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# Ascorbic Acid Decreases the Binding Affinity of the $AT_1$ Receptor for Angiotensin II

Patrice C. Leclerc[1], Christophe D. Proulx[1], Guillaume Arguin[1], Simon Bélanger[1], Fernand Gobeil Jr[1], Emanuel Escher[1], Richard Leduc[1] and Gaétan Guillemette[1]

**BACKGROUND**

Ascorbic acid is an essential vitamin and a powerful antioxidant. Many studies have highlighted the benefits of ascorbic acid for chronic cardiovascular diseases such as hypertension in which angiotensin II (Ang II) plays an significant role. We therefore hypothesized that ascorbic acid could modify the pharmacological properties of the $AT_1$ receptor for Ang II.

**METHODS**

Binding studies and $Ca^{2+}$ mobilization studies were performed with HEK293 cells stably expressing the $AT_1$ receptor for Ang II. Smooth muscle contraction studies were performed with rabbit aorta strips that endogenously express the $AT_1$ receptor.

**RESULTS**

Scatchard analysis revealed that ascorbic acid decreased the binding affinity of the $AT_1$ receptor without modifying its maximal binding capacity. Ascorbic acid did not modify the binding affinity of the $AT_2$ receptor for Ang II or of the UT receptor for urotensin II. In single-cell $Ca^{2+}$ imaging assays, ascorbic acid reduced the frequency of intracellular $Ca^{2+}$ oscillations induced by a low dose of Ang II. In functional assays, ascorbic acid significantly diminished the contraction of rabbit aorta pre-contracted with Ang II but not those pre-contracted with urotensin II.

**CONCLUSIONS**

Ascorbic acid decreases the binding affinity of the $AT_1$ receptor. These results offer a mechanistic explanation for the reported blood pressure lowering effect of ascorbic acid.

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Angiotensin II (Ang II) is an important hormonal regulator of blood pressure. It elicits a variety of physiological reactions, including vascular contraction, aldosterone secretion, sodium and water retention, neuronal activation, and cardiovascular cell growth and proliferation. While Ang II interacts with two angiotensin receptors ($AT_1$ and $AT_2$), most of its cardiovascular effects are mediated through the $AT_1$ receptor, which belongs to the G protein–coupled receptor (GPCR) superfamily. It couples primarily to the heterotrimeric G protein $G_{q/11}$, which activates phospholipase C, which in turn generates the second messengers inositol-1,4,5-trisphosphate and diacylglycerol. Inositol-1,4,5-trisphosphate triggers the release of $Ca^{2+}$ from the endoplasmic reticulum while diacylglycerol activates protein kinase C (for review, see ref. 1).

Ascorbic acid (vitamin C) is an essential nutrient involved in many metabolic pathways. It is a cofactor for several enzymes involved in the biosynthesis of collagen, carnitine, and neurotransmitters.[2] Ascorbic acid is also an important water-soluble antioxidant in biological fluids,[3] scavenging reactive oxygen and nitrogen species such as superoxide, peroxynitrite, and nitrogen dioxide.[4] Many studies have suggested that ascorbic acid may be of benefit in chronic diseases such as cardiovascular disease, cancer, and cataracts (for review, see ref. 2). It has been directly implicated in the regulation of blood pressure. Several studies have shown that the plasma concentration of ascorbic acid is inversely related to blood pressure,[5–9] while an increased intake of ascorbic acid has been associated with lower blood pressure.[10–13]

The binding affinity of the $AT_1$ receptor is sensitive to redox reagents such as such β-mercaptoethanol and dithiothreitol.[14,15] The binding affinity of the $AT_1$ receptor is also sensitive to S-nitrosylation,[16] another redox modification that targets the free thiols of cysteine residues. Because ascorbic acid has antioxidant properties, and also because ascorbic acid was shown to open disulfide bridges in certain proteins,[17] we hypothesized that it could modulate the pharmacological properties of the $AT_1$ receptor. In the study presented here, we describe a series of experiments suggesting that ascorbic acid decreases the binding affinity of the $AT_1$ receptor. These results offer a mechanistic explanation for the reported blood pressure lowering effect of ascorbic acid.

