Stimulation by Proinsulin of Expression of Plasminogen Activator Inhibitor Type-I in Endothelial Cells

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In patients with non-insulin-dependent diabetes mellitus, concentrations in plasma of insulin and its precursors, proinsulin and split proinsulin, are increased. Because increased concentrations of plasminogen activator inhibitor type-1 (PAI-1) occur also, we hypothesized that proinsulin and split proinsulin may augment endothelial cell PAI-1 expression, thereby potentially attenuating endogenous fibrinolysis and accelerating atherosclerosis. Proinsulin increased PAI-1 activity in conditioned media of endothelial cells as did split proinsulin, paralleled by increased expression of PAI-1 mRNA. These effects of proinsulin were not dependent on its conversion to insulin nor on its interactions with the insulin receptor. The proinsulin stimulation of PAI-1 expression was not attenuated by either anti-insulin receptor antibodies or a 100-fold excess of insulin. Furthermore, proinsulin-mediated increases in PAI-1 expression were not inhibited by a 500-fold excess of insulinlike growth factor I. In addition, inhibition of tyrosine kinase, which mediates many of the diverse effects of insulin and insulinlike growth factor I, did not attenuate the effect of proinsulin. These results indicate that proinsulin augments PAI-1 expression, potentially contributing to vasculopathy in patients with non-insulin-dependent diabetes mellitus.
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Diabetes mellitus often has been considered a manifestation of insulin deficiency. However, glucose intolerance precedes overt diabetes by decades (1). Subjects with impaired glucose tolerance have increased concentrations of immunoreactive insulin in plasma (2). Alternatively, hyperinsulinemia may reflect inadequate suppression of endogenous hepatic glucose output and its impact on pancreatic islet cell function (3).

Although insulin secretion in normal subjects generally is accompanied by a release of modest amounts of proinsulin and split proinsulin, the affinity of both for insulin receptors is markedly less than that of insulin (4–7). Proteolytic conversion of proinsulin to intermediates (i.e., 32–33 split proinsulin and 65–66 split proinsulin) and to insulin can occur in the secretory granules of pancreatic β-cells and in the circulation (8). The primary intermediate is 32–33 split proinsulin.

Patients with non-insulin-dependent (type II) diabetes mellitus manifest elevated concentrations of insulin precursors in plasma. Proinsulin and split proinsulin, which account for 10–20% of plasma immunoreactive insulin levels in normal subjects, can account for up to 67% of plasma immunoreactive insulin levels in type II diabetic patients (8–12).

The incidence of cardiovascular disease increases significantly in diabetic patients (13). Furthermore, concentrations in plasma of plasminogen activator inhibitor type-1 (PAI-1), a serine protease inhibitor that attenuates fibrinolytic activity and potentiates thrombosis (14–19), are augmented (20–25). We have shown recently that insulin stimulates production of PAI-1 in a human hepatoma cell line (HepG2) and that insulin plus insulin-like growth factor I (IGF-I) act synergistically (26). In this study, we demonstrate that proinsulin increases elaboration of PAI-1 by endothelial cells, independent of insulin and IGF-I, thereby potentially contributing to attenuation of fibrinolysis, increased thrombosis, and atherogenesis.

RESEARCH DESIGN AND METHODS

Cell culture. Endothelial cells were isolated from aortas (27) obtained from 30–60 kg farm pigs that were anesthetized with ketamine and xylacine and anticoagulated with heparin before cardiac arrest was induced with KCl.

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The procedure was consistent with a protocol approved by the Washington University Animal Studies Committee. A segment of aorta was perfused with collagenase (Worthington, Freehold, NJ) and incubated for 20 min at 37°C. Effluents were collected and centrifuged at 250 *g*. The cells were suspended in M199 with 20% fetal bovine serum (Gibco, Grand Island, NY), 50 μg/ml endothelial cell growth supplement (Collaborative Research, Waltham, MA), 30 μg/ml streptomycin, and 30 U/ml penicillin (Gibco, Grand Island, NY). They were plated (coating C, Tekmat) and stained immunocytochemically with a mouse monoclonal antibody directed against von Willebrand antigen (American Diagnostica, Greenwich, CT). Detectable von Willebrand antigen was expressed in >93% of cells.

