Elevated plasma viscosity in extreme hemodilution increases perivascular nitric oxide concentration and microvascular perfusion

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Submitted 29 September 2004; accepted in final form 29 November 2004

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The dependence of FCD on capillary pressure was demonstrated by the study of Cabrales et al. (10), which showed the linear relationship between these parameters. Consequently, it is plausible to assume most processes that lower capillary pressure will reduce FCD. One of these processes is extreme hemodilution [hematocrit (Hct) 11%] with a low-viscosity (LV) plasma expander (39), which causes a viscosity dependent fall of central and peripheral vascular resistance due to a >50% decrease of blood viscosity from 4.2 to 2.1 cp. In this situation, plasma viscosity is around 1.3 cp, the heart is unable to maintain central blood pressure, and a reflex vasoconstriction further lowers capillary pressure.

A different outcome is obtained in extreme hemodilution if plasma viscosity is augmented to about 2.0 cp, causing blood viscosity to be 2.9 cp. As shown by Tsai and Intaglietta (40), in this situation, hemodilution of the central blood lowers Hct-dependent blood pressure losses in the systemic circulation while making a greater pressure head available to the capillary circulation. This redistribution of viscosity-dependant pressure losses in the circulation pressurizes the capillary circulation, as shown by Cabrales et al. (10), maintaining FCD.

A paradoxical result found in comparing LV and high-viscosity (HV) extreme hemodilution was that the latter also induces vasodilatation, as evidenced by the significantly increased blood flow relative to LV hemodilution and control conditions. This result cannot be reconciled with the overall increase of blood viscosity relative to the LV hemodilution and therefore the increased peripheral vascular resistance inherent to the increase in plasma viscosity. This finding, however, could be explained if increasing plasma viscosity increases shear stress in the microcirculation relative to the level of shear stress prevalent during LV hemodilution, leading to the increase of the shear stress-dependant release of nitric oxide (NO) from the endothelium, which is a potent vasodilator.

Shear stress in the microcirculation has been shown to be regulated within a narrow margin (25) in many studies where portions of the arterial tree were subjected to chronic changes of flow. Several studies have also shown that this regulation is present in the arteriolar microcirculation subjected to sudden changes in blood flow (3, 37). Shear stress is a consequence of the flow configuration and local blood viscosity. However, the local blood viscosity has been invariably assumed to be constant, although it progressively decreases in the microcirculation due to the Fahraeus effect (19, 30). Hct tends to equalize
in the circulation during hemodilution; however, the use of either HV or LV plasma expanders may significantly affect the acute distribution of shear stress.

To test the hypothesis that differences in plasma viscosity in extreme hemodilution may lead to different outcomes in terms of microvascular regulation, we measured the NO concentration in the vicinity of arterioles, venules, and the interstitium of the tissue in the hamster window preparation in conditions of extreme hemodilution (Hct 11%) using LV and HV plasma expanders. In addition, we related this measurement to the calculation of the local vessel wall shear stress (WSS).

MATERIALS AND METHODS

Animal preparation. Investigations were performed in 55- to 65-g golden Syrian hamsters (Charles River Laboratories; Boston, MA). Animal handling and care were provided following the procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The study was approved by the local Animal Subjects Committee.

The hamster window chamber model is widely used for microvascular studies in the unanesthetized state, and the complete surgical technique has been described in detail elsewhere (11, 13). Briefly, the animal was prepared for chamber implantation with a 50 mg/kg ip injection of pentobarbital sodium anesthesia. After hair removal, sutures were used to lift the dorsal skin away from the animal, and one frame of the chamber was positioned on the animal’s back. A chamber consisted of two identical titanium frames with a 15-mm circular window. With the aid of backlighting and a stereomicroscope, one side of the skin fold was removed following the outline of the window until only a thin layer of retractor muscle and the intact subcutaneous skin of the opposing side remained. Saline and then a coverglass were placed on the exposed skin held in place by the other frame of the chamber. The intact skin of the other side was exposed to the ambient environment. The animal was allowed at least 2 days for recovery; its chamber was then assessed under the microscope for any signs of edema, bleeding, or unusual neovascularization. Barring these complications, the animal was anesthetized again with pentobarbital sodium. Arterial and venous catheters (polyethylene-50) were implanted in the carotid artery and jugular vein. Catheters were tunneled under the skin and exteriorized at the dorsal side of the neck, where they were attached to the chamber frame with tape.

Inclusion criteria. Animals were suitable for the experiments if 1) systemic parameters were within normal range, namely, heart rate (HR) > 320 beats/min, mean arterial blood pressure (MAP) > 80 mmHg, systemic Hct > 45%, arterial PO2 (Pao2) > 50 mmHg; and 2) microscopic examination of the tissue observed under ×650 magnification did not show signs of edema or bleeding.

Systemic parameters. MAP and HR were recorded continuously (MP 150, Biopac Systems; Santa Barbara, CA) except during the actual blood exchange. Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes.

