Modulation of vascular development and injury by angiotensin II

Howard G. Hutchinson\textsuperscript{a,b}, Lutz Hein\textsuperscript{a,c}, Masahiko Fujinaga\textsuperscript{a,d}, Richard E. Pratt\textsuperscript{a,e,*}

\textsuperscript{a} Falk Cardiovascular Research Center, Division of Cardiovascular Medicine, Stanford University School of Medicine, Palo Alto, CA 94304, USA
\textsuperscript{b} Medical Affairs, Zeneca Pharmaceuticals, 1800 Concord Pike, PO Box 15437, Wilmington, DE 19850, USA
\textsuperscript{c} Department of Pharmacology, University of Wuerzburg, Versicherer Strasse 9, 97078, Wuerzburg, Germany
\textsuperscript{d} Stanford University School of Medicine, VA Palo Alto Health Care System, 38081 Miranda Avenue, Palo Alto, CA 94304, USA
\textsuperscript{e} Laboratory of Genetic Physiology, Brigham and Women’s Hospital, Thorn-13, 75 Francis Street, Boston, MA 02115, USA

Received 16 April 1998; accepted 6 August 1998

Abstract

Objective: To examine the exact profile of expression and to determine the functional significance of the angiotensin II (Ang II), type 1 (AT\textsubscript{1}) and type 2 (AT\textsubscript{2}) receptors during rat aortic development and following rat carotid artery balloon injury. Methods: AT\textsubscript{1} and AT\textsubscript{2} mRNA levels in rat aortae were measured using a quantitative reverse transcription polymerase chain reaction technique. Ang II receptor function was assessed by quantitating the effects of AT\textsubscript{1} (DuP753) and AT\textsubscript{2} (PD123319) receptor antagonists during these processes. Results: During aortic development, AT\textsubscript{1} expression was detected on gestational day 14, increased until embryonic day 16 (E16), after which, levels were similar throughout postnatal development. Conversely, AT\textsubscript{2} mRNA first appeared at E16, reached maximal levels between E19 and neonatal day 1, and decreased thereafter. DNA synthesis rates decreased with aortic development (high at E15, 73.8±3.1%; dropping to 37.5±2.3% by E21). Whereas AT\textsubscript{1} receptor antagonism accelerated this developmentally regulated decrease in DNA synthesis, AT\textsubscript{2} receptor antagonism blunted this decrease. Because activated adult medial smooth muscle cells express a neonatal phenotype after vascular injury, we assessed Ang II receptor levels and function after carotid artery balloon injury. Both receptor subtypes increased; however, AT\textsubscript{2} receptor mRNA expression peaked earlier than AT\textsubscript{1} (48 to 72 h after injury). As with aortic development, DNA synthesis occurring between 24 to 48 h after injury (when AT\textsubscript{2} receptors constitute 10% of the Ang II receptor population) decreased in DuP753-treated animals and increased in PD123319-treated animals. Conclusion: These results indicate that Ang II receptors play a role in vascular development by promoting opposing effects on vascular smooth muscle cell growth. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Protein coupled receptors; Quantitative RT-PCR; Neointima; Developmental biology; Smooth muscle; Growth control

1. Introduction

Until recently, it was believed that angiotensin II (Ang II) exerts most of its actions via the G-protein-coupled type 1 (AT\textsubscript{1}) receptors, which either activate phosphoplipase C or inhibit adenylate cyclase. This receptor belongs to the superfamily of seven transmembrane domain receptors [1–5]. A second receptor subtype, the AT\textsubscript{2} receptor, was initially defined by its selective binding to ligands PD123319 and CGP42112 [6,7]. Molecular cloning of this receptor demonstrated that it too belongs to the same seven transmembrane superfamily [8–10]. However, the AT\textsubscript{1} and AT\textsubscript{2} subtypes are only 34% identical in sequence. As shown by binding assays, the AT\textsubscript{2} receptor is highly expressed in embryonic tissue, including the vasculature, but is expressed at low levels (if at all) in the adult, where the AT\textsubscript{1} receptor subtype predominates [8–12].

In vitro and in vivo studies confirm that Ang II plays an important role in the growth of vascular smooth muscle cells [13–18]. Because the AT\textsubscript{1} receptor subtype predominates in adult vasculature, the growth effects of Ang II were hypothesized to be mediated via the AT\textsubscript{1} receptor. In fact, all in vitro and in vivo growth-promoting effects of
Ang II in adult smooth muscle cells can be blunted by antagonists specific for the AT$_1$ receptor [17,18]. Moreover, Powell et al. [16] and others [14,17–19] reported that angiotensin converting enzyme (ACE) inhibitors can prevent neointimal formation following balloon injury in rats. This effect of ACE inhibitors on neointimal formation is due, in part, to blockade of Ang II production, as evidenced by the ability of the angiotensin receptor antagonist, DuP753, to prevent neointimal hyperplasia [17]. However, evidence suggests that the increase in kinins observed with ACE inhibitors may also play a role [18].

