Improved Islet Morphology after Blockade of the Renin-Angiotensin System in the ZDF Rat

Christos Tikellis,¹ Peter J. Wookey,² Riccardo Candido,¹ Sof Andrikopoulos,³ Merlin C. Thomas,¹ and Mark E. Cooper¹

The renin-angiotensin system (RAS) has an important role in the endocrine pancreas. Although angiotensin II has significant effects on cell proliferation and apoptosis, the contribution of the RAS to changes in islet structure and function associated with type 2 diabetes is yet to be defined. This study examined the specific effects of RAS blockade on islet structure and function in diabetes. Thirty-six male Zucker diabetic fatty (ZDF) rats, 10 weeks of age, were randomized to receive the angiotensin-converting enzyme inhibitor perindopril (8 mg/l in drinking water; n = 12), irbesartan (15 mg/kg via gavage; n = 12), or no treatment (n = 12) for 10 weeks. Results were compared with lean littermates (ZL) (n = 12) studied concurrently. ZDF rats had increased intraislet expression of components of the RAS correlating with increased intraislet fibrosis, apoptosis, and oxidative stress. Disordered islet architecture, seen in ZDF rats, was attenuated after treatment with perindopril or irbesartan. Islet fibrogenesis was also diminished, as measured by picrosirius staining and expression of collagen I and IV. Gene expression of transforming growth factor-β1 was increased in the ZDF pancreas (ZL, 1.0 ± 0.1; ZDF, 2.0 ± 0.3; P < 0.05) and reduced after blockade of the RAS (ZDF + P, 1.3 ± 0.2; ZDF + I, 1.5 ± 0.1; vs. ZDF, both P < 0.05). Improvements in structural parameters were also associated with functional improvements in first-phase insulin secretion. These findings provide a possible mechanism for the reduced incidence of new-onset diabetes that has been observed in clinical trials of RAS blockade. Diabetes 53:989–997, 2004

Activity of the local renin-angiotensin system (RAS) is an important determinant of structure and function in a range of organs, including the heart, kidneys, adrenals, and gonads (1). An intrinsic RAS has also been demonstrated in the endocrine pancreas (2,3). Human β-cells express both the angiotensin II (Ang II) type 1 receptor (AT₁) and pro-renin genes (4). Short-term infusion of Ang II impairs first-phase insulin release, possibly through changes in intraislet blood flow (5). However, the effects of chronic exposure in the pancreas to Ang II are largely unknown.

In most tissues, chronic exposure to Ang II increases oxidative stress (6), activates fibrogenesis (7), and promotes apoptosis (8). Each of these processes has also been implicated in the progressive loss of β-cell function observed type 2 diabetes (9). In particular, the maintenance of the specialized islet architecture and regulation of β-cell number through proliferation/neogenesis and apoptosis are important determinants of islet function (10,11). In type 2 diabetes, β-cell mass is reduced during the early stages of diabetes and declines further with disease progression (12). A number of factors seem to influence β-cell loss, including functional overload as a result of chronic hyperglycemia, free fatty acids, oxidative stress, and amylin (islet amyloid polypeptide) (13,14). Although Ang II is known to have significant effects on cell proliferation (15) and apoptosis (8), the contribution of the RAS to the dynamic regulation of islet structure and function is yet to be defined.

Recent evidence points to impaired islet function as the major determinant of oral glucose tolerance (16). At least initially, reduced glucose sensitivity in islet cells seems to predominate over insulin resistance in the genesis of impaired tolerance to oral glucose (16). Several large clinical trials (e.g., Heart Outcomes Prevention Evaluation [HOPE] and Losartan for Interventions for Endpoints in Hypertension [LIFE]) (17,18) have demonstrated that blockade of the RAS protects against the development of diabetes in “at-risk” patients with hypertension. These changes have largely been attributed to improvements in peripheral insulin sensitivity. However, the degree of protection against new-onset diabetes shown in these studies suggests a more profound influence on the pathogenesis of type 2 diabetes. We hypothesized that blockade of the RAS may also attenuate the deleterious actions of Ang II on pancreatic islet structure and function that contribute to the progression of β-cell dysfunction and ultimately to the development of diabetes. This study aimed to determine the effect of chronic blockade of the RAS on islet cell structure and function associated with diabetes, using the well-characterized model of non–insulin-dependent diabetes, the genetically obese leptin receptor–deficient (fa/fa) Zucker diabetic fatty (ZDF) rat.

