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Differential structural adaptation to haemodynamics along single rat cremaster arterioles

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We tested the hypothesis that under physiological conditions, arterioles match their diameter to the level of shear stress. Haemodynamic and anatomical data were obtained in segments of the first-order arteriole of the rat cremaster muscle. Along this segment of ~10 mm in length, local blood pressure decreased from 68 ± 4 mmHg upstream to 54 ± 3 mmHg downstream ($n = 5$). Pulse pressure decreased from 8.2 ± 1.3 mmHg upstream to 4.1 ± 0.6 mmHg downstream. At the same locations, an increase in arteriolar diameter was measured in vivo, from 179 ± 4 μ m upstream to 203 ± 4 μ m downstream ($n = 10$). In vitro pressure–diameter relations of maximally dilated vessels showed that the passive diameter was larger in downstream than upstream segments over a 15–125 mmHg pressure range ($n = 18$). The wall stress was similar for the upstream vs. downstream location: 266 ± 16 vs. 260 ± 14 mN mm⁻². However, shear stress decreased from 30 ± 5 to 21 ± 5 dyn cm⁻² (3.0 ± 0.5 to 2.1 ± 0.5 N m⁻²; $n = 4$) along the artery. In conclusion, these results demonstrate that shear stress is not the only factor in determining vascular calibre. We suggest that arteriolar calibre may rather depend on an interplay between shear stress and the local pressure profile.

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Shear stress is believed to be an important variable in vascular design. This concept is supported by the effect of experimental changes in blood flow, using occlusion or fistulas, on arterial diameter (Langille & O'Donnell, 1986; Unthank *et al.* 1996; Tulis *et al.* 1998; Buus *et al.* 2001) and by the notion that in branching systems vascular calibre is matched to the flow that is carried (Murray, 1926; Mayrovitz & Roy, 1983; Labarbera, 1990). However, the widening effect of shear stress could well be opposed by other mechanisms that cause inward remodelling. Some observations suggest that intravascular pressure forms such an opposing mechanism. Thus, essential hypertension is associated with inward arteriolar remodelling (Mulvany, 1990). Furthermore, using organoid culture of arterioles under zero flow, we found inward remodelling at physiological pressure but not at low pressure (Bakker *et al.* 2002). Finally, the low level of shear stress in veins and venules coincides with a low pressure level. In fact, Pries *et al.* (1995) speculated that throughout the circulation a unique relation between pressure and shear stress exists, reflecting the balance between effects of both mechanical stimuli on vascular structure.

The present study was designed to test whether shear stress can be considered as either the dominant controlling factor or even as the controlled variable in vessel design. If this were the case one would expect that a single, unbranched resistance vessel has a constant diameter along its length, since this is the natural consequence of having a constant flow and shear stress. If on the other hand single unbranched vessels have a substantial change in diameter along their length, other factors exist that form an opposing drive for the determination of diameter. Intravascular pressure could form such a drive. In this case, single resistance vessels that are long enough to have a significant pressure drop would show an increase in diameter along their length. Since flow is equal, such vessels would then have a *decrease* in shear stress towards their distal ends. We found that this is indeed the case in the rat first-order cremaster artery, a long, practically unbranched resistance artery that experiences a substantial drop in pressure along its length. While shear stress may still be an important factor in vascular design, this study suggests that the local pressure profile also forms a strong drive for structural adaptation.

METHODS

All experiments were carried out in accordance with the guidelines on animal experiments of our institutions. The study involved four protocols. First, blood pressure and arterial diameter were measured *in vivo* using an intact cremaster muscle preparation. Second, red blood cell velocity and diameter were measured *in vivo* using an open cremaster muscle preparation. Third, passive pressure–diameter relations were obtained from isolated arterial segments *in vitro*. Data for this protocol were obtained from not only cremaster arterioles but also from small epigastric arteries. Fourth, histology was performed on the cremaster muscle to determine morphological parameters. All measurements were made at an upstream and downstream location along the first-order arteriole. These locations were around 10 mm apart. Starting after a major sidebranch, the arteriole was followed until the next major sidebranch was encountered. Usually, three or less minor sidebranches were present between the locations of measurements. These minor sidebranches did not alter flow detectable (see results), and therefore the segment studied was considered to be essentially unbranched.

