doses of glucose induced a total disappearance of t-PA activity and an increase of t-PA/PAI-1 complex.

Free PAI-1 antigen levels were also increased at each glucose concentration (by 138, 169, 251 and 351 % as compared to the levels observed at 5 mmol/l glucose) (Fig. 1B). Likewise, elevated glucose concentrations induced a dose-dependent increase in PAI-1 activity as measured by reverse fibrin autography (Fig. 1B, inset).

Effect of elevated insulin concentration on t-PA and PAI-1 antigen levels and activity in CM of vSMC. Incubation with insulin for 24 h did not affect cell counts at the end of incubation which was between 197 00 and 215 000 cells per well both in the absence and in the presence of insulin 0.1–10 nmol/l. Insulin actively stimulated t-PA antigen release in our cell system. This effect was already significant at the lowest insulin concentration used (127, 172, 213 % above baseline at 0.1, 0.5 and 1 nmol/l insulin respectively, all $p < 0.05$ vs control) and it was strongly evident at

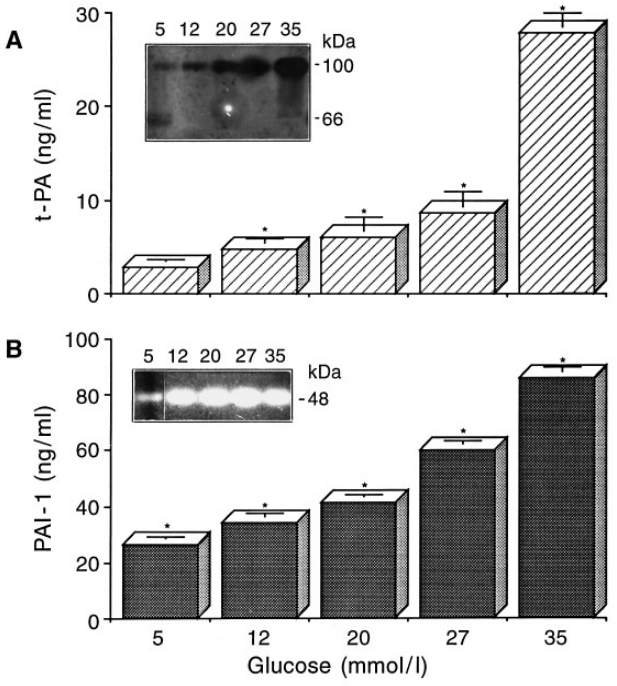

Fig. 1. **A** Effect of glucose on t-PA level in conditioned medium of human vSMC. Each bar represents the mean ± SD of three experiments. *$p > 0.05$ vs 5 mmol/l glucose. Inset: Fibrin autography of the same CM; t-PA /PAI-1 complex migrated at 100 kDa, t-PA migrated at 66 kDa. **B** Effect of glucose on PAI-1 levels in conditioned medium of human vSMC. Each bar represents the mean ± SD of three experiments. *$p < 0.05$ vs 5 mmol/l glucose. Inset: Reverse fibrin autography of the same CM. PAI-1 migrated at 48 kDa

10 nmol/l insulin, which induced a sixfold increase in t-PA antigen concentration in the CM (Fig. 2A). On the other hand, insulin treatment induced a significant reduction (0.1 nmol/l insulin) or total disappearance (0.5, 1 and 10 nmol/l insulin) of t-PA activity (Fig. 2A, inset). A dose-dependent increase of t-PA/PAI-1 complex was observed at the same insulin concentrations (Fig. 2A, inset).

A small but significant increase in PAI-1 protein was detected at 0.1, 0.5 and 1 nmol/l insulin levels (by 121, 128, and 156 % respectively as compared to control, $p < 0.05$) while 10 nmol/l insulin elicited an almost threefold increase in PAI-1 antigen concentration (Fig. 2B).

PAI-1 activity also increased in a dose-dependent manner following insulin treatment (Fig. 2B, inset).

