A New Protocol for Removal of the Endothelium From the Perfused Rat Hind-Limb Preparation

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A new protocol has been developed for selective removal of the endothelium from the arteries of the perfused rat hind-limb preparation. The hind limb was perfused with oxygenated Krebs-Ringer bicarbonate solution with a combination of high flow and timed air bubbles (2 minutes at high flow followed by 5 minutes of air bubbles, followed by a further 2 minutes at high flow). Representative vessels at different diameters of the arterial tree—the femoral artery, the artery supplying the extensor hallucis proprius muscle, and the arteries, arterioles, and capillaries within the hallucis proprius muscle—were taken for examination by transmission and scanning electron microscopy. A graded degree of damage occurred along the length of the hind-limb vasculature, with endothelial cells having been completely removed from the femoral artery, removed from or severely damaged in the artery supplying the hallucis proprius muscle, and partially damaged in arteries within the hallucis proprius muscle. However, no damage occurred to the endothelial cells of the arterioles and capillaries within the hallucis proprius muscle. The integrity of the vascular bed after endothelial removal according to the above protocol was confirmed by the demonstration of no diminution, but in fact an increase, in contractile responses to bolus injections of α,β-methylene ATP, resulting in a shift to the left of the dose-response curve. The viability was further confirmed by the fact that the conductance (flow/perfusion pressure) of the preparation during the periods of high flow was no different before and after removal of the endothelium. In conclusion, the high flow/air bubbles/high flow protocol can be used to selectively remove endothelial cells from the arteries of the rat hind-limb preparation without damage to the endothelium of arterioles and capillaries or to smooth muscle cells. (Circulation Research 1989;64:1190–1196)

Numerous techniques have been used to remove the endothelium from isolated vessels and from isolated vascular beds in vitro. These techniques encompass a broad range of mechanical, physical, chemical, and enzymatic methods such as rubbing, laser irradiation, exposure to 40 mM KCl, exposure to collagenase, air drying, or perfusion with detergents such as saponin and sodium deoxycholate. To our knowledge, there have been no reports of removal of the endothelium from an in vivo vascular bed such as the perfused rat hindquarters. In this study, we demonstrate that perfusion with flow approximately three times the basic level before and after a period of perfusion with controlled air bubbles can selectively remove the endothelium from the rat hind-limb arteries, while leaving it intact in arterioles and capillaries. This method can be used to provide a useful model to study the vascular responses of an in vivo preparation with and without endothelium in the conduit arteries.

Materials and Methods

Thirteen male Wistar rats (300–400 g) were anesthetized by injection with sodium pentobarbitone (60 mg/kg i.p., diluted 1:1 with saline). The right jugular vein was cannulated for administration of heparin, and the animal’s blood pressure was monitored by a pressure transducer (P23, Gould Instruments, Cleveland, Ohio) from a cannula to the right carotid artery. The trachea was also cannulated. The abdomen was opened, and the left renal artery and vein and the vessels supplying the body wall, the scrotum, and all visible arterial and venous side branches in the pelvic and abdominal region were ligated. After intravenous administration of heparin

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(1,000 units), the aorta was cannulated to allow perfusion of the hindquarters and the vena cava was cannulated to direct outflow away from the animal. The hindquarters were initially flushed with 0.9% saline containing heparin (100 units/ml) and then perfused with a regulated pulsatile flow by a Watson-Marlow pump, with a Krebs-Ringer–bicarbonate solution (gassed with 95% O2–5% CO2 and maintained at 37°C) containing (mM) NaCl 118, KCl 4.7, KH2PO4 1.2, MgSO4 • 7H2O 1.2, NaHCO3 25.0, d-glucose 5.5, CaCl2 • 2H2O 2.5, with added bovine serum albumin (10 g/l) and insulin (10 units/l) to improve the condition of the preparation. Perfusion pressure was measured by a pressure transducer from a side arm of the inflow cannula and was matched so as not to exceed the animal’s blood pressure.

To remove the endothelium, a protocol of high flow/air bubbles/high flow was produced by initially increasing the output of the pump rapidly so as to treble resting flow for 2 minutes. This was followed by the introduction of air into the perfusion line as a bubble over 5 seconds by allowing air to be drawn into the perfusion line; this procedure was repeated at 10-second intervals for a total duration of 5 minutes. The air treatment was followed by another 2-minute period of high flow. The whole high flow/air bubbles/high flow procedure was completed within about 10 minutes.

The viability of the rat hind-limb vasculature before and after removal of the endothelium was investigated using the vasoconstrictor agent αβ-methylene ATP. Bolus injections of αβ-methylene ATP (100 µl injections of 3 µM to 1 mM) were given via an injection port of neoprene rubber tubing proximal to the aorta. Responses were measured as changes in perfusion pressure (mm Hg). Data for the dose-response curves were plotted as the means (±SEM) of the responses at each dose. Statistical significance was evaluated using the Student’s t test (p<0.05 was taken to be significant).

