Proapoptotic and Growth-Inhibitory Role of Angiotensin II Type 2 Receptor in Vascular Smooth Muscle Cells of Spontaneously Hypertensive Rats In Vivo

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Abstract—Angiotensin type 2 (AT_2) receptors for angiotensin II suppress cell growth and induce apoptosis in vitro, but their role is poorly defined in vivo. We reported that transient induction of smooth muscle cell (SMC) apoptosis precedes DNA synthesis inhibition and aortic hypertrophy regression in spontaneously hypertensive rats treated with the AT_1 antagonist losartan or the converting-enzyme inhibitor enalapril. Although both drugs are equipotent in reducing SMC number, apoptosis occurs significantly earlier with losartan than enalapril. To examine the role of AT_2 receptors in this model, spontaneously hypertensive rats were given valsartan, an AT_1 antagonist, or enalapril, in combination or not with the AT_2 antagonist PD123319 for 1 or 2 weeks. Control rats received vehicle. Systolic blood pressure was reduced similarly by valsartan and enalapril but it was not significantly affected by PD123319. Angiotensin II plasma levels were increased (6-fold) with valsartan and reduced (80%) with enalapril but unaffected by PD123319. Valsartan significantly increased internucleosomal DNA fragmentation indicative of apoptosis at 1 week only (2.7-fold) and significantly reduced aortic mass (18%), SMC number (33%), and DNA synthesis (24%, measured by ^3_H-thymidine incorporation) at 2 weeks. These valsartan-induced changes were prevented by PD123319. In contrast, enalapril-induced DNA fragmentation (2-fold increase at 2 weeks) was not affected by PD123319. PD123319 given alone did not affect growth or apoptosis. AT_1 and AT_2 receptor mRNAs were detected in the aorta by reverse transcription–polymerase chain reaction. Together, these results provide the first evidence that AT_2 receptors mediate vascular mass regression by stimulating SMC apoptosis in vivo, an effect seen during AT_1 receptor blockade but not during converting-enzyme inhibition. (Hypertension. 2000;35:1069-1073.)

Key Words: angiotensin II n muscle, smooth, vascular n losartan n enalapril

Two main subtypes of specific cell membrane receptors for angiotensin II (Ang II) have been pharmacologically defined and cloned, namely angiotensin type 1 (AT_1) receptors, which are blocked specifically by antagonists such as valsartan, and angiotensin type 2 (AT_2) receptors, which are blocked specifically by PD123319. AT_1 receptors represent the predominant receptor subtype in the adult rat arterial wall, in which they stimulate smooth muscle cell (SMC) contraction and growth. Although AT_2 receptors are predominantly expressed in SMC during fetal development, low levels of expression are found in the aorta of adult rats including spontaneously hypertensive rats (SHR). A growing body of evidence suggests that AT_1 and AT_2 receptors elicit counter-acting influences on the cell growth-death balance. In cultured SMC, AT_1 receptors promote growth and inhibit apoptosis, whereas AT_2 receptors elicit opposite effects. However, AT_2 receptor regulation of SMC number by apoptosis has never been examined in vivo. Moreover, the role of AT_2 receptors in vascular remodeling in vivo is controversial.

The regression of vascular hypertrophy is a potential therapeutic target for the reduction of complications associated with hypertension. In SHR, aortic hypertrophy is associated with increased vascular mass and DNA content. SHR SMC show enhanced growth in vitro and in vivo and an increased propensity to undergo apoptosis in response to growth factor withdrawal in vitro. Recently, we reported that blockade of the Ang II pathway in SHR induces a transient increase in aortic SMC apoptosis at the onset of vascular hypertrophy regression, an effect that is not secondary to blood pressure reduction. Although the AT_1 receptor antagonist losartan and the angiotensin-converting enzyme (ACE) inhibitor enalapril are equipotent in reducing SMC number in the SHR aorta, SMC apoptosis occurs significantly earlier with losartan (at 1 week) than with enalapril (at 2 weeks). Inhibition of SMC DNA synthesis is also observed, but it is sustained and temporally dissociated because it occurs after the early time window of SMC apoptosis. AT_1 receptor blockade in vivo increases Ang II plasma levels.
and allows unopposed activation of AT<sub>2</sub> receptors, whereas suppression of Ang II production by ACE inhibitors reduces activation of both AT<sub>1</sub> and AT<sub>2</sub> receptors. Thus, we hypothesized that Ang II acting through AT<sub>2</sub> receptors may be involved in SMC apoptosis stimulation in response to AT<sub>1</sub> receptor blockade but not in response to ACE inhibition. To the best of our knowledge, this is the first report of AT<sub>2</sub> receptor–dependent cell deletion by apoptosis in the cardiovascular system in vivo.