Time-dependent effect of high glucose and high insulin concentrations on t-PA and PAI-1 antigen levels and activity in CM of vSMC. When the effect of 20 mmol/l glucose on t-PA antigen concentration in the CM of smooth muscle cells was followed over a 24 h period, a distinct time-dependent effect was observed (Table 1). On the other hand, under the same conditions, t-PA activity in the CM exhibited a reduction after 6 h and totally disappeared after 18 h of

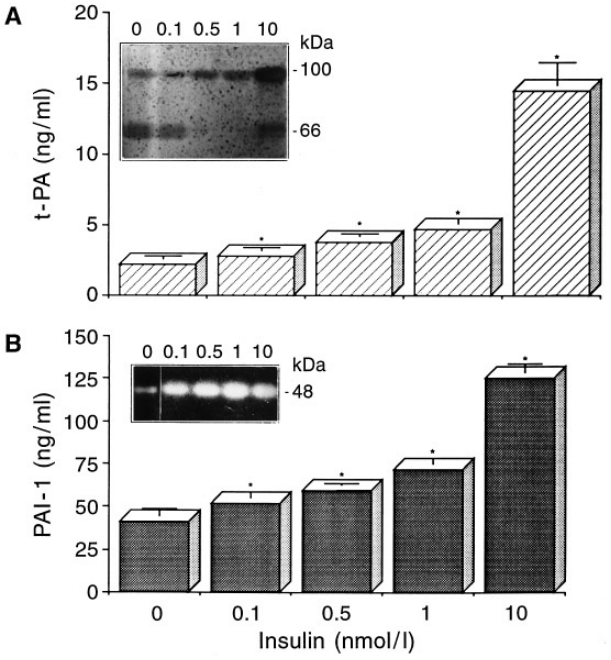

Fig. 2. **A** Effect of insulin on t-PA levels in conditioned medium of human vSMC. Each bar represents the mean ± SD of three experiments. *$p > 0.05$ 0 nmol/l insulin. Inset: Fibrin autography of the same CM; t-PA /PAI-1 complex migrated at 100 kDa, t-PA migrated at 66 kDa. **B** Effect of insulin on PAI-1 levels in conditioned medium of human vSMC. Each bar represents the mean ± SD of three experiments. *$p < 0.05$ vs 5 mmol/l glucose. Inset: Reverse fibrin autography of the same CM. PAI-1 migrated at 48 kDa

incubation. The progressive decrease observed in t-PA activity was paralleled by a time-dependent increase in the activity of t-PA/PAI-1 complex. No time effect on t-PA and t-PA/PAI-1 complex activity was observed in the control experiment (Fig. 3 A).

PAI-1 antigen levels in the CM also increased in a time-dependent manner during 24 h incubation with 20 mmol/l glucose (Table 1). PAI-1 activity was already increased after 6 h of incubation at 20 mmol/l glucose and it kept increasing up to 24 h (Fig. 3B).

When the effect of 10 nmol/l insulin on t-PA antigen concentration in the CM was followed over a 24-h period, a distinct time-dependent effect was also observed (Table 1). Insulin at 10 nmol/l also induced a reduction in t-PA activity after 6 h of incubation and the complete disappearance of t-PA activity after 18 and 24 h of incubation. Similar to that observed with 20 mmol/l glucose incubation, the progressive decrease observed in t-PA activity during 10 nmol/l insulin incubation was paralleled by a time-dependent increase in the activity of t-PA/PAI-1 complex (Fig. 3A). A 24-h incubation with 10 nmol/l insulin also induced a time-dependent increase in PAI-1 antigen concentration in the CM (Table 1). PAI-1 activity was already increased after 6 h of incubation at 10 nmol/l insulin and it kept increasing up to 24 h (Fig. 3B).

Effect of glucose and insulin on PAI-1 mRNA levels. Northern blot analysis of PAI-1 mRNA in cells exposed to 5 mmol/l glucose and no insulin (control), 20 mmol/l glucose and no insulin and 5 mmol/l glucose and 0.5 nmol/l insulin (Fig. 4) showed that both 20 mmol/l glucose and 0.5 nmol/l insulin induced a marked increase in PAI-1 mRNA. Densitometric analysis of the bands revealed that 20 mmol/l glucose induced a 235 % increase and 0.5nmol/l insulin a 191 % increase in PAI-1 mRNA.

