Augmentation of the Synthesis of Plasminogen Activator Inhibitor Type-1 by Precursors of Insulin
A Potential Risk Factor for Vascular Disease

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Background Both vascular disease and elevated concentrations in plasma of plasminogen activator inhibitor type-1 (PAI-1) are prominent in patients with non–insulin-dependent diabetes mellitus (NIDDM). We and others have hypothesized that the increased PAI-1 may contribute to acceleration of atherosclerosis in this condition and in other states characterized by insulin resistance as well. Surprisingly, however, elevations of PAI-1 decrease when type II diabetic patients are treated with exogenous insulin, as do circulating concentrations of the precursor of insulin, proinsulin, in plasma. Accordingly, the increased PAI-1 in patients with NIDDM may reflect effects of precursors of insulin rather than or in addition to those of insulin itself. To assess this possibility directly, this study was performed to identify potential direct effects of proinsulin and proinsulin split products on synthesis of PAI-1 in liver cells, thought to be the major source of circulating PAI-1 in vivo.

Methods and Results Hep G2 cells (highly differentiated human hepatoma cells) were exposed to human proinsulin, des(31,32)proinsulin and des(64,65)proinsulin (split products of proinsulin), or C-peptide. Accumulation of PAI-1 in conditioned media increased in a time- and concentration-dependent fashion in response to the two des-intermediates [3.3-fold with des(31,32)proinsulin and 4.5-fold with des(64,65)proinsulin]. C-peptide elicited no increase. Stimulation was transduced at least in part by the insulin receptor as shown by inhibition of stimulation by insulin receptor antibodies, mediated at the level of PAI-1 gene expression as shown by the 2.2- to 2.9-fold increases in steady-state concentrations of PAI-1 mRNA, and indicative of newly synthesized protein as shown by results in metabolic labeling experiments.

Conclusions Our results are consistent with the hypothesis that precursors of insulin (proinsulin and proinsulin split products), known to be present in relatively high concentrations in plasma in patients with NIDDM and conditions characterized by insulin resistance, may directly stimulate PAI-1 synthesis, thereby attenuating fibrinolysis and accelerating atherogenesis. (Circulation. 1994;89:321-330.)

Key Words • atherogenesis • diabetes mellitus • fibrinolysis • proinsulin split products • atherosclerosis

Non–insulin-dependent (type II) diabetes mellitus (NIDDM) and other conditions associated with insulin resistance, such as hypertension and obesity, are characterized by accelerated vascular disease.1 We and others have hypothesized that one factor that may be responsible is reduced plasma fibrinolytic activity,2,3 which may shift the balance between thrombosis and fibrinolysis toward thrombosis4 and consequently increase exposure of luminal surfaces of vessel walls to clot-associated mitogens. Attenuated fibrinolysis attributable to increased concentrations of plasminogen activator inhibitor type-1 (PAI-1) in plasma, the primary physiological inhibitor of endogenous fibrinolysis, is a risk factor for deep vein thrombosis,5 premature coronary artery disease,6,9 acute myocardial infarction,10-12 and restenosis after percutaneous transluminal coronary angioplasty.13

The “hyperinsulinemia” typically seen in patients with NIDDM reflects contributions to the assayed material, immunoreactive insulin (IRI), not only of insulin but also of proinsulin split product precursors of insulin that are detected in the conventional assays used.14 Split products such as des(31,32)proinsulin and des(64,65)proinsulin constitute a relatively large fraction of IRI in plasma of patients with type II diabetes who are not treated with exogenous insulin.14,15 Administration of proinsulin to patients with NIDDM has been associated with deleterious cardiovascular effects, including acute myocardial infarction,16,17 and elevated concentrations of both proinsulin and des(31,32)proinsulin have been identified as markers of increased cardiovascular risk.15

We and others have reported that PAI-1 activity is elevated in plasma of patients with NIDDM.2,3 In such patients, increases in IRI, one factor implicated in the genesis of the PAI-1 elevations, are attenuated when patients are given exogenous insulin. Furthermore, concentrations in plasma precursors of insulin decline.18 In contrast, concentrations in plasma of precursors of insulin are elevated in patients with NIDDM who are not being treated with exogenous insulin, in part because of the increased stimulation of pancreatic β-cells that results from hyperglycemia secondary to insulin resistance and in part because of the impaired processing of proinsulin to insulin that is typical of the condition. The decline in concentration of proinsulin in plasma induced by exogenous insulin is accompanied by a decline in plasma PAI-1.18 Thus, the increased plasma PAI-1 seen in patients with NIDDM may result from stimulation of PAI-1 synthesis by proinsulin and proin-
insulin split product precursors of insulin rather than by insulin itself.

