Glucocorticoids and Insulin Promote Plasminogen Activator Inhibitor 1 Production by Human Adipose Tissue

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Plasminogen activator inhibitor 1 (PAI-1) is likely to play a role in vascular disease, primarily in subjects with android obesity. It has been demonstrated that PAI-1 is overexpressed in adipose tissue from obese subjects and that visceral adipose tissue produced more PAI-1 than subcutaneous fat. In the present study, the effect of insulin and glucocorticoids, which are key mediators of adipose tissue metabolism, was examined in relation to PAI-1 synthesis by human adipose tissue explants (HAT), collagenase isolated human adipocytes (IHA), cultured human stromal cells (cSC), and differentiated adipocytes from the murine clonal cell line 3T3-F442A. A significant increase in PAI-1 antigen release (1.5-fold) from HAT was detectable after 16 h of treatment with insulin concentrations of at least 10^{-8} mol/l. This was associated with a PAI-1 mRNA increase. Concomitant addition of insulin (10^{-8} mol/l) to forskolin (5×10^{-5} mol/l) reversed the decrease in PAI-1 antigen caused by forskolin alone. No effect on PAI-1 antigen was observed when insulin was incubated with IHA or cSC. 3T3 F442A cells were sensitive to insulin with a four- and twofold increase in PAI-1 antigen and mRNA levels, respectively, after 16 h of stimulation with 10^{-8} mol/l. Dexamethasone (DXM) significantly enhanced PAI-1 antigen and mRNA expression by HAT (1.5- and 2.5-fold increase, respectively) at concentrations of at least 10^{-8} mol/l. A higher stimulation was observed with IHA (sevenfold increase) and with the differentiated 3T3 F442 cell line. Cortisol was found to be less potent than DXM. No effect was observed when glucocorticoids were incubated with cSC. Coincubation of HAT with insulin (10^{-7} mol/l) and DXM (10^{-7} mol/l) led to an additive effect on PAI-1 synthesis. These results support the hypothesis that PAI-1 expression in human adipose tissue is controlled by insulin and glucocorticoids and may help to explain the increase in plasma PAI-1 levels observed in patients with android obesity. *Diabetes* 48:890–895, 1999

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Received for publication 5 August 1998 and accepted in revised form 23 December 1998.

cSC, cultured human stromal cells; DMEM, Dulbecco's modified Eagle's medium; DXM, dexamethasone; FCS, fetal calf serum; HAT, human adipose tissue explants; IHA, collagenase isolated human adipocytes; PAI-1, plasminogen activator inhibitor 1.

Increased plasma plasminogen activator inhibitor 1 (PAI-1) levels have been demonstrated in patients with cardiovascular diseases, and prospective cohort studies of patients with previous myocardial infarction or angina pectoris have underlined the association between plasma PAI-1 levels and the risk of coronary events (1).

Clinical studies have suggested that the insulin resistance syndrome with obesity may be an important regulator of PAI-1 expression, especially for determining plasma PAI-1 levels (1,2). Several studies conducted in mice have underlined the importance of the fat mass for explaining such a relation (3–5). In humans, a significant correlation was observed between the visceral fat area and the plasma PAI-1 level (6,7). We have documented PAI-1 secretion by human adipose tissue and shown that PAI-1 secretion from adipose tissue is more pronounced with visceral fat (8). PAI-1 was also related to cell lipid content and volume of fat cells (9). Besides these relations, however, the observation that plasma PAI-1 falls rapidly with fasting in the absence of changes in adipose tissue mass (10) and is subjected to a circadian variation (11) suggests that other factors influence circulating PAI-1, independent of changes of tissue size or cell depot. Of particular interest is the potential regulatory role of metabolic hormones, such as insulin and glucocorticoids. Several groups have previously reported a positive correlation of insulin with plasma PAI-1 in humans (1,10,12,13), and insulin is able to stimulate PAI-1 synthesis and release by hepatocytes (14–17). The level of PAI-1 activity was significantly higher than that observed in the control group in a large population of Cushing patients (18) and in patients under treatment with steroids (19). Moreover, PAI-1 synthesis has been shown to be positively regulated by dexamethasone (DXM) in several cells in culture (20–22). The present study was performed to assess the effects of insulin and glucocorticoids on PAI-1 synthesis by human adipose tissue and its cell fractions, i.e., isolated adipocytes and cultured stromal cells. This approach was completed by the use of differentiated adipocytes from the murine clonal cell line 3T3-F442A.

