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Autonomic Nervous System and Nitric Oxide in Antihypertensive and Cardiac Inhibitory Effects Induced by Red Mold Rice in Spontaneously Hypertensive Rats

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The aim of this study was to investigate the antihypertensive activity of the ethanol extract (EE) of red mold rice (RMR) and to explore its mechanism of action. In comparison to EE of nonfermented rice, the EE of RMR contained higher levels of total phenolic, total flavonoids, γ-aminobutyric acid, and monacolin K. Intravenous bolus administration of the EE (10−50 mg/kg) resulted in biphasic, dose-dependent antihypertensive effects and decreases in heart rate, cardiac contractility, and sympathetic vasomotor tone in spontaneously hypertensive rats. The initial and delayed antihypertensive responses, and the negative inotropic and chronotropic effects of EE treatment (30 mg/kg, i.v.) were significantly reduced by pretreatment with hexamethonium (30 mg/kg, i.v.) and N^G-nitro-L-arginine methyl ester (20 mg/kg, i.v.). Pretreatment with methylatropine (1 mg/kg, i.v.), however, reversed the initial but not the delayed bradycardiac and negative inotropic effects of EE. We conclude that systemic administration of the EE of RMR elicited both transient and delayed antihypertensive actions that were mediated by the withdrawal of sympathetic tone and the production of nitric oxide (NO). The negative inotropic and chronotropic effects of EE may result from a direct sympathetic inhibition of the heart as well as an activation of the NO-dependent pathway.

KEYWORDS: *Monascus*; red mold rice; autonomic nervous system; nitric oxide; antihypertensive activity; cardiac inhibitory effects; spontaneously hypertensive rats

INTRODUCTION

Hypertension, commonly recognized as a silent killer, is the most common cardiovascular disease and is a major risk factor for atherosclerosis, metabolic syndrome, renal dysfunction, myocardial infarction, heart attack, and stroke, which are the most important causes of death in industrialized countries (*1*). It is, therefore, generally believed that control of blood pressure within recommended normal levels have a direct impact on human longevity. In fact, it is well known that healthy lifestyles such as good eating habits are critical for the prevention and control of hypertension (*1*). In recent years, evidence has accumulated to suggest that many dietary herbs and foods are more effective than allopathic medicines in the prevention and treatment of hypertension (*2*). Traditional pharmacological therapeutics are often accompanied by undesirable effects, which means that safer antihypertensive products of natural origin with fewer side effects and better therapeutic performance are needed imminently.

Among the hundreds of natural products available, *Monascus*-fermented rice, known as red mold rice (RMR), exerts cardiovascular activity by promoting blood circulation and maintaining healthy cardiovascular functions (*3*). RMR has been used in food and folk medicine for thousands of years in Asian nations (*3*). In traditional Chinese medicine, RMR has long been recognized as a medicinal agent for the treatment of hyperlipidemia partly because it contains large amounts of monacolin K (lovastatin), an efficient inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in de novo cholesterol synthesis (*3*). Recently, *Monascus*-fermented products have been used as a supplemental treatment for cardiovascular diseases such as hypertension, coronary heart diseases, and myocardial infarction (*4*, *5*). A number of clinical studies have shown that a Xuezhikang capsule, containing the extract of RMR fermented by *Monascus purpureus*, can efficiently treat patients with hypertension or coronary heart diseases by improving their cardiovascular function (*4*, *5*). In addition, the aqueous extract of RMR has been found to evoke an antihypertensive effect on hypertensive animals (*6*, *7*) and primary hypertensive volunteers (*8*). Although the γ-aminobutyric acid (GABA) component is considered to be one of the active antihypertensive components in *Monascus*-fermented products (*3*, *6*, *7*), the mechanisms underlying the beneficial cardiovascular effects of RMR, however, are still largely unclear. The present study was therefore undertaken to investigate the antihypertensive activity of the ethanol extract (EE) of RMR fermented by

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M. purpureus NTU803 and to delineate its mechanism of action in anesthetized spontaneously hypertensive rats (SHR). We also examined in the present study the bioactive content in the EE of RMR.

MATERIALS AND METHODS

Chemicals. Gallic acid, rutin, monacolin K, GABA, and Folin−Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO). LC grade acetonitrile was purchased from Merck Co. (Darmstadat, Germany). Tryptone, yeast extract, peptone, malt extract, potato dextrose agar broth, and Bacto-agar were purchased from Difco Co. (Detroit, MI). The test agents used in the animal study including acetylcholine chloride (ACh), N^G-nitro-L-arginine methyl ester (L-NAME), atenolol, methylatropine bromide, captopril, hexamethonium bromide, bicuculline methiodine, and CGP 35348, (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid hydrate (TPMPA) were purchased from Sigma Chemical Co. (St. Louis, MO) and were dissolved in isotonic normal saline, which was used as the vehicle control.

Preparation of Ethanol Extracts of Red Mold Rice. The indica rice (*Oryza sativa* L.) purchased from a local market in Taiwan was used as the basic matrix for RMR production by solid-state cultivation. The RMR was produced by *M. purpureus* NTU803 fermentation using the method described in our previous study (*9*). After fermentation, the crushed and dried RMR (280 g) was further extracted by 95% ethanol (1680 mL) at 37 °C for 18 h and then dried under vacuum. The yield of dried EE from RMR was 2.94% of the original weight. The EE was stored at 4 °C in a capped brown bottle before tests. The EE of indica rice served as the blank control for the EE of *Monascus*-fermented rice.

Determination of Total Phenolic Content. Total phenolic contents of the EE were determined by the Folin−Ciocalteu colorimetric method (*10*) using gallic acid as a phenolic compound standard. Briefly, 0.1 g of EE was dissolved in 10 mL of 80% methanol, and the solution (1 mL) was mixed with 2.5 mL of distilled water, 0.5 mL of Folin−Ciocalteu reagent, and 1 mL of saturated sodium carbonate (75 g/L). After incubation at room temperature for 30 min, the absorbance of the resulting blue-colored solution was measured at 765 nm using a spectrophotometer (Model U-2800, Hitachi, Japan). Using a five-point standard calibration curve (0−160 mg/L) of gallic acid, we determined the total phenolic content in EE in triplicate and expressed it as mg gallic acid equivalents/g dry extract.

Determination of Total Flavonoid Content. The aluminum chloride colorimetric method (*11*) was used for the determination of the total flavonoid content in EE, using rutin as the flavonoid compound standard. Briefly, the sample solution (0.5 mL) was mixed with 2 mL of distilled water, 0.15 mL of 10% aluminum chloride hexahydrate, and 2 mL of 4% sodium hydroxide. After incubation at room temperature for 15 min, the absorbance of the resulting red-colored solution was measured at 510 nm. Using a six-point standard calibration curve (0−400 mg/L) of rutin, the total flavonoid content in the EE was determined in triplicate and was expressed as mg rutin equivalents/g dry extract.

