Role of ANG II in coronary capillary angiogenesis at the insulin-resistant stage of a NIDDM rat model

SUBRINA JESMIN,1 YUICHI HATTORI,2 ICHIRO SAKUMA,1
CHISHIMBA N. MOWA,3 AND AKIRA KITABATAKE1

1Department of Cardiovascular Medicine and 2Department of Pharmacology, Hokkaido University Graduate School of Medicine, Sapporo 060-8638; and 3Laboratory of Anatomy, Hokkaido University Graduate School of Veterinary Medicine, Sapporo 060-0818, Japan

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Jesmin, Subrina, Yuichi Hattori, Ichiro Sakuma, Chishimba N. Mowa, and Akira Kitabatake. Role of ANG II in coronary capillary angiogenesis at the insulin-resistant stage of a NIDDM rat model. Am J Physiol Heart Circ Physiol 283: H1387–H1397, 2002; 10.1152/ajpheart.00299.2002.—With the use of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model of human non-insulin-dependent diabetes mellitus (NIDDM), we assessed whether ANG II is involved in coronary capillary angiogenesis at the insulin-resistant stage of NIDDM (20 wk of age). In OLETF rats, ANG II labeling and angiotensin type 1 (AT1) receptor expression in coronary vessels were increased more than in nondiabetic controls. A marked increase in vascular expression of vascular endothelial growth factor (VEGF) at both mRNA and protein levels was found in OLETF rats. The increased expression level of VEGF was associated with accumulation of hypoxia-inducible factor-1 (HIF-1α) and of advanced glycation end products (AGEs). Morphometric analysis showed a significantly increased total coronary capillary density, which was a result of arterIALIZATION of the venular capillary portion in OLETF rats. Treatment of OLETF rats with candesartan, an AT1 receptor blocker, inhibited vascular expressions of VEGF, HIF-1α, and AGEs, and ameliorated the morphometric changes. These results suggest a key role of ANG II in the pathogenesis of the coronary capillary remodeling in this NIDDM model.

vascular endothelial growth factor; hypoxia-inducible factor-1α; advanced glycation end products; coronary capillary remodeling; angiotensin II; non-insulin-dependent diabetes mellitus

UNCONTROLLED ANGIOGENESIS is the major feature of several pathological processes of diabetic complications, such as retinopathy and nephropathy. The molecules that promote cell growth and enhance vascular permeability have been implicated in the pathogenesis of a process referred to as angiogenesis (12). Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic cytokines and promotes all steps in the cascade process of angiogenesis (10). Although VEGF binds to fms-like tyrosine kinase (Flt-1) with a 50-fold higher affinity than to fetal liver kinase 1 [Flk-1/kinase domain region (KDR)], KDR mediates most of the VEGF effects (10, 32, 41). Clinical evidence (1) suggests that VEGF is involved in the development of retinal neovascularization and has a causal role in diabetic retinopathy.

ANG II is well known to be a key factor in cardiovascular homeostasis and to exert multiple actions, thereby playing an important role in many cardiovascular diseases. Clinical studies have demonstrated that angiotensin-converting enzyme (ACE) inhibitors can delay the development and/or progression of diabetic retinopathy and nephropathy (18, 40). The benefit of this therapeutic strategy may be linked to prevention of a potential role of ANG II in angiogenesis leading to diabetic vasculopathy. Indeed, ANG II induces angiogenesis in the rabbit cornea (9), chick embryonic chorioallantoic membrane (22), and rat cremaster muscle (29), although its mechanism remains unknown. ANG II has also been reported (7, 31) to upregulate mRNA expression of VEGF and to potentiate VEGF-mediated angiogenic activity through KDR upregulation in endothelial cells. Interestingly, cardiac tissues from diabetic patients exhibit strong immunoreactivity for ANG II (14). Experimental studies using a rat model of non-insulin-dependent diabetes mellitus (NIDDM) have shown that both the ACE inhibitor and angiotensin type 1 (AT1) receptor blocker prevent the increase in coronary arterial wall thickening and perivascular fibrosis (20). Thus ANG II may play a central role in cardiac remodeling in diabetes. However, the importance of ANG II through the regulation of angiogenesis in coronary capillary network changes in diabetes has not been clarified. Furthermore, despite strong evidence for the causal link between VEGF and the pathogenesis of diabetic retinopathy (1), the possible involvement of VEGF in coronary angiogenesis and the regulatory mechanisms for its expression in the diabetic heart remain to be elucidated.