RESEARCH DESIGN AND METHODS

Tissue acquisition. Subcutaneous adipose tissue was obtained from 17 healthy female patients during elective abdominoplasty with BMI (weight [in kilograms] divided by height [in meters] squared) from 21 to 42 kg/m^2 and age ranging from 24 to 64 years. Informed consent was obtained from each patient. Investigations were conducted according to the principles expressed in the Declaration of Helsinki. After resection, adipose tissue was transported within 30 min to the laboratory in Kreb's bicarbonate buffer, pH 7.4, with 1.5% bovine serum albumin and 5 mmol/l glucose.

Human cell culture conditions. Adipose tissue explants and collagenase isolated adipocyte fractions were prepared and incubated as previously described (8). Incubation media were collected at different times and frozen at –80°C.

The culture of the adipose tissue–derived stromal cells was prepared as described previously (23). The stromal vascular cell fraction was first incubated for 10 min with an erythrocyte lysing buffer (0.154 mol/l NH_4Cl, 10 mmol/l $KHCO_3$, 0.1 mmol/l EDTA); after washing and centrifugation steps, cells were resuspended in a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (1:1, vol/vol) supplemented with 8% fetal calf serum (FCS), 66 µmol/l biotin, 17 µmol/l pantothenate, 15 mmol/l $NaHCO_3$, 15 mmol/l HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin and plated at a density of 5×10^4 cells/cm^2. At confluency, cells were trypsinized and plated at 5,000 cells/cm^2 in 12-well plates. Then, the medium was renewed every 48 h until confluency.

3T3-F442A culture conditions. Murine 3T3-F442A cells were plated at 2×10^3 cells/cm^2 and maintained in DMEM supplemented with 8% FCS (Gibco Life Technologies, Cergy-Pontoise, France), 100 U/ml penicillin, 100 µg/ml streptomycin, 66 µmol/l biotin, 17 µmol/l pantothenate, 15 mmol/l $NaHCO_3$, and 15 mmol/l HEPES (referred to as standard medium).

Differentiated 3T3-F442A cells were obtained by feeding confluent cells with standard medium supplemented with 2 nmol/l triiodothyronin and 3 nmol/l insulin (differentiation medium) for 14 days. Media were changed every 2 days. Insulin removal from 14 days postconfluent adipose cells was performed by rinsing the cells two times at 37°C (30 min each) with DMEM containing 8% FCS. Insulin-deprived cells were then maintained for 24 h in standard medium supplemented with 2 nmol/l T3 (basal medium) before addition of insulin or DXM at concentrations as indicated in the figures.

PAI-1 and leptin antigen determinations. PAI-1 antigen from conditioned media was assayed using enzyme-linked immunosorbent assays specific for human or mouse PAI-1, as previously described (24,25). All measurements were performed in duplicate.

Leptin antigen was assayed with a radioimmunoassay (Human leptin RIA kit; Wak-chemie, Hamburg, Germany). The assay was adapted for culture medium: measurements using 50-µl samples and a standard curve ranging from 0.25 to 50 ng/ml. All measurements were performed in duplicate.

RNA isolation. Cellular RNAs were extracted according to the method of Chomczynski and Sacchi (26). At least two dishes of 3T3-F442A cells were pooled for each condition. RNA from human specimens was prepared as previously described (8).

Northern blot. A 436-bp human PAI-1 fragment subcloned in pSP65 was digested with *Hind*III and *Eco*RI endonucleases and used to prepare antisense RNA probes using SP6 RNA polymerase. A linearized plasmid containing a human β-actin gene fragment inserted downstream from the SP6 promoter (pTRI β actin human; Ambion, Austin TX.) was used to make an antisense RNA (334 bp). Antisense cRNA probes were transcribed in vitro from linearized recombinant plasmids using digoxigenin-labeled UTP as previously described (8). The probe for mouse PAI-1 was a 900-bp *Nco*I-*Hind*III fragment of the PAI-1 cDNA (27). The cDNAs for mouse adipsin and angiotensinogen were obtained from B.M. Spielgelman (Boston, MA) and A. Fukamizu (Tsukata, Japan), respectively, and labeled with [^{32}P]dCTP using the random priming kit of Amersham (Les Ulis, France). Northern blot analysis was performed as previously described (8). Quantitative analysis was performed using the Visiolab 100 software for image capture and the Phoretix software for calculation (Biocom, Les Ulis, France).

