Modification of the Effects of Hypertension on Lysosomes and Connective Tissue in the Rat Aorta

By Harvey Wolinsky, Sidney Goldfischer, Bernice Schiller, and Lisa E. Kasak

ABSTRACT
The relationship of lysosomes to the vascular effects of hypertension and the possible modification of these effects by anti-inflammatory agents (methylprednisolone and aspirin), vitamin E, and estrogen were studied. Each of these agents was given to a group of hypertensive rats; untreated hypertensive rats and normotensive rats served as controls. Vessel wall morphology and dimensions of aortas from hypertensive rats were unaffected by treatment. Usual connective tissue accumulations seen in hypertensive vessels were suppressed to normotensive levels in the methylprednisolone- and estrogen-treated hypertensive groups. Two lysosomal enzymes, acid phosphatase and N-acetyl-β-D-glucosaminidase (NAGA), increased over normal levels in hypertensive vessels from 1.03 ± 0.08 (SE) to 2.04 ± 0.19 μmoles/aortic pair hour⁻¹ and from 1.10 ± 0.24 to 4.57 ± 0.38 μmoles/aortic pair hour⁻¹ for the respective enzymes. Normal enzyme levels in estrogen-treated hypertensive rats (1.14 ± 0.15 μmoles/aortic pair hour⁻¹ for acid phosphatase and 2.04 ± 0.44 μmoles/aortic pair hour⁻¹ for NAGA) and intermediate levels in methylprednisolone-treated hypertensive rats (1.35 ± 0.10 μmoles/aortic pair hour⁻¹ for acid phosphatase and 2.48 ± 0.54 μmoles/aortic pair hour⁻¹ for NAGA) were found. Other treated groups showed the usual elevations associated with hypertension. These group differences were also seen after cytochemical staining for lysosomal acid phosphatase and NAGA. The parallel changes in aortic connective tissue and lysosomal enzymes in hypertension and their modification by two drugs suggest that these events are related.

KEY WORDS
N-acetylglucosaminidase
elastin
N-acetyl-β-D-glucosaminidase (NAGA)
collagen
acid phosphatase
aspirin
vitamin E
methylprednisolone
vascular smooth muscle

Apart from its proven accelerating effect on the development of atherosclerosis (4, 5), hypertension is a useful model for the study of vascular injury; it causes cellular hypertrophy (6), proliferation (7), and degeneration (8, 9) and accumulation of intramural connective tissue (10). Wolinsky (11) has shown that administration of drugs such as estrogen significantly inhibits this response to hypertension. Peters et al. (12) have recently isolated a lysosomal fraction from rabbit aortic wall, and we (13) have recently identified the lysosomes in vascular smooth muscle by ultrastructural cytochemistry. The present study was intended to explore further the relationship of lysosomes to the vascular effects of experimental hypertension and to study possible modifications of these effects by anti-inflammatory agents (methylprednisolone and aspirin), vitamin E, and estrogen.

Methods
Male Carworth (CF-N) rats weighing 170–180 g at the outset of the experiment were used. Hypertension ensued 3 weeks after placement of a left renal artery clip under light ether anesthesia. Systolic blood pres-
sures were measured using a tail cuff and a transducer by an amplification technique (model 79 Grass amplifier); hypertension was defined as a systolic blood pressure greater than 150 mm Hg. Rats were given Purina rat chow and water ad libitum. Six groups of 30 rats each were used: group N = untreated normotensive rats, group H = hypertensive rats untreated except for weekly intramuscular injections (0.05 ml) of the vehicle (29 mg of polyethylene glycol 4000, 8.7 mg of sodium chloride, and 0.19 mg of myristyl-γ-picolinium chloride per 1 ml of vehicle) for methylprednisolone acetate (40 mg/ml), group M = hypertensive rats given weekly intramuscular injections of 2 mg of methylprednisolone (0.05 ml), group V = hypertensive rats given intramuscular injections of 200 IU of vitamin E (dl-α-tocopheryl acetate, lot 22304, USV Pharmaceutical Corp.) (0.2 ml) on alternate days, group A = hypertensive rats given aspirin (Merck) in their chow at a daily dose increased as aspirin (Merck) in their chow at a daily dose increased from approximately 40-60 mg at the outset to 70-100 mg by 3 weeks and maintained at that level thereafter, and group E = hypertensive rats given weekly subcutaneous injections of 100 μg of estradiol cypionate in cottonseed oil (0.1 ml). All drugs were begun 1 week after clipping (and therefore 2 weeks before the onset of hypertension) and were continued for the duration of the experiment, which lasted through an 8-week period of documented hypertension. Blood for serum salicylate determinations were made as previously described (10). Circumferential wall tension was calculated according to the Law of Laplace (wall tension = blood pressure × radius); wall stress was determined by dividing the tension by wall thickness. All measurements were corrected for tissue shrinkage during preparation as determined previously (10).