In the present study, we used the Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which have...
been established as an animal model of congenital diabetes by selective mating (19). This strain displays stable clinical and pathological features that resemble human NIDDM, and one of its characteristics is an early manifestation of the existence of insulin resistance. First, we determined whether expressions of ANG II and its receptors are altered in coronary vessels of this diabetic animal model. Second, we examined coronary expressions of VEGF, its receptors (KDR and Flt-1), and the molecules that can regulate VEGF expression in OLETF rats. Third, we examined whether angiogenic alterations in coronary capillary network occur in OLETF rats. Finally, we tested the therapeutic effect of candesartan, an AT1 receptor blocker, on the angiogenic molecule expression changes and the morphometric changes seen in OLETF rats.

MATERIALS AND METHODS

Animals and drug treatment. The experimental design was approved by the Hokkaido University School of Medicine Animal Care and Use Committee. Male OLETF rats, which are established as spontaneously long-term hyperglycemic rats with NIDDM (19), were obtained from the Tokushima Research Institute, Otsuka Pharmaceutical (Tokushima, Japan). Male Long-Evans Tokushima Otsuka (LETO) rats, which were developed from the same colony by selective mating but did not develop diabetes, served as control animals. Animals were maintained under constant temperature (23°C) and lighting conditions (lights on from 6 AM to 6 PM) with free access to food and water. At 8 wk of age, OLETF rats were randomly divided into two groups. One group was kept on a standard diet, whereas the other group was supplemented with candesartan (1 mg·kg⁻¹·day⁻¹). This treatment period was 12 wk. Body weight, blood pressure, heart rate, plasma glucose and insulin, urinary sugar, and urinary volume were measured at an interval of 4 wk from 7 wk of age. Rats were euthanized at 20 wk of age under 3% halothane in room air, followed by 1% halothane in 21% O₂-79% N₂ mixture through a gas mask connected to a Harvard respirator. The weights of the whole heart and of the left ventricle (LV) were then measured. Some portions of LV were embedded in optimum cutting temperature (OCT) compound (Sakura Finetechnical; Tokyo, Japan) and immediately frozen in liquid nitrogen. The remaining portion was preserved at −80°C without OCT compound.

Immunohistochemistry. Immunohistochemical studies were performed with the following commercially available antibodies: anti-human ANG II rabbit polyclonal antibody (Biogenesis; Poole, UK), AT₁ receptor rabbit polyclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA), AT₂ receptor goat polyclonal antibody (Santa Cruz Biotechnology), VEGF rabbit polyclonal antibody (Immunobiological Laboratories; Fujioka, Japan), KDR rabbit polyclonal antibody (Santa Cruz Biotechnology), Flt-1 rabbit polyclonal antibody (Santa Cruz Biotechnology), hypoxia-inducible factor-1α (HIF-1α) monoclonal antibody (Novus Biologicals; Littleton, CO), and advanced glycation end product (AGE) monoclonal antibody (clone no. 6D12, Transgenic; Kumamoto, Japan). The AGE antibody shows a positive reaction to AGE-samples obtained either from proteins, lysine derivatives or monoamino-carboxylic acids, indicating the immunospecificity to a common structure among AGE structures. Moreover, this antibody is an N-carboxymethyllysine protein adduct. Frozen cryostat sections (8 μm thick) were fixed in acetone for 10 min at 4°C and air dried. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide for 15 min. The sections were then blocked by normal goat serum to prevent non-specific staining by the secondary antibody. After incubation with primary antibodies overnight at 4°C, the sections were exposed to the secondary goat anti-rabbit antibody, goat anti-mouse antibody, or rabbit anti-goat antibody conjugated with horseradish peroxidase. Bound antibody was visualized by a light microscopy with diaminobenzidine. Omission of primary antibodies and staining with nonimmune IgG served as negative controls for each antibody in the present study. At least 12 sections were taken from each sample and at least 40 microscopic fields per sample were examined. We confirmed that protein expressions of the molecules studied herein were not substantially different between the transmural and subendocardial regions of LV myocardium in the same sample. Furthermore, all of these molecules were expressed more abundantly in coronary vessels compared with cardiomyocytes, and we did not find a clear variation in positive staining among intima, media, and adventitia of the vessel wall. Quantitation of immunoreactivity by pixel intensity was analyzed with the use of image-analysis software (Microcomputer Imaging Device, Imaging Research; St. Catherine, Ontario, Canada).