In vivo blood pressure and diameter

Male Wistar rats (250–350 g) were anaesthetized with urethane (1.5 g kg⁻¹ i.p.). Additional doses (20%) of urethane were given when necessary, as judged from toe pinch reflex and respiration. The right cremaster muscle was exposed, but not opened, through

Figure 1. Overview of the preparation for *in situ* measurements

The cremaster muscle was exposed through a dorsal incision of the skin. The first-order arteriole (left) has one small side branch in this example (arrow). Locations of measurement for combined intravascular pressure and lumen diameter or combined red blood cell velocity and diameter are indicated. Note that the diameter of the arteriole increases gradually along the vessel.

a dorsal incision of the skin. Connective tissue was removed and the muscle was superfused with warm (34 °C) bicarbonate-buffered physiological saline solution (PSS; for composition see below) supplemented with succinylcholine (10⁻⁵ M), equilibrated with 95% N₂ and 5% CO₂, pH 7.4. The dorsal approach exposes the first-order arteriole without further manipulation. This preparation ('closed preparation') leaves the circulation of the cremaster muscle intact, including the deferential vessels (Hill *et al.* 1992). The rat was placed on the stage of a stereomicroscope (Zeiss; magnification × 200) equipped with a video camera. The muscle was illuminated by epiluminescence. Images of the first-order arteriole were recorded on tape. The diameter of the arteriole was measured at two locations, located between two major branching points (see Fig. 1). Measurements were made at least 0.2 mm from the actual branching points. Small incisions in the muscle were made just above the locations of diameter measurement, to allow *in situ* measurement of blood pressure using the servo-null method. The incisions were made to expose the arteriole and minimize the chance of breaking the pipettes. At the end of the experiment, rats were killed with an overdose of nembutal (i.p.).

Intravascular pressure (*P*) was measured using a servo-null pressure system (model 5A, Instrumentation for Physiology & Medicine Inc., San Diego, CA, USA) based on the original technique developed by Wiederhielm *et al.* (1964). We used relative large pipettes (diameter > 1 μm) which are very sensitive to changes in pressure but not sensitive to plugging (Fox & Wiederhielm, 1973). Details of the technique used have been described before (Heslinga *et al.* 1997; Versluis *et al.* 2001). Briefly, micropipettes (GC100T-10, Clark Electromedical Instruments, Pangbourne, UK) were pulled in a two-step protocol using a micropipette puller (BB-CH, Mecanex SA, Geneva, Switzerland). The tip diameter (o.d.) of the pipettes was typically 2–5 μm, while the length of the tip was around 200 μm. The pipettes were filled with a 2 M NaCl solution and mounted on an oil-driven micromanipulator (MM0-203, Narishige Co. Ltd, Tokyo, Japan) allowing precise movements in three dimensions.

The validity of the recorded pressure was tested by changing the gain of the servo-null system. Once in the lumen of a vessel, the mean pressure recorded did not change when the gain of the servo-null system was increased. An increase in system gain only induced high frequency oscillations around the mean pressure. When the pipette was clogged or pressed against the wall of the vessel, an increase in gain led to an increase in recorded pressure. Measurements were accepted when a stable signal was obtained for at least 10 s. After approximately 30 s, signals were usually lost because of clot formation in the tip of the pipette. Upstream and downstream measurements were made in random order.

In vivo shear stress

To estimate shear stress, red blood cell velocity and diameter were measured at similar locations to those in the first *in vivo* protocol. The measurement of red blood cell velocity requires transillumination of the preparation. Therefore, the cremaster was opened through a ventral incision and the muscle was pinned down on a silicon pedestal ('open preparation'). The deferential feed vessels were left intact and the testicle was placed aside using a suture. Connective tissue was removed and the muscle was superfused with warm (34 °C) bicarbonate-buffered PSS (mm: NaCl 128, NaHCO₃ 20, KCl 4.7, NaH₂PO₄ 0.42, MgCl₂ 1.1, CaCl₂ 1.4) supplemented with succinylcholine (10⁻⁵ M), equilibrated with 95% N₂ and 5% CO₂. The animal was placed under a microscope and diameter (*d*) was measured on-line with a

