Smooth Muscle Apoptosis During Vascular Regression in Spontaneously Hypertensive Rats

Denis deBlois, Bun-Seng Tea, Than-Vinh Dam, Johanne Tremblay, Pavel Hamet

Abstract We previously reported that apoptosis is increased in smooth muscle cells cultured from the aorta of spontaneously hypertensive rats versus normotensive controls. As an initial in vivo exploration, we now examined smooth muscle cell apoptosis regulation during the regression of vascular hypertrophy in the thoracic aorta media 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. Three criteria were used to evaluate smooth muscle cell apoptosis: (1) oligonucleosomal fragmentation of the extracted aortic DNA, (2) reduction in aortic DNA content, and (3) depletion of smooth muscle cells in the arterial media. Arterial DNA synthesis was evaluated by [³H]thymidine incorporation in vivo. After 4 weeks of treatment, systolic blood pressure was reduced significantly by >42% with losartan, enalapril, and hydralazine; by 23% with nifedipine, versus control values of 220±5 mm Hg. However, these agents affected vascular growth and apoptosis differently. Losartan and enalapril stimulated smooth muscle cell apoptosis threefold to fivefold before there was a significant reduction in DNA synthesis (>25%), vascular mass (>19%), or vascular DNA content (>38%), and these treatments markedly reduced (by 38% to 50%) medial cell number as measured at 4 weeks by the three-dimensional disector method. Losartan and nifedipine stimulated smooth muscle cell apoptosis before reducing blood pressure. In contrast, hydralazine did not affect vascular mass, apoptosis, or DNA synthesis, although blood pressure was lowered. Propranolol or hydrochlorothiazide failed to affect hypertension or vascular growth. Thus, smooth muscle cell apoptosis represents a novel therapeutic target for the control of hypertensive vessel remodeling in response to therapeutic agents (Hypertension. 1997;29(part 2):340-349).

Key Words • apoptosis • smooth muscle cell • angiotensin II • calcium channel antagonist

Studies in humans and in experimental models of hypertension have clearly demonstrated the importance of vascular structure in the regulation of blood pressure. Increased vascular mass is an important feature of hypertensive vessels. At the level of small muscular arteries, vascular hypertrophy accompanied by smooth muscle cell (SMC) hypertrophy or hyperplasia acts as an amplifier for elevated vascular resistance and blood pressure. At the level of the aorta and its main branches, increased mass and rigidity of the arterial wall contribute to systolic hypertension and represent an independent risk factor for left ventricular hypertrophy. The remodeling of vascular structure, including the regression of vascular hypertrophy, is now considered a key therapeutic target in the effort to reduce mortality and morbidity associated with high blood pressure.

Increased vascular mass associated with the replication and accumulation of smooth muscle DNA, as in genetically determined or secondary hypertension, represents a mode of structural remodeling that is less readily reversible than vascular hypertrophy that is solely due to increased protein synthesis without de novo DNA synthesis. Thus, the DNA content of the vessel wall (due to SMC hyperplasia or polyploidy) may be considered as a record of past episodes of vascular growth, contributing to the persistence of the vascular disease. Apoptosis is a highly regulated form of programmed cell death that is involved in tissue morphogenesis and homeostasis as the essential counterpart of cell replication. In this context, apoptosis is potentially involved in the regulation of vascular remodeling, via the deletion of SMC in the vessel wall. We have previously reported that the heightened DNA replication of SMC cultured from the aorta of spontaneously hypertensive rats (SHR) occurs in parallel to an increase in apoptosis. These studies suggested that increased apoptosis may in part counterbalance the heightened cell growth in hypertensive vessels. In this view, the balance between apoptosis and cell (or DNA) replication would constitute a key determinant of vascular mass and a potential therapeutic target for achieving long-term regression of vascular hypertrophy.

The present studies were designed to investigate whether SMC apoptosis contributes to the regression of vascular hypertrophy during antihypertensive therapy in the SHR. Agents tested belong to several of the major classes of drugs used in the clinic to control elevated blood pressure. Our results suggest that SMC apoptosis is a novel therapeutic target for the pharmacological control of vascular structure in hypertension.

