Evaluation of Medial Hypertrophy in Resistance Vessels of Spontaneously Hypertensive Rats

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SUMMARY The role of smooth muscle cell hypertrophy, hyperploidy, and hyperplasia in medial hypertrophy of mesenteric resistance vessels of 107- to 111-day-old spontaneously hypertensive rats (SHR) was examined using a combination of morphometric, biochemical, and immunological techniques. Mesenteric arteries were classified on the basis of branching order for comparative purposes. Branch level I vessels were those that directly enter the jejunal wall, while Branches II to IV represented more proximal vessels; Branch IV vessels were those that branch from the superior mesenteric artery. Medial hypertrophy was assessed in perfusion-fixed vessels by morphometric evaluation of medial cross-sectional area and smooth muscle content. Medial cross-sectional area and smooth muscle content were significantly increased in larger (Branches III and IV) but not smaller (Branches I and II) mesenteric resistance vessels of SHR compared with control normotensive Wistar-Kyoto rats (WKY). Smooth muscle cell hypertrophy and hyperploidy were evaluated in isolated cells obtained by enzymatic dissociation of mesenteric resistance vessels. Approximately 80% of the cells in these preparations were identified as smooth muscle cells using a smooth muscle-specific isoactin antibody. Feulgen-DNA microdensitometric evaluation of isolated cells showed that ploidy cells were present in mesenteric resistance vessels but at very low frequencies, and no differences were apparent between SHR and WKY. Likewise, no differences in cellular protein content or relative smooth muscle cell size (i.e., area profile) were observed between cells obtained from SHR and WKY vessels. These results demonstrate that the increase in medial smooth muscle content observed in larger mesenteric resistance vessels of SHR cannot be accounted for by smooth muscle hypertrophy and hyperploidy, inferring that hyperplasia must be present. Results indicate that studies of the initiating mechanisms for medial smooth muscle hypertrophy in SHR resistance vessels, at least relatively early in hypertension, should focus on examination of factors that induce true cellular proliferation rather than hypertrophy and hyperploidy. (Hypertension 11: 198-207, 1988)

KEY WORDS • smooth muscle growth • smooth muscle cell • polyploidy • cellular hypertrophy • cellular hyperplasia • hypertension • resistance vessels

FOLKOW et al. and others have suggested, based on hemodynamic evidence, that an increase in vascular smooth muscle mass in resistance vessels may play an important role in the maintenance of increased peripheral resistance in chronic hypertension. Results of morphometric studies have demonstrated an increase in vessel and smooth muscle mass in both large and small vessels (> 100 μm in diameter) of chronically hypertensive animals and humans compared with their normotensive counterparts. There is considerable debate as to whether this increase in medial mass, which we will call medial hypertrophy, is a cause or the result of hypertension. There is even uncertainty about whether changes in the hypertensive media encroach on the lumen diameter at maximal vasodilatation. Nonetheless, there is good evidence that increased smooth muscle mass confers a functional advantage by 1) increasing maximal force-developing capability, although the force development per unit contractile mass appears to be unaltered, and 2) conferring a geometric advantage, since thick-walled vessels undergo proportionately larger diameter changes than thin-walled vessels for a given level of smooth muscle activation. Whether this functional advantage would itself result in an increase in vascular resistance, as suggested by Folkow

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Supported by Grants ROI HL-29960, PO1 HL-03174, ROI HL-26405, and PO1 HL-19342 from the National Institutes of Health.

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Received February 27, 1987; accepted September 8, 1987.
et al.\textsuperscript{1} or whether it is an adaptive response to normalize wall stress, is controversial. In either case, it would be an extremely important component of a chronic elevation in vascular resistance.

These studies\textsuperscript{1-12} underscore the importance of understanding the cellular mechanisms responsible for accelerated smooth muscle cell (SMC) growth in resistance vessels of hypertensive animals. It may be particularly important to understand the relative role of processes involving cell replication (i.e., an increase in cell number or hyperplasia) versus processes that involve enlargement of existing SMCs (i.e., cellular hypertrophy) without replication since there is good evidence suggesting that the signals for these processes are different.\textsuperscript{13, 14} Smooth muscle replication has been studied extensively in vitro, suggesting possibly important mechanisms in replicative responses in vivo. Perhaps more importantly, increases in the DNA content of a tissue, unlike changes in other macromolecules, are likely to be irreversible unless cell death occurs. This factor could be important if an increase in DNA content increases the capacity of the cell to synthesize contractile proteins.

Our previous studies\textsuperscript{4, 5, 13} and those of Olivetti et al.\textsuperscript{15} showed that the increased mass of smooth muscle in aortas of spontaneously hypertensive rats (SHR) or Goldblatt hypertensive rats compared with normotensive controls was due principally to SMC hypertrophy rather than to hyperplasia. Aortic SMC hypertrophy was accompanied by an increase in DNA ploidy, implying that DNA replication was a requisite part of a change in mass. However, results in a large conduit vessel may not be relevant to changes in smaller vessels responsible for control of vascular resistance. Lee et al.\textsuperscript{6, 16} have presented evidence that the nature of the growth response varies in different size mesenteric resistance vessels, with cellular hypertrophy predominating in large vessels and cellular hyperplasia in smaller vessels, although their morphometric technique may be compromised by untested assumptions regarding SMC size, shape, and orientation. The studies by Mulvany et al.\textsuperscript{17} present the most convincing evidence for SMC hyperplasia in resistance vessels. However, these studies did not address the possibility of SMC polyploidy and were limited to examination of one branch level of mesenteric vessels. In addition, their morphometric method for determination of numerical density, although clearly superior to most methods, is nevertheless subject to limitations relating to small sample sizes and relatively large experimental variances (see Discussion and the report by Mulvany et al.\textsuperscript{17} for a further discussion).

The principal objectives of the present study were 1) to study the distribution of medial smooth muscle hypertrophy in intermediate size mesenteric resistance vessels of SHR and Wistar-Kyoto rats (WKY), and 2) to explore the relative role of SMC hypertrophy and hyperplody versus hyperplasia in medial hypertrophy of mesenteric resistance vessels of SHR. We studied 107- to 111-day-old SHR and WKY since animals are hypertensive at this time and others have reported the presence of marked medial hypertrophy in mesenteric arteries at this age.\textsuperscript{6, 9} We chose to examine mesenteric arteries between 70 and 250 \( \mu m \) in diameter since these vessels contribute to vascular resistance\textsuperscript{18} and are readily accessible for isolation, a necessary requirement for technical reasons in many of the studies described here.

