Angiotensin II Receptor Antagonist Attenuates Expression of Aging Markers in Diabetic Mouse Heart

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Background Diabetes mellitus is an independent risk factor for heart failure. Diabetes mellitus causes other age-related cardiovascular diseases. We assessed the hypothesis that hearts from diabetic animals are associated with accelerated aging processes. We also examined the effect of an angiotensin II receptor blocker (ARB) on the expression of senescence-associated molecules.

Methods and Results We administered an ARB (candesartan 10 mg/kg per day) or saline to diabetic db/db or control db/+ mice. The treatment was started when mice were 10-weeks-old, and continued for 15 weeks. Systolic function was impaired in db/db mice and candesartan improved cardiac function. The amount of phosphorylated Akt and S6 was decreased in saline-treated db/db mice, and candesartan treatment partially preserved phosphorylation. The amount of p21, p27, p53 or Rb was increased in the heart tissue of saline treated db/db mice. Candesartan treatment completely suppressed the increases of p21, p27, p53 and Rb.

Conclusions An ARB improved cardiac function of diabetic animals, and this was accompanied by decreases of senescence-associated molecules in the myocardium. ARB may be a modality for heart failure patients with diabetes mellitus. (*Circ J* 2006; 70: 482–488)

Key Words: Aging; Diabetes; Heart failure; Signal transduction

Epidemiologic studies have clearly demonstrated that diabetic patients are at increased risk for cardiovascular morbidity and mortality.^{1,2} As diabetes mellitus is associated with several age-related disease, such as atherosclerosis, hypertension and heart failure, diabetes mellitus is considered to be a model of accelerated aging.³ Diabetes is an independent risk factor for heart failure.⁴ Although coronary artery disease is likely to be a major cause of heart failure in diabetes, abnormalities of the myocardium are also suggested to be implicated as a cause of heart failure in diabetic patients.^{5,6} Congestive heart failure is an age-related disorder, and age is an independent risk factor for heart failure.⁷

The renin-angiotensin system has been shown to be increased in tissues of aged animals.⁸ Inhibition of the renin-angiotensin system prevented the development of age-related cardiovascular diseases.⁹ Blockade of the renin-angiotensin system decreased the number of newly onset diabetic patients.^{10,11} Thus, it might be possible that angiotensin II is involved in the development of diabetes and aging.

We assessed the hypothesis that hearts from diabetic animals are associated with an accelerated aging process.

To examine the hypothesis, we measured the amount of cell cycle inhibitors, which are known to be increased because of the association with cellular senescence and organismal aging.^{12–14} We also examined the effect of an angiotensin II receptor blocker (ARB) on cardiac function and expression of senescence-associated molecules in the hearts of diabetic animals.

Methods

Animals

Adult obese male mice (C57BL/6J db/db) at 8 weeks old were purchased from Charles River (Kanagawa, Japan). Animal care and experimentation methods were approved by the Institutional Animal Care and Use Committee of Kitasato University. Both db/+ mice and db/db mice were given saline or candesartan cilexetil (10 mg/kg per ml, Takeda, Osaka, Japan) in drinking water beginning when they were 10 weeks old; this continued for 15 weeks.

Hemodynamics

Systolic blood pressure and heart rate was recorded by the tail cuff method without anesthesia (Softron BP-98A, Tokyo, Japan).¹⁵ For measuring systolic blood pressure, mice were placed in a warm chamber maintained at 40°C for 10 min in an individual restrainer. The tail cuff device was placed around the mouse's tail. Three pressure measurements were recorded for each mouse, and the average systolic blood pressure was calculated for each mouse.

Echocardiography

Transthoracic echocardiographic analysis was performed with ProSound SSD-4000 (ALOKA, Tokyo, Japan), with a 7.5-MHz imaging transducer. Briefly, mice were anesthetized with an intraperitoneal injection of ketamine

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Table 1 Hemodynamic Data of db/db Mice Treated With Candesartan

	Saline		Candesartan	
	db/+	db/db	db/+	db/db
No. animals	10	10	10	10
Blood pressure (mmHg)	130±7	127±8	115±12 [†]	124±10
Heart rate (beats/min)	548±123	591±78	620±65	596±53

Hemodynamic data of db/+ and db/db mice without anesthesia. Values are presented as mean±SEM. [†]p<0.05 vs saline treated mice of the same genotype.

