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Fermented garlic extract decreases blood pressure through nitrite and sGC-cGMP-PKG pathway in spontaneously hypertensive rats

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ABSTRACT

Garlic (*Allium sativum* L.) has long been used as an anti-hypertensive. The study investigated the effect of fermented garlic extract (Fgarlic) on systolic blood pressure (SBP) and its molecular basis using spontaneously hypertensive rats (SHRs). Fgarlic contained high content of stable nitrite (0.975 mg nitrite/ml). Acute feeding of different amounts (0.3, 0.6, and 0.9 ml) of concentrated Fgarlic (9.75 mg nitrite/ml) reduced SBP dose-dependently. Chronic feeding of Fgarlic (containing 27 mg nitrite/day) for 12 days reduced SBP with increased expressions of eNOS and PKG proteins in aortic tissues, which were attenuated by a soluble guanylyl cyclase (sGC) inhibitor. The relaxation responses of thoracic aorta to acetylcholine and sodium nitroprussside were improved in SHRs fed Fgarlic. These results suggest that nitrite in Fgarlic, which converts to NO in the body, functions as an anti-hypertensive molecule and its effect is mediated through sGC-cGMP-PKG pathway.

1. Introduction

Garlic (*Allium sativum* L.) has been used widely as an important medicinal in many ancient civilizations (Pittler & Ernst, 2007). Garlic exhibits a variety of biological functions, including antioxidant, anti-atherosclerotic, anti-microbial, anti-cancer, anti-diabetic, anti-thrombotic, and anti-hypertensive activities (Ali, Al-Qattan, Al-Enezi, Khanafer, & Mustafa, 2000; Fleischauer & Arab, 2001; Ide & Lau, 2001; Jalal, Bagheri, Moghimi, & Rasuli, 2007; Silagy & Neil, 1994). There are at least 100 volatile and non-volatile sulphur-containing bioactive compounds with medicinal values in garlic. Manufacturing processes significantly affect the chemical constituents of garlic preparations and some of garlic bioactives have a low stability and short bioavailability. The major organosulphur bioactive

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compounds are allicin formed by allinase from the precursor alliin in a raw garlic extract (Agarwal, 1996; Iberl, Winkler, Muller, & Knobloch, 1990) and S-allyl-L-cysteine (SAC) in aged garlic extract (AGE) (Harauma & Moriguchi, 2006; Kim, Chang, Kim, & Chun, 2006).

In particular, numerous studies on anti-hypertensive activity have been carried out with raw garlic extract, AGE and garlic-based preparations using various hypertensive models such as spontaneously hypertensive rats (SHRs) (Harauma & Moriguchi, 2006; Kim et al., 2006), renal hypertensive rats (Al-Qattan, Alnaqeeb, & Ali, 1999; Al-Qattan, Khan, Alnaqeeb, & Ali, 2003), L-nitro-arginine methyl ester-treated rats (Pedraza-Chaverri, Tapia, Medina-Campos, de los Angeles Granados, & Franco, 1998), and hypertensive patients (Ashraf, Khan, Ashraf, & Qureshi, 2013; Kapil et al., 2010; Ried, Frank, & Stocks, 2010). Recent studies have shown that garlic exerts its therapeutic effect by increasing nitric oxide (NO) (Al-Qattan et al., 2006; Maslin, Brown, Das, & Zhang, 1997) and hydrogen sulphide production (Benavides et al., 2007), inhibiting angiotensin converting enzyme (ACE) activity (Asdaq & Inamdar, 2010) and synthesis of prostanoids (Al-Qattan, Khan, Alnaqeeb, & Ali, 2001). However, inconsistent and controversial results have been shown (Duda, Sulibursk, & Pupek-Musialik, 2008; Simons et al., 1995), and some adverse effects have been reported, such as anaemia, growth failure (Nakagawa, Masamoto, Sumiyoshi, Kunihiro, & Fuwa, 1980), and a decreased intestinal flora by chronic feeding of raw garlic to rodents (Shashikanth, Basappa, & Sreenivasa Murthy, 1984). The possible reason for these discrepancies may be due to differences in garlic preparations, which affect the stability and toxicity of bioactive compounds in garlic. To address the problems, many investigators have tried to develop the garlic preparation to improve stability and toxicity. Recently, Chun H. (2014) has developed a fermentation method, which increases stability and content of nitrite in garlic. In the present study, we evaluated the anti-hypertensive effect of fermented garlic extract (Fgarlic), specifically focusing on nitrite, and determined the molecular mechanism using SHRs.

2. Materials and methods

2.1. Rats and diet

Male SHR and Wistar-Kyoto rats (WKY), 7 weeks of age, were obtained from the Japan SLC Inc. (Shizuoka, Japan) and housed in individual cages in a room kept temperature-controlled environment with 12-hour light/12-hour dark cycle. The rats were allowed free access to standard laboratory chow (Seoul Feed, Republic of Korea) and water during the acclimation period of one week prior to the experiment. The proximate composition of the diet was a follows: moisture, 10.0%; crude protein, 20.8%; crude fat, 3.9%; crude fibre, 4.3%; crude ash, 6.3%; and nitrogen free extract, 53% (w/w). After stabilization for one week, systolic blood pressure (SBP) was measured using tail cuff plethysmography (Power Lab 2/20, ADInstruments, Dunedin, New Zealand). All animal experimentations described in this study were conducted in accordance with the Guiding Principles in the Care and Use of Animals by the American Physiological Society. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the Chonbuk National University.