**Materials and Methods**

**Animals**

Male SHR and WKY used in this study were supplied by the Core Breeding Colony (National Institutes of Health Program Project PO1 HL 19242) of the University of Virginia. This colony was derived from stock provided by the National Institutes of Health, and the animals have growth characteristics and pressure relationships comparable to those of other SHR colonies.\textsuperscript{18} All rats were between 107 and 111 days of age. A total of 20 SHR and 21 WKY were used in these studies. All animal use protocols were approved by the University of Virginia Animal Research Committee.

Systolic blood pressure was determined in conscious rats by a photoelectric tail-cuff pulse detector (ITTC, Landing, NJ, USA). Ambient temperature was 27° C. Animals were conditioned to restraining cages before pressure measurements were attempted. Animals were identified by number, and the blood pressure technician was not aware of experimental groupings. A minimum of three blood pressure measurements were obtained before the rats were killed. In addition, systolic and diastolic blood pressures as well as heart rates were measured in anesthetized rats with an indwelling catheter just before perfusion fixation in morphometric studies.

**Evaluation of Medial Hypertrophy**

Rats were anesthetized with sodium pentobarbital (25 mg/kg), and the left carotid cannulated for pressure and heart rate determinations using a Beckman Type RB dynagraph (Schiller Park, IL, USA) equipped with a Statham pressor transducer (Hato Rey, Puerto Rico). Animals were then perfusion-fixed at their mean pressure through the carotid cannula using 2% glutaraldehyde/1% paraformaldehyde in Hanks balanced salt solution (HBSS; pH 7.40). The femoral vein was cut to provide a venous outflow. Rats were perfusion-fixed for 5 minutes with close monitoring of pressure. After perfusion fixation, the abdominal aorta, the mesenteric artery, its branches, and the small intestine were removed and immersion fixed for an additional 12 hours and then stored in HBSS at 4° C until used (i.e., <48 hours).

An important consideration in these studies was to have a systematic means of classifying vessels for comparative purposes. Furthermore, our classification scheme had to be applicable to nonfixed vessels, which were needed for a number of the studies to be described. We chose to do this on the basis of branching order, as illustrated in Figure 1. A limitation of this classification scheme was that not all vessel arcades were identical (i.e., some arcades had only three
branch levels, while others had four or more). Since the majority of arcades had four branch levels in both SHR and WKY, we chose to sample vessels only from these. Branch I vessels corresponded to those that directly enter the intestinal wall; Branches II to IV represented the increasing size of vessels. Branch IV vessels represented the first branch from the superior mesenteric artery.

Four to eight vessels of each branch level were dissected from four different arcades from each animal under a stereomicroscope. To facilitate handling of these small vessels, dissected vessels were processed in Flo-Thru specimen capsules (Normco, Silver Spring, MD, USA), which make transfer of vessels much easier and eliminate the possibility of loss, because specimens remain in the capsules while solutions are changed. Vessels were processed and embedded in Epon for transverse sections, as previously described. 14 One-micrometer-thick sections were cut and stained with toluidine blue for morphometric evaluations.

Medial cross-sectional area was determined from transverse sections using a Zeiss videoplan system (Thornwood, NY USA) as previously described. 14 The major advantage of cross-sectional area determinations, as compared with measurements of wall thickness to lumen ratios, is that measurements are not influenced by changes in the activation state of the vessels. 16 Between four and six vessels of each branch level were analyzed per animal. Vessels that were not sectioned transversely (i.e., wall thickness was asymmetrical) were not analyzed. At least two sections were analyzed from each vessel. The coefficient of variation for determinations on different sections of the same vessel was less than 5%. Representative micrographs of vessels are shown in Figure 2. The volume fraction for determinations on different sections of the same vessel was 14.

An estimate of vessel size for each branch level was obtained in two ways. First, luminal area was determined with a Zeiss videoplan system, and lumen diameter was calculated as: diameter = \( \sqrt{\text{area}/\pi} \). Note that this evaluation is only valid with circular sections, and therefore all collapsed (i.e., noncircular) vessels were excluded from analysis. Second, the length of the internal elastic lamina was measured by planimetry, and vessel diameter was calculated as diameter = circumference/\( \pi \). The latter measurement provides a relative index of vessel size that is not profoundly influenced by the activation state of the vessel, 12 but it is not necessarily relevant for hemodynamic purposes.

Isolation of Cells from Mesenteric Resistance Vessels

Animals were killed by CO\(_2\) asphyxia, and four to eight vessels of each branch level were immediately dissected as already described. Care was taken to remove as much adhering connective tissue as possible without damaging the vessels. Cells were then dissociated from pooled vessels of each branch level from each rat using collagenase and elastase digestion as previously described. 28 Dissociation was monitored by phase microscopy. Identical digestion times were used for SHR and WKY vessels from the same class, but slightly different times were necessary for isolation of sufficient cells from the different classes of vessels. Freshly dispersed cells were cytotoxic for 150 g; 5 minutes) onto glass slides. Cell smears were then fixed either in 4% paraformaldehyde in HBSS for subsequent DNA and protein determinations or in cold methanol for staining with a smooth muscle-specific isoactin monoclonal antibody 2 for assessment of the fraction of SMC and relative cell area. Cell viability, determined by trypan blue exclusion, was greater than 90% in all instances.

Cellular Ploidy Measurements

Cellular DNA determinations were done on cell smears by Feulgen-DNA microdensitometry, as previously described. 4, 20 Measurements were made using either a Zeiss-Zonax scanning integrating microdensitometer or a custom-designed digital microdensitometer (Leitz, Rockleigh, NJ, USA). Between 200 and 400 cells were evaluated per branch level per animal. Chicken erythrocytes were used as standards. Lysed cells were excluded from analyses.

Cellular Protein Evaluations

Cellular protein determinations were done as previously described using naphthol yellow S-stained cell smears. 4 Absorption measurements were made at a wavelength of 460 nm. A random sample of at least 50 cells were evaluated per branch level per animal. All measurements were made using the Zeiss-Zonax microdensitometer.

