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Physiology

The angiotensin II type 2 (AT_2) receptor antagonizes the growth effects of the AT_1 receptor: Gain-of-function study using gene transfer

MASATOSHI NAKAJIMA, HOWARD G. HUTCHINSON, MASAHIKO FUJINAGA, WATARU HAYASHIDA, RYUICHI MORISHITA, LUNAN ZHANG, MASATSUGU HORIUCHI, RICHARD E. PRATT, AND VICTOR J. DZAU

Falk Cardiovascular Research Center, Stanford University School of Medicine, Stanford, CA 94305

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ABSTRACT **The type 1 angiotensin II (AT_1) receptor is well characterized but the type 2 (AT_2) receptor remains an enigma. We tested the hypothesis that the AT_2 receptor can modulate the growth of vascular smooth muscle cells by transfecting an AT_2 receptor expression vector into the balloon-injured rat carotid artery and observed that overexpression of the AT_2 receptor attenuated neointimal formation. In cultured smooth muscle cells, AT_2 receptor transfection reduced proliferation and inhibited mitogen-activated protein kinase activity. Furthermore, we demonstrated that the AT_2 receptor mediated the developmentally regulated decrease in aortic DNA synthesis at the latter stages of gestation. These results suggest that the AT_2 receptor exerts an antiproliferative effect, counteracting the growth action of AT_1 receptor.**

The peptide angiotensin II (Ang II) exerts a range of actions on the kidney, heart, vascular system, adrenal gland, and central nervous system in physiological and pathophysiological states. Ang II also regulates growth of vascular smooth muscle cells (VSMCs) (1–5). *In vitro*, Ang II stimulates growth and migration through the induction of autocrine growth factors (1). *In vivo*, continuous infusion of Ang II enhanced VSMC proliferation in the injured rat arterial wall (2). Powell *et al.* (3) reported that angiotensin-converting enzyme (ACE) inhibitors can prevent neointimal proliferation following balloon injury in rats. However, evidence also suggests that the increase in kinins observed with ACE inhibitors may also play a role (5, 6).

Ligand binding studies have demonstrated the existence of at least two distinct types of Ang II receptors (AT_1 and AT_2 subtypes) (7, 8). The AT_2 receptor is abundantly and widely expressed in fetal tissues (9–11) but is present at low levels, if at all, in adult tissues (7–13). Interestingly, this receptor is reexpressed in wound repair and cardiac hypertrophy and after vascular injury (14, 15). The highly abundant expression of this receptor during embryonic and neonatal development has led to the suggestion that this receptor may be involved in tissue growth and/or differentiation. The recent cloning of the AT_2 receptor (16–18) will provide the opportunity to examine in more detail the physiologic effects of this receptor.

VSMCs may be an appropriate model to examine the function of the AT_2 receptor. The AT_2 receptor is highly expressed in the embryonic and neonatal aorta but is markedly reduced in the adult vessel (10, 11). In this study, we used a "gain-of-function" approach to examine by *in vivo* gene transfer (19) the effect of overexpression of the AT_2 receptor in vascular smooth muscle *in vivo* and in cell culture in order to test the hypothesis that this receptor can modulate the growth of these cells.

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EXPERIMENTAL PROCEDURES

Gene Transfer Techniques. The rat AT_2 receptor cDNA (16) was inserted into an expression vector (pUC-CAGGS containing the cytomegalovirus enhancer and the chicken β-actin promoter). The parental vector was used as a control vector. *In vivo* gene transfer into balloon-injured carotid arteries or cultured VSMCs was performed using DNA–liposome complex coated with UV-inactivated hemagglutinating virus of Japan (HVJ) as described (19–22). We have previously determined that the transfection efficiency using this approach is 20–30% *in vivo* as well as in cell culture (19).

Reverse Transcription PCR (RT-PCR). Total RNA was extracted 3 days after transfection. RT-PCR analysis of RNA (1 μg) was performed using a GeneAmp PCR reagent kit (Perkin–Elmer/Cetus). PCR primers for AT_2 receptor are as follows: 5′-ATTCCTGTTCTCTCTACTAC-3′ and 5′-GTAACACGTTGCTATGAA-3′. PCR primers for rat glyceraldehyde-3-phosphate dehydrogenase were purchased from Clontech.