Methods

Drug Treatments

Male SHR weighing 250 to 275 g were purchased from Charles-River (St Constant, Que) and housed for at least 10 days before initiation of drug treatment at 10 to 11 weeks of age. Treated rats were given one of the following drugs: the calcium channel blocker nifedipine (35 mg kg⁻¹ d⁻¹), the angiotensin-converting enzyme inhibitor enalapril (30 mg kg⁻¹ d⁻¹), the angiotensin receptor antagonist losartan (30 mg kg⁻¹ d⁻¹), the smooth muscle relaxant hydralazine (40 mg kg⁻¹ d⁻¹), the β-adrenoceptor blocker propranolol (50 mg kg⁻¹ d⁻¹), or the diuretic hydrochlorothiazide.
rothiazide (HCT; 75 mg·kg\(^{-1}\)·d\(^{-1}\)). The doses of antihypertensive drug were chosen on the basis of published reports of others or our own preliminary data showing effectiveness in reducing hypertension or cardiovascular hypertrophy in the SHR. All drugs were dissolved in the drinking water except for nifedipine, which was mixed with powdered food. The subsets of control rats receiving powdered rat chow without nifedipine showed similar results as the control rats given regular chow in pellets, hence the data from these control groups were pooled for analysis. Food and water, with or without drug, were renewed three times weekly and administered ad libitum. The rats were weighed each week and the dosage of drug adjusted according to their body weight and the amount of water or food consumed. All drugs were from Sigma, except nifedipine, which was provided courtesy of Bayer Canada, Toronto, and losartan and enalapril, which were courtesy of Merck Frosst, Montreal, Canada. The ability of the antihypertensive drugs to reduce aortic hypertrophy was initially examined after 4 weeks of therapy. Drugs that proved effective at 4 weeks were selected for the studies at 2 weeks and 1 week after 4 weeks of therapy. Drugs that proved effective at 4 weeks were selected for the studies at 2 weeks and 1 week. The PhosphorImager data were used to construct a regression line for each sample and radioactivity per unit area (cpm per pixel) was plotted as a function of DNA loaded on the gel (pg DNA). We found this approach to be useful in increasing reproducibility of the measurements. Thus, the slope of the linear regression was

**Blood Pressure Measurements**

Systolic blood pressure was measured between 8 and 12 AM in each rat by use of the tail cuff plethysmograph method (ITC). Measurements were started 1 week before treatment and performed on conscious restrained rats previously trained for the procedure. Pressure measurements were made at weekly intervals during the treatment period and at least 2 days before animal sacrifice for tissue isolation to avoid interfering with the vascular growth response.

**Tissue Isolation and Vascular Mass Measurements**

To evaluate DNA synthesis in vivo, rats were injected IP with [methyl-\(^{3}H\)]thymidine (0.5 mCi/kg, New England Nuclear) at 17, 9, and 1 hour before death. Rats were anesthetized with a single IM injection of ketamine (80 mg/kg), xylazine (4 mg/kg), and acepromazine (2 mg/kg), and killed by perfusion of 200 mL isotonic saline via the abdominal aorta. The thoracic aorta was isolated from the diaphragm to above the first intercostal artery and cut longitudinally to allow removal of the endothelium by scrubbing the intimal surface with a cotton tip applicator. The thoracic aorta was isolated from the diaphragm to above the first intercostal artery and kept at -80°C until further processing. To further confirm changes in vascular mass, we also measured the cross-sectional area of aortae isolated after 4 weeks of effective antihypertensive therapy (a blindly selected subset of tissues was used for the control and enalapril groups). A 3-mm-long ring of the whole aortic media was homogenized in liquid nitrogen using a mortar and pestle. An aliquot of the pulverized aortic media was weighed and total tissue DNA was extracted by the phenol and chloroform procedure, following tissue digestion steps with proteinase K and RNase A in the presence of EDTA, as described previously. DNA concentration was determined by spectrophotometry and total DNA content per unit of aortic length was calculated using the following equation: total aortic DNA content=(μg of DNA/mg of aorta)×(mg of aorta/cm of aorta).

Oligonucleosomal DNA fragmentation into 180- to 200-bp integer fragments is a hallmark of apoptosis. This specific pattern of DNA fragmentation appears as a "ladder" of DNA fragments after conventional agarose gel electrophoresis in various cell types undergoing apoptosis. Including SMC, To quantify the degree of oligonucleosomal DNA fragmentation in the aortic media, 1 μg of extracted DNA was radiolabeled using terminal deoxynucleotidyl transferase and [\(^{32}P\)]dCTP as described previously. Because DNA molecules from apoptotic nuclei have an increased number of free 3'OH ends as a result of enhanced endonuclease activity, these show greater incorporation of labeled dCTP. Increasing quantities of radiolabeled DNA (0.025, 0.05, 0.1, 0.2, and 0.4 μg) were loaded in adjacent lanes of 1.5% agarose gels. After electrophoresis, DNA was transferred onto nylon membrane (Hybond N; Amersham) and the radioactivity associated with 150 to 1500 bp DNA fragments was quantified using a PhosphorImager (Molecular Dynamics) (Fig 1A). The PhosphorImager data were used to construct a regression line for each sample and radioactivity per unit area (cpm per pixel) was plotted as a function of DNA loaded on the gel (μg DNA). We found this approach to be useful in increasing reproducibility of the measurements. Thus, the slope of the linear regression was

**DNA Extraction and Characterization**

The whole aortic media was homogenized in liquid nitrogen using a mortar and pestle. An aliquot of the pulverized aortic media was weighed and total tissue DNA was extracted by the phenol and chloroform procedure, following tissue digestion steps with proteinase K and RNase A in the presence of EDTA, as described previously. DNA concentration was determined by spectrophotometry and total DNA content per unit of aortic length was calculated using the following equation: total aortic DNA content=(μg of DNA/mg of aorta)×(mg of aorta/cm of aorta).

