Effects of Estrogen and Progestogen Treatment on the Response of the Aorta of Male Rats to Hypertension

MORPHOLOGICAL AND CHEMICAL STUDIES

By Harvey Wolinsky

ABSTRACT

The effects of estrogen and progestogen treatment on the response of the thoracic aortic wall of male rats to hypertension were studied. Hypertension levels induced by clipping the renal artery were similar in untreated (H), estrogen-treated (E), and progestogen-treated (P) hypertensive groups. Hypertension of 8 weeks' duration caused increased wall thickness, increased calculated tangential tension, and increased medial area in groups H and P compared to normotensive controls (C), but wall thickness and medial area in groups E and C were similar despite the striking elevation in calculated tension and wall stress in group E. Chemical studies also showed that the absolute amounts of aortic collagen and elastin in groups H and P were similarly increased over group C values, but absolute amounts of both these fibrous proteins in vessels from group E were no different from those of group C. Noncollagenous alkali-soluble proteins in these vessels were present in absolute and relative amounts corresponding to the following rank: P > H > E > C. Thus, it appears that estrogen-treatment has a distinctly inhibitory effect and progestogen-treatment a slightly stimulatory effect on the aortic wall response to hypertension. Since hypertension-induced morphological and chemical changes have many similarities to features of arteriosclerotic plaque growth, recognition of these differences in hormonal effects on the response of the vessel wall to hypertension may have implications beyond their physiological role as sex hormones.

KEY WORDS

female sex hormones  elastin  collagen  vascular disease
vessel wall tension  blood vessels  arterial sclerosis

Two prominent features of the developing arteriosclerotic plaque are proliferation of smooth muscle cells and accumulation of extracellular fibrous proteins (1, 2). The initiating causes of these changes have been speculated on at length but remain unproved. Hypertension, both spontaneous and experimental, results in striking medial smooth muscle hypertrophy and hyperplasia (3–5) and accelerated fibrous protein synthesis (6); in an experimental model we have shown that the degree of these changes is predictably and linearly related to the level of calculated wall tension (6). Experimental hypertension may therefore be a useful prototype for studying modifying influences on these cellular and metabolic common features.

Considerable evidence exists to suggest that the female sex hormones are important modifying factors on the development of spontaneous or experimental arteriosclerotic lesions (7, 8). The protection from disease evidenced by premenopausal women has suggested a beneficial effect of estrogen, and

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this effect has been strongly supported by experimental animal studies (7–9) and by results of estrogen therapy in man (7, 10, 11). However, the protective effect does not seem to be wholly explainable by the lipid changes induced by estrogen treatment. The role of vessel wall factors in this protection has been postulated (8, 12).

It is clear that the female sex hormones, particularly estrogen, exert profound influence on the morphology and metabolism of uterine smooth muscle cells (13, 14) and skin fibroblasts (15, 16). However, detailed information of this type is not available for the smooth muscle cells of the blood vessel wall. A study of the effects of an estrogen and a progestogen on the morphology and chemistry of the thoracic aorta of the hypertensive male rat was therefore undertaken.

Methods

Male Carworth (CFN) rats, 7 weeks of age and weighing 130–140 g at the start of the experiment were used. All animals were housed two per cage and were given laboratory rat chow and drinking water ad libitum.

Hypertension was induced by clipping the left renal artery with a silver clip of aperture 0.25 mm. A systolic blood pressure value of 150 mm Hg or more was the criterion for hypertension, and this level was achieved in 3–4 weeks after clipping; diastolic hypertension is known to occur as well with this method. Controls underwent sham operations at the time of initial clipping. The hormones used were Depo-Estradiol Cypionate (17-beta-cyclo-pentyl-proprionate ester of estradiol-17β) in a dose of 20 μg once weekly or Depo-Provera (medroxy-progesterone acetate) in a dose of 5 mg once weekly.1 Both medications were given subcutaneously and were begun 1 week after clipping of the renal artery. Estrogen-treated animals showed marked testicular atrophy. Controls were injected subcutaneously with distilled water once weekly.

