Relative Contribution of Insulin and Its Precursors to Fibrinogen and PAI-1 in a Large Population With Different States of Glucose Tolerance

The Insulin Resistance Atherosclerosis Study (IRAS)

Andreas Festa, Ralph D’Agostino, Jr, Leena Mykkänen, Russell P. Tracy, Daniel J. Zaccaro, C. Nick Hales, Steven M. Haffner

Abstract—Hyperinsulinemia is associated with the development of coronary heart disease. However, the underlying mechanisms are still poorly understood. Hypercoagulability and impaired fibrinolysis are possible candidates linking hyperinsulinism with atherosclerotic disease, and it has been suggested that proinsulin rather than insulin is the crucial pathophysiological agent. The aim of this study was to investigate the relationship of insulin and its precursors to markers of coagulation and fibrinolysis in a large triethnic population. A strong and independent relationship between plasminogen activator inhibitor-1 (PAI-1) antigen and insulin and its precursors (proinsulin, 32-33 split proinsulin) was found consistently across varying states of glucose tolerance (PAI-1 versus fasting insulin [proinsulin], \( r = 0.38 \ [r = 0.34] \) in normal glucose tolerance; \( r = 0.42 \ [r = 0.43] \) in impaired glucose tolerance; \( r = 0.38 \ [r = 0.26] \) in type 2 diabetes; all \( P < 0.001 \)). The relationship remained highly significant even after accounting for insulin sensitivity as measured by a frequently sampled intravenous glucose tolerance test. In a stepwise multiple regression model after adjusting for age, sex, ethnicity, and clinic, both insulin and its precursors were significantly associated with PAI-1 levels. The relationship between fibrinogen and insulin and its precursors was significant in the overall population (\( r = 0.20 \) for insulin and proinsulin; each \( P < 0.001 \)) but showed a more inconsistent pattern in subgroup analysis and after adjustments for demographic and metabolic variables. Stepwise multiple regression analysis showed that proinsulin (split products) but not fasting insulin significantly contributed to fibrinogen levels after adjustment for age, sex, clinic, and ethnicity. Decreased insulin sensitivity was independently associated with higher PAI-1 and fibrinogen levels. In summary, we were able to demonstrate an independent relationship of 2 crucial factors of hemostasis, fibrinogen and PAI-1, to insulin and its precursors. These findings may have important clinical implications in the risk assessment and prevention of macrovascular disease, not only in patients with overt diabetes but also in nondiabetic subjects who are hyperinsulinemic. (Arterioscler Thromb Vasc Biol. 1999;19:562-568.)

Key Words: non–insulin-dependent diabetes mellitus ■ plasminogen activator inhibitor-1 ■ fibrinogen ■ insulin ■ proinsulin

Considerable epidemiological evidence indicates that hyperinsulinemia is associated with the development of coronary heart disease, a hallmark of atherosclerosis. Hyperinsulinemia has been related to insulin resistance. The relationship between insulin resistance, as measured by a frequently sampled intravenous glucose tolerance test, and atherosclerosis, as determined by carotid ultrasound, was recently reported in a large, multiethnic population. In this study (Insulin Resistance Atherosclerosis Study [IRAS]), an inverse association between insulin sensitivity and the intimal-medial thickness of the carotid artery was demonstrated both in Hispanics and in non-Hispanic whites. However, the mechanisms linking hyperinsulinemia to atherosclerosis are still poorly understood, and the direct effects of insulin and/or its indirect effects via established cardiovascular risk factors have been proposed. Hyperinsulinemia and insulin resistance are associated with factors known to increase cardiovascular risk, namely, glucose intolerance, dyslipidemia, and hypertension.

More recently, additional factors have been related to the insulin resistance syndrome, such as hypercoagulability and hypofibrinolysis. Fibrinogen is a strong and independent predictor of myocardial infarction and stroke in nondiabetic subjects, and high fibrinogen levels have been associated with the risk of
macrovascular complications in diabetic patients. An independent relationship between fibrinogen and insulin has been demonstrated previously in nondiabetic subjects. However, data on a possible relationship in subjects with impaired glucose tolerance (IGT) or type 2 diabetes are scarce.

High levels of plasminogen activator inhibitor type 1 (PAI-1) have been consistently associated with increased insulin concentrations and decreased insulin sensitivity. Juhan-Vague et al have suggested that increased PAI-1 levels may be a link between insulin resistance and coronary heart disease.

