Augmentation of Synthesis of Plasminogen Activator Inhibitor Type-1 in Arterial Endothelial Cells by Glucose and Its Implications for Local Fibrinolysis

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Because of the frequent occurrence of premature cardiovascular disease in patients with non–insulin-dependent, type II diabetes mellitus (NIDDM), the attenuated fibrinolytic activity of plasma from type II diabetic patients with increased concentrations of plasminogen activator inhibitor type-1 (PAI-1), and the fact that insulin stimulates synthesis of PAI-1 by human hepatic cells in vitro, we and others have hypothesized that accelerated vascular disease in type II diabetes may result in part from impaired fibrinolysis secondary to excessive elaboration of PAI-1 stimulated by insulin. Alternatively, the hyperglycemia associated with type II diabetes could influence the synthesis and secretion of PAI-1 directly. The present study was performed to determine whether PAI-1 secretion is or is not sensitive to the prevailing concentration of glucose in the conditioned medium of endothelial and liver cells, which are thought to be the major sources of circulating PAI-1 in vivo. Confluent cells were exposed to 0, 2.8, 5.6, 11.1, or 22.2 mmol/L (0, 50, 100, 200, or 400 mg/dL) glucose in medium without serum and subsequently to media with or without insulin (7.3 nmol/L). Secretion of PAI-1 by highly differentiated human hepatoma (Hep G2) cells did not increase as a function of increasing concentrations of glucose, whether or not insulin was present. In contrast, with pig aortic endothelial cells, the secretion of PAI-1 increased significantly with extracellular glucose with or without insulin. The increases in PAI-1 were specific (as shown by metabolic labeling experiments) and not attributable to osmotic effects (as shown by replacement of glucose by sorbitol). Furthermore, the changes were paralleled by a specific, significant increase in the concentration of PAI-1 mRNA. These results indicate that increases in PAI-1 activity in type II diabetic patients are likely to be attributable to direct effects of glucose on the synthesis of PAI-1 by arterial endothelial cells, in addition to the effects of insulin on the synthesis of PAI-1 by liver cells. The effects of glucose on endothelial cells may contribute to reduced local fibrinolysis, thereby exacerbating atherogenesis.

KEY WORDS • hyperglycemia • fibrinolysis • atherogenesis • atherosclerosis • cardiovascular disease • type II diabetes • non–insulin-dependent diabetes mellitus

Accelerated atherosclerosis is associated with diverse conditions in which insulin resistance and hyperinsulinemia are prominent, including type II, non–insulin-dependent diabetes mellitus (NIDDM). Because the activity of plasminogen activator inhibitor type-1 (PAI-1), the primary physiological inhibitor of tissue-type plasminogen activator (TPA) in plasma, is increased in NIDDM and because pathophysiological concentrations of insulin stimulate PAI-1 synthesis in human hepatic cells (although not in human endothelial cells), we and others have hypothesized that the accelerated atherosclerosis may be secondary, in part, to an unfavorable shift in the balance between thrombosis and fibrinolysis. Under conditions of such an imbalance, cells lining the vessel walls may be exposed inordinately to clot-associated mitogens that can potentiate the migration and proliferation of vascular smooth muscle cells, chemotaxis and activation of macrophages, and, accordingly, atherogenesis. Complex atherosclerotic plaques frequently contain components of thrombi, a phenomenon consistent with this mechanism.

If impaired fibrinolysis is a contributor to accelerated atherosclerosis in patients with NIDDM and other conditions characterized by insulin resistance, then elucidation of the mechanisms responsible should facilitate the development of effective strategies for retarding the otherwise accelerated atherosclerosis. Unfortunately, however, it is not clear whether the increases in activity of PAI-1 and its concentration in plasma are a consequence of augmentation of PAI-1 synthesis by factors other than hyperinsulinemia per se.

We and others have shown that insulin increases PAI-1 synthesis directly in Hep G2 cells in vitro in concentrations that are seen in the plasma in patients...
with NIDDM.\(^4\)\(^5\) The present study was performed to determine whether or not increases in the concentration of glucose consistent with the hyperglycemia seen in NIDDM can influence PAI-1 synthesis directly. Because arterial and venous, fetal and adult endothelial cells may differ,\(^7\) we used adult aortic endothelial cells to simulate human arterial endothelial cells that are thought to be pivotal determinants of atherogenesis.