Discussion

The present study was performed to assess the effect of elevated concentrations of glucose and insulin on the fibrinolytic balance of human vSMC in culture. Our results show that both agents are able to decrease the fibrinolytic potential in this system.

The rationale for these series of experiments stems from the fact that both hyperglycaemia and hyperinsulinaemia have been implicated in the pathogenesis of macrovascular complications in diabetes [12, 13]. Impaired fibrinolysis is likely to play a role in the increased risk for atherosclerosis in diabetes and, as a matter of fact, increased levels of circulating PAI-1 have been found in patients with both NIDDM

Table 1. Time course of t-PA and PAl-1 antigen levels in conditioned medium of human vSMC cultured for 24 h in the presence of 20 mmol/l glucose or 10 nmol/l insulin

	Time (h)	Glucose (20 mmol/l)		Insulin (10 nmol/l)	
		Control	Treated	Control	Treated
t-PA (ng/ml)	3	2.44 ± 0.28	3.60 ± 0.28	4.09 ± 0.41	5.29 ± 0.42
	6	2.50 ± 0.32	5.68 ± 0.48[a]	4.23 ± 0.38	7.08 ± 0.61[a]
	18	3.12 ± 0.28	18.52 ± 0.47[a]	4.56 ± 0.25	8.71 ± 0.74[a]
	24	3.40 ± 0.36	24.40 ± 1.20[a]	4.75 ± 0.39	13.33 ± 1.11[a]
PAl-1 (ng/ml)	3	12.0 9145 1.2	13.7 ± 1.4	23.0 ± 3.0	32.1 ± 6.0
	6	14.3 ± 1.5	41.4 ± 3.4[a]	28.1 ± 4.0	40.2 ± 5.0[a]
	18	16.8 ± 1.1	71.2 ± 9.0[a]	31.0 ± 4.8	76.1 ± 9.0[a]
	24	18.0 ± 1.4	82.0 ±12.5[a]	33.2 ± 4.1	91.3 ± 9.0[a]

Data are mean ± SD

[a] $p < 0.05$ vs control experiment at 5 mmol/l glucose and no insulin

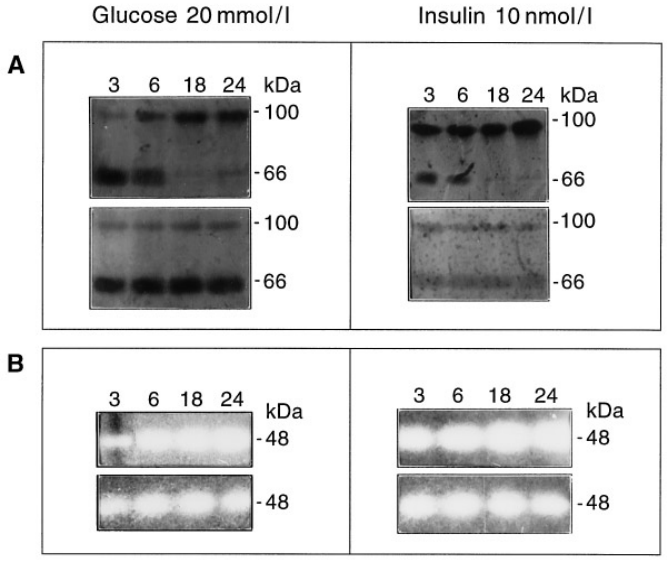

Fig. 3. **A** Time course effect of 20 mmol/l glucose (Left lane) or 10 nmol/l insulin (Right lane) on t-PA/PAI-1 and free t-PA activity in conditioned medium of human vSMC. t-PA /PAI-1 complex migrated at 100 kDa, t-PA migrated at 66 kDa. **B** Time course effect of 20 mmol/l glucose (Left lane) or 10 nmol/l insulin (Right lane) on PA-1 activity in conditioned medium of human vSMC. PAI-1 migrated at 48 kDa. The lower gel in each panel represents the control experiments (5 mmol/l glucose, no insulin added)

