Apoptosis During Regression of Cardiac Hypertrophy in Spontaneously Hypertensive Rats
Temporal Regulation and Spatial Heterogeneity

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Abstract—We previously reported that increased apoptosis participates in the regression of aortic hypertrophy in spontaneously hypertensive rats. To further document the potential role of apoptosis in cardiovascular therapy, we examined apoptosis during regression of hypertrophy in the heart of spontaneously hypertensive rats receiving the antihypertensive drug enalapril (30 mg · kg⁻¹ · d⁻¹), losartan (30 mg · kg⁻¹ · d⁻¹), nifedipine (35 mg · kg⁻¹ · d⁻¹), hydralazine (40 mg · kg⁻¹ · d⁻¹), propranolol (50 mg · kg⁻¹ · d⁻¹), or hydrochlorothiazide (75 mg · kg⁻¹ · d⁻¹) for 1 to 4 weeks, starting at 10 to 11 weeks of age. Systolic blood pressure and heart rate were measured by the tail-cuff method. Markers of apoptosis included oligonucleosomal DNA fragmentation in extracted cardiac DNA or in situ in ventricular cross sections labeled with terminal deoxynucleotidyl transferase. Cardiac DNA synthesis was evaluated by [³H]-thymidine incorporation in vivo. All drugs reduced cardiac workload, defined as the product of blood pressure and heart rate, by >20% at 4 weeks. However, only nifedipine, enalapril, losartan, and propranolol reduced cardiac mass (>19%) within 4 weeks. Regression of cardiac hypertrophy was accompanied by a 50% to 300% increase in DNA fragmentation and a >20% reduction in DNA synthesis, resulting in a >20% reduction in cardiac DNA content after 4 weeks. Apoptosis induction occurred early and was transient within 4 weeks of nifedipine, enalapril, or losartan administration. With all regression-inducing drugs, the increase in DNA fragmentation occurred mainly in the subepicardium. Thus, transient induction of apoptosis in the subepicardium appears to be a characteristic feature of the early response to drug-induced regression of cardiac hypertrophy in spontaneously hypertensive rats. (Hypertension. 1999;34:229-235.)

Key Words: β-adrenergic antagonist ■ calcium channel blocker ■ AT₁ antagonist ■ ACE inhibitor

Although cardiac hypertrophy is an independent risk factor for cardiovascular morbidity and mortality, the mechanisms regulating cardiac mass remain poorly defined. Different classes of antihypertensive drugs are not equally effective at reducing left ventricular mass, suggesting a blood pressure–independent regulation.¹ Cardiac hypertrophy involves both cellular hyperplasia (mainly in nonmyocytes) and hypertrophy (mainly in myocytes).² A potential mechanism contributing to the suppression of cardiac hypertrophy is the gene-regulated process of physiological cell self-destruction called apoptosis.³ Cardiac apoptosis has been documented during normal neonatal maturation,⁴ aging,⁶ hypertension,⁷⁻¹⁰ ischemia, and failure.¹¹

The spontaneously hypertensive rat (SHR) is a model of genetically determined cardiac hypertrophy with increased cardiac mass and DNA content at birth,¹² further suggesting blood pressure–independent regulation. In this model, cardiac alterations evolve from concentric hypertrophy to dilated cardiomyopathy and heart failure.¹³ Results from our group suggest that neonatal cardiac hypertrophy in SHRs might be due in part to an imbalance between cell growth and apoptosis favoring DNA accumulation.¹⁴ As the heart of untreated adult SHRs adapts to hypertension and progresses toward failure, cardiac internucleosomal DNA fragmentation (a hallmark of apoptosis) increases notably in cardiomyocytes.⁷,⁹,¹⁴ Regulation of the cell growth/cell death balance during antihypertensive therapy, particularly during the process of cardiac hypertrophy regression, is poorly understood. Díez et al⁹ and Fortuno et al¹⁰ recently reported that cardiomyocyte apoptosis is reduced after prolonged inhibition of the angiotensin pathway during the established phase of hypertension in SHRs. The same group also reported evidence suggesting increased susceptibility to apoptosis in coronary smooth muscle cells (SMCs) of SHRs that received long-term treatment with an angiotensin I–converting enzyme (ACE) inhibitor.¹⁵ These data are consistent with those in our previous report showing that the stimulation of SMC apoptosis precedes regression of aortic hypertrophy in SHRs.¹⁶ Thus, we...
examined the balance between cell replication and apoptosis during the early phase of cardiac mass reduction in response to several of the major classes of antihypertensive drugs in SHRs. To the best of our knowledge, the present study provides the first evidence that apoptosis is stimulated during the reversal of cardiac hypertrophy.

