Molecular Medicine 6(8): 680–692, 2000

Molecular Medicine
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Insulin Continues to Induce Plasminogen Activator Inhibitor 1 Gene Expression in Insulin-Resistant Mice and Adipocytes

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Accepted April 26, 2000

Abstract

Background: Although the association between insulin resistance and cardiovascular risk is well established, the underlying molecular mechanisms are poorly understood. The antifibrinolytic molecule plasminogen activator inhibitor 1 (PAI-1) is a cardiovascular risk factor that is consistently elevated in insulin-resistant states such as obesity and non-insulin-dependent diabetes mellitus (NIDDM). The strong positive correlation between this elevated PAI-1 and the degree of hyperinsulinemia not only implicates insulin itself in this increase, but also suggests that PAI-1 is regulated by a pathway that does not become insulin resistant. The data in this report supports this hypothesis.

Materials and Methods: We show that insulin stimulates PAI-1 gene expression in metabolically insulin-resistant ob/ob mice and in insulin-resistant 3T3-L1 adipocytes. Moreover, we provide evidence that glucose transport and PAI-1 gene expression are mediated by different insulin signaling pathways. These observations suggest that the compensatory hyperinsulinemia that is frequently associated with insulin-resistant states, directly contribute to the elevated PAI-1.

Conclusions: These results provide a potential mechanism for the abnormal increases in cardiovascular risk genes in obesity, NIDDM, and polycystic ovary disease.

Introduction

Obesity is a major public health problem in Western societies primarily because of the increased risk for cardiovascular disease, dislipidemia, insulin resistance/hyperinsulinemia, hypertension and non-insulin-dependent diabetes mellitus (NIDDM) (1). In spite of the magnitude and cost of this problem, the molecular mechanisms that promote the hypercoagulable state and increased cardiovascular risk in obesity remain to be defined. Epidemiological studies suggest that elevations in the plasma levels of hemostatic factors like fibrinogen, factor VII, and plasminogen activator inhibitor 1 (PAI-1) in obese patients (2–4) may be involved. PAI-1 is the primary physiological inhibitor of plasminogen activation in vivo, and increases in PAI-1 may compromise normal fibrin clearance mechanisms, thereby promoting thrombosis (5). Although plasma PAI-1 is dramatically up-regulated in human obesity (2–4), little is known about the origin of this inhibitor or about the signals that control its biosynthesis. Recent evidence suggests that the adipose tissue itself may be an important source. For example, relatively high levels of PAI-1 mRNA were detected in murine adipose tissues (6), and adipocyte PAI-1 expression and

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plasma PAI-1 levels are significantly increased in genetically obese (ob/ob) mice (7). Significant expression of PAI-1 mRNA also has been demonstrated in cultured 3T3-L1 adipocytes, in the visceral and subcutaneous fat of obese rats, and in adipose tissues from human subjects (8–10).

The strong independent correlation between plasma PAI-1 and insulin levels (11) suggests that insulin may be one of the mediators that elevate PAI-1in obesity. This hypothesis is supported by the observation that conditions that increase endogenous plasma insulin levels (e.g., a high-calorie, carbohydrate-rich meal [12]) are associated with increases in plasma PAI-1, while conditions that reduced endogenous insulin (e.g., fasting, exercise, treatment with metformin or troglitazone) are associated with decreases in plasma PAI-1 (11,13,14). Moreover, direct administration of insulin to rabbits (15), mice (7), and the forearm vascular beds of human volunteers (16) also significantly increases the level of plasma PAI-1. Interestingly, the major effect of insulin on PAI-1 gene expression in the mouse was in the adipose tissue, and specifically in the adipocyte (7). In this respect, PAI-1mRNA and antigen were induced by insulin in cultured 3T3-L1 adipocytes (7), with the level of induction being considerably higher than that reported for other cells types (17,18). Insulin also has been shown to promote PAI-1 production by subcutaneous human adipose tissue (19). These studies raise the possibility that the adipose tissue may be a primary insulin-responsive tissue in terms of PAI-1, and suggest that the induction of PAI-1 by insulin in the adipose tissue is relatively specific for the adipocyte.

In this study, we used both normal and metabolically insulin-resistant mice and adipocytes to more precisely define the cellular and molecular mechanisms by which insulin regulates PAI-1 gene expression in the adipose tissue. We tested the hypothesis that PAI-1 is elevated in metabolically insulin-resistant states because the PAI-1 gene does not become "insulin resistant" like glucose transport. Rather, it continues to respond to insulin through alternative insulin-signaling pathways. Our results support this hypothesis and thus provide a molecular basis and rationale for the continued induction of PAI-1 by insulin in obesity and other metabolically insulin-resistant states associated with hyperinsulinemia.

Materials and Methods

Animals and Tissue Preparation

Obese strains of mice used in these studies include C57BL/6J-ob/ob, C57BLKs/J-db/db, KK/Upj-A<y>/J, C57BL/6J-tub/tub, and C57BLKs/-fat/fat. These mice and their lean counterparts (C57BL/6J and C57BLKs/J) were obtained from the Jackson Laboratory (Bar Harbor, ME). To prepare plasma, mice were anesthetized with metofane (Pitman Moore, Mundelein, IL) and blood was collected into 20 mM EDTA (final concentration) to prevent clotting. For in vivo insulin experiments, mice were injected intraperitoneally with 10 units of regular human insulin (Himulin R; Eli Lilly, Indianapolis, IN); the controls were injected with an equivalent volume of saline. At the conclusion of the experiment, mice were killed by overdose inhalation of metofane and cervical dislocation. Tissues were rapidly removed and immersed in chilled 4% paraformaldehyde (for in situ hybridization) or frozen in liquid nitrogen and powdered for preparation of total RNA. The paraformaldehyde-fixed (overnight) tissues were embedded in paraffin blocks, sectioned at 2–5 μm thickness using a microtome, and used for in situ hybridization analysis.

Determination of PAI-1, Glucose, and Insulin Levels in Plasma

Active PAI-1 antigen in plasma was determined by employing the tPA binding assay as previously described (20). Results were compared to a standard curve constructed using recombinant mouse PAI-1. Plasma glucose levels were determined using the Glucometer Elite glucose monitoring system (Bayer Corporation, Elkhart, IN). Finally, plasma insulin levels were determined using a rat insulin enzyme-linked immunosorbent assay (ELISA) from Crystal Chem. Inc. (Chicago, IL) together with a mouse insulin standard according to the manufacturer's instructions.