Immunofluorescent staining. After overnight incubation with each antibody in the same way as mentioned above, the sections were exposed to the fluorescence secondary antibody, Cy3-conjugated AffiniPure anti-rabbit IgG or fluorescein-conjugated AffiniPure goat anti-rabbit, anti-goat, or anti-mouse IgG (Jackson Immuno Research Laboratories; Westgrove, PA), for 2 h according to the manufacturer’s instructions. The samples processed without primary antibodies served as negative controls. Immunofluorescent images were observed under the Laser Scanning Confocal Imaging System (model MRC-1024, Bio-Rad).

In situ hybridization protocol. Tissue sections (10 to 15 μm thick) were prepared and mounted on glass slides precoated with 3-aminopropyltriethoxysilane. The sections were then fixed by 4% paraformaldehyde for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0). The hybridization procedure was performed as previously described (28). The probes were complementary to nucleotide residues 721 to 766 of AT1 receptor cDNA (accession no. M90065), 2,161 to 2,206 of AT2 receptor cDNA (accession no. D43778), 61 to 106 of VEGF cDNA (accession no. AF227799), 541 to 586 of KDR cDNA (accession no. U93306), and 961 to 1,016 of Flt-1 cDNA (accession no. D28498). The oligonucleotides were labeled with [35S]dATP with the use of terminal deoxyribonucleotidyl transferase. The radiolabeled probes were hybridized to the tissue in a prehybridization buffer for 10 h at 42°C. The sections were exposed to Hyperfilm-βmax (Amersham; Bucks, UK) for 4 wk or dipped in Kodak NTB2 nuclear track emulsion and exposed for 4–8 wk. The specificity of in situ hybridization was confirmed by the disappearance of signals when excessive doses of the corresponding cold oligonucleotides were added to the hybridization fluid. The mRNA grains per blood vessel were quantified with the use of image-analysis software (Microcomputer Imaging Device).

Expression of mRNAs by RT-PCR. Extraction of total RNA from tissues was carried out using an RNA isolation kit (Isogen, Nippon Gene; Tokyo, Japan). Total RNA (1 μg) was used for cDNA synthesis according to the manufacturer’s instructions. The mRNAs of VEGF and KDR were quantitatively determined by the RT-PCR method with the use of an RT-PCR kit (Takara Shuzo; Otsu, Japan). The primer pairs used for VEGF (sense, 5’ CCGAATTCAATGAACTTCTGCTCTTCTT 3’, and antisense, 5’ GAGGAAGTCTTCTGCTGCCAGCTTG

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3') and for KDR (sense, 5' CAGAAAGGAGATGCCCAGAC 3', and antisense, 5' TCAGAGTTTTCAGCCTTTC 3') yielded a 600- and 300-bp PCR product, respectively. The PCR conditions were 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 90 s. The PCR products were subjected to agarose gel electrophoresis and ethidium bromide staining. To standardize the amount of the target molecule, the amount of β-actin mRNA, a ubiquitously expressed housekeeping gene, was determined with the primer pair (sense, 5' GTGGGGGCCTCCAGGCACCA 3', and antisense, 5' GTCCCTAATGTCAAGCGAGAATTCGTTTC 3').

Morphometric analysis. Sections (16 μm thick) were cut from the frozen LV at the widest part. Double staining of sections was carried out to discriminate arteriolar and venular capillaries (21). Arteriolar capillaries with endothelial cells containing alkaline phosphatase were stained blue, whereas venular capillaries containing dipeptidylpeptidase IV were stained red. Intermediate capillaries were stained violet because they contain both enzymes. The number of each type of capillaries was counted in a given microscopic field. At least 16 sections were examined at each sample and at least 64 microscopic fields were examined at ×400 magnification. All sections were encoded and analyzed by two skilled observers blinded to the experimental design. Capillary density, proportions of different capillary portions, myocyte number, capillary-to-myocyte ratio, capillary domain area, and myocyte scan area were calculated as previously described (2).

Collagen in tissue samples was detected using a Collagen Staining kit (Collagen Research Center; Tokyo, Japan). The percentage ratio of collagen to noncollagen protein was then calculated according to a previous study (25).

Statistical analysis. Values are shown as means ± SD. Statistical analysis was performed by ANOVA with multiple comparisons by Fisher’s protected least-significant difference t-test. Nonparametric data were analyzed by the Mann-Whitney’s U-test or Wilcoxon signed-rank test. A P < 0.05 was considered to be statistically significant.

RESULTS

Characteristics of experimental animals. As shown in Table 1, heart and LV weights of OLETF rats were significantly greater than those of LETO rats at 20 wk of age. Candesartan treatment had no influence on weight gain seen in OLETF rats. Plasma glucose and insulin levels were higher in OLETF rats than in LETO rats regardless of whether candesartan was given or not. Urinary volume and urinary sugar excretion were significantly increased in OLETF rats compared with LETO rats. Candesartan tended to suppress the increases in urinary volume and urinary sugar excretion observed in OLETF rats, but no significant differences in these values were found between animals untreated and treated with candesartan. Blood pressure and heart rate did not differ significantly between OLETF and LETO rats. Treatment of OLETF rats with candesartan for 8 wk significantly reduced blood pressure but did not change heart rate.