Table 2 Postmortem Analysis of db/db Mice Treated With Candesartan

	Saline		Candesartan	
	db/+	db/db	db/+	db/db
No. animals	19	20	20	17
Body weight (g)	31.5±0.5	45.5±1.5*	29.9±0.4	43.3±1.8*
Heart weight (mg)	142.7±1.5	130.3±2.7*	132.7±2.0 [†]	132.7±2.0
Liver weight (mg)	1,210.9±42.0	2,384.1±75.2*	1,266.3±27.2	2,354.4±104.5*
Lung weight (mg)	143.3±5.5	135.1±3.4	140.7±3.6	134.5±7.0
Tibial length (mm)	18.2±0.2	17.5±0.1	18.3±0.1	17.5±0.1
Liver weight/bodyweight (mg/g)	38.5±1.7	53.1±1.8*	42.6±1.2	54.5±1.7*
Lung weight/bodyweight (mg/g)	4.6±0.2	3.1±0.1	4.7±0.1	3.2±0.1
Heart weight/bodyweight (mg/g)	4.5±0.1	2.9±0.1*	4.4±0.1	3.0±0.1*
Liver weight/tibial length (mg/mm)	66.7±2.3	136.3±4.4*	69.3±1.5	126.8±9.1*
Lung weight/tibial length (mg/mm)	7.9±0.3	7.7±0.2	7.7±0.2	7.2±0.6
Heart weight/tibial length (mg/mm)	7.9±0.1	7.4±0.1*	7.3±0.1 [†]	6.9±0.4*

Values are presented as mean±SEM. *p<0.05 vs db/+ mice with the same treatment. [†]p<0.05 vs saline treated mice of the same genotype.

(50 mg/kg) and xylazine (10 mg/kg). An M-mode echocardiogram was obtained at the papillary muscle level, and left ventricular end-diastolic dimension, end systolic dimension and left ventricular posterior wall thickness were measured. Fractional shortening was calculated as described.¹⁶

Blood and Plasma Glucose Concentration

Before ARB treatment, blood glucose after 12 h of fasting was measured using Medisafe Mini (TERUMO Co, Tokyo, Japan). At the end of the treatment, mice were anesthetized after 12 h of fasting and blood was drawn from the inferior vena cava. Plasma glucose concentration was measured by using the Glu-DH method (SRL Inc, Tokyo, Japan).

Plasma Insulin Concentration

To measure the plasma insulin concentration in mice, a mouse insulin ELISA kit (Ultra-sensitivity) was used (Shibayagi Co, Shibukawa, Gunma, Japan).

Western-Blotting

The hearts of test mice were removed and immediately frozen in liquid nitrogen. Five mice hearts were used for each group and tissue lysate were individually prepared as described.¹⁶ For analysis of p70 S6 kinase (S6K1) phosphorylation, the blots were probed with anti-phospho-S6K1 (Thr 389) antibody (1:200; Cell Signaling, Beverly, MA, USA) or anti-S6K1 antibody (1:200; Santa Cruz, Santa Cruz, CA, USA). For analysis of S6 phosphorylation, the blots were probed with anti-phospho-S6 protein (Ser 235/236) antibody (1:500; Cell Signaling), or anti-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH) (1:5,000; Research Diagnostics, Flanders, NJ, USA). For analysis of Akt phosphorylation, the blots were probed with anti-phospho-Akt (Ser 473) antibody (1:500; Cell Signaling) or anti-Akt antibody (1:500; Cell Signaling).

For analysis of extracellularly responsive kinase (ERK) phosphorylation, the blots were probed with anti-phospho-ERK antibody (1:200; Santa Cruz) or anti-ERK2 antibody (1:200; Transduction Lab, Lexington, KY, USA). For analysis of p21 expression, the blots were probed with anti-p21 antibody (1:500; Pharmingen, San Diego, CA, USA) or anti-GAPDH antibody. For analysis of p27 expression, the blots were probed with anti-p27 antibody (1:200; Santa Cruz) or anti-GAPDH antibody. For analysis of p53 expression, the blots were probed with anti-p53 antibody (1:500; Cell Signaling) or anti-GAPDH antibody. For analysis of Rb expression, the blots were probed with Rb antibody (1:500; Santa Cruz) or anti-GAPDH antibody.

