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ORIGINAL RESEARCH ARTICLE

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Epoetin beta pegol ameliorates flow-mediated dilation with improving endothelial nitric oxide synthase coupling state in nonobese diabetic rats

Kenichi Serizawa[1] | Kenji Yogo[1] | Yoshihito Tashiro[2] | Ryohei Kawasaki[2] | Koichi Endo[3] | Yasushi Shimonaka[2] | Michinori Hirata[2]

[1]Product Research Department, Chugai Pharmaceutical Co., Ltd, Shizuoka, Japan

[2]Product Research Department, Chugai Pharmaceutical Co., Ltd, Kanagawa, Japan

[3]Medical Science Department, Chugai Pharmaceutical Co., Ltd, Tokyo, Japan

Correspondence
M. Hirata, Product Research Department, Chugai Pharmaceutical Co., Ltd, Kamakura, Kanagawa, Japan.
Email: hiratamcn@chugai-pharm.co.jp

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Summary

Background/Aims: Patients with diabetic nephropathy have a high cardiovascular mortality. Epoetin beta pegol (continuous erythropoietin receptor activator, C.E.R.A.) is a drug for the treatment of renal anemia. In this study, we investigated the effect of C.E.R.A. on vascular endothelial function as evaluated by flow-mediated dilation (FMD) and the relationship between hematopoiesis and FMD in diabetic nephropathy rats.

Methods: Male Spontaneously Diabetic Torii rats (SDT, 22 weeks old) were used. C.E.R.A. (0.6, 1.2 μg/kg) was administered subcutaneously once every 2 weeks for 8 weeks. At 1 week after last administration (31 weeks old), we assessed FMD in the femoral arteries of anesthetized rats using a high-resolution ultrasound system. FMD was also measured 1 week after single C.E.R.A. treatment (5.0 μg/kg) to examine the influence of hematopoiesis.

Results: Flow-mediated dilation was significantly decreased in SDT rats before the start of C.E.R.A. treatment (22 weeks old). Repeated administration of C.E.R.A. dose-dependently improved FMD in SDT rats (31 weeks old) without changing blood glucose, nitroglycerin-induced vasodilation, or kidney function. Long-term administration of C.E.R.A. improved the state of endothelial nitric oxide synthase uncoupling in the femoral arteries of SDT rats, which showed a positive correlation with FMD. On the other hand, there was no correlation between FMD and Hb or Hct in SDT rats. Furthermore, at 1 week after single administration of C.E.R.A., FMD was not significantly improved although hemoglobin levels were comparable with levels following long-term C.E.R.A. treatment.

Conclusion: Long-term treatment with C.E.R.A. improved FMD in SDT rats even after onset of endothelial dysfunction.

KEYWORDS

Chronic kidney disease, Diabetic nephropathy, Endothelial dysfunction, Epoetin beta pegol, Erythropoietin-stimulating agents, Flow-mediated dilation

1 | INTRODUCTION

Diabetes mellitus is a risk factor for the development of chronic kidney disease (CKD) and cardiovascular disease.[1,2] Endothelial dysfunction represents the earliest change in the process of atherosclerosis, which is a major complication and the factor most correlated with mortality in CKD,[3] and is known to be a powerful surrogate marker of cardiovascular events.[4] Recently, endothelial function as evaluated by flow-mediated dilation (FMD) has been recognized as an independent predictor of future cardiovascular events in clinical settings.[5]

Erythropoietin (EPO) is a hematopoietic hormone, and its receptor is widely distributed in both hematopoietic cells and nonhematopoietic cells, including endothelial and smooth muscle cells.[6] Epoetin beta pegol, commonly known as continuous erythropoietin receptor activator (C.E.R.A.), is a unique erythropoiesis-stimulating agent (ESA) for the treatment of anemia in patients with CKD. C.E.R.A. has been modified by integration of methoxypolyethylene glycol into the molecule, endowing it with a long half-life and slow clearance rate.[7] A previous report demonstrated that C.E.R.A. prevented the reduction of FMD in CKD rats.[8] However, there is as yet no evidence about the effect of C.E.R.A. on diabetes-related reduction in FMD.

There are several diabetic model rats, such as Goto-Kakizaki rats, Otsuka Long-Evans Tokushima Fatty rats, and Zucker Diabetic Fatty rats. These rats all develop obesity, hyperinsulinemia, and hypertension. The Spontaneously Diabetic Torii (SDT) rat is also a diabetic nephropathy model, but does not exhibit obesity, hyperinsulinemia, or hypertension.[9] Therefore, SDT rats enable us to evaluate hyperglycemia-induced endothelial dysfunction without those other influences.[10,11]

Erythropoiesis-stimulating agents are able to directly prevent endothelial dysfunction via endothelial nitric oxide synthase (eNOS) activation and their antioxidative effects,[12,13] and it is thought that ESAs can also indirectly influence FMD via the stimulated hematopoiesis. Increased hematocrit (Hct) can augment fluid viscosity and, consequently, wall shear stress,[14] leading to increasing NO production by the endothelium.[15,16] Because FMD is evaluated by shear stress-induced vasodilation, it is possible that upregulation of hemoglobin (Hb) or Hct by ESAs can increase FMD independently of improving endothelial function. However, to date there have been no reports about the relationship between Hb levels and FMD.