RESEARCH DESIGN AND METHODS

Thirty-six male obese ZDF rats, 10 weeks of age, were randomized to the angiotensin-converting enzyme (ACE) inhibitor perindopril (8 mg/l in drinking water; n = 12), irbesartan (15 mg/kg via gavage; n = 12), or no treatment (n = 12) for 10 weeks. Results were compared with lean littermates (ZL) (n = 12) studied concurrently. ZDF rats had increased intraislet expression of components of the RAS correlating with increased intraislet fibrosis, apoptosis, and oxidative stress. Disordered islet architecture, seen in ZDF rats, was attenuated after treatment with perindopril or irbesartan. Islet fibrogenesis was also diminished, as measured by picrosirius staining and expression of collagen I and IV. Gene expression of transforming growth factor-β1 was increased in the ZDF pancreas (ZL, 1.0 ± 0.1; ZDF, 2.0 ± 0.3; P < 0.05) and reduced after blockade of the RAS (ZDF + P, 1.3 ± 0.2; ZDF + I, 1.5 ± 0.1; vs. ZDF, both P < 0.05). Improvements in structural parameters were also associated with functional improvements in first-phase insulin secretion. These findings provide a possible mechanism for the reduced incidence of new-onset diabetes that has been observed in clinical trials of RAS blockade. Diabetes 53:989–997, 2004.

From the ¹Danielle Alberti Memorial Centre for Diabetic Complications, Baker Medical Research Institute, Melbourne, Victoria, Australia; the ²Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Melbourne, Victoria, Australia; and the ³Department of Medicine, Royal Melbourne Hospital, Parkville, Victoria, Australia.

Address correspondence and reprint requests to Professor Mark E. Cooper, Baker Medical Research Institute, P.O. Box 6492, Melbourne, VIC 8008, Australia. E-mail: mark.cooper@baker.edu.au.

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ACE, angiotensin-converting enzyme; Ang II, angiotensin II; AT₁, Ang II type 1 receptor; DAB, 3,3’-diaminobenzidine; PCNA, proliferating cell nuclear antigen; RAS, renin-angiotensin system; TGF-β, transforming growth factor-β, TUNEL, transferase-mediated dUTP nick-end labeling.

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water), the AT1 receptor antagonist irbesartan (15 mg/kg via gavage), or no treatment for 10 weeks. Previous studies have demonstrated that the doses of perindopril and irbesartan used in this study are associated with end-organ protection in diabetic animals (19,20). ZL rats were studied concurrently and acted as further controls. The end point of 20 weeks was selected as the objective. A total of 12–20 islets per rat pancreas (n = 8 rats/group) were analyzed.

Pancreatic β-cell mass was estimated by multiplying the mean density of staining for proinsulin in the islet sections (estimated over 12–20 islets as detailed above) multiplied by the mean area per islet of pancreas (five sections per organ). This was expressed in arbitrary units adjusted for the number of animals evaluated.

Intravascular glucose tolerance test. To assess the first-phase insulin response in ZDF rats, we performed glucose tolerance testing (23). Animals were fasted overnight before the test was commenced. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbitone (Nembutal, Rhone Merieux, Australia). The left carotid artery was then cannulated, using a Silastic catheter (100 g, inner diameter 0.127 inch, outer diameter 0.254 mm; Dow Corning, Midland, MI) filled with heparinized saline (20 units/ml), and the animals were allowed to stabilize for 20 min. A bolus of glucose at 1 g/kg was injected, using a syringe attached to the cannula, through the carotid artery, and 200 μl of blood was sampled at 0, 2, 5, and 10 min. The syringe and cannula were flushed with saline between sampling. Plasma insulin was measured using a rat-specific radioimmunoassay kit (Linco Research, El Paso, IL). Plasma glucagon was measured using a glucagon analyzer (YSI, Yellow Springs, OH).

Picrosirius red staining. Four-micron paraffin sections were prepared from 4% parafformaldehyde-fixed, paraffin-embedded rat pancreas sections. Sections were stained with 0.1% Sirius red (Direct red 80; Fluka Chemika, Buchs, Switzerland) in saturated picric acid (picrosirius red) for 1 h and mounted.