Expressions of ANG II and its receptors. As can be seen in Fig. 1A, immunofluorescent studies showed more abundant ANG II protein expression in LV cross sections from Long-Evans Tokushima Otsuka (LETO) and Otsuka Long-Evans Tokushima Fatty (OLETF) rats. A: representative immunofluorescent findings for ANG II protein. Positive staining was focused on coronary vessels. Magnification ×400. B: quantitation of immunoreactivity shown by pixel intensity. The average pixel intensity was calculated in 20 randomly selected coronary vessels (50–100 μm ID) per sample. Values are means ± SD, n = 10. *P < 0.001 vs. LETO.

Table 1. Characteristics of LETO, OLETF, and candesartan-treated OLETF rats at 20 wk of age

<table>
<thead>
<tr>
<th></th>
<th>LETO</th>
<th>OLETF</th>
<th>Candesartan</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>431 ± 31†</td>
<td>592 ± 30</td>
<td>611 ± 48</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>1,109 ± 99†</td>
<td>1,374 ± 61</td>
<td>1,367 ± 131</td>
</tr>
<tr>
<td>LV weight, mg</td>
<td>886 ± 106*</td>
<td>1,066 ± 55</td>
<td>1,056 ± 105</td>
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<tr>
<td>Plasma glucose, mM</td>
<td>6.0 ± 0.2*</td>
<td>8.7 ± 0.3</td>
<td>9.6 ± 0.1</td>
</tr>
<tr>
<td>Plasma insulin, pM</td>
<td>6.7 ± 1.2*</td>
<td>19.3 ± 5.4</td>
<td>26.4 ± 5.0</td>
</tr>
<tr>
<td>Urinary volume, ml/day</td>
<td>22.3 ± 1.0*</td>
<td>30.3 ± 3.1</td>
<td>27.5 ± 2.7</td>
</tr>
<tr>
<td>Urinary sugar, μmol/l</td>
<td>29.4 ± 0.4*</td>
<td>37.2 ± 3.3</td>
<td>31.1 ± 2.8</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>406 ± 9</td>
<td>428 ± 11</td>
<td>427 ± 13</td>
</tr>
<tr>
<td>Systolic pressure, mmHg</td>
<td>152 ± 2</td>
<td>156 ± 3</td>
<td>131 ± 4</td>
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<tr>
<td>Mean pressure, mmHg</td>
<td>116 ± 2</td>
<td>119 ± 4</td>
<td>105 ± 5</td>
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<tr>
<td>Diastolic pressure, mmHg</td>
<td>98 ± 4</td>
<td>100 ± 5</td>
<td>88 ± 5</td>
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Data are means ± SD of 10 animals. LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty; LV, left ventricle. Blood samples were collected after 24 h of fasting. Candesartan was given to OLETF rats from 8 wk of age for 12 wk.

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that its expression was apparently similar in OLETF and LETO rats (Fig. 2A). Both AT₁ and AT₂ receptors were observed mainly in coronary vessels, although some staining was seen also in cardiomyocytes. The results of quantitative analysis showed that the AT₁ receptor expression level was ~2.1-fold higher in diabetic rats than in nondiabetic controls, whereas no significant difference was found in the AT₂ receptor expression level between the two groups (Fig. 2B). In situ hybridization studies indicated that the increase in AT₁ receptor protein expression in OLETF rats occurred at the level of gene expression. As depicted in Fig. 3A, mRNA expression of AT₁ receptor was evidently higher in OLETF than in LETO rats. When the numbers of mRNA grains per coronary vessel were calculated, AT₁ receptor mRNA in OLETF rats was increased 2.4-fold compared with LETO rats (Fig. 3B). On the other hand, there was no significant difference in the gene expression level of AT₂ receptor between the two groups (Fig. 3, A and B).