Four groups, each consisting of ten animals, were studied: (1) 8 weeks of documented normal blood pressure, (2) 8 weeks of documented hypertension, (3) 8 weeks of documented hypertension plus estrogen treatment, and (4) 8 weeks of documented hypertension plus progestogen treatment. Systolic blood pressures were taken weekly during the entire experimental period using a tail cuff and amplifier with the animal under light anesthesia.

The heart was removed from each animal immediately after it was killed by cutting the great vessels close to the respective valves; the heart was blotted and the total heart weight was obtained.

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Four animals from each group were killed by exsanguination and the thoracic aortic segment between left subclavian and celiac arteries was cannulated at both ends after all branches were ligated and was then transferred to a special frame on which in vivo length was restored. The segment was then distended to the previously measured blood pressure of that animal with a perfusion mixture containing 5% gelatin, 5% India ink and 50% barium sulfate at 40°C. After immersion of the entire apparatus in cold formalin at 10°C, the perfusion mixture gelled quickly so that the segment could be removed from the pressure system without loss of distention. Special holders were then used to maintain appropriate length of the segment during fixation in formalin over the next 48 hours. Further details of these methods can be obtained elsewhere (6, 17).

Diameters at midpoints of the segments were measured on x-rays as has been described in detail (17). Cross sections of the fixed distended segments were taken from the midpoint and were prepared for light microscopy. Histological sections 4μ thick and stained with hematoxylin and eosin and Weigert-van Gieson stains were used for measurements.

Micrometric techniques used to determine medial thickness, internal diameter, and medial lamellae have been described elsewhere (17). The aortic media was defined as that portion of the wall which contained complete, concentric elastic lamellae. Cross-sectional area of the media was calculated according to the formula: area of a ring between two circles of radius r₁ and r₂ is equal to π (r₁² − r₂²).1 Both medications were given subcutaneously and were begun 1 week after clipping of the renal artery. Estrogen-treated animals showed marked testicular atrophy. Controls were injected subcutaneously with distilled water once weekly.

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exactly delimited by the left subclavian artery and celiac artery was removed for study. The segment was opened longitudinally and the media and intima of the entire segment were removed from the adventitia (19).

Fragments of the intima-media strips were dehydrated and the lipid was removed through two changes of ethanol-ether and one change of ether over a total of 3.5 hours. After the ether was driven off by immersion of the sample-containing tubes in water at 50°C, they were transferred to a vacuum oven at 50-55°C where they were dried for 72 hours, at which time constant dry weight was achieved. The samples were then weighed individually and were transferred to 10-ml hydrolysis vials; the total initial dry de-fatted weight of each segment was thus obtained (6, 20).

Preparation of soluble and residue fractions were carried out using a previously described modification (6) of the method of Lansing (21). After hydrolysis in 0.1N NaOH for 50 minutes at 95°C, soluble and insoluble (residue) fractions were obtained and separated as described previously (6). These fractions were then hydrolyzed in 6N HCl for 48 hours at 105-110°C. Known aliquots of these fractions of each aorta were analyzed on an amino acid analyzer (Beckman Instruments model 120) as described by Spackman et al. (22). The amino acid composition of the residue fraction always corresponded to the known amino acid composition of pure elastin. The amino acid profile of the soluble fraction suggested the presence of collagen and considerable amounts of other alkali-soluble proteins. The amount of collagen present was calculated from the hydroxyproline peak on the chromatogram. The amounts of noncollagenous alkali-soluble proteins were obtained after subtraction of the contribution of collagen from the total soluble fraction. Details concerning calculation of the weights of elastin, collagen, and noncollagenous alkali-soluble proteins in each aliquot are described in detail elsewhere (20). The total weights of elastin, collagen, and noncollagenous alkali-soluble protein in each aorta were then calculated from the values computed from the known samples.