## METHODS

*Materials.* Dulbecco's modified Eagle's medium, fetal bovine serum, and penicillin–streptomycin were purchased from Gibco Life Technologies (Gaithersburg, MD). Fugene 6 was purchased from Roche Diagnostics (Indianapolis, IN). $^{125}$I-Ang II

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(1000 Ci/mmol) was prepared using Iodogen (Pierce Chemical, Rockfield, IL) as previously described and purified by high-performance liquid chromatography on a C-18 column.[18] Bovine serum albumin and bacitracin were purchased from Sigma (St. Louis, MO). Ascorbic acid was purchased from Fisher Scientific (Nepean, Ontario, Canada).

*Cell cultures and transfections.* HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 50 IU/ml of penicillin, and 50 µg/ml of streptomycin. At 70% confluency the cells were transfected in serum-free Dulbecco's modified Eagle's medium containing 4 µg of DNA and 8 µl of Fugene 6. Forty-eight hours post-transfection, the cells were used for binding experiments. The HEK-293 cell line stably expressing the human $AT_1$ receptor was developed in our laboratory as previously described.[19]

*Ang II binding experiments.* Cells were frozen in liquid nitrogen, thawed in ice-cold phosphate-buffered saline and broken by five cycles of aspiration–expulsion with a 10 ml serological pipette tightly apposed to the bottom of the dish. Broken cells were centrifuged at 2500*g* for 15 min at 4 °C and resuspended in binding buffer (25 mmol/l Tris–HCl pH 7.4, 100 mmol/l NaCl, 5 mmol/l $mgCl_2$, 0.1% bovine serum albumin, and 0.01% bacitracin). Broken cells were incubated for 1 h at room temperature in binding buffer containing increasing concentrations of $^{125}$I-Ang II, in the presence or absence of ascorbic acid. Bound and free ligands were separated by filtration through GF/C filters presoaked for 2 h in binding buffer. Nonspecific binding was measured in the presence of 1 µmol/l unlabeled Ang II.

*Dynamic video imaging of cytosolic $Ca^{2+}$.* HEK-293 cells stably expressing the $AT_1$ receptor were grown for 24 h on glass coverslips (no. 1) in complete Dulbecco's modified Eagle's medium. They were washed twice with HEPES-buffered physiological saline (Hank's-balanced salt solution: 20 mmol/l HEPES pH 7.4, 120 mmol/l NaCl, 5.3 mmol/l KCl, 0.8 mmol/l $mgSO_4$, 1.8 mmol/l $CaCl_2$, and 11.1 mmol/l dextrose) and incubated with 0.2 µmol/l fura 2-acetoxymethyl ester for 20 min in the presence or absence of 100 mmol/l ascorbic acid. The cells were then washed and bathed in fresh Hank's-balanced salt solution for 20 min in the presence or absence of 100 mmol/l ascorbic acid to ensure complete hydrolysis of the fura 2-acetoxymethyl ester before mounting the Teflon chamber onto the stage of a Axiovert inverted microscope (Carl Zeiss, Thornwood, NY) fitted with an Attofluor Digital Imaging and Photometry System (Attofluor, Rockville, MD). Fluorescence was monitored at excitation wavelengths of 334 and 380 nm and emission wavelength of 510 nm. Data acquisition was typically at 3-s intervals and lasted for 30 min.

*Rabbit aorta strip contraction.* New Zealand White rabbits (1.5–2.5 kg) were anesthetized by intramuscular injection of a mixture of ketamine (48 mg/kg) and xylazine (7 mg/kg). All experimental procedures conformed to Canadian Council on Animal Care guidelines and were approved by the Ethics Committee for Animal Research of the University of Sherbrooke Medical School. Helical strips of aorta devoid of endothelium were prepared as previously described.[20] The tissues were suspended in 10-ml organ baths containing warm (37 °C) oxygenated (95% $O_2$/5% $CO_2$) Krebs' (118 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l $KH_2PO_4$, 1.2 mmol/l $mgSO_4$, 25 mmol/l $NaHCO_3$, and 5.5 mmol/l dextrose). The aorta strips were stretched at an initial tension of 2 g and were allowed to equilibrate for 1–2 h. Isometric contractions were recorded using a Grass Instrument Model 7D polygraph.

