Stimulation of Cardiac Apoptosis in Essential Hypertension
Potential Role of Angiotensin II

Arantxa González, Begoña López, Susana Ravassa, Ramón Querejeta, Mariano Larman, Javier Díez, María A. Fortuño

Abstract—We investigated whether cardiac apoptosis is stimulated in the heart of hypertensive patients and whether angiotensin II plays a role in such alteration. The study was performed in 28 patients with essential hypertension and no evidence of either ischemic cardiomyopathy or heart failure. After randomization, 14 patients were assigned to losartan and 14 patients to amlodipine treatment. At baseline and after 12 months, right septal endomyocardial biopsies were performed, and the number of apoptotic nuclei was assessed by DNA end-labeling (TUNEL). In addition, immunostaining for the active form of caspase-3 was also performed to assess apoptosis. Compared with normotensive autopsied hearts, both cardiomyocyte and noncardiomyocyte apoptosis were increased ($P<0.001$) in hypertensive hearts. Time-course changes in blood pressure during treatment were similar in the 2 groups of patients. In losartan-treated patients, both cardiomyocyte and noncardiomyocyte apoptosis decreased ($P<0.05$). Neither cardiomyocyte nor noncardiomyocyte apoptosis changed significantly in amlodipine-treated patients. These findings indicate that apoptosis is abnormally stimulated in the heart of patients with essential hypertension. Our data also suggest that the ability of antihypertensive treatment to inhibit cardiac apoptosis is independent of its antihypertensive efficacy. We propose that angiotensin II may participate in the stimulation of cardiac apoptosis in essential hypertension.

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Key Words: amlodipine ■ angiotensin II ■ apoptosis ■ heart ■ hypertension, essential ■ losartan

Numerous hypotheses have been considered to explain the fundamental mechanisms for the development of systolic dysfunction and heart failure in animals and humans with arterial hypertension. Besides contractile disturbances of cardiomyocytes and interstitial and perivascular fibrosis, cardiomyocyte loss is now being considered as one of the determinants of the maladaptive process implicated in the transition from compensated to decompensated left ventricular hypertrophy (LVH). A body of experimental evidence suggests that exaggerated apoptosis may account for the loss of cells in the hypertensive left ventricle (see Diez et al for a review).

Apoptosis is a physiologically active, tightly regulated process in which cell death follows a programmed sequence of events. This process regulates cell mass and architecture in many tissues. Increased cardiomyocyte apoptosis has been recently demonstrated in the hypertrophied left ventricle of spontaneously hypertensive rats (SHR) and patients with essential hypertension. Although the available evidence suggests that apoptosis can be induced in cardiomyocytes by a variety of insults, including pressure overload, findings in SHR suggest that an exaggerated local production of angiotensin II can be critically involved in cardiac apoptosis in this model. This possibility is further supported by in vitro and in vivo findings indicating that angiotensin II induces apoptosis of rat cardiomyocytes through a mechanism triggered by the interaction of the peptide with type 1 (AT1) receptors.

We have hypothesized that, in essential hypertension, angiotensin II may also play a role in the stimulation of cardiac apoptosis. To test this hypothesis, the present study was designed with 2 goals: (1) to compare apoptosis of cardiomyocytes and noncardiomyocytes in hypertensives and normotensives and (2) to compare changes in apoptosis in hypertensives receiving either the AT1 receptor antagonist losartan or the calcium channel blocker amlodipine as treatment.

Methods

Subjects
All subjects gave written informed consent to participate in the study, and the local committee on human research approved the study protocol. The study conformed with the principles of the Declaration of Helsinki. Twenty-eight white patients (17 men...
and 11 women, mean age 60 years, range 37–75), referred to our clinic for evaluation and treatment of essential hypertension, were included in the study. All patients had repeated, documented elevated systolic and diastolic blood pressure (SBP and DBP) values (>139 and >89 mm Hg, respectively) and normal ejection fraction. Secondary hypertension and coronary artery disease were excluded as previously described.12 No patient had received previous treatment with angiotensin-converting enzyme inhibitors, AT1 receptor antagonists, or calcium channel blockers.

No washout phase was performed to assure continuous antihypertensive treatment (required by the ethics committee). After randomization, 14 patients were assigned to losartan and 14 patients to amlodipine treatment. After titration all patients in the losartan and amlodipine groups were receiving daily dosages of 50 mg and 10 mg, respectively, during 12 months.

Eight hearts collected from a total of 100 autopsies performed at the University Clinic of Navarra served as controls for cardiac apoptosis after cardiovascular disease was excluded.

