In Vivo Study of AT\textsubscript{1} and AT\textsubscript{2} Angiotensin Receptors in Apoptosis in Rat Blood Vessels

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Abstract—In vitro experiments suggest that angiotensin II (Ang II) may cause growth via angiotensin type 1 (AT\textsubscript{1}) receptors and apoptosis via angiotensin type 2 (AT\textsubscript{2}) receptors. To answer the question of whether AT\textsubscript{1} or AT\textsubscript{2} receptor activation could induce apoptosis in the vasculature in vivo, Wistar rats were infused for 7 days with Ang II (120 ng \cdot kg\textsuperscript{-1} \cdot min\textsuperscript{-1} subcutaneously) and treated with the AT\textsubscript{2} receptor antagonist PD 123319 (30 mg \cdot kg\textsuperscript{-1} \cdot d\textsuperscript{-1} subcutaneously) or the AT\textsubscript{1} receptor antagonist losartan (10 mg \cdot kg\textsuperscript{-1} \cdot d\textsuperscript{-1} orally). Apoptosis in thoracic aorta was quantified by radiolabeled DNA laddering and by terminal deoxynucleotide transferase–mediated dUTP nick end-labeling. The expression of p53, bax, bcl-2, and caspase-3, which play critical roles in apoptotic signaling, was examined by Western blot analysis. The mRNA expression of AT\textsubscript{1} and AT\textsubscript{2} receptors was determined by reverse transcription–polymerase chain reaction. The increase in systolic blood pressure and aortic growth induced by Ang II infusion was completely prevented by losartan alone or losartan given with PD 123319, whereas PD 123319 resulted in a greater increase in systolic blood pressure and aortic growth than Ang II alone. Radiolabeled DNA laddering showed that Ang II infusion ± losartan or PD 123319 significantly increased apoptosis (147±8\%, 178±20\%, and 238±41\%, respectively, \(P<0.05\) compared with control). Expression of bax and active forms of caspase-3 was increased in the Ang II + PD 123319 group, whereas the expression of p53 and bcl-2 was not significantly different in all groups. The expression of AT\textsubscript{1} and AT\textsubscript{2} receptor mRNA was downregulated by losartan and PD 123319, respectively. Thus, when AT\textsubscript{1} or AT\textsubscript{2} receptors are stimulated in vivo, apoptosis is enhanced in the media of blood vessels. In the case of AT\textsubscript{1} receptor stimulation, this may occur secondary to vascular growth and modulate the latter. Both bax and caspase-3 participate in the pathways of apoptosis triggered by in vivo AT\textsubscript{1} receptor stimulation. (Hypertension. 1999;34:617-624.)

Key Words: muscle, smooth, vascular \(\square\) apoptosis \(\square\) angiotensin II \(\square\) p53 \(\square\) cysteine proteases

Angiotensin (Ang) II, the most important peptide that mediates the effects of the renin-angiotensin system, may play an important role in hypertension via its effects on vascular smooth muscle cell (VSMC) growth.\textsuperscript{1} Interestingly, Ang II exerts positive or negative effects on cell growth depending on which subtype of its seven-transmembrane domain receptors the peptide binds. There are 2 main subtypes of Ang II receptors, and these may be blocked by specific antagonists; losartan for angiotensin type 1 (AT\textsubscript{1}) receptors and PD 123319 for angiotensin type 2 (AT\textsubscript{2}) receptors.\textsuperscript{2–4} Most of the known functional effects of Ang II on VSMCs are mediated via AT\textsubscript{1} receptors.\textsuperscript{5} Although it has been hypothesized that AT\textsubscript{2} receptor activation may be involved in the control of cell differentiation, proliferation, and apoptosis,\textsuperscript{6–8} the roles of AT\textsubscript{2} receptors in vivo are poorly understood.

Apoptosis is an active, gene-directed process that is distinguished from necrosis by a number of morphological and biochemical criteria.\textsuperscript{9–13} Morphologically, apoptosis is defined by cell shrinkage, membrane blebbing, condensation and fragmentation of the nuclei, and chromatin condensation.\textsuperscript{9–11} The key biochemical feature of apoptosis is DNA fragmentation at internucleosomal units induced by an endogenous endonuclease.\textsuperscript{12,13} DNA fragmentation can be detected by agarose gel electrophoresis (DNA laddering) and by terminal deoxynucleotide transferase–mediated dUTP nick end-labeling (TUNEL).