**Methods**

**Cell Cultures**

Hep G2 cells were acquired from the American Type Culture Collection (Rockville, Md) and grown to confluence in minimum essential medium with Earle's salts and L-glutamine (Life Technologies, Grand Island, NY) supplemented with 10% NuSerum (Collaborative Biomedical, Bedford, Mass), 30 U/mL penicillin, and 30 \(\mu\)g/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 and \(N\)-2-hydroxyethylpiperazine-\(N'\)-2-ethanesulfonic acid (DME, Washington University Medical School Tissue Culture Support Center, St Louis, Mo) for 16 hours to permit PAI-1 synthesis to decline to basal levels. Cells were exposed to selected concentrations of glucose in glucose-free DME supplemented with cell culture-grade D-glucose (Sigma Chemical, St Louis, Mo). After serum starvation of the cells was complete, the medium was changed so that cells could be exposed to medium constituted with cell culture-grade bovine insulin (Sigma) first dissolved in distilled water with 1% glacial acetic acid (at 10 mg/mL insulin, according to the manufacturer's recommendation) and then further diluted in DME with 10% fetal bovine serum (FBS, Life Technologies). The FBS was used to minimize the adherence of insulin to the storage and transfer devices. Its final concentration was 0.1%. Control experiments were performed with vehicle alone. All solutions and components were verified to be endotoxin free with the Limulus amebocyte assay (Pyrotell assay, Behring Diagnostics, Somerville, NJ) and the use of limulus amebocyte lysate (LAL, Cambrex, Walkersville, Md) according to the manufacturer's recommendation. Monolayers of confluent cells were serum starved in Dulbecco's modified Eagle's medium with Ham's nutrient mixture F-12 and \(N\)-2-hydroxyethylpiperazine-\(N'\)-2-ethanesulfonic acid (DME, Washington University Medical School Tissue Culture Support Center, St Louis, Mo) for 16 hours to permit PAI-1 synthesis to decline to basal levels.

In conditioned media of both Hep G2 and pig endothelial cells, the concentrations of glucose were verified spectrophotometrically in assays with hexokinase and glucose-6-phosphate dehydrogenase (SVR Glucose Test, Behring Diagnostics, Somerville, NJ) and the use of a glucose autoanalyzer (Gementi, Electro-Nucleonics, Fairfield, NJ). The concentrations of glucose in cell culture medium decreased slightly (by 0.9 to 1.3 mmol/L, or 17 to 23 mg/dL) over the 24 hours of incubation of endothelial cells. In conditioned media of Hep G2 cells, it declined by an average of 3.3 mmol/L (60 mg/dL) over 24 hours.

Experiments with pig endothelial cells were performed with cells in primary culture from one animal and repeated with primary cell cultures from different animals. Each type of experiment was performed in triplicate (ie, \(n=3\) for each condition).

**Quantification of PAI-1**

Conditioned media from Hep G2 and endothelial cells were supplemented with Tween 80 (final concentration, 0.01%). Cellular debris was removed by centrifugation at 4°C. Samples of conditioned media were stored at \(-20°C\) until assay.

The concentration of PAI-1 antigen in conditioned medium from Hep G2 cells (active, latent, and TPA-complexed PAI-1) was measured by enzyme-linked immunosorbent assay (ELISA) (TintElize PAI-1, Biopool, Umea, Sweden). Pig aortic endothelial cell PAI-1 antigen is not detected well with the ELISA used for the Hep G2 cell experiments. Because a suitable antibody for assay of porcine PAI-1 was not available, results with pig endothelial cells were obtained by spectrophotometric assay of PAI-1 activity with a chromogenic substrate, S-2251 (KabiVitrum, Stockholm, Sweden), plasminogen (Kabi, Mölndal, Sweden), and TPA (KabiVitrum).\(^8\)\(^9\)

