Effect of removal of adventitia on vascular smooth muscle contraction and relaxation

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Received 10 October 2000; accepted in final form 29 January 2001

Gonzalez, M. C., S. M. Arribas, F. Molero, and M. S. Fernandez-Alfonso. Effect of removal of adventitia on vascular smooth muscle contraction and relaxation. Am J Physiol Heart Circ Physiol 280: H2876–H2881, 2001.—The aim of the present study was to determine whether the adventitia of large arteries modulates vascular function. We developed a method to obtain functional vascular rings devoid of adventitia. Carotid and iliac arteries from 3-mo-old Sprague-Dawley rats were denuded from adventitia after treatment with collagenase followed by gentle peeling. Adventitia removal and integrity of the media was demonstrated by optical and confocal microscopy. Arterial rings with or without adventitia and with or without endothelium were mounted in an organ bath for isometric tension recording. Responses to 75 mM KCl or norepinephrine (0.1 nM–1 mM) were significantly reduced in segments without adventitia. Acetylcholine-induced relaxation (0.1 μM–0.1 mM) was enhanced in arteries without adventitia, whereas sodium nitroprusside-induced responses were not modified. These results demonstrate that the combination of stripping with a previous collagenase treatment allows us to obtain functional rings devoid of adventitia and that this layer plays a role in contractile capacity and in endothelium-modulated responses.

vascular layers; carotid arteries; confocal microscopy; endothelium

BLOOD VESSEL WALL is composed of three layers: adventitia, media, and intima. The media, formed by smooth muscle cells (SMC) and elastic lamella, is responsible for vasomotor function, which is modulated by contractile and relaxant factors released by the endothelium (10) and by the adventitia.

The now well-known influence of the endothelium on vascular function has been mainly assessed in the last two decades. Before, it was considered mainly a barrier between the media and the circulating blood. It was not until the development of a successful and easy method to remove the endothelium that the role of this layer on vasomotor function could be extensively studied (5).

Adventitia is a very complex layer formed by different types of fibers, cells, and nerve endings. It is a structural support for the media, and its main physiological role known, until now, is that mediated by innervation (11). In pathological conditions, where vascular function is altered, structural and biochemical changes are observed in the adventitia (for a review, see Ref. 7). In a model of neointima formation, the adventitia remodels, increasing in thickness and changing the phenotype of adventitia fibroblasts to myofibroblasts (12, 13). Similarly, adventitia remodeling has been reported in arteries from hypertensive rats, where increases in thickness and adventitia cell number were observed (2). In addition, biochemical changes have been described in the adventitia during septic shock, where the marked elevation in nitric oxide contributing to cardiovascular failure seemed to be due to the induction of inducible nitric oxide synthase recently shown in this vascular layer (14).

In view of these structural and biochemical alterations of the adventitia, the question is: does adventitia influence vascular function and how? To answer this question, it is necessary to develop a method to remove the adventitia completely, without medial functional damage.

Previous attempts to remove adventitia by stripping have been made (1, 3, 8, 9). However, these methods do not remove adventitia completely. Kemler et al. (8) compared different methods of adventitia stripping in conduit arteries and demonstrated, with histological methods, that stripping alone did not result in complete removal of this layer. Moreover, the above-mentioned studies do not assess both structurally and functionally possible medial damage after adventitia removal.

This study has two aims. The first was to develop a simple method for removing the adventitia layer of conduit vessels that 1) allows for complete adventitia removal, 2) preserves medial function of vascular rings, and 3) is reproducible. The second was to analyze whether the adventitia contributes to vascular tone by means other than innervation. The establishment of this method would open a new field of research similar to the way endothelium removal...
Fig. 1. A: images from rat carotid arteries after different incubation times (5, 15, and 30 min) with collagenase. Images were taken with a Zeiss Axiosvert 25 inverted microscope with a ×5 objective. Arrow indicates point of medial rupture. B and C: images from rat carotid arteries taken with a confocal microscope (model MRC 1024, Bio-Rad with Nikon microscope, ×20 air objective; numerical aperture 0.45; image size 475 × 475 μm). Arteries were stained with the nuclear dye propidium iodide (PI) and visualized with the 488/515-nm (elastin autofluorescence, green) and 488/615-nm (PI fluorescence, red) wavelengths. B: rings of paraformaldehyde-fixed arteries. Left, artery with adventitia (+A). Right, artery without adventitia (−A). C: longitudinal sections of unfixed arteries. Left, external elastic lamina of an adventitial-denuded artery. Right, internal elastic lamina of an endothelium-denuded artery. Dead cells are shown in red (arrows show some dead cells). L, lumen; M, media; A, adventitia.

