Original Article

Reduction of NO-mediated Relaxing Effects in the Thoracic Aorta in an Experimental Chronic Kidney Disease Mouse Model

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Aim: Chronic kidney disease (CKD) is known to frequently cause cardiovascular events. However, it is unclear how renal dysfunction affects the vascular response. We herein studied the effects of renal dysfunction on the aortic behavior in adenine-fed mice, investigating mechanisms underlying the occurrence of cardiovascular events in CKD patients.

Methods: Biochemical analyses of the plasma creatinine, blood urea nitrogen (BUN) and glucose levels and measurements of the blood pressure were performed using C57BL/6 mice fed with and without an adenine-containing diet. The relaxing effects of acetylcholine (ACh) or sodium nitroprusside (SNP) and effects of NO synthase (NOS) inhibitors on the contractions induced by phenylephrine (PE) were measured in endothelium-intact aortas obtained from both mice.

Results: The mice fed 0.25% adenine for four weeks showed greater plasma creatinine and BUN concentrations than the control mice, suggesting that adenine-fed mice are a useful CKD model. Furthermore, ACh relaxed the PE-stimulated, endothelium-intact aortas, the effect of which was less potent in the adenine-fed mice than in the control mice. In contrast, the degree of SNP-induced relaxation of the aortas was the same in the adenine-fed mice and control mice. The α_1 -adrenergic agonist, PE, induced more potent absolute tension of the endothelium-intact aortas in the CKD model mice than in the control mice, while the NOS inhibitors, N-nitro-L-arginine (LNA) and asymmetric dimethylarginine (ADMA) enhanced the contraction effects of PE in both mice.

Conclusions: The findings of this study indicate that spontaneous and stimulated NO release from the endothelium is decreased in the CKD model mouse aorta. The NO-mediated correlation between renal and elastic arterial endothelial dysfunction is suggested to be a cause of cardiovascular events in patients with CKD.

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Key words: Chronic kidney disease, Endothelium, Aorta, Relaxation, NO

Introduction

It is well known that the incidence of cardiovascular events increases in patients with chronic kidney disease (CKD)¹⁻⁴. Epidemiological cases suggest that renal dysfunction may result in arterial calcification, stiffness and impairment of endothelium-dependent

actions. Therefore, the possibility of injury to the vascular system has been considered in cases of CKD. However, it is currently unclear how renal dysfunction affects the vascular system. Cardiovascular disease (CVD) has been reported to cause dysfunction of the endothelium, inducing changes in the contraction/relaxation of smooth muscle⁵⁻¹¹. In this study, we aimed to examine contraction/relaxation in endothelium-intact aortas using an experimental CKD model of renal dysfunction.

Methods to prepare experimental CKD models have employed the surgical removal of kidney tissue 5/6 nephrectomy, electrocautery of the kidneys and the dietary administration of adenine¹². In the latter

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method, it is thought that 2, 8-dihydroxyadenine produced from adenine via oxidization through xanthine dehydrogenase is precipitated in the kidney tubules as kidney stones^{13, 14}. It has been reported that adenine administration induces chronic tubulointerstitial injury, causing increased plasma creatinine and blood urea nitrogen (BUN) concentrations¹⁵⁻¹⁸.

Aim

In this study, in order to investigate how renal dysfunction affects the function of elastic vessels, we prepared an adenine-induced CKD mouse model. We then tested the relaxation effects induced by acetylcholine (ACh) and sodium nitroprusside (SNP) in endothelium-intact aortas from the CKD model mice and compared with that observed in control mice. In addition, we studied the contraction effects of an adrenergic α_1 -agonist, phenylephrine (PE), in aortas from both the model mice and control mice and evaluated the influence of NO synthase (NOS) inhibitors on the effects of PE in order to examine spontaneous NO release from the endothelium. The results of this study may assist in elucidating the mechanisms responsible for the onset of cardiovascular events in CKD patients, allowing for the identification of targeted molecular signals in CKD therapy.

