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Spontaneously Hypertensive Rats Develop Pulmonary Hypertension and Hypertrophy of Pulmonary Venous Sphincters

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This study explored the spontaneously hypertensive rat as an animal model of pulmonary hypertension and sought to identify anatomic changes in its pulmonary microvasculature, especially focal constrictions of pulmonary veins (sphincters). The average systemic and pulmonary artery blood pressures were 172/139 (±9/9) and 36/14 (±4/3), respectively, for spontaneously hypertensive Wistar Kyoto rats (SHR), and 134/83 (±8/2) and 20/10 (±2/2) for normotensive Wistar Kyoto rats (WKY) (P < 0.01 for both). Light microscopy of the lungs in SHR showed muscularization of both arteries and veins, but this was more pronounced in the small pulmonary veins. Perivascular edema was also present. There were 20 (±4) leukocytes per 100 μm of capillary length in SHR and 9 (±2) in WKY (P < 0.0001). Transmission electron microscopy showed focal venous smooth muscle was greater in SHR than in WKY. Scanning electron microscopy of vascular casts showed the average maximal focal venous contraction (sphincter) was 54% (±10) of its diameter in SHR, but was only 6% (±4) in WKY (P < 0.01). Arterial contraction occurred in the hypertensive rats as hourglass narrowings of the casts, but was less conspicuous than venous constrictions. The mean alveolar capillary diameter was 8.1 μm (±1.6) in SHR, compared with 6.3 μm (±1.0) in WKY (P < 0.01). The central interspace between capillaries was 3.2 μm (±1.6) in SHR and 6.0 μm (±3.6) in WKY (P < 0.01). The venous contraction, capillary size, and capillary interspace distance correlated with the pulmonary blood pressure. The spontaneously hypertensive rat can be a model of pulmonary hypertension with its most notable structural change being increased muscularity in the small pulmonary veins. (Am J Pathol 1996, 148:281–290)

Ideally an animal model of human pulmonary hypertension would develop pulmonary hypertension without requiring extrinsic damage to the lung, production of a high flow state, or obstruction of pulmonary blood vessels. Okamoto and colleagues[1] bred a strain of Wistar Kyoto rats that spontaneously developed systemic hypertension (SHR). This study aimed to evaluate their spontaneously hypertensive rat as a model of pulmonary hypertension. McMurtry and colleagues[2] found this animal had increased pulmonary vascular reactivity and pulmonary hypertension when exposed to hypoxia, and Janssens and colleagues[3] found the animal developed pulmonary hypertension in normoxic conditions. Other investigators have found muscular hyperplasia of the pulmonary blood vessels and other changes indicating pulmonary hypertension,[4–6] but a detailed, three-dimensional, anatomic study of the pulmonary vas-

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culature has not been carried out. Scanning electron microscopy of casts of pulmonary veins has identified focal ring-shaped constrictions called sphincters.[7] They are caused by circular smooth muscle bundles[8–10] that constrict with various stimuli.[7,11–12] Aharinejad and Böck[13] showed that pulmonary veins of SHR had increased focal smooth muscle tufts. We sought to further clarify the pulmonary hypertension that developed in this animal strain and to learn about the anatomic changes that occurred in the pulmonary vasculature, which may underlie the physiological events leading to this process. We compared SHR with control normotensive Wistar Kyoto rats (WKY), measuring blood pressures and tissue and vascular cast parameters by light microscopy, transmission electron microscopy, and scanning electron microscopy.

Materials and Methods

This experiment was approved by the Animal Care Committee at the University of Massachusetts Medical Center at Worcester. The animals were handled humanely according to Public Health Service guidelines. The SHR were 14 to 18 weeks old and weighed 300 (±18) g; the normotensive control rats were 10 to 14 weeks old and weighed 295 (±70) g.

Blood Pressure Measurements

Eight male, spontaneously hypertensive, Wistar Kyoto rats (SHR) (Charles River, Boston, MA) and eight male, control normotensive WKY (Charles River, Boston, MA) were anesthetized with dimethyl ether (Metofane, Fisher Chemicals, Chicago, IL). We opened the abdomen and cannulated the aorta. The cannula was connected to a transducer that recorded blood pressure on a Grass polygraph (Grass Instrument Co., Quincy, MA). The trachea was exposed and cannulated with a plastic tube. The tube was connected to another Y-shaped tube that was connected to a reservoir filled with dimethyl ether at one end and to a Harvard ventilator at the other. The animals were ventilated with room air at a tidal volume of 1.5 ml and rate of 100 breaths/minute. Next, we placed a 24-gauge Teflon catheter with an "R crook" tip, connected to another transducer, into a metal cannula.[14] The cannula was introduced into the right jugular vein and we advanced its tip into the right ventricle until the typical right ventricular pressure wave form was obtained. After measuring the pressure, the catheter was advanced into the pulmonary artery. The pressure was recorded again.[12] After finishing the pressure recordings, the animals were used for casting.

Tissue Preparation

In 10 additional SHR and 10 WKY, the hearts were removed and the atria separated from the ventricles. The outer right ventricular wall was removed and weighed. The left ventricle and septum were also weighed and a ratio of the right ventricle to the left ventricle and septum was made according to the method of Fulton et al.[15] We also compared the heart weights with body weight.