MORPHOLOGY

Thoracic aortic segments from four rats per group were prepared for dissection to the blood pressure of the donor rat as described elsewhere; the dissection mixture contained barium sulfate, gelatin, and carbon (10). After fixation, diameters were measured on x-rays, and after paraffin embedding micrometric measurements were made as previously described (10). Circumferential wall tension was calculated according to the Law of Laplace (wall tension = blood pressure × radius); wall stress was determined by dividing the tension by wall thickness. All measurements were corrected for tissue shrinkage during preparation as determined previously (10).

CYTOCHEMISTRY

Aortic segments, removed immediately after death, were fixed for 3 hours in ice-cold 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4 (14). Specimens were labeled with a code that was not made available to the tissue laboratory until the slides were processed and reported. After fixation tissues were rinsed in buffer containing 7.5% sucrose, and frozen sections 10μ thick were cut with a Sartorius freezing microtome for light microscopic study. Freely floating sections were incubated for acid phosphatase activity (20, 30, and 45 minutes at 37°C) in a modified Gomori medium with β-glycerophosphate as substrate (15) and for N-acetyl-β-d-glucosaminidase (NAGA) activity (60, 75, and 90 minutes at 37°C) in a hexazonitized pararosanilin medium (16) with naphthol AS-BI N-acetyl-β-d-glucosamine (Sigma) as a substrate. After incubation, acid phosphatase preparations were rinsed, visualized in dilute ammonium sulfide and mounted on slides with a water-soluble medium. NAGA preparations were dehydrated and mounted in Permount. For electron microscopy, frozen sections 40μ thick were incubated for acid phosphatase activity, as above, rinsed in buffer, and postfixed for 1 hour in 1% osmium tetroxide in 0.1M phosphate buffer, pH 7.4 (15). Specimens were then dehydrated in graded alcohols and embedded in Epon. Thin sections were cut with a Sorvall MT-2 ultramicrotome and examined in a Siemens Elmskop.

BIOCHEMISTRY

All studies were carried out on intima-media fragments which were stripped from the adventitia of complete thoracic aortic segments.

Connective Tissue and Cellular Proteins.—Six rats per group were used, and the intima-media fragments of an entire aorta were analyzed. After delipidation and dehydration, soluble and residue fractions were prepared according to a modification of Lansing’s method (10). After acid hydrolysis, total elastin and collagen and noncollagenous alkali-soluble proteins (NCASP) per aorta were also determined. The latter component is felt to represent mainly the cellular protein component of the vessel wall (10).

Enzymes.—Determinations were carried out on five pairs of aortas per group. Intima-media fragments from each pair were placed in distilled water and homogenized for 6 minutes with a Teflon mortar and pestle; temperature was maintained at 4°C throughout this procedure. Centrifugation at 4°C at 1,000 g for 10 minutes was followed by separation of the supernatant fluid for enzyme analyses; the pellet was used for DNA determinations. Completeness of this separation was confirmed by “reverse” analyses of some pellets for enzyme activities and of some supernatant samples for DNA; only trace amounts of each were found. Acid phosphatase activity was assayed using β-glycerophosphate (Sigma) as the substrate and following the method of Barrett (17) after appropriate reduction of volumes of reagents. Total NAGA activity was assayed using the appropriate p-nitrophenyl substrate (Sigma) and the method outlined by Barrett (17); this method was modified again for the small amounts of tissue. Time
curves were evaluated to determine optimal conditions, and appropriate reagent and tissue blanks were checked for base-line activity. Enzyme activities are based on micromoles of nitrophenol released by the NAGA (18) and micromoles of inorganic phosphorus released by the phosphatase, measured using the modified method of Fiske and Subbarow (19). Activity is expressed in terms of total micromoles of each substrate released per aortic pair per hour.