Determination of the GABA Content. The GABA content was determined by a modified protocol according to Rossetti and Lombard (*12*). The EE (0.1 g) was obtained by extraction with 5 mL of distilled water at 70 °C for 2 h. After centrifugation at 3000*g* for 10 min, 0.2 mL of supernatant was dried under nitrogen gas. The residue was reconstituted in 40 μL of a mixture of ethanol−triethylamine−water (2:1:2) and was then dried and dissolved in 60 μL of a mixture of ethanol−triethylamine−phenylisothiocyanate−water (7:1:1:1). After incubation at room temperature for 20 min, the sample solution was dried at room temperature under vacuum. The residue was dissolved in 0.2 mL of mobile phase and filtered with a 0.45-μm pore size filter, and its GABA content was determined by reversed-phase high-performance liquid chromatography (HPLC, Model L-2130, Hitachi, Japan) analyzer on a C_{18} column (250 mm × 4.6 mm inner diameter, 5 μm, Mightysil RP-18, Kanto Chemical, Japan). The 80:20 mixture of sodium acetate−triethylamine−acetic acid−acetonitrile−water (8.2:0.5:0.7:5:1000) and acetonitrile−water (60:40) were used as the mobile phase, at the flow rate of 0.6 mL/min. The GABA content was determined by an ultraviolet detector (Model L-2420, Hitachi, Japan) at 254 nm.

Determination of the Monacolin K Content. The EE (0.1 g) was obtained by extraction with 10 mL of 95% ethanol at 60 °C for 30 min. After centrifugation at 3000*g* for 10 min, 1 mL of the supernatant was filtered with a 0.45-μm pore size filter and analyzed by HPLC (Model L-2130, Hitachi, Japan) on a C_{18} column (250 mm ×4.6 mm inner diameter, 5 μm, Discovery C18, Bellefontia, PA). The mixture of acetonitrile−water−trifluoroacetate (55:45:0.05) was used as the mobile phase, at a flow rate of 1.0 mL/min. The content of monacolin K was detected by an ultraviolet detector (Model L-2420, Hitachi, Japan) at 238 nm.

Animals. Animal studies were carried out using adult male normotensive Wistar−Kyoto (WKY) rats and SHR (250−300 g) purchased from Experimental Animal Center of the National Science Council (Taipei, Taiwan). All rats were kept in an animal holding room under conditions of constant temperature (23 ± 2 °C) with a standard light/dark cycle (12 h light/12 h dark). The animals were allowed to acclimatize for at least 7 days with free access to standard laboratory rat chow (Purina) and tap water. All animal experimental procedures were approved by Institutional Animal Care and Use Committee of Tajen University (IACUC, Pingtung, Taiwan) and were conducted in compliance with the guidelines of our institutional animal care committee. All efforts were made to reduce the numbers of animals used and to minimize animal suffering during the experiment.

Measurement of Arterial Pressure, Heart Rate, and Left Ventricular Pressure. The rats were anesthetized initially with pentobarbital sodium (50 mg/kg, i.p.) for surgical preparation and thereafter received continuous infusion of propofol (Zeneca Pharmaceuticals, Macclesfield, UK) at 25−30 mg/kg/h for the maintenance of anesthesia. The routine surgical operation included the intubation of the trachea to facilitate ventilation, cannulation of the left femoral artery to measure systemic arterial pressure (SAP), and cannulation of the left femoral vein to administer test chemicals. In addition, the right common carotid artery was exposed, and a 2 French ultraminiature pressure catheter (SPR-320, Millar Instruments Inc., Houston, TX) was placed in the left ventricle through the right common carotid artery to measure left ventricular pressure and the maximal first derivative of developed pressure (dP/dt_{max}). The correct position of the catheter tip in the left ventricle was confirmed by the waveform of left ventricular pressure. The arterial and ventricular catheters were connected to a pressure transducer (MLT844, ADInstruments, Australia) coupled to a PowerLab 4/25 acquisition system (ADInstruments, Australia). The right femoral vein was also cannulated for the continuous infusion of propofol. This scheme provides satisfactory anesthetic maintenance and preserves the capacity for neural control of cardiovascular functions (*13*).

The animals were thereafter placed on a thermostatically controlled pad to maintain a rectal temperature of 37 ± 0.5 °C. Pulsatile and mean SAP (MSAP), heart rate (HR), and dP/dt_{max} were determined using a real-time data acquisition and analysis system (ADInstruments, Australia) and displayed on a monitor during the experiment. The animals were ventilated mechanically by the use of a small rodent ventilator (SAR-830A, CWE Inc., Ardmore, PA) to maintain the end-tidal CO_2 within 4.0−4.5%, as monitored by a capnograph (Datex Normocap, Helsinki, Finland). This procedure was conducted to minimize possible confounding cardiovascular changes secondary to respiratory perturbation. All data were collected from animals with a steady baseline MSAP above 90 mmHg before administration of the test agents.

Evaluation of Sympathetic Vasomotor Tone. For the evaluation of sympathetic neurogenic vasomotor activity, animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) to perform cannulation of the femoral artery and vein. The arterial catheter was connected to a pressure transducer BD P23XL (Becton, Dickinson and Company, Franklin Lakes, NJ) and in turn to a pressure processor amplifier Biopac DA100C (Biopac Systems, Inc., Goleta, CA) via which SAP signals were amplified and filtered (frequency range: DC to 100 Hz). The SAP signals were simultaneously subject to online power spectral analysis as described previously (*14*). We were particularly interested in the very low-frequency (0−0.25 Hz) and low-frequency (0.25−0.8 Hz) components in the SAP spectrum. The power density (mm Hg^2) of these frequency bands of SAP signals has been used as noninvasive indexes to reflect the prevailing sympathetic neurogenic vasomotor tone (*15*).

Preparation of Test Agents. The EE of RMR was freshly prepared immediately before use by dissolving the extracts in isotonic normal saline (0.9% w/v) to make a stock solution with a concentration of 60 mg/mL. Four different doses at 10, 20, 30, or 50 mg/kg were systemically injected

into the animals. Additional test agents used in this study included a cholinergic receptor agonist, ACh (5 μg/kg); a nonselective nitric oxide (NO) synthase (NOS) inhibitor, L-NAME (20 mg/kg); a selective β-1 adrenoceptor antagonist, atenolol (1.5 mg/kg); a muscarinic cholinergic receptor antagonist, methylatropine bromide (1 mg/kg); an angiotensin-converting enzyme (ACE) inhibitor, captopril (2.5 mg/kg); a ganglionic blocker, hexamethonium bromide (30 mg/kg); a $GABA_A$ receptor antagonist, bicuculline methiodine (1 mg/kg); a $GABA_B$ receptor antagonist, CGP 35348 (5 mg/kg); or a $GABA_C$ receptor antagonist, TPMPA (0.5 mg/kg). All of the test agents were freshly prepared immediately before use and administered in a volume of 1 mL/kg body weight, while ACh was given in a volume of 100 μL. Normal saline served as the vehicle and volume control for all agents, and has been tested to exert no significant effect on baseline hemodynamic parameters during the 120 min observation period.