Blood chemistry and rheological properties. Arterial blood was collected in heparinized glass capillaries (0.05 ml) from the carotid catheter and immediately analyzed for Pao2, arterial PCO2 (Paco2), and pH (Blood Chemistry Analyzer 248, Bayer; Norwood, MA). The comparatively low Pao2 and high Paco2 of these animals was a consequence of their adaptation to a fossorial environment. Blood samples for viscosity and colloid osmotic pressure measurements were taken with a heparinized 5-ml syringe at the end of the experiment for immediate analysis.

The viscosity of plasma and whole blood were determined with a cone and plate viscometer at 37°C (DV-II+ Viscometer, Brookfield Engineering Laboratories; Middleboro, MA). Although viscosity dependence on shear rate is a minor effect at the high levels of dilution used in these experiments, the shear rate of 160 s⁻¹ was used because it is representative of the average value for the vasculature. Blood samples were centrifuged, and colloid osmotic pressure in the plasma was measured using a membrane colloid osmometer (model 420, Wescor; Logan, UT). Calibration of the osmometer was made with a 5% albumin solution using a 30-kDa cutoff membrane (Amicon; Danvers, MA) (42).

Microhemodynamics. Arteriolar and venular blood flow velocities were measured on-line using the photodiode cross-correlation method (20) (Photo Diode/Velocity Tracker model 102B, Vista Electronics; San Diego, CA). The measured centerline velocity (V) was corrected according to vessel size to obtain the mean RBC velocity (31). A video image-shearing method was used to measure vessel diameter (D) (21). Blood flow (Q) was calculated from the measured values as \( Q = V \times \pi (D/2)^2 \). Changes in arteriolar and venular diameter from baseline were used as indicators of a change in vascular tone.

WSS was defined as \( WSS = WSR \times \eta \), where WSR is the wall shear rate given by \( 8VD^2 \) and \( \eta \) is blood viscosity.

In conditions of extreme hemodilution with Hct ~11%, the contribution of RBCs to the total viscosity of blood is linear and amounts to about 0.70 cp, which is the difference between blood and plasma viscosity. According to Lipowsky and Firrell (30), the ratio between arteriolar-venular and systemic Hct is illustrated by a tendency toward equilibrium during extreme hemodilution, converging to an average value of the ratio between microvascular and systemic Hct of about 0.7 for Hct ~ 10%. Therefore, we corrected our extreme hemodilution viscosity data by linearly reducing the viscosity RBC contribution by 70%. The same procedure was used for the normal blood data, where the Hct reduction factor is 0.58 for arterioles and 0.68 for venules (30); however, because at normal Hct blood viscosity is not linearly proportional to hematocrit, we used actual viscosity versus Hct (dilution with hamster blood) data to obtain the corrected value for blood viscosity, which is referred to as microvascular viscosity (10). Errors related with this correction can be estimated by evaluating the maximum change in blood viscosity that would be present in extreme hemodilution using the extreme of the range of the Hct correction, namely, the reduction of Hct by 58% for arterioles, and adding this to the variability found in the measurements of blood viscosity. Carrying out this calculation, we found that LV blood viscosity in the arteriolar microcirculation is 1.78 ± 0.14 versus 1.97 ± 0.09 cp for the measured blood viscosity and 2.53 ± 0.19 versus 2.76 ± 0.15 cp for HV blood.

FCD. Capillaries were considered functional if RBCs transitted through the capillary segments during a 45-s period. FCD was tabulated from the capillary lengths with RBC transit in an area comprised of 10 successive microscopic fields (420 × 320 \( \mu m^2 \)). FCD (cp⁻¹) is the total length of RBC-perfused capillaries divided by the area of the microscopic field of view (17, 26, 39).

Acute isovolemic hemodilution. Progressive stepwise hemodilution to a final systemic Hct level of 25% of baseline was accomplished with three isovolemic exchange steps. The volume of each exchange was a percentage of the animal’s total blood volume, estimated at 7% of the body weight. The level 1 exchange of 40% of the blood volume was followed by level 2 and 3 exchanges of 35% of the blood volume. Level 1 exchange results in a 40% reduction in Hct. Level 2 and 3 exchange result in extreme hemodilution, where systemic Hct is reduced by 60% and 75% of normal, respectively. With the use of an infusion pump, the exchange solution was first passed through an in-line 0.22-\( \mu m \) syringe filter and then into the animal via the jugular vein catheter at a rate of 100 \( \mu \)l/min. Because of the size of the animals, a rate of exchange was chosen to ensure a stable blood pressure during the exchange period. Each hemodilution step was performed 1 h apart beginning with the exchange and followed by systemic and microvascular observation. The animal was given a 5-min recovery period before data acquisition.

Experimental design. The unanesthetized animal was placed into a restraining tube where it had free access to wet food pellets during the entire experiment. The animal was given 30 min to adjust to the tube.
environment before the baseline systemic parameters were measured (MAP, HR, blood gases, Hct). The tube containing the conscious animal was then affixed to the microscopic stage of a transillumination intravital microscope (Olympus BX51WI). The tissue image was projected onto a charge-coupled device camera (COHU, San Diego 4815-2000) connected to a video cassette recorder and viewed on a monitor. The baseline FCD was assessed using a ×40 (numerical aperture = 0.7) SW objective. For easier detection of RBC passage, the contrast between RBCs and tissue was enhanced with a BG12 (420 nm) bandpass filter. Arterioles and venules chosen for study (2–3 of each type) were characterized by their blood flow velocity and diameter. Vessels were chosen by their visual acuity.

