Effects of Hypertension and Its Reversal on Aortic Intima Lesions of the Rat

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SUMMARY A combined transmission (TEM) and scanning (SEM) electron microscopic study was performed on aortae of deoxycorticosterone-salt (DOC-salt)-treated rats and spontaneously hypertensive rats (SHR) to compare the effects of hypertension as well as its reversal on the aortic intima. To best reproduce the in vivo state of the vasculature, rats were perfusion-fixed at pressures corrected for each individual animal (30 mm Hg below measured systolic pressure). The intimal alterations were focal and thus were best appreciated with the combined use of SEM and TEM. Qualitatively, both models of hypertension showed similar intimal changes, which consisted of subintimal thickening due to an accumulation of both extracellular material and cells. Subendothelial cells with a morphology indicating a blood-borne origin were present simultaneously with cells derived from the vessel wall. The increased subendothelial extracellular material included precipitated plasma proteins, reticulated basement membrane, collagen fibers, and fragments of elastin. Increase in the height of endothelial cells with distortion of nuclear shape was prominent. Withdrawal of DOC-salt combined with low-salt diet for 11 weeks did not result in a discernible regression of these intimal changes despite normalization of blood pressure. We conclude that vascular injury, once induced, may be difficult to reverse and suggest that areas of prior damage may serve as foci for later vascular complications.

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KEY WORDS • endothelium • DOC-salt hypertension • spontaneously hypertensive rat • aorta • intima • electron microscopy

MANY of the pressure-related complications of hypertension, such as congestive heart failure and strokes, have been shown to be reduced substantially by reduction of blood pressure,1, 2 but the effects of therapy on intimal lesions of large vessels remain uncertain.3-4 Previous studies of the influence of hypertension on vascular morphology have suggested various abnormalities including increased intimal permeability, intimal thickening, thickening of the entire vascular wall, and the appearance of subendothelial cells.5-6

The current study was performed to characterize in detail the effects of hypertension on the aortic intima and the development and progression of these changes in two different experimental models of hypertension,3 and to examine whether reversal of these changes occurs with correction of the hypertension. This investigation represents the morphologic correlate of our recently published study17 on hypertension and aortic metabolism. Both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been used in this work, and serial studies have been carried out in both the deoxycorticosterone-salt (DOC-salt)-treated rat and the spontaneous hypertensive rat (SHR). The results have indicated that focal intimal changes occur, even after brief periods of hypertension, and that the changes are qualitatively similar in both models. The findings also demonstrate that many of the changes may persist even after control of the hypertension.

Methods

Animal Models

Male SHR and Wistar-Kyoto (WKY) rats from the Okamoto-Aoki strain were obtained from either Taconic Farms or Charles River Breeding Laboratories. Two established models of hypertension were used, the DOC-salt-treated WKY rat18 and the SHR.19

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DOC-salt hypertension was induced in the following manner: 11-week-old WKY rats were anesthetized with ether and one kidney was removed. Weekly treatment with deoxycorticosterone pivalate (1.5 mg/100 g body weight) was begun 7 days later, with 1% saline added to the drinking water. Three to five animals were sacrificed after 1, 2, 4, and 7 weeks of DOC-salt treatment. An additional group of five uninephrectomized animals was treated with DOC-salt for 7 weeks; following cessation of DOC-salt treatment, they were maintained for an additional 11 weeks on a low-salt diet (sodium-deficient diet, Nutritional Biochemicals Co.). Controls for the DOC-salt studies were groups of three WKY rats uninephrectomized at 11 weeks of age and maintained on 1% saline for identical periods (1, 2, 4, and 7 weeks), as well as age-matched WKY rats receiving neither treatment nor surgery. In addition, three uninephrectomized rats were maintained on 1% saline for 7 weeks and then given a sodium-deficient diet for 11 weeks.

Groups of four to six SHR rats were sacrificed at 11, 16, 18, and 21 weeks of life along with control WKY rats of comparable age. In other studies performed in our laboratory using Wistar rats, we have not observed significant morphologic differences from the WKY in the age range studied. We therefore have used only the WKY as our experimental control.