The aim of this study was to examine the effect of C.E.R.A. on FMD in SDT rats. In addition, we also investigated the influence of Hb levels on FMD.

2 | MATERIALS AND METHODS

2.1 | Animals

Male SDT rats (22 weeks old; CLEA Japan, Inc., Tokyo, Japan) were used as nonobese type 2 diabetic rats. Male Sprague-Dawley rats (SD, 22 weeks old; CLEA Japan, Inc.) were used as age-matched control rats because SDT rat is an inbred strain of SD rat. All rats were fed ordinary laboratory chow and allowed free access to water under a constant light and dark cycle of 12 hours. All animal procedures were conducted in accordance with the *Guidelines for the Care and Use of Laboratory Animals* at Chugai Pharmaceutical Co., Ltd, and all experimental protocols were approved by the Animal Care Committee of the institution and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National institutes of Health.

2.2 | Experimental design

C.E.R.A. (0.6 or 1.2 μg/kg) or its vehicle (PBS-T, containing 0.02% Tween-80) was subcutaneously administered once every 2 weeks for 8 weeks. At 1 week after last administration (31 weeks old), endothelial function was evaluated by measuring FMD in the femoral arteries of anesthetized rats with a high-resolution ultrasound system. Furthermore, to examine the influence of Hb upregulation on FMD, FMD was evaluated 1 week after a single injection of C.E.R.A. at 30 weeks of age (5 μg/kg, s.c.).

2.3 | Measurement of FMD

Flow-mediated dilation was measured in rats as described previously.[17,18] Briefly, rats were anesthetized with thiobutabarbital (120 mg/kg, i.p.) with constant monitoring of rectal temperature and monitoring of adequate anesthetic depth by pinching the toe. The animals were kept stable with a heated sheet and warming lamps directed at each rat. Femoral arterial diameter was determined from images of longitudinal sections of the femoral artery obtained with a high-resolution ultrasound system (Vevo 2100; VisualSonics, Toronto, ON, Canada). Experiments were started after a 15-minutes equilibration period and when the body core temperature (37±1°C) was stable. Ischemia and reperfusion of the hindlimb were achieved with a snare occluder positioned upstream from the site to be visualized, around the common iliac artery, through a transabdominal access. The snare occluder consisted of a 5-0 nylon surgical suture filament passed around the artery and through a 4-cm PE-200 tube, and the skin was closed with surgical clips. Hindlimb ischemia was achieved by pulling on the filament through the tube and clamping with a clamp. After the equilibration period, baseline recordings were taken and the common iliac artery was then occluded with the snare occluder. After 5 minutes of ischemia, the hindlimb was reperfused by releasing the occluder, and changes in the diameter of the femoral artery were monitored. FMD was taken to be the peak change in femoral arterial diameter after reperfusion, as the clinical guidelines for assessment of FMD define the peak change in brachial arterial diameter as an endothelial function to be that which occurs approximately 1 minute after reperfusion.[19] After measuring FMD, endothelium-independent vasodilation was also evaluated by monitoring the vasodilation induced by intravenous injection of nitroglycerin (NTG, 5 μg/kg).

2.4 | Measurement of plasma and urine parameters

Urine collection was performed by utilizing metabolic cages. Plasma samples were obtained under anesthesia with thiobutabarbital