2.2. Preparation of Fgarlic

The Fgarlic used in this study was manufactured by HtO Life Co., Ltd. (Wanju-gun, Republic of Korea) in the following steps (Chun, 2014): Fresh garlic purchased from the local market was peeled, and garlic cloves were soaked and sterilized with ozonized water. Twenty hours after shattering to pieces using a crusher, crushed garlic was incubated with *Bacillus subtilis* pre-activated in water in a 1:9 (w/v) ratio at 37 °C for one month under aerobic condition. Supernatant was separated by ultrafiltration and sterilized. The analysis of nitrite (NO_2^-) and nitrate (NO_3^-) levels were performed using an APHA standard method at Korea Testing & Research Institute (Gwacheon-si, Republic of Korea). The analysis of SAC, alliin, and allicin was performed using Thermo ACCELA high performance liquid chromatography and TSQ Quantum Access Max system (Thermo Fisher Scientific Inc., Waltham, MA, USA) at Namhae Garlic Research Institute (Namhae, Republic of Korea) (Jung et al., 2014).

2.3. Animal experimental protocols

2.3.1. Study 1: acute feeding of Fgarlic

To evaluate acute effect of Fgarlic on BP and to determine its dose in SHR, 2 ml of distilled water containing 0.3, 0.6, and 0.9 ml of concentrated Fgarlic (9.74 mg/ml) was orally given using a needle in unanaesthetized SHRs. BP was directly measured in the abdominal artery implanted with a telemetric pressure-recording transmitter (Data Sciences International, St. Paul, MN, USA) before and after feeding for 6 hours ($n = 3$ each).

2.3.2. Study 2: chronic feeding of Fgarlic

Chronic experiment was conducted with eight groups ($n = 6$ each). Groups 1 and 2 were SHRs freely fed water or Fgarlic (0.97 mg/ml) for 25 days, respectively. The total amount of nitrite fed in a day was around 27.0 mg nitrite/day. Groups 3 and 4 were SHRs fed water in the absence or presence of 1H-[1,2,4] oxadiazolo [4,3,-α] quinoxalin-1-one (ODQ; Enzo Life Science, Plymouth Meeting, PA, USA), an inhibitor of soluble guanylyl cyclase (sGC), for 12 days, respectively. Groups 5 and 6 were SHRs fed Fgarlic (27.0 mg nitrite/day) in the absence or presence of ODQ, respectively. ODQ was infused 2 days before Fgarlic feeding for 14 days at a dose of 2 mg/kg/day via a mini-osmotic pump (Alzet 2002, Cupertino, CA, USA) implanted subcutaneously between the scapula. Groups 7 and 8 were WKYs fed water or Fgarlic for 12 days, respectively.

Body weight and SBP were measured periodically. Rats were sacrificed by decapitation, and blood was collected into vials containing 50 μl of 0.1M ethylenediamine tetraacetic acid and centrifuged at 4 °C at 10,000 × *g* for 15 min. Plasma was kept at –70 °C for hormone assay as described below. After the measurement of both left ventricular and septal weight (LV + S), and right ventricular (RV) weight, tissues including aorta and ventricles were rapidly removed, and kept at –70 °C.

2.4. Measurement of BP

In order to measure BP directly in conscious rats, SHRs were anaesthetized with a mixture of ketamine and xylazine (3:1, 2 ml/kg) by intraperitoneal injection. After a midline incision, a telemetric pressure-recording transmitter (Data Sciences International) was implanted in the abdominal aorta according to the manufacturer's protocol, and the abdominal muscle and skin layers were closed. After surgery, rats were housed individually at room temperature (25 ± 2 °C, temperature-controlled) and allowed to recover for 5 days to regain their preoperative body weight. After recovery, BP and the heart rate were continuously monitored online in the conscious unrestrained rats (Data Sciences International), and the data were later analysed offline (Windaq, DATAQ Instruments: Akron, OH, USA) (Yuan et al., 2009). SBP was also measured indirectly by tail cuff plethysmography (Power Lab 2/20, ADInstruments).

2.5. Radioimmunoassay of ANP, renin, and cGMP

Concentration of plasma atrial natriuretic peptide (ANP) extracted using Sep-Pak C_{18} cartridge was measured using specific radioimmuoassay (RIA) as described previously (Cho et al., 1989). Plasma renin concentration (PRC) (Cho et al., 1989) and cGMP levels in plasma and aortic tissue (Lee et al., 2000) were measured by RIA as described previously.

2.6. Real time-PCR for BNP, AT1R, AT2R, ACE, and ACE2 mRNAs

Total RNA was extracted from ventricle using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed using Superscript II and 18-mers Oligo-dT (Invitrogen). Specific primers were designed using primer express software (Applied Biosystems, Foster, CA, USA) and their primer sequences were as follows:

rat brain natriuretic peptide (BNP) (accession NM_031545.1), 5′-GCAGAAGCTGC;
TGGAGCTGC-3′ (forward) and 5′-GATCCGCGGAAGGC GCTGTCT-3′ (reverse);
rat angiotensin type 1 receptor (AT1R) (accession NM_030985.4), 5′-GCCAAAGTC ACCTGCATCAT-3′ (forward) and 5′-AATTTTTTCCCCAGAAAGCC-3′ (reverse);
rat angiotensin type 2 receptor (AT2R) (accession NM_012494.3), 5′-GCATGAGTG TTGATAGGTACCAATCGG-3′ (forward) and 5′-CCCATAGCTATTGGTCTTCA GCAGATG-3′ (reverse);
rat ACE (accession NM_012544.1), 5′-GCCTCCCCAAC AAGACTGCCA-3′ (forward) and 5′-CCACATGTCTCCCCAG CAGATG-3′ (reverse);
rat ACE2 (accession NM_001012006.1), 5′-GGAGAATGCC CAAAAGATGA-3′ (forward) and 5′-CGTCCAATCCTGGTT CAAGT-3′ (reverse);
rat actin (accession NM_031144.2), 5′-ACCAG TTCGCCAT GGATGAC-3′ (forward), and 5′-TGCCGGAGCCGTTGTC-3′ (reverse).

