**AT₂ Receptor and Vascular Smooth Muscle Cell Differentiation in Vascular Development**

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**Abstract**—The angiotensin II type 2 (AT₂) receptor is transiently expressed at late gestation in the fetal vasculature, but its expression rapidly declines after birth. We have previously demonstrated that the expression of this receptor mediates decline in vascular DNA synthesis that occurs at this stage of vascular development. To examine further the role of the AT₂ receptor in vasculogenesis, we have focused on the effect of the AT₂ receptor on vascular smooth muscle cell (VSMC) differentiation. In this study, we examined the time-dependent expression of differentiation markers for VSMCs in the aorta of wild-type and AT₂ receptor–null mice.

- **α-Smooth muscle actin** was expressed at the early stage of differentiation and exhibited unchanged expression before and after the peak of AT₂ receptor expression, which was observed at embryonic day 20, neonatal day 1, and thereafter. No difference in α-smooth muscle actin expression was observed between the wild-type and AT₂ receptor–null mice. In contrast, the mRNA levels for calponin, expressed in the late stage of VSMC differentiation, were significantly higher in the wild-type mouse aorta as compared with the AT₂ receptor–null mice, which correlates with expression of the AT₂ receptor. Moreover, the protein levels of calponin and high-molecular-weight caldesmon (h-caldesmon) showed lower expression in the aorta of AT₂ receptor knockout mice at 2 and 4 weeks after birth. Taken together, our results suggest that the AT₂ receptor promotes vascular differentiation and contributes to vasculogenesis. ([Hypertension. 1999;33:1414-1419.](http://hyper.ahajournals.org/))

**Key Words:** angiotensin || cell differentiation || receptors, angiotensin II || muscle, smooth, vascular || human development

Angiotensin II (Ang II), a key regulator of cardiovascular homeostasis, exerts various actions in its diverse target tissues, that controls vascular tone, hormone secretion, tissue growth, and neuronal activity. Two major isoforms of the Ang II receptor, type 1 (AT₁) and type 2 (AT₂), have been defined on the basis of their ligand selectivity. Most of the known effects of Ang II in the adult tissues are attributable to the AT₁ receptor. The AT₂ receptor is abundantly and widely expressed in fetal tissues, and its expression declines rapidly after birth, which suggests that the AT₂ receptor is involved in growth, development, and/or differentiation.

The expression of the AT₂ receptor in rat fetal blood vessel is “turned on” at late gestation (embryonic days 16 to 21) and in the early neonatal period but decreases rapidly to very low levels in the adult vessel. We have demonstrated that this receptor exerts growth-modulatory effects in vascular smooth muscle cells (VSMCs) such as the inhibition of DNA synthesis and the induction of apoptosis. The spatial and temporal patterns of the rat vascular AT₂ receptor expression, together with the growth modulatory action, have led us to hypothesize that this receptor plays an important role in vascular development and remodeling in late gestation. Indeed, pharmacological blockade of the vascular AT₂ receptor by use of the specific antagonist PD123319 in the rat fetus during rat embryonic days 16 to 21 (E16-E21) has resulted in decreased aortic DNA synthesis, which supports a contribution of the AT₂ receptor to vascular development.

We and others have obtained, by use of a homologous recombination, AT₂ receptor knockout mouse strains that exhibit enhanced pressor response to acute Ang II infusion as compared with the wild-type strain. Because the vascular AT₂ receptor is minimally expressed at the time the blood pressure and Ang II infusion studies were performed, the data suggest that the transient and developmentally regulated AT₂ receptor expression exerts a long-term effect on blood pressure, possibly through its influence on vascular structure and/or function. These results support the notion that the AT₂ receptor modulates the growth of the developing blood vessel and thus contributes to vascular remodeling in late gestation.