**Expressions of VEGF and its receptors.** As shown in Fig. 4A, VEGF and its receptor KDR, which mediates the angiogenic effects of VEGF (10), were weakly stained in LV sections from LETO rats. However, these molecules were strongly expressed in OLETF rats. The results of quantitative analysis revealed that VEGF and KDR expressions were ~1.9- and 2.0-fold higher in OLETF than in LETO rats, respectively (Fig. 4B). Positive staining for VEGF and KDR was mainly in coronary vessels, whereas it was very weak in cardiomyocytes. In OLETF rats treated with candesartan, protein expressions of VEGF and KDR were dramatically reduced nearly to the levels seen in nondiabetic LETO rats (Fig. 4, A and B). Protein expression of another VEGF receptor Flt-1, which is devoid of angiogenic activities (10), was modestly detected in coronary vessels of both LETO and OLETF rat LV sections. In contrast to the KDR level, the expression level of Flt-1 did not differ between the two groups (data not shown).

The increase in positive staining for VEGF and KDR proteins in coronary vessels of OLETF rats correlated with an increase in their mRNAs, which was obtained from in situ hybridization experiments (Fig. 5A). The results of quantitative analysis showed a 2.3- and 3.0-fold increase in VEGF and KDR mRNA expression in OLETF compared with LETO rats, respectively (Fig. 5B). Treatment of OLETF rats with candesartan significantly reduced the expression levels of their mRNAs nearly to those of nondiabetic controls (Fig. 5, A and B).
A and B). Again, the gene expression level of Flt-1 was unaffected by diabetes (data not shown).

The mRNA expression levels of VEGF and KDR were also analyzed by the RT-PCR method (Fig. 6). The mRNA levels of VEGF and KDR were very low in LVs of LETO rats, and they were evidently upregulated in those of OLETF rats. Candesartan treatment suppressed their increased expressions to the levels obtained in LETO rats.

Expression of HIF-1α protein. Immunofluorescent staining for HIF-1α showed that its protein expression was markedly enhanced in coronary vessels of LV sections from OLETF compared with those from LETO rats (Fig. 7A). Diffuse and marked immunoreactivity for the molecule seen in OLETF rats indicates that HIF-1α protein was increased not only at the nuclear level but also at the cytoplasmic level. On the basis of quantitation of immunoreactivity using pixel intensity, HIF-1α protein was increased 2.2-fold compared with LETO rats (Fig. 7B). This marked increase in HIF-1α expression was completely blocked by candesartan treatment (Fig. 7, A and B).

Expression of AGEs. Strongly increased immunofluorescent staining for AGEs was detected in LV sections from OLETF rats (Fig. 8A). The location of positive staining for AGEs was largely within coronary vessels. The expression of AGEs was very faint in LETO rats. The results of quantitative analysis showed a 2.2-fold increase in AGEs expression in OLETF compared with LETO rats (Fig. 8B). Candesartan treatment significantly but incompletely reduced the expression level of AGEs in OLETF rats (Fig. 8, A and B).

Fig. 4. Protein expressions of vascular endothelial growth factor (VEGF) and kinase domain region (KDR) in LV cross sections from LETO, OLETF, and candesartan-treated OLETF (CAN) rats. A: representative immunofluorescent findings for VEGF (top) and KDR (bottom) proteins. Positive staining was focused on coronary vessels. Magnification ×400. B: quantitation of immunoreactivity shown by pixel intensity. The average pixel intensity was calculated in 20 randomly selected coronary vessels (50–100 μm ID) per sample. Values are means ± SD; n = 10. *P < 0.001 vs. LETO; †P < 0.001 vs. OLETF.

Fig. 5. Gene expressions of vascular VEGF and KDR in LV cross sections from LETO and OLETF rats. A: representative in situ hybridization findings for VEGF (top) and KDR (bottom) mRNAs. Nuclei in coronary vessels were stained with hematoxylin as bluish violet. The presence of mRNA is shown by the black grains. Magnification ×400. B: quantitation of mRNAs analyzed by the in situ hybridization method. The numbers of mRNA grains per coronary vessel are shown. For this quantitation, coronary vessels having the same size (~50 μm ID) were selected. Values are means ± SD, n = 10. *P < 0.001 vs. LETO; †P < 0.001 vs. OLETF.
Morphometric changes. Figure 9A shows representative micrographs of coronary capillaries of LV sections in LETO, OLETF, and candesartan-treated OLETF rats obtained by the double-staining method. The venular capillary portion, which was stained red, was evidently remarkable in LETO rats, whereas the intermediate and arteriolar capillary portions, which were stained violet and blue, respectively, were much pronounced in OLETF rats. The total capillary density was significantly higher in OLETF than in LETO rats (Fig. 9B). This was largely due to increases in the intermediate and arteriolar capillary portions in OLETF rats (Fig. 9C). Treatment of OLETF rats with candesartan markedly suppressed the increased proportions of intermediate and arteriolar capillaries, resulting in a significant decrease in the total capillary density (Fig. 9, B and C). Candesartan treatment significantly but incompletely improved the decreased proportion of venular capillaries in OLETF rats (Fig. 9C).

