Sympathoexcitation by Oxidative Stress in the Brain Mediates Arterial Pressure Elevation in Obesity-Induced Hypertension

Ai Nagae, MD, PhD*; Megumi Fujita, MD, PhD*; Hiroo Kawarazaki, MD; Hiromitsu Matsui, MD, PhD; Katsuyuki Ando, MD, PhD; Toshiro Fujita, MD, PhD

Background—Obesity is one of the major risk factors for cardiovascular disease and is often associated with increased oxidative stress and sympathoexcitation. We have already suggested that increased oxidative stress in the brain modulates the sympathetic regulation of arterial pressure in salt-sensitive hypertension, which is often associated with obesity. The present study was performed to determine whether oxidative stress could mediate central sympathoexcitation in the initial stage of obesity-induced hypertension.

Methods and Results—Four-week-old male Sprague-Dawley rats were fed a high-fat (45% kcal as fat) or low-fat (10% kcal as fat) diet for 6 weeks. Fat loading elicited hypertension and sympathoexcitation, along with visceral obesity. In urethane-anesthetized and artificially ventilated rats, arterial pressure and renal sympathetic nerve activity decreased in a dose-dependent fashion when 53 or 105 μmol/kg tempol, a membrane-permeable superoxide dismutase mimetic, was infused into the lateral cerebral ventricle. Central tempol reduced arterial pressure and renal sympathetic nerve activity to a significantly greater extent in high-fat diet–fed hypertensive rats than in low-fat diet–fed normotensive rats. Intracerebroventricular apocynin or diphenyleneiodonium, a reduced NADPH oxidase inhibitor, also elicited markedly greater reductions in arterial pressure and renal sympathetic nerve activity in the high-fat diet–fed rats. In addition, fat loading increased NADPH oxidase activity and NADPH oxidase subunit p22phox, p47phox, and gp91phox mRNA expression in the hypothalamus.

Conclusions—In obesity-induced hypertension, increased oxidative stress in the brain, possibly via activation of NADPH oxidase, may contribute to the progression of hypertension through central sympathoexcitation. (Circulation. 2009;119:978-986.)

Key Words: brain hypertension obesity oxidative stress sympathetic nervous system

Metabolic syndrome, a complex of highly debilitating disorders that consist of hypertension, diabetes mellitus, and dyslipidemia, is associated with the development of visceral obesity.1 All of these features are risk factors for atherosclerosis; therefore, the metabolic syndrome results in a high incidence of cardiovascular events. Although hypertension is one of the major components of the metabolic syndrome, the mechanisms through which obesity contributes to hypertension have not been fully elucidated.

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Sympathetic activation is often associated with obesity. For example, studies using microneurography have consistently shown increased muscle sympathetic nerve activity in obese subjects.2 Furthermore, muscle sympathetic nerve activity is closely associated with the level of abdominal visceral fat.3 As expected, a number of studies indicated that sympathetic activation may be involved in obesity-induced hypertension.4 In young, nondiabetic Japanese men, body mass index correlated with not only mean blood pressure but also pulse rate and plasma norepinephrine.5 In an animal study, obese Zucker rats, which display hyperphagia-induced obesity caused by a mutation of the leptin receptor, have elevated sympathetic nerve activity and arterial pressure (AP).6 Autonomic ganglionic blockade decreased mean AP (MAP) to a much greater extent in obese Zucker rats compared with lean rats.7 In addition, bilateral renal denervation prevented the hypertension and sodium retention associated with obesity in dogs fed a high-fat diet (HF).8 Thus, sympathetic activation may play a major role in the pathogenesis of obesity-induced hypertension.

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Bodies of evidence have suggested associations of obesity with high oxidative stress in human and animal models. Male Sprague-Dawley rats have exhibited hypertension, along with increased oxidative stress, when fed an HF beginning at 3 weeks of age. Furthermore, several studies have demonstrated increased oxidative stress in the kidney and aorta of Fischer rats fed an HF diet with high refined sugar and in the aorta of obese Zucker rats. Thus, reactive oxygen species (ROS) may be the unifying mechanism underlying the development of hypertension in obesity, although it is unknown how ROS increases AP.