The real time-PCR reaction contained in a final volume of 10 μl, 10 ng of reverse transcribed total RNA, 200 nM of forward and reverse primers and 2x PCR master mix. PCR reaction was carried out in 384-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) (Yuan et al., 2010). All reactions were performed in triplicate.

2.7. Western blotting of sGCα1, sGCβ1, PKG, and eNOS proteins in aortic tissue

Total proteins were extracted from abdominal aorta. The samples were placed in lysis buffer (M-PER, Thermo, Rockford, IL, USA) containing protease inhibitor, homogenized, incubated on ice for 30 min and then centrifuged at 16,000 × *g* for 15 min. After determining protein concentrations in supernatant using a modified Bradford assay, 30 μg of total protein was boiled in loading buffer for 5 min and loaded onto gradient SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to an immobilon-polyvinylidine fluoride membrane. Membranes were blocked with TBS-T skim milk powder for one hour at room temperature. The membrane was incubated with primary antibodies against sGCα1, sGCβ1, cGMP-dependent protein kinase (PKG) (Abcam, Cambridge, MA, USA), and endothelial nitric oxide synthase (eNOS, Enzo Life Science). Proteins were detected with horseradish peroxidase conjugated secondary antibody (Enzo Life Science) for 1 hour at room temperature. Immune reactivity was detected by chemiluminescence (Park, Gao, Cha, Park, & Kim, 2013).

2.8. Changes in relaxation response of thoracic aorta to acetylcholine

SHR and WKY fed water or Fgarlic for 12 days were sacrificed, and the thoracic aorta was rapidly removed and immersed in ice-cold Krebs's solution (pH 7.4) containing (in mM) 118.0 NaCl, 1.1 $MgSO_4$, 4.7 KCl, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 10.0 glucose, and 1.5 $CaCl_2$. The thoracic aorta removed of connective tissue and fat was cut into rings of approximately 2–3 mm wide. The aortic rings were suspended in organ chambers containing 5 ml Krebs solution (pH 7.4) at 37 °C, while being continuously bubbled with 95% O_2–5% CO_2. After an equilibration period of 60 min, under a basal tension of 1 g, the changes in isometric tension were recorded via a transducer (Grass FT 03, Grass Instrument Co., Quincy, MA, USA) connected to a Grass Polygraph recording system (Model 7E, Grass Instrument Co.). The relaxation responses of aortic rings precontracted by phenylephrine (PE, 1 μM) to various doses of acetylcholine (Ach) and sodium nitroprusside (SNP, 1 nM–1 μM) were performed.

2.9. Statistical analysis

The results are presented as means ± SEMs. Statistical differences were assessed using one-way analysis of variance followed by the Bonferroni multiple comparison test or Duncan multiple range test (GraphPad Prism 4). The Student's t test was also used. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Comparison of composition of Fgarlic with raw garlic extract

As shown in Table 1, Fgarlic contained higher level of nitrite (9.745 ± 1.177 mg/g of garlic, 0.975 mg/ml of Fgarlic extract, $p < 0.01$) and lower level of alliin (0.008 ± 0.008 mg/g of garlic, $p < 0.01$) and allicin (not detected) compared to raw garlic (nitrite, 0.043 ± 0.040 mg/g of garlic; alliin, 10.650 ± 0.030 mg/g of garlic; allicin, 1.450 ± 0.010 mg/g of garlic). SAC was not detected in Fgarlic. Therefore, we used nitrite as a marker compound for the standardization of Fgarlic feeding in this study.

3.2. Changes in SBP in response to acute feeding of Fgarlic

To evaluate acute effect of Fgarlic on BP, SHRs implanted with a blood transducer received orally 2 ml distilled water containing 0.3, 0.6, or 0.9 ml of concentrated Fgarlic (9.745 mg of nitrite/ml), and SBP was continuously measured for six hours under an unrestrained conscious condition. By intake of Fgarlic, SBP was gradually decreased from 155 to 125 mmHg, from 150 to 123 mmHg, and from 140 to 85 mmHg by 0.3, 0.6, and 0.9 ml of Fgarlic, respectively (Fig. 1A). The time to reach maximum decrease of SBP was around 30–50 min after administration of Fgarlic. Thereafter, SBP was gradually increased and recovered to control level. The time to reach control level was two to three hours depending on dosage.

3.3. Changes in SBP in response to chronic feeding of Fgarlic

Based on the above results, the highest dose of Fgarlic (0.9 ml containing 9 mg nitrite) was selected for chronic experiments. After the measurement of SBP, rats drank freely water or Fgarlic (27 mg nitrite/27 ml/day) for 25 days, and SBP and body weight were measured every 5 days. SBP was gradually increased from 177.6 ± 9.7 to 190–200 mmHg in SHR fed water but was decreased from 174.9 ± 5.3 to 155–170 mmHg in SHR fed Fgarlic (Fig. 1Ba). The difference in SBP between two groups was around 20 mmHg.

No significant differences in heart weight and LV + S were found between two groups (Fig. 1Bc). However, the ratio of RV to LV + S weight was significantly decreased in SHR fed Fgarlic compared to the ratio in SHR fed water (Fig. 1Bc). The expression of BNP mRNA, a marker of cardiac hypertrophy, was also decreased in RV but not in LV by Fgarlic feeding (Fig. 1Bd). Aortic cGMP level was higher in SHR fed Fgarlic compared to the level in SHR fed water (Fig. 1Be). Plasma cGMP level in both groups was not different (176.5 ± 6.3 vs 178.4 ± 9.43 fM).

