Overexpression of Bax Protein and Enhanced Apoptosis in the Left Ventricle of Spontaneously Hypertensive Rats: Effects of AT1 Blockade With Losartan

María Antonia Fortuño, Susana Ravassa, Juan Carlos Etayo, Javier Díez

**Abstract**—An association of increased apoptosis with overactivity of the local angiotensin-converting enzyme has been reported in cells from the left ventricle of adult rats with spontaneous hypertension (SHR). To gain insight into the regulation of cardiac apoptosis in arterial hypertension, we investigated the expression of the proteins Bcl-2 (an inhibitor of apoptosis) and Bax (an inducer of apoptosis) in the left ventricle of 30-week-old normotensive Wistar-Kyoto rats (WKY), SHR, and SHR treated with the angiotensin II type 1 receptor (AT1) antagonist losartan (20 mg · kg⁻¹ · d⁻¹) during 14 weeks before death. The density of apoptotic cells was assessed by direct immunoperoxidase detection of biotin-labeled deoxyuridin nucleotides. The expression of Bcl-2 and Bax was assessed by Western blot analysis. Compared with WKY, untreated SHR exhibited increased (P<0.05) apoptosis, increased (P<0.01) Bax, and similar Bcl-2. The Bcl-2/Bax ratio (an inverse index of cell susceptibility to apoptosis) was lower (P<0.05) in untreated SHR than in WKY. The chronic administration of losartan was associated with the normalization of apoptosis, Bax expression, and the Bcl-2/Bax ratio in treated SHR. No changes in the expression of Bcl-2 were observed in these rats after treatment. No significant changes in the apoptotic density were observed between treated SHR with normal blood pressure and treated SHR with abnormally high blood pressure at the end of the treatment period. These results suggest that an association exists between increased apoptosis and overexpression of Bax oncoprotein in cells from the left ventricle of adult SHR. Chronic blockade of AT1 receptors prevents Bax overexpression and normalizes apoptosis in the left ventricle of SHR independently of its hemodynamic effect. On the basis of our findings, it can be proposed that the interaction of angiotensin II with its AT1 receptors may participate in the stimulation of Bax protein, which in turn renders cells from the left ventricle of SHR more susceptible to apoptosis. (Hypertension. 1998;32:280-286.)

**Key Words:** angiotensin II ■ apoptosis ■ Bax ■ Bcl-2 ■ losartan ■ rats, inbred SHR

Apoptosis is a physiological, active, and tightly regulated process in which cell death follows a programmed sequence of events.¹ Apoptosis plays a role in the regulation of cell mass and architecture in many tissues.² A number of genes have been identified that regulate the apoptotic process. The Bcl-2 proto-oncogene family is critical for the regulation of apoptosis.³ Bcl-2 family members come in 2 functional categories: those that inhibit apoptosis (ie, Bcl-2)⁴ and those that induce apoptosis (ie, Bax).⁵ The relative abundance of proapoptotic and antiapoptotic proteins determines the susceptibility to cell death.⁶ Thus, it has been proposed that cell viability after an apoptotic stimulus may depend on the ratio of the level of Bcl-2 to that of Bax.⁷ A high level of Bcl-2 relative to Bax promotes survival, whereas an excess of Bax relative to Bcl-2 promotes death.⁸

Increased apoptosis has been demonstrated recently in the hypertrophied left ventricle of young,² adult,⁹ and aged¹⁰ SHR. Although the available evidence suggests that apoptosis can be induced in cardiac cells by a variety of insults including pressure overload,¹¹ it appears that cardiac apoptosis in adult SHR results from an exaggerated local production of Ang II.⁹ This possibility is further supported by the findings that Ang II induces apoptosis of adult rat ventricular cells in vitro through a mechanism triggered by the interaction of the peptide with AT1 receptors.¹²,¹³ Furthermore, it has been reported recently that the AT1 receptor antagonist losartan prevents p53-induced apoptosis in rat ventricular myocytes.¹⁴

We therefore hypothesized that increased susceptibility to apoptosis may be present in cells from the left ventricle of SHR, and this in turn facilitates programmed cell death. To test this hypothesis, we analyzed the expression of the oncoproteins Bcl-2 and Bax in ventricular cells from adult normotensive WKY and adult SHR. In addition, the effects of AT1 blockade on left ventricle apoptosis and the expression of Bcl-2 and Bax were also analyzed in adult SHR chronically treated with losartan.

