Angiotensin AT1B Receptor Mediates Calcium Signaling in Vascular Smooth Muscle Cells of AT1A Receptor–Deficient Mice

Zhiming Zhu, Sunny H. Zhang, Charlotte Wagner, Armin Kurtz, Nobuyo Maeda, Thomas Coffman, William J. Arendshorst

Abstract—Our studies on angiotensin II receptor subtype 1A (AT1A) knockout mice define how endogenous receptors other than AT1A receptors stimulate changes in cytosolic calcium concentration ([Ca2+]i) in cultured aortic vascular smooth muscle cells (VSMCs). Wild-type cells have a 1.7 ratio of AT1A/AT1B receptor mRNA as determined by semiquantitative reverse transcriptase–polymerase chain reaction. Mutant cells express AT1B receptor mRNA but not that for the AT1A receptor. In wild-type cells with AT1A present, Ang II (10⁻⁷ mol/L) produces a characteristic rapid peak increase in [Ca2+]i of 150 to 180 nmol/L, followed by a plateau phase characterized by a sustained 70 to 80 nmol/L increase in [Ca2+]i. An unexpected finding was that the magnitude and time-dependent pattern of [Ca2+]i changes produced by Ang II were similar in cells that lacked AT1A receptors but possessed AT1B receptors. The response in mutant cells indicates effective coupling of an Ang II receptor to one or more second messenger systems. The similarity of response patterns between cells with and without AT1A receptors suggests that non-AT1A receptors are functionally linked to similar signal transduction pathways in mutant cells. The fact that mutant and wild-type cells exhibit similar patterns of calcium mobilization and entry supports the notion that AT1A and non-AT1A receptors share common signal transduction pathways. The AT2 receptor ligands PD-123319 and CGP-42112 do not alter Ang II effects in either VSMC type, suggesting a paucity of AT2 receptors and/or an absence of their linkage to [Ca2+]i pathways. The nonpeptide AT1 receptor blocker losartan antagonizes Ang II–induced [Ca2+]i increases in both cell groups, supporting mediation by native AT1B receptors and effective coupling of this subtype to second messenger systems leading to calcium entry and mobilization. Our results demonstrate that Ang II causes calcium signaling in AT1A−deficient VSMCs that is mediated by an endogenous losartan-sensitive AT1B receptor. (Hypertension. 1998;31:1171-1177.)

Key Words: muscle, smooth, vascular • angiotensin II • receptors, angiotensin • calcium channels • antagonists • losartan • fura 2

Angiotensin II, a potent hormone or paracrine substance, stimulates cell surface receptors to produce a variety of regulatory actions in the cardiovascular, renal, endocrine, and neural systems.1–3 The multiple actions of Ang II are mediated by different receptors and various combinations of cell-specific signaling mechanisms. Based on pharmacological criteria, Ang II exerts its actions via two classes of receptors. Radioligand receptor-binding studies using nonpeptide ligands such as losartan and CGP-42112 have defined two distinct types, with losartan having high affinity for AT1 receptors, whereas CGP-42112 is a selective ligand for the AT2 receptor.1,3 AT1 receptors can be subdivided into AT1A and AT1B subtypes, which differ in distribution and regulation among tissues and cell types.1,3 The AT1A receptor is the major subtype in the cardiovascular and renal systems.4,5 The degree to which the biological effects of Ang II are mediated by the predominant and minority receptor subtype in each cell awaits clarification. Although the current generation of nonpeptide receptor ligands allows differentiation between the major classes of AT1 and AT2 receptors, it has not been possible to discriminate between AT1A and AT1B subtypes.2,3,6–7 Thus, it is not known whether AT1A and AT1B receptors are functionally different or similar in natural cells; also unknown are the functional consequences of different relative densities of these receptors in multiple cell types.

Mouse models in which a single receptor is completely eliminated by gene targeting provide an alternative approach to investigate receptor regulation and function.8 Coffman, Smithies, Sugaya, and associates (Ito et al,9 Sugaya et al10) have developed and used such a model to investigate ligand binding and functional characterization of endogenous angiotensin receptors other than AT1A in the AT1A knockout mouse.