Several components of the renin angiotensin system are highly expressed during embryonic development. In addition to AT$_1$ and AT$_2$ receptor expression in the developing rat, ACE, renin and high levels of Ang II have been observed [11,12,20–22]. Functional studies of the role of these components are few; however, reports suggest that they may play a role in pulmonary vessel muscularization as well as nephrovascular development [20,21]. During prenatal growth, several processes that may be modulated by Ang II are necessary for appropriate development of the vessel wall. Following angiogenesis, migration and differentiation of mesenchymal cells occurs to form the immature vessel. During later embryonic and fetal stages, rapid proliferation of smooth muscle is initially observed, followed by a developmentally regulated decrease in growth. Also observed during fetal and neonatal development is the differentiation and maturation of vascular smooth muscle, a reorientation of the smooth muscle layer from a longitudinal to circumferential orientation, and the production of the elastic lamellae. Interestingly, we recently showed that the AT$_2$ receptor can mediate an antiproliferative action during prenatal vascular development and may be responsible for a developmentally regulated decrease in vessel growth [23]. However, the role of the AT$_1$ receptor during development is unclear. Moreover, the importance of functional interactions between angiotensin receptors during vascular development has not been examined.

Although the AT$_1$ receptor subtype predominates in the adult blood vessel, the potential involvement of the AT$_2$ receptor in regulation of adult vascular smooth muscle cannot be discounted. For example, neonatal smooth muscle cells are phenotypically distinct from the medial smooth muscle, as evidenced by the expression in the neointimal cells of a subset of the proteins normally expressed in embryonic or neonatal smooth muscle cells. These include the re-expression of neonatal isoforms of certain cytoskeletal and extracellular matrix proteins, as well as expression of certain growth factors and growth factor receptors [9,24–28], raising the possibility that the AT$_2$ receptor may be re-expressed following injury. Interestingly, Janiak et al. [29] reported that in vivo administration of CGP42112, a specific AT$_2$ receptor ligand, attenuates development of the neointimal lesion in rats. This observation supports the possibility that the AT$_2$ receptor may play a role in the development of the neointimal lesion. However, there are serious caveats to this observation. Because the compound was delivered periadventitial-ly, the local concentration of the compound in the injured vessel could not be assessed and may be quite high. Therefore, a nonspecific biochemical effect of CGP42112 or a crossover effect of this AT$_2$ ligand to the AT$_1$ receptors cannot be ruled out, especially in light of the assumption that the AT$_1$ receptor is the predominant receptor subtype expressed in the adult.

To better understand the potential function of the Ang II receptors during fetal growth, as well as growth following vascular injury, we have established a competitive, quantitative reverse transcription polymerase chain reaction (PCR) assay to measure the mRNA encoding these receptors. Using this assay, we examined the expression pattern of the Ang II receptor during normal pre- and postnatal aortic development, and in the carotid artery following injury. Ang II receptor function was then assessed during these processes by quantitating the effects of the AT$_1$ receptor antagonist, DuP753, and the AT$_2$ receptor antagonist, PD123319, on vascular DNA synthesis.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.1. Animals

Male Sprague-Dawley rats (400 to 450 g, 12 weeks old) and pregnant female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories.

2.2. Arterial injury model

The left carotid artery was injured by balloon catheter as described previously [14,23] The right uninjured carotid artery served as a control. Tissues were harvested at indicated times after injury. During the harvesting procedure, the adventitia was physically removed from both the injured and non-injured control vessel. In this model, the vessel does not become re-endothelialized. Therefore, to better compare the characteristics of the smooth muscle in the vessels, the endothelial cells were removed from the control vessel by water perfusion.

2.3. RNA isolation

Total RNA was isolated from embryonic [days 14 to 17 (E14 to E17)], fetal (E18 to E21), postnatal (days 1 to 4, 7, 8, 12), and adult (12 weeks) rat aortae, and control and injured carotid arteries using RNAzol® B (Friendswood,
TX, USA). For RNA isolation from injured and control carotid arteries, typically 10–20 vessels were pooled. For RNA isolation from developing aorta, typically 10–20 vessels were pooled. Total RNA was subsequently treated with RNase-free DNase I (Gibco BRL, Gaithersburg, MD, USA) to remove any contaminating genomic DNA that may have been found in the sample. One unit of DNase was added per μg of RNA in 20 mM Tris–HCl, 50 mM KCl and 2.5 mM MgCl₂ and incubated at room temperature for 15 min. The reaction was terminated with the addition of 1 μl of 20 mM EDTA (pH 8.0) and incubation at 65°C for 10 min. The solution was re-extracted with RNAzol® B. In preliminary studies, this procedure had no effect on the PCR product generation from RNA but abolished the generation of product from genomic DNA (data not shown).

2.4. Polymerase chain reaction

AT₁-specific primers were chosen so that both AT₁ₐ and AT₁ₐ receptor isoforms would be co-amplified [1,4]. The forward primer spans a region containing the first transmembrane domain, 5’ TATTTGAAAACAGCTTGGTG 3’ [AT₁(I), positions 128 to 148, Ref. [1]]. Two reverse primers were synthesized, primer AT₁(V), 5’ GGTGAGAATGATAAGGAAAGGG 3’ and primer AT₁(VI), 5’ AGAAGAAGAAACACAATCGC 3’, which are located in the regions of the fifth and sixth transmembrane domains, respectively (positions 618 to 639 and 729 to 750). PCR with the AT₁ forward primer and primer AT₁(V) gives a 512-bp product, whereas PCR with the forward primer and primer AT₁(VI) gives a 623-bp product. PCR product generation from RNA but abolished the generation of product from genomic DNA (data not shown).

