Inhibitory effects of alpha-lipoic acid on oxidative stress in the rostral ventrolateral medulla in rats with salt-induced hypertension

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Abstract. Oxidative stress in the rostral ventrolateral medulla (RVLM) plays an important role in the pathophysiology of hypertension. Alpha-lipoic acid (ALA) is widely recognized for its potent superoxide inhibitory properties, and it can safely penetrate deep into the brain. The aim of this study was to explore whether ALA supplementation attenuates hypertensive responses and cardiac hypertrophy by decreasing the NAD(P)H oxidase (NOX)-derived overproduction of reactive oxygen species (ROS) in the mitochondria in the RVLM, and thus attenuating the development of salt-induced hypertension. For this purpose, male Wistar rats were randomly divided into 2 groups and either fed a high-salt diet or not. After 8 weeks, the rats were either administered ALA or an equal volume of the vehicle for 8 weeks. The rats fed a high-salt diet exhibited higher mean arterial pressure (MAP) and higher plasma noradrenaline (NE) levels, as well as cardiac hypertrophy, as evidence by the increased whole heart weight/body weight (WHW/BW) ratio, WHW/tibia length (TL) ratio and left-ventricular weight (LVW)/TL ratio. Compared with the rats in the NS group, the rats in the HS group only exhibited increased levels of superoxide, NOX2, NOX4 and mitochondrial malondialdehyde (MDA), but also decreased levels of copper/zinc (Cu/Zn)-superoxide dismutase (SOD), mitochondrial SOD and glutathione (GSH) in the RVLM. The supplementation of ALA decreased MAP, plasma NE levels and the levels of cardiac hypertrophy indicators. It also decreased the levels of superoxide, NOX2, NOX4 and mitochondrial MDA, and increased the levels of Cu/Zn-SOD, mitochondrial SOD and GSH in the RVLM compared with the rats fed a high-salt diet and not treated with ALA. On the whole, our findings indicate that long-term ALA supplementation attenuates hypertensive responses and cardiac hypertrophy by decreasing the expression of NAD(P)H subunits (NOX2 and NOX4), increasing the levels of mitochondrial bioenergetic enzymes, and enhancing the intracellular antioxidant capacity in the RVLM during the development of hypertension.

Introduction

Hypertension is one of the major risk factors for cardiovascular diseases and an important healthcare concern worldwide (1). A growing body of evidence indicates that oxidative stress plays an important role in the pathophysiology of high-salt-induced hypertension (2-4). High-salt intake is a significant environmental factor, strongly associated with blood pressure (BP) regulation and hypertensive responses and may increase oxidative stress, thus affecting the pathophysiological procedure of adverse events in the sympathetic nervous system (5,6). This breaks the balance between reactive oxygen species (ROS) generation and the antioxidant defenses, and triggers oxidative stress in central and peripheral tissues (7). ROS, such as superoxide anion, hydrogen peroxide and hydroxyl radical, not only participate in numerous cellular signaling pathways, but also modulate systemic vascular resistance and balance in salt and water homeostasis (8,9). Moreover, a previous study demonstrated that the overproduction of ROS in the central nervous system is extremely critical for arterial pressure regulation by enhancing renal sympathetic nerve activity (RSNA) (10). The rostral ventrolateral medulla (RVLM) is one of the main active regions for the central regulation of resting BP and sympathetic outflow (11-13). Therefore, the overproduction of ROS in the RVLM plays a key role in high BP and sympathetic overactivity in salt-induced hypertension.

Alpha-lipoic acid (ALA), chemically known as 1,2-dithiolane-3-pentanoic acid ($C_8H_{14}O_2S_2$), is widely recognized for its potent superoxide inhibitory properties both as natural diet constituent and a synthetic isolate. It is soluble in aqueous and lipid portions of the cell (14,15). The antioxidant capacity of ALA is more potent than that of vitamins C and E, and glutathione (16). ALA and its reduced form, dihydrolipoic acid (DHLA), have been shown to be potent naturally occurring antioxidants by scavenging a variety of ROS (14). Furthermore, ALA appears to regenerate other endogenous antioxidants, including vitamins C and E and glutathione, and has the salubrious property of promoting the body's antioxidant capacity. In addition, it is also a key regulator of

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energy metabolism in the mitochondria, which is a naturally occurring dithiol compound synthesized enzymatically in the mitochondrion from octanoic acid (16). Thus, ALA is closely related to the body's antioxidant activity. This suggests that ALA may be a possible candidate as a protective agent against the risk factors of hypertension. It is also possible that ALA may decrease BP in hypertension by resisting the superoxide damage and protecting the body's biological systems from cardiovascular diseases. Therefore, in the present study, we aimed to explore whether ALA supplementation attenuates oxidative stress in the RVLM, thus decreasing BP and sympathetic nerve activity in salt-induced hypertension.

