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umerous studies have demonstrated that excessive salt intake causes cardiovascular damage and that this was counteracted by potassium supplementation. According to the earlier report, salt loading reduced the survival rate of Dahl salt-sensitive (DS) rats, a model of salt-sensitive hypertension, whereas potassium supplementation alleviated this salt-induced premature mortality, independent of its hypotensive action. It has been speculated that this may be a result of the vasoprotective effect of potassium, because potassium supplementation has been shown to ameliorate vascular endothelial dysfunction in salt-loaded DS rats. Also, Ma et al clearly demonstrated that high-potassium intake inhibited neointimal formation in several vascular injury models, such as a balloon injury of rat carotid and swine coronary arteries from animals without hypertension. Thus, dietary potassium may have vasoprotective mechanisms beyond the blood pressure (BP)–lowering effect. Although it has been shown in vitro experiments that potassium inhibited migration and proliferation of vascular smooth muscle cells, a factor(s) mediating vasoprotective effects of dietary potassium has not yet been clarified by in vivo studies.

Arterial damage could cause intimal thickening, which originates from accumulation of vascular smooth muscle cells and deposition of the extracellular matrix, followed by vascular reactive oxygen species (ROS) overproduction.

Abstract—Hypertensive cardiovascular damage is accelerated by salt loading but counteracted by dietary potassium supplementation. We suggested recently that antioxidant actions of potassium contribute to protection against salt-induced cardiac dysfunction. Therefore, we examined whether potassium supplementation ameliorated cuff-induced vascular injury in salt-sensitive hypertension via suppression of oxidative stress. Four-week–old Dahl salt-sensitive rats were fed a normal-salt (0.3% NaCl), high-salt (8% NaCl), or high-salt plus high-potassium (8% KCl) diet for 5 weeks, and some of the rats fed a high-salt diet were also given antioxidants. One week after the start of the treatments, a silicone cuff was implanted around the femoral artery. Examination revealed increased cuff-induced neointimal proliferation with adventitial macrophage infiltration in arteries from salt-loaded Dahl salt-sensitive rats compared with that in arteries from non–salt-loaded animals (intima/media ratio: 0.471±0.070 versus 0.302±0.037; P<0.05), associated with regional superoxide overproduction and reduced nicotinamide-adenine dinucleotide phosphate oxidase activation and mRNA overexpression. On the other hand, simultaneous potassium supplementation attenuated salt-induced neointimal hyperplasia (intima/media ratio: 0.205±0.012; P<0.001), adventitial macrophage infiltration, superoxide overproduction, and reduced nicotinamide-adenine dinucleotide phosphate oxidase activation and overexpression. Antioxidants, which decrease vascular oxidative stress, also reduced neointima formation induced by salt excess. In conclusion, high-potassium diets seems to have a protective effect against the development of vascular damage induced by salt loading mediated, at least in part, through suppression of the production of reactive oxygen species probably generated by reduced nicotinamide-adenine dinucleotide phosphate oxidase. (Hypertension. 2008;51:225-231.)

Key Words: hypertension ■ sodium ■ potassium ■ antioxidants ■ arteries
taneously hypertensive rats. However, these earlier reports did not evaluate the role of ROS in vascular injury in detail and, to the best of our knowledge, the effect of dietary potassium on the ROS-generating system has never been demonstrated in in vivo studies, although the role of NADPH oxidase has been hypothetically proposed.

In the present study, therefore, we investigated the effect of dietary salt and potassium on the progression of vascular damage and NADPH oxidase–related ROS generation using a cuff-injured model of the femoral artery in DS rats. We also examined the effects of a membrane-permeable superoxide dismutase mimetic, 4-hydroxy-2,6,6-tetramethyl-piperidine-N-oxyl (tempol), and an NADPH oxidase inhibitor, 4-hydroxy-3-methoxy-acetophenone (apocynin), on neointima formation in a cuffed artery prepared from salt-loaded DS rats to clarify the role of ROS on vascular damage.