Blood pressures were measured at 1- to 3-week intervals using the tail-cuff method described by Brecher et al.11

Perfusion and Fixation Procedure

The rats were anesthetized with ether, and a cannula (connected to a perfusion apparatus fitted with a manometer to measure perfusion pressure) was inserted into the aortic arch through the left ventricle. The rat was then perfused with approximately 400 ml of fixative under pressure maintained at 30 mm Hg below theystolic pressure measured for each individual rat before fixation. Insertion of the cannula took less than 5 seconds. Animals were perfused for 15 minutes with either phosphate-buffered 1% glutaraldehyde-4% formaldehyde fixative or cacodylate-buffered glutaraldehyde (2.5%). Perfusate drainage occurred through severed intercostal and epigastric vessels. After fixation in situ for an additional 15 minutes, each aorta was carefully excised and periaortic adipose tissue dissected away. Aortae were immersed in fixative for 1 hour and then stored in 0.1 M sodium cacodylate-7% sucrose, pH 7.4.

Processing for Transmission Electron Microscopy

Rings of the midthoracic aortae were cut and post-fixed with 1% aqueous osmium tetroxide, stained en bloc with 0.1% aqueous uranyl acetate, dehydrated through increasing concentrations of ethanol followed by propylene oxide, and embedded in Epon 812. Sections for light microscopy were cut 1 μ thick and stained with toluidine blue and basic fuchsin.

Ultrathin sections of selected areas were cut using a Reichert OMU2 microtome, stained with uranyl acetate and lead citrate, and examined with a Philips EM 300 electron microscope at 80 kV.

Processing for Scanning Electron Microscopy

Rings of midthoracic aortae were rehydrated with increasing concentrations of acetone and critical-point dried with CO2. Rings were then cut in half and mounted, intimal side up, on aluminum discs using copper tape, sputter-coated with gold, and examined in either a Jeol JSM-35 or an AMR 1000 scanning electron microscope at 15 kV.

Results

Blood Pressure

Blood pressures of the groups from which the animals were taken for morphologic evaluation are given in detail in the previous report from our laboratory.17 Briefly, systolic blood pressure rose gradually during DOC-salt treatment to levels ranging from 220 to 263 mm Hg. Pressure decreased progressively after DOC-salt was withdrawn, reaching normotensive levels (137 ± 4 mm Hg) within 6 weeks after withdrawal; it remained within the normal range for a minimum of 5 weeks thereafter. Blood pressures in SHR rats gradually rose with increasing age to 192 ± 5.1 mm Hg at the age of 21 weeks.

Aortic Intima in Control Animals

The thoracic aortae of untreated WKY rats up to 28 weeks of age showed a flat intima. The endothelial cells were located in close apposition to the internal elastic lamina (IEL), and minimal amounts of extracellular matrix, including reticulated basement membrane, were present in the subendothelial zone (fig. 1A). Cellular elements were virtually absent in the subendothelium, except for occasional foci in close proximity to branches. By SEM, elevations of the intima toward the lumen were found to be almost exclusively formed by the thicker perinuclear cytoplasm of individual endothelial cells (fig. 1B). The IEL was straight in all aortae (fig. 1A). The medial smooth muscle cells showed smooth contours and contained predominantly myofilaments, while other organelles were sparse. Uninephrectomized control animals on high-salt diets showed an essentially similar aortic intima except that foci of intimal thickening, resembling those to be described in the hypertensive animals, were observed on rare occasions.

Development of Hypertensive Intimal Changes in the DOC-Salt Model

After 1 week of DOC-salt administration, the subendothelial space enlarged and assumed an edematous appearance. Although the amount of reticulated basement membrane appeared slightly increased, most of the intimal thickening at this time was due to a widening of the subendothelial zone. Only thin endothelial
cytoplasmic extensions remained in close proximity to the IEL (fig. 2A). These were the positions where most of the semidesmosomes were found (fig. 2A, insert). The membranes constituting the intercellular cleft appeared in their usual close apposition.

