Selective Insulin Resistance Affecting Nitric Oxide Release But Not Plasminogen Activator Inhibitor-1 Synthesis in Fibroblasts From Insulin-Resistant Individuals

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Objectives—Insulin activates several processes potentially dangerous for the arterial wall and hyperinsulinemia might be atherogenic. However, other insulin effects are protective for the vessel wall and thus anti-atherogenic. Aim of this study was to investigate whether insulin effects on potentially pro-atherogenic and anti-atherogenic processes were differently affected in cells from insulin-resistant individuals.

Methods and Results—We determined insulin effect on nitric oxide (NO) production and plasminogen activator inhibitor (PAI)-1 synthesis in 12 fibroblast strains obtained from skin biopsy samples of 6 insulin-sensitive (IS) (clamp \( M > 7 \) mg/kg body weight per minute) and 6 insulin-resistant (IR) (clamp \( M < 5 \) mg/kg body weight per minute) healthy volunteers. Insulin effects on NO release and Akt phosphorylation were significantly impaired in fibroblasts from IR as compared with IS individuals. Conversely, there was no difference between IR and IS strains in insulin ability to increase PAI-1 antigen levels and, after 24-hour insulin incubation, PAI-1 mRNA increase in IR strains was only slightly less than in IS strains. Insulin ability to induce MAPK activation was also comparable in IR and IS cells.

Conclusions—we conclude that in cells from IR individuals, insulin action on anti-atherogenic processes, such as NO release, is impaired, whereas the hormone ability to stimulate atherogenic processes, such as PAI-1 release, is preserved.

Key Words: diabetes mellitus ■ fibroblasts ■ insulin resistance ■ nitric oxide ■ plasminogen activator inhibitor type 1

Metabolic syndrome is characterized by insulin resistance, hyperinsulinemia, and a cluster of risk factors for cardiovascular disease. Because insulin can activate several processes potentially dangerous for the vascular wall, hyperinsulinemia might contribute to the high cardiovascular risk associated with the syndrome.1-5 As a matter of fact, insulin promotes vascular smooth muscle cells migration,6 enhances leukocyte adhesion molecules expression,7 stimulates endothelin8 and plasminogen activator inhibitor-1 (PAI-1)9-11 synthesis and expression. However, several insulin effects could be considered protective for the vessel wall and thus anti-atherogenic; for instance, insulin inhibits platelet aggregation12 and stimulates nitric oxide (NO) release.13,14

In insulin-resistant subjects, molecular pathways leading to the potentially protective insulin effects on the vessel wall could be insulin-resistant as well, thus contributing to the accelerated atherosclerosis associated with insulin resistance. As a matter of fact, different intracellular cascades can transduce insulin signaling15 and it appears that most of the potentially proatherogenic insulin effects (cell growth stimulation, increased PAI-1 synthesis and expression, etc.) are mainly mediated through a MAPK-dependent signaling pathway.16-19 whereas insulin effects on glucose transport and glucose uptake as well as insulin stimulation of NO synthesis and release are mediated through a PI3K-dependent signaling pathway.20 The presence of a pathway-specific insulin resistance has been shown in vascular tissue in a rat model of insulin resistance.21 Furthermore, Cusi et al.22 have shown that in skeletal muscle biopsy specimens from insulin-resistant type 2 diabetic and obese Mexican American subjects, PI3K activity was drastically impaired following insulin stimulation in vivo, whereas MAPK activity was not different as compared with muscle tissue from lean, nondiabetic subjects. However, it remains to be determined whether in human tissues a pathway specific insulin resistance exists affecting insulin actions on the vessel wall. Therefore, in the present

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study, we investigated fibroblasts obtained from healthy non-diabetic subjects characterized to be insulin-resistant or insulin-sensitive on the basis of an euglycemic hyperinsulinemic clamp. In these cells, we confirmed insulin-resistance at the level of glucose metabolism and then investigated, in the same cell strains, insulin ability to modulate NO release and PAI-1 synthesis, 2 processes exemplifying, respectively, potentially protective or harmful insulin actions on the vessel wall. We also investigated whether in our cellular model, in which we have previously demonstrated impaired insulin-mediated PI3K activation, insulin resistance affected insulin ability to activate Akt and/or MAPK-dependent signaling pathways.