Little is known about the ability of the AT₂ receptor to modulate VSMC differentiation. Recent studies suggest that AT₂ receptor activation may enhance differentiation in PC12W cells, a rat pheochromocytoma cell line, and modifying the AT₂ receptor expression...
NG 108-15 cells. The middle-sized neurofilament sub-unit expression was reduced in PC12W cells by AT receptor stimulation. In quiescent PC12W cells, AT receptor stimulation upregulated microtubule components and polymerized β-tubulin and MAP2, but downregulated MAP1B protein levels and, in PC12W cells differentiated by nerve growth factor, AT receptor stimulation elevated polymerized β-tubulin and reduced MAP1B. The morphological changes of AT receptor–mediated neurite outgrowth in NG 108-15 cells were correlated with an increase in the level of polymerized tubulin and in the level of the microtubule-associated protein. These results suggest a specific role of AT receptors in neuronal cell differentiation through regulation of the cytoskeleton. Thus it is conceivable that the AT receptor exerts a similar effect on smooth muscle cell differentiation. The AT receptor–null mouse model provides a unique opportunity to address the physiological role of the AT receptor. According to this hypothesis, activation of the AT receptor would facilitate VSMC differentiation in the wild-type animal, whereas the disruption of the AT receptor in the knockout animals will result in the delayed differentiation of VSMCs.

Methods

Animals

Female mice heterozygous for the AT receptor mutant allele were mated with WT FVB/N male mice (Jackson Laboratory, Bar Harbor, Maine). Pregnant mice were killed with an overdose of anesthesia, and the fetuses were dissected from uterine decidua. The day when a vaginal plug was observed was considered E1. In addition, and the fetuses were removed and prepared for genotyping, as previously described, pregnant mice were anesthetized with an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (4 mg/kg) at 13, 15, 18, and 20 days of gestation (E13, E15, E18, and E20). Fetuses were removed and their thoracic aortas were carefully dissected under stereomicroscopy, frozen in liquid nitrogen, and kept at −80°C before use. Aortas were also prepared from mice at 1, 7, 14, and 28 days after birth. Total RNA was prepared from 4 to 7 pooled thoracic aortas for each group with the use of TRIzol reagent (GIBCO BRL). Total RNA was first treated with RNase-free DNase (0.02 U/µL). After treatment for 5 minutes at 94°C, the samples were subjected to reverse transcription with random hexamer primers and reverse transcriptase (GeneAmp RNA PCR Kit, Perkin Elmer). We performed other sets of reverse transcription–polymerase chain reaction (RT-PCR) without RNA samples to confirm that there was no artificial amplification caused by the contaminations in the reagents. PCR primers were as follows: calponin, 5′-CACCAACAGTGTGCGGACG-3′ and 5′-GGTTGCGAGTGTCCAT-C′; α-SM actin, 5′-GGAGGCCCAGGCACTGCG-3′ and 5′-CCCTGTGCTCCGTTCA-3′; AT receptor, 5′-GGAAACAGCTGTGGTGTT-3′ and 5′-CTGATTTCAGAAGCTCCTTC-3′; 16 AT receptor, 5′-AGTGATCGCCGGAGCTG-3′ and 5′-GCAACAAACAAGCTGACG-3′; and GAPDH, 5′-ATGTTGAAAGCTGTGGTG-3′ and 5′-ACCGTAGGATGCAGGAT-3′. PCR reactions for α-SM actin, calponin, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed with 25 cycles of 1 minute of denaturation at 94°C, 1 minute of annealing at 53°C, and 2 minutes of extension at 72°C followed by 10 minutes of final extension step. PCR reactions for AT or AT receptor were carried out with 30 cycles. We observed a linear increase in amplification of PCR products with an increased amount of RNA up to 2 µg and as well as the increase in PCR cycles until 30 cycles for α-SM actin, calponin, or GAPDH and until 35 cycles for AT and AT receptors. PCR products were separated by 1% agarose gel electrophoresis. To verify the identity of the PCR products, we sequenced PCR products and confirmed that the sequences of PCR products matched up to the predicted sequences.

Blood Pressure Measurements

Male mice were anesthetized and a PE10 tube (Becton Dickinson) was inserted into the left common carotid artery. The next day, the PE50 tube was connected to the carotid artery cannula. The other end of the tube was connected to the blood pressure transducer (model TRN050, Kent Scientific Corp), which was connected to a transducer amplifier (model ETH-400, CB Sciences, Inc) and a MacLab 4/s (Division of AD Instruments, Inc). Blood pressure and heart rate were measured with rats in a conscious, unrestrained condition.

