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高血圧モデルラットの酸化ストレスおよび炎症における アズキ(*Vigna angularis*)ポリフェノールの生理的役割

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２００９年度青森県立保健大学大学院博士論文要旨

Physiological roles of azuki bean (*Vigna angularis*) polyphenols in oxidative stress and inflammation in rats with hypertension

（高血圧モデルラットの酸化ストレスおよび炎症におけるアズキ(*Vigna angularis*)ポリフェノールの生理的役割）

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Ⅰ　はじめに

近年、高血圧とそれに伴う血管内皮における酸化ストレスや慢性炎症が動脈硬化の発症に繋がることが明らかになってきた。一方、アズキ（*Vigna angularis*）は古くから伝統的に食され健康に良いと言われているが、その科学的エビデンスはほとんどない。アズキは、特にその種皮部分に抗酸化作用を有するポリフェノール（PP）を多く含むことが知られている。しかし、高血圧状態における血管内皮や臓器の酸化ストレスや炎症に及ぼすアズキ PP の影響に関する知見はほとんどない。

本研究では、高血圧に伴う酸化ストレスおよび炎症による臓器障害におけるアズキ PP の生理的役割を明らかにするために、アズキ PP を高血圧モデルラットに与え、①血圧上昇を抑制するかを検討し、②活性酸素（特にスーパーオキシド; O_2^-）および活性酸素産生酵素ならびに消去酵素の発現、③血管弛緩作用のある一酸化窒素（NO）の産生および NO 合成酵素（NOS）の発現、④炎症細胞の遊走・活性化を促進する因子の発現、⑤炎症時に活性化される酵素の発現、に及ぼす影響を検討した。

Ⅱ　研究方法と対象

アズキ粒より、エタノール抽出（ABE）または剥皮（ABSC）により得たアズキ PP を実験に供した。高血圧自然発症ラット（SHR）および対照として Wistar Kyoto（WKY）ラットを各々2 群に分けアズキ PP 無添加食または添加食を 8 週間摂取させ、尾部カフ法により収縮期血圧を測定した。24 時間尿を採取し、尿中 NO 代謝物（NOx）をグリース法により測定した。投与終了後大動脈および腎臓を摘出し、O_2^-量をルシゲニンを用いた化学発光法により測定した。O_2^-の主たる産生源である NADPH オキシダーゼのサブユニットおよび単球・マクロファージ系細胞の遊走・活性化を引き起こす MCP-1 の mRNA 発現量をリアルタイム RT-PCR により解析した。O_2^-消去酵素であるスーパーオキシドジスムターゼ（SOD）および内皮型 NOS（eNOS）、ならびに炎症時に過剰に発現する誘導型 NOS（iNOS）およびシクロオキシゲナーゼ-2（COX-2）のタンパク質発現量をウエスタンブロット法により解析した。

Ⅲ　結　果

①SHR の収縮期血圧は加齢に伴い上昇したが、アズキ PP 投与 SHR の血圧の上昇は非投与群に比べて抑制された。②アズキ PP 投与 SHR の大動脈では O_2^-産生が抑制され、NADPH オキシダーゼのサブユニット p47phox の mRNA 発現の抑制が認められた。O_2^-の消去酵素 SOD のタンパク質発現は、アズキ PP 投与 SHR の腎臓において増加した。③アズキ PP 投与 SHR の尿中 NOx 量は高値であり、体内 NO 量が増加したことが示唆された。eNOS の発現は、非投与 SHR で補償的に増加したがアズキ PP 投与により減少した。④アズキ PP 投与 SHR の大動脈では MCP-1 の発現が抑制された。⑤非投与 SHR で過剰に発現した iNOS および COX-2 のタンパク質量はアズキ PP 投与により減少した。

Ⅳ　考　察

以上の結果から、アズキ PP は血圧上昇抑制作用を有し、その作用機序として、O_2^-量を減少させ NO 量を増加させて酸化ストレスを軽減すること、および血管内皮の炎症を抑制することが考えられた。

PHYSIOLOGICAL ROLES OF AZUKI BEAN (*Vigna angularis*) POLYPHENOLS IN OXIDATIVE STRESS AND INFLAMMATION IN RATS WITH HYPERTENSION

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Abstract

The aim of the present study was to examine whether azuki bean polyphenols would contribute to attenuation of hypertension, if so, to clarify the physiological role in oxidative stress and inflammation with hypertension.

Male spontaneously hypertensive rats/Izumo (SHR) and control normotensive WKY/Izumo (WKY) rats were randomly divided into 2 groups each, treated or untreated with polyphenols-containing azuki bean seed coats (ABSC) or extract (ABE). When the systolic blood pressure of SHR reached approximately 200 mmHg, they were administered each diet for 8 weeks. The blood pressure and heart rate were measured once every 2 weeks by the tail-cuff plethysmography method. The nitric oxide (NO) content in 24-h urine was measured by Griess method. After sacrifice, superoxide (O_2^-) production in aorta was determined using a lucigenin-enhanced chemiluminescence technique. Vascular mRNA levels of NADPH oxidase subunits, Nox4, p47phox and p22phox, and monocyte chemoattractant protein 1 (MCP-1) and its receptor CCR2 were respectively analyzed by real-time RT-PCR. Vascular or renal protein levels of superoxide dismutase (SOD), endothelial NO synthase (eNOS), cavelin-1, inducible NOS (iNOS) and cyclooxygenase 2 (COX-2) were respectively analyzed by western blotting.

Azuki bean polyphenols such as procyanidin and catechin, contained in ABSC and

ABE, reduced the elevated blood pressure in long-term treatment of SHR. ABSC treatment decreased the vascular production of O_2^-, and suppressed the mRNA expression of the p47phox subunit of NADPH oxidase in the aorta. ABE treatment increased the renal Cu/Zn-SOD expressions. ABE treatment increased the urinary NOx content, indicating an increased NO production. ABE treatment modulated the eNOS and caveolin-1 expressions in the aorta and kidney of SHR. ABSC treatment suppressed the mRNA expressions of MCP-1, its receptor CCR2, and the protein expression of MCP-1. ABSC treatment suppressed the protein expressions of iNOS and COX-2 in the aorta of SHR. In conclusion, azuki bean polyphenols were showed to contribute to attenuation of oxidative stress and inflammation with hypertension. These effects may lead to prevention of NO inactivation and immune cells infiltration, associated with amelioration of endothelial dysfunction.

Chapter 1. General Introduction

Nowadays, worldwide prevalence estimates for hypertension may be as much as 1 billion individuals [1]. In the case of Japanese, the prevalence estimates for hypertension (≧140/≧90 mmHg) combined with prehypertension (130-139/85-89 mmHg) may be approximately 5.5 million individuals, which is equivalent to 43 percent of the total population in Japan [2].

Epidemiological investigations clearly showed that hypertension is one of the most important risk factors for lifestyle-related diseases and cardiovascular diseases [3-5]. Hypertension causes to promote vascular endothelial dysfunction and remodeling, leading to atherosclerosis. Atherosclerosis leads to ischemic cardiovascular events, such as myocardial infarction, stroke, and renal damage. Therefore, prevention, treatment, and control of hypertension are pivotal procedures for prevention and/or improvement of atherosclerosis.

Evidence of increased oxidative stress and inflammation has been shown in several hypertensive models and humans [6-11]. Oxidative stress is considered to be caused by an excessive production of reactive oxygen species (ROS) or a decline in antioxidant ability against ROS. In the endothelial and vascular smooth muscle cells of renal and vascular tissues with hypertension, ROS, especially superoxide (O_2^-), are excessively produced by overexpressed nicotinamide adenine dinucleotide phosphate (NADPH)

oxidase. Vasoreactivity is maintained by a balance between vasodilators such as nitric oxide (NO) [12] and prostaglandin I_2 [13], and vasoconstrictors such as angiotensin II (AngII) [14] and endothelin 1 (ET-1) [13]. Although endothelium-derived NO particularly plays a pivotal role in regulation of blood pressure, excess O_2^- can interact with NO, forming peroxinitrite ($ONOO^-$), thereby reducing NO bioavailability for vascular relaxation [15, 16]. Excess O_2^- and $ONOO^-$ are strong oxidants that induce endothelial dysfunction and promote inflammatory responses by activating transcriptional factors such as nuclear factor kappa B (NF-kB) [17] and activator protein 1 (AP-1) [18] via mitogen-activated protein (MAP) kinase [19]. In addition, the development of severe hypertension and endothelial dysfunction are associated with activation of renin-angiotensin system (RAS) [14]. Renin secreted by kidney generates angiotensin I from angiotensinogen, which are converted to AngII by angiotensin I converting enzyme (ACE). AngII acts as not only a potent vasoconstrictor but also an activator of ROS generation and inflammation via transcriptional factors. In inflammatory process, several proinflammatory cytokines such as interleukin 1β (IL-1β) [20] and monocyte chemoattractant protein 1 (MCP-1) [21], and adhesion molecules such as intercellular cell adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [20] are expressed by damaged endothelial cells, and induce recruitment of immune cells such as monocyte/macrophage and lymphocyte, to arterial vessel wall [21]. Intimal macrophages express proinflammatory enzymes such as

inducible NO synthase (iNOS) [16] and cyclooxygenase 2 (COX-2) [22], as well as ingest modified lipid particles; oxidized low-density lipoprotein (ox-LDL), and become lipid-laden foam cells, leading to formation of atherosclerotic lesions [23]. As described above, oxidative stress and inflammation in hypertension are synergistically activated and leading to endothelial dysfunction. Therefore, ameliorating oxidative stress and associated inflammation with hypertension are expected to provide beneficial effects on prevention of atherosclerosis.

Azuki bean (*Vigna angularis*) is indigenous to the tropical regions of Asia; currently, it is one of the most important crops in Japan, China, and South Korea. Azuki bean has several breeds differing in the color of the seed coat, such as red, black, speckled purple, brown, green and white [24]. In Japan, red azuki bean is an important and popular legume used as an essential ingredient for ‘an’, a sweetened paste (bean jam), and ‘sekihan’, a festive rice dish in traditional celebrations and sacred rites.

Azuki bean is rich in polyphenols such as proanthocyanidins and quercetin glycoside [25, 26]. Proanthocyanidins are natural antioxidants that exert their antioxidant effects in pathological conditions such as cardiovascular diseases [27], inflammation [28], and diabetes [29]. Recently, it was reported that several plant polyphenols such as quercetin [30] and red wine polyphenols [11] suppressed the vascular expression of NADPH oxidase subunits and decreased vascular oxidative stress, mediated by the improvement in vascular reactivity. The treatement of red azuki bean

seed coats ameliorated the renal fibrosis via suppressing the increase of infiltrating macrophages and chemokine mRNA expression in cisplatin-induced renal damage and streptozotocin-induced diabetic nephropathy [31, 32]. The ethanol-extracted azuki bean with antioxidant activity inhibited the increase in serum cholesterol by suppressing of cholesterol micellization and reducing of cholesterol synthase activity in high cholesterol-fed rats [33]. In terms of hypertension, the azuki bean extract was found to attenuate the elevation of blood pressure with reduction of superoxide production and macrophage infiltration into the heart and kidney in hypertensive model of young rats [34]. However, little is known about the beneficial effects of plant polyphenols including azuki bean polyphenols on oxidative stress and inflammation associated with hypertension.

The aim of the present study was to examine whether azuki bean polyphenols would contribute to attenuation of hypertension, if so, to clarify the physiological role of azuki bean polyphenols in oxidative stress and inflammation with hypertension. In Chapter 2, the effect of azuki bean polyphenols on vascular and renal oxidative stress was discussed. In Chapter 3, the effect of azuki bean polyphenols on vascular inflammation was discussed.

Chapter 2. Suppression of vascular and renal oxidative stress

2.1 Effect of azuki bean seed coats (ABSC) on superoxide production in spontaneously hypertensive rats

Introduction

Excessive production of reactive oxygen species (ROS), such as superoxide (O_2^-) and hydroperoxide, causes oxidative stress in the renal and vascular tissues with hypertension [35-37]. Excess O_2^- forms peroxynitrite ($ONOO^-$), interacting with nitric oxide (NO), and induces endothelial dysfunction and change in vascular response [15, 16].

The potential sources of O_2^-, in vessel, are nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, cyclooxygenase, lipoxygenase, and uncoupled endothelial NO synthase [8, 35, 38, 39]. NADPH oxidase, originally characterized in neutrophils, is present in the endothelium and smooth muscle cells of the arteries and is the principal source of O_2^- production. NADPH oxidase in vascular tissues consists of multiple subunits: cell membrane components (Nox4 and p22phox), cytoplasm components (p47phox and p67phox), and Rac1 proteins. There are several reports demonstrating the increase of NADPH oxidase activity and elevation of O_2^- production with increased mRNA levels of NADPH oxidase subunits in hypertensive

humans [35] and animal models [8, 38, 40]. It was demonstrated that azuki bean extract could suppress O_2^- production by NADPH oxidase in the heart and kidney of young SHR [34]. However, the physiological role of azuki bean polyphenols in NADPH oxidase subunits expression and O_2^- production has yet been unclear.

In the present section, in order to clarify the physiological function of azuki bean polyphenols in elevation of blood pressure and vascular O_2^- production, it was investigated whether polyphenols-containing azuki bean seed coats (ABSC) influence the levels of O_2^- production and mRNA expressions of NADPH oxidase subunits in the aorta of rats with hypertension.