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**Fig 1.** A, DNA laddering obtained by gel electrophoresis of DNA extracted from the aorta of control SHR (CON) or SHR treated for 1 week with nifedipine (NIF; 25 mg·kg\(^{-1}\)·d\(^{-1}\)) or losartan (LOS; 30 mg·kg\(^{-1}\)·d\(^{-1}\)). Increasing amounts of DNA (0.025, 0.05, 0.1, 0.2, and 0.4 μg) radiolabeled with terminal deoxynucleotidyl transferase were fractionated on a 1.5% agarose gel. B, Quantification of radioactivity associated with the 150- to 1500-bp fragments as a function of the amount of DNA loaded, using a PhosphorImager as described in "Methods." The slope of the linear regression was defined as the "DNA fragmentation index" (cpm per pixel per microgram DNA), indicative of apoptosis.
TABLE 1. Effect of Antihypertensive Therapy for 4 Weeks on SHR Body Weight, and Aortic Medial Hypertrophy, DNA Content, DNA Fragmentation, and DNA Specific Activity

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Final Body Weight</th>
<th>Aorta:Body Weight Ratio, mg mm⁻¹ g⁻¹</th>
<th>DNA Content, μg/mg</th>
<th>DNA Fragmentation Index, cpm pixel⁻¹ μg⁻¹</th>
<th>DNA Specific Activity, cpm/μm² μg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control (6)</td>
<td>288 ± 5</td>
<td>5.2 ± 0.4</td>
<td>1.31 ± 0.24</td>
<td>2.4 ± 0.5</td>
<td>134 ± 6</td>
</tr>
<tr>
<td></td>
<td>Losartan (6)</td>
<td>282 ± 6</td>
<td>4.2 ± 0.6</td>
<td>1.2 ± 0.23</td>
<td>3.0 ± 0.5</td>
<td>136 ± 10</td>
</tr>
<tr>
<td>B</td>
<td>Control (9)</td>
<td>306 ± 6</td>
<td>5.0 ± 0.5</td>
<td>1.25 ± 0.09</td>
<td>2.9 ± 0.8</td>
<td>110 ± 4</td>
</tr>
<tr>
<td></td>
<td>Enalapril (12)</td>
<td>200 ± 0</td>
<td>3.0 ± 0.4</td>
<td>0.42 ± 0.07*</td>
<td>16.7 ± 4.1*</td>
<td>76 ± 6*</td>
</tr>
<tr>
<td></td>
<td>Nifedipine (5)</td>
<td>321 ± 7</td>
<td>3.7 ± 0.2*</td>
<td>0.94 ± 0.07</td>
<td>12.0 ± 0.7</td>
<td>126 ± 9</td>
</tr>
<tr>
<td>C</td>
<td>Control (5)</td>
<td>291 ± 14</td>
<td>4.6 ± 1.2</td>
<td>1.17 ± 0.26</td>
<td>9.6 ± 2.7</td>
<td>112 ± 8</td>
</tr>
<tr>
<td></td>
<td>Propranolol (5)</td>
<td>291 ± 4</td>
<td>4.4 ± 1.8</td>
<td>1.14 ± 0.28</td>
<td>10.3 ± 5.3</td>
<td>119 ± 6</td>
</tr>
<tr>
<td></td>
<td>HCT (5)</td>
<td>292 ± 10</td>
<td>4.2 ± 2.3</td>
<td>0.56 ± 0.10</td>
<td>5.0 ± 0.1</td>
<td>129 ± 7</td>
</tr>
</tbody>
</table>

The innucleosomal DNA fragmentation index indicative of apoptosis is expressed as the DNA fragmentation index, as described in *Methods.* The DNA specific activity reflects the incorporation of tritiated thymidine into the smooth muscle DNA over the last 24 hours in vivo. Data were analyzed by ANOVA followed by Student’s t test with Bonferroni correction for multiple comparisons, except for DNA fragmentation index, which was analyzed using the nonparametric tests (Kruskal-Wallis followed by Mann-Whitney). *P < 0.05 was considered statistically significant.*

Results

The ability of the antihypertensive drugs to reduce vascular hypertrophy and growth in the thoracic aorta was initially examined after 4 weeks of therapy in the SHR (Table 1). Drugs that proved effective in reducing vascular hypertrophy at 4 weeks were selected for the studies at 2 weeks and 1 week of treatment (Table 2). In addition, to facilitate intergroup comparisons, selected changes in vascular growth parameters are also presented in Fig 3, where the data are expressed as a percentage of control values (for paired animals without treatment) and plotted as a function of time after initiation of therapy with selected drugs.