Materials and Methods
An expanded Materials and Methods can be found online at http://atvb.ahajournals.org.

Characteristics of Cells Donors
Fibroblasts were obtained from human volunteers whose insulin sensitivity had been characterized by a euglycemic insulin clamp (40 mU/m² per minute). All subjects signed an informed consent before inclusion in the study and the investigation was conducted according to the Helsinki Declaration principles. Subjects were healthy, non-diabetic, non-obese individuals who were not using medications. Six cellular strains were obtained from 6 insulin-sensitive (IS) subjects (age 31 ± 1 years, male/ female 4/2, body mass index 24 ± 2 kg/m², total glucose disposal during the last 30 minutes of the insulin clamp [M] > 7 mg/kg body weight per minute, average 8.8 ± 2.4 mg/kg body weight per minute), and the other 6 cellular strains were obtained from 6 insulin-resistant (IR) subjects (age 34 ± 4 years, male/female 4/2, body mass index 25 ± 2 kg/m², total glucose disposal during the last 30 minutes of the insulin clamp [M] < 5 mg/kg body weight per minute, average 3.9 ± 0.3 mg/kg body weight per minute) (Table).

Cell cultures were established from skin excision performed on the forearm surface within 1 week from the clamp study. Fibroblasts were grown and subcultured as previously described. Glucose uptake was measured by determining the [H]-2-deoxyglucose (DOG) uptake. Glycogen synthesis evaluation was obtained by measuring incorporation of [14C]-glucose from UDP-[U-14C] glucose into glycogen as previously described. Insulin effect on constitutive NO synthase (NOS) activity was evaluated in cultured fibroblasts (IS and IR), by measuring the conversion of L-[3H] arginine into L-[3H] citrulline, expressed as pmol/min per mg total protein. Western blot analysis for Akt and MAPK phosphorylation were performed as described.4

Statistical Analysis
Results are expressed as mean±SD of at least 3 different experiments. Differences were assessed by Student t test and by 2-way ANOVA test. Significance was defined as P<0.05.

Results
Effect of Insulin on Glucose Uptake and Glycogen Synthesis
Inulin ability to enhance both glucose uptake and glycogen synthesis was impaired in IR fibroblasts, which showed a lower [H]-2-deoxyglucose uptake (125 ± 9 and 188 ± 40 pmol/mg per minute, IR and IS, respectively; P<0.05) and a reduced glycogen synthase activity (0.58 ± 0.02 and 0.74 ± 0.03 nmol/min per mg protein, IR and IS, respectively; P<0.05) after exposure to insulin.

Effects of Insulin on NOS Activity
NOS basal activity was not different in IS and IR cells (L-[H] citrulline synthesis=0.11 ± 0.01 versus 0.11 ± 0.02 pmol/min per mg total protein in IS and IR, respectively). However, after insulin stimulation, NOS activity increased significantly in IS fibroblasts (0.19 ± 0.02 pmol/min per mg total protein; P<0.01 versus basal), whereas it was not statistically different from basal in IR fibroblasts (0.13 ± 0.01 pmol/min per mg total protein, P=not significant versus basal, P<0.01 versus IS) (Figure 1). Ionomycin rapidly and significantly increased L-[H] citrulline production from L-[H] arginine in both fibroblast cultures to a rate of 0.20 ± 0.02 and 0.22 ± 0.03 pmol/min per mg total protein in IS and IR fibroblasts, respectively.

Preincubation with L-NAME induced a significant inhibition in insulin-stimulated NOS activity in IS fibroblast (P<0.01) and in ionomycin-stimulated NOS activity in IS and IR fibroblasts (P<0.01). Preincubation with LY294002 suppressed insulin ability to increase NOS activity, confirm-
ing that, in our cell model, PI3K activity is necessary for insulin-induced NOS activation.