Radioligand Binding Studies. Isolation of crude membrane fractions and binding of ^{125}I-labeled (^{125}I)CGP42112A or ^{125}I-[Sar^1,Ile^8]Ang II was performed as described (16, 17). Nonspecific binding was determined in the presence of unlabeled CGP42112A (10^{-6} M) or [Sar^1,Ile^8]Ang II (10^{-6} M).

Measurements of DNA Synthesis *in Vivo*. *Balloon injury model.* Bromodeoxyuridine (BrdUrd) was injected 3 days after injury and transfection [100 mg/kg s.c. and 30 mg/kg i.p. at 18 h prior to sacrifice and then 30 mg/kg i.p. at 12 h prior to sacrifice (23, 24)]. Immunohistochemistry by using anti-BrdUrd antibodies (Amersham) was performed as described.

Fetal aortic model. Two sets of experiments were performed. In the first, Alzet miniosmotic pumps, implanted i.p. into pregnant rats (day 17), delivered vehicle (PEG/saline, 1:1) or PD123319 (20 mg/kg per day). PD123319 readily crosses the placenta (J. Keiser, personal communication). In addition, the fetuses were injected i.p. with 10 μl of vehicle (PEG/saline, 1:1) or PD123319 (10 μl of 5 mg/ml solution) once a day for 3 days. The pregnant animals were injected with BrdUrd (24) (100 mg/kg) at 17, 9, and 1 h before sacrifice, which occurred on gestational day 20. Nuclear labeling of BrdUrd in fetal thoracic aortae was determined as above. Experiment 2 was performed in an identical fashion with the exception that the embryos were not injected with the drug. Tissues were harvested at embryonic days 15, 18, 19, and 21.

This dose of PD123319 resulted in a plasma drug level of 248 $\pm$ 38 nM (n = 9) in the adult circulation, as determined by radioreceptor assay. In fetal homogenates, drug levels were <50 nM. These drug levels are sufficient to block selectively

Abbreviations: Ang II, angiotensin II; AT_1 receptor, angiotensin II type 1 receptor; AT_2 receptor, angiotensin II type 2 receptor; VSMC, vascular smooth muscle cell; HVJ, hemagglutinating virus of Japan; MAP kinase, mitogen-activated protein kinase; RT, reverse transcription; E, embryonic day.

the AT_2 receptors but have no effects on the AT_1 receptor (7, 8).

Cell Culture. Adult rat (WKY) aortic VSMCs (passages 4–8) were maintained in Waymouth's medium with 5% calf serum (25). For experiments, cells were plated in 24-well plates, grown to confluence, and transfected with 3 μg of DNA per well. The following day, the cells were fed medium containing insulin (5×10^{-7} M), transferrin (5 μg/ml), and ascorbate (0.2 mM) for 2 days to induce quiescence (25). At this point, time 0, the cell densities were $0.79 \pm 0.04 \times 10^5$ and $0.77 \pm 0.02 \times 10^5$ cells per cm^2, control vector- and AT_2 receptor-transfected VSMCs, respectively. Thus, at the start of the experiment, the different wells are at essentially the same cellular density. At this time, Ang II (3×10^{-7} M) alone, with PD123319 (10^{-5} M), or with DuP753 (10^{-5} M) were added daily for 3 days. Cell numbers were determined by Coulter Counter. We have previously determined that the transfection efficiency using this approach is 20–30% (19).

Assay of Mitogen-Activated Protein (MAP) Kinase Activity. MAP kinase was assayed for its ability to phosphorylate myelin basic protein as described (26) with modification.

RESULTS

The AT_2 receptor mRNA was highly expressed in the AT_2 receptor cDNA-transfected injured vessels but only at low levels in the control vector-transfected injured vessels and was not detectable in the untransfected uninjured vessels (Fig. 1*A*). Using a quantitative competition-based RT-PCR technique, we examined the levels of the AT_2 receptor mRNA; the level of the AT_2 receptor mRNA in the AT_2 receptor-transfected injured vessel at 3 days was 80 fmol/mg of total RNA as compared with 4 fmol/mg of total RNA found in the injured, control vector-transfected vessel (Table 1). The PCR signal was due to amplification of RNA since the samples were treated with DNase prior to amplification. Moreover, treatment of the sample with RNase abolished the signal (data not shown). Consistent with the low levels of AT_2 receptor mRNA in the nontransfected, injured artery (Table 1) examined at 4 days, Scatchard analysis (Fig. 1*B*) of membranes from control vector-transfected, injured carotid arteries demonstrated a single class of binding sites for ^{125}I-[Sar^1,Ile^8]Ang II. No specific binding of ^{125}I-CGP42112A, an AT_2 receptor ligand, was observed. In contrast, specific binding sites for ^{125}I-CGP42112A were observed 4 days after AT_2 transfection, showing that one-fourth of the total Ang II receptors was of the AT_2 subtype. The expression of the AT_2 receptor transgene as measured by RT-PCR and ligand binding studies reveals that the levels achieved in the vessel wall are 5% of that found in the neonatal vessel and only 25% of the total Ang II binding in vessel membrane preparations (Table 1 and Fig. 1). *In situ* autoradiography demonstrates that the AT_2 receptor expression is primarily in medial smooth muscle (data not shown).