**Methods**

Nine- to 10-week-old male SHR were purchased from Charles River (St Constant, Canada) and housed for ≥10 days before study. Food and water were administered ad libitum. Rats (n=5 to 8 per group) were treated for 1 to 2 weeks with the selective AT<sub>1</sub> antagonist valsartan (30 mg/kg per day; gift of Novartis, Toronto, Canada) in the drinking water, the selective AT<sub>2</sub> antagonist PD123319 (30 mg/kg per day; gift of Parke-Davis, Ann Arbor, Mich) by continuous subcutaneous infusion with an osmotic minipump (Alzet, model 4 ML1 or 4 ML2), or a combination of both drugs. PD123319 was dissolved in saline (pH 3.0, adjusted with 0.1 mol/L sodium citrate).

A subgroup of rats received enalapril (30 mg/kg per day PO, Sigma Chemical Co) in combination or not with PD123319 for 2 weeks. Control animals received vehicle. All animal manipulations were conducted according to institutional guidelines.

Systolic blood pressure was determined in conscious, restrained rats by the tail-cuff method as we described previously. Rats were killed, and the aorta media was prepared for the following measurements as we described previously: vascular cross-sectional area, SMC number (using the 3-dimensional dissector method), DNA synthesis (in vivo ³H-thymidine incorporation into DNA during the last 2 hours before death), and the oligonucleosomal DNA fragmentation index (indicative of apoptosis). To examine aortic gene expression for AT<sub>1</sub> and AT<sub>2</sub> receptors, 4 untreated SHR were killed, and the thoracic aortas were isolated, dissected, and cut longitudinally into 2 halves. One half was immediately frozen; the other was denuded of endothelium as described above before freezing. Total mRNA was extracted, and reverse transcription–polymerase chain reaction (RT-PCR) was used as we described previously, with minor modifications. Briefly, RT was performed in a reaction volume of 30 μL containing 0.55 μg RNA, 1.5 μL of 10 mmol/L dNTP, 6 μL of BRL 5x buffer, 0.6 μL Oligo (dT)₁₂₋₁₈ primer (0.5 μg/μL), 1.5 μL of 200 U/μL M-MLVRT, 0.9 μL RNasin (RNAse inhibitor) 40 U/μL, and 3 μL of DTT 0.1 mol/L at 37°C for 1 hour. The reaction was inactivated at 95°C for 5 minutes. After first-strand synthesis of RNA, 2 μL cDNA was amplified with specific primers as described previously.

The amplification profile involved denaturation at 95°C for 5 minutes, annealing at 57°C for 60 seconds, and extension at 72°C for 60 seconds for 30 cycles. After amplification, PCR products were electrophoresed on a 1.5% agarose gel for 1 hour at 9 V/cm gel. Bands corresponding to RT-PCR products were visualized by UV light after agarose gel electrophoresis, and their intensities were measured by densitometry.

**Statistics**

Values are presented as mean±SEM. Data from treated groups were compared with those of the control group by ANOVA and an unpaired Student’s t test with Bonferroni correction for multiple comparisons. A value of P<0.05 was considered statistically significant.

**Results**

Expression of mRNA of both AT<sub>1</sub> and AT<sub>2</sub> receptors was evident in denuded aorta, suggesting that both receptor subtypes are expressed in aortic SMC (Figure 1). The nondenuded aorta showed similar levels of AT<sub>1</sub> receptor expression as compared with the denuded vessel but a tendency toward higher levels of AT<sub>2</sub> receptor expression, possibly caused by the presence of endothelium.

