Scanning Microscopy

Volume 6 | Number 3 Article 21

8-20-1992

# Appearance of Venous Sphincters in the Pulmonary Microvascular Bed of Normotensive and Spontaneously Hypertensive Rats

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Scanning Microscopy, Vol. 6, No. 3, 1992 (Pages 865-875) 0891-7035/92$5.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

# APPEARANCE OF VENOUS SPHINCTERS IN THE PULMONARY MICROVASCULAR BED OF NORMOTENSIVE AND SPONTANEOUSLY HYPERTENSIVE RATS

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(Received for publication February 8, 1992, and in revised form August 20, 1992)

## Abstract

The appearance of pulmonary venous sphincters was studied in normotensive and spontaneously hypertensive rats using scanning electron microscopy of microvascular corrosion casts and transmission electron microscopy of tissue sections. Vascular casts were prepared either after lavage with Tyrode solution or after glutaraldehyde prefixation. Pronounced pulmonary venous sphincters were more frequently identified in spontaneously hypertensive rats as compared to corresponding circular indentations in normotensive rats. Tissue sections established venous sphincters in hypertensive animals as consisting of multiple layers of smooth muscle cells in the venous walls. We did not observe any autonomic nerve terminals in close proximity to these bundles of smooth muscle cells. The effect of various casting procedures on the appearance of venous sphincters is discussed. It is concluded that glutaraldehyde prefixation is an appropriate method to demonstrate sphincter functioning, because it causes deepening of sphincter indentations. Pulmonary vascular sphincters are thought to be governed by blood-born substances, vasoactive metabolites, or by tension of oxygen and carbon dioxide. Venous sphincters may influence microvascular flow in general and probably substitute for venous valves in the pulmonary vascular bed where valves are missing.

**Key words**: Pulmonary venous sphincters, spontaneously hypertensive rats, normotensive rats, corrosion casting, Mercox, scanning electron microscopy, transmission electron microscopy.

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## Introduction

Venous valves (Morris and Bevan, 1984) and arterial cushions (Fourman and Moffat, 1961) are well known regulatory devices of blood flow in the circulatory system. Recently, venous sphincters have been described as novel devices being involved in blood flow regulation (in the rat exocrine pancreas: Aharinejad *et al.*, 1990; and in the rat pulmonary vasculature: Schraufnagel and Schmid, 1988a, b; Schraufnagel and Patel, 1990; Aharinejad *et al.*, 1991, 1992), using scanning electron microscopy of vascular corrosion casts. Their involvement in blood flow regulation seems now to be established (Aharinejad *et al.*, 1990, 1991, 1992), particularly in the pulmonary microvascular bed which is evidently devoid of venous valves (von Hayek, 1970; Schraufnagel, 1990). Schraufnagel and Patel (1990) concluded that pulmonary venous sphincters are under neural control, whereas Aharinejad *et al.* (1991, 1992) felt that hormones and locally acting agents govern their function.

In spontaneously hypertensive rats systemic blood pressure increases up to 184 mm Hg during the first three postnatal months (Chen *et al.*, 1991), a genetically determined phenomenon that is accompanied by hypertrophy of virtually all peripheral arteries (Amenta *et al.*, 1991). Surprisingly, pulmonary arterial pressure remains normal (19 mm Hg) in adult spontaneously hypertensive rats (Chen *et al.*, 1991), although morphological findings indicate progressive hypertrophy of smooth muscle cells also in the tunica media of pulmonary arteries (Amenta *et al.*, 1991). When assuming that such morphological changes in arterial walls are genetically determined (Okamoto *et al.*, 1973), it would be of interest to prove whether venous wall structures are also hypertrophic, in particular sphincters of small pulmonary veins. Therefore, we studied pulmonary venous sphincters in spontaneously hypertensive rats to examine their probable morphological alterations. Different preparation protocols were performed in order to get additional information on the possible influence of preparation procedures on the casts surface structure. Scanning electron microscopy of vascular corrosion casts in

combination with transmission electron microscopy of tissue sections was felt to be optimum for these studies.

## Material and Methods

### Cast preparation

**Spontaneously hypertensive rats.** Animals of both sexes (SHR-NCrlBR, Charles River Wiga, Sulzfeld, Germany), weighing 200 to 220 g, were randomly divided in two groups consisting of ten rats each.

### Group one, Tyrode perfusion

In group one, rats were anesthetized by intraperitoneal injection of sodium pentobarbital, 40 mg/kg body weight. The abdomen and thorax were opened by a median cut and the abdominal aorta and caudal vena cava were cannulated at the level of renal vessels. The cannula (Argyle 0.8 x 19 mm, Sherwood Medical, St. Louis, MO, USA) was attached to a two-way connector (LS-2, B. Braun-Melsungen, Germany) to allow removing of air bubbles. The pulmonary circulatory system was rinsed by injection of 42 °C warmed heparinized (5,000 IU/l) Tyrode solution into the caval catheter until the efflux of the cannulated abdominal aorta was clear.

### Group two, Tyrode perfusion and fixation with glutaraldehyde

In group 2, animals were treated as described for animals of group 1, however, the pulmonary system was additionally perfused with 2.5% glutaraldehyde (in 0.1 M phosphate buffer) after Tyrode lavage.

### Casting in groups one and two

Following the above steps, animals of both groups were injected with 16 ml Mercox CL-2B (Dainippon Ink & Chemicals, Tokyo, Japan) diluted with 4 ml monomeric methylmethacrylate (Hodde, 1981), through the caudal vena cava using manual pressure. The perfusion pressure was not measured because the pressure measured at the injection site is not equal to the microvascular pressure (Lametschwandtner *et al.*, 1990). Animals bodies were left at room temperature for 2 hours and in a 60 °C water bath overnight to allow polymerization of the resin. Lungs were removed and macerated in a 15% KOH-solution at 40 °C for two days or longer. Thereafter, specimens were rinsed in 5% formic acid for 15 minutes and in several passages of distilled water and then frozen in the latter. All specimens were cut at -20 °C into 5 mm thick slices with a specially adapted circular saw and freeze-dried. Five slices were mounted onto copper foils with silver paste, using the conductive bridge method of Lametschwandtner *et al.* (1980), evaporated with carbon for 3 seconds and sputtered with gold for 600 seconds (Aharinejad *et al.*, 1990). Examination was performed with a Cambridge Stereoscan 90B SEM at an accelerating voltage of 10 kV.