Apoptosis plays a critical role in both the normal development and the pathology of a variety of tissues.\textsuperscript{14} Apoptosis is recognized as a contributing cause of cardiac myocyte loss with ischemia/reperfusion injury, myocardial infarction, and vascular wall remodeling. In VSMCs, apoptosis has been detected after serum and growth factor removal\textsuperscript{15,16} and after cells have been exposed to reactive oxygen species.\textsuperscript{17} Apoptosis has been detected in cultured VSMCs after exposure to nitric oxide donors,\textsuperscript{18} whereas Ang II may offer a protective effect. However, Ang II has been shown to induce apoptosis in human endothelial cells, whereas nitric oxide provides a
protective effect.\textsuperscript{19} It has been proposed that apoptosis may represent an important process that contributes to vascular remodeling in hypertension. However, the molecular mechanisms responsible for VSMC apoptosis remain largely unknown. Because some of the genes involved mechanistically in growth and apoptosis (such as immediate early genes like\textit{c-myc}) are activated in both processes, increased apoptosis may accompany enhanced growth as a controlling and fine-tuning mechanism.\textsuperscript{20}

The present study was designed to examine the hypothesis that AT\(_1\) receptor–induced VSMC growth in vivo could be associated with increased apoptosis. We also asked whether AT\(_2\) receptor–mediated apoptosis may occur and be demonstrated in an in vivo experimental paradigm. The association of administration of Ang II with a selective Ang receptor subtype antagonist was used to enhance cellular responses to Ang II at the subtype receptor that was not blocked. Because there may be non–AT\(_1\), non–AT\(_2\) Ang receptors (AT\(_4\), AT\(_x\)), Ang II was also infused into rats treated simultaneously with both AT\(_1\) and AT\(_2\) antagonists. Furthermore, to study the pathways of apoptosis induced by Ang II without and in the presence of blockade of AT\(_1\) and AT\(_2\) receptors, we measured gene expression of different proapoptotic and antiapoptotic proteins in the vasculature.

**Methods**

**Animal Experiments**

The study was approved by the Animal Care Committee of the Clinical Research Institute of Montreal and was performed according to the guidelines of the Canadian Council for Animal Care. As previously described,\textsuperscript{21} male Wistar rats 7 weeks of age (200 g, n=7 to 8) were infused subcutaneously with the use of osmotic minipumps (Alzet, Alza Corp) with Ile-Ang II\(_5\) (Peninsula) at a dose of 120 ng \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) or PD 123319 (AT\(_2\) receptor antagonist was a gift from Dr Joan Keiser, Parke-Davis, Ann Arbor, Mich) at the dose of 30 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\). Losartan (AT\(_1\) receptor antagonist) was given in the drinking water at a dose of 10 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\). The dose of Ang II used in the present study is the pharmacological dose used previously by others\textsuperscript{22} and us.\textsuperscript{21} After 7 days of treatment, systolic blood pressure (SBP) was taken by the tail-cuff method. Rats were killed by decapitation. A segment of thoracic aorta was dissected out and immediately frozen in dry ice and kept at \(-70^\circ\)C until use.

**Morphometry of Aorta**

The cross-sectional area (CSA) of the media was evaluated as previously described.\textsuperscript{21} The growth index was calculated as (CSA\(_{\text{tr}}\) – CSA\(_{\text{wt}}\))/CSA\(_{\text{wt}}\), where CSA\(_{\text{tr}}\) and CSA\(_{\text{wt}}\) were the CSA of aorta from normotensive and treated rats, respectively.

**Measurement of Apoptosis in Aorta**

Fat, adventitia, and endothelium were removed from the aorta, and DNA was extracted from the media and nick end radiolabeled as previously described.\textsuperscript{23} Radiolabeled DNA was loaded in 1.5% agarose gel. After electrophoresis, DNA was transferred onto a nylon membrane and the radioactivity associated with 100- to 1500-bp DNA fragments was quantified with a phosphorimager (Molecular Dynamics). Apoptosis was also evaluated histologically by the TUNEL technique as previously described.\textsuperscript{23}

**Western Blot Analysis of p53, bax, bcl-2, and Caspase-3**

Protein was extracted from frozen tissue as previously described.\textsuperscript{23} Protein concentration was determined with a protein assay (BioRad Laboratories, Inc). Thirty micrograms of total protein was separated by electrophoresis on a 15% polyacrylamide gel at 100 V for 1 hour and transferred onto a PVDF membrane in the cooling system at 100 V for 1 hour. Membranes were incubated with specific antibody to p53 (Calbiochem-Novabiochem International), bax, and bcl-2 (both from Santa Cruz Biotechnology, Inc) at a dilution 1:1000, 1:2000, and 1:1600, respectively, for 1 hour at room temperature. To evaluate the expression of inactive (M\(_I\)=32 kDa) and active caspase-3 (20 kDAs and 17 kDa), membranes were incubated overnight with rabbit antiserum to human CPP32 (antibody against caspase-3 was a gift from Dr Rafick Pierre Sekaly, Clinical Research Institute of Montreal, Canada) at a dilution 1:1000.\textsuperscript{23} Signals were revealed with chemiluminescence and visualized by autoradiography.