Assessment of Left Ventricular Mass

Two-dimensional, targeted M-mode ultrasound recordings were obtained from each patient as previously described.13 Left ventricular mass and interventricular septal thickness (IVST) were measured, and left ventricular mass index (LVMI) was calculated by dividing left ventricular mass by body surface area. The presence of left ventricular hypertrophy (LVH) was defined as LVMI >104 g/m² in women and >116 g/m² in men, and/or IVST >11 mm.13

Tissue Samples

Transvenous endomyocardial biopsies were taken from the middle area of the interventricular septum with a Cordis 96 cm (7F) bioprobe under fluoroscopic guidance after angiographic examination, at baseline and after 1 year of treatment in patients. The biopsy procedure was well tolerated and no complications were recorded. In control hearts, septal specimens were taken to assess apoptosis. Samples were fixed in 10% buffered formalin, embedded in paraffin, and serially sectioned in 4-μm-thick sections.

In Situ Detection of Apoptosis

Apoptosis was assayed by the terminal deoxynucleotidyl transferase (TdT) reaction and confirmed by caspase-3 immunostaining. DNA end-labeling (TUNEL) methodology was performed as recently described,14 with small modifications (see online supplement). The discrimination of TUNEL-positive nuclei between cardiomyocytes and noncardiomyocytes was performed according the cytological characteristics of the different cell types (Figure 1).15 Three apoptotic indexes were evaluated: a global index for total cells, an index for cardiomyocytes, and an index for noncardiomyocyte cells.

Western Blot Analysis

Immunoblot assay of Bax-α and Bcl-2 was performed as recently described,13 with small modifications (see online supplement). The ratio Bax-α/Bcl-2 was calculated as an index of susceptibility to apoptosis.16

Statistical Analysis

Values are expressed as mean±SEM. The Student’s t test for unpaired or paired data, the Wilcoxon test, and the χ2 Fisher test were employed to assay differences (see online supplement). A value of P<0.05 was considered statistically significant.

The expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results

Baseline Findings

No significant differences in baseline clinical, hemodynamic, and echocardiographic parameters for all patients in either treatment group were observed (Table 1).

The 3 apoptotic indexes were higher (P<0.001) in hypertensive patients than in normotensive controls (Table 2). In accordance with this, caspase-3 immunostaining was more marked in sections from hypertensive patients compared with sections from normotensive subjects (Figure 2, panels A and B). The baseline apoptotic indexes were not related with the levels of blood pressure, the values of LVMI and IVST in hypertensive patients.

Although baseline apoptotic indexes did tend to be higher in the losartan group than in the amlodipine group, the differences did not reach statistical significance (Table 2). Furthermore, none of the values obtained for these parameters in the losartan group was considered as out-layer after statistical evaluation.

Effects of Treatment on Blood Pressure and Left Ventricular Mass

Time-course changes in blood pressure were similar in the two groups of patients. Furthermore, final values of blood
Effects of Treatment on Apoptosis

The global apoptotic index decreased \( (P<0.05) \) after treatment with losartan (Table 2). Thus, in 79% of losartan-treated patients, the global apoptotic index decreased after treatment. In addition, tissue sections obtained after treatment with losartan presented a smaller area of immunostaining for caspase-3 than tissue sections obtained before treatment (Figure 2, panels B and C). To determine whether changes in apoptosis were accompanied by changes in cellularity, we analyzed the cellular density in the losartan group of patients (Table 2). Accordingly, the extent of immunostaining for caspase-3 did not change with amlodipine treatment (Figure 2, panels B and D). When analyzing individual responses to treatment, we found that the global apoptotic index decreased in 36% of patients, increased in 50% of patients, and remained unchanged in 14% of patients. No differences were found in clinical or biochemical parameters among the 3 subgroups of amlodipine-treated patients.

Effects of Treatment with Losartan on Apoptosis Regulatory Proteins

Two representative Western blots of myocardial Bax-α and Bcl-2 proteins are shown in Figure 3. Bax-α protein levels diminished by 18% after treatment with losartan \((4.01 \pm 1.08 \text{ AU})\). On the contrary, Bcl-2 protein levels increased by 77% after treatment with losartan \((2.09 \pm 0.76 \text{ versus } 3.71 \pm 0.47 \text{ arbitrary units [AU]})\). As a consequence, the ratio Bax-α:Bcl-2 decreased by 60% after treatment with losartan \((2.09 \pm 0.76 \text{ versus } 3.71 \pm 1.98 \text{ AU})\). A non-significant percentage of patients \((50\% \text{ versus } 2.98 \pm 1.19 \text{ AU})\). However, these differences did not reach statistical significance, probably because of the small number of cases studied \((n=4)\).