Methods

Animals

Four-week-old male DS and Dahl salt-resistant (DR) rats were obtained from SLC. The DS rats were randomly assigned to rat chow containing a normal salt (0.3% NaCl), a high-salt (8% NaCl), or a high-salt plus high potassium (8% KCl; standard and high-salt rat chow containing 1.3% KCl; Oriental Yeast Co, Ltd) diet. DR rats were randomly divided into normal- and high-salt chow. Tempol (1 mmol/L) or apocynin (1.5 mmol/L), dissolved in tap water, was administered orally to some of the salt-loaded DS rats. The animals were maintained in a humidity- (60%/H11006 1.5°C), light cycle- (7AM to 7PM) regulated room and were allowed free access to the designated diets and water for 5 weeks. During the last week of the treatment, the rats were placed in individual metabolic cages, and 24-hour urine samples were collected. Systolic BP (SBP) was measured using a tail-cuff method (BP-98A, Softron) in unanesthetized animals every week. On the last day of the study, the femoral artery was cannulated under ether anesthesia. Mean arterial pressure was measured after waiting for recovery of the animals from anesthesia for ≥3 hours. All of the animals were handled in an accredited institute facility in accordance with the institutional animal care policies, and all of the research protocols conformed to the guiding principles for animal experimentation as laid down by the ethics committees on animal research of the University of Tokyo.

Cuff Placement

One week after the start of the treatments, a silicone cuff (ID: 0.64 mm; OD: 1.24 mm; length: 5 mm) was placed around femoral artery, as described previously. The rats were anesthetized with an injection of sodium pentobarbital (50 mg/kg IP). The femoral artery was exposed and cleaned of connective tissue, and a longitudinally cut silicone rubber tube was loosely placed around the artery. The contralateral femoral artery was only isolated without cuff placement (sham-operated artery).

Tissue Harvesting and Histological Examination

Four weeks after the cuff placement, the animals were anesthetized by sodium pentobarbital, and blood samples were obtained from the abdominal vein. Then, the rats were euthanized by an overdose of pentobarbital and perfused with PBS. The cuffs were removed, and arteries were cleaned of connective tissue, fixed with 4% paraformaldehyde, and embedded in paraffin. Sections of the middle portion of the cuffed artery were stained with elastic-Verhoeffen-von Gieson method. The cross-sectional areas of the intima and media were measured in micrometers squared using Image-ProPlus 5.0 software.

Measurement of Vascular Superoxide Production

The superoxide (O2·−) production was measured by the bis-N-methylacridinium nitrate (lucigenin)–enhanced chemiluminescence assay. Our previous study showed that O2·− production from the cuff-injured femoral artery increased in a time-dependent fashion until 3 days after the operation and subsequently decreased. Therefore, in this study, the femoral artery was harvested on the third postoperative day for the measurement of O2·− production. For lucigenin chemiluminescence, DS rats were perfused with PBS, the cuff was gently removed, and the femoral artery was excised and freed of surrounding tissue. After 30-minute equilibration in modified Hepes buffer at 37°C, the specimen was transferred into test tubes containing 5 μmol/L of lucigenin. The chemiluminescence was measured using a scintillation counter (Lumat LB9507, Berthold Technologies). To examine the direct effect of antioxidants, cuffed femoral arteries in salt-loaded DS rats were treated for 30 minutes with tempol (0.1 mmol/L or 1 mmol/L) or apocynin (1 mmol/L or 10 mmol/L). For assessment of NADPH oxidase activity, NADPH (100 μmol/L) was added as a substrate, and the measurement was conducted. Repeated measurements of the specimens were interpreted every 30 seconds, and the average value over a 5-minute period was reported. The fresh femoral arteries were weighed, and the lucigenin counts were normalized by the wet weights (milligrams).

Real-Time Quantitative RT-PCR Analysis of NADPH Oxidase Subunit Expression

Total RNA was prepared from the pooled samples of femoral arteries of DS rats on the third postoperative day, and mRNA expression levels of NADPH oxidase subunits, p22phox, gp91phox, and p47phox, were measured. RNA was isolated using RNaseFree Fibrous Tissue Mini Kit (Qiagen Inc) and reverse transcribed. For real-time PCR, primers of TaqMan probe from PE Biosystems were used. An Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems) was used with the default thermal cycle program (95°C for 10 minutes, followed by 40 cycles of 95°C for 20 seconds and 60°C for 1 minute). The transcript levels of components of NADPH oxidase were normalized with those of the 18S RNA transcript.