**Methods**

**Animals and Design**

The rats were provided by Harlan UK Limited (Bicester, England). The SHR and their normotensive genetic controls, WKY, were studied in the following manner: (1) untreated 16-week-old SHR...
Preparation of Tissue Samples

All animals were killed at the age of 30 weeks. They were manipulated in 2 different ways for different investigations. Half the animals in each group were perfused fixed for the in situ detection of apoptosis and the immunohistological investigations. They were anesthetized with 30 mg/kg IP sodium thiopental and perfused via the abdominal aorta retrogradely. The hearts were arrested in diastole by injection of potassium chloride (1 mM/L) into the cardiac artery; a siliconized cannula was inserted into the abdominal aorta and connected to a perfusion pump, and the right atrium was incised to allow the drainage of blood and perfusate. Perfusion was performed first with normal saline buffer to wash out the blood and with 10% buffered formalin for 24 hours and embedded in paraffin.

In Situ Detection of Apoptosis

The TUNEL methodology performed for in situ end-labeling of DNA fragments was adapted from the method of Gavrieli et al. which is based on the preferential binding of terminal deoxynucleotidyl transferase (TdT) to the 3'-hydroxyl ends of DNA. Tissue sections (5 μm) were deparaffinized, transferred to xylene, and rehydrated in descending concentrations of alcohol. After rehydration, the slides were incubated with 20 μg/mL proteinase K (Sigma) in water for 10 minutes at room temperature and washed twice with deionized water. Endogenous peroxidase was inactivated by 3% hydrogen peroxide. After the washing, slides were preincubated in TdT buffer (140 mmol/L sodium cacodylate, 30 mmol/L Tris [Sigma], pH 6.6) for 5 minutes at room temperature. Tissue sections were then covered with the reaction buffer composed of 140 mmol/L sodium cacodylate, 30 mmol/L Tris, 1.5 mmol/L cobalt chloride (Sigma), 0.25 mmol/L deoxytymidine triphosphate, 0.25 mmol/L bovine-16-deoxyuridine triphosphate, and 0.25 U/μL TdT (Boehringer-Mannheim), and they were incubated for 1 hour at 37°C. The slides were then incubated for 5 minutes in the stop buffer (150 mmol/L sodium chloride, 15 mmol/L sodium citrate, pH 7.0) at room temperature. For negative controls, deionized water was used instead of TdT. After end-labeling, the sections were incubated with avidin-biotin complex containing horseradish peroxidase (Vector), stained with diaminobenzidine, and counterstained with hematoxylin.

Western Blot Analysis

For immunoblot assay of Bcl-2 and Bax proteins, left ventricles were homogenized in a lysis buffer (0.1% β-mercaptoethanol, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, and 50 mmol/L Tris, pH 7.4). After centrifugation at 20,000g for 15 minutes, protein concentration was determined using the Bradford method. Aliquots containing 150 μg protein were resuspended in the same volume of 2X sample buffer (20% β-mercaptoethanol, 4% SDS), 20% glycerol, 0.0125% bromophenol blue, and 0.125 mmol/L Tris, pH 6.4), and they were boiled for 5 minutes. Proteins were size-fractionated on 12% polyacrylamide gels by electrophoresis using a Mini-Protean II Dual Slab Cell (Bio-Rad), and they were electrotransferred to nitrocellulose membranes in the presence of a glycine/methanol buffer (250 mmol/L glycine, 20% methanol, and 25 mmol/L Tris, pH 7.5). The filters were blocked with 0.1% Tween and 1% dry skim milk in PBS (100 mmol/L sodium chloride, 80 mmol/L disodium phosphate, 25 mmol/L monobasic sodium phosphate, pH 7.5) for 1 hour at room temperature. Then membranes were incubated with the specific antibodies for 1 hour at room temperature. For Bcl-2 detection, a rabbit polyclonal anti-mouse Bcl-2 antibody (Pharmingen) was used at 1:1000 in the blocking solution; for Bax immunodetection, a rabbit polyclonal anti-mouse Bax antibody (Pharmingen) was used at 1:4000 in the blocking solution. After washing, Bcl-2- and Bax-bound antibodies were detected by peroxidase-conjugated anti-rabbit IgG (Amersham) at 1:10,000 and 1:7500 in PBS, respectively. Finally, the ECL-Plus chemiluminescence detection system (Amersham) was used to visualize the bands. For negative controls, membranes were incubated with normal goat serum without specific antibodies. The specificity of the bands was checked by preabsorbing anti-Bcl-2 and anti-Bax with the corresponding synthetic peptides (Figure 1). Autoradiograms were analyzed using an automatic densitometer (Pharmacia). The concentration of Bcl-2 and Bax was calculated from the densitometry values obtained from each problem sample using the slope and intercept of a calibration curve generated for each experiment. The calibration curve was performed, including in each experiment different known protein concentrations of an internal standard, and measuring the densitometric signals corresponding to those protein concentrations. The densitometry values increased linearly with protein concentration (r=0.997).
Statistical Analysis