Materials and methods

Animals. Adult male Wistar rats (n=56; aged 7 weeks; weighing 180-220 g) were obtained from the Experimental Animal Center of Wuhan University of Science and Technology. All rats were housed in a room with a temperature-controlled (23±2°C) environment with a normal 12-h light-dark cycle and allowed access to normal rat chow *ad libitum*.

All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (the US National Institutes of Health Publication no. 85-23, revised 1996) and approved by the Committee on the Ethics of Animal Experiments of Wuhan University of Science and Technology, Wuhan, China.

General experimental protocol. The male Wistar rats were randomly divided into 2 groups (n=28) as follows: the normal salt diet group administered 0.3% NaCl (NS group) as a control, the high-salt diet group administered 8% NaCl (HS group) in their food for 8 weeks to induce hypertension, as previously described (5,6). After 8 weeks, the rats in the NS and HS group were respectively administered ALA (60 mg/kg) dissolved in the vehicle (0.9% saline) or an equal volume of the vehicle daily by gastric perfusion for 8 weeks, as previously described (17). Thus, there were now 4 groups of rats (HS + vehicle, HS + ALA, NS + vehicle and NS + ALA) with 14 rats in each group.

BP measurements. In the rats from all the chronic feeding groups, arterial pressure was measured non-invasively using a tail-cuff instrument and a recording system (Kent Scientific Corp., Torrington, CT, USA), as previously described (18). Briefly, unanesthetized rats from each group were warmed to an ambient temperature of 30°C by placing them in a holding device mounted on a thermostatically controlled warming plate. Tail cuffs were placed on the animals, and each rat was allowed to become accustomed to the cuff for 10 min prior to performing the BP measurements. All measurements were taken within the same 2-h time window each day. Each session consisted of 30 cycles. BP was measured on 5 consecutive days each week, and values were averaged from ≥6 consecutive cycles. BP was measured at baseline (7 weeks of age) and then weekly until the end of either chronic study period.

Collection of blood and tissue samples. Each group of rats (n=14) was anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture via intraperitoneal (i.p.) injection.

From each group, 7 rats were perfused with 4% paraformaldehyde for immunofluorescence and immunohistochemistry. The remaining rats were decapitated and trunk blood was collected for high performance liquid chromatography (HPLC) fresh tissue was obtained for western blot analysis, ELISA and other experiments. The RVLM tissue was isolated following Palkovits' microdissection procedure as previously described (19). Plasma and tissue samples were stored at -80°C until analysis.

Immunofluorescence and immunohistochemistry. The rats were anesthetized and perfused through the heart with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). The brains were dehydrated in graded sucrose, and OCT-embedded. The RVLM was identified as the region extending caudally 500-700 μm from the caudal pole of the facial nucleus. Serial coronal sections (14-μm-thick) were cut and mounted on glass slides, which were stored at -80°C until use for measurements, as previously described (20).

Immunohistochemical and immunofluorescence staining was carried out on brain sections as described previously to identify NAD(P)H oxidase (NOX2 and NOX4) expression in the RVLM using respective antibodies [NOX2, sc-20782, 1:200; NOX4, sc-5827, 1:200; and copper/zinc (Cu/Zn)-superoxide dismutase (SOD), sc-11407, 1:200; all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA] (19). The brain sections were washed in PBS, permeabilized in 0.5% Triton, blocked using 5% normal goat serum and incubated with the primary antibodies in blocking buffer at 4°C overnight. Following incubation with the primary antibodies (anti-NOX4 and anti-Cu/Zn-SOD antibodies), the sections were incubated with secondary antibodies for immunofluorescence [Alexa 488-labeled anti-rabbit (1:200, green fluorescence) or Alexa 594-labeled anti-rabbit (1:200, red fluorescence); Invitrogen Life Technologies, Carlsbad, CA, USA] for 60 min at 37°C.

For immunohistochemistry, the brain sections were incubated with anti-NOX2 primary antibody and then with anti-rabbit secondary antibody from a Histostain™-Plus kit (SP-9001; ZSGB-Bio, Beijing, China) for 60 min. Antibody binding was visualized using a 3,3'-diaminobenzidine (DAB) kit (AR-1002; Boster Bio-Engineering, Wuhan, China) according to the manufacturer's instructions. Follwing a 10-min wash in tap water, the slices were stained in Harris' hematoxylin solution for 8 min and then differentiated in 1% acid alcohol for 30 sec. Processing was terminated with H_2O and the sections were imaged using a Nikon camera (Tokyo, Japan), as previously described (21).