Methods

Plant materials

ABSC was obtained as follows: Japanese azuki beans harvested in Hokkaido, Japan, were immersed overnight in distilled water at 25ºC. The seed coats were slipped off, collected, dried, and ground in a Waring blender. Approximately 80 g of powdered ABSC was collected from 1 kg of azuki beans. Nutrients in the ABSC were analyzed according to the standard procedure; moisture was measured by the air oven method, protein by the Kjeldahl method, fat by the acid hydrolysis method, ash by the ignition

method (at 550ºC), and dietary fiber by the enzymatic-gravimetric method. Carbohydrate was calculated by the formula of Notification No. 176 (2003), standard for nutrition labeling, Ministry of Health, Labor and Welfare of Japan (http://www.fukushihoken.metro.tokyo.jp/anzen/hoei/image/kkijyun.pdf). The composition of ABSC was as follows: 10.2% moisture, 6.5% protein, 0.9% fat, 7.2% ash, and 73.3% fiber (carbohydrate). The total phenolic compounds measured by the Folin-Ciocalteu method [41] were found to be 103 mg of *d*-catechin equivalent/g of ABSC. The catechin, procyanidins B_1, and rutin contents were measured by high performance liquid chromatography (LC) or LC/mass spectrometry. The ABSC contained 0.30 mg/g catechin, 0.22 mg/g procyanidins B_1, and 0.51 mg/g rutin.

Diets

ABSC was mixed with a standard commercial laboratory diet (MF diet; Oriental Yeast Co. Ltd., Tokyo, Japan). According to the information provided by the manufacturer, this diet contained 7.7% moisture, 23.6% crude protein, 5.3% crude fat, 6.1% crude ash, 2.9% crude fiber, 54.4% nitrogen-free extract (including carbohydrates), minerals (Ca, 1.12 g; P, 0.90 g; Mg, 0.26 g; Na, 0.21 g; K, 0.99 g; Mn, 5.89 mg; Fe, 10.8 mg; Cu, 0.82 mg; and Zn, 5.28 mg/100g of the diet), and vitamins (retinol, 2,160 IU; B_1, 2.12 mg; B_2, 1.24 mg; B_6, 0.87 mg; B_{12}, 5.3 μg; C, 4 mg; E, 11.0 mg; pantothenic acid, 2.73 mg; niacin, 10.4 mg; folic acid, 0.20 mg; choline, 0.22 g; biotin, 23.2 μg; and

inositol, 578 mg/100 g of the diet).

Experimental animals

All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation provided by Aomori University of Health and Welfare. Four-week-old male spontaneously hypertensive rats/Izumo (SHR) and control normotensive WKY/Izumo (WKY) rats were purchased from Japan SLC Inc. (Shizuoka, Japan). They were maintained at a temperature of 23 ± 1°C under a 12-h light/dark cycle and provided tap water *ad libitum*. At 18 weeks of age, the SHR and age-matched WKY rats were randomly divided into 2 groups each. One group was fed 0% ABSC (i.e., the MF diet alone) (n = 8); the other, 1.0% ABSC-containing diet (n = 8). Since the elevation of blood pressure in young SHR was observed to be suppressed by azuki bean extract in the previous study [34], the dose of ABSC was decided to be 1.0% in diet (total polyphenols, approximately 100 mg/100 g diet). The rats were administered each diet for 8 weeks, and systolic blood pressure (SBP) and heart rate of unanesthetized rats were measured once every 2 weeks by the tail-cuff plethysmography method (model MK-1100; Muromachi Kikai Co. Ltd, Tokyo, Japan). Experimental measurements were performed between 0930 and 1300 h. Before sacrificing at week 26, the animals were fasted overnight and then weighed, and blood samples were collected under ether

anesthesia. The heart, kidney, liver, aorta, and carotid artery were immediately removed, rinsed rapidly, and weighed. The aorta tissues were sectioned and kept in saline at 4°C for measurement of O_2^-production, or stored at –80°C for evaluation of mRNA and protein expression.

Blood chemistry

Plasma samples were separated by centrifugation at 800 *g* for 10 min at 4°C, and tested for sodium, potassium, and glucose by using an autoanalyzer for blood chemistry (Dry-Chem 3500 V; Fuji Film, Tokyo, Japan).

O_2^- measurement

O_2^- production was determined using a lucigenin-enhanced chemiluminescence technique. In brief, a ring segment of thoracic aorta (2-3 mm) was incubated in 1 mL lucigenin (5 μM)-phosphate buffer (pH 7.8) at room temperature to determine the response to the addition of 100 μM NADPH for 3 min; then, chemiluminescence was measured for 30 s by using a sensitive luminometer (TD-20/20; Turner Designs, Sunnyvale, CA, US). The results were reported as relative light units per minute.

Real-time RT-PCR

Total RNA in the thoracic aorta was extracted using the SV Total RNA Isolation

System (Promega Corp., WI, US) according to the manufacturer's instructions, and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, US). Vascular mRNA levels of Nox4, p47phox and p22phox were respectively analyzed using the following inventoried primers: TaqMan gene expression assays Rn00580555_m1, Rn00586945_m1 and Rn00577357_m1 (Applied Biosystems). GAPDH mRNA was also analyzed using the TaqMan Rodent GAPDH Control Reagent Kit (Applied Biosystems) as an endogenous control. Real-time PCR was performed using the Universal PCR Master Mix (Applied Biosystems) on an Applied Biosystems 7000 sequence detection system, according to the manufacturer's instructions. The gene expression levels were presented as fold of the levels obtained for untreated WKY, after adjusting for GAPDH by the 2-(-delta delta C (T)) (CT) method.

Statistical analyses

Each value was expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by Tukey's test. $P < 0.05$ was considered statistically significant.

Results

Effect of ABSC treatment on body weight

No mortality was observed in both the ABSC-treated and untreated SHR and WKY rats. Body weights of the SHR were lower than those of the WKY rats during the experimental period. There was no effect of ABSC treatment on the body weights of both the SHR and WKY rats (Figure 1).

Effect of ABSC treatment on blood pressure and heart rate

As expected, the SBP of the SHR groups was elevated (Figure 2). From 2 weeks of ABSC treatment, the SBP of ABSC-treated SHR significantly decreased as compared with that of untreated SHR. These data indicated that ABSC treatment attenuated hypertension in SHR. With regard to DBP, no significant difference was observed between both the ABSC-treated and untreated SHR and WKY rats in the 0 week. At 8 weeks of the ABSC treatment, the DBP of the SHR was found to be significantly higher than that of the age-matched WKY rats (SHR + 0% ABSC, 146 ± 4 mmHg versus WKY rats + 0% ABSC, 77 ± 5 mmHg; $P < 0.05$; mean ± SEM; n = 7–8). On the other hand, the DBP of the ABSC-treated SHR was significantly lower than that of the untreated SHR (SHR + 0% ABSC, 146 ± 4 mmHg versus SHR + 1.0% ABSC, 127 ± 2 mmHg; $P < 0.05$). There was no difference in the heart rates between the ABSC-treated and untreated SHR (SHR + 0% ABSC, 390 ± 18 beats per minute (bpm), SHR + 1.0% ABSC, 393 ± 7 bpm).

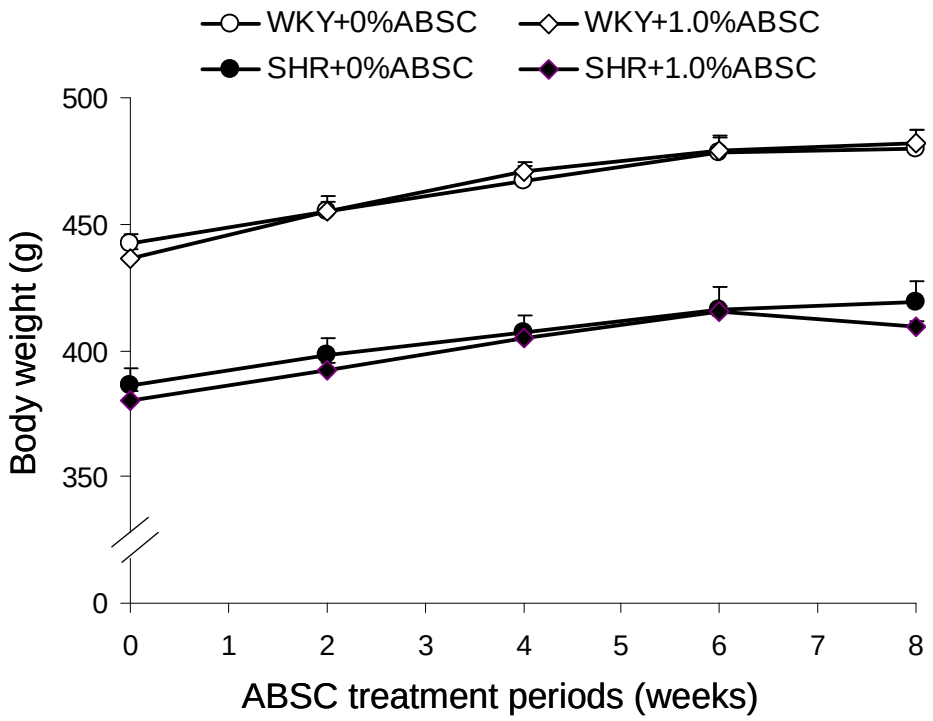

Figure 1 Effect of ABSC treatment on the body weight of SHR and WKY rats. Values are expressed as mean ± SEM (n = 8).

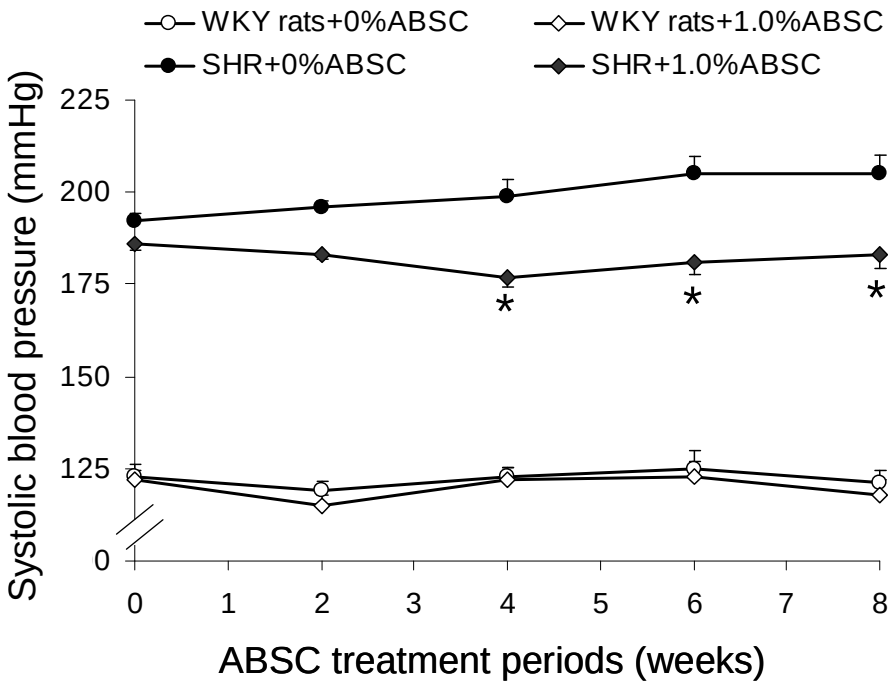

Figure 2 Effect of ABSC treatment on the systolic blood pressure of SHR and WKY rats.

Values are expressed as mean ± SEM ($n = 8$). *$P < 0.05$ compared with untreated SHR.

Morphological characteristics

The absolute and relative kidney, heart and liver weights of ABSC-treated SHR tended to be lower than those of untreated SHR, although no significant difference was found. There was no significant difference in the plasma sodium and potassium levels between the ABSC-treated and untreated SHR groups. On the other hand, the plasma glucose in ABSC-treated SHR was significantly lower than that in untreated SHR (Table 1).

Effect of ABSC treatment on O_2^- production in the aorta

The NADPH-stimulated O_2^- levels in untreated SHR were significantly higher than those in untreated WKY rats (Figure 3). Conversely, O_2^- levels in ABSC-treated SHR decreased significantly compared with those in untreated SHR, indicating that ABSC treatment suppressed O_2^- production. There was no difference between the ABSC-treated and untreated WKY rats.

Effect of ABSC treatment on the mRNA expression of NADPH oxidase subunits

Since ABSC treatment suppressed O_2^- production, the mRNA expression of NADPH oxidase subunits in the thoracic aorta was determined by RT-PCR (Figure 4). The levels of p47phox mRNA in untreated SHR increased significantly compared with those in the

Table 1 Morphological characteristics of azuki bean seed coats (ABSC)-treated and untreated spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats.