**DNA.**—Total DNA content of five pairs of aortas from each group was determined on pellets (see above). The modification by Hubbard et al. (20) of Cerrotti's method was used. DNA standards were corrected for water content with inorganic phosphorus as the criterion, using the method of Fiske and Subbarow (19).

For all statistical comparisons of results, Student's t-test was used. Significance was considered to be present at the 5% level or less.

### Results

Body weights, blood pressures, and heart weight–body weight ratios of rats in each of the groups are shown in Table 1. Final body weights were all similar except for groups M and E, which were significantly lighter than the others (P < 0.001 for both). Final blood pressure levels (average of the last two readings taken at weeks 5 and 7) did not differ significantly among any of the hypertensive groups; all were significantly greater than that of group N (P < 0.001). Similar findings were seen for the heart weight–body weight ratios (Table 1), which reflect cardiac enlargement due to hypertension; all hypertensive groups were similar and differed significantly from group N (P < 0.001).

### Morphology

The results of morphometry and tension calculations are shown in Table 2. All the hypertensive groups in Table 2 had similar aortic diameters which were all significantly greater than those of the normotensive rats in group N (P < 0.01). The same relationship held for each of the other measurements and calculations shown in Table 2: the values for the hypertensive groups were similar to each other regardless of treatment and were significantly greater than those for the normotensive group. For wall thickness and medial area, the significance of this difference between the hypertensive groups and the normotensive group was P < 0.01; for tension per lamellar unit and wall stress the significance was P < 0.001. Thus, for all measurements and calculations made from morphology and blood pressure, no effects from the various treatments of the hypertensive groups were detected.

### Biochemistry

Biochemical analyses of the aortas, however, did show differences among the hypertensive groups. Total aortic dry weight (Fig. 1) for each of the hypertensive groups was greater than that of the normotensive group (P < 0.001) except for group M, which had an intermediate level heavier than that of group N (t = 2.63, P < 0.05) and lighter than that of the other hypertensive groups (P < 0.001). Wolinsky (11) has previously reported that estrogen treatment of hypertensive rats reduces aortic dry weight to normotensive levels, so the values are not given in this paper.

Analysis of individual components (Fig. 2) showed that the hypertensive groups H, V, and A all had significant increases in absolute weights of elastin and collagen in the aorta compared with normotensive group N (all comparisons P < 0.001). However, aortas from methylprednisolone-treated hypertensive rats (group M) had amounts of these two proteins which were no different from those in the normotensive rats (t = 0.82, 0.5 > P > 0.4 for elastin, t = 1.74, 0.2 > P > 0.1 for collagen). Wolinsky (11) has previously reported that estrogen treatment of hypertensive rats reduces aortic dry weight to normotensive levels, so the values are not given in this paper.

### Table 1

<table>
<thead>
<tr>
<th>Characteristics of Groups Studied</th>
<th>Final body wt (g)</th>
<th>Final systolic blood pressure (mm Hg)</th>
<th>Heart wt/body wt (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>370 ± 11</td>
<td>111 ± 1</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>H</td>
<td>377 ± 11</td>
<td>192 ± 7</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>M</td>
<td>268 ± 10</td>
<td>165 ± 7</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td>V</td>
<td>358 ± 17</td>
<td>185 ± 5</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>A</td>
<td>353 ± 15</td>
<td>193 ± 8</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>E</td>
<td>277 ± 9</td>
<td>179 ± 6</td>
<td>0.39 ± 0.02</td>
</tr>
</tbody>
</table>

All values are means ± SE. N = normotensive rats, H = hypertensive rats, M = methylprednisolone-treated hypertensive rats, V = vitamin E-treated hypertensive rats, A = aspirin-treated hypertensive rats, and E = estrogen-treated hypertensive rats.
Total aortic dry weights of the groups studied. All of the values for the hypertensive groups are significantly greater than that for the normotensive group (group N) except for the value for group M, which had an intermediate weight.