Superoxide anion levels in the RVLM were determined by fluorescent-labeled dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA) staining. The coronal sections (14-μm-thick) were incubated with 1 μmol/l DHE at 37°C for 10 min as previously described (20).

Western blot analysis. Protein extracted from the RVLM tissues was prepared as previously described (21). For NOX2, NOX4 and Cu/Zn-SOD detection, protein extracts (5 μl) from the RVLM were resolved by 10-15% SDS-polyacrylamide gels, and electroblotted onto nitrocellulose membranes (Immobilon-P; EMD Millipore, Billerica, MA, USA) that were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% bovine serum albumin for 1 h at room temperature (20). The blots were incubated overnight at 4°C with the primary
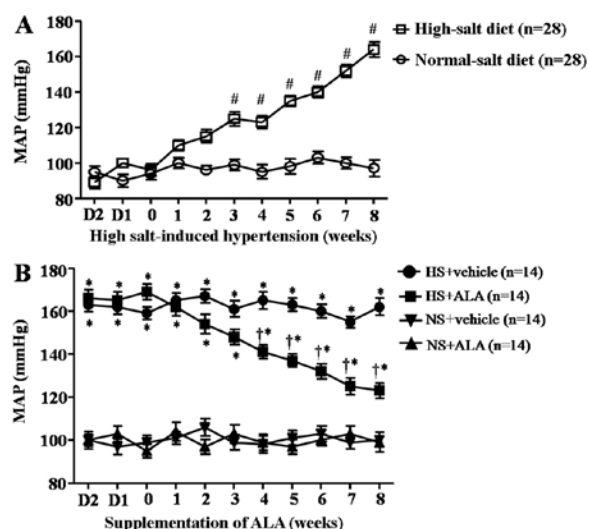
Figure 1. Measurement of mean arterial pressure (MAP). (A) Rats were fed a high-salt diet group containing 8% NaCl (HS) for 8 weeks to induce hypertension. D2 and D1 indicate 2nd and 1st day before the salt diet, respectively. (B) Effects of ALA supplementation or vehicle on MAP of high salt-infused rats and control rats. High-salt infusion induced an increase in MAP compared with the controls. ALA supplementation attenuated high salt-induced pressure response. D2 and D1 indicate the 2nd and 1st day prior to ALA supplementation, respectively. Values are expressed as the means±SEM. #P<0.05 vs. normal-salt diet; *P<0.05 vs. control (NS + ALA or NS + vehicle); †P<0.05 HS + ALA versus HS + vehicle.

antibodies to NOX2 (sc-20782, 1:400), NOX4 (sc-5827, 1:400) and Cu/Zn-SOD (sc-11407, 1:200) (all form Santa Cruz Biotechnology, Inc.) to determine the relative expression levels in the RVLM. After washing with wash buffer (1X TBS, 0.1% Tween-20), the blots were then incubated for 1 h with the secondary antibody (1:10,000 dilution; Santa Cruz Biotechnology) labeled with horseradish peroxidase. Protein loading was controlled by probing all blots with β-actin antibody (Thermo Fisher Scientific, Waltham, MA, USA) and normalizing their protein intensities to those of β-actin. The immunocomplexes were visualized using an ECL-immunoblotting detection kit (PerkinElmer, Inc., Waltham, MA, USA). Band densities were analyzed using NIH ImageJ software, as previously described (21).

Preparation of mitochondria matrix fraction in RVLM. Mitochondrial matrix (stroma) was prepared by applying the method described as follows: brain tissues were rapidly removed and washed with 0.86% cold normal saline, then chopped into small sections, and placed into ice-cold isolation buffer for mitochondria (10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 0.5 M methylene diamine tetra-acetic acid (EDTA), and 0.5 % bovine serum albumin). After being homogenized, the homogenate was centrifuged at 750 x g for 10 min. The supernatant was then centrifuged at 10,000 rpm for 10 min at 4°C. Mitochondrial pellets were washed twice with isolation buffer and then resuspended in the same buffer solution. The mitochondrial matrix was extracted from freshly prepared mitochondria by freezing and defrosting with repeated homogenization in order to burst mitochondria. Following centrifugation at 10,000 rpm for 10 min, the supernatant was the source of SOD, glutathione (GSH) and malondialdehyde (MDA), as previously described (22).