Immunohistochemistry. The expression of the α-cell marker proinsulin II was examined by immunohistochemistry using an anti-proinsulin II antibody (1:1,000; Biogenesis, Technology RD, Poole, U.K.). Slides were then incubated with the primary antibody for 1 h at room temperature. After washing, a secondary antibody (1:500, biotin-conjugated goat anti-rabbit IgG; Dako, Copenhagen, Denmark) was applied for 30 min at room temperature.

For assessing the activity of the RAS in islet sections, staining was performed for ACE (1:500; Chemicon, Temecula, CA) and AT1 receptor (1:250; Immunotech, Birmingham, AL) and a polyclonal goat anti-human type IV collagen antibody (diluted 1:100; Dako, Santa Cruz Biotechnology, Santa Cruz, CA). Recent studies suggest that the novel enzyme ACE2 is involved in the regulation of the local RAS and may also be modified in diabetes (24). Therefore, sections were stained for ACE2 using a primary antibody donated by Millennium Pharmaceuticals (Cambridge, Boston, MA).

Islet expression of type I and IV collagens was assessed using a polyclonal goat anti-human type I collagen antibody (diluted 1:100; Southern Biotech, Birmingham, AL) and a polyclonal goat anti-human type IV collagen antibody (diluted 1:200; Southern Biotech). Pretreatment of the sections to be stained for collagen phenotypes involved serial incubations with 0.4 and 0.2% pepsin consecutively (Sigma, St. Louis, MO). After nonspecific blocking in 1% normal horse serum, sections were incubated with the primary antibodies overnight at 4°C. Biotinylated horse anti-goat immunoglobulin (diluted 1:500; Vector Laboratories, Burlingame, CA) was then applied as a secondary antibody.

Ang II exerts many of its profibrotic effects via the upregulation of transforming growth factor-β (TGF-β) (25). Islet expression of TGF-β was localized using anti-TGF-β antibody (1:500; Santa Cruz Biotechnology). The primary antibody was incubated on slides for 1 h at room temperature. The secondary antibody (1:500, biotin conjugated goat anti-rabbit IgG; Dako) was then applied for 60 min.

Previous studies have shown that islet cell injury in the ZDF rat is associated with increased NO synthase activity and the accumulation of nitrate/nitrite within the islet (20). As a marker of nitrosative stress, we stained for the presence of nitrotyrosine within the islets using a polyclonal antibody for the presence of nitrotyrosine within the islets using a polyclonal antibody (1:100; Upstate Biotechnology, Waltham, MA) for 60 min. The secondary antibody (1:125, multilink biotin-conjugated swine anti-goat mouse rabbit immunoglobulin; Dako) was then applied for 30 min.

Amylin (islet amyloid polypeptide) has a number of effects in the rodent islet, including growth promotion and paracrine effects on insulin secretion (27). Human diabetes (PE AID, Depots and Immune Therapeutics, Germany) in Trizol (Life Technologies, Gathersburg, MD). cDNA was then synthesized with a reverse-transcriptase reaction carried out using standard techniques (Superscript First Strand Synthesis System for RT-PCR; Life Technologies) with random hexamers, dNTPs, and total RNA. For assessing genomic DNA contamination, controls without reverse transcriptase were included. The oligonucleotides and probes (Table 1) were designed using Primer Express (PE Applied Biosystems). cDNA was then amplified with the forward and reverse primer and 50 mmol/l FAM/TAMRA or FAM/MGB probe and VIC/TAMRA 188

Real-time quantitative RT-PCR. Gene expression of ACE, ACE2, TGF-β, AT1 receptor, proinsulin, and amylin in pancreas extracts was determined using real-time quantitative RT-PCR performed using the TaqMan system (ABI Prism 7700; Perkin-Elmer, PE Biosystems, Foster City, CA), as previously described (24). Total RNA was isolated from snap-frozen pancreatic tissue after homogenization using the Ultra-Turrax (Janke & Kunkel IKA, Laborotechnik, Germany) in Trizol (Life Technologies, Gathersburg, MD). cDNA was then synthesized with a reverse-transcriptase reaction carried out using standard techniques (Superscript First Strand Synthesis System for RT-PCR; Life Technologies) with random hexamers, dNTPs, and total RNA. For assessing genomic DNA contamination, controls without reverse transcriptase were included. The oligonucleotides and probes (Table 1) were designed using Primer Express (PE Applied Biosystems). cDNA was then amplified with the forward and reverse primer and 50 mmol/l FAM/TAMRA or FAM/MGB probe and VIC/TAMRA 188

