Original Articles

High-Salt Diet Inhibits Expression of Angiotensin Type 2 Receptor in Resistance Arteries

Magdalena Gonzalez, Lorena Lobos, Felipe Castillo, Lorna Galleguillos, Nandy C. Lopez, Luis Michea

Abstract—Recent studies suggested that type 2 angiotensin receptor (AT2R) could contribute to regulation of blood pressure and/or vascular remodeling. A key question relates to the effects of potential modulators of vascular AT2R expression. In the present work, we evaluated if high salt intake (70 mmol/L NaCl in drinking water) could modulate rat mesenteric artery AT2R function and expression. Angiotensin II dose-response curves were studied in rat perfused pressurized small-diameter arteries in the presence of losartan (AT1R antagonist). Arteries were precontracted with phenylephrine, yielding ≈30% decrease in resting diameter. AT2R activation by angiotensin-induced dose-dependent relaxation of precontracted arteries (60.1±9.1% of phenylephrine-induced contraction, \( P<0.05 \)). In contrast, AT2R-dependent relaxation was not observed in arteries obtained from rats on high-salt diet. Semi-quantitative reverse-transcription polymerase chain reaction experiments demonstrated reduced amount of AT2R mRNA in arteries of rats on high-salt diet (65.5±7.5% of control levels, \( P<0.05 \)). Western blot studies demonstrated decreased AT2R in mesenteric artery protein fractions of high-salt diet rats (60.0±18.0 of control levels, \( P<0.05 \)). In a second set of experiments, adrenalectomy (4 days) blunted AT2R-mediated vasorelaxation and decreased AT2R mRNA (72.0±11.0% of control levels, \( P<0.05 \)). AT2R abundance in protein fractions of mesenteric arteries of ADX rats was also diminished (64.0±13% of control levels, \( P<0.05 \)). Both, AT2R mRNA and protein downregulation were prevented by mineralocorticoid replacement therapy. Finally, physiological concentrations of aldosterone caused a dose-dependent increase in AT2R mRNA of small diameter mesenteric artery explants. The results are consistent with aldosterone-mediated upregulation AT2R. (Hypertension. 2005;45:853-859.)

Key Words: mineralocorticoid ■ sodium ■ hypertension ■ vascular remodeling ■ apoptosis

The renin-angiotensin-aldosterone system (RAAS) regulates vascular tone, body fluid volume, electrolyte balance, hormonal secretion, and neuronal activity. The biological effects of angiotensin II (Ang II), the main effector peptide in the vasculature, are mediated by at least 2 receptor isoforms.\(^1,2\) The type 1 receptor (AT1R) mediates vasoconstriction, sympathetic facilitation, and trophic effects. The type 2 receptor (AT2R) is widely expressed during fetal development, whereas in the adult its expression has been detected in many different vessel types, including mesenteric, coronary, and renal arteries.\(^3-6\) AT2R has opposite effects to those of AT1R, ie, it promotes cell apoptosis and inhibits cell proliferation.\(^6,7\) AT2R also attenuates the pressor action of Ang II and mediates vasodilatation.\(^8,10\) Recently, it has been shown that Ang II relaxes small mesenteric arteries via AT2R when AT1R are blocked.\(^11-13\) Interestingly, the expression of AT2R is increased in several pathologic conditions such as vascular injury,\(^14\) cardiac remodeling, congestive heart failure, and myocardial infarction.\(^15,16\) It has been suggested that in adults the presence of AT2R in vascular tissues may be playing a role in vascular tone and/or tissue remodeling.\(^17-19\) Therefore, a key and complex question that arises is how AT1R and AT2R expression are modulated. Several studies indicate interaction between Ang II and aldosterone, affecting the expression of ATRs. The expression of AT1R appears to be induced by Ang II in vascular smooth muscle,\(^4\) and mineralocorticoids potentiate the action of Ang II in cultured rat vascular smooth muscle cells (VSMCs) by increasing the number of AT1R.\(^20,21\) However, there are few data concerning the physiological regulation of AT2R expression. Dietary sodium depletion, which increases RAAS activity, enhances renal AT2 receptor function\(^20,22\) and expression in both young and mature adult rats, mainly in the glomeruli and interstitial cells.\(^23\) Induction of AT2R-mediated modulation of blood pressure was described in rats fed with a synthetic diet, an effect attributed to the stimulation of the RAAS.\(^9\) Bonnet et al\(^24\) have shown that Ang II infusion in the rat induces the expression of AT2R in the mesenteric vasculature. Nevertheless, it is not clear whether Ang II directly mediates the increased AT2R expression or if it is secondary to direct aldosterone action on arteries.