The ratio of capillary to myocyte, which was defined as the number of capillaries assigned to one myocyte, was significantly higher in OLETF than in LETO rats (Fig. 10A). Furthermore, the capillary domain area, which shows an area supplied by a single capillary and was defined as the area where one capillary provides oxygen, was significantly decreased in OLETF rats (Fig. 10B). Treatment of OLETF rats with candesartan returned the capillary to myocyte ratio and the capillary domain area to the levels obtained in LETO rats (Fig. 10, A and B). Myocyte scan area showed cardiomyocyte hypertrophy in OLETF rats, which was restored by candesartan treatment (Fig. 10C). The percent ratio of collagen to noncollagen protein was significantly increased in OLETF rats, and this change was completely blocked by candesartan treatment (Fig. 10D).

DISCUSSION

The OLETF strain of rats used in this study is well established as an animal model of human NIDDM (19). On the basis of previous reports (15, 27, 44), OLETF rats exhibit the prediabetic phase characterized by postprandial hyperglycemia and insulin resistance from 10 to 20 wk of age, the NIDDM phase showing impaired glucose tolerance with hyperglycemia at 30 wk of age, and the insulin-dependent diabetes mellitus (IDDM) phase after 40 wk of age. Accordingly, our
subjects (20 wk of age) were in the stages of insulin-resistant diabetes. Indeed, we observed that plasma glucose and insulin levels after 24 h of fasting were modestly and prominently higher in OLETF rats than in age-matched LETO rats, which is in good agreement with the results of other investigators (27, 44).

In the current study, we showed that the localization of ANG II increased in coronary vessels of diabetic...
hearts. ANG II labeling in coronary vessels was ∼2.3-fold higher in OLETF than in LETO rats. Recent work has pointed to upregulation of local renin-angiotensin system (RAS) and enhanced synthesis of ANG II in streptozotocin-induced diabetic rats (11). Thus both NIDDM and IDDM appear to activate the local RAS and enhance generation of ANG II in vascular tissues. We also found that AT₁ receptors, but not AT₂ receptors, were more abundantly expressed in coronary vessels of OLETF compared with LETO rats. The increase in the expression level of AT₁ receptors correlated with an increase in the mRNA level, which was obtained from in situ hybridization experiments, indicating that insulin-resistant diabetes causes an increase in AT₁ receptor synthesis at the level of transcriptional regulation. The present study did not address the mechanism activating the local RAS in OLETF rats. Angiotensinogen is known to be the limiting factor in the synthesis of ANG II, and p53 has been shown to promote transcription of angiotensinogen and AT₁ receptors and enhance ANG II formation and ANG II-mediated responses in myocytes (23). Hyperglycemia may result in p53 glycosylation and expression of angiotensinogen and AT₁ receptors (11).

Beyond its hemodynamic effects, ANG II has been considered to be centrally involved in vascular remodeling in diabetes through its growth-promoting actions (6). In the present study, a significant increase in the total capillary density was found in the hearts of OLETF rats. As a result of the increased total capillary density, the ratio of capillary to myocyte was significantly increased and the capillary domain area was shortened as a compensatory change. The increased total capillary density was strongly associated with an increase in the proportion of intermediate and arteriolar capillaries. The transition from the venular to intermediate/arteriolar type in the capillary proportion is consistent with the finding obtained in streptozotocin-induced diabetic rat hearts (30). On the basis of the finding that ANG II and AT₁ receptors were highly expressed in coronary vessels of the OLETF rat, we suggest that ANG II, through activation of AT₁ receptors, may play a key role in the remodeling of coronary capillary network that occurred in this animal model of NIDDM. Indeed, treatment of candesartan, an AT₁ receptor blocker, significantly prevented coronary structural remodeling in OLETF rats. The increased coronary capillary density was reversed to the nondiabetic control level after treatment of OLETF rats with candesartan. The decreased proportion of venular capillaries was significantly inhibited by candesartan treatment. However, the ratio of venular to total capillaries did not completely return to the control level and remained modestly low despite the complete normalization of the total number of capillaries. It may be assumed that transition from venular to intermediate/arteriolar capillaries is partly mediated through mechanisms other than the angiogenic effect caused by activation of AT₁ receptors with ANG II.

