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

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Abstract

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 [^{3}H]-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 iinhibitor) 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^+/Ca^{2+} 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.

Keywords

angiotensin II; intracellular pH; NHE-1; sodium; vascular remodeling

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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 cerebrovas-cular 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 infiltration[10] 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 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% CO_2/95% 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 iinhibitor) for 30 min, 100 nmol l^{-1} of candesartan (Ang II type 1 (AT_1) receptor blocker) for 3 h, 10 μmol l^{-1} of SN-6 (Na^+/Ca^{2+} 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-AT_1 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).

[^{3}H]-thymidine incorporation assay for cell proliferation

Cell proliferation was determined by [^{3}H]-thymidine incorporation, as described previously.[18] The VSMCs were counted and seeded into six-well culture plates (1×10^5 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} [^{3}H]-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 [^{3}H]-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 *AT_1a receptor* (TaqMan Gene Expression Assays: Life Technologies) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers (F: 5′-TGAACGGGAAGCTCACTGG-3′ and R: 5′-TCCACCACCCTGTTGCTGTA-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 $CaCl_2$ 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 AT_1 receptor and ERK 1/2 activation in high-sodium/Ang II-induced cell proliferation by using candesartan (AT_1 receptor blocker) and PD98095 (ERK 1/2 kinase inhibitor). Candesartan and PD98095 inhibited ERK 1/2 phosphor-ylation 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 [^{3}H]-thymidine incorporation. After treatment with Ang II (100 nmol l^{-1}) for 24 h, the [^{3}H]-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 AT_1 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^+/Ca^{2+} exchanger inhibitor), ouabain (Na^+/K^+ ATPase inhibitor) and HMA (NHE-1 inhibitor) on ERK 1/2 phosphorylation and cell proliferation. Whereas HMA completely 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 ouabain[23] 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 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 [^{3}H]-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 AT_1 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 AT_1 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 effect of sodium concentration on intracellular pH in Ang II-treated VSMCs. Ang II (100 nmol l^{-1}, 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 AT_1 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-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 AT_1 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 Ca^{2+} dependent, and the other is Ca^{2+} 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 Ca^{2+} though the sodium–calcium exchange, we used SN-6, a recently developed sodium–calcium exchange inhibitor, to decrease intracel-lular Ca^{2+}.[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 Ca^{2+} independent.

NHE-1 is a member of a multigene family whose activities are increased in the tissues of hypertensive patients[37] 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 cells[40] 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 (Km 17.5±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 AT_1 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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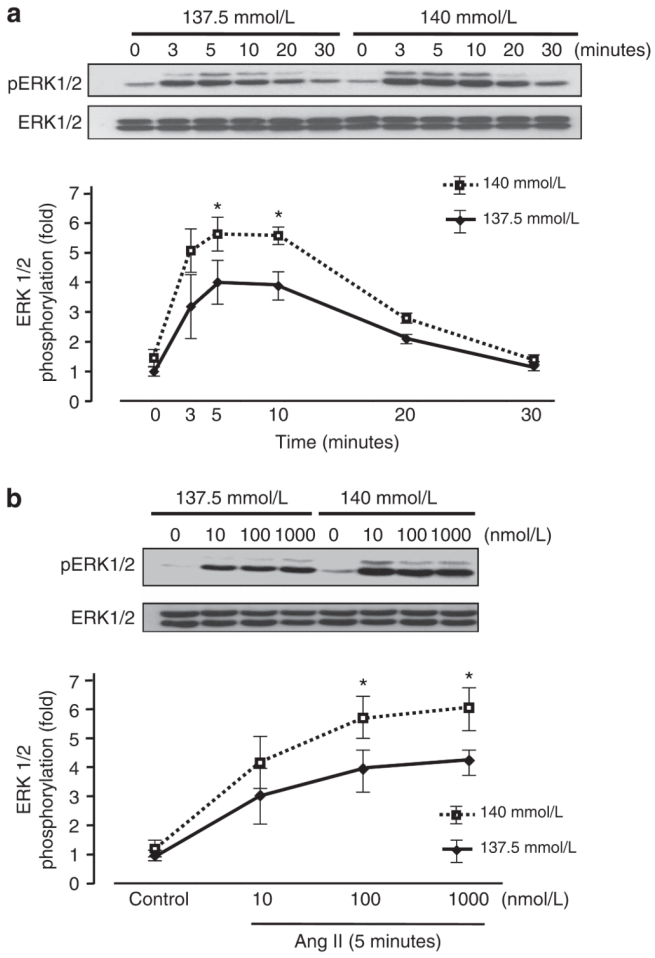

Figure 1.

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±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.