Concentrations of PAI-1 in plasma are thought to be determined primarily by hepatic synthesis, although diverse cell types can express the PAI-1 gene. In preliminary experiments (see "Results"), proinsulin appeared to increase PAI-1 synthesis in liver cells in vitro. Accordingly, we performed the present study to determine whether liver cell PAI-1 synthesis was influenced directly by diverse precursors of insulin, including proinsulin and proinsulin split products, all of which are known to contribute to conventionally measured IRI in plasma of patients with NIDDM.

Methods

Cell Cultures

Studies were performed with Hep G2 cells, highly differentiated human hepatoma cells, because the liver appears to be the primary site for synthesis of circulating PAI-1 and because changes in hepatocyte function are likely to be critical in modulating prevailing concentrations of multiple constituents responsible for net fibrinolytic activity in blood. The Hep G2 cells were acquired from the American Type Culture Collection (ATCC) Rockville, Md), seeded at a concentration of 3.0 × 10^3 cells per well (24-well plate) and grown to confluence in minimum essential medium with Earle’s salts supplemented with L-glutamine (2 mmol/L) (Life Technologies, Grand Island, NY) and 10% NuSerum (Collaborative Biomedical, Bedford, Mass), 30 μM penicillin, and 30 μg/mL streptomycin (Life Technologies). Monolayers of confluent cells were serum-starved in Dulbecco’s modified Eagle’s medium with Ham’s nutrient mixture F-12 supplemented with HEPES buffer (DME medium, Washington University Medical School Tissue Culture Support Center, St Louis, Mo) for at least 16 hours, an interval we found to be sufficient for a decline of PAI-1 synthesis to basal levels. In independent control experiments, we showed that the relatively high concentration of insulin in NuSerum did not affect the elaboration of PAI-1 in these cells after serum starvation.

After serum starvation, cells were exposed to fresh media constituted with selected agents, including human recombinant insulin (Sigma Chemical Co, St Louis, Mo); human proinsulin, human des(31,32)proinsulin, or human des(64,65)proinsulin (Eli Lilly, Indianapolis, Ind); or human synthetic C-peptide (Sigma) dissolved in 0.9% sodium chloride (Abbott, North Chicago, Ill) with 0.5% bovine albumin (fraction V, low endotoxin, cell culture tested, Sigma). Siliconized tubes and pipette tips were used to minimize the adherence of these agents to storage or transfer devices. Control experiments were performed with vehicle alone.

Contamination with endotoxin was excluded in all media and reagents by testing with Limulus amebocyte lysate (Pyrotell assay; Associates of Cape Cod, Woods Hole, Mass). The sensitivity for detection of endotoxin was 0.0125 ng/mL. Stock solutions, reagents, and vehicle contained no detectable endotoxin. DME medium contained as much as 0.025 ng/mL endotoxin (calibrated with lipopolysaccharide [Sigma] from Escherichia coli 0111:B4). However, lipopolysaccharide (0.0001 to 1000 ng/mL) exerted no discernible effects on the secretion of PAI-1 by Hep G2 cells as opposed to effects in endothelial cells.\(^\text{19}\) Infection of the cells with mycoplasma was excluded with the Gen-Probe Mycoplasma Rapid Detection System (Life Technologies, Grand Island, NY).

Human umbilical vein endothelial cells were acquired from Endotech (Indianapolis, Ind) and grown to confluence in M199 medium (Sigma) supplemented with 10% NuSerum, 50 μg/mL endothelial cell growth supplement (ECGS), 10 ng/mL epithelial growth factor (EGF), 1 μg/mL hydrocortisone (all Collaborative Biomedical), 10 μM heparin (Sigma), 100 μM penicillin, and 100 μg streptomycin. Serum starvation was performed in Endothelial-SFM medium (Life Technolo-

pies) supplemented with ECGS, hydrocortisone, and heparin for 24 hours. The endothelial cells exhibited typical cobblestone morphology and were used in passage two.

Human aortic smooth muscle cells were prepared from segments of fresh human thoracic aorta obtained through the Tissue Division of Mid-America Eye and Tissue Bank (St Louis, Mo) using explants. The cells were grown to confluence in RPMI-1640 medium (Sigma), supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic solution (100×, Sigma) and serum-starved in RPMI-1640 medium for 24 hours. They exhibited typical hill-and-valley morphology, stained homogeneously with mouse monoclonal anti-α-actin antibody and goat fluorescein conjugated anti-mouse immunoglobulin (IgG) antibody, and were used in passages three to eight.

Quantification of PAI-1

Conditioned media from Hep G2 cells were supplemented with Tween 80 (final concentration, 0.01%). Cellular debris was removed by centrifugation at 13,000g at 4°C for 1 minute. Samples of conditioned media were stored at −20°C until assay. The concentration of PAI-1 antigen in conditioned media was measured by ELISA with a monoclonal goat antibody, MA-7DB7, and a polyclonal conjugated antibody (TintElize PAI-1, Biopool, Umea, Sweden). Active, latent, and TPA-complexed forms of PAI-1 were detected with equal sensitivity.