Many of the studies describing the association between insulin resistance and/or hyperinsulinemia with cardiovascular disease and its risk factors have been limited by the fact that insulin was measured with an assay that cross-reacts with proinsulin. Temple et al have suggested that proinsulin and 32-33 split proinsulin may comprise the majority of circulating immunoreactive insulin in subjects with type 2 diabetes. Recent data suggest that proinsulin rather than insulin might determine PAI-1 expression in diabetic and nondiabetic subjects. Experimental data support this hypothesis, showing a direct stimulation of PAI-1 synthesis by proinsulin and proinsulin split products. Proinsulin has been related to cardiovascular risk factors, such as dyslipidemia and hypertension, in diabetic and nondiabetic subjects, whereas there are few data on the association of proinsulin and/or its split products with the hemostatic system, especially in subjects with IGT or type 2 diabetes. Currently, no information exists about the impact of insulin sensitivity on the association of insulin and its precursors with hemostasis.

The aim of our study was to investigate the relationship of insulin and its precursors (intact proinsulin, proinsulin split products) with markers of coagulation and fibrinolysis (fibrinogen and PAI-1) in a large, triethnic population across different states of glucose tolerance.

Methods

Study Subjects

The IRAS is a multicenter epidemiological study aiming to explore relationships between insulin resistance, cardiovascular risk factors, and disease across different ethnic groups and varying states of glucose tolerance. A full description of the design and methods of the IRAS has been published previously. In brief, this study was conducted at 4 clinical centers. Clinical centers in Oakland and Los Angeles, Calif, studied non-Hispanic whites and African-Americans recruited from Kaiser Permanente, a nonprofit health maintenance organization. Clinical centers in San Antonio, Tex, and the San Luis Valley, Colo, studied non-Hispanic whites and Hispanics recruited from 2 ongoing population-based studies (the San Antonio Heart Study and the San Luis Valley Diabetes Study). Recruitment was tailored to yield approximately equal numbers of participants with regard to ethnicity, sex, and glucose tolerance categories (type 2 diabetes, IGT, and normal glucose tolerance [NGT]). The IRAS protocol was approved by local institutional review committees, and all subjects gave informed consent.

This report includes data on 1551 subjects in whom proinsulin (intact and split products), PAI-1, and fibrinogen levels were assessed. Demographic and metabolic data of the study subjects are shown in Table 1. Race and ethnicity were assessed by self-report. Ethnicity was defined by the US census question: Are you of Spanish or Hispanic descent? Height and weight were measured by following a standardized protocol. Body mass index (BMI; weight/height² [kg/m²]) was used as an estimate of overall adiposity. The IRAS examination required 2 visits. Patients were asked before each visit to fast for 12 hours, to abstain from heavy exercise and alcohol for 24 hours, and to refrain from smoking on the morning of the examination. Blood was collected in the fasting state for measurements of all reported parameters on the same day that the oral glucose tolerance test (OGTT) was performed.

For the OGTT, a 75-g glucose load (Orangedex, Customs Laboratories) was administered over a period of <10 minutes. Blood was drawn immediately before ingestion and 2 hours after the glucose load. Glucose tolerance status was based on the World Health Organization criteria.

Laboratory Measurements

Glucose and insulin levels in all samples were measured at the central IRAS laboratory at the University of Southern California, Los Angeles, Calif. Plasma glucose was measured with the glucose oxidase technique on an automated autoanalyzer (Yellow Springs Equipment Co). Insulin was measured using the dextran-charcoal radioimmunoassay. This insulin assay cross-reacts with proinsulin. The split pair coefficient of variation (CV) for the insulin radioimmunoassay was 19% (n = 163).

Fasting serum intact proinsulin and 32-33 split proinsulin were determined from samples stored at –70°C for an average of 3.3 years (range, 35 to 44 months) by means of highly specific, 2-site monoclonal antibody–based immunoradiometric assays. The split pair CV was 14% for proinsulin (n = 5). Intact proinsulin and 32-33 split proinsulin may comprise the majority of circulating immunoreactive insulin in subjects with type 2 diabetes. Recent data suggest that proinsulin rather than insulin might determine PAI-1 expression in diabetic and nondiabetic subjects. Experimental data support this hypothesis, showing a direct stimulation of PAI-1 synthesis by proinsulin and proinsulin split products. Proinsulin has been related to cardiovascular risk factors, such as dyslipidemia and hypertension, in diabetic and nondiabetic subjects, whereas there are few data on the association of proinsulin and/or its split products with the hemostatic system, especially in subjects with IGT or type 2 diabetes. Currently, no information exists about the impact of insulin sensitivity on the association of insulin and its precursors with hemostasis.

