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# Torasemide Inhibits Angiotensin II–Induced Vasoconstriction and Intracellular Calcium Increase in the Aorta of Spontaneously Hypertensive Rats

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***Abstract***—Torasemide is a loop diuretic that is effective at low once-daily doses in the treatment of arterial hypertension. Because its antihypertensive mechanism of action may not be based entirely on the elimination of salt and water from the body, a vasodilator effect of this drug can be considered. In the present study, the ability of different concentrations of torasemide to modify angiotensin II (Ang II)–induced vascular responses was examined, with the use of an organ bath system, in endothelium-denuded aortic rings from spontaneously hypertensive rats. Ang II–induced increases of intracellular free calcium concentration ($[Ca^{2+}]_i$) were also examined by image analysis in cultured vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats. A dose-response curve to Ang II was plotted for cumulative concentrations (from $10^{-9}$ to $10^{-6}$ mol/L) in endothelium-denuded aortic rings ($pD_2=7.5\pm0.3$). Isometric contraction induced by a submaximal concentration of Ang II ($10^{-7}$ mol/L) was reduced in a dose-dependent way by torasemide ($IC_{50}=0.5\pm0.04$ $\mu$mol/L). Incubation of VSMCs with different concentrations of Ang II (from $10^{-10}$ to $10^{-6}$ mol/L) resulted in a dose-dependent rise of $[Ca^{2+}]_i$ ($pD_2=7.5\pm0.3$). The stimulatory effect of $[Ca^{2+}]_i$ induced by a submaximal concentration of Ang II ($10^{-7}$ mol/L) was blocked by torasemide ($IC_{50}=0.5\pm0.3$ nmol/L). Our findings suggest that torasemide blocks the vasoconstrictor action of Ang II in vitro. This action can be related to the ability of torasemide to block the increase of $[Ca^{2+}]_i$ induced by Ang II in VSMCs. It is proposed that these actions might be involved in the antihypertensive effect of torasemide observed in vivo. **(*Hypertension*. 1999;34:138-143.)**

**Key Words:** angiotensin II ■ calcium ■ furosemide ■ irbesartan ■ muscle, smooth, vascular ■ rats, inbred SHR ■ torasemide

Recent long-term clinical studies have demonstrated the beneficial effects of low-dose diuretics in reducing cardiovascular morbidity and mortality in the treatment of hypertension.[1,2] Therefore, diuretics continue to be recommended as first-line agents in the treatment of hypertension.[3] It has been suggested that the long-term antihypertensive effectiveness of diuretics is due to its ability to reduce peripheral resistance through a direct arteriolar vasodilator action.[4] Animal experiments have shown that diuretics reduce the vascular responsiveness to vasoconstrictor agonists like norepinephrine and angiotensin II (Ang II).[5,6] In addition, in hypertensive patients it was shown that long-term use of diuretics desensitizes the vasculature in its response to endogenous vasoconstrictor hormones.[7]

Torasemide is a pyridil sulfonil urea class compound whose chemical structure places it between loop diuretics and $Cl^-$ channel blockers.[8] The main tubular site of action of torasemide is the ascending limb of the loop of Henle, where it interacts with the $Na^+$, $2Cl^-$, $K^+$ cotransporter localized in the luminal surface.[8,9] By this interaction, at high doses, torasemide inhibits NaCl reabsorption which provokes diuresis.[10] Torasemide is used as a first-line therapy in hypertension, and the results of several prospective therapeutic trials demonstrate that this compound reduces blood pressure at dosages that do not result in documented natriuresis.[11–13] Therefore, the antihypertensive mechanism of action of torasemide may be based not only on the elimination of salt and water from the body, but also on an arterial vasodilator action. This possibility is in agreement with previous in vivo and in vitro experiments showing that loop diuretics, such as furosemide,[14,15] and $Cl^-$ channel blockers, such as diphenylamine-2-carboxylate,[16,17] exert vasorelaxation effects.