Weights of individual aortic components in each of the groups studied. Groups H, V, and A had significantly more collagen and elastin than did group N; group M did not differ from group N in amount of connective tissue. All hypertensive groups (H, M, V, and A) had significantly more noncollagenous alkali-soluble proteins (NCASP) than did the normotensive group (N).

Enzymes.—Cytochemical studies showed that only a few lysosomes could be stained in the medial smooth muscle cells of aortas isolated from normotensive rats and incubated for acid phosphatase (45 minutes) or NAGA (90 minutes) activities (Fig. 3a). In aortas of untreated hypertensive rats, numerous lysosomes were readily demonstrable in the perinuclear cytoplasm after relatively brief incubations of 30 minutes for acid phosphatase activity and 60 minutes for NAGA activity (Fig. 3b).

### TABLE 2

Dimensions of and Calculated Stresses on the Aortic Wall

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>H</th>
<th>M</th>
<th>V</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (mm)</td>
<td>2.23 ± 0.08</td>
<td>2.68 ± 0.04*</td>
<td>2.60 ± 0.03*</td>
<td>2.58 ± 0.06*</td>
<td>2.75 ± 0.07*</td>
</tr>
<tr>
<td>Wall thickness (mm)</td>
<td>0.883 ± 0.024</td>
<td>1.140 ± 0.048*</td>
<td>1.172 ± 0.058*</td>
<td>1.120 ± 0.026*</td>
<td>1.163 ± 0.051*</td>
</tr>
<tr>
<td>Tension/lamellar unit (dynes/cm × 10^3)</td>
<td>2.03 ± 0.11</td>
<td>4.20 ± 0.16†</td>
<td>4.44 ± 0.29†</td>
<td>3.84 ± 0.18†</td>
<td>4.05 ± 0.13†</td>
</tr>
<tr>
<td>Wall stress (dynes/cm² × 10^5)</td>
<td>1.85 ± 0.09</td>
<td>2.89 ± 0.07†</td>
<td>2.87 ± 0.08†</td>
<td>2.71 ± 0.10†</td>
<td>2.71 ± 0.12†</td>
</tr>
<tr>
<td>Medial area (mm²)</td>
<td>0.646 ± 0.037</td>
<td>0.999 ± 0.053*</td>
<td>1.023 ± 0.056*</td>
<td>0.945 ± 0.010*</td>
<td>1.047 ± 0.058*</td>
</tr>
</tbody>
</table>

All values are means ± SE. N = normotensive rats, H = hypertensive rats, M = methylprednisolone-treated hypertensive rats, V = vitamin E-treated hypertensive rats, and A = aspirin-treated hypertensive rats.

*P < 0.01 (paired analysis) compared with group N.
†P < 0.001 (paired analysis) compared with group N.
LYSOSOMES, FIBROSIS, AND HYPERTENSION

All light micrographs (a, b, d, and e) are of 10μ frozen sections of glutaraldehyde-fixed rat thoracic aortas incubated for N-acetyl-β-d-glucosaminidase activity. The electron micrograph (c) is of a 40μ frozen section incubated for acid phosphatase activity and processed for ultrastructural examination. a and b: Only a few reactive lysosomes (arrows) are barely detectable, even after 90 minutes of incubation, in the normal aorta (a). In the hypertensive vessel (b) reactive lysosomes (arrows) are abundant in the perinuclear cytoplasm of medial smooth muscle cells after 60 minutes of incubation. c: Electron micrograph of hypertensive rat thoracic aorta. Reaction product is deposited on lysosomes (L). A lysosome of the autophagic vacuole type (AV) is also identified. Mitochondria (M) are unreactive. Clusters of lysosomes are common in hypertensive vessels but are rarely seen in the normotensive vessel. d and e: Oil immersion photomicrographs show clusters of darkly stained lysosomes in the aspirin-treated hypertensive rats (d) but not in the estrogen-treated hypertensive rats (e).
Identification of the reactive sites as lysosomes was confirmed by examining acid phosphatase preparations in the electron microscope. The product of the lead phosphate reaction was deposited on lysosomes of the dense body and autophagic vacuole types (Fig. 3c).