The RT-PCR took place with 500 mmol/l forward and reverse primer and 50 mmol/l FAM/TAMRA or FAM/MGB probe and VIC/TAMRA 188

IMPROVED ISLET MORPHOLOGY IN THE ZDF RAT

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Immunohistochemistry. The expression of the α-cell marker proinsulin II was examined by immunohistochemistry using an anti-proinsulin II antibody (1:1,000; Biogenesis, Technology RD, Poole, U.K.). Slides were then incu-
Table 1
Probes and primers used for real-time RT-PCR in pancreas extracts

<table>
<thead>
<tr>
<th>Probe</th>
<th>Primer 1</th>
<th>Primer 2</th>
<th>Probe 1</th>
<th>Probe 2</th>
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<tr>
<td>ACE</td>
<td>3' primer: 5'-CAACGGCAAGGTCTGTT</td>
<td>5' primer: 5'-CTTGCCATAGTTCTGAGGAA</td>
<td>probe FAM5'-CAACAGACTGCGCCTGTTG</td>
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<td>ACET2</td>
<td>3' primer: 5'-CCCTTCTTACATGCGGACTCT</td>
<td>5' primer: 5'-TTTCTTACTCCCTCCCATAT</td>
<td>probe FAM5'-ATGCTTCCCTGCTTTGCTT</td>
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<tr>
<td>AT1R</td>
<td>3' primer: 5'-CGGCTTTCGGGATACATG</td>
<td>5' primer: 5'-GGTGCTACCTCCACCTCAAA</td>
<td>probe FAM5'-CTCAAGAGGCCTGCTTCGTT</td>
<td></td>
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<tr>
<td>Proin II</td>
<td>3' primer: 5'-TGGGTTCATGTTGGAAGCT</td>
<td>5' primer: 5'-GACATGGGTGTGTAGAATCC</td>
<td>probe FAM5'-CCCACACCCAGTAC</td>
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Ribosomal probe, in Taqman universal PCR master mix (PE Biosystems). Each sample was run and analyzed in triplicate.

Statistical analysis. Data are shown as means ± SE. Incremental area under the insulin curve was determined using the trapezoidal rule with subtraction of the basal values. Differences between mean values for variables within individual experiments were compared statistically by two-way ANOVA. Comparisons were performed using Statview SE (Brainpower Calabasas). P < 0.05 was viewed as statistically significant. For the analysis of RT-PCR results, expression values for ZL animals were given an arbitrary value of 1. Results from ZDF study groups were expressed as a ratio compared with this control group.

RESULTS

Animal model. ZDF animals were heavier than their ZL littermates (P < 0.01; Table 2). Neither perindopril nor irbesartan significantly influenced body weight in ZDF animals. Systolic blood pressure in ZDF rats was also higher in ZDF than in ZL rats (P < 0.01). Treatment with either perindopril or irbesartan reduced the systolic blood pressure to levels even lower than that seen in untreated ZL rats (P < 0.01).

Glycemic control. Fasting plasma glucose levels were elevated in ZDF animals, consistent with the presence of diabetes (Table 2). Similarly, GHb levels were elevated, and the first-phase insulin secretion in response to glucose, calculated as the incremental area under the insulin curve between 0 and 10 min after the intravenous injection of glucose, was significantly reduced in ZDF animals (Fig. 1). Treatment with perindopril or irbesartan did not significantly influence fasting hyperglycemia or glycemic control as assessed by GHb. However, both treatments significantly improved first-phase insulin secretion in ZDF animals (both vs. ZDF, P < 0.05). First-phase insulin secretion was negatively correlated with the extent of islet cell apoptosis (R = 0.68, P = 0.02) and nitrotyrosine staining (R = 0.59, P = 0.04) but was not significantly associated with gene expression of insulin, amylin, or components of the RAS (all P > 0.05).

Islet architecture and fibrosis. Islets in untreated ZDF animals were significantly enlarged with associated islet cell hypertrophy, disarray of islet architecture, and irregular islet boundaries. Treatment with perindopril and irbesartan largely attenuated these changes (Fig. 2). Fibrosis of the pancreatic islets was significantly increased in ZDF rats as demonstrated on picrosirius staining (Fig. 2C). Increased picrosirius staining was apparent both within the islet and at the disrupted islet boundary.