A second set of primers that would selectively amplify AT₁ₐ or AT₁ₐ was synthesized. These primers, reported by Matsubara et al. [30], were as follows: AT₁ₐ forward primer, 5’ GCACAGTGCAATGTAATGC 3’, AT₁ₐ reverse primer, 5’ GTTGAAACAAACAAGTGG 3’, AT₁ₐ forward primer, 5’ GCCTGCAAGTTGAAGTGTATT 3’ and AT₁ₐ reverse primer, 5’ TTAAACAGTGCTTGGCTCC 3’.

Primers for the AT₂ receptor cDNA were based on the cDNA sequence for the rat AT₂ receptor [8]. The forward primer [primer AT₂(I)] is located within the first transmembrane domain, 5’ATTCCTGTTCCTACTACATC 3’ (positions 289 to 309). Two reverse primers were synthesized, primer AT₂(III) 5’ TGCTTGCACAGATGTCTCA 3’ and primer AT₂(V) 5’ GTAACACGTTGGCATATGAA 3’, which are located in the third and fifth transmembrane domains, respectively (positions 527 to 544 and 826 to 843). The forward primer and reverse primer AT₂(III) give a product of 256 bp, whereas the forward primer and reverse primer AT₂(V) give a 555-bp product. The specificity of the 555 bp AT₂ cDNA fragment was assessed by digestion with Ava II, which resulted in expected products of 199 and 356 bp (Data not shown).

As controls, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used to compare carotid injury RNA samples, and α-tubulin was used to compare developmental samples. The G3PDH primers were taken from the rat sequence forward primer, 5’ AAGCTAAGTGAGATCATCAACA 3’, and reverse primer, 5’ ATGCCTAATTGGGTCTGCA 3’ [31]. α-Tubulin primers were also obtained from a rat sequence [32]: forward primer, 5’ CAAAGGCTGACATCAACTCC 3’, and reverse primer, 5’ TTTGACATGATAGGACTG 3’. The G3PDH primers yielded a 418-bp product and α-tubulin primers a 336-bp product. Both primer sets spanned an intron.

Total RNA (0.2 to >1 μg) was reverse transcribed using 2.5 mM random hexamers and 50 U of MMLV reverse transcriptase (Perkin Elmer Cetus, Norwalk, CT, USA) in the buffer recommended by the supplier. The reaction mixture was incubated at 42°C for 1 h and at 99°C for 10 min followed by cooling to 5°C for 5 min before PCR.

The reverse transcription product (10 μl) was added to 39.5 μl of PCR master mix (Perkin Elmer Cetus), which contained 0.4 μM each of forward and reverse primers. PCR was initiated using the hot start method. After incubation at 94°C for 5 min, the samples were cooled to 80°C, at which time, 2.5 U of Taq DNA polymerase were added. Subsequent PCR conditions are noted in the text. Semi-quantitative PCR was performed by varying the cycle number and comparison relative to α-tubulin, which was reported to remain constant during development [33]. In preliminary experiments, we varied the cycle number and documented that the amplification was in the exponential range. Confirmation that RNA was being amplified in reverse transcription (RT)-PCR was verified by treating samples pre- and post-RT with DNase-free RNase A.

2.5. Quantitative RT-PCR

The basis of the quantitative RT-PCR technique is that a competition is set up between the cDNA resulting from the amplification of the endogenous AT₂ receptor mRNA and the cDNA originating from an exogenously added RNA. Exogenously added RNA is identical to endogenous mRNA, except for a deletion that allows separation of the two sequences by gel electrophoresis. The two sequences compete for reagents in the PCR reaction; the greater the amount of exogenously added RNA, the lower the amount of PCR product originating from the endogenous mRNA. The PCR products are resolved by gel electrophoresis, and the point at which the signal from the endogenous mRNA equals that of the exogenously added RNA is defined visually. At this point, the two RNAs are present in equal
concentrations, thus allowing quantitation of the endogenous sequence.

Quantitative RT-PCR was performed using a competitive recombinant (rc)RNA internal standard that was synthesized using a modification of the method of Heuvel et al. [34] (see Fig. 1). The forward rcRNA primer (5’TAAATACGACTCATATAGGTATTTTGAAACAGCTTGGTTG 3’) contains a T7 promoter sequence (single underlined) 5’ to the AT1(I) forward primer (double underline). The reverse rcRNA primer (5’T TTTTTTTTTTTTTTTTT AGAAGAAGAAAGCACAATCGCGGTGAGAATGATAAGGAAAGGG 3’) was synthesized by linking 5’→3’ poly d(T) tail (dotted underline), reverse primer AT1(VI) (single underline) and AT1(V) (double underline). Fig. 1 summarizes the procedure for synthesis of the AT1 RNA standard. PCR was performed on AT1 cDNA in a reaction mixture consisting of 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM MgCl2, 0.6 mM forward and reverse rcRNA primers and 1 ng of AT1 cDNA. Taq DNA polymerase (2.5 U) was added during a hot start. PCR was performed at 94°C for 1 min, 56°C for 2 min and at 72°C for 2 min for 35 cycles followed by 72°C for 15 min. A cDNA band of the expected length (571 bp) was obtained following electrophoresis on a 1.5% agarose gel. This product was diluted 1:100 in water and 2 ml were re-amplified using the conditions stated above. PCR products from several amplifications were pooled and purified using the Magic™ Prep DNA purification system (Promega, Madison, WI, USA). The purified cDNA was transcribed into RNA by the T7 promoter using the Riboprobe® Gemini II in vitro transcription system (Promega). Following treatment with RNase-free DNase to remove the remaining cDNA (25°C for 15 min followed by 65°C for 10 min), the RNA was re-extracted using RNAzol® B. The rcRNA product has a predicted size of 534 bp when amplified using the forward [AT1(I)] and reverse [AT1(VI)] AT1 primers, whereas the same primers yielded a 623-bp fragment when endogenous AT1 mRNA was amplified. For quantitative RT-PCR analysis, the sample RNA and increasing amounts of the rcRNA internal standard were mixed together before addition to the reverse transcriptase reaction mixture.