manual video tracking system. Spatial resolution was $\sim 2 \mu\text{m}$. Centreline maximal red blood cell velocity (V_{RBC}) was measured with an optical Doppler velocimeter (Texas A & M Instruments, College Station, TX, USA) and recorded on a Power Lab system. From these measurements and using radius $r = d/2$, mean velocity (V_{m}), flow (Q) and shear stress were calculated as:

$$V_{\text{m}} = V_{\text{RBC}}/1.6, \quad (1)$$

$$Q = \pi r^2 V_{\text{m}}, \quad (2)$$

$$\tau = 4\eta Q/(\pi r^3), \quad (3)$$

where η , the viscosity of blood, was taken as $3.8 \times 10^{-3} \text{ Pa s}$.

In vitro measurements

Rats were decapitated after sedation with 100% CO_2 and cremaster muscles were excised and placed in cold Mops buffer (mm: NaCl 145, KCl 4.7, NaH_2PO_4 1.2, MgSO_4 1.2, CaCl_2 2, Mops 3, glucose 5 and pyruvate 2, pH 7.4). From each rat, segments from upstream and downstream origin of 3 to 4 mm length were dissected and mounted in a pressure myograph under sterile conditions. Vessels were superfused with Dulbecco's modified Eagle's medium (DMEM) containing L-proline 40 mg l^{-1} , ascorbic acid 50 mg l^{-1} , penicillin 100 i.u. ml^{-1} and streptomycin 0.1 mg ml^{-1} . The solution was equilibrated with 19% O_2 , 76% N_2 and 5% CO_2 . The perfusate was supplemented with 10% heat-inactivated fetal calf serum (FCS). The preparation was kept at 34°C , the *in vivo* temperature of the cremaster muscle. Images were obtained using a $\times 10$ objective and a monochrome CCD camera. Images were digitized and inner and outer diameters were recorded using Matlab software. After mounting, vessels were fully dilated with 10^{-4} M papaverin and a passive pressure–diameter relation was recorded. After these measurements, the vessels were used for organoid culture experiments not related to this study, as described elsewhere (Bakker *et al.* 2002).

To test for the specificity of the findings made in the cremaster arterioles, segments of the epigastric artery were excised from the abdominal wall musculature. These segments usually contained 2–4 small sidebranches over an approximate length of 7 mm. Single segments were cannulated in Ca^{2+} -free PSS with 10^{-4} M papaverin. Measurements of pressure–diameter relations were made at the downstream and upstream end of these small arteries.

Histology

For histology, the cremaster muscle was excised, placed in Mops buffer containing 0.1 mM papaverin and fixed in a mixture of formaldehyde (4%) and glutaraldehyde (1%) in phosphate buffer, at room temperature. This procedure yields arterial dimensions under dilated conditions at 0 mmHg. Strips of the muscle containing either an upstream part or a downstream part of the first-order arteriole were dissected and embedded in paraffin. Images of elastin–van Gieson-stained sections were recorded using a $\times 40$ objective and a 1712 pixel $\times$ 1368 pixel digital camera. Morphometry was performed using ImageJ (National Institutes of Health). We quantified medial cross-sectional area (mCSA), exact internal circumference including all folds (eCirc, i.e. the length of the internal elastic lamina), and a 'shortcut' internal circumference skipping the folds (sCirc). The rationale of using these two quantities is that proximal and distal segments showed different degrees of folding. From these measurements, radius (r_0) and intima–media thickness (h_0) at zero pressure were calculated as:

$$r_0 = 0.5\text{sCirc}/\pi, \quad (4)$$

$$h_0 = \text{mCSA}/\text{sCirc}. \quad (5)$$

Data analysis

Using the *in vivo*, *in vitro* and histological data, wall stress was calculated as follows: first, the *in vivo* media thickness (h), which could not be observed directly, was determined from:

$$h = \sqrt{(m\text{CSA}/\pi + r^2) - r}. \quad (6)$$

Then, using Laplace's law, the *in vivo* wall stress was estimated as:

$$\sigma = Pr/h. \quad (7)$$

As P , r and h were not determined in the same experiments, mean $\pm$ S.E.M. values were used for these variables. Using standard error progression analysis, the error in the estimation of σ was calculated as:

Error (σ) =

$$\sqrt{((r/h)^2 \times \text{S.E.M.}(P)^2 + (P/h)^2 \times \text{S.E.M.}(r)^2 + (Pr/h^2) \times \text{S.E.M.}(h))}. \quad (8)$$

Data are given as means $\pm$ S.E.M. Paired Students *t* tests were used to compare means. Analysis of variance (ANOVA) was used to compare pressure–diameter relations and paired *t* tests for individual pressure levels. Differences were considered significant at the $P < 0.05$ level.