Experiments were performed with confluent monolayers of primary culture cells within 7 days. After becoming confluent, cells were washed with phosphate-buffered saline and placed in serum-free media, Dulbecco's modified Eagle's medium with Ham's nutrient mixture F12 (DME) with 50 μg/ml endothelial cell growth supplement. After 2 days in serum-free media, an interval sufficient to lower secretion of PAI-1 mRNA to basal levels, the cells were exposed to selected agents in changed media. In each case, results pertain to multiple cell cultures from numerous animals.

Human recombinant proinsulin (Sigma, St. Louis, MO) and bovine insulin (Sigma, St. Louis, MO) were resuspended in DME with 10% fetal bovine serum. Concentrations of insulin and proinsulin were kept high to minimize the effects of adding fetal bovine serum (≤0.2%). Human recombinant IGF-I (Collaborative Research, Waltham, MA) was resuspended in phosphate-buffered saline. Split proinsulin was produced as previously described (8) by exposing proinsulin to trypsin (Gibco, Grand Island, NY) at 37°C for 15 min.

Polyclonal rabbit anti-human insulin receptor antibody (α subunit; Upstate Biotechnology, Inc., Lake Placid, NY) was used to block the insulin receptor. Genistein (Upstate Biotechnology, Inc.) was used as a specific inhibitor of tyrosine kinase (40 μg/ml for 30 min; 28).

Quantification of PAI-1 protein. Conditioned media supplemented with 0.01% Tween 80 was centrifuged to remove cellular debris, and the supernatant fraction was stored at −20°C until assay. Functional activity of PAI-1 was measured spectrophotometrically with the chromogenic substrate S-2251 (KabiVitrum, Uxbridge, Middlesex, UK) (29).

Metabolic labeling was performed to verify that proinsulin did not cause a general increase in protein synthesis. DME with [^{35}S]methionine (ICN, Montreal, Quebec, Canada) was added after cells had been maintained in serum-free media for 2 days. Conditioned media was harvested 24 h after exposure to proinsulin, split proinsulin, or vehicle alone for precipitation of protein with trichloroacetic acid and assay of overall protein synthesis (30).

Determination of PAI-1 mRNA. Total cellular RNA was isolated by chloroform/phenol purification (RNAzol, Teltest Industries, Friendswood, TX). RNA (5 μg) was size-fractionated with 1.5% formaldehyde agarose gels and assayed by Northern blotting (31). Prehybridization and hybridization were performed (26) with a 0.9 kb cDNA probe for human PAI-1 (EcoRI, SalI digestion) and a 0.6 kb cDNA probe for human glyceraldehyde-3-phosphate dehydrogenase (XbaI, HindIII digestion). Hybridization with glyceraldehyde-3-phosphate dehydrogenase was performed to verify equal loading and transfer of RNA. Total radioactivity of hybridized bands was quantified by radioisotopic scanning (AMBIS Scanner, Automated Micribiology Systems) and autoradiography.

Radiolabeling of proinsulin. Proinsulin was labeled with Na^{125}I (Amersham, Arlington Heights, IL) with chloramine T (Sigma, St. Louis, MO). Free ^{125}I was removed by Sephadex G-25 chromatography (Sigma). Radiolabeled proinsulin was added to serum-free media. Uptake and degradation of proinsulin were delineated by immunoprecipitation of the conditioned media with guinea pig anti-insulin antibody (Biospacific, Emeryville, CA) after 1, 3, 6, and 24 h. The antigen-antibody complexes were isolated with protein A sepharose (Pharmacia, Piscataway, NJ). ^{125}I-proinsulin was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), sensitivity = 0.1 ng).