The role of central ROS in sympathetic regulation of AP has been suggested in some types of hypertensive animal models. The ROS level was increased in the brain of stroke-prone spontaneously hypertensive rats. The pressor and sympathoexcitatory effects of centrally administered angiotensin II were completely abolished by adenosinergic vector-mediated expression of superoxide dismutase and by central administration of 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol), a membrane-permeable superoxide dismutase mimetic, or polyethylene glycol-superoxide dismutase. Furthermore, we have previously demonstrated that salt loading stimulated an increase in AP induced by central sympathoexcitation possibly through brain ROS overproduction in adenomedullin-knockout mice, an animal model of increased oxidative stress. We have also suggested that increased ROS in the brain elevates AP possibly through central sympathoexcitation in a salt-sensitive hypertensive model, Dahl salt-sensitive rats.

Obesity-induced hypertension showed enhancement of salt sensitivity of blood pressure. Conversely, a number of studies, including ours, have demonstrated that salt-sensitive hypertension exhibits the metabolic disorder and sympathoexcitation. In a manner similar to salt-sensitive hypertension, increased oxidative stress and sympathoexcitation may be important pathogenic mechanisms in the progression of obesity-induced hypertension. High dietary fat has been reported to actually induce reduced NADPH oxidase–associated oxidative stress and inflammation in the brain of rats. However, the exact role of increased brain ROS in obesity remains to be elucidated. We hypothesized that ROS overproduction in the brain might contribute to central sympathetic activation and AP elevation in obesity-induced hypertension. In the present study, to clarify our hypothesis, we examined the response of renal sympathetic nerve activity (RSNA) and AP to intracerebroventricular administration of antioxidant agents and the level of NADPH oxidase activity and mRNA expression in obesity-induced hypertensive model rats fed an HF.

### Methods

#### Animals

Four-week-old male Sprague-Dawley rats (Tokyo Laboratory Animals Science, Tokyo, Japan) were randomly assigned to receive an HF (45% kcal as fat; Research Diets, New Brunswick, NJ) or a low-fat diet (LF; 10% kcal as fat) for 6 weeks. All rats were housed in a room maintained at 23°C to 25°C with a 12-hour light/dark cycle and were given food and water ad libitum. All experimental procedures were approved by the Ethics Committees on Animal Research of Faculty of Medicine, University of Tokyo.

#### Measurement of Blood Pressure and Biochemical Parameters

- Body weight and systolic blood pressure measured by the tail-cuff method (P-98A, Softron, Tokyo, Japan) were recorded every week.
- Urinary norepinephrine excretion was measured at 4 and 6 weeks of dietary loading as an indicator of sympathetic nerve activity. At 6 weeks of treatment, in some rats anesthetized with sodium pentobarbital (50 mg/kg IP), blood samples were obtained by cardiac puncture to measure the following metabolic parameters after an overnight fast. Blood glucose concentrations were measured with a Quickauto NEO GLU-HK kit (Shino-Test Inc, Tokyo, Japan). Serum insulin and leptin concentrations were measured with an insulin and a leptin ELISA kit (Morinaga Institute of Biological Science, Inc, Yokohama, Japan), respectively. Serum cholesterol, triglycerides, and free fatty acids were assayed with routine enzymatic assays.
- At the end of the study, the retroperitoneal, epididymal, and total mesenteric fats were harvested, and their wet weights were measured to evaluate visceral fat.

#### Ganglionic Blockade With Hexamethonium Hydrochloride

In some conscious rats, we examined the response of MAP to ganglionic blockade to evaluate sympathetic nerve activity. The femoral artery and vein were cannulated under ether anesthesia. The experiment was begun at least 3 hours after recovery from anesthesia. After the baseline MAP measurement, 30 mg/kg hexamethonium was administered into the femoral vein. The maximal decrease in the MAP was considered an index of sympathetic activity.