Therefore, we hypothesized that torasemide exerts a vasorelaxation effect by interfering with the vascular actions of Ang II. To test this hypothesis we examined the in vitro effects of torasemide on both Ang II–induced isometric contraction in aortic rings from spontaneously hypertensive rats (SHR) and Ang II–induced intracellular free calcium concentration ($[Ca^{2+}]_i$) transients in cultured vascular smooth muscle cells (VSMC) from the aorta of SHR.

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# Methods

## Animals

The study was performed with 30-week-old male SHR obtained from RCC Biotechnology & Animal Breeding (Fullinsdorf, Switzerland). The rats were housed in cages maintained in a humidity- and temperature-controlled room and fed a standard diet. They had access to tap water ad libitum. The investigation was performed according to the European Community guidelines for animal ethical care and use of laboratory animals (Directive 86/609). Before the rats were euthanized by decapitation, the systolic blood pressure was measured by the standard tail-cuff method. Recorded systolic blood pressure values were in agreement with those previously described in this animal model at this age (214±5 mm Hg, mean±SEM of 15 rats).[18]

## Preparation of Isolated Endothelium-Denuded Aortic Rings

The thoracic aorta was removed immediately after euthanization, cleaned, and placed into Krebs-Heinseleit solution of the following composition (mol/L): 118.4 NaCl, 4.74 KCl, 1.18 $MgSO_4$, O·7 $H_2O$, 1.19 $KH_2PO_4$, 2.52 $CaCl_2{\cdot}2H_2O$, 25 $NaHCO_3$, 11.5 glucose. The endothelium of the aorta was removed by gently rubbing the intimal surface with the tip of a small steel probe. The aorta was cut into ring segments (2 to 3 mm in length, 6 per rat) that were placed in a vessel containing 10 mL of Krebs-Heinseleit solution oxygenated with a 95% $O_2$, 5% $CO_2$ mixture and maintained at 37°C in a thermostated bath (LE 13206 Thermostate, Letica Scientific Instruments). Two stainless steel wires were inserted into the vascular lumen; 1 was anchored to a stationary support, and the other was connected to a force-displacement isometric transducer (Tri 110, Letica Scientific). Changes in isometric forces were analyzed and recorded by an Isolated Organs Data Acquisition program (Proto5, Letica Scientific Instruments). The rings were incubated to equilibration at a resting tension of 2*g* for 60 minutes, with buffer changes every 15 minutes during this period. The lack of a functional endothelium was confirmed by demonstrating the complete absence of relaxation induced by acetylcholine ($10^{-5}$ mol/L) in norepinephrine ($10^{-7}$ mol/L)-precontracted aortic rings. The rings were then washed and stretched, if necessary, until a stable baseline force was obtained. After equilibration, each ring was exposed to 120 mmol/L KCl to test the contractile response of the tissue.

## Study of Vasoactive Responses

Cumulative concentration-response curves for the response to $10^{-9}$ to $10^{-6}$ mol/L Ang II were obtained in order to determine a concentration of submaximal contraction and the $pD_2$ value. In a second group of experiments, the aortic rings were incubated for 30 minutes with different concentrations of torasemide, furosemide, and irbesartan ($10^{-10}$, $10^{-8}$, $10^{-6}$ mol/L). After the incubation, a contraction induced by a submaximal concentration of Ang II was tested. Each preparation was exposed to only 1 concentration of the drugs used and only to a single concentration of the agonist, to avoid possible receptor desensitization. One aortic ring per rat was reserved to obtain control responses (100%) in the presence of the solvent.