Methods

Reagents

PE, ACh, N-nitro-L-arginine (LNA), asymmetric dimethylarginine (ADMA) (Sigma Chemical Co., St. Louis, MO, USA), SNP and papaverine hydrochloride (Wako Pure Chemical, Osaka, Japan) were purchased. All other chemicals were of the highest reagent grade available.

Animals

Male C57BL/6 mice were obtained from a local supplier (Japan SLC, Inc., Shizuoka, Japan). The experimental procedures were approved by the animal experiment and welfare committee of Nagoya City University Graduate School of Medical Sciences. The animal care and research protocols were conducted in accordance with the guidelines of our university. Eleven-week-old mice received either a normal chow diet (0% adenine) or a diet containing either 0.125% or 0.25% adenine (CE-2-based diet manufactured by CLEA, Japan) *ad libitum*. After feeding the mice these diets for several days or four weeks, plasma was obtained via heart puncture and stored at -80°C until the assays. The mice were killed with sevoflurane and

subsequently weighed, after which the aortic tension was measured.

Measurement of Plasma Parameters

The concentrations of plasma creatinine, BUN and glucose were measured using a clinical autoanalyzer.

Measurement of Blood Pressure

Systolic, diastolic and mean blood pressure (BP) and heart rate were measured using a tail cuff BP analyzer (BP-98A; Softlon, Tokyo, Japan)¹⁹.

Measurement of Tension

The mice were anesthetized with sevoflurane, and the thoracic aortas were removed and dissected into ring strips (2-mm length). Each strip of the aorta was placed in a 5-mL organ bath containing normal Krebs solution (Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.6, HCO_3^- 15.5, H_2PO_4^- 1.2, Cl^- 134, glucose 11.5 mM; pH 7.4) bubbling with 95% O_2 and 5% CO_2 . After an equilibration period of 60 minutes, the mechanical activity was measured via isometric recording under a resting tension of 300 mg²⁰. PE was added in increments to study the concentration-dependent effects. The contracting response is shown as the mg tension or a percentage of the PE-induced maximal effect. Aortic rings with endothelium were exposed to PE (10^{-6} M or 10^{-7} M) to obtain the submaximal contraction and then relaxed with increasing concentrations of ACh or SNP. In some experiments, the aortic rings were pretreated with LNA (10^{-4} M) or ADMA (3×10^{-4} M) for 30 minutes before the injection of PE. At the end of the experiment, papaverine (10^{-4} M) was added to obtain maximal relaxation. The ACh- and SNP-induced relaxation responses were evaluated as the percentage of the maximal papaverine-induced effect.

Statistical Analysis

The results are expressed as the mean $\pm$ SE. Differences between the mean values were analyzed using Student's *t*-test or the Tukey-Kramer multiple comparison test. Correlation coefficients were obtained using a simple regression analysis. A *p*-value of < 0.05 was considered to be statistically significant.

Results

Organ Weights and Plasma Analyses in the Adenine-Fed Mice (Table 1)

Organs and plasma samples from the control and adenine-fed mice were weighed and analyzed. The

Table 1. Organ weights and plasma analyses

Adenine-diet (%)	0	0.25
Body weight, g (n)	28.5 ± 0.9 (10)	21.8 ± 0.6 (12)*
Kidney weight, g (n)	0.427 ± 0.028 (10)	0.366 ± 0.015 (12)
Kidney weight/Body weight (n)	0.015 ± 0.001 (10)	0.017 ± 0.001 (12)
Creatinine, mg/dL (n)	0.06 ± 0.01 (8)	0.25 ± 0.03 (9)*
BUN, mg/dL (n)	22.0 ± 1.1 (8)	72.4 ± 7.4 (9)*
Glucose, mg/dL (n)	242.5 ± 22.0 (8)	192.0 ± 8.2 (5)

Values are means ± SE.