Data analysis. Results are expressed as means ± SD or SE. n represents the number of independent cell preparations. Means comparison was tested by two-tailed paired Student's t test. Significance was defined as a probability of ≤0.05.

RESULTS

Effect of insulin on PAI-1 antigen and mRNA accumulation. Human adipose tissue explants were incubated for 16 h with increasing doses of insulin. A moderate increase in PAI-1 antigen accumulation (1.5-fold) was observed, which was significant ($P < 0.01$) at a concentration of at least 10^{-8} mol/l (Fig. 1) and was associated with an increase in the two PAI-1 mRNA transcripts (Fig.1 *inset*). Interestingly, concomitant addition of insulin 10^{-8} mol/l to forskolin 5×10^{-5} mol/l significantly reversed the decrease in PAI-1 antigen accumulation caused by forskolin alone (percent of control [$n = 6$]: 72 ± 24 vs. 41 ± 17; $P = 0.006$). Insulin up to 10^{-7} mol/l did not cause any significant change in the abundance of PAI-1 protein in the conditioned medium from collagenase isolated human adipocytes, even on incubation up to 67 h (without [137 ng/ml] vs. with [114 ng/ml] insulin 10^{-7} mol/l; $n = 2$). Cultured human stromal cells constituted by a majority of smooth muscle cells, as assessed by the labeling of α-actin (data not shown), did not respond to insulin stimulation (data not shown).

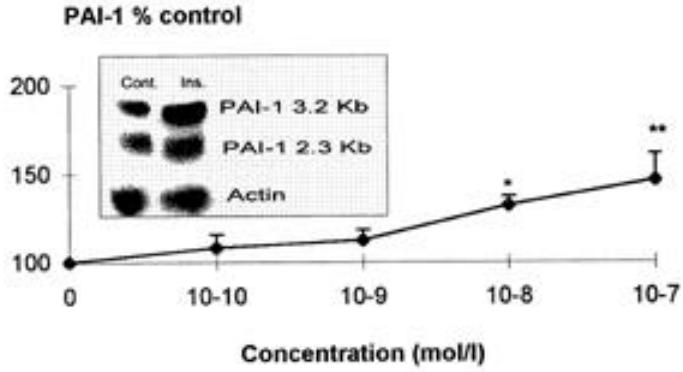

FIG. 1. Effect of increasing concentrations of insulin on PAI-1 antigen in the conditioned medium of human adipose tissue explants incubated for 16 h. Data represent means ± SE of 10 separate experiments and are expressed as percent of the control value (100%). *$P < 0.05$, **$P < 0.01$, compared with control values. *Inset*: A representative Northern blot analysis of PAI-1 mRNA compared with β-actin mRNA without (Cont.) and with (Ins.) 10^{-7} mol/l insulin.

In the culture media of 3T3 F442A, when adipocytes were fully developed, the replacement of the adipogenic medium with a medium without insulin for 24 h leads to a decrease in PAI-1 antigen production from 44 to 16 ng/ml (data not shown). Readdition of insulin at increasing concentrations for 24 h increased PAI-1 antigen and mRNA production in a dose-dependent manner, as observed at concentrations not lower than 10^{-10} mol/l. A four- and twofold increase in PAI-1 antigen and mRNA, respectively, was observed (Fig. 2) when medium was supplemented with 10^{-8} mol/l insulin for 24 h. Adipsin was used as a control because its mRNA level is known to decrease progressively under insulin stimulation (28).

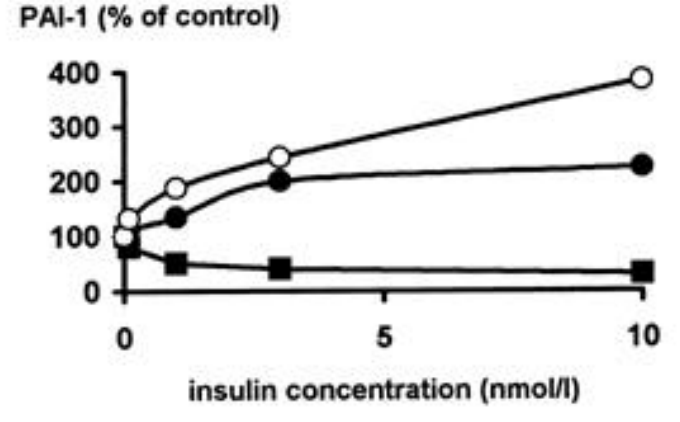

FIG. 2. Effect of increasing concentrations of insulin on PAI-1 antigen (○) in the culture medium and mRNA content (●) of newly differentiated 3T3F442A cells. After differentiation, insulin was removed for 24 h and then added again for another 24 h. Data are means ± SD of one experiment performed in triplicate and are expressed as percent of the control value. mRNA content was expressed in arbitrary units. Adipsin (■) was used as a control because its mRNA is known to be decreased under insulin stimulation. The probes were hybridized to 20 µg total RNA.