## RESULTS

### Ascorbic acid decreases $AT_1$ receptor binding affinity

HEK-293 cells stably expressing the $AT_1$ receptor were treated with increasing concentrations of ascorbic acid and their Ang II binding properties were analyzed. **Figure 1a** shows that ascorbic acid decreased the specific binding of $^{125}$I-Ang II in a concentration-dependent manner. Binding was decreased by ~20% with 1 µmol/l ascorbic acid. The maximal inhibitory effect (~50%) was obtained with 100 µmol/l ascorbic acid. The inhibitory effect of

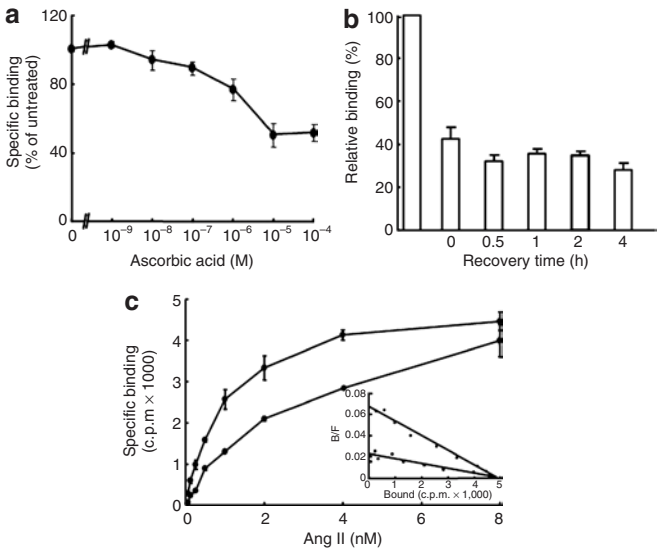


**Figure 1 |** Ascorbic acid decreases the binding affinity of the $AT_1$ receptor. (**a**) Broken HEK-293 cells stably expressing the $AT_1$ receptor (25 µg of protein) were incubated with 1 nmol/l $^{125}$I-angiotensin II (Ang II) for 1 h at room temperature in the presence of increasing concentrations of ascorbic acid. (**b**) Broken HEK-293 cells stably expressing the $AT_1$ receptor (10 µg of protein) were incubated at room temperature with 1 nmol/l $^{125}$I-Ang II in the presence of 100 nmol/l ascorbic acid. After 30 min, the broken cells were centrifuged and resuspended in binding buffer without ascorbic acid and incubated for different periods of time. Results are expressed as percentage of binding to untreated broken cells. The histograms represent the mean ± s.d. of values obtained in three independent eriments. (**c**) Broken HEK-293 cells stably expressing the $AT_1$ receptor (10 µg of protein) were incubated (filled symbols) or not (open symbols) with 100 µmol/l ascorbic acid for 60 min at room temperature in the presence of increasing concentrations of $^{125}$I-Ang II. Scatchard analysis of the binding data is shown in the inset. Nonspecific binding was assessed in the presence of 1 µmol/l unlabeled Ang II. Each data point represents the mean ± s.d. of triplicate values (representative of three independent experiments).