In the present study, we investigated if high-salt diet has an effect on AT2R expression and function in rat small-diameter
mesenteric arteries. Therefore, we studied angiotensin receptor mRNA and protein abundance in arteries obtained from high-salt diet and control rats. Also, AT2R-mediated vasodilation in phenylephrine precontracted arteries was measured. The potential role of mineralocorticoids in AT2R regulation was analyzed in adrenalecтомized (ADX) rats, and ADX rats treated with deoxycorticosterone (DOCA). Finally, we studied the effect of aldosterone on the abundance of AT2R mRNA in mesenteric artery explants.

Materials and Methods

Experimental Animals

Male Sprague-Dawley rats weighing 180 to 220 grams with free access to water and standard rat chow were divided into high-salt diet (70 mmol/L NaCl drinking water, 4 days) and control group. In a second set of experiments, rats were divided into ADX, ADX-DOCA, and control group. Animals were anesthetized (Ketamine/Xilazine) and adrenalecтомized.28 The ADX-DOCA group received DOCA (0.5 mg/100 grams body weight) daily.29 The Ethics Committee of the Faculty of Medicine approved all protocols, according to National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Blood samples for biochemical measurements were taken by vein puncture in anesthetized animals. Electrolytes were determined using the Roche 9180 analyzer and aldosterone was determined by radioimmunoassay (DPC, Los Angeles, Calif).

BP Measurement

Systolic blood pressure was measured by the tail-cuff method in anesthetized rats.

AT2R Function in Small Mesenteric Arteries

The mesenteric arcade was excised and small mesenteric arteries (170 to 245 μm diameter) were prepared in modified Krebs-Ringer bicarbonate equilibrated with 5% CO2–95% O2, as described.26 Arteries were loaded with the calcium indicator FURA2-AM (1.0 μmol/L) in Krebs-Ringer bicarbonate; Molecular Probes) for 75 minutes at 37°C, 2.5 mL/min, and perfused (35 mmHg) for 75 minutes.26 Emitted fluorescence (520 nm) was acquired after excitation at 340/380 nm.26,27 AT2R function was studied according to Widdop et al.13 Artery viability was checked with 10 μmol/L phenylephrine. Thereafter, the tissue was washed, pre-incubated for 15 minutes with losartan 10 μmol/L9 and then re-exposed to phenylephrine (3 to 3.5 μmol/L) to cause ~30% reduction in resting diameter. The integrity of endothelium was assessed at the end of each experiment (acetylcholine 10 μmol/L) after precontraction with phenylephrine (5 μmol/L) and arteries where Ach induced a 60% vasodilation or more were analyzed. Ang II, acetylcholine, and phenylephrine were purchased from Sigma. Losartan was from Laboratorio Chile. Results are expressed as the change in the fluorescent ratio relative to the resting ratio,26 calculated as the average of 7 determinations (28 seconds) at the end of each experimental period. Images obtained at 380 nm excitation were used to estimate vessel diameter.

RNA Isolation

Small-diameter mesenteric arteries were washed in cold Krebs-Ringer bicarbonate and processed for total RNA extraction with TRIzol (GIBCO, Life Sciences). RNA concentration was determined by spectrophotometry and integrity of the RNA was assessed by gel electrophoresis.