Immunoblot Analysis

Mice were anesthetized with an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (4 mg/kg); the thorax was opened and the aorta was removed. The connective tissues around the thoracic aorta were carefully dissected away in ice-cold phosphate-buffered saline (pH 7.40) under stereomicroscopy. Tissue samples were homogenized in 50 mmol/L Tris-HCl buffer, pH 7.5, which contained 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L PMSF, 1.5 µmol/L aprotinin, 5.5 µmol/L leupeptin, and 5.5 µmol/L pepstatin A. Tissue lysates were centrifuged at 10 000g for 20 minutes, boiled in Laemmli loading buffer for 3 minutes, resolved by 8% SDS-PAGE, electroblotted onto nitrocellulose membrane, and immunoblotted with antibodies against high-molecular-weight caldesmon (h-caldesmon) (clone C21),13 calponin (clone hCP),14 or α-smooth muscle actin (α-SM actin) (clone 1A4)15 (Sigma Chemical Co). Antibodies were detected by horseradish peroxidase–linked secondary antibody with the use of an enhanced chemiluminescence system (Amersham Life Science Inc). Densitometric analysis was performed by scanning densitometer (GS800, Hoeffer) and NIH image software.

Reverse Transcription–Polymerase Chain Reaction

Pregnant mice were anesthetized with an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (4 mg/kg) at 13, 15, 18, and 20 days of gestation (E13, E15, E18, and E20). Fetuses were removed and their thoracic aortas were carefully dissected under stereomicroscopy, frozen in liquid nitrogen, and kept at −80°C before use. Aortas were also prepared from mice at 1, 7, 14, and 28 days after birth. Total RNA was prepared from 4 to 7 pooled thoracic aortas for each group with the use of TRIzol reagent (GIBCO BRL). Total RNA was first treated with RNase-free DNase (0.02 U/µL). After treatment for 5 minutes at 94°C, the samples were subjected to reverse transcription with random hexamer primers and reverse transcriptase (GeneAmp RNA PCR Kit, Perkin Elmer). We performed other sets of reverse transcription–polymerase chain reaction (RT-PCR) without RNA samples to confirm that there was no artificial amplification caused by the contaminations in the reagents. PCR primers were as follows: calponin, 5′-CACCAACAGTGTGCGGACG-3′ and 5′-GGTTGCGAGTGTCCAT-C′; α-SM actin, 5′-GGAGGCCCAGGCACTGCG-3′ and 5′-CCCTGTGCTCCGTTCA-3′; AT receptor, 5′-GGAAACAGCTGTGGTGTT-3′ and 5′-CTGATTTCAGAAGCTCCTTC-3′; 16 AT receptor, 5′-AGTGATCGCCGGAGCTG-3′ and 5′-GCAACAAACAAGCTGACG-3′; and GAPDH, 5′-ATGTTGAAAGCTGTGGTG-3′ and 5′-ACCGTAGGATGCAGGAT-3′. PCR reactions for α-SM actin, calponin, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed with 25 cycles of 1 minute of denaturation at 94°C, 1 minute of annealing at 53°C, and 2 minutes of extension at 72°C followed by 10 minutes of final extension step. PCR reactions for AT or AT receptor were carried out with 30 cycles. We observed a linear increase in amplification of PCR products with an increased amount of RNA up to 2 µg and as well as the increase in PCR cycles until 30 cycles for α-SM actin, calponin, or GAPDH and until 35 cycles for AT and AT receptors. PCR products were separated by 1% agarose gel electrophoresis. To verify the identity of the PCR products, we sequenced PCR products and confirmed that the sequences of PCR products matched up to the predicted sequences.

Data Analysis

All values are expressed as mean±SE. Statistical significance for the developmental changes in differentiation marker expression in each strain of mice was assessed by ANOVA followed by Bonferroni test. Student’s t tests were used for statistical comparisons between 2 strains of mice. A value of P<0.05 was considered to be significant.

Results

To study the effect of AT receptor gene disruption on VSMC differentiation in the aorta, we examined the VSMC-specific contractile proteins as differentiation markers at different ages before birth and up to 8 weeks after birth. As shown in Figure 1, the protein contents of h-caldesmon and calponin increase age dependently. These protein levels were significantly lower in AT receptor–null mice than those in wild-type mice at 2 and 4 weeks of age (Figure 1, A, B, and C). Interestingly, at 8 weeks of age there were no differences in these marker proteins between both strains. On the other hand, there were no differences in α-SM actin expression
between the strains throughout development (Figure 1, A and D).