RNA Analysis

The concentration of PAI-1 mRNA in tissues was determined by quantitative reverse transcription polymerase chain reaction (RT-PCR) by using a competitor cRNA standard containing upstream and downstream primers for PAI-1 and β-actin (internal control) as described (7,8). After reverse transcription and PCR amplification in the presence of ^{32}P end-labeled 5′

primers, PCR products were electrophoresed on 1.8% agarose gels. The appropriate bands corresponding to the internal standard cRNA product and the target mRNA product were excised from the gel, and the incorporated radioactivity was quantified using a scintillation counter. A standard curve for the internal control cRNA was constructed and used to determine the specific activity of the target mRNA as described (7,8,21). Variations in sample loading were assessed by direct comparison to β-actin mRNA. In situ hybridization on paraffin-embedded tissue sections was performed as described previously using ^{35}S-labeled antisense or sense riboprobes. Slides were exposed in the dark for 2–3 weeks. After developing the slides, they were counterstained with hemotoxylin and eosin.

Cell Culture

3T3-L1 mouse embryo fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). The culturing of these cells (in 6-well plates) and their differentiation from preadipocytes to mature adipocytes was carried out as described previously (22). To render adipocytes "insulin resistant," fully differentiated 3T3-L1 adipocytes were treated with 3 ng/ml recombinant murine TNF-α (Genzyme Diagnostics, Cambridge, MA) for 3 days, with daily change of medium. Insulin treatment (bovine insulin, Sigma Chemical) of normal and insulin-resistant adipocytes was carried out after a 24-hr pretreatment in serum-free medium containing 0.2% bovine serum albumin (BSA). In some experiments, the cells were pretreated with increasing amounts of the inhibitors wortmannin (Calbiochem, La Jolla, CA), rapamycin (Bachem, Torrence CA), or PD98059 (Calbiochem, La Jolla, CA) for 15 min prior to the performance of glucose transport assays, and for 1 hr prior to the administration of insulin for the PAI-1 gene expression experiments. Total RNA was isolated 3 hr after insulin treatment using the Ultraspec RNA isolation system according to manufacturer's directions (Biotecx Laboratories, Inc., Houston, TX) and the level of PAI-1 mRNA was determined using quantitative RT-PCR.

Measurement of 2-Deoxyglucose Uptake

Glucose transport was measured by assaying ^{3}H-labeled 2-deoxyglucose uptake as previously described with minor modifications (23). Briefly, 24 hr prior to measurement of glucose transport, fully differentiated normal or insulin-resistant adipocytes in 6-well culture plates were placed in serum-free high glucose DMEM containing 0.2% BSA. On the day of the experiment, the cells were washed twice with phosphate-buffered saline (PBS) and incubated in serum free media with 0.2% BSA for 2 hr. This washing/incubation procedure was repeated twice more but with 1-hr incubations. To initiate glucose transport, the media was removed and the cells were incubated in 1 ml/well Krebs-Ringer phosphate buffer containing 100 nM insulin (sigma) for 20 min at 37°C. Radiolabeled glucose (1 μCi/ml 2-deoxy-d-[1,2-^{3}H] glucose; Dupont NEN), was added and the cells were incubated another 20 min at 37°C. For inhibitor experiments, the cells were pretreated with the specific inhibitor for 15 min prior to incubation with insulin. In all cases, assays were terminated by removing the media and rapidly washing the cells three times with ice cold PBS. The monolayers were solubilized in 1 ml of 10% SDS and the cpm in the extracts was determined by liquid scintillation counting. Nonspecific glucose uptake was determined in parallel samples treated with 100 μM cytochalasin B, and this background cmp was subtracted from the total cpm.

Statistical Analysis

Statistical comparison of results was performed using the unpaired Student *t* test.

Results

Effect of Insulin on Plasma Insulin, Glucose, and PAI-1 Levels in ob/ob and Lean Mice

We previously demonstrated that intraperitoneal administration of insulin into lean mice increased plasma PAI-1 levels (7). This observation, together with the strong correlation between plasma PAI-1 and insulin levels in various insulin-resistant states (11) suggested that insulin itself was inducing PAI-1 in ob/ob mice. Experiments were therefore performed to determine whether metabolically insulin-resistant ob/ob mice were sensitive to insulin in terms of PAI-1 induction. Plasma was collected from saline or insulin-treated lean or ob/ob mice and plasma insulin (Fig. 1A), glucose (Fig. 1B), and PAI-1 activity (Fig. 1C) were determined. Figure 1A shows that the

12- to 14-week-old ob/ob mice were slightly hyperinsulinemic when compared with the lean mice (compare insulin levels at time = 0), while the 24- to 28-week-old mice were extremely hyperinsulinemic. The lean mice responded to exogenous insulin with the expected significant decrease in plasma glucose levels at 3 hr ($p < .02$) and this decrease continued for 6 hr (Fig. 1B, $p < .04$). In contrast, the 12- to 14-week-old ob/ob mice showed only a modest and transient decrease in plasma glucose 3 hr after insulin treatment (Fig. 1B) and this decrease was not significant ($p < .06$). As expected, insulin had no significant effect on plasma glucose levels at any times in the more insulin-resistant older ob/ob mice. In spite of their decreased sensitivity to insulin in terms of glucose homeostasis, the ob/ob mice continued to respond to insulin in terms of PAI-1 induction (Fig. 1C). Interestingly, the older most insulin-resistant ob/ob mice seemed to be hyperresponsive to exogenous insulin because the PAI-1 response in these mice was greater and more sustained than that of the lean mice (Fig. 1C). These studies clearly demonstrate that insulin increases plasma PAI-1 levels in metabolically insulin-resistant mice. They raise the possibility that the insulin-signaling pathway that governs PAI-1 gene expression may differ from the insulin pathway that controls glucose homeostasis.

