ORIGINAL ARTICLE

High sodium augments angiotensin II-induced vascular smooth muscle cell proliferation through the ERK 1/2-dependent pathway

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Angiotensin II (Ang II)-induced vascular injury is exacerbated by high-salt diets. This study examined the effects of high-sodium level on Ang II-induced cell proliferation in rat vascular smooth muscle cells (VSMCs). The cells were cultured in a standard medium containing 137.5 mmol l⁻¹ of sodium. The high-sodium medium (140 mmol l⁻¹) contained additional sodium chloride. Extracellular signal-regulated kinase (ERK) 1/2 phosphorylation was determined by western blot analysis. Cell proliferation was evaluated by [³H]-thymidine incorporation. Ang II (100 nmol l⁻¹) significantly increased ERK 1/2 phosphorylation and cell proliferation in the both medium containing standard sodium and high sodium. High-sodium level augmented Ang II-induced ERK 1/2 phosphorylation and cell proliferation compared with standard sodium. Pre-treatment with candesartan (1 μmol l⁻¹, Ang II type 1 receptor blocker) or PD98095 (10 μmol l⁻¹, ERK kinase inhibitor) abolished the proliferative effect induced by high sodium/Ang II. Pre-treatment with 5-*N,N*-hexamethylene amiloride (30 μmol l⁻¹, Na⁺/H⁺ exchanger type 1 (NHE-1) inhibitor), but not SN-6 (10 μmol l⁻¹, Na⁺/Ca²⁺ exchanger inhibitor) or ouabain (1 mmol l⁻¹, Na⁺/K⁺-ATPase inhibitor) attenuated ERK 1/2 phosphorylation or cell proliferation. Osmotic pressure or chloride had no effect on Ang II-induced proliferative changes. High-sodium level did not affect Ang II receptor expression. Ang II increased intracellular pH via NHE-1 activation, and high-sodium level augmented the pH increase induced by Ang II. These data suggest that high-sodium level directly augments Ang II-induced VSMC proliferation through NHE-1- and ERK 1/2-dependent pathways and may offer new insights into the mechanisms of vascular remodeling by high-sodium/Ang II.

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INTRODUCTION

Sodium homeostasis is a critical morbidity and mortality factor in patients with cardiovascular diseases. Clinical studies have shown that a high-salt diet increases the risks of cardiovascular and cerebrovascular diseases, independent of other risk factors including blood pressure.^{1,2} In addition, experimental studies have demonstrated that a high-salt treatment exaggerates the development of hypertension and cardiovascular complications in hypertensive animals.^{3,4} In normotensive rats, chronic treatment with high-salt diets elicited structural changes in the arterioles, reductions in the microvessel density and impaired relaxation of the skeletal muscle resistance vessels in response to a variety of vasodilator stimuli.^{5–7}

Hansen-Smith *et al.*⁸ reported that microvascular rarefaction and profound ultrastructural alterations occur in hypertensive and normotensive animals after only 3 days on a high-salt diet. High-salt-induced vascular complication has been considered to be

predominantly caused by hypertension; blood pressure-independent vascular injury has also been reported.^{1,2,9} Possible molecular mechanisms of high blood pressure-independent salt-induced vascular remodeling have been proposed, such as transforming growth factor-β and nitric oxide production in endothelial cells,¹⁰ hypertrophy of cardiovascular cells,¹¹ macrophage infiltration¹⁰ and activation of the local renin–angiotensin (Ang) system.¹² The mechanism of high-salt-induced vascular smooth muscle cell (VSMC) proliferation, which is involved in the progression of atherosclerosis,¹³ is unclear.

Ang II has crucial roles in the pathogenesis of vascular remodeling.¹⁴ A growing body of evidence indicates that salt treatment augments Ang II-induced vascular remodeling.^{15,16} We hypothesized that increased extracellular levels of sodium directly augment Ang II-induced pro-atherogenic changes in VSMCs. To test this hypothesis, we examined the effects of sodium on Ang II-induced proliferation in cultured

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VSMCs. We investigated Ang II-induced intracellular signaling in a medium with different sodium concentrations.