Semi-quantitative Reverse-Transcriptase Polymerase Chain Reaction

Total RNA (0.5 μg) was reverse-transcribed (random hexamers, Invitrogen II Reverse Transcriptase System; Promega), cDNAs were amplified using 2 μL and 4 μL of template for AT1R and AT2R, respectively, with 2.5 U of TaqDNA polymerase (Fermentas). AT1R primer sequences were performed according to Dimitropoulos et al.12 AT2R sense primer (5’ GCC TGC ATT TTA AGT AGT GC 3’) and antisense primer (5’-AG trichloroacetic acid GCC ACA GCC AGA TT- 3’) amplified a product of 380 bp. Polymerase chain reaction (PCR) of the 18S ribosomal RNA served as an internal control29 added to the tubes in which ATRs were amplified (9 last cycles). All reactions were in the exponential phase (data not shown). PCR products from duplicates were subjected to gel electrophoresis with Gelstar and densitometric analyses were performed (EDAS 120; Kodak Digital Science).

Membrane Preparation and Western Blot Analysis

Membrane fractions were prepared from a pool of 4 mesenteric arteries per group (repeated 3 and 4 times, 72 animals) as previously described.25 Rodent protein was separated on 10% SDS-PAGE and Western blot was performed as described.25 Primary antibody used was rabbit polyclonal anti-AT2 (H-143) diluted 1:200 (Santa Cruz Biotechnology, Inc). Densitometry analysis results were expressed as the relative band intensity compared with the control paired sample.25

Statistical Analysis

Values are reported as mean±SEM. Differences between mean values were assessed by ANOVA or Student t test. Differences were considered statistically significant for values of P<0.05.

Results

Effect of High-Salt Diet in Plasma Electrolytes, Aldosterone, and Blood Pressure

Adult male rats were maintained under control or a high-salt diet (70 mmol/L NaCl added to the drinking water) during 4 days. There were no significant differences in plasma electrolytes or blood pressure among the groups (Table). In contrast, aldosterone levels decreased from 31.8 to ng/dL to 12.1 ± 2.3 ng/dL (P<0.05).

Effect of the High-Salt Diet on the Angiotensin Type 2 Receptor Function in Mesenteric Arteries

ATR2 function was analyzed in pressurized small-diameter mesenteric arteries kept under constant perfusion. Resting diameter of arteries from control and high-salt diet rats was similar (210.8±10.5 μm and 221.1±5.4 μm, respectively). Vasodilation via AT2R was tested in arteries precontracted with phenylephrine (5 μmol/L) and arteries where Acetylcholine induced a 60% reduction in resting diameter of arteries from control and high-salt diet rats.
with a submaximal dose of phenylephrine, which reduced the arterial resting diameter to 73.6±1.6% and 73.7±2.0% in vessels obtained from high-salt diet rats and control animals, respectively (not significant, n=5 each group). To evidence the vasodilatory action of the AT2R activation, angiotensin type I receptors were blocked with losartan (10 μmol/L). Ang II induced a significant vasodilation in arteries from control animals, up to 60.1±9.1% of the phenylephrine-induced contraction (Figure 1; P<0.05). In contrast, no vasodilatory effects of Ang II were observed in phenylephrine-contracted arteries obtained from high-salt diet animals (Figure 1B).

Intracellular calcium was estimated using the FURA2 340/380 fluorescent emission ratio. Resting fluorescent ratio in arteries from control and high-salt diet rats was similar (2.3±0.3 control versus 2.2±0.2 high salt, not significant). Phenylephrine induced a transient increase of intracellular calcium followed by a plateau phase. The plateau was 118.1±3.7% of resting fluorescence ratio in control rats and 118.9±3.0% of resting fluorescence ratio in high-salt diet rats (n=5, not significant versus control). The addition of Ang II (10⁻¹¹ to 10⁻⁷ M) did not modify the fluorescence levels compared with those observed in the presence of phenylephrine only in any experimental group (data not shown).
Effect of High-Salt Diet on Angiotensin Receptor Expression in Small-Diameter Mesenteric Arteries

To analyze if the blunted vasodilatory AT2R response in high-salt diet could be related to decreased receptor expression, we isolated total RNA from small mesenteric arteries and determined AT1R and AT2R mRNAs abundance by semi-quantitative reverse-transcription PCR. High-salt diet decreased AT2R mRNA to 65.5 ± 7.5% of control levels (P < 0.05, n = 5). AT1R mRNA levels had a tendency to increase with the high-salt diet, but the differences were not significant (Figure 2A).