Analysis of data. Effects of insulin plus proinsulin and IGF-I plus proinsulin on PAI-1 activity in conditioned media were delineated using two mathematical models—the isobole (32) and the median effect model (33). With the isobole model, synergy, noninteraction, and antagonism are defined for two agents, *a* and *b*, as $a/A_e + b/B_e = r$, where *a* is the dose of *a* used in combination with *b*, A_e is the equieffective dose of *a* alone, *b* is the dose of *b* in combination with *a*, B_e is the equieffective dose of *b* alone, and *r* is the sum of the ratios ($r < 1$ identifies synergy, $r = 1$ identifies noninteraction, and $r > 1$ identifies antagonism).

The median effect model analyzes the combination of two agents for synergy, antagonism, or noninteraction regardless of whether the dose effect curves are first order or higher order (33). With the median effect model, the nature of an interaction for two agents (1 and 2) is defined as follows

$$\frac{D_1}{DM_1} + \frac{D_2}{DM_2} + \frac{D_1}{DM_1} + \frac{D_2}{DM_2} = r$$

where *D* is the dose of either agent necessary to produce ED_{50} in a fixed molar ratio with both agents, and *DM* is the dose of either agent 1 or 2 required to produce the same ED_{50} when the agent is used alone. The ED_{50} is defined with the plot of the log of the fraction affected divided by the fraction unaffected versus the log of the concentration of agents added. A maximal PAI-1 activity is arbitrarily defined to derive the fraction affected and unaffected. Once again, $r < 1$ identifies synergy, $r = 1$ identifies noninteraction, and $r > 1$ identifies antagonism.

Data were expressed as means ± SE. Univariate and multivariate analyses were used to compare results in control cells with those in cells exposed to selected agents. One-tailed Student's *t* tests were used to define significance ($P < 0.05$) with the isobole and median effect models.

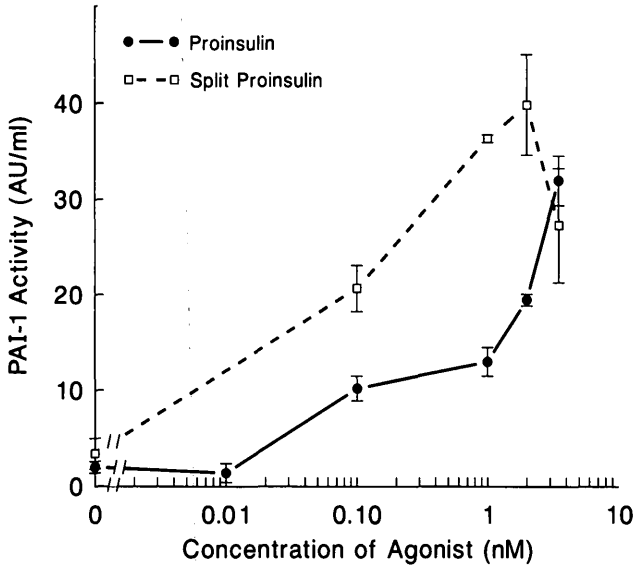

FIG. 1. PAI-1 activity in porcine aortic endothelial cells in conditioned media 24 h after adding proinsulin or split proinsulin (means ± SE). All increases over control (0 nM) were significant ($P < 0.05$) except for those seen with 0.01 nM proinsulin.