Identification of Smooth Muscle Cells in Cell Smears

The frequency of SMCs and non-SMCs in isolated cell preparations was determined using a smooth muscle–specific isoactin monoclonal antibody provided by Drs. Allen Gown and David Gordon (University of Washington). This antibody shows specific reactivity with both the \( \alpha \)-smooth muscle and \( \gamma \)-smooth muscle isoactin forms but does not react with \( \alpha \)-skeletal muscle actin, \( \alpha \)-cardiac muscle actin, or with \( \beta \)-nonmuscle and \( \gamma \)-nonmuscle isoactins. 21 It has been shown to stain SMCs from a variety of sources. Of most importance for this investigation, it does not stain endothelial cells or fibroblasts, which are the most likely nonmuscle cells in our isolated cell preparations.

Methanol-fixed cell smears were stained using an avidin-biotin-peroxidase procedure (Vectastain, Vector Laboratories, Burlingame, CA, USA). The smooth muscle isoactin antibody was used as ascites fluid diluted 1:250 in phosphate-buffered saline (pH 7.40). Controls were stained with nonimmune mouse serum or with an antineurofilament monoclonal antibody unreactive to SMC. In addition, some smears were
stained with a rabbit anti-chicken gizzard polyclonal actin antibody, which stains both muscle and nonmuscle isoactins as a positive control. At least 200 cells were evaluated per animal per branch level for frequency determinations.

Assessment of Relative Smooth Muscle Cell Size: Area Profile Determinations

The relative size of SMCs in isolated cell preparations was evaluated by determining the area of cells stained with the smooth muscle isoactin antibody as previously described.20 Cell area measurements were determined at a magnification of 1000× using a Leitz microscope equipped with a video camera and interfaced to a Zeiss videoplan digitizing board. Cell parameters were traced with an electronic pen. A minimum of 50 SMCs were evaluated per branch level per animal.

Statistical Analysis

Since multiple vessels were analyzed from each rat in determination of medial cross-sectional area, VVSMC, vessel diameter, and smooth muscle content, comparison of differences between SHR and WKY for these parameters was done by analysis of variance with a repeated-measures term that takes into account both between-animal and within-animal variance for intergroup comparisons. This was done as a single hierarchical analysis of variance (including a term for branch level), followed by a multicomparison test using a t statistic on least-square means (General Linear Models Procedure, SAS Institute, Cary, NC, USA). Note that the repeated-measures term was used since it is not appropriate to use a single mean value for these parameters for each branch level for each animal since such a test would not account for the variation between different vessels within an animal and since preliminary analyses showed that this intra-animal variation was as great as the inter-animal variance. Statistical evaluation of cellular protein content and area profile was done in a similar manner but without the repeated-measures term. An α level of 0.05 was considered significant.

A simultaneous combined linear model (General Linear Models Procedure, SAS Institute) was used for regression analyses. This model provides a more precise estimate of the relationship between variables than can be obtained by separate linear regression analyses in SHR and WKY. The model yields separate slope and y intercept terms for SHR and WKY respectively, but by definition the simultaneous combined model provides a single r² value for both lines that reflects the overall fit of the data to their respective lines. The significance of the slope and intercept (i.e., testing the null hypothesis that they equal zero) was evaluated using a t statistic (General Linear Models Procedure, SAS Institute). Differences in slopes were compared by analysis of covariance (General Linear Models Procedures, SAS Institute).

Results

Blood Pressures and Heart Weights

The mean (± SE) conscious systolic blood pressures of the SHR and WKY used in these studies during the 4 weeks preceding death were 186 ± 4 (n = 20) and 128 ± 2 (n = 21) mm Hg, respectively. Heart weights and heart weight/body weight ratios were also significantly increased (p < 0.001, by analysis of variance) in SHR compared with WKY, while body weights were not different between groups.

Evaluation of Medial Hypertrophy

Our initial objective was to determine whether medial smooth muscle hypertrophy occurred in mesenteric resistance vessels of SHR by comparing medial cross-sectional area and smooth muscle content measurements of vessels from the same branch level (see Figure 1) between SHR and WKY. Results of initial analyses showed that the medial cross-sectional area was significantly greater in SHR vessels than in WKY vessels for Branches III and IV, but not for Branches I and II (Table 1). To determine whether the increases in medial cross-sectional area in these vessels were due to an increase in the smooth muscle or extracellular component of the wall, smooth muscle volume densities (VVSMC) were determined. Results showed no differences in VVSMC between SHR and WKY vessels within a given branch level (see Table 1), indicating that medial hypertrophy in SHR is associated with proportionate increases in the cellular and extracellular components of the vessel. Consistent with cross-sectional area data, smooth muscle content was significantly increased in vessels from Branches III (23% increase;
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TABLE 1. Morphometric Analysis of Perfusion-Fixed Mesenteric Resistance Vessels of 107- to 111-Day-Old SHR and WKY

<table>
<thead>
<tr>
<th>Branch level</th>
<th>Medial cross-sectional area (µm²)</th>
<th>Lumen diameter* (µm)</th>
<th>Vsmc</th>
<th>SM content (µm³/µm length)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>SHR</td>
<td>1,788 ± 51</td>
<td>73.7 ± 2.1†</td>
<td>0.824 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>1,871 ± 54</td>
<td>91.4 ± 3.0</td>
<td>0.801 ± 0.007</td>
</tr>
<tr>
<td>II</td>
<td>SHR</td>
<td>4,928 ± 254</td>
<td>125.3 ± 4.9</td>
<td>0.776 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>4,372 ± 240</td>
<td>126.9 ± 4.3</td>
<td>0.769 ± 0.013</td>
</tr>
<tr>
<td>III</td>
<td>SHR</td>
<td>8,435 ± 414†</td>
<td>139.8 ± 6.1†</td>
<td>0.719 ± 0.023</td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>7,436 ± 244</td>
<td>164.5 ± 6.6</td>
<td>0.668 ± 0.008</td>
</tr>
<tr>
<td>IV</td>
<td>SHR</td>
<td>14,962 ± 1,005†</td>
<td>175.6 ± 9.0†</td>
<td>0.698 ± 0.023</td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>11,392 ± 522</td>
<td>210.7 ± 8.1</td>
<td>0.660 ± 0.014</td>
</tr>
</tbody>
</table>

Values are means ± SE of six animals per group. Four to eight vessels of each branch level were analyzed per animal. 