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Autoradiographic characterization of receptors reveals a markedly reduced density of losartan-sensitive \[^{[125]}\text{I}\text{Ang II}\] binding in the kidneys of AT\textsubscript{1A} mutant mice. Furthermore, the importance of AT\textsubscript{1A} receptors in blood pressure control is suggested by the reduction in basal arterial pressure, attenuated acute pressor responses to administered Ang II, and increased plasma renin activity.\(^{5,10}\) Thus, Ang II appears to exert most, if not all, of its effects on the cardiovascular and renal systems via the AT\textsubscript{1A} receptor, the predominant subtype in these systems. Little is known about the function of the native AT\textsubscript{1B} receptor in cells that normally possess this receptor subtype. AT\textsubscript{1B} receptors are assumed to have a minor influence on the smooth muscle function and the regulation of arterial pressure. The mouse model deficient in AT\textsubscript{1A} receptors affords a unique opportunity to investigate Ang II effects mediated by the remaining receptor subtypes, including the AT\textsubscript{1B}.

The present studies were conducted on VSMCs obtained from wild-type control mice and mutant mice with absent AT\textsubscript{1A} receptor. We investigated the ability of Ang II to stimulate \([\text{Ca}^{2+}]\text{i}\), in cultured aortic VSMCs. A major finding was that Ang II produced changes in \([\text{Ca}^{2+}]\text{i}\), in VSMCs lacking the AT\textsubscript{1A} receptor that were similar in magnitude and overall pattern to changes in cells with AT\textsubscript{1A} receptor present. Thus, Ang II stimulated signal transduction mechanisms by a native receptor(s) other than AT\textsubscript{1A} in mutant cells. Losartan (DuP 753) effectively antagonized the Ang II–induced increase in \([\text{Ca}^{2+}]\text{i}\), reflecting mediation by AT\textsubscript{1B} receptors in control cells and AT\textsubscript{1B} receptors in VSMCs lacking AT\textsubscript{1A} receptors. Possible mediation by AT\textsubscript{2} receptors was eliminated by observations that AT\textsubscript{2} receptor ligands had no effect on Ang II–induced changes in \([\text{Ca}^{2+}]\text{i}\), in VSMCs with or without AT\textsubscript{1A} receptors. Our studies provide new information about the mediation of calcium stimulation by Ang II acting on endogenous AT\textsubscript{1B} receptors in mouse aortic VSMCs.

**Methods**

**Culture of Aortic VSMCs**

Four-month-old adult AT\textsubscript{1A} homozygous knockout mice with C57B/6 and 129 mixed genetic background were used; age-matched wild type mice with C57B/6 and 129 F1 backgrounds served as controls.\(^4\) Mice were anesthetized with Avertin, and the thoracic and abdominal aorta was isolated, extirpated, and cut longitudinally. The internal surface was gently scraped with watchmaker forceps to remove endothelial cells, and the adventitia was removed by stripping with the aid of microscopy and forceps. Aortic VSMCs were cultured by an explant method using standard methods.\(^11\) Briefly, the aortic media was cut into 1- to 2-mm pieces and put into 24-well plates with 100 to 200 \(\mu\)L DMEM-H medium (Gibco BRL) containing 10% fetal calf serum (HyClone), 100 U/mL penicillin, 100 \(\mu\)g/mL streptomycin, 25 \(\mu\)g/mL amphotericin B, and 200 mg/mL 1-glutamine incubated at 37°C, in a humidified 5% CO\textsubscript{2}/95% air incubator. About 0.5 mL of fresh medium was gently added 3 days later to each well. Cells formed a confluent monolayer in 10 to 14 days. The medium was changed twice weekly. The growth rates of wild-type and AT\textsubscript{1A}-deficient cells did not differ appreciably, suggesting that the AT\textsubscript{1A} receptor was not essential for growth. Immunocytochemistry was used to verify the presence of smooth muscle–specific \(\alpha\)-actin using a monoclonal antibody (Clone 1A4; 1:200 dilution, Dako Corp) and to verify the absence of endothelial cell contamination using von Willebrand factor (Dako, 1:50 dilution) as previously described.\(^{12}\) Reactions of the subcultured VSMCs with these antibodies revealed that all cells contained smooth muscle–specific \(\alpha\)-actin with an abundance of filaments. No endothelial cell contamination was evident.