Next, we examined the developmentally regulated calponin, α-SM actin, and AT₂ receptor mRNA expressions in the fetal aorta by RT-PCR. To show the linear increase of RT-PCR products with increased amounts of RNA by use of our PCR conditions described in Methods, total RNA (2, 1, or 0.5 µg of RNA) prepared from thoracic aorta of wild-type mice at the age of E20 was subjected to RT-PCR for AT₁ receptor, AT₂ receptor, calponin, α-SM actin, and GAPDH (Figure 2). In the following experiments shown in Figure 3, we applied 1 µg RNA to RT-PCR. The mouse AT₁ receptor mRNA is expressed at relatively constant levels from the initial age tested (E13) throughout development both in AT₂ receptor–null and wild-type mice (Figure 3A). The AT₂ receptor mRNA expression was observed at E13, increased there-after, and rapidly declined after birth (Figure 3A). As shown in Figure 3B, the mRNA expression of calponin in wild-type mice was observed at E13, associated with the onset of AT₂ receptor mRNA expression, and increased thereafter. The expression and the developmental increase in calponin mRNA expression in AT₂ receptor–null mice were significantly delayed. Aortic calponin mRNA expression in the wild-type and AT₂ receptor–null mouse appeared to be similar at 14 and 28 days after birth (Figure 3C). In contrast, α-SM actin mRNA level did not differ throughout development between the 2 strains.

**Discussion**

AT₂ receptor is expressed in fetal aorta during late gestation; its expression decreases rapidly after birth, and the AT₂ receptor is reexpressed in vascular injury. We have demonstrated that pharmacological blockade of the AT₂ receptor in fetal rats E16 to E21 by use of the specific antagonist PD123319 has resulted in decreased DNA synthesis of the developing aorta. Accordingly, we have hypothesized that the AT₂ receptor is involved with vascular development. In this study we examined the possibility that the AT₂ receptor modulates VSMC differentiation and plays a role in vasculogenesis. Our data demonstrated that the protein levels of h-caldesmon and calponin in VSMC in thoracic aorta of AT₂ receptor–null mice were significantly lower than those in wild-type mice up to 4 weeks of age, whereas the expression of α-SM actin did not show any difference between the 2 strains. Expressions of h-caldesmon and calponin as well as α-SM actin appear in the early stage in vasculogenesis, whereas the expressions of h-caldesmon and calponin begin later and have been used as markers of VSMC differentiation in the late stage of vasculogenesis.

To examine the relation of AT₂ receptor expression with VSMC differentiation, we examined h-caldesmon, calponin, α-SM actin, and AT₂ receptor expression in fetal and neonatal mice. Consistent with previous data on the rat, we demonstrated that mouse AT₂ receptor mRNA expression is low during early development (E13-E15) but
increases significantly during later stage of development (E18-E20) and in the neonate (1 day after birth), whereas the AT1 receptor is constantly expressed through early embryonic stage after birth. The mRNA expression of calponin in wild-type mice aorta was “switched on” at E13 to E15 in both strains of mouse. The level of calponin mRNA increased rapidly thereafter in wild-type mouse aorta, whereas calponin mRNA in the AT2 receptor–null mouse was significantly lower and reached similar levels of calponin mRNA in the wild-type mouse at 14 days after birth. These results suggest that the level of expression of calponin is closely associated with the AT2 receptor expression in the fetal aorta. Moreover, we demonstrated that the protein levels of calponin and h-caldesmon are significantly higher in the AT2 receptor–null mouse aorta up to 4 weeks after birth. In contrast, we did not observe any significant difference in α-SM actin mRNA expression in aorta between the 2 strains during development. α-SM actin expression was already observed at E13, when the AT2 receptor was not expressed, which suggests that α-SM actin is not regulated by the AT2 receptor. These findings suggest strongly that the AT2 receptor plays a role in the phenotypic differentiation of VSMC, especially in the late stage of gestation.