Vsmc = volume fraction of vascular smooth muscle cell in vessels; SM = smooth muscle.

*Lumen diameter was determined on cross-sections of perfusion-fixed vessels by measuring luminal area and calculating the diameter of the circle with that area.

†p < 0.05, compared with respective value for WKY (by analysis of variance with a repeated-measures term reflecting the fact that multiple vessels from each branch level were analyzed from each subject).

p < 0.05) and IV (38% increase; p < 0.001) from SHR as compared with WKY while no differences were observed in vessels from Branches I and II.

There are several potential limitations in assessing medial hypertrophy in vessels solely on the basis of branching level. First, based on visual observation as well as morphometric estimates of vessel size (e.g., cross-sectional area and diameter), there were marked heterogeneities in vessel sizes even within a given branch order, which increase intragroup variances and thereby compromise intergroup comparisons. Second, shifts may occur in the size of vessels present in a given branch level in SHR and WKY that might not be apparent by examination of branching patterns alone. Thus, medial hypertrophy was also assessed in these studies by determining whether SHR vessels of a given size were hypertrophied relative to the corresponding vessels in WKY. Note that this type of comparison is not identical to one based on branching order alone due to heterogeneity of vessel sizes within a given branch level. The major difficulty with such an analysis is determining an appropriate index that accurately reflects a vessel’s size rather than its activation state. Ideally one would like to maximally relax a vessel and measure the undistended vessel diameter. However, this feat would be extremely difficult to accomplish in an intact animal. Furthermore, in these studies animals were perfusion-fixed at their own blood pressure in an attempt to observe the in vivo state of vessels. Thus, we have estimated vessel size based on the length of the internal elastic lamina (IEL) in order to reduce the influence of differences in vessel tone on these analyses. Note that at diameters at which the IEL is not load-bearing, IEL length will be constant with changes in activation state. Although this method does not eliminate the influence of vessel tone on size estimates, it is more advantageous than size estimates based on lumen diameter, which are dramatically influenced by differences in activation state. We want to emphasize that our intent was to assess medial hypertrophy by some additional means other than comparison of vessels of a given branch level. Furthermore, we felt that it is inappropriate to arbitrarily divide vessels into different size classes as has been done frequently in previous studies in this area. To accomplish these goals, a simultaneous combined linear regression analyses (see Materials and Methods) was done of medial cross-sectional areas versus relative vessel size (IEL length) for all SHR and WKY vessels evaluated in these studies.

Results showed a highly significant linear relationship between medial cross-sectional area and IEL length for SHR and WKY vessels (r² = 0.92, p < 0.001 for the slope and y intercept of the curves for both SHR and WKY; Figure 3). Significantly, analysis of covariance showed that the slope of the line for SHR was steeper than that for WKY, indicating that the differences in medial area between SHR and WKY were greater in larger diameter vessels. This supports our findings based on comparison of vessels classified by branching order.

We were also concerned that differences in activation state between SHR and WKY vessels may have influenced medial area determinations. Whereas medial area should not change with changes in diameter, it could change if vessel length is altered with changes in activation. To address this possibility, control studies were done in which rats were infused with high doses of sodium nitroprusside (160 µg/kg/min) to induce vasodilation. This procedure resulted in a rapid and dramatic fall in blood pressure. After a 5-minute nitroprusside infusion, rats were perfusion-fixed at this reduced pressure as described in Materials and Methods. Medial area was determined in Class I vessels from six SHR and six WKY. Medial areas from these animals were nearly identical (p = 0.9) to those obtained in non-nitroprusside-infused animals, indicating that differences in activation state did not influence medial area determinations. These observations are in agree-
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FIGURE 2. Light micrographs showing representative cross-sections of two mesenteric arteries evaluated in these studies. The vessel depicted in A shows much folding of the internal elastic lamina, presumably indicative of vessel contraction. Note that the wall thickness to lumen ratio is high. In contrast, in the vessel depicted in B, the internal elastic lamina is not folded and the wall thickness to lumen ratio is much smaller. Comparison of A and B illustrates the profound influence that vessel activation state can have on wall thickness or wall to lumen ratio measurements and illustrates the importance of assessing wall hypertrophy by measurement of cross-sectional area rather than wall to lumen ratios. (Magnification bars = 25 μm.)

FIGURE 3. Relationship of medial cross-sectional area to relative vessel size (i.e., length of the internal elastic lamina [IEL]) for mesenteric resistance vessels of SHR and WKY. An approximate vessel diameter can be obtained by dividing IEL length by π. The r² value reflects the overall fit of SHR and WKY data to their respective regression lines.

Analysis of Polyploidy in Smooth Muscle Cells from Mesenteric Arteries

Although ideally one would like to evaluate the frequency of polyploid SMC in tissue sections by Feulgen-DNA microdensitometric analysis, pilot studies showed that this was not possible because the considerable overlap of SMC nuclei prevented these measurements on a technical basis. Therefore, the following alternative method was used to determine whether medial hypertrophy in mesenteric resistance vessels was accompanied by increases in SMC polyploidy, as occurs in large conduit vessels. Four to eight vessels of each branch level were dissected from each rat and pooled, and cells were isolated as described in Materials and Methods. Cell smears were prepared and processed for analysis of ploidy (Table 2) or for determination of the frequency of SMCs using a smooth muscle-specific isoactin monoclonal antibody. Ploidy analyses demonstrated that tetraploid cells were present in mesenteric arteries of both SHR and WKY. However, the frequency of tetraploid cells was low in all instances, and no differences were apparent between cells from SHR and WKY for any of the branch levels studied. The percentage of SMCs in cell preparations, based on antibody staining, ranged from 78.9 ± 4.8 to 83.5 ± 4.2%, and no differences were observed between SHR and WKY. This finding indicates that 1) the non-SMC contaminants had a relatively small influence on the frequencies reported in Table
TABLE 2. Frequency of Tetraploidy in Cells Isolated from Mesenteric Resistance Vessels of 107- to 111-Day-Old SHR and WKY

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Branch I</th>
<th>Branch II</th>
<th>Branch III</th>
<th>Branch IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>2.0 ± 0.4 (6)</td>
<td>2.6 ± 0.6 (6)</td>
<td>4.0 ± 1.3 (6)</td>
<td>2.1 ± 0.4 (6)</td>
</tr>
<tr>
<td>WKY</td>
<td>2.2 ± 0.9 (7)</td>
<td>3.7 ± 0.7 (6)</td>
<td>4.5 ± 1.0 (9)</td>
<td>2.7 ± 0.5 (6)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Number of animals is shown in parentheses. Four to eight vessels of each branch level were taken from each rat and pooled, and cells were isolated as described in Materials and Methods. Between 200 and 400 cells per branch level per animal were evaluated. The fraction of SMCs in isolated cell preparations was approximately 80% (see text). No differences were observed between SHR and WKY by analysis of variance.

