Coronary Capillary Remodeling in Non-Insulin-Dependent Diabetic Rats: Amelioration by Inhibition of Angiotensin Converting Enzyme and Its Potential Clinical Implications

Taeko SUGAWARA, Satoshi FUJII, Tarikuz AKM ZAMAN, Daisuke GOTO, Takeaki KANEKO, Tomoo FURUMOTO, Hiroko TOGASHI*, Mitsuhiro YOSHIOKA*, Tomiyasu KOYAMA**, and Akira KITABATAKE

Using Otsuka Long Evans Tokushima Fatty (OLETF) rats, a model of human non-insulin-dependent diabetes mellitus (NIDDM) that exhibits hypertension, obesity, hyperglycemia and hyperlipidemia, the role of local angiotensin II in cardiovascular complications at early stages of NIDDM was characterized. OLETF rats were given an angiotensin converting enzyme (ACE) inhibitor, cilazapril (10 mg/kg/day) or vehicle from the age of 5 weeks to 20 weeks. Arteriolar, intermediate and venular capillary proportions were determined by the double-staining method and levels of collagen and non-collagenous proteins were determined by the selective dye-binding method in heart tissues. In OLETF rats at 20 weeks of age, capillary network remodeling (i.e., an increase in arteriolar portions and a decrease in venular portions) and an increase in collagen content were detected. Cilazapril not only exerted favorable effects on markers of diabetes, but also prevented capillary network remodeling and ameliorated the increase in collagen content. These results suggest that 1) capillary network remodeling and increase in extracellular matrix protein levels precede the onset of overt NIDDM in OLETF rats, and 2) angiotensin II may be involved in the pathogenesis of cardiac complications in the early stages of NIDDM. (Hypertens Res 2001; 24: 75-81)

Key Words: diabetes, ACE inhibitor, capillaries, collagen, heart

Introduction

The prevalence of hypertension is higher in patients with diabetes than in nondiabetic patients. Non-insulin-dependent (type II) diabetes mellitus (NIDDM) and hypertension are known to be closely associated (1). The coexistence of hypertension and diabetes is clinically important because the two conditions constitute multiple risk factors for macrovascular and microvascular diseases. Diabetes and insulin resistance are major risk factors in the pathogenesis of atherosclerotic cardiovascular diseases (2-4), and clinical and experimental investigations suggest that the renin angiotensin system is important for the development of various cardiovascular diseases, including hypertension (4-8). Diabetes is independently associated with left ventri-
cular mass, and angiotensin II and insulin have been suggested to be additive stimuli to left ventricular hypertrophy (9). Angiotensin converting enzyme (ACE) inhibitors can significantly retard the progression of diabetic renal disease (10), but the effects of ACE inhibitors on diabetic cardiovascular complications have not been fully clarified. Otsuka Long Evans Tokushima Fatty (OLETF) rats are an established model of human NIDDM (11), and exhibit hypertension, obesity, hyperglycemia and hyperlipidemia. Cardiac lesions have previously been characterized in advanced-age OLETF rats (12), demonstrating the usefulness of the model in studying the pathogenesis of cardiac complications in NIDDM. In the present study we investigated the effects of an ACE inhibitor on cardiac complications of early stages of NIDDM using OLETF rats. The results suggest that capillary network remodeling and an increase in collagen occur in the early stages of NIDDM, and that inhibition of ACE prevents both these effects.

Materials and Methods

Experimental Animals

All procedures were in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Male OLETF rats and Long Evans Tokushima Otsuka (LETO) rats, genetic controls for OLETF, were supplied by Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). They were given standard chow (Oriental Kobo, Tokyo, Japan) and had free access to tap water.

Experimental Protocol

Five-week-old OLETF rats were divided into two groups: 1) a vehicle-treated control group (n = 5) and 2) a cilazapril (Eisai-Nippon Roche; 10 mg/kg/day, p.o. mixed in chow)-treated group (n = 5). The age-matched LETO rats were treated with vehicle (n = 5). The cilazapril treatment was carried out for 15 weeks (from age 5 to 20 weeks). After the treatment the rats were anesthetized by pentobarbital, the mean blood pressure was measured through the femoral artery, and the heart was rapidly perfused with phosphate buffered saline (pH 7.4). Heart weight was then measured. A portion of the left ventricle was embedded into optimum cutting temperature (OCT) compound (Sakura Finetecnical, Tokyo, Japan), then immediately frozen in liquid nitrogen and stored at −80°C. The remaining portion was fixed in 4% paraformaldehyde and embedded in paraffin.