ascorbic acid was still observed for at least 4 h after washing the broken cells with the binding buffer (**Figure 1b**). To better define the effect of ascorbic acid, $^{125}$I-Ang II saturating binding isotherms were performed in the absence or presence of 100 µmol/l ascorbic acid (**Figure 1c**). Scatchard analysis of the binding data revealed that untreated broken cells had a high binding affinity ($K_d$ of 0.8 ± 0.2 nmol/l) whereas ascorbic acid–treated broken cells had a significantly lower $K_d$ of 1.8 ± 0.5 nmol/l. The treatment with ascorbic acid did not significantly affect the maximal binding capacity of the broken cells (2.4 ± 0.6 pmol/mg of protein vs. 2.2 ± 0.7 pmol/mg of protein for the untreated and the ascorbic acid–treated cells, respectively). These results clearly show that ascorbic acid decreases the binding affinity of the $AT_1$ receptor. Like other GPCRs, the $AT_1$ receptor can adopt a low affinity state when it is uncoupled from its cognate G protein.[21] To verify whether the low affinity state induced by treatment with ascorbic acid was due to the uncoupling of the $AT_1$ receptor from its G protein, we evaluated the binding properties in the presence of the well-known G protein uncoupling agent GTPγS. After treatment with ascorbic acid, the incubation of broken cells with 1.0 nmol/l $^{125}$I-Ang II yielded a specific binding value of 7259 ± 470 c.p.m. Under the same conditions, GTPγS diminished $^{125}$I-Ang II binding to a value of 5287 ± 276 c.p.m., which represents a significant ($P < 0.05$) decrease of 37 ± 6% (mean of three independent experiments). These results show that GTPγS produced an uncoupling effect on ascorbic acid–treated cells, implying that the $AT_1$ receptor was still coupled to its G protein after the treatment with ascorbic acid and therefore the mechanism by which ascorbic acid decreases the binding affinity of the $AT_1$ receptor is not related to G protein coupling.

### The effect of ascorbic acid is specific to the $AT_1$ receptor

Ang II specifically interacts with the $AT_1$ and the $AT_2$ receptors. We verified whether ascorbic acid could also modulate the affinity of the $AT_2$ receptor transiently expressed in HEK-293 cells. Scatchard analysis of $^{125}$I-Ang II saturation binding isotherms showed that ascorbic acid did not modify the $K_d$ of the $AT_2$ receptor (1.5 ± 0.4 nmol/l vs 1.8 ± 0.4 nmol/l for the untreated and the ascorbic acid–treated cells, respectively). As observed for the $AT_1$ receptor, **Figure 2a** shows that ascorbic acid did not modify the maximal binding capacity of the $AT_2$ receptor (7.6 ± 1.1 vs. 6.9 ± 0.3 pmol/mg of protein for the untreated and the ascorbic acid–treated cells, respectively). Similarly, **Figure 2b** shows that ascorbic acid did not modify the $K_d$ (4.3 ± 0.1 nmol/l vs. 3.9 ± 1.7 nmol/l for the untreated cells and the ascorbic acid–treated cells, respectively) or the maximal binding capacity (4.4 ± 0.1 vs. 4.2 ± 0.5 pmol/mg of protein for the untreated and the ascorbic acid–treated cells, respectively) of the UT receptor for urotensin II, another GPCR involved in the regulation of blood pressure. These results show that ascorbic acid modified the binding properties of the $AT_1$ receptor but did not affect the binding properties of two other GPCRs expressed in the cardiovascular system.

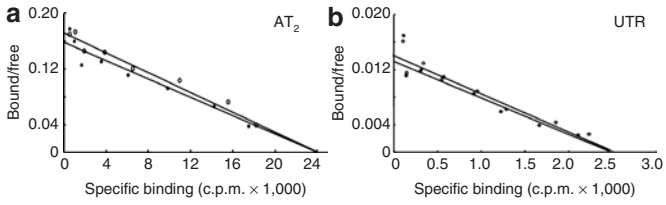


**Figure 2 |** The effect of ascorbic acid is specific to the $AT_1$ receptor. Broken HEK-293 cells transiently expressing the $AT_2$ receptor or the urotensin (UT) receptor (10 µg of protein) were incubated (filled symbols) or not (open symbols) with 100 µmol/l ascorbic acid for 60 min at room temperature in the presence of increasing concentrations of $^{125}$I-Ang II or $^{125}$I-UII. Scatchard analysis of the binding data is shown for (**a**) the $AT_2$ receptor and the (**b**) UT receptor. Nonspecific binding was assessed in the presence of 1 µmol/l unlabeled peptide. Each data represents the mean ± s.d. of triplicate values (representative of three independent experiments). Ang II, angiotensin II; UTR, urotensin II receptor.