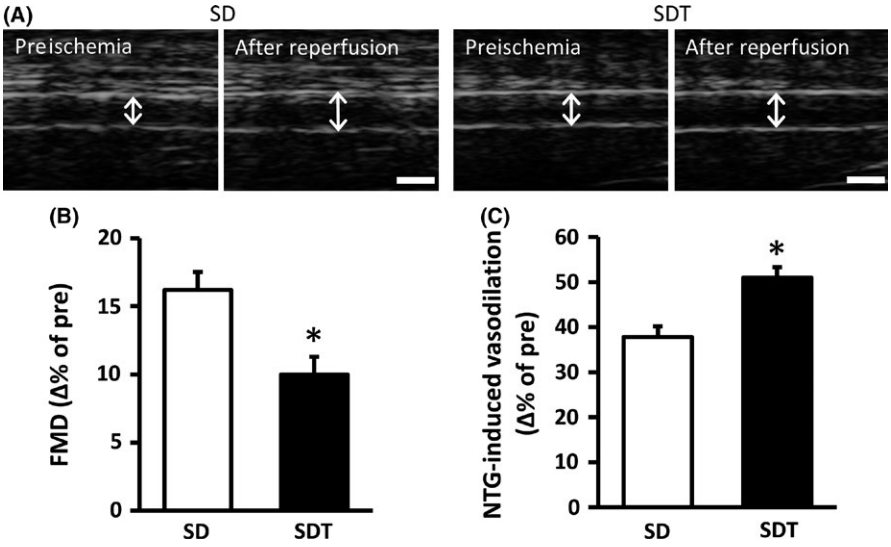


FIGURE 1 Evaluation of flow-mediated dilation (FMD) before the start of C.E.R.A. administration in Spontaneously Diabetic Torii (SDT) rats (22 weeks old). Representative images showing femoral arteries before ischemia and after reperfusion in SD and SDT rats (A). FMD evaluated as the peak change in femoral arterial diameter was significantly decreased before the start of C.E.R.A. treatment in SDT rats (B). Endothelium-independent vasodilation by nitroglycerin (NTG) injection was rather increased in SDT rats (C). Scale bars=0.5 mm, **P*<.05 vs SD by unpaired *t*-test (n=8)

(120 mg/kg, i.p.) after the measurement of FMD. Urinary protein and blood urea nitrogen (BUN) were measured with an automatic chemistry analyzer (TBA-120FR; Toshiba Medical Systems, Tochigi, Japan). Hb levels and Hct were assessed using an automated hematology analyzer (XT-2000iV, Sysmex, Hyogo, Japan).

2.5 | Western blotting analysis

Rats were euthanized by exsanguination under anesthesia with thiobutabarbital, and femoral arteries were harvested and frozen in liquid N_2 immediately after isolation and stored in a freezer at −80°C. To investigate the state of eNOS uncoupling, low-temperature SDS-PAGE was performed.[20] Briefly, protein extracts were mixed with sample buffer (without β-mercaptoethanol), and nonboiled samples were separated on SDS-polyacrylamide gel on ice. Immunoblotting was performed with anti-eNOS antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with antinitrotyrosine antibodies (Santa Cruz Biotechnology).

2.6 | Statistical analyses

All data are expressed as mean±SEM. The n values refer to the number of individual animals in each group on which experiments were performed. The statistical significance of differences was determined using unpaired *t*-test or Dunnett's test. Test of no correlation was also performed. Probability values of less than .05 were considered significant. Statistical analyses were performed using JMP version 11.2.1 software (SAS Institute, Cary, NC, USA).

3 | RESULTS

3.1 | Endothelial function before C.E.R.A. treatment in SDT rats

Before the start of C.E.R.A. treatment, the blood glucose level of SDT rats (22 weeks old) was significantly increased compared to control SD rats (SD: 143.9±12.3 mg/dL, SDT: 484.6±15.9 mg/dL, *P*<.05). At that time, FMD was already decreased in SDT rats (SD: 16.2±1.3%, SDT: 10.0±1.3%, *P*<.05; Figure 1A,B). After the measurement of FMD, we also assessed the vascular smooth muscle function which could influence the vasodilatory potency. NTG-induced vasodilation was rather increased in SDT rats (Figure 1C).

3.2 | Effects of repeated administration of C.E.R.A. on FMD in SDT rats

Repeated administration of C.E.R.A. (1.2 μg/kg) significantly upregulated FMD in SDT rats (SD+vehicle: 17.8±1.7%; SDT+vehicle: 10.4±1.8%; SDT+C.E.R.A. 0.6 μg/kg: 17.0±2.0%; SDT+C.E.R.A. 1.2 μg/kg: 19.2±2.1%, *P*<.05; Figure 2A,B). C.E.R.A. did not affect the endothelium-independent vasodilation by NTG injection (Figure 2C).

To evaluate the state of eNOS uncoupling, which is a cause of endothelial dysfunction, we detected eNOS dimer and eNOS monomer in femoral arteries. The eNOS dimer/monomer ratio was lower in vehicle-treated SDT rats, and C.E.R.A. dose-dependently increased the eNOS dimer/monomer ratio (Figure 3A), which showed a positive correlation with FMD in SDT rats (Figure 3D). Total eNOS expression was upregulated in vehicle-treated SDT rat, and C.E.R.A. showed no influence on it (Figure 3B). Because oxidative stress is a cause of eNOS uncoupling, we evaluated nitrotyrosine accumulation, which is a marker of peroxynitrite. Nitrotyrosine accumulation was increased in femoral arteries of SDT rats, and repeated treatment with C.E.R.A. showed a tendency, but not significantly, to reduce the increase of it (Figure 3C). On the other hand, there was no correlation between FMD and Hb or Hct in SDT rats (Figure 3E,F), although C.E.R.A. (1.2 μg/kg) increased Hb and Hct (Table 1). Baseline femoral artery diameter at the measurement of FMD in vehicle-treated SDT rats was larger than in SD rats, and it was reduced by C.E.R.A. treatment (Table 1). However, there was no correlation between FMD and baseline diameter of femoral artery (Figure 3G). C.E.R.A. showed no influence on blood glucose, kidney function (urinary protein, BUN), or body weight (Table 1).