2 and 2) differences in non-SMC contaminants cannot account for the lack of differences in ploidy levels between SHR and WKY.

Analysis of Smooth Muscle Cell Hypertrophy

The preceding analyses indicate that medial hypertrophy in mesenteric arteries of SHR is not accompanied by changes in SMC ploidy. Given the strong relationship between development of SMC polyploidy and cell hypertrophy observed in our previous studies, this observation alone provides evidence that cellular hypertrophy has not occurred. Nevertheless, the possible contribution of an increase in individual cell mass (or cellular hypertrophy) to medial hypertrophy was directly evaluated in two ways: 1) microdensitometric analysis of protein content of individual cells in isolated cell preparations used in ploidy analyses and 2) planimetric evaluation of the area profile of isolated cells stained with the SMC-specific isoactin antibody. The advantage of protein determinations is that they provide a very quantitative index of cell mass or hypertrophy, since greater than 50% of the dry mass of cells is protein and since the amount of the other major cellular components such as sugar and lipids are dependent on them. Their disadvantage is that it is not possible to distinguish between SMC and non-SMC contaminants in isolated cell preparations. However, the influence of non-SMCs on overall protein measurements would be relatively small, since they account for only 20% of the cells present, and would only affect our comparisons of SHR and WKY if they had a quite different protein content in one strain relative to the other (see further arguments in the next paragraph). The advantage of the area profile determinations is that analysis is restricted to SMCs. However, their major disadvantage is that they are a less reliable index of cell size than are protein measurements because of uncertainties regarding changes in cell shape that occur during cell processing. In addition, comparisons of area profile data between different classes of vessels cannot be made due to inherent differences in the nature of the cell isolation from vessels of different sizes. For example, isolation of cells from Class I vessels required shorter digestion times than did those from large vessels, and the cells obtained tended to be elliptical in shape, while those from large vessels were nearly spherical. Thus, comparison of area profile data between cells from different vessel classes is invalid as a measure of relative cell size due to differences in the area/volume ratio in cells from different vessels. However, within a given vessel class, identical isolation protocols were used for SHR and WKY and no obvious differences in cell shape were apparent in the isolated cells obtained. Thus, it is reasonable to assume that differential shape changes have not occurred and that area profile measurements provide a reasonable estimate of relative cell size for comparison of SHR and WKY cells within a given vessel class. Nevertheless, small differential changes in cell shape that occurred during cell isolation may not have been detected, and for this reason these data are not as reliable as the protein data. However, area profile determinations for cells from larger vessels, because cells were nearly spherical, were largely shape-independent.

No differences in cellular protein content were observed between SHR and WKY cells for each respective branch order (Table 3). It is extremely unlikely that the presence of non-SMCs in protein assay preparations significantly influenced our comparisons of SHR and WKY in these experiments since they were present at the same frequency (i.e., 20%) and would have to have an extremely large difference in protein content in one strain relative to the other to have an appreciable affect on mean protein content. For example, in Class IV vessels we saw a 38 ± 2% increase in smooth muscle content as compared with WKY. If this increase were due to SMC hypertrophy (i.e., 38% increase) rather than to hyperplasia, non-SMCs would have to have a negative protein content (clearly an impossibility) to account for the lack of a difference in

TABLE 3. Evaluation of Protein Contents of Individual Cells Isolated from Mesenteric Arteries and Arterioles of 107- to 111-Day-Old SHR and WKY

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Branch I</th>
<th>Branch II</th>
<th>Branch III</th>
<th>Branch IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>135.3 ± 13.6* (6)</td>
<td>195.1 ± 16.0† (6)</td>
<td>267.2 ± 17.2 (6)</td>
<td>224.9 ± 12.9 (6)</td>
</tr>
<tr>
<td>WKY</td>
<td>178.2 ± 20.7† (7)</td>
<td>228.7 ± 28.2 (5)</td>
<td>233.3 ± 28.9 (6)</td>
<td>193.3 ± 28.2 (4)</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Number of animals is shown in parentheses. Cells were isolated from four to eight mesenteric arteries from each respective branch level and stained for combined protein/DNA determinations as previously described. Protein content was evaluated in a random sample of at least 50 cells per branch level per animal.

* p < 0.005, compared with values for branches II to IV for both SHR and WKY (by analysis of variance.)
† p < 0.05, compared with values for SHR Branch III (by analysis of variance.)
mean protein content in the mixed cell populations. Results of area profile determinations are consistent with results of protein determinations in that no differences were observed between SMCs from SHR and WKY for any of the branch levels evaluated (Table 4). Taken together the results of these two assays provide good evidence that SMC hypertrophy cannot account for the increase in medial smooth muscle content in large (i.e., Branches III and IV) mesenteric resistance vessels of SHR compared with WKY.

Although there were no differences in either protein content or area profile between cells from SHR and WKY within a given branch level, Class I cells from SHR did have a lower protein content than cells from Classes II to IV from either SHR or WKY (see Table 3). Class I WKY cells had a significantly lower protein content than Class III and IV cells from SHR, but they were not significantly different from those from other WKY classes. The importance of these observations is unclear.