Table 1. Relative expression of AT_2 receptor mRNA in the vessel wall

Tissue	*n*	AT_2 mRNA level, fmol/mg of total RNA	Ratio
Uninjured adult carotid artery	5	Undetectable	—
Injured adult carotid artery (4 days)	3	4 ± 2	1
Transfected, injured adult carotid artery (4 days)	3	80 ± 9	20
Neonatal aorta (day 1 postpartum)	3	1500 ± 210	375

Total RNA was isolated from the above vessels and the level of AT_2 mRNA was determined by a competition-based, quantitative RT-PCR assay. RT-PCR was carried out as described in the legend to Fig. 1 with the addition of increasing levels of a competitor RNA that was identical to the targeted sequence in the endogenous AT_2 mRNA with the exception of a 260-bp deletion (expected sizes are 554 bp for the endogenous mRNA and 294 bp for the competitor). The PCR products were resolved by gel electrophoresis; 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 the quantitation of the endogenous sequence. The results are expressed as fmol of AT_2 mRNA per mg of total RNA (mean ± SEM).

Transfection of the AT_2 receptor expression vector resulted in a 70% reduction in neointimal area compared to vessels transfected with the control vector (Fig. 2 *A–E*). This inhibitory effect on neointimal formation was blocked by treatment with the AT_2 receptor antagonist, PD123319 (20 mg/kg per day), administered via osmotic minipump implanted i.p. at the time of vascular injury, suggesting that the action is due to the AT_2 receptor. On the other hand, treatment with PD123319 did not affect the development of the neointimal lesion in

