L-Arginine Reverses p47phox and gp91phox Expression Induced by High Salt in Dahl Rats

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Abstract—Derangements in the production and degradation of reactive oxygen species (ROS) as well as nitric oxide (NO) have been implicated in cardiovascular diseases. We explored how supplementation with L-arginine, an NO synthase substrate, restores such derangements of ROS/NO systems in Dahl salt-sensitive, hypertensive (DS) rats. We detected an increase of NADPH oxidase activity, a key enzyme that produces superoxide, in the membrane fraction of the renal cortex derived from DS rats loaded with high salt for 4 weeks; high salt loading also remarkably increased urinary H2O2, 8-isoprostane, and thromboxane B2 excretion and decreased plasma NO end products. These changes from high salt loading were counteracted by oral L-arginine supplementation. We further examined expression patterns of NADPH oxidase subunits in renal cortex derived from these animals. High salt loading increased gp91phox and p47phox but not p22phox or Rac1 or mRNA abundance, which were counteracted with L-arginine supplementation. Western blot analyses after subcellular fractionation revealed that L-arginine supplementation distinctly decreases membrane localization of p47phox protein, as it decreases total expression of Rac1 protein in DS rats with high salt loading. These results disclose that high salt loading causes a deficiency in available L-arginine amounts for NO synthases and induces NADPH oxidase activation in the renal cortex of DS rats, which L-arginine supplementation markedly restores. Since superoxide rapidly eliminates NO, which inhibits sodium reabsorption in the cortical collecting duct, superoxide production caused by upregulated NADPH oxidase activity in the renal cortex of high salt–loaded DS rats may accelerate sodium reabsorption and hypertension. (Hypertension. 2003;42:1014-1020.)

Key Words: rats, Dahl ■ arginine ■ hypertension, sodium-dependent ■ nitric oxide ■ cardiovascular diseases

Recent studies have revealed significant associations between the pathogenesis of numerous cardiovascular diseases, including hypertensive states, and the increase in reactive oxygen species (ROS) and/or the decrease in nitric oxide (NO).1–3 Complete understanding of such derangements in ROS/NO systems has been prevented by the fact that mammalian organs are provided with multiple layers of complicated enzymatic as well as nonenzymatic machineries that deal with ROS/NO.4,5

Among such machineries, NADPH oxidase, which was originally known to produce a large amount of superoxide anion to kill bacteria in neutrophils,6 has attracted much attention as a potentially important player in a wide array of cardiovascular disorders. Although NADPH oxidase in the nonphagocytic cells has been less well characterized, physiologically relevant low generation of ROS and neutrophil-like expression of NADPH oxidase subunits is present in vascular tissues,7,8 which is activated by pulsatile stretch,9 platelet-derived growth factor,10 and angiotensin II.11–15 These studies, when taken together, suggest that NADPH oxidase may exert broader actions in cardiovascular disorders.

Dahl salt-sensitive (DS) rats, when loaded with high salt diets, have organ failure including nephrosclerosis,16 vascular and cardiac hypertrophy,17 impaired NO-dependent vasodilation,18 and abnormal vasocontractile response.19–21 The characteristics of hypertension in the DS rats are similar in nature to those observed in black Americans.22 The renal system appears to represent a predominant target organ in DS rats, since replacing a kidney in a Dahl salt-resistant (DR) rat with a kidney derived from a DS donor induces hypertension in the recipient DR rat.23 However, regulations of renal NADPH oxidase system in high salt–loaded DS rats are mostly unknown.

High salt loading increases NO generation in Sprague-Dawley rats24,25 and NO enhances urinary sodium chloride excretion, since NO inhibits sodium reabsorption in the cortical collecting duct26 and chloride transport in the thick ascending limb.27 On the contrary, in DS rats, high salt loading decreases NO generation.20,28 Whereas L-arginine, a substrate of NO synthase (NOS), is known to reverse hypertension in high salt–loaded DS rats,20,29 molecular mechanisms whereby L-arginine exerts such favorable actions remain less well clarified.
In the present study, we explored how renal NADPH oxidase system is modulated after high salt loading in DS rats. We demonstrated evidence that NADPH oxidase is upregulated at multiple levels after high salt loading and that l-arginine supplementation remarkably reverses many of such alterations.