Northern Hybridization Analysis

Total RNA was purified from mouse heart tissue using the acid guanidinium-phenol-chloroform method. Five mice hearts were used for each group and total RNA was individually prepared. An equal amount of total RNA was mixed for each group. Twenty microgram of total RNA of each group were electrophoresed in 1.3% denaturing formaldehyde agarose gel and blotted with Hybond N (Amersham, Buckinghamshire, England, UK). The membrane was probed with mouse probes for brain natriuretic peptide (BNP) cDNA as described.¹⁶ Blots were washed twice at 50°C in 2×standard saline citrate (SSC)/1% sodium dodecyl sulfate (SDS) for 5 min, and twice at 50°C in 2×SSC/1% SDS for 10 min.

Immunofluorescence Study

Heart tissue was embedded in O.C.T. compound, snap-frozen on dry ice, and stored at -70°C. Tissue was sectioned on a cryostat at 10 µm and then fixed for 5 min in acetone at 4°C. The sections were incubated with anti-p53 antibody (rabbit IgG, Santa Cruz) and anti-actinin antibody (mouse IgG, Sigma) at 4°C overnight. Next, sections were incu-

Table 3 Echocardiographic Data of db/db Mice Treated With Candesartan

	Saline		Candesartan	
	db/+	db/db	db/+	db/db
No. animals	18	20	20	17
Diastolic anterior wall thickness (mm)	0.9±0.0	0.9±0.0	0.9±0.0	0.8±0.0
Diastolic posterior wall thickness (mm)	0.9±0.0	1.0±0.0	0.9±0.0	0.9±0.0
LV diastolic diameter (mm)	2.4±0.1	2.0±0.1*	2.1±0.1	1.8±0.1
LV systolic diameter (mm)	1.3±0.1	1.2±0.1	1.4±0.1	1.0±0.1
Fractional shortening (%)	44.6±2.0	37.2±1.8*	46.3±2.0	42.5±2.0
Heart rate (beats/min)	306±25	282±21	282±24	345±21

Cardiac function was analyzed by using echocardiography under anesthesia. Values are presented as mean±SEM. * $p<0.05$ vs db/+ mice with the same treatment. LV, left ventricle.

Table 4 Concentrations of Plasma Glucose and Insulin of db/db Mice Treated With Candesartan

	Saline		Candesartan	
	db/+	db/db	db/+	db/db
No. animals	8	10	10	9
Blood glucose (mg/dl)	188.2±26.6	1,090.9±44.9*	110.3±6.24 [†]	1,101.1±34.5*
Insulin (μ U/ml)	2.8±0.2	22.9±6.4*	2.5±0.1	6.5±0.2* [†]

Values are presented as mean±SEM. * $p<0.05$ vs db/+ mice with the same treatment; [†] $p<0.05$ vs saline treated mice of the same genotype.

bated with fluorescent isothiocyanate-labeled anti-rabbit IgG and rhodamine labeled anti-mouse IgG antibodies at room temperature for 30 min. Nuclei were stained using TOTO-3 (Invitrogen, Carlsbad, CA, USA). Sections were analyzed by confocal microscopy (Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

All values are expressed as mean±SEM. Differences between the groups were compared using Student's t-tests. $P<0.05$ was considered as significant.

Results

Hemodynamic Analysis

Blood pressure of saline-treated db/+ mice was not different from that of saline-treated db/db mice (Table 1). The blood pressure of candesartan-treated db/+ mice was decreased by 13% compared with saline-treated db/+ mice (115±12 mmHg vs 130±7 mmHg, $p<0.05$). The blood pressure of candesartan-treated db/db mice was not significantly different from that of saline-treated db/db mice.

