Apoptosis Is Not Increased in Myocardium Overexpressing Type 2 Angiotensin II Receptor in Transgenic Mice

Hiroshi Sugino, Ryoji Ozono, Satoshi Kurisu, Hideo Matsuura, Mari Ishida, Tetsuya Oshima, Masayuki Kambe, Yasuhiro Teranishi, Hiroya Masaki, Hiroaki Matsubara

Abstract—To determine whether angiotensin type 2 (AT₂) receptor stimulation induces apoptosis in cardiomyocytes in vivo, we developed transgenic mice overexpressing the AT₂ receptor in a cardiac-specific manner, using the α-myosin heavy-chain promoter. Ten- to 12-week-old male homozygous transgenic mice (n = 44) and wild-type mice (n = 44) were used. Both transgenic and wild-type mice were given either saline (control), a subpressor dose of angiotensin II (100 ng · kg⁻¹ · min⁻¹), a pressor dose of angiotensin II (1000 ng · kg⁻¹ · min⁻¹) for 14 days, or a pressor dose of angiotensin II for 28 days to investigate the effects of stimulation on both angiotensin type 1 (AT₁) and AT₂ receptors, the AT₁ antagonist L158809 alone, or a combination of angiotensin II (1000 ng · kg⁻¹ · min⁻¹) and L158809 for 14 days to investigate the effects of selective AT₂ receptor stimulation. Apoptosis was analyzed in paraffin-embedded ventricular sections by the terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling (TUNEL) technique. In both transgenic and wild-type mice, administration of a subpressor dose of angiotensin II, L158809, or a combination of angiotensin II and L158809 did not significantly affect the tail-cuff blood pressure or heart-to-body weight ratio, whereas administration of a pressor dose of angiotensin II for 14 or 28 days significantly increased blood pressure and the heart-to-body weight ratio. However, there was no statistical difference between the effects of angiotensin II in transgenic and wild-type mice. The number of TUNEL-positive nuclei was ≈0 to 10 per 100,000 cardiomyocytes, with no difference between transgenic and wild-type mice, regardless of saline infusion or any stimulation. In infarcted canine myocardial tissue sections for positive control, the number of TUNEL-positive nuclei was increased by 13.8 to 19.1 times compared with those in the noninfarcted myocardium. In conclusion, angiotensin II infusion for a period of 28 days failed to induce cardiomyocyte apoptosis regardless of the presence or absence of cardiac AT₂ receptor overexpression. It is unlikely that in mice the AT₂ receptor is a strong signal to induce cardiomyocyte apoptosis in vivo.

(Hypertension. 2001;37:1394-1398.)

Key Words: myocytes, cardiac ■ apoptosis ■ mice, transgenic ■ angiotensin II ■ L158809

Angiotensin (Ang) II has 2 major receptor isoforms: Ang II type 1 receptor (AT₁R) and Ang II type 2 receptor (AT₂R). Most of the well-known Ang II functions, such as increasing blood pressure, stimulating myocyte hypertrophy, and the proliferating effect in the cardiovascular system, are mediated by AT₁R. On the other hand, AT₂R has been implicated in the inhibition of cell growth and proliferation. AT₂R has been shown to mediate induction of apoptosis in the PC₁2 cell line, vascular smooth muscle cells, vascular endothelium, fibroblasts, granulose cells in the rat ovary, neurons from the rat brain, and human fetal adrenal cells. Recently, it has been reported that cardiac remodeling in various heart diseases involves a loss of cardiomyocytes due to apoptosis and that Ang II stimulation induces cardiomyocyte apoptosis in vivo and in vitro. Ligand-binding experiments indicated that AT₁R as well as AT₂R is expressed in the heart and that AT₂R subtype is upregulated in the diseased heart. It has yet to be determined whether AT₂R is involved in the induction of cardiomyocyte apoptosis. We have recently developed transgenic (TG) mice overexpressing AT₂R selectively in cardiomyocytes by using the α-myosin heavy chain promoter. In the present study, we used these TG mice to examine whether AT₂R stimulation enhances Ang II–induced cardiomyocyte apoptosis in vivo.

Methods

Animals

TG mice overexpressing AT₂R selectively in cardiomyocytes were generated using the α-myosin heavy chain promoter. Homozygous littermates and wild-type (WT) littermates were obtained by mating between heterozygous littermates. Mice were housed under climate-controlled conditions with a 12-hour light/dark cycle, and they were

Received August 30, 2000; first decision October 10, 2000; revision accepted December 4, 2000.
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maintained on regular chow and tap water. Northern blot analysis of total RNA from various tissues of adult homozygous TG mice revealed that AT1R was exclusively and abundantly expressed in both atria and ventricles but not in other tissues. Saturation and competitive inhibition experiments using 125I-[Sar1 Ile8]-Ang II as a ligand revealed that the proportions of AT1R relative to AT2R were 41% in atria and 45% in ventricles of TG mice, with no AT2R detected in atria or ventricles of WT mice. There was no significant difference between levels of AT1R in TG and WT mice. This study was performed in accordance with the guidelines for animal experiments of Hiroshima University School of Medicine.