Assay of Plasma Nitrite and Nitrate Levels. NO concentration in plasma was determined using a highly sensitive HPLC analyzer (ENO-20, Eicom, Kyoto, Japan). At preinjection and various postinjection time intervals, 0.8 mL of blood samples was collected from the femoral artery. Any blood withdrawn was immediately replaced by intravenous injection of an equal amount of saline. The blood samples were centrifuged (5000*g* for 5 min) to prepare plasma, which were then mixed with an equal volume of methanol, and were subsequently centrifuged for 10 min at 10000*g* (4 °C). This methanol precipitation procedure was carried out to remove the presence of proteins and improve detection sensitivity. The thus-obtained samples were stored at −80 °C for no more than 2 weeks before the assay. In plasma, NO reacts with molecular oxygen to form nitrite and with oxyhemoglobin and superoxide anion to form nitrate. For analysis, the nitrite and nitrate were separated by a separation column packed with polystyrene polymer (NO-PAK, Eicom, Kyoto, Japan), and nitrate was reduced to nitrite using a copper-coated cadmium column (NO-RED, Eicom, Kyoto, Japan). These nitrites were then reacted with the Griess reagent in a reaction coil to yield red diazo compounds that were quantitatively determined by absorbance at 540 nm. The mobile phase used was 10% methanol containing 0.15 mol/L NaCl-NH_4Cl and 0.5 g/L EDTA-4Na, and the flow rate was 0.33 mL/min. The Griess reagent was delivered at a rate of 0.1 mL/min. Total nitrite and nitrate (NOx) contents were calculated by the sum of nitrite and nitrate levels.

Experimental Protocol. In the first set of experiments, a dose−response curve on cardiovascular effects of EE on WKY rats or SHR was investigated. After obtaining a 20-min basal SAP value, changes in

Table 1. Content of Bioactive Compositions in the EE of Red Mold Rice and Indica Rice[a]

sample	total phenolic	total flavonoids	GABA	monacolin K
EE of red mold rice	58.13 ± 1.99[b]	70.55 ± 1.15[b]	16.01 ± 1.96[b]	36.52 ± 0.65[b]
EE of indica rice	20.7 ± 1.1	52.2 ± 1.1	0.59 ± 0.04	0

[a] Values are presented as the mean ± SEM (mg/g dry extract), n = 4 per experimental group. [b] Statistically significant from the blank control ($P < 0.05$).

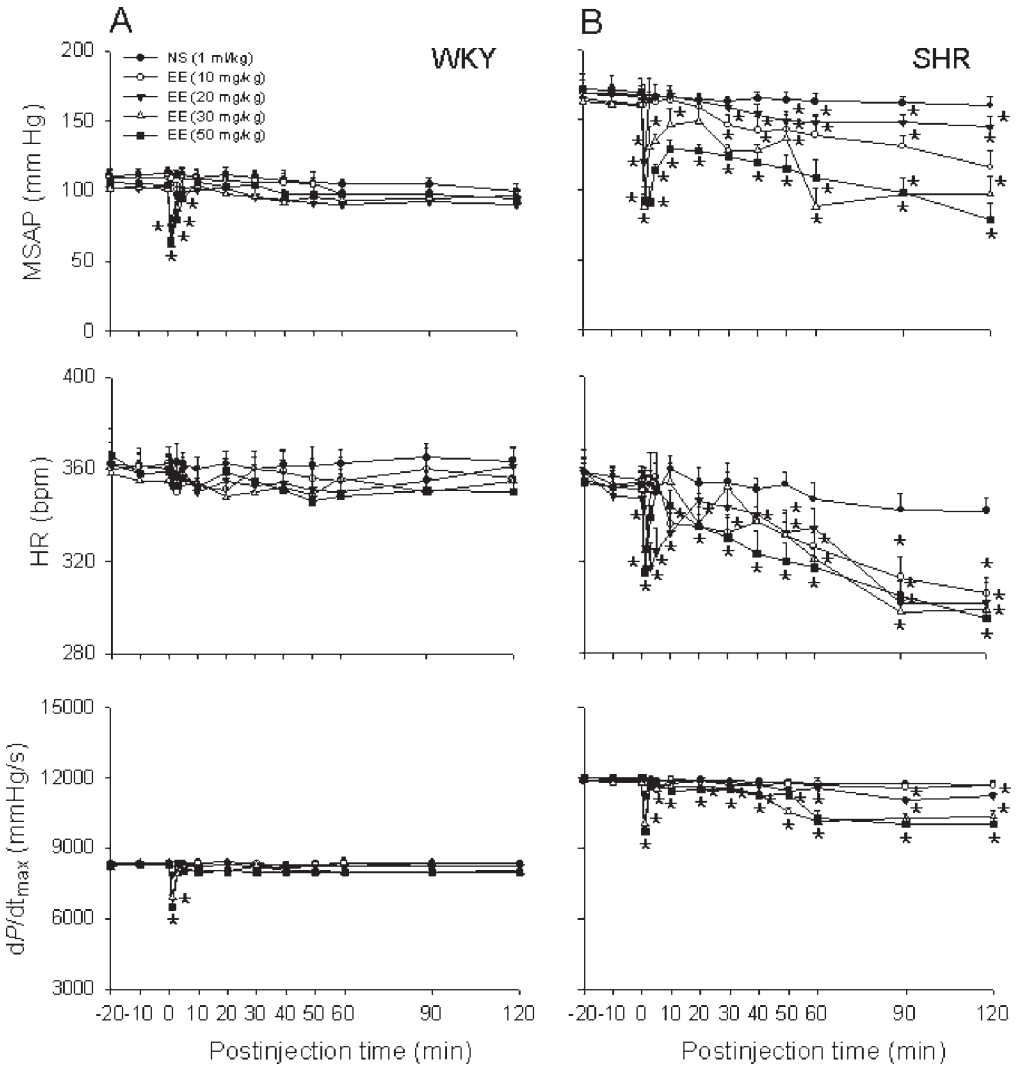

Figure 1. Time-course of the changes in mean systemic arterial pressure (MSAP), heart rate (HR), and maximal first derivative of the developed left ventricular pressure (dP/dt_{max}) in the anaesthetized Wistar−Kyoto (WKY) rats (**A**) or spontaneously hypertensive rats (SHR) (**B**) that received an intravenous injection (at time 0) of normal saline (NS, 1 mL/kg) or ethanol extract (EE, 10−50 mg/kg) of red mold rice. Values are presented as the mean ± SEM; n = 8−9 animals per experimental group. *Statistically significant from the NS group at corresponding time points ($P < 0.05$).