Tissue Distribution of PAI-1 mRNA in Insulin-Treated ob/ob Mice

Experiments were performed to determine the tissue specificity of the induction of PAI-1 by insulin in ob/ob mice. Mice were injected intraperitoneally with 10 units of insulin or with saline alone, tissues were removed 3, 6, and 24 hr later, and total RNA was prepared and analyzed for PAI-1 mRNA by quantitative RT-PCR (Fig. 2A). Insulin increased PAI-1 mRNA in the adipose tissues by approximately 4-fold ($p < .05$) within 3 hr, with a maximum 5- to 6-fold induction ($p < .01$) by 6 hr. The increase in PAI-1 mRNA in the adipose tissues was apparent for at least 24 hr ($p < .04$). PAI-1 mRNA also was elevated in the lung at 6 hr after insulin treatment, but this increase, although reproducible and statistically significant ($p < .02$), was quite small compared to the increase in mRNA in the adipose tissue (Fig. 2A). Although insulin increases PAI-1 mRNA in cultured hepatoma cells (17,18), it had no

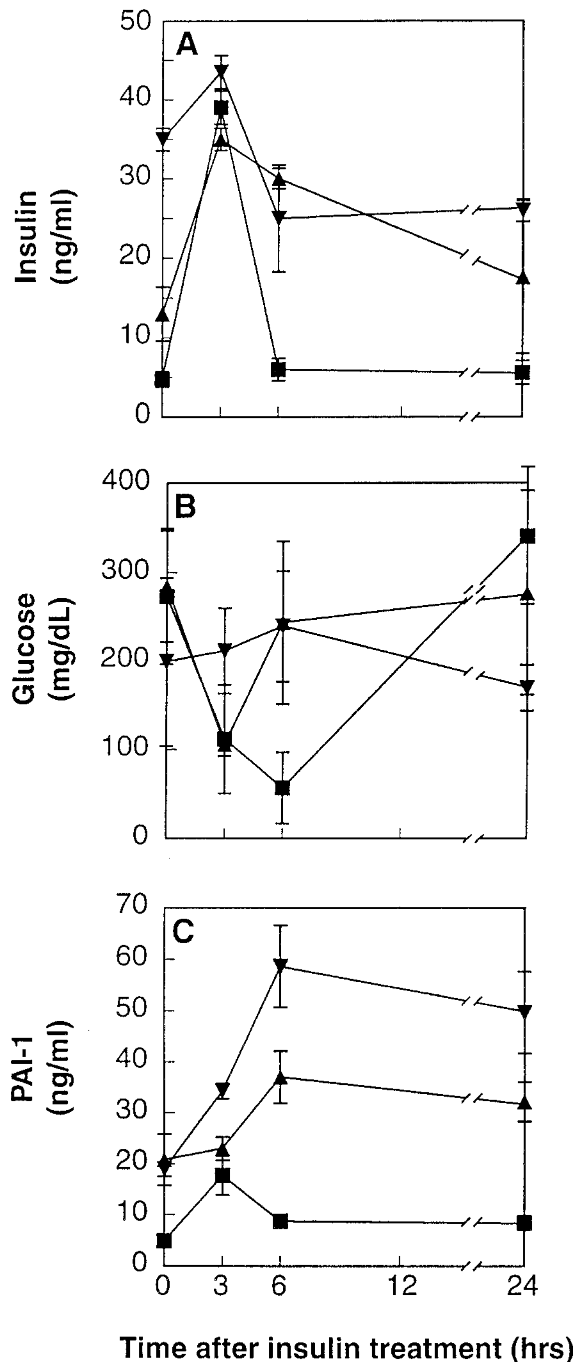
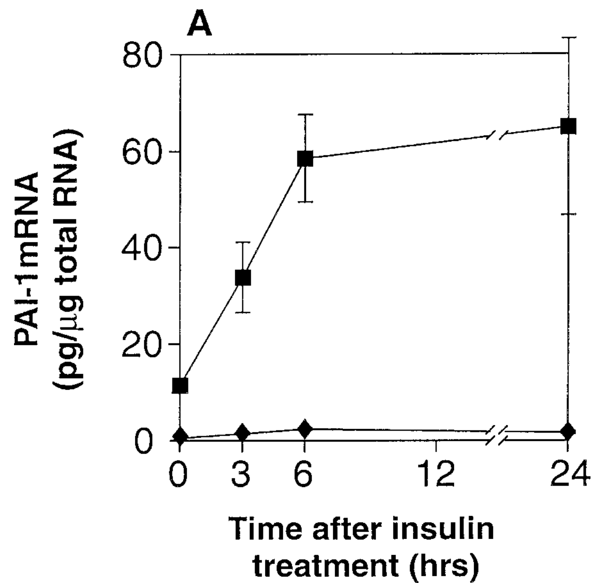


Fig. 1. Changes in plasma insulin, glucose and PAI-1 levels after insulin treatment of obese (ob/ob) and lean mice. ob/ob and lean mice were injected intraperitoneally with saline or 10 units of regular human insulin. The animals were sacrificed at the indicated times and the blood was collected into 20 mM EDTA (final concentration). After centrifugation, the plasma was collected and plasma insulin (panel A), glucose (panel B), and PAI-1 (panel C) levels were determined as indicated in Materials and Methods. Each time point on the graph represents the mean ±SD of six animals. ■: lean, 12–14 weeks; ▲: ob/ob, 12–14 weeks; ▼: ob/ob, 24–28 weeks.



