Asymmetrical Dimethylarginine, an Endogenous Nitric Oxide Synthase Inhibitor, in Experimental Hypertension

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Abstract N0,N0-dimethyl-L-arginine (ADMA) is an endogenously synthesized nitric oxide (NO) synthase inhibitor which has potent pressor/vasoconstrictor effects. Dimethylargininase metabolizes ADMA to L-citrulline and plays a key role in determining the in vivo levels of ADMA. To investigate the role of ADMA in the pathogenesis of hypertension, we measured 24-hour urinary excretion of ADMA (UADMA) and nitrate/nitrite (NOx) in Dahl salt-sensitive hypertensive rats and spontaneously hypertensive rats (SHR). In Dahl salt-resistant rats, high-salt diet increased blood pressure (P<0.05), and UADMA was inversely correlated with NOx excretion (P<0.05) and smaller UADMA than Wistar-Kyoto rats (P<0.05). ADMA was inversely correlated with their mean arterial pressure (r=64, P<0.05). In conclusion, ADMA, independently of the renal dimethylargininase content, may play a role in the pathogenesis in Dahl salt-sensitive hypertensive rats but not in SHR. (Hypertension. 1997;29[part 2]:242-247.)

Key Words • blood pressure • Dahl • sodium • spontaneously hypertensive rats

Nitric oxide is a mediator involved in the regulation of vascular tone, neurotransmission, and host defense. NO is synthetized from L-arginine, and defects in its synthetic pathway have been implicated in several diseases. Attempts to evaluate a role of NO in experimental hypertension have been extensively performed, however, the results of these studies have been highly divergent, depending on animal models and vascular beds examined. In salt-sensitive hypertension, abnormalities of the L-arginine–NO pathway have been demonstrated. Increases in dietary salt raised the NO production (estimated from urinary cGMP) in salt-resistant Dahl/Rapp rats but not in salt-sensitive rats. Parenteral or oral administration of L-arginine abrogated salt-sensitive hypertension and improved pressure natriuresis. These studies suggest that the salt-sensitive Dahl rat possesses a unique defect in the production of NO that manifests as hypertension with high-salt diet and is corrected by administration of the biological substrate for NO synthesis. In contrast to the Dahl rat, L-arginine does not affect the development of hypertension in SHR. Acute studies using NO synthesis inhibitors intravenously have suggested that the NO production is preserved in SHR. Furthermore, recent evidence demonstrated the increased but not decreased production of NO in the heart and aorta of SHR.

The synthesis of NO can be inhibited experimentally by some analogues of arginine including L-NMMA and ADMA, both of which have equally potent vasoconstrictor and pressor actions. Acute administration of ADMA into guinea pigs and rats causes blood pressure elevation partly via elevation of total peripheral resistance. In humans, intra-arterial administration of ADMA lowers forearm blood flow. Although both L-NMMA and ADMA are synthesized and metabolized endogenously, the plasma concentration of dimethylarginine is ten times greater than that of L-NMMA. Since studies in animals and humans suggest that the kidney may be involved in the excretion of ADMA, abnormalities of the ADMA production or elimination have been reported in human kidney diseases. Although recent evidence demonstrated high accumulation of ADMA in plasma from hypercholesterolemic animals and in balloon-injured vessels, the role of endogenous NO synthase inhibitor in the pathogenesis of hypertension has not been elucidated at all.

Accordingly, we hypothesized that endogenous ADMA may play a role in salt-sensitive hypertension by competitive inhibition of NO synthase. To test this hypothesis, urinary and plasma ADMA and NOx were evaluated in Dahl rats on different salt intakes. We also measured urinary and plasma ADMA in SHR as a model of salt-sensitive, NO-independent hypertension to exclude the
Selected Abbreviations and Acronyms

ADMA = \(N^\alpha, N^\alpha\)-dimethyl-L-arginine

DR = Dahl salt-resistant rats

DS = Dahl salt-sensitive rats

L-NMMA = \(N^\alpha\)-monomethyl-L-arginine

MAP = mean arterial pressure

NO = nitric oxide

NOx = nitrate/nitrite

SHR = spontaneously hypertensive rats

UADMA = 24-hour urinary excretion of ADMA

WKY = Wistar-Kyoto rats

possibility that changes in ADMA may be merely an epiphenomenon secondary to blood pressure elevation

