Increased aortic stiffness in the insulin-resistant Zucker fa/fa rat

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Several studies have demonstrated that elevated pulse-wave velocity, a measure of central arterial stiffness, is independently associated with cardiovascular and all-cause mortality and increased risk of stroke (3, 20, 21, 24). Furthermore, aortic stiffness is increased in diabetic compared with nondiabetic individuals (9, 34, 35). Recent evidence indicates increased arterial stiffness, independent of hyperglycemia, even in glucose-intolerant individuals (13, 32, 34).

Impaired insulin-mediated glucose uptake is a cardinal feature of Type 2 diabetes. The insulin-resistant stage begins well before the onset of hyperglycemia and is marked by a compensatory hyperinsulinemia that maintains normal plasma glucose levels. This long, latent stage, referred to here as the prediabetic period, carries significant health risks. High plasma insulin levels in prediabetic individuals have been shown to correlate with hypertension, hypertriglyceridemia, decreased plasma HDL, and increased incidence of coronary artery disease (28).

On the basis of the above-mentioned clinical evidence, we sought to study the process of arteriosclerosis before the onset of hyperglycemia in the Zucker fa/fa (ZF) rat model of Type 2 diabetes. ZF rats are homozygous for a mutation in the leptin receptor, phenotypically resulting in hyperphagia, obesity, and eventual insulin resistance with compensatory hyperinsulinemia. We chose to study these rats at 13–16 wk of age, before the onset of hyperglycemia.

By mechanical testing of freshly isolated aortas, it was determined that ZF vessels developed more strain in the circumferential and longitudinal directions and possessed a reduced isotropy index. Molecular analysis of vessels indicated higher expression of matrix proteins and major components of the transforming growth factor-β1 (TGF-β1) signaling cascade in aortas from ZF rats.

Materials and Methods

Animal handling. Seven male Zucker lean (ZL) and eight ZF rats (Harlan) were maintained on a normal chow diet and housed in a room with a 12:12-h light-dark cycle and an ambient temperature of 22°C. Aortas were harvested and snap-frozen in liquid nitrogen for subsequent RNA isolation, and a subset was fixed in 10% formalin for histological evaluation. These protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University and were carried out in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care. At the time of death, intravenous blood samples were obtained, and plasma glucose, insulin, and triglyceride concentrations were determined as previously described (29).

Real-time quantitative PCR. Snap-frozen aortic samples were homogenized and suspended in Trizol reagent (Life Technologies). RNA was extracted using an RNaseasy isolation kit (Qiagen), and cDNA conversion was performed using a first-strand cDNA kit (Invitrogen).

Table 1 displays a list of the primers and probes for each gene analyzed. Linearity of amplification was verified for each primer-probe set. Reactions were performed using the Applied Biosystems ABI Prism 7700.

Threshold cycles were placed in the logarithmic portion of the amplification curve, and each sample was referenced to the 18S RNA amplification to control for the total amount of RNA. Fold difference between two samples (relative quantification) was determined using the delta-delta method

S1/S2 = 2^-\Delta\Delta C T

where S1 and S2 represent samples 1 and 2 and T1 and T2 represent the threshold cycle of samples 1 and 2.

Immunostaining. Paraffin sections (5 μm) were processed for immunohistochemical analysis by incubation with primary antibodies to collagen IV (Chemicon; 1:50 dilution) or TGF-β1 (Santa Cruz Biotechnology; 1:30 dilution) for 1 h at room temperature. Immunoreactivity was visualized with a standard avidin-biotin-peroxidase technique (Vector Laboratories).