Methods

Animal Procedures
The majority of the 132 male SHRs (Charles-River, St. Constant, Canada) included in the present study were used previously in another study to demonstrate increased apoptosis of aortic SMCs during regression of vascular hypertrophy in response to antihypertensive treatment. In brief, rats were treated for 1 to 4 weeks with the following drugs, starting at 10 to 11 weeks of age: nifedipine (35 mg·kg⁻¹·d⁻¹; gift of Bayer, Toronto, Canada), enalapril (30 mg·kg⁻¹·d⁻¹; gift of Merck-Frosst, Montreal, Canada), losartan (30 mg·kg⁻¹·d⁻¹; gift of Merck-Frosst, Canada), propranolol (50 mg·kg⁻¹·d⁻¹; Sigma Chemical Co., St. Louis, Mo), diuretic hydrochlorothiazide (75 mg·kg⁻¹·d⁻¹; Sigma), and hydralazine (40 mg·kg⁻¹·d⁻¹; Sigma). Systolic blood pressure and heart rate were determined weekly in all rats by the tail-cuff method. Cardiac workload was calculated as the product of blood pressure and heart rate. To evaluate DNA synthesis in vivo, rats were injected intra peritoneally with [methyl-³²P]-thymidine (0.5 mCi·kg⁻¹) 17. 9, and 1 hour before killing. Rats were anesthetized with a single intramuscular injection of a mixture of ketamine (80 mg·kg⁻¹), xylazine (4 mg·kg⁻¹), and acepromazine (2 mg·kg⁻¹). Rats were killed by venous draining with retrograde perfusion of 200 mL of isotonic saline via the abdominal aorta. The whole heart was isolated and weighed, and equatorial 3-mm sections of both ventricles were fixed in 4% paraformaldehyde. The remaining cardiac tissue was immediately frozen in liquid nitrogen and stored at −80°C until analysis. Animal handling protocols were approved by the Animal Care Committee of the University of Montreal Hospital Research Center.

DNA Analysis
Hearts were pulverized in liquid nitrogen with a mortar and pestle. Aliquots of frozen tissue powder were weighed, cardiac DNA was extracted by the phenol-chloroform method, and DNA content per milligram of tissue powder was calculated. Specific activity of extracted DNA aliquots (50 μg), indicative of DNA synthesis in vivo, were measured by liquid scintillation counting. Apoptosis was quantified by use of the oligonucleosomal DNA fragmentation index as previously described. In brief, the extracted cardiac DNA was fragmented in vitro by DNase I to induce the formation of DNA strand breaks. In negative controls, TdT addition was omitted. Sections were counterstained with hematoxylin.

Statistics
Data were analyzed by analysis of variance and unpaired Student’s t test with Bonferroni correction for multiple comparisons when appropriate. The DNA fragmentation index was analyzed with the nonparametric Kruskal-Wallis test followed by the nonparametric Mann-Whitney test because of unequal variance between the groups and because we have no evidence that the index is a normally distributed variable. Values are mean±SEM, and P<0.05 was considered statistically significant.

Results

Systemic and Hemodynamic Changes During Drug Therapy
Pretreatment values of body weight of all rats were 252±2 g (n=132). Body weight gain was not affected by the treatments (not shown). All rats showed high systolic blood pressure before the beginning of treatment (174±4 mm Hg; n=132) and at 4 weeks (220±5 mm Hg; n=25). As previously reported, systolic pressure in these rats was reduced at 4 weeks with hydralazine (44% reduction), losartan (40% reduction), enalapril (44% reduction), and nifedipine (33% reduction), but pressure was unchanged with propranolol or hydrochlorothiazide when treated rats were compared with paired controls. Heart rate was not different between control and experimental groups before treatment (pretreatment values: 354±10 bpm; n=132), and this parameter did not change in untreated animals over the course of the experiment. Heart rate was reduced significantly after 2 weeks with propranolol (16% reduction) and hydrochlorothiazide (13% reduction), after which time these values remained stable. Nifedipine increased heart rate at 2 weeks by 11%, after which time this value remained stable. Heart rate was not affected by losartan, enalapril, or hydralazine (not shown).

Cardiac workload was not different between control and experimental groups before treatment (pretreatment values: 61±2×10⁵ mm Hg·bpm; n=132), and this parameter increased by 33% in untreated animals during the course of the experiment, mainly as a result of the increase in blood pressure. Although the drugs tested showed distinct effects on blood pressure and heart rate, all treatments reduced cardiac workload when treated rats were compared with controls at 4 weeks, eg, with losartan (42% reduction), enalapril (45% reduction), nifedipine (28% reduction), propranolol (35% reduction), hydralazine (40% reduction), and hydrochlorothiazide (38% reduction). All drugs reduced cardiac workload significantly as early as 1 week after the start of treatment except nifedipine, which reduced cardiac workload significantly by 4 weeks.