Animals were randomly divided into three experimental groups: 1) control (no hemodilution); 2) level 3-LV (hemodilution with 6% Dextran 70 to reduce the Hct to 25% of baseline); and 3) level 3-HV (hemodilution with Dextran 70 to reduce the Hct to 35% of baseline followed by hemodilution with 6% Dextran 500 (mean molecular weight: 500,000, Pharmacia) in 0.9% normal saline to further reduce the Hct to 25% of baseline).

The experimental time course is shown in Fig. 1, where exchanges began every hour, i.e., the second exchange commenced exactly 1 h after the first exchange. Blood samples were withdrawn from level 3 exchange animals at the end of the experiment for subsequent analysis of viscosity and colloid osmotic pressure measurements. The duration of the experiments was about 3 h.

The concentration of the high-molecular-mass Dextran 500 solution and the exchange protocol were established in a previous study showing that animals could withstand an entire third exchange with 6% Dextran 500 solution without visible in vivo RBC aggregation (10, 39). Pertinent physical properties of the exchange solutions used in this study are listed in Table 1.

**Electrodes for NO measurements.** The nanomolar NO concentration was measured using carbon fiber electrodes (Carbostar-1, Kation Scientific; Minneapolis, MN) (1, 4). The basic shape of the electrodes and dimensions are shown in Fig. 2A. The tip of the electrode was coated with Nafion (5% in aliphatic alcohols, Sigma; St. Louis, MO) to repel active anions (e.g., nitrate, nitrite, and ascorbate) (16). The process consisted of three individual Nafion coats. The first Nafion coat was applied by immersing the electrode tip in the Nafion solution for 5 days at 25°C followed by drying for 24 h. Two additional coats were applied by leaving the electrodes in the Nafion solution at 50°C for 10 min and then drying at 80°C for 20 min. The microelectrodes were polarized at +0.8 V relative to a silver-silver chloride reference electrode (ee009, Cypress Systems; Lawrence, KS). This reference electrode was chosen for its small active size (diameter 800 μm) and limited leakage due to a protective polyether ketone membrane cover, which provides improved stability during calibration and in vivo measurements. NO measurements were performed using the two electrodes system (working and reference electrode), and the current generated was measured with a potentiostat and electrometer (Keithley model 610C; Cleveland, OH). Amplification of the electrometer was 30 mV/pA.

The microelectrodes were calibrated at 37°C with concentrations of NO gas balanced with N2 of 2.746 nM (1,788 ppm 1%), 466 nM (304 ppm 1%), and 0 nM (100% argon) (Airgas; Los Angeles, CA).

Calibrations for the linearity of response to NO were carried out in 25 ml of PBS (pH 7.4) mixed with distilled and deionized water at 37°C. First, the system was purged with argon gas for a period of 10–15 min delivered at a moderate flow rate (pressure 50–150 mmHg) until a stable zero electrode signal was obtained. Each NO gas concentration was bubbled through the test solution for 5–10 min until a stable signal was obtained. The system was purged with argon between each period of NO bubbling. The results are shown in Fig. 2B.

Microelectrodes had a linear current-NO relationship and 0 nM NO currents were in the range of 5–10 pA (Fig. 2C). Only microelectrodes that generated >1 pA/1,000 nM NO were used. This sensitivity translated to an increase of 1 mV in output of the electrometer for each 4 nM elevation in NO concentration or 80–120 mV above baseline for typical periarteriolar NO concentration. Before measurements, the electrode tip was immersed in the supernatant suffusion solution and the current was registered was set to be the 0 nM NO reference point. Upon introduction into the tissue, the microelectrodes responded with a time constant that was estimated to be of the order of 30 s. A stable reading was obtained within 1 min, and upon reaching the current plateau value, the electrode was extracted from the tissue and the tip maintained within the suffusing saline solution. The current for the next 0 nM NO was usually slightly different from the prior measurement, and the peak measured value was corrected by subtracting the average of the before and after zero reference values (Fig. 2B, difference between point 2 and 1). The reference 0 nM equivalent output voltage and the calibration output voltage-NO relationship were used to calculate NO concentration.

