Heart angiotensin II-induced cardiomyocyte hypertrophy suppresses coronary angiogenesis and progresses diabetic cardiomyopathy

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The rising incidence of type 2 diabetes mellitus raises major public health concerns because of the increased risk of cardiovascular complications (1, 2). Diabetic cardiomyopathy (DCM) is defined as left ventricular (LV) dysfunction that occurs independently of coronary artery disease and hypertension (1, 2). Its functional alterations are LV hypertrophy (LVH) and LV diastolic dysfunction, which may precede the development of LV systolic dysfunction (1, 2). Its pathological features involve cardiomyocyte hypertrophy and apoptosis, microvascular pathology, including abnormal capillary density and permeability, and interstitial fibrosis (1, 2, 6).

The activation of the renin-angiotensin system (RAS) is well recognized in DCM. The circulating RAS is downregulated in diabetes (24, 35). Nevertheless, pharmacological blockade of the RAS with ANG II type 1 receptor blockers (ARBs) or angiotensin-converting enzyme inhibitors prevents cardiac damage in diabetic patients (3, 12). Furthermore, ANG II and the other RAS components are elevated in the diabetic heart (5, 6, 46). Therefore, the local RAS, activated in the diabetic heart, independent of the circulating RAS, could induce LV dysfunction and injury through the ANG II type 1 receptor.

Heart ANG II induces LVH by raising blood pressure (40) and acting as a growth factor on cardiomyocytes in the absence of hypertension (23, 41). Cross-talk between cardiomyocytes and the coronary vasculature during cardiac growth is very important in maintaining cardiac function (37, 45). During adaptive cardiac growth, secretion of angiogenic growth factors such as VEGF from hypertrophied cardiomyocytes under chronic hypoxia is responsible for enhanced angiogenesis (37, 45). This, in turn, contributes to cardiac growth and the maintenance of contractile function by carrying O2 and nutrients into the hypertrophied myocardium (37, 45). However, disruption of the coordination between coronary angiogenesis and cardiac growth results in a transition from adaptive hypertrophy to heart failure with interstitial fibrosis (37, 45). The suppression of angiogenesis associated with cardiac growth is induced by the downregulation of the angiogenic growth factors (37, 45). These reports led us to hypothesize that local ANG II overproduced in the diabetic heart may induce LVH, which may suppress coronary angiogenesis, therefore playing a crucial role in the pathogenesis of DCM. As it stands, direct proof of this hypothesis has been lacking. Furthermore, in the pathogenesis of DCM, it is unclear what factors produced from the hypertrophied cardiomyocytes are involved in the inhibition of coronary angiogenesis.

We used male Spontaneously Diabetic Torii (SDT) rats, a model of human nonobese type 2 diabetes (44), treated with or without the ARB olmesartan medoxomil (Olm) (27) and examined whether and how heart ANG II may disrupt the coordination between LVH and coronary angiogenesis and progress DCM.
To assess LV diastolic function, two-dimensional guided Doppler cross-sections and stored at Finetek, Tokyo, Japan, frozen in 100% ethanol on dry ice for 30 min, embedded in optimum cutting temperature compound (Sakura/H9262) into 4-μm sections also stained with picro-Sirius red. The cross-sectional area of sections also stained with picro-Sirius red. The cross-sectional area of tissue samples was evaluated throughout the inner third of the LV, which as previously described (8). All histological measurements were carried out using image-analysis software (Image Pro Plus 6.2J, Media Cybernetics, Bethesda, MD), as previously described (8, 15). All quantifications were performed in a blinded manner.

Materials and Methods

Experimental animals. The study protocol was approved by the Animal Ethics Committee of Jichi Medical University. Male SDT rats and age-matched male Sprague-Dawley rats (both from CLEA Japan, Tokyo, Japan) were housed under specific pathogen-free conditions. The SDT rat used in the present study is an inbred rat strain from an outbred colony of Sprague-Dawley rats (44). Male SDT rats spontaneously develop gradual impairment of insulin secretion and hyperglycemia without obesity after 20 wk of age, with an incidence of 100% at 40 wk of age, but they survive long term without insulin treatment (44). In our study, plasma glucose levels were measured once weekly from 15 wk of age to the onset of diabetes and every 8 wk after that. The onset of diabetes was defined as a plasma glucose concentration >250 mg/dl, as previously described (26). At the onset of diabetes, which we defined as “0 wk,” SDT rats were randomly divided into two groups: the SDT group and the Olm-treated group (Olm group). Age-matched male Sprague-Dawley rats were used as a control group and the Olm group was given powdered food (CE-2, CLEA Japan) including a dose [0.01% (wt/wt)] of Olm (Daichi SANKyo, Tokyo, Japan) (27) for 0, 8, or 16 wk from the onset of diabetes. Both the SDT and control groups were given the powdered food without Olm for the identical three periods. To confirm that the Olm intake was adequate and that the dose of Olm was not toxic, we measured serum Olm concentrations by HPLC (48). Serum Olm concentrations in SDT rats treated with Olm for 8 and 16 wk after the onset of diabetes were 118.4 ± 31.5 ng/ml (n = 5) and 125.8 ± 18.6 ng/ml (n = 5), respectively. These concentrations were not significantly different from each other and corresponded to approximately one-half of the maximum plasma concentration obtained when healthy human volunteers were given a single oral dose of 10-mg Olm (224 ± 45 ng/ml) (42). No serum Olm was detectable in control rats or SDT rats not given Olm.