## Cell Isolation and Culture

Primary VSMCs were obtained from the thoracic aorta of adult SHR and cultured by the tissue explants method in accordance with previously published procedures.[19] Briefly, the smooth muscle tissue was longitudinally opened and cut in small pieces that were grown in plastic 6-well plates and maintained at 37°C in a humidified incubator with an atmosphere of 95% air, 5% $CO_2$. Tissue explants were cultured in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum and antibiotic-antimycotic (10 000 U/mL penicillin G sodium, 10 000 μg/mL streptomycin sulfate and 25 μg/mL amphotericin B as fungizone R in 0.85% saline). When VSMCs began to emerge from the explants, they were subcultured with 0.05% trypsin to a 25 $cm^2$ flask. After the first subculture, cells were subcultured every week and reached confluence in 5 to 6 days. The medium was changed initially after 24 hours and then every 3 to 4 days. The culture was continued up to 8 passages with 5% fetal bovine serum and antibiotic. Immunocytochemical characterization of isolated VSMCs was performed on primary cultures by use of specific antibodies against smooth muscle specific α-actin (Dako Ltd).

## Measurement of $[Ca^{2+}]_i$ in VSMCs

Measurement of $[Ca^{2+}]_i$ was performed with nonconfluent cultures of VSMCs grown on 12 mm round coverslips that were rendered quiescent by deprivation of serum for 48 hours. Afterward, each coverslip was incubated in a humidified incubator for 45 minutes with fura-2-AM, 4 μmol/L dissolved in DMSO with 0.02% pluronic acid. Unincorporated fura-2 was removed by giving each coverslip two 15-minute washes in modified Hanks' buffer (mmol/L: 137 NaCl, 4.2 $NaHCO_3$, 3 $Na_2HPO_4$, 5.4 KCl, 0.4 $KH_2PO_4$, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 10 glucose, and 5 HEPES, pH 7.4). Coverslips with fura-2 loaded VSMCs were placed in a perfusion chamber with 200 μL of modified Hanks' buffer in a thermostatically controlled stage heater on top of the stage of an inverted microscope equipped for epifluorescence with a ×40 N.A. 1.3 oil immersion objective (Diaphot, Nikon) and attached to an image analysis system (Magiscal Applied Imaging). Excitation light for fluorescence was provided by a 100-watt Xenon lamp, with double and alternating excitatory wavelengths (340 and 380 nm) and a single emission wavelength (510 nm). On binding $Ca^{2+}$, fura-2 exhibits an absorption shift from 340 nm to 380 nm that can be monitored by measuring emission at 510 nm. The $[Ca^{2+}]_i$ was calculated as previously described by Grynkiewicz et al.[20]

## Study of $[Ca^{2+}]_i$ Responses

The effects of various concentrations of Ang II ($10^{-10}$ to $10^{-6}$ mol/L) were examined in 4 to 6 experiments per concentration. After a 10 minute period to equilibrate the preparation, 40 μL of the appropriate Ang II concentration was added to the buffer. Basal and Ang II–stimulated $[Ca^{2+}]_i$ were measured in single cells. To avoid possible desensitization, repetitive determinations were not made. The effects of torasemide, furosemide, and irbesartan on Ang II–evoked $[Ca^{2+}]_i$ responses were also determined. For these experiments, the cells were preincubated for 10 minutes with each drug at 3 concentrations ($10^{-10}$, $10^{-8}$, $10^{-6}$ mol/L). Then, Ang II was added, and the $[Ca^{2+}]_i$ transients were measured. To test their effects in vitro, all drugs were weighed and dissolved in water or in DMSO on the day of the experiment.

## Statistical Analysis

Values are given as mean±SEM. Statistical differences between mean values were determined with 1-way ANOVA followed by Student-Newman-Keuls test. When the number of data was less than 10, the statistical analysis was performed with Mann-Whitney *U* test. A $P$ value $<0.05$ was considered statistically significant. Concentration-response curves were fitted by nonlinear regression, and a concentration giving 50% of the maximal response ($EC_{50}$) was determined and $pD_2$ calculated as $-\text{Log } EC_{50}$ (mol).