Results are presented as mean±SEM computed from the average measurements obtained from each group of rats. Normal distribution of data was checked. Differences among the 3 groups of rats were tested by a 1-way ANOVA. Subsequent analysis for significant differences between 2 groups was performed using the multiple comparison Student-Newman-Keuls test or using the contrast coefficient matrix when variances were not homogeneous from a Levene test. The significance level was assumed at a value of *P*, 0.05. When a normal distribution test was significant, the *x*2 (Kruskal-Wallis) method was used to analyze the differences among the 3 groups of animals.

Results

Blood Pressure and Cardiac Hypertrophy

At the beginning of the experiment, SBP was significantly increased in SHR compared with WKY (Figure 2). Although SBP remained elevated at hypertensive levels in SHR throughout the experimental 16-week period, it decreased progressively to values close to those seen in WKY in SHR-L (Figure 2). Therefore, at 30 weeks of age, SBP was higher (*P<0.001*) in SHR than in WKY and SHR-L (Table 1). At that age, SBP was not significantly different in SHR-L or in WKY (Table 1). However, 5 SHR-L exhibited values of SBP at the end of the experiment above the upper limit seen in WKY (177 mm Hg). The remaining 4 animals of the SHR-L group exhibited final values of SBP within the limits measured in WKY.

Cardiac weight was greater (*P<0.01*) in SHR than in WKY (Table 1). Accordingly, SHR had left ventricular hypertrophy when expressed as the increase (*P<0.01*) in cardiac weight normalized with respect to body weight (Table 1). SHR-L exhibited values of cardiac weight close to those of WKY, and the values of the cardiac index were not significantly different between the 2 groups (Table 1).

Apoptosis of Ventricular Cells

Apoptosis detected by in situ end-labeling was predominantly confined to the cardiomyocytes, which were easily distinguished from other nonmyocyte cells according to their morphology:9,20 well-shaped, elongated, and striated cells. To eliminate the possibility of positive labeling in interstitial cells, those stained cells without the morphological criteria of cardiomyocytes were excluded from the evaluation.

The apoptotic density in the left ventricle was increased in SHR compared with WKY (5.41±1.50 versus 2.14±0.56 apoptotic nuclei/mm², *P<0.05*) (Figure 3). As shown in Table 2, the increase of apoptosis was more predominant in the subendocardium and the mesocardium. Although SHR exhibited values of apoptotic density in the right ventricle that were

<table>
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<th>Parameter</th>
<th>WKY</th>
<th>SHR</th>
<th>SHR-L</th>
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<tr>
<td>SBP, mm Hg</td>
<td>164±3</td>
<td>230±3‡</td>
<td>177±4</td>
</tr>
<tr>
<td>Cardiac weight, g</td>
<td>1.27±0.03</td>
<td>1.51±0.05†</td>
<td>1.34±0.02</td>
</tr>
<tr>
<td>Cardiac index, ×10⁻³</td>
<td>3.26±0.06</td>
<td>3.66±1.13†</td>
<td>3.08±0.04*</td>
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*P<0.05 compared with WKY; †P<0.01 compared with WKY and SHR-L; ‡P<0.001 compared with WKY and SHR-L.