Discussion
Our major objective in these studies was to explore the nature of the SMC growth response that is responsible for the increased content of smooth muscle in mesenteric resistance vessels of SHR versus WKY. Since there was some controversy as to the nature of hypertrophic changes in resistance vessels, our initial aims were to define if medial hypertrophy occurred and what vessels were affected. We found that significant medial hypertrophy was present in large (i.e., third and fourth branch level vessels with mean diameters approximately ≥ 150 μm) but not small (i.e., first and second branch level vessels) mesenteric arteries of SHR compared with WKY at 107 to 111 days of age. Our data are in agreement with those of Mulvany and co-workers,3,9 who reported medial hypertrophy in 150-μm to 200-μm (i.e., second and third branch order) vessels from SHR as compared with WKY in animals between 12 and 24 weeks of age, and are consistent with those of Bohlen and Lobach,7 who found no evidence of medial hypertrophy in small mesenteric resistance vessels of SHR that were approximately 80 to 130 μm in internal diameter. Likewise, our findings are consistent with those of Furuyama,6 who studied medial hypertrophy in human vessels using a morphometric technique that corrects for the lack of distention in immersion-fixed autopsy material. She found that medial hypertrophy was prominent in vessels larger than 100 μm in lumen diameter from subjects with essential hypertension but not present in vessels smaller than this. Lee et al.8 also observed marked medial hypertrophy in large mesenteric arteries of SHR versus WKY. Consistent with previous observations, results of the present study showed that medial hypertrophy was due in part to increased smooth muscle content. In contrast to our observations, however, Lee et al.8 reported that medial hypertrophy was present in first branch order vessels from 12-week-old SHR (see Table 1). Closer comparison of our data with theirs showed that medial cross-sectional area values were nearly identical for SHR vessels but their values for WKY vessels were lower than all other experimental groups. The reason for this difference is not clear, but it may relate to the fact that Lee et al.8 obtained their WKY from a commercial supplier, while their SHR were from a colony raised and maintained in house. Note also that we have not cited studies in which wall thickness or wall thickness to lumen diameter ratios alone were measured since such measurements are profoundly influenced by the activation state of the vessel and may or may not reflect medial hypertrophy. Taken together the cited studies provide good evidence that medial hypertrophy is present in larger but not smaller mesenteric resistance vessels of SHR as well as hypertensive humans compared with normotensive controls.

This increase in medial mass apparently is attributable to SMC hyperplasia. Because of the small size of vessels, we could not directly estimate cell number from DNA content as we have done previously with larger vessels.1,13,14,20 Cellular protein content and relative SMC size, however, could be measured on isolated cells. No differences in either were observed between cells derived from mesenteric arteries of SHR and WKY for any of the branch levels examined. Since the increase in smooth muscle mass observed in third and fourth level branches in SHR in these studies (i.e.,

| Table 4: Relative Size of Smooth Muscle Cells from Mesenteric Arteries and Arterioles of SHR and WKY |
|-----------------------------------------------|---------------|---------------|---------------|---------------|
| Rat strain | Branch I | Branch II | Branch III | Branch IV |
| SHR | 127 ± 6.5 (6) | 137.1 ± 6.8 (7) | 139.1 ± 11.0 (6) | 161.2 ± 8.2 (7) |
| WKY | 128.9 ± 5.4 (10) | 136.9 ± 6.0 (10) | 164.9 ± 7.5 (11) | 183.1 ± 6.6 (11) |

Values shown represent least-square means from three separate replicate experiments done on separate days. Our initial analysis of variance showed a significant effect of the day of experiment on values obtained that was the same for SHR and WKY. No significant differences between SHR and WKY cells were observed in any one experiment for any of the branch levels (by analysis of variance), but the sample sizes were quite small because of methodological limitations in the number of animals that could be done on a given day. Least-square means were calculated using General Linear Model Procedure (SAS Institute) to account for day of experiment effects. The number of animals is shown in parentheses.

Only values for SHR and WKY within a given class should be compared since slightly different cell isolation protocols were used for different classes of vessels, which may have influenced area profile measurements (see text). See Materials and Methods for details regarding cell isolation and analysis.
23 and 38% increases in smooth muscle content) cannot be accounted for by an increase in the mass of individual cells, we conclude that the SHR vessels must have more cells (i.e., cellular hyperplasia). Our findings are in agreement with those of Mulvany et al., who found, using a three-dimensional dissector method for determining numerical density, that SMC hyperplasia, not hypertrophy, was responsible for medial hypertrophy in Branch III mesenteric arteries of SHR compared with WKY. The mutual confirmation of our results and theirs, using totally different experimental approaches, is important, given the potential limitations of methods employed in each of the studies. Their methods have the major advantage of directly counting cell number in the intact vessel but the disadvantage that the method is tedious, producing a limited sample size (<0.2% of the aggregate volume of the vessel was analyzed from each vessel in their studies, and in most instances a single vessel was analyzed from a given animal). Furthermore, the large variances associated with their method are of some concern since their conclusion that medial hypertrophy is due to hyperplasia was based on observations that SMC numerical density was not different between SHR and WKY while smooth muscle content was increased in the SHR. Our methods have the advantage of directly evaluating cell hypertrophy or mass but only indirectly address the question of hyperplasia. Likewise, because of technical limitations, the number of cells that could be obtained from microvessels for protein and size analysis was quite small, resulting in relatively large experimental variances. Taken together, however, the results of Mulvany et al. and those from the present studies provide very strong evidence that medial hyperplasia in large mesenteric resistance vessels of SHR is due to SMC hyperplasia rather than to hypertrophy.

These studies are the first, to our knowledge, to explore SMC hyperplasia in resistance vessels. The data demonstrated that, whereas polyploid cells were present in mesenteric arteries, no differences in the frequency of polyploidy were observed between vessels from SHR and WKY. This observation is important because it suggests that changes in SMC ploidy do not play a major role in the etiology of SHR hypertrophy, although it remains to be determined whether changes in SMC ploidy do not play a major role in the etiology of SHR hypertrophy, although it remains to be determined whether longer periods of hypertension in the SHR might be associated with ploidy changes in microvascular SMCs.