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Table 1. Sequences of primers and probes used for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
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<tbody>
<tr>
<td>TGF-β1</td>
<td>GAG GTG ACC</td>
<td>GGC CAT GAG</td>
<td>6FAM - CAT GAC</td>
</tr>
<tr>
<td></td>
<td>TGG GCA CCA T</td>
<td>GAG CAG GAA</td>
<td>ATG AAC CGA CC</td>
</tr>
<tr>
<td>Smad 2</td>
<td>TGC CGC CTC</td>
<td>GAA ATT TGT</td>
<td>6FAM - ACC CAC</td>
</tr>
<tr>
<td></td>
<td>TGG ATG ACT A</td>
<td>GTT</td>
<td>TCC ATT CCA</td>
</tr>
<tr>
<td>Smad 4</td>
<td>AGT CAG CCG</td>
<td>GAA GCT ATC</td>
<td>6FAM - CTC GCA</td>
</tr>
<tr>
<td></td>
<td>GCC AGC AT</td>
<td>TGC AAC AT</td>
<td>GGC AGC C</td>
</tr>
<tr>
<td>Smad 5</td>
<td>ACC ACC CCG</td>
<td>AGA GGC CCA</td>
<td>6FAM - ACT CCA</td>
</tr>
<tr>
<td></td>
<td>AGG ATG TAA</td>
<td>TGG AGG TGA AT</td>
<td>TGC TGG ATT GA</td>
</tr>
<tr>
<td>Smad 7</td>
<td>TGG ATG GCG</td>
<td>TGG CGG ACT</td>
<td>6FAM - AAC CGC</td>
</tr>
<tr>
<td></td>
<td>TGT GGG TTT A</td>
<td>TGA TGA AGA TG</td>
<td>AGC AGT TAC</td>
</tr>
<tr>
<td>Fibronectin-1</td>
<td>TCT ATC CAG</td>
<td>GTT TGC CAA</td>
<td>6FAM - CAG CTG</td>
</tr>
<tr>
<td></td>
<td>GGA GGG CAG</td>
<td>GGG TCT ATC TC</td>
<td>GTT CAT ATC T</td>
</tr>
<tr>
<td>Collagen IVα3</td>
<td>GAA AAC CTA</td>
<td>ACC TGA CAG</td>
<td>6FAM - CTC CAA</td>
</tr>
<tr>
<td></td>
<td>TTC CAT CGA</td>
<td>CGG CTG ATG</td>
<td>GTC TCC AGC T</td>
</tr>
<tr>
<td></td>
<td>CTG TGA</td>
<td>ATT T</td>
<td></td>
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Tissue culture. Rat vascular smooth muscle cells were harvested from ZL aortas by enzymatic digestion and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and penicillin-streptomycin under standard tissue culture conditions. After 24 h of serum starvation, the cells (passages 2–3) were stimulated with 100 nM insulin or vehicle. RNA isolation and purification were performed as described above, and real-time PCR was used to analyze the relative quantity of TGF-β.

Tissue mechanical properties. Aortic tissue mechanics were analyzed using a custom-made tissue mechanical tester. Thoracic aorta segments with minimal branching vessels were chosen, and any branching vessels were ligated before explanation. Within 4 h of explanation, 15-mm-long cylindrical thoracic aorta specimens were cannulated to barbed-end fittings with suture. Under constant longitudinal strain (held at in vivo length), pressure was gradually increased from 0 to 300 mmHg. Then, under constant pressure (held at mean physiological pressure), length was extended from relaxed to 150% of in vivo length. Constant pressure was maintained by using a large reservoir held approximately 6.5 feet above the specimen as a pressure source, making any leakage out of the explanted vessel negligible. Intraluminal pressure and axial load were recorded through a data source, making any leakage out of the explanted vessel negligible. Statistical significance was considered to be P < 0.05.

RESULTS

ZF aortas show increased stiffness in the circumferential and longitudinal directions. ZF and ZL aortas were mechanically tested to determine the stress-strain relation describing tissue mechanical properties. Figure 1, A and C, displays the average stress-strain relations of ZF and ZL aortas in the circumferential and longitudinal directions, respectively. Averages of the area under the curve for each rat indicate increased developed strain in ZF compared with ZL vessels in the circumferential (Fig. 1B) and longitudinal (Fig. 1D) directions. Modeling of these data with Fung’s soft tissue strain energy equation further demonstrates decreased anisotropy in the ZF aorta as a consequence of the greater increase in the longitudinal than in the circumferential component (Fig. 1E).