Fig. 2. Original recording of the effect of transmural nerve stimulation (TNS) on iliac +A (A) and −A (B) arteries after contraction with 75 mM KCl. Contraction is expressed in milligrams; n = 6 rats. W, washout.
enabled determination of the functional contribution of the intima.

MATERIALS AND METHODS

Three-month-old rats (Harlan Sprague Dawley under specific pathogen-free conditions; \( n = 25 \); weight 350–400 g) were used. All experimental procedures were approved by the Institutional Animal Care and Use Committee according to the guidelines for ethical care of experimental animals of the European Community.

Rats were anesthetized with pentobarbital sodium (50 mg/kg) and bled by cardiac puncture. Carotid and iliac arteries were carefully isolated, placed in oxygenated physiological salt solution (PSS), and cleaned of blood and perivascular fat. PSS was composed of the following: 115 mM NaCl, 4.6 mM KCl, 2.5 mM CaCl\(_2\), 25 mM NaHCO\(_3\), 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 0.01 mM EDTA, 11 mM glucose, 0.8 \( \mu \)M dexamethasone, and 5 \( \mu \)M indomethacin.

Adventitia removal. Arteries were placed in a shaking bath at 37°C for 15 min in PSS containing 2 mg/ml collagenase type II (chlostridiopeptidase A; EC 3.4.24.3). Thereafter, vessels were immediately rinsed and placed for 10 min in PSS at 4°C. The arteries were then fixed with pins at both ends to a Sylgard-based dissecting dish containing cold PSS, and the adventitia was carefully removed by gentle peeling with two pairs of fine forceps under a dissecting microscope. Control arteries with adventitia (+A) were submitted to the same steps and temperature changes as arteries without adventitia (−A). This protocol was established after testing several incubation times with collagenase and cold PSS. In some arteries (both +A and −A), the endothelium was removed (−E). This was performed by gentle scraping with a cotton thread through the vessel lumen. In −A segments, endothelium removal was always performed after incubation in collagenase; otherwise, the media would be seriously damaged.

Histology. Optical and confocal microscopy were used to assess the degree of adventitia removal and to determine histologically possible SMC and external elastic lamina damage.

The degree of adventitia removal was tested at different time points after incubation with collagenase (5, 15, or 30 min). After adventitia peeling, the segments were visualized mounted on a slide with a Zeiss Axiovert 25 inverted microscope with a ×5 objective. This protocol allowed us to determine the optimal time of incubation with collagenase.

The degree of arterial damage was tested with confocal microscopy. +A or −A segments of carotid artery (not used for functional experiments) were fixed in 4% formaldehyde solution for 2 h. Thereafter, the segments were incubated for 15 min in a PSS solution containing 0.1 \( \mu \)M propidium iodide (PI), a nonpermeable fluorescent nuclear dye, and washed three times in PSS. Rings of ∼50 \( \mu \)m were cut and mounted on slides for visualization with a laser scanning confocal microscope (model MRC 1024, Bio-Rad) coupled to a Nikon microscope with a ×20 air objective [numerical aperture 0.45]. PI stains nuclei of all vascular cells (2) that can be visualized using the 488/615-nm line of the microscope. Elastic lamella were visualized simultaneously with the 488/

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Fig. 3. A: effect of 75 mM KCl at different resting tensions in +A and −A segments; \( n = 6 \) rats. B: effect of different KCl concentrations in +A and −A segments; \( n = 6 \) rats. C: effect of 75 mM KCl on carotid arteries after stretching, collagenase incubation for 15 min and adventitia peeling (−A). Contraction is expressed in milligrams as means ± SE; \( n = 6 \) rats. *\( P < 0.05 \) with respect to +A or control. #\( P < 0.05 \) with respect to 1.5 g.
515-nm line due to elastine autofluorescence at this wavelength.

To determine the degree of SMC and external elastic lamella damage after adventitia removal, longitudinal sections of intact and adventitia-denuded rat carotid arteries were incubated without fixation in PI-containing PSS solution following the above-described protocol. The sections were mounted on slides with the adventitia side up and visualized with a 20X20 air objective at the above-mentioned wavelengths. To determine the degree of SMC and internal elastic lamina damage after endothelium removal, the same protocol was performed, and the arteries were visualized with the endothelium side up with a 20X20 air objective.

Functional studies. To determine vascular function, 3-mm-long carotid artery rings were suspended on two intraluminal parallel wires, introduced in an organ bath containing PSS, and connected to a Piodem strain gauge for isometric tension recording. A set of experiments was first performed to establish the optimal resting tension of rat +A and −A carotid arteries. Thus arterial rings were submitted to different tensions between 0.5 and 2 g. This tension was readjusted every 15 min during a 90-min equilibration period. After the equilibration period, the vessels were exposed to 75 mM KCl to check their contractility.

