Ghrelin counteracts salt-induced hypertension via promoting diuresis and renal nitric oxide production in Dahl rats

Hirotaka Aoki¹, Masanori Nakata¹, Katsuya Dezaki¹, Ming Lu¹, Darambazar Gantulga¹, Keiji Yamamoto², Kazuyuki Shimada², Kazuomi Kario² and Toshihiko Yada¹

¹) Department of Physiology, Division of Integrative Physiology, Jichi Medical University School of Medicine, Shimotsuke 329-0498, Japan
²) Department of Internal medicine, Division of Cardiovascular Medicine, Jichi Medical University School of Medicine, Shimotsuke 329-0498, Japan
³) Department of General Internal Medicine, Saitama Medical School, Saitama 350-0495, Japan

Abstract. Ghrelin is the endogenous ligand for the growth hormone-secretagogue receptor expressed in various tissues including the heart, blood vessels and kidney. This study sought to determine the effects of long-term treatment with ghrelin (10 nmol/kg, twice a day, intraperitoneally) on the hypertension induced by high salt (8.0% NaCl) diet in Dahl salt-sensitive hypertensive (DS) rats. Systolic blood pressure (SBP) was measured by a tail cuff method. During the treatment period for 3 weeks, high salt diet increased blood pressure compared to normal salt (0.3% NaCl) diet, and this hypertension was partly but significantly (P<0.01) attenuated by simultaneous treatment with ghrelin. Ghrelin significantly increased urine volume and tended to increase urine Na⁺ excretion. Furthermore, ghrelin increased urine nitric oxide (NO) excretion and tended to increase renal neuronal nitric oxide synthase (nNOS) mRNA expression. Ghrelin did not alter the plasma angiotensin II level and renin activity, nor urine catecholamine levels. Furthermore, ghrelin prevented the high salt-induced increases in heart thickness and plasma ANP mRNA expression. These results demonstrate that long-term ghrelin treatment counteracts salt-induced hypertension in DS rats primarily through diuretic action associated with increased renal NO production, thereby exerting cardio-protective effects.

Key words: Salt-sensitive hypertension, Ghrelin, Nitric oxide, Diuresis, Cardiac protection

GHRELIN is the endogenous ligand for the growth hormone (GH)-secretagogue receptor (GHS-R) [1]. Ghrelin, a 28-amino acids peptide, is acylated at the 3rd serine residue by the GOAT [2, 3], and is produced predominantly in X/A like cells in the stomach [4] and also in the small intestines, heart, pancreas, liver and brain [5]. Secretion of ghrelin is stimulated by fasting and inhibited by feeding [4], resulting in pre-prandial rise of the plasma ghrelin level [6]. The plasma ghrelin level is also correlated inversely with body mass index (BMI), low in obese patients and high in patients with anorexia nervosa and cardiac cachexia [7, 8]. Ghrelin stimulates GH secretion [1], promotes feeding [9], and decreases insulin secretion and increases blood glucose levels [10, 11].

GHS-R is expressed in a variety of tissues, including the heart, blood vessels and kidney [5], suggesting possible cardiovascular effects of ghrelin. It has been reported that ghrelin accelerates the contractile activity of the heart through the release and action of GH both in healthy volunteer and in human patients and rat models with cardiac failure [12, 13, 14]. Regarding the effect on blood pressure (BP), administration of ghrelin reportedly induces hypotensive effect in animal models with normal BP via inhibiting sympathetic nervous system [15] and vasodilatation [16], while fails to influence BP and heart rate (HR) in healthy humans [17]. In the patients with cardiac failure, ghrelin administration fails to influence BP and HR [18]. Thus, the effect of ghrelin on BP is in controversy and remains to be determined. It should be noted that the effect of ghrelin to lower BP has been reported only in healthy humans and
animals but not in those with hypertension. Therefore, it is of particular importance to study the effect of ghrelin on BP in animals and patients with hypertension.