Results

Characteristics of the groups are shown in Table 1. Untreated hypertensive rats had final body weights similar to those of controls, but estrogen-treated hypertensive rats and progestogen-treated hypertensive rats had body weights significantly lower than controls (t = 9.20, P < 0.001 and t = 3.77, P < 0.01, respectively) and significantly different from each other (t = 2.48, P < 0.05 for estrogen-treated vs. progestogen-treated rats). The lower body weights in the hormone-treated groups reflected a reduced rate of body growth rather than a decrease from a previously greater weight; none of the animals was sickly. Final systolic blood pressures (Table 1) are an average of the last four readings prior to death. All the values of hypertensive groups were significantly greater than those of the controls (P < 0.001 for all), and notably all of the hypertensive groups sustained similar levels of hypertension (P at least > 0.3 for all intergroup comparisons). When ratios of heart weight to body weight were compared (Table 1), again all of the hypertensive groups had values significantly greater than those of controls (P < 0.001 for all). Also, the values for estrogen-treated and progestogen-treated hypertensive rats were significantly greater than that for untreated hypertensive rats (t = 2.40, P < 0.05 and t = 3.01, P < 0.01, respectively) but did not differ from each other (t = 1.47, 0.2 > P > 0.1).

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Gross Appearance.—Thoracic aortic segments of untreated hypertensive rats (H) and of progestogen-treated hypertensive rats (P) were markedly stiffer than vessels from controls (C) and did not collapse on removal; vessels from estrogen-treated hypertensive rats (E) were not grossly distinguishable from controls except for their slightly smaller size. No gross intimal disease was seen in any vessel.
Measured diameters of distended vessels are shown in Table 2; groups C, H, and P all had similar mean aortic diameters (P at least > 0.5). The mean diameter of group E appeared to be less than that of the other groups, but none of the intergroup statistical comparisons was significant (t = 1.81, 0.6 > P > 0.5 for E vs. C, t = 2.33, 0.1 > P > 0.05 for E vs. H, and t = 1.58, 0.2 > P > 0.1 for E vs. P).

**Microscopic Appearance.**—The adult mammalian aortic media consists of a series of concentric fibromuscular layers which we have designated as lamellar units; the number of these layers is predictably related to calculated levels of wall tension (17). In this study we confirm a consistent finding in previous studies that the number of these layers does not increase in the presence of hypertension (6, 20). None of the intergroup comparisons of lamellar unit complements (Table 2) was significant (P at least > 0.5). However, when tangential tension per lamellar unit was calculated, this was greatly elevated in all hypertensive groups (H, E, and P) when compared to the control group (C) because of the increased blood pressure (P < 0.01 for H or E vs. C and P < 0.001 for P vs. C). The values for the hypertensive groups were not significantly different from one another.

Medial thicknesses (Table 2) of groups H and P were significantly greater than that of group C (t = 3.64, P < 0.02 and t = 6.80, P < 0.001, respectively) whereas group E was similar to group C (t = 1.61, 0.2 > P > 0.1). Aortas of group E were consequently also significantly thinner than those of groups H and P (P < 0.05 for both). These differences were strikingly apparent when the microscop ic appearances of cross sections of each group were compared. In addition to group differences in thickness and apparent amount of connective tissue as shown by the Weigert-van Gieson stain, medial cell nuclei of groups C and E were similarly dense in appearance whereas the aortic nuclei of groups H and P were vesicular and frequently paired, suggesting recent mitoses, as shown by the hematoxylin and eosin stains.

Wall stress on a vessel reflects the tangential tension per unit wall thickness; if calculated tension and wall thickness increased similarly in hypertensive animals, the resultant calculated wall stress should not differ from control values. This is precisely what happened in groups H and P in this experiment (Table 2); values for wall stress in these groups were not significantly different from that of control vessels (t = 1.40, 0.3 > P > 0.2 and t = 1.75, 0.2 > P > 0.1, respectively) and were similar in groups H and P. However, in the case of group E (Table 2), the thinness of the wall in the presence of hypertension resulted in a calculated wall stress significantly greater than that of controls (t = 3.49, P < 0.02).