The present studies do not define what role medial hypertrophy might play in SHR hypertrophy. Indeed, since our animals were already hypertensive, these studies do not distinguish whether medial changes were the cause or the result of the hypertension. Our observations that lumen diameter was significantly reduced in SHR vessels that were fixed at their own blood pressure is consistent with hemodynamic studies showing increased resistance in this vascular bed.23 However, in our studies lumen diameter was reduced even in Branch I vessels, where there was no medial hypertrophy. Although this finding indicates that medial hypertrophy was not the cause of the reduced diameter in Branch I vessels, it does not exclude the possibility that other types of structural changes (e.g., an anatomically smaller vessel, changes in passive elastic properties) may play a role. It is also possible that active contractile changes occur first but become fixed by structural changes occurring with chronic exposure of vessels to the hypertensive stimulus. Consistent with this idea, Joshua et al. reported that structural changes in cremaster vessels of rats with one-kidney, one clip hypertension gradually extend to smaller and smaller arterioles with increased duration of hypertension. Our studies in this model of chronic hypertension provide little insight as to the contribution of medial hypertrophy to the diameter reductions observed in Branch III and IV vessels, and the influence of "structural" versus "functional" reductions in lumen diameter in resistance vessels is highly controversial. Structural encroachment is supported by hemodynamic studies showing increased resistance in SHR even at "maximal" vasodilatation.1 Consistent with this, Warshaw et al. reported a reduced lumen diameter of Branch II and III vessels under conditions of maximal relaxation in their in vitro studies. In contrast, Lee et al. found no evidence for structural encroachment of lumen diameter in their studies of SHR. In summary, our studies and those of others provide morphological evidence supporting the contention of Folkow et al. that medial hypertrophy occurs in resistance vessels of hypertensive animals and provide a possible explanation for the increased pressor responsiveness observed in the studies of Folkow et al. It remains unclear whether this medial hypertrophy contributes to the initial cause of the hypertension or whether it is an adaptive response involved in the maintenance of the hypertension. However, in either case, it would be an extremely important component of a chronic elevation in vascular resistance.

The demonstration in this and previous studies that medial smooth muscle hypertrophy in resistance vessels of SHR occurs by cellular hyperplasia as opposed to cellular hypertrophy and hyperplasia will be important in attempts to identify the initiating mechanisms for accelerated SMC growth in resistance vessels. The growth response of aortic SMCs depends on the mode of growth stimulation in that cells undergo hypertrophy and hyperplasy in several chronic models of hypertension, but they undergo proliferation in response to traumatic injury with a balloon catheter or induction of acute severe coarctation hypertension. It is intriguing in this regard to note that endothelial denudation and platelet deposition have been described in hypertensive microvessels but not in larger arteries. One interesting possibility consistent with the hypothesis of Folkow et al. is that transient spikes in blood pressure, which precede development of established hypertension, may elicit a SMC proliferative response in a manner similar to that seen when large vessels are injured with a balloon catheter. Indeed, Reidy and Schwartz and others have demonstrated that, following acute increases in blood pressure by infusion of vasoactive substances, transient breaks in the endothelial lining of resistance
vessels occur and platelets are observed attached to the injured surface. In contrast, no breaks were observed in large vessels without endothelium, suggesting that small vessels are more sensitive to pressure-induced injury. A similar phenomenon may occur in resistance vessels of SHR, and the SMC growth response in these vessels may resemble the "response to injury" advocated by Folkow et al. (1985) or that occurs in large vessels, where it is hypothesized that injury-evoked increases in platelet-derived, macrophage-derived, and endothelium-derived growth factors play a role in SMC growth initiation. This speculation obviously remains to be tested, and one must also consider the possibility that there is a genetically determined abnormality in the growth control mechanisms in SMCs from SHR or that other growth mediators are involved. In any case, studies of growth control in microvessels, at least in early hypertension, should focus on factors that induce SMC proliferation rather than hypertrophy, and studies of growth control mechanisms in SMCs from large vessels may provide useful insight into the possible cellular growth control mechanisms operating in small vessels.

Acknowledgments
The authors thank Betty Ferguson for typing the manuscript; Sharon Coleman, An-Thu Phan, Susan Purdy-Ramos, and Donna Lombardi for their technical assistance; the Physiology Electron Microscopy Core Laboratory for preparation of morphology specimens; Drs. Allen Gown and David Gordon (University of Washington) for the CAG7 antibody; and Donald Kaiser (Statistical Computing Laboratory, University of Virginia) for his advice regarding statistical analyses.

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Angioedema of the face, extremities, lips, tongue, glottis, and/or larynx has been reported in patients treated with angiotensin-converting-enzyme (ACE) inhibitors, including VASOTEC (0.2% of patients treated with VASOTEC in clinical trials). In such cases, VASOTEC should be promptly discontinued and the patient carefully observed until the swelling disappears. Angioedema associated with laryngeal edema may be fatal. Where there is involvement of the tongue, glottis, or larynx, likely to cause airway obstruction, appropriate therapy, e.g., subcutaneous epinephrine solution 1:1000 (0.3 ml to 0.5 ml), should be promptly administered.

Excessive hypotension was rarely seen in uncomplicated hypertensive patients but is a possible consequence of enalapril use in severely salt-volume-depleted persons, such as those treated vigorously with diuretics or patients on dialysis. In using VASOTEC, consideration should be given to the fact that another ACE inhibitor, captopril, has caused agranulocytosis, particularly in patients with renal impairment or collagen vascular disease, and the available data are insufficient to show that VASOTEC does not have a similar risk.

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Hypotension. Hypotension was rarely observed in hypertensive patients but is possible in some patients taking VASOTEC. The usual response is to discontinue the drug and to raise the patient's blood pressure. 

Neutropenia/Agranulocytosis: Another ACE inhibitor has been shown to cause agranulocytosis and bone marrow depression, rarely in uncomplicated patients but more frequently in patients with renal impairment. If neutropenia develops and is severe enough to precipitate an infection, VASOTEC, may be associated with oliguria and/or progressive azotemia and rarely with acute renal failure and/or death. When this occurs, VASOTEC should be discontinued. 

Clinical studies in hypertensive patients with unilateral or bilateral renal artery stenosis, results from substituting unknown amounts of ACE inhibitors in clinical trials, and reports of renal functional deterioration in patients with renal disease treated with VASOTEC have been reported in patients with pre-existing renal impairment. Dosage reduction of VASOTEC and/or discontinuation of the drug may be required. 

Evaluation of the hypertensive patient should always include assessment of renal function. (See DOSAGE AND ADMINISTRATION in complete Prescribing Information.) 