METHODS

Cell culture and treatment

Cells were isolated from the thoracic aorta of male Sprague–Dawley rats by enzymatic digestion as previously described.¹⁷ The experimental procedures were performed according to the guidelines for the care and use of animals established by Kagawa University. The cells were grown in 40% minimum essential medium (Na 144.4 mmol l⁻¹; Life Technologies, Carlsbad, CA, USA) plus 60% medium 199 (Na 133.0 mmol l⁻¹; Life Technologies) supplemented with 10% fetal bovine serum (Hyclone Laboratory, Logan, UT, USA), 100 U ml⁻¹ of penicillin (Life Technologies) and 100 µg ml⁻¹ of streptomycin (Life Technologies) at 37 °C under 5% CO₂/95% air in a humidified incubator. This medium was used as the standard medium with a sodium concentration of 137.5 ± 1.0 mmol l⁻¹. Medium containing 140 mmol l⁻¹ of sodium was made by adding sodium chloride (Wako, Osaka, Japan) to the standard medium as previously reported.¹¹

After incubation with Ang II, protein or mRNA was extracted as described previously.¹⁸ In some experiments, the VSMCs were pre-treated with 10 µmol l⁻¹ PD98095 (an extracellular signal-regulated kinase (ERK) kinase inhibitor) for 30 min, 100 nmol l⁻¹ of candesartan (Ang II type 1 (AT₁) receptor blocker) for 3 h, 10 µmol l⁻¹ of SN-6 (Na⁺/Ca²⁺ exchanger inhibitor) for 5 min, 1 mmol l⁻¹ of ouabain (Na⁺/K⁺ ATPase inhibitor) for 5 min or 30 µmol l⁻¹ of 5-*N,N*-hexamethylene amiloride (HMA, Na⁺/H⁺ exchanger type 1 (NHE-1) inhibitor) for 1 h.

Western blot analysis

Cells at 80–90% confluence were made quiescent by incubation in a medium containing 0.1% fetal bovine serum for 24 h. The cells were stimulated with agonists in a serum-free medium and lysed, as described previously;¹⁹ the solubilized proteins were isolated by centrifugation and quantified by Bradford assay. The proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, the blots were incubated with primary antibodies; the blots with embedded infrared dye were visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Antibodies to phospho-ERK 1/2 or ERK 1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA), and anti-AT₁ receptor antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). To confirm equal protein loading, each membrane was reprobed with anti-β-actin antibody (Sigma-Aldrich, St Louis, MO, USA). The band intensities were quantified by immunoblot densitometry using NIH ImageJ software (NIH, Bethesda, MD, USA).

Immunoprecipitation

We determined the NHE-1 phosphorylation by co-immunoprecipitation and western blotting, as reported previously.^{19,20} The protein samples (130 µg) from the VSMCs were immunoprecipitated by overnight incubation with anti-phospho-Ser 14-3-3 antibody (Cell Signaling Technology), followed by western blot analysis with the antibody against NHE-1 (Cell Signaling Technology).

[³H]-thymidine incorporation assay for cell proliferation

Cell proliferation was determined by [³H]-thymidine incorporation, as described previously.¹⁸ The VSMCs were counted and seeded into six-well culture plates (1 × 10⁵ cells per well). After 24 h, the medium was changed to standard medium containing 0.1% fetal bovine serum to ensure their quiescence for 24 h. Ang II (100 nmol l⁻¹) was added to medium containing 137.5 or 140 mmol l⁻¹ of sodium for 24 h. The stimulated cells were labeled with 1 µCi ml⁻¹ [³H]-thymidine during the 24 h of culture. After labeling, the cells were washed twice with phosphate-buffered saline and twice with ice-cold 5% trichloroacetic acid to remove unincorporated [³H]-thymidine, solubilized in 500 µl of 0.25 mol l⁻¹ NaOH containing 0.1% sodium dodecyl sulfate and neutralized with HCl. Aliquots of the samples were added to 10 ml of scintillation fluid and counted in a scintillation counter.