SDT rats treated with and without Olm at 0, 8, and 16 wk after the onset of diabetes as well as age-matched control rats were killed by decapitation at the corresponding time points. Systolic blood pressure was measured in the conscious state by a noninvasive tail-cuff system (224 ng/ml) (42). No serum Olm was detectable in control rats with <8-μm lumen size and with 1 nucleus/field was counted, as previously described (15). Capillary density was expressed as the number of capillaries per field. The ratio of the number of capillaries to cardiomyocytes per field (capillary-to-cardiomyocyte ratio) was also determined. To examine whether there was any evidence of capillary endothelial cell proliferation, double immunostaining was performed with anti-RECA-1 and anti-PCNA antibodies, as previously described (15). For proliferative capillaries, the numbers of proliferative capillaries (anti-RECA-1-positive and anti-PCNA-positive cells) and all capillaries (anti-RECA-1-positive cells) were counted per LV field, and the percentage of proliferative capillaries was expressed as the percentage of proliferative capillaries compared with the total number of capillaries.

Identification of apoptosis. Apoptotic cells, including cardiomyocytes and capillary endothelial cells, were identified based on the presence of fragmented nuclear DNA in histological sections labeled using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method, as previously described (15, 19).

Capillary endothelial cell apoptosis was identified by double labeling using TUNEL and anti-RECA-1, as previously described (15). Slides were viewed with a microscope (BX50, Olympus) equipped with a digital camera (DP71, Olympus), and ≥20 randomly selected LV subendocardial areas (0.141 mm²/field) were blindly quantified at ×400 magnification using image-analysis software (Image Pro Plus 6.2J, Media Cybernetics), as previously described (8, 15). For apoptotic capillaries, the numbers of apoptotic capillaries (anti-RECA-1-positive and anti-PCNA-positive cells) and all capillaries (anti-RECA-1-positive cells) were counted per LV field, and the percentage of apoptotic capillaries was calculated out of the total number of capillaries.

Immunofluorescence. Immunofluorescence staining for ANG II and cardiac myosin in the LV was performed using frozen sections; staining for other proteins in the LV was carried out using formaldehyde-fixed paraffin sections. The tissue was permeabilized using 1% Triton X-100 (10 min, room temperature). Non-specific binding was blocked using 2% BSA plus 5% normal goat serum. The primary antibodies used for the staining were as follows: polyclonal goat
anti-ANG II (1:200, Santa Cruz Biotechnology), monoclonal mouse anti-hypoxia-inducible factor (HIF)-1α (1:500, Chemicon), affinity-purified rabbit anti-VEGF (1:200, Immuno-Biological Laboratories, Gunma, Japan), monoclonal mouse anti-thrombospondin (TSP)-1 (1:50, Santa Cruz Biotechnology), monoclonal mouse anti-heavy chain cardiac myosin (1:100, Abcam, Cambridge, MA), and monoclonal mouse anti-RECA-1 (1:100, Oxford Biotechnology). For quantification of the expression of ANG II in LV tissues, the fluorescence intensity was measured in more than five randomly selected areas of the stained tissue within a given field using a computerized image-analysis system (Image Pro Plus 6.2J, Media Cybernetics), as previously described (43). Expression of ANG II, VEGF, and TSP-1 in SDT rat LV tissues was also performed using double staining with antibodies against each plus cardiac myosin. The percentage of particles expressed in cardiomyocytes was calculated using 500 randomly selected particles. Expression of HIF-1α in cardiomyocytes was measured using triple staining with antibodies against cardiac myosin, HIF-1α, and 4′,6-diamidino-2-phenylindole (a stain for nuclei). The percentage of HIF-1α-positive nuclei was calculated using 1,000 randomly selected nuclei in each group. The fluorescence signal was visualized with a fluorescence microscope equipped with a fluorescein isothiocyanate filter (AX80, Olympus) and was quantified with image-analysis software (Image Pro Plus 6.2J, Media Cybernetics), as previously described (8, 15).