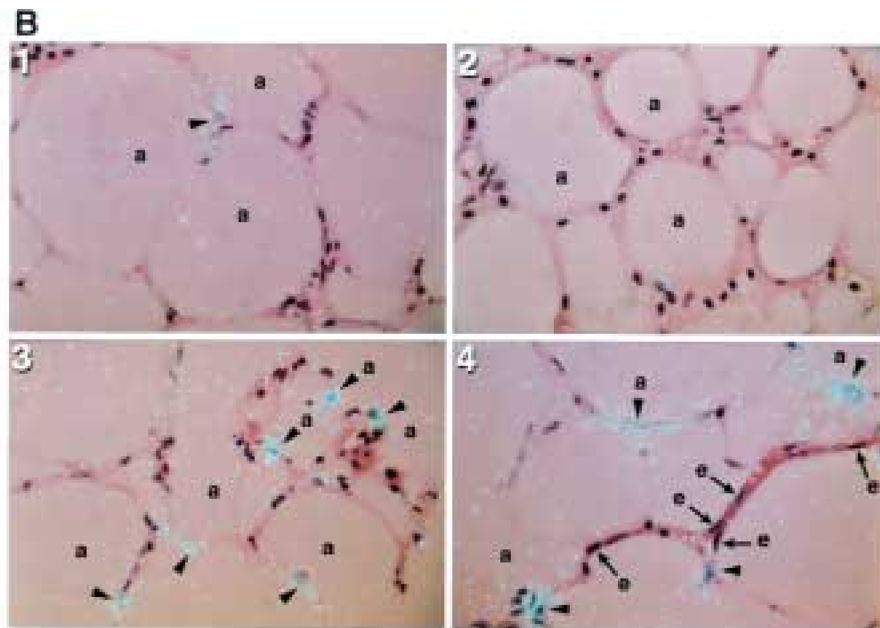


Fig. 2. Changes in PAI-1 mRNA after insulin treatment of ob/ob mice. ob/ob mice (24–28 weeks old) were injected intraperitoneally with 10 units of insulin and the indicated tissues were removed 3, 6, and 24 hr later. (A) Total RNA was prepared and analyzed for PAI-1 gene expression by quantitative RT-PCR as described in Materials and Methods. Each time point on the graph represents the mean ±SD of six animals. ■: adipose tissue; ◆: lung. (B) In situ hybridization was performed on paraffin sections of epididymal fat pads from the untreated ob/ob mice (1 and 2) and the ob/ob mice treated for 6 hr with insulin (3 and 4). Slides were exposed for 3 weeks at 4°C and stained with hematoxylin and eosin. a, adipocyte; e, endothelial cell; arrowheads indicate positive signal for PAI-1 mRNA, and arrows point to endothelial cells. Original magnification: 400 × for all sections.

detectable effect on PAI-1 mRNA expression in the liver of ob/ob mice (data not shown). No significant induction of PAI-1 mRNA by insulin was observed in other tissues including the heart, kidney, and brain (data not shown). Thus, the regulation of PAI-1 gene expression by insulin in vivo in ob/ob mice is fairly specific for the adipose tissue. A similar tissue specificity for PAI-1 induction by insulin was observed in lean mice (7).

In situ hybridization experiments were performed to identify the insulin-responsive

cells in the adipose tissue (Fig. 2B). Although a weak signal for PAI-1 mRNA was detected in the fat from untreated ob/ob mice (Fig. 2B, panels 1 and 2), a much stronger signal was observed after insulin treatment (Fig. 2B, panels 3 and 4), primarily in cells that morphologically resembled adipocytes. To confirm elevated PAI-1 gene expression in adipocytes from insulin-treated obese mice, we separated mature adipocytes from stromal vascular cells by collagenase digestion of the adipose tissue followed by differential centrifugation as previously described (24). Total RNA was extracted from the two cellular fractions and the amount of PAI-1 mRNA associated with each compartment was determined. Cross-contamination of the mature floating adipocyte fraction with microvascular endothelial cells was evaluated by PCR analysis using von Willebrand factor (an endothelial cell marker [25])-specific primers (26). The mature adipocyte and stromal-vascular cell fractions from untreated ob/ob mice contained 6 ± 1.2 and 5 ± 1.5 pg PAI-1 mRNA/μg total RNA, respectively. Insulin-treatment for 6 hr increased PAI-1 mRNA expression to 41 ± 14 and 20 ± 6.3 pg PAI-1 mRNA/μg total RNA in the adipocyte and stromal-vascular fractions, respectively. These results thus confirm the in situ hybridization studies (Fig. 2), and together with them, demonstrate that insulin induces PAI-1 expression in mature adipocytes in the ob/ob mouse. Whether insulin increases PAI-1 expression in microvascular endothelial cells cannot be conclusively determined by these studies. However, a number of capillary endothelial cells (Fig. 2B, panel 4) did not appear to express PAI-1 mRNA in response to insulin. Thus, the adipocyte seems to be the primary cell type in the adipose tissue of ob/ob mice that responds to insulin with elevated PAI-1 gene expression.

Insulin-Mediated Glucose Transport and PAI-1 Induction in Normal and Insulin-Resistant 3T3-L1 Adipocytes

The in vivo experiments performed demonstrate that metabolically insulin-resistant mice remain sensitive to insulin in terms of PAI-1 induction, and that the adipocytes are the primary cell type responsible for this effect. To begin to investigate the molecular mechanisms of insulin-mediated PAI-1 expression in insulin-resistant states, we established an in vitro model of metabolic insulin resistance using tumor necrosis factor alpha (TNF-α)–treated 3T3-L1 adipocytes as described previously (27). These cells were metabolically insulin resistant because they showed significantly decreased glucose uptake in response to a wide range of insulin concentrations when compared to normal adipocytes (Fig. 3A). However, these insulin-resistant adipocytes continued to respond normally to insulin in terms of PAI-1 mRNA induction over the same range of insulin concentrations (Fig. 3B). These studies