### Table 1. Effects of Treatment on Hemodynamic and Left Ventricular Mass Parameters in Hypertensive Patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Losartan Group</th>
<th>Amlodipine Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mm Hg)</td>
<td>Before TX</td>
<td>After TX</td>
</tr>
<tr>
<td></td>
<td>173±2</td>
<td>136±2†</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>94±3</td>
<td>78±3†</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>117±6</td>
<td>98±3†</td>
</tr>
<tr>
<td>PP (mm Hg)</td>
<td>79±6</td>
<td>58±2†</td>
</tr>
<tr>
<td>LVMI (g/m²)</td>
<td>133.36±8.94</td>
<td>104.93±6.2†</td>
</tr>
<tr>
<td>IVST (mm)</td>
<td>11.42±0.43</td>
<td>10.06±0.45*</td>
</tr>
</tbody>
</table>

TX, treatment; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; LVMI, left ventricular mass index; IVST, interventricular septum thickness.

Values are expressed as mean±SEM.

\*\(P<0.05\) compared with before treatment; †\(P<0.01\) compared with values before treatment.

### Table 2. Apoptotic Indexes in Normotensive Subjects and Hypertensive Patients

<table>
<thead>
<tr>
<th>Indexes</th>
<th>Normotensives</th>
<th>Hypertensives</th>
<th>Losartan Group</th>
<th>Amlodipine Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before TX</td>
<td>After TX</td>
<td>Before TX</td>
<td>After TX</td>
</tr>
<tr>
<td>Global</td>
<td>59±29</td>
<td>2501±371*</td>
<td>2860±644*</td>
<td>1320±295*,†</td>
</tr>
<tr>
<td>Cardiomyocytes</td>
<td>45±16</td>
<td>2251±395*</td>
<td>2843±730*</td>
<td>1118±176*,†</td>
</tr>
<tr>
<td>Noncardiomyocytes</td>
<td>73±34</td>
<td>2212±361*</td>
<td>2818±656*</td>
<td>1306±213*,†</td>
</tr>
</tbody>
</table>

TX, treatment.

Apoptotic indexes are expressed as TUNEL-positive nuclei/10⁶ nuclei. Values are expressed as mean±SEM.

\*\(P<0.001\) compared with normotensives; †\(P<0.05\) compared with values before treatment.
Discussion

The main findings of this study are as follows: (1) apoptosis is abnormally stimulated in the myocardium of patients with essential hypertension; (2) the efficacy of antihypertensive treatment in reducing blood pressure does not predict its capacity to reduce myocardial apoptosis in hypertensive patients; and (3) chronic AT₁ blockade is associated with reduction of myocardial apoptosis in essential hypertension.

It has been shown recently by electron microscopy that, not only apoptotic, but also necrotic cardiomyocytes and cardiomyocytes undergoing DNA repair may be labeled by the TUNEL method, leading to falsely high levels of apoptotic cell death. Thus, the interpretation of cell numbers reported here should be made with extreme caution. Nevertheless, the association that we found between changes in DNA fragmentation and changes in expression of the active form of caspase-3 suggests that, beyond its real quantitative magnitude, apoptosis is abnormally increased in different cell types in the hypertensive human heart and can be modified by antihypertensive treatment.

Although, in the present study apoptosis was analyzed in right septal endomyocardial biopsies, some arguments suggest that this location may be representative of the apoptosis existing in the free wall of the left ventricle. Thus, (1) Pearlman et al have reported in postmortem studies in hypertensive human hearts that myocardial remodeling (ie, fibrosis) present in the septum parallels remodeling existing in the free wall; (2) the intensity of DNA fragmentation reported in the current study is similar to that reported by Yamamoto et al in the free wall of autopsied hypertensive human hearts; and (3) we have previously reported that cardiomyocyte apoptosis is increased in the right ventricle of spontaneously hypertensive rats compared with normotensive Wistar-Kyoto rats.

The mechanisms of increased cardiac apoptosis in arterial hypertension are not well known. As suggested by experimental in vitro and in vivo studies, physical forces induce cardiac apoptosis in conditions of experimentally induced pressure overload of the heart. Thus, although no association was found in this study between hypertension or LVH and apoptosis, a role for long-term hemodynamic overload cannot be excluded in enhanced cardiac apoptosis in hypertensive patients.

An alternative explanation is that humoral factors, ie, angiotensin II, stimulate cardiac apoptosis in arterial hypertension. This hypothesis is supported by several experimental findings: (1) in vitro, angiotensin II induces apoptosis of adult and neonatal cardiomyocytes; (2) cardiomyocytes isolated from the left ventricle of SHR exhibit enhanced susceptibility to angiotensin II-induced apoptosis compared...
with cells isolated from the left ventricle of normotensive Wistar-Kyoto control rats; (3) stimulation of cardiac apoptosis in the left ventricle of SHR is related in a temporal way to an increase of local angiotensin-converting enzyme activity and not to the elevation of blood pressure values; (4) pharmacological interference with the renin-angiotensin system normalizes apoptosis in the left ventricle of SHR independently of its ability to reduce blood pressure. In accordance with the above hypothesis, we report here that, whereas a similar reduction in blood pressure was attained in the 2 groups of hypertensives with treatment, only losartan-treated patients exhibited a significant reduction in cardiac apoptosis. Furthermore, changes found here in the cardiac expression of the proapoptotic protein Bax-α and the anti-apoptotic protein Bcl-2 in losartan-treated hypertensives are in agreement with the suppression of angiotensin II-induced susceptibility to apoptosis.