The lungs of another six SHR and six WKY rats were fixed by vascular perfusion with 2.5% glutaraldehyde in 0.2 mol/L phosphate buffer at pH 7.2, through the caudal vena cava. The lungs were removed and, for light microscopy, sliced into sections about 5 mm thick and transferred to a glutaraldehyde solution for at least 24 hours. The tissue slices were dehydrated and embedded in Paraplast (Sigma, Vienna, Austria). The sections were stained with hematoxylin and eosin. For transmission electron microscopy, the lungs were minced into 1 × 1 × 1 mm blocks, washed in phosphate buffer, postfixed with 1% osmium tetroxide for 2 hours, dehydrated in a series of graded alcohols and propylene oxide, and embedded in Epon 812. Semithin and thin sections were cut on a Reichert (Vienna, Austria) OM U2 ultramicrotome. We stained semithin sections with alkaline toluidine blue O, and the thin sections with methanolic uranyl acetate and alkaline lead citrate. The sections were viewed with a Zeiss EM 10 transmission electron microscope (Zeiss, Oberkochen, Germany).

With both light and electron microscopy we examined the tissue for evidence of pulmonary hypertension, such as arterial and venous muscular hypertrophy, intimal damage, and plexiform lesions. With light microscopy, we counted the number of leukocytes per capillary length in randomly selected alveolar areas.

Cast Preparation

The vasculature of the rats used for blood pressure measurements was rinsed through the caudal vena cava with warmed 20% dextran-40 in 0.9% saline and 5000 units/L of heparin until the efflux from the aorta began to clear. This required ~80 ml of solution.[10] We then cast the lungs with 20 ml methyl methacrylate (Mercox, Ladd Industries, Burlington, VT) mixed with 2 g of accelerator. This was injected over 1 minute through a 20-gauge vena caval can-

nula connected to a hand-held syringe. After hardening at room temperature for 2 hours, the lungs were removed and transferred to a 60°C water bath overnight. The tissue was corroded in a 5% potassium hydroxide solution at 40°C for about 2 more days.[10] The specimens were rinsed with tap water for 30 minutes, with 5% formic acid for 10 minutes, and finally with three rinses of distilled water for 2 minutes each.[16] The casts were frozen in distilled water, freeze-dried, cut with scissors into 1 to 2 mm-thick slices, and mounted onto studs with silver paste and conductive bridges.[17] The casts were sputter-coated with palladium gold and studied with a JEOL JSM-35C scanning electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 10 kV.

From each animal used for casting, we placed four slices of the lung casts on aluminum studs. The specimens were coded. On each stud, we studied four arteries, four veins, and four alveolar fields. The fields containing the arteries and veins were selected randomly at 200× magnification. Those containing alveoli were selected randomly at 1000× magnification. We first inspected the blood vessels for changes from the normal structure that we have reported in other studies.[16,18] Arterial casts are distinguishable from venous casts by several features. Arterial endothelial cell nuclei make elongated imprints that run with the long axis of the vessel. Venous endothelial cell nuclei make round impressions without axial orientation.[19] Veins have regular ring-like constrictions that we have termed sphincters.[7] Arteries do not. Arteries generally run with airways; small veins do not. Venous casts usually have less space between their wall and adjacent capillaries than the arterial casts.[18]

We measured the maximum depth of the venous contraction as a percentage of the diameter by taking one minus the smallest diameter of the contracted segment divided by the diameter of the cast at the contraction site.[11] Alveolar capillaries cover most of the alveoli but leave a small space that appears as a hole in the middle of a spoke configuration of the cast.[20] In each alveolar capillary cast basket, the widths of 10 of the capillary interspaces, or holes, were measured.[21] Last, we measured the diameters of four capillaries per field and four fields per stud in separate areas.

Data Analysis

We analyzed the animal body weight; systemic and pulmonary arterial systolic, diastolic, and mean pressures; the number of leukocytes in a capillary segment; the diameter and depth of contraction of the vein (in percentage); the width of the interspaces in the alveolar capillary casts; and the diameters of the capillaries. The differences between the two groups were evaluated by *t*-tests. To account for multiple measurements per animal for the venous contractions, capillary diameters, and capillary interspace measurements, we did nested regression, nesting these measurements with the individual animal.[22] We also used multivariate analysis to find out whether the blood pressure and capillary differences could be accounted for by the venous contraction. We concluded that differences were significant if $P < 0.01$. The standard deviation follows the ± in the text. Statistics were carried out on a Dell 310 microcomputer running SAS statistical software.[23]

Results

In WKY the average systemic blood pressure was 134/83 (±8/2); in SHR it was 172/139 (±9/9), which is 28% higher for the systolic and 67% higher for the diastolic pressure ($P < 0.01$ in both cases). In WKY the average pulmonary blood pressure was 20/10 (±2/2); in SHR it was 36/14 (±4/3), which is 80% higher for the systolic and 40% higher for the diastolic pressure ($P < 0.01$ in both cases). The percentage increase in systolic mean blood pressure was greater in the pulmonary circulation compared with the systemic circulation ($P < 0.01$), but the increase in diastolic pressure was not.