A similar procedure was used to synthesize the AT2 RNA standard, which used the AT2(I) forward primer and AT2(III) and AT2(V) reverse primers.

To assess the efficiency of RT-PCR amplification using

---

**Fig. 1. Generation of AT1 mRNA competitor RNA.** The indicated primers were employed to generate the internal RNA competitor for the quantitative RT-PCR.
the rRNA internal standard, serial dilutions of neonatal RNA samples were made and tested against the rRNA standard. In each case, shifts in the crossover point were observed, consistent with the dilutions in the target RNA. As a second method to standardize our technique, a target sequence was constructed in a fashion similar to that used to create the rRNA internal standard. The forward rRNA primer was used along with a second primer that was constructed by linking the distal primer [i.e., primer AT$_4$(VI) for AT$_2$] to a poly(T) tail. After PCR and transcription, an RNA target is generated that can be quantitated and that amplifies to the size of the native messenger RNA. Competitive RT-PCR assays using the synthesized target against the internal standard revealed that equivalent amounts of each amplified similarly.

2.6. In vivo analysis of DNA synthesis and effects of angiotensin receptor antagonists

Assessments of DNA synthesis in developing aortae were made by quantitating incorporation of bromodeoxyuridine (BrdU, Sigma, St. Louis, MO, USA) into aortae similar to a procedure described by Cook et al. [35]. Briefly, pregnant female Sprague-Dawley rats at various gestational days were injected with 100 mg/kg body weight BrdU at 18, 12 and 1 h before sacrifice. Following sacrifice, whole embryo or dissected aortae were placed in 4% paraformaldehyde for 12 h. Samples were subsequently paraffin embedded, sectioned and stained for BrdU using a kit purchased from Zymed Laboratories (South San Francisco, CA, USA). BrdU incorporation was measured as a percentage of BrdU immunoreactive nuclei/total nuclei present within the medial smooth muscle cell layer. Differentiation of the medial smooth muscle cell layer from the adventitia was made by staining for α-actin with a monoclonal antibody (Zymed Laboratories).

To determine the potential role of AT$_1$ versus AT$_2$ receptor subtypes during development, an AT$_1$ antagonist, DuP753 (provided by Dr. Ronald Smith, Dupont–Merck), and an AT$_2$ antagonist, PD123319 (provided by Dr. Joan Keiser, Parke Davis), were infused into pregnant rats. Rats were anesthetized using NO and halothane gas under carefully controlled conditions. Alzet pumps (Alza, Palo Alto, CA, USA) were then placed subcutaneously to deliver vehicle, DuP753 (20 mg/kg/day), PD123319 (20 mg/kg/day), or a combination of DuP753 and PD123319 (each 20 mg/kg/day). Infusions began four days before sacrifice.

Potential teratogenic effects were assessed by comparing litters with regard to size, malformations, crown–rump length and body weight. No significant differences in these parameters were observed.

Functional analysis of AT$_1$ and AT$_2$ receptor pathways following carotid injury were made in a similar fashion. Receptor antagonists were infused (20 mg/kg/day) subcutaneously for four days before carotid injury and continued until sacrifice. BrdU was injected at 18 h (100 mg/kg subcutaneously and 30 mg/kg intraperitoneal) and at 12 h (30 mg/kg intraperitoneal) before sacrifice. Following sacrifice, the samples were processed as described above.

3. Results

Although it has been reported that the AT$_2$ receptor is prenatally expressed in the vasculature, little is known concerning the profiles of expression (i.e., when expression commences, when expression is at its highest, and when expression decreases). Moreover, little is known about the expression or function of the AT$_1$ receptor during this phase of development. To examine the expression of the receptors, both qualitative and quantitative RT-PCR assays were established. Qualitative RT-PCR analysis of developing aortae for AT$_1$ receptor mRNA expression is shown in Fig. 2A–B. Total RNA (0.2 μg) isolated from aortae removed at various times from E14 to 12 weeks postpartum (adult) was reversed transcribed; the resultant cDNA was amplified by PCR. The 623 bp RT-PCR product of the amplification of the AT$_1$ receptor mRNA was easily detected in the amplification product of RNA isolated from aortae removed from E14 rats. Because of the small size of the aorta, it was difficult to examine the vascular expression of the AT$_1$ receptor at earlier times. However, the amplification product of the AT$_1$ receptor mRNA could be seen in RNA isolated from whole embryo at E9. Interestingly, the levels of expression of the AT$_1$ receptor mRNA remained relatively constant (with minor fluctuations) throughout pre- and postnatal development. In mouse and rat, two isoforms of the AT$_1$ receptor have been reported [3–5] and termed AT$_{1a}$ and AT$_{1b}$. In the vasculature of the adult, AT$_{1a}$ is the predominant form. To examine the pattern of expression of these isoforms during postnatal development, we used primers that would amplify AT$_{1a}$ or AT$_{1b}$ selectively [30]. The expression pattern of the AT$_{1a}$ receptor mRNA appeared to parallel that seen with total AT$_1$ mRNA, whereas levels of the AT$_{1b}$ mRNA appeared unchanged throughout development.