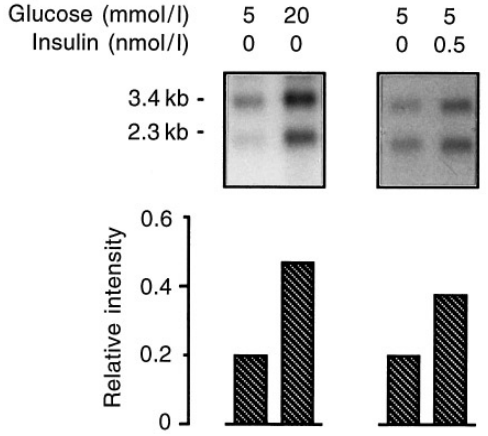

Fig. 4. Northern blot analysis of mRNA extracted from hvSMC cultured in control conditions and exposed to 20 mmol/l glucose or 0.5 nmol/l insulin. Densitometry ratios of PAI-1/GAPD blots are shown in the bar graph

and insulin-dependent diabetes [6, 7]. More recently, increased circulating PAI-1 levels have been reported in offspring of two NIDDM parents [24].

Studies performed on human vascular endothelial cells have shown that an elevated glucose concentration upregulates the expression and secretion of both t-PA and PAI-1 in these cells. Insulin has also been shown to affect t-PA and PAI-1 expression and secretion. This effect, however, has been clearly demonstrated on liver cells (HepG2) [25–28], while the role of insulin in modulating the fibrinolytic system in endothelial cells is still controversial [17, 18, 29]. These results lend support to the hypothesis that hepatocytes and, possibly, endothelial cells might be the source of elevated circulating PAI-1 levels in diabetes.

However, besides a reduction in plasma fibrinolytic potential, alterations in local fibrinolysis might potentially play a role in accelerated atherosclerosis in diabetes. vSMC play a key role in the formation of the atherosclerotic plaques and alterations in the regulation of the fibrinolytic system in these cells might facilitate SMC migration, proliferation and extracellular matrix accumulation [30]. Furthermore, the possibility exists that the accelerated vascular disease in diabetic subjects may be mediated, in part, by increased synthesis of PAI-1 and attenuation of fibrinolytic activity at the site of the arterial lesions [31]. The present study provides support for such a possibility. Our results, indeed, show that both hyperglycaemia and hyperinsulinaemia are able to reduce fibrinolytic potential of vSMC in culture. When glucose was present at concentrations of at least 5 mmol/l, the levels of both PAI-1 and t-PA did not change appreciably during 24 h of incubation. Increases in glucose concentration elicited a significant increase both in PAI-1 and t-PA antigen concentration in the CM, a significant increase in PAI-1 activity and a sharp decrease in t-PA activity which was already totally suppressed at glucose concentrations of 12 mmol/l. These results are similar to those previously obtained by Maiello et al. [14] and Cagliero et al. [16] using higher glucose concentrations (30 mmol/l) in human umbilical vein endothelial cells. A glucose-induced increase in PAI-1 secretion and PAI-1 mRNA concentration in human endothelial cells has also been reported by Nordt et al. [15]. Our results, however, represent, to our knowledge, the first observation that glucose, in concentrations typically found in diabetic patients, can significantly affect fibrinolytic potential in human arterial vSMC, fostering the concept that among the mechanisms by which hyperglycaemia might promote and or exacerbate atherogenesis there is a reduction in local fibrinolysis.

There is evidence that the hyperinsulinaemia which often occurs in NIDDM subjects may also play a significant role in the pathogenesis of diabetic macrovascular complications [12]. To determine whether insulin could affect fibrinolytic potential in cells of the arterial wall, we studied both PAI-1 and t-PA secretion in CM of human vSMC after treatment with different doses of insulin. We observed that insulin was able to stimulate secretion of both PAI-1 and t-PA in these cells, but PAI-1 secretion prevailed under insulin-stimulated conditions, so that the final effect of insulin in this system was to reduce both t-PA activity and fibrinolytic potential in the CM.