**Perivascular NO measurements.** The coverglass of the window chamber was removed at the completion of the microhemodynamics measurements after the third exchange, and then tissue preparation was superfused (~5 ml/min) with a physiological Krebs salt solution. The tissue was maintained at 33–34°C by the heated solution. The solution spread on the tissue as a thin film, drained into a platter, and drawn off by suction. The solution was equilibrated with 95% N2-5% CO2, which maintained the suffusate at a pH of 7.4 and minimized O2 delivery to the tissue (35, 36). NO measurements were initiated 20 min after glass window removal, a period that was found to allow the tissue to stabilize with constant microvascular parameters (10). Perivascular measurements were made by penetrating the perivascular tissue with the micropipette so that the tip was as close as possible to the microvessel without touching the wall, up to the point that no visible wall deformation could be noted. As in the experiments of Bohlen and Nase (3), if the vessel wall was penetrated, there were transient and sustained increases in NO concentration. The reference electrode was placed in the bath, and the carbon electrode was placed in a shielded holder and advanced towards the measurement site with a micromanipulator. The microscope frame was grounded without additional electrical shielding. A long-working distance ×10 Leitz

**Table 1. Viscosity and COP of the exchange solutions**

<table>
<thead>
<tr>
<th>Molecular Mass, Da</th>
<th>Viscosity, cp</th>
<th>COP, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran 70 (6%)</td>
<td>70,000</td>
<td>2.68</td>
</tr>
<tr>
<td>Dextran 500 (6%)</td>
<td>500,000</td>
<td>5.92</td>
</tr>
</tbody>
</table>

Viscosity was measured at 37°C. COP, colloid osmotic pressure at 25°C.
objective was used to direct the electrode to the measurement site. As in the studies of Nase et al. (34), our goal was to achieve the highest possible NO for a given vessel. Perivascular arteriolar and venular NO values obtained with these Nafion carbon fiber microelectrodes were compared with the Nafion-coated, gold-plated, recessed-tip microelectrodes developed by Buerk and colleagues (7) and the group of Bohlen (2, 28). Reproducibility of measurements was on the order of ±10%. Perivascular measurements should be representative of the NO concentration at the blood tissue interface according to Buerk (6), who showed that the models of Butler et al. (9) and Vaughn et al. (41) predict a maximal fall of NO concentration of about 5% at a distance from the endothelium corresponding to the vessel wall thickness.

**Measurement of eNOS activity.** An independent verification of whether NO generation by shear stress is a factor in causing the detected vasodilatation is to measure the degree of gene and protein expression in the endothelium of markers related to the functional capacity of the cells to produce NO. eNOS activity is measured by the conversion of L-[^3H]arginine to L-[^3H]citrulline (8, 14). Two hours (22) after the completion of hemodilution, the hamster was sedated with pentobarbital sodium (50 mg/kg) and heparinized (1,000 IU/kg). Aortic and window preparation tissue was harvested and snap frozen (−70°C). Cells were homogenized and combined with the assay mixture to accentuate L-[^3H]citrulline. As a second enzymatic assay (Dowex AG50W-X8) was used to remove unreacted L-[^3H]arginine. L-[^3H]citrulline production was measured using a liquid scintillation spectrometer. Cells were washed with ice-cold PBS and then lysed in buffer (14). The cell lysate was centrifuged, and the supernatant was collected. The protein concentration was measured using a Bio-Rad reagent. To detect specific protein expression, the membranes were blocked in 5% nonfat milk; incubated with an antibody against NOS-3, PDGF receptor, PY20, cyclin A, cyclin E, p27, p21, or proliferating cell nucleus antigen for 1–1.5 h and then incubated with mouse anti-mouse IgG-horseradish peroxidase for 1 h. Labeled peroxidase activity was detected using an ECL Western blotting detection kit. For multiple blotting for different proteins on the same membrane, the membrane was stripped with occasional agitation (8). The intensities of the bands are quantified by densitometric analysis using a gel scanner, and the results are expressed as the densities of eNOS-specific bands.

**Data analysis.** Results are presented as means ± SD unless otherwise noted. All measurements were compared with their levels at baseline before the blood exchange except for the NO measurements, which were compared between groups of animals. For repeated measurements within a group, time-related changes were assessed by ANOVA. Comparison between different groups of animals was performed with the Mann-Whitney rank sum test. Multiple comparisons were made with Newman-Keuls comparison test (Prism 4.01, GraphPad; San Diego, CA). Changes were considered statistically significant if \( P < 0.05 \).

**RESULTS**

Eighteen animals were entered into this study for the measurement of NO, and all animals tolerated the entire hemodilution protocol without visible signs of discomfort. The animals were assigned randomly to the experimental groups: control (\( n = 7 \)), LV hemodilution (\( n = 5 \)), and HV hemodilution (\( n = 6 \)).

An additional eight animals were used to determine the relative levels of eNOS between LV and HV hemodilution. These animals were also randomly assigned to experimental groups: LV hemodilution (\( n = 4 \)) and HV hemodilution (\( n = 4 \)).

**Baseline and control groups.** One-way ANOVA on these data showed no significant differences in any of the systemic or microcirculatory parameters, therefore allowing for grouping of the data into one representative group for each of the two states: baseline (\( n = 18 \)) and level 2 (\( n = 11 \)). The control group (\( n = 7 \)) was used to directly compare parameters that could not be repeated at each level in the animal, namely, NO levels and blood rheology parameters.