	WKY rats		SHR	
ABSC treatment	0%	1.0%	0%	1.0%
BW at sacrifice (g)	461 ± 6	465 ± 7	400 ± 7*	393 ± 3*
Heart (g)	1.19 ± 0.02	1.20 ± 0.02	1.49 ± 0.04*	1.45 ± 0.02*
Kidney (g)	3.13 ± 0.04	3.12 ± 0.05	3.09 ± 0.06	2.97 ± 0.03
Liver (g)	11.5 ± 0.24	11.2 ± 0.31	13.5 ± 0.38*	12.8 ± 0.15*
H/BW (g/kg)	2.59 ± 0.03	2.58 ± 0.04	3.72 ± 0.05*	3.68 ± 0.04*
K/BW (g/kg)	6.79 ± 0.10	6.75 ± 0.10	7.74 ± 0.08*	7.54 ± 0.05*
L/BW (g/kg)	25.0 ± 0.34	24.0 ± 0.38	33.8 ± 0.40*	32.6 ± 0.29*
pNa (mEq/L)	144.6 ± 0.8	144.7 ± 0.6	146.8 ± 0.3	147.0 ± 0.4*
pK (mEq/L)	5.1 ± 0.2	4.4 ± 0.2	3.7 ± 0.1*	4.0 ± 0.1*
pGLC (mg/dL)	164.3 ± 2.2	150.9 ± 4.5	235.1 ± 11.3*	214.9 ± 7.4*[#]

Data are expressed as mean ± SEM (n = 7–8). BW, body weight; H, heart; K, kidney; L, liver; pNa, plasma sodium; pK, plasma potassium; and pGLC, plasma glucose.

*P < 0.05 compared with WKY rats + 0% ABSC. [#] P < 0.05 compared with SHR + 0% ABSC.

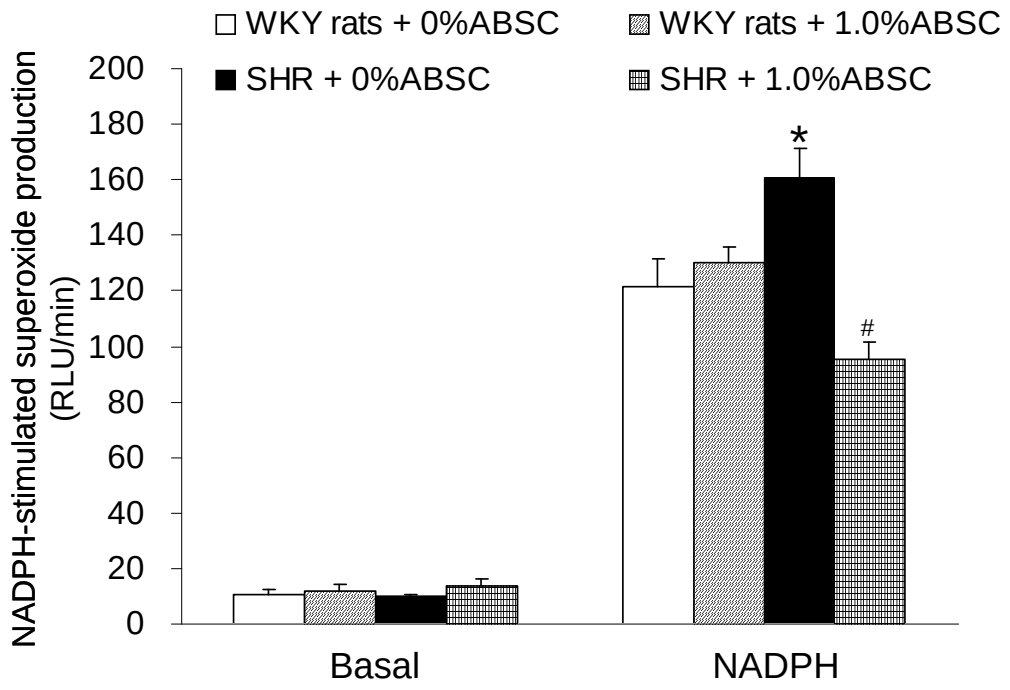

Figure 3 Effect of ABSC treatment on NADPH-stimulated superoxide production in the aorta of SHR and WKY rats. Values are expressed as mean ± SEM (n = 8). *P < 0.05 compared with untreated WKY rats. $^{\#}P$ < 0.05 compared with untreated SHR.

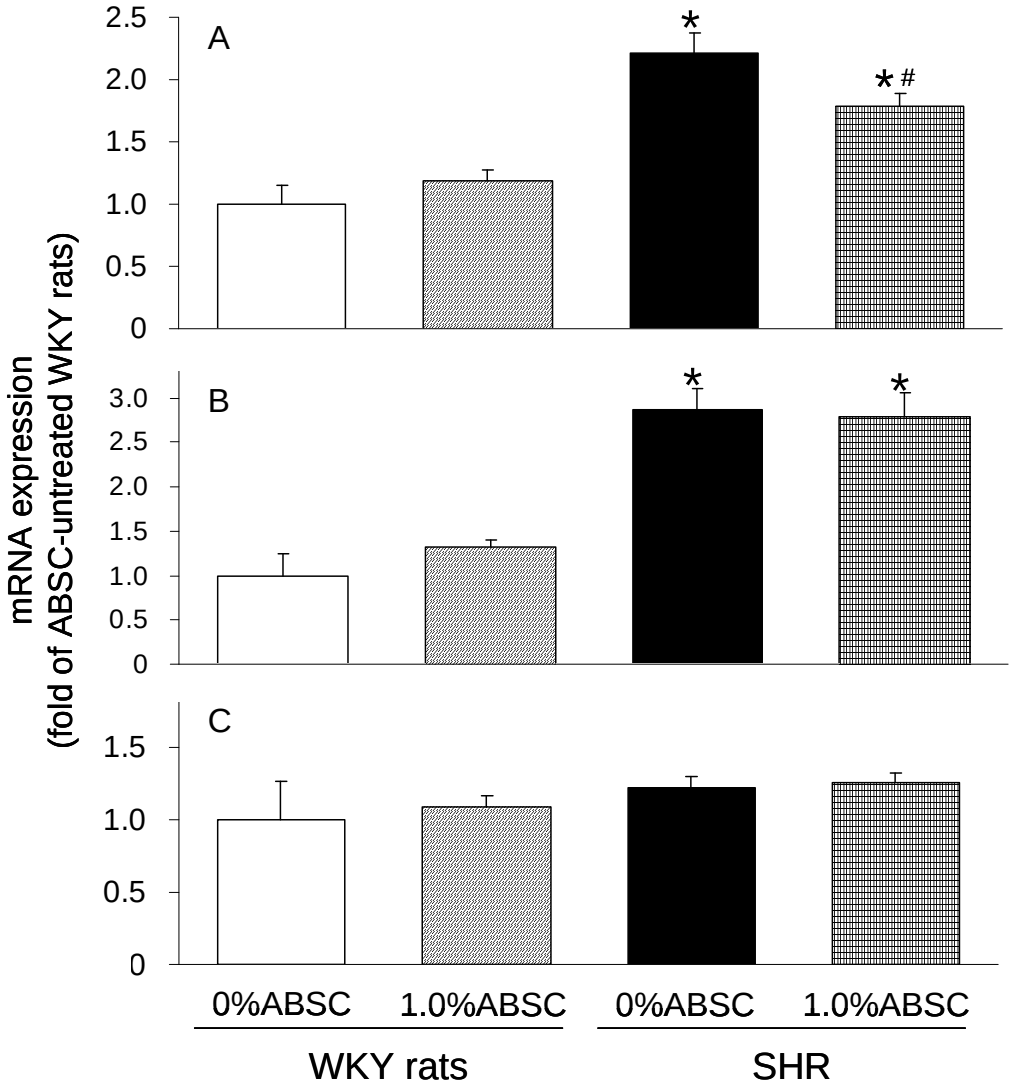

Figure 4 Effect of ABSC treatment on mRNA expression of the p47phox (A), Nox4 (B), and p22phox (C), NADPH oxidase subunits in the aorta of SHR and WKY rats. The gene expression levels were quantified as fold of the levels observed in untreated WKY rats, after adjusting for GAPDH. Values are expressed as mean ± SEM (n = 5–7). *$P < 0.05$ compared with untreated WKY rats. $^{\#}P < 0.05$ compared with untreated SHR.

WKY rats. Conversely, the levels of p47phox mRNA in ABSC-treated SHR were significantly lower than those in untreated SHR. These results indicated that ABSC treatment suppressed the p47phox expression (Figure 4A). The level of Nox4 mRNA expression in untreated SHR was significantly higher than that in WKY rats; however, there was no significant difference between ABSC-treated and untreated SHR (Figure 4B). There was no difference between the p22phox mRNA levels of all groups (Figure 4C).

2.2 Effect of azuki bean extract (ABE) on nitric oxide metabolism in spontaneously hypertensive rats

Introduction

Endothelium-derived nitric oxide (NO) which induces vascular relaxation is known to play numerous physiological roles including the regulation of blood pressure, renal haemodynamics [12], and modulation of platelet function in heart failure [19]. Deficient NO production has been implicated in the pathogenesis of hypertension [42, 43]. NO and the by-product L-citrulline are produced from molecular oxygen (O_2) and the amino acid L-arginine by nitric oxide synthase (NOS) [44]. In blood vessel wall, endothelial NOS (eNOS) protein is constitutively expressed and plays a major role in vascular tone. Moreover, eNOS is known to be localized in caveolae, the invaginations in cell membranes. At rest, eNOS binds to its allosteric regulator, caveolin-1, and inhibits its activity. On physiological stimulation such as share stress, eNOS uncouples from caveolin-1 and translocates to the plasma membrane, where it releases NO [45-47]. In the face of increased oxidative stress and endothelial dysfunction, reduced vascular NO level induces coordinated expressional changes in eNOS and caveolin-1. This phenomenon may even be viewed as a compensatory mechanism to maintain the production of bioactive NO [30, 48].

In the pathogenesis and maintenance of hypertension, a reduction of NO

bioavailability by increased reactive oxygen species, especially superoxide (O_2^-), contributes to elevated vascular resistance [36, 49]. O_2^- reacts rapidly with NO, leading to formation of the strong oxidant peroxynitrite ($ONOO^-$), which can enhance endothelial dysfunction. Thus, increase of NO production and maintenance of bioactive NO seem to be important advantages in the prevention and treatment of hypertension.

Superoxide dismutase (SOD) is an antioxidant enzyme by which O_2^- can be converted to H_2O_2. It was previously reported that the activity of SOD, either as Mn-SOD in mitochondria or as Cu/Zn-SOD in cytoplasm, is reduced in heart of SHR [50]. Plant polyphenols such as flavonoid quercetin [51] and resveratrol [52] were found to increase the O_2^- dismutation activity of SOD in cardiovascular and renal diseases with oxidative stress. However, it is unknown whether azuki bean polyphenols would affect NO production and O_2^- dismutation in the aorta and kidney of hypertensive animals.

In the present section, in order to clarify the physiological function of azuki bean polyphenols in elevation of blood pressure and NO production, it was investigated whether polyphenols-containing azuki bean extract (ABE) influence the levels of NO production and expressions of eNOS, caveolin-1 and SOD proteins in the aorta and kidney of rats with hypertension.

Methods

Plant materials

ABE was obtained as follows: 1 kg Japanese red azuki beans harvested in Hokkaido, Japan, was ground in a Waring blender and then extracted polyphenol in 5 L of 80% ethanol overnight at 25ºC. After centrifugation (at 1,500 g for 10 min), the supernatant was concentrated at 50ºC for 90 min. After incubation with 600 mL distilled water, the supernatant was lyophilized at –80ºC. Approximately 80 g dried ABE was normally collected from 1 kg azuki beans. Nutrients in the ABE were analyzed as mentioned above Chapter 2 Section 1. The composition of the ABE was as follows: 4.9% moisture, 6.7% protein, 17.2% fat, 5.8% ash, and 65.4% carbohydrate. In addition, dietary fiber was 11 g per 1 kg ABE. The total phenolic compounds measured by the Folin-Ciocalteu method [41] were found to be 93 mg of *d*-catechin equivalent/g of ABE. The proanthocyanidins measured by the pro-anthocyanidin method [53] were found to be 90 mg of procyanidin B_1 equivalent/g of ABE.

Diets

ABE was mixed with a standard commercial laboratory diet (CE-2 diet; CLEA Japan Inc., Tokyo, Japan). According to the information provided by the manufacturer, this diet contained 8.6% moisture, 24.9% crude protein, 4.6% crude fat, 6.7% crude ash, 3.7% crude fiber, 51.4 % nitrogen-free extract (including carbohydrates), minerals (Ca,

1.03 g; P, 0.97 g; Mg, 0.33 g; Na, 0.32 g; K, 1.05 g; Mn, 11.26 mg; Fe, 34.35 mg; Cu, 0.79 mg; and Zn, 5.35 mg/100g of the diet), and vitamins (retinol, 0.96 mg; B_1, 1.70 mg; B_2, 1.33 mg; B_6, 1.25 mg; B_{12}, 6.3 μg; C, 13 mg; E, 6.2 mg; pantothenic acid, 2.90 mg; niacin, 16.9 mg; folic acid, 0.28 mg; choline, 0.21 g; biotin, 41.6 μg; and inositol, 616 mg/100g of the diet).