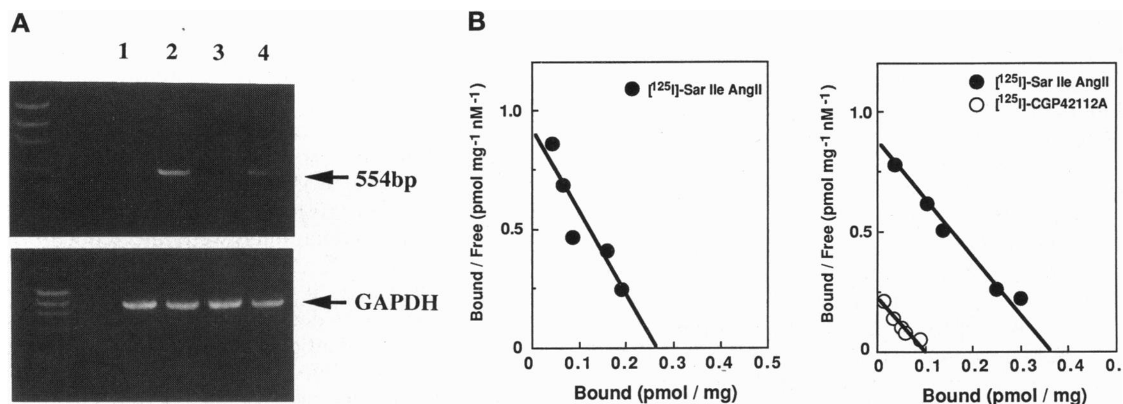

FIG. 1. The AT_2 receptor expression vector directs the synthesis of the AT_2 receptor in transfected arteries. (*A*) RT-PCR (30 cycles) of RNA from rat carotid arteries transfected 3 days earlier with a control vector or an AT_2 receptor expression vector. Lanes: 1, uninjured vessels; 2, injured vessels transfected with AT_2 receptor expression vector; 3, injured vessels transfected with control expression vector; 4, whole rat fetus (16–18 days). (Top) AT_2 receptor. (Bottom) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (When amplification was carried out for 40 cycles, an increase in AT_2 mRNA in the untransfected vessel could be observed following injury.) (*B*) Scatchard plots of ^{125}I-[Sar^1,Ile^8]Ang II and ^{125}I-CGP42112A binding to the membrane fractions prepared from control vector (Left) and AT_2 receptor expression vector (Right) transfected rat carotid arteries. In control vessels, only AT_1 binding was observed (K_d and B_{max}, 0.34 nM and 0.29 pmol/mg of protein, respectively). In AT_2 transfected vessels, AT_1 and AT_2 binding was observed (K_d and B_{max} for ^{125}I-[Sar^1,Ile^8]Ang II, 0.38 nM and 0.34 pmol/mg of protein, respectively; K_d and B_{max} for ^{125}I-CGP42112A, 0.28 nM and 0.085 pmol/mg of protein, respectively). These experiments were repeated three times with essentially identical results.

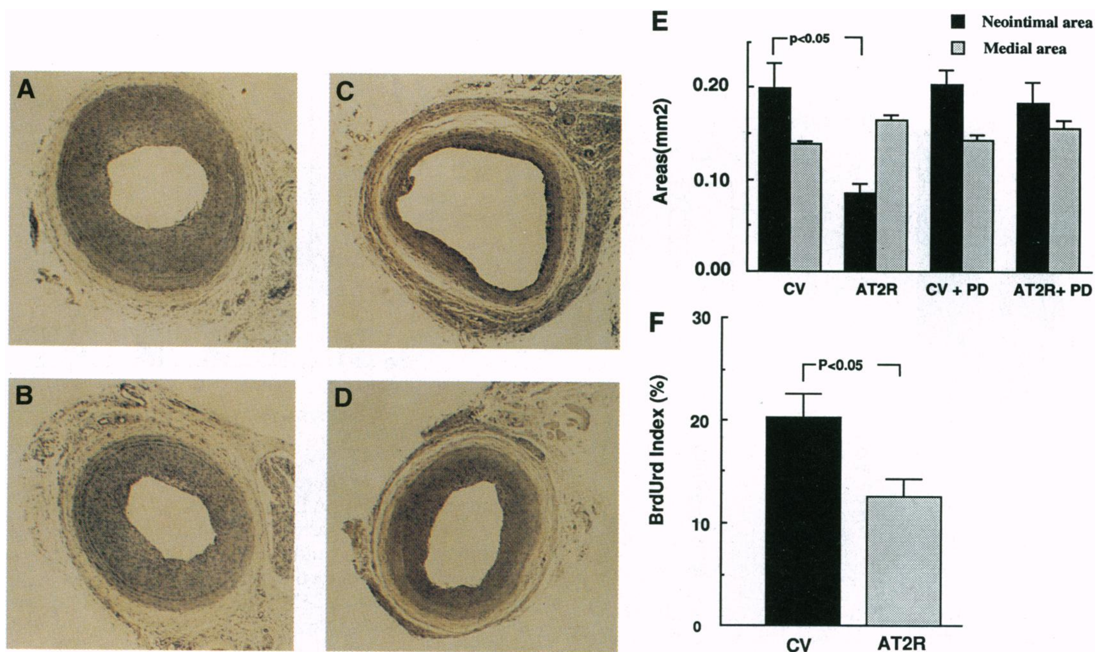

FIG. 2. Overexpression of the AT_2 receptor transgene results in the attenuation of neointimal development. Fourteen days after transfection with the control vector or the AT_2 expression vector, the vessels were isolated and examined by morphometry. (*A*) Control vector-transfected rat carotid artery. (*B*) Control vector-transfected carotid artery from rats treated with PD123319. (*C*) AT_2 receptor expression vector-transfected carotid artery. (*D*) AT_2 receptor expression vector-transfected carotid artery from rats treated with PD123319. (×30.) (*E*) Multiple animals were examined as above and the areas of the neointima and media were measured. Results are expressed as the mean ± SEM; $P < 0.05$ in Scheffe's test. CV, control vector-transfected arteries from rats treated with vehicle ($n = 9$); CV + PD, control vector-transfected arteries from rats treated with PD123319 ($n = 8$); AT2R, AT_2 receptor expression vector-transfected arteries from rats treated with vehicle i.p. ($n = 6$); AT2R + PD, AT_2 receptor expression vector-transfected arteries from rats treated with PD123319 ($n = 6$). Data were obtained in a blinded fashion. (*F*) DNA synthesis was assessed by determining the incorporation of BrdUrd into the vessel wall. Results are calculated as the ratio of BrdUrd-positive nuclei/total nuclei and are expressed as the mean ± SEM; $P < 0.05$ in Student's *t* test. CV, control vector-transfected arteries ($n = 6$); AT2R, AT_2 receptor expression vector-transfected arteries ($n = 6$). Data were obtained in a blinded fashion.