The average weight of the free wall of the right ventricle was not different between SHR (0.15 ± 0.03 g) and WKY (0.14 ± 0.01 g), but the ratio of the weight of the right ventricle to the left ventricle and septum was less in SHR (0.17 ± 0.03) compared with WKY (0.24 ± 0.01) ($P < 0.01$). The ratio of the weight of the right ventricular wall to the body weight was 4.7 (±1.0) × 10^5 in SHR and 5.0 (±0.04) × 10^5 in WKY, which was not different.

In the paraffin sections of WKY, the walls of the veins consisted of an endothelial lining with underlying connective tissue. Smooth muscle cells were hardly discernible. In the semithin sections, single or small groups of smooth muscle cells could be identified between endothelium and elastic lamina of the veins. These smooth muscle cells that indented their lumen corresponded to the sphincters described earlier.[13]

The light microscopy of the hypertensive rats showed that veins up to 350 μm in diameter, had focal aggregations of circumferential, smooth muscle cells inside the elastic lamina just beneath the

endothelium (sphincters). These smooth muscle bundles bulged into the lumens of the pulmonary veins of the hypertensive rats (Figures 1 and 2), but were not found in the normotensive controls. In the hypertensive rats, the muscle cell aggregates stacked to form cuneiform structures and were serially arranged along the course of the veins (Figures 1 and 2). In the pulmonary arterial walls of the hypertensive rats, the tunica media had thickened segments serially arranged along its course, which reduced its diameter (Figure 3). In addition, perivascular edema was regularly present especially in the bronchovascular bundle (Figure 4), and the capillaries were congested with erythrocytes and leukocytes. The average capillary length studied for leukocytes was 132 (±11) μm in WKY and 142 (±14) μm in SHR, which was not different. The number of leukocytes was 20 (±4) per 100 μm of capillary length in SHR *versus* 9 (±2) per 100 μm in WKY ($P < 0.01$).

The transmission electron microscopy showed that pulmonary veins of the normotensive rats had one or two layers of circularly arranged, spindle-shaped, smooth muscle cells just inside the elastic lamina at intervals in the venous wall, structures termed pulmonary venous sphincters.[8] The smooth muscle cells next to the endothelial layer formed myoendothelial junctions. In SHR, the walls of small pulmonary veins between the muscle usually consisted of an endothelial layer resting on connective tissue lamella (Figure 5). Sphincters, by contrast, were isolated cones of smooth muscle aggregates inside the elastic lamina with their tips directed toward the lumen (Figure 6). Here the sphincters consisted of up to 20 spindle-shaped smooth muscles cells arranged in four to five rows over each other (Figure 7). Smooth muscle cells next to the endothelium often shared their basal laminae with that of the endothelium. Moreover, the basal lamina between endothelium and smooth muscle cells was often missing (Figures 7 to 9).

Another characteristic of SHR lungs was the presence of leukocytes in the capillaries. Both neutrophil granulocytes (Figures 10 and 11) and lymphocytes (Figures 12 and 13) remained attached to the endothelial surface after vascular rinsing and perfusion-fixation. The leukocytes were attached to the endothelium with flattened cytoplasmic extensions that formed a gap of constant width (Figures 12 and 13). Adhering leukocytes also contacted opposing capillary walls (Figures 10 and 12) trapping erythrocytes upstream, implying that capillary blood flow was impeded (Figures 11 and 12).

In both strains of rats, scanning electron microscopy of arterial casts showed that their surfaces were smooth and their endothelial nuclear imprints regular. Only in hypertensive animals were regions of arterial casts cylindrically narrowed and accompanied by a longitudinally wrinkled surface (Figures 14 and 15). The veins used for contraction measurements ranged from 20 to 400 μm in diameter. Both groups of rats had ringlike circular constrictions that readily fractured, but the sphincters were deeper in SHR than in WKY (Figures 16 and 17). The average greatest contraction was 54% (±10) of the venous diameter in the hypertensive rats and 6% (±4) of the venous diameter in the normotensive rats ($P < 0.01$).

Alveolar capillaries in the hypertensive rats were larger in diameter than the normotensive rats (Figure 18). The average diameter of capillaries was 8.1 μm (±1.6) in the hypertensive rats and 6.3 μm (±1.0) in the normotensive rats ($P < 0.01$). The width of alveolar capillary interspaces was 3.2 μm (±1.6) in the hypertensive rats (Figure 18) compared with 6.0 μm (±3.6) in the normotensive animals ($P < 0.01$). Of the capillary casts of SHR, 95% had holes caused by trapped blood cells compared with 5% in the normal animals. These holes occurred despite the vascular perfusion for rinsing and casting.