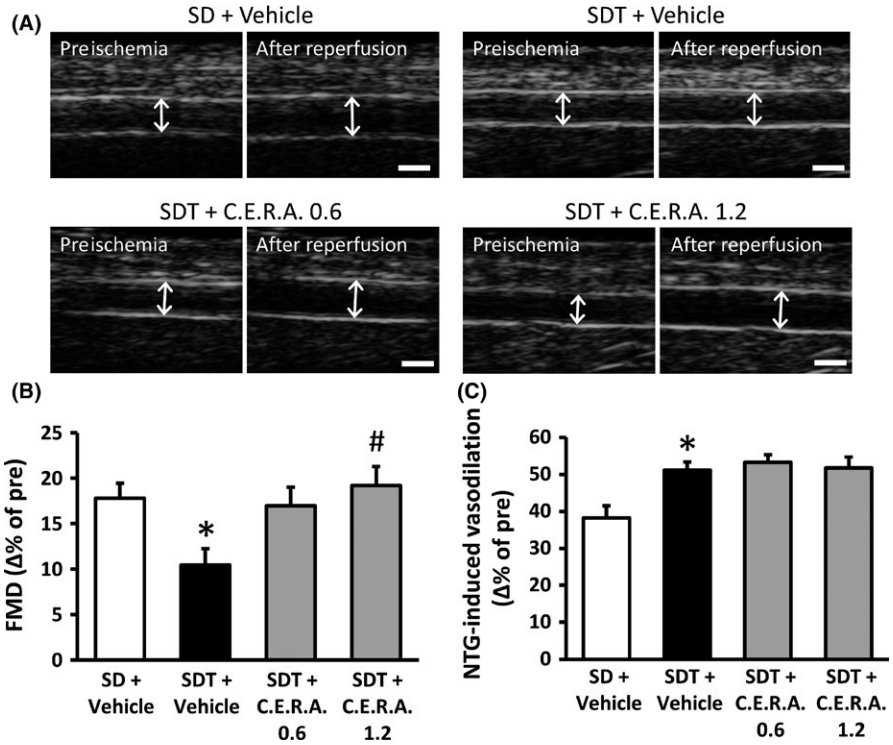


FIGURE 2 Effect of repeated administration of C.E.R.A. on flow-mediated dilation (FMD) in Spontaneously Diabetic Torii (SDT) rats (31 weeks old). C.E.R.A. (0.6 or 1.2 μg/kg) or its vehicle was subcutaneously administered once every 2 weeks from 22 weeks of age to 30 weeks of age. At 1 week after last administration (31 weeks old), endothelial function was evaluated by measuring FMD. Representative images show the femoral arteries before ischemia and after reperfusion in SD and SDT rats (A). C.E.R.A. (1.2 μg/kg) significantly increased FMD in SDT rats (B). C.E.R.A. showed no influence on endothelium-independent vasodilation by nitroglycerin (NTG) injection (C). Scale bars=0.5 mm, *P<.05 vs SD+vehicle by unpaired t-test, #P<.05 vs SDT+vehicle by Dunnett's multiple comparison test (n=7-10)

