Salt Induces Vascular AT₁ Receptor Overexpression
In Vitro and In Vivo

Georg Nickenig, Kerstin Strehlow, Jörg Roeling, Oliver Zolk, Andreas Knorr, Michael Böhm

Abstract—The molecular events governing salt-sensitive hypertension are currently unknown. Because the renin-angiotensin system plays a central role in blood pressure regulation, as well as electrolyte balance, it may be closely involved in the phenomenon of salt sensitivity. Therefore, we examined the effect of a high salt diet (8%) on aortic angiotensin type 1 (AT₁) receptor expression in Sprague-Dawley rats by means of radioligand binding assays and quantitative polymerase chain reaction. High salt intake caused an increase of AT₁ receptor mRNA and AT₁ receptor density to approximately 160% compared with control levels. Northern analysis revealed that incubation of vascular smooth muscle cells (VSMCs) with an increased concentration of sodium chloride (by 10 mmol/L) caused a time-dependent elevation of AT₁ receptor mRNA levels, with a maximum of 241±28% after 24 hours. There was a similar increase in AT₁ receptor density in VSMCs in response to sodium chloride, as assessed by radioligand binding assays. The salt-induced AT₁ receptor upregulation led to an enhanced functional response of VSMCs on stimulation with angiotensin II, since the angiotensin II–elicited intracellular calcium response was significantly increased in cells preincubated for 24 hours with the high salt concentration. Thus, sodium chloride may directly induce AT₁ receptor upregulation in vitro as well as in vivo; this suggests a potential mechanism participating in salt-induced hypertension because the AT₁ receptor activation is tightly coupled to blood pressure regulation. (Hypertension. 1998;31:1272-1277.)

Key Words: angiotensin II ■ sodium, dietary ■ receptors, angiotensin ■ hypertension, essential ■ muscle, smooth, vascular ■ sodium sensitivity

Various epidemiological studies have suggested a correlation of dietary salt intake with blood pressure regulation and the prevalence and progression of essential hypertension. In contrast, several interventional studies have failed to establish a relationship between alterations in sodium intake and blood pressure. Therefore, the salt–blood pressure theory has remained a subject of ongoing controversy.¹ ² Most individuals are not salt sensitive, but there are individuals who develop an elevation in blood pressure in response to an increased dietary salt intake, many of whom are black or elderly. In addition, essential hypertensives as a group tend to have a higher frequency of salt sensitivity than is found in the normotensive population. There is some evidence that salt sensitivity is associated with low plasma renin activity and/or impaired renal function with regard to sodium excretion. However, the mechanisms underlying this phenomenon are poorly understood.³ ⁴

The renin-angiotensin system plays a significant role in controlling cardiovascular functions. Ang II regulates electrolyte balance by enhancing salt retention via aldosterone release, induces vasoconstriction, and enhances blood pressure.⁵ The AT₁ receptor is a G protein–coupled receptor expressed in various tissues that mediates most of the known biological effects of Ang II.⁶ ⁷ In addition to its role in the control of blood pressure, fluid, and electrolyte regulation, Ang II, along with the AT₁ receptor, has been implicated in chronic vascular disease, which may be due to reported growth-promoting effects of Ang II on VSMCs in vivo and in vitro.⁶ ⁸ ⁹

Dietary sodium intake is known to modulate the renin-angiotensin system. A low salt diet leads to elevation of plasma renin and aldosterone activity and consequently to a decreased AT₁ receptor expression via homologous downregulation. High salt intake, which causes hypertension in some individuals, induces a decrease in the activity of the circulating renin-angiotensin system, and this is thought to be involved in the accompanying upregulation of AT₁ receptor expression.⁹ ¹⁰ In any event, upregulation of AT₁ receptor expression may lead to enhanced vasoconstriction and water retention and ultimately to elevated blood pressure levels. In this context, it is not known exactly which mechanisms govern the regulation of the AT₁ receptor. We hypothesized that sodium chloride itself may modulate the renin-angiotensin system on the AT₁ receptor level. To test this hypothesis, we examined the effect of sodium chloride on vascular AT₁ receptor gene expression in Sprague-Dawley rats and cultured VSMCs.

