Increased shear stress–released NO and decreased endothelial calcium in rat isolated perfused juxtamedullary nephrons

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Background. Nitric oxide is an important vasodilator released from endothelial cells by the calcium-dependent endothelial nitric oxide synthase (NOS). We considered it important to investigate how shear stress/perfusion pressure influenced endothelial cell calcium concentration, nitric oxide release, and autoregulation of the afferent arteriole, since this arteriole controls glomerular filtration rate (GFR) and renal autoregulation of the whole kidney (i.e., for the constancy of renal blood flow), at perfusion pressures ranging from 75 to 200 mm Hg. In attempts to account for this process, two mechanisms operating synergistically have been proposed namely: the myogenic vasoconstriction and the tubuloglomerular feedback mechanism [1]. It has been suggested that increased wall tension resulting from an increase in luminal pressure elicits vasoconstriction so that the blood flow remains essentially constant [2]. The tubuloglomerular feedback mechanism, on the other hand, senses the fluid or solute delivery to the macula densa segment of the early distal tubule [3]. An increased load will cause vasoconstriction of the terminal afferent arteriole and a reduced load will lower the tone of the vessel in order to restore tubular delivery.

Many reports have shown that the autoregulatory response is modulated by vasodilators, most important, nitric oxide. Nitric oxide release can be produced by different isoforms of nitric oxide synthase (NOS) within the kidney [4]. It is possible that luminal flow and shear stress deriving from luminal flow in the afferent arteriole cause release of nitric oxide from the endothelium. Changes in nitric oxide production by the macula densa have been shown to modify the sensitivity of the tubuloglomerular feedback mechanism [5]. Opinions diverge regarding the role of endothelial intracellular calcium [Ca2+], in the shear stress-induced production of nitric oxide from endothelial NOS (eNOS). In most studies shear stress has been found to be associated with a rise in intracellular calcium, suggesting that this process is calcium-dependent [6–9]. However, in some more recent studies, the shear stress-associated release of nitric oxide has been shown to be calcium-independent [10–13].

Nitric oxide is being continuously produced by eNOS. It readily diffuses out of the endothelial cell towards the lumen and also in the abluminal direction and is eliminated in a number of reactions. Thus, nitric oxide binds not only to its target molecule, the smooth muscle...
METHODS

The autoregulation of renal blood flow. It has been found that scavenging of nitric oxide by erythrocytes is important in afferent arteriolar reactivity and in the regulation of glomerular hemodynamics [15, 16].

We considered it important to investigate how shear stress and perfusion pressure influences the endothelial cell calcium concentration and nitric oxide release, and in addition how nitric oxide influences the renal vasculature. To make predictions about what happens in the vessels with different nitric oxide concentrations, we used mathematical modeling to make calculations of the events [14].

To be able to visualize the vasculature on the surface of the kidney, we used the juxtamedullary nephron preparation developed by Casellas and Navar [17]. Our results show that increased perfusion pressure with increased shear stress led to an increase in nitric oxide release while the endothelial cell calcium was reduced. Furthermore, that the mathematical modeling indicated that nitric oxide scavenging by the erythrocytes plays a major role in the autoregulation of renal blood flow.

A modified Krebs-Ringer-bicarbonate solution was used as perfusate, containing 4% bovine serum albumin (BSA) (Fraction V) (Sigma, St. Louis, MO, USA) and amino acids (L-methionine 0.33, L-isoleucine 0.3, L-alanine 2.0, glycine 2.3, L-arginine 0.5, L-proline 0.9, L-aspartic acid 0.2, L-glutamine 0.5, and L-serine 1.0 mmol/L (Sigma). The ionic composition of the buffer (in mmol/L) was 105 NaCl, 16 CH₃COONa, 25 NaHCO₃, 5 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes buffer), 5.55 glucose, 4.84 KCl, 5 urea, 1.2 MgSO₄, 0.6 Na₂HPO₄, 0.13 NaH₂PO₄, and 2.22 CaCl₂.