**Effects of Insulin on Akt and Endothelial NOS–ser1177 Phosphorylation**

Insulin-induced Akt and endothelial NOS (eNOS)–ser1177 phosphorylation in IR cells was significantly lower at all time points in IR as compared with IS cell strain (Figure 2). Addition of LY294002 to the culture medium resulted, as expected, in a significant inhibition of insulin effect on Akt phosphorylation in both cell strains.

**Effects of Insulin on PAI-1 mRNA levels**

Insulin stimulation resulted in a significant (4- to 5-fold) PAI-1 mRNA increase in both fibroblast strains (Figure 3A). The time course of insulin stimulation of PAI-1 mRNA appeared, however slightly, different in the 2 cell strains in that at 24 hours, PAI-1 mRNA increase above basal was significantly greater in IS than in IR ($P<0.01$).

Twenty-four hour incubation with PD98059 (25 μmol/L) profoundly inhibited insulin ability to increase PAI-1 mRNA levels in both fibroblast strains (% inhibition 96±7 and 98±5 in IS and IR fibroblasts, respectively), suggesting that MAPK activation is necessary for insulin stimulation of PAI-1 gene transcription.

**Effects of Insulin on PAI-1 Release in Culture Media**

After 24 hours of incubation, insulin significantly stimulated PAI-1 release in both sensible and resistant cell strains at both 10 (58±3 and 51±4 ng/mL, IS and IR, respectively, versus 37±2 and 35±1 ng/mL, IS and IR, respectively, at the basal level) and 100 nmol/L concentrations (98±5 and 89±6 ng/mL, IS and IR, respectively, versus 37±2 and 35±1 ng/mL IS and IR, respectively, at the basal level) (Figure 3B). At 100 nmol/L concentration, a significant insulin effect was detected already after 12-hour incubation in both IS and IR fibroblasts. Addition of PD98059 (25 μmol/L) in the culture medium significantly inhibited the effect of insulin on PAI-1 release in both IS and IR fibroblasts (data not shown).

**Figure 2.** Effects of insulin on Akt and eNOS–ser1177 phosphorylation in cultured IS (A and B, white bars) and IR fibroblasts (C and D, black bars). Cells were treated with or without LY294002 (50 μmol/L) for 1 hour and then stimulated with insulin (100 nmol/L) for the times indicated. The blot was probed with an anti-phospho Akt (60 KDa) and anti-phospho eNOS–ser1177 (132 KDa), and the membrane was reprobed with anti-Akt (60 KDa) and anti-eNOS (140 KDa) antibodies. Histograms show anti-phospho-Akt and anti-phospho eNOS–ser1177 Western blot densitometric analysis. The ratios of phosphorylated Akt and eNOS to total Akt and eNOS are expressed as the relative intensity (A.U., arbitrary units). *$P<0.001$ vs 5’, 10’, and 15’ IS fibroblasts.

**Figure 3.** Time-dependent effect of insulin on mRNA PAI-1 levels and antigenic release in cultured IS (white bars) and IR fibroblasts (black bars). A, Time-dependent effect of insulin (100 nmol/L) on PAI-1 gene expression evaluated by real-time polymerase chain reaction assay. Data are expressed setting the basal level of PAI-1 in both untreated IS and IR fibroblasts as baseline value of 1 and calculating the fold-increase after insulin exposure at different times (*$P<0.001$ vs time 0; **$P<0.01$ vs time 0; # $P<0.01$ IS vs IR). B, PAI-1 concentration in the culture medium at different time points from 0 to 24 hours. Control IS fibroblasts (white bars) and control IR fibroblasts (black bars). * $P<0.05$ vs controls; ** $P<0.01$, # $P<0.001$ vs controls.
Effects of Insulin on MAPK Activity and MAPK Phosphorylation

Because the results obtained on insulin-dependent PAI-1 gene expression and release in the medium in the presence of a MAPK inhibitor suggested a crucial role for MAPK in mediating these effects, we performed experiments aimed at testing whether insulin-induced increased MAPK activation was indeed preserved in IR fibroblasts and whether this entailed intact insulin-induced MAPK phosphorylation in these cells (Figures 4 and 5). Insulin significantly increased MAPK activity in both IS and IR fibroblasts, as documented by the increase in Elk-1 phosphorylated form at all explored time points. Insulin also induced a significant increase in MAPK phosphorylation in both IS and IR fibroblasts. After insulin stimulation, MAPK phosphorylation was significantly greater as compared with basal level at all time points in both cell strains. Addition of PD98059 to the culture medium resulted, as expected, in a significant inhibition of insulin effect on MAPK phosphorylation in both cells groups at all time points (Figure 5).