3.3 | Effects of single treatment with C.E.R.A. on FMD in SDT rats

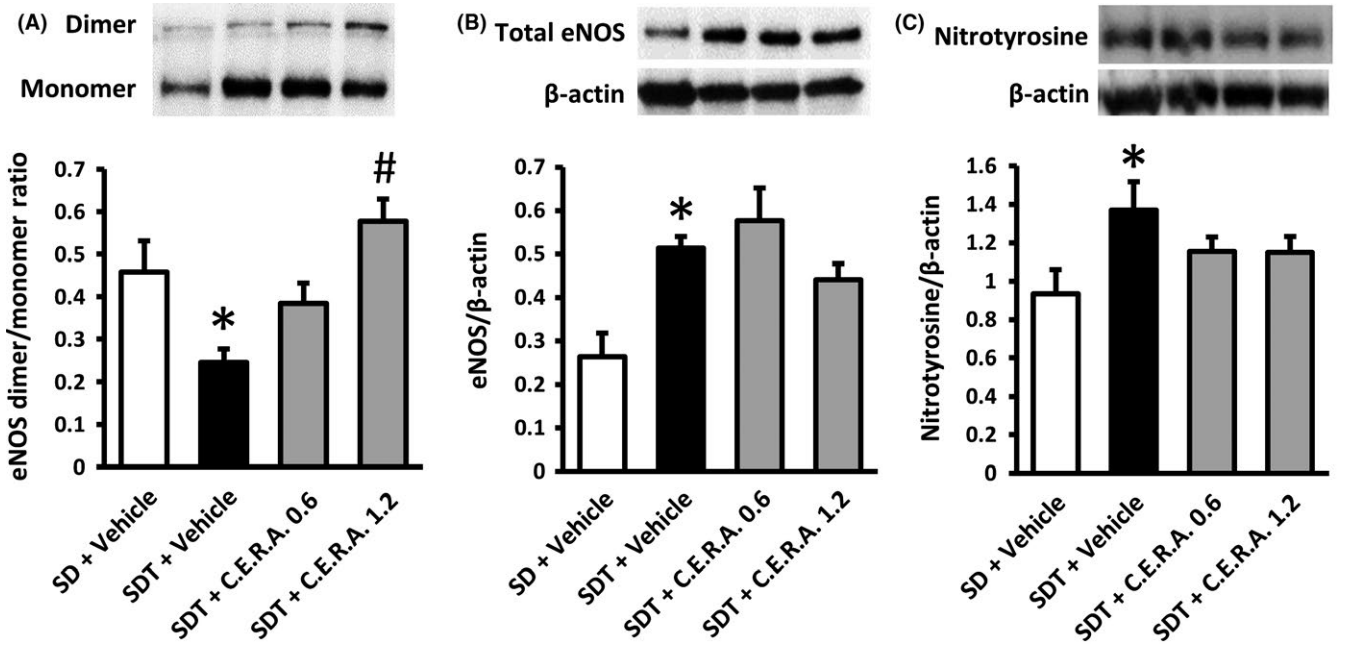
To examine whether Hb upregulation itself could increase FMD, we measured FMD 1 week after single administration of C.E.R.A. (5.0 μg/kg) in SDT rats. At 1 week after treatment with a single dose of C.E.R.A. (5.0 μg/kg), Hb (SDT+vehicle: 15.7±0.3 g/dL; SDT+C.E.R.A. 5.0 μg/kg: 18.4±0.2 g/dL, P<.05) and Hct (SDT+vehicle: 45.2±1.0%; SDT+C.E.R.A. 5.0 μg/kg: 54.9±0.4%, P<.05) were significantly higher than in vehicle-treated SDT rats. Although Hb and Hct levels were comparable with those in SDT rats treated with repeated administration of C.E.R.A. (1.2 μg/kg), single treatment with C.E.R.A. (5.0 μg/kg) did not change FMD (SD+vehicle: 15.5±1.6%, SDT+vehicle: 10.8±0.5%, SDT+C.E.R.A. 5.0 μg/kg: 12.5±2.1%; Figure 4A,B) or NTG-induced vasodilation (Figure 4C).

4 | DISCUSSION

In the present study, C.E.R.A. treatment was started after onset of endothelial dysfunction in SDT rats. Repeated treatment with C.E.R.A. improved FMD and the eNOS dimer/monomer ratio in SDT rats. Furthermore, FMD showed a positive correlation with eNOS dimer/monomer ratio but not with Hb or Hct. In addition, single administration of C.E.R.A. did not affect FMD when given at a dose sufficient to result in comparable Hb levels to that attained in SDT rats treated with repeated administration of C.E.R.A. Earlier basic studies have demonstrated that ESAs confer endothelial protective effects,[12,21] but in those studies, ESAs were administered prophylactically. Previously, we also demonstrated that prophylactic administration of C.E.R.A. prevented the reduction of FMD in 5/6 nephrectomized rats.[8] This study is the first report to indicate a therapeutic effect of C.E.R.A. on diabetic endothelial dysfunction.

Diabetes mellitus is a major independent risk factor for cardiovascular events.[22] Endothelial dysfunction represents the earliest change in the process of atherosclerosis and plays a central role in diabetic vascular diseases.[23] A reduction in NO availability, which reflects an imbalance between NO production and degradation, can cause endothelial dysfunction.[24] One of the most important causes of a reduction in NO availability is the uncoupling of eNOS. Under normal conditions, eNOS exists as dimer and produces NO from arginine. But under oxidative conditions, reactive oxygen species, especially peroxynitrite, can oxidize avidly tetrahydrobiopterin (BH_4) to dihydrobiopterin (BH_2), leading to a monomer eNOS (ie, it becomes uncoupled) with the production of reactive oxygen species (ROS) rather than NO.[25] eNOS uncoupling has been observed in diabetic patients[26] and animals.[18,27] SDT rats,