### Ascorbic acid decreases Ang II–induced $Ca^{2+}$ responses

We previously showed that low concentrations of Ang II induce intracellular $Ca^{2+}$ oscillations in HEK-293 cells stably expressing the $AT_1$ receptor.[19] **Figure 3a** shows a typical trace where a low concentration of Ang II (0.1 nmol/l) elicited repetitive $Ca^{2+}$ oscillations with a frequency of 33 ± 5 oscillations/h (mean ± s.d. of 215 cells, representative of three independent experiments) while a maximal dose of Ang II (1 µmol/l) produced a high amplitude $Ca^{2+}$ transient. Under these conditions, only a small proportion of cells (6%) did not respond to Ang II. **Figure 3b** shows a typical trace where, after a 30-min treatment with ascorbic acid (100 µmol/l), Ang II (0.1 nmol/l) elicited repetitive $Ca^{2+}$ oscillations with a frequency of 22 ± 4 oscillations/h (mean ± s.d. of 224 cells, representative of three independent experiments) while a maximal dose of Ang II

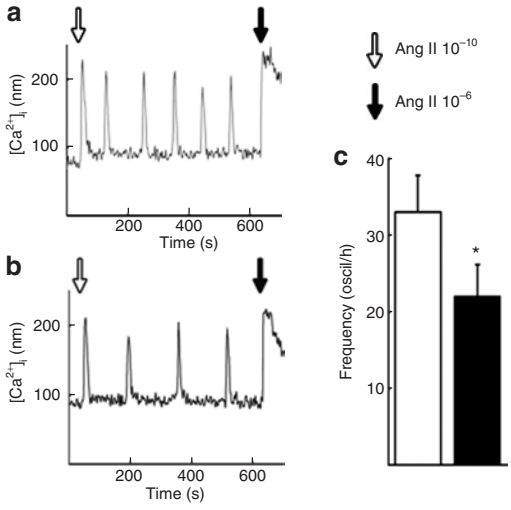


**Figure 3 |** Ascorbic acid slows $Ca^{2+}$ oscillations in single cells. HEK-293 cells stably expressing the $AT_1$ receptor were loaded with fura 2-acetoxymethyl ester and their intracellular $Ca^{2+}$ concentration was monitored as described in "Methods." (**a**) Untreated cells and (**b**) cells treated with 100 µmol/l ascorbic acid were stimulated at the indicated times with different concentrations of Ang II (white and black arrows). These typical traces showing variations of the fluorescence ratio ($F_{334}/F_{380}$) within single cells are representative of more than 200 different cells for each condition. Panel (**c**) shows the quantitative analysis of $Ca^{2+}$ oscillatory rates (*$P < 0.05$ compared with respective untreated cells). Ang II, angiotensin II.

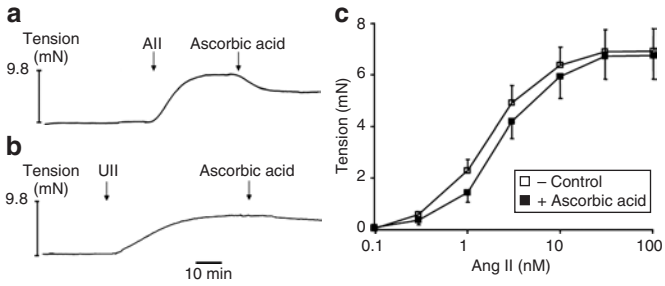


**Figure 4 |** Ascorbic acid decreases the angiotensin II (Ang II)-induced isometric contraction of rabbit aorta. Typical traces showing the effect of ascorbic acid on the contractile retsponse of isolated endothelium-denuded rabbit aorta strips stimulated with (**a**) Ang II or with (**b**) UII. The compounds were added as indicated by arrows. These typical traces are representative of at least six independent experiments. Panel (**c**) shows the concentration–response curves for Ang II–induced rabbit aorta strip contraction in the absence (open symbols) or the presence of ascorbic acid (filled symbols). Each point within the two curves is the average of data issued from eight independent experiments. AII, angiotensin II; UII, urotensin II.