PAI-1 activity was assayed spectrophotometrically. Samples were incubated with exogenous TPA (KabiVitrum, Stockholm, Sweden) at room temperature for 10 minutes under conditions in which PAI-1 but not other low-affinity inhibitors could bind to the TPA. Subsequently, they were acidified and snap-frozen to eliminate residual α-antiplasmin activity. Residual TPA activity was assayed spectrophotometrically by incubation with plasminogen (Kabi, Mönndal, Sweden) and a chromogenic substrate S-2251 (KabiVitrum).\(^\text{20}\)

Metabolic Labeling and Immunoprecipitation of Newly Synthesized PAI-1

After serum starvation and stimulation of the cells in culture, DME was replaced by DME devoid of methionine. After 30 minutes, [\(^3\)H]methionine (TransLabel; ICN, Irvine, Calif) was added at a final concentration of 100 μCi/mL. After an additional 60 minutes, the cells were transferred to fresh DME without radioactive methionine. One hour later, conditioned media, supplemented with 0.01% Tween 80, were harvested and stored at −20°C until immunoprecipitation was performed. The cells were washed with phosphate-buffered saline (PBS) and lysed with ice-cold buffer (10 mmol/L Tris [pH 7.4], 150 mmol/L sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mol/L phenylmethylsulfonyl fluoride, and 1 mol/L iodoacetate). Cell lysates were scraped, disrupted by sonication on ice for 1 minute, and centrifuged at 10,000g for 10 minutes. The supernatant fractions were stored at −20°C until immunoprecipitation was performed.

For immunoprecipitation,\(^\text{21}\) bovine serum albumin (final concentration, 0.1%), used to reduce nonspecific binding of proteins to antibodies, and mouse monoclonal anti-human PAI-1 antibody (American Diagnostica, Greenwich, Conn) in molar excess (which binds to active, latent, and TPA complexed PAI-1) were added to the samples. After incubation and gentle agitation at 4°C for 16 hours and subsequent incubation with agarose-linked goat anti-mouse IgG antibody (HyClone, Logan, Utah) at room temperature for 2 hours, antibody-bound PAI-1 was pelleted by centrifugation of the samples in a microcentrifuge. The pellets were washed in PBS with 0.1% SDS, 0.5% Nonidet P-40, and 0.1% deoxycholic acid twice; in PBS alone once; resuspended in reducing buffer; and heated at 100°C for 3 minutes. The supernatant fractions, which contained PAI-1, were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on stacking gels of 4% and separating gels of 10% acrylamide (BioRad, Richmond,
Calif; Protogel, National Diagnostics, Manville, NJ). After electrophoresis, the gels were stained with rapid Coomassie blue (Diversified Biotech, Boston, Mass), dried (Drygel Jr SE 540; Hoefer, San Francisco, Calif), and subjected to autoradiography at −70°C for selected intervals. Intensity of individual bands was quantified by laser densitometry (Ultrascan XL; Pharmacia LKB, Piscataway, NJ) and by radioisotopic scanning.

**Quantification of PAI-1 mRNA**

To obtain a probe for Northern blotting, plasmids containing human PAI-1 cDNA were purified from transfected E. coli by alkaline lysis and with Qiagen resins (Qiagen Plasmid Maxi Kit; Diagen, Hilden, Germany) and digested with the restriction enzymes, EcoRI and Sal I (Promega, Madison, Wis). The DNA fragments were separated on low melting temperature agarose gels (2% NuSieve GTG agarose; FMC BioProducts, Rockland, Me) in TAE buffer (40 mmol/L Tris acetate, 2 mmol/L EDTA [pH 8.5]). A 0.9-kb fragment containing PAI-1 mRNA was recovered with elutri-d minicolumns (Schleicher & Schuell, Keene, NH), precipitated with ethanol (Quantum, Tuscola, Ill) and glycogen (Boehringer Mannheim, Indianapolis, Ind) as a carrier, rinsed in 70% ethanol, and dissolved in TE buffer (10 mmol/L TrisCl, 1 mmol/L EDTA [pH 7.4]). Before hybridization, the PAI-1 cDNA was labeled with desoxycytidine 5'-[α-32P]triphosphate (Amersham, Arlington Heights, Ill) by the random oligonucleotide primer method (Random Prime DNA Labelling Kit; Boehringer Mannheim).22