Methods

Animals

Dahl/Rapp salt-sensitive rats (DS) and -salt-resistant rats (DR) at 7 weeks of age (Sayawa, Japan) were placed on either 0% or 8% NaCl diet (Oriental Kohbo, Japan) for 1 week (n=8 to 10) SHR and WKY at 7 weeks old (6 each, Charles River Japan, Kanagawa) were fed normal rat chow (CE-2, Clea Japan, Tokyo), which consisted of 110 mmol sodium, 280 mmol potassium, and 15.5 g arginine per kilogram. Throughout the study period, animals were housed in a room at constant temperature (25±C) and humidity (60±5%). The room was lighted automatically from 7 AM to 7 PM. Rats were provided free access to water and rat chow. Animal care and treatment were conducted in conformity with institutional guidelines that are in compliance with international laws and policies (EEC Council Directive 86/609, OJL 358, December 1987, NIH Guide for the Care and Use of Laboratory Animals, NIH publication No 85-23, 1985).

Experimental Protocol

DS or DK fed high- or low-salt diet and SHR or WKY fed normal diet for 1 week were weighed and housed over 4 days in individual metabolic cages to measure water intake, the consumption of rat chow, urine volume, and 24-hour urinary excretion of NOx and ADMA. The metabolic study was performed for only 1 day. After the metabolic experiment, rats were anesthetized with pentobarbital (50 mg/kg), and a catheter (PE-10 connected to PE-50) was passed into the lower abdominal aorta via the right femoral artery to measure MAP directly for 1 hour (model TP-101T, Nihon Koden). Reported pressures represent the average MAP during the last 15 minutes of the recording period. After measurements of MAP, blood was withdrawn for analysis of plasma NOx and ADMA, and all rats were killed immediately after removal, kidneys were frozen in liquid nitrogen for Western blot analysis.

Chemical Analysis

Immediately after sampling, blood was centrifuged in plain tubes without heparin for NOx measurement and in heparinized tubes for ADMA measurement. Urine and plasma samples were stored at -85°C until analysis. The frozen kidneys were rapidly minced and placed on ice in 9 volumes of 10 mmol/L sodium phosphate buffer, pH 7.0, containing 1 mmol/L 2-mercaptoethanol, trypsin inhibitor (10 mg/mL), leupeptin (10 mg/mL), pepstatin (10 mg/mL), and antipain (10 mg/mL), using a Potryn homogenizer. After centrifugation at 10 000 g for 40 minutes, the supernatant was stored at -85°C until analysis. Urine sodium was determined by flame photometer (No 710, Hitachi).

Analysis of NOx and ADMA

NOx were determined by a colorimetric method using the Griess reagent (Wako). Urinary NOx and ADMA excretion were calculated as products of the 24-hour urine volume and urinary NOx and ADMA concentration, respectively. High-performance liquid chromatography was used to measure ADMA using orthophthalaldehyde for fluorescence determination. Immunochemical nitric oxide synthase staining of the kidney was performed with anti-nitric oxide synthase antibodies. Immunohistochemistry was used to measure ADMA using orthophthalaldehyde for fluorescence determination. It was performed on the Hitachi L-6300 system equipped with a F-1080 fluorescence detector for excitation at 348 nm and emission at 450 nm with an L-column ODS (4.6 mm x 250 mm, Chemical Inspection & Testing Institute). Samples are eluted with 75 mmol/L aqueous sodium acetate buffer.

Western Blot Analysis

The supernatant of the kidney was analyzed for dimethylarginase immunoreactivity. In brief, proteins were separated by SDS-polyacrylamide gel electrophoresis using the method of Laemmli \(^{23}\) and then electrophoretically transferred to a nitrocellulose membrane. Immunoblotting analyses were carried out in the manner described previously using Mah-AI raised against denatured dimethylarginase from the rat kidney. \(^{20}\) The immunocomplexes on the membranes were probed with peroxidase-conjugated sheep anti-mouse IgG (1 000 dilution) after the membranes were washed with 1BS/T/wtex. The bound antibodies were visualized by peroxidase reaction with 4-chloro-I-naphthol as a substrate. The relative amounts of the dimethylarginase were assessed by laser densitometry of immunoblots (Densitron PAN-FV, JooKoo Co Ltd). Data were expressed as percent density to non-muscle controls.

