Hypertrophy of Intramyocardial Arteriolar Smooth Muscle Cells in Experimental Renal Failure

JOHANNES TÖRNIG,* MARIE-LUISE GROSS,* AURELIA SIMONAVICIENE,* GERHARD MALL,‡ EBERHARD RITZ,† and KERSTIN AMANN*†

Departments of *Pathology and †Nephrology, University of Heidelberg, Heidelberg, Germany; and ‡Department of Pathology, Städtische Kliniken, Darmstadt, Germany.

Abstract. Wall thickening of intramyocardial arteries in patients with chronic renal failure may contribute to the increased susceptibility of the uremic heart to ischemic injury. In this context, the following questions arise: (1) Is intramyocardial wall thickening in experimental renal failure due to hypertrophy, hyperplasia or both? (2) Which stimuli trigger wall thickening? Using novel stereologic techniques (Nucleator, Selector, hyperplasia or both? (2) Which stimuli trigger wall thickening? Using novel stereologic techniques (Nucleator, Selector, intramyocardial arteries were examined in sham-operated and subtotally nephrectomized (SNX) Sprague Dawley rats with moderate renal failure of 8 wk duration. Systolic BP during the experiment was not significantly different in both groups. Absolute and relative left ventricular weight, wall thickness (5.69 ± 1.11 μm versus 4.42 ± 0.99 μm), and wall-to-lumen ratio of intramyocardial arteries (0.117 ± 0.03 μm/μm versus 0.089 ± 0.01 μm/μm) were significantly greater in SNX than in sham-operated rats. The mean cell and nuclear volume of intramyocardial arteriolar smooth muscle cells was significantly increased in SNX rats (650 ± 230 μm³ versus 430 ± 90 μm³ and 26 ± 4.5 versus 19.9 ± 2.2 μm³, respectively). In parallel, the total arteriolar wall volume was significantly greater in the left ventricle of SNX (+58%) compared with sham rats. In contrast, the total length of all left ventricular arteries was comparable in both groups. The increase in mean cell volume without significant change in cell number indicates that arteriolar wall thickening in the heart of SNX rats is explained by hypertrophy rather than hyperplasia of arterial smooth muscle cells. This finding in a nonhypertensive experimental model of chronic renal failure contrasts with findings in spontaneously hypertensive rats. Independent of BP and left ventricular hypertrophy, specific growth signals must act on cardiac arteriolar smooth muscle cells.

Death of cardiac causes is the leading cause of mortality in patients with chronic renal failure (1,2), and one potential explanation is increased myocardial susceptibility to ischemic injury (3,4) secondary to functional (5–9), metabolic (3,10,11), and structural (12–15) changes of the heart and the vasculature. Structural changes of the heart include: (1) left ventricular hypertrophy; (2) expansion of the nonvascular cardiac interstitium leading to myocardial fibrosis; (3) reduced capillary supply; and (4) wall thickening of intramyocardial arteries (16).

An important functional role of intramyocardial arteriolar wall thickening was suggested by Rostand et al. (17,18) and Rutsky and Rostand (19), who demonstrated that in 30% of uremic patients with angina pectoris, no narrowing of the epicardial conduit arteries was noted on coronarography. In addition, wall thickening of large arteries, i.e., A. carotis, and of the aorta was documented in patients with chronic renal failure (7–9,20).

Wall thickening of intracardiac arteries has been investiga


Correspondence to Dr. Kerstin Amann, Department of Pathology, Im Neuenheimer Feld 220, D-69120 Heidelberg, Germany. Phone: 06221-562668; Fax: 06221-562521; E-mail: kerstin_amann@ukl.uni-heidelberg.de

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Materials and Methods

Animals

Male Sprague Dawley rats (300 g; Invanos Co., Kissleg, Germany) were housed in single cages at a constant temperature (20°C) and humidity (25%). The animals were fed a diet containing 40% protein and 0.6% NaCl (Altromin C 1002/C 1036, Altromin Co., Lage, Germany). After 3 d of adaptation, the animals were randomly allocated to subtotal nephrectomy (SNX) or sham operation (sham). The right kidney was removed under ketamin/diazepam anesthesia (100 mg/kg or 2.5 μg/kg, respectively). Seven days later, the left kidney was subtotally resected by removing two-thirds of the weight of the contralateral pendant from its cortex. The control animals were
sham-operated by decapsulating the kidney, taking special care not to affect the adrenals. Systolic BP was measured under light ether anesthesia by tail plethysmography every 2 wk.