For assay of PAI-1 mRNA, total cellular RNA was extracted with RNAzol B (Tel-Test, Friendswood, Tex) and chloroform (Fishcer), precipitated with isopropanol (Fishcer), washed with 75% ethanol, dissolved in 1 mmol/L EDTA (pH 7.0), and size fractionated (10 to 20 μg) on 1.5% formaldehyde agarose gels containing 0.53 μg/mL ethidium bromide in MOPS buffer (20 mmol/L MOPS, 5 mmol/L sodium acetate, 1 mmol/L EDTA [pH 7.0]). Northern blotting was performed by capillary transfer of RNA to nylon membranes (Biodyne B; Pall Filter, Vienna, Austria) with 20× SSC (3 mol/L sodium chloride, 0.3 mol/L sodium citrate [pH 7.0]). RNA was fixed by baking at 80°C for 1 hour in a vacuum. The integrity, equal loading, and transfer of RNA were verified by ethidium bromide staining of ribosomal RNA.

Prehybridization was performed at 42°C for at least 2 hours, and hybridization was performed at 42°C for 20 to 24 hours. Membranes were prehybridized in a solution of 50% deionized formamide, 10× Denhardt’s solution, 50 mmol/L Tris-HCl (pH 7.5), 1 mol/L sodium chloride, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate, and 100 μg/mL denatured calf thymus DNA. For hybridization, a labeled PAI-1 probe (500 000 cpm/mL) was added.23 Membranes were washed three times at room temperature for 5 minutes each in a solution of 1% SDS and 2× SSC followed by washing at 60°C for 20 minutes in fresh washing solution. Radioactivity of hybridized bands was quantified by radioisotopic scanning (AMBIS; Radioanalytic Imaging System, San Diego, Calif) with results confirmed and documented by autoradiography.

In additional internal control experiments, membranes were boiled in Northern strip buffer (10 mmol/L Tris [pH 8.0], 1 mmol/L EDTA [pH 8.0], 1% SDS) for 10 minutes to strip off probes and were rehybridized as above with a labeled glyceraldehyde-3-phosphate probe (GAP probe, 0.6-kb fragment after Xba I/HindIII dehydrogenase digestion, 200 000 cpm/mL).

**Additional Procedures**

Protein in conditioned media supplemented with 0.01% Tween 80 and in cell lysates was quantified with bicinchoninic acid (Pierce, Rockford, Ill) conventionally. To prepare lysates, cells were washed with PBS three times, incubated in filtered 0.5% Triton X-100 at room temperature for 1 hour, and sonified on ice for 1 minute. The supernatant fractions obtained after centrifugation at 12 000g at 4°C for 10 minutes were stored at −20°C until assay.

Purified mouse monoclonal anti-human insulin receptor antibody (clone MA 20) was purchased from Amersham. The antibody binds to an epitope on the α-subunit of the insulin receptor, inhibits the binding of insulin to its receptor, and induces no significant autophosphorylation of the insulin receptor. Rabbit polyclonal anti-human IGF-1 receptor antibody to an epitope on the α-subunit of the insulin-like growth factor-1 (IGF-1) receptor was obtained from Upstate Biotechnology (UBI, Lake Placid, NY). Both antibodies were added to media bathing the cells at least 1 hour before the addition of an agonist to be tested. IGF-1 was purchased from Collaborative Biomedical. Actinomycin D (from Streptomyces sp), cycloheximide (from Streptomyces griseus), and genistein were obtained from Sigma and dissolved immediately before use.

**Statistical Analysis**

Results are mean±SEM values. The significance of differences between groups of cells was assessed by one-way ANOVA and with Student’s t-tests. Significance was defined as P<.05.

**Results**

**Effects of Proinsulin, des(31,32)Proinsulin, des(64,65)Proinsulin, and C-Peptide on the Concentration of PAI-1 in Conditioned Media of Hep G2 Cells**

Hep G2 cells were exposed to media containing 10 mmol/L proinsulin, 10 mmol/L des(31,32)proinsulin, 10 mmol/L des(64,65)proinsulin, 10 mmol/L C-peptide, or vehicle alone (as a control) (n=3 for each condition). Samples of conditioned media (less than 2.5% of the volume of conditioned media each) were removed every 12 hours for up to 5 days. Stimulation with proinsulin induced a prompt increase in PAI-1 protein in the first 36 hours followed by a plateau for an additional 36 hours (with 25 ng/mL PAI-1 being the average) and a late increase between 72 and 96 hours (with 46 ng/mL as an average) (Fig 1). After 24 hours and at all subsequent intervals, PAI-1 was significantly increased with exposure of the cells to proinsulin or to des(31,32)proinsulin and des(64,65)proinsulin. The two proinsulin split products elicited an increase in PAI-1 that persisted for up to 60 hours with a “rapid” increase occurring between 12 and 36 hours (from 4 to 45 and from 3 to 50 ng/mL, respectively) and a more modest increase seen between 36 and 60 hours (from 45 to 60 and from 50 to 70 ng/mL.