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TABLE 2. Correlation Analysis (Unadjusted) in the Overall Population and by Glucose Tolerance Status (P in Parentheses)

<table>
<thead>
<tr>
<th></th>
<th>BMI</th>
<th>Fasting Insulin</th>
<th>Proinsulin (Intact)</th>
<th>Proinsulin (Split)</th>
<th>S_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall population (n=1551)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>0.38</td>
<td>0.47</td>
<td>0.46</td>
<td>0.50</td>
<td>−0.43</td>
</tr>
<tr>
<td></td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.28</td>
<td>0.20</td>
<td>0.20</td>
<td>0.21</td>
<td>−0.24</td>
</tr>
<tr>
<td></td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
</tr>
<tr>
<td>NGT (n=693)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>0.30</td>
<td>0.38</td>
<td>0.34</td>
<td>0.40</td>
<td>−0.36</td>
</tr>
<tr>
<td></td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.23</td>
<td>0.20</td>
<td>0.16</td>
<td>0.17</td>
<td>−0.21</td>
</tr>
<tr>
<td></td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
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<tr>
<td>IGT (n=348)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>0.27</td>
<td>0.42</td>
<td>0.43</td>
<td>0.43</td>
<td>−0.27</td>
</tr>
<tr>
<td></td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.24</td>
<td>0.07</td>
<td>0.15</td>
<td>0.17</td>
<td>−0.12</td>
</tr>
<tr>
<td></td>
<td>(0.0001)</td>
<td>(NS)</td>
<td>(&lt;0.006)</td>
<td>(&lt;0.002)</td>
<td>(&lt;0.03)</td>
</tr>
<tr>
<td>Type 2 diabetes (n=510)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>0.30</td>
<td>0.38</td>
<td>0.26</td>
<td>0.37</td>
<td>−0.27</td>
</tr>
<tr>
<td></td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.23</td>
<td>0.11</td>
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<td>0.08</td>
<td>−0.15</td>
</tr>
<tr>
<td></td>
<td>(0.0001)</td>
<td>(&lt;0.02)</td>
<td>(NS)</td>
<td>(NS)</td>
<td>(&lt;0.002)</td>
</tr>
</tbody>
</table>

shows results similar to the full protocol and was used because of the large number of subjects. Glucose in the form of a 50% solution (0.3 g/kg body weight) and regular human insulin (0.03 U/kg body weight) were injected through an intravenous line at 0 and 20 minutes, respectively. Blood was collected at −5, 2, 4, 8, 19, 22, 30, 40, 50, 70, 100, and 180 minutes for plasma glucose and insulin concentrations. Insulin sensitivity, expressed as the insulin sensitiv-
ity index (S_i), was calculated by mathematical modeling methods (MINMOD, version 3.0, 1994). This modified version of the FSIGT protocol used in the IRAS has recently been compared with the hyperinsulinemic euglycemic clamp and has been shown to be a valid measure of insulin resistance.

Fibrinogen was measured in citrated plasma with a modified clot-rate assay using the Diagnostica STAGO ST4 instrument, as described previously. This method is based on the original method of Claus, with a CV of 3.0%. PAI-1 was also measured in citrated plasma by using a 2-site immunoassay that is sensitive to free PAI-1 but not to PAI-1 complexed with tissue plasminogen activa-
tor. For quality control purposes, duplicate laboratory measurements were made on an 20% sample of participants for the first 10 weeks of the examination and an 10% random sample of participants thereafter.

Statistical Analysis

Statistical analyses were performed using the SAS statistical software system. Descriptive statistics (mean values±SE) and (n and %) are shown in Table 1. Next, unadjusted Spearman rank correlations for PAI-1 and fibrinogen with BMI, fasting insulin, proinsulin, split proinsulin, and insulin sensitivity were estimated for the overall population and then stratified by glucose tolerance status (Table 2).
significantly contributed to PAI-1 levels in a stepwise multivariate analysis based on univariate analysis revealing a significant relationship weakened but remained still highly significant (23.0±0.9 versus 25.0±1.1 mg/dL in NGT, IGT, and type 2 diabetes, respectively; \( P < 0.01 \) for overall comparison). However, because only the magnitude (and not the direction) of the relationship was different, only the data derived from pooled analyses are reported for PAI-1 as well as for fibrinogen.