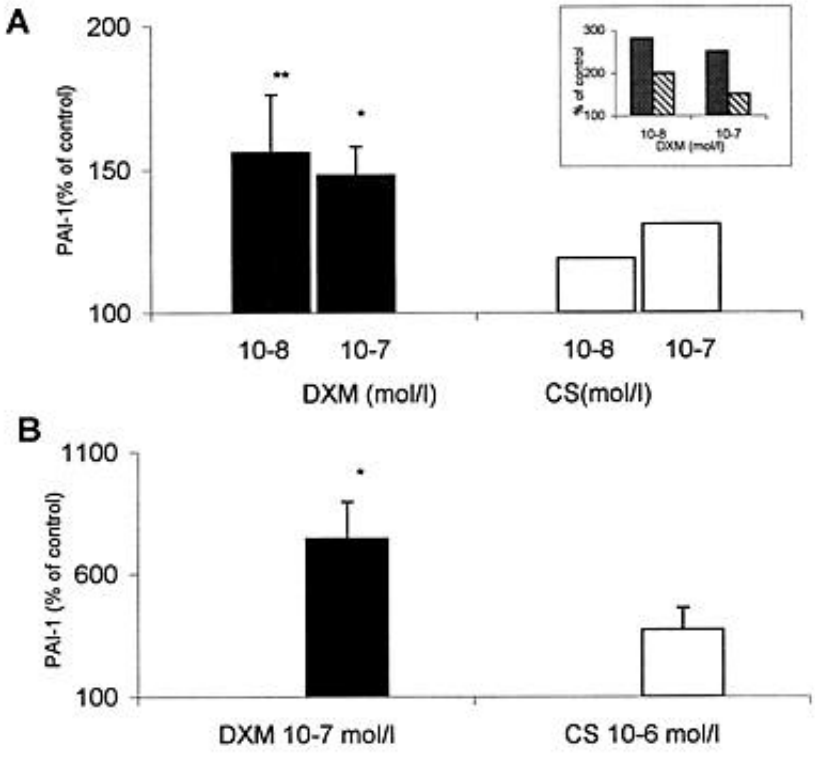

FIG. 3. *A*: **Effect of DXM (■) (n = 5) and cortisol (CS) (□) (n = 2) on PAI-1 antigen content in the conditioned medium of human adipose tissue explants incubated for 16 h. Values represent the means ± SD of separate experiments, each performed in duplicate or triplicate, and are expressed as percent of the control value (100%). *P = 0.04, **P = 0.02, compared with control values.** *Inset*: **A representative result of PAI-1 mRNA determination (3.2 kb species; 2.3 kb species) under DXM stimulation, determined by Northern blot analysis and expressed as percent of the control value after β-actin normalization. The probes were hybridized to 20 µg total RNA.** *B*: **Effect of DXM (n = 5) and CS (n = 2) on PAI-1 antigen accumulation in the conditioned medium of human isolated adipocytes. Values represent the means ± SD and are expressed as percent of the control value (100%). *P = 0.03, compared with control value.**

Effect of glucocorticoids on PAI-1 antigen and mRNA accumulation. When human adipose tissue explants were incubated for 16 h with increasing doses of DXM, a 1.5-fold increase in PAI-1 antigen accumulation was observed ($P \leq 0.04$) (Fig. 3*A*). A 2.5-fold increase was observed with mRNA (Fig. 3 *inset*). Cortisol increased, to a lesser extent, PAI-1 antigen production (Fig. 3*A*). This stimulating effect was already present after 4 h of incubation (data not shown). A more pronounced response was observed when glucocorticoids were tested on isolated human adipocytes. A seven- and fourfold increase in PAI-1 antigen accumulation was noted after 16 h of incubation under DXM 10^{-7} mol/l (P = 0.03) and cortisol 10^{-6} mol/l (Fig. 3*B*). This effect was already present after 2 h of incubation (data not shown). Leptin levels did not show any modification under glucocorticoids stimulation (data not shown). PAI-1 as angiotensinogen (29) mRNA from the differentiated 3T3 F442A was highly stimulated by DXM (10^{-7} mol/l) in a time-dependent manner (Fig. 4) with a 13- and 12-fold increase, respectively, after 16 h of incubation.