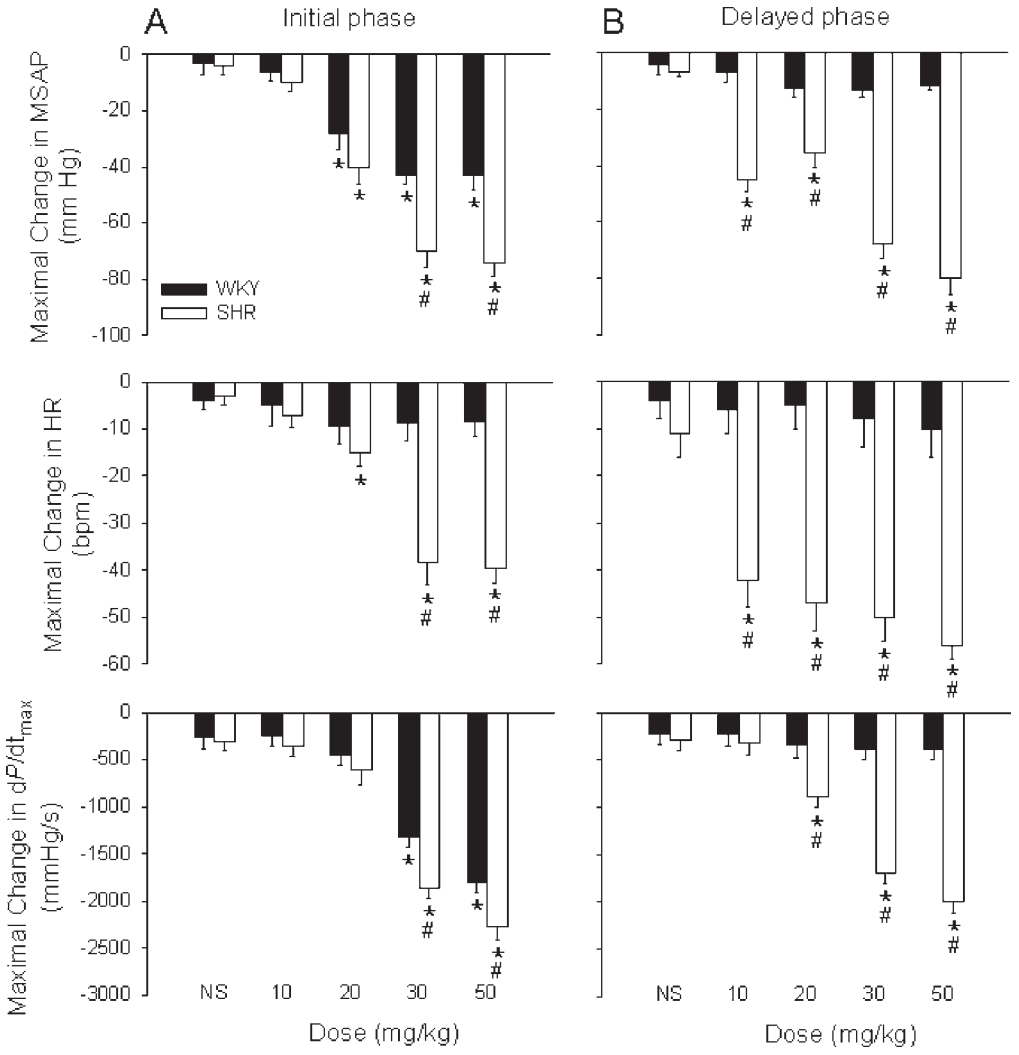

Figure 2. Maximal decreases in mean systemic arterial pressure (MSAP), heart rate (HR), and maximal first derivative of the developed left ventricular pressure (dP/dt_{max}) measured at the initial (0−5 min postinjection in WKY rats or 0−10 min postinjection in SHR) (**A**) or delayed (30−120 min postinjection in SHR) (**B**) phase in the anaesthetized Wistar−Kyoto (WKY) rats or spontaneously hypertensive rats (SHR) that received an intravenous injection of normal saline (NS, 1 mL/kg) or ethanol extract (10−50 mg/kg) of red mold rice. Values are presented as the mean ± SEM; $n = 8-9$ animals per experimental group. *Statistically significant from the corresponding NS group ($P < 0.05$) and #statistically significant from the corresponding WKY group ($P < 0.05$).

MSAP, HR, or dP/dt_{max} in response to systemic administration of EE (10−50 mg/kg) were examined for at least 120 min. Each injection was given at a fixed volume of 0.2 mL to minimize possible volume effects. Intravenous injection of ACh (5 μg/kg) was included as a positive control. In a separate series of experiments, changes in sympathetic neurogenic vasomotor activity after EE (10−50 mg/kg) injection were recorded for 120 min.

To delineate the mechanisms underlying the cardiovascular effects of EE of RMR, maximal change in MSAP, HR, or dP/dt_{max} elicited by intravenous injections of EE (30 mg/kg) was evaluated for 120 min in rats subjected to pretreatment by systemic injections of saline, L-NAME (20 mg/kg), atenolol (1.5 mg/kg), methylatropine bromide (1 mg/kg), captopril (2.5 mg/kg), hexamethonium bromide (30 mg/kg), bicuculline methiodine (1 mg/kg), CGP 35348 (5 mg/kg), or TPMPA (0.5 mg/kg) 10 min prior to EE application. The dose and treatment scheme were adopted from preliminary experiments and a previous study (*16*) that employed the same test agents for the same purpose as that in this study. To avoid confounding effects of drug interactions, each animal received only one treatment scheme or vehicle, given alone or in combination with one test agent.

Statistical Analysis. All values are expressed as the mean ± SEM in each experiment. Baseline changes in the hemodynamic parameters before chemical treatment were assessed using one-way analysis of variance (ANOVA). The time course of the effects of various treatments on the MSAP, HR, dP/dt_{max}, power density of vasomotor components of SAP signals, or plasma NOx levels was assessed using two-way ANOVA with repeated measures for group difference. This was followed by the Scheffé multiple-range test for post hoc assessment of individual means. The contents of EE and the maximal changes in the hemodynamic parameters were evaluated with Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Bioactive Composition in the EE of RMR. In comparison to the blank control (the EE of nonfermented rice), the EE of *Monascus*-fermented rice contained significantly higher levels of bioactive compositions, including total phenolic, total flavonoids, GABA, and monacolin K (**Table 1**).

Effects of EE of RMR on MSAP, HR, and dP/dt_{max} in WKY Rats or SHR. Baseline MSAP, HR, or dP/dt_{max} before chemical treatment was similar among all experimental groups ($P > 0.05$, one-way ANOVA). In anaesthetized normotensive WKY rats and SHR, averaged baseline MSAP was 110 ± 6 ($n = 41$) and 165 ± 8 mm Hg ($n = 43$), averaged baseline HR was 361 ± 9 and 352 ± 8 beats/min, and dP/dt_{max} was 8322 ± 117 and 11775 ± 128 mm Hg/s, respectively. In comparison to the saline control,

Table 2. Effects of GABA and ACh on Baseline MSAP, HR, or Cardiac Contractility in WKY Rats or SHR[a]

treatments	WKY rats			SHR		
	MSAP (mm Hg)	HR (beats/min)	dP/dt_{max} (mm Hg/s)	MSAP (mm Hg)	HR (beats/min)	dP/dt_{max} (mm Hg/s)
			GABA			
basal values	115 ± 6	370 ± 8	8401 ± 102	167 ± 6	357 ± 9	11682 ± 116
0.48 mg/kg	105 ± 5	361 ± 8	8312 ± 88	152 ± 7	351 ± 6	11581 ± 98
0.8 mg/kg	99 ± 9	353 ± 7	8327 ± 52	143 ± 4[b]	347 ± 5	11598 ± 63
			ACh			
basal values	118 ± 6	364 ± 7	8322 ± 190	169 ± 5	359 ± 8	11485 ± 174
5 μg/kg	83 ± 6[b]	362 ± 8	7213 ± 114[b]	73 ± 5[b]	361 ± 6	9921 ± 204[b]

[a] Values are presented as the mean ± SEM, $n = 4$ per experimental group. [b] Statistically significant from basal values ($P < 0.05$).