Correlation of Blood Pressure Measurements and Cast Measurements

The multivariate analysis showed that the venous contraction of the sphincters accounted for a great deal of the variation in pulmonary arterial blood pressures between the two groups (systolic $R^2 = 0.78$, $P < 0.01$, diastolic $R^2 = 0.32$, $P < 0.01$, and mean $R^2 = 0.62$, $P < 0.01$). The univariate correlation showed an association between the capillary interspace distance and both the venous contraction ($r = -0.46$, $P < 0.01$) and the pulmonary blood pressures (systolic $r = -0.44$, $P < 0.01$, diastolic $r = -0.26$, $P < 0.01$, and mean $r = -0.38$, $P < 0.01$). The nested regression showed that the relationships were significant after accounting for more than one measurement within the same animal.

Discussion

Although SHR were bred to be a model of systemic hypertension,[1] they can also serve as a model of pulmonary hypertension. Even though the left ventricular hypertrophy is greater than the right, the pulmonary hypertension in these older rats was as great as the systemic hypertension when measured as a percentage change. This model is attractive because the pulmonary hypertension develops with-

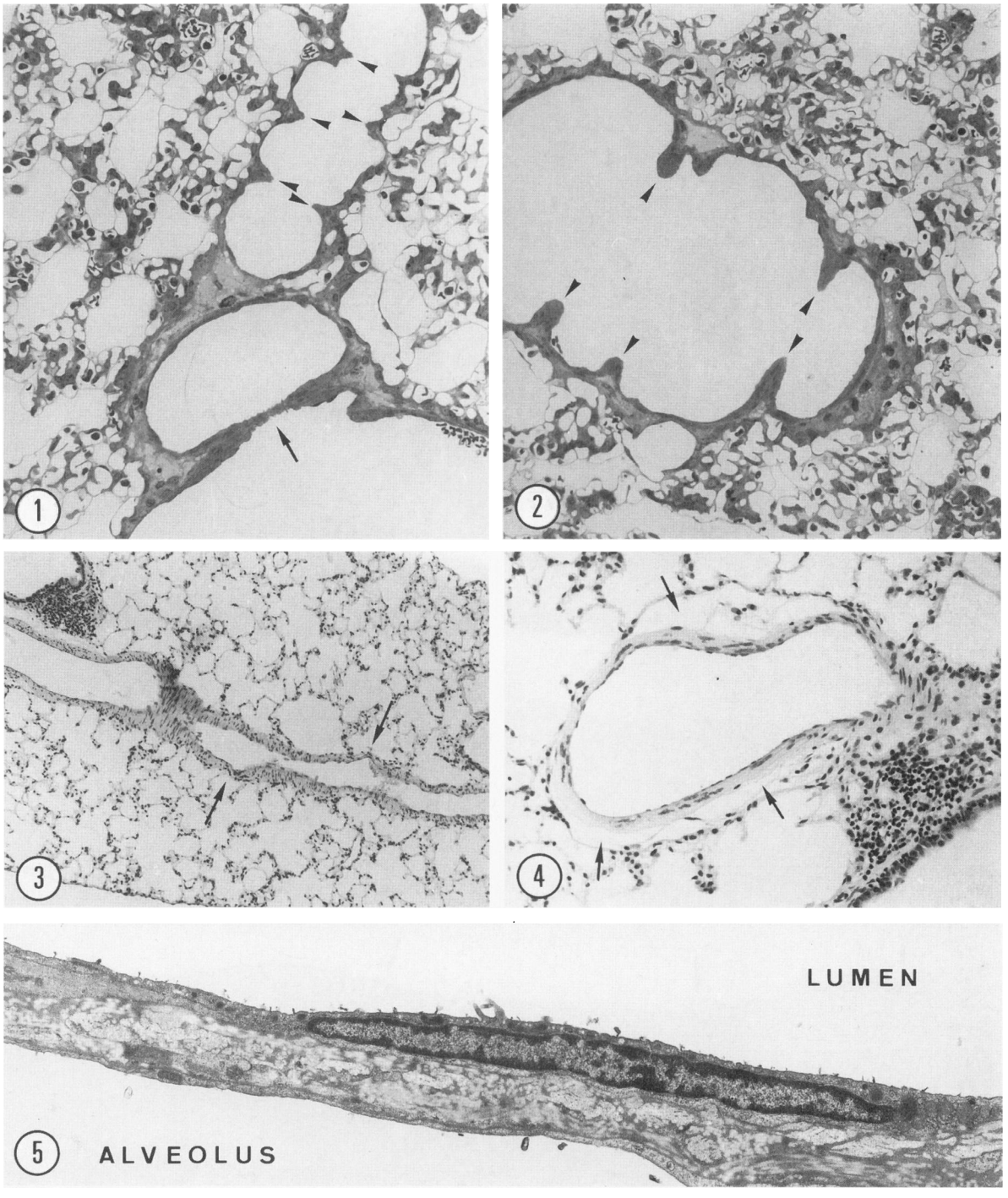


Figure 1. *Light micrographic picture of the junction of two pulmonary veins in the lung of an SHR. The lumens are carinated by bulging smooth muscle sphincters* (**arrowheads**). *Next to the merger, a sphincter is sectioned tangentially* (**arrow**). ×*280.* **Figure 2.** *This light micrograph of a small intrapulmonary vein in an SHR shows sphincters in the wall similar to that shown in Figure 1. These sphincters are composed of grouped smooth muscle cells* (**arrowheads**). ×*280.* **Figures 3 and 4.** *Light micrographs of pulmonary arteries in SHR. Figure 3 shows a longitudinally sectioned artery with a segmentally thickened tunica media narrowing its lumen. Arrows point to those arterial wall regions where the media is not thickened and the lumen in not narrowed.* ×*100. Figure 4 shows edema* (**arrows**) *around a pulmonary artery of a hypertensive rat.* ×*280.* **Figure 5.** *This electron micrograph shows a segment of the wall of a small pulmonary vein in SHR located between venous sphincters. The venous wall is made only of endothelium and underlying connective tissue fibers.* ×*5000*