**Southern Blot Analysis**

To determine cell genotypes, genomic DNA was purified from cultured VSMCs with and without AT\textsubscript{1A} receptor gene mutation and analyzed by Southern blot analysis.\(^7\) After digestion of DNA with HindIII, size separation in a 0.8% agarose gel, and transfer to nylon membrane, previously described probes were used to identify generation of 3.3-kb wild-type and 5.0-kb mutant fragments.

**RT-PCR for AT\textsubscript{1A} and AT\textsubscript{1B} mRNA**

AT\textsubscript{1A} and AT\textsubscript{1B} mRNA were determined by RT-PCR, performed as described previously.\(^11\) Briefly, 2 \(\mu\)g of total RNA was reverse transcribed with an RT mixture consisting of oligo dT (12 to 18) and 200 U of M-MLV RT (Gibco BRL). After reverse transcription, a small aliquot of RT mixture was used for PCR with sense primer (5\'-CCCAAAAGT CACCCTGCACTC-3'\) and antisense primer (5\'-CACCAATGCC CTAATATTCC-3') , which are common for both AT\textsubscript{1A} and AT\textsubscript{1B} receptors.\(^7\) The PCR reaction was carried out in a total volume of 20 \(\mu\)L containing 3 \(\mu\)L RT mixture, 1 \(\mu\)L of each primer (10 pmol \(\cdot\) L\(^{-1}\) \(\cdot\) \(\mu\)L\(^{-1}\)), 5 \(\mu\)L MgCl\textsubscript{2} (25 mmol/L), 2.5 \(\mu\)L 10× PCR buffer, 1 \(\mu\)L dNTP (925 mmol/L), 3 \(\mu\)Ci \(\beta\)-ACTP (64 Ci \(\mu\)mol/L), and 1 \(\mu\)L of the RT mixture (described with an RT mixture consisting of oligo dT (12 to 18) and 200 U) Taq polymerase (Boehringer Mannheim). To distinguish between AT\textsubscript{1A} and AT\textsubscript{1B} receptors, amplification products (1.5 \(\mu\)L EcoRI, 25 \(\mu\)L/mL) were added to 20 \(\mu\)L of the PCR product obtained with the AT\textsubscript{1A} primers. The digestion yielded in fragments of the expected sizes of 128 and 177 bp, as has been visualized with agarose gel electrophoresis and ethidium bromide staining. For quantitative analysis, the PCR products were separated by polyacrylamide gel electrophoresis. \(N\textsubscript{N}\textsubscript{'-methylene-bis-acrylamide} was replaced by dihydroxyethylene-bis-acrylamide. The bands were excised and solubilized in 0.025 mol/L periodic acid, and radioactivity was measured in a liquid scintillation spectrophotometer.

**Measurement of \([\text{Ca}^{2+}]\text{i}\)**

Measurements of \([\text{Ca}^{2+}]\text{i}\) in cultured VSMCs were performed using the calcium-sensitive dye fura 2-AM as previously described.\(^{12,14}\) A monolayer of VSMCs was grown on 22-mm\(^2\) glass coverslips as described above. Confluent cells were rendered quiescent by maintenance in a serum-free medium for 24 hours before an experiment. Calcium determinations were performed on subcultures between the second and sixth passages. On the day of study, the VSMCs were washed twice in physiological salt solution (PSS, in mmol/L: 135 NaCl, 5 KCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 5 1-glucose, 10 HEPES, pH 7.4) and incubated with 4 \(\mu\)mol/L fura 2-AM in 0.02% pluronic F-127 (Molecular Probes Inc) for 60 minutes at room temperature. After fura 2 loading, monolayers were washed twice in PSS, and the cells were centered in the optical field of a \(\times 40\) oil immersion fluorescence objective of an inverted microscope (Olympus IMT-2). The cells were excited alternately with light of 340- and 380-nm wavelength from dual monochromators of a Photon Technology International (PTI) dual-excitation wavelength Deltascan (model RMD). Fluorescence was detected with a photon-counting device after passing through a dichroic mirrored barrier filter (510 nm). Fluorescence signal intensity of 20 to 30 cells was acquired, stored, and processed by an IBM-PC–compatible computer and Felix software (PTI), with calibration of \([\text{Ca}^{2+}]\text{i}\) based on the ratio at 340/380 nm. The \([\text{Ca}^{2+}]\text{i}\) was calculated according to the formula

\[
[N_{\text{Ca}^{2+}}] = \frac{[N_{\text{Ca}^{2+}}]}{[N_{\text{Ca}^{2+}}] + [N_{\text{Ca}^{2+}}]} \times \frac{[N_{\text{Ca}^{2+}}]}{[N_{\text{Ca}^{2+}}] + [N_{\text{Ca}^{2+}}]}
\]
Results