Effect of Drugs on Cardiac Hypertrophy and DNA Content
The effects of drug treatments that affected cardiac mass and growth parameters are summarized in Figure 1, where data are expressed as a percentage of control values from untreated animals and plotted as a function of time after initiation of therapy. Cardiac mass (heart to body weight ratio) was significantly reduced after 2 weeks of nifedipine and 4 weeks
of losartan, enalapril, or propranolol (Figure 1A). In untreated animals, cardiac mass did not increase significantly between 1 week (5.2 ± 0.4 × 10^{-3}; n = 10) and 4 weeks (5.3 ± 0.1 × 10^{-3}; n = 25), suggesting a regression rather than a prevention of cardiac hypertrophy with the treatments. Cardiac mass was not affected after 4 weeks of treatment with hydralazine (4.8 ± 0.1 × 10^{-3}; n = 6) or hydrochlorothiazide (5.1 ± 0.2 × 10^{-3}; n = 6). To further analyze cardiac growth, the total cardiac DNA content normalized for body weight was evaluated (Figure 1B). Cardiac DNA content (μg · g^{-1} body weight) was not affected after 4 weeks of treatment with hydralazine (5.4 ± 0.3; n = 6) or hydrochlorothiazide (4.0 ± 1.0; n = 6) in treated rats as compared with controls (4.8 ± 0.2; n = 25). In contrast, cardiac DNA content was significantly reduced at 1 week with losartan or nifedipine and at 2 weeks with enalapril or propranolol. In summary, regression of cardiac hypertrophy was associated with a reduction in cardiac DNA content.

**Effect of Drugs on DNA Synthesis and Degradation**

Within 24 hours before they were killed, all rats were injected with [3H]-thymidine to evaluate DNA replication in vivo. The specific activity of whole-heart homogenates (cpm · 100 μg^{-1} DNA) was similar in controls at 1 to 4 weeks (eg, at 1 week: 218 ± 9; n = 10), and this parameter was not affected by treatment (eg, with losartan at 1 week: 222 ± 41; n = 5), suggesting similar cardiac uptake of [3H]-thymidine. In contrast to cardiac homogenate, however, cardiac DNA showed a decrease in specific activity (cpm · 100 μg^{-1} DNA) with drug treatment, suggesting an inhibition of cardiac DNA synthesis (Figure 1C). Cardiac DNA synthesis was reduced significantly with losartan (beginning at 1 week) and enalapril (beginning at 2 weeks) and was reduced transiently with nifedipine (at 1 week) and propranolol (at 2 weeks). In contrast, DNA synthesis was not affected by hydralazine (851 ± 41; n = 6) or hydrochlorothiazide (759 ± 50; n = 5) in treated rats as compared with controls at 4 weeks (757 ± 72; n = 25). In controls, DNA synthesis was not different between 1 and 4 weeks (not shown). Autoradiography showed small numbers of [3H]-thymidine–positive cells with no specific localization in the ventricular wall (not shown). In summary, cardiac regression was associated with a reduction in cardiac DNA synthesis.

We tested the hypothesis that apoptosis contributed to the reduction in cardiac DNA content. Oligonucleosomal DNA fragmentation was similar in controls at 1 to 4 weeks (eg, at 1 week: 1.0 ± 0.1 arbitrary units · μg^{-1} DNA; n = 10). However, DNA fragmentation was increased as early as 1 week with nifedipine or losartan (Figure 1D). Enalapril and pro-
Propranolol stimulated DNA fragmentation at 2 and 4 weeks, respectively. In contrast, DNA fragmentation was not affected at 4 weeks with hydralazine (0.6 ± 0.2; n=6) or hydrochlorothiazide (0.5 ± 0.1; n=5) in treated rats as compared with controls (0.8 ± 0.2; n=25). Thus, the regression of cardiac hypertrophy was associated with increased apoptotic activity.