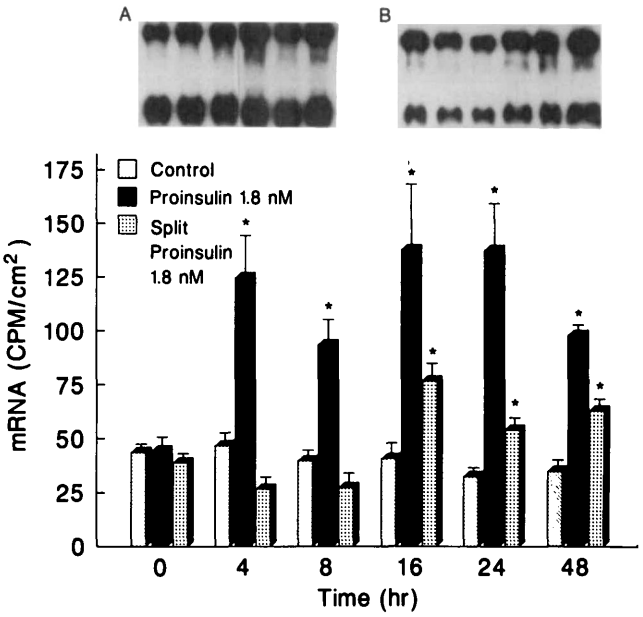

FIG. 2. PAI-1 mRNA expression in porcine aortic endothelial control cells and cells exposed to 1.8 nM proinsulin or 1.8 nM split proinsulin. The inset shows a representative autoradiogram (*A*, proinsulin; *B*, split proinsulin). The top 2 bands are 3.2 and 2.2 kb PAI-1 mRNA that were summed for quantification. The third band is glyceraldehyde-3-phosphate dehydrogenase mRNA. Values are means ± SE. * $P < 0.02$ compared with control mRNA.

RESULTS

Effects of proinsulin on expression of PAI-1 by endothelial cells. Confluent monolayers of endothelial cells that had been in serum-free media for 2 days were exposed to proinsulin and split proinsulin at selected concentrations. Conditioned media were harvested after 24 h. As Fig. 1 shows, cells exposed to proinsulin or split proinsulin elaborated more PAI-1 activity into conditioned media than control cells ($n = 3$ for each condition). Concentrations of proinsulin present in plasma of normal subjects (0.01 nM) did not augment PAI-1 activity. A 10-fold increase, from 2.0 ± 0.6 to 19.5 ± 0.6 AU/ml ($P < 0.001$), was seen when the proinsulin concentration was increased from 0 to 1.8 nM. Similarly, a 10-fold increase in PAI-1 activity was seen when the concentration of split proinsulin was increased from 0 to 1.0 nM (control, 3.4 ± 1.6; split proinsulin 1.0 nM, 36.4 ± 0.2 AU/ml, $P < 0.001$). Metabolic labeling with [^{35}S]methionine demonstrated that neither proinsulin nor split proinsulin increased overall protein production.

Total cellular RNA was harvested 4, 8, 16, 24, and 48 h after adding the proinsulin and split proinsulin ($n = 3$ for each). PAI-1 mRNA was detected as two species (3.2 and 2.2 kb), with the 3.2 kb species comprising 80–90% of total PAI-1 mRNA. RNA from control cells (treated with vehicle only) displayed stable total (3.2 + 2.2 kb) PAI-1 mRNA expression over 16 h, with a slight decrease in PAI-1 mRNA over 48 h (Fig. 2).

PAI-1 mRNA began to increase 4 h after exposure of cells to 1.8 nM proinsulin. The maximal increase in PAI-1 mRNA occurred over a 16- to 24-h interval (Fig. 2). A fourfold increase in total PAI-1 mRNA, from 32.6 ± 2.5 to 137.9 ± 20.2 CPM/cm^2 ($P < 0.005$), was seen 24 h after exposing cells to proinsulin. Split proinsulin augmented PAI-1 mRNA expression as well. PAI-1 mRNA did not increase until 16 h after adding 1.8 nM split proinsulin. A twofold increase in total PAI-1 mRNA, from 40.9 ± 5.7 CPM/cm^2 to 76.9 ± 6.6 CPM/cm^2 ($P < 0.01$), occurred 16 h after adding 1.8 nM split proinsulin. PAI-1 mRNA expression remained significantly elevated over a 48-h interval (Fig. 2). Both proinsulin and split proinsulin augmented concentrations of the 3.2 and 2.2 kb species of PAI-1 mRNA similarly.