control vector-transfected injured vessels, consistent with the low-level expression of the endogenous gene. We examined the rates of DNA synthesis [as assessed by BrdUrd incorporation (23)] 4 days after injury (Fig. 2*F*). In uninjured vessels, <1% of the cells of the media stained positively for BrdUrd while in the control-transfected injured vessel, 20.2% of the medial cells stained positively. AT_2 receptor transfection significantly decreased BrdUrd incorporation to 12.6%, suggesting that one of the actions of the AT_2 receptor is the inhibition of progression of the cells into the S phase.

To examine further this action of the AT_2 receptor, cultured rat aortic VSMCs, which express endogenously only the AT_1 receptor, were transfected with the AT_2 receptor vector or control vector. AT_2 receptor binding was detected in the AT_2 receptor vector-transfected cells but not the nontransfected cells (Table 2). The levels achieved were 20% of the total Ang II binding (Table 1). In confluent, quiescent cells, transfected with the control vector, Ang II (3×10^{-7} M) increased the cell number (25) (Table 2). This increase was mediated by AT_1 receptor, as it was abolished by DuP753. In contrast, in cells transfected with AT_2 receptor, Ang II treatment had little effect on cell number. Furthermore, in AT_2 receptor-expressing cells, treatment with Ang II plus PD123319 for 3 days increased the accumulation of cells to a level comparable to that observed in control vector-transfected cells treated with Ang II. These data demonstrate that the proliferative action of Ang II was mediated by the AT_1 receptor, whereas the antiproliferative effect was mediated by the AT_2 receptor.

We next examined the potential intracellular signaling pathway that may mediate the action of the AT_2 receptor. We hypothesize that the AT_2 receptor may antagonize the MAP kinase pathway since the growth actions of the AT_1 receptor are mediated in part by this signal mechanism (27, 28). We observed that treatment of the control vector-transfected cells with Ang II resulted in an AT_1 receptor-mediated increase in MAP kinase activity (Fig. 3). DuP753, but not PD123319, blocked the effects of Ang II (data not shown). Conversely, in cells transfected with the AT_2 receptor vector, the AT_1-mediated increase in MAP kinase activity was greatly attenuated as compared to the control vector-transfected cells. Furthermore, in the presence of PD123319, Ang II stimulated MAP kinase activity in the AT_2 receptor vector-transfected cells to a value indistinguishable from that of the Ang II-

Table 2. Effect of AT_2 receptor transgene expression on Ang II-induced VSMC growth *in vitro*

Treatment	*n*	% transfection	
		CV	AT_2 receptor
Control	12	100	100
Ang II	12	194 ± 7	128 ± 3*
+ PD123319	12	188 ± 5	175 ± 5
+ DuP753	12	119 ± 2	82 ± 3*

Confluent VSMCs were transfected with either the AT_2 expression vector or a control vector. The following day, the cells were placed in a defined serum-free medium for 2 days to induce quiescence (25). At this time, Ang II (3×10^{-7} M) alone, with PD123319 (10^{-5} M), or with DuP753 (10^{-5} M) were added daily for 3 days. Cell numbers in control medium were taken as 100% [$1.07 \pm 0.04 \times 10^5$ cells per cm^2 from control vector (CV)-transfected VSMCs and $0.98 \pm 0.03 \times 10^5$ cells per cm^2 for AT_2 receptor-transfected VSMCs]. Data show mean ± SEM; *n*, number of wells for each condition; *, statistically significant difference from CV ($P < 0.001$ in Scheffe's test). Transfection of VSMCs with the AT_2 expression vector, performed as described (19, 20, 22), resulted in the expression of AT_2 binding sites (AT_1 receptor: B_{max}, 1.63 pmol/mg of protein; K_d, 0.11 nM. AT_2 receptor: B_{max}, 0.385 pmol/mg of protein; K_d, 0.37 nM).