Experimental animals

All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation provided by Aomori University of Health and Welfare. Five-week-old male spontaneously hypertensive rats/Izumo (SHR) and control normotensive WKY/Izumo (WKY) rats were purchased from Japan SLC Inc. (Shizuoka, Japan). They were maintained at a temperature of 23 ± 1°C under a 12-h light/dark cycle and provided tap water *ad libitum*. At 19 weeks of age, the SHR and age-matched WKY rats were randomly divided into 2 groups each. One group was fed 0% ABE (i.e., the CE-2 diet alone) (n = 8); the other, 0.9% ABE-containing diets (n = 8). Since the elevation of blood pressure in young SHR was observed to be suppressed by azuki bean extract in the previous study [34], the dose of ABE was decided to be 0.9% in diet (total polyphenols, approximately 85 mg/100 g diet). When the systolic blood pressure (SBP) of the 19-week-old SHR reached approximately 200 mmHg, they were administered each diet for 8 weeks. The SBP and heart rate of unanesthetized rats were measured

once every 2 weeks by the tail-cuff plethysmography method (model MK-1100, Muromachi Kikai Co. Ltd, Tokyo, Japan). In addition, the diastolic blood pressure (DBP) was measured in the 0 week (before treatment) and at 8 weeks of the ABE treatment, as mentioned above. Experimental measurements were performed between 0930 and 1300 h. At 26 weeks of age, rats were individually placed in each metabolic cage and urine was collected for 24 hours. Before sacrificing at Week 27, the animals were fasted overnight and then weighed; blood samples were collected under ether anesthesia. The heart, kidney, liver and aorta were immediately removed, rinsed rapidly, and weighed. The tissues were stored at –80°C until processing. Parts of the kidney were homogenized, and the amount of lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substances (TBARS) using a commercially available kit (Wako Pure Chemical Industries, Osaka, Japan) and was expressed as malondialdehyde (MDA) content [54].

Blood chemistry

Plasma samples were separated by centrifugation at 800 *g* for 10 min at 4°C, and tested for blood urea nitrogen (BUN), sodium, and glucose by using an autoanalyzer for blood chemistry (Dry-Chem 3500 V; Fuji Film, Tokyo, Japan).

Measurement of urinary NO excretion

The NO content in 24-h urine was measured by Griess method with NO_2/NO_3 Assay Kit-C II (Dojindo Laboratories, Kumamoto, Japan). Samples were read at 540 nm with a Microplate Reader (Model 680; Bio-Rad Laboratories Inc., Hercules, CA). The creatinine content in 24-h urine was measured by Jaffe method with assay kit (Creatinine-test Wako; Wako Pure Chemical Industries, Osaka, Japan). NO excretion was normalized by creatinine content and expressed as total nitrate and nitrite (NOx).

Western blotting

Tissues of kidney and aorta were homogenized in 6 times its volume of a homogenization buffer (50 mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.5% (v/v) Tween-20, pH 7.4) containing Protease Inhibitor Cocktail Tablets (Roche Applied Science, Indianapolis, IN, US). The homogenates were centrifuged at 5,000 *g* for 45 min at 4°C. Supernatants were collected, and protein concentration was determined using BCA™ Protein Assay Kit (Pierce, Rockford, IL, US). For western blot analysis, the proteins were electrophoresed on SDS-polyacrylamide gel (10% for eNOS and 15% for caveolin-1, Cu/Zn- and Mn-SOD) and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked in 5% skimmed milk in a washing buffer (25 mM Tris-HCl (pH 7.4), 0.8% NaCl, 0.02% KCl, and 0.1% Tween-20). After appropriate blocking, the blot was incubated

with eNOS mouse antibodies (1:500; BD Biosciences, San Jose, CA, US), caveolin-1 rabbit antibody (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, US), Cu/Zn-SOD (SOD-1) rabbit antibody (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, US) or Mn-SOD rabbit antibody (1:1000, Upstate, Lake Placid, NY, US), and β-actin mouse antibody (1:1000; Abcam, Cambridge, UK). After washing, the specifically bound primary antibodies were detected with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG or swine anti-rabbit IgG antibody (1:1000; DAKO, Glostrup, DK) and enhanced with ECL Western Blotting Detection Reagents (Amersham Biosciences) on Hyperfilm (Amersham Biosciences). Quantitative analysis of the band density was performed by densitometry, using ATTO densitometry software (ATTO Corp., Tokyo, Japan). Protein levels were normalized to β-actin expression from the same sample; the results are presented as fold of the levels obtained for untreated WKY rats.

Statistical analyses

Each value was expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by Tukey's test. $P < 0.05$ was considered statistically significant.

Results

Effect of ABE treatment on body weight

No mortality was observed in both the ABE-treated and untreated SHR and WKY rats. Body weights of the SHR were lower than those of the WKY rats during the experimental period. There was no effect of ABE treatment on the body weights of both the SHR and WKY rats (Figure 5).

Effect of ABE treatment on blood pressure and heart rate

The SBP of the SHR groups was higher than those of the age-matched WKY rats throughout the treatment period (Figure 6). During 6–8 weeks of the ABE treatment, the SBP of the ABE-treated SHR significantly decreased as compared with that of the untreated SHR; however, no significant changes were observed in the WKY rats. These data indicated that ABE treatment attenuated hypertension in the SHR. With regard to DBP, no significant difference was observed between both the ABE-treated and untreated SHR and WKY rats in the 0 week. At 8 weeks of the ABE treatment, the DBP of the SHR was found to be significantly higher than that of the age-matched WKY rats (SHR + 0% ABE, 158 ± 3 mmHg versus WKY rats + 0% ABE, 77 ± 4 mmHg; $P < 0.05$; mean ± SEM; n = 7–8). On the other hand, the DBP of the ABE-treated SHR was significantly lower than that of the untreated SHR (SHR + 0% ABE, 158 ± 3 mmHg

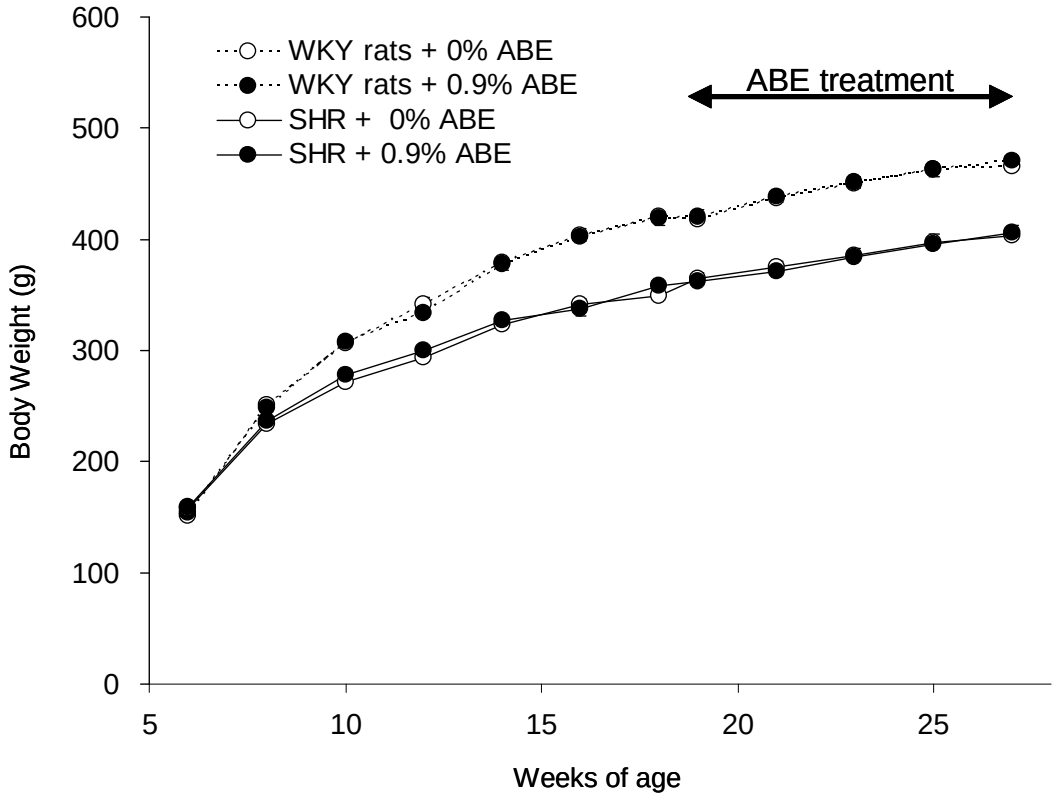

Figure 5 Effect of ABE treatment on the body weight of SHR and WKY rats. Values are expressed as mean ± SEM (n = 8).

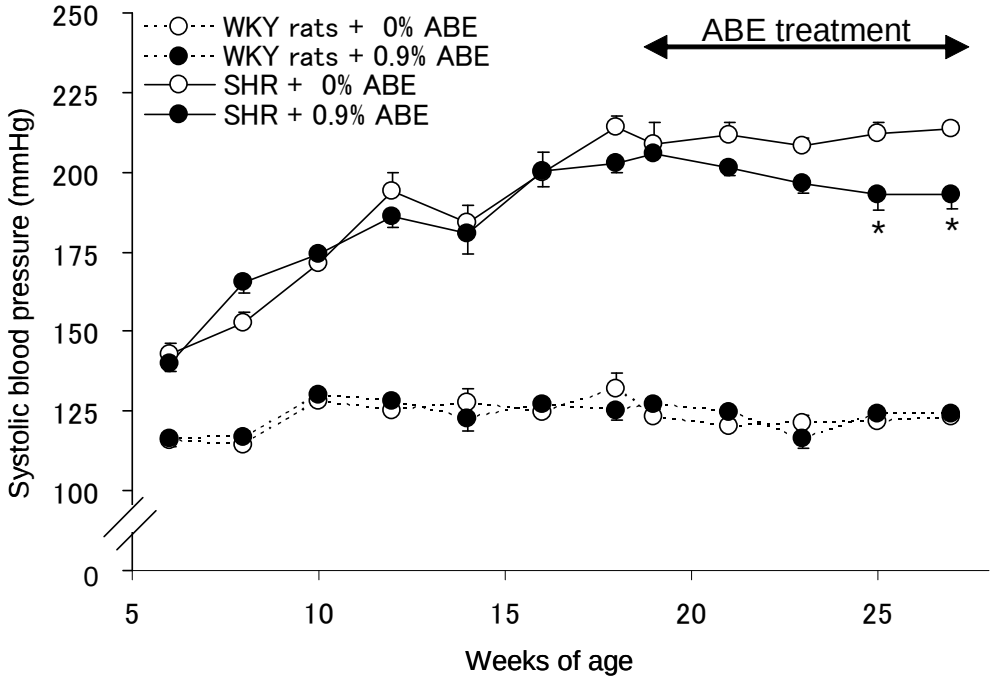

Figure 6 Effect of ABE treatment on the systolic blood pressure of SHR and WKY rats. Values are expressed as mean ± SEM ($n = 8$). $^{*}P < 0.05$ compared with untreated SHR.

versus SHR + 0.9% ABE, 133 ± 8 mmHg; $P < 0.05$). There was no difference in the heart rates between the ABE-treated and untreated SHR (SHR + 0% ABE, 407 ± 19 bpm, SHR + 0.9% ABE, 418 ± 48 bpm).

Morphological characteristics

The absolute and relative heart weights of the 0.9% ABE-treated SHR were lower than those of the untreated SHR (Table 2), indicating improvement in the cardiac hypertrophy in the ABE-treated SHR. Although no significant difference was found, a tendency toward lower plasma BUN levels in the 0.9% ABE-treated SHR was observed (Table 2). There was no significant difference in the plasma sodium and glucose levels, and the 24-h urine volume between the ABE-treated and untreated SHR groups.

Effect of ABE treatment on Cu/Zn- and Mn-SOD protein expressions in the kidney

To examine the effect of ABE treatment on the expressions of Cu/Zn- and Mn-SOD proteins in the kidney, western blot analysis was performed. Immunoreactive bands corresponding to Cu/Zn- and Mn-SOD were seen at 23 and 24 kDa in the proteins extracted from the kidney.

The Cu/Zn-SOD protein abundance in the ABE-treated SHR and WKY rats significantly increased as compared with those in the untreated SHR and WKY rats, while the abundance in the untreated SHR was similar to that in the untreated WKY rats

Table 2 Morphological characteristics of azuki bean extract (ABE)-treated and untreated spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats.

	WKY rats		SHR	
ABE treatment	0%	0.9%	0%	0.9%
BW at sacrifice (g)	455 ± 6	457 ± 6	380 ± 7[a,b]	389 ± 4[a,b]
Heart (g)	1.20 ± 0.02	1.12 ± 0.03	1.72 ± 0.04[a,b]	1.53 ± 0.04[a,b,c]
Kidney (g)	3.50 ± 0.10	3.46 ± 0.07	3.25 ± 0.11	3.26 ± 0.09
Liver (g)	13.76 ± 0.29	13.56 ± 0.41	15.03 ± 0.29	13.59 ± 0.33
H/BW (g/kg)	2.65 ± 0.05	2.45 ± 0.08	4.53 ± 0.11[a,b]	3.93 ± 0.13[a,b,c]
K/BW (g/kg)	7.71 ± 0.25	7.57 ± 0.13	8.57 ± 0.28[b]	8.39 ± 0.25
L/BW (g/kg)	30.3 ± 0.7	29.6 ± 0.6	39.6 ± 0.5[a,b]	35.0 ± 0.7[a,b]
pBUN (mg/dL)	18.2 ± 0.8	22.0 ± 0.8	28.4 ± 1.1	24.6 ± 2.0
pNa (mmol/L)	137.1 ± 1.7	139.4 ± 0.5	136.9 ± 1.9	142.6 ± 0.6
pGLC (mg/dL)	170.1 ± 11.5	162.6 ± 9.8	178.5 ± 9.4	179.9 ± 7.6
Urine volume (mL)	7.1 ± 0.5	6.4 ± 1.4	8.0 ± 0.6	8.0 ± 0.4

Data are expressed as means ± SEM (n = 7–8). BW, body weight; H, heart; K, kidney; L, liver; pBUN, plasma blood urea nitrogen; pNa, plasma sodium; pGLC, plasma glucose.