We next examined the expression of the AT$_2$ receptor during the same pre- and postnatal time periods. Based on the sequence of rat AT$_2$ receptor mRNA [8], we chose a primer pair that would yield a PCR product of 555 bp. In contrast to the pattern of expression of the AT$_1$ receptor, PCR products derived from AT$_2$ mRNA were not abundant until E17. From E17, levels increased to a maximum at postnatal day one and rapidly decreased thereafter.

Several controls were run to demonstrate the validity of the RT-PCR results. To control for input mRNA, co-amplification of an internal housekeeping gene product is usually performed. However, during embryonic and fetal development, the phenotype of the cells changes dramati-
Fig. 2. Developmental regulation of angiotensin receptors in neonatal and adult rat aortae (Panels A and B). Total RNA isolated from aortae of embryonic (E14 to E17), fetal (E18 to E21), neonatal (days 1 to 4, 7, 8 and 12), and adult rats (12 weeks of age) was analyzed by RT-PCR. Comparisons were made during the logarithmic amplification phase of the polymerase chain reaction. The starting amount of RNA and the number of PCR cycles for AT1, AT1a, AT1b, and α-tubulin was 0.5 μg and 30 cycles. For AT2, 0.2 μg and 35 cycles were used. Note that, in Panel A, AT1 levels were detected as early as E14 and maintained at relatively similar levels. Conversely, AT2 levels were detected between E15 and E16 and increased to a maximum at postnatal day 1.

Panel B examines postnatal trends for angiotensin receptor expression. Note that AT1 levels remained relatively similar through day E16 to postnatal day 12 and decreased slightly in the adult. Analysis of AT1 subtype expression reveals that the pattern of expression of the AT1a receptor subtype, the predominant subtype in vascular tissue, parallels that of the total AT1 receptor. Conversely, the levels of expression of the AT1b subtype remained similar throughout development. Analysis of AT2 receptor mRNA levels revealed a dramatic decrease after day 4 postpartum. No significant changes were observed in the product yield from α-tubulin mRNA. (Panel C) Competitive, quantitative RT-PCR analysis of AT1 and AT2 mRNA expression during aortic development was performed as described in Section 2. These quantitations were performed with multiple, separately isolated mRNA preparations and were performed at least twice with each preparation. (Panel D) The results indicate the abundance of angiotensin receptors during pre- and early postnatal development, with a predominance of AT1 receptors during late gestation and early postpartum. Angiotensin receptor levels decrease substantially in adult vessels, with the AT1 subtype being predominant at this time (note the different scale used for the 12-week sample). n=2. This experiment was repeated twice with similar results.
cally, making the choice of such a control difficult. Therefore, we compared the intensities of the 18S and 28S ribosomal RNA bands on an ethidium bromide-stained agarose gel analysis of aortic RNA isolated at different embryonic and fetal times and demonstrated equal intensities (not shown). In addition, we co-amplified α-tubulin mRNA, which was previously reported to have stable expression throughout postnatal development [33]. Consistent with this, the yield of PCR product obtained with the α-tubulin primers (336 bp product) was similar at the time points examined.

Since the genes encoding the AT1 and AT2 receptors do not contain introns within the coding region and, therefore, the above PCR primers would not distinguish cDNA from genomic DNA. Therefore, all RNA samples were treated with RNase-free DNase before amplification. As an added test to ensure that the products generated were a result of amplification of cDNA and not due to amplification of genomic DNA, RNA samples were digested with DNase-free RNase A. When RNase was added before first strand cDNA synthesis, no product was observed following the PCR. However, when the RNase was added after first strand cDNA synthesis, the yield of product was unaffected (data not shown).

Using competitive RT-PCR, the levels of AT1 and AT2 receptor mRNA were quantitated in developing aortae. Internally deleted RNA standards, consisting of truncated AT1 and AT2 sequences, were generated as shown in Fig. 1. Amplification of these standards with the above AT1- and AT2-specific primers yielded products of 534 and 275 bp, respectively. When the standard RNA was added to aortic RNA at appropriate levels, competition resulted (for example, see Fig. 2). The efficiency of the competition reactions was assessed as described in Section 2. In contrast to previous reports [11,12] that suggest that the AT2 receptor predominates during fetal aortic development, we found that, at early gestation (E15), AT1 and AT2 receptor mRNA levels were similar (Fig. 2C). With development, the expression of AT2 mRNA increased dramatically, reaching maximal levels by postnatal day one and decreasing rapidly thereafter. In contrast, AT1 levels remained relatively similar through pre- and postnatal development, but decreased 3.6-fold in the adult vessel. Note that the AT2 receptor mRNA is the predominant isoform during late gestation and early postnatal development, with a switch to AT1 subtype predominance in the mature vessel. For example, about 96% of the receptors are AT2 and 4% are AT1 in the day one neonate. In contrast, about 92% of the receptors are AT1 and only 8% are AT2 in the adult. These quantitations were performed with multiple, separately isolated mRNA preparations and were performed at least twice with each preparation.