Discussion

Insulin-resistance is undoubtedly associated to an enhanced cardiovascular risk1–5; however, whether hyperinsulinemia has a direct harmful effect on the vascular wall is more controversial. As a matter of fact, insulin effects on the vascular wall can be protective (it is known for instance that insulin induces vasodilation by modulating eNOS activity and expression)6,30 as well as potentially harmful (for instance, insulin stimulates mitogenesis and increases endothelin, leukocytes adhesion molecules, and PAI-1 expression).6–9,32,33 Aim of the present study was to observe whether these different insulin actions were equally or differently impaired in a cellular model of insulin resistance, such as fibroblasts obtained from insulin-resistant subjects. We demonstrated that although insulin-induced NO synthesis was impaired in insulin-resistant fibroblasts, in the same cells the hormone’s ability to stimulate PAI-1 expression and release in the culture medium was preserved. We also demonstrated that after insulin stimulation, phosphorylation and activity of MAPK34 were not different in IR and IS cells, whereas phosphorylation of both Akt and eNOS at the serine 1177 residue30,31 was significantly lower in IR as compared with IS fibroblasts. However, ionomycin, which affects eNOS activity by a mechanism calcium/calmodulin-dependent and PI3-K/Akt/eNOS protein phosphorylation-independent, increased NO production to the same extent in IS and IR fibroblasts.

We cultured fibroblasts from skin biopsy specimens of individuals whose insulin sensitivity had been characterized by euglycemic glucose clamp. It needs to be pointed out that none of the subjects had history of diabetes or presented impaired glucose tolerance. As expected, the IR subjects had, as a group, slightly greater fasting blood glucose and fasting plasma insulin values and their blood pressure was slightly but significantly greater than in IS subjects. However, none of the 6 subjects in the IR group had fasting blood glucose levels >110 mg/dL, none had diastolic blood pressure >90 mm Hg, and only 1 had systolic blood pressure >140 mm Hg. In this way we obtained, by an acceptably low invasive procedure, cells in which, as previously shown, we could confirm impaired insulin modulation of glucose metabolism23,35 and at the same time we could investigate insulin actions on molecular pathways typically involved in the modulation of arterial functions, such as NO synthesis and PAI-1 secretion.

As a matter of the fact, insulin’s ability to modulate glucose metabolism and insulin’s ability to stimulate NOS activity was reduced in IR fibroblasts when compared with IS fibroblasts. Several studies have, in fact, demonstrated that insulin resistance causes a reduction in the sensitivity of arterial cells to the vasodilatory effects of insulin30,31 and this has been connected to insulin resistance-mediated impairment in the phosphorylation and activity of NOS30,31. We also demonstrated that the phosphorylation of the serine residue 1177 of eNOS was significantly lower in IR fibroblasts than in IS fibroblasts.

Figure 4. Effect of insulin on MAPK activity in cultured IS (white bars) and IR fibroblasts (black bars). A and C, Representative phosphorylated Elk-1 (62 KDa) bands. B and D, Mean±SD of the phosphorylated Elk-1 bands intensity, quantitated using Molecular Analyst Software. *P<0.001 vs time 0; **P<0.01 vs time 0).