Cross-sectional area of the media represents a synthesis of diameter and wall thickness and corresponds well to total aortic weight (6, 20). Mean values for groups H and P were significantly greater than that of group C (t = 3.16, P < 0.02 and t = 4.69, P < 0.01,

**TABLE 2**

Dimensions of and Calculated Stresses on the Aortic Wall

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>H</th>
<th>E</th>
<th>P</th>
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<tbody>
<tr>
<td>Diameter (mm)</td>
<td>2.49 ± 0.11</td>
<td>2.50 ± 0.07</td>
<td>2.21 ± 0.11</td>
<td>2.41 ± 0.08</td>
</tr>
<tr>
<td>Lamellar units</td>
<td>8.01 ± 0.17</td>
<td>8.06 ± 0.26</td>
<td>8.13 ± 0.13</td>
<td>8.20 ± 0.12</td>
</tr>
<tr>
<td>Tension/lamellar unit (dynes/cm X 10^9)</td>
<td>2.35 ± 0.14</td>
<td>3.60 ± 0.21</td>
<td>3.43 ± 0.14</td>
<td>4.01 ± 0.22</td>
</tr>
<tr>
<td>Wall thickness (mm)</td>
<td>0.102 ± 0.004</td>
<td>0.135 ± 0.008</td>
<td>0.112 ± 0.005</td>
<td>0.152 ± 0.006</td>
</tr>
<tr>
<td>Wall stress (dynes/cm² X 10^9)</td>
<td>1.86 ± 0.14</td>
<td>2.17 ± 0.18</td>
<td>2.48 ± 0.12</td>
<td>2.17 ± 0.11</td>
</tr>
<tr>
<td>Medial area (mm²)</td>
<td>0.831 ± 0.065</td>
<td>1.101 ± 0.056</td>
<td>0.823 ± 0.073</td>
<td>1.218 ± 0.051</td>
</tr>
</tbody>
</table>

Values are means ± SE. C = control; H = untreated hypertension; E = estrogen-treated hypertension; P = progestogen-treated hypertension.
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respectively). Again the value for group E was very similar to that of group C \(t = 0.08, P > 0.9\) and was significantly less than those of groups H and P \(t = 3.02, P < 0.05, t = 4.43, P < 0.01\) for E vs. H and E vs. P. Groups H and P were not significantly different from each other.

CHEMICAL FINDINGS

Total weights of dried de-fatted thoracic aortic segments are shown in Table 3. Vessels of groups H and P were significantly heavier than those of group C \(t = 4.23, P < 0.01\) and \(t = 6.70, P < 0.001\), respectively. Vessels of group E were not significantly different than those of group C \(t = 2.18, 0.1 > P > 0.05, t = 3.34, P < 0.01\) and \(t = 6.13, P < 0.001\), respectively. Groups H and P were not significantly different.

This pattern was maintained when absolute weights of elastin and collagen were compared (Table 3). Vessels from groups H and P contained significantly greater amounts of each fibrous protein than those of group C \(P < 0.01\) for all comparisons. Vessels of group E contained absolute amounts of these proteins similar to those of group C \(P < 0.3\) for elastin and \(P < 0.9\) for collagen and correspondingly also contained less of these proteins than vessels from groups H and P \(P < 0.01\) for all comparisons. Again, groups H and P were similar to each other \(P > 0.5\) for elastin and \(P > 0.6\) for collagen.

Noncollagenous alkali-soluble proteins constitute the third fraction of the vessel wall which was measured (Table 3). Because the vessel wall contains only a limited number of constituents, it has previously been thought very likely that the noncollagenous alkali-soluble proteins primarily reflect the cellular component of the vessel wall with perhaps a minor contribution from the protein moiety of the mucopolysaccharides \(20\). The amounts of these proteins were significantly greater in all hypertensive vessels (groups H, E, and P) than they were in normotensive ones (group C). However, unlike the finding with the fibrous proteins, each of the hypertensive groups differed significantly from one another. Vessels from group E contained significantly more of the soluble proteins compared to group C \(t = 4.10, P < 0.01\) and significantly less than groups H and P \(t = 8.02, P < 0.001\) and \(t = 6.73, P < 0.001\), respectively. Vessels of group H contained the next largest amount of these proteins, significantly more than those of group C and E \(t = 8.02, P < 0.001\) and significantly less than those of group P \(t = 3.10, P < 0.02\). Vessels of group P contained the largest absolute amount of this protein component, significantly more than group C \(t = 9.04, P < 0.001\), group E \(t = 6.73, P < 0.001\), and group H \(t = 3.10, P < 0.02\).