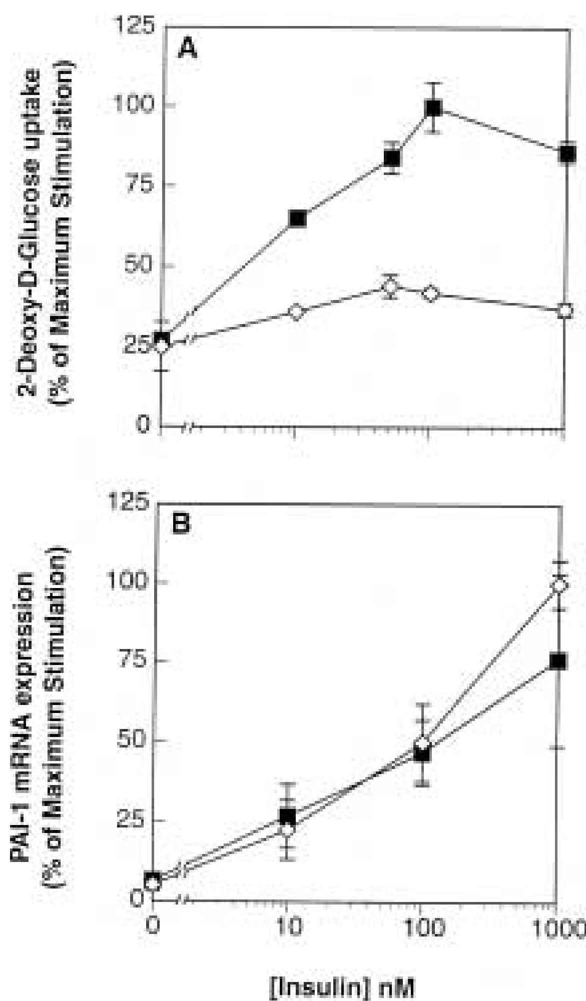


Fig. 3. Effects of insulin on glucose transport and PAI-1 mRNA expression in normal and insulin-resistant 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were treated for 3 days with buffer (normal adipocytes; ■) or with 3 ng/ml recombinant murine TNF-α to render them insulin resistant (◇). (A) The effect of insulin on 2-deoxy-d- [1,2-^{3}H] glucose uptake was determined as described in Materials and Methods. (B) The effect of insulin on PAI-1 mRNA expression was determined 3 hr after exposure to insulin using quantitative RT-PCR. For panels A and B, $n = 6 \pm$ SD.

again suggest that, in insulin-resistant adipocytes, the pathway that governs insulin-mediated glucose transport becomes insulin resistant, while the pathway that regulates PAI-1 gene expression does not.

Effect of Inhibitors of Insulin Signaling on Glucose Transport and PAI-1 Gene Expression in 3T3-L1 Adipocytes

Inhibitors of distinct insulin signaling pathways are available and were used to begin to dissect the molecular mechanisms by which insulin induces PAI-1 gene expression and glucose transport in normal and insulin-resistant 3T3-L1 adipocytes. For example, the role of phosphatidylinositol 3-kinase (PI 3-kinase), p70 S6 kinase, and Ras/MAP kinase was studied using the inhibitors wortmannin, rapamycin, and PD98059, respectively. These inhibitors are relatively specific and have been used successfully in studies evaluating aspects of insulin signaling in a variety of cell systems (including 3T3-L1 adipocytes) with few detectable toxic effects (28,29).

We initially used normal 3T3-L1 adipocytes to test the hypothesis that PAI-1 induction by insulin utilizes different signaling pathways than those employed for glucose transport. 3T3-L1 adipocytes were either left untreated, or were pretreated with the inhibitors at the indicated concentrations as described in Materials and Methods. Uptake of 2-deoxyglucose (Fig. 4, panel A) was measured in the absence or presence of 100 nM of insulin. PAI-1 mRNA (Fig. 4, panel B) was determined in parallel cultures 3 hr after treatment with 1000 nM of insulin. The doses of insulin employed were the optimum doses for glucose transport and PAI-1 gene expression as determined in Fig. 3. Although pretreating the cells with 100 nM of wortmannin reduced the rate of insulin-stimulated glucose transport to approximately the basal levels observed without insulin (Fig. 4A, $p < .002$), this inhibitor had no effect on insulin-mediated PAI-1 gene expression (Fig. 4B). In contrast, pretreating the cells with PD98059 had no effect on insulin-mediated glucose transport (Fig. 4A), but dose-dependently decreased insulin-mediated PAI-1 induction (Fig. 5B; 1μM PD98059, $p < .04$; for 10μM PD98059 and 50 μM PD98059, $p < .0001$). Pretreating cells with rapamycin had no effect on either insulin-stimulated glucose transport (Fig. 4A) or PAI-1 induction (Fig. 4B). None of the inhibitors changed the basal

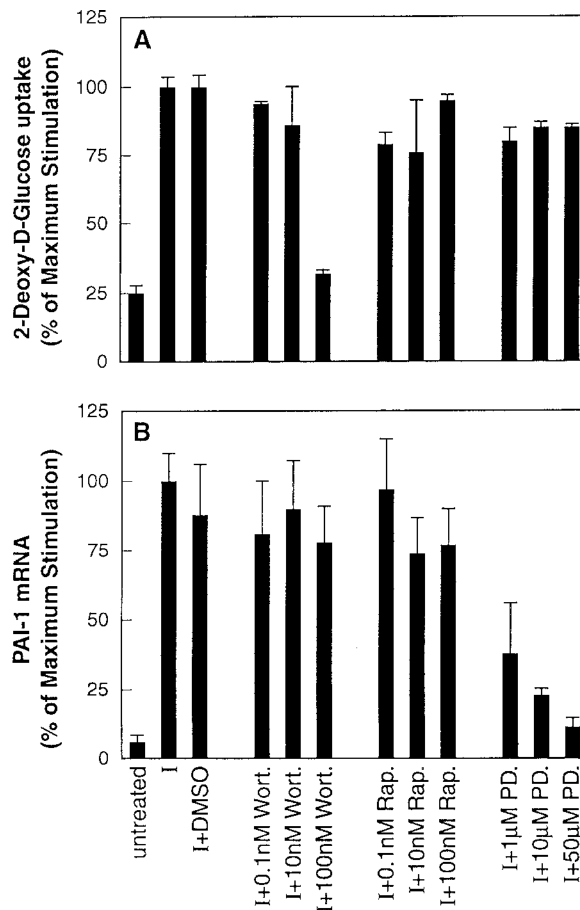


Fig. 4. Effect of inhibitors of insulin signaling on glucose transport and PAI-1 mRNA expression in normal 3T3-L1 adipocytes. (A) Fully differentiated 3T3-L1 adipocytes were left untreated or were pretreated for 15 min with DMSO (vehicle) or with the inhibitors wortmannin, rapamycin, and PD98059 at the indicated doses. Uptake of 2-deoxyglycose was measured in the absence or presence of 100 nM insulin as described in Materials and Methods. (B) Fully differentiated 3T3-L1 adipocytes were left untreated or were pretreated for 1 hr with DMSO or with the inhibitors wortmannin, rapamycin, and PD98059 at the indicated doses. The cells were then treated with 1000 nM insulin for 3 hr and PAI-1 mRNA expression was measured using quantitative RT-PCR. For panels A and B, $n = 6 \pm$ SD. I, insulin; wort, wortmannin; Rap, rapamycin; PD, PD98059.