In order to reach a pH of 7.4 and adequately supply oxygen, the solution was pressurized and equilibrated with a gas mixture containing 95% O₂ and 5% CO₂.

The perfused kidney was then sectioned longitudinally and the ventral half was discarded. The fornices were then cut off and the papilla was reflected back to allow visualization of the pelvic structures. Visible veins were cut open along their courses and the pelvic fat and connective tissue were removed.

In order to reduce the total perfused vascular cross-section and flow, all identifiable branches of the renal artery were ligated with 10-0 sutures. The main interlobar branch was then ligated distally so that only a few afferent arterioles and/or interlobular branches remained perfused. A further advantage of perfusing only a few nephrons is that the perfusion pressure measured with the double-barrel cannula will be approximately the same as in the proximal end of the perfused arterioles. One juxtamedullary nephron with good visibility was chosen for the experiment.

The preparation was then transferred to a chamber fitting the plate of a Zeiss Axioskop fixed stage microscope and the kidney was continuously washed with Krebs-Ringer bicarbonate solution containing 1% BSA preheated to 37°C.

Loading the endothelium with Fura-2 AM and measurement of the endothelial [Ca²⁺]i

The afferent arteriolar endothelial cells were loaded with the calcium-sensitive dye Fura-2 AM [18] at a perfusate concentration of 5 μmol/L. The loading lasted for 45 to 60 minutes and the perfusion pressure was set at 100 mm Hg in all the experiments. The microscope was equipped with a Zeiss Achroplan 40×0.75 W water immersion objective suitable for transmission of ultraviolet light. We used a Heizinger LNX 150-1 ultraviolet and a visible wavelength monochromator as excitation light source. The fluorescent image was intensified with a Video Scope Model KS-1381 (Video Scope International, Ltd., Washington, D.C., USA) image intensifier (set invariably throughout the experiments at 300) and was detected with a DAGE MTI CCD-72EX monochrome charged-coupling device (CCD) camera. The images
were fed to a personal computer. Image-1 (Universal Imaging Corporation, West Chester, PA, USA) image analysis software was used to collect the fluorescent images excited at 340 and 380 nm wavelengths and to analyze them off-line.

The ratio of the two intensity images in each corresponding pixel was calculated. The sampling rate, correspondingly, was about one ratio per 3 seconds.

**Measurements of afferent arteriolar diameter**

The imaging system was calibrated to measure diameters with the 40× magnification objective. Fura-2 fluorescence images, excited at 340 nm, were used for the diameter measurements during off-line analysis. Identical points in the vessel wall were identified and used in the subsequent images. Diameter data of the five arterioles within the experimental groups under corresponding conditions were pooled and are expressed in Table 3 as mean ± SD In a separate series of experiments using 17 arterioles visible light was used to determine diameter using a calliper (Table 2). Diameters were measured at 60 and 120 mm Hg using cell-free perfusate with or without L-arginine analog N-nitro-L-arginine methyl ester (L-NAME).

**Measurement of endothelial nitric oxide production**

In a small series of experiments (N = 3), the nitric oxide production was checked by the use of the nitric oxide-sensitive 4-amino-5 methylamino-2′, 7′-difluorescein diacetate (DAF-FM DA) (10 μmol/L), which was loaded in 45 to 60 minutes. A cooled CCD camera and a personal computer-based imaging system (Applied Imaging QuantiCell 900, Sunderland, UK) were used to detect fluorescence. The excitation wavelength was 488 nm (10 nm bandwidth) and a 515 nm dichroic mirror and 515 nm long pass emission filter were used to separate the emitted fluorescence. Blockade of nitric oxide production rate was achieved by using L-NAME (Sigma).

**Experimental logistics**

*Calcium measurements.* Loading of the endothelial cells with Fura-2 AM was checked from the intensity of the fluorescence in the images.