**Study Design**

Ang II (Sigma Chemical) dissolved in saline was infused subcutaneously via an Osmotic Minipump (Alzet model 2002, Alza Corp). The AT1R antagonist L158809 (a gift from Merck, BANYU Co, Tokyo, Japan) was administered in drinking water at a dose of 1.5 mg · kg\(^{-1}\) · d\(^{-1}\). Both TG and WT mice at 10 to 12 weeks of age were given either (1) normal saline as a control (TG, n=10; WT, n=10), (2) a subpressor dose of Ang II (100 ng · kg\(^{-1}\) · min\(^{-1}\); TG, n=6; WT, n=6), (3) a pressor dose of Ang II (1000 ng · kg\(^{-1}\) · min\(^{-1}\); TG, n=10; WT, n=10) for 14 days and (4) for 28 days (TG, n=6; WT, n=6) to investigate the effects of stimulation on both AT1 and AT2 receptors, (5) AT1 antagonist L158809 (1.5 mg · kg\(^{-1}\) · d\(^{-1}\); TG, n=6; WT, n=6) alone or (6) a combination of Ang II (1000 ng · kg\(^{-1}\) · min\(^{-1}\)) and L158809 (TG, n=6; WT, n=6) for 14 days to investigate the effects of selective AT1R stimulation. In a preliminary experiment, we compared the pressor effects of various doses of Ang II (100, 500, 1000, and 2000 ng · kg\(^{-1}\) · min\(^{-1}\)). Blood pressure was significantly elevated by the infusion of 500, 1000, and 2000 ng · kg\(^{-1}\) · min\(^{-1}\) Ang II, whereas there was no significant difference between the pressor effects of 1000 and 2000 ng · kg\(^{-1}\) · min\(^{-1}\). The subpressor dose of Ang II raised plasma concentration of Ang II from 46.8±20.3 to 97.7±26.7 pg/mL. Systolic blood pressure and heart rate were determined in conscious mice before and every other day during the experimental period by the tail-cuff method (BP98A, Softron Co). Mice were prewarmed in a 37°C temperature-controlled restrainer for 15 minutes. The pulse wave was monitored by an electrophygmomanometer while inflating the cuff, and at least 3 to 5 readings were averaged.

To investigate apoptosis in pressure-overloaded myocardium in both TG and WT mice, we made aortic coarctation by abdominal aortic banding (TG, n=6; WT, n=6). The suprarenal abdominal aorta was constricted with nylon strings by ligating the aorta with a bluntened 26-guage needle, which was removed soon after.

**Histological Analysis**

On the 14th or 28th day of treatment, all mice in each group were euthanized, and the hearts were rapidly excised. The left ventricle was weighed and immersion-fixed in 10% neutral formalin. The left ventricle was horizontally sliced in 3 pieces and embedded in paraffin. Serial sections were sliced to 1 to 2 μm in thickness. Endogenous peroxidase was inactivated by 3% hydrogen peroxide. Sections pretreated with DNase I (10 U/mL) for 20 minutes at 37°C were used as positive controls (Figure 1); the TdT enzyme step was omitted for negative controls. Tissue sections from each myocardial specimen were examined over all fields, and the numbers of TUNEL-positive cardiomyocytes and total cardiomyocytes were counted under a microscope with an eyepiece grid (magnification, ×400 to ×1000). Approximately 10 000 cardiomyocytes per slide were analyzed. Cardiomyocytes were distinguished from nonmyocytes by microscopic appearance; that is, well-shaped, elongated, and striated cells.

**Canine Myocardial Infarction**

To further validate our TUNEL method, we examined whether TUNEL-positive nuclei are increased in infarcted canine myocardial
tissue sections (Figure 1). We ligated the left anterdescending artery of a canine to induce myocardial infarction. Tissue sections from areas of the infarcted zone, border zone, and noninfarcted zone of the myocardium were analyzed by the TUNEL method as described above. The number of TUNEL-positive nuclei was increased significantly by 13.8 times (0.51±0.11%) in the infarcted zone and by 19.1 times (0.71±0.27%) in the border zone of the myocardium compared with the noninfarcted myocardium (0.037±0.017%). The numbers of apoptotic myocytes in the infarcted zone and border zone were consistent with those in a previous report.23

Statistical Analysis
All values are presented as mean±SEM. Statistical analyses were performed with Student’s t test. A value of P<0.05 was interpreted to denote statistical significance.