The femoral artery, the artery supplying the extensor hallucis proprius muscle, and the extensor hallucis proprius muscle were excised from six control and seven experimental animals and immersed in 3% glutaraldehyde in 0.1 M phosphate buffer. This muscle was chosen because it can be fixed in its entire cross section; it weighs typically about 20 mg,7 and the supplying artery can be followed before and immediately after its penetration into the tissue. During fixation (2 hours), arteries were cleaned and cut into blocks. After washing in several changes of 0.1 M phosphate buffer, the blocks were postfixed in 2% OsO4, dehydrated in ascending grades of alcohol, and embedded in spurr resin for electron microscopy. The ultrathin sections were stained with lead citrate and subsequently examined with a Philips 301 electron microscope (Eindhoven, The Netherlands).

Femoral arteries of two experimental animals were examined with scanning electron microscopy. After dehydration, specimens were immersed in amyl acetate before critical point drying and examined in a Hitachi S-530 scanning electron microscope (Reading, England).

### Results

The basal flow through the rat hind-limb vasculature was 7–14 ml/min, with a perfusion pressure of 25–60 mm Hg. A threefold increase in flow was accompanied by a less than threefold increase in the perfusion pressure, and this was reflected in an increase in conductance of the rat hind-limb preparation, indicating a good autoregulation of the whole vascular bed. (A purely passive response to high flow would have resulted in a similar conductance.)

No significant differences were found between comparable values of flow rate, perfusion pressure, and conductance (flow/perfusion pressure) of the vascular bed, either at basal or at high flows, before and after introduction of air (see Table 1). During perfusion with air, the peak rise in perfusion pressure was 141.7±10.03 (n=9).

The P2-purinoceptor agonist, αβ-methylene ATP (100 µl injections of 3 µM to 1 mM), which acts directly on smooth muscle, produced dose-dependent contractions of the rat hind-limb vasculature detected as increases in the perfusion pressure (Figure 1a). Removal of the endothelium greatly potentiated the magnitude of contractions to equivalent doses of αβ-methylene ATP as evidenced by a shift to the left of the dose-response curve (Figure 1b). Thus, the ability of the vascular smooth muscle to contract in response to an agent acting specifi-
Femoral Artery

The endothelial cells of this artery were either completely removed (Figure 2b) or, when still present, totally destroyed (Figure 2c): their cytoplasm contained vacuoles of various sizes, the plasmalemma was disrupted, and usually a nucleus with remnants of the cytoplasm protruded into the lumen. There was no evidence of undamaged endothelial cells. Due to contraction of the artery, the lamina elastica interna was thrown into folds; fragments of endothelial cells with a more preserved structure were found in these folds.

The intercellular matrix under the endothelial cells was destroyed, but the lamina elastica interna was well preserved in all experiments. There were no detectable changes of the smooth muscle cells or of the finger-like processes of the inner layer of these cells penetrating into the holes in the lamina elastica interna (Figure 2c).

Scanning electron microscopic examination of the femoral artery showed a well preserved lamina elastica interna in all vessels (Figure 3a). The subendothelial matrix and what are probably finger-like protrusions of the innermost layer of smooth muscle cells could be seen in the fenestrae of the lamina elastica interna (Figure 3b).

Artery Supplying the Extensor Hallucis Proprius Muscle

The endothelial cells of this artery outside the muscle were damaged in a similar way to those in the femoral artery, but the damage was less extensive. In some cases, large areas of the artery were completely denuded of endothelial cells (Figure 4a) and those that remained were severely damaged. There were no detectable morphological changes of the lamina elastica interna or smooth muscle cells of the tunica media.

Arteries Within the Extensor Hallucis Proprius Muscle

The wall of the arteries was composed of more than one layer of smooth muscle cells, the number of layers depending on which part of the muscle was taken for investigation. The endothelial cells of these arteries showed the smallest changes of all three segments studied (Figure 4b). The entire lumen was always covered with endothelial cells, and these showed different degrees of damage. Some of the endothelial cells were completely destroyed in a similar manner to those in vessels of larger diameter, while others were without detectable ultrastructural changes. The lamina elastica interna, present as patches of elastin, and the smooth muscle cells of the tunica media were without morphological changes.