Serum ANG II and local ANG II in the LV. Levels of serum and LV ANG II were measured by RIA (SRL), as previously described (50). LV ANG II levels were normalized for protein content by the Biuret method, measured in the same samples.

Western blot analysis. LVs were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5% Nonidet P-40, and 0.1% SDS) containing protease inhibitors (Complete Mini, Roche, Mannheim, Germany). Lysates were then centrifuged for 40 min at 14,000 rpm at 4°C. Protein concentrations were determined using BCA Protein Assay Reagent (Thermo Fisher Scientific, Kanagawa, Japan). Ten micrograms of protein were separated by SDS-PAGE (4–12% NuPAGE Novex Bis Tris Gel, Invitrogen, Carlsbad, CA) followed by transfer to a polyvinylidene difluoride membrane (Hybond-P, GE Healthcare, Little Chalfont, UK). The primary antibodies used for Western blot analysis were as follows: polyclonal rabbit anti-VEGF receptor-2 (pVEGFR2) at Tyr1214 (1:200, Santa Cruz Biotechnology), polyclonal rabbit anti-phosphorylated Flk-1 [phosphorylated VEGF receptor (pVEGFR2)] at Tyr1244 (1:200, Santa Cruz Biotechnology), and monoclonal mouse anti-β-actin (1:2,000, Santa Cruz Biotechnology). Bands were detected by enhanced chemiluminescence (GE Healthcare). Membranes were rebotted with anti-β-actin antibody as a loading control. The signal band density was quantified using ImageJ (version 1.63, National Institutes of Health, http://rsb.info.nih.gov/ij/image/) and was normalized to that of the corresponding β-actin band.

Statistical analyses. Results are presented as means ± SE. Data were analyzed using paired or unpaired Student’s t-tests for comparisons of parameters among two groups or ANOVA for comparisons of parameters among three groups, followed by post hoc analysis with the Fisher test, where appropriate. P values of <0.05 were considered statistically significant.

RESULTS

Characteristics of SDT rats as a model of human nonobese type 2 diabetes. Diabetes onset in male SDT rats varied from 17 to 32 wk of age, as previously reported (22, 44). Unlike other studies (7, 25), which used age-matched male SDT rats with different diabetes onsets and durations, we used male SDT rats with the same diabetes onsets and durations and characterized their diabetic features at 0, 8, and 16 wk after diabetes onset. At 0 wk, the ages of the control, SDT, and Olm groups of rats used in the study were 22.7 ± 1.1, 22.2 ± 0.7, and 22.6 ± 0.7 wk (n = 25 rats/group), respectively; this did not differ statistically among the groups. Food intake in both SDT and control rats increased with time; the magnitude was greater in SDT rats at 0, 8, and 16 wk (Fig. 1A). Nonetheless, the body weight of SDT rats was almost identical to that of control rats at 0 wk but decreased at 8 and 16 wk, although the body weight of control rats gradually increased with time (Fig.

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Fig. 1. Clinical features of spontaneously diabetic Torii (SDT) rats as a model of human nonobese type 2 diabetes. A–E: food intake (A), body weight (BW; B), plasma insulin (C), plasma glucose (D), and systolic blood pressure (E) in SDT rats without (SDT group) and with olmesartan medoxomil (Olm group) at 0, 8, and 16 wk after the onset of diabetes as well as age-matched control rats (control group) at the corresponding time points. Data are means ± SE of 5–7 rats/group. *P < 0.05 and **P < 0.001 vs. the control group at the same time point; †P < 0.05 vs. the 0-wk control group; ††P < 0.001 vs. the 0-wk SDT group; §§P < 0.001 vs. the 0-wk Olm group; §§§P < 0.001 vs. the 8-wk SDT group; ||P < 0.001 vs. the 8-wk Olm group.
Insulin secretion in SDT rats was nearly identical to that of control rats at 0 wk but decreased at 8 and 16 wk (Fig. 1C). Plasma glucose in SDT rats was already greater than in control rats at 0 wk, despite similar plasma insulin levels, and increased further at 8 and 16 wk (Fig. 1D). Systolic blood pressure did not differ between the groups (Fig. 1E). Therefore, SDT rats can be used as a model of human nonobese type 2 diabetes, characterized by insulin resistance at diabetes onset, impaired insulin secretion at 8 and 16 wk, but no systolic blood pressure elevation. These parameters were unaffected by Olm (Fig. 1, A–E).

