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Alterations in structure and mechanics of resistance arteries from ouabain-induced hypertensive rats

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Hypertension induced by ouabain administration seems to depend, at least in part, on central mechanisms associated with increased sympathetic tone, subsequent to the activation of the brain renin-angiotensin (16, 40) and endothelin (8) systems. However, it is possible that additional peripheral mechanisms are also involved. Essential hypertension is associated with increased peripheral vascular resistance (27, 34). Its fundamental cause is a decrease in lumen diameter that could be originated by structural, mechanical, and/or functional alterations (6, 18). At peripheral level, ouabain-treatment alters vascular function in conductance and resistance arteries (22, 33, 39). However, whether this altered vascular reactivity is associated with structural changes of the resistance vasculature has never been explored.

The vascular structural alterations that accompany hypertension, known as “hypertensive arterial remodeling,” are now considered to be a complex process that might involve the increase (hypertrophy), decrease (hypotrophy), or rearrangement (eutrophy) of wall material (26). The common feature is the decrease in internal diameter (inward remodeling). However, the contribution of growth to the structural abnormalities is different depending on the vascular bed and model of hypertension studied (18, 26). Thus small arteries from patients with essential hypertension show eutrophic remodeling (27), whereas mesenteric resistance arteries (MRA) and renal afferent arterioles from spontaneously hypertensive rats (SHR) show eutrophic and hypotrophic remodeling, respectively (5, 20, 29). However, secondary forms of human hypertension and experimental models with high circulating renin levels are characterized by inward hypertrophic remodeling (23, 32). A second common feature in hypertension is the alteration in the mechanical properties of resistance arteries. Thus arterial stiffness is increased in essential hypertensive patients and in some rat models of hypertension (18). Alterations in collagen, non-fibrous extracellular matrix proteins, adhesion molecules (18), and, more recently, in elastic fibers (5) have been proposed as determinants of the altered vascular stiffness and hypertensive arterial remodeling found in resistance arteries in hypertension.

The aim of the present study has been to test whether, in addition to the central mechanisms already described, alterations in the structure of resistance arteries might contribute to ouabain-induced hypertension. In particular, we have investigated 1) gross structure and mechanics, 2) cellular distribution,
content and distribution of fibrous extracellular matrix proteins in the vascular wall. To our knowledge, this is the first time that these parameters have been investigated in resistance arteries from this model of hypertension.

MATERIALS AND METHODS

Animal Model

Six-week-old male Wistar rats (Harlam Ibérica, Barcelona, Spain) were obtained from colonies maintained at the Animal Quarters of the Facultad de Medicina of the Universidad Autónoma de Madrid. Rats were housed at a constant room temperature, humidity, and light cycle (12:12-h light-dark) and had free access to tap water and standard rat chow ad libitum. The investigation conforms to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and the current Spanish and European laws (RD 223/88 MAPA and 609/86). The experimental protocol was approved by the Universidad Autónoma de Madrid Ethics Committee.

Rats were anesthetized with diethyl ether, and a small incision was made in the back of the neck to implant a subcutaneous controlled time-release pellet (Innovative Research of America, Sarasota, FL) containing ouabain (0.5 mg/pellet) or vehicle (placebo) as previously described (36). These pellets are designed to release a constant...
amount of either ouabain (~8.0 μg/day) or vehicle for a 60-day period. The length of treatment was 5 wk. Systolic blood pressure (SBP) was measured by the tail-cuff method before the start of treatment and weekly during treatment.

The rats were decapitated, and the mesenteric arcade was removed and placed in Krebs-Henseleit solution (KHS) of the following composition (in mM): 115.0 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.7 MgSO4·7 H2O, 1.2 H2O, 25.0 NaHCO3, 11.1 glucose, and 0.01 mM EDTA. The mesenteric arcade was maintained at 4°C and continuously gassed with 95% O2-5% CO2. The length of treatment was 5 wk. Systolic blood pressure (SBP) was measured by the tail-cuff method before the start of treatment and weekly during treatment.