#### Intracerebroventricular Administration of Antioxidant Agents

- In urethane-anesthetized (1 g/kg) and artificially ventilated rats, MAP, heart rate (HR), and RSNA were recorded, as mentioned in our previous reports. After recording the basal MAP, HR, and RSNA during a 30-minute stabilization period, we infused artificial cerebrospinal fluid (ACSF) or tempol (53 or 105 μmol/kg in 10 μL ACSF) into the lateral ventricle in 10 minutes and recorded changes in the parameters. The dose of tempol was determined as described previously.
- The effect of intracerebroventricular apocynin (1.05 or 2.1 μmol/kg in ACSF) or diphenyleneiodonium (DPI; 13 μmol/kg in ACSF), an inhibitor of NADPH oxidase, was also examined. Accuracy of the intracerebroventricular injection was confirmed by injection of Evans blue dye after each experiment.

#### Measurement of NADPH-Induced Superoxide Production

Production of NADPH-induced superoxide anions was measured by bis-N-methylacridinium (lucigenin) chemiluminescence in the isolated hypothalamus, where several nuclei critically involved in cardiovascular regulation are known to be located, as described previously.

#### Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction for NADPH Oxidase Subunits

NADPH oxidase subunit p22phox, p47phox, gp91phox mRNA expression in the isolated hypothalamus was evaluated by real-time quantitative reverse-transcription polymerase chain reaction according to the procedure described previously. Assay-on-demand primers and probes sets (Applied Biosystems, Tokyo, Japan) were used for the rat p22phox, p47phox, gp91phox, and β-actin.

#### Statistical Analysis

All values are presented as mean±SEM. In the intracerebroventricular administration experiments, the baseline value was defined as the mean value over a 1-minute stabilization period before administration of drugs, and the peak value was defined as a mean value for 10 seconds around the maximum response. Magnitudes of changes...
Effects of HF on Total Body Weight, Visceral Fat Weight, and Blood Pressure

The body weight in HF-fed rats increased significantly after 3 weeks of treatment (3 weeks: 286.4±2.1 versus 264.6±6.4 g for HF- and LF-fed rats, respectively; P=0.004; 6 weeks: 436.2±5.8 versus 391.8±9.0 g; P<0.001; n=10, respectively). The visceral fat weight was significantly greater in HF-fed rats (n=10) than in LF-fed rats (n=8) (retroperitoneal fat, 12.4±0.8 versus 6.1±0.9 g; P<0.001; epididymal fat, 9.0±0.6 versus 5.3±0.6 g; P<0.001; total mesenteric fat, 8.5±0.4 versus 5.6±0.8 g; P=0.003). Thus, the larger body weight in the rats was associated with a greater accumulation of visceral fat mass in the present study.

Like body weight, systolic blood pressure was significantly elevated in HF-fed rats (n=10) compared with LF-fed rats (n=10) from 3 (148±3 versus 126±2 mm Hg; P<0.001) to 6 (148±3 versus 129±3 mm Hg; P<0.001) weeks of treatment. In the conscious state, MAP also was significantly elevated in HF-fed rats (n=4) compared with the LF-fed rats (n=4) by direct measurement (113±2 versus 94±8 mm Hg; P=0.048).

Effects of HF on Metabolic Parameters

Fasting blood glucose tended to be higher in HF-fed rats compared with LF-fed rats (P=0.058; the Table). Moreover, serum insulin was apparently higher in HF-fed rats (P<0.001). Thus, insulin resistance was enhanced in HF-fed rats as indicated by the homeostasis model assessment of insulin resistance (P<0.001). Serum leptin concentrations also were elevated in HF-fed rats (P<0.001). However, no significant differences were found in serum cholesterol, triglycerides, and free fatty acids between HF- and LF-fed rats.

Twenty-Four-Hour Urinary Norepinephrine and Effects of Ganglionic Blockade With Hexamethonium Hydrochloride

Urinary norepinephrine excretion increased in HF-fed rats (n=10) compared with LF-fed rats (n=10) (4 weeks of treatment: 1.1±0.1 versus 0.7±0.1 μg/24 h; P=0.003; 6 weeks: 1.4±0.0 versus 1.1±0.1 μg/24 hours; P=0.032). Moreover, the MAP reduction induced by hexamethonium was significantly greater in HF-fed rats (−62±1.5 versus −46±3.5 mm Hg; P=0.006; n=4). Both results suggest that sympathetic activity increased significantly in HF-fed rats compared with LF-fed rats.