Methods

Materials
Ang II, salts, DRB, and other chemicals were purchased from Sigma Chemical Co., [¹²⁵I]Ang
II were obtained from Amersham. Antibiotics, serum, and cell culture medium were purchased from Gibco-BRL. RNA-clean was from AGS, and losartan was a gift from Merck, Sharp & Dohme. Oligonucleotides were synthesized using Pharmacia chemicals, with an automated DNA synthesizer (Pharmacia LKB, gene assembler plus). Fura 2-AM was obtained from Calbiochem.

**Cell Culture**

VSMCs were isolated from rat thoracic aorta by enzymatic dispersion as described previously and cultured over several passages according to Ross. Cells were grown in a 5% CO2 atmosphere at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1% nonessential amino acids (100x), and 20% fetal bovine serum. Experiments were performed with cells from passages 5 through 15.

**Animals**

Male Sprague-Dawley rats were bred and housed at the central animal laboratories of Bayer AG, Wuppertal-Elberfeld, Germany. The rats received a standard rodent chow with 0.4% or 8% sodium chloride and tap water from the age of 5 to 6 weeks. After 6 weeks, the animals were killed and the aortas excised and cleaned from connective tissue. The animal experiments were approved by the institutional committee and are in accordance with guidelines for experimental research (Nordrhein-Westfalen, Germany).

**mRNA Isolation and Northern Analysis**

Experimental cells were lysed in 1 mL RNA-clean (AGS), scraped, and processed according to the manufacturer’s protocol to obtain total cellular RNA. Aliquots of 2 to 10 μg were electrophoresed through 1.2% agarose–0.67% formaldehyde gels and stained with ethidium bromide to verify the quantity and quality of the RNA. After capillary transfer onto Hybond N membranes in 20% SSA, nonradioactive membranes were prehybridized for 2 hours at 42°C in a buffer containing 50% deionized formamide, 0.5% SDS, 6× SSC, 0.25% bovine serum albumin, and proteinase inhibitors [trypsin inhibitor 0.2 mg/mL, pepstatin A 0.25 mg/mL, and leupeptin 0.25 mg/mL] pH 7.1. [125I]Ang II was used as radiolabeled ligand (0.125 to 2 nmol/L) to assess AT1 receptor density. Dpg753 (10 μmol/L) was used to determine nonspecific binding. The assay was performed in a total volume of 250 μL incubation buffer. The incubation was carried out at 24°C for 60 minutes. These conditions allowed a complete equilibration of the receptor with the radioligand. The reaction was terminated by rapid vacuum filtration through Whatman GF/C filters; the filters were washed immediately three times with 5 mL of ice-cold incubation buffer, and radioactivity was determined in a gamma counter. All experiments were performed in triplicate. The maximal density (Bmax) and apparent affinity (Kd) of binding sites were obtained by nonlinear regression analysis.

**Measurement of Free [Ca2+]i**

VSMCs were cultured on round glass microscope slides (diameter, 12 mm) and at confluence incubated with 2 mmol/L fura 2-AM at 37°C for 30 minutes in 20 mmol/L HEPES, 16 mmol/L glucose, 130 mmol/L NaCl, 1 mmol/L MgSO4, and 0.5 mmol/L CaCl2. Before the measurements, cells were rinsed gently with the same buffer containing 1 mmol/L CaCl2 instead of 0.5 mmol/L CaCl2. The glass slides were positioned diagonally in the cuvette, and the [Ca2+]i was measured in a Hitachi fluorescence spectrofluorometer at excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. Maximum (Rmax) and minimum (Rmin) fluorescence was determined by adding digitonin at a final concentration of 30 mmol/L followed by the addition of Tris-base/EGTA (final concentration, 0.1 mmol/L/25 mmol/L). Fluorescence was corrected for cellular autofluorescence. Fluorescence signals were calibrated according to Grynkiewicz et al.