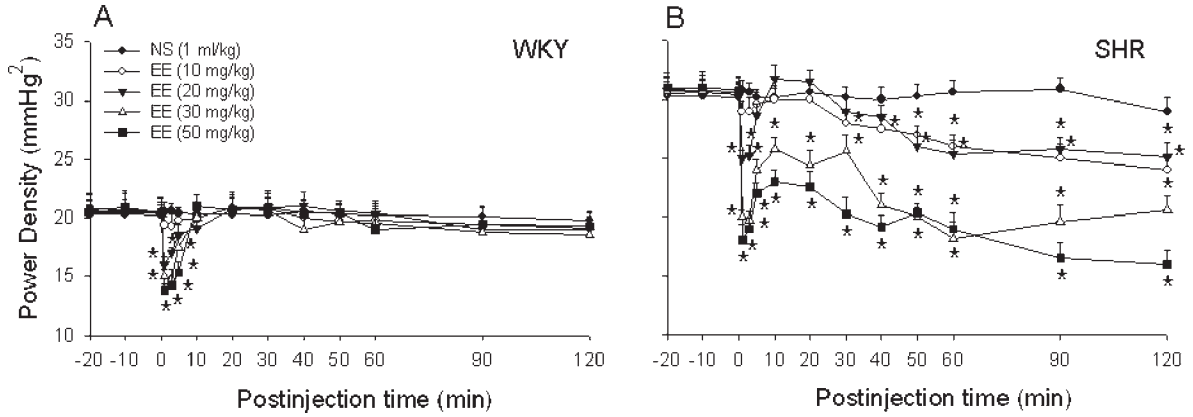

Figure 3. Time-course of the changes in total power density of vasomotor components (0−0.8 Hz) of SAP signals in the anaesthetized Wistar−Kyoto (WKY) rats (**A**) or spontaneously hypertensive rats (SHR) (**B**) that received an intravenous injection of normal saline (NS, 1 mL/kg) or ethanol extract (EE, 10−50 mg/kg) of red mold rice. Values are presented as the mean ± SEM; $n = 8-9$ animals per experimental group. *Statistically significant from the NS group at corresponding time points ($P < 0.05$).

intravenous injections of EE of RMR (10, 20, 30, or 50 mg/kg) resulted in significant biphasic, dose-related antihypertensive responses and decreases in HR and cardiac contractility in SHR (**Figures 1B** and **2**). The EE promoted biphasic cardiovascular response was characterized by an immediate decrease in MSAP, HR, and cardiac contractility (initial phase) within 5−10 min postinjection, followed by a delayed cardiovascular depressive effect (delayed phase) that commenced at 30 min and lasted for at least 120 min postinjection. The EE treatment, however, only evoked the initial hypotensive and negative inotropic effects in WKY rats (**Figures 1A** and **2**). When compared with WKY rats, the initial cardiovascular depressive responses induced by EE were significantly greater in SHR (**Figure 2**). In the delayed response, low-dose EE (10 mg/kg) normalized the MSAP of SHR (163 ± 5 mm Hg, $n = 8$) to a level comparable to that of WKY rats (116 ± 8 mm Hg, $n = 8$) (**Figures 1B** and **2B**). In contrast, intravenous injection of the EE of nonfermented rice (10−50 mg/kg) had no discernible effect on the same cardiovascular parameters in WKY rats or SHR (data not shown). In a separate series of experiments, administration of pure GABA at a dose (0.48 mg/kg) which is equivalent to GABA content in 30 mg/kg EE of RMR had no significant effect on baseline MSAP, HR, or cardiac contractility in WKY rats or SHR (**Table 2**). Whereas the administration of a higher dose of 0.8 mg/kg GABA, which is equivalent to the GABA content in 50 mg/kg EE of RMR, elicited a significant but transient depressor response in SHR (**Table 2**). Control injection of ACh (5 μg/kg), however, induced a significant decrease in MSAP (**Table 2**) that reached its peak within the first 30 s and lasted for less than 5 min postinjection.

Effect of EE of RMR on Sympathetic Vasomotor Activity in WKY Rats or SHR. There is no appreciable difference between the experimental and control groups in basal values of power density of the vasomotor components of SAP signals (WKY rats, 20.2 ± 0.7 mm Hg^2 vs 20.5 ± 0.8 mm Hg^2, $P > 0.05$; SHR, 30.3 ± 0.9 mmHg^2 vs 30 ± 0.5 mmHg^2, $P > 0.05$), our experimental index of the neurogenic sympathetic vasomotor activity. Compared to the saline control, intravenous administration of EE (10−50 mg/kg) produced significant biphasic dose-related decreases in the power density of the vasomotor components of SAP signals in SHR (**Figure 3B**). In WKY rats, EE treatment only evoked a transient decrease in the same parameter that returned to baseline within 10 min postinjection (**Figure 3A**).

Effect of Antagonists on EE-Induced Cardiovascular Depressive Responses. In contrast to saline pretreatment, intravenous injection of hexamethonium (30 mg/kg) or L-NAME (20 mg/kg), 10 min prior to EE (30 mg/kg) administration, significantly attenuated the initial (**Figure 4A**) and delayed (**Figure 4B**) antihypertensive, bradycardiac, and negative inotropic effects promoted by the EE in SHR. Pretreatment with methylatropine (1 mg/kg) reversed the initial but not the delayed cardiac inhibitory effects of the EE (**Figure 4**). Similarly, the EE-induced initial hypotension and negative inotropic effects in WKY rats were also discernibly attenuated by hexamethonium (30 mg/kg) or L-NAME (20 mg/kg) (**Figure 5A**). Pretreatment with captopril (2.5 mg/kg), atenolol (1.5 mg/kg), bicuculline (1 mg/kg), CGP 35348 (5 mg/kg), or TPMPA (0.5 mg/kg), however, did not affect the cardiovascular depressive responses elicited by the EE in WKY rats (**Figure 5**) or SHR (**Figure 4**).