**Methods**

**Animals**
Seven-week-old male DS rats (Yoshitomi Seiyaku, Osaka, Japan) were fed a normal diet (0.24% NaCl), a high salt diet (8% NaCl), or a high salt diet (8% NaCl) plus l-arginine (20 mg/mL drinking water) for 4 weeks. Systolic arterial pressure (SAP) was measured in awake rats by the tail-cuff method. The rats were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt) and killed by exsanguination. Tissues were immediately frozen in liquid nitrogen and stored at −80°C. All animal procedures were in accordance with the institutional guidelines of the Kagawa Medical University.

**Measurement of Urinary H2O2, Plasma Nitrite Plus Nitrate, and l-Arginine**
Protein concentrations were measured by the Bradford method (Bio-Rad Laboratories). Urinary H2O2 levels were measured as degrees of peroxidation of nonfluorescent 2',7'-dichlorodihydrofluorescein (H2DCF) to fluorescent 2',7'-dichlorofluorescein (DCF). We optimized the assay conditions and verified that urinary protein interfered with H2O2 determination, urine and plasma samples were filtered as described. The filtrate was treated without or with 500 U/mL catalase for 10 minutes at room temperature, and DCF fluorescence levels obtained in the presence of catalase was subtracted. Amounts of nitrite plus nitrate were measured by using respective enzyme immunoassay kits (Cayman Chemical Co), following manufacturer’s instructions.

**Preparation of Total Homogenate and Membrane Fraction of Renal Cortex**
The membrane fraction of renal cortex was prepared according to Zou et al. Homogenized renal cortex was centrifuged at 6000g for 10 minutes at 4°C, and the supernatant was stored as “total homogenate” or centrifuged at 2000g for 15 minutes at 4°C; the pellet was washed 3 times, resuspended in the homogenization buffer, immediately frozen in liquid nitrogen as “membrane fraction,” and stored at −80°C.

**Measurement of Superoxide Producing Activity in Renal Cortex**
NADPH activity in the membrane fraction was measured with acetylated cytochrome c reduction assay.

**Reverse Transcription–Polymerase Chain Reaction Analysis**
Reverse transcription–polymerase chain reaction (RT-PCR) was carried out as described previously, with synthetic oligonucleotide primers used for p22phox, gp91phox, p47phox, Rac1, and GAPDH. We optimized the assay conditions and verified that increasing the amounts of starting mRNA sample yield increasing amounts of RT-PCR product under these conditions in each primer pair.

**Western Blot Analyses**
Western blot analyses were performed as described, with the use of primary antibodies directed to gp91phox, p47phox, or Rac1 (Transduction Laboratories), secondary antibody conjugated with horseradish peroxidase, and SuperSignal West Pico substrate (Pierce) with exposure to radiographic films (Fuji).

**Results**
Figure 1 shows the changes in the SAP in DS rats. SAP in the high-salt diet group was significantly higher than that in normal-salt group (Figure 1). Supplementation with l-arginine significantly reduced SAP. We next studied whether or not high salt loading in DS rats increases protein excretion into urine. DS rats fed with high salt had significantly higher amounts of protein excretion, which was suppressed by L-arginine supplementation (Figure 2A).

We sought to explore how high salt loading and l-arginine supplementation in DS rats modulate renal ROS systems. We measured levels of urinary H2O2 based on fluorescence detection after peroxidation of dichlorofluorescein with horseradish peroxidase. High salt loading increased urinary H2O2 excretion, which was suppressed by l-arginine supplementation (Figure 2B). These results demonstrate that high salt loading markedly increases ROS production.

NADPH oxidase represents a key ROS producing system in a wide variety of mammalian organs. We therefore examined NADPH oxidase activity in the renal cortex material derived from animals studied above, by using an acetylated cytochrome c reduction assay. NADPH-dependent, superoxide-producing activity in the membrane fraction was defined as a difference between the reduction rate of acetylated cytochrome c without and with superoxide dismutase (SOD). High salt loading increased NADPH-dependent su-
peroxide generation, which was counteracted by L-arginine supplementation (Figure 3). The NADPH oxidase system comprises several subunit proteins, including gp91phox, p22phox, p47phox, as well as Rac1, each of which represents a potential target of regulation. In the subsequent studies, we explored the expression levels of several of such subunits at a level of mRNA by using RT-PCR analyses in renal cortex. High salt loading for 4 weeks increased gp91phox but not p22phox, mRNA abundance in renal cortex, which was counteracted with oral L-arginine supplement (Figures 4A and 4B). The high salt loading increased p47phox mRNA abundance in renal cortex, which was counteracted with L-arginine supplementation (Figure 4C). The high salt loading did not significantly increase Rac1 mRNA abundance in renal cortex; however, L-arginine supplementation decreased Rac1 mRNA (Figure 4D). These results demonstrate that high salt loading and L-arginine supplementation complexly modulate expression levels of rat renal transcripts that encode NADPH oxidase subunits.