### Fibrinogen

Fibrinogen levels increased with impairment of glucose tolerance (271.4±2.1 versus 287.7±3.1 versus 293.8±2.7 mg/dL in subjects with NGT, IGT, and type 2 diabetes, respectively; \( P < 0.001 \) for overall comparison). However, after adjustment for sex, age, clinic, and ethnic group, fibrinogen levels were no longer significantly different in IGT compared with type 2 diabetes (270.5±2.2 versus 282.6±3.1 versus 289.9±2.6 mg/dL in NGT, IGT, and type 2 diabetes; \( P = 0.0013 \) for NGT versus IGT, \( P < 0.001 \) for NGT versus type 2 diabetes, and \( P = NS \) for IGT versus type 2 diabetes), and further adjustment for BMI and \( S_t \) abolished all differences between the glucose tolerance groups (277.8±2.4 versus 278.2±3.1 versus 282.8±2.8, \( P = NS \) for all comparisons).

Fibrinogen levels were positively related to insulin and its precursors in the overall study population (Table 2). The relationship was strongest in subjects with NGT and weaker or absent in subjects with IGT and type 2 diabetes (Table 2). Again, as for PAI-1, adjustment for \( S_t \) was performed because of a significant inverse relationship between fibrinogen and \( S_t \) in univariate analysis. Multiple adjustments as shown in Table 3 generally weakened the relationships, and after adjustment for age, sex, clinic, ethnic group, BMI, glucose tolerance status, and \( S_t \), only intact proinsulin but not fasting insulin or split products remained significantly related to fibrinogen levels.
by Després et al,3 which used a radioimmunoassay for insulin and proinsulin, respectively, to measure insulin sensitivity.4 In fact, the demonstrated relationships were independent of insulin sensitivity.

In the present study, we have demonstrated a positive relationship between insulin and its precursors to PAI-1 and fibrinogen levels consistently across varying states of glucose tolerance. This effect was independent of BMI.

The associations of variables included in the insulin resistance syndrome,16 such as BMI, waist-to-hip ratio, triglyceride and HDL cholesterol levels, as well as insulin with PAI-1 levels, have been demonstrated previously in obese and nonobese healthy subjects, in patients with type 2 diabetes, and in coronary heart disease.17,21,27,32,50–53 These findings and the corresponding in vitro data30,54 led to the view of impaired fibrinolysis as a possible link between insulin resistance and atherosclerotic disease, as indicated by increased plasma PAI-1 levels.37 The strongest evidence that hyperinsulinemia is prospectively and independently associated with the development of atherosclerosis, as judged by clinically significant coronary artery disease, is a recent study by Després et al,3 which used a radioimmunoassay for insulin that does not cross-react with proinsulin.

In vitro data show that both insulin54 and proinsulin and its conversion intermediates30 are able to stimulate PAI-1 synthesis. However, in those studies, relatively high, conceivably supraphysiological concentrations of insulin precursors were used. The availability and more widespread use of sensitive insulin assays recognizing proinsulin and its conversion intermediates have focused increasing interest on the relative contribution of insulin per se versus its precursors (proinsulin and its conversion intermediates) to atherosclerotic disease. The present study is the first investigation addressing the relationship of insulin and its precursors to PAI-1 levels in a large population of nondiabetic and diabetic subjects.

Similar to our findings, a relationship between insulin and its precursors to PAI-1 activity has been demonstrated recently in a large, healthy population from northern Sweden.55 In that study, in subjects with NGT, partial correlation analysis (adjusted for age and sex) showed a significant relation of both fasting insulin and fasting proinsulin to PAI-1 as well as to fibrinogen. In a multivariate regression model, however, fasting proinsulin but not fasting insulin significantly predicted fibrinogen levels, whereas neither proinsulin nor insulin was predictive of PAI-1 activity. This is in contrast to the findings of the present study. The differences in the results of these 2 studies might be due to differences in the multivariate regression models. In the Swedish study, the regression model included independent variables not included in the present study (triglycerides, postload insulin, diastolic blood pressure, and smoking).

Data on the relationship of PAI-1 and/or fibrinogen and insulin, including its precursors, in subjects with IGT or type 2 diabetes are both scarce and inconsistent. Most previous studies are limited by a relatively small number of subjects. In a recent study by Gray et al,59 PAI-1 was correlated with intact and split proinsulin in nondiabetic (n=76) and diabetic (n=56) subjects but not with fasting insulin. In a triennial population of diabetic (n=261) and nondiabetic (n=314) subjects, PAI-1 activity was significantly correlated with fasting insulin, proinsulin, and des-31,32-proinsulin, irrespective of ethnicity.60 In a small subgroup of subjects with IGT and type 2 diabetes (n=19) of the Sweden MONICA Study, only proinsulin but not fasting insulin was correlated with PAI-1 activity.55 PAI-1 activity was correlated significantly only with 32-33 split proinsulin but not with fasting insulin or intact proinsulin in 51 subjects with type 2 diabetes.32 The results of our study add to the evidence that the deleterious effects of insulin, its precursors, and/or insulin resistance could be at least partly mediated through PAI-1, as suggested previously by Juhan-Vague et al.27 In addition, in our study a consistent relationship of PAI-1 to insulin, its precursors, and insulin sensitivity across varying states of glucose tolerance was shown, thus indicating that similar pathomechanisms might link hyperinsulinemia and atherosclerotic disease in nondiabetic and diabetic subjects.