Matrix protein expression. To determine whether matrix components are altered in the prediabetic state, mRNA expression levels of fibronectin-1, collagen IVα3, and collagen Iα2 were assessed by real-time PCR. Fibronectin-1 and collagen IVα3 expression was elevated five- and eightfold, respectively, in the ZF compared with the ZL aorta. In contrast, collagen Iα2 mRNA expression was unchanged (Fig. 2A). Immunostaining shows a notable increase in collagen IV throughout the media of the ZF aorta (Fig. 2B) compared with the ZL aorta (Fig. 2C). The sections are representative of the differences between all ZL and ZF aortic sections.

Enhanced expression of TGF-β signaling molecules. Because TGF-β1 is a major regulator of matrix protein production, we probed for expression levels of TGF-β and related signaling molecules. Quantitative real-time PCR indicated significant elevation of TGF-β1, Smad 2, Smad 4, Smad 5, and Smad 7 mRNA in ZF compared with ZL aortas (Fig. 3A). TGF-β immunoreactivity was also increased in ZF compared with ZL aortic sections (Fig. 3, B and C). In addition to increased staining of the media, staining of the ZF aortic endothelium was markedly increased.

Insulin upregulates TGF-β1. To determine whether elevated concentrations of insulin could affect TGF-β1 levels, rat vascular smooth muscle cells were isolated from ZL aortas. When exposed to 100 nmol/l insulin for 12–16 h, these cells expressed significantly higher mRNA levels of TGF-β1 (Fig. 4). In contrast, Smad 2 mRNA levels were unaffected by exposure to insulin (data not shown).

DISCUSSION

Arteriosclerosis is most commonly associated with advanced age; however, it is a pathology that affects diabetic individuals...
Although several studies have examined the effect of hyperglycemia on the vascular wall, relatively few studies have explored the process of arteriosclerosis at the insulin-resistant, prehyperglycemic stage. Employing the Otsuka Long-Evans Tokushima fatty rat model of Type 2 diabetes, Noma et al. (26) found increased collagen content and medial wall thickness in prediabetic rats. Here, we report mechanical, structural, and molecular evidence of increased aortic stiffness in ZF rats before the onset of hyperglycemia.

Under physiological conditions, the blood pressure pulse creates diameter expansion and axial extension of the vessel wall. The forces required to create such expansions correspond to the stresses measured in this study during mechanical testing. It is important to note that active smooth muscle contraction was assumed to be negligible in the present study on the basis of the elastic thoracic artery theory (6, 23). Although Gaballa et al. (12) determined an increase in circumferential and longitudinal stress in response to strain in aged rat carotid artery, we demonstrate increased longitudinal and circumferential stiffness in prediabetic adolescent rat aortas. Increased vessel stiffness, in particular increased longitudinal stiffness, corresponds to increased pulse-wave velocity. As mentioned above, elevated pulse-wave velocity has been observed in hyperinsulinemic, glucose-intolerant humans and is independently associated with cardiovascular and all-cause mortality.

In addition to increased stiffness, ZF rat aortas demonstrated a loss of anisotropy. Anisotropy refers to varying tissue properties based on the direction of strain. In nondiseased arteries, more effort is required to stretch the vessel around its circum-