Hypertension is a common health disorder serving as a major risk factor for the cardiovascular disease, stroke, and organ impairment. Recently, a type of hypertension associated with obesity or metabolic syndrome is noticeably increasing, possibly due to the changes in the lifestyle including eating habits and social stresses. The salt-sensitivity is considered one of principal processes that develop hypertension including that associated with obesity. It has recently been reported that plasma ghrelin level is elevated and antagonism of the ghrelin receptor induces earlier onset of salt-sensitive hypertension in Dahl rats, suggesting that endogenous ghrelin serves as an anti-hypertensive hormone [19]. The aim of the present study is to clarify the effect of treatment with ghrelin on the salt-induced hypertension and to elucidate the underlying mechanisms in Dahl salt-sensitive rats, an established model of hypertension.

Methods

Animals

Five-week-old male Dahl-Iwai salt-sensitive (DS) rats were obtained from Japan SLC (Hamamatsu, Japan). Rats were maintained at a constant room temperature (24°C) on a 12-hour light/dark cycle (7:30 light on, 19:30 light off), and took chow and water freely. Rats were fed high salt chow containing 8.0% NaCl (Oriental Yeast Co.), or control salt chow containing 0.3% NaCl (Oriental Yeast Co., Tokyo, Japan).

Treatment with high salt and ghrelin

Rats were fed control chow and accustomed to the environment for one week. They were then divided into 4 groups; high salt chow with intraperitoneal (ip) ghrelin injection, high salt chow with ip vehicle injection, control chow with ip ghrelin injection, and control chow with ip vehicle injection groups (Fig. 1). Ghrelin (Peptide Institute, Osaka, Japan) was administered ip at 10 nmol/kg twice a day.

Measurements of blood pressure and pulse

Systolic blood pressure was measured by tail cuff method (Model MK-2000, Muromachi Co., Tokyo, Japan) in rats without anesthesia at 3 hours after administration of ghrelin or vehicle every week.

Blood and urine collections

Blood samples were collected from coccygeal vein

Fig. 1 Protocols for high salt diet, ghrelin treatment, and assessment of their effects during 3 weeks period. Male Dahl salt-sensitive (DS) rats at 5 weeks of age were fed a control chow for a week and then divided into 4 groups; control low salt (0.3% NaCl) chow, high salt (8.0% NaCl) chow, low salt chow with ghrelin administration, and high salt chow with ghrelin administration groups. Ghrelin was administered ip at 10 nmol/kg twice a day for 3 weeks. Systolic blood pressure (SBP) was measured by tail cuff method every week. Blood samples were collected at 2 weeks and heart and kidney were collected at 3 weeks of the treatment.
at 2 weeks after the beginning of ghrelin administration. Plasma concentrations of atrial natriuretic peptide (ANP) were measured with enzyme immunoassay method (Phoenix Pharmaceutical Inc., CA, USA and Cayman Chemical Company, MI, USA, respectively). Plasma renin activities and angiotensin II concentrations were measured with radioimmunoassay double-antibody method (SRL Inc., Tokyo, Japan). Growth hormone concentrations were measured with radioimmunoassay solid phase method (SRL), and Na\(^+\) and K\(^+\) concentrations with electrode method (SRL). Urinary sample were collected with the rat metabolic cage KN-649 (Natsume Co. Ltd., Tokyo, Japan) at 2 hours after beginning of ghrelin administering. Urinary NO\(_3\) (NO\(_2\)+NO\(_3\)) concentrations were measured with Griess method (Dojindo Laboratories, Tokyo, Japan), urinary catecholamine concentrations with high performance liquid chromatography (SRL), and urinary Na\(^+\) and K\(^+\) concentrations with the electrode method (SRL).

**Echocardiography**

Cardiac function was measured by echocardiography (ProSound SSD-α5, ALOKA Co. Ltd., Tokyo, Japan) under the sevoflurane inhalation anesthetizing at 2 weeks after beginning of ghrelin administration. Left ventricular internal dimension in diastole, left ventricular internal dimension in systole, intraventricular septum thickness, and left ventricular posterior wall thickness were measured.