Assay of Serum and Urine

Sodium and potassium concentrations in serum and urine were measured by flame photometry.

Statistical Analysis

Data are expressed as mean±SEM. In all of the cases, “n” refers to the numbers of animals. Statistical analysis was done by a 1-way and repeated-measurements (SBP; Figure 1A) ANOVA followed by Turkey’s posthoc multiple comparisons. P values <0.05 were considered to be statistically significant.
Results

Effects of Salt Loading on Cuff-Induced Neointima Formation in Femoral Arteries of DS and DR Rats

Urinary sodium excretion suggested successful salt loading (Table). Serum sodium and potassium did not change with salt loading. Salt loading increased SBP (209 ± 6.9 versus 135.7 ± 1.7 mm Hg; P < 0.001; Figure 1A) and mean arterial pressure (164 ± 2.3 versus 111 ± 4.5 mm Hg; P < 0.001; Figure 1B) in DS rats. Intimal hyperplasia developed in the femoral artery by 28 days after the cuff implantation (Figure 2). Because there was no apparent increase in medial thickness, a statistical comparison was conducted using the I/M ratio. Salt loading accelerated neointima formation in DS rats (I/M ratio: 0.471 ± 0.070 versus 0.302 ± 0.038; P < 0.001; Figures 2 and 3). On the other hand, a high-salt diet did not significantly increase the I/M ratio in DR rats (Figure 3), in which salt loading also did not affect SBP (131 ± 1.9 versus 134 ± 2.3 mm Hg; n = 4, respectively).

Effects of Potassium Supplementation on Salt-Induced Acceleration of Neointima Formation in Cuff-Induced Femoral Arteries of DS Rats

As suggested by urinary electrolytes excretion, potassium supplementation did not decrease salt intake (Table). With potassium supplementation, serum sodium was not altered, but serum potassium increased (P < 0.001). Dietary potassium supplementation slightly reduced BP in salt-loaded DS rats (SBP: 187 ± 4.1 mm Hg; mean BP: 147 ± 3.9 mm Hg; P < 0.05, respectively; Figure 1A and 1B). In spite of the small reduction in BP, potassium supplementation almost completely attenuated salt-induced acceleration of neointima proliferation in DS rats (I/M ratio: 0.205 ± 0.012; P < 0.001; Figures 2 and 3).

ROS Production in Cuff-Induced Femoral Arteries After Salt Loading and Potassium Supplementation

High-salt diet increased O_2^- production in the cuff arteries of DS rats (544.0 ± 67.1 versus 164.3 ± 17.7 cpm/mg; P < 0.001; Figure 4A). On the other hand, potassium supplementation reduced salt-induced O_2^- overproduction (219.3 ± 24.4 cpm/mg; P < 0.001; Figure 4A). Also, ex vivo treatment with tempol and apocynin inhibited the O_2^- generation in cuff arteries of salt-loaded DS rats in a dose-dependent fashion (Figure 4B). The potent ROS-inhibitory action of apocynin suggests that the major source of ROS may be NADPH oxidase. Actually, NADPH-induced O_2^- production was increased in the cuff arteries of salt-loaded DS rats than in non–salt-loaded rats (P < 0.001), whereas potassium supplementation reversed the increase induced by salt loading (Figure 4C, right; P < 0.001). In addition, the O_2^- production induced by NADPH from the cuff femoral arteries was significantly increased compared with that produced in the absence of NADPH in DS rats fed high- and normal-salt diets (P < 0.05, respectively), but not in DS rats fed a high-salt plus high-potassium diet (Figure 4C); NADPH oxidase activity was lower in cuffed arteries of DS rats fed a high-salt plus high-potassium diet than in those fed a normal-salt diet (P < 0.05; Figure 4C, right).
mRNA Expressions of NADPH Oxidase Subunits in the Cuffed Femoral Artery

Expression of p22phox (P<0.01), gp91phox (P<0.001), and p47phox (P<0.001) was significantly higher in salt-loaded DS rats than in non–salt-loaded animals (Figure 5). Dietary potassium supplementation restored the expression levels of NADPH oxidase subunits in salt-loaded DS rats (P<0.001, respectively).