# Results

## Responses to Ang II in Endothelium-Denuded Aortic Rings

Ang II evoked concentration-dependent increases in vascular tone in aortic rings from SHR (Figure 1a). The calculated $pD_2$ value was 7.5±0.3. On the basis of these concentration-response curves, a concentration of $10^{-7}$ mol/L was chosen to carry out the next experiments, because this concentration is critically located on the curve. Different levels of maximal tension were obtained either by stepwise cumulative addition of Ang II or by the addition of a single concentration of Ang

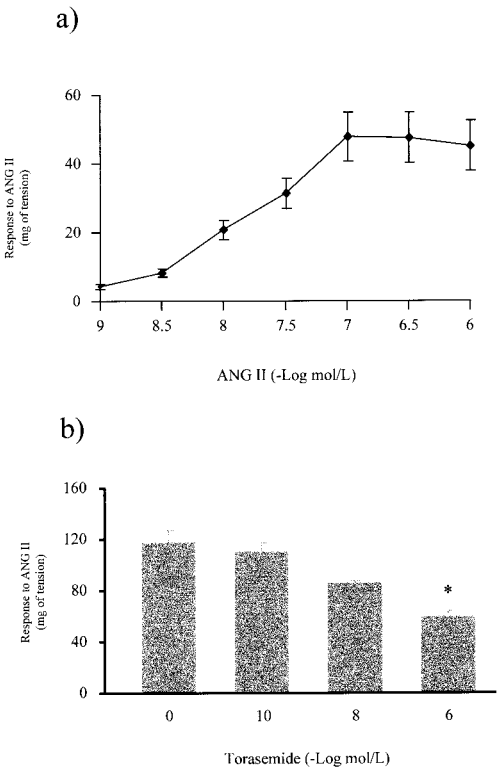


**Figure 1.** a, Dose-response curve to Ang II–induced contractile response in endothelium-denuded aortic rings from SHR. Contractile responses are expressed as Ang II–induced increase of wall tension. Values are mean±SEM from at least 10 determinations per concentration. b, Bar graph shows the Ang II ($10^{-7}$ mol/L)–induced changes in tension after the 30-minute incubation of endothelium-denuded aortic rings from SHR with different concentrations of torasemide. Mean±SEM from 4 to 6 preparations. *$P<0.01$ vs control response to Ang II.

II. These data suggest that there was tachyphylaxis to Ang II responses during the cumulative concentration-response curve in this tissue.

## Effects of Torasemide on Ang II–Induced Responses in Endothelium-Denuded Aortic Rings

The ability of torasemide to induce relaxation of norepinephrine-precontracted rings was tested. The percentage of relaxation (40%) induced by torasemide in rings from SHR was observed only at a high concentration ($10^{-4}$ mol/L); it was considered to be a nonspecific response and probably caused by the solvent, as previously suggested by others.[21]

Torasemide inhibited, in a dose-dependent way, the vasoconstriction induced by Ang II ($10^{-7}$ mol/L) in aortic rings from SHR, $IC_{50}=0.5\pm0.04$ $\mu$mol/L (Figure 1b). The inhibition of Ang II–induced contractile response almost reached 50% at $10^{-6}$ mol/L torasemide (Table). As Figure 2 shows, a single concentration of Ang II ($10^{-7}$ mol/L) caused a prompt and brief contraction of the aortic rings from SHR with a maximum value of 116.6±10 mg (n=22). This value was reached 2 or 3 minutes after the beginning of incubation with the agonist (Figure 2a). A 30 minute period of incubation with torasemide ($10^{-6}$ mol/L) significantly inhibited the Ang II–induced vasoconstriction (Figure 2b).