When sufficient fluorescent intensity was achieved, perfusion of the preparation was continued either with a cell-free, albuminumated perfusate (series 1) (N = 5) or with an erythrocyte-supplemented perfusate (series 2) (N = 5). Homologous blood was freshly harvested and the erythrocytes were separated from the plasma. After resuspending the red blood cells in albumuminated buffer and centrifugation, the red cells were separated twice, and the washed red blood cells were added to the perfusate to a hematoctrit of 20%.

The following protocol was used in both experimental series. In the first step the perfusion pressure was reduced from the control value of 100 to 50 mm Hg. After a 15-minute stabilization period, single cell endothelial calcium values were measured. The measurements were then repeated at 75, 100, 125, and 150 mm Hg and then again at 50 mm Hg; in each step after the same stabilization period of 15 minutes. Fluorescence images were stored for off-line analysis and diameter measurements.

*Measurement of endothelial nitric oxide production.* After loading of the DAF-FM DA with the perfusion pressure was kept at 100 mm Hg and three consecutive measurements were made at 10-minute intervals (i.e., at 10, 20, and 30 minutes). The pressure was then switched to 125 mm Hg and another set of three measurements followed. This was repeated at 150 and 125 mm Hg and then at the control perfusion pressure of 100 mm Hg. A similar series was also carried out at 75 and 50 mm Hg.

**Calculation of shear stress**

Since the production of nitric oxide is supposed to depend on the friction of the blood stream at the wall of the vessel the shear stress was calculated. In the calculation of this force, it is considered that a fluid sheet moving at a high velocity tends to drag with it a surrounding sheet moving at a lower speed. We may then analyze the forces acting on an interface located at a radius (r) separating a central and a peripheral fluid column. The force acting on the central column can be calculated by multiplying the difference (ΔP) between the pressures acting on the two sides of the column by the cross-sectional area of the column (πr²). The shear force [f(r) shear], will obviously be directed opposite to the fluid stream as:

\[ f(r)_{\text{shear}} = \Delta P \cdot \pi r^2 \]  
(equation 1)

Shear stress [τ(r)] is defined as force per unit area. This can be calculated by dividing this shear force [f(r) shear], by the area of the interface separating the central and peripheral columns; the latter area can be obtained by multiplying the circumference (2πr) by the length (Δl) of the vessel segment:

\[ \tau(r) = \frac{\Delta P \cdot \pi r^2}{2\pi r \cdot \Delta l} = \frac{\Delta P \cdot r}{2\Delta l} \]  
(equation 2)

Since the production of nitric oxide depends on the shear stress at the wall of the vessel (τ wall) the corresponding radius (r wall), may be inserted into (equation 3) as:

\[ \tau_{\text{wall}} = \frac{\Delta P \cdot r_{\text{wall}}}{2\Delta l} \]  
(equation 3)

In order to determine the above pressure gradient (ΔP/Δl) the length of the arteriole (Δl) was put at 100 μm and the pressure difference was calculated as perfusion pressure minus glomerular capillary pressure; the latter
Table 1. Net afferent arteriolar driving pressures used for the calculation of shear stress in isolated juxtamedullary nephrons perfused with cell-free and erythrocyte-containing media.

<table>
<thead>
<tr>
<th>Perfusion pressure</th>
<th>Blood perfusion</th>
<th>Cell-free perfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glomerular capillary pressure</td>
<td>Afferent arteriole driving pressure</td>
</tr>
<tr>
<td>75</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>125</td>
<td>55</td>
<td>70</td>
</tr>
<tr>
<td>150</td>
<td>55</td>
<td>95</td>
</tr>
</tbody>
</table>

Glomerular capillary pressures were either taken from the literature (erythrocyte perfusion) or estimated (cell-free perfusion). All pressures are given in mm Hg.

was obtained from previous experiments on the same rat strain (Table 1) valid for blood-perfused nephrons. However, no data from direct measurements are available for the case of nephrons perfused with cell-free perfusate. For this reason, the calculation was made for both the afferent and efferent arterioles, in which case the pressure difference equals the perfusion pressure and the length is assumed to be twice that of the afferent arteriole (i.e., the shear stress will be the average of that in the two arterioles).