(i.e., in the absence of insulin) rate of glucose transport or PAI-1 induction in these cells (data not shown). These studies provide strong evidence for differential insulin signaling pathways for glucose transport and for PAI-1 induction in normal 3T3-L1 adipocytes.

Similar inhibitor experiments were performed to determine whether the insulin signaling pathways for glucose transport and PAI-1

gene expression also differed in insulin-resistant 3T3-L1 adipocytes. Although insulin increased glucose transport in the insulin-resistant adipocytes (Fig. 5A), the response was significantly reduced compared to that of normal adipocytes (see Figs. 3 and 4). As shown in Fig. 5A, pretreating insulin-resistant 3T3-L1 adipocytes with 100 nM of wortmannin, decreased the rate of insulin-mediated glucose transport to approximately basal levels ($p < .03$), whereas pretreatment of the cells with rapamycin or PD98059 had no effect (Fig. 5A). Unexpectedly, a dose-dependent decrease in insulin-mediated PAI-1 mRNA expression was observed in the insulin-resistant cells when they were pretreated with any of the inhibitors (Fig. 5B). Interestingly, in the insulin-resistant adipocytes, wortmannin inhibited insulin-mediated PAI-1 expression at a much lower dose than that required to inhibit glucose transport. For example at 10 nM wortmannin, PAI-1 gene expression was inhibited ~75% with no significant change in glucose uptake. These results suggest either that glucose uptake and PAI-1 gene expression are mediated by separate targets of wortmannin, or that they require different amounts of PI3-kinase activity. In this regard, at least two different wortmannin sensitive targets (high and low affinity) have been identified in 3T3-L1 adipocytes (30). In contrast to normal 3T3-L1 adipocytes, insulin-mediated PAI-1 expression in insulin-resistant adipocytes appears to be mediated by both the MAP kinase and the PI3-kinase signaling cascades. Whatever the mechanism for these differences, our in vitro studies clearly demonstrate that in both normal and insulin-resistant adipocytes, PAI-1 induction by insulin utilizes different insulin signaling pathways than those used for glucose transport.

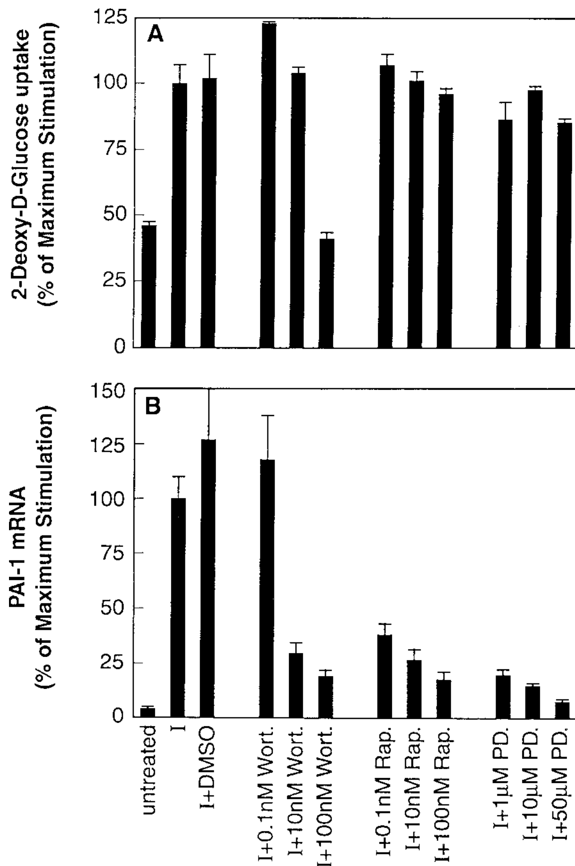


Fig. 5. Effect of inhibitors of insulin signaling on glucose transport and PAI-1 mRNA expression in insulin-resistant 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were treated for 3 days with 3 ng/ml of recombinant murine TNF-α to render them insulin resistant. (A) The insulin-resistant cells were either left untreated or were pretreated for 15 min with DMSO or with wortmannin, rapamycin, and PD98059 at the indicated doses. Uptake of 2-deoxyglucose was then measured in the absence or presence of 100 nM insulin as described in Materials and Methods. (B) Insulin-resistant 3T3-L1 adipocytes were left untreated or were pretreated for 1 hr with DMSO or with wortmannin, rapamycin, and PD98059 at the indicated doses. The cells were then treated with 1000 nM insulin for 3 hr and PAI-1 mRNA expression was measured using quantitative RT-PCR. For panels A and B, $n = 6 \pm$ SD. I, insulin; wort, wortmannin; Rap, rapamycin; PD, PD98059.

Discussion

Although clinical studies consistently demonstrate a strong association between elevated plasma PAI-1 and the insulin-resistant syndrome (11), the underlying mechanisms remain to be defined. A critical question is whether the elevated plasma PAI-1 results from the insulin resistance per se or from the compensatory hyperinsulinemia that usually accompanies this condition. The studies reported here using insulin-resistant mice and adipocytes shed light on this issue and support the latter possibility. For example, we directly demonstrate that exogenous insulin increases PAI-1 expression in both lean (insulin sensitive) and in obese (insulin resistant) mice, and that adipose tissue is the major site of this induction (Figs. 1 and 2). This continued sensitivity to insulin was not a unique property of ob/ob mice because PAI-1 gene expression also was induced by insulin in other obese insulin-resistant mice such as the

Table 1. Plasma insulin, glucose, and PAI-1 levels after insulin treatment of different strains of obese insulin resistant mice