Table 1 – Comparison of components of fermented and raw garlic extracts.

	Fermented garlic extracts (n = 4)	Raw garlic (n = 3)
NO_2^- (mg/g garlic)	9.745 ± 1.177**	0.043 ± 0.040
NO_3^- (mg/g garlic)	0.067 ± 0.078	0.043 ± 0.020
Alliin (mg/g garlic)	0.008 ± 0.008**	10.650 ± 0.030
Allicin (mg/g garlic)	ND	1.450 ± 0.010
SAC (mg/g garlic)	ND	0.030 ± 0.000

Values are the mean ± SE. SAC, S-allyl-L-cysteine; ND, not detected.
** vs raw garlic extract, $p < 0.01$.

3.4. Blockade of anti-hypertensive effect of Fgarlic by sGC inhibitor

Because of increasing aortic cGMP level and high content of nitrite in Fgarlic, we hypothesized that anti-hypertensive effect of Fgarlic is mediated through sGC-cGMP-PKG pathway since it is known that nitrite/nitrate converts to NO in the body (Habermeyer et al., 2015). To block anti-hypertensive effect of Fgarlic, SHRs were pretreated with ODQ, a sGC inhibitor, using osmotic pump 3 days before exposure to Fgarlic and drank either Fgarlic or water for 12 days. SBP was significantly decreased from 183.4 ± 3.5 to 169.5 ± 2.0 mmHg by Fgarlic feeding compared to SHRs fed water (from 182.4 ± 3.2 to 191 ± 2.3 mmHg). Supporting the observations, SBP was not decreased in SHRs pretreated with ODQ and then treated with Fgarlic (from 182. 0 ± 2.8 to 190.9 ± 4.9 mmHg) (Fig. 2Aa). The anti-hypertensive effect of Fgarlic was significantly abolished by the pretreatment with ODQ. Body weight did not differ among groups except Fgarlic + ODQ group (Fig. 2Ab). ODQ itself had no effect on SBP and body weight. These results indicate that nitrite in Fgarlic exhibits anti-hypertensive activity.

The ratio of RV to LV + S weight in SHRs fed Fgarlic was 0.189 ± 0.006, which was significantly lower than that in SHRs fed water (0.225 ± 0.001). Pretreatment with ODQ attenuated anti-hypertrophic effect of Fgarlic (0.213 ± 0.001) (Fig. 2Ba). Plasma concentration of ANP and renin did not differ among the four groups (Fig. 2Bb). Fgarlic caused decreases in AT1R and BNP mRNA expressions in RV, which were blocked by the pretreatment with ODQ (Table 2). However, the expression of ACE and ACE2 mRNA in both RV and LV was not significantly different among groups.

3.5. Changes in sGC, PKG, and eNOS protein expression by Fgarlic

To find out whether eNOS and sGC-cGMP-PKG pathway is activated by Fgarlic, the expression levels of sGC, PKG, and eNOS protein were measured in aortic tissues. The expression of sGCα1 was increased by the treatment with either ODQ or Fgarlic, and both treatments compared to the levels in water-fed group. The expression level of sGCβ1 was not different among groups (Fig. 3B). The expression of PKG and eNOS proteins was substantially increased in aortic tissue by Fgarlic feeding, which was decreased by the pretreatment with ODQ (Fig. 3B). ODQ itself had no significant effect on the expression of both PKG and eNOS protein.

3.6. Comparison of relaxation responses to Ach and SNP in Fgarlic-fed rats

To evaluate whether SBP is decreased in normotensive rats and the relaxation response of aorta is improved by Fgarlic