**Inhibitory Effects of Different Concentrations of Torasemide, Furosemide and Irbesartan on Ang II-Induced Vascular and Intracellular Responses in SHR**

| Effect | Torasemide | Furosemide | Irbesartan |
|---|---|---|---|
| Inhibition of contraction | | | |
| Concentration, −Log mol/L | | | |
| 10 | 5.8±0.4* | 4.6±3.0* | 72.9±4.5 |
| 8 | 27.1±1.4* | 15.5±2.5* | 85.1±8.4 |
| 6 | 49.5±4.3* | 44.7±7.9* | 95.7±1.2 |
| Inhibition of $[Ca^{2+}]_i$ responses | | | |
| Concentration, −Log mol/L | | | |
| 10 | 41.0±6.9 | 31.2±3.1 | 43.1±5.5 |
| 8 | 65.5±6.6 | 20.9±5.7† | 72.1±2.9 |
| 6 | 77.7±4.7 | 27.5±5.8† | 84.7±2.4 |

Results are expressed as percentage of inhibition (%) of the response to Ang II ($10^{-7}$ mol/L) in control conditions. Values are mean±SEM from 4 to 6 preparations. *$P<0.05$ vs irbesartan; †$P<0.05$ vs irbesartan and torasemide.

## Basal and Ang II–Induced $[Ca^{2+}]_i$ Responses in VSMCs

Figure 3a shows the concentration-response curves to Ang II in VSMCs from adult SHR. The basal $[Ca^{2+}]_i$ was determined in 139 cells (83±6 nmol/L). These data are the average of all cells studied in the presence of 1.3 mmol/L $Ca^{2+}$ in the bathing medium. Ang II increased the $[Ca^{2+}]_i$ in VSMCs from SHR in a dose-dependent way (Figure 3a). The calculated $pD_2$ value was 7.5±0.3. A concentration of $10^{-7}$ mol/L of Ang II was chosen to carry out the next experiments, because it induced a submaximal effect (663±69 nmol/L, n=10). Both the resting $[Ca^{2+}]_i$ values and the Ang II–induced increase in $[Ca^{2+}]_i$ obtained here are in agreement with those reported for VSMCs from SHR.[19,22]

## Effects of Torasemide on Ang II–Induced $[Ca^{2+}]_i$ Responses in VSMCs

Torasemide inhibited the Ang II–induced $[Ca^{2+}]_i$ stimulation in a dose-dependent way, $IC_{50}=0.04\pm0.01$ nmol/L (Figure 3b). The inhibitory effect was nearly 80% at $10^{-6}$ mol/L torasemide (Table) and was independent of the cell passage. The variations of $[Ca^{2+}]_i$ are shown as changes on a color scale (Figure 4). In basal conditions, cells are seen in blue. Upon stimulation with Ang II, a rapid rise in $[Ca^{2+}]_i$ occurred that reached its maximum in 10 seconds (yellow-red color), declined rapidly within 30 to 40 seconds, and then gradually decreased in the next 1 to 2 minutes to near resting values (Figure 5). VSMCs from SHR incubated with $10^{-6}$ mol/L of torasemide did not show the response of $[Ca^{2+}]_i$ to Ang II $10^{-7}$ mol/L (Figure 4b).

## Effects of Furosemide and Irbesartan on Ang II–Induced Vascular Responses

Furosemide and irbesartan inhibited, in a dose-dependent way, the vasoconstriction induced by Ang II ($10^{-7}$ M) in aortic rings from SHR (Table). Whereas irbesartan inhibited the Ang II–induced $[Ca^{2+}]_i$ stimulation in a dose-dependent way, furosemide did not exert any effect on this parameter (Table).