Postmortem Analysis

Body weight was increased by 44% in saline-treated db/db mice compared with saline-treated db/+ mice (31.5±0.5 g vs 45.5±1.5 g, $p<0.05$, Table 2). Candesartan treatment did not affect the bodyweight of db/db mice. The heart weight of saline-treated db/db mice was decreased by 9% compared with saline-treated db/+ mice (130.3±2.7 mg vs 142.7±1.5 mg, $p<0.05$). The heart weight/tibial length ratio of saline-treated db/db mice was also decreased compared with saline-treated db/+ mice (7.4±0.1 mg/mm vs 7.9±0.1 mg/mm, $p<0.05$). The heart weight of the candesartan-treated db/db mice was not significantly different from that of saline-treated db/db mice.

Echocardiographic Analysis

The left ventricular end-diastolic dimension of saline-treated db/db mice was significantly decreased compared

with that of saline-treated db/+ mice (2.0±0.1 mm vs 2.4±0.1 mm, $p<0.05$, Table 3). The left ventricular posterior wall thickness of saline-treated db/db mice was not different from saline-treated db/+ mice. Fractional shortening of saline-treated db mice was significantly lower than that of saline-treated db/+ mice (37.2±1.8% vs 44.6±2.0%, $p<0.05$). Fractional shortening of candesartan-treated db/db mice tended to be higher than that of saline-treated db/db mice (42.5±2.0% vs 37.2±1.8%, $p=0.056$).

Blood Glucose and Insulin Concentration

We measured the blood glucose level of the saline-treated db/+ group, saline-treated db/db group, candesartan-treated db/+ group, and the candesartan-treated db/db group ($n=5$ for each group) to check if the db/db mice were diabetic. Before ARB treatment, the blood glucose levels of the saline-treated db/+ group, saline-treated db/db group, candesartan-treated db/+ group, and candesartan-treated db/db group was 123.7±4.5, 479.1±31.1*, 134.0±4.2, and 501.3±18.1 mg/dl*, respectively ($n=5$ for each group, * $p<0.05$ vs saline-treated db/+ group). After the treatment, blood was drawn from the inferior vena cava and plasma glucose and insulin concentrations were determined. Plasma glucose of saline-treated db/db mice was increased compared with saline-treated db/+ mice (1,090.9±44.9 mg/dl vs 188.2±26.6 mg/dl, $p<0.05$, Table 4). Candesartan treatment did not change the plasma glucose level of db/db mice. Plasma glucose of candesartan-treated db/+ mice was lower than that of saline-treated db/+ mice (110.3±6.24 mg/dl vs 188.2±26.6 mg/dl, $p<0.05$). The insulin concentration of saline-treated db/db mice was significantly higher than that of saline-treated db/+ mice (22.9±6.4 μ U/ml vs 2.8±0.2 μ U/ml, $p<0.05$). The insulin concentration of candesartan-treated db/db mice was lower than that of saline-treated db/db mice (6.5±0.2 μ U/ml vs 22.9±6.4 μ U/ml, $p<0.05$). It is reported that db/+ mice were associated with abnormal glucose handling!¹⁷

Phosphorylation of Akt, S6, S6K1 and ERK

We examined the activation of potential downstream

Fig 1. Phosphorylation of Akt, S6 protein, S6K1 or ERK in db/db mice. The phosphorylation of Akt, S6, S6K1 and ERK in the heart tissue was analyzed by Western blotting. Representative Western blots are shown in the upper panels and the results of quantitative densitometry of 5 animals in each group are shown in the lower panels. The phosphorylation of Akt was markedly decreased in db/db mice compared with db/+ mice, and candesartan treatment partially preserved Akt phosphorylation. The phosphorylation of S6 was also decreased in db/db mice, and candesartan partially preserved the phosphorylation. The phosphorylation of S6K1 and ERK did not change in db/db mice. S, saline-treated group; Can, candesartan-treated group; S6K1, S6 kinase; ERK, extracellularly responsive kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Values are presented as mean $\pm$ SEM. * $p < 0.05$ vs db/+ mice with the same treatment. † $p < 0.05$ vs saline-treated mice of the same genotype.