**Systemic and blood gas parameters.** Changes in systemic and blood gas parameters for each experimental group are presented in Table 2. Systemic Hct dropped to 0.26 ± 0.02 and 0.25 ± 0.01 of baseline as a result of the level 3 exchanges with Dextran 70 (LV) and Dextran 500 (HV), respectively (\( P < 0.05 \)). Hct values were not statistically different between level 3 LV and HV groups.

Blood pressure fell to 0.65 ± 0.09 of baseline in the level 3 LV group (\( P < 0.05 \)). In the level 3 HV group, blood pressure fell to 0.82 ± 0.09 of baseline (\( P < 0.05 \)). HR was not affected by either of the hemodilution protocols.
Table 2. Systemic parameters

<table>
<thead>
<tr>
<th></th>
<th>Control/Baseline</th>
<th>Level 2</th>
<th>LV</th>
<th>HV</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>11</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>107.4 ± 9.4</td>
<td>87.5 ± 10.4 (0.84 ± 0.08)*</td>
<td>75.7 ± 9.4 (0.70 ± 0.12)†</td>
<td>84.1 ± 3.4 (0.82 ± 0.05)†</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>422.8 ± 51.1</td>
<td>457.2 ± 20.3 (1.07 ± 0.11)</td>
<td>422.8 ± 28.9 (1.01 ± 0.13)</td>
<td>434.7 ± 23.1 (1.04 ± 0.10)</td>
</tr>
<tr>
<td>Hct, %</td>
<td>5.6 ± 2.5</td>
<td>18.7 ± 0.7 (0.42 ± 0.02)*</td>
<td>11.6 ± 0.6 (0.26 ± 0.02)†</td>
<td>11.1 ± 0.2 (0.25 ± 0.02)†</td>
</tr>
<tr>
<td>P02, mmHg</td>
<td>62 ± 6.8</td>
<td>77.4 ± 12.1 (1.07 ± 0.17)*</td>
<td>90.5 ± 13.6 (1.43 ± 0.24)†</td>
<td>104.9 ± 6.6 (1.74 ± 0.14)†</td>
</tr>
<tr>
<td>Pco2, mmHg</td>
<td>52.1 ± 5.9</td>
<td>54.0 ± 0.4 (1.07 ± 0.17)</td>
<td>46.2 ± 9.8 (1.01 ± 0.20)*</td>
<td>47.6 ± 10.6 (0.67 ± 1.5)*</td>
</tr>
<tr>
<td>pH</td>
<td>7.38 ± 0.03</td>
<td>7.37 ± 0.05 (1.00 ± 0.01)</td>
<td>7.40 ± 0.07 (1.00 ± 0.01)</td>
<td>7.40 ± 0.10 (1.00 ± 0.01)</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of animals. MAP, mean arterial pressure; HR, heart rate; Hct, hematocrit; P02, PCO2, and pH, arterial blood gas analysis. The first numbers represent the absolute values of the parameters, and the numbers in parentheses are the parameters normalized relative to baseline. There were no differences between any of the groups at baseline and at level 2 exchange, and the values were combined. *P < 0.05 relative to baseline; †P < 0.05 relative to level 2.

Arterial blood gas analysis showed a rise in P02 and a fall in Pco2, but there were no statistical differences between the level 3 LV and HV exchange groups. Animals were able to maintain their blood pH, and changes were not significant from baseline.

**Physical properties of blood.** A comparison of rheological properties and the colloid osmotic pressure of the blood after level 3 exchange is presented in Table 3. Changes in blood and plasma viscosity were statistically different from baseline and between the HV and LV groups (P < 0.05). Blood viscosity was reduced to 0.47 and 0.65 of baseline, and plasma viscosity was increased to 1.10 and 1.64 of baseline levels for level 3 LV and HV groups, respectively. Colloid osmotic pressure was statistically unchanged during the progressive hemodilution in both experimental groups. Leukocytes were significantly depleted by the exchange procedure and were not found to be sticking in the window chamber microvessels during the period of window removal, due to short duration of tissue exposure (<1 h).

**Microhemodynamics.** The changes in arteriolar and venular hemodynamics relative to baseline are shown for level 2 and level 3 for LV and HV groups in Fig. 3.

**Level 3 exchange with the LV and HV protocol resulted in no significant change in arteriolar diameter from baseline, level 2, and each other. Venular diameter after level 3 HV was statistically similar to baseline but statistically increased compared with level 2 and level 3 LV (P < 0.05).**

Arteriolar and venular velocity was increased after level 2 exchange relative to baseline (P < 0.05). Level 3 LV resulted in a significant decrease in both arteriolar and venular velocity (P < 0.05 relative to baseline and level 2). Level 3 HV resulted in a statistical increase in arteriolar velocity relative to baseline and level 3 LV and a statistical decrease relative to level 2 (P < 0.05). Venular velocity after level 3 HV found a statistical increase relative to baseline and level 3 LV but no difference from level 2 (P < 0.05).