AT2R protein abundance was evaluated by Western blot of membrane fractions obtained from mesenteric arteries. As shown in Figure 2B, 4 days of high-salt diet decreased AT2R protein abundance to 60.0 ± 18.0% of control levels (P < 0.05).

Effect of Adrenalectomy on the Expression of Angiotensin Receptors and AT2R Function

Figure 3 includes the results on the effect of adrenalectomy in the AT2R mRNA abundance in rat small mesenteric arteries. After 4 days of adrenalectomy, AT2R mRNA decreased (72.0 ± 11.0% of control levels, P < 0.05, n = 5). Hormonal reposition with DOCA prevented the effects of adrenalectomy. No significant changes for AT1R mRNA levels were observed in ADX or ADX-DOCA rats.

In a parallel set of experiments, AT2R protein abundance in membrane protein fractions from mesenteric arteries was evaluated by Western blot. Adrenalectomy induced a significant reduction on AT2R (64.0 ± 13.0% of control levels, n = 4, P < 0.05), which was prevented by DOCA replacement therapy (Figure 4).

AT2R-mediated vasodilation was analyzed in mesenteric arteries from ADX animals in the presence of losartan (Figure 5). Resting diameter of arteries from control and ADX animals was similar (212.8 ± 16.5 μm and 206.7 ± 14.5 μm, respectively). In contrast with the response observed in arteries from adrenal-intact animals, Ang II did not induce vasodilation in arteries of ADX rats.

No significant differences in basal FURA2 fluorescence ratio (2.5 ± 0.1 in control versus 2.6 ± 0.3 in ADX) or phenylephrine-induced increased before or after Ang II were detected.

Effect of Aldosterone on the Abundance of AT2R mRNA in Mesenteric Artery Explants

These included results indicate that low circulating aldosterone levels, induced by high-salt diet or ADX, correlated with reduced levels of AT2R irrespective to renin-angiotensin plasma levels, which suggested that aldosterone could directly modulate AT2R expression. Therefore, mesenteric artery explants were incubated with increasing physiological concentrations of aldosterone for 24 hours. As shown in Figure 6, aldosterone, in vitro, increased AT2R mRNA abundance. Significant differences were detected with 0.1 nmol/L aldosterone, and the maximum increase was observed at 1 nmol/L aldosterone.
Discussion

Results from different studies analyzing the putative functions of vascular AT2R have raised the possibility that type 2 receptors could counterbalance type 1 receptor function and/or may contribute to the beneficial actions of pharmacological type 1 receptor blockade, used for the treatment of hypertension. How the expression and function of these vascular AT2R is modulated is unclear. In the present study, we found that AT2R in small mesenteric arteries is downregulated by a high-salt diet or adrenalectomy and upregulated by mineralocorticoids.

In agreement with previous studies, activation of AT2R in precontracted arteries from control animals induced vasodilation. Although vasodilation plateau between increasing Ang II doses was not evident in our AT2R agonism studies, the experiments demonstrate blunted AT2R-mediated vasodilation in arteries from high-salt diet or ADX animals. We found that the absence of AT2R-mediated vasodilation observed in animals maintained in the high-salt diet correlated with a significant decrease in AT2R abundance. This observation indicates a differential regulation for AT2R as compared with AT1R, because several studies in experimental animals show that the high dietary sodium intake can lead to upregulation of AT1R expression, a tendency that was also observed in our experiments. In the case of AT1R, the effect of high salt intake seems to be independent of RAAS and could imply a NaCl-dependent mechanism acting on the vascular wall. In contrast, a simple interpretation of our results is that the downregulation of AT2R results from low circulating aldosterone induced by the high-salt diet.