Modeling the scavenging of nitric oxide and calculation of the nitric oxide profile across the vessel

In order to analyze the importance of the scavenging of nitric oxide, its concentration profile across the afferent arteriole wall was assessed. The model was based on the following assumptions: (1) that the nitric oxide concentrations at the luminal and abluminal endothelial cell borders are the same; (2) that the transport of nitric oxide by luminal convection is small and the skimming effect is negligible; and (3) that the vessel radii are small in relation to the length of the arteriole.

Theoretically, anywhere within the afferent arteriole or its vicinity (r), the elimination rate can be expressed as a rate constant (k) times the concentration [C(r)] seen in the second term in equation 4. This rate in a dr thick segment at a radius (r) from the center of the vessel lumen is balanced by the difference in diffusion across the two borders of the segment. In the case of cylindrical vessel coordinates and at united arteriolar length we obtain:

\[ \frac{1}{2\pi r} \frac{d}{dr} \left( 2\pi r \frac{dC(r)}{dr} \right) - kC(r) = 0 \]  

(equation 4)

where D is the diffusion coefficient of nitric oxide in water \((3.3 \times 10^{-5} \text{ m}^2 \text{ sec}^{-1})\) or \(3.3 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}\). Since nitric oxide is soluble in fat, the cell membranes do not restrict the diffusion.

Regarding the rate constant k, no accurate methods are available to measure its magnitude in blood and plasma. However, using data on the average nitric oxide activity in blood, Vaughn et al [14] estimated the degradation rate to be 15 s\(^{-1}\), which would correspond to a half-life of \(\ln (2)/15 = 0.05\); this means that half of the nitric oxide in blood will be consumed within five hundredths of a second. It should be noted that the data presented by Vaughn et al [14] refer to normal blood (i.e., to a higher hematocrit than in the present studies). Because of the uncertainties inherent in these data, this difference was not considered, however. It should also be pointed out that the red cell-containing perfusate used in the present studies might also contain some free hemoglobin, which will increase the nitric oxide scavenging to a much larger extent than the red cell hemoglobin at corresponding concentrations. In cell-free medium and in the tissues adjacent to the arteriole, the rate constant (k) (see Vaughn et al [14]), is much smaller at 0.05 s\(^{-1}\) or less, a number that would correspond to a half-life of about 15 seconds. The abluminal consumption of nitric oxide is thus negligibly small in comparison with that in the blood stream.

In order to solve equation 4, we assume that at a large distance in the abluminal direction, the concentration of nitric oxide will approach zero. The concentration gradient will be different from zero, however. By assuming a specific gradient, equation 4 may be used to calculate the abluminal concentration profile and thence the nitric oxide concentration at the borders of the endothelial cell.

At the center of the vessel, the nitric oxide concentration gradient is zero (i.e., on account of vessel symmetry). In contrast, the concentration at the center is different from zero, but can be derived by assuming a series of concentrations such that the concentration at the luminal side of the endothelial cell becomes the same as that at the abluminal side. For this case, equation 4 was adapted to an afferent arteriolar radius of 10 \(\mu\)m, and was solved numerically by subdividing each micrometer in the radial direction into 10 parts (dr = 0.1 \(\mu\)m).

In order to determine the concentration profile in absolute terms, the normal endothelial cell synthesis of nitric oxide, M, of \(5.3 \times 10^{-8} \text{ mol m}^2 \text{ sec}^{-1}\) [14] and, thence, the transport by diffusion across the two boundaries of the cell (with indices lum and ablum) was inserted into the equation:

\[ M = 2\pi r_{\text{lum}} D \frac{dC(r)_{\text{lum}}}{dr} - 2\pi r_{\text{ablum}} D \frac{dC(r)_{\text{ablum}}}{dr} \]  

(equation 5)

Statistical analysis

All values are given as mean ± SD. Values from calcium and nitric oxide measurements were tested with analysis for variance for repeated measures, followed by the
Bonferroni test for pair-wise multiple comparisons. Normally distributed parameters were tested for significance with Student paired or unpaired t test and others with the Mann-Whitney U test. A P value less than 0.05 was accepted for significance.