**RT-PCR**

We removed rat’s heart and kidney by using an isoflurane at 3 weeks after beginning of ghrelin administration, and extracted total RNA by using TRIzol (Invitrogen, WI, USA). We then removed genome DNA by the RNase-free DNase (Promega, WI, USA) processing, and synthesized cDNA by using ReverTraAce (TOYOBO, Osaka, Japan). We analyzed the gene expression by the real time PCR that used SYBR Premix EX taqTM II (TAKARA, Tokyo, Japan). We corrected it by 18S Ribosomal RNA, and analyzed it by the ΔΔCT method. The base sequences of primers used are as follows.

Rat ANP: forward; 5’-TGACACGGATGAGCCAGAC-3’
reverse; 5’-TCGAGCAGATTGGCTTATCTTC-3’

Rat nNOS: forward; 5’-CTATGCAAAGACCTGTGTGGA-3’
reverse; 5’-GTGGCAAAGGTGCGTGTTG-3’

Rat eNOS: forward; 5’-AGCGAGTAGAACCAGCCCG-3’
reverse; 5’-CCTCGTGAGCTTGTCCCTGA-3’

18S ribosomal RNA: forward; 5’-TTCGAACGTCCTGCTATCAA-3’
reverse; 5’-ATGGTAGCAGCGGCGACT-3’

**Statistical analysis**

Data are expressed as mean±SE. Statistical analyses were performed using multiple comparisons after ANOVA. Unpaired t-test was used for a part of result. P values < 0.05 were considered statistically significant.

**Results**

**Blood pressure and pulse rate**

The systolic blood pressure (SBP) in DS rats fed high salt (8%NaCl) chow increased compared to that fed control low salt chow at 1, 2 and 3 weeks after beginning of high salt administration (Fig. 1A). Ghrelin was administered during the same period as that for high salt chow load. Ip injection of ghrelin twice a day attenuated the high salt chow-induced increase in SBP at 1 and 2 weeks, and tended to attenuate it at 3 weeks, after beginning of high salt administration (129.5±1.7 mmHg with ghrelin vs. 150.5±1.7 mmHg with vehicle, at 2 weeks) (Fig. 2A). Ip injection of ghrelin had no effect on SBP in rats fed control chow. On the other hand, high salt chow had no significant effect on the pulse rate (PR) during the 3 weeks period, and administration of ghrelin did not influence PR in both high salt and control chow groups (Fig. 2B). Thus, ghrelin attenuated the high salt-induced hypertension without altering PR.

**Urinary volume and water intake**

The urine volume and amount of water intake were measured under fed conditions at 2 weeks after high salt and/or ghrelin administration. The volume of urine increased in the high salt group, and it was further increased significantly by ghrelin administration in the high salt group (Fig. 3A), while ghrelin had no effect in the low salt group. The water intake tended to increase in response to high salt chow and it was significantly elevated by ghrelin administration (Fig. 3B), while ghrelin had no effect on water intake in the low salt group. Food intake was not different between all groups (Fig. 3C). The body weight gain was decreased by ghrelin administration in the high salt group (Fig. 3D). These results of ghrelin-induced increase in urinary volume and decrease in weight gain in the high salt group suggest that ghrelin promotes diuresis, thereby possibly decreasing preload.
Fig. 2  Systolic blood pressure and pulse rate.
A, Rats fed with high salt chow for 3 weeks showed a progressive increase in systolic blood pressure (SBP). Ip injection of ghrelin twice a day for 3 weeks counteracted the hypertensive effect of high salt. Ghrelin did not influence the blood pressure in rats fed with control chow. B, Ghrelin did not influence PR in both high salt chow and control chow groups.

Fig. 3  Urinary volume and water intake under fed conditions.
A, Urinary volume was increased by high salt chow and further increased by treatment with ghrelin. Ghrelin had no effect on urinary volume in control chow group. B, Water intake tended to be increased by high salt chow, which was further increased by administration of ghrelin. C, Food intake was not different among four groups. D, Body weight gain was reduced by treatment with ghrelin. **p < 0.01, *p < 0.05.
Anti-hypertensive effects of ghrelin

**Urinary excretion and serum concentrations of Na⁺ and K⁺**

Urinary excretion of Na⁺ measured under fed conditions increased in high salt group, and this increased level tended to be further elevated by ghrelin administration (Fig. 4A). Na⁺ concentration in the urine increased in high salt group (Fig. 4B). These data suggested that the increases in urinary volume by high salt and ghrelin were caused by increases in the urinary sodium excretion.