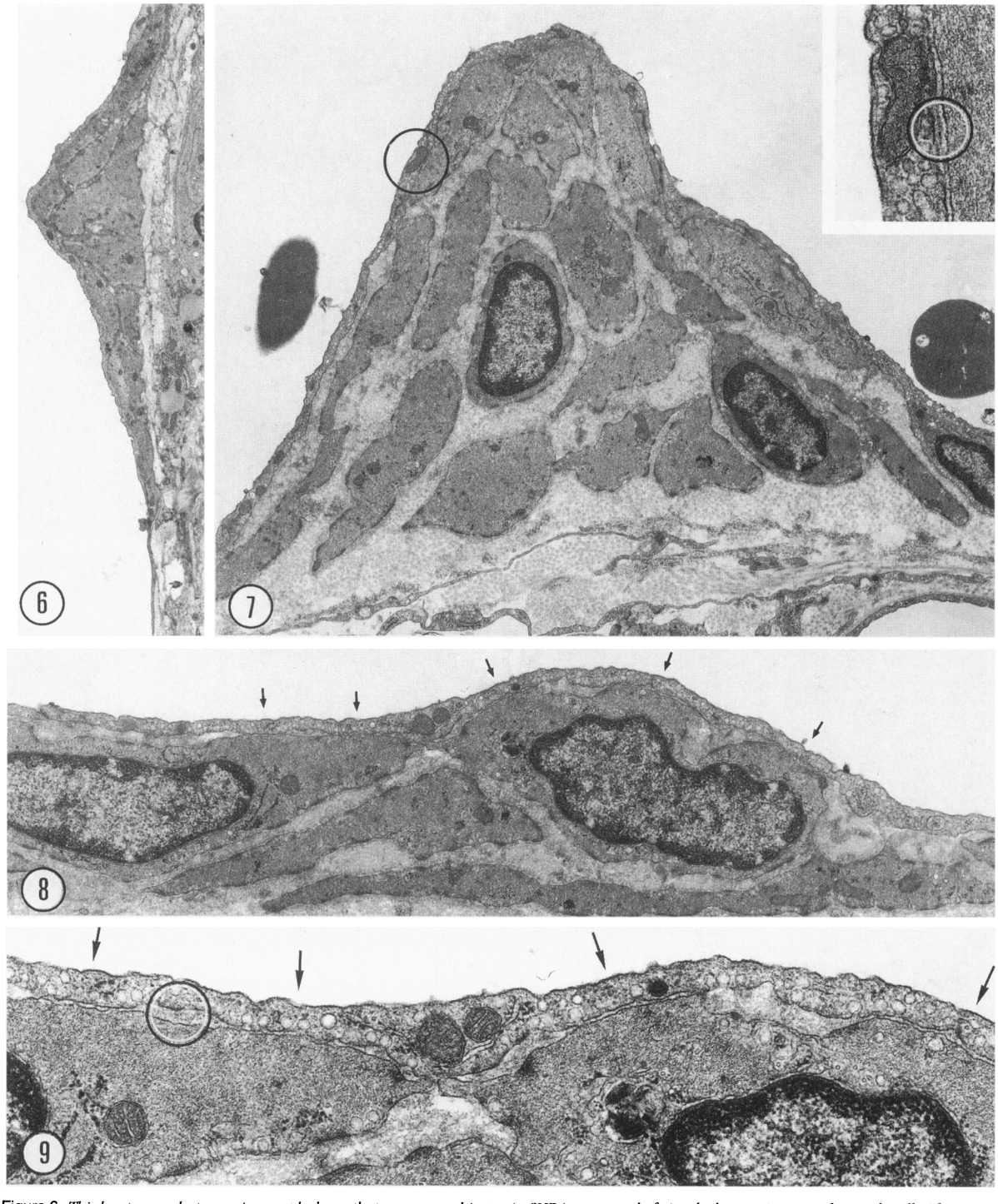
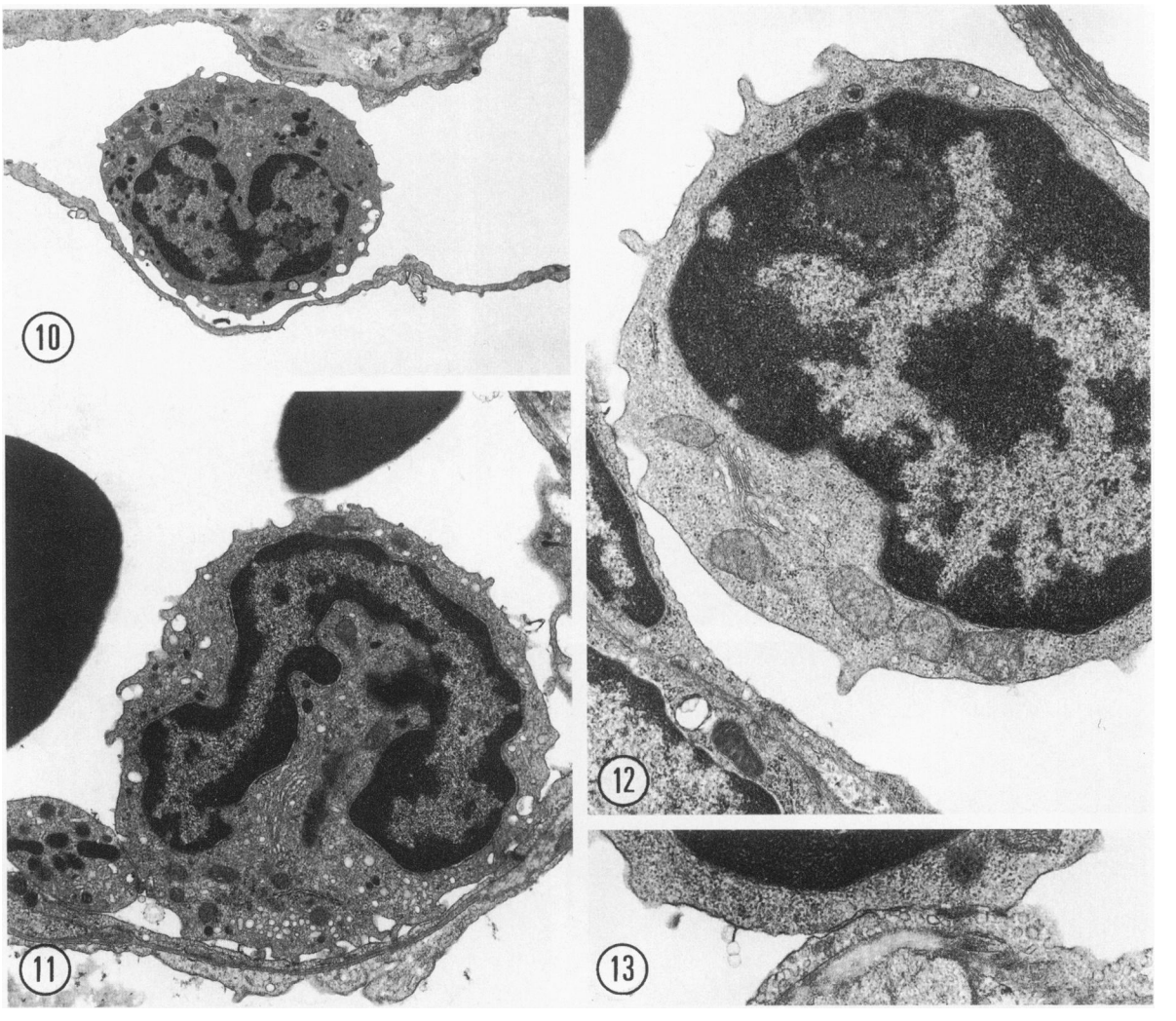