Immunohistochemical Analysis

In addition to animals (n=5, each), in vivo renal fixation was performed with 4% paraformaldehyde (Sigma Chemical Co) in 0.01 mol/L phosphate buffer (pH 7.4), through a 19-gauge needle inserted in the left ventricle, at constant perfusion pressure of 140 mm Hg. After a wash with 10% sucrose solution saline and excision, the fresh samples were immediately embedded in compound and were frozen by liquid nitrogen. Serial 5-μm-thick frozen sections were adhered to poly-L-lysine-coated slides. The labeled streptavidin-biotin method was used for immunohistochemical staining (LSAB Kit, DAKO Co). Briefly, the specimens were treated with 0.3% hydrogen peroxide for 30 minutes to inhibit endogenous peroxidase. All sections were incubated with 1% BSA and then incubated with primary antibody, Mah-A1, raised against denatured dimethylarginase from the rat kidney, \(^{20}\) at 4°C overnight. After three washes in PBS (pH 7.4), biotinylated anti-mouse IgG secondary antibodies were applied, followed by peroxidase-conjugated streptavidin. Peroxidase activity was visualized with 3- amino-9-ethylcarbazole, and the sections were faintly counterstained with Meyer's hematoxylin or methyl green.

Statistical Analyses

Experimental groups were compared by ANOVA and, when appropriate, with Scheffe's test for multiple comparisons. All data are expressed as mean±SEM unless otherwise indicated. Linear regression analyses were performed between the amount of urinary ADMA excretion and mean arterial pressure.

Results

Hemodynamics and Urinary Sodium Excretion

MAP of DS fed high salt diet (DSH) was significantly higher than that of DS fed low-salt diet (DSL) (Fig 1, P<0.1). MAP was similar between DR fed high-salt diet (DRH) and DR fed low-salt diet (DRL). MAP was significantly higher in SHR than in WKY (Fig 2, P<0.005). Although SIIR had significantly higher heart rate compared with WKY (340±14 versus 290±9 bpm, P<0.05), there were no significant differences in heart rate among Dahl rats (DSH, 316±13, DSL, 342±7, DRH, 341±13, and DRL, 314±15 bpm, NS) in Dahl rats fed high-salt diet, urinary sodium excretion was significantly (P<0.01) greater in rats fed low-salt diet, although there was no significant difference in urinary sodium excretion between salt-sensitive and -resistant rats on either high- or low-salt diet.
low-salt diets (DSH, 22.7±2.1; DSL, 0.4±0.1; DRH, 21.0±2.5; and DRL, 0.5±0.1 mEq/d).

NOx and ADMA

DR fed high-salt diet had greater urinary NOx excretion than DR fed low-salt diet (Fig 1, P<.01). However, in DS, there was no significant difference in urinary NOx excretion between high-salt and low-salt diet (Fig 1, NS). The plasma NOx level was similar among groups (DSH, 10.0±0.7; DSL, 9.5±0.5; DRH, 10.2±0.5; and DRL, 9.6±0.7 μmol/L; NS). Urinary ADMA excretion was significantly greater in DS fed high-salt diet than in DS fed low-salt diet (Fig 1, P<.01) and was similar between the two groups of DR rats. The plasma ADMA level was similar among groups (DSH, 0.73±0.03; DSL, 0.66±0.03; DRH, 0.69±0.03; and DRL, 0.67±0.03 pmol/L; NS).

Urinary NOx excretion was significantly greater in SHR than in WKY (Fig 2, P<.05), although there was no difference in the plasma NOx concentration (SHR, 14.4±1.2; WKY, 13.4±2.5 μmol/L; NS). There was no difference in the plasma ADMA concentration (SHR, 0.62±0.03 versus WKY, 0.62±0.03 μmol/L; NS); urinary ADMA excretion, however, was significantly smaller in SHR than in WKY (Fig 2, P<.05).