FIGURE 3 Effect on endothelial nitric oxide synthase (eNOS) uncoupling and relationship between flow-mediated dilation (FMD) and some parameters in Spontaneously Diabetic Torii (SDT) rats. C.E.R.A. dose-dependently increased eNOS dimer/monomer ratio in the femoral arteries of SDT rats (A). C.E.R.A. showed no influence on total eNOS expression in the femoral arteries of SDT rats (B). C.E.R.A. tended to reduce nitrotyrosine accumulation in the femoral arteries of SDT rats (C). Scatter plots show the relationship between FMD and eNOS dimer/monomer ratio (D), Hb levels (E), Hct (F), or baseline femoral artery diameter (G) in SDT rats. eNOS dimer/monomer ratio was correlated with FMD (D). Black circles, SDT+vehicle; gray circles, SDT+C.E.R.A. (0.6 μg/kg); gray squares, SDT+C.E.R.A. (1.2 μg/kg). *P<.05 vs SD+vehicle by unpaired t-test; #P<.05 vs SDT+vehicle by Dunnett's multiple comparison test (n=7-10)


(A) Dimer
Monomer
(B) Total eNOS
β-actin
(C) Nitrotyrosine
β-actin
eNOS dimer/monomer ratio
eNOS/β-actin
Nitrotyrosine/β-actin
SD + Vehicle
SDT + Vehicle
SDT + C.E.R.A. 0.6
SDT + C.E.R.A. 1.2