Systemic Changes During Drug Administration

Rats used in this study were hypertensive before initiation of therapy, with an average pretreatment value of systolic blood pressure of 176 ± 2 mm Hg (n = 110; no difference between control and experimental groups). During the experimental period, systolic pressure increased further in untreated animals, up to 203 ± 5 mm Hg at 2 weeks (n = 39) and 220 ± 5 mm Hg at 4 weeks (n = 25). Fig 2A shows the evolution of systolic blood pressure in control and treated rats over the 4-week experimental period. Systolic blood pressure was not significantly affected in rats receiving propranolol or HCT for 4 weeks (Fig 2B) but it was significantly reduced in rats receiving either hydralazine (47% reduction in final pressure), losartan (42% reduction), enalapril (44% reduction), or nifedipine (23% reduction). Significant reduction in blood pressure was achieved after 1 week with enalapril or hydralazine, after 2 weeks with losartan, and after 3 weeks with nifedipine (Fig 2A). Thus, at the high doses used in this study, the drugs showed the following order of potency for blood pressure reduction: enalapril = hydralazine > losartan > nifedipine > HCT > propranolol.

Pretreatment values of body weight were 260 ± 2 g (n = 110). All rats gained weight during the experimental period but final values of body weight were similar be-
Smooth Muscle Cell Apoptosis in Vascular Regression

**TABLE 2. Effect of Antihypertensive Therapy for 1 or 2 Weeks on SHR Body Weight, and Aortic Medial Hypertrophy, DNA Content, DNA Fragmentation, DNA Specific Activity, and Tissue Specific Activity**

<table>
<thead>
<tr>
<th>Duration of Treatment</th>
<th>Treatment (n)</th>
<th>Final Body Weight, g</th>
<th>Aorta:Body Weight Ratio, mg mm⁻¹ g⁻¹</th>
<th>DNA Content, μg/mm²</th>
<th>DNA Fragmentation Index, cpm/μg²</th>
<th>DNA Specific Activity, cpm/μg</th>
<th>Aortic Tissue Specific Activity, cpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week</td>
<td>Control (5)</td>
<td>270±11</td>
<td>4.8±0.8</td>
<td>1.07±0.29</td>
<td>2.7±0.5</td>
<td>118±8</td>
<td>56±9</td>
</tr>
<tr>
<td></td>
<td>Losartan (5)</td>
<td>264±3</td>
<td>4.2±0.7</td>
<td>0.96±0.24</td>
<td>14.2±3.3*</td>
<td>111±10</td>
<td>42±5</td>
</tr>
<tr>
<td></td>
<td>Nifedipine (5)</td>
<td>271±8</td>
<td>4.3±0.9</td>
<td>1.00±0.31</td>
<td>8.7±3.0*</td>
<td>115±6</td>
<td>41±10</td>
</tr>
<tr>
<td>2 weeks</td>
<td>Control (14)</td>
<td>276±7</td>
<td>6.1±0.6</td>
<td>1.31±0.14</td>
<td>1.8±0.5</td>
<td>125±6</td>
<td>33±4</td>
</tr>
<tr>
<td></td>
<td>Enalapril (6)</td>
<td>277±0</td>
<td>5.2±0.2</td>
<td>1.61±0.18</td>
<td>6.9±3.0</td>
<td>160±10</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Losartan (5)</td>
<td>283±5</td>
<td>3.7±0.7</td>
<td>0.66±0.22*</td>
<td>7.4±2.7*</td>
<td>94±3*</td>
<td>38±5</td>
</tr>
<tr>
<td></td>
<td>Nifedipine (5)</td>
<td>313±8</td>
<td>3.4±0.3</td>
<td>0.55±0.06*</td>
<td>1.0±0.5</td>
<td>72±7*</td>
<td>41±7</td>
</tr>
</tbody>
</table>

Internucleosomal DNA fragmentation indicative of apoptosis is expressed as the DNA fragmentation index, as described in "Methods." The DNA specific activity and the tissue specific activity reflect the incorporation of tritiated thymidine into the smooth muscle DNA or into the whole aortic media, respectively, over the last 24 hours in vivo. Data were analyzed by ANOVA followed by Student's t test with Bonferroni correction for multiple comparisons, except for DNA fragmentation index, which was analyzed using the nonparametric tests Kruskal-Wallis followed by Mann-Whitney.

**P < 0.05**

tween corresponding control and experimental groups at 1, 2, and 4 weeks (not shown). Daily water consumption was increased in rats treated with losartan (by 20%) or enalapril (by 23%). Powdered food consumption was not different between the nifedipine and the corresponding control group (not shown).