* $p < 0.05$ compared with control group.

mice fed the 0.25% adenine-containing diet for four weeks showed significant reductions in body weight. In addition, the kidneys of the 0.25% adenine-fed mice appeared white and nephrosclerosis-like, although the kidney size, as assessed based on the kidney weight to body weight ratio, did not appear to be reduced in the adenine-fed mice compared with the control mice. The plasma creatinine concentration increased approximately four-fold within four weeks after the initiation of 0.25% adenine feeding. Meanwhile, the concentrations of BUN, but not glucose, in the adenine-fed mice were significantly elevated compared with that observed in the control mice (approximately three-fold greater), whereas the administration of the 0.125% adenine-containing diet for four weeks or the 0.25% adenine-containing diet for 3-6 days resulted in no increases in the plasma creatinine or BUN concentrations (data not shown). The mice fed 0.25% adenine for four weeks appeared to constitute a useful early-stage CKD model, as determined according to a renal dysfunction analysis.

Chronological Changes in Blood Pressure in the 0.25% Adenine-Fed Mice

We investigated the cardiovascular effects of this CKD mouse model by measuring and comparing the BP values in the mice fed 0.25% adenine for one, two and three weeks. The mean BP (MBP) results showed no significant differences between the adenine-fed mice and the control mice (**Fig. 1**). In addition, no effects of the 0.25% adenine-diet for one, two and three weeks were observed on the systolic and diastolic BP or heart rate values in the mice (data not shown).

Effects of ACh-Induced Relaxation on PE-Stimulated Contractions in the Endothelium-Intact Aortas of the Adenine-Fed Mice

The administration of adenine (10^{-6} - 10^{-4} M) to the endothelium-intact aortas from the C57/BL6 mice, which were placed in an organ bath, did not induce

either contractions or relaxation (data not shown). We then studied the vascular function using aortic ring preparations from mice fed 0, 0.125 and 0.25% adenine for four weeks. The injection of ACh (10^{-8} - 10^{-4} M) induced the relaxation of the endothelium-intact aortas, which had been submaximally pre-contracted with 10^{-6} M PE, in a concentration-dependent manner (**Fig. 2A**). Relaxation at 10^{-7} - 10^{-4} M of ACh, expressed as the percentage of the 10^{-4} M papaverine-induced maximal effect, was significantly less potent in the 0.25% adenine-fed mice than in the control and 0.125% adenine-fed mice. The maximal relaxation in the mice treated with and without adenine was approximately 35% and 60% of the papaverine-induced maximal effect, respectively. Furthermore, the effect of the 0.25% adenine diet for 3-6 days on ACh-induced relaxation was not different from that observed in the 0% adenine-fed mice (**Fig. 2B**). Pretreatment with the NOS inhibitor, LNA (10^{-4} M), completely inhibited the ACh-induced relaxation in the endothelium-intact aortas (data not shown). These results indicate that endothelium-dependent, NO-mediated relaxation was attenuated in the aortas of the mice fed 0.25% adenine for four weeks.

Effects of SNP-Induced Relaxation on PE-Stimulated Contractions in the Endothelium-Intact Aortas of the Adenine-Fed Mice

The administration of a NO donor, SNP (10^{-9} - 10^{-6} M), resulted in the relaxation of the aortas in the adenine-fed and control groups in a concentration-dependent manner (**Fig. 3**). The concentration-dependent curves for the relaxation effects observed in both groups were similar. The maximal effect for both groups was almost 100%, expressed as a percentage of 10^{-4} M papaverine-induced maximal relaxation. These results indicate that the degree of endothelium-independent relaxation remained unchanged in the aortas from the 0.25% adenine-fed mice compared with the control mice.

Fig. 1. Mean blood pressure in the control and 0.25% adenine-fed mice. Each point represents the mean $\pm$ SE. $\square$: normal diet ($n=5$), $\bullet$: 0.25% adenine ($n=5$). The ordinates show the mean blood pressure (mmHg). The abscissae show the duration of feeding of the diets with or without adenine (weeks).