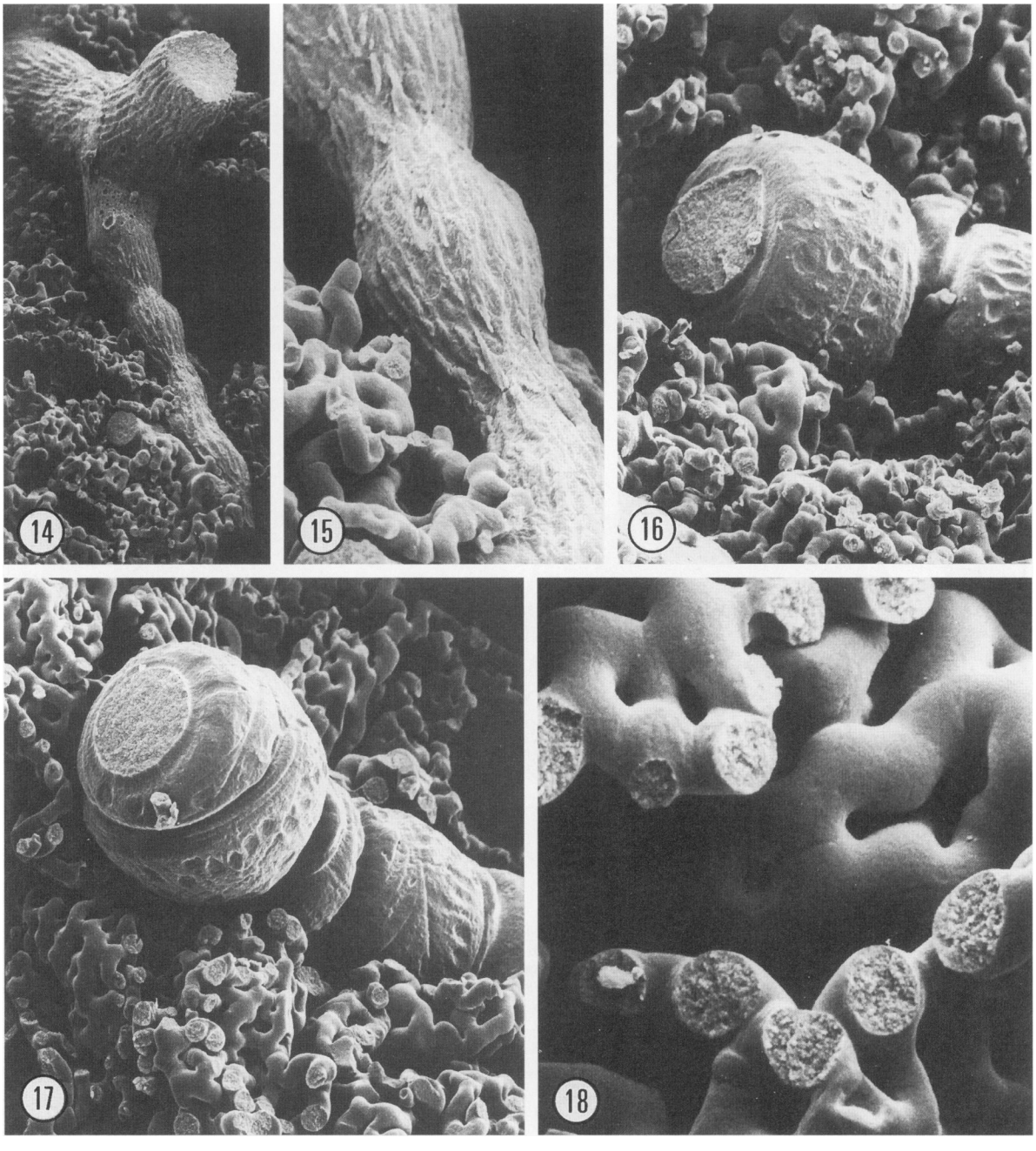
Figure 6. *This low power electron micrograph shows that a venous sphincter in SHR is composed of circularly running smooth muscle cells. The group of smooth muscle cells bulges into the lumen (left side). The sphincter is situated inside the connective tissue layers of the venous wall and is clearly demarcated. ×2800.* **Figure 7.** *This electron micrograph shows a cross-sectioned venous sphincter of an SHR with 18 smooth muscle cells. Delicate collagen fibrils lie between the smooth muscle cells. They become tightly packed toward the luminal tip of the sphincter as they approach the endothelial layer. Here smooth muscle cell membranes border against endothelial cell membranes with only remnants of the basal lamina interposed (encircled region, shown at higher magnification in the insert). ×5600.* **(Inset)** *×20,000.* **Figure 8.** *The venous smooth muscle cells often are in close contact with endothelial cells in SHR. In these regions both endothelial cells and smooth muscle cells lack a basal lamina* **(arrows)**. *SHR ×8000.* **Figure 9.** *Higher magnification of subendothelial region indicated in Figure 8* **(arrows)**. *Note close contact of endothelial and smooth muscle membranes with only small remnants of basal laminal material in between* **(encircled region)**. *An arrow marks the same region shown in Figure 8. ×20,000.*