Double Staining

Remodeling of the coronary capillary network in the left ventricle was evaluated using differential enzyme activities in coronary microvascular endothelial cells, as described in our previous study (13, 14). The coronary capillary network was evaluated in the subendocardium to estimate capillary density from regions of myocytes that are sectioned transversely. Frozen left ventricular tissues were randomly sampled, sectioned with a cryostat (16 μm) and stained for alkaline phosphatase (AP) and dipeptidylpeptidase IV (DPPIV). In brief, the AP reaction was performed using glycyl-l-proline-4-methoxy-β-naphthylamine (Sigma, St. Louis, USA) and fast blue B (Merck, Darmstadt, Germany). The DPPIV reaction was performed using naphthol AS-MX phosphate (Sigma) and variaamine blue salt RT (Sigma). The sections were fixed in chloroform acetone for 5 min, then incubated for 90 min at room temperature to allow DPPIV reaction, and for 25 min at room temperature to allow AP reaction. Postfixation was performed with 4% formalin for 2 h at 4°C. Arteriolar capillary portions, whose endothelial cells contained AP, were stained blue, and venular capillary portions containing DPPIV were stained red. The intermediate capillary portions containing both enzymes were stained violet. Capillary density, the proportion of different capillary portions, capillary to myocyte (C:M) ratio defined as the number of capillaries assigned to one myocyte, and capillary domain area defined as the area to which one capillary provides oxygen, were determined as we previously described (13-15). Image analysis was performed using image analysis software (Microcomputer Imaging Device, Imaging Research, St. Catherine, Ontario, Canada) (15). For each experimental condition, at least 2 sections from each sample were examined, and at least 4 microscopic fields were investigated at ×400 magnification. All sections were encoded and analyzed by two experienced investigators blinded to the experimental conditions.

Collagen Measurements

Collagen content was measured as previously described (16). In brief, frozen left ventricular samples were sectioned into 10-μm thick sections using a cryostat, then placed on silane-coated glass slides. The sections were stained with Sirius red F3BA and fast green FCF. Both dyes were eluted readily and simultaneously with NaOH-methanol, and the absorbances obtained at 530 and 605 nm were used to determine the amount of collagen and protein.

Miscellaneous Measurements

Plasma glucose was measured by the glucose oxidase method. Plasma insulin was measured by an enzyme linked immunosorbent assay (Shibayagi, Tokyo, Japan). Values corresponding to those in homeostasis model assessment (HOMA), which were defined as the product of the fasting glucose (mg/dl) and fasting insulin (ng/ml) values, were calculated and used as an index of insulin sensitivity. The
concentrations of cholesterol and triglyceride were determined enzymatically using commercially available kits (Determiner TC55, Kyowa Medex, Tokyo; Serotech TG5, Serotech, Sapporo, Japan).

Statistical Analysis

Results are expressed as the means ± SD. Statistical analysis was carried out using the Statgraphics statistical package. Comparison was made between the two groups using an unpaired two-tailed Student's t-test. P values less than 0.05 were considered to indicate statistical significance.

Results

Body Weight, Heart Weight and Blood Pressure of OLETF Rats

The body weight of vehicle-treated OLETF rats at 20 weeks of age was significantly greater than that in the LETO rats of the same age (337 ± 13 g vs. 43 ± 13 g, p < 0.05). The body weight of the cilazapril-treated group (468 ± 13) was not significantly different from that of LETO rats. The heart weight of OLETF rats was greater than that of LETO rats (1,284 ± 78 vs. 1,083 ± 261 mg), and was significantly decreased by treatment with cilazapril (1,006 ± 18 mg, p < 0.05). Because the body weight of OLETF rats was substantially greater than that of LETO rats, the heart weight to body weight ratio of OLETF rats tended to be lower than that of LETO rats (0.23 ± 0.01 vs. 0.28 ± 0.06%). and was significantly decreased by treatment with cilazapril (0.22 ± 0.01%, p < 0.05 vs. LETO). The blood pressure of OLETF rats was significantly higher than that of LETO rats (95 ± 4 vs. 81 ± 2 mmHg, p < 0.05). Cilazapril lowered the blood pressure of OLETF rats to a significant extent (59 ± 7, p < 0.05).

Plasma Glucose, Insulin, Cholesterol and Triglyceride

The plasma glucose level of vehicle-treated OLETF rats was significantly greater than that of the LETO rats of the same age (152 ± 4 vs. 126 ± 6 mg/dl, p < 0.05), but was not significantly different from that of the cilazapril-treated OLETF rats (146 ± 10). The plasma insulin level of OLETF rats was greater than that of LETO rats (1.37 ± 0.38 vs. 1.26 ± 0.25 ng/ml) and was significantly decreased by treatment with cilazapril (0.87 ± 0.22, p < 0.05). The HOMA equivalent value was significantly higher in OLETF rats than in