Real-time reverse transcriptase-PCR

For the real-time reverse transcriptase-PCR analysis, the total RNAs were extracted using ISOGEN (Nippon Gene, Tokyo, Japan); the complementary DNA (from 1 µg RNA) was synthesized as described previously.²¹ The expression of mRNA was analyzed using a Light Cycler Fast Start DNA Master SYBR Green I kit (Life Technologies). Reverse transcriptase-PCR was performed using predesigned primers for the *AT₁a receptor* (TaqMan Gene Expression Assays: Life Technologies) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers (F: 5'-TGAACGGAAGCTCACTGG-3' and R: 5'-TCCACCACCTGTTGCTGTA-3').²² The data were normalized to the expression of *GAPDH*.

Intracellular pH measurement

For the intracellular pH imaging, VSMCs were grown on two-well glass chamber slides (BD Bioscience, Franklin Lakes, NJ, USA). The cells were incubated with the acetoxymethyl ester of the pH-reporter dye carboxy-SNARF-1 (Life Technologies) at 10 µmol l⁻¹ for 30 min. Excess extracellular dye was washed away with the medium. The pH_i dye was excited by a 488 nm argon laser, and fluorescence was detected by confocal microscopy (LSM 700, Carl Zeiss, Oberkochen, Germany) at 580 and 640 nm. The 580/640 nm fluorescence ratio was converted to pH_i using a calibration curve. The standard curve was obtained by measuring ratio signals of carboxy-SNARF-1 AM-loading cells in high-potassium buffers (25 mmol l⁻¹ of HEPES, 145 mmol l⁻¹ of KCl, 0.8 mmol l⁻¹ of CaCl₂ and 5.5 mmol l⁻¹ of glucose) at different pH levels (pH 6.7, 7.0, 7.3, 7.6 or 7.9) in the presence of 10 µg ml⁻¹ of nigericin (electroneutral H⁺/K⁺ ionophore).

Statistical analysis

Values are presented as the mean ± s.e. Multiple-group comparisons were made using one-way or two-way analyses of variance, followed by Bonferroni's test. Student's *t*-tests were performed to compare the mean values when the experimental design consisted of two individual groups. Values of *P* < 0.05 were considered statistically significant.

RESULTS

Effect of high-sodium level on Ang II-induced ERK 1/2 phosphorylation and cell proliferation

Phosphorylation of ERK 1/2 induced by Ang II was significantly higher in 140 mmol l⁻¹ of sodium than in 137.5 mmol l⁻¹ of sodium (Figure 1a). In addition, 100 or 1000 nmol l⁻¹ of Ang II (5 min)-induced ERK 1/2 phosphorylation was significantly higher in 140 mmol l⁻¹ of sodium than in 137.5 mmol l⁻¹ of sodium (Figure 1b).

We investigated the potential roles of AT₁ receptor and ERK 1/2 activation in high-sodium/Ang II-induced cell proliferation by using candesartan (AT₁ receptor blocker) and PD98095 (ERK 1/2 kinase inhibitor). Candesartan and PD98095 inhibited ERK 1/2 phosphorylation in 137.5 or 140 mmol l⁻¹ of sodium medium to basal levels (Figure 2a). The Ang II-stimulated cell proliferation was assessed by [³H]-thymidine incorporation. After treatment with Ang II (100 nmol l⁻¹) for 24 h, the [³H]-thymidine incorporation in the 140 mmol l⁻¹ sodium medium was significantly higher than in the 137.5 mmol l⁻¹ sodium medium (Figure 2b). These data suggest that increased extracellular sodium concentration augments Ang II-induced cell proliferation through the AT₁ receptor- and ERK 1/2 activation-dependent pathway in the VSMCs.