**Reverse Transcription–Polymerase Chain Reaction Analysis of AT\(_1\) and AT\(_2\) Receptors**

mRNA expression of AT\(_1\) and AT\(_2\) receptors was measured with reverse transcription–polymerase chain reaction (RT-PCR) as previously described\textsuperscript{23} with some modifications. Total RNA samples were treated with RNase-free DNase (Gibco Life Technologies) and extracted with phenol-chloroform to eliminate possible residual DNA before RT-PCR.

**Statistical Analysis**

Results are presented as mean±SEM. Data were analyzed by 1-way ANOVA followed by a Newman-Keuls test or by a Kruskal-Wallis nonparametric test followed by a \(t\) test for Gaussian populations with different SDs where applicable. \(P<0.05\) was considered statistically significant.

**Results**

**Body Weight, SBP, and Growth Index**

Body weight was greater (\(P<0.001\)) in Ang II–infused rats treated simultaneously with losartan and PD 123319 compared with normotensive rats, whereas body weight was similar in all other groups (Table). The increase in SBP and aortic growth induced by Ang II–infusion (\(P<0.001\) and \(P<0.05\) versus control, respectively) was completely prevented by treatment with losartan alone or losartan given with PD 123319 (Table 1, Figure 1). Interestingly, PD 123319, when given to Ang II–infused rats, resulted in a greater increase in SBP and aortic growth than Ang II alone.
Treatment of normotensive rats with either losartan or PD 123319 separately or together had no effect on SBP and aortic growth.

DNA Laddering and TUNEL

Because we used the 3'-end DNA labeling method, which is more sensitive than ethidium bromide DNA staining, to detect DNA laddering typical of apoptosis, we could detect basal levels of apoptosis in untreated rats. Figure 2 shows a significant increase in apoptosis by increased DNA laddering in the Ang II–infused group (147 ± 6.8%) and groups infused with Ang II that received losartan (178 ± 6.2%) or PD 123319 (238 ± 4.1%) in comparison to control rats (P < 0.05). Losartan and PD 123319 alone did not have a detectable effect on apoptosis. In situ 3'-end labeling with an in situ apoptosis detection kit (ApopTag, Intergan Company) in aorta (Figure 3) demonstrated histologically sparse apoptotic smooth muscle cells in the media of aorta in all treated groups but not in the control group.

Expression of Proapoptotic and Antia apoptotic Proteins

Western blot analysis was performed to quantify the expression of the proapoptotic proteins p53 and bax, the antiapoptotic protein bcl-2, and the inactive and the active fragments of a cysteine protease (caspase-3) critically situated in the proteolytic pathway of apoptosis (M, = 32 kDa for the inactive form and 20 kDa and 17 kDa for the active forms). Figure 4 shows that the expression of bax was increased in Ang II–infused rats treated with PD 123319, whereas expression of p53 and bcl-2 remained unchanged in all groups. Expression of the active forms of caspase-3 (particularly the 20-kDa peptide) was increased relative to the inactive 32-kDa protein only in Ang II–infused rats that received PD 123319 (Figure 5). In losartan-treated rats (infused or not infused with Ang II), absolute amounts of caspase-3, both the 32-kDa inactive peptide and the 17-kDa active form, were increased, but relative amounts of the active form (active/inactive caspase-3) were not.

AT₁ and AT₂ Receptor mRNA by RT-PCR

Expression of AT₁ receptor mRNA was reduced in rats that received losartan with or without Ang II (Figure 6), whereas AT₂ receptor mRNA expression was significantly diminished in the Ang II+PD 123319 group.