**Fig 1.** Plot of concentration of plasminogen activator inhibitor type-1 (PAI-1) protein in conditioned media of highly differentiated human hepatoma cells (Hep G2) exposed to 10 mmol/L concentrations of proinsulin, des(31,32)proinsulin, des(64,65)proinsulin, C-peptide, or vehicle alone as a function of the duration of exposure.
respectively). After 60 hours, no further increase occurred, consistent with a general attenuation of protein synthesis over time in Hep G2 cells in culture under most conditions. Overall, the accumulation of PAI-1 induced by des(64,65)proinsulin appeared to be greater than that with des(31,32)proinsulin. Between 36 and 84 hours, both agonists induced significantly greater accumulation of PAI-1 compared with the increases induced by proinsulin itself. In contrast, Hep G2 cells exposed to C-peptide exhibited no increase in PAI-1.

To define concentration-response relations, Hep G2 cells were exposed to each agonist at concentrations of 0.1, 1, 10, and 100 nmol/L for 24 and 48 hours. Fig 2 shows the concentration-dependent increase of PAI-1 protein in conditioned media seen with proinsulin, des(31,32)-proinsulin, and des(64,65)proinsulin after 24 and 48 hours (n=3 for each). After 48 hours, 100 nmol/L proinsulin had elicited a 2.0-fold increase. des(31,32)Proinsulin (100 nmol/L) and 100 nmol/L des(64,65)proinsulin had elicited a 3.3-fold and a 4.5-fold increase, respectively. The threshold concentration of agonist was 10 nmol/L for proinsulin but only 1 nmol/L for the other two precursors of insulin. In contrast, C-peptide in concentrations as high as 100 nmol/L had no effect.
Effects of Proinsulin, des(31,32)Proinsulin, des(64,65)Proinsulin, C-peptide, or vehicle alone for 24 hours and for 48 hours.

**Effects of Proinsulin, des(31,32)Proinsulin, des(64,65)Proinsulin, and C-peptide on Overall Protein Synthesis**

To determine whether nonspecific effects on protein synthesis had been induced by the agonists studied, Hep G2 cells were exposed to 10 nmol/L proinsulin, des(31,32)proinsulin, des(64,65)proinsulin, C-peptide, or vehicle alone (n=3 each) for 24 and 48 hours. As shown in Fig 3, no significant increase in total protein in conditioned media was seen compared with values in control cells that had not been exposed to the agonists. Total protein increased in a generally linear fashion between 24 and 48 hours (from 0.26±0.02 to 0.55±0.03 mg/mL, P<.0005). Total protein in cell lysates did not increase in response to any of the agonists tested (1.48±0.05 mg/mL at 24 hours, 1.68±0.06 mg/mL at 48 hours). Thus, effects of the insulin precursors on PAI-1 synthesis were not simply a reflection of a general effect on overall protein synthesis.

**Table 1. PAI-1 Activity in Conditioned Media of Hep G2 Cells 24 or 48 Hours After the Addition of 10 nmol/L Proinsulin, 10 nmol/L des(31,32)Proinsulin, 10 nmol/L des(64,65)Proinsulin, 10 nmol/L C-peptide, or Vehicle Alone**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>PAI-1 Activity, arbitrary units/mL</th>
<th>24 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>1.3±0.4</td>
<td>1.1±0.1</td>
<td></td>
</tr>
<tr>
<td>Proinsulin</td>
<td>1.5±0.1</td>
<td>1.0±0.2</td>
<td></td>
</tr>
<tr>
<td>des(31,32)Proinsulin</td>
<td>3.9±0.11</td>
<td>2.0±0.2*</td>
<td></td>
</tr>
<tr>
<td>des(64,65)Proinsulin</td>
<td>3.0±1.1</td>
<td>1.7±0.2*</td>
<td></td>
</tr>
<tr>
<td>C-Peptide</td>
<td>1.6±0.2</td>
<td>1.3±0.2</td>
<td></td>
</tr>
</tbody>
</table>

PAI-1 indicates plasminogen activator inhibitor type-1; Hep G2, highly differentiated human hepatoma cells.

*P<.05, tP<.005.