[a]$P < 0.05$ compared with WKY rats + 0% ABE, [b]$P < 0.05$ compared with WKY rats + 0.9% ABE, [c]$P < 0.05$ compared with SHR + 0% ABE.

(Figure 7A). In addition, the Mn-SOD protein abundance in the ABE-treated WKY rats was significantly higher than that in the untreated WKY rats, while there was no significant difference in the Mn-SOD protein abundance between ABE-treated and untreated SHR (Figure 7B). These results indicated that ABE treatment upregulated Cu/Zn-SOD expressions in the kidney of SHR.

Effect of ABE treatment on TBARS content in the kidney

In order to examine renal oxidative stress, the amount of lipid peroxidation in kidney homogenate was determined by measuring TBARS. The TBARS content in the kidney of the untreated SHR significantly increased as compared with that of the untreated WKY rats (SHR + 0% ABE, 5.55 ± 0.24 nmol/mg protein versus WKY rats + 0% ABE, 4.49 ± 0.26 nmol/mg protein; $P < 0.05$; mean ± SEM; $n = 7–8$). ABE treatment showed a tendency to reduce renal TBARS levels in SHR groups compared with that in the untreated SHR (SHR + 0.9% ABE, 4.77 ± 0.17 nmol/mg protein). There was no significant difference between the ABE treated and untreated WKY (WKY rats + 0.9% ABE; 5.37 ± 0.18 nmol/mg protein).

Effect of ABE treatment on urinary NO excretion

Since NO metabolites reflect the amount of systemic NO, the content of 24-h urinary NOx was measured (Figure 8). The level of 24-h urinary NOx was significantly

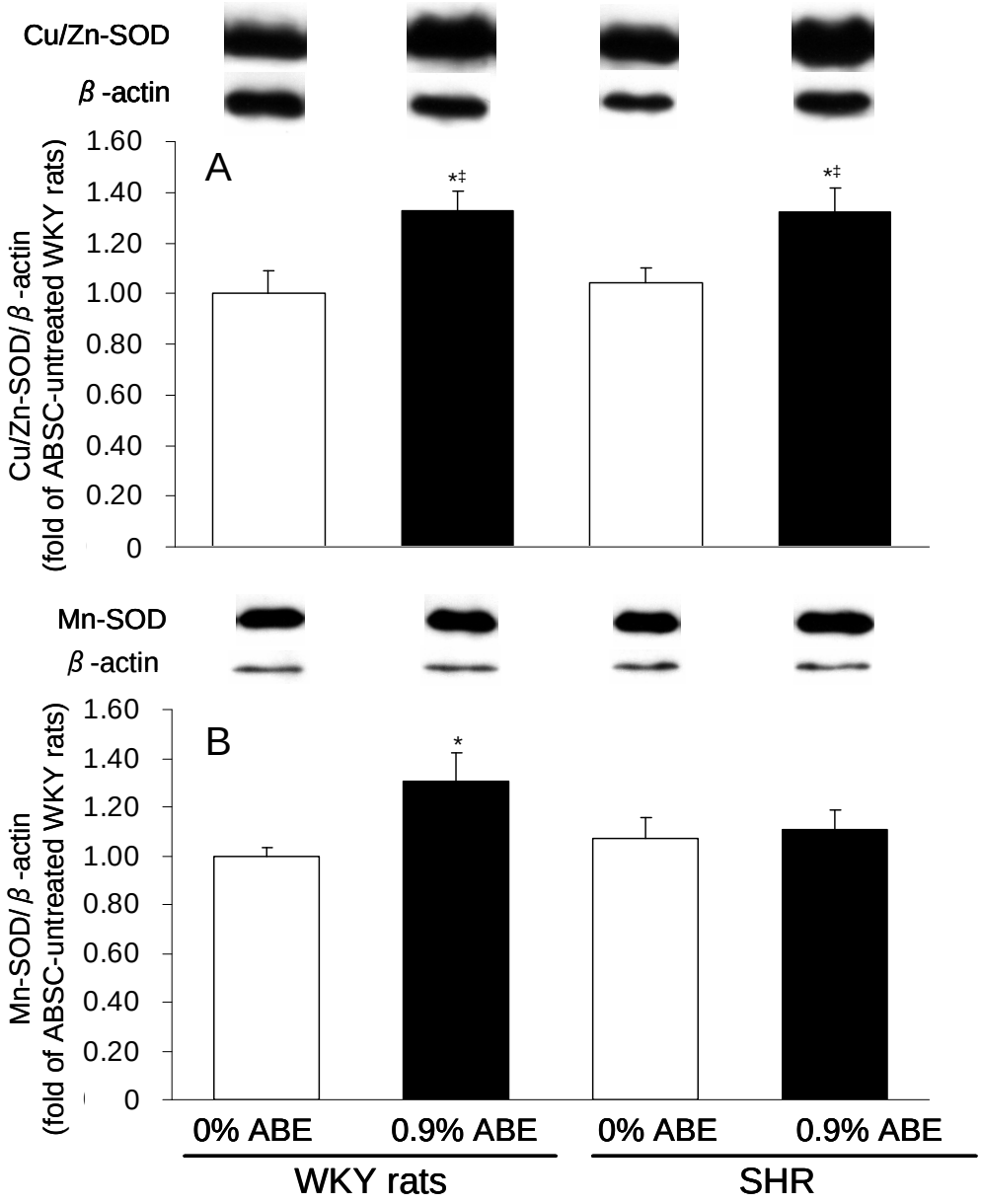

Figure 7 Representative western blot and relative optical densities of the renal (A) Cu/Zn-SOD and (B) Mn-SOD bands in each group. The protein expression levels were quantified as fold of the levels observed in untreated WKY rats, after adjusting for endogenous β-actin. Values are expressed as mean ± SEM (n = 7–8). $^{*}P < 0.05$ compared with untreated WKY rats. $^{‡}P < 0.05$ compared with untreated SHR.

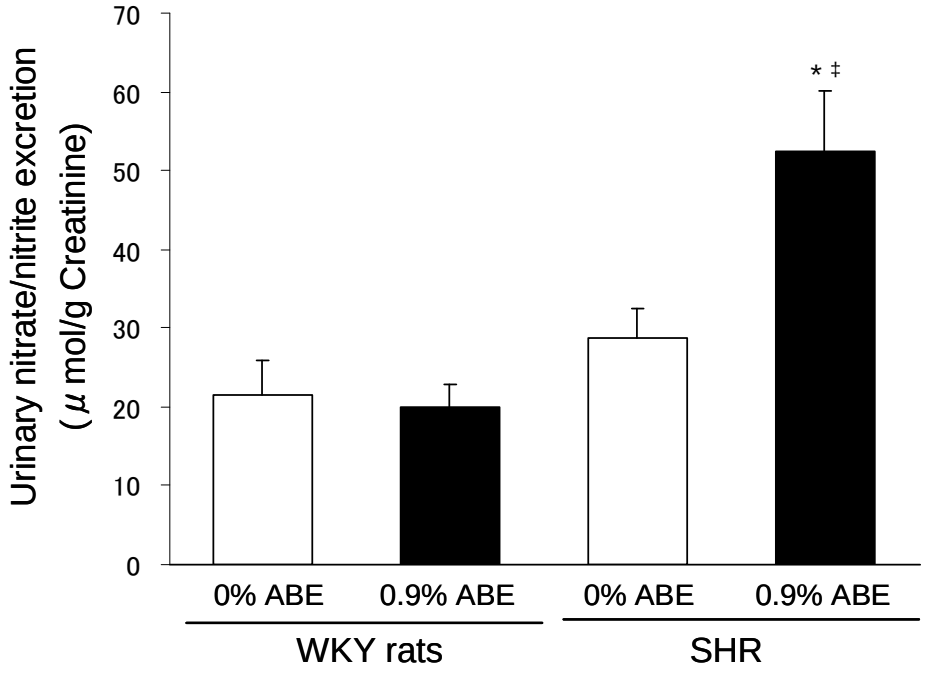

Figure 8　Effect of ABE treatment on the urinary nitrate/nitrite excretion in SHR and WKY rats. Values are expressed as mean ± SEM (n = 7–8). $^{*}P < 0.05$ compared with untreated WKY rats. $^{‡}P < 0.05$ compared with untreated SHR.

higher in the ABE-treated SHR than in the untreated SHR, indicating that ABE treatment upregulated NO production. There was no difference between the ABE-treated and untreated WKY rats.

Effect of ABE treatment on eNOS and caveolin-1 protein expressions in the aorta and kidney

To examine the effect of ABE treatment on the expressions of eNOS and caveolin-1 proteins in the aorta and kidney, western blot analysis was performed. Immunoreactive bands corresponding to eNOS and caveolin-1 were seen at 140 and 22 kDa in the proteins extracted from the aorta and kidney.

In the aorta, increased eNOS protein abundance was observed in the untreated SHR as compared with the WKY rats ($P < 0.05$). The upregulation of eNOS protein expression significantly decreased in the ABE-treated SHR (Figure 9A). While the caveolin-1 protein abundance in the untreated SHR was significantly lower than that in the untreated WKY rats, the ABE-treated SHR recovered the caveolin-1 protein expression to a similar extent as the untreated WKY rats (Figure 9B).

In the kidney, upregulation of eNOS protein expression was observed in the untreated SHR as compared with that in the WKY rats, while the eNOS protein abundance in the ABE-treated SHR was significantly lower than that in the untreated SHR (Figure 10A). While the caveolin-1 protein abundance in the untreated SHR was

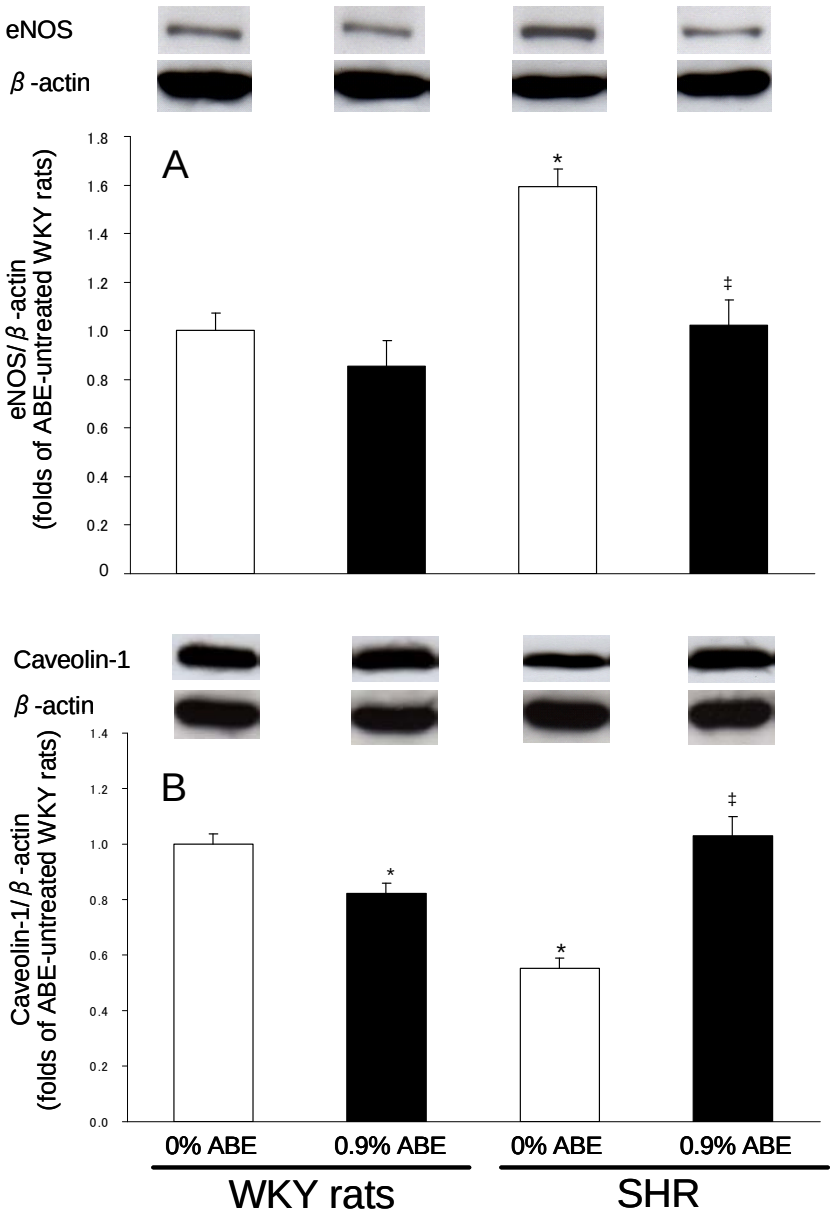

Figure 9 Representative western blot and relative optical densities of the aortic (A) eNOS and (B) caveolin-1 bands in each group. The protein expression levels were quantified as fold of the levels observed in untreated WKY rats, after adjusting for endogenous β-actin. Values are expressed as mean ± SEM (n = 7–8). $^{*}P < 0.05$ compared with untreated WKY rats. $^{‡}P < 0.05$ compared with untreated SHR.