**Normotensive rats.** Sprague Dawley rats of both sexes, weighing 200-250 g, were randomly divided in two groups consisting of ten rats each. The same perfusion (Tyrode and Tyrode/glutaraldehyde) and casting procedures described above were applied to each group.

In cast preparations, depth of sphincter constrictions was compared by viewing micrographs of different treatment groups. The depth was not measured on those veins without fractures because in case of drastic constrictions the deepest point of casts surface often can not be seen, even when tilting the cast. Our values of the constrictions depth are based on observations of fractured venous casts, where the narrowing is clearly seen as circumference of the fracture surface (Aharinejad *et al.*, 1992). The analysis of micrographs was performed before the observer learned which micrograph belonged to which treatment group. The entire surface of all cut corrosion casts was analyzed, all veins exposed in these sections were photographed without regard to the presence or absence of sphincter-like structures. The value of constriction depth was evaluated comparing the outer and inner diameter of the fractured venous cast at the site of venous sphincter constrictions according to Schraufnagel and Patel (1990).

For isolation of pulmonary venous casts, the cut surface of lung specimens was analyzed under the dissection microscope. When pronounced circular indentations were identified on venous casts surface, the adhering alveolar capillaries were removed with a fine needle and an entire length of approximately 3-5 mm was cut and mounted as described above.

### Tissue sectioning

Five normotensive and five spontaneously hypertensive rats of the same weight category described above were used. The animals were fixed by vascular perfusion with 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH = 7.2. Small tissue blocks were cut with razor blades and immersion fixed in the same fixative for additional 2 hours. Thereafter, the specimens were washed in phosphate buffer and postfixed in 1% osmium tetroxide in Michaelis buffer, pH = 7.2, for 2 hours at 4 °C. Dehydration was done in a series of graded ethanol solutions with increasing concentrations of ethanol, and finally in propylene oxide. Embedding was performed in Epon 812. Semi-thin sections were cut on a Reichert OmU2 ultramicrotome and stained with alkaline Toluidine Blue O, ultrathin sections were double stained with methanolic uranyl acetate and alkaline lead citrate. Tissue sections were studied with a Zeiss EM 9-S2 electron microscope.

In each animal 2 tissue blocks were prepared from each lobe on both sides, peripheral and central areas of

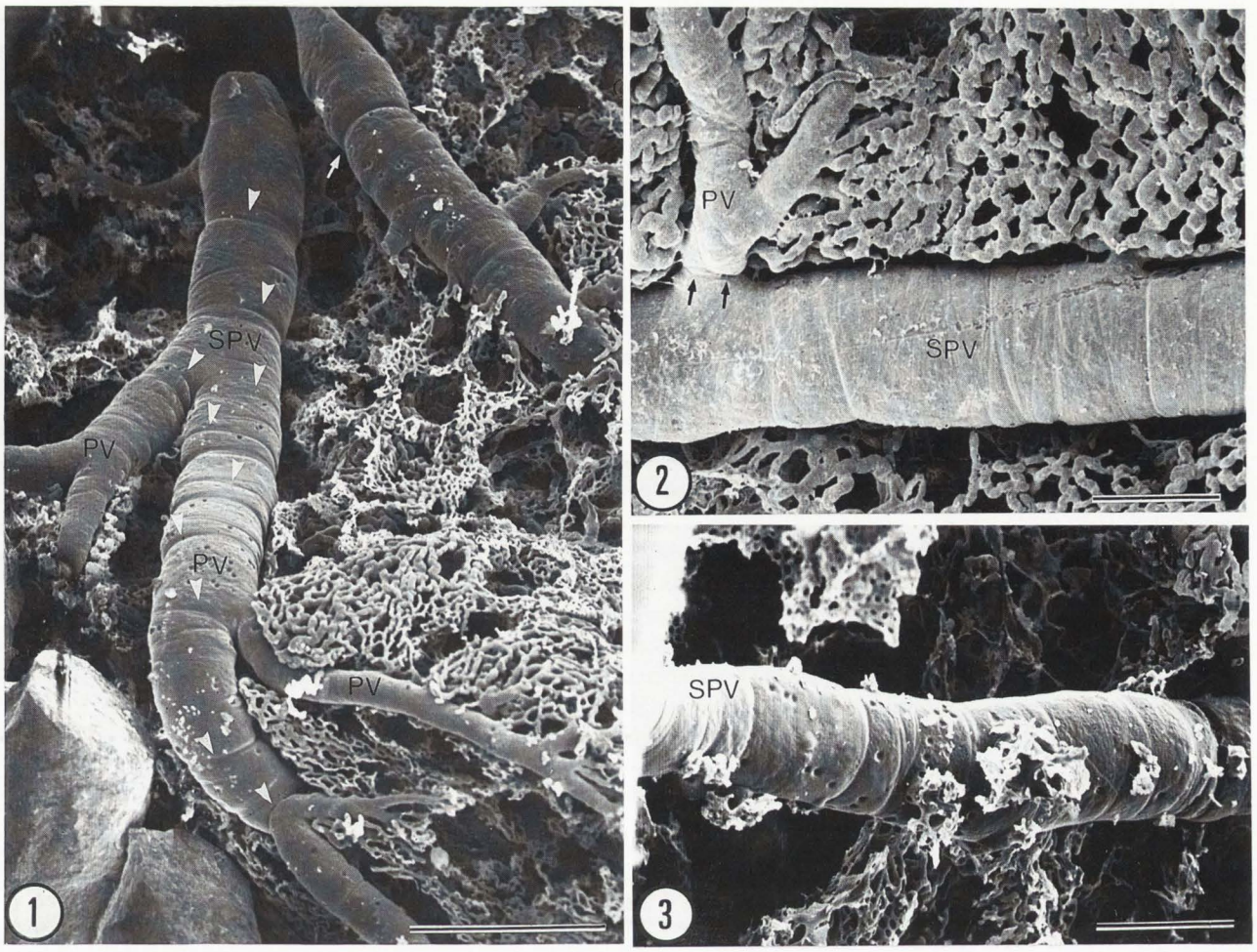


**Fig. 1.** Low magnification scanning electron micrograph of pulmonary venules (PV) forming a small pulmonary vein (SPV). Multiple shallow constrictions are indicated by arrowheads, a deeper constriction is marked by arrows. Normotensive rat perfused with Tyrode solution. Bar = 250 $\mu$m.