**Study With Valsartan**

In rats treated with valsartan alone, high blood pressure was significantly reduced after 1 and 2 weeks (170±3 and 164±3 mm Hg, respectively) as compared with control animals (191±3 and 189±4 mm Hg, respectively). Cotreatment with PD123319 did not affect the antihypertensive effect of valsartan (165±3 mm Hg at 2 weeks). PD123319 alone did not significantly affect blood pressure (178±3 mm Hg at 2 weeks). Final body weights were not significantly affected by valsartan (223±10 g), PD123319 (258±7 g), or valsartan+PD123319 (225±10 g), as compared with control animals (245±7 g). Within 2 weeks, PD123319 alone did not affect SMC DNA fragmentation (Figure 2A) and DNA synthesis (Figure 2B) or final SMC number (Figure 2C), medial cross-sectional area (Figure 2D), and aortic mass (5.24±0.29 mm/mm per gram ×10⁻³ vs control values: 5.14±0.17 mm/mm per gram ×10⁻³). In contrast, administration of valsartan alone significantly increased SMC DNA fragmentation (2.7-fold at 1 week only) and reduced aortic SMC number (by 33% at 2 weeks), suggesting SMC death by apoptosis. Valsartan-induced SMC apoptosis at 1 week was followed at 2 weeks by a significant reduction of SMC DNA synthesis, medial cross-sectional area, and aortic mass (18% reduction). However, coadministration of PD123319 prevented valsartan-induced changes in DNA fragmentation.
DNA synthesis, SMC number, medial cross-sectional area, and aortic mass (5.03±0.25 mg/mm per gram ×10⁻³ at 2 weeks). The total radioactivity in aortic smooth muscle homogenates was not different between treated and untreated animals (eg, 46±3 cpm/10 mg protein in the valsartan group at 2 weeks vs 50±7 cpm/10 mg protein in the control group), thus ruling out differences in tissue uptake of ³H-thymidine. Ang II plasma levels (485±84 pg/mL in the control group) were increased significantly after 2 weeks in the valsartan group (2916±457 pg/mL) or valsartan+PD123310 group (3195±540 pg/mL). PD123319 alone did not affect Ang II levels (561±76 pg/mL).

Study With Enalapril

We showed previously that SMC DNA fragmentation is increased but that DNA synthesis and aortic hypertrophy are not yet reduced after a 2-week treatment with enalapril in the SHR aorta.¹³ We examined whether coadministration of PD123319 affects the induction of SMC apoptosis at 2 weeks of enalapril therapy. Valsartan-treated rats were used as positive controls. In this study, high blood pressure was significantly and similarly reduced by enalapril (143±1 mm Hg) and valsartan (144±1 mm Hg) versus control animals (191±2 mm Hg) at 2 weeks. The antihypertensive effect of enalapril at 2 weeks was not prevented by PD123319 cotreatment (143±1 mm Hg). The significant antihypertensive effects of each treatment were also similar at 1 week (data not shown). Final body weights were not significantly affected by valsartan (275±5 g), enalapril (272±7 g), or enalapril+PD123319 (277±13 g) as compared with control animals (270±4 g). Treatment with enalapril alone caused a significant 2-fold increase in SMC DNA fragmentation (Figure 3). In contrast to valsartan, however, the enalapril-induced increase in DNA fragmentation was not prevented by coadministration of PD123319. As in our previous study,¹³ enalapril administration for 2 weeks did not reduce SMC DNA synthesis (123±2 vs 127±4 cpm/100 µg in control animals), aortic DNA content (0.92±0.05 vs 1.05±0.06 µg/mm in control animals), or aortic mass (4.7±0.1 vs 5.2±0.1 mg/mm per gram ×10⁻³ in control animals). Consistent with the data described above, valsartan administration for 2 weeks significantly reduced SMC DNA synthesis (25%), aortic DNA content (39%), and aortic mass (17%), whereas DNA fragmentation was no longer elevated at that time point. Ang II plasma levels were markedly reduced after 2 weeks of enalapril (87±11 pg/mL) or enalapril+PD123319 (83±13 pg/mL).