After 2 weeks of DOC-salt administration, the thickness of the intima increased further. The thickening consisted of both edematous, loose extracellular material and increased reticulated basement membrane in approximately equal portions (fig. 2B).
FIGURE 2. Development of the aortic intima thickening after DOC-salt treatment, as shown by TEM. A. After 1 week of treatment, the endothelial space (Sp) is edematous. Endothelial cell extensions remain near the internal elastic membrane (IEL). The extension has semidesmosomes (arrowhead) and a Weibel-Palade body (asterisk). Junctional complexes (J) between endothelial cells appear normal. Scale represents 0.25 μm. Insert: Detail of a semidesmosome. Scale represents 0.1 μm. B. After 2 weeks of treatment, the intimal thickening is increased; it consists of edematous spaces (Sp) filled with loose floccular material and denser areas with the structure of reticulated basement membrane (B) and possibly precipitated plasma proteins. Endothelial cells (E) bulge toward the lumen (L) and show intact junctional complexes. Scale represents 1 μm.
After 4 weeks of treatment, denser material, including reticulated basement membrane, precipitated plasma proteins, and collagenous extracellular material, had completely filled in the widened subintimal zone. In addition, the height of the endothelial cells themselves was increased. The endothelial nuclei appeared folded and often showed bizarre configurations (fig. 3A). Additional elevations of the intima were caused by the appearance of subendothelial cells of various origins that often showed swollen...
cytoplasmic extensions and increased amounts of organelles. Together, these three major changes (namely: increased height of endothelial cells, the presence of subendothelial cells and extensions, and increased amounts of extracellular material including reticulated basement membrane) produced multiple elevations of the intima, which were focal and varied in size and shape as seen by SEM (fig. 3B). None of the bulging was due to waves of the IEL since the IEL consistently appeared straight, due to fixation at adequate perfusion pressures.

After 7 weeks of treatment, the intimal thickening was not increased discernibly and showed essentially the same cellular and extracellular elements, including endothelial extensions reaching the IEL. Extracellular collagenous material was more organized; and cells, mature collagen fibers, and fragments of elastin could be identified (fig. 4). Endothelial junctions remained closely apposed throughout DOC-salt treatment.

Development of Hypertensive Intimal Changes in SHR Rats

The aortic intima of the 11-week-old SHR appeared composed of predominantly flat endothelial cells in close apposition to the IEL (fig. 5A). By SEM, very occasional areas of elevation were seen other than the ones due to slight perinuclear cytoplasmic bulges (fig. 5B). By 18 weeks, focal areas of change were present (including distorted endothelial nuclei), as well as increased amounts of extracellular material (including reticulated basement membrane), and subendothelial cells and cell extensions. Occasionally, some necrotic debris was also present (fig. 5C). Qualitatively, the changes were indistinguishable from those seen in DOC-salt-treated rats. In 21-week-old SHR, more foci showing similar changes were found (fig. 5D). The focal nature and extent of these changes were evident on larger intima areas overseen by SEM (fig. 5E).

Effects of Withdrawal of DOC-Salt Treatment

Animals given DOC-salt for 7 weeks followed by 11 weeks on a low-sodium diet with no DOC-salt treatment showed aortic intima changes qualitatively similar to animals after seven weeks on DOC-salt. These changes included distortion of endothelial cell nuclei, presence of subendothelial cells, and increased amounts of extracellular material including reticulated basement membrane (fig. 6A). Such intimal changes occurred most prominently above fenestrations in the IEL. Medial smooth muscle cells showed uneven contours, often with projections extending through IEL fenestrations. The subendothelial zone contained mature collagen fibers, fragments of elastin, and myelineated cell debris (fig. 7). The membranes at the junctions between endothelial cells remained in close apposition. These intimal changes produced the SEM picture of multiple areas of focal bulging (fig. 6B).