**Fig. 2.** Scanning electron micrograph shows a deeper constriction (arrows) at the joining site of a pulmonary venule (PV) into a small pulmonary vein (SPV) at higher magnification. Furrows on the surface of the small pulmonary vein run both in an oblique or circular manner. Alveolar capillaries appear inconspicuous. Normotensive rat perfused with Tyrode solution. Bar = 100 $\mu$m.

**Fig. 3.** Scanning electron micrograph of a small pulmonary vein (SPV). Note numerous deep circular constrictions on the casts surface. Normotensive rat perfused with Tyrode solution and glutaraldehyde. Bar = 100 $\mu$m.

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the parenchyma were used. Venous sphincters could be observed in each animal. Before analyzing ultrathin sections, a semi-thin section was investigated from each block. The block was consecutively cut (every other 20 $\mu$m) for TEM in case of sphincter occurrence. At least 10 ultrathin sections were analyzed from each block.

## Results

### Corrosion casting

**Experimental protocol 1, normotensive rats.** Corrosion casts of the normotensive rats pulmonary vascular bed regularly show constrictions on the surface of veins (pulmonary venules: 45-135 $\mu$m diameter, small pulmonary veins: 135 $\mu$m or larger in diameter). These circular furrows are often at regular intervals (Fig. 1). In particular, where pulmonary venules empty into small pulmonary veins, the constrictions are remarkably deep, reducing the luminal diameter to about 70% the original width (Fig. 2). The alveolar capillary network is inconspicuous, well perfused, and shows no surface indentations or constrictions (Fig. 2).

**Experimental protocol 2, normotensive rats.** Casting after prefixation with glutaraldehyde resulted in

also well perfused vessels, strikingly deepened sphincter constrictions were regularly present in the venous vascular bed (Fig. 3).

**Experimental protocol 1, spontaneously hypertensive rats.** In this group, the venous sphincters were also present as observed in normotensive rats. The number and distribution of sphincter constrictions appeared to be well comparable to the situation described for normotensive rats, while the frequency of pronounced constrictions was higher (Figs. 4, 5).

**Experimental protocol 2, spontaneously hypertensive rats.** Luminal constrictions were seen not only on pulmonary venules but also on artery casts (Fig. 6). The shape of arterial constrictions differs from venous sphincter indentations as they show continuous transition of non-constricted areas toward the constricted luminal segments. In other words, sharply demarcated circular constrictions, as seen on venous casts surfaces, are missing. Additional signs of constriction on arterial casts are wrinkled endothelial cell nuclei indentations and corrugated casts surface, besides of reduced luminal diameter (Fig. 6). Venous sphincter constrictions in spontaneously hypertensive rats were striking. They were observed both on isolated casts (Fig. 7) as well as on small pulmonary veins in situ (Fig. 8). The depth of sphincter indentations in hypertensive rats after glutaraldehyde fixation was remarkable, often narrowing down the venous lumen to about 50% the original width (Fig. 8).

### Tissue sectioning

The wall of small pulmonary veins in normotensive rats merely consists of an endothelial lining, its basal lamina and a delicate layer of connective tissue. In addition, pericytes are loosely interspersed subjacent to the endothelium. These pericytes, however, do not form a continuous layer. Along the course of the veins, circularly running smooth muscle cells abruptly occur which are arranged either solitarily or in groups (Fig. 9). The occurrence of smooth muscle cells most probably correlates to the furrows seen on corrosion casts surface. Although intensive search was performed to detect axonal profiles near to these sphincters, we could not observe any of them.

Electron microscopic observation of pulmonary veins in spontaneously hypertensive rats also revealed the presence of abruptly occurring bundles of smooth muscle cells (Fig. 10). These contractile bundles are either well defined and less extended or they occur over longer distances. In any case the contractile elements are located inside the veins elastic lamina. In hypertensive rats, the sphincters were often seen to be composed of multiple layers of smooth muscle cells, up to four layers could be observed (Fig. 11). Smooth muscle cells of the innermost layer regularly formed myo-endothelial junctions (Fig. 11). Axonal varicosities were never found in close proximity to the sphincters smooth muscle cells.

**Fig. 4.** Confluence of two pulmonary venules (PV) forming a small pulmonary vein (SPV). Constrictions of varying depth are indented into the pulmonary venules cast (arrows). Arrowheads mark the relief of endothelial cell nuclei. Spontaneously hypertensive rat perfused with Tyrode solution. Bar = 50 $\mu$m.

**Fig. 5.** Higher magnification of lower pulmonary venule shown in Fig. 4. Note the deep sphincter relief (white arrowheads) and endothelial cell nuclei imprints (black arrowheads). Spontaneously hypertensive rat perfused with Tyrode solution. Bar = 25 $\mu$m.