Interaction of proinsulin with insulin and IGF-I. Both insulin (Fig. 3) and IGF-I (Fig. 4) independently stimulated PAI-1 expression by porcine aortic endothelial cells. Insulin increased PAI-1 activity by 6- to 12-fold (Fig. 3). IGF-I increased PAI-1 activity by 2- to 7-fold (Fig. 4). The known biological effects of proinsulin are mediated through the insulin receptor, with proinsulin having <20% of the activity of insulin (4–7). Nevertheless, proinsulin and insulin were equieffective in stimulating PAI-1 expression by endothelial cells (Fig. 3). Furthermore, the combination of insulin and proinsulin (1:1 molar ratio) resulted in additive effects. Both isobole and median effect model analyses indicated that proinsulin was stimulating PAI-1 expression through a pathway independent of insulin (isobole, $r = 0.78 \pm 0.15$, NS compared with $r = 1$; median effect method, $r = 0.26 \pm 0.01$, $P < 0.01$ compared with r = 1). In confirmation of this hypothesis, 1.0 nM proinsulin added to media containing 100 nM insulin increased PAI-1 activity (PAI-1 with 100 nM insulin, 27.3 ± 3.5 vs. PAI-1 with 100 nM insulin and 1.0 nM proinsulin, 53.7 ± 6.3 AU/ml, $P < 0.03$).

Proinsulin and IGF-I exhibit ~50% structural homology (34), and proinsulin is more effective in stimulating PAI-1 expression than IGF-I (Fig. 4). When proinsulin was combined with IGF-I in a 1:20 molar ratio, additive effects were seen (Fig. 4). Noninteraction between proinsulin

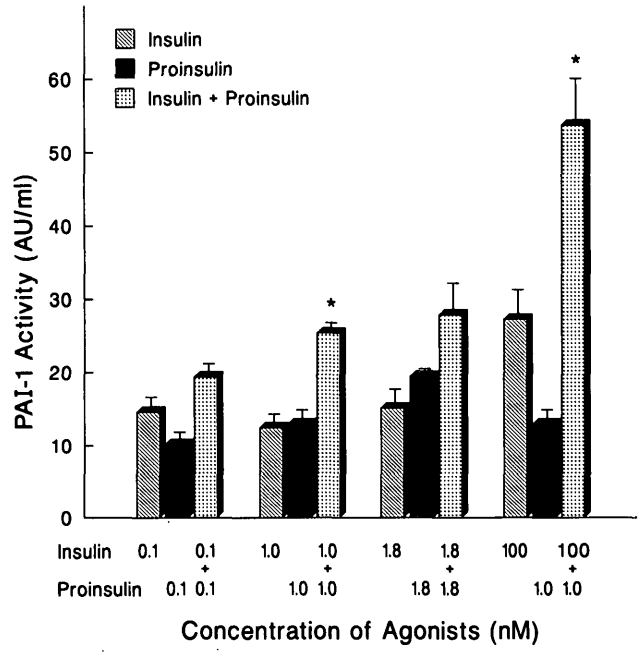

FIG. 3. PAI-1 activity in porcine aortic endothelial cells in conditioned media 24 h after adding of insulin alone, proinsulin alone, and the combination of insulin and proinsulin. PAI-1 activity in media from control cells averaged 2.0 ± 0.6 AU/ml. Values are means ± SE. * $P < 0.02$ compared with insulin alone and with proinsulin alone.

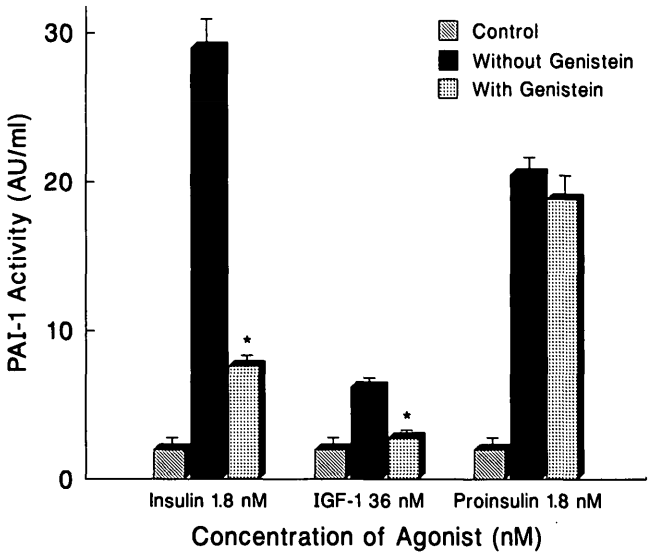

FIG. 5. PAI-1 activity in porcine aortic endothelial cells in conditioned media 24 h after adding insulin, IGF-I, and proinsulin with and without exposure of the cells to 40 μg/ml of genistein for 30 min at time 0. Values are means ± SE. * $P < 0.01$ compared with results in cells that were not exposed to genistein.