**LV function and dimensions in SDT rats.** LV diastolic function was impaired in SDT rats at 8 and 16 wk; they exhibited an increase in deceleration time, with the degree of increase being greater at 16 wk (Fig. 2, A and B). SDT rats also exhibited a decrease in E/A at 16 wk only (Fig. 2, A and C). LV systolic function assessed by LVFS was preserved in SDT rats at 8 wk but was impaired at 16 wk (Fig. 2D). Because brain natriuretic peptide levels were not elevated, no heart failure was observed in SDT rats (data not shown). LVH, as demonstrated by an increase in LVPWT (Fig. 2E), was evident in SDT rats at 8 and 16 wk. Olm treatment reversed these abnormalities in SDT rats (Fig. 2, A–E). In control rats, LVPWT was greater at 16 wk than at 0 wk (Fig. 2E). Heart rate or interventricular septum thickness did not differ among the groups (data not shown). Thus, SDT rats after diabetes onset developed LV diastolic dysfunction at 8 wk, followed by moderate LV systolic dysfunction at 16 wk, similar to the pattern observed in human DCM (1, 2), and LVH at 8 and 16 wk.

**Cardiomyocyte hypertrophy and interstitial fibrosis in SDT rats.** Because LVH includes cardiomyocyte growth (hypertrophy) and/or extracellular matrix accumulation, we measured the cross-sectional area of single cardiomyocytes and the interstitial collagen volume fraction in LVs stained by picrosirius red. In SDT rat LVs, single cardiomyocyte cross-sectional areas were increased at 8 and 16 wk compared with those in control rats, with a greater degree at 16 wk (Fig. 3, A and B). The interstitial collagen volume fraction was increased at 16 wk only (Fig. 3, A and C). Both were completely normalized by Olm (Fig. 3, A–C). Thus, SDT rats after diabetes onset developed cardiomyocyte hypertrophy at 8 wk, followed by interstitial fibrosis at 16 wk; both were Olm sensitive. On the other hand, similar to the change in LVPWT, single cardiomyocyte cross-sectional areas in control rats were greater at 16 wk than at 0 wk (Fig. 3B), suggesting a possibility that these may be age-associated changes (13, 51).

**LV ANG II in SDT rats.** Circulating and cardiac ANG II induce cardiac hypertrophy and fibrosis in hypertensive and normotensive animals (16, 23, 36, 40, 52). It has been reported that heart ANG II induces cardiac hypertrophy and fibrosis in diabetic patients (6) and streptozotocin-induced (STZ) diabetic rats (5). Thus, we measured the concentrations of serum and LV ANG II. At 8 and 16 wk, but not at 0 wk, serum ANG II levels were lower in SDT rats than in control rats, but both LV tissue ANG II levels and LV tissue immunofluorescence intensity were greater in SDT rats; all of these were completely reversed by Olm (Fig. 4, A–C). Almost all (99.6%) ANG II-positive particles colocalized with cardiac myosin, a cardiomyocyte marker, in the SDT rat LV at 16 wk (Fig. 4D). Therefore, local ANG II was overproduced by SDT rat LV.

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**Fig. 2. Echocardiographic findings in the three groups of rats.** A: representative Doppler flow measurements of mitral inflow obtained from the three groups of rats at 16 wk. DT, deceleration time; E, E-wave; A, A-wave. B–E: E-wave DT (B), E-wave-to-A-wave ratio (E/A; C), left ventricular (LV) fractional shortening (LVFS; D), and LV posterior wall thickness (LVPWT; E) in the three groups of rats at 0, 8, and 16 wk. Data are means ± SE of 5–7 rats/group. *P < 0.05 and **P < 0.001 vs. the control group at the same time point; †P < 0.05 vs. the SDT group at the same time point; ‡P < 0.05 vs. the 0-wk control group; ‡‡P < 0.05 vs. the 0-wk SDT group; ¶P < 0.05 vs. the 8-wk SDT group.
cardiomyocytes at 8 and 16 wk, and this was completely reversed by Olm. These findings indicate that local ANG II overproduced in SDT rat LV cardiomyocytes induced both cardiomyocyte hypertrophy at 8 and 16 wk and interstitial fibrosis at 16 wk.

Factors involved in cardiomyocyte hypertrophy and interstitial fibrosis in SDT rats. Because EGFR transactivation (49) and NHE1 activation (4) are important downstream signals for ANG II-induced LVH, we next examined the expression of pEGFR, total EGFR, and NHE1 in the LV. At 8 and 16 wk, there were increased protein levels of pEGFR and NHE1 in SDT rats, both of which were completely suppressed by Olm, although the expression of total EGFR did not differ among the groups (Fig. 5, A and B). Thus, these findings indicate that both enhanced EGFR phosphorylation and NHE1 upregulation were involved in the local ANG II-induced cardiomyocyte hypertrophy in SDT rats at 8 and 16 wk.