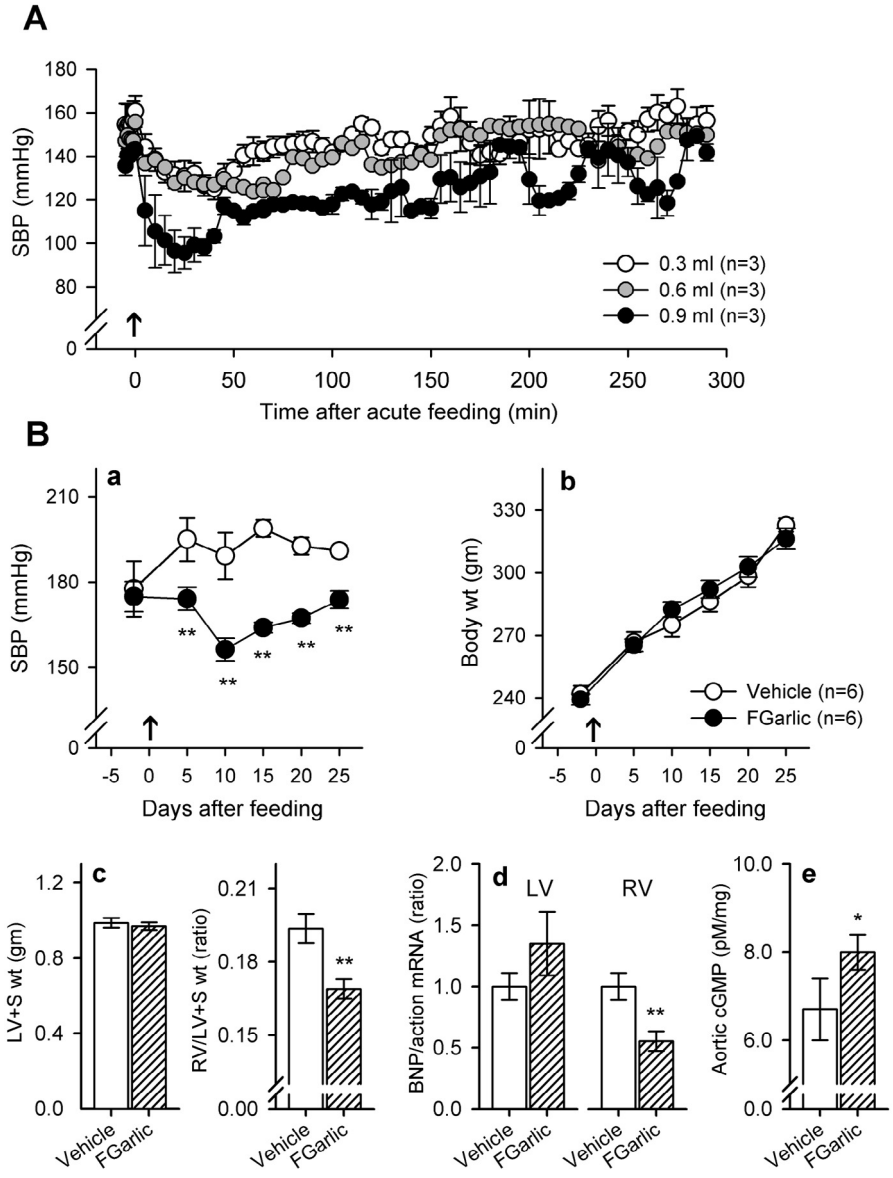

Fig. 1 – (A) Changes in systolic blood pressure (SBP) by acute feeding of water or different doses of fermented garlic extract (FGarlic) in conscious spontaneously hypertensive rats *n* = 3. (B) Changes in SBP (a), body weight (b), ventricular weight (c), BNP mRNA level (d) and aortic cGMP level (e) by chronic feeding of water or FGarlic for 25 days in SHR. Values are means ± SEMs. *n* = 6. Vehicle, SHR fed water; FGarlic, SHR fed diluted FGarlic; LV + S, left ventricular and septal weight; RV, right ventricular weight. * vs SHR fed vehicle, $p < 0.05$, **$p < 0.01$.

feeding, WKY and SHR received Fgarlic or water for 12 days, and vascular strips from thoracic aorta were prepared. SBP was decreased by 10 mmHg in WKY and by 20 mmHg in SHR by Fgarlic feeding (Fig. 4A). The pre-contracted thoracic aortic strip with PE was relaxed in response to Ach and SNP dose-dependently in both WKY and SHR (Fig. 4B). The relaxation responses to Ach and SNP were markedly attenuated in SHR fed water compared to that WKY fed water. By intake of Fgarlic, the relaxation responses to Ach and SNP were significantly accentuated in WKY and tended to accentuate in SHR (Fig. 4B).

4. Discussion

We demonstrated here that Fgarlic with high nitrite content has anti-hypertensive effect accompanied with increases in expression of PKG and eNOS protein in SHRs. Moreover, these Fgarlic effects were blocked by a sGC inhibitor. The aortic relaxation by Ach and SNP was also accentuated in Fgarlic-fed SHRs. These results suggest that Fgarlic exhibits anti-hypertensive effect mediated through sGC-cGMP-PKG pathway in SHR and that the major compound with anti-hypertensive activity in Fgarlic is nitrite.