Correlation Between MAP and ADMA

In DS, the amount of urinary ADMA excretion was positively correlated with MAP (r=−.65, P<.01) (Fig 3a), whereas in SHR and WKY the amount of urinary ADMA excretion was inversely correlated with MAP (r=−.64, P<.05; Fig 3b).

Dimethylargininase Immunoblot Analysis

Fig 4 shows Western blot analysis of dimethylargininase in kidney extracts. Either between SHR and WKY or among Dahl rats on different diets, there were no significant differences in protein levels of dimethylargininase in the kidney. Accordingly, laser densitometric scan of Western blots revealed no differences between SHR and WKY (SHR 112±12%; WKY 100±0%;) and among four groups of Dahl rats (DSH, 114±14%; DSL, 144±23%; DRH, 118±20%; and DRL, 100±0%).

Dimethylargininase Immunohistochemical Analysis

Fig 5 shows representative photomicrographs of dimethylargininase immunohistochemical staining in kidney of DS fed high-salt diet (top) and SHR (bottom). Dimethylargininase was localized in the luminal side of tubular epithelium, whereas no immunoreactivity was detected in the vasculature and glomeruli. These results were similar among strains and between low- and high-salt diet (data not shown).

Discussion

The purpose of this study was to examine the role of ADMA, an endogenous NO synthase inhibitor, in experimental hypertension. High-salt diet raised blood pressure.
dominantly affect the NOx production. Because the dietary intake of NOx and tubular reabsorption of NOx has been considered to reflect the systemic NO production, we evaluated the 24-hour urinary NOx production as an index of the NO production in this study. Although urinary NOx excretion was a significant correlation between the amount of urinary ADMA excretion in SHR and WKY. However, we have not complete ADMA excretion than WKY. Although we have not likely to be a secondary phenomenon in response to blood pressure elevation, because SHR had lower urinary ADMA excretion in response to increases in urinary ADMA excretion in DS. There was a significant correlation between the amount of ADMA excretion and arterial pressure. Because high-salt diet increased urinary NOx excretion in DR but not in DS, our results may be interpreted to suggest that the NO production is compensatorily increased during high-salt intake to attenuate blood pressure elevation in DR, whereas the increased production of ADMA during high-salt intake elevates blood pressure by inhibiting the compensatory increases in the NO production in DS. In contrast to Dahl rats, high blood pressure in SHR was associated with increased urinary NOx excretion and decreased ADMA excretion compared with WKY. Our results suggest that ADMA may play a role in the pathogenesis of hypertension in DS but not in SHR and that the increase in ADMA in DS during high-salt intake may not be just an epiphenomenon of hypertension.

The in vivo activity of NO cannot be directly monitored because NO is very unstable and rapidly metabolized by oxidation initially to nitrite (NO2) and ultimately to the stable end product, nitrate (NO3). We therefore evaluated the 24-hour urinary NOx production as an index of the NO production in this study. Although urinary NOx excretion has been considered to reflect the systemic NO production, dietary intake of NOx and tubular reabsorption predominantly affect the NOx production. Because the 24-hour consumption of rat chow measured simultaneously with the urine collection was similar among rat groups (data not shown), the greater NOx excretion in DR on high-salt diet was not due to the greater NOx intake. It is possible that the renal tubular handling of NOx among Dahl rats is different and may have caused greater excretion of NOx in DR on high-salt diet. The recent study from the laboratory of Dr Baylis (Süttö et al) has shown that NOx is reabsorbed extensively in the proximal tubule and that inhibition of proximal reabsorption leads to an increase in urinary NOx. They suggested that measurements of acute changes in urinary NOx should be interpreted cautiously, since it may reflect altered tubular handling of NOx rather than the changes in the systemic and/or renal NO production. However, our studies were conducted in the chronic setting of 1 week, and the changes in the tubular handling were less likely to account for our results, although the possibility remains.