Correlations between the Plasma Creatinine and BUN Concentrations and ACh-Induced Relaxation

The relationship between renal dysfunction and endothelium-dependent relaxation was examined using a simple regression analysis. ACh-induced relaxation (10^{-6} M) negatively correlated with the plasma BUN concentration ($r = -0.515$, $p = 0.034$, $n = 17$). In contrast, the relationship between the relaxing effects of ACh (10^{-6} M) and the plasma creatinine concentration was not statistically significant ($r = -0.391$, $p = 0.207$, $n = 17$).

Effects of NOS Inhibitors on PE-Induced Contractions in the Endothelium-Intact Aortas of the Adenine-Fed Mice (Fig. 4)

PE administration (10^{-9} – 10^{-5} M) induced concentration-dependent contractions of the endothelium-intact aortas in the mice fed 0 and 0.25% adenine. The absolute tension induced by PE administration (10^{-7} M) was significantly greater in the aortas of the mice fed 0.25% adenine than in the control mice (45.8 ± 6.4 ($n=13$) vs. 25.8 ± 7.1 ($n=6$) mg in the 0.25% adenine-fed and control mice, respectively, $p < 0.05$). In order to study endogenous NO release from the endothelium-intact aortas, the effects of pretreatment with a NOS inhibitor, LNA or ADMA, on 10^{-7} M PE-induced contractions were tested. As a result,

pretreatment with LNA (10^{-4} M) or ADMA (3×10^{-4} M) for 30 minutes significantly enhanced the contraction effects of PE (10^{-7} M) expressed as a percentage of the 10^{-5} M PE-induced maximal effect in the aortas from both groups of mice compared with the respective control groups.

Discussion

In this study, we found that the CKD mouse model developed using adenine feeding induced less potent endothelium-dependent relaxation following ACh administration and more potent aortic contractions via the α_1 -adrenergic receptor compared with that observed in the control mice. We also noted that spontaneous NO release from the aorta endothelium was decreased in the CKD model mice compared with the control mice. These results show that adenine-fed mice exhibiting renal dysfunction may develop disorders of the elastic vessels, such as the aorta.

Animal models of CKD have been previously reported, mostly using rats^{12, 15–18}. The CKD mouse model employed in the current study was induced by feeding the mice a diet containing 0.25% adenine for four weeks, which resulted in increases in the plasma creatinine and BUN concentrations to approximately four and three times that seen in the control mice. In

Fig. 2. Effects of adenine feeding on ACh-induced relaxation in the endothelium-intact thoracic aortas of the mice. The relaxant response to these drugs was determined in aorta strips pretreated with PE (10^{-6} M). The ordinates show the degree of relaxation expressed as the percentage of the 10^{-4} M papaverine-induced maximal effect. The abscissae show the concentrations of the applied drugs (log M). A □: 0% ($n=7$); ●: 0.125% ($n=6$); ▲: 0.25% ($n=10$) adenine diet for four weeks. B □: 0% adenine diet for four weeks ($n=7$); ●: 0.25% adenine diet for 3-6 days ($n=5$). Each point represents the mean $\pm$ SE. * $p < 0.05$ compared with the 0% adenine diet (control) mice.

addition, feeding with the 0.25% adenine-diet for eight weeks caused greater increases in the plasma levels of creatinine and BUN than the levels noted after four weeks (data not shown). Significant excretion of urinary protein was not detected in the adenine-fed mice in this study. Moreover, images of the kidneys from the mice fed 0.25% adenine showed apparent morphological changes, although the BP values did not change during feeding of the diet. Histopathological changes induced by adenine feeding have been reported in the kidneys of mice¹⁷⁾ as well as rats. In the present study, feeding with 0.25% adenine decreased the body weights of the mice, as noted in previous reports¹⁷⁾. However, the level of food intake in the 0 and 0.25% adenine diet groups were almost the same, suggesting that the adenine-fed mouse model is not related to cachexia. Feeding the mice the 0.125% adenine-containing diet for four weeks or the 0.25% adenine-containing diet for several days resulted in no

increases in the plasma creatinine or BUN concentrations and no changes in the degree of endothelium-dependent relaxation induced by ACh. Furthermore, the administration of adenine to the endothelium-intact aortas placed in an organ bath induced neither contractions nor relaxation. Based on these results, we used mice fed the 0.25% adenine diet for four weeks as an early-stage CKD model. However, the results of this study using adenine-fed mice do not rule out the direct vascular effects of adenine. In the future, further research is needed to examine the effects of incorporated adenine on endothelial cells in order to determine whether the effects of adenine in this CKD mouse model are due not to direct actions on aortic tension, but rather the onset of renal dysfunction.