Discussion

We previously reported that losartan and enalapril are equipotent in reducing aortic SMC number in SHR.¹³ One interpretation of these results is that reduced AT₁ receptor activity with both treatments decreased SMC growth and survival. However, the effects were initiated significantly earlier with losartan than with enalapril. Because AT₁ blockade increases Ang II plasma levels,¹⁴ we hypothesized that the suppression of SMC growth-survival may involve AT₂ receptors in SHR treated with an AT₁ antagonist but not an

Figure 2. Changes in A, internucleosomal DNA fragmentation; B, aortic SMC DNA synthesis; C, SMC number; and D, medial hypertrophy in aorta of SHR treated for 1 or 2 weeks with valsartan (VAL), PD123319 (PD), or a combination of VAL and PD. Stimulation of DNA fragmentation and suppression of DNA synthesis induced by VAL were prevented by cotreatment with PD. Reduction in aortic SMC number and medial cross-sectional area induced by VAL were prevented by cotreatment with PD. PD given alone did not affect these parameters. *Significantly different (P<0.05) from control group (n=5 to 8 per group).

Figure 3. Changes in internucleosomal DNA fragmentation in aortic SMC in SHR treated for 2 weeks with enalapril, PD123319 (PD), or a combination of enalapril and PD. Stimulation of DNA fragmentation induced by enalapril was not prevented by cotreatment with PD. *Significantly different (P<0.05) from control group (n=5 per group).
ACE inhibitor. In the present study, we confirmed that mRNA for both receptor subtypes are expressed in the SHR aorta. Recently, we reported a correlation between levels of AT1 and AT2 receptor protein and mRNA in SHR vessels.\textsuperscript{15} Consistent with our previous findings,\textsuperscript{13} AT1 receptor blockade induced a transient burst of SMC apoptosis at the onset of the regression of aortic hypertrophy and before SMC DNA replication was suppressed. In rats receiving enalapril, SMC apoptosis was stimulated at 2 weeks, but DNA synthesis and aortic hypertrophy were not yet reduced as previously shown.\textsuperscript{13}

The significant new finding is that PD123319 blocked the proapoptotic and growth-inhibitory effects of valsartan, suggesting a role for AT2 receptors in regulating SMC fate in vivo. The lack of effect of PD123319 administered alone suggests that AT2 receptors do not elicit tonic proapoptotic or antigrowth effects on SMC under basal conditions in the aorta of young adult SHR. This is in contrast to late gestation, when SMC DNA replication is stimulated by AT1 blockade.\textsuperscript{16} Interestingly, cotreatment with valsartan and PD123319 did not affect SMC DNA replication and apoptosis. These data suggesting that basal AT1 and AT2 receptor activity do not regulate SMC growth-survival are possibly related to the short treatment period examined. Alternatively, AT1 receptors may downregulate the AT2 pathway, which suggests that the countervailing influence of these 2 receptors is an important determinant of SMC fate. The latter interpretation is more consistent with the known role of AT1 receptors in Ang II–dependent vascular disorders.\textsuperscript{1,2,17} Such a paradigm also may explain the significant antigrowth effect of AT2 receptors in aortic SMC before birth,\textsuperscript{16} because AT2 receptors predominate over AT1 receptors at that time.\textsuperscript{18} Possible nonspecific effects of PD123319 cannot be ruled out. It should be mentioned, however, that the present dose of PD123319 results in plasma antagonist concentrations that are widely regarded as highly specific for AT2 receptors (250 nmol/L).\textsuperscript{19} Ang II plasma levels were markedly elevated by valsartan but not affected by PD123319. Therefore, the simplest explanation for the present results is that AT1 receptor blockade rapidly reduced aortic SMC accumulation by favoring Ang II–dependent AT2 receptor activity, with proapoptotic and antigrowth consequences. Consistent with this, PD123319 did not block SMC apoptosis induced by enalapril (that is, in a model in which Ang II production was markedly reduced). That ACE inhibitors and AT1 antagonists elicit convergent effects in part through different pathways has been documented in rat models of cardiovascular remodeling.\textsuperscript{20–22} In these studies, increased activity of the kinin B2 receptor pathway has been implicated in the beneficial effects of ACE inhibitors. It should be mentioned that although both B2 and AT2 receptors stimulate endothelial production of nitric oxide,\textsuperscript{23} a known stimulant of SMC apoptosis,\textsuperscript{24} only ACE inhibitors prevent kinin degradation and B2 receptor desensitization.\textsuperscript{25,26} These added effects may help increase SMC apoptosis with enalapril. Reduced Ang II levels during ACE inhibition may contribute further by correcting endothelial dysfunction in SHR.\textsuperscript{27} In contrast, AT2 receptors are able to stimulate SMC apoptosis independent of endothelial cells.\textsuperscript{2}