**Quantitative PCR**

The aorta was isolated, quickly frozen in liquid nitrogen, and homogenized. RNA was isolated with RNA-clean (AGS) according to the manufacturer’s protocol to obtain total cellular RNA. The original AT1 receptor cDNA was digested with MSCI and self-ligated. The resulting plasmid lacking the region from base 446 to 734 (mutAT1) was linearized by digestion with Sac I, and a deletion-mutated AT1 receptor mRNA was synthesized using the Megascript Kit (Ambion) following the manufacturer’s instructions. Two micrograms of the isolated total RNA and 10 pg of the mutAT1 mRNA were mixed and reverse transcribed using random primers. The single-stranded cDNA was amplified by PCR using Taq DNA polymerase (Boehringer). Twenty-eight cycles were performed under the following conditions: 94°C, 30 seconds; 55°C, 45 seconds; 72°C, 45 seconds. The sequencing reactions for AT1 receptor sense and antisense primers were 5'-ACCUCCTACAGCATTTTGTGTTGTTGGGGA-3' and 5'-GGGAAGGCTGAATCTCCATAC-3', respectively. The same samples were used for GAPDH cDNA amplification to confirm that equal amounts of RNA were reverse transcribed. The primers used were 5'-ACCAAGGCTCAC-3' and 5'-TCGCCACCCCTTGGCCTGTA-3'. PCR amplification gave 479 bp, 191 bp, and 452 bp of fragments originated from the AT1 receptor mRNA, the mutated AT1 receptor mRNA, and GAPDH mRNA, respectively. PCR reactions were separated through 1.5% agarose gels, and DNA was visualized by ethidium bromide staining. For quantification, DNA was transferred by vacuum blotting to nylon membranes, which were then hybridized with a radiolabeled AT1 receptor cDNA probe. Autoradiograms were analyzed by laser densitometry.
Statistical Analysis
Data are presented as mean±SE. Statistical analysis was performed using ANOVA.

Results
To assess the influence of high salt intake on vascular AT1 receptor expression, male Sprague-Dawley rats were fed a 0.4% or 8% sodium chloride diet for 6 weeks before the aortas were excised and used for measurement of AT1 receptor density and mRNA level. The blood pressure level was not different between groups (data not shown). After RNA isolation, the AT1 receptor mRNA was detected by means of a quantitative PCR. The reverse transcription and PCR reactions of the AT1 receptor mRNA were monitored by including an internal standard. This deletion-mutated AT1 receptor mRNA yielded a substantially shorter PCR product (191 bp), enabling distinction of the wild-type and mutated AT1 receptor mRNA (479 bp). Quantity and quality of the included RNA was controlled by an additional PCR reaction from the same reverse transcription samples using an external standard (GAPDH). The exponential phase for the amounts of wild-type and mutated RNA used was found to be in a range between 20 and 36 cycles (data not shown). Therefore, 31 cycles were used in our experimental setup. Figure 1 illustrates a representative ethidium bromide–stained agarose gel loaded with PCR reactions generated from aortic RNA of rats fed a 0.4% or 8% sodium chloride diet for 6 weeks before the aortas were excised and used for measurement of AT1 receptor density and mRNA level. The blood pressure level was not different between groups (data not shown). After RNA isolation, the AT1 receptor mRNA was detected by means of a quantitative PCR. The reverse transcription and PCR reactions of the AT1 receptor mRNA were monitored by including an internal standard. This deletion-mutated AT1 receptor mRNA yielded a substantially shorter PCR product (191 bp), enabling distinction of the wild-type and mutated AT1 receptor mRNA (479 bp). Quantity and quality of the included RNA was controlled by an additional PCR reaction from the same reverse transcription samples using an external standard (GAPDH). The exponential phase for the amounts of wild-type and mutated RNA used was found to be in a range between 20 and 36 cycles (data not shown). Therefore, 31 cycles were used in our experimental setup. Figure 1 illustrates a representative ethidium bromide–stained agarose gel loaded with PCR reactions generated from aortic RNA of rats fed a 0.4% or 8% sodium chloride diet, indicating that the AT1 receptor mRNA expression was markedly increased in aortas isolated from rats on a high salt diet. Figure 2 shows the quantitative analysis, indicating that the AT1 receptor mRNA was increased to 171% in rats on a high salt diet (AT1 receptor mRNA/internal standard ratio, 5.7±1.5 versus 9.8±2.3).