**Quantification of PAI-1 mRNA**

After serum starvation for 2 days and subsequent incubation of the endothelial cells in fresh culture medium for an additional 24 hours, total cellular RNA was extracted with RNAzol B (Tel-Test, Friendswood, Tex) and chloroform (Fisher, Fair Lawn, NJ), precipitated by isopropanol and ethanol, and fractionated by size on 1.5% formaldehyde agarose gels (5 \(\mu\)g). Northern blots were performed by prehybridization at 42°C for 6 hours and hybridization at 42°C for 20 to 24 hours.\(^5\) The 0.9-kb cDNA probe used for PAI-1 (EcoRI and Sal I digestion) and the 1.1-kb cDNA probe used for 18S ribosomal RNA (18S rRNA) (EcoRI and BamHI digestion) were random-prime labeled with \(\alpha\)-\(^32\)P]dCTP (Amersham, Arlington Heights, Ill, and Boehringer Mannheim, Indianapolis, Ind). To ensure equal loading and transfer of RNA, staining with ethidium bromide was performed. The radioactivity of hybridized bands was quantified by radioisotopic scanning (AMBIS, Radioanalytic Imaging System, San Diego, Calif). Results were confirmed by autoradiography. To strip the probes, membranes were boiled in 10 mmol/L tris(hydroxymethyl)aminomethane (Tris), pH 8.0; 1 mmol/L EDTA, pH 8.0; and 1% sodium dodecyl sulfate (SDS) for 10 minutes.

**Quantification of Cellular DNA**

After serum starvation for 2 days and incubation for an additional 24 hours, the endothelial cells were trypsinized, washed in ice-cold phosphate-buffered saline, and resuspended in digestion buffer (100 mmol/L NaCl, 10 mmol/L Tris-Cl, pH 8.0; 25 mmol/L EDTA,
pH 8.0; 0.5% SDS; and 0.1 mg/mL proteinase K from Sigma). After incubation with gentle agitation at 50°C for 16 hours, DNA was extracted by phenol/chloroform/isooamyl alcohol (25:24:1, vol/vol/vol, saturated with 10 mmol/L Tris, pH 8.0, and 1 mmol/L EDTA; Sigma) and precipitated with 7.5 mol/L ammonium acetate and 100% ethanol. The pellets were rinsed with 70% ethanol and resuspended in TE buffer (10 mmol/L Tris-Cl and 1 mmol/L EDTA, pH 8.0).

The concentrations of solubilized DNA were measured fluorometrically (TKO 100 Mini Fluorometer, Hoefer, San Francisco, Calif; excitation wavelength, 365 nm; emission wavelength, 460 nm) in TEN buffer (10 mmol/L Tris, 1 mmol/L EDTA, and 100 mmol/L NaCl, pH 7.4) with bis-benzimidazole (a fluorochrome from Hoechst, No. 33258, highly specific for DNA, acquired from Calbiochem-Novabiochem, La Jolla, Calif) and calf thymus DNA as a standard (activated type XV, Sigma).

**Metabolic Labeling of Protein Synthesized by Cells in Culture**

To assay the rates of synthesis of cellular protein, cells were metabolically labeled. After serum starvation for 2 days and stimulation of the cells in culture for 23 hours, DME was replaced by DME devoid of methionine. After 30 minutes, the cells were exposed to fresh medium containing 50 μCi/mL [35S]methionine (Tran35S-Label, ICN, Irvine, Calif). After an additional 60 minutes, the cells were transferred to fresh DME with the usual concentration of unlabeled methionine but no radioactively labeled methionine. Protein in conditioned medium, supplemented with Tween 80, was precipitated with 12.5% (wt/vol) trichloroacetic acid. The cells were washed with phosphate-buffered saline and lysed with ice-cold buffer (10 mmol/L Tris, pH 7.4; 150 mmol/L NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS; 1 mmol/L phenylmethylsulfonyl fluoride; and 1 mmol/L iodoacetate). Cell lysates were scraped, disrupted by sonification on ice for 1 minute, and centrifuged at 10 000g for 10 minutes. The supernatant fractions were precipitated with 12.5% trichloroacetic acid. Radioactivity in the precipitates from conditioned media and cell lysates was assayed by liquid scintillation spectrometry.

**Statistical Analysis**

Results are means±SE. Differences were assessed with one-way ANOVA and Student's t tests. Significance was defined as P<.05.