The segments were set to the optimal resting tension and responses to agonists were tested as follows: single doses of KCl (25, 50, and 75 mM), and concentration–response curves to NE (0.1 nM–1 µM), acetylcholine (ACh, 1 nM–0.1 mM), and sodium nitroprusside (SNP, 0.1 nM–0.01 mM). The responses to the vasodilator agents ACh and SNP were tested on vessels precontracted with 0.1 µM NE. Segments with more than 60% relaxation to 0.1 mM ACh were considered with endothelium (+E) and segments with <10% relaxation to 0.1 mM ACh were considered endothelium free (−E).

Both +A and −A carotid and iliac artery segments were submitted to transmural nerve stimulation (TNS; 200 mA, 0.2 ms, 30 s at 0–32 Hz) in addition to the previous protocol.

Analysis of data. Contractions are expressed as the percentage of contraction produced by 75 mM KCl. Relaxations are expressed as the percentage of contraction induced by 0.1 µM NE. The half-maximal effective concentration (EC50) was calculated choosing as maximal response (Emax) the maximum value of the control curve (+A/+E). Statistical significance was analyzed by one-way ANOVA followed by the Newman-Keuls test or Student’s t-test. P < 0.05 was considered significant.

RESULTS

Assessment of adventitia removal. Adventitia removal was confirmed histologically by optical microscopy. Denudation was dependent on the collagenase incubation time (Fig. 1A). Incubation periods in collagenase solution shorter than 15 min did not allow an adequate and complete removal of the adventitia. Incubation periods longer than 15 min produced artey rupture on peeling (Fig. 1A) and impaired vascular function. In addition, periods in cold PSS shorter or longer than 10 min made the process of peeling difficult, inducing medial damage (results not shown).

Adventitia removal was also confirmed by confocal microscopy after PI staining (Fig. 1, B and C). Rings +A showed stain of SMC and adventitia cells (PI fluorescence, red) and elastic lamella (elastine autofluorescence, green). In −A rings, adventitia had been completely removed, as shown by the lack of red fluorescence in the outer part of the ring.

In addition, confocal microscopy was useful for analyzing vascular damage. Adventitia removal does not damage external elastic lamella and SMC underneath, as shown by confocal images of unfixed arteries (Fig. 1C, left). Only dead or broken cells exhibit staining with PI. The degree of cell death was similar, or even smaller, to that produced by mechanical endothelium denudation (Fig. 1C, right).

Adventitia removal was confirmed functionally by analyzing the response of iliac arteries to TNS. TNS-induced frequency-dependent contractions in iliac arteries were abolished in −A arteries (Fig. 2). TNS produced no modification of vascular tone on carotid artery segments.

Effect of adventitia removal on responses to vasoconstrictor agents. Responses to 75 mM KCl were analyzed in carotid arteries in tensions ranging 0.5–2.0 g. Arteries (+A) elicited a similar maximal contraction at resting tension values between 1 and 2 g. Arteries (−A) showed a maximal contraction at 1.5 g and the contractile response was significantly reduced at 2 g (Fig. 3A). An optimal resting tension of 1.5 g was chosen for subsequent experiments.

The effect of different KCl concentrations was analyzed in +A and −A arteries. KCl-induced contractions were significantly lower in −A arteries at all concentrations studied (Fig. 3B).

To analyze whether the reduction in response to KCl was due to enzymatic treatment, several segments were incubated with collagenase without removing the...
Table 1. $E_{\text{max}}$ and EC$_{50}$ values for ACh-induced relaxation and norepinephrine-induced contraction in rat carotid arteries with and without adventitia and with and without endothelium

<table>
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<tr>
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<th>Norepinephrine</th>
<th>ACh</th>
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<tr>
<td></td>
<td>$E_{\text{max}}$</td>
<td>EC$_{50}$ nM</td>
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<tr>
<td>+A +E</td>
<td>90.5 ± 6.3</td>
<td>6.6(3.8, 11)</td>
</tr>
<tr>
<td>-A +E</td>
<td>65 ± 5.2*</td>
<td>21(4.6, 100)</td>
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<tr>
<td>+A -E</td>
<td>117.5 ± 7.1</td>
<td>2(1.1, 3.6)*</td>
</tr>
<tr>
<td>-A -E</td>
<td>108.7 ± 6.4</td>
<td>3.1(1.3, 7.1)</td>
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Values are means ± SE. Norepinephrine maximal response ($E_{\text{max}}$) values are expressed as percentage of contraction with 75 mM KCl. ACh $E_{\text{max}}$ values are expressed as percentage of relaxation of a previous contraction with 0.1 mM norepinephrine. EC$_{50}$ values are expressed as geometric means with confidence limits. +A, with adventitia; -A, without adventitia; +E, with endothelium; -E, without endothelium. *$P < 0.05$ compared with +A +E arteries.