Activities are minuscule with respect to total PAI-1 protein (which has a specific activity of approximately 0.5 arbitrary units/ng when fully active) because of the rapid conversion of active PAI-1 to latent PAI-1 and to inactive (cleaved) PAI-1 in conditioned media in the absence of addition of an exogenous binding protein such as vitronectin.42

**Effects of Proinsulin, des(31,32)Proinsulin, des(64,65)Proinsulin, and C-peptide on PAI-1 Activity**

After cells had been incubated with 10 nmol/L proinsulin, 10 nmol/L des(31,32)proinsulin, 10 nmol/L des(64,65)proinsulin, 10 nmol/L C-peptide, or vehicle alone for 24 and 48 hours, respectively, conditioned media were harvested and assayed for PAI-1 activity (n=3 for each condition). PAI-1 activity was low in each case (Table 1), although values were somewhat higher in cells exposed to the proinsulin split products. The dichotomy between activity and concentration of PAI-1 protein in conditioned media is consistent with rapid conversion of PAI-1 to a latent form and to inactive (cleaved) forms in conditioned media of cultured cells in the absence of exogenous vitronectin (t½ <2 hours at 37°C).24

**Effects of Proinsulin, des(31,32)Proinsulin, des(64,65)Proinsulin, and C-peptide on PAI-1 Synthesis**

Metabolic labeling of Hep G2 cells was performed to determine whether the precursors of insulin stimulated synthesis as well as secretion of PAI-1. The midpoint of the interval of incubation with labeled methionine corresponded to the 24- and 48-hour interval after the addition of proinsulin, des(31,32)proinsulin, or des(64,65) proinsulin (10 nmol/L each, n=6 each for conditioned media, n=3 each for cell lysates).

As shown in Fig 4, labeled PAI-1 was detectable in a band of approximately 50 kd.25 In conditioned media harvested 24 hours after addition of agonists, newly synthesized PAI-1 increased from 107±6 (control) by 1.6-fold to 174±15 (P=.002) with proinsulin, 1.5-fold to 165±12 (P=.002) with des(31,32)proinsulin, and 2.0-fold to 216±22 cpn (P<.001) with des(64,65)proinsulin. These increases were consistent with results in the concentration-response experiments [1.3-fold increase of PAI-1 protein with proinsulin, 1.7-fold with des(31,32)proinsulin, and 1.8-fold with des(64,65)proinsulin] and with increased plasma concentrations of PAI-1 in type II diabetic patients in comparison with those in nondiabetic subjects.2 After 48 hours, differences compared with values in controls were still present, consistent with the persistent elevation of PAI-1 mRNA (see later).

In lysates of cells incubated for 24 hours, the increases in newly synthesized PAI-1 seen after exposure of the cells to proinsulin and to proinsulin split products paralleled increases seen in conditioned media after 24 hours. Increases seen with des(64,65)proinsulin were most prominent (1.7-fold increase from 0.44±0.06 to 0.75±0.05 arbitrary units, P=.014). Thus, changes in accumulation of PAI-1 protein in conditioned media appeared to reflect changes in the rate of PAI-1 synthesis. This interpretation is supported by results of control experiments with cycloheximide, an inhibitor of protein synthesis, in which Hep G2 cells exposed to the agonists exhibited no increase in PAI-1 in conditioned media after 24 hours (Table 2).

**Effects of Proinsulin, des(31,32)Proinsulin, des(64,65)Proinsulin, and C-peptide on Steady-State Concentrations of PAI-1 mRNA**

The increases in PAI-1 synthesis could reflect changes in gene expression (transcription or degradation of mRNA), changes in translation, or both. To
assess potential effects on gene expression, Hep G2 cells were exposed to 10 nmol/L concentrations of the agonists tested (n=4 for each condition). After 24 and 48 hours, total cellular RNA was extracted, and PAI-1 mRNA was assayed. Two forms of PAI-1 mRNA were detected: a 3.2-kb and a 2.2-kb form. The ratio of the 3.2- to the 2.2-kb form was constant (0.9) under basal conditions and with stimulation (Fig 5). Steady-state levels of PAI-1 mRNA were increased 2.3-fold in 24 hours with exposure of cells to proinsulin (P=.005), 2.2-fold to des(31,32)proinsulin (P=.004), and 2.9-fold to des(64,65)proinsulin (P<.0005). C-peptide did not elicit any increase. Steady-state levels of GAP mRNA (used as an internal control) did not change significantly. Increases in PAI-1 mRNA persisted for 48 hours. Accordingly, the effects of the insulin precursors appeared to be mediated in part at the level of gene expression. Control experiments with actinomycin D, a specific inhibitor of transcription, were confirmatory in that this inhibitor completely attenuated the increases in PAI-1 protein in conditioned media over 24 hours (Table 2).

Mediation of Observed Effects by Insulin Receptors

To determine whether the effects observed were mediated in whole or in part by insulin receptors, a
monoclonal antibody against an epitope on the α-subunit of the insulin receptor capable of inhibiting the binding of insulin to its receptor yet not acting as a partial agonist or autophosphorylating the receptor was used. Hep G2 cells were exposed to 10 nmol/L concentrations of each agonist for 24 hours with 0, 1, 5, or 10 μg/mL of the insulin receptor antibody (n=3 for each condition). Insulin (10 nmol/L) was used as a control.