Biochemical evaluation of MDA, GSH, and SOD in RVLM mitochondria. Lipid peroxidation product in the RVLM was determined by measuring the MDA content in tissue homogenates according to the method of Begue and Aust spectrophotometrically at 532 nm (23). Values were expressed as mm/g protein. SOD activity was determined by following the method of Kono at 550 nm (24). Values were expressed as U/mg protein. The level of reduced GSH was measured as protein-free sulfhydryl content by the method of Sedlak and Lindsay at 412 nm and values were expressed as μm/g protein (25).

According to the manufacturer's instructions, the standards or sample diluents were added to the appropriate well of a microtiter plate pre-coated with specific antibodies and incubated. Conjugate was added followed by incubation at 37°C for 1 h and then washing. The reactions were terminated with stop solution and read at 450 nm for MDA, GSH and SOD measurements using a microtiter plate reader (MK3; Thermo Fisher Scientific).

Measurement of plasma levels of norepinephrine (NE). Plasma NE levels were measured by HPLC as described previously with minor modifications in plasma sample preparation Plasma samples were prepared by adding activated alumina, Tris buffer, EDTA, and internal standard 3,4-dihydrobenzylamine, along with 0.5 ml of rat plasma. The samples were centrifuged, and supernatant was separated, rinsed 2 times in ultrapure water, and filtered through a Millipore filter (Ultrafree MC UFC30GV00; Millipore). The composition of the mobile phase was as follows: monochloroacetic acid (14.14 g/l), sodium hydroxide (4.675 g/l), octanesulfonic acid disodium salt (0.3 g/l), ethylenediaminetetraacetic acid (0.25 g/l), acetonitrile (3.5%) and tetrahydrofuran (1.4%). The mobile phase was carried out in pyrogen-free water and then filtered and degassed through the Millipore filter and pumped at a flow rate of 1.8 ml/min. The sensitivity of the detector was 1 nA full scale, and the potential of the working electrode was 0.65 V. The column oven maintained the temperature of the column at 37°C. At the time of HPLC analysis, tissue samples were homogenized in 150 μl of 0.1 M $HClO_4$ using a micro-ultrasonic cell disruptor (Kontes, Vineland, NJ, USA) and centrifuged at 10,000 x g for 10 min. 50 μl of the supernatant along with 25 μl of the internal standard (0.05 M dihydroxybenzylamine) were injected into the HPLC system (26,27).

Statistical analysis. All data are expressed as the means ± standard error of the mean (SEM). The significance of differences between mean values was analyzed by ANOVA followed by Tukey's test. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Mean arterial pressure (MAP). A high-salt diet induced a significant increase in MAP compared with the control rats after 8 weeks prior to the supplementation of ALA (Fig. 1A). Fig. 1B presents the MAP trends for each group of rats treated with ALA for 8 weeks. The MAP of rats fed a high-salt diet was significantly higher compared to that of the control animals (NS group). The supplementation of ALA decreased the MAP in the rats with high salt-induced hypertension.
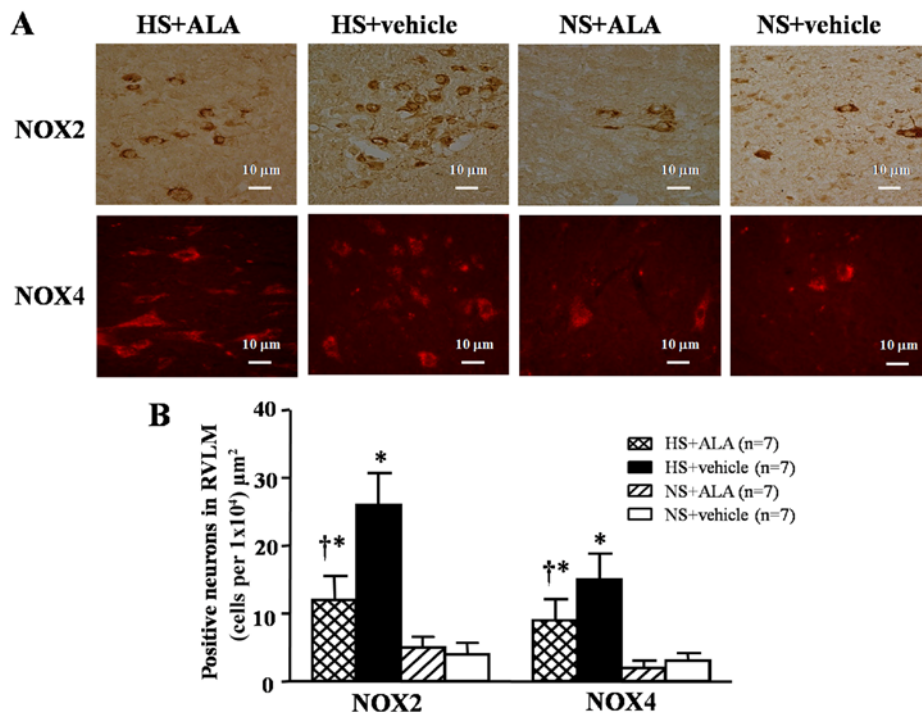
Figure 2. Effects of alpha-lipoic acid (ALA) supplementation or the vehicle on NOX2- and the NOX4-positive neurons in the rostral ventrolateral medulla (RVLM) of rats with high salt-induced hypertension and control rats. The numbers of RVLM NOX2- and NOX4-positive neurons in rats with high salt-induced hypertension were higher than those in rats fed a nomal salt diet. The supplementation of ALA reduced the numbers of NOX2- and NOX4-positive neurons in the RVLM of rats fed a high-salt diet. (A) Immunofluorescence for NOX2 (brown) and immunohistochemistry for NOX4 (bright red) in different groups. (B) Column diagram showing the effects of ALA supplementation on the positive neurons in different groups. Values are expressed as the means ± SEM. *P<0.05 vs. control (NS + ALA or NS + vehicle); †P<0.05 HS + ALA vs. HS + vehicle.