Discussion

To evaluate the hypothesis that AT₁ receptor–induced smooth muscle cell growth is associated with secondarily increased apoptosis and that AT₂ receptor–mediated apoptosis of smooth muscle cells demonstrated previously in vitro could be shown to occur in vivo, we examined the following paradigm. Ang II–infused rats treated with the AT₂ receptor antagonist PD 123319 were used as a model of AT₁ receptor stimulation, and Ang II–infused rats treated with the AT₁ receptor antagonist losartan were used as a model of AT₂ receptor stimulation. Ang II–infused rats treated simultaneously with the AT₁ and AT₂ antagonists were used as a model of AT₁- (non-AT₁, non-AT₂) mediated effects. Our results show that both AT₁ and AT₂ receptor stimulation were associated with enhanced apoptosis of smooth muscle cells in
Figure 3. Representative photomicrographs of in situ end-labeling detection of fragmented DNA with TUNEL from sections of aorta from some of the groups studied: control group (A and B), after treatment with Ang II (C and D), Ang II+PD 123319 (E and F), and losartan (G and H). Because of space constraints, figures for Ang II+losartan, Ang II+losartan+PD 123319, and PD 123319 alone, which are all similar to figures G and H, are not shown. Sparse apoptotic cells labeled by TUNEL are shown in the left insets (arrows in A, C, E, G), whereas all nuclei are labeled with Hoechst 33258 in the right insets (B, D, F, H). Original magnification ×80.
aorta of rats. AT₁ receptor activation was accompanied by apoptosis, which may modulate the proliferation of smooth muscle cells, whereas AT₂ receptors in the present paradigm only induced apoptosis and thus may exert an antigrowth effect in vivo. No effects except for a greater body weight could be attributed to non-AT₁, non-AT₂ receptors in this experimental paradigm. However, this need not be attributed to a non-AT₁, non-AT₂ (AT₄, AT₅) effect. Blood pressure elevation is often associated with lower body weight in some rat models. Blockade of blood pressure elevation and of AT₁- and AT₂-mediated apoptosis could presumably, particularly in combination, be associated with greater body weight gain.

Figure 4. Left, Bar graph representation of mean±SEM of results from 4 Western blots of p53, bax, and bcl-2. *P<0.05 vs control, †P<0.05 vs Ang II group. Right, Representative photograph of Western blots of p53, bax, and bcl-2.

Figure 5. Top, Representative photograph of Western blots of caspase-3. Bands of the 32-kDa inactive proenzyme and the 20-kDa and 17-kDa active forms are visible. Bottom, Bar graph shows the proenzyme (black, 32 kDa) and 2 active forms (light gray, 20 kDa; and dark gray, 17 kDa) as a percentage of the total caspase-3. Error bars indicate SEM, n=4. *P<0.05 vs control.
However, this remains speculative. Furthermore, we also show that AT1 receptor activation (Ang II–infused rats receiving PD 123319) induces apoptosis in blood vessels via a bax-dependent pathway and via activation of the caspase cascade, as evidenced by proteolytical cleavage of caspase-3 into its active 20-kDa and 17-kDa subunits. These findings extend our understanding of the role of Ang II and its receptor subtypes, particularly AT1 receptors as important contributors and regulators of remodeling of blood vessels in hypertension, which is shown by the increase in DNA fragmentation, bax expression, and caspase-3 activation in the Ang II group (AT1 stimulation).

Cell hypertrophy, proliferation, or both are mediated by AT1 receptors, whereas stimulation of AT2 receptors has been proposed to lead to inhibition of cell proliferation or to apoptosis.5–8 The distribution of AT1 and AT2 receptors in tissues varies depending on the cell type or tissue considered. In rat aorta, 60% of Ang receptors are AT1, whereas AT2 receptors are abundantly and widely expressed in aorta of fetal and young rats but present only to a limited extent in adult tissues.26 Several in vitro studies have demonstrated different effects of Ang II on different cell types.6–8,18–19,27–29 In addition, Ang II may induce growth in VSMCs.27 However, recent studies in non–smooth muscle cells have demonstrated that Ang II is also capable of inducing apoptosis via stimulation of AT2 receptors.7,8 It has been suggested that the antigrowth effect of Ang II on endothelial cells is mediated by AT2 receptors,6 although the underlying mechanisms have not been elucidated. Pollman et al18 failed to induce apoptosis by Ang II in VSMCs, which may be explained by the fact that the expression of AT2 receptors in VSMCs disappears in passaged cells during cell culture. Interestingly, these authors showed that Ang II directly antagonized nitric oxide donor–induced apoptosis via activation of AT1 receptors. In contrast, Dimmelner et al19 demonstrated that activation of programmed cell death by Ang II clearly involves the stimulation of AT2 receptors in human endothelial cells. In contrast to the antiproliferative effect of AT2 that has been shown in endothelial cells and in PC12W cells that overexpress AT2 receptors, AT1 receptors play a more important proapoptotic role in cardiomyocytes of both neonatal and adult rats.28,29 In these studies, the AT1 receptor antagonist losartan completely blocked Ang II–induced apoptosis, whereas the AT2 receptor antagonist PD 123319 failed to reduce Ang II–induced apoptosis. Moreover, Leri et al30 showed that the increase in Ang II–induced apoptosis triggered by stretch was abolished by losartan, which indicated that apoptosis in cardiomyocytes is mediated by AT1 receptors. Taken together, these data suggest that Ang II induces apoptosis via different receptor subtypes depending on the cell type and the ratio between the expression of AT1 and AT2 receptors. When PD 123319 was given to Ang II–infused rats, AT1 receptor mRNA expression was reduced as shown by RT-PCR (Figure 6), and any expressed AT2 receptors were furthermore presumably blocked by the AT2 receptor antagonist. Growth in this experimental condition was stimulated via unblocked AT1