Figures 10 to 13. *These electron micrographs show white blood cells in pulmonary capillaries in SHR. The leukocytes adhere to the endothelium either with small bands* (Figure 10) *or junctional complexes extending over a broader surface* (Figures 11 to 13). *The neutrophils may contact opposing sides of the capillary wall* (Figures 10 and 12). *Both granulocytes* (Figures 10 and 11) *and lymphocytes* (Figures 12 and 13) *are involved.* (Figure 13) *Adherence is shown between a lymphocyte and capillary endothelium at higher magnification.* Figure 10: *×2,800;* Figure 11: *×5,600;* Figure 12 : *×8000;* Figure 13: *×16,000.*

out extrinsically damaging the lung, which may make it better suited to study the biological and pharmacological aspects of clinical pulmonary hypertension.[24] Although it has histological features of human pulmonary hypertension, such as medial hypertrophy of pulmonary arteries, the arterial changes are less, and we found no plexiform or angiomatoid lesions and no fibrinoid necrosis. Venous contraction and venous hypertension have not been thought to cause primary (arterial) pulmonary hypertension in humans, but recent evidence shows that the pulmonary veins are also involved.[25] We believe the pathogenesis of human pulmonary hypertension requires a great deal more investigation. It is possible that idiopathic human pulmonary hypertension has several mechanisms and etiologies.