We aimed to investigate mechanisms underlying signal transduction incidental to contraction/relaxation of aortic smooth muscles in CKD model mice, examining the correlation between kidney dysfunction

and changes in the vascular system. As a result, ACh-induced relaxation in the endothelium-intact aortas of the CKD mice was less potent than that observed in the control mice. The NOS inhibitor, LNA, completely blocked the ACh-induced relaxation in the endothelium-intact aortas of the mice, suggesting that the relaxing effect on the aorta was the result of NO release from the endothelium. In contrast, the capacity for NO-mediated relaxation did not differ, as the degree of SNP-induced relaxation was the same in both the adenine-fed and control mice. In addition, the degree of ACh-induced relaxation correlated with the plasma levels of BUN. These results suggest that the endothelial mechanisms associated with NO release do not function correctly in the aorta in this CKD mouse model. As reported previously^{1-3, 7, 10}, epidermal data have demonstrated endothelial dysfunction in CKD patients. Furthermore, the results of experiments using animal CKD models^{12, 16, 21-24} indicate that endothelium-dependent relaxation is attenuated in these animals, as in atherosclerosis models^{5, 6, 25, 26}. Our results align with the findings of previous reports. Impairment in the endothelial function for NO release may be responsible for the onset of cardiovascular events in patients with CKD. However, contrary to our results, it has been reported that the endothelium-dependent relaxation induced by ACh does not result in any damage in adenine-fed rats¹⁵. This discrepancy may be due to differences in the duration of administration and concentrations of the adenine diets.

In the current study, endothelium-dependent relaxation was inhibited more potently in the aortas of the mice fed the 0.25% adenine diet for eight weeks (data not shown). The significant correlation observed between endothelium-dependent relaxation and the increases in the plasma BUN concentrations in the current study suggests that renal dysfunction may cause decreases in stimulated NO release in the aorta.

PE administration induced concentration-dependent contractions in the endothelium-intact aortas from the 0.25% adenine-fed mice. The absolute values of tension induced by 10^{-7} M PE were significantly greater in the adenine-fed mice than in the control mice. The increase in tension values suggest the following possibilities. As PE results in the contraction of vascular smooth muscle through the α_1 -adrenergic subtype²⁷, the upregulation and/or signal transduction of these receptors may occur in cases of CKD²⁸. Alternatively, the current results may be associated with sympathetic hyperactivity in the setting of CKD, which has been demonstrated in both experimental and clinical studies^{29, 30}. In addition, pretreatment with the NOS inhibitor, LNA or ADMA, potentiated

Fig. 3. Effects of adenine feeding on SNP-induced relaxation in the endothelium-intact thoracic aortas of the mice. The relaxant response to these drugs was determined in aorta strips pretreated with PE (10^{-6} M). The ordinates show the degree of relaxation expressed as the percentage of the 10^{-4} M papaverine-induced maximal effect. The abscissae show the concentrations of the applied drugs (log M). ○: 0% ($n=5$); ●: 0.25% ($n=5$) adenine diet for four weeks. Each point represents the mean $\pm$ SE.

the 10^{-7} M PE-induced contractions in the CKD model mice as well as the control mice. The results suggest that NO is released spontaneously in the aortas of 0 and 0.25% adenine-fed mice.