and IGF-I was evident with both the isobole ($r = 0.94 \pm 0.44$, NS compared with $r = 1$) and the median effect models ($r = 0.95$, NS compared with $r = 1$). Even with a 500-fold excess of IGF-I, proinsulin augmented PAI-1 activity (500 nM IGF-I, 16.4 ± 0.8 AU/ml vs. 500 nM IGF-I and 1.0 nM proinsulin, 40 ± 1.3 AU/ml, $P < 0.001$).

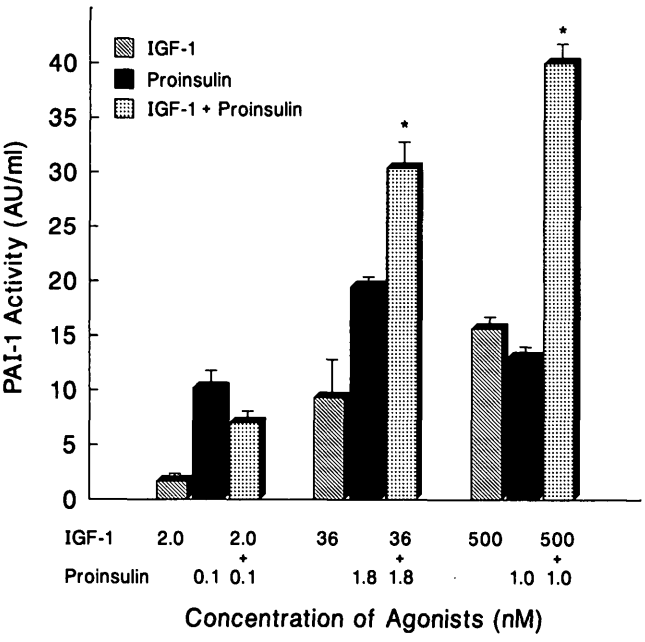

FIG. 4. PAI-1 activity in porcine aortic endothelial cells in conditioned media 24 h after adding IGF-I alone, proinsulin alone, and the combination of IGF-I and proinsulin. PAI-1 activity in media from control cells averaged 2.0 ± 0.6 AU/ml. Values are means ± SE. * $P < 0.01$ when compared with IGF-I alone and with proinsulin alone.

Effects of proinsulin despite inhibition of insulin and IGF-I receptors. A polyclonal insulin receptor antibody directed against the external (α) subunit of the insulin receptor did not prevent augmentation of PAI-1 expression in the presence of 1.0 nM insulin with up to 30 nM concentration. In fact, a 300 nM concentration of the insulin receptor antibody independently increased PAI-1 expression. However, the high concentration of antibody did inhibit the effect of 1.0 nM insulin (control, 0%; receptor antibody 300 nM, 28.4 ± 8%; insulin 1.0 nM, 100%; antibody plus insulin, 30.8 ± 1.8%, $P < 0.001$; $n = 3$ for each). In contrast, when cells were exposed to high concentrations of the insulin receptor antibody (300 nM), proinsulin (1.0 nM) induced a threefold increase (276 ± 18%, $P < 0.001$) in PAI-1 activity. Both monoclonal and polyclonal IGF-I receptor antibodies directed against the α-subunit increased PAI-1 expression in a manner similar to that seen with IGF-I alone. The effects of proinsulin and insulin were additive to effects of the antibody alone; thus, the effect of the insulin receptor antibody appeared to be specific for insulin.