RESULTS

Diameter of the afferent arteriole

The relations between the measured afferent arteriolar diameters and the perfusion pressure are summarized in Figure 1, where the upper curve refers to cell-free perfusion and the lower observation relate to perfusion with 20% red blood cells.

It is evident from this figure that during cell-free perfusion, an increase in perfusion pressure was accompanied by a parallel increase in the arteriolar diameter (i.e., the nephron showed no sign of autoregulation). After addition of red blood cells to a hematocrit of 20%, increases in the perfusion pressure above 75 mm Hg resulted in a progressive reduction in arteriolar diameter. In order to analyze the autoregulatory capacity, the line seen in the lower curve has been drawn through the markers up to 75 mm Hg, which is generally considered to be the lower pressure limit in the autoregulatory range. From that point on the line has been drawn such that the blood flow should remain the same as that at 75 mm Hg as based on Poiseuille’s law. It is evident that the experimental points fall very close to the theoretic line (i.e., as for a perfectly autoregulating nephron).

Table 2. Afferent arteriolar diameter at 60 and 120 mm Hg pressure using cell-free perfusion media with and without L-arginine analog N-nitro-L-arginine methyl ester (L-NAME)

<table>
<thead>
<tr>
<th>Perfusion pressure</th>
<th>Cell-free perfusion</th>
<th>Cell-free perfusion+L-NAME</th>
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<tbody>
<tr>
<td></td>
<td>60 mm Hg</td>
<td>120 mm Hg</td>
</tr>
<tr>
<td>Diameter µm</td>
<td>24.4 ± 6</td>
<td>28 ± 6</td>
</tr>
<tr>
<td>Diameter %a</td>
<td>123.0 ± 17</td>
<td>141 ± 19</td>
</tr>
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</table>

*a% measured at 60 mm Hg perfusion with L-NAME. Values are mean ± SD (N = 17).

Shear stress

The calculations show that irrespective of vasoactivity and actual diameter, elevations in perfusion pressure result in increased shear stress (Fig. 2). In the presence of red cells, however, the smaller diameter at high perfusion pressures will result in a somewhat smaller number. This means that the increase in diameter consequent to a rise in nitric oxide (due to, for example, decreased scavenging), results in a larger shear stress and thence stress-driven nitric oxide production such as to augment the primary nitric oxide-driven vasodilation.

Scavenging of nitric oxide

The nitric oxide concentration profiles estimated for an afferent arteriole are shown in Figure 3, where in
Fig. 3. Calculated nitric oxide (NO) concentration profiles in afferent arterioles during perfusion with a cell-free medium (upper curve) and a medium containing erythrocytes (lower curve). Note that the elimination of nitric oxide by scavenging decreases its concentration in the blood stream, whereby the gradient at the wall is increased with consequently larger transport from the endothelium, which also reduces nitric oxide at the level of the smooth muscle cells.

both cases, the synthesis of nitric oxide was set at $5.3 \times 10^{-8}$ mol m$^{-2}$ sec$^{-1}$ [14, 19]. The concentrations of nitric oxide within the endothelium (indicated by the two vertical lines) were assumed to be identical at the inner and outer borders of the endothelial cell and throughout the cell. The figure shows that with movement to higher radii in the abluminal direction there is a decline in nitric oxide concentration, but this is the simple consequence of the increase in radius. Towards the lumen, scavenging by red cell hemoglobin in the blood stream will result in a large concentration gradient at the vessel wall. The consequently larger transport in the luminal direction will then lower the concentration within both the endothelial cells and the smooth muscle cells. During perfusion with the cell-free medium, the luminal scavenging will be insignificant. For this reason the nitric oxide concentration at the level of the smooth muscle cells will be relatively high at 800 nmol/L, as against 400 nmol/L in the case of red cell perfusion. These estimates correlate very well with the previously proposed equilibrium dissociation constant of the smooth muscle guanylyl cyclase (250 nmol/L) [20].