After 8 wk, the experiment was terminated by perfusion fixation via the abdominal aorta under ketamin/diazepam anesthesia (see above). Blood samples were taken and the viscera were fixed by retrograde perfusion at a controlled pressure. Before fixation, the vascular system was rinsed with dextran solution 10% containing 0.5 g/L Procain-HCl for 2 min. Ten seconds after starting the aortic perfusion, the vena cava was incised to drain the blood. After dextran infusion, the vascular system was perfused with 0.2 M phosphate buffer containing 3% glutaraldehyde for 12 min (25).

Afterward, the heart of each animal was taken out for determination of weight and volume. Eight pieces of the left ventricular muscle including the septum were randomly sampled as described by Amann et al. (22) and processed according to the orientator method and embedded in Epon-Araldite (27). Semithin sections (1 μm) were stained with methylene blue and basic fuchsin.

Stereologic Techniques

Various definitions of arteries are in use today; several of them use vessel diameter for classification of arteries, arterioles, and capillaries. However, diameters depend on the arterial size as well as on the contractile state of VSMC. In the present study, a qualitative definition of arteries was preferred: Intramyocardial arteries were identified according to Wiest et al. (28), as vessels having a complete layer of VSMC separated from the endothelium by a continuous basement membrane, i.e., without any myoendothelial contact. A subclassification into arterioles, terminal arterioles, and precapillary sphincters was not made. Most of the vessels investigated were between 23 and 155 μm in diameter.

**Total Length of Intramyocardial Arteries**

Length density, $L_a$, (length of structures per unit reference volume), was derived from $L_a = 2 \times Q_a$, $Q_a$ is the number of arterial sectional transects per unit sectional area. $Q_a$ of arteries was determined at a magnification of 160:1 using a Zeiss eyepiece (integrated square area: 0.468 mm$^2$). Squares were superimposed on all histologic sections (mean total sectional area: 500 mm$^2$). An average of at least 500 arteries per animal were counted.

The total length ($L_{art}$) of the arterial tree was determined as $L_{art} = L_a [mm] = L_a [mm/mm^2] \times V [mm^3]$. The left ventricular volume $V$ [mm$^3$] was derived from the left ventricular weight (LVW) divided by the specific weight of perfusion-fixed hearts (1.04 mg/mm$^3$) according to $V$ [mm$^3$] = LVW/1.04 mg/mm$^3$ (28).

**Wall Thickness, Wall-to-Lumen Ratio, and Total Arterial Wall Volume of Intramyocardial Arteries**

Wall thickness, and wall and lumen area of intramyocardial arteries were determined planimetrically on eight isotropic uniform random sections per animal (magnification 400:1, phase contrast), using an automatic image analyzing system (Videoplan, Kontron Co., Eching, Germany) (25,27). The contours of the arterial profiles were marked manually with a cursor, and the maximal and minimal diameter and wall and lumen area were calculated. Wall thickness of intramyocardial arteries was determined as the mean of the measurements of the two opposite walls in the direction of the minimal diameter (because this is the direction where measurements are least affected by sectioning angle). Wall/lumen ratio was calculated by dividing the mean wall thickness by the minimal lumen diameter.

The ratio of sectional area of arterial walls per reference sectional area of the myocardium ($A_w$) was determined. Because $A_w$ (area per reference area) = $V_v$ (volume per reference volume) (29,30), the total volume of the left ventricular arterial wall ($V_{wall}$) could be derived from the total left ventricular volume ($V$) according to $V_{wall} = V_v \times V$.

**Mean Cell Volume and Mean Nuclear Volume of Arterial Smooth Muscle Cells**

**Mean Cell Volume.** For determination of the mean volume of arterial smooth muscle cells (SMC) ($V_{cell}$) by the Nucleator method, isotropic uniform random sections were used (22,27,31). This allows an unbiased estimation of the mean volume from a three-dimensional random sample of nuclei that was gained by a series of equally distanced semithin histologic sections. For practical reasons, a set of eight parallel section planes at an interval of 1 μm per orientator tissue probe was gained. The investigations were performed on eight orientator probes per animal, resulting in a total of 64 sections per animal. The sections were analyzed at a final magnification of 1000:1 using oil immersion and a Videoplan computer (Kontron, Eching, Germany). Preparation of parallel sections and stereologic measurements were performed as described in the Appendix (see also reference 22).