This model of pulmonary hypertension was first explored by McMurtry and colleagues,[2] who used 2- to 4-month-old animals and kept them at normal and low atmospheric pressure corresponding to high altitude. They found an elevated pulmonary artery pressure in SHR kept at altitude; these animals also had marked right ventricular hypertrophy and were more responsive to angiotensin II and prostaglandin $F_{2\alpha}$. The lack of a consistent changes in animals kept at low altitude may have resulted from testing younger animals because the hypertension develops as the animals age. Although we did not address the question of age, the changes we found are not expected in animals under 12 weeks old.[26] McMurtry and associates[2] found a higher hematocrit in SHR, which is in keeping with our finding of pulmonary



Figures 14 and 15. *Scanning electron microscopic images of cast pulmonary arteries show the surface of the artery in SHR is wrinkled indicating contraction.* (**Figure 14**) *The branch of the artery directed toward the bottom of the figure shows many hourglass-shaped narrowings, shown at higher magnification* (**Figure 15**). **Figure 14:** *× 140;* **Figure 15:** *× 500.* **Figures 16 and 17.** *Scanning electron microscopic images of cast pulmonary veins show deep surface constrictions in SHR. These sphincters reduce the vascular lumen to 50% of the original width. Almost round imprints of endothelial cell nuclei lie between the reliefs of smooth muscle cell furrows. ×440 for both micrographs.* **Figure 18.** *Cast alveolar capillaries in the lung of SHR were larger than those of WKY. × 1800.*

edema and cast lymphatics. Janssens and colleagues[3] found pulmonary hypertension at normoxia in 12- to 14-week-old SHR and an increased response to hypoxia. They found no difference between SHR and WKY in the medial thickness of the pulmonary arteries by light microscopy. The differences we found were primarily at the electron microscopic level. In fact, we "discovered" our light microscopic finding only after we found the changes on the scanning electron microscopy of the vascular casts.

Other investigators have explored other models of pulmonary hypertension. Peiro and coworkers[27] found vascular smooth muscle proliferation in hypertensive transgenic rats, which are similar to SHR.

Others have used the fawn-hooded rat as a model of spontaneous pulmonary hypertension.[28] Although its defect is different, it also is a useful model.[29]

Aharinejad and Böck[13] showed that focal tufts of smooth muscle increased in the veins of SHR. This study examined more closely the role of these sphincters in pulmonary hypertension. The surprising finding was the magnitude of their constriction. Pulmonary veins normally constrict little, ~5% of their diameter, depending on the age and sex of the animal.[30] With head injury severe enough to cause pulmonary edema, the sphincters contract more, but generally <10%.[7] For SHR in this study the average maximal venous constriction without any stimulus was >50%. We believe this could account in part for the pulmonary hypertension in these animals. It could create resistance and be a stimulus for arterial contraction and hypertrophy that was present. Contraction of small veins would increase the tension upstream and cause bulging of capillaries, which was shown on light microscopy as capillary congestion. The finding of edema also correlates with the idea that the lesion is in the veins, which may constrict and cause capillary transudation. Another mechanism to explain the increased capillary diameter in SHR could be the increased intravascular leukocytes. These leukocytes adhered to the vascular walls in SHR after vascular rinsing, perfusion fixation, and resin instillation, showing the tightness of their attachment. We have seen with intravital microscopy that adhering leukocytes reduce alveolar blood flow. Schmid-Schönbein and associates[31] proposed that hypertension-induced organ damage in SHR might be related to an elevated blood leukocyte count and more spontaneously activated granulocytes circulating in these rats compared with WKY. Arndt and coworkers[32] found that leukocyte-endothelial cell adhesion is higher in the lung but lower in the mesentery of SHR compared with normotensive rats. Increased intravascular macrophages have been found in the pulmonary arteries of rats with cirrhosis induced by bile duct ligation but pulmonary hypertension was not found in that model.[33]

The correlation of increased pulmonary blood pressure and hypertrophic venous sphincters supports the assumption that their contraction has functional significance. Faint contraction would reduce local capillary blood flow and increase time for oxygenation. Deeper contraction could route blood away from the contracting segment. If venous contraction were caused by a decreased oxygen content of its blood either through nitric oxide[34] or endothelin,[12] a method of precise matching of ventilation and perfusion is evident. Plasma levels of endothelin and its production in the lung are increased in spontaneously hypertensive rats.[28,35]

Sato and colleagues[29] found the weight of the right ventricle divided by the weight of the left ventricle, and septum was 0.27 in 6-week-old control rats and 0.46 in fawn-hooded rats at the altitude of Denver, CO, but stayed near normal, 0.29, if the animals were kept in a 25% oxygen environment. The ratio of the weight of the right ventricle to left ventricle and septum in our hypertensive animals was less than normal, indicating greater hypertrophy of the left ventricle. Although the pressure of the pulmonary circulation rose proportionately more than the systemic circulation, it did not reach the high levels of the systemic circulation. It appears that the high pressures required by the left ventricle are a greater stimulus for hypertrophy.

Although hypertrophy of the pulmonary arteries was present, the veins had proportionally greater muscularization. Zhao and colleagues[36] found that veins contract more than arteries to hypoxia in rats. With this model of pulmonary hypertension it may be possible to test vasoactive drugs that could reverse the hypertrophic process. In addition, the contribution of venous muscle contraction to hypertension may be underestimated and is likely to reverse with vasodilators.

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