NOX2- or NOX4-positive neurons in the RVLM. Imunohisto-chemisty and immunofluorescence staining revealed that the high-salt diet induced a significant increase in the expression of NOX2 and NOX4 in the RVLM compare to the control rats. The supplementation of ALA decreased the number of NOX2- and NOX4-positive neurons in the hypertensive rats (Fig. 2).

Superoxide- and Cu/Zn-SOD-positive neurons in the RVLM. Immunofluorescence staining revealed that the high-salt diet induced a significant decrease in Cu/Zn-SOD levels, and an increase in fluorescence-labeled DHE compared with the control rats. The supplementation of ALA decreased the DHE fluorescence intensity and increased the number of Cu/Zn-SOD-positive neurons in the hypertensive rats (Fig. 3).

Protein expression levels of NOX2, NOX4 and Cu/Zn-SOD in the RVLM. The results of western blot analysis indicated that the rats fed a high-salt diet exhibited significantly increased levels of NOX2 and NOX4, and decreased expression levels of Cu/Zn-SOD in the RVLM compared with the control rats. The supplementation of ALA decreased the levels of NOX2 and NOX4, and increased the Cu/Zn-SOD expression levels in the hypertensive rats (Fig. 4).

Levels of MAD, SOD and GSH in the RVLM mitochondria. The MDA levels in the RVLM mitochondria were significantly higher in the rats fed a high-salt diet than in those in the normal control group. The supplementation of ALA decreased the levels of MDA as compared with the respective control group (HS + vehicle; Fig. 5A). On the other hand, the results revealed that the levels of SOD and GSH in the RVLM mitochondria were decreased in the rats fed the high-salt diet. The supplementation of ALA increased the levels of SOD and GSH (Fig. 5B and C). The GSH level in the ALA-treated rats was similar to that in the control groups (Fig. 5C).

Effect of ALA supplementation on cardiac hypertrophy and plasma NE levels. Whole heart weight/body weight (WHW/BW) ratio, WHW/tibia length (TL) ratio and left-ventricular weight (LVW)/TL ratio were measured as indicators of cardiac hypertrophy. Plasma NE presents the activity of the sympathetic nervous system. The rats fed a high-salt diet exhibited increased cardiac hypertrophy as indicated by the increased WHW/BW ratio, WHW/TL ratio, and LVW/TL ratio, which were decreased by ALA supplementation (Fig. 6A-C). In addition, the plasma NE levels in the rats fed a high-salt diet were higher than those in the control group. The supplemenation of ALA decreased the levels of plasma NE in the hypertensive rats (Fig. 6D).