The profibrotic growth factor TGF-β1 plays an important role in cardiac fibrosis, and ANG II is known to enhance TGF-β1 expression in the myocardium (52). Moreover, ANG II infusion in mice enhances cardiomyocyte apoptosis (16). Thus, we examined the expression of TGF-β1 and measured the number of TUNEL-positive cardiomyocytes in LV tissues. In conjunction with interstitial fibrosis in the LV tissue, the SDT rat LV tissue at 16 wk alone exhibited increases in TGF-β1 expression and in the number of TUNEL-positive cardiomyocytes, both of which were completely reversed by Olm (Fig. 5, C and D). Therefore, these results suggest that both TGF-β1 upregulation and cardiomyocyte apoptosis may contribute to the local ANG II-induced interstitial fibrosis in the SDT rat LV tissue at 16 wk only.

Coronary capillary angiogenesis associated with cardiomyocyte hypertrophy in SDT rats. To test whether the LV ANG II-induced cardiomyocyte hypertrophy in SDT rats at 8 and 16 wk affects coronary capillary angiogenesis, we examined the capillary density and capillary-to-cardiomyocyte ratio by staining endothelial cells with anti-RECA-1. We found that in SDT rat LVs after the onset of diabetes, coronary capillary angiogenesis associated with LV ANG II-induced cardiomyocyte hypertrophy was enhanced at 8 wk but was suppressed at 16 wk.

We next examined whether either the proliferation and/or apoptosis of capillary endothelial cells contribute to the...
coronary angiogenesis associated with the LV ANG II-induced cardiomyocyte hypertrophy at 8 and 16 wk. In SDT rat LVs, the percentages of PCNA-positive capillaries and TUNEL-positive capillaries increased at 8 and 16 wk, respectively, and these increases were completely reversed by Olm (Fig. 7, A and B). On the other hand, the percentages of TUNEL-positive capillaries and PCNA-positive capillaries in SDT rat LVs were unchanged at 8 and 16 wk, respectively (Fig. 7, A and B).

**Chronic hypoxia in hypertrophied cardiomyocytes is a trigger for coronary capillary angiogenesis in SDT rats at 8 wk.** HIF-1α is a key transcription factor for the hypoxic induction of angiogenic growth factors, such as VEGF. To test whether chronic hypoxia in hypertrophied cardiomyocytes is a trigger for coronary capillary angiogenesis, we examined the expression of the two hypoxia-responsive proteins: HIF-1α and VEGF. In accordance with the degree of cardiomyocyte hypertrophy, the percentage of HIF-1α-positive nuclei in SDT rat LV cardiomyocytes increased at 8 and 16 wk, with a higher percentage at 16 wk (Fig. 8A). VEGF expression in the LV was also upregulated in SDT rats at 8 and 16 wk (Fig. 8B). Almost all (97.1%) of VEGF-positive particles colocalized with cardiac myosin in the SDT rat LV at 16 wk (Fig. 8C). The upregulation of HIF-1α and VEGF was completely abolished by Olm (Fig. 8, A and B). Therefore, hypertrophied cardiomyocytes in SDT rat LVs at 8 and 16 wk were subjected to chronic hypoxia that was completely reversed by Olm.

VEGF possesses angiogenic activity through binding to its tyrosine kinase receptors, including VEGFR2, which is expressed almost exclusively in endothelial cells (31), and thereafter through pathways involving their phosphorylation (33). Thus, we next examined whether VEGFR2 phosphorylation is involved in the coronary angiogenesis associated with cardiomyocyte hypertrophy in SDT rat LVs at 8 and 16 wk. In parallel with the upregulation of VEGF in SDT rat LV cardiomyocytes at 8 wk, there were increased protein levels of pVEGFR2 compared with those in control cardiomyocytes, which were completely normalized by Olm (Fig. 8D). In contrast, at 16 wk, despite the upregulation of VEGF in SDT rat LV cardiomyocytes, the protein levels of pVEGFR2 were not increased significantly compared with those in control cardiomyocytes (Fig. 8D). These findings indicate that, in SDT rat LVs, VEGF produced from hypertrophied cardiomyocytes under chronic hypoxia and...
VEGFR2 phosphorylation contribute to the increased coronary angiogenesis at 8 wk only.

Mechanisms of suppression of coronary angiogenesis associated with cardiomyocyte hypertrophy in SDT rats at 16 wk. The above finding, that the angiogenesis in SDT rats at 16 wk was suppressed despite the upregulation of the angiogenic factor VEGF, suggests the possibility that antiangiogenic factors may overcome the stimulatory effects of VEGF on coronary angiogenesis. To test this, we examined the expression of TSP-1 in the LV tissue, because TSP-1 inhibits angiogenesis by inducing endothelial cell apoptosis (10, 17) and suppressing endothelial cell proliferation (14). At 16 wk alone, expression of TSP-1 in LVs was upregulated in SDT rats and could be completely suppressed by Olm (Fig. 9, A and B). Notably, almost all (92.2%) TSP-1-positive particles colocalized with cardiac myosin in the SDT rat LV at 16 wk (Fig. 9C). These results indicate that, in SDT rat LVs, TSP-1 produced from hypertrophied cardiomyocytes under chronic hypoxia is involved in the reduced coronary angiogenesis at 16 wk only.