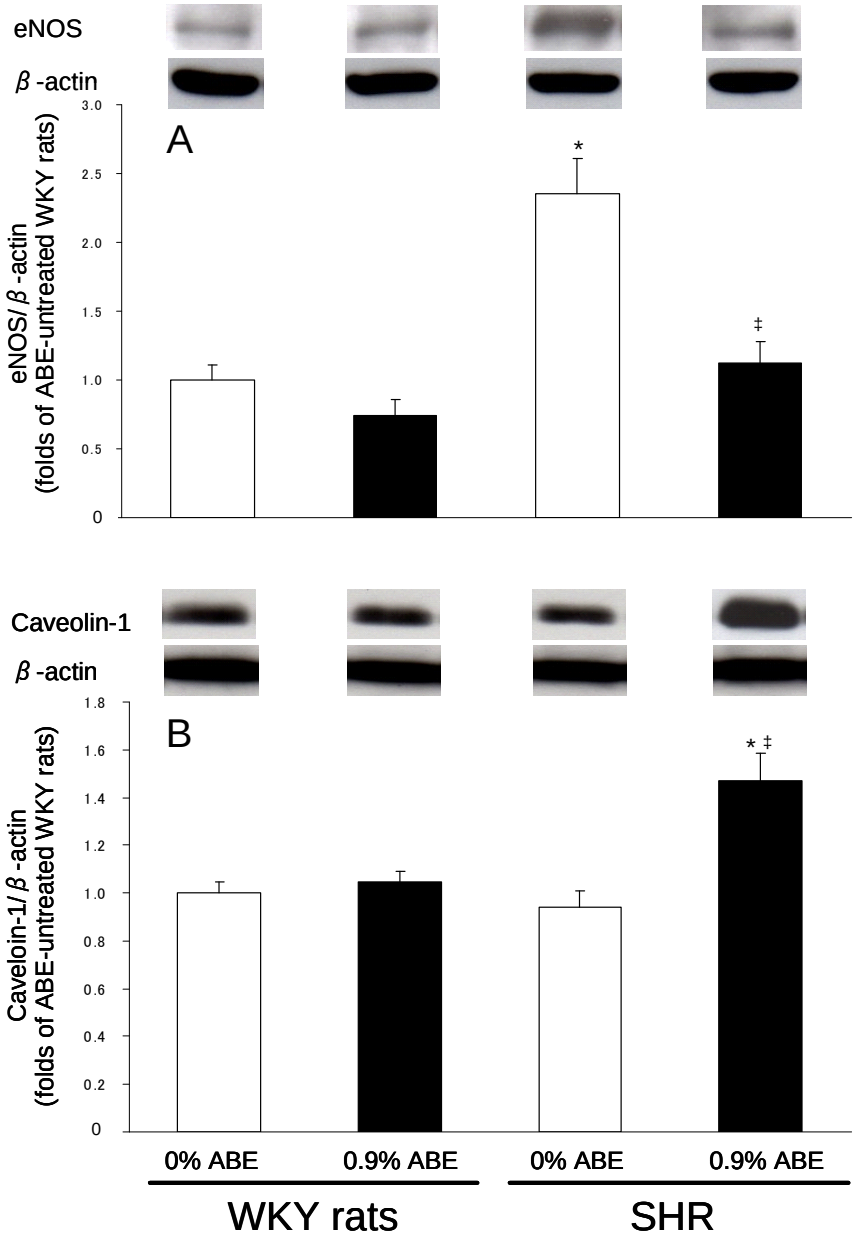

Figure 10 Representative western blot and relative optical densities of the renal (A) eNOS and (B) caveolin-1 bands in each group. The protein expression levels were quantified as fold of the levels observed in untreated WKY rats, after adjusting for endogenous β-actin. Values are expressed as mean ± SEM (n = 7–8). $^{*}P < 0.05$ compared with untreated WKY rats. $^{‡}P < 0.05$ compared with untreated SHR.

similar to that in the WKY rats, the abundance in the ABE-treated SHR significantly increased as compared with that in the untreated SHR (Figure 10B). These results indicated that ABE treatment suppressed the upregulated eNOS expression, and enhanced the caveolin-1 expression in both aorta and kidney of SHR.

2.3 Discussion

The effect of polyphenols-containing azuki bean seed coats (ABSC) and extract (ABE) on the vascular and renal oxidative stress during development of hypertension was discussed in this chapter. The major findings were as follows: (i) both ABSC and ABE, containing polyphenols such as procyanidin and catechin, suppressed the elevated blood pressure in long-term treatment of SHR, while no effect was observed in the normotensive WKY rats; (ii) ABSC treatment decreased the vascular production of O_2^-, and suppressed the mRNA expression of the p47phox subunit of NADPH oxidase in the aorta; (iii) ABE treatment increased the renal Cu/Zn-SOD expression; (iv) ABE treatment increased the urinary NOx content, reflecting an increased NO production; and (v) ABE treatment modulated the eNOS and caveolin-1 expressions in the aorta and kidney of SHR.

Several studies reported that treatments with plant polyphenols such as tea [55] and wine [56] polyphenols suppressed the development of hypertension. Azuki bean extract was found to suppress the elevation of blood pressure in young SHR [34]. In this study, it was confirmed that azuki bean polyphenols attenuated the elevated blood pressure throughout the treatment period (Figures 2 and 6).

In the pathogenesis and maintenance of hypertension, several studies have shown that ROS, in particular O_2^-, are excessively produced by endothelial and vascular

smooth muscle cells in hypertensive humans and animals [35-37]. The increased O_2^- levels can enhance NO inactivation and reduce NO bioavailability [57]. In this study, NADPH-stimulated O_2^- levels increased in the aorta of untreated SHR compared with that in the aorta of untreated WKY rats (Figure 3). In contrast, the NADPH-stimulated O_2^- produced in the aorta of ABSC-treated SHR was less than that of untreated SHR, suggesting that ABSC suppressed excess O_2^- production. This seems to be associated with the prevention of blood pressure elevation. Consistent with this result, red wine polyphenols were demonstrated to reduce hypertension and vascular dysfunction through reduction of aortic O_2^- production [48].

One of the most important producers of O_2^- in vascular cells is NADPH oxidase [36, 58]. NADPH oxidase in vascular wall is composed of multiple subunit proteins, such as Nox4, p22phox, p47phox, and Rac1 [58]. Previous studies revealed that NADPH oxidase activity was abnormally increased in the vascular wall of hypertensive rats, and that this abnormality was associated with upregulated mRNA and protein expression of NADPH oxidase subunits [38, 40]. As expected, it was found that the expression of p47phox and Nox4 mRNA increased in the aorta of untreated SHR in the present study. Conversely, this upregulated expression of p47phox mRNA was suppressed in ABSC-treated SHR (Figure 4). These results were supported by several studies that polyphenols such as red wine polyphenols [11] and quercetin [30], anthocyanidins and catechin origomer [59], suppressed the

overexpression of NADPH oxidase subunits in the aorta in several models of hypertension. Therefore, ABSC seems likely to attenuate O_2^- production via reduction of p47phox expression.

In the model studied here, ABE treatment upregulated the renal expression of Cu/Zn-SOD in the ABE treated SHR (Figure 7). Previous studies demonstrated that some antioxidants such as ascorbic acid and tocopherol [44], and resveratrol [52] significantly enhance the expression and activation of SOD, leading to increased dismutation of O_2^- in arteries of model rats of cardiovascular disease. On the basis of these results, it is hypothesized that, at least in part, azuki bean polyphenols may reduce O_2^- level via activation of Cu/Zn-SOD.

NO is an important molecule that functions as an endogenous vasodilator and is associated with endothelial function [49]. Total body NO production reflects in urinary excretion of nitrate and nitrite (NOx), and blood pressure elevation was accompanied by a decline of urinary excretion of NOx in hypertensive animal model [60]. In this study, the urinary NOx excretion increased significantly in the ABE-treated SHR as compared with that in the untreated SHR (Figure 8). This result indicated that the ABE treatment increased the systemic NO content, suggesting that the increased NO production may prevent elevation of blood pressure.

Excessively produced O_2^- can react with NO and reduce bioavailability of NO. Therefore, NO inactivation by increased O_2^- has been considered to induce the

compensatory upregulation of eNOS protein expressions in the vascular and renal tissues of animal models with hypertension [48, 61-63]. The upregulation of the eNOS protein expressions in the aorta and kidney of the untreated SHR (Figures 9A and 10A) was consistent with the results obtained in the previous studies, suggesting it to be the compensatory response to the reduction of NO bioavailability via increase of ROS in the untreated SHR. On the other hand, the upregulation of the eNOS protein expression was significantly reduced in both the aorta and kidney of the ABE-treated SHR as compared with that of the untreated SHR (Figures 9A and 10A). These findings were supported by several studies that treatments with antioxidants such as quercetin [30], vitamin E [61], and tempol [64] reversed the compensatory upregulation of eNOS protein expressions. On the basis of these findings and the previous reports, it is hypothesized that ABE may, at least in part, play a role in moderating the vascular and renal expressions of the eNOS proteins.

In this study, the vascular expression of caveolin-1, a negative regulatory protein of eNOS, decreased in the untreated SHR, while it was restored in the ABE-treated SHR (Figure 9B). The results were consistent with a previous report which stated that caveolin-1 expression increased in the aorta of SHR by treatment of quercetin [30] and red wine polyphenols [48]. Therefore, ABE treatment may affect the expressional changes of eNOS and caveolin-1 proteins in the present animal model with hypertension.

In summary, treatment of azuki bean polyphenols may decrease O_2^- level and increase NO bioavailability by modulation of expressions of NADPH oxidase subunits, SOD, eNOS and caveolin-1.

Chapter 3. Suppression of vascular inflammation

3.1 Effect of azuki bean seed coats (ABSC) on vascular inflammation in spontaneously hypertensive rats

Introduction

Clinical and experimental investigations have shown that long-term hypertension is associated with initiation of atherosclerosis, which is relevance to inflammation as well as oxidative stress in the arterial wall [4]. It is known that several inflammatory events occur with the development of hypertension; for example, the expressions of proinflammatory enzymes and cytokines are regulated, and the infiltration of immune cells increases, mainly in the kidney and vessels [16, 65]. One of the central mediators of the inflammatory response in hypertensive vascular disease is monocyte chemoattractant protein-1 (MCP-1), the chemotactic factors produced by damaged endothelial cells which induces the migration and attachment of monocytes/macrophages, lymphocytes [66], endothelial cells [67], and vascular smooth muscle cells [68] through the activation of C-C chemokine receptor 2 (CCR2). Expression of CCR2 which is the receptor for MCP-1 is upregulated in monocytes and endothelial cells of hypertensive patients and rats [68, 69]. Previous reports demonstrated the accumulation of immune cells in accordance with the elevated

MCP-1/CCR2 expression in hypertensive humans and animal models [21, 70].

Inducible nitric oxide synthase (iNOS) is known to be a proinflammatory enzyme and its expressions of protein and mRNA are upregulated by proinflammatory stimuli such as ischaemia-reperfusion and LPS [12]. Also in hypertension, iNOS activity and/or protein expression increased in macrophages infiltrated into the aorta and kidney of hypertensive rats, accompanied by reduction of vascular tone [15, 16]. Moreover, cyclooxygenase 2 (COX-2), which is one of the main enzymes of the arachidonic acid metabolism, plays an important role in the pathogenesis of inflammation. It was described that COX-2 expression upregulated in hypertensive rats could contribute to renal inflammation, and that endothelial dysfunction and inflammation were ameliorated by administration of COX-2 inhibitor [22, 71]. Although several plant polyphenols such as resveratrol [72] and proanthocyanidin [73] were demonstrated to suppress the inflammatory process with regulation of MCP-1, iNOS and COX-2 expressions, it is unknown about the beneficial effects of azuki bean polyphenols on inflammatory responses associated with hypertension.

In the present section, in order to clarify the physiological function of azuki bean polyphenols in vascular inflammation with elevated blood pressure, it was investigated whether polyphenols-containing azuki bean seed coats (ABSC) influence the levels of MCP-1, CCR2, iNOS, and COX-2 expression in the aorta of rats with hypertension.

Methods

Plant materials

ABSC was prepared as mentioned above Chapter 2 Section 1.

Experimental design

All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation provided by Aomori University of Health and Welfare. Experimental design was mentioned above Chapter 2 Section 1.

Western blotting

Homogenate of abdominal aorta tissue was prepared and applied to western blot analysis as mentioned above Chapter 2 Section 1. The primary antibodies used in the present experiment were anti-COX-2 (1:200; Cayman Chemical, Ann Arbor, MI, US) or anti-iNOS antibodies (1:200; BD Biosciences, San Jose, CA, US), and β-actin mouse antibody (1:1000; Abcam, Cambridge, UK). Protein levels were normalized to β-actin expression from the same sample; the results are presented as fold of the levels obtained for untreated WKY rats.

Real-time RT-PCR

As mentioned above Chapter 2 Section 2, after total RNA in the thoracic aorta was extracted, vascular mRNA levels of MCP-1 and CCR2 were respectively analyzed by using real-time RT-PCR. The inventoried primers used were as follows: TaqMan gene expression assays Rn00585380_m1 and Rn00573193_s1 (Applied Biosystems). The gene expression levels were presented as fold of the levels obtained for untreated WKY, after adjusting for GAPDH by the 2-(-delta delta C (T)) (CT) method.