Effects of Intracerebroventricular Tempol on MAP, HR, and RSNA

MAP, integrated RSNA, and HR values started decreasing a few minutes after intracerebroventricular temporal administration and reached their lowest level within ≈15 minutes, after which they gradually returned to the respective control levels within ≈30 minutes (Figure 1). However, in some cases of HF-fed rats, the reduction continued for >30 minutes. In all of the rats, MAP, HR, and RSNA values decreased in a dose-dependent fashion (Figure 2). The reduction in MAP was significantly (P<0.001) greater in HF-fed rats than in LF-fed rats (−13.3±2.1% versus −5.5±2.0% at 53 μmol/kg tempol and −32.7±3.0% versus −13.4±2.4% at 105 μmol/kg tempol). Similarly, the reduction in RSNA was significantly (P<0.001) greater in HF-fed rats (−8.8±2.4% versus −1.9±0.5% and −13.4±1.7% versus −5.1±1.0%). The reduction in HR also was stimulated (P=0.004) in HF-fed rats (−7.9±1.8% versus −0.8±1.7% and −13.4±3.1% versus −4.5±0.6%). Thus, downward shifts were observed in the dose-response curves of the MAP, RSNA, and HR values for intracerebroventricular tempol in HF-fed rats compared with LF-fed rats (Figure 2).

Effects of Intracerebroventricular Apocynin or DPI on MAP, HR, and RSNA

Intracerebroventricular apocynin or DPI, an NADPH oxidase inhibitor, also elicited remarkably greater reductions in MAP and RSNA in HF-fed rats. As with tempol, intracerebroventricular apocynin decreased MAP and RSNA in a dose-dependent fashion (Figure 3). The sample size of the intracerebroventricular apocynin experiment was too small (n=3) to analyze statistically. Therefore, we have presented the scatterplot of the percentage change in MAP and RSNA against dose with a line joining the results of each rat in the online-only Data Supplement (Figure I). The rats from the same group demonstrated very similar responses by dose, and the dose-response curves of the MAP and RSNA values showed markedly greater downward shifts in HF-fed rats compared with LF-fed rats. Intracerebroventricular DPI also decreased MAP and RSNA values in HF-fed rats but not in LF-fed rats; intracerebroventricular DPI-induced reductions in the MAP and RSNA values were significantly greater in
HF-fed rats (MAP, \( P=0.004 \); RSNA, \( P=0.015 \); Figure 4). The response of HR to intracerebroventricular apocynin or DPI varied with each animal even in the same groups and did not show any difference between HF- and LF-fed rats.

**Measurement of NADPH-Induced Superoxide Production in the Isolated Hypothalamus**

NADPH-induced superoxide production increased significantly in the isolated hypothalamus from HF-fed rats compared with LF-fed rats (3.30\( \pm \)0.28\( \times \)10^6 versus 2.20\( \pm \)0.11\( \times \)10^6 relative light units per 10 minutes per 1 g; \( P=0.005 \); Figure 5).

**mRNA Expression for NADPH Oxidase Subunits in the Hypothalamus**

The mRNA expression levels for the NADPH oxidase subunits p22^phox, p47^phox, and gp91^phox were significantly increased in the hypothalamus from HF-fed rats compared with LF-fed rats (p22^phox, \( P=0.039 \); p47^phox, \( P=0.036 \); gp91^phox, \( P<0.001 \); Figure 6).

**Discussion**

In the present study, we have demonstrated 3 major findings. First, high fat loading caused sympathoexcitation and hyper-
tension, along with obesity-related metabolic disorder. Second, reductions in RSNA and AP values elicited by intracerebroventricular administration of the antioxidant tempol, apocynin, or DPI were significantly greater in HF-fed rats than in LF-fed rats. Third, hypothalamic NADPH oxidase activity and mRNA expression were higher in HF-fed rats than LF-fed rats. These results suggest that obesity enhances ROS generation in the brain via activation of NADPH oxidase, which leads to central sympathoexcitation and hypertension. To the best of our knowledge, these are the first findings providing evidence that there may be a close association between oxidative stress and sympathoexcitation in the brain of obesity-induced hypertensive subjects.