Immunohistochemical and immunofluorescent studies showed abundant protein expressions of VEGF and KDR in coronary vessels of the hearts from OLETF rats. There was a significant correlation between increases in their protein and mRNA levels, as determined by in situ hybridization analysis. This parallel behavior of protein and mRNA expressions implies that coronary expression levels of VEGF and KDR in OLETF rats are regulated in a transcriptional manner. VEGF is known to be among the most potent and specific angiogenic factors (10). Because KDR mediates most of the VEGF effects, including angiogenesis (10, 32, 41), KDR overexpression could potentiate the angiogenic effect of VEGF. Thus upregulation of VEGF and its receptor KDR suggests that VEGF may participate in promoting the formation of new capillaries from existing coronary vessels in the OLETF rat heart. Treatment of OLETF rats with candesartan dramatically reduced VEGF and KDR expressions nearly to those seen in LETO rats at both protein and mRNA levels. This suggests that endogenous ANG II can stimulate the transcription of these molecule genes in coronary vessels of OLETF rats mediated through AT₁ receptors. Therefore, in OLETF rats, enhanced local generation of ANG II and increased number of AT₁ receptors result in increases in VEGF and KDR expressions in coronary vessels, thereby leading to the angiogenic changes in coronary capillary network.

We found that HIF-1α protein was much highly expressed in coronary vessels of OLETF rats. HIF-1α is one of the subunits composing HIF-1, which is a transcriptional factor under hypoxic conditions (42). Under hypoxic conditions, HIF-1α protein is stabilized without being degraded through oxygen-dependent proteolysis and initiates a multistep pathway of activation, including dimerization with its partner (43). Activation of HIF-1 regulates the VEGF gene by its binding to a hypoxia-responsive element in the 5′-flanking region of the VEGF gene (13). Thus HIF-1 is a strong inducer of VEGF mRNA expression. Therefore, it seems most likely that the increased expression level of HIF-1α protein may have contributed to increased VEGF expression in coronary vessels of OLETF rats.

There could be factors other than hypoxia involved that induce HIF-1α protein expressions. During diabetes, AGEs are generated by nonenzymatic reactions between glucose and free amino reactive group of proteins and lipids (4). Recent work (39) has demonstrated that AGEs stimulate VEGF expression through the accumulation of HIF-1α and the subsequent activation of HIF-1. This may explain the possible mechanism for involvement of HIF-1α in increased transcription of VEGF in the OLETF rat, because the accumulation of AGEs was found to be very pronounced in coronary vessels of this strain. Candesartan treatment significantly suppressed coronary accumulation of AGEs in diabetic rats without affecting the blood glucose and insulin concentrations. Consistently, long-term treatment with AT₁ receptor blocker has been reported to exert salutary effects on AGEs levels in the rat remnant kidney model (34). AGEs are known to bind to receptors for AGE (RAGE) to exert their effects. AGEs increase RAGE protein expression, and AGE-induced
RAGE expression can be inhibited by the ACE inhibitor captopril (17). Thus blockade of AT\(_1\) receptors might modify the interaction between AGEs and RAGE by attenuating RAGE expression, leading to suppression of AGE accumulation. Candesartan treatment did incompletely decrease AGEs accumulation in coronary vessels of OLETF rats despite the complete return of HIF-1\(\alpha\) expression to the normal level. This might be associated with the AGE-independent mechanism for induction of HIF-1\(\alpha\). Indeed, evidence has been provided that ANG II itself can also induce HIF-1\(\alpha\) through the production of reactive oxygen species (33). Furthermore, with respect to the effect of candesartan on coronary VEGF and KDR expressions in OLETF rats, it cannot be entirely excluded that activation of AT\(_1\) receptors directly upregulates mRNA levels VEGF and KDR, thereby potentiating VEGF-induced angiogenic activity (8, 31).

Contrary to the present findings in OLETF rats, Chou et al. (7) have shown that with the use of RT-PCR or Northern blot, gene expressions of VEGF and its receptors KDR and Flt-1 in the myocardium are significantly decreased in streptozotocin-induced diabetic rats as well as established diabetic patients (of both types) with hypertension and/or myocardial infarction. They have also demonstrated twofold increased expression levels of VEGF and its receptors in retina and glomeruli from streptozotocin-induced diabetic rats and suggested that differential regulation of VEGF and its receptors may exist between microvascular and cardiac tissues, which could be regulated by insulin (7). The apparent discrepancy between their results and ours cannot be solely attributed to differences in techniques for detecting expressions of target molecules. Our results using the RT-PCR method revealed that upregulation of VEGF and KDR mRNAs was evident in LVs from OLETF rats. In our experiments, OLETF rats were used at 20 wk of age, which were in the early insulin-resistant stage of NIDDM. At the late stage (\(\geq 40\) wk of age) this strain represents the conversion from NIDDM to IDDM with prominent hyperglycemia (19, 44). Our preliminary experiments showed that OLETF rats exhibited a significant reduction in the coronary capillary density at the late stage (unpublished observation), probably due to declined expressions of VEGF and KDR. Indeed, the progressive decreases in the capillary density in myocardium resulting from severe, chronic diabetes has been demonstrated in alloxan-induced IDDM rats (38). Furthermore, chronic diabetic patients suffering from ischemic heart disease show significantly low capillary densities in the hearts (45). Therefore, we assume that whereas VEGF and KDR could be highly expressed in coronary vessels at early insulin-resistant stage of NIDDM, their coronary expression levels may be declined with disease progression.