Discussion

The results of our study demonstrated that ALA supplementation for 8 weeks markedly alleviated high salt-induced hypertensive responses, as evidenced by the reduction in MAP and plasma NE levels, that represent the activity of the sympathetic nervous system. Moreover, ALA supplementation not only decreased the expression of NAD(P)H subunits (NOX2 and NOX4) in the RVLM and attenuated the overproduction of ROS in the
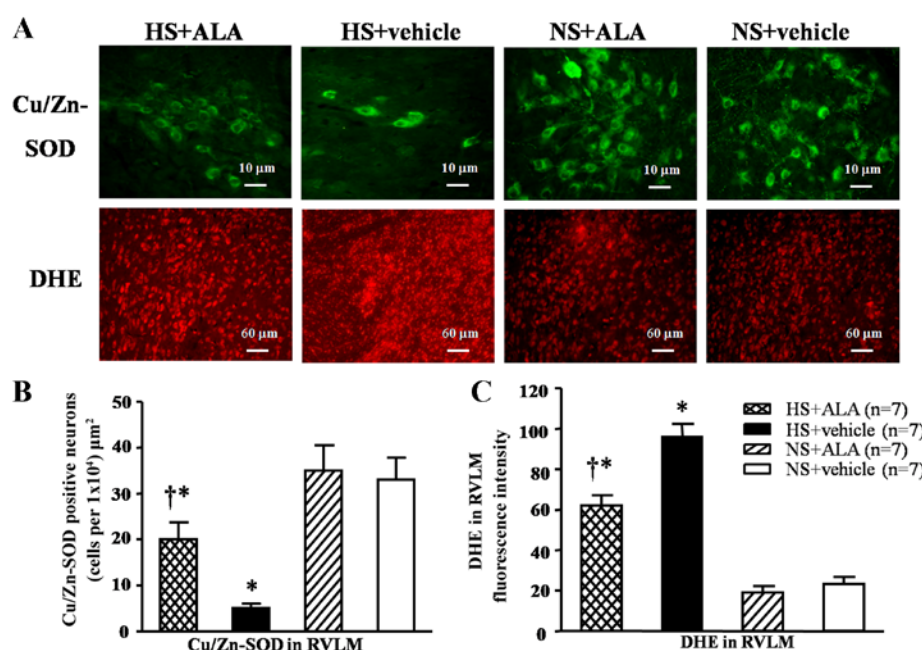
Figure 3. Effects of alpha-lipoic acid (ALA) supplementation or the vehicle on superoxide- and Cu/Zn-SOD-positive neurons in the rostral ventrolateral medulla (RVLM) of rats with high salt-induced hypertension and control rats. The number of superoxide anions (shown by DHE) in the RVLM was increased in rats with high salt-induced hypertension, and the number of Cu/Zn-SOD-positive neurons was lower than that in rats fed a normal salt diet. The supplementation of ALA reduced the levels of superoxide anions, and increased the number of Cu/Zn-SOD-positive neurons in the RVLM of rats with high salt-induced hypertension. (A) Immunofluorescence for Cu/Zn-SOD (bright green) and superoxide (bright red) in different groups. (B) Bar graph of Cu/Zn-SOD positive neurons and (C) superoxide in different groups. Values are expressed as the means ± SEM. *$P<0.05$ vs. control (NS + ALA or NS + vehicle); †$P<0.05$ HS + ALA vs. HS + vehicle. SOD, superoxide dismutase; DHE, dihydroethidium.
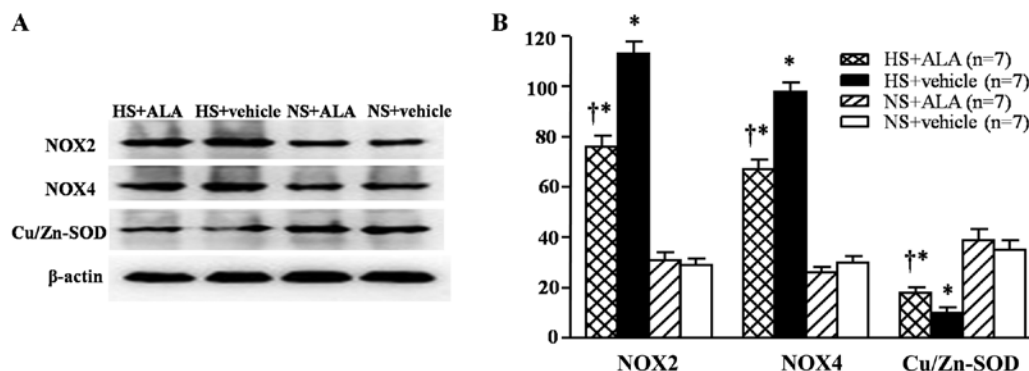

Figure 4. Effects of alpha-lipoic acid (ALA) supplementation or the vehicle on the protein expression levels of NOX2, NOX4 and Cu/Zn-SOD in the rostral ventrolateral medulla (RVLM) of rats with high salt-induced hypertension and control rats. The protein expression levels of NOX2 and NOX4 in the RVLM in rats with high salt-induced hypertension were higher, and Cu/Zn-SOD protein expression was lower compared to the control rats. The supplementation of ALA reduced the levels of NOX2 and NOX4, and augmented Cu/Zn-SOD protein expression in the RVLM of rats with high salt-induced hypertension. (A) A representative immunoblot; and (B) densitometric analysis of protein expression of NOX2, NOX4 and Cu/Zn-SOD in different groups. Values are expressed as the means ± SEM. *$P<0.05$ vs. control (NS + ALA or NS + vehicle); †$P<0.05$ HS + ALA vs. HS + vehicle. SOD, superoxide dismutase.