A number of reports suggest that ROS overproduction in the kidneys, heart, and arteries is involved in obesity-induced hypertension. However, the role of ROS in the brain has never been elucidated, although high-dietary-fat has been reported to induce NADPH oxidase–derived oxidative stress and inflammation in the brain. Our viewpoint that oxidative stress in the brain exerts an effect on central sympathoexcitation in obesity-induced hypertension is plausible because recent studies have also implicated the contribution of ROS overproduction in the brain to activation of the sympathetic nervous system. For example, microinjection of tempol into the dorsomedial hypothalamus or the rostral ventrolateral medulla attenuated the pressor and sympathoexcitatory responses to emotional stress in rabbits. Microinjection of superoxide dismutase into rostral ventrolateral medulla of anesthetized pigs reduced AP, HR, and RSNA values. Intracerebroventricular infusion of antioxidants, including in this study, elicited a direct sympathoinhibitory effect, suggesting that ROS might directly stimulate central sympathetic nerve activity. Moreover, in stroke-prone spontaneously hypertensive rats, the ROS level was increased in the rostral ventrolateral medulla, and bilateral microinjection of tempol into the rostral ventrolateral medulla decreased AP.

Intracerebroventricular administration can act in the hypothalamic area, which is supposed to contain sodium sensors and osmosensors and several nuclei critically involved in the cardiovascular regulation system such as the subfornical organ, paraventricular hypothalamic nuclei, and organum vasculosum of the stria terminalis. Other regions of the brain such as the rostral ventrolateral medulla have also been recognized as key areas in the normal and reflex control of AP. In contrast, previous studies, including those in our laboratory, with intracerebroventricular infusion of antioxidants suggest that the hypothalamus is a critical area in the ROS generation and maintenance of sympathetic control of the cardiovascular system. These results are compatible with the present data although the region-related functions of the brain are complicated and remain unknown.

Although the present study has demonstrated that ROS generation may be mediated through the activation of NADPH oxidase, the potential mechanisms underlying ROS generation in the brain of obese rats remain unclear. Emerging bodies of evidence suggest that several possible factors...
contribute to the excessive formation of ROS in obesity, although the mechanism may be complicated. Several studies have shown that the central renin-angiotensin system mediates ROS generation, and it is well known that adipose tissue can secrete angiotensinogen. In obese Zucker rats, brain angiotensin II type 1 receptor mRNA expression and pressor response to intracerebroventricular angiotensin II were partially increased compared with lean rats. Although centrally administered angiotensin II exhibited a sympathoexcitatory action possibly through its ROS-generating effect, the central renin-angiotensin system has not been fully investigated in obese hypertensive animals. On the other hand, insulin resistance may be critical in ROS overproduction and sympathoexcitation because HF-fed rats exhibited significantly elevated homeostasis model assessment of insulin resistance. Rats fed a high fructose diet, a model of insulin resistance, have in fact increased ROS generation and attenuated baroreflex function. In addition, a peroxisome proliferator-activated receptor-γ agonist, pioglitazone, which ameliorates insulin resistance, prevented hypertension and oxidative stress in diet-induced obese rats. Although no reports have suggested that insulin acts as an antioxidant, insulin resistance may be related to ROS generation through several mechanisms. For example, one of the plausible candidates to stimulate ROS generation in the brain may be leptin, a polypeptide hormone mediator produced by adipocytes, because serum leptin concentrations were elevated in HF-fed rats. Leptin can generate oxidative stress, as has been reported for endothelial cells in culture. In addition, leptin indirectly stimulates production of inflammatory cytokines such as interleukin-6.

Figure 4. Percentage changes in MAP (A), RSNA (B), and HR (C) values from HF- and LF-fed rats in response to intracerebroventricular DPI, an NADPH oxidase inhibitor. Intracerebroventricular DPI decreased MAP and RSNA in HF-fed rats but not in LF-fed rats. The intracerebroventricular DPI-induced changes in the MAP and RSNA values were significantly different between HF- and LF-fed rats. Data are presented as mean±SEM.