To investigate whether this AT1 receptor mRNA upregulation was associated with a similar change in receptor density, radioligand binding assays on aortic cell membranes isolated from animals on a regular or high salt diet were conducted. The representative saturation binding assay with [125I]Ang II in Figure 3 shows that the 8% salt diet in Sprague-Dawley rats led to an increase of AT1 receptor density (Bmax=8.7±1.0 fmol/mg protein, Kd=0.31 [0.05 to 0.6] nmol/L in rats on 8% salt; Bmax=5.5±0.3 fmol/mg protein, Kd=0.21 [0.09 to 0.31] nmol/L in rats on 0.4% salt). These data demonstrate that high salt intake is associated with an upregulation of vascular AT1 receptor expression in vivo.

At this point, we hypothesized that sodium chloride itself may influence the cellular AT1 receptor expression. Therefore, we studied the effect of sodium chloride on AT1 receptor expression in VSMCs, which express most if not all of the AT1 receptors in the vessel wall. Cells were grown to confluence, and serum was removed from the culture medium 24 hours before initiation of experimental treatments to

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Figure 1. Effect of high salt diet on AT1 receptor mRNA expression. Representative ethidium bromide–stained agarose gel of a reverse-transcription PCR of RNA isolated from aortas excised from Sprague-Dawley rats on regular and high salt diets. The 496-bp DNA fragment corresponds to the AT1 receptor (AT1-R) mRNA, and the 191-bp DNA fragment results from the mutated AT1 receptor (mutAT1-R) mRNA (internal standard). GAPDH mRNA was used as internal standard.

Figure 2. Effect of high salt diet on AT1 receptor mRNA levels. The relative AT1 receptor expression in aorta from rats subjected to regular or high salt diet is illustrated. The AT1 receptor (AT1-R) mRNA level is expressed in relation to the mutated AT1 receptor (mutAT1-R) mRNA, which was used as internal standard. Also shown is GAPDH mRNA expression. Each point represents data±SE from five separate experiments.

Figure 3. Effect of high salt diet on AT1 receptor density is shown by radioligand binding assays on aortic tissue. Saturation binding assay with [125I]Ang II in aortas isolated from rats on 0.4% control diet and 8% salt diet. Each point represents the mean±SE of three separate experiments.
obviate its effects. Preliminary experiments revealed that the AT1 receptor expression remains stable in this setting for at least 48 hours (data not shown). Figure 4 illustrates autoradiographic results from Northern hybridization of a rat vascular AT1 receptor cDNA probe to 10 μg of electrophoretically separated, total cellular RNA extracted from VSMCs at the indicated time points after increase of the sodium chloride concentration in the culture medium by 10 mmol/L. This autoradiogram reveals a time-dependent elevation of the transcript level. The AT1 receptor mRNA signal appeared significantly increased 12 hours after exposure to high salt concentrations, and this increase was sustained for up to 48 hours. Figure 4 also shows hybridization of a GAPDH cDNA probe to the same Northern blot. GAPDH mRNA appeared stable over the time course of the experiment. Autoradiographic data, generated from three separate experiments, were analyzed by laser densitometry. Figure 5A shows the salt-induced upregulation of AT1 receptor mRNA hybridization signal relative to control levels at 0 hours. A 24-hour incubation with an increased salt concentration caused an upregulation of AT1 receptor mRNA levels to 241 ± 28%. After 48 hours of incubation in high salt concentration, the AT1 receptor mRNA signals were measured at 211 ± 12% relative to the control level at 0 hours (100%). GAPDH mRNA expression was not significantly regulated. In a set of control experiments, cells were exposed to an increase of potassium chloride concentration by 10 mmol/L to exclude unspecific osmotic effects. As demonstrated in Figure 5B, neither the AT1 receptor mRNA nor the GAPDH mRNA was significantly altered during the time course of the assay, which suggests that the AT1 receptor mRNA expression is specifically upregulated by sodium chloride.