The percents of each of the wall components described above in absolute terms are shown in Table 4. The total percent of the initial dry aortic weight recovered was similar in groups C and E and similar in groups H and P, but the values of each pair of groups differed very significantly with the much higher percents obtained in groups H and P \(P < 0.01\) for all comparisons. For elastin, groups C, H, and E did not differ significantly among themselves \(t = 2.17, 0.1 > P > 0.05\) for C vs. H, \(t = 1.67, 0.2 > P >

### TABLE 3

<table>
<thead>
<tr>
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<th>C</th>
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<th>E</th>
<th>P</th>
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<tbody>
<tr>
<td>Total aortic dry weight</td>
<td>6.02 ± 0.39</td>
<td>9.27 ± 0.66</td>
<td>6.96 ± 0.19</td>
<td>10.44 ± 0.53</td>
</tr>
<tr>
<td>Elastin (mg)</td>
<td>2.61 ± 0.11</td>
<td>3.77 ± 0.26</td>
<td>2.77 ± 0.12</td>
<td>3.60 ± 0.16</td>
</tr>
<tr>
<td>Collagen (mg)</td>
<td>1.29 ± 0.09</td>
<td>1.77 ± 0.11</td>
<td>1.28 ± 0.07</td>
<td>1.84 ± 0.09</td>
</tr>
<tr>
<td>Alkali-soluble protein (mg)</td>
<td>0.86 ± 0.04</td>
<td>2.22 ± 0.11</td>
<td>1.19 ± 0.07</td>
<td>3.16 ± 0.28</td>
</tr>
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Values are means ± SE. C = control; H = untreated hypertension; E = estrogen-treated hypertension; P = progestogen-treated hypertension.
TABLE 4

Percents of Aortic Wall Components

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<tbody>
<tr>
<td>Elastin</td>
<td>42.39 ± 0.77</td>
<td>39.86 ± 0.87</td>
<td>39.75 ± 1.38</td>
<td>34.65 ± 1.29</td>
</tr>
<tr>
<td>Collagen</td>
<td>20.87 ± 0.84</td>
<td>18.78 ± 0.39</td>
<td>18.39 ± 0.75</td>
<td>17.66 ± 0.34</td>
</tr>
<tr>
<td>Alkali-soluble protein</td>
<td>13.93 ± 0.54</td>
<td>24.24 ± 0.61</td>
<td>17.08 ± 0.90</td>
<td>30.00 ± 1.83</td>
</tr>
<tr>
<td>Total</td>
<td>77.19 ± 0.76</td>
<td>82.88 ± 0.98</td>
<td>75.22 ± 1.32</td>
<td>82.40 ± 0.92</td>
</tr>
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Values are means ± SE. C = control; H = untreated hypertension; E = estrogen-treated hypertension; P = progestogen-treated hypertension.

It seems clear from the results of this study that estrogens have a distinctly inhibitory effect on the response of the vessel wall to hypertension and that progestogens slightly enhance the effects of hypertension. The aortic medial cells of the control and the estrogen-treated hypertensive groups had a similar "nonactivated" appearance, whereas those of the untreated hypertensive and the progestogen-treated hypertensive groups had an "activated" vesicular nuclear appearance with frequent paired cells (23).

The reasons for using the hypertension-stimulated wall for studies of the effects of sex hormones are several. First, both estrogens and progestogens have unpredictable effects on blood pressure in normal man and other animals (24, 25), which would add another variable to any study of their effects. This variable was avoided in the present study in view of the similar levels of hypertension attained by groups H, E, and P. Second, the difference in growth rate of the estrogen-treated animals was anticipated (12). We have previously shown that the degree of changes in the vessel wall induced by hypertension are so striking that they overwhelm any growth-related differences in vessel wall morphology and chemistry (6, 20). The relative unresponsiveness of the vessel wall of group E to hypertension in the present study cannot therefore be attributed to the slower growth rate of this group. Indeed, the progestogen-treated animals, which were also growth-retarded, nonetheless manifested a vascular response to hypertension which exceeded that of the untreated hypertensive animals. Also, we have previously shown no difference between the responsiveness to hypertension of vessel wall from rapidly growing males and nongrowing females (20). Of course, no hormonal treatment was given in that study. Finally, the strikingly greater, but qualitatively similar, changes induced by hypertension alone compared to those occurring with normal growth might permit the detection of more subtle influences acting on the vessel wall.