**Effect of Drugs on Vascular Hypertrophy and DNA Content**

At 4 weeks the aorta:body weight ratio was not affected by hydralazine, propranolol, or HCT (Table 1). In contrast, there was a significant reduction in aortic mass at 4 weeks in rats given losartan (19% reduction), enalapril (22% reduction), or nifedipine (26% reduction). Further studies at earlier times revealed a significant reduction in aortic mass as early as 2 weeks with losartan or nifedipine but not enalapril (Table 2 and Fig 3A). The aorta of untreated animals showed no increase in vascular mass between tissues isolated at 1, 2, and 4 weeks, suggesting that the drug-induced reductions in vascular mass represented a regression rather than a prevention of vascular hypertrophy. As a further confirmation that vascular mass was decreased, the aortic cross-sectional area was significantly decreased by losartan, enalapril, or nifedipine but not hydralazine for 4 weeks (Table 3).

To further analyze vascular growth, aortic DNA content was evaluated per length of vessel. The length of the freshly isolated thoracic aorta was 30 ± 1 mm (n = 26) in untreated animals at 4 weeks, and this value was not affected by any of the treatments studied (not shown). Among all the drugs studied, only losartan and enalapril reduced aortic DNA content significantly, by 63% and 66%, respectively, in the aorta after 4 weeks of treatment (Table 1). In contrast, DNA content at 2 weeks showed a significant 60% to 65% reduction with nifedipine and losartan, and no evidence of reduction with enalapril (Table 2 and Fig 3B). Administration of losartan or nifedipine for a shorter period of time (1 week) did not affect total DNA content in the aorta. Because the enalapril-induced reduction in DNA content required more than 2 weeks of treatment, this drug was not included in the 1-week studies designed to examine apoptosis.

**Effect of Drugs on SMC Apoptosis**

The therapy-induced reduction in DNA content in the thoracic aorta suggested a stimulation of apoptosis and a corresponding reduction in SMC number. To test this hypothesis, the DNA fragmentation index was evaluated as described in "Methods." Losartan caused a threefold to fivefold increase in internucleosomal DNA fragmentation. This effect of losartan was highest at 1 week and sustained as late as 4 weeks after beginning of treatment (Tables 1 and 2, and Fig 3C). Enalapril also stimulated internucleosomal DNA fragmentation by threefold to fivefold, although the highest effect was observed at 4 weeks. In nifedipine-treated rats, internucleosomal DNA fragmentation was increased at 1 week only. The DNA fragmentation index for the large 20- to 30-kbp DNA fragments showed no significant change with treatment (not shown), compared with control values of 1.8 ± 0.1 cpm per pixel per microgram DNA (n = 40).

The three-dimensional disector method was used to determine whether the reduced DNA content corresponded to a reduced SMC number in the arterial wall (Table 3). In the aorta of untreated rats, we found 116 ± 6 SMC per micrometer of vessel length. This SMC number is comparable to what Owens reported in the aorta of untreated 3-month-old SHR, using a different method of evaluation. We found that the SMC number per length of aorta was significantly reduced by 50% with losartan, by 47% with enalapril, and by 38% with nifedipine. In contrast, the potent antihypertensive effect of hydralazine did not result in reduced SMC number in the arterial wall. Thus, there was a highly significant correlation between the aortic DNA content and the number of SMC in the arterial wall (P < 0.001; r = 0.6). Although losartan, enalapril, and nifedipine significantly reduced the cross-sectional area and therefore the total mass of the aorta, the reduction in SMC number was large enough so that the SMC numerical density was also significantly decreased in the arterial wall (Table 3). None of the treatments under study with the disector method affected significantly the average nuclear length of arterial SMC, which was 15 ± 1 μm in the untreated aorta, a value comparable with the results obtained by Mulvany et al in SHR small mesenteric arteries using the same method.

**Effect of Drugs on DNA Synthesis**

As shown in Table 2, several treatments caused a significant reduction in arterial DNA specific activity without affecting radioactivity levels in the whole vascular media (at least at 2 weeks), thus suggesting an inhibition of SMC DNA synthesis. Within 4 weeks, the specific activity of aortic DNA was decreased by losartan, enalapril, and nifedipine but not by hydralazine, propranolol, or HCT (Tables 1 and 2). The kinetic but not the magnitude of the...
**Conclustion**

The inhihitory effect varied between losartan, enalaprl, and nifedipine (Fig 3D). Thus, DNA specific activity was significantly reduced by losartan at 2 and 4 weeks (25% and 31% reduction, respectively), by nifedipine at 2 weeks only (42% reduction), and by enalaprl at 4 weeks only (32% reduction).

**Effects of a Lower Dose of Losartan**

The regulation of aortic growth was examined in rats treated with a lower dose of losartan for 4 weeks. As shown in Table 4, administration of losartan (10 mg kg⁻¹ d⁻¹) to SHR was unable to reverse the pre-existing hypertension but prevented the further development of high blood pressure. Interestingly, this treatment did not result in any significant change in aortic mass, DNA fragmentation, DNA specific activity, or DNA content at 4 weeks.