Figure 5. Effects of insulin on MAPK phosphorylation in cultured IS (white bars) and IR fibroblasts (black bars). Cells were treated with or without PD98059 (PD, 25 μmol/L) for 1 hour and then stimulated with insulin (100 nmol/L) for the times indicated. A to C, The blot was probed with an anti-phospho p44/42 MAPK, and the membrane was reprobed with anti-MAP kinase antibody. B to D, Anti-phospho-MAPK Western blot densitometric analysis. The ratio of phosphorylated pp44/42MAPK to total p44/42MAPK is expressed as the relative intensity. *P<0.0001 and #P<0.001 vs control.
activity was impaired in cells from IR subjects; after insulin stimulation, NOS activity doubled in cells from insulin sensitive, whereas it was not different from baseline in cells from IR individuals. The positive control obtained by stimulation with ionomycin showed that NO synthetic pathway was functionally preserved in IR cells albeit not responsive to insulin stimulation. We have previously demonstrated that primary cultures of endothelial cells carrying the G972R polymorphism of the IRS-1 gene, known to be associated with insulin resistance, show impaired NO synthesis in response to insulin as compared with wild-type cells; however, the results of the present study represent, to our knowledge, the first evidence that insulin modulation of NO synthesis is impaired in cells of IR individuals. This is consistent with the observation obtained in the same cellular model we used in the present study that insulin ability to stimulate PI3-K activity is impaired in fibroblasts from IR subjects. Because it is amply known that insulin modulates eNOS activity and expression through activation of the IR/IRS-1/PI3-K/PDK-1/Akt signaling cascade, and because it is also known that the same signaling cascade is involved in insulin stimulation of glucose transport, it is not surprising that, in the cell model we used, we observed impaired insulin ability to promote Akt and eNOS-ser1177 phosphorylation and hence to stimulate both glucose metabolism and NO synthesis.

On the contrary, when we looked at the ability of insulin to stimulate PAI-1 expression and release in the culture medium, in both cell strains, we observed a dose-dependent, time-dependent effect of insulin in increasing both PAI-1 mRNA and PAI-1 antigen levels. The effect of insulin on these parameters was comparable in the 2 cell strains, with the exception of a slight difference in the time course pattern of insulin stimulation of PAI-1 mRNA, presumably related to a difference in PAI-1 mRNA post-transcriptional modifications. Thus, apparently IR cells showed a preserved insulin effect on PAI-1 synthesis. Because insulin stimulation of PAI-1 synthesis is thought to be mediated mostly through a MAPK-dependent signaling pathway, we investigated the effect of PD98059 (a selective MAPK inhibitor) on insulin stimulation of PAI-1 gene transcription and PAI-1 release. In both IS and IR fibroblasts, addition of PD98059 drastically blunted insulin action on PAI-1 mRNA and PAI-1 antigen levels. On the basis of this result, we investigated insulin ability to activate the MAPK-dependent signaling pathway by determining MAPK phosphorylation and MAPK activity after insulin stimulation. The insulin-induced increase in MAPK activity was of the same magnitude in both cell strains and, consistently, insulin stimulated MAPK phosphorylation to the same extent in both IS and IR cells. Thus, in fibroblasts from IR subjects, in whom insulin stimulation of PI3K is blunted, insulin stimulated Akt and eNOS phosphorylation is impaired and so are PI3K/Akt-mediated insulin effects such as stimulation of glycogen synthase and NO synthesis. On the other hand, insulin’s ability to modulate the MAPK signaling pathway is preserved as it is preserved in its ability to induce PAI-1 gene transcription and PAI-1 release in the medium. These findings are consistent with the in vivo observations by Cusi et al who, in a different population of IR subjects, such as obese and/or diabetic Mexican American individuals, demonstrated that after a 2-hour euglycemic hyperinsulinemic clamp, stimulation of the PI3K signaling pathway was impaired, whereas activation of the MAPK signaling pathway was unaffected in skeletal muscle tissue homogenates.

Our cell model was suitable to investigate molecular features of high insulin concentration actions relevant to the hormone effect on vascular wall; as matter of the fact, selective IR at the vascular wall cell level has been demonstrated in an animal model of insulin resistance (Zucker fa/fa rats) by Jiang et al who showed impaired PI3K but intact MAPK activity stimulation in microvessels tissue homogenates after insulin (10 mU/kg per minute for 1 hour) administration in vivo. Our observations are consistent with these results and demonstrate the existence of such selective IR in cells obtained from nondiabetic nonobese IR individuals.