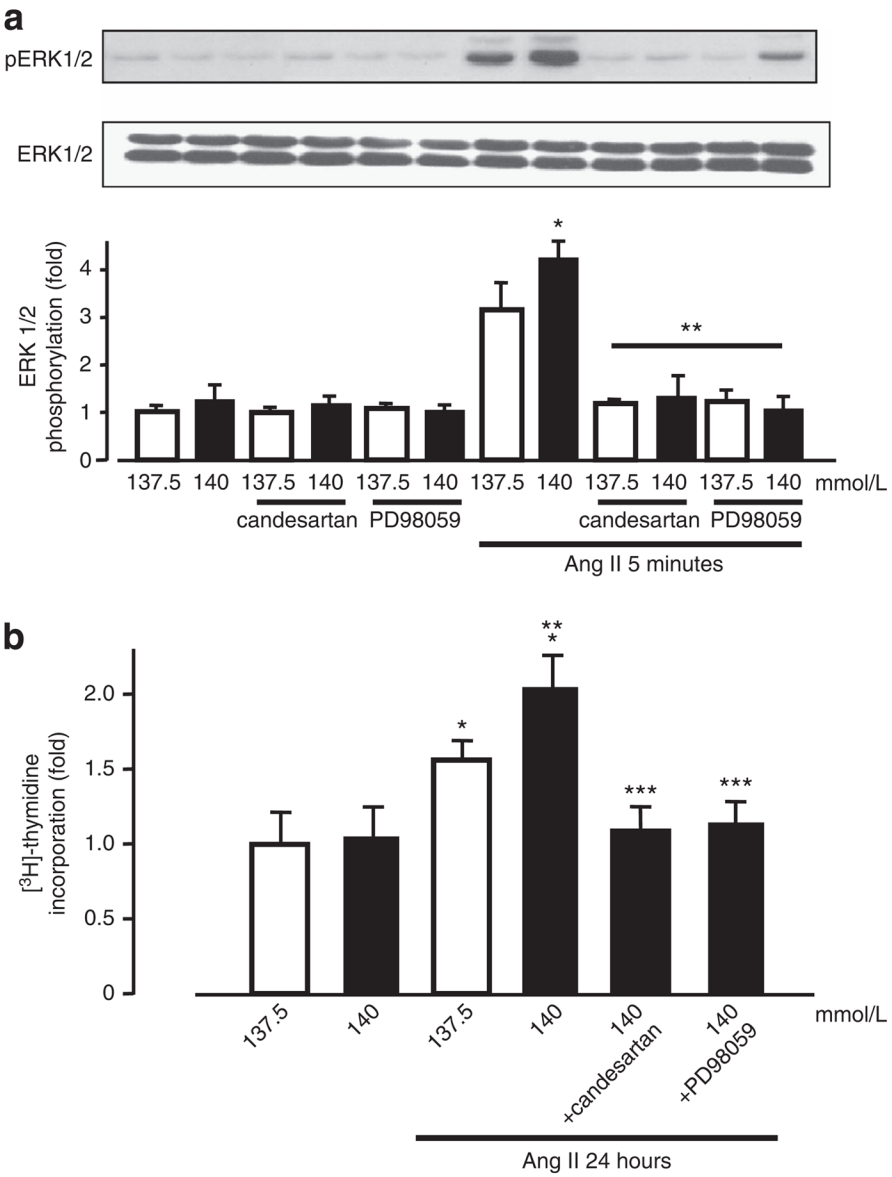

Figure 2.

Effects of Ang II type 1 (AT_1) 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 [^{3}H]-thymidine incorporation. Data represent the mean±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.

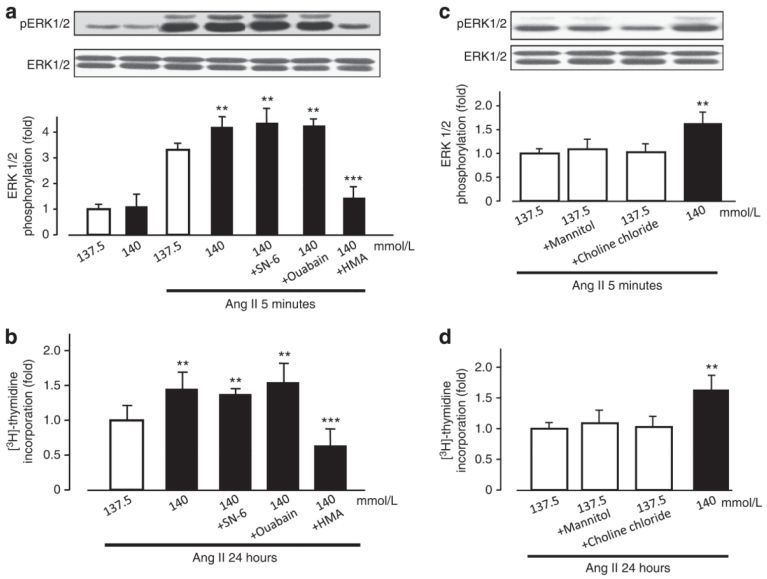

Figure 3.

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^{-1} 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}H]-thymidine incorporation. Data represent the mean±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.