RVLM mitochondria, but it also enhanced the antioxidant capacity and attenuated cardiac hypertrophy, as indicated by the decreased WHW/BW ratio, WHW/TL ratio and LVW/TL ratio in the hypertensive rats administered ALA. Therefore, the novel findings of this study are that the long-term administration of ALA attenuates MAP, decreases sympathetic nervous system activity and body oxidative damage in rats with high salt-induced hypertension by decreasing the expression of NAD(P)H subunits (NOX2 and NOX4), increased the levels of mitochondrial bioenergetic enzymes, and enhancing the intracellular antioxidant capacity in the RVLM during the development of hypertension.

It is well known that a high-salt intake is responsible for the development of high BP in human communities (28,29).

Moreover, studies over the past decade have demonstrated that a high-salt diet increases oxidative stress in brain regions, such as the hippocampus and cerebral cortex, which contributes to the pathological mechanisms of hypertension (30-32). The RVLM is considered to be a cardiovascular center that determines basal sympathetic tone, and to be responsible for activating the sympathetic nervous system (5). In the present study, we found that a high-salt diet not only increased sympathetic nervous system activity, but also elevated arterial BP. We also observed that the production of superoxide was significantly increased, whereas the antioxidant capacities (SOD and GSH in RVLM mitochondria) were significantly decreased in the RVLM in the rats with high salt-induced hypertension. Consistent with
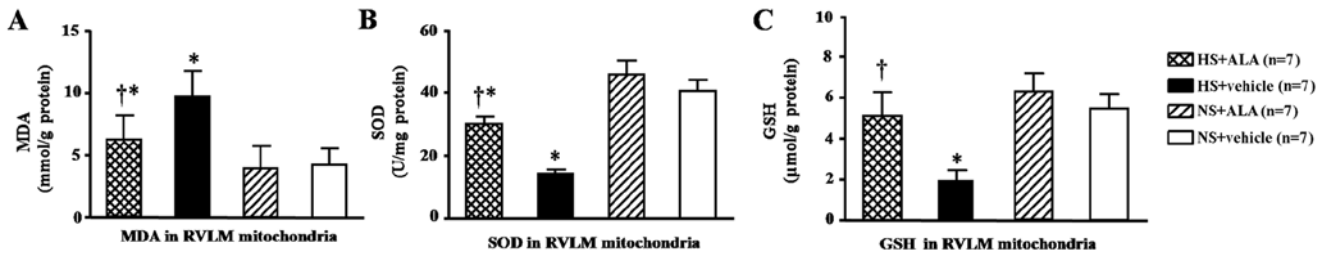
Figure 5. Effect of alpha-lipoic acid (ALA) supplementation or the vehicle on the rostral ventrolateral medulla (RVLM) mitochondrial levels of (A) malondialdehyde (MDA), (B) superoxide dismutase (SOD) and (C) glutathione (GSH) in rats with high salt-induced hypertension and control rats. RVLM mitochondrial levels of MDA in the rats with high salt-induced hypertension were higher, while the SOD and GSH levels were lower than in the control rats. The supplementation of ALA reduced the levels of MDA, and increased the levels of SOD and GSH in the RVLM mitochondria of rats with high salt-induced hypertension. Values are expressed as the means ± SEM. *P<0.05 vs. control (NS + ALA or NS + vehicle); †P<0.05 HS + ALA vs. HS + vehicle.
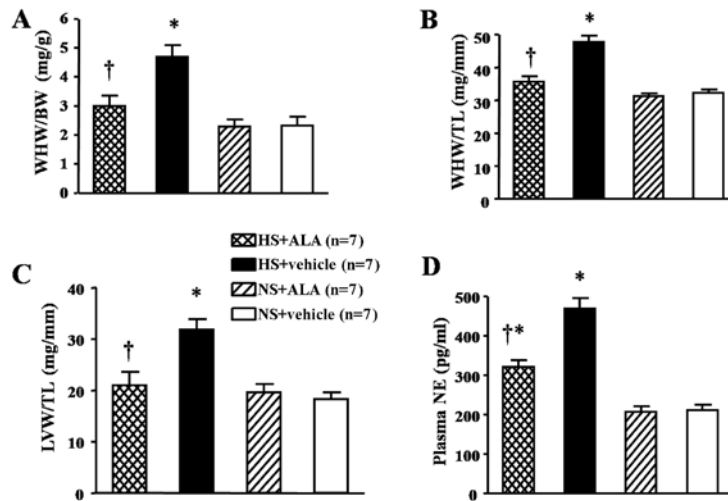