We did not directly measure VSMC differentiation or vasculogenesis. h-caldesmon and calponin, thin filament–associated proteins, have been suggested to modulate smooth muscle contractility. Binding of h-caldesmon and calponin to actin inhibits actomyosin ATPase, which is necessary for initiation of contraction of VSMC and appears to be involved in vascular contraction in vivo. Moreover, h-caldesmon and calponin have been reported to inhibit contraction by providing a basal resting inhibi-

Figure 2. RT-PCR showing semiquantitative evaluation of mRNAs for AT1 receptor, AT2 receptor, calponin, α-SM actin, and GAPDH. Total RNA was prepared from thoracic aorta of wild-type mice at the age of E20; 2 μg (lane 1), 1 μg (lane 2), or 0.5 μg (lane 3) of RNA was subjected to RT-PCR. M, 100-bp ladder DNA marker.

Figure 3. Developmental changes in mRNA expression of (A) AT1, and AT2 receptors, (B) calponin, α-SM actin, and AT2 receptor, and (C) calponin in mouse aorta. Total RNA (1 μg) was prepared from thoracic aortas of AT2 receptor–null (Null) and wild-type (WT) mice at the ages of E13, E15, E18, E20, N1, 1, 2, and 4 weeks and subjected to RT-PCR. Results show representative data of 3 separate experiments. GAPDH was used as internal control to standardize the amount of total RNA used.

tion of vascular tone in smooth muscle cells. To examine the possibility that delayed expression of h-caldesmon and calponin in AT2 receptor–null mice influences the resting vascular tone and blood pressure, we measured blood pressure and heart rate in conscious mice at 4 and 8 weeks of age. Basal mean blood pressure in 4-week-old AT2 receptor–null mice was significantly higher than that in wild-type mice (105.2±4.5 versus 87.5±3.9 mm Hg, n=10, P<0.05), whereas there was no difference in blood pressure at 8 weeks of age (109.2±5.7 versus 107.7±4.8 mm Hg, n=10). There were no significant differences in heart rate between both strains. Mean blood pressure of AT2 receptor–null mice was significantly higher than that in wild-type mice (105.2±4.5 versus 87.5±3.9 mm Hg, n=10, P<0.05), whereas there was no difference in blood pressure at 8 weeks of age (109.2±5.7 versus 107.7±4.8 mm Hg, n=10). There were no significant differences in heart rate between both strains. Mean blood pressure of AT2 receptor–null mice was significantly higher compared with wild-type mice at the age of 4 weeks, when h-caldesmon and calponin contents are lower in AT2 receptor–null mice. Along with the increase in h-caldesmon and calponin in AT2 receptor–null mouse aorta, the blood pressure difference disappeared at age 8 weeks. These results suggest the possibility that the
delayed expression of these VSMC markers in AT2 receptor null–mice plays some role in vasculogenesis and influence vascular contractility.

We have demonstrated that the AT2 receptor exerts antiproliferation and proapoptotic effects in VSMC in vitro and in vivo.\textsuperscript{4,6,10} The antiproliferative effect of the AT2 receptor in endothelial cells and cardiomyocytes have also been reported.\textsuperscript{31,12} In this report, we postulate that the AT2 receptor enhances the differentiation of VSMC in aorta. Indeed, it has been reported that the AT2 receptor promotes differentiation of neuronal cells through regulation of cytoskeletal proteins.\textsuperscript{9–12} The AT2 receptor has been reported to stimulate protein tyrosine phosphatase (PTPase) activity,\textsuperscript{4,6,33–37} which plays an essential role in hematoipoetic cell differentiation.\textsuperscript{8} We have reported that the intracellular third loop of the AT2 receptor is important for its PTPase activation.\textsuperscript{30} Interestingly, recent studies suggest that dopamine receptors, which have a similar amino acid motif in the intracellular third loop,\textsuperscript{39} promotes morphogenesis of developing neurons.\textsuperscript{40,41} These findings suggest a possible mechanism whereby AT2 receptor–regulated PTPase is involved in the differentiation of VSMC. Our results also suggest that AT2 receptor stimulation did not modify the expression of housekeeping genes such as GAPDH.\textsuperscript{42,43} In summary, our results suggest that the AT2 receptor participates in mouse vasculogenesis.

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