Urinary K⁺ excretion measured under fed condition tended to decrease in high salt group as compared to low salt group, in which the change was statistically significant only in the presence of ghrelin, and in both high and low salt groups it tended to be increased by administration of ghrelin (Fig. 4C). The urinary K⁺ concentration tended to decrease in high salt groups, in which the change was statistically significant only in the presence of ghrelin (Fig. 4D). Together with the result that ghrelin enhanced the high salt action to increase Na⁺ excretion, it was suggested that ghrelin might suppress the counter transport of Na⁺ reabsorption and K⁺ excretion in the kidney. Plasma Na⁺ and K⁺ concentrations were unaltered by high salt and by ghrelin (Fig. 4E, F).

**Urinary NOₓ excretion and NOS expression in the kidney**

The amount of the urinary NOₓ (NO₂⁺NO₃⁻) excretion measured under fasting conditions was markedly elevated by administration of ghrelin in the high salt,
endothelial NOS (eNOS) mRNA was changed neither by high salt nor by ghrelin administration in the renal cortex and medulla (Fig. 5E, F). Similarly, inducible NOS (iNOS) was not altered by high salt and ghrelin administration (data not shown).

**Plasma angiotensin II and catecholamine levels and renin activity**

Plasma renin activity decreased (Fig. 6A) and plasma angiotensin II concentration tended to decrease (Fig. 6B) in high salt group. Administration of ghrelin did not alter the plasma renin activity and angiotensin II level in high salt, as well as low salt, groups. Urinary NOx concentration in the high salt and ghrelin-treated group was significantly elevated compared to the low salt and vehicle-treated control group (Fig. 5B).

We measured the mRNA expression of nitric oxide synthase (NOS) in the renal cortex and medulla. The expression of neuronal NOS (nNOS) mRNA tended to be increased by high salt chow loading and by ghrelin administration in the renal medulla and cortex (Fig. 5C, D). The nNOS mRNA expressions in the renal medulla and cortex were both significantly elevated in the high salt, ghrelin-treated group compared to the low salt, vehicle-treated control group (Fig. 5C, D). The endothelial NOS (eNOS) mRNA was changed neither by high salt nor by ghrelin administration in the renal cortex and medulla (Fig. 5E, F). Similarly, inducible NOS (iNOS) was not altered by high salt and ghrelin administration (data not shown).

**Fig. 5** NOx in the urine and NOS profiles in the kidney.

A, Ghrelin significantly increased urinary NOx excretion in high salt chow group but not low salt chow group. B, High salt plus ghrelin treatment group showed a significantly higher level of urinary NOx concentration ([NOx]) than low salt control group. C and D, High salt plus ghrelin groups showed significantly higher mRNA expressions of neural nitric oxide synthase (nNOS) in the renal medulla (C) and cortex (D) compared with low salt control groups. E and F, Ghrelin did not influence mRNA expressions of endothelial nitric oxide synthase (eNOS) in the renal medulla (E) and cortex (F). *p < 0.05, **p < 0.01.
adrenaline and noradrenalin excretions decreased in high salt group, and ghrelin influenced neither of them (Fig. 6C, D).

Assessment of cardiac hypertrophy

The intraventricular septum thickness was larger in high salt than low salt group, and this hypertrophy was significantly (p<0.01) attenuated by administration of ghrelin (Fig. 7A), while it had little effect on the thickness in the low salt group.

The plasma concentration of atrial natriuretic peptide (ANP), a maker of the cardiac dysfunction, tended to be increased by high salt loading (Fig. 7B), and this change was abolished by administration of ghrelin, while it was without effect on the plasma ANP level in low salt group.