Figure 6. Left, Bar graph shows expression of mRNA of AT1 receptor mRNA (top) and AT2 receptor mRNA (bottom) by RT-PCR. Error bars indicate SEM, n=4. *P<0.05 vs control, †P<0.05 vs Ang II, ‡P<0.05 vs Ang+PD, §P<0.05 vs Los, and ||P<0.05 vs Ang+Los by 1-sided t test for Gaussian populations with different SDs. Right, Representative photograph shows bands that correspond to RT-PCR amplification of mRNA of AT1 receptors (top), GAPDH (middle), and AT2 receptor (bottom).
receptors, and secondary apoptosis may have occurred as evidenced by a 2.5-fold increase in DNA laddering (Figure 2b), indicating a compensatory balance between cell replication and deletion in the maintenance of tissue homeostasis. When both losartan and PD 123319 were given to Ang II–infused rats, there were no demonstrable additional effects except for a greater body weight gain. In this group, both AT1 and AT2 receptors are blocked, and Ang II could be acting on non-AT1, non-AT2, Ang receptors or degraded to Ang IV and act on AT1 receptors. However, the present results do not provide definitive evidence of non-AT1, non-AT2–mediated effects, because as mentioned earlier, this could result from a combined effect of blockade of blood pressure increase and of inhibition of AT1– and AT2–mediated apoptosis. AT1 and AT2 receptor mRNA was evaluated by RT-PCR, which is only semiquantitative, and thus the reported levels of mRNA can only be considered as an approximation. However, previous work from our laboratory has already demonstrated a correlation between levels of AT1 and AT2 receptor protein and mRNA.25

In this study, we investigated molecular steps involved in the mechanisms that trigger apoptosis in aorta. Cell death is controlled in part by a complex interplay between regulatory proteins. The bax protein, a member of the Bcl family, is one of these regulatory proteins and is found in various tissues.31 The present study shows bax expression even in tissues from normotensive rats. Overexpression of bax was found in aorta of Ang II–infused rats after treatment with PD 123319 despite the absence of changes in the expression of p53. Thus, p53 is not necessarily the main regulator of bax expression. Bcl-2 is a suppressor of apoptosis that homodimerizes or forms heterodimers with the homologous protein bax. A previous study from our laboratory has shown that both bax and bcl-2, another member of the Bcl family, are involved in pathways of apoptosis in aorta.23 In the present study, we extended those observations and demonstrated that caspase-3 also participates in the mechanisms that trigger apoptosis of VSMCs in rat aorta. Recent work has supported a central role for the caspase family of cysteine proteases, especially caspase-3 (CPP32, YAMA, or apopain) has been considered for the caspase family of cysteine proteases, especially caspase-3 (20 kDa and 17 kDa) present in SMCs in aorta of angiotensin II–infused rats. However, the present results do not provide definitive evidence of non-AT1, non-AT2–mediated apoptosis. AT1 and AT2 receptor mRNA was evaluated by RT-PCR, which is only semiquantitative, and thus the reported levels of mRNA can only be considered as an approximation. However, previous work from our laboratory has already demonstrated a correlation between levels of AT1 and AT2 receptor protein and mRNA.25

In conclusion, the balance between growth and apoptosis in blood vessels, which may play an important role in vascular remodeling in hypertension, may be regulated in vivo by Ang receptors, particularly by AT1 receptors. Activation of AT1 receptors in vivo results in SBP increase, blood vessel growth, and associated VSMC apoptosis, which may modulate the degree of growth. Whether apoptosis that is induced by AT1 receptors in VSMCs is primary, or secondary as we believe, awaits clarification. Thus, the present results extend our knowledge on the essential role of AT1 receptors in blood pressure control, VSMC growth, and apoptosis, as shown by increases in blood pressure, aortic growth, DNA fragmentation, bax expression, and caspase-3 activation.

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References


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