Hyperkalemia: Elevated serum potassium (greater than 5.7 mEq/L) was observed in approximately 1% of patients treated in clinical trials; serum potassium levels were observed in 20% of patients. This effect was reversed on discontinuation of VASOTEC. In patients with moderate to severe renal impairment, serum potassium levels may rise to toxic levels. 

The recommended initial dose of enalapril in patients receiving VASOTEC is 10 mg daily. This dosage may be increased or decreased, depending on the patient's response, at 1-week intervals. If the dose is increased, the dosage should be increased at 2-week intervals. 

Dosage and Administration: In patients who are currently being treated with a diuretic, hypotensive hypertension occasionally may occur following the initial dose of VASOTEC. The diuretic should be discontinued 2 or 3 days before beginning therapy with VASOTEC to reduce the likelihood of hypotension. If the patient's blood pressure is not controlled with VASOTEC alone, diuretic therapy may be resumed. If the diuretic cannot be discontinued, an initial dose of 2.5 mg (break the 5-mg tablet) should be used. 

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CARDIOTEM (diltiazem HCI) 30 mg, 60 mg, 90 mg, and 120 mg Tablets

CONTRAINDICATIONS
Cardiac and vascular syndromes in (1) patients with sick sinus syndrome except in the presence of a functioning ventricular pacemaker, (2) patients with second- or third-degree AV block except in the presence of a functioning ventricular pacemaker, and (3) patients with hypertension (less than 90 mm Hg systolic)

WARNINGS
1. Cardiovascular. CARDIOTEM prolongs AV node refractoriness periods without significantly prolonging sinus node recovery time, except in patients with sick sinus syndrome. This effect may rarely result in abnormally slow heart rates (particularly in patients with sick sinus syndrome) or second or third degree AV block (six of 1,243 patients (0.48%). Concomitant use of diltiazem with beta blockers or digoxin may result in additive effects on cardiac conduction. A patient with Prinzmetal's angina developed periods of asystole (2 to 5 seconds) after a single dose of 60 mg of diltiazem.

2. Congestive Heart Failure. Although diltiazem has a negative inotropic effect in isolated animal tissue preparations, hemodynamic studies in humans with normal venricular function have not shown a reduction in cardiac index nor consistent negative effects on contractility (dp/dt).

Experience with the use of CARDIOTEM alone or in combination with beta blockers in patients with impaired ventricular function is very limited. Caution should be exercised when using the drug in such patients.

3. Hypotension. Decreases in blood pressure associated with CARDIOTEM therapy may occasionally result in symptomatic hypotension.

4. Acute Hepatic Injury. In rare instances, significant elevations in enzymes such as alkaline phosphatase, ALT, (LD) SGOT, SGPT and other symptoms consistent with acute hepatic injury have been noted. These reactions have been reversible upon discontinuation of drug therapy. The relationship to CARDIOTEM is uncertain in most cases, but probable in some. (See PRECAUTIONS)

PRECAUTIONS
General: CARDIOTEM (diltiazem hydrochloride) is extensively metabolized by the liver and excrated by the kidneys and in bile. As with any new drug given over prolonged periods, laboratory parameters should be monitored at regular intervals. The drug should be used with caution in patients with impaired renal or hepatic function. In subacute and chronic dog and rat studies designed to produce toxicity, high doses of diltiazem were associated with hepatic damage in special subacute hepatic studies. Oral doses of 125 mg/kg and higher in rats were associated with histological changes in the liver which were reversible when the drug was withdrawn. In dogs, doses of 20 mg/kg were also associated with hepatic changes, however, these changes were reversible with continued dosing.

Drug Interactions: Pharmacologic studies indicate that there may be additive effects in prolonging AV conduction when using beta blockers or digoxins concomitantly with CARDIOTEM. (See WARNINGS)

Controlled and uncontrolled domestic studies suggest that concomitant use of CARDIOTEM and beta blockers or digoxins is usually well tolerated. Available data are not sufficient, however, to predict the effects of concomitant therapy, particularly in patients with atrioventricular dysfunction or cardiac conduction abnormalities. In healthy volunteers, diltiazem has been shown to increase serum digoxin levels up to 20%.

Carcinogenesis, Mutagenesis, Impairment of Fertility: A 24-month study in rats and a 21-month study in mice showed no evidence of carcinogenicity. There was also no mutagenic response in in vitro bacterial tests. No intrinsic mutagenic effects were observed in rats.

Pregnancy: Category C. Reproduction studies have been conducted in mice, rats, and rabbits. Administration of doses ranging from five to ten times higher (on a mg/kg basis) than the daily recommended therapeutic dose has resulted in embryo and fetal lethality. These doses, in some studies, have been reported to cause fetal abnormalities. In the perinatal/postnatal period, there was some reduction in early individual pup weights and survival rates. There was an increased incidence of stillbirths of doses of 20 times the human dose in guinea pigs. There are no well controlled studies in pregnant women. Therefore, use of CARDIOTEM in pregnant women only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: Diltiazem is excreted in human milk. The manufacturer suggests that breastfeeding infants be substituted with another milk. The safety of use of CARDIOTEM is deemed essential, an alternative method of infant feeding should be instituted.

Pediatric Use: Safety and effectiveness in children have not been established.

ADVERSE REACTIONS
Serious adverse reactions have been rare in studies carried out to date, but it should be recognized that patients with impaired ventricular function and cardiac conduction abnormalities have usually been excluded.

In domestic placebo-controlled trials, the incidence of adverse reactions reported during CARDIOTEM therapy was not greater than that reported during placebo therapy. The following represent occurrences observed in clinical studies which may persist or recur, but have not been included in the following list.

Nervous System: Dizziness, headache, nervousness, paresthesia, paresthesia, somnolence, tremor, urticaria.

Other: Amblyopia, dysphasic aphasia, eye irritation, hypertension, hyperglycemia, nasal congestion, narcolepsy, pericardial pain, polynuropathy, sexual difficulties.

The following postmarketing events have been reported infrequently in patients receiving CARDIOTEM: alopecia, gingival hyperplasia, erythema multiforme, vasculitis. However, a definitive cause and effect between these events and CARDIOTEM therapy is yet to be established.

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References:

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045357
Evaluation of medial hypertrophy in resistance vessels of spontaneously hypertensive rats.
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Hypertension. 1988;11:198-207
doi: 10.1161/01.HYP.11.2.198

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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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