Endothelial nitric oxide production during pressure manipulations

The changes in nitric oxide level observed on analysis of the fluorescence of DAF-FM are illustrated in Figure 5. Interestingly, in the time control, where the pressure was kept at 100 mm Hg (middle curve), the signal was fairly stable over time; after 2 hours, for instance, the fluorescence showed only a 10% decline. The fact that the fluorescence was relatively constant at this pressure most probably indicates that the elimination rate and the rate of de novo formation of fluorophore-nitric oxide complex are similar.

It is also evident that elevation of the perfusion pressure both in the erythrocyte-perfused and the cell-free-perfused groups ($P < 0.001$). A notable difference between the calcium levels in the two models is that during blood perfusion endothelial calcium is shifted to higher levels. During blood perfusion the endothelial calcium value was 185.8 $\pm$ 39.2 nmol/L at 50 mm Hg and 76.2 $\pm$ 24.6 nmol/L at 150 mm Hg, while during perfusion with cell-free medium the corresponding values were 115.6 $\pm$ 33.2 nmol/L and 56.3 $\pm$ 20.6 nmol/L, respectively.

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It is also evident that elevation of the perfusion pressure to 125 and 150 mm Hg resulted in increases in DAF fluorescence. By contrast, a reduction of the perfusion pressure to 75 and 50 mm Hg was followed by a reduced signal. In all these cases the changes were relatively small although highly significant ($P < 0.01$).
DISCUSSION

The present studies were carried out on the isolated perfused juxtamedullary nephron preparation model, which since its introduction has proved to be an excellent tool for visualization of the afferent and efferent arterioles and subsequent studies on their reactivity. In comparison with isolated perfused arterioles, the major advantage of the technique employed derives from the fact that it preserves not only the pre- and postglomerular vessels, but also the functional integrity of the entire nephron [17]. Intact myogenic and tubuloglomerular feedback responses and consequently a full autoregulatory capacity, as well as seemingly adequate responses to a number of vasoactive substances, have been reported from many earlier studies carried out with this preparation [21–25].

Nitric oxide is an important vasodilator in many respects. It is tonically released by the endothelium and thereby contributes to a basal vascular tone. Nitric oxide modulates the autoregulation of the kidney and since the endothelial production of nitric oxide is flow-dependent, it modifies the constriction elicited by vasoconstrictors or the myogenic mechanism.

DAF-FM has been newly developed for real-time measurement of nitric oxide, with a detection limit of 5 nmol/L [26-28]. DAF-FM selectively traps nitric oxide in its molecule and yields a stable compound which emits green fluorescence when excited at about 490 nm. Fluorescent DAF-FM is not formed in the absence of nitric oxide. Stable forms of nitric oxide (e.g., NO\textsuperscript{−} and NO\textsuperscript{−−}), reactive oxygen species, such as superoxide (O\textsuperscript{2−}), H\textsubscript{2}O\textsubscript{2}, and peroxynitrite (ONOO\textsuperscript{−}) do not react with DAF-FM to yield a fluorescent product [26].

From the slope of the curves in Figure 5, the nitric oxide production rate could be estimated. At the lowest perfusion pressure levels of 50 and 75 mm Hg there were decreases in fluorescence with time indicating that the rate of formation of the stable fluorescent compound was lower than the loss of the compound out of the cells or photobleaching of the fluorophore.