Radioligand binding assays were performed to assess whether the increased level of AT1 receptor mRNA coincided with an elevation of AT1 receptor protein expression. Therefore, AT1 receptor binding sites were measured after a 24-hour treatment of VSMCs with the high salt concentration (10 nmol/L increase). Figure 6 shows graphically the

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\text{Figure 4. Representative Northern hybridization autoradiography shows effect of salt on AT1 receptor mRNA expression in VSMCs. VSMCs were grown to confluence, serum-deprived for 24 hours, and exposed to a salt concentration increased by 10 mmol/L over normal. Hybridization of an AT1 receptor cDNA probe to Northern blots of 10 μg total RNA extracted from VSMCs at the indicated time points (top) and a GAPDH cDNA probe to the same blot stripped of the AT1 receptor cDNA probe (bottom) are shown. Blots are representative of three separate experiments.}
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\text{Figure 5. Quantification of Northern hybridization signal intensity shows effect of sodium chloride (A) and potassium chloride (B) on mRNA levels in VSMCs. A, Time course of the AT1 receptor and GAPDH mRNA in the presence of high salt concentration. Northern hybridizations were performed as described in "Methods." Each point represents the relative hybridization signal (mean ± SE) normalized to the 0-hour treatment with vehicle (100%) from three separate experiments.}^{*} P < .05. \text{B, AT1 receptor mRNA and GAPDH mRNA levels in VSMCs that were serum-deprived for 24 hours and then exposed to a potassium chloride concentration increased by 10 mmol/L over normal. RNA was isolated at the indicated time points and analyzed as described in "Methods." Each point represents the relative hybridization signal (mean ± SE) of three separate experiments.}
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\text{Figure 6. Effect of salt on membrane receptors by saturation binding with [125I]Ang II. Confluent cells on 24-well culture plates were exposed to either regular (control) or high (salt) salt concentration. Saturation binding assays using [125I]Ang II were performed on isolated membranes. The AT1 receptor antagonist losartan (10 μmol/L) was used to define nonspecific binding. Each curve represents specific binding of the radioligand.} \text{Kd and Bmax values reported in the text were derived from nonlinear regression of the specific binding vs free data. Each point represents binding data ± SE of three independent experiments.}
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radioligand ($K_d$ value, 0.6 [0.04 to 1.12] nmol/L). These binding data indicate that salt directly enhances AT$_1$ receptor mRNA and protein expression.

We further reasoned that upregulation of AT$_1$ receptor gene expression should consequently lead to an enhanced functional response of VSMCs on Ang II stimulation. To test this theory, we examined Ang II–induced elevation of $[\text{Ca}^{2+}]_i$. Figure 7 illustrates a representative time course of $[\text{Ca}^{2+}]_i$ of VSMCs pretreated for 24 hours with either regular salt concentration or a 10-mmol/L increased salt concentration. Basal $[\text{Ca}^{2+}]_i$ was measured at approximately 30 nmol/L. After 1 minute, the cells were challenged with 100 nmol/L Ang II. Calculation of four separate experiments revealed that 100 nmol/L Ang II induced in vehicle-treated VSMCs a maximal $[\text{Ca}^{2+}]_i$ increase of 165±20 nmol/L, whereas the same dose of Ang II caused in salt-pretreated VSMCs a maximal $[\text{Ca}^{2+}]_i$ increase of 263±28 nmol/L. These data suggest that the salt-induced upregulation of AT$_1$ receptor mRNA and protein leads to the expected elevated functional response of VSMCs with respect to AT$_1$ receptor–mediated Ang II stimulation.

To gain insights into mechanisms involved in salt-induced AT$_1$ receptor regulation, the effect of NaCl on AT$_1$ receptor half-life was investigated. Therefore, cells were preincubated for 24 hours in either regular or high salt medium before 50 $\mu$g/mL DRB was added to block transcription. AT$_1$ receptor mRNA was detected after 0 to 8 hours by Northern blotting. Figure 8 shows that salt has no significant effect on AT$_1$ receptor mRNA half-life, suggesting that transcriptional rather than posttranscriptional mechanisms are involved in NaCl-induced AT$_1$ receptor upregulation.