The insulin receptor antibody inhibited the increased accumulation of PAI-1 protein in conditioned media completely when proinsulin was the agonist and almost completely (86%) when des(31,32)proinsulin was the agonist (Fig 6). With des(64,65)proinsulin, the inhibition was 68%. Inhibition was even greater than that seen with insulin (47%). Thus, effects of the precursors of insulin appeared to be mediated, largely or exclusively, through the insulin receptor. This interpretation is corroborated by our finding that stimulation by the precursors was not additive to that induced by insulin. In fact, insulin attenuated the effects. In control experiments with a polyclonal receptor antibody to an epitope on the α-subunit of the IGF-1 receptor, no inhibition was seen. Effects of genistein,26 an inhibitor of tyrosine protein kinase that mediates diverse effects of both the insulin and the IGF-1 receptor, were not characterized because the conditions required were found to damage the Hep G2 cells.

Quantitative differences in the effects on synthesis of PAI-1 by the different precursors of insulin seen were not likely to be attributable to different affinities of the precursors proinsulin, des(31,32)proinsulin, and des(64,65)proinsulin to the insulin receptor as judged from the comparable effects of equimolar concentrations of each on the time course of increase and steady-state levels of PAI-1 mRNA. They do not appear to be attributable to conversion of any of the precursors to insulin as judged from the lack of appearance of insulin in the conditioned media based on SDS-PAGE studies and with autoradiography after addition of labeled proinsulin to the conditioned medium incubated over 48 hours (data not shown).

Vascular Endothelial and Smooth Muscle Cells

Human umbilical vein endothelial cells were exposed to 1 and 10 nmol/L concentrations of proinsulin, des(31,32)proinsulin, des(64,65)proinsulin, C-peptide, or vehicle alone (as a control) (n=3 each) for 24 hours. None of the agonists increased PAI-1 protein in conditioned media in comparison with results under control conditions. Human aortic smooth muscle cells were incubated with 0.1, 1, 10, and 100 nmol/L concentrations of the agonists used above for 24 and 48 hours (two different donors, n=3 each). Again, none of the agonists increased PAI-1 protein in conditioned media.

Discussion

Hep G2 cells were used in this study because hepatocytes appear to be the primary source of circulating PAI-1 and because Hep G2 cells are highly differentiated human cells with gross morphological and functional properties similar to those in hepatocytes in primary culture, including interactions of cell surface receptors with insulin27 and insulin-induced secretion of PAI-1.28-30

Conversion of proinsulin to insulin in vivo occurs primarily in immature secretory granules of pancreatic β-cells in reactions catalyzed by two types of calcium-dependent endopeptidases (trypsin-like and carboxypeptidase B-like endopeptidase) through a branched pathway. This type of proteolytic cleavage, involved in the processing of many eukaryotic secretory proteins, occurs on the carboxyl side of the two dibasic sections of the proinsulin molecule at the Arg31Arg32 and at Lys66Arg67 sites. Proinsulin is processed either to (32,33)split proinsulin and des(31,32)proinsulin or to (65,66)split proinsulin and des(64,65)proinsulin. Subsequently, each split product can be converted to insulin. The first pathway [formation of des(31,32)proinsulin] dominates. Because of the high activity of carboxypeptidase B in the pancreatic β-cell secretory granules, neither of the split proinsulins (in contrast to the des-split products) is readily detected within the pancreatic cells themselves in situ.31,32

Proinsulin has been given intravenously to human subjects. Over intervals as long as 24 hours, it is not converted appreciably to intermediates or to insulin, with 99% of the material remaining intact in plasma as proinsulin.31 In normal human subjects, the typical concentration of proinsulin in plasma is 0.002 nmol/L under fasting conditions and 0.010 nmol/L after an oral glucose load. Corresponding values for des(31,32)proinsulin are 0.002 nmol/L and 0.020 nmol/L. The two moieties together account for only

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**Table 2. PAI-1 Protein in Conditioned Media of Hep G2 Cells 24.5 Hours After the Addition of 2.5 μmol/L Cycloheximide or 0.2 μmol/L Actinomycin D and 24 Hours After the Addition of 10 nmol/L Proinsulin, 10 nmol/L des(31,32)Proinsulin, 10 nmol/L des(64,65)Proinsulin, or Vehicle Alone**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Control</th>
<th>Cycloheximide, 2.5 μmol/L</th>
<th>Actinomycin D, 0.2 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>37±4</td>
<td>10±2</td>
<td>16±1</td>
</tr>
<tr>
<td>Proinsulin</td>
<td>59±2*</td>
<td>11±1</td>
<td>20±1</td>
</tr>
<tr>
<td>des(31,32)Proinsulin</td>
<td>60±3*</td>
<td>12±1</td>
<td>18±1</td>
</tr>
<tr>
<td>des(64,65)Proinsulin</td>
<td>70±2†</td>
<td>12±1</td>
<td>20±2</td>
</tr>
</tbody>
</table>

PAI-1, plasminogen activator inhibitor type-1. n=3 for each, mean±SEM.