**Results**

**Accumulation of PAI-1 by Hep G2 Cells Exposed to Selected Concentrations of Glucose**

The concentrations of glucose were selected to be in the range between mild hypoglycemia (2.8 mmol/L) and severe hyperglycemia (22.2 mmol/L) in NIDDM. It was recognized prospectively that medium devoid of glucose might compromise PAI-1 synthesis nonspecifically because of deprivation of a substrate needed for energy for cell metabolism. Accordingly, our analysis of the potential response of PAI-1 synthesis to augmentation of the concentration of glucose focused on the changes elicited by concentrations >2.8 mmol/L (50 mg/dL).

Monolayers of confluent Hep G2 cells were exposed to 0, 2.8, 5.6, 11.1, 16.7, or 22.2 mmol/L (0, 50, 100, 200, 300, or 400 mg/dL) glucose in conditioned medium devoid of serum for 16 hours and in fresh media with or without insulin during subsequent incubations for 24 hours. PAI-1 synthesis was assayed after exposure of the cells either to 7.3 nmol/L (1000 μU/mL) insulin, a concentration to which hepatocytes are likely to be exposed in vivo by portal venous blood draining directly from the pancreas into the liver, or to vehicle alone. PAI-1 was assayed in the conditioned medium to define the magnitude of increases induced by glucose or insulin.

The concentration of PAI-1 protein in conditioned medium did not increase when the concentration of glucose was increased from 2.8 to 22.2 mmol/L (Fig 1).
The decreases in concentrations of glucose observed (decreases of 1.3 mmol/L with 2.8 and 5.6 mmol/L glucose, 1.2 mmol/L with 11.1 mmol/L, and 0.9 mmol/L with 22.2 mmol/L) were similar to those seen by others.

As shown in Fig 2, PAI-1 activity was 12.0±2.4 arbitrary units (AU)/mL under basal conditions when glucose was absent from the conditioned medium. With 2.8 mmol/L glucose, PAI-1 activity increased to 16.6±0.8 AU/mL, presumably because of the greater availability of energy for protein biosynthesis. Increases in the concentration of glucose to 5.6, 11.1, and 22.2 mmol/L led to further increases in PAI-1 activity in the presence and absence of insulin.

Thus, values were consistently close to the concentrations of PAI-1 seen with 2.8 and 5.6 mmol/L glucose (41±3 ng/mL). Insulin did not elicit accumulation of PAI-1 in the absence of glucose (29±3 ng/mL). PAI-1 without insulin and 32±4 ng/mL, respectively, did not increase PAI-1 mRNA compared with that seen by others in Hep G2 cells.

At the highest concentration of glucose (22.2 mmol/L), PAI-1 actually declined modestly in the presence and absence of insulin.
in which DNA was measured. The results are shown in the Table, with those of PAI-1 mRNA values normalized for DNA. The total amount of DNA per culture flask remained virtually constant regardless of the concentration of glucose. This observation is consistent with the results by Kaiser et al, who exposed confluent calf aortic endothelial cells to selected concentrations of glucose for 24 to 48 hours and saw no change in cell number. A decrease in cell number and thymidine incorporation was seen by Maiello et al, in human umbilical vein endothelial cells exposed to high concentrations of glucose. This decrease may have occurred because of the long incubations in medium (13±4 days for primary cultures).

To verify the consistency of loading and transfer of RNA to the membranes, additional experiments were performed in which 18S rRNA was quantified. The membranes with the size-fractionated RNA used for PAI-1 mRNA assay were stripped first because the 18S rRNA bands (at 1.9 kb) would have interfered with the 2.2-kb PAI-1 bands. Thus, after stripping, the membranes were rehybridized with a cDNA probe for 18S rRNA. No significant glucose-dependent changes in 18S rRNA concentrations were evident (Table). In fact, the PAI-1 mRNA to 18S rRNA ratio increased with increasing concentrations of glucose, indicative of a relatively specific effect of glucose on the concentration of PAI-1 mRNA.