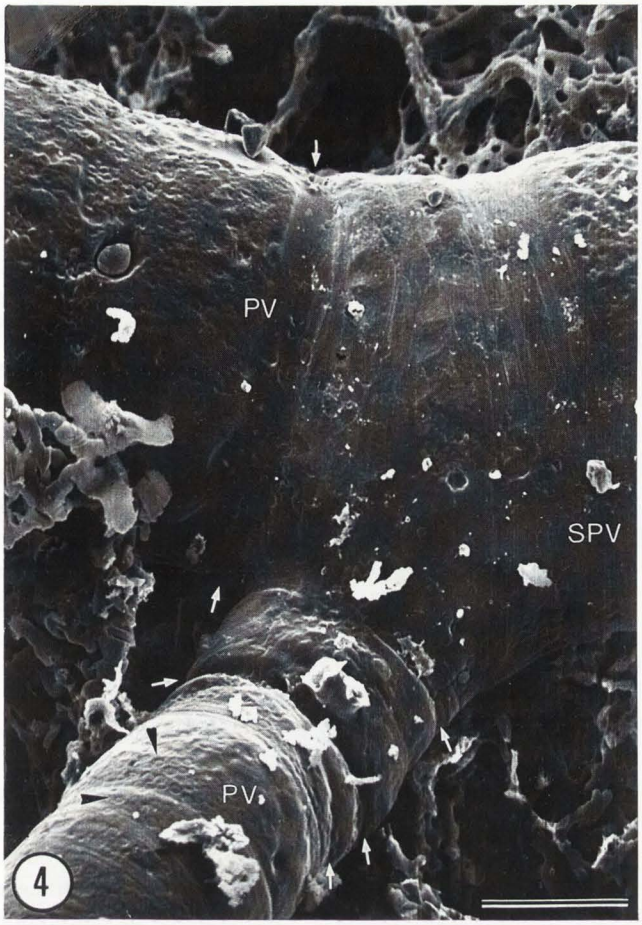
**Fig. 6.** Corrosion cast of a pulmonary artery narrowed by smooth muscle constriction (arrows). Note multiple longitudinally arranged parallel folds in areas where the artery is narrowed. These structures are caused by folded endothelium. Spontaneously hypertensive rat perfused with Tyrode solution and glutaraldehyde. Bar = 100 $\mu$m.

**Fig. 7.** High magnification scanning electron micrograph of an isolated small pulmonary vein. Note deeper constrictions indented into the casts surface. Spontaneously hypertensive rat perfused with Tyrode solution and glutaraldehyde. Bar = 100 $\mu$m.

**Fig. 8.** High magnification scanning electron micrograph of a small pulmonary vein (SPV) joined by pulmonary venules (PV). Remarkably deep constrictions are seen on the casts surface (arrows; see Fig. 2). Arrowheads indicate reliefs of erythrocytes. Spontaneously hypertensive rat perfused with Tyrode solution and glutaraldehyde. Bar = 50 $\mu$m.

## Discussion

Indentations and circular crests were observed in casts of small pulmonary veins, both in normotensive and spontaneously hypertensive rats. Our previous studies have shown the presence of venous sphincter crests on casts surface of rat pulmonary veins (Aharinejad *et al.*, 1991) without any special pre-treatment, whereas Schraufnagel and Patel (1990) favored the importance of a neurogenic stimulus for the demonstration of venous sphincters in pulmonary corrosion casts (application of a sharp head blow after resin injection). The present results substantiate the view that such a treatment is not mandatory in case of normotensive rats (Aharinejad *et al.*, 1991), and the same holds true for spontaneously hypertensive rats. The localization of sphincter smooth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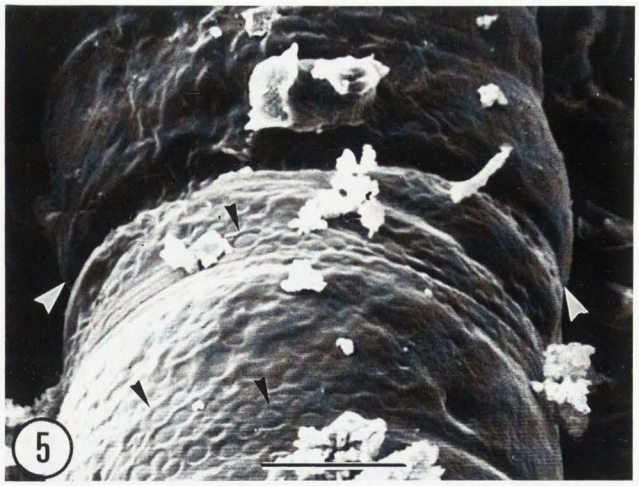
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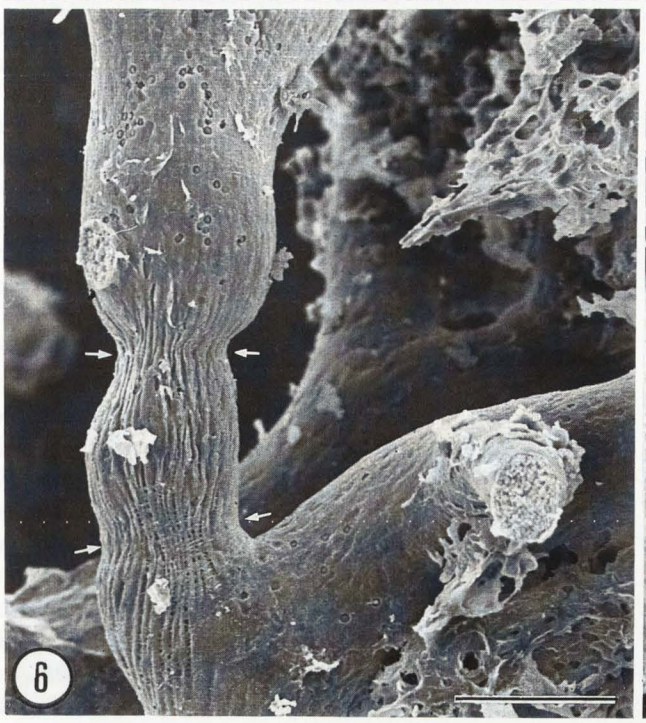
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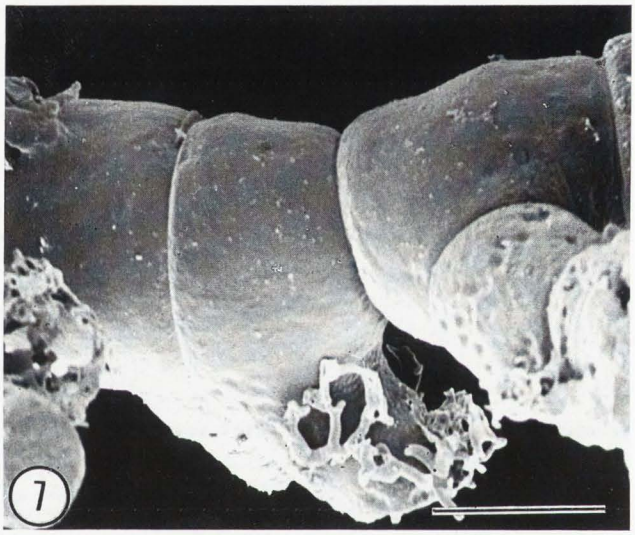
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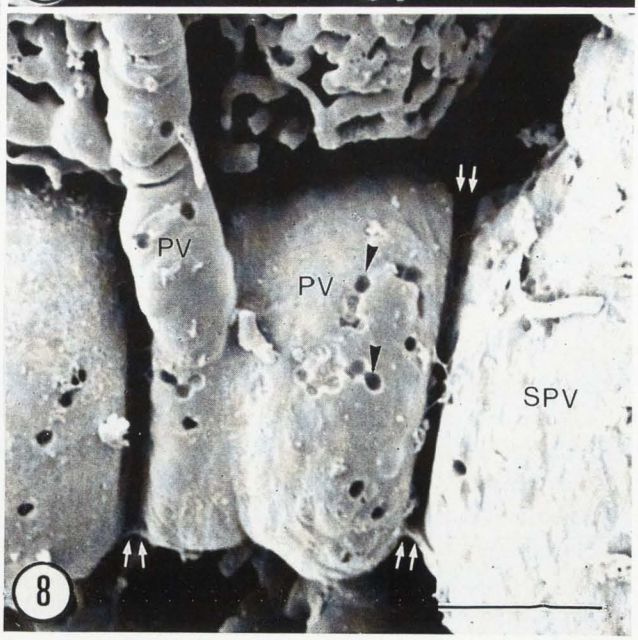
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muscle cells inside the elastic lamina that defines the boundary between tunica media and adventitia, assigns these cells as belonging either to the tunica intima or media. A clear demarcation of the latter two layers is not easily done in small veins, as a distinct inner elastic lamina is missing. However, it should be stressed that smooth muscle cells of the sphincters next to the endothelium form far-reaching myo-endothelial junctions (a finding which has been already noted by previous investigators: Rhodin, 1978; Aharinejad *et al.*, 1992), where basal lamina material or connective tissue fibers are not interposed. Therefore, we tend to interpret the sphincters as belonging to the intimal layer, although we do not insist on such a classification. The sphincter regions may well be categorized as regions where a clear separation of tunica intima and media is not possible, similar to that situation described for intra-arterial cushions (Gorgas and Böck, 1975). Circular bundles of smooth muscle cells in venous walls which we interpret as sphincters may well correspond to the so-called "small cushions" described by De Almeida *et al.* (1975) in the rat small pulmonary veins. These cushions were subendothelially located accumulations of smooth muscle cells which pushed the endothelium toward the lumen. In addition, in the media of the rat larger pulmonary veins cardiac muscle fibers occur which extend until the vessels attain about 100 $\mu$m in diameter (De Almeida *et al.*, 1975). Kay (1983) reported on a medial coat of cardiac muscle in the rat intrapulmonary veins exceeding 80 $\mu$m in diameter. In such veins, it may be difficult to envisage the detection of venous sphincters, i.e., to deduce whether corrosion casts constrictions are caused by smooth muscle cells or by cardiac muscles. According to our experiences, however, cardiac muscle fibers occur only in larger pulmonary veins near to the lung hilum. In tissue sections of the pulmonary veins obtained from the central lobular areas, we did not observe cardiac muscle fibers. The combination of corrosion casting and tissue sectioning now enables us to obtain three-dimensional views and information on the functioning of smooth muscle bundles, as well as to correlate different pulmonary vessel types with corresponding cast specimens, so allowing the interpretation of smooth muscle cells accumulation as venous sphincters.