Effect of Tempol and Apocynin Administration on Salt-Induced Acceleration of the Cuff Injury

Tempol suppressed neointima formation in salt-loaded DS rats (I/M ratio: 0.276±0.042; P<0.01; Figure 3), associated with decreased vascular O$_2^-$ production (P<0.001; Figure 4A), although it did not decrease BP (SBP: 203±2.1 mm Hg; mean BP: 157±3.9 mm Hg; Figure 1A-B). Apocynin, which reduced BP (SBP: 186±4.9 mm Hg; P<0.05; mean BP: 141±3.1 mm Hg; P<0.01; Figure 1A and 1B) and O$_2^-$ production (P<0.001; Figure 4A), potently suppressed neo-

Discussion

In the present study, salt loading exacerbated cuff-induced intimal thickening in the femoral artery of DS rats, accompanied by regional ROS overproduction. Dietary potassium supplementation alleviated a salt-induced increase of neointima proliferation and ROS generation. The vascular redox status may contribute to both salt-induced acceleration of neo-intima formation (I/M ratio: 0.145±0.021; P<0.001; Figure 3) in salt-loaded DS rats.

Macrophage Infiltration in the Cuffed Femoral Arteries

In DS rats fed a normal-salt diet, immunohistochemical staining for ED1 was slightly increased in the adventitia of cuffed arteries compared with that in adventitia of a sham-operated artery (Figure 6). Salt loading further increased the adventitial macrophage infiltration, whereas simultaneous potassium supplementation and Tempol decreased it.
NADPH oxidase may be the major source of ROS. Actually, the changes in ROS production caused by salt loading and potassium supplementation were parallel to changes in regional NADPH oxidase activity and expression.

A cuff implanted around arteries works as a foreign body, activating inflammation in the affected area. In the present study, macrophage infiltration was predominantly observed in the adventitia of cuffed arteries. Adventitial macrophage infiltration was enhanced in salt-loaded DS rats, which was attenuated by potassium supplementation. Similarly, in the previous study, a high-potassium diet ameliorated macrophage infiltration of the aorta in stroke-prone, spontaneously hypertensive rats. Vascular injuries by cuff placement and balloon angioplasty have been shown to be associated with adventitial inflammation characterized by accumulation of leukocytes, leading to subsequent intimal hyperplasia. Because there is in vivo evidence implicating that proliferating adventitial fibroblasts might migrate into the intima after arterial injury, adventitial inflammation may play a key role in neointimal hyperplasia. In the present study, tempol suppressed both salt-induced enhancement of adventitial infiltration by macrophages and neointimal proliferation in cuffed arteries. Conversely, intimal thickening with enhanced adventitial macrophage infiltration was observed in cuffed arteries of mice with intrinsic antioxidant deficiency. Moreover, adequate anti-ROS therapy directed at adventitial cells can potentially counteract neointima formation. Thus, numerous macrophages infiltrating adventitia could generate excessive ROS and play an important role in the inflammatory responses in the vasculature.

In the present study, dietary potassium supplementation counteracted salt-induced acceleration of vascular injury, possibly via its antioxidant effect mediated through inactivation of NADPH oxidase. Recently, it has been postulated that an increase in extracellular potassium can boost the membrane sodium pump activity, hyperpolarizing the cell membrane and “turning off” NADPH oxidase activation, because membrane depolarization upregulates the activity of this enzyme complex. However, whereas potassium supplementation slightly increased serum potassium in salt-loaded DS rats, a mere increase of serum potassium by 0.8 mEq/L (Table) cannot fully explain the ~60% decrease in ROS production observed in this study (Figure 4A); in another study, a 1-mEq/L increase of serum potassium caused only a 10% to 20% reduction in oxidative stress. On the other hand, negative sodium balance may contribute to potassium-induced suppression of ROS overproduction, because potassium supplementation causes natriuresis. Recently, in relation to salt-induced stimulation of oxidative stress, it has been suggested that endogenous cardiotonic steroids, which have been shown to be increased because of volume expansion, caused cardiomyopathy through ROS overproduction associated with Src phosphorylation. Thus, changes in volume status might play an important role in the antioxidant effect of dietary potassium, because the cardiovascular protective effect of dietary potassium was certainly apparent in salt-loaded hypertensive mice.18-21 Actually, the antioxidant effect of potassium supplementation has been demonstrated in vivo only in salt-loaded, stroke-prone, spontaneously hypertensive rats, although dietary potassium depletion, which may be more critical in potassium homeostasis compared with potassium supplemen-
tation, has been shown to induce ROS overproduction in the non–salt-loaded state.\textsuperscript{15}