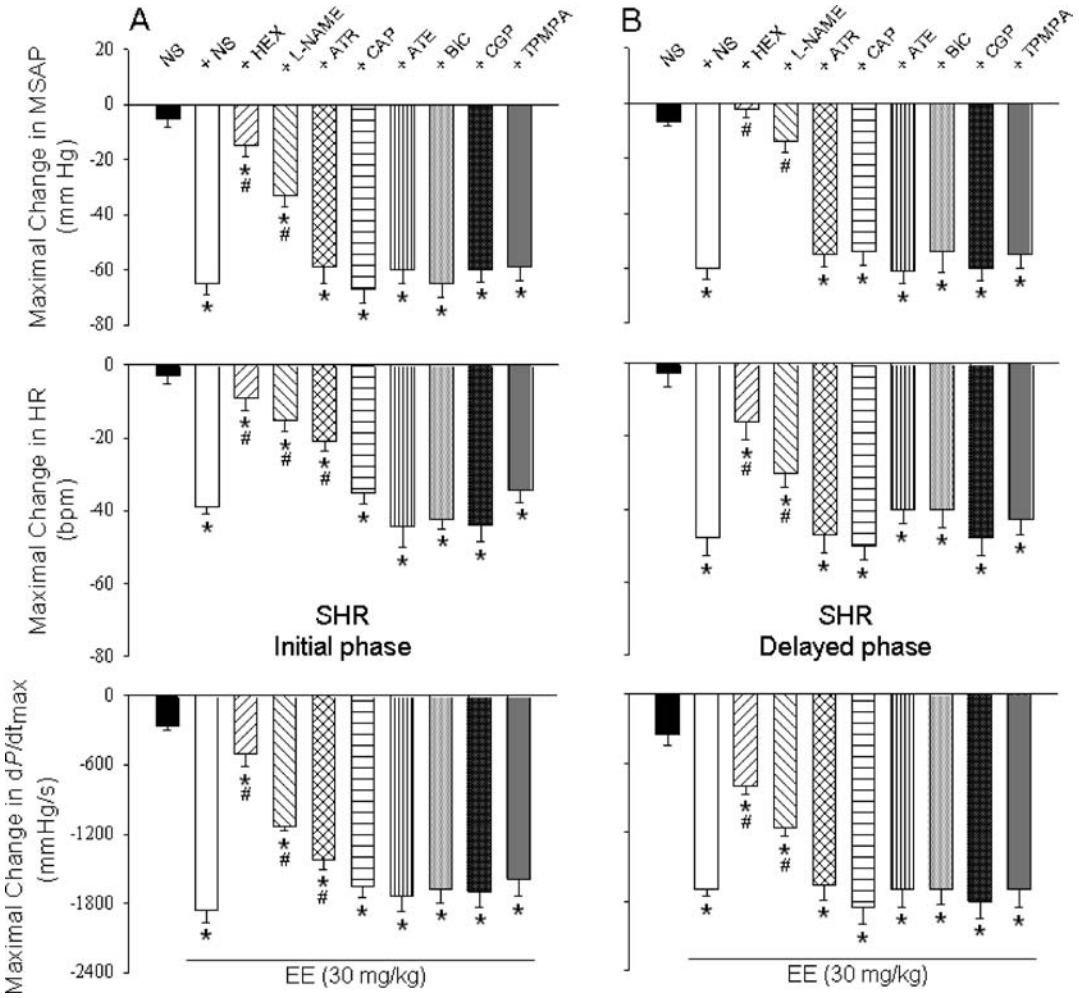

Figure 4. Maximal changes in mean systemic arterial pressure (MSAP), heart rate (HR), and cardiac contractility (dP/dt_{max}) measured at the initial (**A**) or delayed (**B**) phase in the anaesthetized spontaneously hypertensive rats (SHR) that received an intravenous injection of ethanol extract (EE, 30 mg/kg) of red mold rice. These animals received an additional intravenous pretreatment of normal saline (NS, 1 mL/kg), hexamethonium (HEX, 30 mg/kg), N^G-nitro-L-arginine methyl ester (L-NAME, 20 mg/kg), atropine (ATR, 1 mg/kg), captopril (CAP, 2.5 mg/kg), atenolol (ATE, 1.5 mg/kg), bicuculline (BIC, 1 mg/kg), CGP 35348 (CGP, 5 mg/kg), or (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid hydrate (TPMPA, 0.5 mg/kg) 10 min prior to the administration of the extract. Values are presented as the mean ± SEM; $n = 6-8$ animals per experimental group. *Statistically significant from the corresponding NS group ($P < 0.05$) and #statistically significant from the corresponding EE+NS group ($P < 0.05$).

Effect of EE of RMR on Plasma NOx Level in WKY Rats or SHR. Basal levels of plasma NOx for experimental and saline control animals were similar (WKY rats, 140 ± 18 pmol vs 132 ± 12 pmol, $P > 0.05$; SHR, 101 ± 13 pmol vs 95 ± 12 pmol, $P > 0.05$). In WKY rats, the EE of RMR (10−50 mg/kg) only caused a short-lasting increase in plasma NOx level measured at 1 min postinjection (**Figure 6A**). In SHR, intravenous injection of EE caused a significant increase in plasma NOx level in a dose- and time-dependent manner that lasted for at least 120 min postinjection (**Figure 6B**).

DISCUSSION

In the present study, we used SHR, an animal model for the study of human primary hypertension, to evaluate the potential beneficial effect of EE of *Monascus*-fermented rice on cardiovascular functions in vivo. Our study provides novel observations to demonstrate that systemic administration of EE from RMR fermented with *M. purpureus* NTU803 elicited biphasic and potent hypotensive and cardiac inhibitory effects, as well as a significant decrease in sympathetic vasomotor activity. The initial cardiovascular depressive effects promoted by EE not only were significantly greater in SHR than normotensive WKY rats but also were followed by delayed and sustained antihypertension, bradycardiac and negative inotropic effect, and inhibition of the sympathetic neurogenic vasomotor tone, which were not observed in WKY rats. This differential response between SHR and WKY rats provides novel evidence for potential therapeutic applications of this RMR extract in the treatment of hypertension. In the delayed response, low-dose EE (10 mg/kg) almost normalized the MSAP of the SHR to a level comparable in WKY rats suggesting that even low dose EE may exert a potent antihypertensive activity.

In a number of animal and clinical studies, extracts or preparations of RMR (*4*, *6*−*8*) and its bioactive constituent, GABA, have been reported to possess antihypertensive effects (*3*, *6*, *7*). The mechanisms of these cardiovascular effects, however, are not fully understood. Therefore, the major contribution of the present study is to reveal the autonomic nervous systems as sites of action for the cardiovascular depressive activities of EE. We demonstrated that antihypertensive, negative inotropic and chronotropic effects induced by the EE of RMR were mediated, at least in part, by a sympatholytic mechanism. We also noted that these cardiovascular depressive effects elicited by EE were eliminated by pretreatment with a ganglionic blocker,