Figure 6. Effect of alpha-lipoic acid (ALA) supplementation or the vehicle on cardiac hypertrophy and plasma noradrenaline (NE) levels in control and in rats with high salt-induced hypertension. The rats with high salt-induced hypertension exhibited (D) increased plasma levels of NE and increased cardiac hypertrophy, as assessed by (A) the ratio of whole heart weight/body weight (WHW/BW), (B) the ratio of WHW/tibia length (TL) and (C) the ratio of left ventricular weight (LVW)/TL, which was reduced following ALA supplementation for 8 weeks. Values are expressed as the means ± SEM. *P<0.05 vs. control (NS + ALA or NS + vehicle); †P<0.05 HS + ALA vs. HS + vehicle.
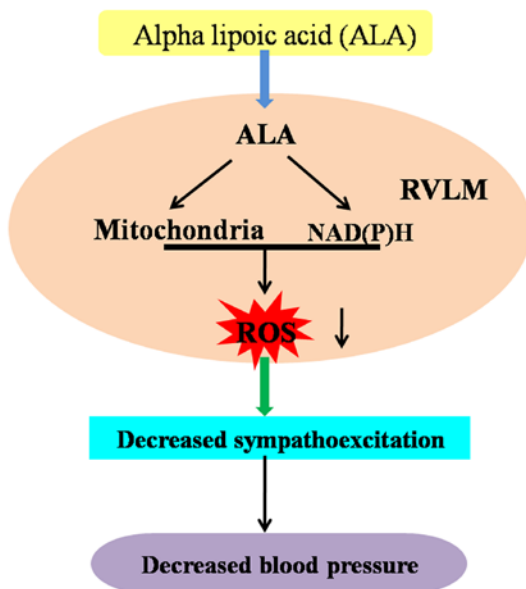

Figure 7. Schematic showing the proposed pathways of the effects of alpha-lipoic acid (ALA) on regulating the NAD(P)H oxidase- and mitochondria-derived reactive oxygen species (ROS) within the rostral ventrolateral medulla (RVLM) in high salt-induced hypertension.

previous studies (33-35), the findings of our study demonstrated that a high-salt diet enhanced superoxide generation in the RVLM, and activated the sympathetic nervous system during the development of hypertension.

ALA has been described as a potent biological antioxidant and an essential co-factor for mitochondrial bioenergetic enzymes, which has extensively been applied as a therapy for preventing diabetic polyneuropathies, and restoring intracellular glutathione levels (36-38). It is also unique among antioxidants that could be soluble in both lipid and aqueous environments (36,38). Therefore, ALA can safely penetrate deep into the brain, helping to scavenge free radicals and reversing the damaging effects of ROS overproduction. Our present study demonstrated that the long-term supplementation of ALA decreased MAP, delayed the progress of cardiac hypertrophy, and reduced the levels of NAD(P)H subunits (NOX2 and NOX4) and mitochondrial superoxide in RVLM in rats with high salt-induced hypertension. These results provide sufficient evidence that ALA can cross the blood-brain barrier, reach the RVLM, and scavenge free radicals derived from NAD(P)H in the mitochondria. Thus, in this study, we hypothesized that ALA supplementation may decrease oxidative stress in the RVLM by decreasing NOX2

and NOX4 expression, increasing the levels of mitochondrial bioenergetic enzymes, and enhancing the intracellular antioxidant capacity in the RVLM, finally leading to reduced BP and cardiac hypertrophy in rats with high salt-induced hypertension.

In conclusion, the present findings suggest that the long-term consumption of a high-salt diet augments BP and induces the overproduction of ROS derived from NAD(P)H in the mitochondria in the RVLM, which plays an important pathophysiological role in the development of hypertension. More importantly, our results indicate that the long-term supplementation of ALA attenuates hypertensive responses and attenuates cardiac hypertrophy by decreasing the expression of NAD(P)H subunits (NOX2 and NOX4), increasing the levels of mitochondrial bioenergetic enzymes, and enhancing intracellular antioxidant capacity in the RVLM during the development of hypertension. The mechanisms responsible for the effects of ALA on hypertension are presented in Fig. 7.

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