**Discussion**

Inhibited vascular mass is an important feature of hypertensive vessels. In the adult SHR, the thoracic aorta shows medial hypertrophy and a higher SMC DNA content and synthesis rate as compared to normotensive animals. 17 18 34-38 In the absence of high blood pressure, SHR SMC cultured in vitro maintain an increased growth behavior, including hyperresponsiveness to growth factors, accelerated entry into S phase of the cell cycle, reduced cell-contact inhibition, and high incidence of polyploidy, suggesting a pressure-independent dysregulation of growth. 39-44 We have reported that SMC cultured from SHR aorta also exhibit a heightened propensity to undergo apoptosis, e.g., in response to growth factor withdrawal. 21 We recently reviewed 45 the possible role of apoptosis in regulating vascular mass and DNA content, a hypothesis that has never been examined in hypertensive vessels. The balance between SMC DNA synthesis and degradation determines the total DNA content of the arterial media, which in turn is a primary determinant of vascular mass. Thus, the aim of the present studies was to investigate the regulation of SMC apoptosis during the regression of aortic medial hypertrophy induced by antihypertensive therapy in the SHR.

**Time Window of Apoptosis**

Within 4 weeks of effective antihypertensive therapy, we observed a reduction in aortic mass in rats treated with losartan, enalapril, or nifedipine but not hydralazine. Several lines of evidence suggest that the regression of vascular hypertrophy was associated with increased SMC apoptosis. First, there was a marked increase in internucleosomal fragmentation of the DNA in the aortic media. This specific pattern of DNA is a hallmark feature of apoptosis observed in several types of cells 21-30-33 Second, there was a significant reduction in aortic DNA content in the weeks following the initial burst of internucleosomal DNA fragmentation. Finally, the number of SMC in the aortic wall was reduced after 4 weeks of losartan, enalapril, or nifedipine but not hydralazine, in correlation with the effects of these drugs on aortic DNA content. The present data on the kinetics of arterial SMC apoptosis during drug therapy are reminiscent of our recent observations in the heart adapting to pressure overload caused by aortic coarctation.46 In this model, the development of cardiac hypertrophy is preceded by a transient increase in apoptosis, notably in cardiomyocytes. We suggested that the design of therapeutic approaches aimed at controlling cardiovascular remodeling should take into account the presence of time windows of apoptosis during pathogenesis. 47 The present studies provide evidence that windows of rapid change in apoptosis also occur in response to drug therapy.

**SMC Apoptosis and Blood Pressure**

The stimulation of SMC apoptosis could be dissociated from the antihypertensive effects of the drugs tested. Although hydralazine was very effective at reducing hypertension, it did not affect DNA fragmentation, DNA content, SMC number, or vascular mass at 4 weeks. Furthermore, the stimulation of SMC apoptosis was more rapid with losartan or nifedipine (1 week) than with enal-
Fig 3  Changes in growth-related parameters in the aortic media of SHR receiving losartan (30 mg kg⁻¹ d⁻¹), enalapril (30 mg kg⁻¹ d⁻¹), nifedipine (35 mg kg⁻¹ d⁻¹), or hydralazine (40 mg kg⁻¹ d⁻¹) for 1 to 4 weeks A, Vascular mass expressed as the aorta body weight ratio B, Aortic DNA content C, Aortic DNA fragmentation index, indicative of apoptosis D, Aortic DNA specific activity, indicative of DNA synthesis in vivo. The data were taken from Tables 1 and 2, expressed as a percentage of the corresponding control values and plotted as a function of time after initiation of drug therapy.

April (2 weeks), whereas blood pressure reduction was more rapid and pronounced with enalapril (1 week) than with losartan (2 weeks) or nifedipine (>2 weeks) Taken together, these data strongly suggest that drug-induced SMC apoptosis was not a secondary response to blood pressure reduction. Consistent with this, calcium channel blockers stimulate apoptosis independently of hemodynamic changes in cultured SMC,⁴⁷

Several groups reported a dissolution between blood pressure reduction and aortic mass regression in the SHR

TABLE 3. Effect of 4-Week Antihypertensive Therapy on Medial Cross-Sectional Area, SMC Nuclear Length, SMC Numerical Density, and SMC Number in the Aortic Media of SHR