**Discussion**

This study demonstrates that increased concentrations of sodium chloride elevate vascular AT$_1$ receptor gene expression in vivo and in vitro. Because most of the known biological effects of Ang II are mediated by the AT$_1$ receptor subtype, regulation of the responsivity of this receptor has been a prominent subject of recent research. Indeed, it is well established that the AT$_1$ receptor is regulated in vivo as well as in vitro. Conditions of increased renin-angiotensin system activity cause downregulation of AT$_1$ receptors, whereas a decrease in the activity of the renin-angiotensin system upregulates the AT$_1$ receptor. $^9$,$^{10}$,$^{15}$,$^{16}$ It is well known that changes in osmolarity in the sense of hypotonic or hypertonic stress induce second-messenger pathways such as calcium, cAMP, inositol phosphate, and the MAP kinase cascade. $^{17}$–$^{19}$ Moreover, it has recently been reported that hypotonic stress induces ERK and c-fos expression in cardiac myocytes. $^{20}$ Nevertheless, to date, the intracellular pathways mediating salt-induced modulation of AT$_1$ receptor expression are unknown. It has recently been shown that various growth factors, as well as Ang II, induce a profound downregulation, $^{21}$–$^{23}$ whereas LDL causes upregulation of AT$_1$ receptor gene expression in cultured VSMCs. $^{24}$,$^{25}$ Again, the underlying intracellular mechanisms are poorly understood, but there is increasing evidence that the adenylate cyclase/cAMP pathway may participate in the regulation of AT$_1$ receptor expression. $^{22}$,$^{23}$

As already outlined, increased dietary salt intake causes a decrease in the activity of the circulating renin-angiotensin
system. Because of the concept of homologous regulation of G protein–coupled receptors, the decreased Ang II levels are thought to be involved in the compensatory upregulation of the AT$_1$ receptor.$^{9,10,15,16}$ Based on our data, there are obviously additional factors that may influence the expression level of the AT$_1$ receptor in this setting. Sodium chloride itself is capable of upregulating the vascular AT$_1$ receptor gene expression independently of Ang II in cultured VSMCs, suggesting that an increased intake of sodium chloride may cause elevated blood pressure by means of enhanced AT$_1$ receptor expression. This is especially likely because activation of AT$_1$ receptors by Ang II leads to vasoconstriction and has also been implicated in abnormal growth of VSMCs.$^{7,8}$ The observation that elevation of [Ca$^{2+}$]$_i$ in VSMCs on Ang II stimulation, which closely correlates to vasoconstriction, is significantly increased by treatment with salt strengthens this notion.

In any event, other mechanisms such as increased water retention, expansion of the intravascular volume, and impaired excretion of sodium and water may also participate in the salt-induced elevation of blood pressure. Interestingly, many epidemiological studies suggest that only some individuals are susceptible to salt with regard to an increase in blood pressure, giving rise to the term “salt sensitivity.” Among these individuals, black persons and the elderly are predominantly, as well as persons who have already developed a hypertensive disease.$^{1–4}$ The key question is which molecular events are involved in this differential susceptibility toward dietary salt intake with respect to hypertension? One might speculate that a differential upregulation of the vascular AT$_1$ receptor participates in this individual salt sensitivity, might speculate that a differential upregulation of the vascular AT$_1$ receptor gene expression independently of Ang II in cultured VSMCs, suggesting that an increased intake of sodium chloride may cause elevated blood pressure by means of enhanced AT$_1$ receptor expression. This is especially likely because activation of AT$_1$ receptors by Ang II leads to vasoconstriction and has also been implicated in abnormal growth of VSMCs.$^{7,8}$ The observation that elevation of [Ca$^{2+}$]$_i$ in VSMCs on Ang II stimulation, which closely correlates to vasoconstriction, is significantly increased by treatment with salt strengthens this notion.

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**References**

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