Chemicals

Salts were purchased from Merck (Darmstadt, Germany). Urethane, papaverin and succinylcholine chloride were from Sigma (St Louis, MO, USA).

RESULTS

In vivo experiments

From attempts in a total of seven rats, pressures were recorded successfully both upstream and downstream in five animals. A substantial decrease in blood pressure along the arteriole was found. Recordings from a typical experiment are shown in Fig. 2.

Mean upstream and downstream pressures were 68 ± 4 and $54 \pm 3 \text{ mmHg}$ respectively ($P = 0.0007$; Fig. 3A). In addition, the systolic–diastolic pressure pulse was

Figure 2. Examples of intravascular pressure recordings using the servo-null method

Tracings are plotted along the same time scale but were recorded non-simultaneously. Note the decrease in both mean pressure and amplitude of pressure oscillation from upstream to downstream.

Table 1. Histological data

	Lumen (μm)	Media (μm)	mCSA (μm^2)	eCirc (μm)	sCirc (μm)	Degree of folding
Upstream	69 ± 4	6.5 ± 0.3	1739 ± 89	269 ± 9	216 ± 9	0.20 ± 0.04
Downstream	$80 \pm 4^*$	6.5 ± 0.3	1810 ± 81	280 ± 9	$252 \pm 11^*$	$0.10 \pm 0.03^*$

Lumen diameter, media thickness, medial cross-sectional area (mCSA), exact internal circumference including all folds (eCirc, i.e. the length of the internal elastic lamina), and a 'shortcut' internal circumference skipping the folds (sCirc) were quantified at $P = 0$ mmHg. The degree of folding was calculated as $(\text{eCirc} - \text{sCirc})/\text{eCirc}$. * $P < 0.05$ for upstream vs. downstream location.

markedly higher upstream vs. downstream: 8.2 ± 1.3 vs. 4.1 ± 0.6 mmHg ($P = 0.007$).

Diameter of the arterioles at the upstream location was smaller than that at the downstream location for all individual experiments in both the closed preparation (Fig. 3B, filled circles) and the open preparation (Fig. 3B, open circles). Mean upstream and downstream diameter were 179 ± 4 and 203 ± 4 μm , respectively ($P < 0.001$; $n = 10$). The increase in diameter along individual segments was found to be gradual, from upstream to downstream, as shown in Fig. 3C. Based on equal flow, the

increase in diameter reflects a $49 \pm 8\%$ higher level of shear stress upstream as compared to the downstream site. In order to estimate the absolute levels of shear stress, velocity and diameter were simultaneously measured in four vessels. These data are indicated in Fig. 3D. Flow was observed to be in the downstream direction at both locations in all experiments. Shear stress (eqn (3)) was estimated at 30 ± 6 dyn cm^{-2} at the upstream location and 21 ± 6 dyn cm^{-2} at the downstream location ($P = 0.03$; $n = 4$). Flow was calculated and found to be identical upstream and downstream: 23 ± 3 and 22 ± 4 $\mu\text{l min}^{-1}$, respectively ($P = \text{n.s.}$, $n = 4$).

Figure 3. Overview of data obtained *in vivo*

A, intravascular pressure measurements of individual experiments. Upstream and downstream measurements were made in five rats. A significant decrease in pressure was found along the vessel ($P = 0.0007$). B, diameter measurements of individual experiments. Upstream and downstream lumen diameter was measured *in situ* in 10 rats in the closed and open cremaster preparation as indicated. A significant increase in diameter between the upstream and downstream location was found ($P < 0.001$). C, diameter measurements along the length of an additional four individual segments *in situ*. D, calculated shear stress of individual experiments. Mean shear stress decreased significantly along the vessel ($P < 0.05$).