DISCUSSION

Using functional and morphological investigation of SDT rats for 16 wk after the onset of diabetes, we focused on the cross-talk between cardiomyocytes and the coronary vasculature during cardiac growth and analyzed the pathogenesis of DCM that mimics human type 2 diabetes.

SDT rats at 8 wk exhibited LVH because of LV cardiomyocyte hypertrophy, and this was accompanied by moderate LV diastolic dysfunction. Since the hypoxia-responsive transcription factor HIF-1α was upregulated in SDT rat LV cardiomyocytes at 8 wk, the hypertrophied cardiomyocytes were under chronic hypoxia, which triggered VEGF production in the cardiomyocytes. This promoted coronary angiogenesis by enhancing capillary endothelial cell proliferation via VEGFR2 phosphorylation, leading to an increase in the capillary-to-cardiomyocyte ratio. Therefore, SDT rats at 8 wk showed an early adaptive phase with coordinated coronary capillary angiogenesis and cardiomyocyte hypertrophy. Similarly, coordinated coronary angiogenesis associated with cardiac hypertrophy has been described in the acute phase in mice after Akt transgene induction (45) and in the early adaptive phase in mice receiving a severe transverse aorta constriction (37). In these animals, coronary angiogenesis was also induced by the upregulation of hypoxia-induced angiogenic factors, including VEGF, expressed in their hearts (37, 45).

In SDT rat LVs at 16 wk, in addition to sustained cardiomyocyte hypertrophy, interstitial fibrosis with cardiomyocyte apoptosis and TGF-β1 upregulation was observed, and this was
accompanied by progressive LV diastolic dysfunction and moderate LV systolic dysfunction. Furthermore, the degree of cardiomyocyte hypertrophy was significantly greater at 16 wk. The degree of chronic hypoxia in hypertrophied cardiomyocytes was also significantly greater at 16 wk, because the upregulation of HIF-1α in cardiomyocytes was greater at 16 wk than at 8 wk. Moreover, in accordance with HIF-1α upregulation, the upregulated VEGF was maintained. Nevertheless, the SDT rat LV at 16 wk showed the suppression of coronary capillary angiogenesis, resulting in a decrease in the capillary-to-cardiomyocyte ratio. This led to deteriorating chronic hypoxia in extensively hypertrophied cardiomyocytes and therefore to cardiomyocyte apoptosis and consequently to the development of interstitial fibrosis. Therefore, SDT rats at 16 wk represented a maladaptive late phase with a loss of coordination between coronary angiogenesis and cardiomyocyte hypertrophy. Also in the chronic phase in the mice mentioned above (37, 45), coronary angiogenesis associated with cardiomyocyte hypertrophy was inhibited, and the loss of coordinated coronary angiogenesis with cardiomyocyte hypertrophy progressed from adaptive cardiac hypertrophy to heart failure. In the chronic phase in these animals, downregulation of VEGF led to impaired coronary angiogenesis and contractile dysfunction without changing the expression of antiangiogenic factors such as TSP-1. In sharp contrast to these reports, in the late phase of our diabetic model, we observed TSP-1 upregulation in extensively hypertrophied cardiomyocytes with increased capillary endothelial cell apoptosis and reduced capillary endothelial cell proliferation. Furthermore, VEGFR2 phosphorylation was not increased. Similarly, in diabetic Zucker rats, increased expression of TSP-1 in the adventitia of the aorta and a reduced number of vasa vasorum in the aorta have been reported, and a potent antiangiogenic effect of TSP-1 on the vasa vasorum has been suggested (47). TSP-1, a 450-kDa glycoprotein secreted into the extracellular matrix by many cell types, is a naturally occurring antiangiogenic factor (10, 14, 19). It has been reported that TSP-1 inhibits angiogenesis by inducing endothelial cell apoptosis (10, 19) and suppressing endothelial cell proliferation (14). Our laboratory (15) previously reported in the kidney cortex of DOCA/salt hypertensive rats that the upregulation of TSP-1 in their cortical tubule cells occurred despite the upregulation of HIF-1α and VEGF, and this led to peritubular capillary loss and the development of tubulointerstitial fibrosis. Kaur et al. (20) recently reported that TSP-1 inhibited VEGF-stimulated VEGFR2 phosphorylation and its downstream signaling in...
cultured endothelial cells, although TSP-1 alone had no effect on VEGFR2 phosphorylation or its downstream signaling. Accordingly, TSP-1 upregulated in the extensively hypertrophied cardiomyocytes of SDT rat LVs at 16 wk could both induce capillary endothelial cell apoptosis and suppress capillary endothelial cell proliferation via the inhibition of VEGF-stimulated VEGFR2 phosphorylation and thereby overcome the stimulatory effect of VEGF on coronary angiogenesis, leading to coronary capillary loss. These findings are compatible with those in a recent study (28) showing that in cultured human microvascular endothelial cells, despite the induction of VEGF in hypoxia-conditioned media, the increased levels of TSP-1 were able to override the protective effects of VEGF on their survival and proliferation. Taking these previous reports together with our findings, the signals from the hypertrophied cardiomyocytes under chronic hypoxia to inhibit coronary angiogenesis differ markedly between the above-mentioned models (37, 45) and our own, and we suggest an important role for TSP-1 as an antiangiogenic factor in the diabetic heart of SDT rats.