The synthesis of nitric oxide by eNOS may be stimulated by different routes: besides the classic, calcium-calmodulin-dependent pathway (used for example by the agonists bradykinin and acetylcholine), shear stress-evoked nitric oxide production has been shown to take intracellular signaling pathways other than intracellular...
free calcium. Physical forces are most probably sensed by structures on the luminal wall of the endothelial cell and are probably through rearrangement of its cytoskeleton. This process is coupled to eNOS phosphorylation, which modifies the synthetic activity of eNOS [13]. Since, as shown in the present paper, shear stress is directly proportional to the pressure gradient across the vessel, elevations in the perfusion pressure in the current experiments supposedly stimulated the production of nitric oxide and resulted in vasodilation. This would be counteracted by the myogenic mechanism, where a rise in perfusion pressure would increase wall tension and initiate active vasoconstriction. The physiologic significance of this dual system is not known, but the damping effect of the nitric oxide system may serve to avoid an oscillatory response and may support a steady state.

In the present study, perfusion with blood (or rather a red cell suspension) was associated with seemingly good autoregulation, which was very similar to the autoregulatory adjustments in a hypothetic, perfectly autoregulating afferent arteriole (Fig. 2) as estimated by Poiseuille’s law. During perfusion with cell-free medium, however, the arterioles were unable to reduce their diameter in response to a rise in perfusion pressure. On the contrary, any increase in pressure resulted in vasodilation. This does not necessarily mean that the myogenic mechanism was absent; rather, we believe that myogenic vasoconstriction was overridden by the vasodilatory action of the nitric oxide excess. The build-up of inappropriately high nitric oxide levels would be due to the negligible scavenging by the perfusing medium in the absence of red cell hemoglobin. Furthermore, in Table 2, it was shown that a blockade of the nitric oxide system with L-NAME also returned autoregulation. Excessive dilation due to high nitric oxide levels will lead to further increases in shear stress (Fig. 3), which will force the system to produce even more nitric oxide. In this vicious circle maximal dilation of the vessel ensues. The comparison of the cell-free–perfused and blood-perfused arterioles offers a model for studying effects solely attributable for differences in nitric oxide levels in the vessel wall. It should be noted that the difference in viscosity between plasma and blood does not invalidate this reasoning. The fact that the viscosity of plasma is about one third of that of whole blood obviously means that for any given shear rate, the shear stress will also be one third of that in blood-perfused vessels. This is compensated for by the fact that the lower viscosity causes a threefold increase in flow. Consequently, the magnitude of shear stress does not depend on the actual viscosity of the perfusing medium.

The notion that erythrocytes in the perfusate potentiate autoregulation is familiar from the literature. Carmines and Inscho [15], from studies using a similar experimental model, concluded that perfusing the preparation with a cell-free medium may abolish the myogenic response of the interlobular artery. Imig et al [16] found that a small amount of erythrocytes, in the order of 1% to 5% hematocrit, is sufficient to restore near-maximal autoregulatory constriction of the afferent arteriole.

Mathematic models of the vascular production and subsequent degradation and elimination of nitric oxide date back to the early 1990s [29] and have become increasingly elaborate, taking more factors into account. The one we chose to use [14] was directly applicable to the case of afferent arterioles, being based on a similar vessel size and geometry, and with the concept of luminal scavenging as a major means of elimination. We used the above model by inserting our own experimental data to estimate nitric oxide profiles and concentrations in afferent arterioles of isolated juxtamedullary nephrons perfused either with cell-free or with erythrocyte-containing perfusate. Using this simplified model of vascular wall nitric oxide production, diffusion, and elimination, and assumptions about the rate of endothelial nitric oxide synthesis taken from the literature, we have estimated nitric oxide concentrations in erythrocyte-perfused (autoregulating) and in cell-free medium-perfused (passive, nonautoregulating) vessels of the size of an afferent arteriole. The predicted concentrations at the site of the smooth muscle cells ranged from about 400 to about 800 nmol/L (Fig. 1). These estimates correlate well with the proposed equilibrium dissociation constant of the soluble guanylyl cyclase (250 nmol/L) [20]. However, the concentration range is higher than that in control animals studied with electrode measurements by Levine et al [30], who found values in the renal tubules of 100 to 200 nmol/L. It is possible that an increase in the hematocrit to the normal level from the 20% used in the present study to 40% to 45% might reduce the nitric oxide concentration to this range. In a recent study, Smith, Moore, and Layton [31] have suggested that in vessels like the renal arterioles, with transit times of 3 to 30 msec, the scavenging of nitric oxide by red cell hemoglobin will in fact be smaller than its transport by advection (transport by fluid flow). This leads to the interesting conclusion that locally produced nitric oxide is sufficient to significant nitric oxide-concentrations along much of the afferent arteriole. It may perhaps even be so that afferent arteriolar tone, to a significant part, is governed by nitric oxide formed in the interlobular artery.