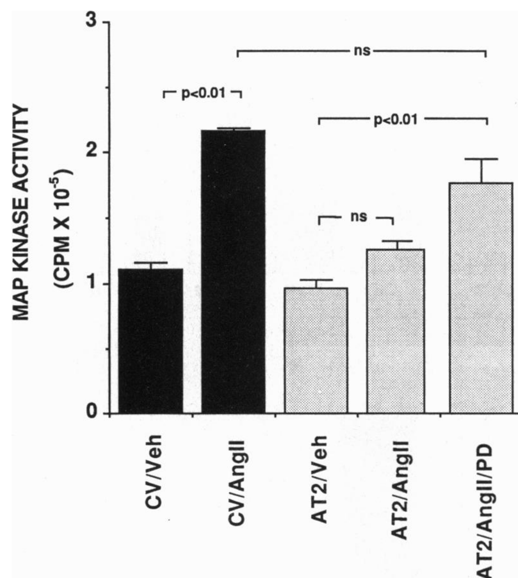

FIG. 3. The Ang II receptors mediate opposing effects on MAP kinase activity. VSMCs were transfected with a control vector or the AT_2 receptor expression vector as in Table 2. Cells were then treated with Ang II (3×10^{-7} M) and the effects on MAP kinase activity were assessed. In the control transfected cells, Ang II induces MAP kinase activity, whereas in the cells expressing the AT_2 receptor transgene, Ang II has no significant effect. In these cells, blockade of the AT_2 receptor with PD123319 (10^{-5} M) allows the Ang II-induced increase in MAP kinase activity. Data are expressed as cpm and were analyzed by ANOVA followed by the Scheffe F test (n = three plates per condition). ns, Nonsignificant ($P > 0.05$).

treated control cells. Taken together, these results demonstrate that the two Ang II receptor subtypes exert opposing effects on MAP kinase activity and suggest that antigrowth action of AT_2 receptor is mediated by its inhibitory effect on MAP kinase.

To examine further the physiological significance of the AT_2 receptor-mediated antigrowth effects, we examined the effects of Ang II receptor blockade on the rates of DNA synthesis in the developing aorta during the period of high AT_2 receptor expression. We have shown that the levels of AT_2 receptor expression are low during early embryonic development [embryonic day 15 (E15)] but are high during the later stages of development (E16–E21) and in the neonate (Table 1). The physiologic role of the AT_2 receptor in the developing fetal aorta was examined *in utero* using PD123319. Drug was administered for 3 days prior to tissue harvest and the rates of DNA synthesis in the fetal aortae *in utero* were measured by incorporation of BrdUrd during a 24-h period prior to tissue harvest. As previously shown (24), the rates of DNA synthesis in the fetal aorta are developmentally regulated (Fig. 4). At early times, when the AT_2 receptor is not expressed and aortic DNA synthesis rates are at near maximum (E15, prior to the developmentally regulated decrease in DNA synthesis), PD123319 has no effect on DNA synthesis. However, between E16 and E21, the growth rates in the fetal aorta are declining as the AT_2 receptor is expressed. PD123319 treatment attenuates significantly this reduction in aortic DNA synthesis. Based on these data, one would conclude that the AT_2 receptor modulates the growth of the blood vessel, perhaps by counteracting the growth-stimulatory effects of developmentally regulated growth factors or by some other mechanisms such as apoptosis.

DISCUSSION

The above data demonstrate that in three models of smooth muscle cell growth the AT_2 receptor exerts an inhibitory effect

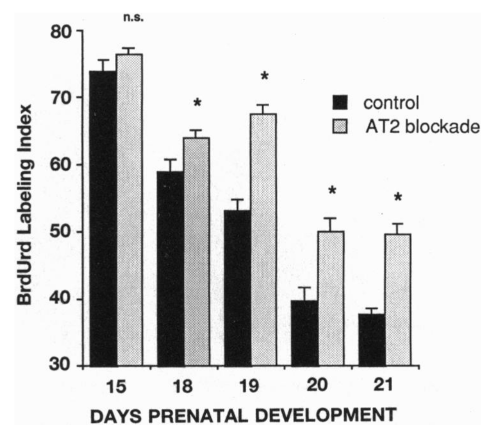

FIG. 4. The AT_2 receptor modulates DNA synthesis in the fetal aorta. Pregnant rats were implanted with Alzet minipumps delivering either vehicle or the AT_2 antagonist, PD123319, for 3 days prior to harvesting of fetal aortae. One day prior to harvest, BrdUrd was administered. DNA synthesis rates in the fetal aortae were assessed by immunochemical detection of the BrdUrd into nuclei. Note that administration of PD123319 attenuates the developmentally regulated decrease in DNA synthesis. Data are expressed as % nuclei that were BrdUrd positive and were analyzed by ANOVA followed by the Fisher PLSD test (n = three embryos at day 15 and five embryos at the other time points). n.s., Nonsignificant ($P > 0.05$); *, $P < 0.05$.

on cell proliferation. The data also show that AT_2 receptor activation blocks the growth response to AT_1 receptor activation (1–6, 25). The opposing effects of these receptors suggest an important antagonistic interaction between the two receptor subtypes.