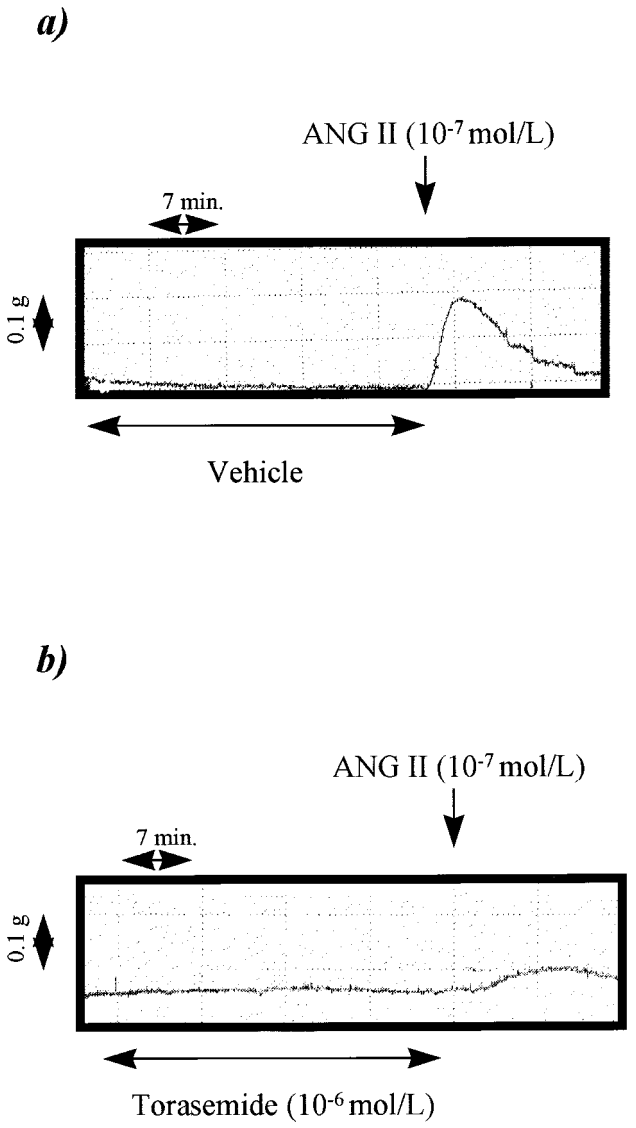


**Figure 2.** The recording shows the contractile response induced by Ang II ($10^{-7}$ mol/L) in endothelium-denuded aortic rings from SHR. Recordings were obtained after 30 minutes incubation with vehicle (a) or torasemide $10^{-6}$ mol/L (b). Each recording shows tension variation through time.

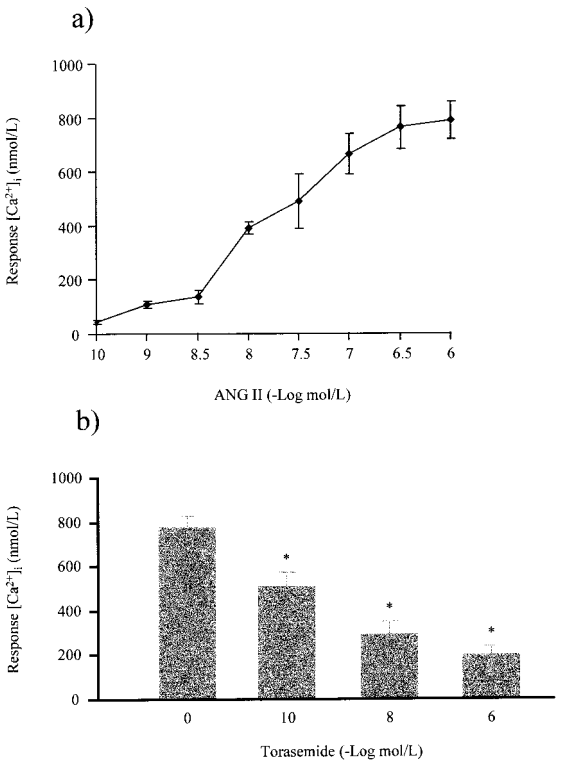


**Figure 3.** a, Dose-response curve to Ang II–induced increase in $[Ca^{2+}]_i$ in VSMCs from SHR. Responses are expressed as Ang II–induced increase of $[Ca^{2+}]_i$. Values are mean±SEM from at least 10 determinations per concentration. b, Bar graph shows the Ang II ($10^{-7}$ mol/L)–induced changes in $[Ca^{2+}]_i$ after 30-minute incubation of VSMCs from SHR with different concentrations of torasemide. Mean±SEM from 10 to 15 determinations. *$P<0.01$ vs control response to Ang II.