**Effects of Glucose on Overall Protein Synthesis in Endothelial Cells**

Metabolic labeling of all newly synthesized protein was performed to identify potentially nonspecific effects of glucose on the synthesis of protein. Metabolic labeling was preferred to chemical quantification of total protein content in conditioned medium because of the possible interference by the protein in FBS in the conditioned medium that was used to avoid unspecific binding. Confluent pig aortic endothelial cells were serum starved for 2 days and stimulated with 7.3 mmol/L insulin or vehicle alone for the subsequent 23 hours in media with 2.8, 5.6, 11.1, or 22.2 mmol/L (50, 100, 200, or 400 mg/dL) glucose, 0.5 hour in methionine-free medium, and 1 hour in the same medium spiked with labeled methionine. These concentrations of glucose were maintained throughout the incubations with methionine-free medium and with the medium containing labeled methionine. At the midpoint of the incubation with labeled methionine, the duration of exposure of the cells to medium corresponded to that preceding harvesting of conditioned medium for assay of PAI-1 and harvesting of RNA in the experiments described above. Assay of cell lysates 1 hour and of conditioned media 24 hours after the addition of labeled methionine did not indicate any influence of changes in the concentration of glucose on protein biosynthesis. Exposure of the cells to insulin elicited a modest, nonsignificant increase in overall protein biosynthesis.

**Discussion**

The present study was performed to identify the potential contribution of hyperglycemia, as opposed to hyperinsulinemia, to the increases in PAI-1 in plasma seen in hyperinsulinemic, type II diabetic patients. Because the liver and endothelium are considered to be the primary sources of plasma PAI-1, we studied Hep G2 cells and pig aortic endothelial cells as model systems. The Hep G2 cell line is an “immortal,” well-differentiated human hepatoma cell line that responds similarly to human hepatocytes in primary culture in terms of induction of PAI-1 synthesis by insulin. The pig aortic endothelial cells were used in primary culture only because secretion of PAI-1 by endothelial cells is consti-

<table>
<thead>
<tr>
<th>Glucose, mmol/L</th>
<th>PAI-1 mRNA, cpm</th>
<th>DNA, μg</th>
<th>PAI-1 mRNA to DNA Ratio, cpm</th>
<th>18S rRNA, cpm</th>
<th>PAI-1 mRNA to 18S rRNA Ratio, cpm</th>
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<tbody>
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<td>2.8</td>
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<td>19±2</td>
<td>159±10</td>
<td>548±130</td>
<td>159±10</td>
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<td>220±3</td>
<td>525±21</td>
<td>242±3</td>
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<td>20±2</td>
<td>247±8</td>
<td>571±64</td>
<td>250±8</td>
</tr>
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<td>22±3</td>
<td>241±10</td>
<td>525±117</td>
<td>292±11</td>
</tr>
</tbody>
</table>

PAI-1 mRNA activator inhibitor type-1 mRNA in pig aortic endothelial cells was exposed to 2.8, 5.6, 11.1, or 22.2 mmol/L (50, 100, 200, or 400 mg/dL) glucose during serum starvation for 48 hours followed by 24-hour incubations. Results are means±SE. PAI-1 mRNA values that are normalized for DNA contents and concentrations of 18S rRNA are shown in columns 4 and 6.
factor for atherosclerotic vascular disease. In 1979, the International Collaborative Group concluded that asymptomatic hyperglycemia should not be considered an independent risk factor for coronary artery disease or other major types of adult cardiovascular disease. Subsequently, increased mortality attributable to coronary artery disease has been attributed to hyperglycemia (Tecumseh Study). Some have reported that both fasting and postprandial hyperglycemia are independent risk factors. Elevated HbA1c, an index of hyperglycemia over time, appears to be associated with an increased risk of coronary disease in women but not men in the Framingham Heart Study.

Microvascular complications of diabetes such as retinopathy are associated with hyperglycemia. However, the pathophysiological link between NIDDM and microvascular manifestations such as coronary artery disease remains obscure. We and others have considered the possibility that the hyperinsulinemia typical of NIDDM may be a significant contributor to microvascular complications. Because insulin can increase expression of PAI-1 in cells of hepatic origin, even at high concentrations of glucose as shown in the present study, hyperinsulinemic subjects may be prone to excessive deposition or persistence of microthrombi secondary to attenuated fibrinolysis. This in turn may exacerbate atherosclerosis. In addition, as shown in the present study, glucose can increase the expression of PAI-1 in arterial endothelial cells. Thus, hyperglycemic diabetic patients may be at additional risk for accelerated atherosclerosis as a consequence of impaired fibrinolysis at the interface between the blood and the arterial wall. Accordingly, our results suggest that normalization of endogenous fibrinolysis may be possible by interdiction of potentially deleterious effects of both hyperinsulinemia and hyperglycemia on the fibrinolytic system.

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