Fig 2. Expression of senescence markers in db/db mice. The amount of senescence-associated molecules, p21, p27, p53 or Rb was analyzed by Western blotting. Representative Western blots are shown in the upper panels and the results of quantitative densitometry of 5 animals in each group are shown in the lower panels. The amount of p21, p27, p53 or Rb was increased in the heart tissue of saline-treated db/db mice. The candesartan treatment completely suppressed the increases of p21, p27, p53 and Rb. S, saline-treated group; Can, candesartan-treated group; ERK, extracellularly responsive kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Values are presented as mean $\pm$ SEM. * $p < 0.05$ vs db/+ mice with the same treatment. † $p < 0.05$ vs saline-treated mice of the same genotype.

Fig3. Brain natriuretic peptide (BNP) mRNA expression in the heart tissue of db/db mice. BNP mRNA of saline-treated db/db mice was decreased compared with saline-treated db/+ mice. Candesartan did not affect BNP gene expression. S, saline-treated group; Can, candesartan-treated group.

targets of an insulin receptor. Representative Western blots are shown in the upper panels of Fig 1 and the results of quantitative densitometry of 5 animals in each group are shown in the lower panels. Akt (or protein kinase B) is one of the best characterized targets of the insulin receptor.¹⁸ Activation of Akt in the heart tissue was assessed by measuring the amount of Akt phosphorylated at Ser473 using a phosphospecific antibody. The amount of phosphorylated Akt was decreased in saline-treated db/db mice, and candesartan treatment increased the Akt phosphorylation in db/db mice. S6 phosphorylation was decreased in db/db mice and candesartan partially preserved the S6 phosphorylation in db/db mice. The phosphorylation of S6K1 or ERK

did not change between db/db and db/+ mice. And candesartan treatment did not affect the phosphorylation of S6K1 or ERK.

Protein Expression of p21, p27, p53 and Rb

The amount of cell cycle inhibitors such as p21, p27, p53 or Rb increase in cellular senescence.¹⁴ The increase in number of these molecules are also described in the heart tissue of aged animals.¹³ The amount of senescence-associated molecules, p21, p27, p53 or Rb, was analyzed by Western blotting. Representative Western blots are shown in the upper panels of Fig 2 and the results of quantitative densitometry of 5 animals in each group are shown in the lower panels. The amount of p21, p27, p53 or Rb was significantly increased in the heart tissue of saline-treated db/db mice. Candesartan treatment almost completely suppressed increases of p21, p27, p53 and Rb.

BNP mRNA Expression

The BNP mRNA expression of saline-treated db/db mice was decreased compared with that of saline-treated db/+ mice. Candesartan did not affect the BNP gene expression in db/+ or db/db mice.

Localization of p53 Protein in the Heart Tissue of db/db Mice

Localization of the p53 protein was analyzed by immunofluorescence analysis (Fig 4). Several nuclei of cardiac myocytes were stained with the anti-p53 antibody in a transverse myocardial section of db/db mice. No significant signal was observed in the sections of db/+ mice.

Discussion

In the present study, the cardiac systolic function was significantly decreased in db/db mice, and candesartan

Fig4. Localization of the p53 protein in the heart tissue of db/db mice. Localization of p53 in the myocardium of db/db mice was examined by immunofluorescence study. p53 protein was localized in the nuclei of cardiac myocytes (arrows). A, p53; B, actinin; C, nuclei; D, merged image.

treatment improved cardiac function. Cardiac insulin signaling, assessed by the phosphorylation of Akt or S6, was attenuated in db/db mice, and candesartan partially preserved insulin signaling. Senescence-associated molecules were increased in the myocardium of db/db mice and candesartan completely suppressed the increase.

The absolute heart weight of db/db mice was lower compared with db/+ mice in the present study. Although there has been a report that ob/ob mice at 6-months of age are associated with an increase in heart weight, the heart weight of db/db mice aged 2 months is lower in that report.¹⁹ There was another report that the absolute heart weight of db/db mice is not different from db/+ mice.²⁰ We have previously reported that the phosphoinositide 3-kinase pathway is necessary and sufficient to promote heart growth.¹⁶ Thus, a decrease in Akt phosphorylation observed in our db/db mice might cause a decrease in heart weight.