**Nuclear Volume.** The mean nuclear volume ($V_{nucleus}$) was estimated by using the Selector method as described in detail (32,33). This method is appropriate for determination of the mean volume of particles without clearly visible subcomponents, i.e., cell nuclei.

**Total Number of Arterial SMC**

In addition to the volume of arterial SMC, the total number of SMC ($n_{cell}$) of left ventricular arterial walls was derived from the total volume of nonendothelial wall cells ($V_{wall}$) and the mean volume of SMC ($v_S$) using the equation $n_{cell} = V_{wall}/v_S$. This equation can be applied because the wall of intramyocardial arteries was found to consist mainly (>95%) of SMC and only in a neglectable percentage of extracellular matrix (22).

**Statistical Analyses**

Data are given as mean ± SD. All data have been proven to be normally distributed (assessed by Kolmogorov–Smirnov test and Shapiro–Wilk statistics). Lilliefors niveau of significance (for distribution) was 5%. Mann–Whitney U test was chosen to determine whether the differences between the two groups were significant. The results were considered significant when $P$ was <0.05.

**Results**

**Description of the Model**

After 8 wk of renal failure, plasma urea concentration was significantly higher in SNX than in sham rats (Table 1). Despite significantly lower hematocrit concentrations in SNX, we emphasize that the animals were not severely anemic. Absolute weight of the perfused left ventricle (LVW) and relative LVW, i.e., left ventricular weight/body weight ratio, were significantly increased after SNX. Body weight and mean systolic BP were comparable in both groups.

**Quantitative Measurements of Intramyocardial Arteries**

In our experimental study, nine to 46 intramyocardial arteries per animal with a mean of 270 ± 11 (sham) and 225 ± 8 (SNX) cells per animal were analyzed using the Nucleator method. Lumen diameters of intramyocardial arteries ranged from 23 to 286 μm, with 95% of arteries ranging from 23 to...
The mean lumen diameters were comparable in both groups (55.2 ± 13.4 μm in sham rats and 58.7 ± 22.4 μm in SNX rats, respectively) (Table 2).

In the left ventricle, arteriolar wall thickness (+28%) and wall-to-lumen ratio (+31%), as well as the total arteriolar wall volume (+58%), were significantly greater in SNX than in sham rats. In contrast, the total length of all left ventricular arteries was comparable in both groups (2402 ± 729 mm in sham rats versus 2724 ± 736 in SNX rats). Nuclear (+30%) and cellular volume (+51%) of intramyocardial VSMC was significantly higher in SNX animals than in sham. The total number of VSMC as well as the number of VSMC per arterial length in the myocardium of the left ventricle were comparable in both groups. Representative examples of intramyocardial arteries of sham and SNX rats are given in Figures 1 and 2.

Figure 3 shows the frequency distribution of intramyocardial arteriolar lumen diameters in both groups. No significant differences in the distribution of lumen diameters were found between sham and SNX rats. This finding excludes sampling artifacts from assessment of vessels of different sizes in both experimental groups.

The frequency distribution of different classes of wall thickness is shown in Figure 4. The figure documents a systematic shift to the right for the SNX animals, indicating a significantly higher percentage of thicker intramyocardial arteries in this group.

### Discussion

In the present study, significantly increased wall thickness (+28% versus sham-operated controls), wall-to-lumen ratio (+31%), and arteriolar wall volume (+58%) were noted in the left ventricle of nonhypertensive SNX rats with moderate renal failure of short duration and moderate left ventricular hypertrophy. Using stereologic techniques (Nucleator and Selector method), elevated mean nuclear (+30%) and cellular (+51%) volume of VSMC without a significant change in the total cell number accounted for wall thickening of cardiac arteries. These findings indicate that VSMC hypertrophy rather than hyperplasia is responsible for remodeling of myocardial arteries in renal failure. One requirement for obtaining data on the cell volume of VSMC using the Nucleator method is that all profiles of a VSMC can be referred to the same cell. This may be difficult if the VSMC are shaped like a “banana.” The use of electron microscopy and serial sections in our previous study (22) suggest that this is not the case. This problem may be overcome by making an indirect estimate of the cell volume of VSMC, using a combination of the dissector method (34) and the point-counting method (29,30). The use of the dissector method, however, requires the estimation of section thickness, which may also bear possible confounders. Therefore, in the present study we decided to use the Nucleator technique for estimation of mean cell volume.