Fig. 1. A: circumferential stress-strain relation in Zucker fa/faq (ZF, thick line) and Zucker lean (ZL, thin line) rats. Note increased stiffness of ZF specimens at all strain values. B: area under the curve (AUC) analysis for circumferential stress data. C: longitudinal (axial) stress-strain relation in ZF and ZL rats. Note increased stiffness of ZF specimens at all strain values. D: AUC analysis for longitudinal stress data. E: isotropy index for ZL and ZF aortas. Smaller isotropy index (a1/a2) indicates a loss of anisotropy in ZF aortas. Error bars, SE. P < 0.05 for B, D, and E.
ference than along its length because of the preferential alignment of collagen fibers in the circumferential direction. Prediabetic specimens showed increased stiffness in both directions; however, there was a preferential increase in the longitudinal direction, thus diminishing the tissue’s isotropy index, $a_1/a_2$. The numerator represents the slope of the circumferential stress-strain curve, and the denominator represents the slope of the longitudinal stress-strain curve (11). Given that increased longitudinal stiffness is specifically associated with elevated pulse-wave velocity, a loss of anisotropy may correspond with higher systolic blood pressure.

Much research has linked hyperglycemia to advanced glycation end-product cross-link production in diabetic vessels (2). These cross-links are thought to result in increased stiffness. However, in the absence of hyperglycemia, the mechanisms responsible for increased stiffness in the setting of insulin resistance have yet to be elucidated. We therefore hypothesized that modification of the extracellular matrix was one of the processes involved in altering the tissue mechanical properties of the ZF aorta. The present study shows a substantial and significant increase in the mRNA expression levels of fibronectin and collagen IV, as well as increased staining for collagen IV, in the ZF aortic media. The distribution of collagen IV as seen by immunohistochemistry correlates with the distribution of collagen IV described by Dingemans et al. (10) in their systematic examination of the aortic wall’s extracellular components. They found that fibronectin and collagen IV are concentrated around smooth muscle cells in the aortic media. These proteins constituted the major components of the multilayered lamina that encased the smooth muscle cells and surrounded gap junctions connecting the cells within the media. It is conceivable that the increased levels and the organization of these matrix proteins within the media contributed to the altered mechanical properties of the ZF aorta.

In contrast to collagen IV levels, collagen I and III levels were unchanged (data not shown for collagen III). Collagens I and III are produced abundantly by endothelial and smooth muscle cells, so increases in mRNA expression may not be observed because of near-maximal transcription of the genes. To explore the molecular basis for this change in the extracellular matrix, we examined TGF-β expression. This protein has been implicated in several sclerotic processes, including myocardial fibrosis, diabetic nephropathy, and scleroderma (4, 15). It plays an active and important role during wound healing by elaborating extracellular matrix components, particularly
the various collagen isoforms and fibronectin (36). TGF-β overexpression in targeted organs of transgenic mice leads to excessive matrix deposition and fibrosis of those organs (19, 33). In culture, the cytokine has been shown to upregulate the production of fibronectin and collagen from smooth muscle cells and fibroblasts (18).

The TGF-β signaling cascade that results in the production of matrix proteins has been well characterized (25, 37). Activation of the TGF-β receptor complex results in phosphorylation and activation of Smad 2, Smad 3, and Smad 5. These receptor-activated Smads then couple with Smad 4, and the complex is translocated to the nucleus, where it combines with

Table 2. Biochemical data for ZF and ZL rats

<table>
<thead>
<tr>
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<th>Glucose, mg/dl</th>
<th>Insulin, μU/ml</th>
<th>Triglycerides, mg/dl</th>
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<tbody>
<tr>
<td>ZF</td>
<td>161±11</td>
<td>71.4±7.4*</td>
<td>119.4±12*</td>
</tr>
<tr>
<td>ZL</td>
<td>154±6</td>
<td>32.6±8.1</td>
<td>89.6±6.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. ZF, Zucker fa/fa rat; ZL, Zucker lean rat. *P < 0.05.
other factors to induce transcription of downstream targets such as extracellular matrix proteins. The inhibitory Smad 6 and Smad 7 are structurally similar to receptor-activated Smads but lack the phosphorylation domain and are therefore thought to inhibit TGF-β transcriptional activity.