In this regard it should be pointed out that the ELISA assay we used to measure PAI-1 antigen in

the CM does not detect t-PA complexed PAI-1. This is the reason why PAI-1 increase in the CM did not exceed t-PA increase. Previously, an effect of insulin in stimulating PAI-1 synthesis and activity had been demonstrated by Schneider and Sobel on HepG2 cells [27] and in pig aortic endothelial cells [18]. On the other hand, other studies failed to observe an effect of insulin, in concentrations comparable to those used in the present study, on PAI-1 secretion, activity and expression [29, 32] in human umbilical vein endothelial cells. Differences in the cell lines used might explain these seemingly controversial results. Studies performed on the effect of acute hyperinsulinaemia on circulating PAI-1 concentrations and activity in vivo in man have shown either no effect of insulin on these paramenters [33], or even a reduction in circulating PAI-1 concentration and activity during insulin infusion [34]. However, the duration of hyperinsulinaemia might have been too short in the in vivo studies (up to 3 h, while in the time course experiment we observed a significant effect of insulin on PAI-1 antigen concentration and PAI-1 activity after 6 h of incubation) and in vivo acute hyperinsulinaemia might have increased the clearance of circulating PAI-1 [34].

The design of our experiments only allows speculations about the potential mechanism(s) by which glucose and insulin affected fibrinolytic potential in vSMC in culture. It is obvious from our data that both agents are able to induce an overproduction of both PAI-1 and t-PA which results, however, in an increased amount of PAI-1 available to complex t-PA, thus reducing its activity. As far as elevated glucose concentrations are concerned, hypertonicity per se has been reported to contribute to PAI-1 and t-PA overexpression in human umbilical endothelial cells [14]: However, a series of experiments was performed where medium osmolarity was increased by addition of mannitol and we were not able to detect an effect of hypertonicity per se on PAI-1 and t-PA production and activity in our cells (data not shown). Several studies have shown a strong correlation beetween PAI-1 and t-PA gene expression and protein secretion in the culture media [14, 15]: in agreement with these findings, in the present study we evaluated the effect of 20 mmol/l glucose and of 0.5 nmol/l insulin on PAI-1 mRNA and observed that the increase in PAI-1 secretion was paralleled by an increase in PAI-1 gene expression. Therefore, it is likely that both glucose and insulin can regulate the transcription of the genes for these proteins in vSMC. On the basis of our data, however, it is not possible to establish whether this is a direct or an indirect effect. It could be argued that the observed effect of glucose and insulin on PAI-1 and t-PA secretion is not a specific effect of these two agents, but the non-specific result of cellular proliferation, since it has been proposed that both insulin [35] and glucose [36] can stimulate cellular proliferation in vSMC. However, Hiroishi et al. [37] have shown that 28 mmol/l glucose, a concentration very close to the maximal glucose concentration used in the present study, has no competent effect and cannot stimulate cell cycle progression in vSMC in the G0 phase. Furthermore, in the experiments by Takagi et al. [38], 10 nmol/l insulin, the maximal insulin concentration used in the present study, did not induce any stimulation of [^{3}H]thymidine incorporation into the DNA of rat aortic vSMC in culture. Finally, in our experiments, we did not observe any increase in cell counts at the end of incubations with high glucose or insulin as compared to the counts obtained after incubation with 5 mmol/l glucose; moreover, flow cytometric analysis revealed that 48 h of serum deprivation resulted in a complete synchronization of the cell cycle in G0/G1 phase in 95–98 % of the vSMC. Neither 20 mmol/l glucose nor 10 nmol/l insulin added to the culture medium had any stimulating effect on cell growth or resulted in any progression of the cell cycle to the S and M phase when evaluated after 24 h (data not shown).

In conclusion, our data provide the first evidence that both glucose and insulin can reduce the fibrinolytic potential in human vSMC in culture. If the response of vSMC to these agents was to be the same in vivo, then hyperglycaemia and hyperinsulinaemia would independently induce reduced local fibrinolysis in the arterial wall. This might represent one of the factors responsible for the increased risk of atherosclerosis observed in NIDDM patients. Further studies are needed to clarify the mechanisms by which glucose and insulin induced the observed alterations in PAI-1 and t-PA secretion in vSMC.

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