The expression of ANP mRNA in the ventricular myocardium was markedly increased by high salt loading, and this increase was significantly (p<0.01) counteracted by ghrelin treatment (Fig. 7C), while it had little effect in low salt group.

Since plasma ANP level reflects the cardiac preload, our data suggested that the cardiac preload increased under high salt loading, and that ghrelin treatment reduced it by increasing the urinary volume.

Growth hormone

Plasma growth hormone (GH) concentration did not change upon high salt loading (Fig. 7D), but tended to increase in response to ghrelin administration in both high salt and low salt groups.

Discussion

This study revealed antihypertensive effect of ghrelin against high salt chow-induced hypertension in rats. By contrast, ghrelin fails to affect the blood pressure

![Fig. 6](image_url) Levels of renin, angiotensin and catecholamines.

A, High salt chow decreased plasma renin activity, and ghrelin did not influence it in both high and low salt chow groups. B, High salt chow tended to decrease plasma angiotensin II concentration, and ghrelin did not influence it in both high and low salt chow groups. C, High salt chow in the absence of ghrelin decreased urinary adrenaline excretion, and ghrelin did not influence it in both high and low salt chow groups. D, High salt chow decreased urinary noradrenalin excretion, and ghrelin did not influence it in both high and low salt chow groups. *p < 0.05, **p < 0.01.
in normotensive rats fed with control chow. Ghrelin increases the urinary volume via renal NOx system, which may serve as the mechanisms underlying ghrelin’s antihypertensive action. Furthermore, ghrelin attenuates the high salt-induced elevation of plasma ANP levels and cardiac wall thickness, showing a cardioprotective effect.

The effects of ghrelin to decrease blood pressure have been reported in the animals with normal blood pressure [15], the healthy individuals [13], and the patients with heart failure [14]. In animals and individuals with hypertension, in contrast, the effect of ghrelin to lower blood pressure has not been documented by now. In addition, previous reports examined only acute effects of ghrelin, in the range of several hours after single administration. In this study, we used a rat model of high salt-induced hypertension and examined the effect of ghrelin administration twice a day for 3 weeks. We found that ghrelin attenuates the high salt-induced hypertension during the 3 weeks period of treatment, revealing a novel long-lasting antihypertensive effect of ghrelin. This finding provides a basis for a clinical application of ghrelin or its derivatives to treat patients with hypertension.

Ghrelin increased the urinary volume and tended to increase the sodium excretion in high-salt treated hypertensive rats. It is suggested that these diuretic effects may largely account for the reduction of weight gain by ghrelin treatment, since ghrelin had no effect on food intake. It has been reported that iv administration of ghrelin in the patients with heart failure had no effect on the urinary volume at 60 min after administration [14]. The apparent discrepancy between their and our results could be due to the different method of administration of ghrelin; they observed the acute effect of single infusion of ghrelin whereas we observed the chronic

![Fig. 7](image_url)

**Fig. 7** Cardiac hypertrophy, plasma ANP and growth hormone levels, and ANP mRNA level in the heart.
A, High salt chow thickened the intraventricular septum. The high salt chow-induced cardiac hypertrophy was attenuated by ghrelin. B, High salt chow tended to increase plasma ANP concentration and ghrelin tended to attenuate the change. C, High salt chow increased the ANP mRNA expression, and this increase was blocked by ghrelin. In low salt control group, ghrelin did not influence ANP mRNA level. D, Ghrelin tended to increase plasma GH concentration in high and low salt chow groups. *p < 0.05, **p < 0.01.
effect of ghrelin administered twice a day for 3 weeks. Therefore it is suggested that some mechanism elicited only by a long-term administration, but not a single infusion, of ghrelin could promote diuresis to decrease blood pressure. Alternatively, ghrelin could decrease blood pressure only under hypertensive states.