Expression of collagen I and IV protein was also significantly increased in untreated ZDF rats, correlating closely with picrosirius staining (vs. collagen I, r² = 0.48; vs. collagen IV, r² = 0.58, both P < 0.01) and with each other (r² = 0.53, P < 0.01). Both perindopril and irbesartan reduced expression for collagen I and IV protein, although perindopril reduced the picrosirius staining to a greater extent than irbesartan (Table 3).

The profibrotic growth factor TGF-β was also significantly increased in pancreatic islets from ZDF (Fig. 2D). TGF-β mRNA levels were increased twofold in untreated ZDF animals (ZL, 1.0 ± 0.1; ZDF, 2.0 ± 0.3; P < 0.05), and protein expression was increased by nearly 50% (P < 0.05; Table 3). The increase in gene expression of TGF-β seen in the ZDF animals was largely confined to within the pancreatic islets, as demonstrated by in situ hybridization (data not shown). As seen with respect to collagen expression, both treatments significantly reduced TGF-β mRNA and protein expression (ZDF + P, 1.3 ± 0.2; ZDF + I, 1.5 ± 0.1; vs. ZDF, both P < 0.05).

RAS. The intracellular expression of components of the RAS...
mRNA were strongly correlated ($r^2 = 0.43, P < 0.01$). In untreated ZDF rats, ACE2 expression was increased at both a protein and an mRNA level (Table 4). Increased expression of ACE2 mRNA in ZDF animals was strongly correlated with TUNEL staining ($r^2 = 0.41, P < 0.01$), collagen IV expression ($r^2 = 0.36, P < 0.01$), and picrosirius staining ($r^2 = 0.52, P < 0.01$). Unlike ACE, the expression of ACE2 was also correlated with TGF-$\beta_1$ expression. The expression of ACE2 was reduced by both perindopril and irbesartan. However, the effect of perindopril on gene expression did not reach statistical significance ($P = 0.07$).

Pancreatic staining for the AT$_1$ receptor was weak in the islets of ZL animals, and expression was localized to ducts and vessels (Fig. 3C). Untreated ZDF animals had greater islet-specific staining for AT$_1$ receptor (Fig. 3F) compared with ZL rats. Pancreatic gene expression of AT$_1$ receptor mRNA was also significantly greater in ZDF rats (Table 4). Again both treatments significantly reduced the expression of AT$_1$ receptor protein in islets and AT$_1$ receptor mRNA (Table 4).

**Proinsulin and $\beta$-cell mass.** Immunohistochemical staining for $\beta$-cell marker proinsulin II was strong and intense in ZL islets (Fig. 4A). By comparison, staining for proinsulin in ZDF islets was diffused, with focal trapping of $\beta$-cells as “islands” within matrix boundaries (Fig. 4C) associated with an overall reduction in the percentage of proportional area staining positively for proinsulin (ZL, 54.0 ± 1.8; ZDF, 23.0 ± 1.9; $P < 0.01$). This reflected increases in interstitial material (predominantly collagen) in the islet and an overall increase in islet size. Treatment with perindopril or irbesartan significantly increased staining density for proinsulin (ZDF + P, 42.7 ± 1.3; ZDF + I, 45.0 ± 1.4; vs. ZDF, $P < 0.01$). This was largely coincident with the reduction in islet fibrosis.

Pancreatic $\beta$-cell mass was estimated by multiplying the mean islet density of staining for proinsulin by the mean islet area per area of pancreas (five sections per organ). This was expressed in arbitrary units adjusted for the pancreatic wet weight for individual animals. ZDF rats had more than twice the $\beta$-cell mass of ZL animals (ZL, 71 ± 6; ZDF, 194 ± 25; $P < 0.05$). Blockade of the RAS further increased total pancreas insulin content (ZDF + P, 322 ± 35; ZDF + I, 347 ± 39; vs. ZDF, $P < 0.05$).

Consistent with hyperinsulinemia observed in this model, the expression of proinsulin II mRNA in pancreas extracts was also significantly increased in ZDF rats (ZL, 1.0 ± 0.2; ZDF, 10.6 ± 3.2; $P < 0.05$). Treatment with perindopril or irbesartan was associated with a reduction in proinsulin II mRNA in pancreas extracts (ZDF + P, 2.2 ± 0.8; ZDF + I, 3.9 ± 0.6; vs. ZDF, $P < 0.05$).