Results

Effects of Treatment on Hemodynamics
There were no significant differences in systolic blood pressure, heart rate, and body weight among the 12 groups before treatment. Systolic blood pressure in the 12 groups of mice at days 0, 14, and 28 are shown in Table 1. Systolic blood pressure was significantly higher in TG and WT mice treated with a pressor dose of Ang II than in the saline control mice (P<0.01). However, there was no significant increase in systolic blood pressure in TG and WT mice treated with a subpressor dose of Ang II. The elevation of systolic blood pressure mediated by the pressor dose of Ang II was canceled by concomitant administration of the AT1R antagonist L158809. There was no significant change in heart rate in any group.

Effects of Treatment on Heart-to-Body Weight Ratio
There was no difference in body weight during any treatment among the 12 groups. As shown in Figure 2, heart-to-body weight (HW/BW) ratio was significantly higher in TG and WT mice treated with a pressor dose of Ang II than in the saline control mice (P<0.05). However, there was no significant difference between the ratio in TG and WT mice. The weight (HW/BW) ratio tended to be higher in mice groups treated with a subpressor dose of Ang II, but the difference was not statistically significant.

Cardiomyocyte Apoptosis
Figure 1 shows photomicrographs of apoptotic cardiomyocytes. TUNEL-positive cardiomyocytes were sparsely distributed from the endocardial side to the epicardial side with no focal concentration. The number of apoptotic cells in the saline control group was 1·100 000 myocytes, which is consistent with recent reports that apoptosis was almost undetectable in the rodent heart in the basal state,24–26 whereas the number of apoptotic cells increased to as high as 20 to 50/100 00014,24 in the failing heart. Table 2 shows the percentages of apoptotic cardiomyocytes. The number of apoptotic cardiomyocytes was not significantly increased over the control either by Ang II infusion, which stimulates both AT1R and AT2R, or by infusion of L158809/Ang II plus

<table>
<thead>
<tr>
<th>TABLE 1. Hemodynamic Changes of Each Groups</th>
<th>Systolic Blood Pressure, mm Hg</th>
<th>Heart Rate, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Day 0</td>
<td>Day 14</td>
</tr>
<tr>
<td>Saline control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>92±2</td>
<td>97±1</td>
</tr>
<tr>
<td>TG</td>
<td>97±1</td>
<td>101±1</td>
</tr>
<tr>
<td>Ang II subpressor dose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>95±4</td>
<td>104±6</td>
</tr>
<tr>
<td>TG</td>
<td>102±3</td>
<td>107±2</td>
</tr>
<tr>
<td>Ang II pressor dose 14 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>94±1</td>
<td>151±4*</td>
</tr>
<tr>
<td>TG</td>
<td>98±1</td>
<td>152±3*</td>
</tr>
<tr>
<td>Ang II pressor dose 28 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>98±2</td>
<td>152±9*</td>
</tr>
<tr>
<td>TG</td>
<td>101±3</td>
<td>159±13*</td>
</tr>
<tr>
<td>AT1 antagonist</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>104±9</td>
<td>94±4</td>
</tr>
<tr>
<td>TG</td>
<td>105±5</td>
<td>99±3</td>
</tr>
<tr>
<td>AT1 antagonist + Ang II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>99±3</td>
<td>102±11</td>
</tr>
<tr>
<td>TG</td>
<td>105±11</td>
<td>96±2</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
*P<0.05 vs saline control.
L158809, which stimulates AT₂R selectively. Notably, although Ang II and L158809 treatment in TG mice was thought to maximally stimulate the pathway via AT₂R, cardiomyocyte apoptosis was not increased at all. As determined by localization and histological features, all of the counted TUNEL-positive cells were judged to be cardiomyocytes on the basis of the characteristic appearance.

Because none of the Ang II infusions induced apoptosis, we tested an acute hemodynamic overload as a possible stimulator for apoptosis. After the surgery, the mean pressure gradients between the carotid and femoral arteries were 24±8 mm Hg in TG mice and 24±2 mm Hg in WT mice. The HW/BW ratio was significantly elevated both in TG and WT mice compared with sham-operated controls, but there was no significant difference between the ratios in TG mice and WT mice (Figure 3). However, the acute hemodynamic overload failed to increase the number of apoptotic myocytes both in TG mice and WT mice. (Table 3)

**Discussion**

We hypothesized that AT₂R counteracts the growth-promoting effect of Ang II mediated by AT₁R via apoptosis in the myocardium. To evaluate the effects of emphasized AT₂R stimulation in vivo, we used transgenic mice overexpressing AT₂R in a cardiac-specific manner. We evaluated both the sole Ang II effects (subpressor dose of Ang II) and the effects of Ang II and hemodynamic overload mediated by Ang II (pressor dose of Ang II) on cardiomyocyte apoptosis. Furthermore, we used L158809, a specific AT₁R antagonist, to eliminate the effects through AT₁R. This AT₁R antagonist also causes upregulation of endogenous Ang II, which