Roles of sodium channels in Ang II-induced ERK 1/2 phosphorylation and cell proliferation

To investigate the roles of different sodium transporters in high-sodium/Ang II-induced ERK 1/2 phosphorylation, we examined the effects of SN-6 (Na⁺/Ca²⁺ exchanger inhibitor), ouabain (Na⁺/K⁺ ATPase inhibitor) and HMA (NHE-1 inhibitor) on ERK 1/2 phosphorylation and cell proliferation. Whereas HMA completely

Figure 1 Time course of angiotensin II (Ang II)-induced vascular smooth muscle cell (VSMC) extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in media containing 137.5 and 140 mmol L⁻¹ of sodium (a). VSMCs were incubated with 100 nmol L⁻¹ of Ang II for the indicated times. Dose-dependent change of Ang II-induced ERK 1/2 phosphorylation (b). VSMCs were incubated with the indicated concentrations of Ang II for 5 min. Western blotting was performed with anti-phospho-ERK 1/2 or anti-ERK 1/2 antibody. Data represent the mean $\pm$ s.e. ($n=4$), expressed as fold change compared with the unstimulated cells. Two-way analysis of variance (ANOVA) showed that the time- or dose-dependent increase of ERK 1/2 phosphorylation induced by Ang II was significantly higher in 140 mmol L⁻¹ of sodium than in 137.5 mmol L⁻¹ of sodium. * $P<0.05$ vs. control VSMCs.

attenuated high-sodium/Ang II-induced ERK 1/2 phosphorylation (Figure 3a) and cell proliferation (Figure 3b), SN-6 and ouabain showed no effect. A number of studies report that inhibition of ouabain²³ or NHE-1,²⁴ is involved in VSMC proliferation. As shown in Supplementary Figure 1, these inhibitors alone have no effect on cell proliferation. Ang II increased NHE-1 phosphorylation, although the sodium concentration did not affect Ang II-induced NHE-1 phosphorylation (Supplementary Figure 2). These data suggest that NHE-1 has a role in the augmentation of Ang II-induced ERK 1/2 phosphorylation with high-sodium level.

Effects of osmolarity and chloride on Ang II-induced ERK 1/2 phosphorylation and cell proliferation

To evaluate the effects of osmolarity on Ang II-induced ERK 1/2 phosphorylation, we used mannitol to obtain the identical osmolarity in the media. The changes in osmolarity by the addition of mannitol

Figure 2 Effects of Ang II type 1 (AT₁) receptor blocker and extracellular signal-regulated kinase (ERK) 1/2 kinase inhibitor on angiotensin II (Ang II)-induced vascular smooth muscle cell (VSMC) ERK 1/2 phosphorylation (a) and cell proliferation (b) in media containing 137.5 and 140 mmol L⁻¹ of sodium. (a) Western blotting was performed with anti-phospho-ERK 1/2 or anti-ERK 1/2 antibody. (b) Cell proliferation was evaluated by [³H]-thymidine incorporation. Data represent the mean $\pm$ s.e. ($n=4$), expressed as fold change compared with unstimulated cells. * $P<0.05$ vs. control VSMCs. ** $P<0.05$ vs. Ang II-treated VSMCs in normal sodium medium. *** $P<0.05$ vs. Ang II-treated VSMCs in high-sodium medium.

showed no effect on Ang II-induced ERK 1/2 phosphorylation and cell proliferation in a normal sodium medium (Figures 3c and d), suggesting that high-sodium level augments Ang II-induced cell proliferation in an osmotic pressure-independent manner.

We measured the effect of chloride concentration on Ang II-induced VSMC proliferation. Choline chloride did not affect ERK 1/2 phosphorylation (Figure 3c) or [³H]-thymidine incorporation (Figure 3d), suggesting that sodium, but not chloride, induces cell proliferation by Ang II.