We next studied expression patterns of NADPH oxidase subunits at a level of protein. Western blot analyses show that high salt loading increased gp91phox protein, which was reversed by L-arginine supplement both in the total homogenates and in the membrane fractions (Figure 5A). High salt loading powerfully increased p47phox protein 14-fold in the membrane fractions, which was counteracted near to the baseline levels by L-arginine supplementation (Figure 5B). In contrast, in the total homogenates, the response appeared to be relatively small and was insensitive to L-arginine supplementation. The difference in the expression pattern between gp91phox and p47phox protein reminds us that the former is membrane-localized and the latter translocates from cytosol to membrane after stimulation in neutrophils. High salt loading did not change Rac1 protein expression levels. However, L-arginine supplementation decreased Rac1 protein expression (Figure 5C).

Previously, we reported the increase in urinary nitrate and cGMP excretion after L-arginine supplementation in the hypertensive salt-sensitive Dahl rats. In the present study, we examined plasma nitrate plus nitrite. High salt loading decreased plasma nitrate plus nitrite levels, which were restored by L-arginine supplementation (Figure 6). Plasma L-arginine concentrations in the normal-salt, high-salt, and high-salt plus L-arginine groups were \(100\)\(\mu\)mol/L, \(120\)\(\mu\)mol/L, and \(331\)\(\mu\)mol/L, respectively.

We next examined urinary excretion of 8-isoprostane (8-iso-prostaglandin \(F_2\)), a noninvasive index of in vivo lipid peroxidation. High salt loading increased urinary 8-isoprostane excretion, which was counteracted by L-arginine supplementation (Figure 7A). Enhanced formation of \(F_2\)-isoprostanes has been found to correlate with in vivo thromboxane \(A_2\) biosynthesis. High salt loading increased urinary thromboxane \(B_2\) (a stable metabolite of thromboxane \(A_2\)) excretion in hypertensive Dahl salt-sensitive rats. Urinary protein (A) and \(H_2O_2\) (B) excretion in hypertensive Dahl salt-sensitive rats. Urinary protein concentrations were determined by the Bradford method. Urinary concentrations of \(H_2O_2\) were determined based on the increase in fluorescence of dichlorofluorescein as described in the Methods section. Each data point represents mean±SEM (n=4 to 9). *\(P<0.0001\) vs normal rats; †\(P<0.0001\) vs high salt-fed rats; ‡\(P<0.05\) vs high salt-fed rats.
Discussion

Our present studies revealed that in DS rats, high salt loading for 4 weeks increased blood pressure; NADPH-oxidase activity in the membrane fraction; urinary excretion of H2O2, 8-isoprostane, as well as thromboxane B2; whereas it decreased plasma NO end products. A high salt diet increased mRNA abundance of NADPH oxidase subunits gp91phox and p47phox in the renal cortex, as it increased gp91phox protein both in the total homogenates as well as in the membrane fractions in the renal cortex, whereas the high salt diet increased the p47phox protein specifically in the membrane fractions of the cortex. All these changes induced by high salt loading were counteracted by concomitant L-arginine supplementation.

Our results show that in Dahl salt-sensitive rats, high salt loading decreases NO concentration (Figure 6) while increasing NADPH oxidase activity in their renal cortex (Figure 3), associated with the augmentation of urinary H2O2 excretion (Figure 2B). Such a decrease in NO and an increase in superoxide, when combined, can accelerate sodium reabsorption and hypertension, since NO inhibits sodium reabsorption
These results are also consistent with the so-called “L-arginine paradox,” that is, L-arginine supplementation stimulates NO synthesis despite saturating intracellular L-arginine concentrations. In fact, we have previously observed that ischemia causes a deficiency of L-arginine available for NOS and that L-arginine supplementation ameliorates the vasodilation and contraction responses impaired in hypertensive DS rats. Thus, the conditions in which the “L-arginine paradox” occurs may have broader implications in the pathogenesis of cardiovascular diseases, including hypertensive states as well as ischemia/reperfusion.