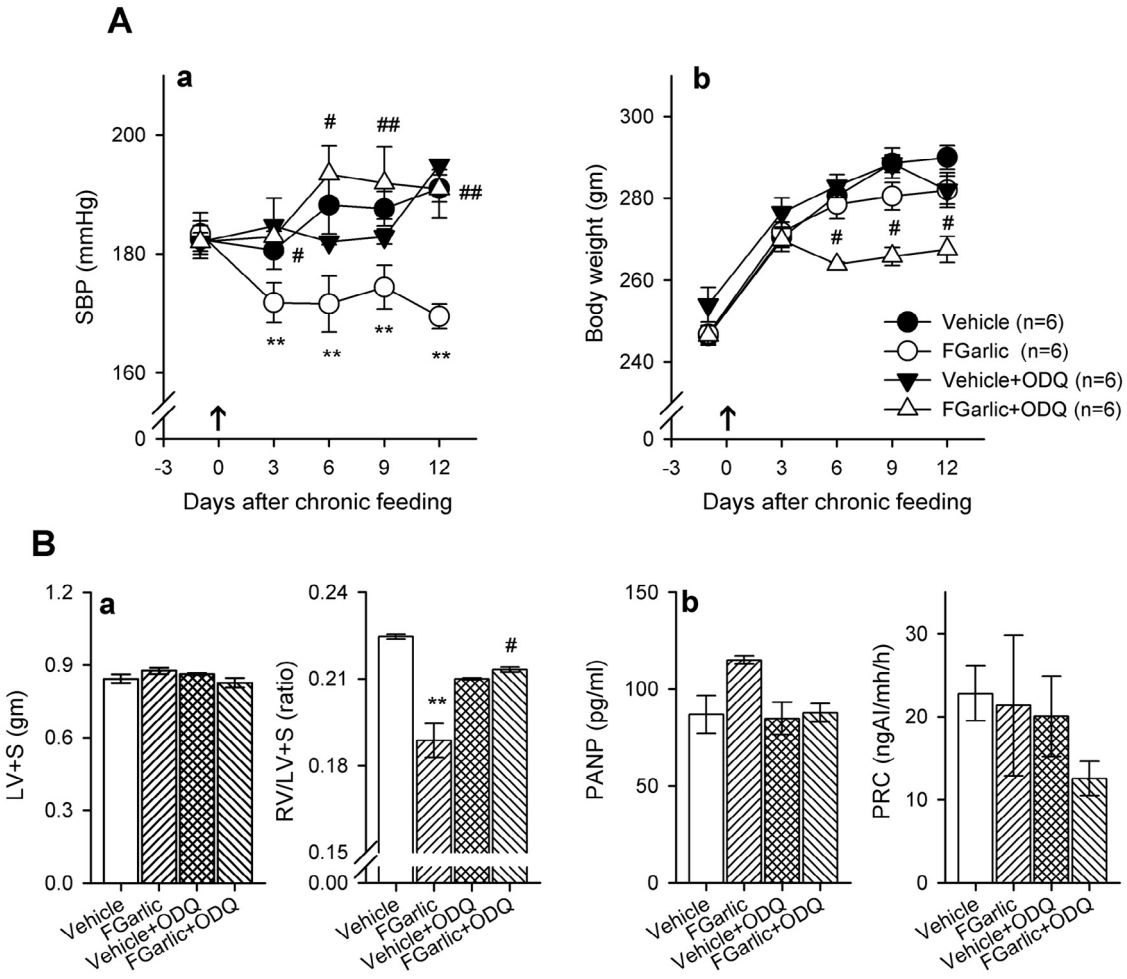

Fig. 2 – Changes in SBP (Aa) and body weight (Ab), ventricle weight (Ba), and plasma concentrations of ANP (PANP) and renin (PRC) by chronic feeding of water or FGarlic with or without ODQ in SHR for 12 days. Values are means ± SEMs. *n* = 6. FGarlic + ODQ, SHR fed FGarlic in the presence of ODQ; Vehicle + ODQ, SHR fed water in the presence of ODQ. * vs SHR fed vehicle, p < 0.05; # vs SHR fed FGarlic p < 0.05, ##p < 0.01.

The preparation methods including crushed raw garlic, dehydrated garlic powder, garlic oil, and AGE may yield different outcomes because of differences in the amount of various biologically active compounds. AGE contains more water-soluble organic sulphur-containing compounds such as SAC (Kim et al., 2006) and S-allyl-mercaptocysteine and raw garlic contains more alliin (Jung et al., 2014) whereas Fgarlic used in the present study contains high level of stable nitrite and less alliin compared to raw garlic extract. It has been reported that nitrite is formed naturally at rather low steady-state concentration in the nitrogen cycle by nitrogen fixation and is subsequently converted to nitrate (Habermeyer et al., 2015). After dietary nitrate is rapidly absorbed in the upper gastrointestinal tract and reaches the salivary glands, salivary nitrate can be reduced to nitrite by com-

Table 2 – Comparison of expression of pressure-related mRNAs in left and right ventricles among four groups.

		BNP	AT1R	AT2R	ACE	ACE2
LV	Vehicle	1.00 ± 0.10	1.00 ± 0.30	1.00 ± 0.17	1.00 ± 0.31	1.00 ± 0.07
	FGarlic	1.66 ± 0.23	0.78 ± 0.37	1.93 ± 0.27	1.20 ± 0.11	1.39 ± 0.10
	Vehicle + ODQ	1.57 ± 0.47	1.39 ± 0.16	2.43 ± 0.91	1.10 ± 0.00	1.31 ± 0.07
	FGarlic + ODQ	1.90 ± 0.45	1.51 ± 0.45	2.92 ± 0.56	1.18 ± 0.08	1.41 ± 0.15
RV	Vehicle	1.00 ± 0.11	1.00 ± 0.13	1.00 ± 0.26	1.00 ± 0.21	1.00 ± 0.05
	FGarlic	0.55 ± 0.08*	0.49 ± 0.10*	1.39 ± 0.38	1.44 ± 0.48	1.21 ± 0.38
	Vehicle + ODQ	1.43 ± 0.35	1.39 ± 0.25	0.50 ± 0.11*	1.28 ± 0.08	1.04 ± 0.29
	FGarlic + ODQ	1.03 ± 0.27#	1.20 ± 0.10#	1.33 ± 0.44	1.29 ± 0.15	0.94 ± 0.26

Values are mean ± SEM of 5 experiments. LV, RV, left and right ventricle, respectively; BNP, brain natriuretic peptide; AT1R, AT2R, angiotensin II type 1 and type 2 receptor, respectively; ACE, angiotensin II converting enzyme. *p < 0.05 vs SHR fed vehicle; #p < 0.05 vs SHR fed FGarlic.