Effect of high-sodium level on expression of the AT₁ receptor

After Ang II stimulation for 24 h, protein expression of the AT₁ receptor in the normal and high-sodium media did not significantly differ (Figure 4a). The mRNA expression of the AT_{1a} receptor was not affected by sodium (Figure 4b). These data suggested that high-sodium level augments Ang II-induced cell proliferation through the AT₁ receptor, but does not change the receptor expression.

Effect of sodium on intracellular pH in Ang II-treated VSMCs

NHE-1 participates in the regulation of intracellular pH by exchange of Na⁺ for H⁺ across the plasma membrane.²⁵ We examined the

Figure 3 Effects of inhibitors, osmolarity and chloride concentration on angiotensin II (Ang II)-induced vascular smooth muscle cell (VSMC) extracellular signal-regulated kinase (ERK) 1/2 phosphorylation (a, c) and cell proliferation (b, d) in media containing 137.5 and 140 mmol/L of sodium. (a, c) Western blotting was performed with anti-phospho-ERK 1/2 or anti-ERK 1/2 antibody. (b, d) Cell proliferation was evaluated by $[^3\text{H}]$ -thymidine incorporation. Data represent the mean $\pm$ s.e. ($n=4$), expressed as fold change compared with unstimulated cells. * $P<0.05$ vs. control VSMCs. ** $P<0.05$ vs. Ang II-treated VSMCs in normal sodium medium. *** $P<0.05$ vs. Ang II-treated VSMCs in high-sodium medium.

Figure 4 Vascular smooth muscle cell (VSMC) protein expression of angiotensin II type 1 (AT₁) receptor in media containing 137.5 or 140 mmol/L of sodium (a). Western blotting performed with anti-AT₁ receptor antibody. Representative data from three samples are shown. Effect of high sodium on VSMC mRNA expression of AT_{1a} receptor (b). AT_{1a} receptor mRNA was measured by reverse transcriptase-PCR (RT-PCR). The data represent the mean $\pm$ s.e. ($n=4$), normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

effect of sodium concentration on intracellular pH in Ang II-treated VSMCs. Ang II (100 nmol/L, 5 min) significantly increased intracellular pH, and high-sodium level augmented the pH increase induced by Ang II, whereas HMA or candesartan completely inhibited Ang II-induced intracellular pH changes (Figure 5). To confirm the effect of sodium on Ang II-induced VSMC proliferation through NHE-1, we measured the effect of HMA on Ang II-induced changes under normal sodium concentrations. Pre-treatment with HMA inhibited Ang II-induced ERK phosphorylation (Supplementary Figure 3A), thymidine incorporation (Supplementary Figure 3B) and pHi (Supplementary Figure 3C) under normal sodium concentrations; the inhibition is similar to that seen with high-sodium concentrations.

DISCUSSION

The relationship between dietary salt intake and Ang II-induced cardiovascular remodeling has been previously demonstrated in animal and clinical experiments.^{15,16,26} In Dahl salt-sensitive rats that were fed high levels of salt, treatment with Ang-converting enzyme inhibitors or AT₁ receptor blockers did not alter blood pressure, but it did reduce cardiac and/or renal dysfunction.^{27–29} The precise molecular mechanism by which salt augments Ang II-induced vascular injury has been fully elucidated. In this study, we hypothesized that high-sodium level directly augments Ang II-

Figure 5 Effect of sodium on intracellular pH in angiotensin II (Ang II)-treated vascular smooth muscle cells (VSMCs). Cells were incubated with the pH-reporter dye carboxy-SNARF-1. The pH_i dye was excited with a 488 nm argon laser, and fluorescence was detected by confocal microscopy at 580 and 640 nm. Intracellular pH was evaluated by the fluorescence ratio using a standard curve. Data represent the mean $\pm$ s.e. ($n=4$). * $P<0.05$ vs. control VSMCs. ** $P<0.05$ vs. Ang II-treated VSMCs in normal sodium medium. A full color version of this figure is available at the *Hypertension Research* journal online.

induced pro-atherogenic changes. Our *in vitro* study provides evidence that an increased sodium concentration in medium augments Ang II-induced VSMC proliferation through the ERK 1/2-dependent pathway via the AT₁ receptor and NHE-1. These findings have implications regarding the molecular mechanisms of Ang II-dependent development and the progression of vascular remodeling in a high-salt diet.