*P<.05, †P<.005.
The concentration of plasminogen activator inhibitor type-1 (PAI-1) mRNA in highly differentiated human hepatoma cells (Hep G2) exposed to 10 nmol/L concentrations of proinsulin, des(31,32)proinsulin, des(64,65)proinsulin, C-peptide, or vehicle alone for 24 hours and for 48 hours (*P < .05, **P < .005, ***P < .0005). Hybrized bands were quantified by radioisotopic scanning. A, Representative autoradiograms and the same membrane after ethidium bromide staining (24 hours), and B shows the total amount of PAI-1 mRNA (the sum of that in the 3.2- and 2.2-kb bands).

10% to 20% of immunoreactive insulin-like molecules (IRI). In nonobese patients with NIDDM, the fractional contributions increase to 14% for proinsulin and up to 45% for des(31,32)proinsulin under both fasting conditions [0.018 nmol/L for proinsulin and 0.056 nmol/L for des(31,32)proinsulin] and after an oral glucose load. In obese subjects with NIDDM, the two precursors of insulin account for as much as 66% of total IRI. Thus, much of the conventionally measured IRI reflects contributions of precursors rather than insulin per se in patients with NIDDM who are not being treated with insulin. The concentrations of agonists used in the present study were selected to be consistent with concentrations of precursors seen in patients with NIDDM. In vivo, hepatocytes may be exposed to even higher concentrations (as high as 7 nmol/L) of the precursors of insulin because portal venous blood drains directly from the pancreas into the liver.

"Hyperinsulinemia" (actually increased IRI), known to occur in subjects with NIDDM or other conditions characterized by insulin resistance including obesity and hypertension, appears to be a risk factor for coronary artery disease. In patients with NIDDM, the increased concentrations of IRI in plasma are paralleled by increased PAI-1. Such patients exhibit dysfunction of pancreatic β-cells characterized by elaboration of increased quantities of precursors of insulin that can comprise more than 50% of IRI in blood. Proinsulin is cleared more slowly from the circulation than insulin, thereby intensifying hyperproinsulinemia.

Some investigators have obtained information suggesting that the "hyperinsulinemia" in NIDDM actually reflects hyperproinsulinemia and hyperdes(31,32)proinsulinemia as judged from results of assays with highly specific immunoassays for each moiety.
Thus, “hyperinsulinemic” patients may, in fact, be insulin deficient. In view of these considerations, it is of particular interest that plasma PAI-1 declines (sic) in patients with type II diabetes given exogenous insulin, perhaps because concentrations in plasma of proinsulin and other precursors of insulin decline as stimulation of the pancreatic β-cells by hyperglycemia declines. In fact, concentrations of PAI-1 in plasma in type II diabetic patients correlate with concentrations of proinsulin and des(31,32)proinsulin rather than with concentrations of insulin per se.

The markedly (up to 18-fold) increased frequency of cardiovascular events observed when proinsulin was administered to patients for at least 1 year is consistent with a deleterious effect of hyperproinsulinemia on fibrinolysis mediated by increased PAI-1. Under such conditions, the concentration of proinsulin in plasma in the proinsulin-treated patients was as high as 8 nmol/L, a concentration similar to that used in the present study.

The results of the present study are consistent with the hypothesis that both the increased risk of cardiovascular disease associated with insulin resistance and “hyperinsulinemia” (defined in terms of increased IRI) and the association of increased PAI-1 with increased IRI may be attributable, in part, to effects of precursors of insulin on the fibrinolytic system and specifically on PAI-1 synthesis. They are consistent with the dichotomy between plasma PAI-1 in patients with NIDDM who are given exogenous insulin compared with plasma PAI-1 in those who are not and with the parallel changes in plasma PAI-1 and plasma proinsulin seen in patients with NIDDM not given exogenous insulin. They are consistent also with the possibility that precursors of insulin may contribute to vascular disease by impairing fibrinolysis, consequently increasing exposure of vessel walls to intermittent platelet activation and release of platelet- and clot-associated mitogens. They suggest that reduction of atherogenesis in patients with NIDDM and in those with other conditions characterized by insulin resistance may be possible by suppression of elaboration of precursors of insulin or by pharmacologic antagonism of the direct effects of precursors of insulin on PAI-1 synthesis.

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Augmentation of the synthesis of plasminogen activator inhibitor type-1 by precursors of insulin. A potential risk factor for vascular disease.

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