Although cardiac apoptosis diminished in losartan-treated hypertensives, apoptotic indexes still remained abnormally increased after treatment. Because angiotensin II has been shown to induce apoptosis in cardiomyocytes and other cell types, ie, fibroblasts, through stimulation of AT1 receptors, the possibility exists that blockade of AT1 receptors allows the peptide to interact with AT2 receptors in cardiac cells, thus maintaining apoptosis partially stimulated. This possibility can be of relevance because AT2 receptor expression has been reported to be increased in fibroblasts of hypertensive human hearts with fibrosis.

The role of calcium channel blockers, particularly dihydropyridines, in apoptosis has been controversial. Nifedipine was shown to block calcium-mediated apoptosis in isolated chick cardiomyocytes. In contrast, Tea et al reported that cardiac apoptosis transiently increased in SHR chronically treated with nifedipine but returned to baseline values at the end of the treatment period. Thus, our data in amlodipine-treated hypertensives would suggest that dihydropyridines do not exert long-term effects on cardiac apoptosis in hypertension. In this respect, amlodipine has been demonstrated to stimulate the sympathetic nervous system during chronic treatment of essential hypertension, and it is well established that beta-adrenergic stimulation enhances myocardial apoptosis. Thus, the possibility exists that the lack of effect of amlodipine in cardiac apoptosis reported here may be mediated by its effect on sympathetic activity.

The pathophysiological meaning of the increased apoptosis of cardiac cells in arterial hypertension remains a matter of discussion. It has been proposed that apoptosis-induced cardiomyocyte loss precedes the impairment in ventricular pump function and may be implicated in the initiation of ventricular maladaptation and the transition to heart failure in hypertensive heart disease. In support of this possibility are recent findings showing that loss of cardiomyocytes caused by apoptosis increases in parallel with the deterioration of cardiac function in SHR. On the other hand, Olivetti et al documented a 30% and 16% loss of these cell types in the left and right ventricle, respectively, of hypertensive patients with LVH and no anatomic evidence of myocardial infarction and necrosis. In this context, our data would support the possibility that apoptosis is involved in the long-term loss of cardiomyocytes in patients with essential hypertension.

Previous experimental evidence indicates that cardiac fibroblasts and cells in the wall of the cardiac vessels (ie, endothelial cells and vascular smooth muscle cells) die by apoptosis in arterial hypertension. Even a contributory role of noncardiomyocyte apoptosis to cardiac remodeling that occurs in the transition from compensated hypertrophy to heart failure in SHR has been proposed. Furthermore, the number of cardiac fibroblasts has been shown to be increased in the heart of SHR. Although we did not assess fibroblast proliferation in the present study, the possibility exists that apoptosis in these cells may be increased as a consequence of stimulated cell turnover in the myocardium of hypertensive patients.

In this study we found that losartan regressed LVH and diminished cardiac apoptosis in treated hypertensives. Thus, it seems that the treatment of hypertensives with losartan resulted in decreased cardiac mass despite the preservation of cardiac cellularity. This apparent discrepancy can be explained by the following considerations: (1) because cardiomyocytes can replicate in some conditions in the adult human heart, and proliferation of cardiac fibroblasts is increased in hypertensive heart disease, further studies are required to assess whether losartan, given long-term, modifies the balance between cell apoptosis and replication in hypertensive heart disease; (2) because individual cardiomyocyte enlargement is the most important determinant in hypertensive LVH, and it has been shown that angiotensin II promotes cardiomyocyte hypertrophy via the AT1 receptor, the possibility exists that losartan-induced LVH regression may be the consequence of changes in cardiac cell dimensions; and (3) it is possible that part of left ventricular mass decrease observed in losartan-treated hypertensives is due to a decrease in extracellular matrix. In fact, we have reported recently that the deposition of collagen fibers was significantly reduced in the hearts of hypertensive patients chronically treated with losartan.

In summary, our findings show that enhanced apoptosis affecting all cardiac cell types is present in human hypertensive heart disease. The presented data suggest that the interaction of angiotensin II with its AT1 and AT2 receptors, in combination with the impact of arterial hypertension and other humoral factors, may facilitate cardiac apoptosis in hypertensive patients. Finally, from our observations it can be proposed that pharmacological interventions in hypertension should be applied, not only to normalize growth, but also to control apoptosis in the heart and other target organs.

Acknowledgments
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