<table>
<thead>
<tr>
<th>Measurements on cross-sections</th>
<th>Control (n=9)</th>
<th>Losartan (n=6)</th>
<th>Enalapril (n=7)</th>
<th>Nifedipine (n=5)</th>
<th>Hydralazine (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal cross-section, mm²</td>
<td>0.32±0.01</td>
<td>0.23±0.01*</td>
<td>0.26±0.01*</td>
<td>0.27±0.01*</td>
<td>0.32±0.01</td>
</tr>
<tr>
<td>Total volume of disectors, mm³×10⁻⁶</td>
<td>238±27</td>
<td>322±34</td>
<td>256±19</td>
<td>164±24</td>
<td>204±20</td>
</tr>
<tr>
<td>No. of nuclei in top sections</td>
<td>123±15</td>
<td>115±10</td>
<td>107±7</td>
<td>71±12</td>
<td>126±8</td>
</tr>
<tr>
<td>No. of nuclei still present in bottom sections</td>
<td>37±7</td>
<td>38±6</td>
<td>47±6</td>
<td>27±6</td>
<td>49±9</td>
</tr>
<tr>
<td>Average nuclear length, μm</td>
<td>15±1</td>
<td>15±1</td>
<td>18±1</td>
<td>16±1</td>
<td>15±1</td>
</tr>
<tr>
<td>Cell numerical density, mm⁻³×10⁻⁶</td>
<td>26±1</td>
<td>26±2*</td>
<td>24±2*</td>
<td>26±2*</td>
<td>26±2*</td>
</tr>
<tr>
<td>Combined measurements</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cells/μm of aorta</td>
<td>116±6</td>
<td>58±6*</td>
<td>61±4*</td>
<td>72±5*</td>
<td>122±11</td>
</tr>
<tr>
<td>% reduction in cell number</td>
<td>50%</td>
<td>47%</td>
<td>46%</td>
<td>38%</td>
<td></td>
</tr>
</tbody>
</table>

Medial cross-sectional area was measured using a computerized image analysis system, as described in *Methods* A blindly selected subset of tissues was used for the control and enalapril groups. *P<0.05 by ANOVA followed by Student's t test with Bonferroni correction for multiple comparisons.
on the basis of comparative studies between hydralazine and losartan, a calcium channel blocker, or an ACE inhibitor. The present results extend these findings by showing that mifepristone induced aortic mass regression before systolic pressure reduction (ie, at 2 and 3 weeks, respectively). Increased SMC apoptosis may account for this early structural effect of the calcium channel blocker. SMC apoptosis may also contribute, along with vasodilatation and decreased extracellular matrix accumulation, to increase the compliance of large conduit arteries in response to ACE inhibitors, AT1 antagonists or calcium channel blockers, a major beneficial effect of these drugs against left ventricular hypertrophy and systolic hypertension.

The available evidence from previous studies suggests that in SHR the antihypertensive and antihypertrophic effects of β-blockers and diuretics are modest, slow to develop, and may be more pronounced when the drugs are administered before hypertension is established. Thus, although propranolol and HCT did not significantly affect blood pressure or vascular structure at this established phase of SHR hypertension, we cannot rule out that an earlier or more prolonged schedule of administration may affect arterial SMC apoptosis.

### SMC Apoptosis and DNA Synthesis

Heightened DNA replication rates are often associated with and even counterbalanced by an increase in SMC apoptosis. Cho et al. reported that high rates of DNA synthesis in specific arteries of the neonatal lamb do not translate to a corresponding increase in vascular DNA content. In these arteries, which show regression after birth, the high rates of SMC DNA synthesis are counterbalanced by heightened apoptosis. SMC apoptosis is also markedly increased in the developing neointimal lesion after balloon catheter injury in rats. Apoptosis and DNA replication are both found mainly among SMC located near the luminal surface of the neointima, suggesting that apoptosis may regulate the cellularity of the lesion in the face of chronic cell proliferation. Apoptosis and DNA replication are both found mainly among SMC located near the luminal surface of the neointima, suggesting that apoptosis may regulate the cellularity of the lesion in the face of chronic cell proliferation.

Detailed studies by Owens did not detect a change in vascular DNA content or SMC number in the aorta of SHR treated with the ACE inhibitor captopril. Differences in experimental design may explain the discrepancies between the latter and present studies. First, captopril was administered to SHR for 3 months beginning at 2 months of age, ie, before the onset of hypertension. In contrast, rats used in the present study were already hypertensive when drug therapy was initiated and their aorta was examined at earlier times thereafter. Second, we observed a marked reduction of the preexisting hypertension (at least with inhibitors of the angiotensin II pathway), whereas the treatment with captopril mainly prevented the development of high blood pressure over time. It is intriguing to
speculate that stimulation of SMC apoptosis in the arterial media may require aggressive antihypertensive therapy causing abrupt yet specific changes in the homeostatic environment of the cells in the arterial wall. In support of this view, we showed that a lower dose of lisinopril that prevented, but did not reverse, the progression of hypertension in SHR failed to affect aortic mass. DNA fragmentation index, or DNA content at 4 weeks. Previous studies support the view that rates of blood pressure change over time may be critical in determining SMC growth behavior. For instance, the gradual increase in blood pressure in SHR, Goldblatt rats, and DOCA-salt and norepinephrine-induced models of hypertension results in increased aortic SMC polyploidy. In contrast, the abrupt increase in intra-aortic blood pressure that follows suprarenal aortic coarctation is associated with the rapid and significant increase in aortic SMC hyperplasia. The regulation of SMC apoptosis in normotensive rats, which show less prominent hemodynamic responses to antihypertensive drugs than SHR, remains to be determined. An additional though nonexclusive possibility is that the depletion of SMC in the arterial wall is transient and reversible within the first months of drug therapy. Indeed, our data with nifedipine at 2 and 4 weeks (Fig 3) are consistent with such a complex regulation of SMC growth in the arterial wall. Ultimately, it is conceivable that vascular remodeling without a net change in vascular mass or cell number (eutrophic vascular remodeling) may result from the reversible depletion of SMC via a balanced process of SMC apoptosis and replication.