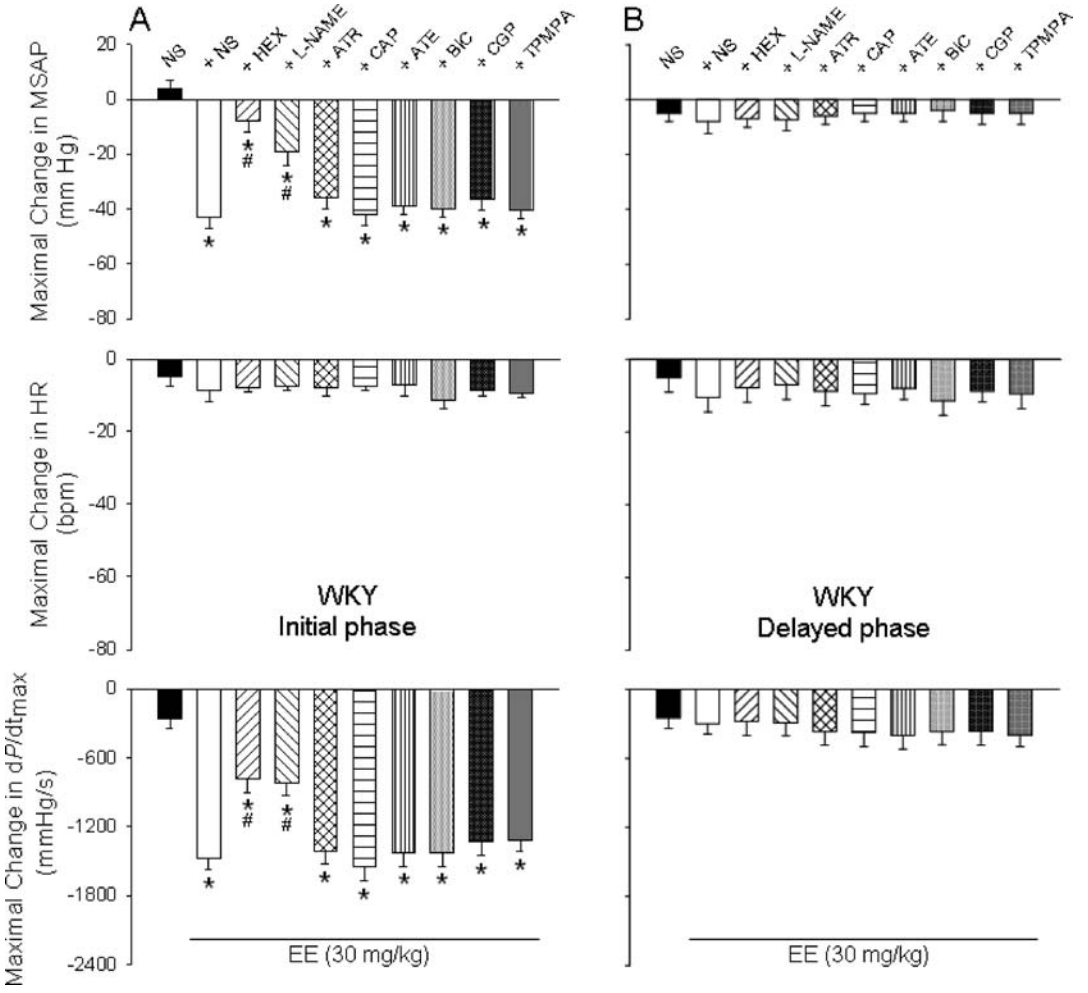

Figure 5. Maximal changes in mean systemic arterial pressure (MSAP), heart rate (HR), and cardiac contractility (dP/dt_{max}) measured at the initial (**A**) or delayed (**B**) phase in the anaesthetized Wistar−Kyoto (WKY) rats that received an intravenous injection of ethanol extract (EE, 30 mg/kg) of red mold rice. These animals received an additional intravenous pretreatment of normal saline (NS, 1 mL/kg), hexamethonium (HEX, 30 mg/kg), N^G-nitro-L-arginine methyl ester (L-NAME, 20 mg/kg), atropine (ATR, 1 mg/kg), captopril (CAP, 2.5 mg/kg), atenolol (ATE, 1.5 mg/kg), bicuculline (BIC, 1 mg/kg), CGP 35348 (CGP, 5 mg/kg), or (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid hydrate (TPMPA, 0.5 mg/kg) 10 min prior to the extract administration. Values are presented as the mean ± SEM; $n = 6-8$ animals per experimental group. *Statistically significant from the corresponding NS group ($P < 0.05$) and #statistically significant from the corresponding EE+NS group ($P < 0.05$).

hexamethonium. In addition, we found that the EE of RMR reduced the power density of the vasomotor components of SAP signals, our experimental index for sympathetic neurogenic vasomotor outflow (*15*, *16*), in a temporal profile that correlated positively with its antihypertensive effect. Taken together, these observations suggest that the antihypertensive effect of the EE of RMR is attributable to the withdrawal of sympathetic vasomotor tone.

Another major contribution of this study is to demonstrate the involvement of NO in both the initial and delayed phases of EE-induced cardiovascular depressive responses. Many plant foods or purified drugs derived from botanical medicinal herbs have been reported to affect the NO signaling pathway (*17*). In the present study, we found that both the initial and delayed phases of EE-induced cardiovascular depressive responses were attenuated by pretreatment of the nonselective NOS inhibitor, L-NAME. These results indicate that NO is engaged in cardiovascular depression induced by the EE of RMR and further imply that cardiovascular depressive responses of EE may be mediated via the interaction between EE and NO signals. Such speculation is supported by our observations in which the EE of RMR increased plasma NO contents in a temporal profile that correlated positively with the EE-induced cardiovascular depressive responses. More importantly, our preliminary results showed that such an increase in plasma NO level at the initial and late stages of antihypertensive response was significantly suppressed by pretreatment of L-NAME (data not shown). Although we did not directly assess the putative endothelium-dependent relaxation mechanisms underlying the EE-induced antihypertensive effect, we found in the present study that the time course of the initial antihypertensive response induced by EE is similar to that induced by ACh. It is well established that ACh causes generalized vasodilation by releasing NO from the vascular endothelial cells (*18*). As such, it is reasonable to speculate that an endothelium-dependent NO signaling pathway might mediate the initial antihypertensive effect of RMR extract. Such speculation is further supported by a previous in vitro study, which reported that the vasodilatory effects of an aqueous extract of RMR fermented with *Monascus rubber* is mediated by endothelium-dependent NO production in rat thoracic aorta (*19*). Additionally, it has been observed that activation of endothelial NOS induces short-term releases of NO, whereas inducible NOS causes prolonged generation of NO (*20*, *21*). Whether these different enzyme kinetics may participate differentially in EE-induced