We examined the effects of the AT_2 receptor on vascular smooth muscle growth *in vivo* using the model of balloon injury of rat carotid artery for several reasons. First, this is a simple model of *in vivo* smooth muscle proliferation and migration (29) that will permit the examination of the "antigrowth" effects of the AT_2 receptor *in vivo*. Furthermore, it has been shown in the rat that this process involves the participation of the AT_1 receptor, which stimulates the production of autocrine growth factors (1–5). Thus, this model provides an ideal situation to study potential mitogenic/antimitogenic actions induced by the AT_1 vs. the AT_2 receptor.

The development of a neointimal lesion in this model can be broadly divided into three phases: replication of medial smooth muscle cells, migration of the medial smooth muscle cells into the intima, and further proliferation in the intima (4, 29). Our data suggest that overexpression of the AT_2 receptor transgene in the medial smooth muscle cells in the first and early phase inhibits the subsequent later development of the neointima, at least in part by a reduction in the rates of DNA synthesis in the medial cells during the first phase.

Cultured adult rat aortic VSMCs, which express only AT_1 receptor, provide an opportunity to study the interaction of AT_1 and AT_2 receptors using *in vitro* gene transfer of the AT_2 receptor. With these cells, we demonstrated that the expression of the AT_2 receptor blocked the AT_1 receptor-mediated cell growth through an inhibition of MAP kinase activity. Potential mechanisms include direct inhibitory actions on AT_1 receptor signal transduction (e.g., via MAP kinase phosphatase). Cellular mechanisms including direct influences on cellular differentiation and/or the induction of apoptosis are also possible. Clearly, more studies concerning the intracellular actions and consequences of AT_2 receptor stimulation are warranted.

The use of gene transfer to examine the actions of the AT_2 receptor does not fully address potential physiologic effects of the receptor. For this reason, we examined the effects of AT_2 receptor blockade on the growth of vascular smooth muscle

under a physiological condition in which the receptor is highly expressed—i.e., the developing aorta. We chose to study the embryonic aorta (E16–E21) since the level of AT_2 receptor expression is increased. During embryonic and fetal development, the rates of proliferation of the vascular smooth muscle are extremely high with 75–80% of the cells incorporating BrdUrd in a 24-h period. Cooke *et al.* (24) reported (confirmed in Fig. 4) that there is a developmentally timed decrease in these rates of proliferation around day E18–E19, which is at a time when the AT_2 receptor is also highly expressed, leading us to hypothesize that the decrease may be mediated in part by the AT_2 receptor. Indeed, our observation that AT_2 receptor blockade resulted in a significant increase in aortic DNA synthesis strongly supports this hypothesis. These results should be viewed in light of the fact that the maximum observed rate of DNA labeling during the embryonic/fetal period is ≈74% per day; thus, an effect of PD123319 increasing the labeling index from 53% to 68% on day 19 may be mathematically only a 28% increase but is actually achieving a 71% recovery of the maximum observed *in utero* value.

In summary, the results of this study demonstrate a functional antagonism between AT_2 and AT_1 receptors with respect to VSMC growth. In addition, these results provide insight into a possible broader antagonistic role of AT_2 receptor on AT_1 receptor actions as well as AT_2 receptor's overall contribution to the growth, differentiation, and development of other tissues. For example, during the course of these studies, Stoll *et al.* (30) reported the inhibition of endothelial cell growth mediated by the AT_2 receptor. Moreover, antagonism between the AT_1 and AT_2 receptors with respect to phosphatidylinositol hydrolysis has been reported in rat skin fibroblasts (31). Additional information is needed concerning the potential actions of the AT_2 receptor and interactions with the AT_1 receptor during pathophysiological situations such as during neointimal hyperplasia or heart failure, situations in which the AT_2 receptor is expressed (14). These data should have implications in vascular biology and in future drug development.

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