![Representative micrographs of double-stained cardiac tissue sections. Panel A: A cross-section of the subendocardium of a LETO rat at 20 weeks of age. Capillaries with AP activity are stained blue and DPPIV are stained red. Capillaries reactive to both enzymes are stained violet. Panel B: A cross-section of the subendocardium from an OLETF rat at 20 weeks of age. The number of blue-stainable AP-reactive capillaries is increased and that of red-stainable DPPIV-reactive capillaries is decreased compared to those in panel A. Panel C: A cross-section of the subendocardium from an OLETF rat treated with cilazapril from 5 weeks to 20 weeks of age. The number of blue-stainable AP-reactive capillaries is decreased compared to that in panel B, and the number of red-stainable DPPIV-reactive capillaries is increased compared to that in panel B. Bar indicates 50 μm.](image-url)
Fig. 2. Capillary density (number/mm²) for the arteriolar, intermediate and venular capillary portions in the subendothelialum of LETO (n=5) and OLETF (n=5) rats at 20 weeks of age, and in OLETF rats treated with cilazapril from 5 to 20 weeks of age (n=5). Results are presented as the means±SD. *p<0.05 compared to LETO rats. †p<0.05 compared to OLETF rats.

LETO rats (213±63 vs. 162±37, p<0.01) and was significantly decreased by treatment with cilazapril (132±46, p<0.01). The plasma cholesterol level of OLETF rats was significantly higher than that of LETO rats (84±9 vs. 66±11 mg/dl, p<0.05). Cilazapril treatment did not lower the cholesterol level of OLETF rats (94±12). The plasma triglyceride level was significantly higher in OLETF than LETO rats at 20 weeks of age (146±34 vs. 36±6 mg/dl, p<0.05), and cilazapril significantly lowered the triglyceride level of the former (44±7, p<0.05).

Capillary Network Remodeling in OLETF Rats

Remodeling of the capillary network was quantitated at 20 weeks using the double-staining technique. A representative double stain is shown in Fig. 1. The arteriolar capillary portion containing AP was stained blue and the venular capillary portion containing DPP IV was stained red. The intermediate capillary portion was stained violet. The total capillary density was significantly increased in OLETF rats compared with control LETO rats (2.075±155 vs. 2.057±322 capillaries/mm², p<0.01) (Fig. 2). The red-stained area of the venular capillary portion was significantly smaller in OLETF rats than in LETO rats (OLETF 715±159 vs. LETO 1,295±122, p<0.05). In contrast, the violet-stained area of the intermediate capillary portion was significantly increased (1,285±114 vs. 542±148, p<0.01). And the blue-stained area of the arteriolar capillary portion was also significantly increased (672±158 vs. 226±105, p<0.05). Treatment with cilazapril in OLETF rats significantly inhibited the increase in total capillary density (2.326±215, p<0.05) and somewhat ameliorated the decrease in venular capillary density (761±221), the increase in intermediate capillary density (1,089±242), and the increase in arteriolar capillary density (476±197). The capillary domain area was significantly decreased in OLETF rats compared to the values in LETO rats (374±22 vs. 490±65 mm², p<0.01). Treatment with cilazapril ameliorated the decrease of capillary domain area in OLETF rats (430±42). There was no difference in the C:M ratio between OLETF and LETO rats (1.08±0.13 vs. 1.18±0.12). Cilazapril did not alter the C:M ratio of OLETF rats (1.00±0.07).

Collagen Content

The collagen/non-collagen protein ratio in heart tissue of vehicle-treated OLETF rats was significantly greater than that in age-matched LETO rats (21.4±0.8 vs. 7.4±1.9 mg collagen/mg protein, p<0.05). The increase was significantly inhibited in the cilazapril-treated OLETF group (8.2±3.8, p<0.05) (Fig. 3). On Elastica-Masson's trichrome staining, neither the perivascular fibrosis nor the increase in vessel wall thickness previously reported in aged OLETF rats (II) were noted around arterioles less than 100 μm diameter in either 20-week-old OLETF or LETO rats, suggesting that perivascular fibrosis and an increase in vessel wall thickness did not occur in the early stages of NIDDM in the present study.

Discussion

The renin angiotensin system participates in the pathogene-
sis of cardiac hypertrophy and fibrosis induced by hypertension (17) and myocardial infarction (18). The cardiac renin angiotensin system may be activated in streptozotocin-induced diabetes (19). It remains uncertain, however, whether the renin angiotensin system is involved in the pathogenesis of cardiac complications in NIDDM. OLETF rats characterized by early increase in serum insulin, late onset of hyperglycemia and mild course of diabetes mellitus (11) also exhibit hypertension (20). Thus, they are suitable for elucidating the role of the renin angiotensin system in cardiac complications observed in NIDDM. Although it has been reported that aged OLETF rats show an increase in the cardiac expression of transforming growth factor (TGF)-β, a potent growth factor involved in fibrosis, as well as an increase in perivascular fibrosis (12), the early changes in cardiac tissues in OLETF rats have not yet been characterized. Accordingly, we here investigated whether alterations in coronary microvascular remodeling and/or in the levels of extracellular matrix components were present in young OLETF rats before the onset of overt diabetes.