Here, we demonstrate increased TGF-β, Smad 2, Smad 4, Smad 5, and Smad 7 mRNA expression in the aortic wall of insulin-resistant, prediabetic rodents. Although Hosomi et al. (14) found increased staining for the TGF-β receptor II in Otsuka Long-Evans Tokushima fatty rat aortas, we demonstrate increased staining for TGF-β itself. The distribution of TGF-β as seen by immunohistochemistry correlates with the expected distribution within the aortic media, inasmuch as the protein is stored in the extracellular matrix before cleavage and activation (4, 7). The intense staining seen in the endothelium of ZF aortas suggests that endothelial cells, in addition to smooth muscle cells, may be contributing to the elevated levels of TGF-β.

Although the activating Smad 2, Smad 4, and Smad 5 were significantly upregulated, so too was Smad 7, the inhibitory Smad. It is possible that this latter upregulation represents a negative-feedback mechanism. Smad 7 is upregulated to a lesser degree than the others, suggesting that the stoichiometry of the gene profile may favor an active Smad pathway. Although we did not assay for phosphorylated Smad proteins, there is convincing evidence that transcriptional regulation of the Smad proteins plays an essential role in activation of the pathway. Overexpression and attenuation of Smad gene expression lead to an increase and a decrease, respectively, in the activity of the TGF-β signaling cascade (16, 17).

Several characteristics of the ZF rat potentially contribute to the mRNA and protein expression patterns observed in our study. One physiological explanation is that ZF rats are markedly more hyperinsulinemic than NZL rats, as demonstrated in Table 2 and in other studies (22). Anderson et al. (1) reported that insulin and angiotensin II act additively to increase expression of TGF-β from mesangial cells. Previous in vitro experiments have demonstrated that insulin has a proliferative, migratory, and secretory effect on smooth muscle cells and can induce collagen expression (27, 30, 31). To address the effects of hyperinsulinemia, we cultured smooth muscle cells with insulin and observed increased TGF-β mRNA expression. This upregulation may be a consequence of insulin-induced intracellular hyperglycemia. Elevated intracellular glucose levels result in increased flux through the hexosamine biosynthesis pathway, which has been shown to upregulate TGF-β (8). In contrast to TGF-β mRNA expression, Smad 2 mRNA expression was unaffected by exposure to insulin. This observation is likely due to the fact that the complicated metabolic milieu in vivo cannot be adequately replicated in cell culture. Interestingly, TGF-β itself has been shown to upregulate Smad 2 expression. However, the level of TGF-β stimulated by insulin in the present study may not have been sufficient to induce such a change.

Metabolic consequences of obesity may also play a role in the profibrotic gene profile observed. In obese humans and rodent models, plasma levels of TNF-α and leptin are increased. Liu et al. (22) reported significantly elevated plasma TNF-α and leptin levels in 15-wk-old ZF rats compared with lean controls. These circulating factors may have a role in upregulating TGF-β. TNF-α has been linked to insulin resistance (5), so its potential effects on TGF-β expression may be mediated by hyperinsulinemia. Although other cytokines and growth factors may be contributing to the functional changes seen in the ZF aorta, the upregulation of TGF-β in this rat model of type 2 diabetes may provide insight into one of the mechanisms by which prediabetic individuals develop increased aortic stiffness.

Arteriosclerosis is a vascular pathology that has important consequences for diabetic and prediabetic individuals. Aortic stiffness is a strong and independent predictor of overall mortality and cardiovascular mortality, and aortic stiffness is more prevalent in diabetic and prediabetic than in nondiabetic individuals. Numerous cardiovascular pathologies have been identified as beginning before the onset of hyperglycemia, and the metabolic syndrome is now considered a major cardiovascular risk factor. The evidence presented here and in other studies suggests that arteriosclerosis is another pathological process that begins before the onset of frank diabetes, making an even stronger case for the identification of prediabetic individuals. The molecular data presented here offer potential avenues for the treatment of arteriosclerosis in this population.

REFERENCES

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