## Discussion

The main finding of the current study is that pharmacological concentrations of torasemide inhibit Ang II–induced fast contraction in endothelium-denuded aortic rings from SHR. Furthermore, we did observe that pharmacological concentrations of torasemide block Ang II–induced rapid increase in $[Ca^{2+}]_i$ in cultured VSMCs of the aorta from SHR.

Central to the direct vasoconstrictor action of Ang II on smooth muscle is its capacity to increase $[Ca^{2+}]_i$. Following the interaction with the Ang II type 1 receptor ($AT_1$), Ang II mobilizes $Ca^{2+}$ by releasing $Ca^{2+}$ sequestered intracellularly and by increasing $Ca^{2+}$ influx from the extracellular compartment. The former event may occur by way of phosphoinositide turnover, and the latter may occur in association with voltage-sensitive $Ca^{2+}$ channels.[23,24] As a consequence, $[Ca^{2+}]_i$ increases and contraction in VSMCs ensues. The occurrence of this sequence of events is confirmed by our results with the $AT_1$ antagonist irbesartan. Because of its physicochemical properties, it is unlikely that torasemide directly antagonizes the binding of Ang II to the $AT_1$ in VSMCs.[25] Therefore, other mechanisms may be influenced by the drug.

Sodium and other ions influence Ang II binding and, therefore, modulate hormone-receptor signaling.[26] The $Na^+$, $2Cl^-$, $K^+$ cotransport system is but one of several means by which $Na^+$ transport regulates Ang II–receptor interaction. An $Na^+/H^+$ antiporter, an $Na^+/Ca^+$ antiporter, and an $Na^+$ channel have also been described.[27,28] Torasemide might inhibit the $Na^+$, $2Cl^-$, $K^+$ cotransport system in VSMCs as it does in tubular cells.[29,30] Thus, the blockade of this cotransporter would reduce the cytosolic $Na^+$ activity, and this, in turn, would interfere with Ang II–$AT_1$ signaling in VSMCs. This is further supported by our observation that another inhibitor of the cotransporter, furosemide, also interferes with the vasoconstrictor activity of Ang II. Interestingly, the activity of the $Na^+$, $2Cl^-$, $K^+$ cotransport system has been reported to be abnormally increased in VSMCs[31] and erythrocytes[32] from SHR. Furthermore, it has been found that torasemide derivatives, which inhibit the activity of the $Na^+$, $2Cl^-$, $K^+$ cotransport system, prevent the development of hypertension in SHR.[33] Whether the cotransporter abnormality is also present in VSMCs from hypertensive patients, as it is in erythrocytes from them,[34] and whether this can be

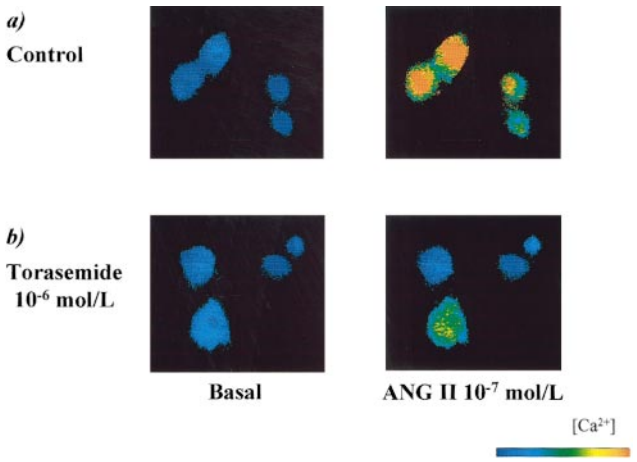


**Figure 4.** Temporal changes of fura-2–associated fluorescence intensity in the individual VSMC was automatically calculated by the digital imaging processor. The fluorescence changes were clearly visualized by the camera. The 340/380 nm fura-2–associated fluorescence ratio on a pixel by pixel basis was calculated and used as $[Ca^{2+}]_i$ measure. The recordings were obtained after 10-minute incubation of VSMCs from SHR with vehicle (a) or torasemide $10^{-6}$ mol/L (b). The left panels show the cells in basal conditions and the right panels show the Ang II ($10^{-7}$ mol/L)–stimulated cells.

corrected by torasemide and other loop diuretics in vivo deserve further investigation.