Plasma renin level and angiotensin II concentration were markedly reduced in high salt-diet hypertensive rats compared to control normotensive rats irrespective to the ghrelin treatment. This suppression of the renin-angiotensin system associated with hypertension is in agreement with the previous report [20] and could reflect the compensatory response to the rise in the blood pressure caused by high salt diet. Urine levels of catecholamines were reduced in high salt-diet hypertensive rats, which may also reflect compensatory response to hypertension.

In humans, the half-life of total plasma ghrelin was reported to be around 27-31 min, possibly reflecting the rapid degradation of acylated active ghrelin in the plasma [21]. This half-life of the plasma ghrelin appears to be too short to explain the longer-lasting blood pressure-lowering effect of ghrelin. Hence, the effect of ghrelin on blood pressure may involve a chronic action, including a change in gene expression. In fact, ghrelin increased the urinary NO\textsubscript{x} excretion and tended to elevate urinary NO\textsubscript{x} concentration and nNOS mRNA expressions in the medulla and cortex of kidney. These results suggest that ghrelin increases NO\textsubscript{x} possibly by promoting nNOS in the kidney. It has been reported that nNOS takes part in the pathogenesis of the salt sensitive hypertension [20]. It was reported that nNOS is localized most abundantly in the macula densa of the adult kidney where this molecule plays a critical role in the tubuloglomerular feedback mechanism [22], though it is also distributed within the pre- and postglomerular resistance vessels and tubules. Moreover, selective blockade of nNOS in the macula densa amplifies the TGF response and induces hypertension [23], and that nNOS in the macula densa is impaired in Dahl salt-sensitive rats [24]. Furthermore, nNOS inhibits Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} co-transporter at the ascending limb of Henle loop in the kidney [25]. These nNOS-related mechanisms could mediate the effects of ghrelin to increase urinary sodium and water excretion and to eventually ameliorate the high salt-induced hypertension. Although the mechanism underlying the diuretic effect of ghrelin is yet uncertain, it is a unique action of ghrelin. Further study is definitely required to identify the mechanism of ghrelin action.

It has been reported that ghrelin promotes eNOS mRNA expression [26]. In our study, however, neither high salt nor ghrelin altered the eNOS mRNA expression and the NO\textsubscript{x} concentration in the blood (data not shown).

It is well known that the plasma ANP, as well as BNP, levels increase in association with the left ventricular hypertrophy, cardiac failure, and an increase of the circulatory blood flow, and that the increased ANP level is attenuated upon successful treatment in rats and humans [27, 28]. Therefore ANP is considered a useful marker for the increased circulatory blood flow, cardiac hypertrophy, and cardiac failure [27, 28]. In the present study, expression of ANP mRNA in the ventricular myocardium was markedly elevated by the salt loading, and this change was prevented by co-administration of ghrelin. Moreover, ultrasonographic analysis demonstrated that the salt loading induced the left ventricular hypertrophy and it was inhibited by co-administration of ghrelin. Our results suggest that the salt loading induces the circulatory blood flow increase associated with left ventricular hypertrophy reflected by a rise in ANP, and co-administration of ghrelin increases the urinary volume and thereby counteracts the circulatory blood flow increase and left ventricular hypertrophy associated with a fall in ANP. Our results suggest that the ghrelin treatment reduces the preload and prevents the cardiac hypertrophy, thereby improving cardiac functions.

In the present study, ghrelin administration tended to elevate plasma GH levels. It is known that GH, directly and/or secondarily via releasing insulin-like growth factor-1, increases cardiac muscle contraction [29] and blood glucose levels [8]. In the present study, neither cardiac hypertrophy nor hyperglycemia was observed during the 3 weeks period of administration (data not shown).

This study has suggested that the long-term ghrelin treatment counteracts salt-induced hypertension in DS rats primarily through the diuretic action associated with elevated renal NO system, and that all these effects collectively act as cardioprotective. Our data suggest the preventive and therapeutic abilities of long-term ghrelin treatment against salt-sensitive hypertension and cardiac hypertrophy. Toward its clinical application, it is required to further study the effects of long-term ghrelin treatment on cardiovascular and renal functions, as well as on glycemia.
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Conflict of Interests

None of the authors have any conflicts of interest associated with this study.

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