We next addressed the functional significance of the prenatal expression of the Ang II receptors. It has previously been demonstrated that AT2 receptor signaling inhibits vascular growth in utero [23] but the effects of AT receptor signaling are unknown. Because angiotensin II, via the AT1 receptor, has been shown to stimulate the growth of adult vascular smooth muscle cells both in vitro and in vivo [13–18], we postulated that this receptor may have similar functions during prenatal development. To examine the functional significance of the prenatal expression of the AT1 and AT2 receptors, we examined the effects that the specific antagonists, DuP753 and PD123319, had on DNA synthesis in the aorta (Fig. 3). Drugs were administered maternally using Alzet minipumps for four days before tissue harvest. On the last day, BrdU was administered maternally by intraperitoneal injection. As shown previously by Cook et al. [35], a developmentally regulated decrease in DNA synthesis was observed. During early gestation (E15), 73% of the aortic nuclei displayed positive immunoreactivity for BrdU following an 18-h exposure to the compound, demonstrating the rapid growth of the embryonic aorta. This rate dropped progressively such that, on E21, the rate of DNA synthesis was 37%. As we showed previously [23], this developmentally regulated decrease in DNA synthesis is mediated, in part, by the action of the AT1 receptor. At days E18, E19 and E21, administration of the AT2 receptor antagonist increased DNA synthesis towards the maximum rate observed on day E15. In contrast, infusion of the AT1 receptor antagonist had opposing effects on DNA synthesis. A significant decrease in BrdU incorporation was observed with infusion of the AT1 receptor antagonist on
days E18, E19 and E21. Interestingly, in animals receiving both receptor antagonists, the rates of DNA synthesis were indistinguishable from the rates observed in the AT$_2$ receptor antagonist-treated animals, suggesting that the antiproliferative effects of the AT$_1$ receptor antagonists may be mediated, in part, by the unopposed action of Ang II at the AT$_2$ receptor.

Next, we examined the interaction of the two receptor subtypes in another model of vascular smooth muscle cell growth, the balloon injured rat carotid artery (Fig. 4, Table 1). After injury, the smooth muscle cells of the carotid artery respond with a well described increase in DNA synthesis and cell proliferation. While it has been shown that AT$_1$ receptor levels increase following injury, the expression patterns of the AT$_2$ receptor are unknown. Because activated medial smooth muscle cells and neoint...

<table>
<thead>
<tr>
<th>Receptor subtype expression (fmol/mg total RNA)</th>
<th>AT$_1$</th>
<th>AT$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57</td>
<td>Not detected</td>
</tr>
<tr>
<td>Injured day 1</td>
<td>57</td>
<td>0.1</td>
</tr>
<tr>
<td>Injured day 2</td>
<td>57</td>
<td>5.8</td>
</tr>
<tr>
<td>Injured day 3</td>
<td>142</td>
<td>5.8</td>
</tr>
<tr>
<td>Injured day 4</td>
<td>142</td>
<td>2.9</td>
</tr>
<tr>
<td>Injured day 14</td>
<td>199</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Total RNA was isolated from control carotid arteries or carotid arteries injured with a balloon catheter at the indicated times. AT$_1$ and AT$_2$ transcript levels were determined using the quantitative RT-PCR. For each RNA sample, 10–20 aortae were pooled. Results presented are representative of two experiments.

Fig. 4. Re-expression of the AT$_2$ receptor following carotid artery injury. (Panel A) Total RNA (1 μg) isolated from control carotid arteries (C) or carotid arteries injured one–four, seven or 14 days before isolation was analyzed by RT–PCR. After injury, re-expression of the AT$_2$ receptor was detected as early as day 1 and was still evident at day 14. G3PDH was used as a control. This experiment was performed twice using separate isolations of RNA from carotid arteries injured on separate occasions. (Panel B) Quantitative RT/PCR was performed on the RNA isolated from the normal and injured carotid arteries. Shown here is a typical competition result for AT$_2$ mRNA following carotid artery balloon injury. This experiment was repeated twice with similar results. (Panel C) RNase A digestion of the RNA [from vessels injured for three (lane 1) and 14 (lane 2) days] prior to the RT-PCR (Pre-RT) abolished the generation of PCR product, whereas RNase digestion after the reverse transcriptase (Post-RT) had no effect on PCR product generation.
After injury, amplification products from the Ang II at the AT receptor. sical DNA engineering to PCR-based methods. Our tech-2 
blockade may be due, in part, to the unopposed action of synthesize competitive RNA standards, ranging from clas-
interval. Simultaneous infusion of both antagonists again employing an exogenous RNA template as an internal
mRNA levels that were 20-fold greater than that observed for the endogenous receptor (i.e., at levels comparable to the AT receptor mRNA) could inhibit DNA synthesis in the balloon injured vessel [23]. However, the biological effects of the much lower levels of expression of the endogenous receptor are unknown. Therefore, the functional significance of these changes in angiotensin receptor expression was again assessed using BrdU incorporation studies (Fig. 5). The angiotensin receptor antagonists were administered by Alzet minipumps continuously from four days before injury until two days after injury. On the last day, BrdU was adminis-
ted to measure the rates of DNA synthesis. This time interval was chosen because, during this time, the AT2 receptor levels increased to 10% of the total Ang II receptor population, while the AT1 receptor levels remained at control levels. Consistent with the results of Prescott et al. [17], AT1 receptor antagonist reduced BrdU labeling indices to 35% of that of the nontreated injured vessel. Similar to results seen in the prenatal aorta, the AT1 and AT2 receptor pathways appeared to mediate opposing functions. AT2 receptor antagonism increased synthesis (7.9±0.4 versus 12.2±0.8, P<0.01) during this time interval. Simultaneous infusion of both antagonists again increased DNA synthesis compared to DuP753 treatment levels (2.8±0.3 versus 7.6±0.5, P<0.01), again suggest-
ing that the antiproliferative effects of the AT1 receptor blockade may be due, in part, to the unopposed action of Ang II at the AT2 receptor.