Variability of Structures Causing Intimal Thickening

The consistent picture of intimal thickening and surface bulging described above was found to have several causes. We found subendothelial cells of various morphologic characteristics suggesting different sites of origin, including cells resembling...
FIGURE 5. Development of focal thickenings in the thoracic aortic intima of SHR rats. A. TEM of aortic intima of an 11-week-old SHR. Flat endothelial cell (E) is in close apposition to the internal elastic lamina (IEL). Scale represents 1 μ. B. SEM of aortic intima of the same animal. Only minimal changes can be seen. Scale represents 10 μ. C. TEM of aortic intima of an 18-week-old SHR. A focus of intimal thickening is shown which consists of an endothelial cell with an infolded nucleus (N), a subendothelial cell (S), and increased extracellular material including neutotic cellular debris (D), and myelinated figures (M). Scale represents 1 μ. D. TEM of aortic intima of a 21-week-old SHR. A subendothelial cell (S) causes the endothelium to bulge toward the lumen (L). Increased amounts of dense extracellular material are interspersed between endothelium and internal elastic membrane. Scale represents 1 μ. E. SEM of aortic intima of the same animal shown in fig. 5D. The focal nature of the intimal changes can be seen. Areas with flat endothelium (F) interchange with areas showing intimal bulging by endothelial and subendothelial cells (T). Scale represent 10 μ.
modified smooth muscle cells (fig. 8A), cells containing granules found in blood monocytes (fig. 8B), and red blood cell fragments (fig. 8C). Bizarre folding of endothelial nuclei was also found to cause dramatic increase in height of the entire endothelial cell (fig. 8D). The subendothelial zone often contained one or multiple swollen membrane-bound cytoplasmic extensions of subendothelial cells (fig. 8E), or partially necrotic extensions or fragments (fig. 8F), sometimes exhibiting myelin whirls. Often, a combination of cellular and extracellular material constituted the intimal thickening.

Discussion
These investigations performed in conjunction with our recently published metabolic studies (Brecher et al.17) were designed to characterize the morphologic changes induced by high DOC-salt treatment followed by withdrawal. A TEM shows that the focal intimal thickening persists and consists of endothelial cells with folded nuclei (N), swollen extensions of subendothelial cells (S), and increased amorphous material (M). The internal elastic lamina (IEL) is straight. Scale represents 1 μ. B. SEM shows multiple focal thickenings (T). Most of the bulging areas involve several endothelial cells (E), indicating focal accumulation of subendothelial cells and extracellular material. Scale represents 10 μ.
changes occurring in the aortic intima during the development and regression of hypertension.

The combined use of SEM and TEM was very helpful in giving an accurate assessment of hypertensive vascular changes, since aortic intima lesions in both the DOC-salt and SHR groups were highly focal in nature. For this reason, the use of SEM was mandatory because it allowed evaluation of large intimal areas, thereby giving proper perspective to the frequency and distribution of the intimal changes and avoiding sampling errors inherent in many TEM investigations. The concomitant use of TEM allowed the precise characterization of the surface changes detected by SEM, revealing the multitude of subendothelial structures that produced these lesions. Proper fixation at an adequate pressure was crucial for both SEM and TEM. The importance of perfusion fixation of vasculature for TEM was emphasized by Haudenschild et al. and, subsequently, for SEM, by Clark and Glagov. Insufficient attention to this particular methodologic problem has raised questions about the morphologic findings of earlier studies of the vascular intima. The issue becomes even more complex in studies of hypertensive animals where blood pressure is the major variable. Our preparatory fixation studies at various pressures indicated that perfused fixation at 30 mm Hg below the systolic blood pressure, measured prior to fixation in each animal, produced an image which we consider to represent most closely the status of the vessel in vivo, as judged by the consistently straight appearance of the IEL in all our specimens. After waviness of the IEL and other fixation artifacts are eliminated, the remaining focal elevations of the aortic intima seen by SEM can be interpreted with greater confidence as being true lesions. Indeed, animals that showed multiple thickenings by SEM consistently exhibited endothelial cell bulging, subendothelial cells, and extracellular material located on a straight IEL when viewed by TEM.