Our findings that, in SDT rat LVs at 16 wk, the expression of VEGF protein was upregulated but VEGFR2 phosphorylation did not increase, are consistent with those obtained from diabetic patients (39) and rats (38). Sasso et al. (39) showed that type 2 diabetic patients with chronic coronary heart disease had significantly higher myocardial VEGF mRNA and protein levels and lower VEGFR2 mRNA and protein levels than nondiabetic subjects, along with a downregulation of VEGF-dependent intracellular signaling, including reduced VEGFR2 phosphorylation. It has been reported that, in STZ diabetic rat hearts 90 days after the induction of diabetes, the expression of VEGF mRNA was increased, but the expression of VEGFR2 mRNA was unaffected (38). These two reports proposed several potential mechanisms for their observations but did not investigate the relationship between the expression of VEGF and its downstream signaling, coronary capillary angiogenesis associated with cardiomyocyte hypertrophy, and cardiac function. The present study represents a novel mechanism responsible for their observations. In sharp contrast to the data from STZ diabetic rats mentioned above (38), Yoon et al. (54) performed serial clinicopathological investigation of STZ diabetic rats over 1 yr and reported that a progressive decrease of myocardial VEGF expression was the initial event, followed by reduced coronary capillary angiogenesis with decreased phosphorylation of VEGFR2 and increased capillary endothelial cell apoptosis, resulting in the progression of DCM with...
interstitial fibrosis. Similarly, both VEGF expression and microvessel density were decreased in diabetic mouse myocardia 5 wk after a single peritoneal injection of STZ (11). However, these reports did not address the cross-talk between cardiomyocytes and the coronary vasculature during cardiac growth. The reason for the discrepancy in the expression of VEGF in cardiac tissues of the STZ diabetic animals is unclear at present, but these reports and the present findings may suggest different mechanisms for the coronary capillary loss observed in diabetic hearts.