Demonstration of such selective insulin resistance has important implications for the possible pathophysiological links between insulin resistance, hyperinsulinemia, and atherosclerosis. In vascular wall cells, insulin has been shown to be able to increase leukocytes adhesion molecule expression, endothelin synthesis, vascular smooth cells migration, and PAI-1 transcription and release. All these effects can be considered as proatherogenic. However, several insulin actions can be viewed as antiatherogenic, such as the NO-dependent insulin-mediated vasodilation. Insulin resistance is characterized by compensatory hyperinsulinemia. In these conditions, in which the proatherogenic insulin actions to be mediated by signaling pathways affected by the insulin resistance, this would result in a sort of protection of the vascular wall from hyperinsulinemia. However, were the insulin resistance to be selectively confined to potentially antiatherogenic effects, such as induction of NO release (as our study suggests it is the case), compensatory hyperinsulinemia would result in a great increase in atherosclerotic potential in IR states. On the basis of the observed insulin effects on NO synthesis and PAI-1 release, our study, strongly suggests that molecular pathways leading to proatherogenic and antiatherogenic insulin effects are differently affected by insulin resistance in humans. Although our study has been performed in vitro and thus at supraphysiological insulin concentrations, its results allow to speculate that pathway-specific insulin resistance in the vessel wall might contribute to atherosclerosis in hyperinsulinemia and IR states.

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References

Insulin Resistance, NO, and PAI-1 Release


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EXPANDED MATERIAL AND METHODS

Chemicals
Dulbecco’s Modified Eagle Medium (DMEM), Fetal Calf Serum (FCS), glutamine, Phosphate Buffered Saline (PBS) and 0.05% trypsin/0.02% EDTA were purchased from Mascia Brunelli (Milan, Italy). Insulin (Actrapid HM 100 UI/ml) was from Novo Nordisk A/S (Bagsvaerd, Denmark). [3H]2-deoxyglucose (DOG), 14C-glucose, UDP-[U-14C] glucose and L-(3H)-arginine were purchased from PerkinElmer Italia S.p.a. (Milano, Italy). PD98059 (2’-amino-3’-m ethoxyflavone), LY294002, N-nitro l-arginine methyl ester (L-NAME), Ionomycin, Tris/HCl, KF, EDTA, glycogen, glucose-6-P and Dowex AGWX8-200 were from Sigma Chemicals (St. Louis, MO, USA). All chemicals and instruments for Real-Time PCR were purchased from PE Applied Biosystems (Foster City, CA, USA). Anti-p44/42 MAPK antibody was from Promega (WI, USA), anti-phospho p44/42 MAPK, anti-Akt and anti-phospho Akt antibodies were from Cell Signaling (Beverly, MA, USA), anti-eNOS was from BD Transduction Laboratories (Lexington, KY, USA) and anti-phospho-eNOS (ser1177) was from Upstate (Lake Placid, NY, USA). Tissue-culture disposable were purchased from Hiwaki Glass (Tokio, Japan).

Cell cultures.
Cell cultures were established from skin escission performed on the forearm surface within one week from the clamp study. Fibroblasts were grown and subcultured as previously described (23). For experiments, fibroblasts were used between the 6th and 12th passages. Confluent IS and IR fibroblasts were resuspended (10x10^4 cell/ml) and plated in twelve-well plates (PAI-1 release study), in six-well plates (Nitric Oxide Synthase activity) and T75 flasks (Real Time PCR, MAPK activity and Western blot analysis). All cell cultures were grown to sub-confluency for 5 days in DMEM plus 10% FCS. Sub-confluent fibroblasts were washed three times with PBS and then were exposed for 72 hours to DMEM plus 0.1% FCS to allow both NO and PAI-1 to decline to basal levels. After 72 hours cells were confluent and quiescent.
Glucose uptake.

Glucose uptake after 1 hour stimulation with insulin 5µmol/l was measured by determining the $[^3]$H2-deoxyglucose (DOG) uptake (24). For these studies, confluent fibroblasts were treated with 10 µmol/l $[^3]$H2-DOG (4 µCi/ml) for 5 minutes. Because transport of tracer amounts of $[^3]$H2-DOG is linear between 2 to 30 minutes in vascular smooth muscle cells (25), glucose transport was assessed after 10 minutes as previously described (24).