Although the mechanisms of signal transduction for insulin and IGF-I receptors remain obscure, tyrosine kinase appears to be the transducing pathway (35,36). Inhibition of tyrosine kinase by the protein genistein significantly reduced augmentation of PAI-1 activity by 1.8 nM insulin (82% reduction in PAI-1 activity, $P < 0.01$). In addition, it reduced the augmentation of PAI-1 expression by 36 nM IGF-I (100% reduction in PAI-1 activity, $P < 0.01$). In contrast, genistein did not significantly modify the effect 1.8 nM proinsulin has in stimulating PAI-1 activity (Fig. 5).

Uptake and degradation of ^{125}I-proinsulin by endothelial cells. The ^{125}I-proinsulin used was not contam-

inated with insulin or C-peptide judging from results of SDS-PAGE. During the formation of split proinsulin, <10% of the proinsulin was converted to insulin and C-peptide. Immunoprecipitation of conditioned media with an anti-insulin antibody 1, 3, 6, and 24 h after adding either 18 nM or 1.8 nM proinsulin resulted in no detectable conversion of the proinsulin to insulin ($n = 3$ for each). The half-life of ^{125}I-proinsulin, determined by trichloroacetic acid precipitation of conditioned media, was 79 h. After 24 h, 78% of the ^{125}I-proinsulin was still present and intact in the conditioned media.

DISCUSSION

In this study, porcine aortic endothelial cells in primary culture were used for several reasons, including: *1*) proinsulin, insulin, and IGF-I are highly homologous in pigs and humans (37,38); *2*) atherosclerosis develops similarly in pigs and humans (39); *3*) pigs, like humans, become hyperinsulinemic with increasing body weight (40); and *4*) endothelial cells exhibit specialized functions related to their site of origin that may decay when cells are maintained in culture for prolonged intervals (41). Our results indicate that proinsulin and split proinsulin increase endothelial cell expression of PAI-1 mRNA with marked increases in PAI-1 activity.

Although concentrations of proinsulin and split proinsulin in blood are typically elevated in type II diabetic patients, the biological activity of these precursors has been considered limited because of limited interaction with insulin receptors (4–12). Alessi et al. (17) found that proinsulin exhibited only 20% of insulin's stimulation of PAI-1 expression in HepG2 cells. In contrast, as shown in this study, insulin and proinsulin are equally effective in stimulating expression of PAI-1 activity by porcine aortic endothelial cells. Neither agent augmented PAI-1 expression in concentrations present in plasma of normal subjects, but both increased PAI-1 activity by approximately 10-fold in concentrations seen in plasma of type II diabetic patients.

Our results indicate that the proinsulin effect on PAI-1 expression is independent of the insulin receptor and of contamination of the proinsulin used by insulin or its conversion to insulin in vitro. The additive effect of proinsulin combined with insulin, despite a 100-fold excess of insulin, is consistent with these observations, as is the failure of insulin receptor antibodies and inhibition of tyrosine kinase by genistein to attenuate the proinsulin effect.

Proinsulin exhibits considerable structural homology with IGF-I (34), which we have found can stimulate expression of PAI-1. Gruppuso et al. (42) showed that the affinity of proinsulin for the IGF-I receptor is only 0.01% of that of IGF-I and that the affinity of split proinsulin is <5% of that of IGF-I. Our observation that adding proinsulin to IGF-I augments PAI-1 expression further, even with a 500-fold excess of IGF-I, indicates that mechanisms independent of the IGF-I receptor are involved. Furthermore, although inhibiting tyrosine kinase precluded the stimulation of PAI-1 expression by IGF-I, it did not attenuate stimulation mediated by proinsulin.

Patients with elevated plasma immunoreactive insulin, particularly those with type II diabetes, have an increased incidence of cardiovascular disease (13). Elevated plasma PAI-1 has been shown to correlate with immunoreactive insulin levels in obese, hypertensive, and diabetic subjects (21–26) and with split proinsulin levels (10). Our results suggest that these correlations reflect a direct effect of proinsulin on endothelial cells in the arterial wall. Because the effect of proinsulin we observed is independent of both insulin and IGF-I receptors, targeted pharmacological therapy aimed against it may decrease the otherwise augmented PAI-1 expression seen in diabetic patients, potentially ameliorating progression of vascular disease.

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