Insulin signaling in the heart of db/db mice is evaluated by measuring the amount of phosphorylated Akt, S6 or S6K1. Phosphorylation of Akt and S6 was decreased in db/db mice compared with db/+ mice, and S6K1 phosphorylation was not different from that of db/+ mice. Akt is one of the best characterized targets of the insulin receptor.¹⁸ S6K1 is also a downstream of the insulin receptor, but it is also activated in an insulin signal independent pathway.^{21,22} S6K1 is not necessary for S6 phosphorylation in intact tissue.²³ In this experiment, S6K1 activity may be controlled in an insulin signaling independent manner.

Candesartan increased Akt phosphorylation of diabetic db/db mice. Recently, a series of studies has revealed a tight connection between the insulin signal transduction pathway and the angiotensin signal transduction pathway. Several studies have suggested that angiotensin II might negatively modulate insulin-mediated actions by regulating several levels of the insulin signaling cascade such as the insulin receptor, insulin receptor substrate, and phosphoinositide 3-kinase.^{24,25} Therefore, candesartan might increase Akt phosphorylation by suppressing the angiotensin II-induced negative regulation of insulin signaling. Alternatively, candesartan may increase Akt phosphorylation in an insulin signaling independent manner because angiotensin II is reported to modulate Akt phosphorylation in a phosphoinositide 3-kinase independent manner.²⁶

In the present study, candesartan treatment effectively attenuated the increase of senescence-associated molecules in the heart tissue of db/db mice. Angiotensin II decreases the telomerase activity of the endothelial progenitor cell and the effect is reversed by superoxide dismutase, suggesting that angiotensin II may induce senescence like phenotype via oxidative stress.²⁷ Angiotensin II is involved in the generation of oxidative stress^{8,28} and oxidative stress is thought to play a critical role in the aging process.²⁹ Candesartan may decrease markers of senescence by reducing oxidative stress. In addition, overexpression of insulin-like growth factor-1 in the heart causes an increase in Akt phosphorylation and a decrease in the amount of p21, p27 and p53.¹³ Thus, it is also possible that preservation of insulin signaling by candesartan may be involved in the decrease of p21, p27, p53 and Rb in the heart tissue of db/db mice.

An increase in the amount of the angiotensin-converting enzyme, angiotensinogen, in the tissue of diabetic animals has been reported.^{30,31} An angiotensin-converting enzyme inhibitor decreased the inflammatory response in the myocardium of diabetic animals.³² An ARB improved cardiac

diastolic function and decreased fibrosis of diabetic rats.³³ In the present study, cardiac function was impaired in db/db mice, which is what was found in previous reports,^{34,35} and candesartan treatment improved cardiac function.

The amount of senescence-associated molecules, such as p21, p27, p53 and Rb, were increased in the myocardium of diabetic db/db mice. p53 has been reported to be involved in myocardial damage induced by stretch,³⁶ tachycardia³⁷ or renin-angiotensin system activation.³⁸ p53 knockout mice are associated with reduced doxorubicin-induced myocardial dysfunction.³⁹ Thus, the increase of senescence-associated molecules may be involved in the cardiac dysfunction of db/db mice, and candesartan may improve cardiac function, in part, by reducing the amount of these senescence-associated molecules.

BNP mRNA is decreased in the myocardium of db/db mice. BNP levels were recently shown to be inversely proportional to the body mass index in individuals without heart failure^{40,41} and in those with chronic heart failure.⁴² Obesity is associated with the impaired atrial natriuretic peptide mRNA expression in the hearts of Zucker diabetic rats,⁴³ suggesting that a decrease in the production of BNP in the myocardium may explain the lower BNP level in the obese population.

Diabetes is associated with several age-related disorders such as cataracts, osteoporosis, or atherosclerosis, raising speculation that diabetes and physiological aging have a common process.³ Heart failure is an age-associated disorder.⁷ In the present study, candesartan partially preserved insulin signaling as measured by the phosphorylation of Akt and S6. In contrast, candesartan completely suppressed the senescence markers. Thus, angiotensin II may be involved in the development of the aging process itself, in addition to the prevention of diabetes.

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