**TABLE 3**

|         | Picosirius | Collagen I | Collagen IV | TGF-$\beta_1$
|---------|------------|------------|-------------|-----------
| ZL      | 2.5 ± 0.1  | 14.0 ± 0.8 | 14.2 ± 0.8  | 11.0 ± 0.7 |
| ZDF     | 13.7 ± 0.8*| 24.0 ± 0.9*| 24.4 ± 0.9* | 16.5 ± 1.3*|
| ZDF + P | 3.5 ± 0.2*†| 16.8 ± 0.9†| 16.9 ± 0.9† | 6.3 ± 0.6*†|
| ZDF + I | 7.5 ± 0.5† | 16.2 ± 0.6†| 16.2 ± 0.6† | 4.5 ± 0.3*†|

Data are proportional area (%) of each islet occupied by cells staining positively for specific islet proteins, shown as means ± SE. *$P < 0.05$ vs. ZL; †$P < 0.05$ vs. ZDF.
Amylin. Like the expression of proinsulin II, staining for amylin in ZDF islets was less intense and diffusely distributed throughout the islet (Fig. 4D). Treatment with perindopril or irbesartan increased the proportional area staining for amylin protein, again reflecting the associated reduction in interstitial matrix. However, pancreatic amylin gene expression was significantly increased in ZDF rats (ZL, 1.0 ± 0.1; ZDF, 12.8 ± 2.3; P < 0.05). Notably, the level of expression of amylin mRNA was also closely correlated with the expression of proinsulin mRNA (R² = 0.8) in ZDF animals. Both treatments reduced the pancreatic amylin mRNA, although not to control levels (ZDF + P, 7.7 ± 1.2; ZDF + I, 4.8 ± 0.7; vs. ZDF, P < 0.05). The percentage reduction in amylin protein and mRNA achieved after blockade of the RAS was greater than the reduction in staining for proinsulin II.

Apoptosis and proliferation. TUNEL staining was used to identify apoptotic cells within islet boundaries. Intraislet cell death was significantly greater in ZDF rats, in which there was approximately two to three apoptotic cells per islet section compared with infrequent apoptotic

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<th>n</th>
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<td>ZDF + I</td>
<td>8</td>
<td>1.6 ± 0.2**</td>
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Data are proportional area (%) of each islet occupied by cells staining positively for specific islet proteins, shown as means ± SE (for immunohistochemistry), and data are results from ZDF study groups expressed as a ratio compared with ZL, shown as mean expression ± SE (for real-time RT-PCR). *P < 0.05 vs. ZL; †P < 0.05 vs. ZDF.
cells seen in ZL islets (ZL, 0.3 ± 0.1/islet; ZDF, 2.5 ± 0.3/islet; P < 0.01). Blockade of the RAS reduced TUNEL staining within islet cells to control levels (ZDF + P, 0.9 ± 0.1; ZDF + I, 0.6 ± 0.1; both vs. ZDF, P < 0.01).

There was no significant difference in the number of cells staining positively for the PCNA marker between the ZL and ZDF islets (Fig. 5). However, blockade of the RAS significantly increased the number of PCNA-positive staining within islet boundaries compared with both ZL and ZDF animals. This increase was almost totally explained by an increase in cells staining positively for both insulin and PCNA (Fig. 5).

**Nitritative stress.** Within the pancreatic islets of ZDF rats, there were increased levels of oxidative stress as measured by percentage of proportional staining for nitrotyrosine (Fig. 6). Staining for nitrotyrosine was increased more than fourfold in ZDF compared with ZL rats (ZDF, 30.1 ± 2.7; ZL, 7.3 ± 0.7; P < 0.01). Blockade of the RAS with perindopril or irbesartan significantly reduced staining for nitrotyrosine (ZDF + P, 10.1 ± 0.9; ZDF + I, 7.0 ± 0.7; vs. ZDF, P < 0.01). Nitrotyrosine staining was strongly correlated with islet fibrosis (vs. picrosirius, $R^2 = 0.82$, $P < 0.01$), apoptosis ($R^2 = 0.62$, $P < 0.01$), and staining for proinsulin ($r = -0.72$, $P < 0.01$). Increased expression of the AT$_1$ receptor mRNA in the pancreas of ZDF rats was also strongly correlated with staining for nitrotyrosine ($R^2 = 0.45$).