We were impressed by the variety of structures that can cause intimal thickening. In accordance with recent reports, we find both blood-borne and vessel wall-derived cells in the intimal lesions. When cytoplasmic swellings produced intimal thickening, the nature of these cells often could not be determined. We noted that the swellings usually lacked organelles and almost always involved peripheral portions of the cytoplasm. Both endothelial cell pseudopods and ex-
Figure 8. TEM showing various causes of intimal thickening and endothelial bulging in the intima of hypertensive rats. Figs. B, C, and E are after 4 weeks of DOC-salt treatment. Figs. A, D, and F are after 7 weeks of DOC-salt treatment followed by 11 weeks of withdrawal. Note that the internal elastic lamina is straight in all micrographs. Scales represent 1 μm. A. A subintimal cell resembling a modified smooth muscle cell. B. Abundant amorphous material and a subendothelial cell containing granules resembling those often found in monocytes. C. Red blood cell fragments located in the subendothelial space, surrounded by dense amorphous extracellular material. D. Bulging cytoplasm of an endothelial cell containing a folded nucleus of bizarre shape. Such cells have been interpreted as being contracted.

Early in the development of intimal hypertensive changes, the subendothelial zone widens. Initially this zone contains very sparse material, suggesting that edema causes endothelial cell lifting. Increased water content of the entire vascular wall was described as an early event in hypertension, and the alteration in endothelial cell permeability associated with hypertension in different models would support this suggestion. With the widening of the subendothelial zone, the adhesion sites of the endothelial cells may be reduced to the few semidesmosomes that were found primarily on endothelial extensions remaining near the IEL. With the continuation of hypertension, the extracellular material in the subendothelial space becomes denser; subsequently, structures resembling collagen and elastin are found. The persistence of this extracellular material after reversal of the elevated tension of subendothelial cells of various origins can be suspected as the source of these enlarged structures, but serial sectioning will be required to demonstrate the connections between the extensions and the cell bodies.

In addition to the presence of subendothelial cells and increased extracellular material, the endothelial cells themselves were involved in the formation of intimal changes. The bizarre, folded shapes of the endothelial cell nuclei were found invariably in endothelial cells of greatly increased height. Majno et al. first interpreted this degree of nuclear folding as being a morphologic equivalent of endothelial cell contraction. With the use of antiactin antibodies, Gabbiani et al. noted increased amounts of contractile elements in endothelial cells in acutely hypertensive rats.
blood pressure may possibly be related to the inability of local mechanisms to remove it once it has reached a certain degree of organization.

This observation corresponds with our biochemical data on the same group of rats as well as on SHR treated with a combination of chlorothiazide, reserpine, and hydralazine, indicating that the total amount of connective tissue protein was increased in hypertension and remained elevated following its reversal. This also supports the prior studies of Wolinsky who demonstrated that, after reversal of experimental renovascular hypertension by removal of the renal artery clip, elastin and collagen content remained elevated in amounts similar to those in animals with sustained hypertension. These results are in contrast to those of Spector et al., who observed, after chlorothiazide or reserpine treatment of DOC-salt rats, a decrease in the elevated collagen content.

Although the clinical significance of the persistence of some vascular changes after the correction of hypertension is uncertain, the findings may have relevance with respect to recent reports in man suggesting that certain complications of hypertension may not be prevented by antihypertensive therapy. Despite major reductions in the incidence of strokes and congestive heart failure observed with antihypertensive therapy, the major risks relating to ischemic heart disease and, presumably, coronary artery atherosclerosis appear to be either uninfluenced or only reduced somewhat by treatment. There has been speculation that these findings might be due to an adverse influence of antihypertensive treatment on the status of other cardiovascular risk factors. However, our studies indicate that certain vascular effects will persist even after reduction of blood pressure by nonpharmacological means. The findings are not unlike those reported in cholesterol-fed animals where complete regression of atherosclerotic lesions has not been achieved over prolonged periods of time. Vascular injury, once induced, may be difficult to reverse, and it is interesting to speculate that areas of prior damage may serve as foci for later vascular complications.

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