The blood flow was increased from baseline after level 2 in both arterioles and venules due in part to the increase in arteriolar and venular velocity (P < 0.05). Level 3 LV decreased arteriolar blood flow relative to baseline and level 2 (P < 0.05); venular flow was unchanged from baseline but lower than level 2. Level 3 HV increased both arteriolar and venular flow relative to baseline and level 3 LV (P < 0.05).

The high venular flow achieved after level 2 was maintained after level 3 HV.

FCD. FCD decreased to 0.90 ± 0.09 of baseline (P < 0.05) after level 2 exchange. This 10% drop in FCD has been found to be within the normal physiological fluctuation over a period of 6 h for this tissue bed (17) and should not be considered a significant change in this parameter from baseline. A comparison between FCD after level 3 LV and HV found a decrease to 0.54 ± 0.06 and 0.80 ± 0.06, respectively, both statistically lower relative to baseline and different from each other (P < 0.05).

WSS. WSS was calculated following the scheme set forth in MATERIALS AND METHODS using the diameter and velocity for each vessel and viscosity. The viscosities used in the calculation of WSS were 1) whole blood viscosity, 2) blood viscosity in the microcirculation corrected according to Lipowsky and Firrell (30), and 3) plasma viscosity. Figure 4 compares WSS using the different viscosities for each study group and vessel type.

**Perivascular and tissue NO concentration.** The results of NO concentration measurements for each experimental group are shown in Fig. 5. There were no statistical differences between arteriolar and venular perivascular NO levels within each of the study groups. Within the control and level 3 HV groups, there were statistically significant differences between perivascular and tissue NO levels, which were not found in the level 3 LV group.

Perivascular arteriolar NO levels during control were not significantly different to those measured in level 3 LV. Level 3 HV perivascular NO was statistically significantly increased relative to control and level 3 LV. Comparison of perivascular venular and tissue NO levels found a similar pattern with no difference between control and level 3 LV, whereas level 3 HV had a significant increase in the NO level. NO levels perivascular to arterioles and venules and in the tissue were signifi-

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Table 3. Viscosity and COP after level 3 exchange

<table>
<thead>
<tr>
<th>Blood (control)*</th>
<th>n</th>
<th>ηpb, cP</th>
<th>ηpb, mmHg</th>
<th>COP, cP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV (Dextran 70)</td>
<td>5</td>
<td>1.97 ± 0.09†</td>
<td>1.33 ± 0.04†</td>
<td>17.2 ± 1.0</td>
</tr>
<tr>
<td>HV (Dextran 500)</td>
<td>6</td>
<td>2.76 ± 0.15†</td>
<td>1.99 ± 0.11†</td>
<td>17.9 ± 1.9</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of animals. ηpb, whole blood viscosity; ηpb, plasma viscosity. *Cabral et al. 2004 (10). †P < 0.01 between low-viscosity (LV) and high-viscosity (HV) plasma after level 3 exchange.
level 3 HV was compared with level 3 LV (P < 0.05). In this study, in conditions of extreme hemodilution (Hct = 11%) perfusion was maintained above baseline (normal Hct) values when plasma viscosity was elevated to 1.99 ± 0.11 cp (vs. 1.21 ± 0.08 cp in control).

The changes in perfusion, in terms of FCD and microvascular flow, were similar to those obtained previously with the same protocol by Tsai et al. (39) and Cabrales et al. (10). Arteriolar and venular diameters were not different from baseline for HV plasma; however, arteriolar flow with HV plasma was 20% higher than baseline (P < 0.05). This is the conse-

Fig. 3. Arteriolar (A) and venular (B) hemodynamics relative to baseline for level 2, level 3 LV, and level 3 HV. The baseline mean and standard deviation for 1) arteriolar diameter and velocity are 54.9 ± 17.7 μm and 5.5 ± 2.7 mm/s, respectively; and, 2) venular diameter and velocity are 61.9 ± 18.3 μm and 1.8 ± 0.9 mm/s for venules, respectively. The dotted line denotes the baseline level 1.0; changes above and below this line are increases and decreases relative to baseline. *P < 0.05 from baseline; **P < 0.05 from level 2; ***P < 0.05 between level 3 LV and level 3 HV.

Fig. 4. Wall shear stress (WSS) in arterioles and venules for each of the study groups: control, level 3 LV, and level 3 HV. A–C: WSS calculated using different viscosities in the calculation, namely, whole blood viscosity (A), microvascular viscosity (B), and plasma viscosity (C). Solid bars, arterioles; shaded bars, venules. *P < 0.05; ***P < 0.01; ****P < 0.001.

DISCUSSION

The principal finding of this study was that the increased perfusion found in conditions of extreme hemodilution using HV plasma corresponds to increased perivascular NO concentrations in both arterioles and venules of 50- to 60-μm diameter in the tissue of the hamster window preparation, which are significantly greater than in control conditions and in conditions of extreme hemodilution with LV plasma. Tissue NO, however, was statistically unchanged from control with LV

eNOS expression and blood viscosity. Analysis of the relative differences in aortic eNOS protein expression is shown by the pattern of Western blot and quantitated by measuring their optical density (Fig. 6). The optical density of the eNOS band was statistically higher in level 3 HV relative to level 3 LV animals (P < 0.001). Control eNOS-specific proteins were used to establish a band specific location on the Western blot. The animals were maintained at the hemodiluted condition for 2 h before their tissue was harvested. The eNOS activity of the window tissue was not detectable; therefore, the results are only from harvested aortic tissue.