Enzyme-linked Immunosorbent Assay for MCP-1 protein

Rat carotid artery was crushed into powder and resuspended in 100 μL of lysis buffer (20 mM HEPES, 0.4 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1mM EDTA, 1% Triton X-100, and 20% glycerol) conteining Protease Inhibitor Cocktail Tablets (Roche Applied Science, Indianapolis, IN, US). The homogenates were centrifuged at 13,000 *g* for 30 min at 4°C. MCP-1 in the supernatant was quantified using an ELISA kit (Biosource^{TM}, Invitrogen, Carlsbad, CA, US). The values were corrected by protein concentrations measured by BCA^{TM} Protein Assay Kit (Pierce, Rockford, IL, US).

Statistical analyses

Each value was expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by Tukey's test. $P < 0.05$ was

considered statistically significant.

Results

Effect of ABSC treatment on the mRNA expression of MCP-1 and CCR2

The expressions of MCP-1 mRNA significantly increased in the thoracic aorta of untreated SHR, as compared with untreated WKY rats (Figure 11A). Conversely, the levels of MCP-1 mRNA in ABSC-treated SHR were significantly lower than those in untreated SHR. In response to MCP-1, the expression of CCR2 mRNA in the aorta of untreated SHR was higher than that in WKY rats (Figure 11B). In contrast, the CCR2 mRNA expression in ABSC-treated SHR was significantly decreased compared with untreated SHR, indicating that ABSC treatment suppressed the expression of MCP-1/CCR2 in the arterial walls of SHR.

Effect of ABSC treatment on the expression of MCP-1 protein

The expressions of MCP-1 protein in the carotid artery of untreated SHR increased significantly, as compared with untreated WKY rats (Figure 12). Conversely, the levels of MCP-1 protein in ABSC-treated SHR were significantly lower than those in untreated SHR. The results indicated that ABSC treatment suppressed the expression of MCP-1

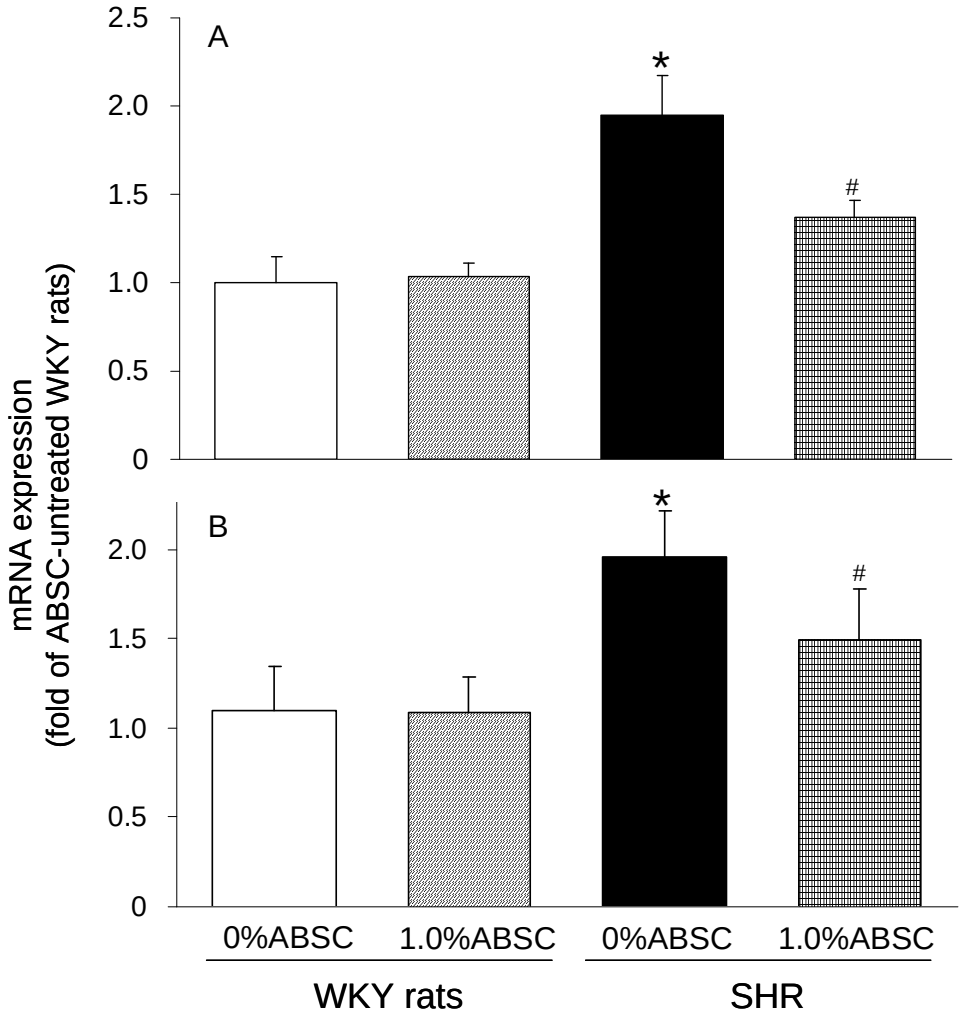

Figure 11 Effect of ABSC treatment on the mRNA expression of the MCP-1 (A) and CCR2 (B) in the aorta of SHR and WKY rats. The gene expression levels were quantified as fold of the levels observed in untreated WKY rats, after adjusting for GAPDH. Values are expressed as mean ± SEM (n = 5–7). *P < 0.05 compared with untreated WKY rats. $^{\#}P$ < 0.05 compared with untreated SHR.

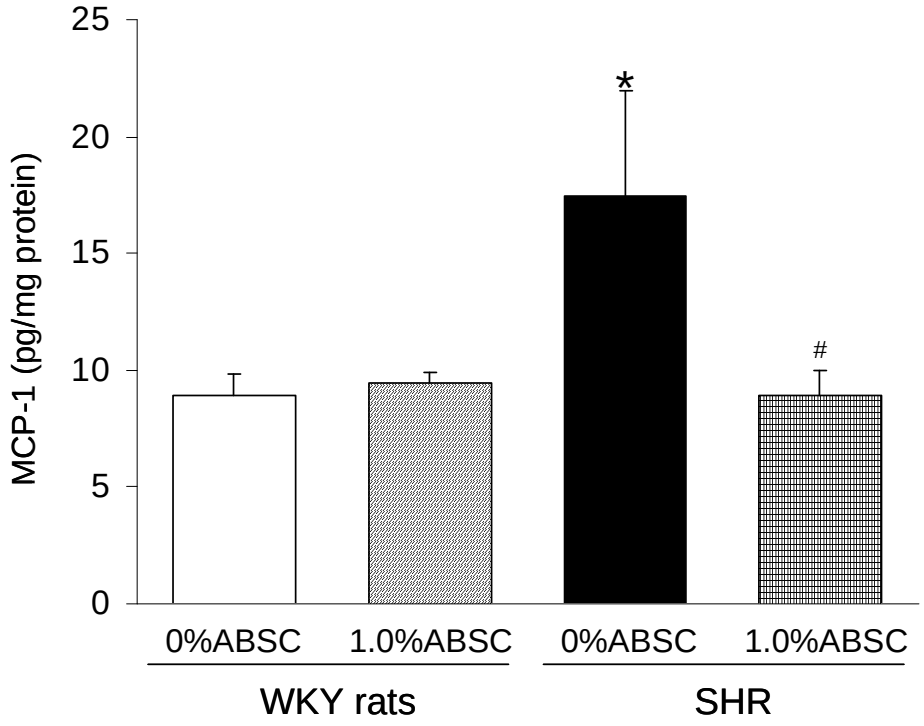

Figure 12 Effect of ABSC treatment on MCP-1 protein expression in the carotid artery of SHR and WKY rats. Values are expressed as mean ± SEM (n = 4–7). *P < 0.05 compared with untreated WKY rats. $^{\#}P$ < 0.05 compared with untreated SHR.

protein in the carotid artery of SHR.

Effect of ABSC treatment on the expression of iNOS and COX-2 proteins

Since iNOS and COX-2 are known to be pro-inflammatory enzymes [16, 71], to examine the effect of ABSC treatment on inflammation, the expression of these proteins in the abdominal aorta was determined by western blot analysis (Figures 13 and 14). Immunoreactive bands corresponding to iNOS and COX-2 were seen at 130 kDa and 72 kDa, respectively, in the proteins extracted from the aorta. The protein abundance of both iNOS and COX-2 in untreated SHR increased as compared with those in untreated WKY rats. In contrast, iNOS and COX-2 protein expressions decreased significantly in the aorta of ABSC-treated SHR.

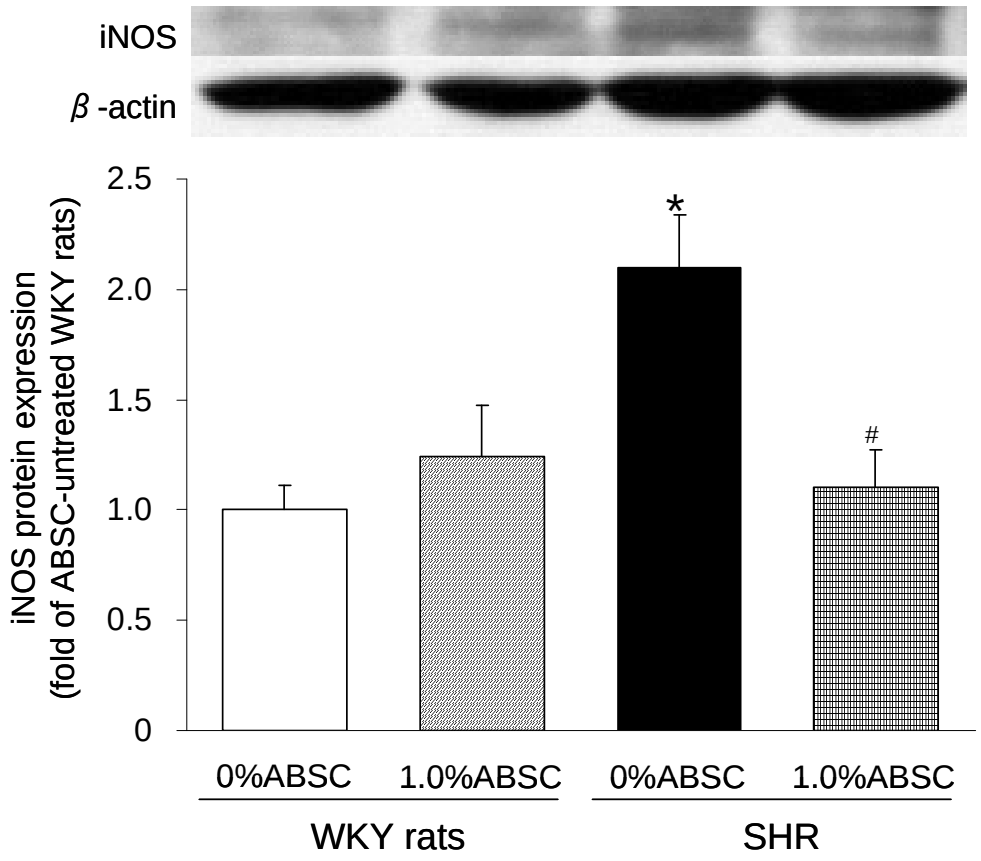

Figure 13 Representative western blot and relative optical densities of the aortic iNOS bands in each group. The protein expression levels were quantified as fold of the levels observed in untreated WKY rats, after adjusting for endogenous β-actin. Values are expressed as mean ± SEM (n = 7–8). *P < 0.05 compared with untreated WKY rats. $^{\#}P$ < 0.05 compared with untreated SHR.

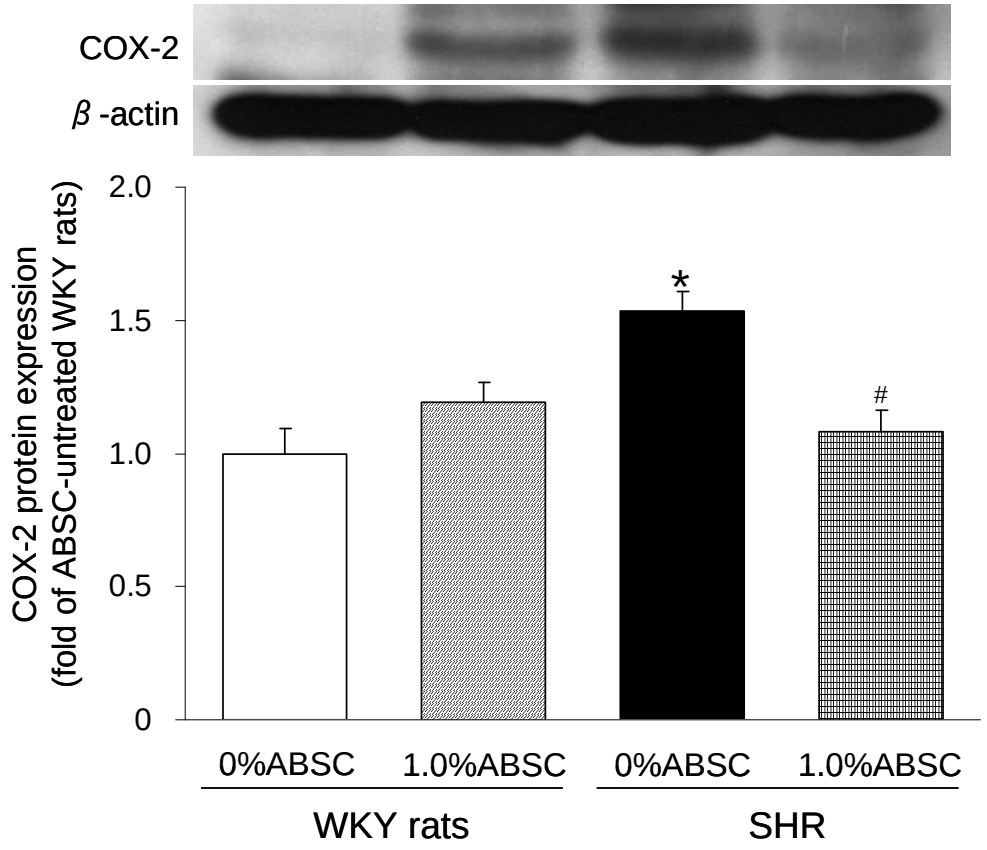

Figure 14 Representative western blot and relative optical densities of the aortic COX-2 bands in each group. The protein expression levels were quantified as fold of the levels observed in untreated WKY rats, after adjusting for endogenous β-actin. Values are expressed as mean ± SEM (*n* = 8). **P* < 0.05 compared with untreated WKY rats. $^{\#}P < 0.05$ compared with untreated SHR.