The observation of VSMC hypertrophy in a nonhypertensive experimental model of renal failure with moderate left ventricular hypertrophy is in contrast to what was described in spontaneously hypertensive rats. The extent to which this is due to elevated BP in spontaneously hypertensive rats, to their genetic makeup, or other factors is unknown. In the present experiment, we compared SNX with normotensive controls. The findings are compatible with BP-independent mechanisms in uremia.

We acknowledge that BP measurements by sphygmomanometry may not reliably reflect 24-h changes in BP. This is of potential importance because intermittent fluctuations in BP have been documented in experimental renal failure (35). A potential role of BP elevation can not formally be excluded, but it is likely that the vascular changes are (at least in part) independent of BP, because they could not be prevented by nonspecific antihypertensive treatment (13). In the SNX rats, other factors may influence arterial walls, particularly changes in hematocrit and shear stress, which were not measured or controlled in the present study. We emphasize that the above findings are proven only for intramyocardial arteries. Apparently, the behavior of blood vessels is quite heterogeneous in uremia, because in former studies examining windkessel arteries we found marked hyperplasia with only modest hypertrophy (36).

In this model, however, we found that treatment with the calcium antagonist nifedipine, the angiotensin-converting enzyme inhibitor ramipril, and endothelin antagonists completely prevented intramyocardial arterial wall thickening, whereas treatment with the central sympatholytic agent moxonidine did not (25,37).

The observation of wall thickening in experimental renal failure is in line with findings in patients with chronic renal failure. Rostand et al. documented absence of stenosing lesions of the epicardial arteries in 30 to 50% of uremic patients with angina pectoris (18). Schwartzkopff et al. found wall thickening of intramyocardial arteries in nonuremic patients with syndrome X, i.e., angina pectoris and reduced coronary reserve...
despite normal coronarography (21). In a manner analogous to syndrome X, wall thickening of intramyocardial arteries exists in uremic patients as well, as we showed at post mortem and in heart biopsies collected at cardiac surgery (unpublished data).

Studies of London et al. (6,7,9) and Barenbrock et al. (20) demonstrated increased wall thickness of extracardiac arteries, e.g., the aorta and the carotid artery. In patients with chronic renal failure, a close relationship was found between wall thickness of the carotid artery and plasma endothelin (ET-1) levels (38). This finding is of note, because VSMC are a known target of ET-1 (39). Interaction of angiotensin II (AngII) and ET-1 was documented in rat aortic SMC; ET-1 modulates the renin-angiotensin system (RAS) and stimulates VSMC proliferation (40–42). In line with this finding, in experimental renal failure wall thickening of arteries was completely prevented by selective and unselective ET-1 receptor antagonists (26).

Which cellular mechanisms are responsible for the altered vessel wall structure? Using immunohistochemistry, a significantly increased number of proliferating cell nuclear antigen-
positive VSMC was demonstrated comparing hearts of SNX rats with short-term renal failure with sham-operated controls and rats with renovascular hypertension and comparable left ventricular hypertrophy (1C-2K) (24). In view of the unchanged total cell number in this study, however, the increased number of proliferating cell nuclear antigen-positive cardiac VSMC argues for cell activation rather than for cell proliferation. Indeed, electron microscopic investigations of the heart demonstrated increased intracellular actin filament content and signs of cell activation in VSMC, i.e., expansion of the Golgi apparatus, increased number of intracellular vesicles, etc. (13). This observation is in line with increased protein expression of \(\alpha\)-smooth muscle cell actin and collagen I through III in the wall of cardiac arteries (24). On the ultrastructural level, degenerative changes of the myocardium or myocyte necrosis were never encountered.