Our studies revealed decreased AT2R expression in the mesenteric arteries of ADX rats, which was prevented by DOCA replacement. The decrease in mRNA abundance correlated with a similar reduction in AT2R protein abundance and impaired AT2R-mediated vasodilation of small mesenteric arteries. In the present study, we did not analyze vasomotor function in arteries from ADX animals with DOCA replacement therapy, but it is plausible that the recovery of AT2R expression correlated with restored vasodilation. Further, physiological concentration of aldosterone increased AT2R mRNA abundance in artery explants. These results suggest direct genomic regulation of AT2R expression by circulating mineralocorticoids acting on the vascular wall. The present results suggest that aldosterone is involved in the reported upregulation of AT2R protein in mesenteric arteries of rats after Ang II infusion. Further, the increased AT2R-dependent production of cyclic GMP in the aorta of spontaneously hypertensive rats with elevated Ang II levels could also imply aldosterone action.

VSMCs express the mineralocorticoid receptor (MR) and the enzyme 11βHSD-2 that protects the MR from activation by circulating glucocorticoids. Considering that the adventitia was removed from the arteries used in the present study, and that AT2R mRNA and protein are expressed in the VSMCs of
the mesenteric arteries, it is plausible that adrenalectomy or the high-salt diet decreased AT2R present in the VSMCs. Recent reports indicate the presence of AT2R in coronary and pulmonary endothelial cells. Also, aldosterone actions on human endothelial cells has been demonstrated, making the endothelium another potential target of aldosterone-regulated AT2R expression.

Activated MRs act as transcription factors that transactivate glucocorticoid-responsive elements in the promoters of target genes. The presence of glucocorticoid-responsive elements in the human AT2R has been reported previously. Alternatively, MRs could indirectly modulate AT2R expression, ie, via activation of activating protein-1 (Fos/Jun). This mechanism has been postulated for the aldosterone-dependent induction of epidermal growth factor receptor expression and for the cardiac actions of aldosterone.

Numerous potential AP1 binding sites were identified in the promoter region of the human, mouse, and rat AT2R genes. Clarification of these or other potential mechanisms mediating AT2R mRNA abundance changes will need further studies.

We have not detected significant changes of [Ca\textsuperscript{2+}] associated with AT2R-mediated vasodilation. The comparison of fluorescence obtained from endothelium-denuded and intact arteries (data not shown) demonstrated no significant differences, suggesting that VSMCs are the major compartment of the arterial wall where FURA is distributed. AT2R-dependent activation of phosphatases could modulate the phosphorylation of contractile proteins and their sensitivity to increases in [Ca\textsuperscript{2+}], leading to vasodilation without a marked reduction of intracellular calcium. However, considering the technical limitations of the present study, this and other potential mechanisms implicated in the regulation of contractile proteins and their sensitivity to increases in [Ca\textsuperscript{2+}] need further investigation.

Although the physiological role of AT2R is still controversial, several studies suggest that AT2R activation could lower blood pressure. We speculate that AT2R down-regulation could contribute in increasing blood pressure in the presence of a high-salt diet, a condition that can lead to increased AT1R expression. Conversely, increased RAAS activity by low-salt diet would upregulate AT2R expression and decrease AT1R expression by homologous downregulation. It has been shown that AT2R activation in conscious rats can decrease systolic blood pressure. This effect is blocked by inhibition of nitric oxide synthase, implying that AT2R acts via a vasodilator pathway. Interestingly, the AT2R-mediated decrease in systolic blood pressure is more pronounced in rats on a sodium-restricted diet. Also, AT2R-null mice have greater susceptibility to increase in blood pressure in response to DOCA than wild-type controls. It is possible that the absence of vascular AT2R, which normally would be upregulated by mineralocorticoids, is involved in the greater sensitivity of AT2R-null mice to DOCA.

**Perspectives**

Several studies indicate interaction between Ang II and aldosterone. Aldosterone increases AT1R in rat VSMCs and vessels. An increasing body of evidence indicates that AT2R exerts antigrowth, antihypertrophic, and pro-apoptotic effects that may counteract the growth stimulation mediated by AT1R. We speculate that the upregulation of AT2R expression by mineralocorticoids could be relevant in the modulation of vascular actions of Ang II. AT2R downregulation with a high-salt intake could represent an additional factor in vascular remodeling that can be related to hypertension, a possibility that merits further study.

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**References**


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