Reducing arterial wall tension stimulates SMC apoptosis in rabbit vessels, suggesting that blood pressure–dependent apoptotic pathways are possible.\textsuperscript{28} However, we previously reported that blood pressure reduction with hydralazine does not induce aortic SMC apoptosis in SHR.\textsuperscript{13} In the present study, PD123319 suppressed apoptosis, even though the antihypertensive effect of valsartan was not affected. Thus, the causal relation between blood pressure and SMC apoptosis is complex and modulated by endocrine factors. As previously observed,\textsuperscript{13} apoptosis induction and growth suppression were temporally dissociated during treatment. Because PD123319 blocked both events, it is not possible to determine whether growth suppression was secondary to apoptosis induction or, alternatively, a primary effect of AT2 stimulation. That AT2 receptors suppress growth-associated signaling pathways and ultimately DNA replication, however, is supported by several in vitro studies.\textsuperscript{2} Previous reports have shown contrasting effects of PD123319 on vascular mass regulation in vivo. Some\textsuperscript{5–7} but not all\textsuperscript{29} groups observed that PD123319 administration for 10 weeks attenuates aortic mass in SHR or rats given long-term infusions of Ang II. None of these studies examined the balance between SMC DNA replication and apoptosis. It is known, however, that long-term Ang II infusion stimulates SMC DNA replication through AT1 receptors.\textsuperscript{29,30} Interestingly, short-term Ang II infusion in normotensive rats was recently shown to induce SMC apoptotic activity through both AT1 and AT2 receptors, although effects on SMC number or DNA replication were not determined.\textsuperscript{31} In contrast, the present results clearly show that short-term treatment with PD123319 in SHR potently suppresses the induction of SMC apoptosis, the inhibition of SMC growth, and the rapid regression of SMC number and vascular mass induced by an AT1 receptor antagonist. The discrepancies with the studies mentioned above may reflect differences in experimental models and/or schedule of drug administration. For instance, the documented suppression of endothelial cell growth by AT2 receptors\textsuperscript{32} raises the possibility that long-term blockade of this receptor with PD123319 may alter endothelial cell behavior in vivo, an important determinant of vascular mass.\textsuperscript{33} Our RT-PCR data in vessels with or without endothelium suggest that AT2 receptors are expressed in SHR aortic endothelial cells, as previously shown in smaller rat vessels.\textsuperscript{34} Thus, a role for endothelial AT2 receptors in the present model may not be ruled out. In the short time period examined, however, we consider a direct effect on AT2 receptors in SMC more likely because of the rapidity of the responses observed. Whether the present observations are vessel specific remains to be determined. Aortic hypertrophy is important in isolated systolic hypertension and left ventricular hypertrophy,\textsuperscript{35} although arterioles are the major site of increased vascular resistance in hypertension.\textsuperscript{36}

In conclusion, this study in SHR provides the first evidence that AT2 receptors induce arterial SMC deletion by apoptosis in vivo. AT2 receptors also reduced SMC growth and vascular mass but did not affect blood pressure during AT1 receptor blockade. In comparison, enalapril-induced apoptosis was not affected by AT2 receptor blockade, suggesting different mechanism(s) of action for ACE inhibitors and AT1 antago-
nists. Although the therapeutic significance of these findings remains to be established, we speculate that AT2-mediated SMC apoptosis may contribute to the potent inhibitory effects of AT1 antagonists on vascular hypertrophic remodeling in cardiovascular disorders.

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