**DISCUSSION**

The development of diabetes in the ZDF rat is associated with progressive histopathological changes in pancreatic islets, including selective loss of β-cells and fibrosis. We describe for the first time the attenuation of these changes after chronic blockade of the RAS. Previous studies have attributed progressive islet damage seen in this model to chronic hyperglycemia (glycotoxicity) (21). Although treatment with ACE inhibitors or AT$_1$ receptor antagonists improves peripheral insulin sensitivity and glucose disposal in ZDF rats (31), the beneficial effects on islet morphology described here were independent of any direct effects on glycemic control. These findings suggest that activation of the local RAS may represent an independent mechanism for progressive islet damage in the ZDF model. In addition, these findings provide a possible explanation for the markedly reduced incidence of new onset of diabetes observed in clinical trials involving ACE inhibitors and Ang II receptor antagonists (17,18).

The mechanisms that lead to activation of the RAS in the pancreas of ZDF rats have not been established. It has previously been demonstrated that chronic hyperglycemia and hyperlipidemia activate the local RAS (32). Even before the development of diabetes, ZDF rats have increased pressor responsiveness to Ang II (33). In the kidney, this may be related to increased AT$_1$ receptor abundance and function (34). Consistent with this finding, expression of AT$_1$ receptor protein was increased more than threefold in the islets of ZDF rats, correlating with fibrosis and β-cell loss. Studies in obese patients suggest that weight gain itself activates the RAS (35). Pancreatic inflammation may also result in upregulation of the local RAS as demonstrated in animal models of pancreatitis.
However, we saw no evidence of insulinitis in this model. In addition, in the ZDF model, early extracellular matrix remodeling in the kidney seems to be independent of any inflammatory process (37). Finally, hypertension in the ZDF rat may also contribute to activation of the RAS and potentially to progressive β-cell dysfunction in this model (38). It is conceivable that excellent blood pressure control after blockade of the RAS may have contributed to their observed benefit. If this were true, then other antihypertensive agents, such as calcium channel blockers, may also confer benefits on islet function and structure. However, it should be noted that previous studies with the calcium channel blocker verapamil showed a paradoxical worsening of hyperinsulinemia despite an equivalent antihypertensive effect to captopril (38). Phenomenologically, this is similar to the differential effects of blockade of the RAS on proteinuria in this model (39).

The maintenance of the specialized architecture of the pancreatic islet is important for continuing function. Disruption of contacts between β-cells reduces the secretory efficiency of islets (40). Loss of cell-to-cell communication associated with increased intraislet fibrosis may also promote islet cell apoptosis (41). The RAS has been linked to increased fibrosis in a variety of tissues, including the heart (42), kidney (43), and liver (44). Locally generated Ang II is thought to activate the AT1 receptor, resulting in upregulation of the fibrogenic cytokines and growth factors, including TGF-β1 (25). In addition to stimulating the deposition of increased amounts of extracellular matrix, TGF-β1 is known to regulate cell growth, differentiation, and function in the pancreas (45). We report here the increased expression of TGF-β mRNA and protein in the islets of ZDF rats (Table 3). TGF-β has been previously associated with fibrosis after pancreatitis (36,46) and autoimmune diabetes (47). However, this is the first description of increased islet expression of TGF-β associated with a model of type 2 diabetes. In other tissues, blockade of the RAS seems to result in reduced fibrosis through inhibition of TGF-β1 (20). In models of rodent pancreatitis, blockade of the RAS results in lower levels of TGF-β and fibrosis (36). It is conceivable that the reduction in islet fibrosis observed in this study after treatment with perindopril and irbesartan may be mediated through a similar pathway.

The regulation of islet cell apoptosis and proliferation is also important in maintaining β-cell mass, particularly in the setting of concomitant insulin resistance. In particular, the pivotal role of apoptosis has been demonstrated by the development of diabetes in the animals with partial pancreatic duodenal homeobox-1 deficiency (an animal model of maturity-onset diabetes of the young, type 4) (10). In this model, increase β-cell apoptosis leads to β-cell depletion and an abnormal islet architecture similar to that described here (10). Although Ang II is known to influence growth and proliferation in the endocrine pancreas (5), the contribution of these changes to islet dysfunction in diabetes has not been previously investigated. In cultured rat renal proximal epithelial cells, Ang II triggers cell death after the upregulation of TGF-β (8). In addition, the tubular expression of TGF-β and tubular apoptosis are both significantly increased in the diabetic transgenic (mRen-2)27 rat associated with overactivity of the local RAS (48). Both of these changes can be attenuated after blockade of the RAS (49). Consistent with these renal