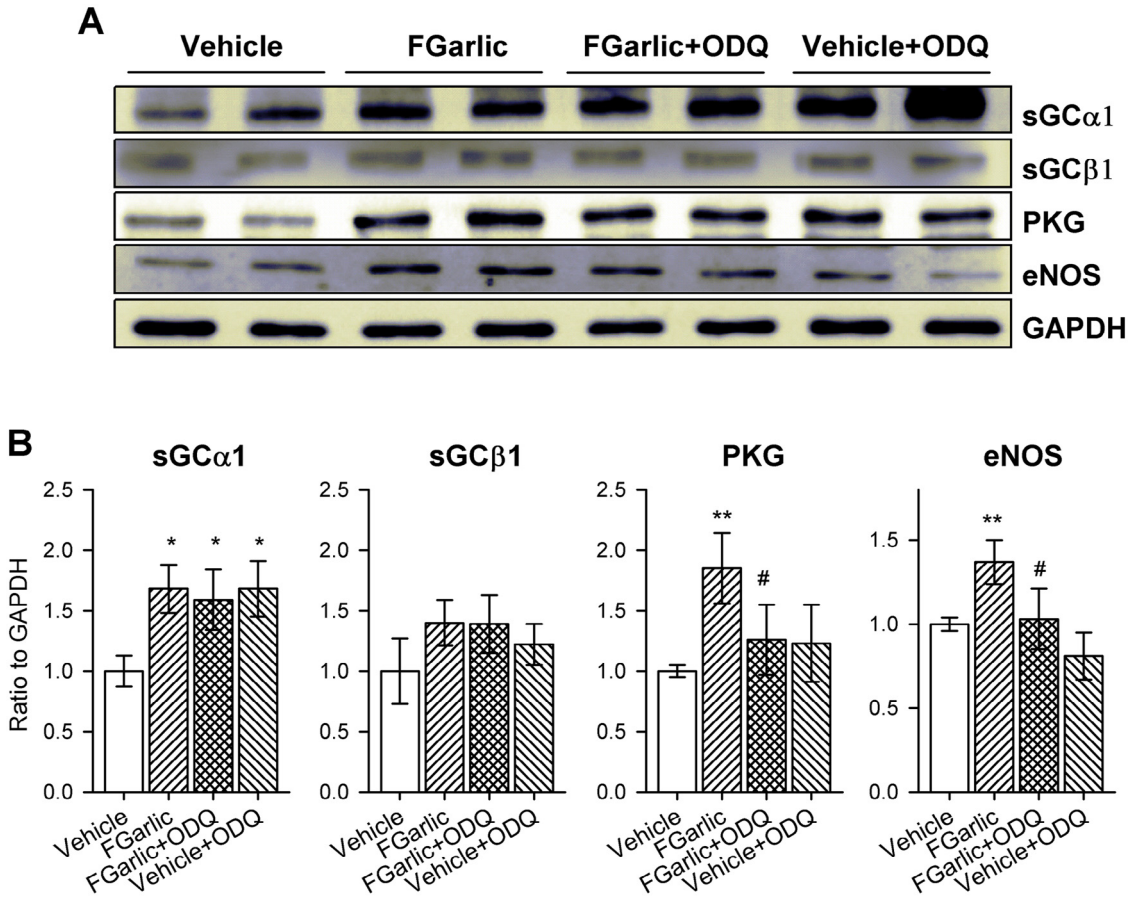

Fig. 3 – Representative western blots (A) and densitometric analysis (B) of soluble guanylyl cyclase (sGC) α1, sGCβ1, cGMP-dependent protein kinase (PKG), and endothelial nitric oxide synthase (eNOS) protein expressions as compared to GAPDH in aortic tissue of SHR fed vehicle or FGarlic in the presence or absence of ODQ for 12 days. Values are means ± SEMs. *n* = 6. * vs SHR fed vehicle, $p < 0.05$; # vs SHR fed FGarlic, $p < 0.05$.

mensal bacteria, thereby reentering to the gastrointestinal tract (Cosby et al., 2003; Dejam et al., 2007). To some extent, saliva-derived nitrite also contributes NO formation under acidic condition in the stomach (Modin et al., 2001). Therefore, nitrite and nitrate have been taken into consideration as an alternative source for endogenous NO (Habermeyer et al., 2015).

Acute administration of Fgarlic caused a decrease in BP in SHR with short half-life characterized by the peak effect at 30 min and recovery within 2–3 hours after feeding.

SBP was decreased around 30 mmHg by Fgarlic feeding containing 0.3 ml of Fgarlic (9.74 mg of nitrite/ml), which shows relatively more potent anti-hypertensive effect than ingestion of 4 or 12 mmol inorganic nitrate (KNO_3) in human (Kapil et al., 2010). In the present study, whenever freely fed Fgarlic chronically for 14 days or 25 days, SBP was decreased by 20 mmHg at 3 days after feeding, which was consistently maintained throughout the experiment. A decrease in SBP by Fgarlic feeding was accompanied with decreases in right ventricular hypertrophy and in the expression of BNP and AT1R mRNAs. However, we did not find any significant changes in AT1R, AT2R, ACE and ACE2 mRNA expressions in ventricles by Fgarlic. These results are consistent with the report showing a decrease in SBP by 20 mmHg by chronic feeding of AGE or raw garlic in SHR (Harauma & Moriguchi, 2006), but it takes 4 weeks to exert anti-hypertensive effect after feeding. Reinhart et al. (Reinhart, Coleman, Teevan, Vachhani, & White, 2008) have also reported that garlic reduced SBP by 16.3 mm Hg and DBP by 9.3 mm Hg compared with placebo in patients with elevated SBP, while garlic had no effect in normotensive patients. In addition, there are still debates on anti-hypertensive effect of garlic, including a decrease in DBP only in humans by sodium nitrate (Larsen, Ekblom, Sahlin, Lundberg, & Weitzberg, 2006), no change in SBP by oily garlic in primary hypertensive patients (Duda et al., 2008), or a decrease in SBP only by AGE (Harauma & Moriguchi, 2006). The possible reason for inconsistent and controversial results may be due to differences in garlic preparations, dosage, treatment duration, and species used in studies. It is not easy to compare anti-hypertensive effect of Fgarlic quantitatively to other reports, but anti-hypertensive activity induced by Fgarlic in the present study appears to be more potent than others (Harauma & Moriguchi, 2006; Reinhart et al., 2008).