Aortas from hypertensive rats treated with aspirin reacted in the same fashion as those from the untreated hypertensive rats (Fig. 3d); the reaction of vitamin E-treated hypertensive vessels was also similar. Aortas from hypertensive rats treated with estrogen closely resembled those from the normotensive controls in their paucity of acid hydrolase-reactive lysosomes (Fig. 3e). Aortas from methylprednisolone-treated hypertensive rats had more reactive lysosomes than did those from normotensive rats but far fewer than those from untreated hypertensive rats. These group distinctions were more pronounced in NAGA than in acid phosphatase preparations.

Results of analyses of aortas for two lysosomal enzymes are shown in Figure 4. Compared to normotensive rats (group N), untreated hypertensive rats (group H) had significantly greater activities of both acid phosphatase and NAGA \( (t = 4.85, P < 0.001 \text{ and } t = 7.68, P < 0.001, \text{ respectively}) \). Aspirin-treated hypertensive rats (group A) had increased activities similar to those of untreated hypertensive rats. Vitamin E-treated hypertensive rats (group V) showed marked increases in both enzymes, significantly greater even than those found in group H \( (t = 2.58, P < 0.05 \text{ and } t = 3.98, P < 0.01, \text{ respectively}) \). Methylprednisolone-treated (group M) and estrogen-treated (group E) hypertensive rats both showed suppressed levels compared with those found in untreated hypertensive rats (group H). In the case of group M, acid phosphatase was at an intermediate level, very significantly less than that in group H \( (t = 3.19, P < 0.02) \) and significantly more than that in group N \( (t = 2.82, P < 0.05) \). NAGA levels in group M were also very significantly less than those in group H \( (t = 3.15, P < 0.02) \) but were not quite significantly above those in group N \( (t = 2.16, 0.1 > P > 0.05) \). In group E, activities of both enzymes were suppressed to levels not different than those for normotensive rats \( (t = 0.67, 0.6 > P > 0.5) \) for acid phosphatase, \( t = 1.65, 0.2 > P > 0.1 \) for NAGA).

DNA.—Determinations of total DNA in aortic pairs showed no significant differences between any of the groups, hypertensive or normotensive. The actual values expressed as \( \gamma \) per aortic pair \( (\text{mean} \pm \text{SE}) \) were: group N 35.4 ± 1.3, group H 36.0 ± 1.7, group M 34.6 ± 2.6, group V 39.5 ± 2.1, group A 37.2 ± 3.4, and group E 34.5 ± 1.4.

Discussion

We found parallel changes in connective tissue accumulation and lysosomal enzyme activity in hypertensive rat aortic walls. These changes were not related to either cell number (DNA) or cell bulk (NCASP). Treatment with estrogen and methylprednisolone (groups E and M) suppressed both the connective tissue and the lysosomal responses in hypertensive rats but did not affect aortic DNA levels. In these rats, NCASP was also comparable to (group M) or reduced (group E) relative to the levels in other hypertensive rats. Treatment of hypertensive rats with aspirin and vitamin E had little or no effect on cell number, bulk, lysosomal acid hydrolase activities, or connective tissue accumulations.

Before proceeding with a discussion of these findings, the possible objection that suppression of vascular connective tissue seen in the aortas of groups E and M reflected their slower growth rate (Table 1) must be considered. Hypertension was originally chosen as our experimental model, because we could show that the vascular response to increased pressure was far greater than any
vascular effects from changes in body weight of the magnitude found in this study. Severe hypertension alone might cause retarded body growth (Table 2, ref. 21) and increased aortic connective tissue. Dissociation of growth and vascular effects has also been shown in studies of drugs, such as progesterone, which suppress growth but do not affect the vascular effects of hypertension (11). In a recent study that used controlled feeding, Wolinsky (22) has shown that the vascular effects of estrogen administration to normotensive oophorectomized rats occur independently of its effects on body growth. Optimally, possible body growth effects on aortic composition in groups E and M of the present study should have been controlled by pair-feeding of all groups. Nevertheless, we believe that the evidence suggests that the major findings described in the paper are not growth related.