Student-Newman-Keuls 1-way ANOVA test was applied to assess statistical significance of differences among groups in SBP and cardiac index analysis. A Kruskal-Wallis test was applied to assess the statistical significance of differences among groups in cardiac weight analysis.

**Figure 2.** Longitudinal changes in SBP in the 3 groups of rats during the experimental period. A Student-Newman-Keuls 1-way ANOVA test was applied to assess the statistical significance of differences among groups (*P<0.001* compared with WKY and SHR-L).

**Figure 3.** Values of the apoptotic density measured in the left ventricle in each experimental group. Bars represent the mean±SEM of 10 animals in WKY and SHR groups and 9 animals in the SHR-L group. A Student-Newman-Keuls 1-way ANOVA test was applied to assess the statistical significance of differences among groups (*P<0.05* compared with WKY and SHR-L).
higher than those in WKY, the mean values of this parameter measured in the 2 groups were not significantly different (2.14 ± 0.61 versus 1.47 ± 0.42 apoptotic nuclei/mm²).

After treatment with losartan, the apoptotic density in the left ventricle of SHR-L decreased significantly to values close to those measured in WKY (2.92 ± 0.50 apoptotic nuclei/mm²) (Figure 3). The decrease in the apoptotic density was more pronounced in the subendocardium and the mesocardium (Table 2). No significant differences in this parameter were observed between SHR-L still hypertensive at the end of the experiment (3.03 ± 0.87 apoptotic nuclei/mm²) and SHR-L with normal blood pressure after treatment (2.80 ± 0.47 apoptotic nuclei/mm²). The chronic administration of losartan did not modify significantly the apoptotic density in the right ventricle of SHR-L (data not shown).

Expression of Bcl-2 and Bax

As shown in Figure 4A, the intensity of the signal corresponding to Bcl-2 was greater in ventricles from SHR than in ventricles from WKY. However, no significant differences were found in the amount of Bcl-2 protein between the 2 groups of rats (WKY, 0.32 ± 0.06 μg/mg total protein; SHR, 0.49 ± 0.05 μg/mg total protein) (Figure 5A). Treatment with losartan did not significantly modify the amount of Bcl-2 protein in the ventricles of SHR-L (0.52 ± 0.08 μg/mg total protein) (Figures 4A and 5A).

Figure 4B shows that the signal corresponding to Bax was more intense in ventricles from SHR than in ventricles from WKY. Accordingly, the amount of Bax protein was higher in SHR than in WKY (0.80 ± 0.10 versus 0.27 ± 0.04 μg/mg total protein, *P < 0.01) (Figure 5B). The intensity of the signal corresponding to Bax was less intense in SHR-L than in SHR (Figure 4B). Accordingly, the amount of Bax protein (0.41 ± 0.06 μg/mg total protein) was significantly diminished (*P < 0.05) in SHR-L compared with SHR (Figure 5B). No significant differences in Bax expression were observed between SHR-L and WKY.

The Bcl-2/Bax ratio was decreased (*P < 0.05) in SHR (0.61 ± 0.11) compared with WKY (1.18 ± 0.15) and SHR-L (1.26 ± 0.13) (Figure 6). No significant differences were found in the ratio of Bcl-2 to Bax between WKY and SHR-L (Figure 6).

These data suggest that susceptibility to apoptotic stimuli is abnormally increased in ventricular cells from SHR and that this abnormality is prevented by losartan in treated SHR.

Discussion

The observations of the present study confirm previous findings that increased cardiac apoptosis present in SHR is localized preferentially in the hypertrophied left ventricle. In addition, these results provide the first indication of an overexpression of the proapoptotic Bax protein in the left ventricle of adult SHR. Interestingly, these alterations are not observed in the left ventricle of adult SHR chronically treated with the AT1 antagonist losartan.