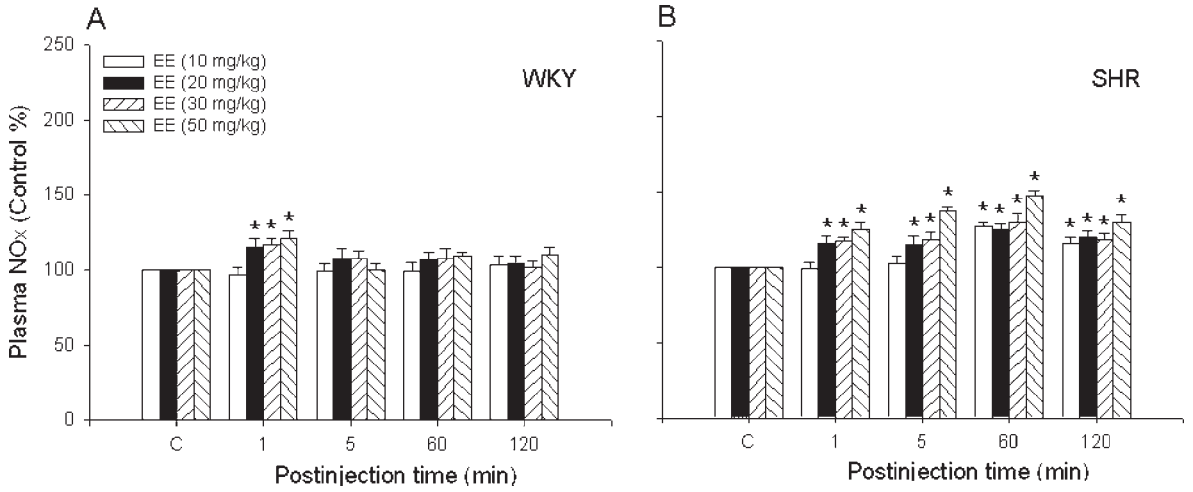

Figure 6. Time-course of the changes in the plasma nitrate/nitrite (NOx) levels in the anaesthetized Wistar−Kyoto (WKY) rats (**A**) or spontaneously hypertensive rats (SHR) (**B**) that received an intravenous injection of ethanol extract (EE, 10−50 mg/kg) of red mold rice. Values are presented as the mean ± SEM; $n = 5-6$ animals per experimental group. *Statistically significant from the pretreatment control (C) group ($P < 0.05$).

initial and delayed phases of cardiovascular depressive effects also required further investigation.

Similar to the mechanisms underlying antihypertensive effect, negative chronotropic and inotropic effect of the EE of RMR may be mediated by activation of the NO-dependent pathway. In fact, NO may act as an important mediator in the autonomic regulation of cardiac excitability (*22*). Previous in vitro studies also demonstrated that the NO-cGMP pathway inhibits norepinephrine release, as well as the positive chronotropic and inotropic effects during cardiac sympathetic nerve stimulation (*23*, *24*) via modulation of ion channels (*22*). Therefore, the EE-induced negative chronotropic and inotropic effect appears to be dependent upon the presence of an operational autonomic drive to the heart, as demonstrated by its significant attenuation in L-NAME-pretreated rats. These data indicate the possibility that the cardiac inhibitory effects of EE might be mediated by a NO-dependent cardiac sympathetic inhibition. Moreover, we found that in addition to attenuating EE-induced antihypertension, the ganglionic blocker, hexamethonium, almost completely reversed the initial and delayed phases of cardiac inhibitory effects by the extract. The pretreatment with the parasympathetic muscarinic receptor antagonist, methylatropine, at the dose exerting minimal effect on EE-induced antihypertension, resulted in partial attenuation of the initial cardiac inhibitory effects. These results further indicate a dominant contribution by the sympathetic rather than the parasympathetic system on the cardiac effects of the RMR extract.

It is well recognized that ACE plays an important role in the regulation of cardiovascular homeostasis (*25*), and ACE blockers promote antihypertensive effects (*26*). We demonstrated, however, that the antihypertensive and cardiac inhibitory effects induced by EE were not related to the action of ACE since the specific ACE inhibitor, captopril, had no significant effect on the cardiovascular depressive responses evoked by EE. Moreover, our preliminary results of the in vitro studies showed that the same extract had no inhibitory effect on ACE activity (data not shown). Similarly, we observed no effect of atenolol on EE-induced cardiovascular depressive responses. These results suggest that EE-induced cardiovascular depressive responses were not dependent on the inhibition of ACE activity and β-1 adrenoceptors.

In the present study, several bioactive constituents, such as monacolin K, GABA, flavonoids, and phenolic compounds, are found in the EE of RMR. Although GABA has been previously reported as one of the active antihypertensive constituents in the RMR extract (*3*, *6*, *7*), the selective GABA receptor antagonist did not significantly attenuate the EE-induced cardiovascular depressive responses in the present study. In addition, direct injection of pure GABA (0.48 mg/kg), at a dose equivalent to the GABA content in the EE (30 mg/kg) of RMR, had no discernible effect on these same circulatory parameters in WKY rats or SHR. In contrast to the low dose (< 0.48 mg/kg) GABA, a transient hypotensive effect in the SHR was only observed at a high dose (0.8 mg/kg) of pure GABA, which is equivalent to the GABA content in 50 mg/kg EE of RMR. Meanwhile, we noted that the intravenous administration of pure monacolin K at a high dose (1.8 mg/kg) that is equivalent to the monacolin K content in 50 mg/kg EE of RMR did not alter the baseline circulatory parameters (data not shown). Together, these results strongly suggest that under our experimental conditions, GABA and monacolin K may not play an important role in the cardiovascular depressive responses of the RMR extract. Flavonoids and phenolics found in the EE of RMR exhibit NO-dependent vasorelaxation (*17*, *27*) and antihypertensive activity (*28*), which this class of bioactive metabolites are known to do. Recent chemical constituent analysis reported that in addition to GABA and monacolin K, many functional secondary metabolites such as azaphilonoid pigments (e.g., ankaflavin, monascin, rubropunctatin, monascorburin, rubropunctamine, and monascorburamine), dimerumic acid, isoflavones, phytosterols, and unsaturated fatty acids have also been found in the *Monascus*-fermented product (*3*). To better understand the cardiovascular depressive effect of the extracts from RMR, further studies are required to isolate the active compounds from this extract.

Secondary metabolites produced from the *Monascus* species have been demonstrated to contain citrinin, a mycotoxin known to cause some adverse effects such as nephropathy and hepatopathy (*29*). In our previous study, animals fed with *Monascus* powder containing trivial levels of citrinin (939 μg/kg) for 8 weeks did not show any changes in plasma liver index analysis or microscopic examination of liver biopsy (*30*). These observations deem unlikely that cardiovascular depressive responses of RMR extract result indirectly from the possible toxic effects of citrinin. In our preliminary acute toxicity assay, the EE demonstrated a good safety profile, as indicated by the lack of any behavioral abnormality as well as the absence of morbidity or mortality after

oral administration of the extract up to 2 g/kg (WKY rats and SHR, $n = 5$).

In summary, our results demonstrate for the first time that the EE of RMR exhibits a transient followed by delayed antihypertensive and cardiac inhibitory effects in SHR. Antihypertensive effect of EE in SHR was mediated by the withdrawal of sympathetic neurogenic vasomotor tone and the production of NO. The negative inotropic and chronotropic effects may result predominantly from a direct sympathetic inhibition to the heart as well as activation of the NO-dependent pathway. These findings provide mechanistic insights for the therapeutic uses of *Monascus*-fermented products in folk medicine and for the development of a new therapeutic strategy in the treatment of hypertension.

ABBREVIATIONS USED

dP/dt_{max}, the maximal first derivative of developed pressure; EE, ethanol extract; HPLC, high-performance liquid chromatography; HR, heart rate; L-NAME, N^G-nitro-L-arginine methyl ester; MSAP, mean SAP; NO, nitric oxide; NOS, NO synthase; NOx, total nitrite and nitrate; RMR, red mold rice; SAP, systemic arterial pressure; SHR, spontaneously hypertensive rats; TPMPA, (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid hydrate; WKY, Wistar−Kyoto.

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