Sodium homeostasis is strictly regulated, particularly the blood sodium concentration. In this study, we assumed high-sodium concentration to be 140 mmol L⁻¹, which is frequently seen in physiological conditions. A recent clinical report clearly revealed that the plasma sodium concentration significantly increased after salt intake,³⁰ suggesting that a high-sodium condition (140 mmol L⁻¹) may be observed *in vivo* as similar as in this *in vitro* study. A previous *in vivo* experiment demonstrated that interstitial sodium concentration differs from the blood sodium concentration and is altered by a high-salt diet.³¹ The vascular interstitial concentration of sodium might be increased by a high-salt diet.

Ang II increases intracellular calcium ions in the VSMCs.³² The ERK 1/2 activation induced by Ang II has been previously shown to be mediated by at least two pathways; one pathway is Ca²⁺ dependent, and the other is Ca²⁺ independent.^{33–35} To determine whether the augmented effect of high-sodium level on Ang II-induced ERK 1/2 phosphorylation depends on increased intracellular Ca²⁺ though the sodium–calcium exchange, we used SN-6, a recently developed sodium–calcium exchange inhibitor, to decrease intracellular Ca²⁺.³⁶ The results showed no effect by SN-6 on proliferation or ERK 1/2 phosphorylation, indicating that the augmented effect of high-sodium level on Ang II-induced VSMC proliferation might be Ca²⁺ independent.

NHE-1 is a member of a multigene family whose activities are increased in the tissues of hypertensive patients³⁷ and animal models.³⁸ As NHE-1 is activated by hyperplastic agonists, it has been proposed that abnormal NHE-1 function is involved in the pathophysiology of hypertension and its complications. Bobik *et al.*²⁴ and Mitsuke *et al.*³⁹ have shown that the growth of cultured VSMCs is attenuated by NHE-1 inhibition with pharmacological agents. Despite the increasing interest in the potential role of ERK 1/2 in the

activation of NHE-1, several reports suggest that NHE-1 has a role in regulating ERK 1/2 activation. Some evidence supports NHE-1 inhibition suppressing ERK 1/2 phosphorylation in human gut cells⁴⁰ and cardiomyocytes.⁴¹ In this study, Ang II increased NHE-1 phosphorylation and inhibition of NHE-1, and HMA completely attenuated ERK 1/2 phosphorylation and cell proliferation induced by high-sodium/Ang II, suggesting that these changes are regulated by NHE-1. These data are in agreement with previous studies that indicate intracellular pH change via NHE-1 to be a crucial factor in high-salt-induced vascular remodeling.^{41,42} Although the affinity of NHE-1 for sodium (K_m 17.5 $\pm$ 3.4 mmol L⁻¹)⁴³ is lower than the sodium concentration in this study (137.5–140 mmol L⁻¹), our results indicate a novel mechanism for NHE-1 activation under high-sodium conditions.

Our study shows that high-sodium concentration augments Ang II-induced VSMC proliferation. A possible molecular mechanism of the upregulated proliferative effect of high-sodium concentration is ERK 1/2 activation via the AT₁ receptor and NHE-1. These findings may offer new insights into the molecular mechanisms of atherosclerosis induced by high-sodium/Ang II. Inhibition of NHE-1 might be a novel therapeutic target for vascular remodeling in patients with salt-sensitive hypertension.

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