We next examined the effect of receptor blockade between days three and four, when AT2 receptor levels were dropping to 2% of the total population and AT1 receptor levels were increasing by 2.5-fold over control values. Again, AT1 receptor antagonism decreased BrdU labeling indices, in this case, to 50% of that of the nontreated, injured controls. However, during this interval, AT2 receptor blockade had no effect on the BrdU labeling index or inhibition due to AT1 receptor blockade.

4. Discussion

Recent reports suggest that the Ang II receptors, AT1 and AT2, mediate opposing effects on growth [23,37], with AT1 pathways promoting growth and AT2 pathways inhibiting growth. However, the majority of the studies have concentrated on cell culture observations. In the present study, we extend these observations by showing that the angiotensin receptors play an important role in vivo in regulating developmental maturation of blood vessels and vascular response to injury. During both of these processes, AT1 and AT2 receptors have reciprocal effects on growth.

To assess AT2 expression during development and after carotid injury, we used a competitive RT-PCR technique, employing an exogenous RNA template as an internal competitor. Because both the target mRNA and the internal standard compete for the same primer sequences, amplification is truly competitive. Several methods exist to synthesize competitive RNA standards, ranging from classical DNA engineering to PCR-based methods. Our tech-
ique, as outlined in Fig. 1, is a simplified version of that...
proposed by Heuvel et al. [34]. The technique is simple, rapid and can be completed in a few days. The technique differs from that of Heuvel et al. [34] in that the target mRNA and exogenous competitor RNA are identical, except for the deletion. Therefore, the efficiencies for RT and PCR steps should be essentially identical between target and competitor RNAs. Competitive RT-PCR is a sensitive technique that can discern differences in mRNA levels as little as two-fold or less. The sensitivity could be increased further by narrowing the concentration range and using $^{32}$P or radiolabeled primer in the PCR reaction.

The ratio of $\text{AT}_1$:$\text{AT}_2$ receptors varied dramatically during early vascular development. Previous studies reported that the $\text{AT}_2$ receptor is the predominant angiotensin receptor during vascular development, with little $\text{AT}_1$ receptor present [11,12]. This is not entirely accurate. We have found that $\text{AT}_1$ receptor mRNA is present to a significant degree during vascular development. In early whole embryo specimens (i.e., E11), only $\text{AT}_1$, not $\text{AT}_2$, receptor mRNA could be detected. At E15, when the aorta can be successfully isolated, similar levels of both $\text{AT}_1$ and $\text{AT}_2$ receptor expression were observed. Furthermore, $\text{AT}_1$ levels are three times higher at this time than in the adult vessel. Not until days 16 to 18 do we begin to see the dramatic increases in $\text{AT}_2$ receptor and, during this time, $\text{AT}_1$ expression remains at levels similar to those found on E15. The increase in $\text{AT}_2$ expression after E16 is noteworthy because of the developmentally regulated decrease in DNA synthesis that occurs subsequent to E17.

A potential role for the renin–angiotensin system during development is suggested by previous studies [23,37]. Our laboratory has previously shown that ACE is found in high concentrations in the vascular media during development [23]. Moreover, Jones et al. [22] found that circulating Ang II levels are much higher in the fetus than in the adult rat. Using BrdU incorporation as a measure of DNA synthesis, we found that the $\text{AT}_1$ and $\text{AT}_2$ receptors mediate opposing growth-related functions. $\text{AT}_1$ pathways are growth-promoting, whereas $\text{AT}_2$ pathways are growth-inhibitory. Infusion of DuP753 decreased BrdU incorporation on E18, E19 and E21, while PD123319 infusion increased incorporation on each of these days [23]. Of note, the rates of DNA synthesis in the aortas of fetal mice receiving both DuP753 and PD123319 increased incorporation to levels that were indistinguishable from those observed with infusion of PD123319 alone, suggesting that some of the growth inhibitory effects of $\text{AT}_1$ receptor antagonists may be due to unopposed action of Ang II at the $\text{AT}_2$ receptor site. It is also noteworthy that while blockade of either receptor could influence the rates of vascular DNA synthesis, the developmentally regulated decrease of growth still occurred, albeit at either an enhanced ($\text{AT}_1$ receptor blockade) or blunted ($\text{AT}_2$ blockade) rate. This is not surprising since numerous other growth modulators are present during development and these can almost certainly influence the rates of vascular growth during these time periods.

One may argue that the effects of the angiotensin receptor antagonists during development are a result of effects on placental blood flow. Although placental flow could have been altered, several lines of evidence suggest that differences in the rates of vascular growth were not due to alterations in flow. First, no differences were observed between treated and control groups with regard to the gross parameters of growth (crown–rump length, fetal weight, litter size or congenital malformations), parameters that would certainly have been affected if significant effects on blood flow had occurred. Second, the alterations in BrdU incorporation observed with receptor blockade were restricted to the time period where the normal developmentally regulated decrease was observed. No effects of receptor blockade were seen prior to or after this phase, even though placental circulation is firmly established by day 15.