Fig. 3. Arteriolar (A) and venular (B) hemodynamics relative to baseline for level 2, level 3 LV, and level 3 HV. The baseline mean and standard deviation for 1) arteriolar diameter and velocity are 54.9 ± 17.7 μm and 5.5 ± 2.7 mm/s, respectively; and, 2) venular diameter and velocity are 61.9 ± 18.3 μm and 1.8 ± 0.9 mm/s for venules, respectively. The dotted line denotes the baseline level 1.0; changes above and below this line are increases and decreases relative to baseline. *P < 0.05 from baseline; **P < 0.05 from level 2; ***P < 0.05 between level 3 LV and level 3 HV.

AJP-Heart Circ Physiol • VOL 288 • APRIL 2005 • www.ajpheart.org

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quency of the significant decrease in blood viscosity due to hemodilution, which was 65% of baseline (Table 3), and the moderate lowering of blood pressure, which was 78% of baseline. HV microvascular flow was significantly higher than the flow attained with LV plasma, which was 40% below baseline. These results were similar for both arterioles and venules.

The values of perivascular NO concentration found in this study for control conditions are somewhat higher than those reported by Bohlen and Nase (3) in their study of the anesthetized rat intestine. They found that the periarteriolar NO concentration in vessels averaging 523 ± 16 μm in diameter was 522 ± 33 nM, whereas in our study the periarteriolar concentration for similar vessels was 632 ± 36 nM (means ± SE). This difference is probably accounted for by the difference in species, tissue, and our animals not being anesthetized. It is also possible that the difference in electrodes may account for the discrepancy in results, because the electrodes used in the Bohlen and Nase study (3) were based on those of the Whalen et al. (43) electrodes modified according to Buerk et al. (7) and Bohlen (2). In our study, we used Nafion-coated carbon fiber microelectrodes, which we found to be more reproducible and to exhibit less noise in our preparation compared with the Nafion-coated gold-plated recessed-tip microelectrode. Another factor that we found to introduce a considerable variability in the measurements was the changes in temperature in our exposed preparation. Thus all of our measurements are individually corrected according to the simultaneously measured temperature of the preparation in the vicinity of the point of measurement via a thermocouple.

While the increase in perivascular NO concentration was significant during level 3 HV perfusion, it is also apparent that the dispersion of the data significantly increased in this condition. This indicates that the generalized increase of NO concentration is not a uniform phenomenon common to all microvessels. These experiments do not allow differentiation between upregulation of production of eNOS versus inducible NO synthase; however, because the only contact of the visco-
genic Dextran 500 is at the surface of the endothelium, and because this material does not extravasate, the effects should be mostly contributed to generation of NO by the endothelium. This assumption is supported by the finding of increased aortic eNOS protein expression. Tissue NO levels were the same for control and level 3 LV hemodilution; however, level 3 HV tissue NO levels were statistically higher than level 3 LV tissue NO levels (P < 0.05), a result that is probably due to the increased NO diffusion from the perivascular tissue into the tissue.

According to Nase et al. (34), hypoxia increases the perivascular NO concentration, which would explain the findings with HV hemodilution; however, LV hemodilution, which produces an identical level of both microvascular and tissue PO2 (39), did not exhibit increased NO concentrations. Thus the only variables that are different between HV and the LV experiments are flow and plasma/blood viscosity.

In these experiments, arterioles and venules were characterized individually, in such a fashion that flow velocity and local diameter were determined before removal of the chamber window for measurement of perivascular NO concentration in the same vessel. We have previously shown that changes after this manipulation did not alter these parameters in control conditions (10). Viability of the preparation was evaluated in terms of maintenance of flow, which was of the order of 2 h; therefore, the same vessels could not be measured for perivascular NO concentration at baseline and then again when the animal was hemodiluted and the plasma viscosity was changed. Consequently, each NO measurement is only paired relative to the local flow and shear stress, but we are unable to relate the change in flow and shear stress relative to the change in perivascular NO concentration as in the study of Bohlen and Nase (3).

Shear stress and perivascular NO were found not to be statistically related. This finding is in contrast with previous work showing a direct correlation between shear stress and NO production by the endothelium by Frangos et al. (15), who measured NO production in endothelial cell cultures. De Wit et

![Graph showing perivascular and tissue NO concentration levels for each of the study groups: control, level 3 LV, and level 3 HV. A, arteriole; V, venule; T, tissue. *P < 0.05; **P < 0.01; ***P < 0.001; ns, no significance.]