Endothelial production of nitric oxide was found to correlate with pressure/shear stress (Fig. 5), as expected. The role of endothelial calcium in the mediation of shear stress-dependent nitric oxide release is controversial. It has been suggested that a rise in endothelial calcium is essential for release of nitric oxide [8]. In some more recent studies, however, the shear stress-associated release of nitric oxide has been shown to be calcium-independent. Schilling, Mo, and Eskin [10], who measured intracellular calcium in cultured pulmonary artery endothelial cells by Fura-2 fluorescence, clearly showed that the synthesis
of nitric oxide was independent of intracellular calcium. Neither was the efflux of Rb86, which was used to study outward transport of K\(^+\) via Ca\(^{2+}\)-activated K\(^+\) channels, affected by the channel-specific inhibitor tetrabutylammonium, as would be expected if shear stress were to increase cytosolic [Ca\(^{2+}\)]. Macarthur et al [11] reached the same conclusion, showing that N-ethylmaleimide and dithiodipyridine (interfering with the intracellular mobilization of calcium), or thapsigargin (a specific inhibitor of Ca\(^{2+}\)-ATPase), blocked the agonist-induced but not the shear stress-induced formation of nitric oxide. In fact, some studies suggests the opposite: that the synthesis and release of nitric oxide may be accompanied by a reduction in cellular calcium. Oliver and Chase [12] noted that decreased shear stress in bovine aortic cultured cells increased cytosolic Ca\(^{2+}\) and vice versa. They proposed that increased stress causes closure of Ca\(^{2+}\) channels at the apical side of the endothelial cell and, since calcium is continuously pumped out by Ca\(^{2+}\)-ATPase, that this will lead to a decrease in intracellular calcium.

Endothelial calcium and perfusion pressure (consequently also shear stress) (see Fig. 3) were found to be inversely related in our studies. This in itself supports the notion that shear stress acts via calcium-independent signaling mechanisms to elevate nitric oxide [13]. If we take for granted that elevations in shear stimulate nitric oxide production and per se result in a diameter increase, then in terms of autoregulation (i.e., in a model where the input is pressure and the output is a steady flow-adjusted diameter), shear-evoked nitric oxide production is a self-amplifying mechanism. Theoretically, intracellular calcium, being inversely related to pressure or shear stress, may serve as a feedback by modulating calcium-dependent regulation of the endothelial nitric oxide synthesis and thereby counteracting the shear-dependent stimulation. Since during cell-free perfusion the arterioles do not autoregulate owing to the diminished luminal scavenging in this situation, the vessel wall nitric oxide levels are higher than in vivo and the endothelial nitric oxide homeostasis operates out of range. It is also possible that the endothelial calcium relates inversely to the nitric oxide levels as part of a putative autocrine feedback mechanism involved in the endothelial production of nitric oxide [32].

**CONCLUSION**

The present study shows that the autoregulatory response of the afferent arteriole perfused with a medium containing 20% erythrocytes can be inhibited by removal of the red blood cells. By using a mathematical model for vascular nitric oxide production, it was found that absence of nitric oxide scavenging by red blood cells drastically increases the nitric oxide concentration in the endothelium but also, that cell-free perfusate produces higher shear stress that could release more nitric oxide.

Measurements of nitric oxide in endothelial cells in the afferent arteriole using DAF-FM showed that increased pressure/shear stress increased nitric oxide release, while at the same time the endothelial cell calcium decreased. This may suggest the existence of a feedback control of endothelial cell calcium by nitric oxide.

**REFERENCES**