Figure 4. Pressure–diameter relations of arteries *in vitro*

A, pressure–diameter relation of maximally dilated arterioles from cremaster muscle tested *in vitro* ($n = 18$). Upstream segments were significantly smaller than their paired downstream counterparts at all pressures tested ($P < 0.001$). B, pressure–diameter relation of maximally dilated small epigastric arteries *in vitro* ($n = 6$). A significant increase in diameter was found along the artery at all pressure levels, with exception of the lowest pressure level.

***In vitro* experiments**

Passive pressure–diameter relations of cremaster arterioles were made *in vitro* after full dilation with 0.1 mM papaverin. In the range 15–125 mmHg, diameters were significantly larger in downstream vs. upstream segments ($P < 0.001$ for all pressures). Fig. 4A shows mean data of 18 paired vessel segments.

Although this was not addressed directly in the present study, comparison of the diameter *in vitro* with the measurements *in situ* suggests that the arteriole has little or no tone in the *in situ* situation. Thus, at 75 mmHg the diameter of segments from the upstream location measured $181 \pm 3.5 \mu\text{m}$, while the diameter *in situ* was $179 \pm 4 \mu\text{m}$ at the *in situ* pressure of 68 ± 4 mmHg.

The diameter of the epigastric artery was larger and more variable than the cremaster arteriole, $268 \pm 27 \mu\text{m}$ at 75 mmHg ($n = 6$). Therefore, normalized data are depicted (Fig. 4B). With exception of the lowest pressure

level, a significant increase in the diameter of the epigastric artery was found from upstream to downstream.

Histology

In sections of cremaster muscle containing the first-order arteriole, the media cross sectional area, lumen diameter, inner circumference (sCirc, not taking the folds into account) and length of the internal elastic lamina (eCirc) were determined. From the latter two measurements, the degree of folding was calculated (Table 1). While the media CSAs were similar, a significantly smaller lumen diameter was found at the proximal location as compared to the distal location. In addition, the degree of folding of the internal elastic lamina (IEL) was significantly higher at the proximal location. Typical sections illustrating these features are shown in Fig. 5.

Wall stress

From the average data on *in vivo* pressure and diameter measurements and the calculated wall thickness at local

Figure 5. Example of cross-sections of the cremaster muscle at $P = 0$ mmHg, stained with elastin–van Gieson

The first-order arteriole at the upstream location (left) and downstream location (right). At the upstream location, the arteriole has a smaller diameter and the vessel wall is more folded.

pressure, *in vivo* wall stress was calculated. The level of wall stress was found to be identical within 2% at both locations: 266 ± 16 vs. 260 ± 14 mN mm⁻² for upstream vs. downstream ($P = \text{n.s.}$), despite differences in pressure, diameter and wall thickness of, respectively, -11 ± 1 , $+14 \pm 2$ and -7 ± 4 %.

DISCUSSION

We showed that: (1) a substantial decrease in blood pressure and pulse pressure occurred along the length of first-order rat cremaster muscle arterioles, (2) the passive arterial diameter increased by ~ 14 % between the upstream and downstream location within this single segment, (3) a similar level of wall stress was present at both locations, (4) shear stress was ~ 50 % higher in the upstream as compared to the downstream location in this single vessel. These results falsify the hypothesis that shear stress is the single dominating factor driving arteriolar calibre. We speculate that the profound change in pressure profile along this single vessel plays an important role in the design of the arteriole.