This study showed that insulin and its precursors are related to PAI-1 independently of insulin sensitivity. Insulin sensitivity has been independently related to PAI-1 levels in the present study and previously in patients with type 2 diabetes57 and obese diabetic and nondiabetic subjects.25 Including S I in the analysis made it possible to discern direct effects of insulin from indirect effects of insulin sensitivity, as derived from the finding that fasting insulin levels may also serve as a proxy for insulin sensitivity.4 In fact, the demonstrated relationships were independent of insulin sensitivity,

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### Table 6. Stepwise Regression Analysis With Fibrinogen as the Dependent Variable

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>B</th>
<th>SE (B)</th>
<th>P</th>
<th>Partial R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>2.18</td>
<td>0.29</td>
<td>0.0001</td>
<td>6.9%</td>
</tr>
<tr>
<td>S I</td>
<td>-2.17</td>
<td>0.90</td>
<td>0.003</td>
<td>0.54%</td>
</tr>
<tr>
<td>Proinsulin (split)</td>
<td>0.22</td>
<td>0.11</td>
<td>0.047</td>
<td>0.23%</td>
</tr>
</tbody>
</table>

B indicates the regression coefficient; SE (B), standard error of the regression coefficient. R² for the model=15.2%. After adjustment for age, sex, ethnicity, and clinic, independent variables entered into the model were as follows: BMI, glucose tolerance status, insulin sensitivity (S I), fasting insulin, and proinsulin (intact) or proinsulin (split). Only variables significantly contributing to PAI-1 levels are shown.
thus favoring the hypothesis that insulin per se is independently involved in PAI-1 metabolism. Furthermore, our results do not support the hypothesis that the link between the insulin resistance syndrome and atherosclerotic disease is mediated by insulin precursors rather than "true" insulin, as shown by univariate and multivariate analyses. These findings are in keeping with in vitro data, suggesting a dose-dependent and additive impact of both insulin and proinsulin on PAI-1 synthesis in endothelial cells.\textsuperscript{58}

The relationship of fibrinogen with insulin and its precursors is less clear. There is still controversy as to whether fibrinogen levels are generally elevated in type 2 diabetic patients or only in those with macrovascular disease\textsuperscript{19,20,59} or in the presence of increased urinary albumin excretion, as a marker of macrovascular disease.\textsuperscript{60} However, an independent relationship between insulin levels and fibrinogen levels has been reported in nondiabetic subjects.\textsuperscript{21–23} Mohamed-Ali et al\textsuperscript{23} showed a strong correlation of proinsulin, its split products, and insulin to fibrinogen in a large number of subjects with NGT. According to the present study, fasting insulin and proinsulin do not seem to contribute to fibrinogen levels in IGT and type 2 diabetes, whereas a weak but significant relationship was found in subjects with NGT. This might be explained by the fact that glucose-tolerant subjects represent a population with a relatively low prevalence of preexisting atherosclerosis compared with subjects with IGT or type 2 diabetes. Additional mechanisms, such as inflammation and oxidative stress, which are involved in enhanced fibrinogen synthesis and atherogenesis, may contribute more importantly to fibrinogen levels in subjects with preexisting atherosclerosis. Accordingly, in patients with type 2 diabetes, high fibrinogen levels have been related to the presence of macrovascular disease.\textsuperscript{20} At least in healthy subjects, insulin seems to act as a general risk marker, with fibrinogen being predictive of clinical macrovascular disease, such as coronary heart disease and stroke\textsuperscript{61} and peripheral vascular disease.\textsuperscript{62}

In summary, we showed a strong and consistent relationship of 2 crucial factors of hemostasis, fibrinogen and PAI-1, to insulin and its precursors. This association was only partially explained by insulin sensitivity. Our data provide no evidence that the demonstrated relationship is markedly stronger for proinsulin than for "true" insulin. Our findings may have important clinical implications in the risk assessment and prevention of macrovascular disease, not only in patients with overt diabetes but also in nondiabetic subjects who are hyperinsulinemic. The implications might be even more important in light of recent data emphasizing the importance of potentially modifiable metabolic factors, as opposed to genetic factors, in determining PAI-1 expression.\textsuperscript{63}

Acknowledgments
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