Glycogen synthase activity.

An estimation of glycogen synthesis was obtained by measuring incorporation of $^{14}$C-glucose from UDP-[U-$^{14}$C] glucose into glycogen as previously described (26). The activity was expressed as nanomoles of UDP-glucose incorporated into glycogen · mg protein$^{-1}$ · min$^{-1}$, and was referred to 4 mM UDP-glucose.

Nitric Oxide Synthase activity.

Insulin (100 nmol/l) effect on constitutive NOS activity was evaluated in cultured fibroblasts (IS and IR) by measuring the conversion of L-(3H)-arginine into L-(3H)-citrulline as described by Pandolfi et al (27). In some experiments, L-NAME (1 mmol/l) was added 40 minutes before adding L-(3H)-arginine. In further experiments 50 µmol/l LY294002, an inhibitor of the PI3K pathway, were added to culture medium 60 minutes before starting insulin stimulation.

PAI-1 mRNA quantification by Real Time PCR.

Quiescent cells were incubated with 5 ml/flask serum free medium (5mM glucose) with or without addition of insulin (100 nmol/l) for 2, 6, 12, 24, 36 and 48 hours. Several experiments were also performed in the presence of 25 µmol/l PD98059, an inhibitor of the MAPK pathway, added to culture medium 60 minutes before starting insulin stimulation.

RNA preparation. Total RNA was isolated from cultured fibroblasts using NucleoSpin RNAII (Macherey-Nagel GmbH & Co. D-52313 Duren, Germany).
**RT reaction.** Two micrograms total RNA was used for the synthesis of first strand cDNA using the High-Capacity cDNA Archive Kit.

**Real Time PCR.** A single-tube Real-Time PCR assay was optimized for the quantitation of PAI-1 gene expression vs GAPDH or 18s rRNA with specific primers and probes by using TaqMan™ technology on the ABI Prism 9700HT Sequence Detection System Instrument, connected to Sequence Detector Software (SDS version 2.0) for analysis of data.

According to manufacturer recommendations, PAI-1 gene expression evaluation was performed by Assay-on-Demand™ Gene Expression Product 20X for PAI-1 target gene (TaqMan MGB probe, FAM™ dye-labeled) as previously described (28).

**PAI-1 release studies.**

Quiescent cells were incubated with or without addition of insulin (10-100 nmol/l). After 24 hours, conditioned media were stored at –80°C. In each well total protein content was measured by Bradford method (29). PAI-1 antigen (active and latent) concentration in conditioned medium was measured by means of a double monoclonal antibody enzyme-linked immunosorbent assay (ELISA, Zimutest PAI-1 Antigen, Hypen BioMed, Neuville sur Oise, France). In several experiments 25 µmol/l PD98059, an inhibitor of MAPK activity, were added to the culture medium 60 minutes before starting insulin stimulation.

**In vitro p44/42 MAPK assay.**

Lysates from cells exposed to the different experimental conditions were immunoprecipitated by phosphospecific p44/42 MAPK antibody; the resulting active p44/42 MAPK was then allowed to phosphorylate Elk-1 fusion protein and MAPK activity was assayed by Western blot analysis detection of phosphorylated Elk-1. Kinase assay were performed as described by New England Biolabs (Beverly, MA, USA). All of the assays were repeated three times. Densitometric analysis was used to quantify phosphorylated Elk-1 bands intensity.
Western blot analysis for Akt, eNOS and MAPK phosphorylation.

Quiescent cells were treated with or without LY294002 (50 µmol/l) or PD98059 (25µmol/l) for 1 hour and then stimulated with 100 nmol/l insulin or 10% FCS for 5, 10 and 15 minutes at 37°C.

Cell lysis, Western Blot were performed as described (14). eNOS phosphorylation on Ser1177 was assessed after total eNOS immunoprecipitation. Proteins phosphorylation levels were normalized to the matching densitometric values of non-phosphorylated proteins. Autoradiographs were quantified using a Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA, USA).