![FIG. 6. Immunostaining for nitrotyrosine in ZL islets (A), untreated ZDF islets (B), ZDF islets treated with perindopril (C), and ZDF islets treated with irbesartan (D). Magnification ×850 in A, ×430 in B–D.](image-url)
findings, we demonstrate increased apoptosis and expression of TGF-β in the ZDF islets, both of which were prevented after treatment with perindopril or irbesartan. Previous studies in the ZDF rat have demonstrated that islet cell proliferation is increased early but is not significantly different from lean littermates by 12 weeks of age (50). Consistent with this finding, we found no significant difference between ZL and ZDF rats at 20 weeks of age, a time when diabetes is established. Although the local RAS has independent antiproliferative effects in many tissues (1), an effect on islet cell proliferation and neogenesis has not been previously documented. In our experiments, both perindopril and irbesartan were associated with a significant increase in intraislet proliferation as demonstrated by positive staining for PCNA. This increase was almost entirely explained by the presence of proliferating β-cells. Along with reduced apoptosis, these changes may significantly contribute to enhanced β-cell mass in this model after blockade of the RAS. Although a role for the RAS in islet cell regeneration and neogenesis is plausible, further research is required to delineate its significance in the onset and progression of type 2 diabetes.

Pancreatic islets are highly susceptible to oxidative injury, owing to low endogenous antioxidant activity (51). The ZDF model is associated with elevated levels of oxidative stress in the pancreas (52). In addition, prooxidant challenge in vivo provokes the onset of diabetes in the ZDF rat (53). In β-cells, glucose stimulates the production of reactive oxygen species in islets through protein kinase C–dependent activation of NAD(P)H oxidase (54). Hyperglycemia (and, subsequently, advanced glycation and hyperinsulinemia) further increases the generation of reactive oxygen species. Ang II also increases tissue NAD(P)H oxidase activity (55). ACE inhibitors and angiotensin receptor antagonists indirectly inhibit NAD(P)H oxidase by preventing activation of the AT1 receptor (6). Direct effects on NAD(P)H oxidase activity have also been attributed to some ACE inhibitors and angiotensin receptor antagonists (56). It is also possible that reduction of oxidative stress (as measured by staining for nitrotyrosine) contributes to the preservation of islet architecture and reduction in fibrosis seen after blockade of the RAS in the ZDF model. Notably, changes in intraislet oxidative stress in this study were the strongest predictor of islet morphology and function.

Although islet architecture was maintained after blockade of the RAS, these agents did not influence long-term glycemic control or protect against the onset of diabetes in this model. This may be a result of the comparatively late intervention with RAS blockade used in this study. Although this time point may potentially be better representative of the high-risk patients shown to benefit from RAS blockade, ZDF rats at 10 weeks of age already have substantial β-cell loss and glucose intolerance. It is conceivable that an earlier intervention (e.g., from 6 to 7 weeks of age) may have proved more beneficial. Indeed, studies using rosiglitazone have suggested that a therapeutic window may exist for islet structural and functional improvement, its end corresponding with the development of persistent hyperglycemia (50). Nonetheless, the first-phase insulin response was significantly improved after blockade of the RAS, suggesting an improvement in islet function. Some of this improvement may reflect increased β-cell mass as well as changes in intraislet blood flow (5), improved glucose delivery, and insulin washout. Intraislet amylin may also act as a local inhibitor of stimulated β-cell secretion (27) and was significantly reduced after blockade of the RAS in ZDF rats. In addition, the disruption of the integrity of contiguous anatomical structures associated with islet hypertrophy in the ZDF rat interferes with coordinated insulin secretion (57). However, the importance of these functional and structural changes to the impairment of first-phase insulin release remains to be determined.

In summary, the local RAS is upregulated in the islets of ZDF rats associated with the disruption of islet architecture, fibrosis, and apoptosis. This is consistent with observations in other tissues, where locally released Ang II has an important role in the regulation of both organ structure and function. In the ZDF model, blockade of the RAS significantly attenuated islet damage and augmented β-cell mass, possibly by a reduction in oxidative stress, apoptosis, and attenuation of profibrotic pathways. These findings provide a novel mechanism that could partly explain the reduced incidence of new-onset diabetes that has been observed in clinical trials involving ACE inhibitors and Ang II receptor antagonists.

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