Smooth muscle cells of venous sphincters in normotensive rats appeared to be devoid of nervous supply (Aharinejad *et al.*, 1991, 1992). In hypertensive rats, the sphincters were identified as striking bundles of smooth muscle cells which do not show spatial relationship to vascular nerves. Although axonal varicosities are absent, the well developed venous sphincters in spontaneously hypertensive rats cause remarkably deep surface indentations, already under standard casting conditions. These observations suggest that the depth and

**Fig. 9.** Low magnification transmission electron micrograph of a cross-sectioned venous sphincter, consisting of grouped smooth muscle cells (S) running parallel to each other. Smooth muscle cells are inside the elastic lamina (E) and cause folding of the endothelial lining. Normotensive rat, glutaraldehyde fixation. Bar = 4 $\mu$m.

**Fig. 10.** Low magnification transmission electron micrograph showing an abruptly occurring sphincter, consisting of grouped true smooth muscle cells (S) inside the elastic lamina (E) of a small pulmonary vein. Spontaneously hypertensive rat, glutaraldehyde fixation. Bar = 4 $\mu$m.

**Fig. 11.** Higher magnification of a complex venous sphincter consisting of four layers of smooth muscle cells (1-4). Note the folded endothelial lining atop the smooth muscle cells, protruding into the lumen. Axonal varicosities in close relationship to the sphincter are missing. Arrowhead marks a myo-endothelial junction. Spontaneously hypertensive rat, glutaraldehyde fixation. Bar = 2 $\mu$m.

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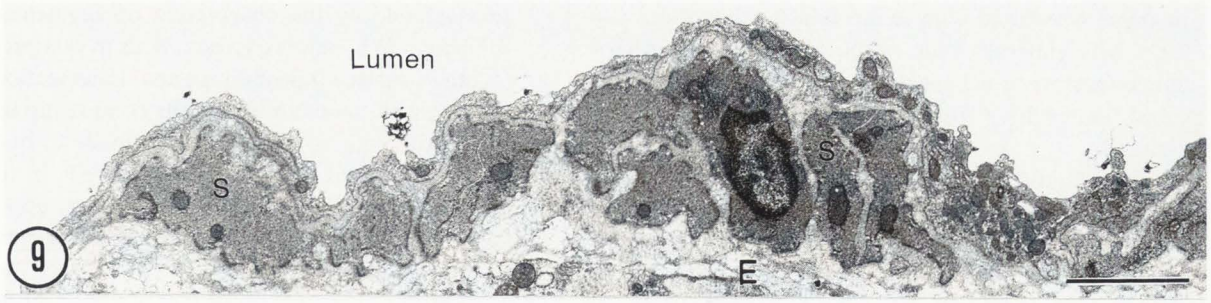
orientation of surface constrictions on cast preparations are related to position and size of smooth muscle bundles only, independent of a neurogenic stimulation of the latter.