### Table 1. Laser scanning confocal microscope measurements of mesenteric resistance artery morphology in control and ouabain-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>Ouabain (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adventitia thickness, μm</td>
<td>6.5±0.3</td>
<td>5.9±0.5</td>
</tr>
<tr>
<td>Media thickness, μm</td>
<td>11.1±0.6</td>
<td>11.6±0.9</td>
</tr>
<tr>
<td>LSA, mm²</td>
<td>1.02±0.03</td>
<td>0.91±0.03*</td>
</tr>
<tr>
<td>Wall volume, mm³</td>
<td>0.034±0.001</td>
<td>0.029±0.002*</td>
</tr>
<tr>
<td>Total number of cells</td>
<td>7,381±272</td>
<td>6,407±345*</td>
</tr>
<tr>
<td>Total number of AC</td>
<td>1,421±58</td>
<td>1,269±109</td>
</tr>
<tr>
<td>Total number of SMC</td>
<td>4,515±220</td>
<td>3,859±201*</td>
</tr>
<tr>
<td>Total number of EC</td>
<td>1,445±37</td>
<td>1,279±110</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. All calculations were performed on the basis of 1-mm-long segments. LSA, luminal surface area; AC, adventitial cells; SMC, smooth muscle cells; EC, endothelial cells. *P < 0.05 with respect to control rats.

Pressure-induced myogenic responses. The myogenic index (MI) was calculated as follows (12):

\[
MI = (100 \times \Delta D/D_o) / \Delta IP,
\]

where \(D_i\) is the internal diameter at any given pressure, \(D_o\) is the diameter at the previous intraluminal pressure, and \(\Delta IP\) is the change in intraluminal pressure (\(\Delta IP\) were made in 20-mmHg steps).

To quantify the active vasoconstriction of the arteries for each intraluminal pressure, some results were expressed as a percentage of the diameter of each artery under passive conditions at each intraluminal pressure.

### Calculation of passive structural and mechanical parameters

From internal and external diameter measurements in passive conditions, the following structural parameters were calculated: wall thickness \((e_0Ca^++KHS)\) was calculated as follows (12):

\[
e = (D_{ioCa} - D_{oCa})/2; \quad \text{cross-sectional area (CSA)} = (\pi/4) \times (D_{ioCa}^2 - D_{oCa}^2); \quad \text{wall/lumen} = (D_{oCa} - D_{ioCa})/2D_{oCa}.
\]

The following mechanical parameters were calculated according to the method of Baumbach and Heistad (3): circumferential wall strain \((\varepsilon) = (D_{ioCa} - D_{oCa})/D_{oCa}\), where \(D_{oCa}\) is the internal diameter at 3 mmHg and \(D_{ioCa}\) is the observed internal diameter for a given intravascular pressure both measured in 0Ca²⁺-KHS medium; and circumferential wall stress \((\sigma) = (P \times D_{oCa})/(2WT)\), where \(P\) is the intraluminal pressure (1 mmHg = 133.4 N/m²) and WT is wall thickness at each intraluminal pressure in 0Ca²⁺ medium.

Arterial stiffness independent of geometry is determined by Young’s elastic modulus \((E = \text{stress/strain})\). The stress-strain relationship is nonlinear; therefore, it is more appropriate to obtain a tangential or incremental elastic modulus \((E_{inc})\) by determining the slope of the stress-strain curve \((E_{inc} = \delta \varepsilon / \delta \sigma)\) (10). \(E_{inc}\) was obtained by fitting the stress-strain data from each animal to an exponential curve by using the equation

\[
\sigma = \sigma_{orig} e^{\beta \varepsilon}
\]

where \(\sigma_{orig}\) is the stress at the original diameter (diameter at 3 mmHg) and \(\beta\) is the slope of the stress-strain curve in 0Ca²⁺-KHS. Data are expressed as means ± SE. Number of animals in each case is indicated in parenthesis. D, internal diameter at 3 mmHg; Do, observed internal diameter for a given intravascular pressure.
Taking derivatives on the above equation, we see that $E_{\text{inc}} = \beta r$. For a given $\sigma$ value, $E_{\text{inc}}$ is directly proportional to $\beta$. An increase in $\beta$ implies an increase in $E_{\text{inc}}$, which means an increase in stiffness.