Figure 5. Reduced NADPH-dependent superoxide anion production assessed by lucigenin chemiluminescence in the isolated hypothalamic sections from HF- and LF-fed rats. The chemiluminescence value was significantly higher in HF- than LF-fed rats. Data are represented as mean±SEM. RLU indicates relative light units.
and tumor necrosis factor-α, which increase NADPH oxidase production. However, no data suggest that leptin stimulates ROS generation in the brain. High blood glucose, which marginally increased in HF-fed rats, could generate ROS through synthesis of advanced glycation end product (AGE). The receptor for AGE also exists in neural cells, and AGE has been shown to stimulate p47phox expression in neuroblastoma cells. However, no reports exist on AGE or the receptor for AGE in the brain of obesity-induced hypertensive animal models. Moreover, any abnormality in the ROS-scavenging system might contribute to ROS upregulation in obesity. Thus, further study is needed to clarify the mechanisms underlying ROS generation in the brain of obese hypertensive animals.

Obesity-induced hypertension has been demonstrated to elicit salt-sensitive hypertension and vice versa. Both exhibit increased oxidative stress and sympathoexcitation. We have recently suggested that in salt-sensitive hypertension, increased oxidative stress in the brain, possibly via activation of NADPH oxidase, might mediate AP elevation through central sympathetic activation, which is quite similar to the HF-fed rats. Thus, our series of studies, including the present one, suggest a possible common pathogenic background: increased ROS production in the brain and central sympathoexcitation in obesity-induced and salt-sensitive hypertension.

Dobrian et al demonstrated that HF caused obesity in approximately half of the Sprague-Dawley rats but not in the other half. However, in our study, all Sprague-Dawley rats fed HF gained more weight compared with LF-fed rats. This discrepancy may be due to the difference of age (adulthood versus childhood) at the beginning of HF loading. In the former studies, rats with a body weight of 300 to 350 g were used at the start of fat loading, whereas the mean body weight of rats used in the present study was 79.4 g (range, 67 to 87 g). In all rats, which began to eat HF at 3 weeks of age and had been loaded for 10 weeks, body weight gain was accelerated in a manner similar to that in our study. These findings suggested that HF feeding from a juvenile age may induce a greater risk for the progression of obesity and hypertension beyond any individual genetic difference. Currently, hypertension in young children is increasing in prevalence, along with an obesity epidemic. Moreover, juvenile hypertensive patients with left ventricular hypertrophy have a greater body mass index and a greater number of metabolic syndrome components. Juvenile hypertension with obesity is associated with an increased risk of cardiovascular disease. Therefore, the present study involving HF-fed young rats implies that strategies to control body weight in youth are critical to control sympathetic overactivity possibly caused by ROS upregulation in the brain, which leads to adult hypertension and resultant cardiovascular disease.

Conclusions
The present study suggests for the first time that in obesity-induced hypertension, increased oxidative stress in the brain, possibly via activation of NADPH oxidase, may mediate AP elevation through central sympathetic activation. Based on our new insights, a novel strategy such as administration of an antioxidant factor with a sympathoinhibitory effect may be useful for preventing and managing hypertension associated with the metabolic disorder.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

Metabolic syndrome, a highly predisposing condition for cardiovascular disease caused by visceral obesity, requires appropriate management. However, the detailed mechanisms have not been fully elucidated. High salt intake increases blood pressure to a greater degree in patients with metabolic syndrome than in those without it. In the present study, we have demonstrated that sympathoexcitation via oxidative stress in the brain may mediate arterial pressure elevation in obesity-induced hypertension. We have also demonstrated the similar mechanisms in salt-sensitive hypertension in a previous study. Therefore, our series of findings suggest that sympathoexcitation via oxidative stress in the brain could be a possible common pathogenic background in metabolic syndrome. This finding is also supported by bodies of clinical studies that suggest that sympathoexcitation and oxidative stress are associated with metabolic syndrome, as well as salt-sensitive and obesity-induced hypertension. An antioxidant with a sympathoinhibitory effect may become a candidate for a new therapeutic strategy to manage the patient with metabolic syndrome.
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Online Data Supplements

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Figure legend

Figure S1: Appendix of Figure 3. The scatter plot of percent change in mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA) against dose with a line joining the results of each rat in the experiment of intracerebroventricular apocynin. The rats from the same group have similar responses, and the dose-response curves of MAP and RSNA showed apparently greater downward shifts in HF compared with LF. LF: low-fat diet-fed rat; HF: high-fat diet-fed rat.