On the other hand, the unchanged total number of cardiac VSMC after SNX could theoretically also be explained by cellular proliferation accompanied by apoptosis. Activation of the \(c\)-myc proto-oncogene can lead to either proliferation or apoptosis, depending on the availability of growth factors (43). In this context, it is of interest that AngII activates the \(c\)-myc proto-oncogene and is a potent mitogen for VSMC in vitro, inducing both hypertrophy and hyperplasia of VSMC (44). In experimental renal failure, increased renin mRNA in the cardiac interstitium was documented, pointing to activation of the local renin system (23,45).

In the same experimental model, significantly higher cardiac vascular expression of the growth factors platelet-derived growth factor and vascular endothelial growth factor and a permissive effect of parathyroid hormone (PTH) for intramyocardial arterial wall thickening were found (24,46). The latter finding is of interest because AngII is known to stimulate PTH/PTHrp receptor expression (47,48). In uremia, the interaction may be complicated by downregulation of the PTH/PTHrp receptor (49) and possible hyper-responsiveness to PTH. On the other hand, it has been suggested that downregulation of the PTH/PTHrp receptor may enhance the trophic actions of AngII (47).

The observation of isolated hypertrophy of intramyocardial VSMC in rats with renal failure narrows down potential pathogenic mechanisms, further investigations of which may lead to potential therapeutic strategies to prevent arteriolar wall thickening in uremic patients.

Figure 3. Frequency of lumen diameters in sham-operated controls (sham) and SNX rats. No significant difference in the frequency distribution is noted between the two groups.

Figure 4. Frequency of different classes of wall thickness in sham-operated controls (sham) and SNX rats. Note a systematic shift to the right for the SNX animals, indicating a higher percentage of thicker intramyocardial arterial walls in SNX.
Appendix: Nucleator Technique

Cell Volume

The Nucleator is a so-called unbiased stereologic technique that allows estimation of three-dimensional structures from measurements on two-dimensional sections.

With this technique, an isotropic uniform random section plane is chosen as the so-called lookup plane, and a parallel section plane, the so-called measure or reference plane (i.e., a second histologic section at a certain distance from the first one). Comparison of the serial sections was performed by using a set of two microscopes and a video camera for permanent visualization of the lookup plane and measurements on the reference plane. A cell profile was sampled on the measure plane if its nucleus is visible, provided that it was not detectable on the lookup plane. Whenever a sampled nucleus is hit by a test point (100-point grid; Zeiss, Oberkochen, Germany), the length of a random cell intercept (l+) through this point is determined. The measurement was repeated three times per cell yielding l1+, l2+, l3+, and l3−. The mean intercepts for each direction l1, l2, and l3 were determined as:

\[ l_1 = \frac{1}{2}(l_{1+} + l_{1-}) \]

\[ l_2 = \frac{1}{2}(l_{2+} + l_{2-}) \]

\[ l_3 = \frac{1}{2}(l_{3+} + l_{3-}) \]

The mean intercept length in the third power per cell (\(l_O^3\)) was then determined by the equation \(l_O^3 = \frac{1}{3}(l_{1+}^3 + l_{2+}^3 + l_{3+}^3)\). Afterward, the mean cell volume (\(v_O\)) and the estimated mean cell volume in the numerical distribution (\(v_N\)) were determined according to Gundersen (31) by the following equations (n = number of sampled cells):

\[ v_O = \frac{4}{3} \pi l_O^3 \]

\[ v_N = \frac{(v_1 + v_2 + v_3 + \ldots + v_n)}{n} = \frac{4/3 \pi \times (l_{1+}^3 + l_{2+}^3 + \ldots + l_{n}^3)}{n} \]

Nuclear Volume

The mean nuclear volume (\(V_{nucleus}\)) was estimated by using the Selector method as described in detail (32,33). This method is appropriate for determination of the mean volume of particles without clearly visible subcomponents, i.e., cell nuclei.

The mean nuclear volume (\(v_O\)) was derived from \(v_O = \pi l_O^3 \times \frac{n}{3}\). For n measurements, \(v_N\) was determined as:

\[ v_N = \frac{\pi}{3} \times (l_{1+}^3 + l_{2+}^3 + \ldots + l_{n}^3)/n. \]

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References

19. Rutzky E, Rostand SG: The management of coronary artery


Cruz-Orive LM: Particle number can be estimated using a dissector of unknown thickness: The selector. *J Microsc* 145: 121–142, 1987