(1 µmol/l) produced a high amplitude $Ca^{2+}$ transient that was similar to that obtained with untreated cells. After the treatment with ascorbic acid, the proportion of cells that did not respond to 0.1 nmol/l Ang II increased to 15%. These results are consistent with a decreased binding affinity of the $AT_1$ receptor caused by ascorbic acid and suggest that the integrity of the $Ca^{2+}$ signaling machinery is unaffected by ascorbic acid.

### Ascorbic acid decreases the Ang II–induced contraction of smooth muscle

The $AT_1$ receptor is endogenously expressed in the smooth muscle of rabbit aorta. **Figure 4a** shows a classic experiment where Ang II elicited a sustained contraction of the rabbit aorta strip. In a typical experiment, the force of contraction elicited by Ang II (1 nmol/l) was 9.1 ± 2.6 mN (mean ± s.d.). At the peak of the contraction, the addition of 100 µmol/l ascorbic acid relaxed the tissue by 35 ± 10% (mean ± s.d. of three experiments). Like the results obtained with the binding experiments, ascorbic acid had no effect on the urotensin II–induced contraction of the rabbit aorta (**Figure 4b**). Furthermore, ascorbic acid had no significant effect on the basal tension of rabbit aorta strips (data not shown). These results show that ascorbic acid decreased the contraction induced by the $AT_1$ receptor but not the contraction induced by the UT receptor. **Figure 4c** shows that ascorbic acid did not modify the maximal response, but caused a rightward shift (from 1.8 ± 0.1 nmol/l to 2.6 ± 0.3 nmol/l, $P < 0.05$) of the concentration–response curve for Ang II–induced contraction of the rabbit aorta. Together, these results suggest that all the components of the signaling pathway downstream from the UT and the $AT_1$ receptors are not affected by ascorbic acid. Also, these results are consistent with a direct effect of ascorbic acid on the $AT_1$ receptor resulting in a decreased binding affinity.

## DISCUSSION

This study provides evidence that ascorbic acid dose-dependently decreases the binding affinity of the $AT_1$ receptor without modifying its level of expression at the cell surface. This is not a common effect of ascorbic acid on GPCRs because the $AT_2$ receptor and the UT receptor were not affected. Ascorbic acid is a well-known cofactor for several enzymes involved in many metabolic pathways in the body.[2] Ascorbic acid is also one of the most effective antioxidants in biological fluids.[3] In humans, the antioxidant activity is involved in the regulation of lipid, DNA, and protein oxidation.[2] The binding affinity of the $AT_1$ receptor can be regulated by redox mechanisms. Reducing agents such as β-mercaptoethanol and dithiothreitol have been used to discriminate between the $AT_1$ and the $AT_2$ receptor because they decrease the binding affinity of the $AT_1$ receptor[14,15] but do not affect the $AT_2$ receptor.[22] These effects are related to the disruption of critical intramolecular disulfide bridges within the $AT_1$ receptor that are essential for Ang II binding. Another study reported that N-acetylcysteine, another antioxidant, decreases Ang II binding in vascular smooth muscle cells.[23] The authors reported that N-acetylcysteine affect receptor density at the cell surface without affecting the binding affinity. The authors suggested that N-acetylcysteine mimicked the effect of dithiothreitol by disrupting a disulfide bridge in the $AT_1$ receptor. However, our results indicate that ascorbic acid only decreased the binding affinity of the $AT_1$ receptor without affecting the receptor density at the cell surface. The $AT_1$ receptor contains two disulfide bridges whose disruption causes a complete loss of binding activity.[24] However it was shown that mild reducing conditions do not abolish the binding properties of the $AT_1$ receptor but cause a significant decrease in binding affinity.[14] These results imply that one disulfide bridge in the $AT_1$ receptor is more labile than the other and that the disruption of this labile disulfide bridge decreases the binding affinity. It is thus likely that ascorbic acid acts by reducing a labile disulfide bridge within the $AT_1$ receptor. Other determinants of the $AT_1$ receptor can be regulated by a redox mechanism. We previously demonstrated that the affinity of the $AT_1$ receptor can be decreased by S-nitrosylation of one of its endogenous transmembrane cysteine residues ($Cys^{289}$).[16] However, a mutant receptor whose $Cys^{289}$ was replaced by an Ala remained sensitive to ascorbic acid (data not shown), suggesting that another determinant of the $AT_1$ receptor is involved in the ascorbic acid effect. Further studies are warranted to identify the exact molecular mechanism by which ascorbic acid modulates $AT_1$ receptor binding affinity.