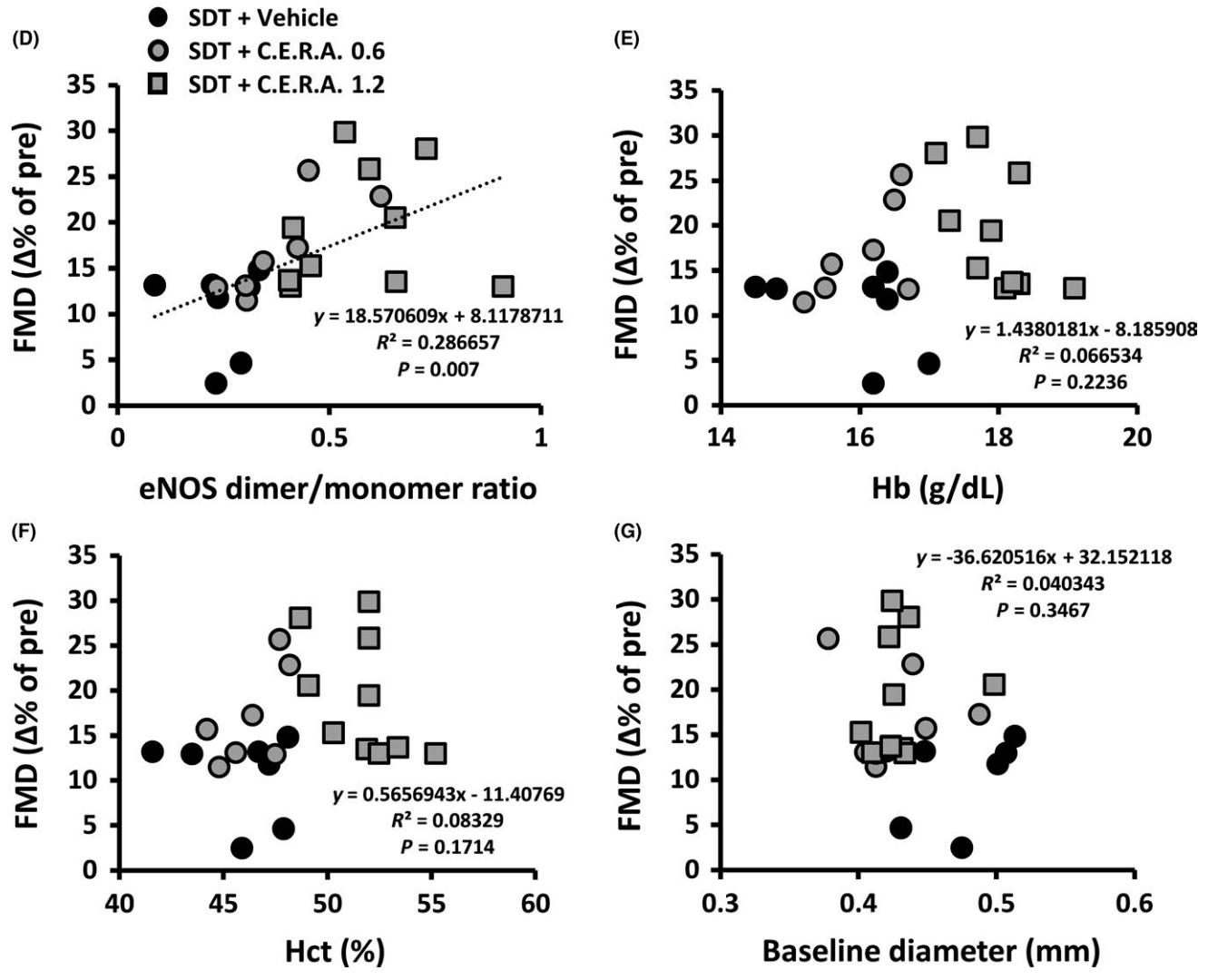

(D)
SDT + Vehicle
SDT + C.E.R.A. 0.6
SDT + C.E.R.A. 1.2
FMD (Δ% of pre)
y = 18.570609x + 8.1178711
R² = 0.286657
P = 0.007
eNOS dimer/monomer ratio
(E)
y = 1.4380181x - 8.185908
R² = 0.066534
P = 0.2236
Hb (g/dL)
(F)
y = 0.5656943x - 11.40769
R² = 0.08329
P = 0.1714
Hct (%)
(G)
y = -36.620516x + 32.152118
R² = 0.040343
P = 0.3467
Baseline diameter (mm)

TABLE 1 Physiological parameters in vehicle-treated SD and Spontaneously Diabetic Torii (SDT) rats and in SDT rats treated with repeated administration of C.E.R.A. (all rats were 31 wk old)

	SD+vehicle	SDT+vehicle	SDT+C.E.R.A. 0.6	SDT+C.E.R.A. 1.2
Blood glucose (mg/dL)	134.0±4.5	511.4±27.5*	526.0±22.9	477.7±14.0
Hb (g/dL)	15.1±0.3	15.9±0.3	16.0±0.2	18.0±0.2#
Hct (%)	41.5±0.6	45.8±0.9*	46.3±0.6	51.7±0.6#
Urinary protein (mg/d)	6.0±0.3	80.1±19.2*	103.8±19.4	110.8±20.1
Blood urea nitrogen (mg/dL)	17.9±0.9	24.2±1.1*	23.0±0.4	24.0±1.3
Baseline femoral artery diameter (mm)	0.42±0.01	0.47±0.01*	0.43±0.01#	0.43±0.01#
Body weight (g)	715.1±16.3	462.9±9.3*	447.9±12.5	450.5±8.1

*P<.05 vs SD+vehicle by unpaired t-test; #P<.05 vs SDT+vehicle by Dunnett's multiple comparison test (n=7-10).

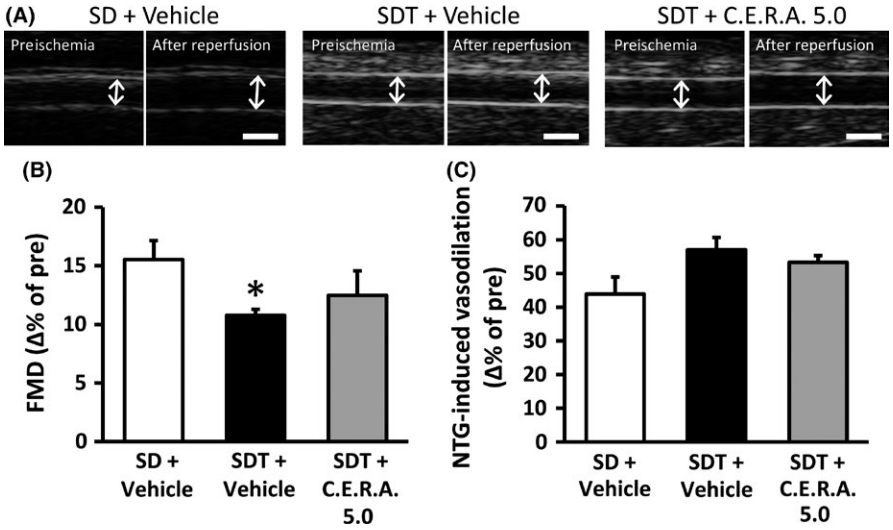


FIGURE 4 Effect of single administration of C.E.R.A. on flow-mediated dilation (FMD) in Spontaneously Diabetic Torii (SDT) rats (31 weeks old). SDT rats were treated with single administration of C.E.R.A. (5.0 μg/kg, s.c.) at 30 weeks of age. At 1 week after C.E.R.A. treatment (31 weeks old), endothelial function was evaluated by measuring FMD. Representative images show the femoral arteries before ischemia and after reperfusion in SD and SDT rats (A). C.E.R.A. showed no influence on FMD (B) or endothelium-independent vasodilation by nitroglycerin (NTG) injection (C). Scale bars=0.5 mm, *P<.05 vs SD+vehicle by unpaired t-test (n=6-8)