However, the results of Chou et al. (7), with the use of insulin-resistant Zucker and insulin-resistant spontaneous-diabetic Zucker rats, provide evidence that insulin-resistant glucose-intolerant states may also decrease expressions of VEGF and its receptors in myocardium. There appears to be an important difference in insulin-resistance of Zucker and OLETF rats. Whereas candesartan treatment did not alter hyperinsulinemia in OLETF rats, Henriksen et al. (16) have demonstrated that the insulin resistance in obese Zucker rats can be reduced by chronic AT\(_1\) receptor antagonism. At present, it remains controversial as to whether ACE inhibitors and AT\(_1\) receptor blockers can improve glucose tolerance and insulin sensitivity in human diabetes (26, 35, 37, 46). Nonetheless, there are significant limitations with the use of myocardium of insulin-resistant NIDDM patients at the early stage. It is beyond the scope of this study to answer the question of whether changes in coronary capillary morphology associated with expressions of angiogenic molecules seen in early insulin-resistant NIDDM of human can be mimicked by those found in Zucker rats, OLETF rats, or both.

In addition to coronary capillary remodeling, OLETF rats exhibited a significant increase in cardiac collagen deposition. Consistent with this finding, cardiac fibrosis was reported by Mizushige et al. (27) to be promoted from the prediabetic state in OLETF rats. The reversal of cardiac collagen deposition after AT\(_1\) receptor blockade with candesartan indicates that ANG II stimulates cardiac collagen synthesis via AT\(_1\) receptors in OLETF rats. Although the primary autocrine and paracrine mediators of ANG II effects on fibrillar collagen synthesis remain to be elucidated, the prosceloretic cytokine, transforming growth factor-\(\beta\) (TGF-\(\beta\)), has been implicated as a key mediator of extracellular matrix expansion in diabetes (3). We found that TGF-\(\beta\) was highly expressed in coronary vessels of the OLETF rat heart at both protein and mRNA levels and that its overexpression was completely suppressed by candesartan treatment (unpublished observations). This supports that activation of AT\(_1\) receptors with ANG II stimulates cardiac collagen production by promoting TGF-\(\beta\) synthesis (36). It should be noted that cardiac fibrosis may be an explanation for the LV diastolic dysfunction, including a prolonged deceleration time and a decreased amplitude of peak velocity of the early diastolic filling wave, seen from the early stage of developing diabetes in OLETF rats (27).

The beneficial effects of AT\(_1\) receptor blockade with candesartan may be due in part to activation of AT\(_2\) receptors. Thus treatment with AT\(_1\) receptor antagonists could result in an uninhibited stimulation of AT\(_2\) receptors because circulating and tissue ANG II levels would be increased after blockade of AT\(_1\) receptors (5). Although little is known regarding how AT\(_2\) receptors may counteract actions of AT\(_1\) receptors, there is evidence that AT\(_2\) receptors are a negative regulator of collagen synthesis. AT\(_2\) receptors have been shown to inhibit collagen synthesis by stimulating kinin production (24).

In conclusion, OLETF rats showed prominent increases in local generation of ANG II and expression of AT\(_1\) receptors in coronary vessels at the insulin-resistant stage of NIDDM. The morphometric analysis showed arterialization of the venular capillary portion...
and resulting increased capillary density in the OLETF rat heart. Such structural changes would have occurred as compensatory adaptation under the pathological condition, which could lead to impaired physiological coronary vessel regression. This coronary capillary remodeling appeared to result from overexpression of VEGF through the induction of HIF-1α by the pathways involving ANG II and AGEs. Our present findings that candesartan treatment ameliorated the structural changes in coronary capillary network and suppressed coronary expressions of molecules contributing to angiogenesis in the OLETF rat suggest a key role of ANG II in the pathogenesis of the coronary capillary remodeling at the insulin-resistant stage of NIDDM in this strain.

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