3.2 Discussion

The effect of polyphenols-containing azuki bean seed coats (ABSC) on the vascular inflammation during development of hypertension was discussed in this chapter. The major findings were as follows: (i) ABSC treatment suppressed the mRNA expressions of MCP-1, its receptor CCR2, and the protein expression of MCP-1; and (ii) ABSC treatment suppressed the protein expressions of iNOS and COX-2 in the aorta of SHR.

Hypertension gradually develops significant severe inflammation marked by a large number of infiltrating macrophages via regulation of expression of adhesion molecules and cytokines, demonstrated in humans [74] and hypertensive models, such as SHR [20], AngII-induced [65], and Dahl salt-sensitive rats [71]. MCP-1 is one of the chemokines that binds to CCR2 and induces migration and activation of immunocompetent cells during the development of atherosclerosis. In this study, the expressions of MCP-1 and CCR2 mRNA significantly increased in the aorta of SHR (Figure 11). In contrast, the upregulated expression of MCP-1 and CCR2 mRNA decreased in the aorta of ABSC-treated SHR compared with that of untreated SHR. Moreover, the level of MCP-1 protein was also significantly lower in ABSC-treated SHR than those in untreated SHR (Figure 12). Recent studies showed that plant polyphenols, such as resveratrol [75], catechin [72], and those found in cava [76], grape seed [77], and green tea [78], attenuated MCP-1 expression *in vitro*, in experiments *in vivo*, and in humans. Taken

together with the present results and previous studies, it is suggested that ABSC could suppress the infiltration of macrophages in the aorta by suppressing the expression of MCP-1/CCR2 mRNA during the progression of hypertension.

In this study, iNOS protein expression in the aorta of ABSC-treated SHR decreased compared with the expression in the aorta of untreated SHR (Figure 13). iNOS is known to be one of the pro-inflammatory enzymes, and its expression increases in response to various cellular stresses [15, 16]. Several reports demonstrated that in hypertension, iNOS was overexpressed in the aorta and epithelial cells of the kidney [15, 16, 61, 62, 79]. Excess NO derived from iNOS reacts with enhanced O_2^- to generate $ONOO^-$, which is responsible for vascular dysfunction [15, 16, 80]. Therefore, long-term treatment with ABSC might be effective in decreasing $ONOO^-$ concentration and preventing endothelial dysfunction through the suppression of iNOS expression and O_2^- production. Moreover, COX-2 protein expression reduced in the aorta of ABSC-treated SHR compared with that of untreated SHR (Figure 14). Increased activity and expression of COX-2 was described in animal models of hypertension *in vivo* [22, 71] and in vascular cells from hypertensive rats *in vitro* [81]. Therefore, ABSC may, at least in part, play a role in suppressing the vascular expression of COX-2, upregulated during the progression of hypertension. These hypotheses are supported by the earlier study demonstrated that proanthocyanidin regulated the expression of proteins related to inflammation such as iNOS, COX-2, and NF-kB p65 in diabetic rats with oxidative

stress [73].

In summary, treatment of azuki bean polyphenols may attenuate vascular inflammation during hypertension, evidenced by suppressing MCP-1, CCR2, iNOS, and COX-2 expressions in the aorta of SHR.

Chapter 4. General Discussion

Hypertension is one of the typical risk factors associated with the development of life style-related diseases such as atherosclerosis and myocardial infarction. Azuki bean (*Vigna angularis*) contains polyphenols such as proanthocyanidins that exhibit remarkable radical scavenging activities *in vitro* [25]. Proanthocyanidins exert preventive effects against oxidative damage associated with a variety of diseases, including inflammation, cardiovascular diseases, atherosclerosis, and diabetes [27-29]. So far, it was found that azuki bean extract suppressed the elevation of blood pressure in young SHR [34], however, the mechanisms underlying the beneficial effects of azuki bean on the progression of hypertension have been unclear.

The aims of the present study were to examine whether azuki bean polyphenols would attenuate oxidative stress and inflammation with hypertension. In addition, the physiological role of azuki bean polyphenols in oxidative stress and inflammation with hypertension is discussed.

In this study, polyphenols-containing azuki bean seed coats (ABSC) and extract (ABE) reduced the elevated blood pressure in long-term treatment of SHR, indicating the anti-hypertensive effect of azuki bean polyphenols.

As seen in Figure 15, azuki bean polyphenols decreased vascular O_2^- level and increased NO bioavailability in SHR. First, treatment of ABSC or ABE including

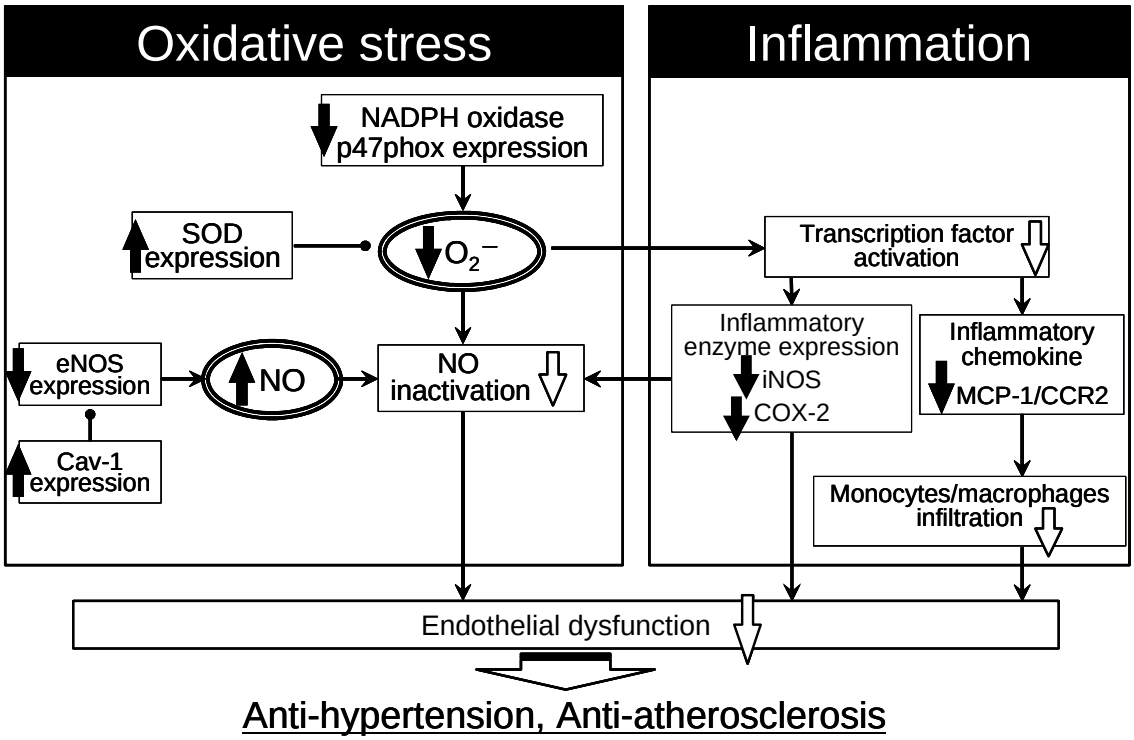

Figure 15 Proposed mechanisms, whereby azuki bean polyphenols affect oxidative stress and inflammation associated with hypertension. Closed arrows indicate up/down-regulation of expression or increase/decrease of production by azuki bean polyphenols.

polyphenols suppressed the mRNA expression of p47phox subunit of NADPH oxidase and enhanced the protein expression of Cu/Zn-SOD, suggesting the decreased O_2^- production. In fact, O_2^- production by NADPH oxidase in the aorta of azuki bean polyphenols-treated SHR was significantly suppressed compared with that in the untreaed SHR. It is hypothesized that azuki bean polyphenols may not only reduce O_2^- production by NADPH oxidase but also enhance O_2^- dismutation by SOD. This hypothesis is supported by several studies that reported that treatments with flavonoids such as quercetin [82, 83], and tea [55] and wine [56] polyphenols suppressed the development of hypertension associated with a reduced oxidant status.

Second, NOx content in 24-h urine was higher in the treated SHR than that in the untreated SHR, suggesting that azuki bean polyphenols may increase the level of bioavailable NO, which is an endogenous vasodilator and is associated with endothelial function [49]. NO production increased in rat aorta treated with red wine polypheols *ex vivo* [84], and in vascular endothelium treated with green tea polyphenols *in vitro* [85]. Therefore, it is considered that azuki bean polyphenols may be associated with the prevention against blood pressure elevation by increasing NO vasorelaxation activity.

Third, eNOS expression was significantly reduced and caveolin-1 expression was increased in the aorta and kidney of SHR treated with azuki bean polyphenols. Since excess O_2^- produced by vascular tissues in hypertensive animals enhanced NO inactivation to form $ONOO^-$ [57], the upregulation of eNOS expression in the aorta and

kidney of untreated SHR was considered to be a compensatory response to the reduction of NO bioavailability. When treated with azuki bean polyphenols, the expressions of eNOS and caveolin-1 protein may be reversed due to the reduction of excess O_2^- production. This phenomenon was consistent with earlier observations that demonstrated the effect of polyphenol quercetin [30] and tocopherol [61] on the reduction of upregulated eNOS expression in hypertensive rats. On the basis of these findings, azuki bean polyphenols may, at least in part, play a role in moderating the vascular and renal expressions of eNOS and caveolin-1.

Fourth, the mRNA expressions of MCP-1 and its receptor CCR2 increased in the aorta of untreated SHR and decreased in the SHR treated with azuki bean polyphenols. Excess O_2^- level is known to have deleterious effects on stimulating NF-kB via MAP kinase [18, 19, 71]. NF-kB is the general transcription factor for a variety of pro-inflammatory cytokines and chemokines, and its activation has been found to account for upregulations of MCP-1 and ICAM-1 expressions in SHR [86, 87]. MCP-1 is one of the chemotactic factors that induce activation, attachment, and migration of immune cells such as monocytes/macrophages [65]. Therefore, the fact that treatment of azuki bean polyphenols suppressed the overexpressions of MCP-1 and CCR2 in the aorta may suggest the suppression of infiltrating immune cells.

Fifth, iNOS and COX-2, which expressions increase in inflammatory processes, were also suppressed in the aorta of treated SHR, indicating that azuki bean polyphenols

may ameliorate the vascular inflammation in the present hypertensive model. This fact is supported by several studies that proanthocyanidin [73] and plant flavonoids, such as cocoa [88] and soy nuts [89], attenuated the inflammation associated with diabetes and hypertension in humans and animals.

Another factor, by which oxidative stress and inflammation are mediated, is angiotensin II (AngII) in cardiovascular tissues in hypertension. AngII was shown to promote NF-kB activation in renal and vascular cells [17]. Several studies demonstrated the upregulation of systemic renin-angiotensin system (RAS) in some models of hypertension [14, 71, 90]. On the other hand, the augmentation of RAS was prevented by antioxidant substances such as tempol in hypertensive rats [90], and the activity of angiotensin I converting enzyme, which contributes to Ang II generation, was inhibited by various beans extract including azuki bean *in vitro* [91]. Although the level of AngII was not examined in this study, it is suggested that azuki bean polyphenols might affect RAS activity.

Chapter 5. Conclusion

On the basis of the results in this study, azuki bean polyphenols were showed to contribute to attenuation of hypertension. The physiological roles of azuki bean polyphenols in oxidative stress and inflammation were to decrease O_2^- level and increase NO bioavailability by modulation of expressions of NADPH oxidase subunits, SOD, eNOS and caveolin-1, and to suppress inflammatory responses such as MCP-1, iNOS and COX-2 upregulations. These effects may lead to prevention against NO inactivation and immune cells infiltration, associated with amelioration of endothelial dysfunction. Therefore, azuki bean polyphenols should be useful as a new preventive strategy of anti-hypertension and anti-atherosclerosis.

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