which are used in this study, also show endothelial dysfunction accompanied by eNOS uncoupling in the artery.[10] Our results indicated that C.E.R.A. improved FMD and the eNOS dimer/monomer ratio without changing in total eNOS expression in the femoral arteries of SDT rats. Furthermore, there is a positive correlation between FMD and the eNOS dimer/monomer ratio. In this study, we mainly focused on the endothelial function in SDT rats. Unexpectedly, vascular smooth muscle function as evaluated by NTG-induced vasodilation was enhanced in SDT rats. This result corresponds to the previous findings that the response to NO donor was increased with the reduction of phosphodiesterase (PDE) activity in the diabetic rats.[28] These results may demonstrate that diabetes can indicate compensatory upregulation of NO reactivity at a certain time. In spite of NO hyperactivity in SDT rats, FMD was decreased by the reduction of NO availability. Although the details were unknown, the effect of C.E.R.A. on PDE activity might be a cause of no influence on the endothelium-independent vasodilation by NTG injection. In any case, our results suggest that FMD reflected endothelial function and C.E.R.A. ameliorated endothelial function but not smooth muscle function by improving NO availability. d'Uscio et al.[29] reported that treatment with EPO prevented endothelial dysfunction caused by eNOS uncoupling in GTP-cyclohydrolase I-deficient mice via antioxidative effects. Previous studies suggest some mechanisms of antioxidative effects by EPO. For example, EPO could increase Cu/Zn-SOD and heme oxygenase-1 expression via PI3K/Akt pathway.[13] EPO could also induce the expression of SOCS-1, which reduces NADPH oxidase activity, depending on STAT5 signaling.[13,30] Our previous report showed that repeated treatment with C.E.R.A. reduced oxidative stress in the femoral arteries of 5/6 nephrectomized rats.[8] In addition, it was also reported that treatment with C.E.R.A. could ameliorate oxidative stress with enhancing the antioxidant defense system and reducing ROS production in patients with CKD.[31] In the present study, repeated treatment with C.E.R.A. showed a nonsignificant

tendency towards reduction of the nitrotyrosine accumulation in the femoral arteries of SDT rats. Although our Western blotting analysis could not demonstrate the significant reduction of oxidative stress by C.E.R.A., this slight antioxidative effect for a long time might contribute to improving the state of eNOS uncoupling in SDT rats. Further experiments are needed to clarify the involvement of antioxidative effect by C.E.R.A. on improving the state of eNOS uncoupling in SDT rats.

Rapid increases in Hct and blood viscosity increase vessel wall shear stress[14] and the production of NO by the endothelium.[15,16] Because FMD is evaluated by shear stress-induced vasodilation, it is possible that upregulation of Hb or Hct by ESAs can increase FMD independently of improving endothelial function. On the other hand, long-term change in hemodynamics can be regulated by coupled physical and biochemical mechanisms including the myogenic response, shear stress-induced NO production, and the sympathetic nervous system.[32-34] In this study, repeated treatment with C.E.R.A. (1.2 μg/kg/2 wk) significantly increased Hb levels and FMD in SDT rats. The value of FMD showed a positive correlation with eNOS function, as indicated by the eNOS dimer/monomer ratio, but showed no correlation with Hb or Hct levels. Because any persistent change in hemodynamics can be autoregulated by the myogenic response and so on, it is more than probable that FMD strictly reflects endothelial function without any influence from upregulated Hb. In fact, arterial diameter before the measurement of FMD was not changed by upregulation of Hb levels in C.E.R.A.-treated SDT rats. Furthermore, single treatment with C.E.R.A. (5 μg/kg) did not result in any significant increase in FMD despite the upregulation of Hb and Hct in SDT rats.

There is a limitation in the present study. SDT rats did not develop anemia, and C.E.R.A. improved diabetes-induced endothelial dysfunction under plethoric conditions. Under these conditions, FMD in SDT rat was not correlated with hemoglobin and Hct levels. However, it might be possible that the relationship between FMD and hematopoiesis in anemic condition is different from the result of our present study because tissue ischemia could influence the endothelial function. Furthermore, there is undeniable possibility that chronic increase in hemoglobin functionally affects the endothelial function. Therefore, it may be controversial whether hematopoiesis is not completely associated with FMD value. Further detailed examinations may be needed to clarify the effect of C.E.R.A. on endothelial function and the relationship between FMD and hematopoiesis in anemia patients with diabetic nephropathy.

In conclusion, we demonstrated that repeated administration of C.E.R.A. improved FMD in SDT rats even after onset of endothelial dysfunction. In addition, FMD was correlated with eNOS dimer/monomer ratio but not with Hb and Hct levels. Our results are expected to clarify the effect of C.E.R.A. on endothelial or cardiovascular function in patients with diabetic nephropathy.

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CONFLICTS OF INTEREST

All authors are employees of Chugai Pharmaceutical Co., Ltd.

AUTHOR CONTRIBUTIONS

Kenichi Serizawa was involved in the conception of the study, data acquisition, and drafting of the article. Kenji Yogo, Yoshihito Tashiro, and Ryohei Kawasaki were involved in the conception and data acquisition. Koichi Endo, Yasushi Shimonaka, and Michinori Hirata were involved in the conception. Michinori Hirata is the corresponding author and takes responsibility for the integrity of the study. All authors have reviewed the article and have approved the final manuscript.

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