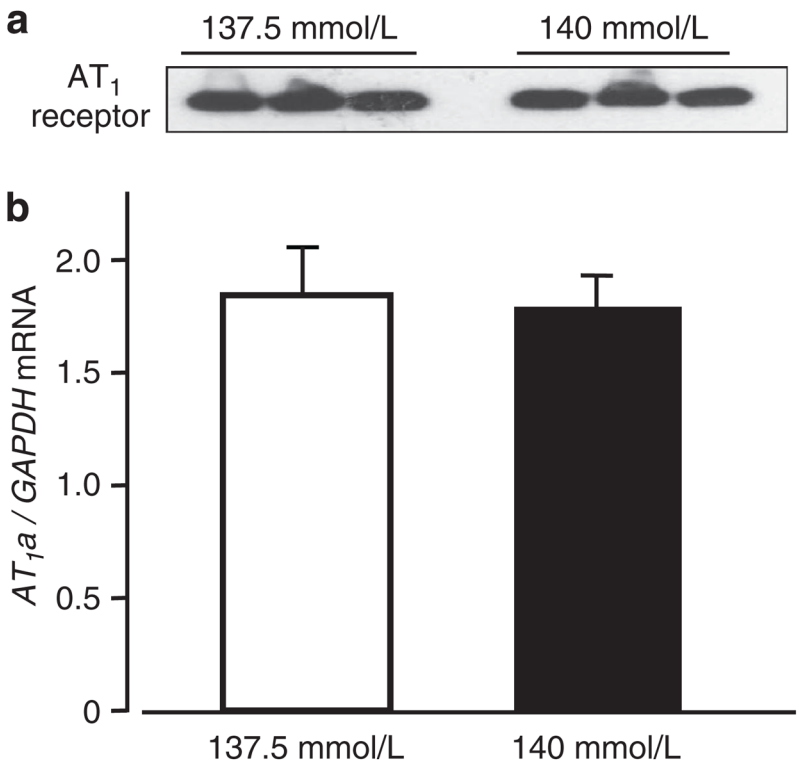

Figure 4.

Vascular smooth muscle cell (VSMC) protein expression of angiotensin II type 1 (AT_1) receptor in media containing 137.5 or 140 mmol l^{-1} of sodium (**a**). Western blotting performed with anti-AT_1 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±s.e. (n = 4), normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels.

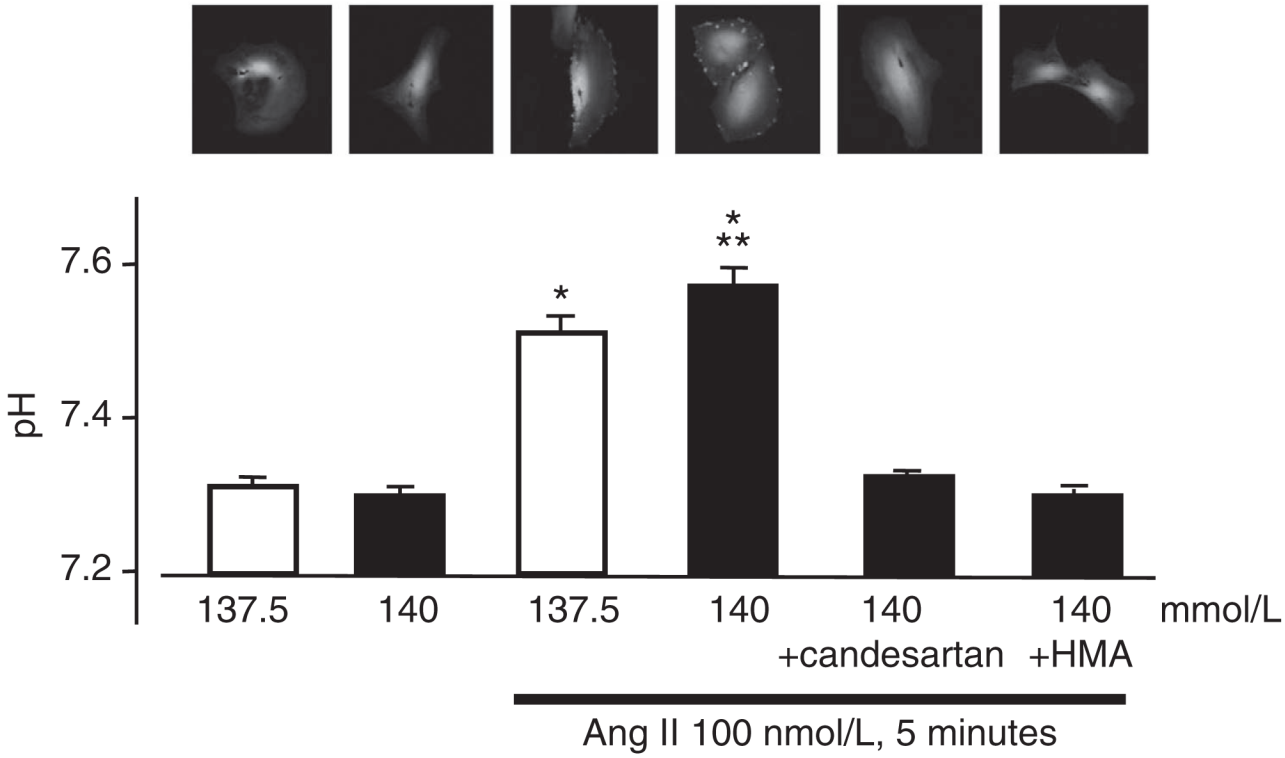

Figure 5.

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±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.