Statistical Methods

Results

Cell genotypes were confirmed by Southern blot analysis of cultured VSMCs. Fig 1 shows a 5.0-kb band for cells lacking the AT₁A receptor, which contrasts with the 3.3-kb band present in wild-type control cells. AT₁ receptor subtype mRNA was analyzed using RT-PCR methodology. The results in Fig 2 demonstrate the presence of the AT₁B receptor mRNA in both groups of VSMCs, whereas the AT₁A mRNA was absent in the gene-knockout mice. The ratio of mRNA for AT₁A/AT₁B receptors was 1.7 in wild-type mice. A relative ratio does not apply to cells from knockout mice with only AT₁B mRNA present. Light microscopy showed no discernible differences between the general appearance of subcultured VSMCs derived from control and mutant mice.

Functional activity of endogenous Ang II receptors was evaluated by signal transduction in aortic VSMCs. Fura 2 fluorescence was used to determine the ability of different concentrations of Ang II to produce a rapid change in [Ca²⁺]. Before Ang II addition, the basal [Ca²⁺], was greater in AT₁A mutant cells than in wild-type control cells: 96±2 (n=74) versus 74±2 (n=68) nmol/L, P<.001. The reason for this difference is not known, but it may reflect changes in the relative importance of various calcium entry versus mobilization pathways under basal conditions. As noted below, the resting control values persist when the external medium is rendered calcium free for a brief period to minimize calcium entry and suggest involvement of intracellular sources.

Stimulation of VSMCs with Ang II caused concentration-dependent increases in [Ca²⁺]. After the basal [Ca²⁺], was recorded for 50 seconds, Ang II was added to maintain stimulation for the duration of a recording period of 250 seconds. A peak [Ca²⁺], increase was observed within the initial 30 seconds following addition of Ang II. Fig 3 presents the summarized data for control and mutant cells. A major finding was that the maximum [Ca²⁺], response was as large in mutant cells as it was in wild-type cells studied under identical conditions. Each preparation was challenged only once with a given concentration of Ang II to eliminate uncertainty that may result from receptor desensitization and increased variability with repeated applications.

The time-dependent changes in [Ca²⁺], showed a characteristic peak response immediately after Ang II addition, followed by a decline that plateaued at a sustained level for

**Figure 1.** Genotype analysis of purified genomic DNA from cultured aortic VSMCs from mice with and without AT₁A receptor gene mutation assayed by Southern blot analysis. After HindIII restriction enzyme digestion, the wild-type allele was detected as a 3.3-kb fragment, and the disrupted AT₁A allele appeared as a 5.0-kb fragment.

**Figure 2.** Determination of AT₁A and AT₁B mRNA expression in VSMCs with and without AT₁A gene using PT-PCR. Ethidium bromide-stained DNA is shown. This was generated from a total of 2 µg RNA derived from wild-type and AT₁A knockout mice. The PCR was run for 35 cycles with use of AT₁A- and AT₁B-specific primers as described in “Methods.” Lanes 1 and 2 show the expression of the AT₁A and AT₁B mRNA in wild-type cells; lanes 3 and 4 show the expression of the AT₁B mRNA in AT₁A knockout cells; lanes 4 and 6 are internal standard for wild-type cells; and lanes 7 and 8 are internal standard for AT₁A knockout cells.