In this study two different preparation methods were used, which allow to compare the effect of either monomeric methylmethacrylate (MMA) or glutaraldehyde combined with MMA on the functioning of vascular smooth muscle cells. After prefixation with glutaraldehyde the resin contacts vascular walls with smooth muscle cells which are no longer capable to contract, the shape of the resulting cast preparation reflects the situation of the fixed vascular wall structures. However, one should be well aware that glutaraldehyde per se may stimulate contractile cells. When injecting the resin into unfixed vessels, the capability of smooth muscle cells to contract is sustained over a certain period of time after contact with MMA. Our results during the present study in particular, as well as those of previous studies in general (Aharinejad *et al.*, 1991), show a deepening effect of glutaraldehyde prefixation on venous sphincter crests. It should be considered, however, that glutaraldehyde fixation of smooth muscle cells not only prevents further constriction of smooth muscle cells but also prevents relaxation of constricted smooth muscle cells. Therefore, glutaraldehyde prefixation and consecutive casting is the method of choice to demonstrate sphincter structures because only contracted sphincters can be readily identified on casts surface. Concerning the effect of glutaraldehyde on sphincter smooth muscle cells, our findings are in contrast to those of Schraufnagel and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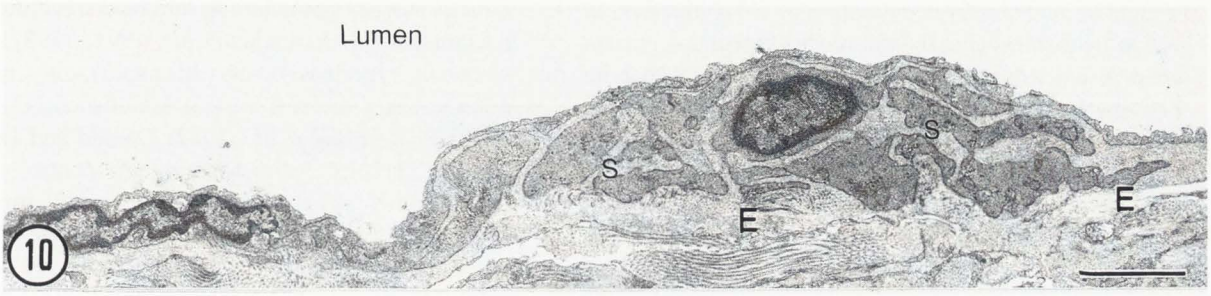
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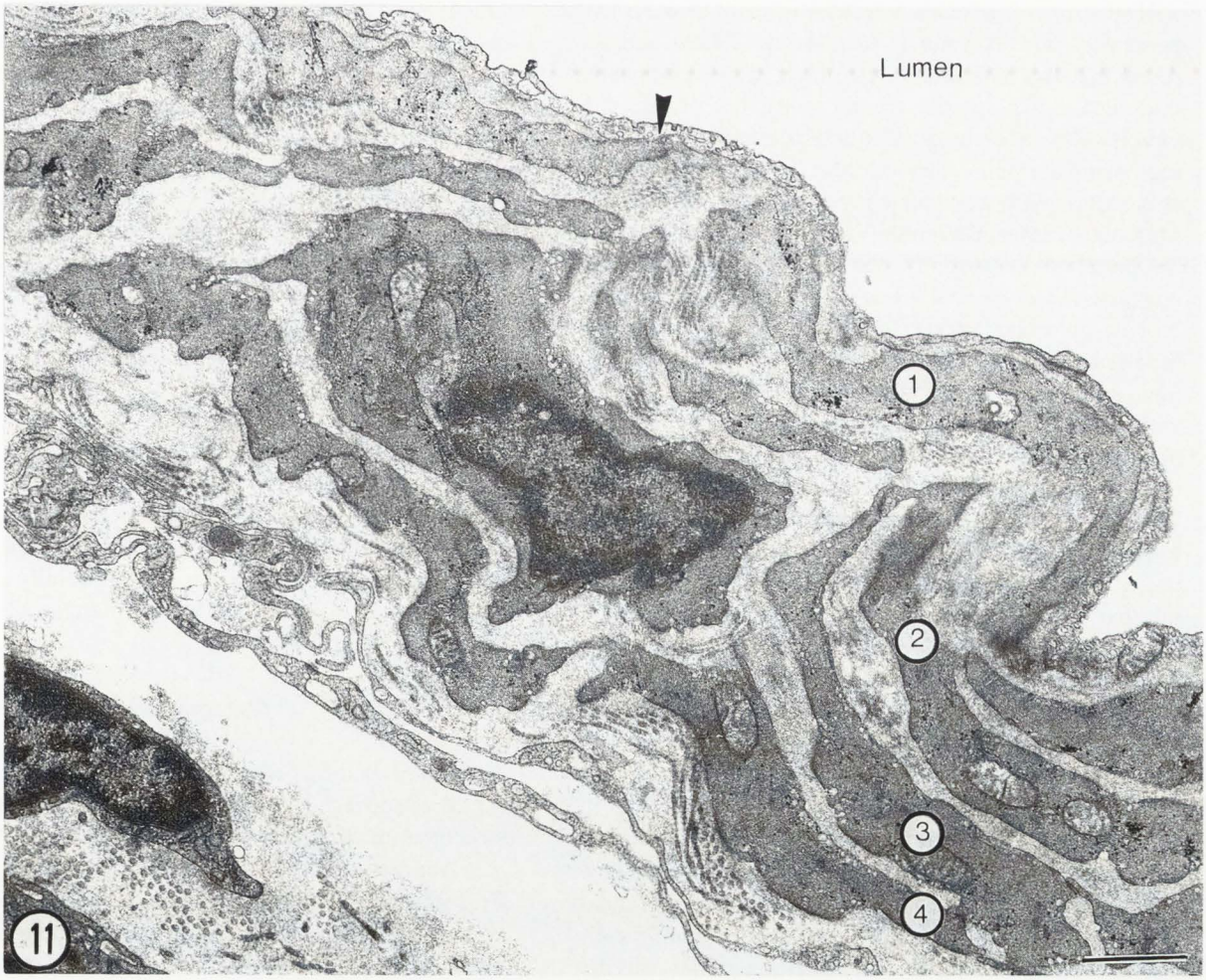
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Schmid (1988a), who found the sphincters to be deeper in animals perfused with saline solution than those perfused with glutaraldehyde. Probably, other parameters, unknown to us or not considered as yet, may have influenced the results. This matter deserves further investigations.