	Plasma Insulin (ng/ml)		Plasma Glucose (mg/dL)		Plasma PAI-1 (ng/ml)	
Strain[a]	(−)	(+)	(−)	(+)	(−)	(+)
Lean						
C57BL/6J	4.7 ± 1	39 ± 2.1	273 ± 77	76 ± 56	4.9 ± 3	18 ± 4
C57BLKs/J	5.5 ± 2	42 ± 3.2	140 ± 16	19 ± 3	7.6 ± 1.5	35 ± 1
Obese						
C57BL/6J-ob/ob	35 ± 1.4	43.5 ± 2.1	199 ± 95	211 ± 48	19 ± 1.4	58 ± 8
C57BLKs/J-db/db	24.6 ± 7.5	52.1 ± 4.3	203 ± 4.9	148 ± 4.2	24 ± 4.9	103 ± 3.5
KK/Upj-A<y>/J	34 ± 2.8	47.9 ± 4.5	401 ± 5.6	416 ± 16.2	25 ± 2.8	69 ± 12
C57BL/6J-tub	24 ± 13	46 ± 1.4	292 ± 7.7	107 ± 8.6	22 ± 7	49 ± 6
C57BLKs/-fat/fat	8.9 ± 1.5	54 ± 1.9	211 ± 5.6	25.5 ± 6.3	19 ± 1.6	53 ± 29

[a]ob/ob mice: 24–28 weeks old; all other strains: 12–14 weeks old.
(−): before insulin treatment; (+): after insulin treatment. The (+) values for insulin and glucose are 3 hr after insulin treatment. The (+) values for PAI-1 are 6 hr after insulin treatment.
$n = 4 \pm$ SD for all mice except the ob/ob, where $n = 6 \pm$ SD.

db/db, tub/tub, fat/fat and KK/Upj-A<y>/J mice (Table 1). Moreover, insulin increased PAI-1 biosynthesis in normal and insulin-resistant 3T3-L1 adipocytes (Fig. 3).

Although plasma insulin levels were markedly elevated in the older (21–28 weeks) compared to the younger (12–14 weeks) mice, their plasma PAI-1 levels were similar (Fig. 1C, 0 hr). This lack of correlation between PAI-1 and insulin at these ages may reflect the fact that in addition to insulin, TNF-α and TGF-β also induce plasma PAI-1 expression in obese mice (21,31). Thus, it is possible that these cytokines decrease in very old mice. It is also possible that older obese mice produce higher levels of tPA and thus have a higher proportion of PAI-1 complexed to it. PAI-1/tPA couples would not be not be detected in the tPA-binding assay used to measure PAI-1 activity in these experiments. It is perhaps surprising that the maximal increases in plasma PAI-1 antigen (Fig. 1C) and in adipose tissue PAI-1 mRNA (Fig. 2A) occur at approximately the same time (6 hr) after insulin treatment. On the surface, one might expect a lag period between the increase in mRNA and antigen. However, PAI-1 mRNA is significantly increased in the adipose tissue even at 3 hr after insulin treatment (Fig. 2A). Moreover, although the adipose tissue may be a significant contributor to plasma PAI-1, the contribution of other tissues cannot be excluded. In this regard, we have shown that PAI-1 mRNA is induced in several other tissues in normal mice after insulin-treatment, and in this case the maximum induction is at 3 hr (7). Although glucose can regulate PAI-1 expression in some cells (32), it is unlikely that the observed effect on PAI-1 expression by insulin in our studies is mediated indirectly by a change in glucose levels. This conclusion is based on the observation that injection of insulin significantly induced PAI-1 gene expression in the 24- to 28-week-old ob/ob mice (Fig. 1C), but had no effect on plasma glucose levels (Fig. 1B). Taken together, these results argue that insulin can stimulate PAI-1 biosynthesis irrespective of the metabolic insulin-resistant state. The fact that plasma PAI-1 activity (Table 1) and adipose tissue PAI-1 mRNA (data not shown) levels also were elevated in other strains of obese insulin-resistant/hyperinsulinemic mice (Table 1), not only supports this hypothesis, but suggests that elevated PAI-1 is a common feature of these disorders.

The pleotrophic effects of insulin (e.g., regulation of cell growth, metabolic activity, gene expression, etc.) are initiated through its interactions with its plasma membrane receptor and with the subsequent sequential tyrosine phosphorylation of the insulin receptor and intracellular substrates such as insulin receptor substrate (IRS) proteins, or Shc (28). These substrates bind to the Src homology 2 (SH2) domains of several cytoplasmic signal proteins through

these tyrosine phosphorylation sites, thereby initiating multiple signaling cascades. Two of the best-studied molecules that bind activated intracellular substrates include phosphatidylinositol (PI) 3-kinase and the growth factor receptor binding protein-2 (GRB-2). Activation of these molecules, and the subsequent activation of other intracellular proteins, such as $p21^{ras}$, raf-1 mitogen activated protein kinase, or S6 kinase, is believed to be responsible for many of the biological responses to insulin. Thus, divergent and multiple signaling pathways appear to mediate the many actions of insulin, and it is possible that insulin resistance in terms of glucose transport may reflect the selective resistance of some of these pathways but not others. Thus, some actions of insulin (e.g., induction of PAI-1 and perhaps of other cardiovascular risk genes) may not become insulin resistant, but rather may be increased by the compensatory hyperinsulinemia. Our studies certainly suggest the existence of such a mechanism for the induction of PAI-1 in insulin-resistant states associated with hyperinsulinemia. In addition to PAI-1, insulin affects the expression of more than 100 genes (33) including leptin, the product of the ob gene, which is also elevated in obesity (34,35) and strongly associated with PAI-1 levels (36). Other insulin-regulated genes encode proteins involved in a variety of biological phenomena. Several of these are enzymes that have a well-established metabolic connection to insulin, whereas others represent major secretory proteins/hormones, integral membrane proteins, oncogenes, transcription factors and structural proteins (33).