**Figure 3.** Dose-dependent increases in [Ca²⁺] produced by Ang II (10⁻¹³ to 10⁻⁰ mol/L). Each mean represents 5 to 30 measurements. The changes in [Ca²⁺] were similar in AT₁A knockout and wild-type control VSMCs.
the duration of the recording period. The averaged responses to Ang II (10^{-7} mol/L) in all cell preparations are presented in Fig 4. Interestingly, the maximum change in [Ca^{2+}]_{i} did not differ between mutant and control cells [180±24 (30) versus 155±28 (20) nmol/L, \( P > .1 \)]. Likewise, the steady-state increases recorded at 200 seconds were similar in both groups of cells [70±9 (30) in mutant versus 79±11 (20) nmol/L in control cells, \( P > .2 \)]. The plateau levels averaged 36±21% and 57±6% of the maximum response \( (P > .1) \). The general shape of the calcium response is similar to that previously reported for an Ang II effect on rat aortic VSMCs.\(^{12} \)

To determine the contribution of calcium entry versus mobilization from internal stores after activation of native AT_{1} receptor subtypes in VSMCs, the [Ca^{2+}]_{i} response was evaluated in a calcium-free medium, achieved by adding 5 mmol/L EGTA to an otherwise normal solution containing 1 mmol/L CaCl\(_{2}\) at 10 seconds before starting a recording. Preliminary studies showed that short-term exposure to EGTA had no effect on basal [Ca^{2+}]_{i}. Calcium responses to Ang II were strongly dependent on calcium entry in both control and AT_{1A} mutant cells (Fig 5). In the calcium-free medium, stimulation by Ang II (10^{-7} mol/L) produced smaller changes in [Ca^{2+}]_{i}, with attenuation of both the peak and plateau phases. Ang II produced a peak increase in [Ca^{2+}]_{i} of 103±29 (7) nmol/L in cells with only AT_{1B} receptors compared with a 82±25 (10)–nmol/L increase in control cells with both AT_{1} receptors. These peak responses in calcium-free medium (line with circles) were 57±16% and 53±16% of the changes observed in mutant and control cells, respectively, compared with responses when calcium was present in the bathing medium (line with triangles). In addition, the sustained plateau phase was abolished as the initial calcium transient returned to baseline levels. This observation indicates that calcium entry is responsible for approximately one half of the peak response and almost all of the sustained increase in [Ca^{2+}]_{i}, that follows stimulation of AT_{1} receptors. On the other hand, about 50% of the initial [Ca^{2+}]_{i} response was independent of external calcium, suggesting a major contribution of calcium mobilization.

In other studies we evaluated the effect of an AT_{1} receptor antagonist on Ang II–induced [Ca^{2+}]_{i} responses. In control cells (Fig 6, left panel), losartan (10^{-5} or 10^{-7} mol/L) markedly antagonized the response to Ang II (10^{-7} mol/L). The degree of inhibition was about 80%. An important observation was that losartan likewise caused marked inhibition of Ang II–induced changes in [Ca^{2+}]_{i}, in mutant cells lacking AT_{1A} receptors. Furthermore, we demonstrated that the AT_{1} receptor is either absent or nonfunctional in calcium signaling in mouse VSMCs. The AT_{1} receptor ligand CGP-42112 had no discernible effect on the ability of Ang II to elicit [Ca^{2+}]_{i} increases in cells with the AT_{1A} receptor present or absent [84±17% (6) versus 91±22% (13) of Ang II effect, respectively]. Likewise, another AT_{1} receptor ligand, PD-123319, failed to influence the ability of Ang II to stimulate [Ca^{2+}]_{i}; in the presence of the PD compound, Ang II elicited a normal response averaging 93±33% (7) in control and 94±31% (7) in mutant cells.

**Discussion**

The present study provides new information about the ability of Ang II to produce rapid changes in [Ca^{2+}]_{i}, via stimulation of endogenous Ang II receptors in cultured mouse aortic VSMCs. In control cells obtained from wild-type mice, Ang II initiates a calcium response by acting on receptors inhibited by the AT_{1} receptor antagonist losartan. In contrast, the AT_{2} receptor agents PD-123319 and CGP-42112 have no discern-
able effect on either cytosolic calcium before addition of Ang II or the calcium response to Ang II. Our observations agree with previous reports that these agents act as specific antagonists without any partial agonist effects in rat VSMCs. Earlier studies on rat aortic VSMCs establish that losartan effectively and almost completely attenuates the \([Ca^{2+}]_i\) response to Ang II\(^{14,17–19}\).