### Confocal Microscopy

**Confocal microscopy study of nuclei distribution.** Pressure-fixed intact arteries were incubated with the nuclear dye Hoechst 33342 (0.01 mg/ml) for 15 min. After being washed, arteries were mounted on slides with a well made of silicon spacers to avoid artery deformation. They were viewed with a Leica TCS SP2 confocal system fitted with an inverted microscope and argon and helium-neon laser sources with oil immersion lens ($\times 63$) (excitation wavelength 488 nm and emission wavelength 400–500 nm) (38). The experiments were performed in intact pressure-fixed MRA with a Leica TCS SP2 confocal system. Serial optical sections from the adventitia to the lumen (z step = 0.3 μm) were captured with a $\times 63$ oil objective (numerical aperture 1.3) using the 488-nm line of the confocal microscope. A minimum of two stacks of images of different regions was captured in each arterial segment. All the images were taken under identical conditions of laser intensity, brightness, and contrast.

Quantitative analysis was performed with MetaMorph Image Analysis Software, as previously described (5). From each stack of serial images, individual projections of the internal elastic lamina (IEL) were reconstructed, and IEL thickness, total fenestrae number, fenestra area, and relative area occupied by elastin were measured. Fluorescent intensity values were used as an estimate of the elastin concentration as previously described (5), based on the assumption that the concentration of elastin has a linear relationship with fluorescence intensity (4).

### Collagen Determination by Picrosirius Red

In a separate set of experiments, segments of MRA were removed from the mesentery and immediately fixed in 4% PFA in phosphate buffer for 1 h, transferred to a cryomold containing optimum cutting temperature embedding medium (Tissue Tek, Sakura), and frozen in liquid nitrogen. Frozen transverse sections (10 μm) were incubated with picrosirius red [0.1% (wt/vol) Sirius red 3FB in saturated aqueous picric acid] for 30 min with gentle agitation for collagen staining (21). Color images were captured with a microscope (Nikon Eclipse TE 2000-S, $\times 40$ objective) by using a digital camera (Nikon DXM 1200F). Quantitative and qualitative analyses of collagen content and distribution were performed with MetaMorph Image Analysis Software (Universal Imaging). Original images were transformed to grayscale level. Thereafter, collagen content was estimated separately in the adventitial and medial layers by subtracting the background from the intensity values obtained in the medial or adventitial area of each cross section. In addition, grayscale images from control and ouabain-treated rats were thresholded at the same level, and images showing the distribution of collagen in the vascular wall were obtained.

### Western Blot Analysis

Collagen I/III protein expression was determined in homogenates (50 μg protein) from MRA of control and ouabain-treated rats. Proteins were separated by 7.5% SDS-PAGE and then transferred to nitrocellulose membranes for 1.5 h. Membranes were incubated with rabbit polyclonal antibody for collagen I/III (1:500 dilution; Calbiochem, San Diego, CA) and mouse monoclonal antibody for α-actin (1:10,000, Sigma Chemical, St. Louis, MO). After being washed, membranes were incubated with anti-rabbit (1:2,000) or anti-mouse (1:10,000) IgG antibodies conjugated to horseradish peroxidase (BioRad, Hercules, CA). The immunocomplexes were detected with the use of an enhanced horseradish peroxidase-luminol chemiluminescence system (ECL Plus; Amersham International, Little Chalfont,
Fig. 4. A: representative images of collagen staining with picrosirius red of transversal sections obtained from mesenteric resistance arteries from control and ouabain-treated rats. A, top: original images. A, bottom: same images after thresholding. Images were captured with a light microscope (×40 objective, ×1 zoom). B: quantitative analysis of collagen content in adventitia and media. *P < 0.05 vs. control. C: confocal projections of the internal elastic lamina of mesenteric resistance arteries from control and ouabain-treated rats. Vessels were pressure-fixed at 70 mmHg and mounted intact on a slide. Projections were obtained from serial optical sections captured with a fluorescence confocal microscope (×63 oil immersion objective, ×2 zoom). Data are expressed as means ± SE. Number of animals in each case is indicated in parenthesis.
Results are expressed as means ± SE, and n denotes the number of animals used in each experiment. The dependency of either vascular structure or mechanics on rat treatment group and intraluminal pressure was studied by two-way ANOVA. For specific two-means comparison, an unpaired Student’s t-test was used. A value of P < 0.05 was considered significant.