The anatomic distribution of cells undergoing apoptosis is presented in Figure 2A to 2H, which shows the density of radiolabeled DNA fragments across the ventricular wall in hearts from rats given placebo or treatment, as detected with a PhosphoImager. The time points selected for losartan, enalapril, and propranolol (in Figure 2B to 2D) correspond to the peak increase in internucleosomal fragmentation observed in the DNA extracted from the whole heart (Figure 1D). Bar graphs in the lower panels of Figure 2 show the mean density of radiolabeled DNA fragments as quantified separately in the epicardial and endocardial areas. In control hearts, the epicardial and endocardial areas showed higher DNA fragmentation in both ventricles (Figure 2A). In hearts undergoing regression, however, the main increase in DNA fragmentation appeared in the area of the epicardium in both ventricles (Figures 2B to 2E). In contrast, there was no change in signal in hearts from rats treated for 4 weeks with hydralazine (Figure 2H) or hydrochlorothiazide (not shown). The time-dependent changes in in situ DNA fragmentation are shown for nifedipine in Figures 2E to 2G. The use of the nonradioactive digoxigenin–based TUNEL method confirmed the nuclear localization of the labeling signal in the subepicardial and subendocardial areas (not shown). Few, if any, TUNEL-positive cells were found to be associated with vascular structures at the time points examined. Levels of DNA fragmentation in the whole heart (Figure 1D) correlated significantly with DNA fragmentation in the epicardial area (P < 0.001; r=0.8) but not the endocardial area. Thus, regression of cardiac hypertrophy was associated with apoptosis in the subepicardial area.
**Discussion**

The significant new finding of the present study is that, regardless of the class of antihypertensive drug administered to SHRs, those drugs able to induce cardiac hypertrophy regression were also found to induce a localized increase in apoptosis in the subepicardium. Drugs active in this regard included a calcium channel blocker, an ACE inhibitor, an AT₁ receptor antagonist, and a β-adrenoreceptor antagonist. Evidence of apoptosis includes a rapid increase in cardiac DNA fragmentation coupled with a reduction in cardiac DNA content within 4 weeks of therapy.

The induction of apoptosis was transient, a feature most evident with nifedipine. Losartan and enalapril did not cause a steady decline in cardiac DNA content after the initial burst of DNA fragmentation and drop in DNA content, also suggesting temporary activation of apoptosis around 1 week. The more sustained elevation of the DNA fragmentation index may reflect the persistence of DNA fragments in the cardiac tissue beyond the early period of active apoptosis. Transient episodes of apoptosis induction were previously observed during normal, pathological, or therapy-induced cardiovascular remodeling.8,11,16,18 A phenomenon we referred to as “time window of apoptosis.”18 For instance, we reported that regression of aortic hypertrophy in SHRs is accompanied by a time window in SMC apoptosis in response to losartan or nifedipine, with a peak increase within 1 week of treatment.16 With enalapril, apoptosis in the aorta is slower to develop, with the highest levels reached after 2 weeks. In the present study, the induction of cardiac apoptosis followed a similar time course during regression of hypertrophy with these 3 drugs. The faster onset of apoptosis activation with losartan versus enalapril may implicate the activation of AT₂ receptors for angiotensin II in animals treated with an AT₁ antagonist but not an ACE inhibitor.19 It is noteworthy that propranolol increased cardiac apoptosis in SHRs, considering our previous observation that the β-blocker does not affect aortic apoptosis within 4 weeks in the same animals.16 Thus, these results strongly suggest that apoptosis is regulated in a time- and organ-specific manner by cardiovascular drugs in vivo.

With losartan or propranolol, the suppression of cardiac DNA synthesis was dissociated in time from the induction of apoptosis. With losartan, there was a sustained inhibition of DNA synthesis over 4 weeks, whereas the DNA fragmentation index was transiently increased at 1 and 2 weeks only. With propranolol, DNA synthesis was decreased transiently at 2 weeks and DNA fragmentation was increased at 4 weeks only. Together, these data suggest that cardiac DNA content is determined by a dynamic balance between DNA synthesis and degradation and that these 2 parameters may be regulated independently in the heart during drug treatment.

Cardiac workload was reduced by all drugs tested, including hydralazine and hydrochlorothiazide. The latter drugs, however, did not affect cardiac mass, growth, or apoptosis within 4 weeks even though hydralazine showed a potent antihypertensive effect. Large doses of β-blockers and diuretics, such as in the present study, are known to be poorly effective in reversing hypertension in SHRs.20,21 We cannot rule out a possible effect of these drugs on cardiac apoptosis with an earlier or more prolonged schedule of drug administration. In contrast, losartan and nifedipine stimulated apoptosis at 1 week, before blood pressure was significantly reduced. With enalapril, apoptosis was stimulated at 2 weeks, ie, after blood pressure was significantly reduced (at 1 week). Although nifedipine did not reduce blood pressure as much as enalapril or losartan, these 3 drugs were equipotent in reducing cardiac mass and DNA content at 4 weeks. Together, these results suggest that the trophic changes in the heart were not secondary to hemodynamic changes. We previously reached similar conclusions in our study of SMC apoptosis in the SHR aorta undergoing regression.16 Although it is conceivable that the antihypertensive response to therapy was underestimated because of the stress induced in rats during immobilization for blood pressure measurements by the tail-cuff method, the results with hydralazine clearly demonstrate that cardiac growth and apoptosis can be fully dissociated from blood pressure regulation.