The interpretation of sphincter constrictions on vascular casts basically remains a problem. Sphincter constrictions on luminal casts appear as differences in luminal diameter between sphincter region and preceding and following vascular segments. Such differences can be brought about by active contraction of the sphincters, as well as by distension of the interposed vascular segments whereby the sphincters hold passive resistance, or both these mechanisms may be involved.

The observation of remarkably narrowed arterial lumen in hypertensive rats (Fig. 6) becomes of particular interest. Narrowing of arteries might well reflect the *in vivo* situation in hypertensive rats and might be stabilized by glutaraldehyde fixation. Such constrictions of arterial casts may mimic the histologically found changes in the wall structure of pulmonary arteries in spontaneously hypertensive rats (Amenta *et al.*, 1991). These authors found that the medial muscular layer was significantly thickened in hypertensive rats by hypertrophy of smooth muscle cells. Meyrick *et al.* (1978) described four main regions of the pulmonary arteries: **1.** Muscular arteries at the hilum with a complete layer of circular muscle; **2.** Muscular arteries, decreasing in caliber, continue toward the periphery in partially muscular arteries where the complete muscular coat gives way to a spiral of muscle; **3.** Non-muscular arteries in the periphery, where the muscle totally disappears; and **4.** Arteries with the oblique muscle segment are those vessels within the region with a complete muscle coat where the muscle fibers run obliquely. It should be considered that luminal constrictions on pulmonary artery casts may correspond to the thick walled oblique muscle segments described by Meyrick *et al.* (1978) in normotensive rats. Hypertrophy of pulmonary arteries in spontaneously hypertensive rats has been interpreted as an adaptation mechanism against "hypertension" (Amenta *et al.*, 1991). However, it is a discrepancy between the obviously normal pulmonary pressure in spontaneously hypertensive rats (19 mm Hg; Chen *et al.*, 1991), and the hypertrophy of arterial smooth muscle cells as evidenced by Amenta *et al.* (1991). Accepting that Amenta *et al.* (1991) referred to hypertension of systemic vascular system and not to the pulmonary circulation, they did not precisely state how this hypertension may influence the pulmonary circulation. In our view, hypertrophy of smooth muscle cells and thickening of arterial wall structures is not causally related to blood pressure of the pulmonary circulatory system but may rather reflect a genetic peculiarity of spontaneously hypertensive rats. This assumption is substantiated by the observation of augmented venous sphincters in hypertensive rats, both in cast preparations (depth of surface indentations) and tissue sections (multiple layers of smooth muscle cells comprising the sphincter). Although no attempts were made to quantify the number and size of smooth muscle cells in tissue sections, on a first view, venous sphincters appear better developed in spontaneously hypertensive rats than in normotensive rats.

Our TEM results show that nervous supply of pulmonary venous sphincters should be disregarded, both in normotensive (Aharinejad *et al.*, 1991, 1992) and spontaneously hypertensive rats (this paper), only innervation of pulmonary arteries is well established (El-Bermani, 1978; El-Bermani *et al.*, 1982; Donald and Lillywhite, 1988). Therefore, direct neurogenic control of sphincters becomes rather unlikely. This view supports the hypothesis that pulmonary venous sphincters are governed by blood-born or endothelium-derived substances (Matsuse *et al.*, 1990; McCormack, 1990; Cremona *et al.*, 1991; Hemsen, 1991). Moreover, the possibility should be stressed that the sphincters are influenced by vasoactive metabolites released from neighboring tissues, e.g., adenosine nucleotides, as well as by oxygen and carbon dioxide tension.

Evidently the pulmonary vascular bed is devoid of venous valves (Schraufnagel, 1990). In accordance with Schraufnagel (1990), we interpret pulmonary venous sphincters as substitutes of venous valves. This hypothesis becomes plausible if it is considered that as yet venous sphincters have been only described in organs where venous valves are missing (e.g., pancreas; Aharinejad *et al.*, 1990).

## Acknowledgments

The authors wish to express their special thanks to Mr. Helmut Oslansky and Mr. Richard Reichhart for their photographic assistance. We gratefully appreciate the financial support of "Anton Dreher-Gedächtnisschenkung, Nr. 173/90".

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## Discussion with Reviewers

**J.M. Kay**: The results are inconsistent with published accounts of the anatomy of pulmonary veins in rats. Pulmonary venules are intra-acinar vessels less than 20 $\mu$m in diameter and are identical in structure with arterioles. Their walls consist of endothelium and single elastic lamina. The small pulmonary veins measure less than 80 $\mu$m in diameter. They consist of an adventitia of loose collagen fibers, a single elastic lamina and an intima composed of fibromuscular pads covered by endothelium. Presumably, these intimal fibromuscular pads correspond to the sphincters identified by scanning electron microscopy.