Conversely, glucocorticoids did not cause any significant increase in the abundance of PAI-1 antigen in the conditioned medium from cultured human stromal cells (data not shown).

Effect of simultaneous addition of insulin and DXM on PAI-1 production by human adipose tissue explants. When DXM (10^{-7} mol/l) was added to insulin (10^{-7} mol/l), an additive stimulatory effect was observed (Fig. 5). This effect was not observed when isolated adipocytes were used instead of tissue explants (data not shown).

DISCUSSION

We previously reported that human adipose tissue, when maintained in culture, is able to synthesize PAI-1. We demonstrate in the present study that both insulin and glucocorticoids stimulate PAI-1 synthesis from human adipose tissue.

Insulin was able to increase PAI-1 antigen accumulation in the conditioned medium from human adipose tissue explants at high concentrations. Forskolin, an activator of adenylate cyclase, has been shown to downregulate PAI-1 expression in several cell lines (30–32). The present study has demonstrated the same effect in human adipose tissue. Adenylate cyclase is a key regulator of adipocyte metabolism (33), and the capacity of insulin to reverse the action of forskolin on PAI-1 release by human adipose tissue explants suggests a potential role of insulin in regulating PAI-1 expression in human adipose tissue. In comparison with glucocorticoids, insulin exhibited a lower capacity to induce PAI-1 synthesis, since no effect was observed when insulin was tested with primary isolated human adipocytes. An acute effect of insulin on PAI-1 production was described, however, by the differentiated murine 3T3 F442 cells, confirming the results previously observed with 3T3L1 (34). The absence of effect of insulin on primary isolated adipocytes could be due to a degradation of the insulin receptor during the cell isolation procedure, as has been previously shown (35,36). Interestingly, the same discrepancy in insulin effect between freshly isolated human adipocytes and adipocyte cultured cells has already been demonstrated for leptin synthesis (37–40). The assumption that insulin may be involved in the regulation of PAI-1 expression is only partly supported by in vivo studies. A clear correlation between plasma PAI-1 and insulin concentration has been demonstrated in humans (1,7,10,13,41). Acute injection of insulin into lean mice increased PAI-1 mRNA six- to eightfold in the epididymal fat in cells that morphologically resembled adipocytes (34). Increased plasma PAI-1 activity was shown to occur after proinsulin or insulin administered to euglycemic conscious rabbits (42); however, acute injection of insulin in humans using the euglycemic-hyperinsulinemic clamp technique failed to show an increase in plasma PAI-1 (43). This does not

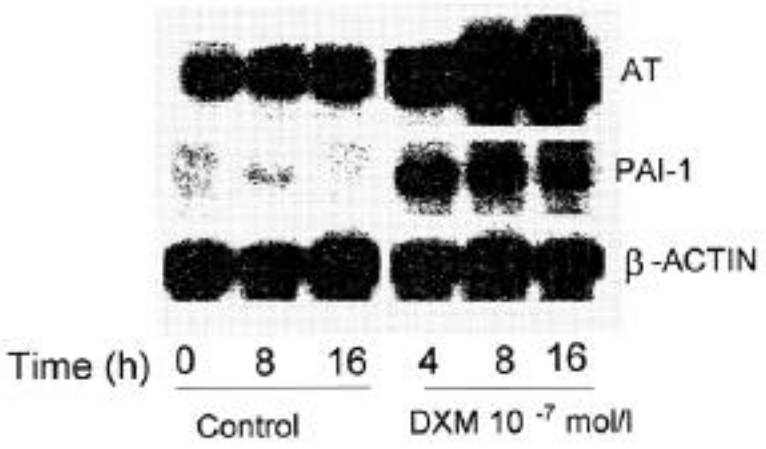

FIG. 4. A representative time course of PAI-1 mRNA during incubation of differentiated 3T3 F442A with 10^{-7} mol/l DXM. The mRNA was extracted at the indicated time period. PAI-1, angiotensinogen (AT) and β-actin were tested with the same membrane. The probes were hybridized to 20 µg total RNA.

exclude the possibility that chronic hyperinsulinemia, which is associated with android obesity, prevents the decrease in PAI-1 levels in some conditions, such as that observed when insulin was used simultaneously with forskolin.