In rats with streptozotocin-induced insulin dependent diabetes mellitus (IDDM), captopril has been shown to partially prevent diabetic cardiomyopathy (21), suggesting that ACE inhibitors may be useful for preventing cardiomyopathy in IDDM. In the present study cilazapril prevented increases in heart weight and collagen protein content in OLETF rats, suggesting that inhibition of the renin angiotensin system may be beneficial in preventing cardiomyopathy in NIDDM as well as in IDDM. Because ACE inhibitors lead to inhibition of bradykinin degradation and bradykinin inhibits cardiac remodeling (22), cilazapril may exert its effects at least partially through bradykinin. Furthermore, angiotensin type 2-receptors have been shown to exert an antiproliferative action on endothelial and smooth muscle cells (23, 24). Further investigations will be needed to determine the potential involvement of bradykinin and angiotensin type 2 receptors in protecting against cardiovascular complications in OLETF rats.

We have previously reported that significant capillary network remodeling can occur in rats subjected to coronary artery occlusion or nitric oxide depletion (13-15), suggesting that the capillary network can undergo substantial adaptation under various pathological conditions. Indeed, in OLETF rats the red-stained area of the venular capillary portion was significantly decreased compared with the area in LETO rats. In contrast, the violet-stained area of the intermediate capillary portion was significantly increased. And the blue-stained area of the arteriolar capillary portion was also significantly increased. These results suggest that, in the early stages of NIDDM, arteriolarization of the venular capillary portion and intermediate capillary portion may be induced. There was a decrease in capillary domain area — defined as the area to which one capillary provided oxygen (14) — in young OLETF rats relative to that in LETO rats, suggesting an adaptation process for more efficient delivery of oxygen in response to metabolic alterations, microvascular dysfunction or myocyte atrophy. This capillary network remodeling was prevented by cilazapril. Because angiotensin II can potentiate angiogenic activity in microcapillary endothelial cells (25) and ACE inhibitor may suppress tumor angiogenesis (26), inhibition of capillary network remodeling by cilazapril is caused at least partly by its direct action in addition to its hypotensive action. Indeed, angiotensin II can upregulate vascular endothelial growth factor, a potent mitogen for endothelial cells, in rat heart endothelial cells (27). In IDDM model rats treated with streptozotocin, a reduction has been observed in vascular surface area, as well as in reduction in length and diameter of capillaries and post capillary venules (28). The structural changes may underlie the reduced function of the diabetic heart and limited angiogenesis in diabetes. Because the blood pressure was measured through the femoral artery under anesthesia, the blood pressure values were generally lower than those previously reported (20). The question of whether or not the hypotensive action provided by other antihypertensive drugs can also alter coronary capillary remodeling in OLETF rats will require further investigation.

Although perivascular fibrosis was not observed in young OLETF rats in the present study, the collagen/non collagen protein ratio in heart tissue was increased, suggesting that extracellular matrix remodeling occurred. In streptozotocin-treated rats, an increase in the extracellular spaces between cardiac myocytes — as well as depositions of collagen fibrils and amorphous components of ground substances — have been reported (28). The extracellular matrix remodeling could increase the distance between capillaries and myocytes, increase the stiffness of the ventricular wall, and reduce both oxygen delivery and left ventricular contractility. In young OLETF rats, activation of the renin angiotensin aldosterone system may include a progressive remodeling of the heart mediated, in part, by the induction of various cytokines and growth factors as in patients with heart failure (29). Cardiac aldosterone may also be increased in heart and coronary blood vessels, thereby leading to autocrine or paracrine activity (30).

Although cilazapril did not significantly affect plasma glucose or cholesterol, there were significant reductions in plasma insulin, HOMA equivalent values, body weight and triglyceride levels, suggesting that cilazapril had minor but beneficial effects on diabetes itself. Administration of ACE inhibitors has been shown to result in improved glucose metabolism and insulin resistance in fructose fed rats (31). Thus, it cannot be completely ruled out that the effects of cilazapril may be at least partly mediated through the improvement of insulin resistance.

In summary, angiotensin II appears to be involved in the development of the cardiovascular complications observed in the early stages of NIDDM in OLETF rats, and inhibition of ACE ameliorated myocardial hypertrophy.
coronary capillary network remodeling, and increase in collagen content. Our results support the concept that ACE inhibition may be useful not only in the treatment but also in the prevention of cardiac complications in patients with NIDDM. Amelioration of coronary capillary network remodeling may at least partly explain the favorable anti-ischemic effects of ACE inhibitors in high-risk patients (32).

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