There is clear evidence for the sensitivity to shear stress of the arteriole used in the present study. Thus, in isolated cremaster muscle arterioles, flow-dependent dilation occurs over a physiological range of shear stresses (Koller *et al.* 1993). Effects of experimentally induced chronic changes in blood flow on vascular structure were shown in this preparation by Wang & Prewitt (1991). These authors performed unilateral orchidectomy and found that wall shear rate was unchanged despite a more than 50% reduction in blood flow. Thus, these studies demonstrate the ability of the cremaster arterioles to adapt their diameter in order to normalize shear stress upon acute and chronic changes in flow. However, the current study now demonstrates that the level of shear stress is not constant along the length of the arteriole. As both pressure and pulse pressure change profoundly along the arteriole, we speculate that the effect of shear stress is modulated by the local intravascular pressure profile. Some previous observations provided initial clues for this hypothesis. Thus, structural narrowing of resistance vessels is seen in various hypertension models (Mulvany, 1990). Shear stress has not regularly been measured in hypertensive animals, but based on similar cardiac outputs as compared to normotensive animals, the inward remodelling and rarefaction in the resistance vasculature is, at least on average, necessarily accompanied with increasing shear stresses. As regards the current preparation, the elevated local pressure in the cremaster muscle arterioles of renal hypertensive rats is accompanied by a structural reduction in diameter (Imig & Anderson, 1991). Independent manipulation of pressure and flow *in vivo* could have provided further evidence for the importance of both parameters in vessel design. However, currently no such

approach is available. Using an *in vitro* approach, we previously found that in cultured, cannulated rat cremaster arterioles maintained at zero flow, ~ 15 % inward remodelling occurs in 3 days when kept at 75 mmHg, while remodelling is absent when kept at 2–3 mmHg (Bakker *et al.* 2002). In the same study it was demonstrated that this difference is a direct consequence of the continuing presence of tone at the higher pressure. Whether such a direct relation between tone and remodelling also exists *in vivo* remains to be established, but it seems not unrealistic to assume that the smaller proximal diameter, where pressure is higher, is a structural consequence of the negative slope of the active, myogenic pressure–diameter relation seen in these vessels (Falcone *et al.* 1991).

The hypothesis that vascular calibre results from an interactive effect of pressure and shear stress was addressed earlier by Pries *et al.* (1995). Based on observations and calculations of local haemodynamics in the mesenteric bed, these authors found that pressure and shear stress decrease concomitantly along the arterioles and venules. A concern in such analysis over several branching orders however, is that other variables than pressure are modulating the shear stress in these networks. Obviously, many functional and structural differences are present between arterioles, capillaries and venules. In addition, large differences exist in the chemical environment of the different compartments of a vascular network. The current study excludes many of the possible confounding concerns on network behaviour since measurements of pressure, velocity, and diameter were done along a single vessel. While we cannot rule out that the chemical environment may gradually change along the arteriole, we believe that the pressure profile is the most likely parameter linked to the differential structural adaptation along the arteriole.

Acute and structural effects of pressure have commonly been attributed to the resulting wall stress (VanBavel & Mulvany, 1994; Bund, 2001). The level of wall stress was not significantly different at both sites, despite substantial differences in pressure and diameter. While this suggests very strong regulation of wall stress by structural adaptation, further unravelling of the cellular pathways and the study of effects of interventions herein will be needed to substantiate this point. In theory, the vessels could have maintained both wall stress and shear stress constant along their length by independent variation of internal diameter and wall thickness. However, the observed differences in local shear stress along these single, healthy arterioles make clear that such a view is incorrect.

It remains to be established whether, in addition to differences in mean pressure, the 50% difference in amplitude of the pressure pulse could be related to the larger distal diameter. Some evidence presented in the

literature is in support of this view. Large arteries are known to show increased stiffening with increased pulse pressure (Safar *et al.* 2001). In small arteries, pulse pressure was found to correlate most closely of all pressure parameters studied to the vessel media/lumen ratio (Christensen, 1991). As a putative mechanism, smooth muscle cells, which are the main cell type exposed to the cyclic mechanical strain during the cardiac cycle, may respond with an increase in deposition of matrix elements upon stimulation (Williams, 1998). Similarly, human smooth muscle cells show enhanced deposition of collagens and fibronectin, as well as an increase in matrix degrading metalloproteinase activity in response to cyclic strain (O'Callaghan & Williams, 2000). Associated with a gradual change in pulse pressure, a quantitative shift in these processes could then result in differential remodelling and stiffening along the length of an artery.

In conclusion, we found that along the length of a resistance artery, wall stress is maintained while shear stress decreases downstream as a consequence of an increase in diameter. A profound change in the pressure profile may play an important role in this paradoxical increase in diameter. Hence, not only shear stress but also the local pressure profile may play a key role in structural regulation of microvascular diameter.

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