In this study, we observed that the tension induced by PE administration was greater in the 0.25% adenine-fed mice than in the 0% adenine-fed mice. This result is different from the findings of a previous experiment with adenine-fed rats¹⁵, suggesting the current observation to be controversial. After treatment with LNA or ADMA, the PE-induced contractions increased similarly in both groups of mice. These results suggest that endogenous NO release is decreased under pathological conditions, such as CKD. Furthermore, it has been reported that the plasma levels of ADMA, an endogenous NOS inhibitor^{31, 32}, are increased in CKD patients^{3, 33, 34}. Our results suggest the possibility that the plasma ADMA levels are also increased in this CKD mouse model. More experiments are needed to determine the plasma ADMA levels in 0.25% adenine-fed mice.

Fig. 4. Effects of NOS inhibitors on PE-induced contraction in the endothelium-intact thoracic aortas from the 0 and 0.25% adenine-fed mice. A: 0% adenine diet for four weeks; B: 0.25% adenine diet for four weeks. The ordinates show 10⁻⁷ M PE-induced contractions expressed as the percentage of the 10⁻⁵ M PE-induced maximal effect. control (□, A: *n*=6; B: *n*=5), 10⁻⁴ M LNA (▨, A: *n*=3; B: *n*=3) or 3 × 10⁻⁴ M ADMA (■, A: *n*=3; B: *n*=3) at 30 minutes before the application of PE, followed by the determination of the degree of PE-induced contractions in the presence of LNA or ADMA. Each value represents the mean ± SE. Comparisons among groups were made using the Tukey-Kramer test. **p* < 0.05 compared with the control group.

Although many studies³⁵⁻³⁷ have indicated that CKD is associated with oxidative stress and NO deficiency, a CKD mouse model induced by nephrectomy was reported to show no effects with respect to PE-induced contractions²⁴. This discrepancy in the results for PE-induced contractions may be the result of differences in the preparation methods in the CKD models. The endothelium-intact aorta is considered to release both endothelium-derived relaxing and contracting factors. It is therefore possible that another factor influences the degree of aortic tension associated with PE administration.

In the current study, the BP values did not change at 1-3 weeks after 0.25% adenine feeding. This result is contradictory to the results obtained in an adenine-fed rat study^{15, 18} and 5/6 nephrectomy CKD model³⁸. Animal models of CKD may involve the induction of various stages of the disease depending on the duration and concentration of the adenine diet and/or type of animal. It is possible that the method used to induce CKD in this study resulted in the development of a stage of CKD in which the BP does not change. In the future, measurements of the BP should be obtained using radiotelemetry. As reported

previously¹⁵⁾, we observed the water intake to be increased in the adenine-fed CKD model mice (data not shown). We hypothesize that rises in BP may be inhibited by regulation of the body fluid volume.

There are few studies reporting on experimental CKD mouse models. The combined use of various transgenic or knock-out mice treated with the administration of an adenine diet may help to clarify the mechanisms causing progression of CKD.

In conclusion, the results of the current study suggest that the current CKD mouse model, developed by feeding mice adenine, elicits less potent endothelium-dependent relaxation associated with ACh and more potent absolute tension associated with PE via the aortic α_1 -adrenergic receptor. Furthermore, the NOS inhibitors, LNA and ADMA, potentiated the PE-induced contractions up to similar levels in the CKD mouse model and the control mice. These results suggest that spontaneous and stimulated NO release from the endothelium is decreased in the aorta in adenine-fed CKD model mice. Aortic dysfunction of the endothelial NO-releasing system may be a mediator associated with the onset of CKD and CVD. A close association has been previously reported between atherosclerosis and heart failure in CKD patients³⁹⁻⁴³⁾. Therefore, improved understanding of the endothelial NO-signaling pathway may play an important role in the prevention and treatment of cardiovascular events in patients with CKD.

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Disclosures

The authors declare that they have no conflicts of interest in association with this study.

Author Contributions

M. Mori-Kawabe designed the study and conducted the experiments. M. Ito analyzed the data. M. Mori-Kawabe, Y. Yasuda and S. Matsuo organized the data and wrote the paper.

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