Our studies using inhibitors of specific insulin-signaling pathways in normal and insulin-resistant adipocytes clearly demonstrate that insulin regulates glucose transport by separate and distinct signaling pathways from those that it employs to regulate PAI-1 expression (Figs. 4 and 5). Consistent with previous studies (29,37,38), the regulation of glucose transport in adipocytes by insulin appears to require the activation of PI3-kinase but not MAP kinase or p70 S6 kinase (Figs. 4A and 5A). In contrast, for insulin-mediated PAI-1 expression, the MAP kinase pathway, a pathway not implicated in the glucose transport function of insulin, seemed to be most significant (Figs. 4B and 5B). Interestingly, the induction of PAI-1 by insulin in insulin-resistant adipocytes was more complex than this. For example, besides the MAPK pathway, these cells also appeared to utilize a signaling cascade that required the activation of PI3-kinase and p70 S6 kinase (Fig. 5B). Thus, insulin-resistant adipocytes were capable of using multiple signaling pathways, and these were quite distinct from the pathway used for glucose transport in these systems. These observations raise the intriguing possibility that insulin may activate alternate and additional compensatory signaling pathway(s) for PAI-1 gene expression in insulin-resistant states. Alternately, it could also mean that there may be some crossing over between the PI3-kinase and MAPK pathways in insulin-resistant adipocytes or that the coordinated activation of the two pathways is necessary for PAI-1 induction by insulin in these cells. Whatever the mechanism, it is obvious that additional studies are required to resolve this issue. It should be noted that a similar set of complex signaling pathways involving the activation of PI3-kinase, p70 S6 kinase, and MAP kinase was also recently reported for insulin induced secretion of leptin in rat adipocytes (39).

Of the multiple insulin signaling pathways described so far, the pathway leading to the activation of MAP kinase does not seem to contribute to the metabolic (short-term effects) of insulin, but has been implicated in the regulation of gene expression and the more long-term effects of insulin (40). Our data demonstrating that PAI-1 induction in adipocytes is MAP kinase dependent (Fig. 4B, 5B) are certainly consistent with this idea. In this respect, insulin-mediated expression of immediate early genes such as erg-1 and c-fos was also shown to require the activation of MAP kinase but not PI3-kinase (41). Interestingly, PAI-1 is classified as an immediate early gene (42), and may be a member of a family of immediate early genes whose expression is induced by the activation of MAPK. Activation of MAP kinase also mediates the stimulation of glycogen synthesis by insulin (33), while the induction of hexokinase II gene transcription by insulin is through a PI3-kinase/p70 S6 kinase-dependent pathway (43). Recent studies demonstrate that hyperinsulinemia increases the cellular pool of farnesylated p21 Ras, thereby potentiating the activation of p21 Ras by growth factors including insulin (44). Because activation of the Ras pathway is a major determinant of MAP kinase activation, one can speculate that the hyperinsulinemia associated with insulin-resistant states

may cause an exaggerated activation of this pathway, leading to the subsequent increase in PAI-1 and perhaps other genes that contribute to increased cardiovascular risk.

One potential complication of the above studies is that the induction of PAI-1 by insulin in 3T3-L1 adipocytes may be mediated both by the insulin receptor and by the insulin-like growth factor-1 (IGF-1) receptor. In preliminary studies to test this possibility, we attempted to block the IGF-1 receptor using commercially available antibodies (Upstate Biotechnology, Lake Placid, NY). We observed that pretreating the cells for 1 hr with 10 μg/ml of the IgY fraction of the antisera had little effect on the induction of PAI-1 by insulin over a range of insulin concentrations (10–1000 nM). However, pretreatment of the cells with one-half the amount of antibody (5 μg/ml) caused a 65% decrease in the induction of PAI-1 by IGF-1 itself. Thus, it seems unlikely that the IGF-1 receptor contributes significantly to the insulin-mediated induction of PAI-1 biosynthesis by mature 3T3-L1 adipocytes.

Another possible complication of studies of insulin action, particularly in vivo, are the effects of the so-called counterregulatory hormones (catacholamines, glucagon, glucocorticoids, etc.) that are secreted in response to insulin-induced hypoglycemia (33). Many of these hormones may also regulate the gene in question, making it difficult to distinguish between direct and indirect effects of insulin. Studies in which hypoglycemia is prevented by the use of a glucose clamp may obviate some of these problems and this approach can be used to analyze whether glucose metabolism is also required for an insulin response. Clearly, tissue culture or tissue explant studies are the systems of choice, because they allow the cellular environment to be exactly controlled. In our in vivo studies, injection of insulin into lean mice was followed by a brief period of hypoglycemia (Fig. 1B), while injection of insulin into insulin-resistant ob/ob mice (Fig. 1B) or into db/db and Kky/Upj mice (data not shown) did not induce significant hypoglycemia. Yet PAI-1 was induced by insulin in all of these mice. These observations, as well as our in vitro results showing induction of PAI-1 by insulin in cultured 3T3-L1 adipocytes (Fig. 3B), support our hypothesis that the observed increases in PAI-1 in our study result from direct effects of insulin. In this respect, infusion of insulin into rabbits under conditions of euglycemic control also increased plasma PAI-1 activity (15).

It should be noted that although the effects of insulin on PAI-1 gene expression in the mouse have been extremely consistent (e.g., this study; 7,45), the literature on the effects of insulin on PAI-1 biosynthesis in human adipose tissue/adipocytes is limited and contradictory. For example, while Morange et al. (19) showed that insulin increased PAI-1 expression in human adipose tissue explants, they were unable to detect changes in isolated adipocytes. Moreover, other investigators were unable to detect changes in PAI-1 expression in human adipose tissues after insulin treatment (46). The absence of an effect of insulin on isolated human adipocytes may be due to the degradation of insulin receptors during the cell isolation procedure as has been previously shown (47). A similar discrepancy in the effect of insulin on isolated adipocytes also has been demonstrated for leptin synthesis (48–50). Based on these rather limited studies, it is difficult to draw valid conclusions about the role of insulin in the induction of PAI-1 in human adipocytes.

In summary, we demonstrate that insulin induces PAI-1 in both lean and obese mice, and that the adipose tissue is a major site of this induction. We also provide novel information regarding the signaling mechanisms by which insulin regulates PAI-1 gene expression in normal and insulin-resistant adipocytes and mice. These latter studies indicate that the insulin signaling pathways that control PAI-1 biosynthesis differ from the pathways that govern glucose homeostasis. These observations thus provide a novel molecular mechanism for the observed (11) overproduction of PAI-1, and perhaps of other cardiovascular risk genes, in insulin-resistant states associated with hyperinsulinemia, such as obesity, NIDDM, and polycystic ovary disease (13).

Acknowledgments

This work was supported in part by grants from the NIH (HL 47819 and HL 59549) and Novartis to DJL. The authors thank T. Thinnes for technical assistance and M. McRae for her expert secretarial skills. TSRI Manuscript No. 12576-VB.

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