Corticosteroids stimulate PAI-1 expression by human tissue explants and by collagenase isolated adipocytes, as well as by differentiated 3T3 F442A cells. The response of collagenase isolated adipocytes to glucocorticoids was higher than that observed with adipose tissue explants. The response observed for PAI-1 was faster than that observed for leptin production. This is in agreement with the data (44,45) showing a time lag of 24 h before the difference in leptin production became evident. Besides the promoting effect of glucocorticoids on the differentiation of human adipose precursor cells (46,47), it has been suggested that they are involved in the syndrome of abdominal obesity (48). Several studies have demonstrated the hyperactivity of the hypothalamus-pituitary-adrenal axis during the development of visceral obesity (49). Moreover, a local production of cortisol has been documented in visceral adipose tissue (50), and visceral fat has a higher density of glucocorticoid receptors (51), suggesting that the net effects of hypercortisolemia would be more pronounced with the visceral fat. The relevance of glucocorticoids in PAI-1 regulation was emphasized by clinical studies showing that Cushing's disease and steroids treatment are associated with increased plasma PAI-1 levels (18,19). However, no relationship was found between cortisolemia and PAI-1 levels during the day (11,52) or after ACTH administration in healthy subjects (53). These latter results, compared with those showing a relationship between body fat and urinary cortisol excretion rather than blood cortisol levels (54), could underline the relevance of local generation of active cortisol by omental adipose stromal cells (50).

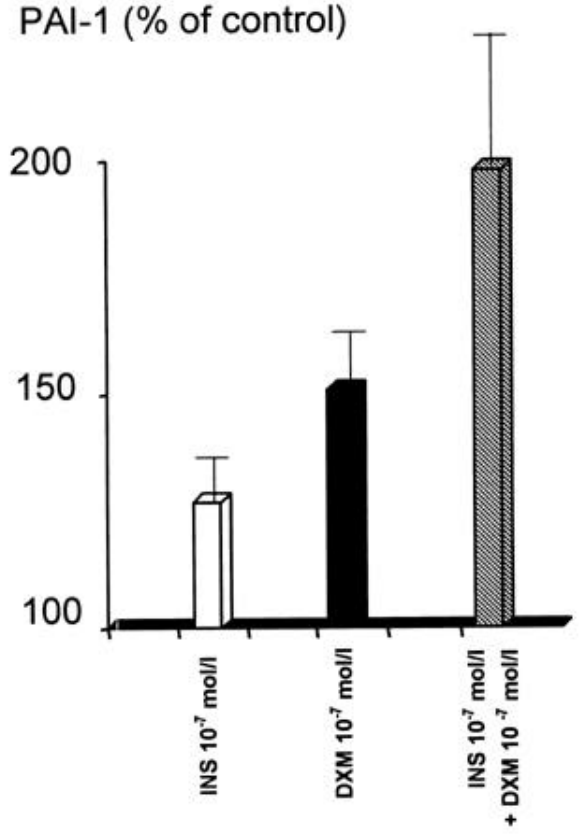

FIG. 5. Effect of simultaneous addition of DXM and insulin (INS) on PAI-1 antigen produced by human adipose tissue explants incubated for 16 h. Values represent the means ± SD of two separate experiments, each performed in triplicate, and are expressed as percent of the control value (100%).

Upon isolation, we have previously shown that the stromal cell compartment of adipose tissue contained a larger amount of PAI-1 mRNA than the amount of adipocytes (8); thus, it was interesting to analyze the response of stromal cells to hormones. This cell compartment is highly heterogeneous, including mainly endothelial, mesothelial, and smooth muscle cells (55). The conditions we have used to culture stromal cells led to selective proliferation of smooth muscle cells. These cells were unable to increase their production of PAI-1 in response to insulin or glucocorticoids stimulation.

Our results, therefore, support the concept that changes in local corticosteroids and, to a lesser extent, in insulin concentrations may contribute to the increase in PAI-1 levels observed in subjects with android adiposity.

ACKNOWLEDGMENTS

This work was supported by grants from Institut National de la Santé et de la Recherche Médicale (INSERM), from the Foundation pour la Recherche Médicale, and from the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de l'Insertion Professionnelle (Contrat Quadriennal).

The authors wish to thank O. Geel and B. Bonardo for their skillful technical assistance.

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