![Image of Western blot analysis showing increased aortic endothelial NO synthase (eNOS) protein expression when animals were hemodiluted with a HV versus LV plasma expander. The optical density of the eNOS-specific bands in each group shown below the x-axis were statistically significantly different from each other (**P < 0.001).]
blood. If we assume that shear stress is directly related to the extreme hemodilution; however, this is not the case for whole assumptions converge to similar values for shear stress in 

The differences in perivascular NO concentration between control conditions and hemodilution may also be due in part to NO scavenging by hemoglobin and other materials. Current models for NO transport, as reviewed by Buerk (5), predict that a decrease in hemoglobin concentration due to hemodilution lowers NO scavenging. A simulation performed with the model of Buerk et al. (6) to examine the reduction in NO scavenging that would be predicted with a reduction in Hct from 45 to 11% shows that peak NO in the endothelium increases by 1.52 times, with NO at the outer wall having a similar increase. Furthermore, NO reacts also with other materials having -SH groups in blood (such as albumin, glutathione, and cysteine), because -SH reacts with NO to produce NO S-nitrosothiol (SNO). Therefore, because extreme hemodilution with dextran not only reduces Hct but also dilutes all blood components, NO scavenging should be significantly reduced (33). This could account for the lower perivascular NO found in extreme hemodilution, even though shear stress is considerably higher than with HV hemodilution if either whole blood or microvascular blood viscosity are used to compute vessel WSS.

Regardless of the viscosity chosen for evaluating shear stress in the microvascular wall, our results show that for control conditions hemodilution arteriolar wall shear stress is always higher than at the venular wall. However, the perivascular NO concentration was the same for arterioles and venules for all groups. Another factor to be considered is the difference in wall thickness between arterioles and venules, which could affect the amount of measured perivascular NO. The differences in wall thickness between arterioles and venules are presumed to be substantial, maybe a factor of 2, but in vivo data are not readily available and there is scarce in vivo evidence for this. The analysis of Buerk, previously referred to, shows that a factor of 2 in wall thickness, namely, the difference between 10 and 20 μm in microvessels of 100 μm in diameter may account for about 5% in the difference between NO concentration, well within the margin of error in these experiments.

Venules could be considerably more sensitive to shear stress than arterioles; however, the prominent role of the arterioles as suppliers of NO has been shown by Kashiwagi et al. (24), who found that arterioles constitute a source of NO that is not present in venules. An alternative explanation is that NO produced in the arterioles can be transported downstream by the blood as proposed by Jia et al. (23) and as shown by Kosaka and Seiyama (27). According to this hypothesis, SNO is transported in the vasculature by reacting with Cysβ93 on the hemoglobin molecule, with an affinity that is governed by the local oxygen concentration.

NO concentration is to some extent dependent on oxygen concentration because NO synthase produces NO using arginine and O2 and finally NO converts to NO2 by the reaction with O2. In a previous study (39), we measured the distribution of oxygen tension using the identical protocol as in the present study; however, there were no statistical differences in oxygen distribution between arterioles, venules, and tissue. Therefore, in these experiments, NO concentrations appear to be independent of blood and tissue Po2, which was the same with HV and LV extreme hemodilution.

The finding that aortic eNOS protein expression was detectable with HV but not with LV supports the finding that HV is associated with conditions that promote NO production compared with LV.

In conclusion, maintenance of microvascular perfusion in extreme hemodilution obtained by increasing the viscosity of plasma is directly associated with increased perivascular NO concentration, an effect related to vasodilatation and increased perfusion and FCD compared with the same
procedure using normal viscosity plasma. This effect may be due to HV plasma maintaining shear stress at the baseline level, an effect not attainable with normal viscosity plasma. Shear stress at the microvascular wall with HV plasma is definitely higher than that found with LV plasma in extreme hemodilution, leading to a significantly higher concentration of perivascular NO in the arterioles and venules. Perivascular NO concentration in extreme hemodilution with HV plasma is higher than baseline, although shear stress at baseline is not statistically different from that attained during HV hemodilution when blood viscosity is corrected for the decrease in hematocrit as blood advances in the microcirculation. This finding suggests that NO scavenging by hemoglobin in whole blood is significantly greater by comparison with diluted blood. The finding that arterioles and venules with either whole blood or reduced RBC (hemodiluted) perfusion have the same levels of NO suggests that NO is transported from the high shear stress arterioles to the venules, an effect that may significantly contribute to the dispersion of the data, because locally measured effects are dependent on distal activation of NO production. Finally, the effects due to the increase of viscosity plasma induced by the relatively inert molecular species Dextran 500, which does not extravasate, should be restricted to direct effects due to shear stress acting the endothelium; therefore, it appears that the arteriolar endothelium is a prodigious source of NO when stimulated by increased shear stress.

ACKNOWLEDGMENTS

The authors thank Froilan Allan Barra and Cynthia Walser for excellent animal preparations and technical assistance.

GRANTS

This research was conducted with the financial support from National Heart, Lung, and Blood Institute (NHBLI). Bioengineering Research Partnership Grant R24-HL-64395 and Grants R01-HL-62354 and R01-HL-62318 (to D.G. Buerk) supported in part by NHLBI Grant R01-HL-62318 to A. G. Tsai. D. G. Buerk supported in part by NHLBI Grant R01-HL-68164.

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