It has been reported that nitrite-rich saliva from orally ingested nitrate is entered into the circulation via the stomach, and then, once within the circulation, nitrite is converted to the potent vasodilator NO (Cosby et al., 2003; Dejam et al., 2007). Because Fgarlic used in this study contains high level of stable nitrite (9.75 ± 1.18 mg/g of garlic), nitrite may be converted to

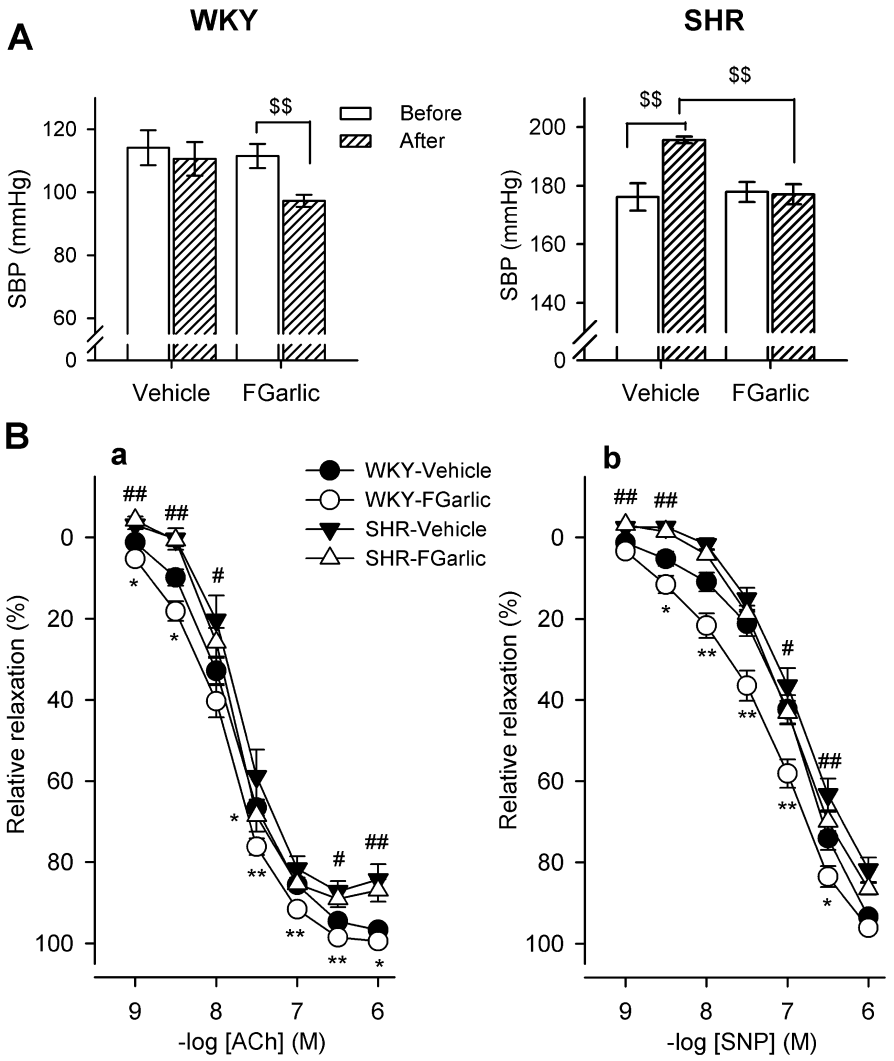

Fig. 4 – (A) Changes in SBP by chronic feeding of vehicle or FGarlic for 12 days in Wistar-Kyoto rats (WKY) and SHR. (B) Comparison of relaxation responses to acetylcholine (Ach) and sodium nitroprusside (SNP) in thoracic aorta precontracted by phenylephrine in WKY and SHR fed vehicle or FGarlic. Values are means ± SEMs. n = 6. $$ vs corresponding group, $p < 0.01$; * vs rats fed vehicle, $p < 0.05$, **$p < 0.01$; # vs WKY fed vehicle, $p < 0.05$, ##$p < 0.01$.

NO followed by the activation of sGC and the production of cGMP. To identify the molecule in Fgarlic that exhibits anti-hypertensive effect in SHR, an inhibitor of sGC was pretreated and then fed Fgarlic. Decreases in SBP and right ventricular hypertrophy induced by Fgarlic were attenuated by the pre-treatment with the sGC inhibitor. Additionally, the expressions of PKG and eNOS protein were highly up-regulated, and aortic cGMP content was increased in aortic tissues from SHR fed Fgarlic compared to those in aortic tissues from SHR fed water. These findings suggest that nitrite in Fgarlic is the functional molecule and exerts anti-hypertensive effect through sGC-cGMP-PKG pathway in SHR.

In addition, to define whether anti-hypertensive effect by Fgarlic feeding is accompanied with the alteration of endothelium-dependent vasorelaxation, the responses to Ach and SNP were examined in thoracic aorta from WKY and SHR. The relaxation responses to Ach and SNP accentuated or tended to accentuate in WKY- or SHR-fed Fgarlic, respectively, compared to those fed water. Our data are consistent with other reports showing anti-hypertensive effect of garlic mediated through the NO pathway (Al-Qattan et al., 2006; Kapil et al., 2010; Maslin et al., 1997).

5. Conclusions

Since Fgarlic used in the present study contains high level of stable and natural nitrite produced during the fermentation process and has more potent anti-hypertensive effect compared to raw garlic extract, and aged garlic extract, Fgarlic appears to become a kind of functional food. The results of the present study showing anti-hypertensive effect with enhancement of PKG and eNOS protein expressions in aortic tissues and the augmentation of aortic relaxation in response to Ach and SNP in Fgarlic-fed rats suggest that Fgarlic shows anti-hypertensive effect through sGC-cGMP-PKG pathway in SHR and the major compound with anti-hypertensive activity in Fgarlic is nitrite.

Conflict of interest

None.

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