The large pulmonary veins exceeding 80 $\mu$m in diameter have a fibrous adventitial coat and a media composed of striated muscle continuous with the cardiac muscle which extends from the left atrium to ensheath the major pulmonary veins. This medial coat of cardiac muscle consists of an external longitudinal layer and an internal circular layer. It is difficult to envisage the detection of sphincters in pulmonary veins measuring

more than 80 $\mu$m in diameter using SEM of casts, when the tunica media of such vessels has a thick layer of cardiac muscle. The venous sphincters illustrated by Schraufnagel and Patel (1990) were in vessels measuring from 37-78 $\mu$m. Information about the structure of pulmonary veins in normal rats is included in the following publications [Kay, 1983; De Almeida *et al.*, 1975; Kay JM, Heath D (1969) *Crotalaria spectabilis*: The Pulmonary Hypertension Plant, Charles C. Thomas, Springfield, Illinois, chap. 5; and Kay JM, Heath D (1966) Observations on the pulmonary arteries and heart weight of rats fed on *Crotalaria spectabilis* seeds, J. Pathol. Bacteriol. **92**: 385-394].

**Authors**: The localization of sphincter smooth muscle cells inside the elastic lamina that defines the boundary between tunica media and adventitia, assigns these cells as belonging either to the tunica intima or media. A clear demarcation of the latter two layers is not easily done in small veins, as a distinct inner elastic lamina is missing. However, it should be stressed that smooth muscle cells of the sphincters next to the endothelium form far-reaching myo-endothelial junctions (a finding which has been already noted by previous investigators: Rhodin, 1978; Aharinejad *et al.*, 1992), where basal lamina material or connective tissue fibers are not interposed. Therefore, we tend to interpret the sphincters as belonging to the intimal layer, although we do not insist on such a classification. The sphincter regions may well be categorized as regions where a clear separation of tunica intima and media is not possible, similar to that situation described for intra-arterial cushions (Gorgas and Böck, 1975). Circular bundles of smooth muscle cells in venous walls which we interpret as sphincters may well correspond to the so-called "small cushions" described by De Almeida *et al.* (1975) in the rat small pulmonary veins. These cushions were subendothelially located accumulations of smooth muscle cells which pushed the endothelium toward the lumen.

In the media of the rat larger pulmonary veins cardiac muscle fibers occur which extend until the vessels attain about 100 $\mu$m in diameter (De Almeida *et al.*, 1975). You reported on a medial coat of cardiac muscle in the rat intrapulmonary veins exceeding 80 $\mu$m in diameter (Kay, 1983). In such veins, it may be difficult to envisage the detection of venous sphincters, i.e., to deduce whether corrosion casts constrictions are caused by smooth muscle cells or by cardiac muscles. According to our experiences, however, cardiac muscle fibers occur only in larger pulmonary veins near to the lung hilum. In tissue sections of the pulmonary veins obtained from the central lobular areas, we did not observe cardiac muscle fibers. The combination of corrosion casting and tissue sectioning now enables us to obtain three-dimensional view and information on the functioning of smooth muscle bundles, as well as to correlate different pulmonary vessel types with corresponding cast specimens, so allowing the interpretation of smooth muscle cells accumulation as venous sphincters.

**J.M. Kay**: Constrictions of pulmonary arteries were also noted by Schraufnagel and Patel (1990). You should consider the possibility that these constrictions correspond to the thick walled oblique muscle segments described by Meyrick *et al.* (1978).
**Authors**: We agree. Meyrick *et al.* (1978) described four main regions of the pulmonary arteries: **1**. Muscular arteries at the hilum with a complete layer of circular muscle; **2**. Muscular arteries, decreasing in caliber, continue toward the periphery in partially muscular arteries where the complete muscular coat gives way to a spiral of muscle; **3**. Non-muscular arteries in the periphery, where the muscle totally disappears; and **4**. Arteries with the oblique muscle segment are those vessels within the region with a complete muscle coat where the muscle fibers run obliquely. We consider that luminal constrictions on pulmonary artery casts observed in our material may correspond to the thick walled oblique muscle segments described by Meyrick *et al.* (1978) in normotensive rats.

**F. Amenta**: The authors used a normotensive reference group Sprague Dawley rats and spontaneously hypertensive rats from Wistar origin. Is it possible to compare two different strains? In addition, the authors did not measure the blood pressure values in hypertensive animals they used.
**Authors**: Spontaneously hypertensive rats are known to have an increased systemic blood pressure (Okamoto *et al.*, 1973). For our corrosion casting studies, we used spontaneously hypertensive rats in which the increased systemic blood pressure was evidenced by the company they were provided by. Basically, we agree that the measuring of the pulmonary blood pressure in these animals would be of benefit, but this will be the subject of a coming study in our laboratory. In other words, the morphology of pulmonary vessels was the main subject of the present study in hypertensive and normotensive animals.

**O. Matthew-Costello**: Constriction in Fig.2 is described as "remarkably deep" and in Fig. 3 as "strikingly deepened". This seems to be clear for some constrictions but not others. Also, there is a likely effect of the viewing angle on the perception of depth. The authors should indicate how they dealt with this.
**Authors**: The comparison between Figs. 2 and 3 indicates that venous sphincters at the merging site of two

venous vessels are almost always deeper than the others. This observation has been made by Schraufnagel and Patel (1990) and later confirmed by Aharinejad *et al.* (1992). It goes without saying that the estimation of the depth of venous sphincters as described by Schraufnagel and Patel and Aharinejad *et al.* (1992) i.e., measuring the outer and inner diameter of the sphincter at the site where the sphincter is fractured, is the method of choice. However, if the fractured sphincter cast should serve as the basis of measurements, it is not readily possible to tell whether the area under consideration belongs to a merging site or not. Hence, where sphincters are present both at the joining site and on the surface of merging veins, the comparison of sphincter depth can be only roughly done.

**O. Matthew-Costello:** How did the authors conclude that the frequency of pronounced constrictions is higher in spontaneously hypertensive rats than that in normotensive rats?

**Authors:** While studying the casts of hypertensive rats, we found no specimen without sphincter indentations, whereas the specimens of normotensive rats occasionally lacked pronounced sphincters. Concerning the number of animals we studied for each strain, i.e., 20 hypertensive and 20 normotensive animals and concerning the number of stubs studied in each animal, i.e., at least four studs, we concluded that the occasional lack of pronounced sphincters in normotensives and the regular presence of them in hypertensives indicate that the number of pronounced sphincters in hypertensives is higher.