Norepinephrine, ACh, papaverine, Hoechst 33342, and Sirius red were obtained from Sigma-Aldrich. All other chemicals were of reagent grade or better and obtained from Sigma Chemical or other commercial suppliers.

RESULTS

Ouabain treatment progressively increased SBP from the first week of treatment, as previously described by our group (31, 36). At the end of the treatment, control and ouabain-treated rats showed the following SBP values; control, 118 ± 1.5 (n = 8); ouabain, 149 ± 2.0 mmHg (n = 9) (P < 0.05). The body weight was similar in both experimental groups [control, 327 ± 10 (n = 8); ouabain, 339 ± 17 g (n = 9)].

Vascular Structure and Mechanics

Figure 1 shows the structural parameters of MRA from control and ouabain-treated rats under fully relaxed conditions (0Ca2+-KHS). Internal diameter (Fig. 1A) and external diameter (data not shown) were diminished in arteries from ouabain-treated rats compared with controls. However, wall thickness was similar in both groups at all pressures studied (Fig. 1B). As a consequence, wall-to-lumen ratio was higher and CSA was smaller in MRA from ouabain-treated rats compared with controls (Fig. 1, C and D).

In active conditions, i.e., in the presence of extracellular calcium, external and internal diameters were also significantly reduced in ouabain-treated rats compared with controls (data not shown). MRA had a small level of myogenic tone at all pressures tested that was similar in both groups (data not shown). Similarly, there was no statistical difference in intrinsic tone between control and ouabain-treated rats (Fig. 1E).

Figure 2 shows the mechanical parameters of MRA from control and ouabain-treated rats. Media stress was significantly smaller in arteries from ouabain-treated rats than in control animals (Fig. 2A). In addition, MRA from ouabain-treated rats showed decreased elasticity, as shown by the larger value of β [control, 4.17 ± 0.07 (n = 8); ouabain, 4.67 ± 0.14 (P < 0.05; n = 9)] and a leftward shift of the stress-strain relationship (Fig. 2B).

Table 2. Characteristics of IEL in pressurized segments of mesenteric resistance arteries from control and ouabain-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ouabain</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>IEL thickness, μm</td>
<td>3.5 ± 0.1</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>Total number of fenestrae</td>
<td>3,437 ± 409</td>
<td>3,265 ± 428</td>
</tr>
<tr>
<td>Fenestra area, μm²</td>
<td>13.8 ± 1.3</td>
<td>12.7 ± 0.9</td>
</tr>
<tr>
<td>Relative area of elastin/image</td>
<td>0.80 ± 0.01</td>
<td>0.79 ± 0.01</td>
</tr>
<tr>
<td>Intensity/pixel</td>
<td>113.80 ± 1.32</td>
<td>116.90 ± 1.52</td>
</tr>
<tr>
<td>Total fluorescence (×10⁶), intensity/pixel⁻¹vol⁻¹</td>
<td>4.085 ± 195</td>
<td>3.658 ± 131</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. IEL, internal elastic lamina.
Morphology of Vascular Wall

Adventitia and media thickness were similar in control and ouabain-treated rats (Table 1). As expected, luminal surface area and wall volume were significantly smaller in arteries from ouabain-treated rats (Table 1). The total number of cells (adventitial plus smooth muscle plus endothelial cells) was diminished in ouabain-treated rats compared with control rats. When each cell type was analyzed separately, only the SMC number was statistically different (Table 1).