The adventitia is a very complex layer formed by different types of fibers, cells, and nerve endings. A well-known physiological role of the adventitia is that mediated by innervation (11). To study vascular reactivity in vessels lacking adventitia, it was necessary for us to exclude possible interferences of innervation on vascular function. We have, therefore, chosen the rat common carotid artery as an artery lacking functional innervation, and we compared the results with a well-innervated vessel, like the rat iliac artery (6).

Kemler et al. (8) compared different methods of adventitia stripping in conduit arteries and demonstrated histologically that stripping alone did not result in a complete removal of this layer. In the present work, the combination of stripping with a previous collagenase treatment allowed complete removal of the adventitia, as confirmed histologically by optical and confocal microscopy in carotid arteries and functionally by the abolishment of the response to TNS (but not to KCl) in iliac arteries.

Because sympathetic fibers are harbored within the adventitia, removal of this layer would interrupt sympathetic modulation of vascular tone. In fact, in densely innervated iliac arteries, TNS-induced frequency-dependent contractions were abolished after removal of the adventitia (6). We suggest that this approach may serve as an easy confirmation of adventitia removal in innervated vessels.

Carotid arteries exhibited diminished contractions to KCl after removal of the adventitia. One possibility was that removal of the adventitia might induce a shift of the optimal resting tension. This was excluded because we set the vessels at 1.5 g, which was the optimal resting tension for both +A and -A vessels. However, we found that in +A segments the optimal contraction was maintained at 2 g, whereas in -A segments contraction dropped at this tension. This might be due to a change in mechanical properties of the vessels after adventitia removal. A second possible explanation for the reduction in contractility in -A vessels is that manipulation and/or collagenase treatment could be impairing contractile function, although there seemed

Fig. 5. A: effect of acetylcholine (ACh) on carotid arteries with (+A) and without (-A) adventitia in the presence or absence of endothelium. $E_{\text{max}}$ and EC$_{50}$ values are shown in Table 1. B: effect of sodium nitroprusside (SNP) on carotid arteries with (+A) and without (-A) adventitia. Relaxation is expressed in % of a previous contraction to 0.1 μM NE. Results are means ± SE; n = 13–23 rats. *$P < 0.05$ with respect to control.

**DISCUSSION**

We developed an enzymatic method for removing the adventitia layer of conduit vessels that 1) allows complete adventitia removal, 2) preserves medial function of vascular rings, and 3) is reproducible in different vessels. The present work also demonstrates that removal of the adventitia reduces contractile capacity and potentiates endothelium function in rat carotid arteries.
to be no histological damage. However, neither incubation with collagenase nor stretching of the vessels modified responses to KCl. Another indication that the smooth muscle layer was well preserved after adventitia removal was the fact that the relaxation to SNP was not different between +A and −A vessels. These results suggest that manipulation and collagenase treatment for adventitia removal do not impair smooth muscle functionality. We suggest that differences in contractility observed in adventitia-denuded rings might be due to a possible influence of the adventitia on vascular function and has to be further studied.

The possible interrelationship between endothelium and adventitia on vascular function was analyzed using +E and −E arteries in addition to adventitia removal. Sensitivity to ACh was higher in −A carotid arteries, suggesting first, that collagenase treatment did not affect the endothelium layer and second, that endothelium-dependent relaxations might be affected by the presence of adventitia. In addition, in −A/+E segments, the concentration-response curve to NE was reduced in $E_{\text{max}}$. Removal of the endothelium significantly increased responses to NE, as previously described for several vasoconstrictors in numerous vascular beds after endothelium removal (10) in both +A and −A carotid arteries. It is interesting to note that endothelium removal abolished the differences in $E_{\text{max}}$ observed between +A and −A arteries. These results suggest that in the absence of adventitia, endothelium function is potentiated. In fact, it has been suggested that adventitia-derived superoxide anions might shorten the half-life of nitric oxide (4).

In conclusion, we developed an enzymatic method for removing the adventitia layer of conduit vessels, which is reproducible in different vessels and preserves the contractile and relaxant function of the smooth muscle layer. This method will allow to study in more detail the influence of this layer on vascular function.

We are grateful to Dr. C. Fernández Criado for the care of the animals and to P. Arnaiz Sánchez for technical assistance.

This work was supported by Fondo de Investigaciones Sanitarias Grant 98/0736.

REFERENCES