If one considers the association of increased lysosomal enzyme activity with connective tissue accumulation to be other than fortuitous, several possible mechanisms, which are not necessarily exclusive, might be invoked to relate these events in the aortic wall. Permeability of the intima to a variety of plasma constituents (9, 23-25) is enhanced in the hypertensive vessel. Exposure of the mural smooth muscle cells to an influx of proteins (24) and lipids (25) that is far in excess of normal should result in increased endocytosis and intracellular digestion within secondary lysosomes. Circulating proteins and lipids are conveyed into the cytoplasm by endocytic vacuoles that fuse with lysosomes, and their contents are subsequently degraded by the lysosomal acid hydrolases (26). A direct destructive effect on the lysosome by a specific lipid (27) or protein (7) serum component or an overwhelming of the cellular disposal system (28) could result in cell degeneration and necrosis. Release of cellular and lysosomal contents into the vessel matrix would then perpetuate the lytic process and elicit further proliferative and reparative responses.

In addition to the response of lysosomes to endocytosis and their involvement in degenerative aspects of injury, deDuve (26) has suggested that they may be activated during repair of damaged tissue, including connective tissue synthesis. The accelerated connective tissue and mucopolysaccharide synthesis seen in the hypertensive vessel in response to increased tension (1) would therefore necessitate increased metabolic and lysosomal activity. Whether the stimulus to lysosomal activity is enhanced endocytosis, increased synthesis, or both, the background of excessive mechanical stress would further serve to increase cell degeneration and death.

Of the drugs administered, aspirin and vitamin E showed little or no effect; estrogen and methylprednisolone showed distinct suppressive effects on both lysosomal enzymes and connective tissue accumulation in hypertensive vessels. The different findings for aspirin and methylprednisolone suggest that nonspecific anti-inflammatory activity of these agents cannot be implicated in these effects on the vessel. Glucocorticoids are believed to be potent stabilizers of membranes, particularly those of lysosomes (29). Estrogen has also recently been shown to inhibit release of enzymes from lysosomes of polymorphonuclear leukocytes (30) and to stabilize artificial lipid spheres (29); in contrast, aspirin has little or no effect on lysosomal stability (31). Vitamin E in high doses (as given in this study) is thought to be a lysosomal membrane labilizer (32). It is of interest that testosterone, also considered a lysosomal labilizer (29), has been shown by Wolinsky (33) to result in increased vascular connective tissue when it is administered to normotensive male rats. Drug effects on membrane stability are obtained from in vitro systems; the extent to which these results are transferrable to in vivo circumstances is a point of active controversy. Evidence that destruction of lysosomal membranes and subsequent leakage of hydrolases into the cytoplasm leads to tissue necrosis and repair has been obtained in studies of silica toxicity (34) and gouty arthritis (35).

It is also possible that the effective drugs exert their primary moderating action not on the lysosome but rather on vascular permeability. Reduction of endothelial permeability by estrogen (36) or corticosteroids (37) might result in decreased needs for phagocytosis and secondary lysosomal stimulation. Other alternative sites of drug action undoubtedly are possible.

Somewhat analogous to the findings in this paper are those by Currerri et al. (38), who showed that lysosomal enzymes are markedly increased in atherosclerotic plaque-laden aortic tissue of rabbits fed an atherogenic diet. Administration of cortisone in addition to the diet aggravated the hypercholesterolemia of these rabbits but resulted in complete protection from disease and maintenance of activities of lysosomal enzymes at normal levels. From that experiment and from ours, it appears that despite the continued presence of hypercholesterolemia or hypertension—two of the
most potent risk factors for development of atherosclerotic vascular disease (5)—it is possible to modify certain aspects of the vascular response to those stimuli. Although their precise role remains to be determined, it seems that lysosomes are an important element of that response.

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References

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