**Molecular Mechanisms Regulating SMC Apoptosis**

It is important to note that the triggering of apoptosis is dependent on a balance of environmental cues that are far from being specific to apoptosis. In vitro studies have identified several factors that can modulate, in parallel or in opposition, SMC DNA replication and apoptosis. For instance, DNA replication is inhibited and apoptosis is increased via the cAMP pathway, the nitric oxide pathway, interferon-γ, or calcium channel blockers. In contrast, platelet-derived growth factor or insulin-like growth factor-1 inhibits apoptosis and promotes DNA replication. In addition, overexpression of the transcription factor c-myc or its adenoviral functional homologue E1A increases both DNA replication and apoptosis in SMC. Finally, basic fibroblast growth factor and epidermal growth factor stimulate DNA replication without affecting SMC apoptosis.

Angiotesin II acting via AT₁ receptors may act as a survival factor for SMC. Both the ACE inhibitor enalapril and the angiotensin II AT₁ receptor antagonist losartan potently increased SMC apoptosis in the aorta. Angiotesin II binding to AT₁ receptors may inhibit SMC apoptosis either directly or indirectly by stimulating the production of autocrine survival factors, including platelet-derived growth factor and insulin-like growth factor-1, or by stimulating the production of specific extracellular matrix molecules, such as osteopontin, in the arterial wall. Disruption of binding to the α₂β₃ integrin receptor for osteopontin induces apoptosis in angiogenic blood vessels. In fact, a growing body of evidence suggests that extracellular matrix proteins regulate apoptosis by interacting with cellular integrin receptors and modulating intracellular protein tyrosine phosphatase activity. Stimulation of the AT₂ receptor subtype for angiotensin II is a potential pathway for the rapid stimulation of SMC apoptosis in losartan-treated animals, a condition where plasma levels of angiotensin II are markedly increased. Recently, the angiotensin AT₂ receptor subtype has been implicated in the reduction of high blood pressure, and the inhibition of arterial SMC DNA replication, and the induction of apoptosis in fibroblasts, ovarian cells and neuronal cells via the activation of protein tyrosine phosphatase pathways.

Enalapril effects on SMC apoptosis, DNA synthesis, and vascular mass were comparable in magnitude though delayed compared with losartan, which was the most potent inducer of apoptosis or inhibitor of DNA synthesis. The faster onset for blood pressure reduction with enalapril may involve the reduced breakdown of endogenous vasodilatory kinins. Effects of kinins on apoptosis are unknown, but it is possible that kinin-induced secretion of NO by endothelial cells may contribute to stimulate SMC apoptosis in vivo.

Nifedipine had a transient effect on arterial growth and apoptosis. Recent retrospective epidemiological studies suggested that treatment of hypertensive patients with rapidly acting formulations of nifedipine (as in the present study) might increase the risk of coronary mortality or cancer. In the latter study, inhibition of apoptotic cell death by nifedipine was proposed as a putative mechanism for the promotion of neoplastic growth. The present study does not provide evidence supporting this hypothesis. Clearly, these clinical issues remain to be clarified within the framework of prospective studies. Notably, it remains unclear whether the putative risk increases might represent a class effect of calcium channel blockers, a secondary effect related to sympathetic activation with short acting formulations of nifedipine (in the case of coronary mortality), or simply a medication-independent association between hypertension and cancer. The present data suggest that antihypertensive medications have complex effects on growth and apoptosis which need to be explored further in the long term. It has been suggested that long-term benefits from antihypertensive therapies may depend on the remodeling of vascular structure with the normalization of wall-to-lumen ratio. Recent studies in hypertensive humans indicated that blood pressure reduction is accompanied with significant remodeling of small muscular arteries in patients treated with ACE inhibitors or a calcium channel antagonist but not β-blockers. The present studies suggest that SMC apoptosis is one of the earliest events in the sequence of changes taking place during drug-induced regression of vascular hypertrophy. It remains unclear what is the long-term significance of apoptosis on SMC population dynamics in the arterial wall. Another critical question is whether the regulation of apoptosis is vessel specific, as it has been suggested for SMC growth behavior.

In summary, nifedipine and inhibitors of the angiotensin pathway stimulated SMC apoptosis early during regression of vascular hypertrophy in the thoracic aorta, before SMC DNA replication was inhibited, and independently of blood pressure reduction. Thus, a major goal of research should be to better characterize mechanisms regulating
apoptotic SMC death in vivo and its putative role in hypertensive vessel remodeling in disease and in response to therapeutic agents.

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