Fig. 8. Expression of hypoxia-inducible factor (HIF)-1α, VEGF, and phosphorylated VEGF receptor-2 (pVEGFR2) in the three groups of rat LVs. A, top: immunofluorescence triple staining for cardiac myosin, HIF-1α, and 4',6-diamidino-2-phenylindole (DAPI) in the three groups of rat LVs. Arrowheads show HIF-1α-positive nuclei expressed in cardiomyocytes. Scale bar = 20 μm. Bottom, summary of HIF-1α-positive nuclei in the three groups of rat LVs. Data are means ± SE of 6 rats/group. B, top: Western blot analyses of VEGF in the three groups of rat LV tissues at 8 and 16 wk. Bottom, densitometric analyses of the expression of VEGF protein in the three groups of rats at 8 and 16 wk relative to that in the control group at 8 and 16 wk, respectively. Data are means ± SE of 6 rats/group. C: immunofluorescence double staining for cardiac myosin and VEGF in the LV tissue of the SDT group at 16 wk. Arrowheads show VEGF expressed in cardiomyocytes. Scale bar = 20 μm. D, top: Western blot analyses of pVEGFR2 in the three groups of rat LV tissues at 8 and 16 wk. Bottom, densitometric analyses of the expression of pVEGFR2 in the three groups of rats at 8 and 16 wk, respectively. Data are means ± SE of 6 rats/group. *P < 0.05 and **P < 0.001 vs. the control group at the same time point; #P < 0.05 and ##P < 0.001 vs. the SDT group at the same time point; ‡P < 0.05 vs. the 8-wk SDT group.
In SDT rats, both phases were characterized by ANG II overproduction in LV cardiomyocytes, without systolic blood pressure elevation. In contrast, at the onset of diabetes, there was no LV ANG II overproduction or LV functional or morphological alteration—only significant hyperglycemia. As reported in diabetic patients and animals (24, 35), circulating ANG II levels were also decreased in SDT rats at 8 and 16 wk only. Treatment of SDT rats with Olm for 8 and 16 wk after diabetes onset completely suppressed both LV ANG II overproduction and LV dysfunction and morphological abnormalities, without influencing hyperglycemia or systolic blood pressure. These findings are in good agreement with a report by Mazzolai et al. (23), in which mice overexpressing angiotensinogen specifically in the heart displayed increased heart ANG II levels and cardiac hypertrophy without an elevation of either plasma ANG II levels or blood pressure, and these were abolished by treatment with losartan, an ARB. Similarly, elevated ANG II levels have been demonstrated in cardiomyocytes of STZ diabetic rats, which were inhibited by treatment with ARBs (5, 46). Moreover, the SDT rat LV at 8 and 16 wk exhibited enhanced EGFR phosphorylation and NHE1 upregulation, both being downstream signals of ANG II-induced LVH (4, 49); these were completely suppressed by Olm treatment. Therefore, these findings indicate that during both phases under persistent hyperglycemic conditions, sustained LV cardiomyocyte ANG II overproduction, but not chronic pressure overload, induces prolonged LVH through its growth factor effect on cardiomyocytes, which respectively stimulates and suppresses coronary capillary angiogenesis in the early and late phases. It should be noted that the local ANG II overproduction by SDT rat LV cardiomyocytes continued in the late phase. In the late phase alone, we detected these events, including interstitial fibrosis, cardiomyocyte apoptosis, TGF-β1 upregulation in LV tissues, decreased coronary capillary angiogenesis associated with cardiomyocyte hypertrophy, enhanced apoptosis and suppressed proliferation of capillary endothelial cells, and upregulation of TSP-1 in hypertrophied cardiomyocytes. However, it is presently unknown why these events, especially the TSP-1 upregulation, were limited to the late phase. The events observed in the late phase alone were accompanied by more persistent hyperglycemia and longer ANG II overproduction and more severe and longer hypoxia in the hypertrophied cardiomyocytes than in the early phase, all of which (except for hyperglycemia) were reversed by Olm. Thus, these Olm-sensitive events observed in the late phase alone must be closely linked to each other independently of hyperglycemia. It has been reported that ANG II upregulates TSP-1 protein expression in cultured rat cardiac fibroblasts independently of high-glucose media (55). It has been shown that hypoxia induced HIF-1α-dependent expression of TSP-1 mRNA and protein in cultured human coronary artery smooth muscle cells (34). Therefore, the longer ANG II production and/or more severe and longer hypoxia in hypertrophied cardiomyocytes at 16 wk may be involved in processes involving TSP-1 upregulation. TGF-β1 is a known regulator of TSP-1 expression in a variety of cell types (29, 30, 32). It has been reported that TGF-β1 overproduced under more severe chronic hypoxia at 16 wk may contribute to the TSP-1 upregulation. These possibilities need further investigation. Taken together, in SDT rats after diabetes onset, sustained local ANG II overproduction actually contributes to all the events observed in both phases of DCM in a time-dependent manner. Conversely, from a therapeutic point of view, our findings suggest that persistent inhibition of LV ANG II overproduction with
Olm may provide protective effects against the progression of DCM in diabetic patients, independently of hyperglycemia and systolic blood pressure.

Our study has several important limitations that deserve mention. First, although our findings indicate that Olm-sensitization process, especially LV cardiomyocyte ANG II overproduction, correlate well with the events observed in both phases, we did not directly demonstrate that LV ANG II developed and progressed DCM. Second, we did not evaluate how persistent hyperglycemia induced the sustained LV cardiomyocyte ANG II overproduction or how the sustained LV cardiomyocyte ANG II overproduction was inhibited by the ARB Olm. These currently unresolved issues should be addressed in future studies.

In conclusion, the present findings suggest that LV ANG II in SDT rats at 8 and 16 wk after the onset of diabetes induces cardiomyocyte hypertrophy without affecting hyperglycemia or systolic blood pressure, which promotes and suppresses coronary angiogenesis, respectively, via VEGF and TSP-1 produced from hypertrophied cardiomyocytes under chronic hypoxia. TSP-1 may play an important role in DCM progression in this model. These findings reveal a novel mechanism for the pathogenesis of DCM, in terms of the cross-talk between cardiomyocytes and the coronary vasculature during diabetic heart ANG II-induced cardiac growth and suggest the clinical significance of long-term heart ANG II inhibition in patients with diabetes.

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DISCLOSURES

No conflicts of interest financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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