In the present studies, gp91phox mRNA and protein were upregulated in the renal cortex derived from high salt–loaded DS rats. p47phox protein was also upregulated by high salt loading, notably limited within the membrane fractions. Present studies demonstrate alterations also in the subcellular distribution of NADPH oxidase components. Several of these components, including p40phox, p47phox, and p67phox, exist in the cytosol, whereas others, including p22phox and gp91phox, reside in the membranes in the resting vascular smooth muscle cells. When stimulated, the former group translocates to the membrane fractions to achieve full activation of the enzyme. Since translocation of the p47phox subunit has been shown to play a pivotal role in the regulation of NADPH oxidase, our results suggest that remarkable upregulation of p47phox protein in the membrane fraction (Figure 5B) may contribute to the increase of NADPH oxidase activity. Rac1, which belongs to the small GTPase protein superfamily, participates in the regulation of NADPH oxidase in numerous cell types. We were surprised to find that L-arginine decreases Rac1 at the levels of mRNA as well as protein in renal cortex of DS rats (Figures 4D and 5C). Taken together, these actions of L-arginine, which appear to interfere with translocation of p47phox to the membrane fraction and to downregulate total expression levels of gp91phox as well as Rac1, may contribute to decrease overall enzyme activity of renal NADPH oxidase in high salt–loaded animals. The molecular basis whereby L-arginine exerts these actions remains to be fully elucidated.

In the present studies, we examined urinary excretion of 8-isoprostane, a noninvasive index of in vivo lipid peroxidation. A recently discovered series of prostaglandin F2-like compound, 8-isoprostane, are produced in vivo nonenzymatically by free radical catalyzed peroxidation of arachidonic acid in cell membranes and in circulating LDL.
salt loading increased urinary 8-isoprostane excretion, which was counteracted by L-arginine supplementation (Figure 7A). Isoprostane can exert potent biological activity such as vasoconstriction,\(^4\) stimulate thromboxane receptors, and contribute to atherosclerotic lesion development.\(^4\) Enhanced formation of 8-isoprostane has been associated with several cardiovascular risk factors\(^6\) and has been found to correlate with in vivo thromboxane A2 biosynthesis.\(^3,9,45\) In line with these reports, we previously reported that high salt induces acetylcholine-induced contraction caused by endothelium-derived contraction factor, which may be thromboxane A2 and/or prostaglandin H2, in carotid and renal arterial rings but not aorta of hypertensive DS rats,\(^7\) and L-arginine supplementation diminished the contraction.\(^20\) Actually, we showed in this study that high salt loading increases urinary thromboxane B2 (a stable metabolite of thromboxane A2) excretion (Figure 7B), which was reduced by L-arginine supplementation. These results suggest that such actions of ROS to increase vasoconstrictive agents, including 8-isoprostane and thromboxane A2, may contribute to the progression of hypertensive states in this model. Since NO rapidly inactivates superoxide,\(^40\) higher amounts of NO production observed in L-arginine–supplemented animals (Figure 6) may lead to decreasing amounts of “biologically active” ROS, associated with the decrease in ROS-induced vasoconstrictive agents such as 8-isoprostane, and ultimately may reverse hypertensive effects exerted by high salt loading.

In summary, high salt loading in Dahl salt-sensitive rats for 4 weeks increased urinary H2O2 excretion, NADPH-dependent superoxide producing activity, NADPH oxidase subunits gp91phox and p47phox proteins and mRNA expressions in renal cortex, urinary 8-isoprostane and thromboxane B2 excretion, and decreased plasma NO end products, which were alike counteracted by L-arginine supplement.

**Perspectives**

Our results revealed that high salt loading increases ROS through NADPH oxidase activation and decreases NO production, probably as the result of a decrease in L-arginine availability. L-Arginine supplementation ameliorates NO deficiency and the hypertensive state and decreases ROS through downregulation of NADPH oxidase. These actions of L-arginine may represent another potential point of control in human cardiovascular disorders associated with hypertensive states and/or oxidative stress.

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**References**

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