Analytical Biochemistry 499 (2016) 71–77

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Adventitial ablation technique that permits the assessment of adventitial-dependent contribution to microvascular contractile function

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ARTICLE INFO

Article history:
Received 30 September 2015
Received in revised form
7 January 2016
Accepted 11 January 2016
Available online 25 January 2016

Keywords:
Microcirculation
Adventitia
Vasoconstriction
Vasorelaxation
Pressure autoregulation

ABSTRACT

Resistance arteries have been implicated as a major contributing factor in the sequela of disease conditions such as hypertension and diabetes and, as such, are a major focus of cardiovascular research. The paracrine influence of the intimal endothelial layer of resistance arteries is well established. Considering the growing body of evidence substantiating a functionally relevant vascular adventitia, in this study we have established a technique that permits determination of the functional influence of the adventitial layer on resistance artery tone. Isolating adventitial-dependent function, analogous to isolating endothelial function, has potentially significant implications for studying the as yet unexplored role of the microvascular adventitial layer in modulating acute vascular contractile function.

Blood vessels comprise three anatomically and physiologically distinct layers: the intimal endothelium, the medial vascular smooth muscle, and the outer adventitial layer. To date, vascular research has focused largely on the endothelium and smooth muscle-dependent function and the interplay between both layers in the regulation of vascular tone in health and disease [1]. Historically, the adventitial layer has been considered as a passive encapsulating structure containing perivascular sympathetic nerves and in large blood vessels a vasa vasorum. However, an increasing number of studies have demonstrated a distinct physiological role for the adventitia [2]. Studies have demonstrated that the adventitia in large caliber arteries plays a part in atherogenesis and more recently as a modulator of agonist-dependent responses [3,4]. Moreover, the adventitial layer has been proposed to be potentially crucial in vascular remodeling phenomena and has intriguingly been reported as the “first responder” during early vascular disease development [4]. Many studies have documented structural adaptation of the medial vascular smooth muscle layer in disease conditions; however, the adventitial layer similarly undergoes structural adaptation [1,5]. Human subcutaneous and skeletal muscle resistance arteries have undergone hypotrophic remodeling, with the greatest structural loss being observed within the adventitial layer [6]. Similar to large caliber arteries, it is feasible that adventitial-derived signaling molecules such as reactive oxygen species may modulate underlying vascular smooth muscle contractile function [2]. Excluding trans-nerve stimulation studies, there are no other studies focusing on specific adventitial-derived cell signaling intermediaries in small resistance arteries. Considering the importance of small resistance artery function in health and disease and the growing body of evidence pointing toward a likely significant role of the adventitial layer as an important contributor to both acute (contractile) and chronic (structural) vascular function in larger caliber arteries, the development of a method for isolating vascular adventitial-dependent function analogous to endothelial removal in small resistance arteries would permit the advancement of focused adventitial-dependent microvascular research. Thus, the aim of this study was to establish a reliable and reproducible method of functionally isolating adventitial-dependent function in small resistance arteries.

Abbreviations used: MCA, middle cerebral artery; PSS, physiological saline solution; FBS, formaldehyde-buffered saline; TNS, trans-nerve stimulation; ROS, reactive oxygen species; DETCA, diethyldithiocarbamate; RLU, relative light units; ANOVA, analysis of variance.

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http://dx.doi.org/10.1016/j.ab.2016.01.009
0003-2697/

Materials and methods

Isolation of rat middle cerebral arteries

All procedures were performed under the U.K. Animals (Scientific Procedures) Act of 1986 and were approved by the ethical review committee of the University of Strathclyde. Adult male Sprague–Dawley rats (12 weeks, 250–300 g) were killed by cervical dislocation. The brain was removed, and the middle cerebral arteries (MCAs; outer diameter = 170 ± 2.8 μm) were dissected from surrounding connective tissue under a dissection microscope (10 × /22; model SSZ, WPI, UK) and placed in ice-cold physiological saline solution (PSS).

Adventitial removal following collagenase digest

Following isolation, both ends of resistance arteries (5-mm lengths) were closed by tying sutures around the ends to protect the luminal compartment. Arteries were placed in Eppendorf tubes filled with Krebs solution containing collagenase type II in a 37 °C shaking water bath [3]. The protocol was repeated with incubation times of 5, 7.5, 10, and 15 min and collagenase concentrations of 1, 1.5, and 2 mg ml^{-1}. Following collagenase exposure, arteries were immediately rinsed with ice-cold PSS. The arteries were then pinned at both ends to a Sylgard-based dissecting dish, and the adventitia was carefully removed by gentle stripping with Dumont no. 5 fine forceps (WPI) under a dissecting microscope.