In this study, cardiomyocytes were not isolated from other cellular components of the myocardium. Thus, we cannot conclude that all the apoptotic cells were of myocytic origin. However, the observed occurrence of TUNEL staining in cells with characteristic histological features of ventricular fibers (ie, well-shaped, elongated, and striated cells) suggests that the majority of apoptotic cells were from cardiomyocytes. Analysis of the distribution of apoptotic cells, albeit without reference to the total number of cells in each location, showed an increase in the subendocardium and the mesocardium of the left ventricle of SHR. This pattern of distribution corresponds to the pattern of wall stress in the chronic pressure-overloaded left ventricle. A recent study has shown apoptosis in pressure overload-induced left ventricular hypertrophy in the rat. Furthermore, stretch of cardiac myocytes in vitro, which mimics an elevation of diastolic stress in vivo, induces apoptosis of these cells. However, in a previous study we reported that cardiomyocyte apoptosis in the left ventricle of SHR is not related in a temporal way to blood pressure. Furthermore, in the present work, we found that left ventricular apoptosis is normalized even in those treated SHR that remained hypertensive at the end of the losartan treatment period. It thus appears that although arterial hypertension cannot be excluded as an additional contributing factor in the development of left ventricular apoptosis in adult SHR, other mechanisms play a more critical role in this process.

Because the regulation of apoptosis has been found to be altered in smooth muscle cells of adult SHR, we investigated the regulation of cell susceptibility to apoptosis in the left ventricle of SHR. The Bcl-2 family comprises death-inhibitory and death-inducing members, and they regulate apoptosis by competitive homodimerization or heterodimerization. In fact, the ratio of death antagonist to agonist...
determines whether a cell will respond to apoptotic stimuli. This way of apoptosis regulation also affects cardiomyocytes. The Bax protein has been shown to act as an accelerator of apoptosis in ventricular cells, and the Bcl-2 protein prevents apoptosis of ventricular cells. Therefore, the ratio of Bcl-2 to Bax may be considered as a determinant for survival or apoptosis of ventricular cells. Therefore, the ratio of Bcl-2 to Bax may be considered as a determinant for survival or death of ventricular cells after an apoptotic stimulus.

The main finding of the present study is that compared with that in WKY, the expression of Bax is increased in the left ventricle of SHR. However, left ventricular expression of Bcl-2 was similar in SHR and WKY. Thus, the Bcl-2/Bax ratio was abnormally decreased in these rats. These findings suggest that cells of the left ventricle are highly susceptible to apoptosis in adult SHR. What are the stimuli responsible for the overexpression of Bax in cardiomyocytes of SHR? p53 is a transcriptional regulator of the Bcl-2 and Bax genes. It has been recently shown that infection of adult rat ventricular myocytes with a replication-deficient adenoviral vector containing wild-type p53 results in downregulation of Bcl-2, upregulation of Bax, decrease in the Bcl-2/Bax ratio, and death of 34% of the cells. However, several data argue against a role for p53 in Bax overexpression in SHR. First, p53 is expressed at high levels in embryonic heart and during the early phases of postnatal development, p53 transcripts are barely detectable in the adult myocardium and do not seem to increase with cardiac hypertrophy. Second, no documentation of elevated p53 labeling by immunohistochemistry of heart muscle after stretch-induced cardiomyocyte cell death has been obtained thus far. Finally, Bcl-2 expression was unchanged in SHR compared with WKY. Thus, the participation of p53 to promote left ventricular Bax overexpression in SHR seems unlikely. Nevertheless, further studies are necessary to assess its precise role in cardiac apoptosis in these rats.

Beside p53, other factors stimulate cell apoptosis, ie, c-myc, tumor necrosis factor-α, wild-type p53 activated fragment-1 (WAF-1), and Fas/Apo-1. For instance, it has been recently reported that an increase in WAF-1 expression accompanies an increase in apoptotic cells in the hearts of SHR, without any change in the expression of Bcl-2. The question of whether an enhanced expression of WAF-1 induces overexpression of Bax in the heart of SHR requires additional studies.

One alternative mechanism is that Ang II stimulates Bax expression and apoptosis in the left ventricle of adult SHR. This possibility is supported by several findings. First, we observed that Bax expression is normalized in SHR treated with the AT1 receptor antagonist losartan. Second, we reported recently that enhanced apoptosis is closely related to exaggerated local ACE activity in the left ventricle of adult SHR. Third, it has been shown in vitro that ligand binding of AT1 receptors on adult and neonatal ventricular cells triggers apoptosis by a mechanism involving protein kinase C–mediated increases in cytosolic calcium and the stimulation of calcium-dependent endogenous endonuclease. Finally, Pierzchalski et al reported that losartan and Ang II antibodies prevented p53-induced apoptosis in rat cardiomyocytes. Thus, it can be hypothesized that the interaction of Ang II with its AT1 receptor may induce not only left ventricular growth and fibrosis but also programmed myocardial cell death in animals and humans with arterial hypertension.