Results of smooth muscle and endothelial cells nuclei morphology are shown in Fig. 3. There was no difference in either the length or width of cell nuclei between both groups. Consequently, nuclei area was similar in control and ouabain-treated rats (Fig. 3).

Collagen and Elastin Quantification

As shown in Fig. 4A, collagen was present throughout the whole vascular wall, being more densely distributed in the adventitia. Ouabain treatment did not affect collagen content in the adventitia. However, ouabain treatment significantly increased collagen content in the media (Fig. 4B). Western blot experiments showed increased collagen I/III expression in MRA from ouabain-treated rats compared with controls (Fig. 5).

Figure 4C shows maximal intensity projections of the IEL from control and ouabain-treated rats, and Table 2 resumes the quantification of the IEL characteristics. We did not find any differences in the structure of elastin in the IEL between arteries from normotensive and hypertensive rats, because both fenestrae area and number were similar. Average fluorescence intensity per pixel was not significantly different between control and ouabain-treated rats, indicating no evidence for a difference in the total amount of elastin in a 1-mm length of artery.

Vascular Reactivity

KCl (120 mM) evoked similar contractions in vessels from ouabain-treated and control rats [control, 9.4 ± 0.7 (n = 11); ouabain-treated, 9.3 ± 0.6 mN (n = 14)]. The endothelium-dependent relaxation to ACh and contractile responses to norepinephrine also remained unchanged after ouabain treatment (Fig. 6).

DISCUSSION

Elevated levels of endogenous ouabain or a closely related isomer are implicated in rat and human hypertension and in associated cardiovascular complications. Thus, in ~30% of patients with uncomplicated essential hypertension, plasma endogenous ouabain levels are increased. Several findings indicate that endogenous ouabain, in addition to directly influencing blood pressure, may be involved in the development of cardiovascular complications (cardiac hypertrophy, heart failure, and myocardial infarction) associated with hypertension. Endogenous ouabain may therefore play a direct role in vivo as a prohypertrophic hormone and thus may affect cardiovascular function and structure, being responsible for cardiac remodeling that contributes to an increased risk of morbid events (13, 14, 15, 36). Furthermore, exogenous ouabain induces hypertension when chronically administered to normotensive rats (24, 39). Central and peripheral mechanisms have been suggested as possible participants in the hypertension induced by ouabain (33, 39, 40). However, the contribution of structural alterations of resistance arteries to this model of hypertension has not been analyzed so far.

Hypertension is characterized by increased peripheral vascular resistance to blood flow. According to Poiseuille’s law, flow resistance is inversely related to the fourth power of the vessel’s radius. Therefore, small decreases in the lumen of small arteries and arterioles will significantly increase resistance (27, 35). We observed that MRA from ouabain-treated rats showed decreased internal and external diameters in active conditions. At the functional level, both an increase in the contractile responses or a decrease in relaxant responses might increase peripheral resistance by reducing internal diameter. However, our experimental data seem to exclude functional changes as responsible for the observed decrease in vessel size in this model of hypertension. This is based on three observations: 1) no difference between treatment groups in either MI or intrinsic tone; 2) unaltered vasoconstrictor responses to K+ or norepinephrine; and 3) unaltered ACh-induced relaxation. Moreover, we have previously reported that the modulation of norepinephrine responses by endothelial nitric oxide was higher in ouabain-treated rats compared with controls (39).