Although arterial NADPH oxidase activity was suppressed in potassium-supplemented and salt-loaded DS rats compared with normal-salt diet–fed animals in this study, ROS generation from cuffed arteries was similar in these animals. Thus, NADPH oxidase–independent ROS-producing or scavenging systems, eg, mitochondrial ROS generation\textsuperscript{30} and microvascular superoxide dismutase,\textsuperscript{31} which have been reported to be stimulated by salt loading but might not be inhibited by dietary potassium, may also contribute to ROS production in cuffed arteries of salt-loaded DS rats.

In this study, potassium supplementation slightly inhibited the salt-induced rise of BP. Because hypertension is one of the major determinants of vascular injury, changes in BP induced by dietary salt and potassium may have contributed to the observed vascular effects. However, tempol ameliorated intimal hyperplasia in cuffed arteries of salt-loaded DS rats without significant BP reduction, suggesting that ROS may, nonetheless, be the key mediator of influences of

\begin{figure}
\centering
\includegraphics[width=\textwidth]{mRNA_expression.png}
\caption{mRNA expression of p22phox, p47phox, and gp91phox, NADPH oxidase subunits by real-time RT-PCR analyses in cuffed arteries from DS rats (n=3, respectively). High-salt diet (closed bar) greatly increased these expressions compared with normal-salt diet (open bar), whereas potassium supplementation (shaded bar) attenuated the increase in the expression. †P<0.01, ‡P<0.001. Abbreviations are the same as those shown in the legend for Figure 1.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure_6.png}
\caption{Representative immunoreactive photomicrographs of macrophage infiltration in sham-operated (top left) and cuffed femoral arteries from DS rats. ED1 staining was more prominent in arteries of salt-loaded DS rats (top right) vs those in normal-salt diet–fed animals (top middle) or salt-loaded animals with potassium supplementation (bottom left). Tempol attenuated ED1 staining (bottom middle) in cuffed arteries from salt-loaded DS rats. Original magnification, \times 200. Bar=100 \mu m. Abbreviations are the same as those shown in the legend for Figure 1.}
\end{figure}
sodium and potassium balance on vascular damage. Actually, in spite of higher BP, the I/M ratio was slightly, but nonsignificantly, reduced in DS rats fed a high-salt plus high-potassium diet compared with animals fed a normal-salt diet. In conclusion, we demonstrated that excessive salt intake enhanced neointimal hyperplasia in the cuffed femoral artery in DS rats, accompanied by ROS overproduction mediated via upregulation of NADPH oxidase, and that potassium supplementation protected against salt-induced intimal thickening by suppressing NADPH oxidase activity. Thus, suppressive action of potassium on NADPH oxidase may be important in the attenuation of vascular injury in salt-sensitive hypertension.

Perspectives
Our results showed that potassium supplementation could blunt the progression of vascular injury, possibly through attenuation of vascular injury in salt-sensitive hypertension.

Sodium and potassium intakes are important determinants of BP levels (7–9). In general, sodium intake is thought to be the main source of BP elevation (10); however, potassium intake may have a significant effect on BP levels (11). In this research, potassium intake might reduce macrophage adherence to the vascular wall in stroke-prone spontaneously hypertensive rats (12). However, dietary potassium may involve mainly its counteracting action against salt-induced NADPH oxidase activation, a harmful effect of salt excess, but potassium supplementation might not inhibit some other vascular ROS-generating systems. Thus, potassium supplementation might be more effective for cardiovascular protection than a simple antioxidant.

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Disclosures
None.

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Effect of Dietary Potassium on Vascular Injury

Protective Effect of Dietary Potassium Against Vascular Injury in Salt-Sensitive Hypertension

Makiko Kido, Katsuyuki Ando, Maristela L. Onozato, Akihiro Tojo, Masahiro Yoshikawa, Teruhiko Ogita and Toshiro Fujita

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