Immunoblotting of Bcl-2 protein in the present study failed to detect significant differences in the expression of this protein between SHR and WKY. This is in agreement with data previously reported by Li et al in SHR. Because Bcl-2 has been found to protect various cell types from apoptosis, our finding would suggest that the appearance of apoptosis in the SHR left ventricle might not be accompanied

Figure 6. Ratio of Bcl-2 to Bax (an inverse index of cell susceptibility to apoptosis) in the left ventricle from normotensive WKY, SHR, and SHR-L. A Kruskal-Wallis test was applied to assess the statistical significance of differences among groups (*P<0.05 compared with WKY and SHR-L).
by compensatory changes in this protein in an attempt to maintain survival of myocardial cells. Accordingly, increased apoptosis might result in a significant loss of myocardial cells along the time. In this setting, it should be noted that the absolute number of apoptotic cells found in the left ventricle of the SHR studied here was small; nevertheless, the loss of cells can be significant because it is known that the time course for apoptotic cell death is only a few hours.31

A reduction in the number of cardiomyocytes has been found in the hypertrophied left ventricle of SHR34 and hypertensive patients.35 Increased apoptosis is present in failing SHR hearts compared with nonfailing SHR hearts.10 Some recent reports have indicated that the loss of myocytes due to apoptosis occurs in human end-stage heart failure.36 37 All these data reinforce Bing’s38 proposal that apoptosis may be a mechanism for loss of viable cardiomyocytes, myocardial dysfunction, and the transition to heart failure associated with chronic pressure overload.

Another finding reported here is that chronic treatment with losartan normalizes susceptibility to apoptotic stimuli and prevents the exaggerated apoptosis of left ventricular cells in SHR. However, because we did not assess the kinetics of cardiac apoptosis during losartan therapy, we cannot exclude an initial wave of cardiomyocyte apoptosis, as shown by deBlois et al.39 in aortic smooth muscle cells of SHR chronically treated with this drug. Furthermore, administration of losartan was associated with the regression of left ventricular hypertrophy in treated SHR. Similar results have been reported previously in SHR chronically treated with an ACE inhibitor, which exhibited normalization of left ventricular growth and apoptosis.9 Together, these data provide experimental support to the idea of Hamet et al8 that pharmacological interventions in hypertension can be useful not only to normalize cell growth but also in apoptosis in target organs.

On the other hand, the above observations may be consistent with the possibility that therapeutic strategies interfering with the formation of Ang II or ligand binding to its AT1 receptors may improve cell survival in the left ventricle of animals and humans with arterial hypertension. Further studies are necessary to establish whether the beneficial effects of ACE inhibitors and AT1 receptor antagonists in patients with heart failure40,41 are due in part by the ability of these drugs to suppress the Ang II–dependent apoptotic loss of cardiomyocytes. The findings of Li et al10 support this possibility, showing that administration of the ACE inhibitor captopril to SHR with heart failure is associated with reduction of cardiomyocyte apoptosis and improvement of ventricular function.

In conclusion, our findings show that increased cell susceptibility to apoptotic stimuli is associated with enhanced apoptosis in the left ventricle of adult SHR. The presented data suggest that the long-term impact of arterial hypertension in combination with local mechanisms (ie, the interaction of Ang II with its AT1 receptors) may facilitate left ventricular apoptosis in these rats via stimulation of the Bax protein, an inducer of apoptosis. Nevertheless, the lack of response of the Bcl-2 protein, an inhibitor of apoptosis, cannot be excluded as an additional facilitating factor in the development of left ventricular programmed cell death in SHR. Finally, our results confirm that cell death dysregulation is a novel target for antihypertensive agents that interfere with the renin-angiotensin system in hypertension.

References


36. Bing OHL. Hypothesis: apoptosis may be a mechanism for the transition to heart failure with chronic pressure overload. *J Mol Cell Cardiol*. 1994;26:943–948.


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