Structural and mechanical changes in resistance arteries may result in decreased lumen size (35). In passive conditions, we
also observed decreased internal and external diameters in MRA from ouabain-treated rats. In other models of hypertension, the reduction in internal diameter is associated with thickening of the vessel wall (6, 18, 27); in our experimental model, wall thickness remained unchanged. However, we observed diminished CSA. This alteration, together with decreased vessel diameter, is a typical feature of hypotrophic inward remodeling (26), which has been previously described in afferent arterioles from SHR (29). Mechanisms underlying hypotrophic remodeling are unclear. Atrophy of one or more layers of the vascular wall due to decreased cellular number might be implicated in the development of hypotrophic arterial remodeling. In this sense, Coats et al. (7) reported atrophy in both the medial and adventitial layers of the artery wall of distal ischemic arteries due to hypoplasia. We found a decrease in the total number of cells, mainly due to decreased SMC number, in arteries from ouabain-treated rats. Thus some signs of hypoplasia seem to appear after ouabain treatment. In this line, Orlov et al. (31) recently reported that long-term exposure to ouabain culminates in porcine aortic endothelial cell necrosis mediated by the interaction of ouabain with Na\(^+\)-K\(^+\)-ATPase. Another possibility to explain hypotrophic arterial remodeling could be a decrease in cell size. Because we and other authors have demonstrated changes in the morphology of endothelial or SMC nuclei in other models of hypertension (1, 2, 9) and there is evidence that SMC nuclear size correlates with SMC length (9), we analyzed possible changes in endothelial or SMC nuclei morphology. However, the size of nuclei from both cell types was not modified by ouabain treatment, pointing to the reduction in cell number as responsible for hypotrophic remodeling observed in ouabain-treated rats.

The decrease in internal diameter could be originated by a structural modification, i.e., “true hypertensive remodeling” or by a mechanical alteration of the vessels (6, 18). We observed increased stiffness in MRA from ouabain-treated rats as shown by the leftward shift of the stress-strain relationship and the increased β value (which reflects arterial stiffness independent of vessel geometry). Stiffness is increased in MRA from SHR (5, 20), whereas no differences in intrinsic elastic properties were found in MRA from other models of hypertension, such as Dahl salt-sensitive (17) and mRen-2 transgenic rats (11). In resistance arteries, enhanced vascular stiffness in hypertension has been generally attributed to an increase in collagen content (19, 20, 37), nonfibrous extracellular matrix proteins and adhesion molecules (18), and, more recently, to changes in elastic fiber organization of the IEL (5). Arteries from ouabain-treated rats showed increased collagen deposition in the media but not in the adventitia. Fibrillar type I/III collagens are the most abundant in the vascular wall (28), and they play an important role controlling arterial expansion (10). Therefore, we explored expression of these types of collagens. Our results show augmented expression of type I/III collagens in arteries from ouabain-treated rats, suggesting increased synthesis as the mechanism responsible of the increased collagen deposition. In our experimental model, we did not observe differences in either the organization of the IEL or in elastin fluorescence, which provides an estimate of the elastin concentration (4). Our results, therefore, point to an increase in collagen deposition as responsible for the changes in vascular mechanics and, possibly, in inward remodeling in this model of hypertension.

In summary, the present work demonstrates hypotrophic inward remodeling of MRA from ouabain-treated rats that seems to be the consequence of a combination of decreased cell number and impaired distension of the vessel, possibly due to a higher stiffness associated with collagen deposition. We suggest that the narrowing of resistance vessels could play a role in the pathogenesis of hypertension in this model. There are several reports analyzing, in different cultured cell types, the effect of sustained inhibition of the Na\(^+\)-K\(^+\) pump in macromolecular synthesis and cell proliferation (30, 31). The present experimental model seems to represent a good experimental approach to look into the geometry of resistance arteries after administration of ouabain. However, to assess the role of endogenous ouabain in hypertensive vascular remodeling, it would be useful to study the effect of antibodies against endogenous Na\(^+\)-K\(^+\) pump inhibitors in other experimental models of hypertension. Another possibility would be the use of the ouabain antagonist PST 2238, a new antihypertensive compound that selectively antagonizes the pressor effect and the alteration of the renal Na-K pump and ouabain-induced organ hypertrophy (13).

ACKNOWLEDGMENTS

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