**Table 2. Percentage of TUNEL-Positive Cardiomyocytes**

<table>
<thead>
<tr>
<th>Group</th>
<th>TUNEL-Positive Cardiomyocytes, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline control</td>
<td>0.006±0.003</td>
</tr>
<tr>
<td>Ang II subpressor dose</td>
<td>0.003±0.002</td>
</tr>
<tr>
<td>Ang II pressor dose 14 days</td>
<td>0.009±0.004</td>
</tr>
<tr>
<td>Ang II pressor dose 28 days</td>
<td>0.019±0.033</td>
</tr>
<tr>
<td>AT₁ antagonist</td>
<td>0.003±0.002</td>
</tr>
<tr>
<td>AT₁ antagonist+Ang II (Pressor)</td>
<td>0.002±0.001</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

**Table 3. Percentage of TUNEL-Positive Cardiomyocytes**

<table>
<thead>
<tr>
<th>Group</th>
<th>TUNEL-Positive Cardiomyocytes, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.003±0.002</td>
</tr>
<tr>
<td>Aortic coarctation</td>
<td>0.007±0.003</td>
</tr>
</tbody>
</table>

selectively stimulates overexpressed AT₁R. Therefore, in this experimental system, we could stimulate AT₁R selectively and maximally. In none of the conditions, however, did the number of TUNEL-positive nuclei in TG mice differ from that in WT mice, indicating that Ang II infusion for 28 days did not induce apoptosis in the mouse heart. At least, considering that we probably stimulated the cardiac AT₂R maximally, it appears that stimulation of the cardiac AT₁R is not a strong signal to induce cardiomyocyte apoptosis in the mouse heart.

On the other hand, recent in vitro17,18 as well as in vivo16 studies have demonstrated that Ang II–induced apoptosis was inhibited by treatment with an AT₁R antagonist, indicating that AT₂R rather than AT₁R is more important for induction of cardiomyocyte apoptosis. Cardiomyocyte apoptosis has been reported to be induced in vitro by the stimulus of Ang II added to the medium17 or by mechanical stretch.19 It has been shown that Ang II is involved in the induction of cardiomyocyte apoptosis in vivo in failing hearts such as those in aged (30 weeks old) spontaneously hypertensive rats13,28 or in dogs that underwent cardiac pacing for over 3 months during which the apoptosis was inhibited by an AT₁R antagonist16 or an ACE inhibitor.13,14 In the present study, however, the number of apoptotic cardiomyocytes in WT mice was not significantly increased by administration of Ang II over a period of 1 month at maximum. Such a treatment caused cardiac hypertrophy but not heart failure, because we did not observe ventricular dilatation or marked interstitial fibrosis in the Ang II–treated heart. Our results indicate that Ang II–induced hypertrophy is not a stimulus for apoptosis in the mouse heart. However, because studies show that apoptosis is evident in heart failure, it is possible that Ang II elevation that occurs with chronic hemodynamic overload, not in its early stage, could contribute to the apoptotic process. Moreover, there may be species-specific differences in the effect of Ang II. It is possible that Ang II does not induce apoptosis in the mouse heart. To the best of our knowledge, the present study is the first to investigate the Ang II–induced cardiomyocyte apoptosis in the mouse heart.

We also failed to detect a prominent increase in the number of apoptotic cardiomyocytes after aortic coarctation, which was inconsistent with the report by Teiger et al15 who demonstrated that banding at the ascending aorta for 4 days in the rat caused a marked increase in cardiomyocyte apoptosis. This difference between the results of the current study and the study by Teiger et al15 may be related to the difference in the severity of aortic coarctation; aortic banding by Teiger et al caused an increase in ventricular weight to a level 50% higher than that of the control in 3 days, whereas only a 15% increase was observed in our study. Apoptosis may play a role in cardiac remodeling in such an extreme condition but...
not in moderate myocardial hypertrophy such as that observed in the present study. In addition, the length of treatment may also be an important factor in the induction of apoptosis because aortic coarctation for an extended period of time resulted in an increase in the number of apoptotic cells.25,26

In conclusion, Ang II infusion for a period of 28 days failed to induce cardiomyocyte apoptosis, regardless of the presence or absence of cardiac AT2R overexpression. It is unlikely that in mice the AT2R is a strong signal to induce cardiomyocyte apoptosis in vivo.

Acknowledgments
This study was supported by a Grant-in Aid for Scientific Research (Nos. 08457639, 07407065, 11470518, and 11771511). The authors thank Katsunari Ogawa for the excellent technical assistance.

References
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Hypertension. 2001;37:1394-1398
doi: 10.1161/01.HYP.37.6.1394

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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