Unlike furosemide and other loop diuretics, the chemical structure of torasemide is similar to that of the $Cl^-$ channel blockers. It has recently been described that Ang II stimulates the opening of $Cl^-$ channels in VSMCs resulting in a $Cl^-$ efflux and consequent membrane depolarization.[16] This process would drive the opening of voltage-gated $Ca^{2+}$ channels, $Ca^{2+}$ influx, and vasoconstriction.[35] It has been shown that torasemide inhibits $Cl^-$ conductance in epithelial cells.[10] Interestingly, sulfonic derivatives have been shown to block $Cl^-$ channels in VSMCs from the rat aorta.[36] Our observation that torasemide inhibits Ang II–induced $[Ca^{2+}]_i$ increase in VSMCs more potently than does furosemide suggests that the

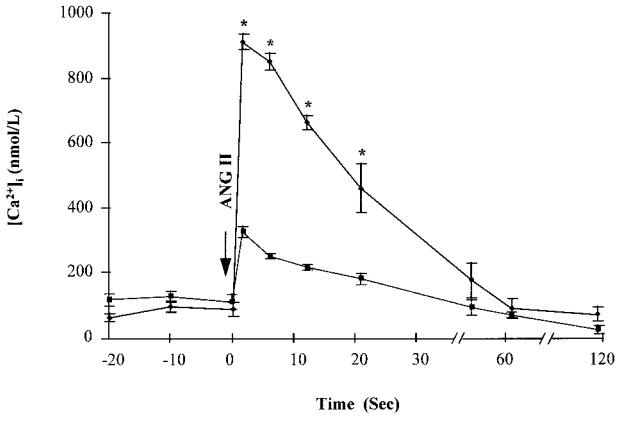


**Figure 5.** Representative line graph shows the differences in the time course of Ang II–induced $[Ca^{2+}]_i$ increase in the absence (upper curve) and presence (lower curve) of $10^{-6}$ mol/L torasemide. Maximal stimulated $[Ca^{2+}]_i$ was taken at 1 to 3 seconds and recovery time to basal was measured thereafter. Mean±SEM from 10 to 15 determinations per concentration. *$P<0.01$ vs curve obtained in presence of torasemide.

blockade of $Cl^-$ channels may be an additional mechanism involved in the ability of torasemide to interfere with the vasoactive actions of Ang II.

Results presented here indicate that torasemide alone is not able to induce relaxation of precontracted endothelium-denuded aortic rings from SHR. Because the endothelium was removed in our preparation, this finding does not exclude the possibility that torasemide exerts a direct vasorelaxation effect on the intact wall. This is supported by previous experiments which show that torasemide increased the content of cGMP in VSMCs of the aorta from renal hypertensive rats.[37] In addition, torasemide has been shown to enhance prostacyclin secretion in primary cultured human endothelial cells.[38]

In summary, the results of the present study presented here indicate that torasemide inhibits Ang II–induced contraction of aortic strips from SHR. Our findings also suggest that torasemide interferes with Ang II–dependent mechanisms which lead to the stimulation of $[Ca^{2+}]_i$ in VSMCs. Further experiments are necessary to make the roles of $Cl^-$ channels and/or the $Na^+$, $2Cl^-$, $K^+$ cotransporter in these vascular actions of torasemide more precise. The in vitro data presented here may add further information to the in vivo antihypertensive effects of nonnatriuretic dosages of torasemide.

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