Arterioles and Capillaries of the Extensor Hallucis Proprius Muscle

All arterioles (i.e., vessels with only one layer of smooth muscle cells) showed no detectable ultrastruc-
FIGURE 2. 
a: Femoral artery of the control rat after surgical preparation of the hind limb. Endothelial cells (EC) showing well-preserved ultrastructure. A protrusion (P) of smooth muscle penetrates into the fenestra of the lamina elastica interna (I). Calibration bar, 2 μm. b: Femoral artery after high flow/air treatment. The lamina elastica interna (I) is completely denuded of endothelial cells. On its surface there are remnants of intercellular matrix (arrow). The whole lamina elastica interna is without morphological changes. M, smooth muscle cells of the innermost layer of the tunica media. Calibration bar, 5 μm. c: Femoral artery after experimental conditions. Endothelial cells (EC) are completely destroyed. Relatively well-preserved cytoplasm occurs in folds of the lamina elastica interna (I). M, smooth muscle cells with finger-like processes (arrow). Calibration bar, 5 μm.
FIGURE 3. **Two parts of the same femoral artery after experimental conditions, with (a) and without (b) folds of the lamina elastica interna.** In all places endothelial cells were completely removed. Some holes in the lamina elastica interna are probably filled with protrusions (P) of the innermost layer of smooth muscle cells. Calibration bars, 50 μm (a), 20 μm (b).

Discussion

This study describes a new protocol for the removal of endothelial cells from the perfused rat hind-limb preparation by a combination of high flow together with a controlled application of air. High flow alone did not produce any obvious ultrastructural changes. Ultrastructural analysis of the femoral artery, the artery supplying the extensor hallucis proprius muscle, and the arteries within the hallucis proprius muscle after the high flow/air bubbles/high flow procedure demonstrated that this regime successfully removed or severely damaged endothelial cells of the arteries along the vascular tree, to the level of arteries composed of more than one layer of smooth muscle cells. The degree of damage was graded along the vascular tree according to the size of the vessels, with greatest damage always occurring in the largest arteries.

In spite of the destruction or complete removal of endothelial cells, the other components of the arterial wall were well preserved. As seen by transmission and especially by scanning electron microscopy, in the femoral artery and the artery supplying the hallucis proprius muscle the lamina elastica interna partially isolates the inner layer of smooth muscle cells. Its function is probably retained even after removal of endothelial cells: transmission electron microscopic examination of the tunica media of the smooth muscle cells revealed no signs of damage. The integrity of the vascular smooth muscle was further verified by measurement of conductances before and after removal of the endothelium, as well as from in vivo responses to the vasoconstrictor α,β-methylene ATP. The whole vascular bed showed a good degree of autoregulation in response to high flow, and this was not affected by the removal of the endothelium from the arteries.

The vasoconstrictor agent α,β-methylene ATP was used to investigate the viability of the rat hind-limb preparation before and after loss of the endothelium, since it acts directly on the smooth muscle via P2X-purinoceptors. Removal of the endothelium resulted in a large increase in evoked contractile responses to equivalent doses of α,β-methylene ATP with an overall shift to the left of the dose-response curve, thus demonstrating that the hind-limb preparation was still viable after using the high flow/air bubbles/high flow procedure. Furthermore, this suggests that the endothelium had indeed been removed, as it has previously been observed that removal of the endothelium in vitro may enhance the responsiveness of vessels to α,β-methylene ATP and to other contractile agents. This may be due to the removal of a diffusion barrier to α,β-methylene ATP. Alternatively, endo-
**FIGURE 4.** a: Artery supplying extensor hallucis proprius muscle after experimental conditions. I, lamina elastica interna; M, smooth muscle of tunica media. Calibration bar, 5 μm. b: Artery of extensor hallucis proprius muscle after experimental conditions. Some of the endothelial cells are severely damaged (arrow); others are without morphological changes (arrowhead). Lamina elastica interna is present as patches of elastin (*). Two layers of smooth muscle are present (M). TA, tunica adventitia. Calibration bar, 5 μm. c: Arteriole of extensor hallucis proprius muscle after experimental conditions. All parts of the arteriole are without morphological changes. EC, endothelial cells. Calibration bar, 5 μm. d: Capillary of extensor hallucis proprius muscle after experimental conditions. Endothelial cell (EC) is without morphological changes. Calibration bar, 2 μm.
The present experiments indicate that loss of the endothelium from larger arteries can considerably change peripheral resistance in response to vasoconstrictor stimuli. On the other hand, if the endothelium is in any way involved in the autoregulatory response, this response appears to be manifested within the small arteries and arterioles because, overall, the change in conductance in response to increased flow was not affected by endothelium removal.

Removal of the endothelium from isolated vessels and isolated vascular beds is now a routine procedure, and this is now extended to include removal of the endothelium from the perfused rat hindquarters in vivo. A technique of air drying has been used by other workers to remove the endothelium from an in vivo preparation, but this was confined to a part of a single vessel, the rat carotid artery, and relied on initial exposure of the vessel segment. We suggest that a procedure using a combination of high flow and air bubbles may be used to remove the endothelium from the rat hind limb and that this may be preferred in cases where further studies are to be carried out, since it relies neither on harsh abrasive methods nor on chemicals.

The perfused hindquarters provides a useful model to study vascular reactivity, and, in view of the increasing interest in endothelial cells, we suggest that the high flow/air bubbles/high flow technique of endothelium removal in the hindquarter preparation, and maybe in other vascular beds, will facilitate the study of the role of endothelial cells in blood flow regulation.

References


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