Our results suggest that the renin–angiotensin system may play an important role in vascular development and that alterations of the components of this system may influence this process. Indeed, vascular abnormalities (mainly renovascular) have been reported in ACE- and angiotensinogen knockout mice [38,39]. Individually, $\text{AT}_{1a}$ [40] and $\text{AT}_{1b}$ [41] receptor knockout mice do not exhibit abnormalities, while the dual $\text{AT}_{1a}/\text{AT}_{1b}$ knockout animals [42] exhibit abnormalities that are similar to that seen in the ACE and angiotensinogen knockouts. The $\text{AT}_2$ knockout mouse does not show vascular abnormalities under basal conditions [43]. One reason for the lack of vascular abnormalities in the $\text{AT}_2$ knockout animal (and the lack of more widespread abnormalities in the ACE, angiotensinogen and $\text{AT}_{1a}/\text{AT}_{1b}$ animals) may be the redundancy and compensatory regulation of the systems responsible for controlling growth and development. For example, pharmacologic blockade of the $\text{AT}_2$ receptor during development (Fig. 3) did not result in the long-term prolongation of the high rates of growth seen at day E15. What was observed was a rightward shift in the decrease by one–two days. Similarly, pharmacologic blockade of the $\text{AT}_1$ receptor did not immediately shut off DNA synthesis, but shifted the decrease leftward, again by one–two days. These results suggest that there are probably multiple regulatory pathways involved in the regulation of vascular development. Similarly, the knockout mice must have other pathways to compensate for the loss of the $\text{AT}_1$ or $\text{AT}_2$ receptors.

It should be mentioned that what appears to be ‘normal’ development in a knockout animal may be a lack of sensitivity to an alteration. For example, in a recent study, Akishita et al. [44] have shown that, in $\text{AT}_2$ receptor knockout mice undergoing vascular injury (by cuff placement on the femoral artery), the neointimal response is accentuated. This result suggests that the importance of the...
AT<sub>2</sub> receptor in the control of vascular growth can be unmasked in knockout animals when reparative systems are stressed.

The re-expression of a neonatal phenotype after tissue injury and in pathological conditions appears to be a consistent paradigm in biology. We and others have shown that the neointimal smooth muscle cells re-express a fetal program of gene expression involving contractile, cytoskeletal and extracellular matrix proteins as well as membrane bound proteins and receptors [24–28,33]. We therefore evaluated the effect of carotid artery injury on Ang II receptor expression. Our results were quite fascinating. First of all, the AT<sub>2</sub> receptor was re-expressed after injury and expression was maximal by 48 to 72 h. In contrast, AT<sub>1</sub> levels did not begin to elevate until 72 to 96 h, a time when the first wave of cell migration occurs. Of note, AT<sub>2</sub> levels comprised 10% of the receptors at 48 h after injury and dropped to less than 2% of the receptors by 96 h. We chose the 48-h time point to assess BrdU incorporation because of the favorable AT<sub>2</sub>:AT<sub>1</sub> ratio. Once again, our results indicated that the AT<sub>1</sub> and AT<sub>2</sub> receptors mediated opposing pathways. The fact that AT<sub>2</sub> antagonism increased BrdU incorporation is consistent with previous work by Janiak et al. [29], which showed that the compound CGP42112, an AT<sub>2</sub> receptor agonist [45], decreased neointimal development.

Our results also suggest that AT<sub>2</sub> receptor expression differs in various vascular beds. The expression of the AT<sub>2</sub> receptor in the adult aorta is consistent with the results of Viswanathan et al. [36], who reported that up to 20% of the Ang II receptors in the adult aorta were of the AT<sub>2</sub> subtype. However, these investigators failed to observe an increase in AT<sub>2</sub> receptor expression 15 days after aortic injury. The reason for the discrepancy between our observations is unknown but may be related to the different vessels examined, the timing of examination of the tissue, or the techniques employed. Our study used a competitive RT-PCR analysis of mRNA isolated from the vessel wall, whereas Viswanathan et al. [36] employed film autoradiography. Regardless of the relative merits of the various techniques, the expression of the AT<sub>2</sub> receptor in the injured vessel in our study provides important confirmation of the observation of Janiak et al. [29] and additional insight into the potential role of the AT<sub>2</sub> receptor in the regulation of vascular growth.

In summary, these data demonstrate a specific time course of expression of the AT<sub>1</sub> and AT<sub>2</sub> receptors during vascular development and after vascular injury. Our data suggest that both receptors have functional significance during these processes. It appears that the AT<sub>2</sub> receptor mediates cellular differentiation and/or inhibits cellular proliferation. Given the limited tissue distribution of the AT<sub>2</sub> receptor in the adult, this receptor may be a viable target in the treatment of the clinical disorders of vascular growth, such as restenosis and atherosclerosis. In light of the clinical problems associated with restenosis, further investigation into the function and role of the AT<sub>2</sub> receptor is warranted.

Acknowledgements

This research was funded by NIH grants HL42663 and HL58516. Howard Hutchinson was funded by an NIH Training Grant HL07708 and was a fellow of the American Heart Association Bugher Center for Molecular Biology. Lutz Hein was a recipient of the Deutsche Forschungsgemeinschaft. The authors thank Drs. Victor J. Dzau, Masa Horiuichi and Gary H. Gibbons for helpful discussions.

References