Although cardiovascular and renal cells in rodents express both AT\(_{1A}\) and AT\(_{1B}\) receptors, the AT\(_{1A}\) subtype predominates. AT\(_2\) receptors are prevalent in young animals but sparse in adult animals.\(^1–3\) We observed a ratio of 1.7 for mRNA for AT\(_{1A}/AT_{1B}\) receptors in VSMCs of wild-type mice by RT-PCR method. This value does not differ appreciably from previous reports for AT\(_{1A}/AT_{1B}\) mRNA ratios of 1 to 2 for rat aortic VSMCs.\(^4,5\) By means of gene targeting, VSMCs of mutant mice lack the native AT\(_{1A}\) receptor. The absence of mRNA for this receptor is confirmed by RT-PCR. Semiquantitative RT-PCR suggests that AT\(_{1B}\) receptor expression is upregulated in the absence of AT\(_{1A}\) receptors.

Our results demonstrate that Ang II produces increases in \([Ca^{2+}]_i\) in mouse VSMCs by stimulating two basic signal transduction pathways. About one half of the calcium response is mediated by calcium entry across the plasma membrane, as evidenced by attenuated responses to Ang II when calcium entry is prevented by short-term EGTA addition to the medium. The fact that roughly one half of the \([Ca^{2+}]_i\) change persists in a nominally calcium-free medium implicates a second major hormone-responsive calcium-regulating site involving calcium mobilization from intracellular reserves. These results contrast with previous studies on cultured rat aortic VSMCs that generally point to a predominant, if not exclusive, role for calcium mobilization.\(^19,20\) Recent evidence, however, supports calcium entry as a more important mechanism in renal resistance vessels examined in isolation and in vivo.\(^19–21\)

The present findings provide new evidence that a losartan-sensitive Ang II receptor can elicit rather normal changes in \([Ca^{2+}]_i\) in mouse VSMCs by stimulating two basic signal transduction pathways. About one half of the calcium response is mediated by calcium entry across the plasma membrane, as evidenced by attenuated responses to Ang II when calcium entry is prevented by short-term EGTA addition to the medium. The fact that roughly one half of the \([Ca^{2+}]_i\) change persists in a nominally calcium-free medium implicates a second major hormone-responsive calcium-regulating site involving calcium mobilization from intracellular reserves. These results contrast with previous studies on cultured rat aortic VSMCs that generally point to a predominant, if not exclusive, role for calcium mobilization.\(^19,20\) Recent evidence, however, supports calcium entry as a more important mechanism in renal resistance vessels examined in isolation and in vivo.\(^19–21\)

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receptor is reported to elicit a weaker and less reproducible response. In some cases, only 14% of the preparations display calcium responses.\textsuperscript{6} AT\textsubscript{1B} receptor stimulation is characterized by a rapid \([\text{Ca}^{2+}]\), increase that subsequently decays to the baseline without a plateau phase.\textsuperscript{6,7} Another apparent AT\textsubscript{1} subtype difference is noted in steady-state dose-response curves. AT\textsubscript{1A} receptors usually trigger a typical sigmoidal-shaped concentration-\([\text{Ca}^{2+}]\) response curve indicative of saturation.\textsuperscript{7,8,9} In contrast, AT\textsubscript{1B} receptor stimulation can produce a biphasic or inverted bell-shaped response, with low Ang II concentrations stimulating \([\text{Ca}^{2+}]\), and high concentrations producing less stimulation, suggesting receptor desensitization.\textsuperscript{7,8,9} The biphasic response to AT\textsubscript{1B} receptor stimulation is reminiscent of the dual stimulatory and inhibitory effects of Ang II on proximal tubular reabsorption.\textsuperscript{7}

While informative, it is doubtful that an inserted foreign receptor couples or interacts with the plasma membrane and all intracellular intermediates in naive host cells in a functional manner identical to those stimulated by an endogenous receptor in its natural effector cells. Studies on natural cells are required to establish whether such information about transfected receptors pertains, and to what extent, to specific cell types that normally possess the functional receptors subject to physiological coupling and control. In this regard, animal models with genetic engineering provide an attractive alternative approach to investigate receptor regulation and function in a wide range of normal target cells.