Studies are under way in our laboratory to determine the relative importance of myocyte and nonmyocyte apoptosis in the present model of cardiac regression. It is tempting to speculate that apoptosis occurred preferentially in noncardiomyocytes, notably fibroblasts, during the early phase of cardiac regression. Fibrosis is an important factor in the decreased distensibility, causing impairment of cardiac performance during left ventricular hypertrophy. Angiotensin II and catecholamines stimulate fibroblast hyperplasia, and blockade of these pathways in vivo suppresses cardiac fibrosis.22 In contrast, previous studies showed that the number of cardiomyocytes is not reduced after cardiac regression in response to long-term treatment with ACE inhibitors,23 calcium channel blockers,24 or β-blockers,25 suggesting that these treatments do not stimulate apoptosis predominantly in cardiomyocytes. Moreover, these treatments improve cardiac function and reduce cardiovascular mortality.26,27 ACE inhibition suppresses cardiomyocyte apoptosis in the hypertrophic heart of SHRs9,10,14 and dogs.28 In contrast, angiotensin II acting via AT₁ receptors and β-adrenergic agonists stimulate cardiomyocyte apoptosis in vivo and in vitro.29–32 At this point, however, the possibility of increased cardiomyocyte apoptosis during the early phase of cardiac regression cannot be totally excluded. Panizo-Santos et al15 reported that administration of quinapril decreased the percentage of tetraploid cardiomyocytes in SHRs, suggesting that either nuclear division or apoptosis was induced in polyploid cardiomyocytes. Thus, evaluation of the global myocardial response will likely be complex, in part because of the time-dependent and cell-specific apoptotic responses.

A striking feature of apoptosis during cardiac regression is its specific localization in the subepicardium, suggesting heterogeneity in cardiac cell phenotype or local environment across the ventricular wall. Several studies have documented a negative transmural gradient in the percentage of myocardial shortening and strain from the subendocardium to the subepicardium.34 Heterogeneity in cardiomyocyte phenotype is also well documented. Subepicardial and subendocardial cardiomyocytes differ in terms of intracellular free ion concentrations,35,36 kinetics of ion channel activity,37–40 and receptor expression.41 Lukas and Antzelevitch37 reported that
in vivo differences in repolarization rates between epicardial and endocardial cardiomyocytes are maintained in vitro, further supporting the hypothesis of phenotypical heterogeneity in these cells. Fortuno et al recently reported a heterogeneous distribution of cardiomyocyte apoptosis in hearts of 16-week-old SHRs, with the highest levels in the subendocardium and mesocardium. Losartan reduced subendocardial and mesocardial apoptosis without affecting subepicardial apoptosis measured after 14 weeks of treatment. The specific induction of apoptosis in the subepicardium in the present study is in contrast to the typically diffuse distribution of apoptosis in adult hearts during pathological remodeling in response to a metabolic, hemodynamic, or immune insult. Previous studies in the ischemic rat heart suggested that ventricular function is more resistant to myocyte cell loss if it is localized rather than diffuse. Whether phenotypic heterogeneity exists in cardiac fibroblasts is currently unknown. Additional studies are needed to determine the mechanisms underlying the heterogeneous distribution of apoptotic cells in the heart undergoing rapid regression in response to antihypertensive therapy. It is possible that apoptosis in subepicardial cells during therapy-induced regression of hypertrophy may help preserve cardiac function by decreasing ventricular wall thickness without increasing ventricular cavity. Indeed, an increase in ventricular cavity would tend to increase wall stress and precipitate ventricular dysfunction.

In summary, we provide evidence that apoptosis is induced in the subepicardium of the hearts of SHRs during the first 4 weeks of antihypertensive drug therapy, regardless of the class of drug able to induce regression of cardiac hypertrophy and independently of blood pressure reduction. In contrast to the time window of apoptosis, there was a sustained reduction in cardiac mass, DNA content, and DNA synthesis. These results reinforce the suggestion that apoptosis is a potential therapeutic target in controlling, and perhaps initiating, cardiovascular remodeling. Caution should be exercised, however, when translating the present findings to the human population that receives long-term treatment with antihypertensive medication.

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