The effects of the sex hormones are clearly tissue-dependent and may also vary with species (9). In several species, estrogen alone produces a profound stimulatory effect on
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uterine smooth muscle morphology and metabolism (13, 14). In the rat, progesterone causes the cells to increase greatly in size, but little change in synthetic activity is evident (26). When both hormones are present, as in pregnancy, uterine smooth muscle cell and fibrous protein components increase absolutely severalfold (27). Estrogen also stimulates collagen synthesis by skin fibroblasts (15) and by cells in granulomas (16), but because catabolism is even more greatly accelerated the net amount of collagen in these tissues actually decreases compared to controls (15, 16). In turnover studies with labeled proline of collagen from aortas, granulomas, and skin from guinea pigs, estrogen treatment increased the specific activity of soluble collagen fractions, suggesting that maturation to more highly cross-linked, stable insoluble collagen was interfered with (16). Our findings of decreased absolute amounts of elastin and collagen in aortas of estrogen-treated animals are basically in agreement with the above. However, our findings suggest that inhibition of fibrous protein synthesis was responsible for the decreased amounts of protein. It should be noted that a previous study of the chicken aorta showed a decreased concentration of elastin and collagen in females and estrogen-treated males compared to untreated males (28). Unfortunately interpretation of these findings is limited by the lack of data on the absolute amounts of these proteins (29). In that study, testosterone treatment restored female vessel concentrations of the fibrous protein to male levels, which suggests an effect separate from that of mere estrogen lack. Some support for a stimulatory effect of androgen on collagen synthesis comes from studies of skin biopsies in women with osteoporosis who were found to have increased absolute amounts of collagen after androgen therapy (30); furthermore, women with hirsutism were found to have striking increases in skin collagen compared to controls (31).

A remarkable finding was the minimal response of the estrogen-treated vessel wall to hypertension. In spite of an elevated calculated wall stress, elastin and collagen were not increased over control values and only a slight increase in the fraction which presumably reflects smooth muscle was seen, far less than that seen with hypertension alone. Others have found a marked reduction of aortic oxygen consumption (32) and a marked suppression of 35S-sulfate incorporation into ground substance of aortas of male rats (12) after treatment with estradiol. Parallel with our finding of a stimulatory effect of progestogen on cellular and fibrous components is the finding by Gore et al. (33) that treatment of rabbits with norethynodrel, a progestogen, resulted in marked accumulation of acid mucopolysaccharides in the aortas. These findings taken together with evidence that vascular changes associated with pregnancy parallel in many ways those seen with progestogens (34-36) suggest that a circumspect posture toward current interest in developing progestogen-only contraceptives should be assumed (37, 38).

The development of a fibromuscular subintimal layer in vessels early in life seems to be closely related to later development of atheromata (39, 40). Whereas some have attributed formation of this layer to excessive stress (40), this currently is only conjectural. However the two cardinal structural features of this layer are smooth muscle proliferation and accumulation of extracellular fibrous proteins (39, 40). In fact, the vessel wall responds similarly to a variety of types of direct trauma (41, 42); indeed, even hypercholesterolemia causes sustained proliferation of aortic explants (43). Although a precise explanation for the etiology of the atherosclerotic plaque is lacking, the hope of achieving specific inhibition of those features of the lesion responsible for its growth and subsequent complications is stimulated by this study.

If the sequence is such that lipid accumulates in a wall matrix of increased thickness and with an increased tangle of fibrous proteins, efforts directed toward reduction of elevated serum cholesterol should be additive in beneficial effect (7, 44) to measures directed toward the cellular and fibrous
components. In addition to all of the above, prevention of increased thickness of the vessel wall (as seen with estrogen) of hypertensive animals would possibly have benefits with respect to vessel wall nutrition (45). The possible relation of excessive thickening of the avascular media of the human abdominal aorta to its special susceptibility to vascular disease has been discussed previously (46).

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Effects of Estrogen and Progestogen Treatment on the Response of the Aorta of Male Rats to Hypertension: MORPHOLOGICAL AND CHEMICAL STUDIES

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