Adventitial ablation by paraformaldehyde exposure

As above, both ends of the resistance artery were tied off. The adventitial layer was ablated by dipping the artery in 4% formaldehyde-buffered saline (FBS) for 5 s and then immediately rinsed in ice-cold PSS. In this study, arteries undergoing treatment to remove or ablate the adventitial layer are referred to as ADV− (experimental), and intact adventitial arteries are referred to as ADV+ (control). As a control measure, ADV+ arteries were exposed to an identical processing method as ADV− arteries, with the only difference being that ADV+ arteries were dipped in PSS as opposed to 4% FBS.

Functional confirmation of resistance artery function

Successful removal/ablation of the adventitia was immediately measured by trans-nerve stimulation (TNS) of adventitial perivascular nerve-dependent modulation of vascular tone. Disruption of adventitial-dependent function was confirmed by loss of TNS-dependent contraction (1–16 Hz/50–130 v) and loss of TNS/nicotine-dependent relaxation (in the presence of guanethidine).

Resistance artery function: wire myography and TNS

Arterioles were bathed in PSS and gassed with 20% O_2/5% CO_2/75% nitrogen maintaining pH 7.4 at 37 °C. MCAs were mounted on a Danish MyoTech 4 channel myograph model 410P (Aarhus, Denmark) using the standard technique [7]. Recordings of isometric tension/force generated by the arteries were measured using an ADI PowerLab 4/25 bridge amplifier/digitizer (Chalgrove, UK) and intercept chart software. Two electrodes were placed on either side of wire myograph mounted arteries. TNS was applied using a S44D Grass stimulator (Grass Technologies, Slough, UK) with the following parameters: 40–130 V, 0.3–0.5 ms pulse duration, and increasing frequency (1, 2, 4, 8, and 16 Hz). The neurogenic nature of the TNS response was confirmed following 30 min of incubation with the postganglionic sympathetic nerve terminal blocker guanethidine (10 μM).

Resistance artery function: pressure myography

Isolated arterioles were studied using previously described techniques [6]. Briefly, arterioles were mounted on a Danish MyoTech P110 pressure myograph system and secured with two 17-μM nylon sutures to size-matched micro-cannulas. Pressure-independent responses to contractile agonists were measured at an intraluminal pressure of 40 mmHg. Pressure-dependent myogenic responses were studied by increasing intraluminal pressure from 40 to 120 mmHg in 40-mmHg steps for a period of 5 min at each pressure step. Myogenic tone (%) was calculated using the formula

$$\text{LD } Ca^{2+} \text{ free PSS} - \text{LD } Ca^{2+} \text{ PSS/LD } Ca^{2+} \text{ free} \times 100,$$

where LD Ca^{2+} is the lumen diameter in the presence of Ca^{2+} and LD Ca^{2+} free is the lumen diameter in the absence of Ca^{2+}.

Chemiluminescence measurements of ROS

Reactive oxygen species (ROS) produced from MCAs was measured by superoxide production by control and collared carotid artery ring segments measured using 5 μmol/L lucigenin-enhanced chemiluminescence. Vessel segments (dry weight = 10 ± 0.5 μg) were preincubated in PSS at 37 °C with $NADH^+$ (substrate for Nox, 100 μM) and diethyldithiocarbamate (DETCA, 10 mM), a superoxide dismutase inhibitor, for 30 min. Arteries were transferred into Krebs–Hepes buffer containing angiotensin II (0.1 μM), $NADH^+$ (100 μM), and lucigenin in plastic cuvettes. Luminescence was measured in relative light units per second (RLU/s) every 10 s over a period of 200 s using a Sirius L luminometer (Sirius, Germany).

Assessment of vascular mechanics

Circumferential stress (σ), force generated per unit area of the arteriole wall thickness (WT) as a product of intraluminal pressure (IP), was calculated using the formula

$$\sigma = (\Delta \text{IP} r_i)/(2\lambda \text{WT}),$$

where ΔP is the transmural pressure difference and r_i is the luminal radius at a given IP. Circumferential strain (ε), fractional changes in lumen diameter (LD) as a product of pressure, was calculated from arteriole lumen diameter measurements using the formula

$$\varepsilon = (\text{LD} - D_o)/D_o,$$

where D_o is the original diameter and 5 mmHg was used as the original diameter in the calculation of ε. To determine the passive stiffness (resistance to pressure-dependent stretch) of the artery under study, the stress–strain data for individual arteries were fitted to an exponential curve ($y = ae^{bx}$) to obtain the tangential elastic modulus

$$E = \sigma_{orig} e^{\beta\varepsilon},$$

where σ_{orig} is the stress at the original diameter (5 mmHg) and β is the slope of the tangential elastic modulus.