It has been reported that high-salt intake induces NO synthase in normotensive rats, and functional defects of such adaptive response have been suggested as a pathogenesis of salt-sensitive hypertension. Indeed, Chen and Sanders reported that the increase in urinary cGMP excretion in response to high-salt diet was significantly lower in Dahl/Rapp salt-sensitive rats, suggesting impaired NO synthase as a maladaptation to high-salt stress. In accordance with the previous study, we demonstrated that high-salt diet increased urinary NOx excretion in normotensive Dahl salt-resistant rats, whereas salt-sensitive hypertensive rats did not show any change in NOx in responses to high-salt diet. Thus, our results also suggest the impaired NO production in DS on high-salt intake. The mechanisms of this impaired NO synthase synthesis of salt-sensitive rats on high-salt diet are unknown. Because ADMA is an endogenous L-arginine analogue that has potent vasoconstrictor/pressor effects by inhibiting NO synthase and because it has been demonstrated that chronic L-arginine administration abrogates salt-sensitive hypertension and improves pressure natriuresis, we thought that endogenous ADMA may play a role in salt-sensitive hypertension by competitive inhibition of NO synthase. Indeed, in contrast to NOx, urinary ADMA excretion increased in only salt-sensitive rats fed high-salt diet but not in salt-resistant rats. Furthermore, the amount of urinary ADMA excretion of the endogenous NO synthase inhibitor was positively correlated with blood pressure in salt-sensitive rats. The increase in urinary ADMA excretion in DS on high-salt intake is not likely to be a secondary phenomenon in response to blood pressure elevation, because SHR had lower urinary ADMA excretion than WKY. Although we have not...
proven that the NO synthesis decreased in the Dahl S rat by competitive inhibition, the correlation between arterial pressure and ADMA excretion in the DS may be important for understanding the pathophysiology of salt-sensitive hypertension.

Mechanisms by which high-salt intake increased urinary ADMA excretion are not clarified in this study, but several possibilities are considered. Although the metabolic pathway of ADMA in physiological conditions has been extensively investigated by the laboratories of Ogawa and of Vallance, we have little information regarding the altered metabolism in pathological conditions. ADMA is produced in several organs, released into blood, and excreted from the kidney. However, the plasma level and urinary excretion are mainly determined by the renal production and excretory capacity of ADMA. It is unlikely that the excretory capacity of ADMA from the kidney was augmented in DS on high-salt intake because the plasma level of ADMA was similar among strains. It is possible that greater urinary ADMA excretion may reflect augmented renal production of ADMA in DS, since our results, as well as those of others, demonstrated the impaired renal production of NO in salt-sensitive rats, which was reversed by L-arginine supplement. Since dimethylargininase has been considered as a key limiting enzyme of ADMA and reported to distribute dominantly in the kidney, we conducted Western blot and immunohistochemical analyses of this enzyme in the kidney to examine whether dimethylargininase in the kidney accounts for the augmented urinary ADMA excretion in DS on high-salt intake. Both protein levels and immunoreactivities of dimethylargininase were similar among Dahl rats and similar between SHR and WKY. Thus, it is unlikely that the renal degradation of ADMA was attenuated in DS on high-salt intake. Finally, it is also possible that the increased urinary excretion was due to the systemic overproduction of ADMA. Dimethylargininase is localized not only in the kidney but also in the pancreas and
In the present study, Dahl rats were fed high- or low-salt diet only for 1 week. Nonetheless, high-salt-fed DS showed significantly higher blood pressure elevation. Chronic high-salt exposure to salt-sensitive rats is known to cause vascular damages, which may affect NO production non-specifically secondary to hypertension. To avoid this possibility, we fed them for only 1 week in this study. The longer feeding in Dahl rats may have produced different results for NOx and ADMA. Our results may suggest that ADMA is involved in the development of salt-sensitive hypertension. However, additional experiments in which measurements are made of MAP and NOx excretion during chronic administrations of ADMA or of an inhibitor of dimethylarginase in Dahl rats during high-salt intake may be necessary to verify our conclusion. These studies should be performed in the future.

In summary, DS fed high-salt diet had significantly higher blood pressure, lower NO production, and greater excretion of endogenous NO synthase inhibitor than DSS fed low-salt diet. Urinary ADMA excretion was significantly correlated with arterial blood pressure in DS SHR had significantly greater NO production and lower ADMA excretion than WKY. Our results may suggest the role of ADMA, an endogenous NO synthase inhibitor, in the pathogenesis of salt-sensitive hypertension in DS but not in SHR.

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