We provide new evidence that endogenous Ang II receptors other than the AT\textsubscript{1A} can play a critical role in calcium signaling in VSMCs of rodents. A functional role of non-AT\textsubscript{1A} receptors is indicated by the recent observations showing that inhibition of Ang II production by angiotensin-converting enzyme inhibition reduces arterial pressure in AT\textsubscript{1A}-deficient mice and that administered Ang II can elicit an acute pressor response after reducing endogenous levels of Ang II.\textsuperscript{10} This pressor response to Ang II is probably mediated by the AT\textsubscript{1B} receptor as it is inhibited by losartan.\textsuperscript{10} Nevertheless, it is noteworthy that the in vivo pressor response to exogenous Ang II in AT\textsubscript{1A}-deficient mice is considerably attenuated, producing smaller effects than might be predicted based on the relatively normal ability of Ang II to stimulate \([\text{Ca}^{2+}]\), in VSMCs in vitro. This apparent discrepancy may reflect one or more differences. One explanation relates to differences in vessel type between conduit arteries and resistance beds in the integrated arterial pressure and total peripheral vascular resistance responses to Ang II. Another possibility is that in vitro observations in isolated cells do not accurately predict integrated functional responses in vivo. As a corollary, \([\text{Ca}^{2+}]\), changes in cultured aortic VSMCs may not directly translate to contractile function of resistance vessels. Also, we cannot rule out the possibility that cultured aortic VSMCs undergo phenotypic alterations that are expressed in calcium signaling. For example, culturing may induce or magnify calcium coupling of the AT\textsubscript{1B} receptor. However, there is no obvious reason to suspect differential changes in aortic cells cultured from two mouse strains of close genetic background under identical conditions. Further investigations are required to address these issues and provide more insight into extrapolation from changes in \([\text{Ca}^{2+}]\), in aortic VSMCs and cultured arterial or arteriolar VSMCs to functional roles in regulating resistance in specific arteries.

Previous evidence suggests that humans have no AT\textsubscript{1B} receptor. In rodents the AT\textsubscript{1B} may be redundant and share functional properties with the AT\textsubscript{1A} receptor. Our recent studies on VSMCs from renal resistance arterioles demonstrate parallel regulation of AT\textsubscript{1A} and AT\textsubscript{1B} receptors in response to changes in salt intake and activity of the renin-angiotensin system.\textsuperscript{27} On the other hand, several lines of evidence suggest different AT\textsubscript{1} receptor subtype distribution and regulation in rats and mice.\textsuperscript{1,2,6–9,12} In addition, arterial pressure in mice is affected differently by gene targeting of either the AT\textsubscript{1A} or AT\textsubscript{1B} receptor.\textsuperscript{9,10,32} A pathophysiological role is suggested by cosegregation studies that indicate a link between the AT\textsubscript{1B} receptor and models of hypertension.\textsuperscript{33} Another potentially important function of an AT\textsubscript{1B} or undefined receptor may be proliferation and maturation of renal cells in general and in VSMCs in particular.\textsuperscript{14,35} To the extent that this variation reflects physiological differences, humans may have a heretofore unrecognized receptor that shares similarities with the AT\textsubscript{1B}.

In summary, we show that Ang II can activate an endogenous AT\textsubscript{1B} receptor to elicit changes in \([\text{Ca}^{2+}]\), in VSMCs isolated from AT\textsubscript{1A} knockout mice. This Ang II receptor is functionally coupled to at least two calcium mechanisms leading to increased calcium entry and mobilization. The relative contributions of these two pathways appear to be similar in VSMCs with native AT\textsubscript{1A} receptors present or deleted. Losartan blocks Ang II–induced \([\text{Ca}^{2+}]\), increases in mutant VSMCs expressing AT\textsubscript{1B} receptors, as well as wild-type VSMCs having AT\textsubscript{1A} receptors. Thus, endogenous losartan-sensitive AT\textsubscript{1B} as well as AT\textsubscript{1A} receptors can be coupled to pathways leading to calcium entry and calcium mobilization in VSMCs.

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**References**


Angiotensin \( \text{AT}_{1B} \) Receptor Mediates Calcium Signaling in Vascular Smooth Muscle Cells of \( \text{AT}_{1A} \) Receptor−Deficient Mice
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