The $AT_1$ receptor is a $G_q$-coupled GPCR that can raise the intracellular $Ca^{2+}$ concentration. We previously showed that low concentrations of Ang II increased the frequency of $Ca^{2+}$ oscillations within spontaneously oscillating HEK-293 cells, whereas high concentrations of Ang II–induced a high amplitude $Ca^{2+}$ transient within these cells.[19] The frequency of $Ca^{2+}$ oscillations is therefore dependent (up to a certain point) on the amount of receptors occupied by the agonist. In the present study, we show that ascorbic acid slowed the rate of $Ca^{2+}$ oscillations induced by a low dose of Ang II but did not affect the amplitude of the $Ca^{2+}$ transient induced by a high dose of Ang II. These results are consistent with the notion that ascorbic acid decreases the binding affinity of the $AT_1$ receptor. Because the maximal $Ca^{2+}$ response can still be obtained with high concentrations of Ang II, these results also suggest that the $Ca^{2+}$

signaling machinery downstream from the $AT_1$ receptor is not affected by ascorbic acid.

We also show that ascorbic acid decreased the Ang II–induced but not the urotensin II–induced isometric contraction of the rabbit aorta strip. These results suggest that ascorbic acid may be involved in the regulation of vascular tone. Regulation of vascular tone by ascorbic acid has been inferred from several epidemiological studies that demonstrated that there is an inverse correlation between the plasma concentration of ascorbic acid and mean blood pressure.[5–9] In a randomized, double-blind, placebo-controlled study, Duffy *et al.*[10] showed that a dietary supplement of ascorbic acid lowers the blood pressure of hypertensive patients,[10] with the plasma concentration of ascorbic acid reaching 100 µmol/l after 1 month of daily intake. Another study showed that a 1-month treatment with a daily dose of 500 mg of ascorbic acid lowers the brachial blood pressure and arterial stiffness of type 2 diabetes patients,[11] with the plasma concentration of ascorbic acid reaching ~80 µmol/l.

The mechanism by which ascorbic acid regulates blood pressure is not well understood. In blood vessels, endothelial cells constitutively produce nitric oxide, a vasodilator that is inactivated by complexing with superoxide, a product of normal oxidative metabolism.[25,26] Several studies have suggested that ascorbic acid scavenges superoxide, thus increasing the availability of nitric oxide in blood vessels.[27,28] A recent study showed that Ang II attenuated endothelium-dependent forearm vasodilation in healthy young men. Because the effect was reversed by ascorbic acid, the authors suggested that Ang II acted by increasing the production of superoxide anion.[29] However, it appears that millimolar concentrations of ascorbic acid are required to scavenge superoxide.[30] These concentrations are much higher than those required to lower blood pressure. It is interesting to note that the concentration at which ascorbic acid modulates the binding affinity of the $AT_1$ receptor is approximately 100 µmol/l, which is well within the range of concentrations at which ascorbic acid is reported to reduce blood pressure. In summary, the data presented here indicate that ascorbic acid is a negative modulator of the $AT_1$ receptor affinity. This effect is specific to the $AT_1$ receptor. These results offer a mechanistic explanation for the reported blood pressure lowering effect of ascorbic acid.

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