Oxygen-Dependent Vasomotor Responses Are Conducted Upstream in the Mouse Cremaster Microcirculation

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Key Words

Arteriole • Conducted vasodilation • Cremaster • Hypoxia • Microcirculation • Vasomotor responses

Abstract

Background: A new method was evaluated where local changes in oxygen tension were induced in a tissue while being studied under a microscope in vivo. We tested whether hypoxic vasodilation and hyperoxic vasoconstriction in arterioles in striated muscle are being propagated upstream, and whether the endothelium and smooth muscle cell layers are necessary components in the signaling pathway. **Methods:** The study was performed in mouse cremaster muscle superfused with Krebs buffer. A section of the capillary bed was then superfused with human red blood cell suspension equilibrated with either 95% nitrogen or 95% oxygen, and 5% carbon dioxide. **Results:** The superfusions caused a $12.9 \pm 2.4\%$ ($p < 0.01$) dilation and a $12.3 \pm 2.7\%$ ($p < 0.01$) constriction of the supplying non-exposed arteriole. Vasomotor responses could be detected 1 mm upstream of the stimulation site. The responses to hypoxia and hyperoxia were not affected by inhibition of nitric oxide (NO) synthases by L-NAME. Damage to the wall of an intervening segment of the arteriole abolished upstream changes. **Conclusions:** The

method is capable of changing the oxygen tension locally in a membranous tissue and elicits NO-independent local and upstream vasomotor responses. Upstream responses were transmitted by a conducted vascular response.

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Introduction

Regulation of blood flow in relation to exercise and hypoxia has been studied intensively for almost a century [1–3], but the exact mechanisms involved and the way they interact remain obscure. Tissue hypoxia is followed immediately by increased blood flow. It is believed that local sensing of changed oxygen tension is transmitted upstream and causes changes in the diameter of the supplying arteriole to adjust blood flow. Two mechanisms have been proposed for this upstream vasodilation. One mechanism is flow-induced vasodilation, which is thought to be a result of increased flow in more proximal artery segments due to downstream local vasodilation [4]. The increased flow and shear stress cause release of vasoactive autacoids from the endothelium and a subsequent vasodilation in the proximal arterioles and arteries. The second mechanism is conducted vasodilation [5],

i.e. the spread of local vasomotor signals within the arterial wall, most likely transmitted through gap junctions [6, 7] between endothelial cells and/or vascular smooth muscle cells. Also important in this respect could be heterocellular myoendothelial gap junctions [8] that form channels between endothelial and vascular smooth muscle cells through the internal elastic lamina.

A common perception is that appropriate local circulatory adjustments to exercise and changes in oxygen levels in the blood depend on an intra- or extracellular oxygen sensor, which is able to initiate adequate alterations in muscular blood flow [9–11]. It is well known that several metabolites from active muscle fibers, such as carbon dioxide, potassium, lactic acid and adenosine, can induce vasodilation [12–14]. So far, solid experimental evidence demonstrating the actual mechanism underlying oxygen sensing is lacking. It has been suggested that the red blood cell (RBC) itself might function as a mobile oxygen sensor [15] via an adenosine triphosphate (ATP)-mediated pathway [16, 17]. This theory is intriguing because RBC are mobile and can therefore function where they are needed, and because of the very well documented relationship between the amount of circulating oxygen and blood flow in exercising skeletal muscle [18–20].

In the present study, we established a method to induce local hypoxia and hyperoxia in membranous tissue thereby enabling the study of vasomotor responses induced in arterioles supplying the affected area. The local changes in oxygen tension were induced by selective local superfusion of a capillary bed in the membranous cremaster muscle from the C57 mouse with oxygenated or deoxygenated suspended human RBC. This is a novel approach to induce local changes in the oxygen level. To examine the mechanism of propagation, the experiments were repeated after damage was inflicted on an intervening segment of the arteriole, or after administration of N ω -nitro-L-arginine methyl ester hydrochloride (L-NAME), a nitric oxide (NO) synthase (NOS) inhibitor.

In the present paper, we investigated the mechanisms underlying the upstream vascular responses to changes in tissue oxygen tension. Specifically, it was the aim to test (1) whether localized changes in oxygen tension elicit a vasomotor response in vessels not exposed to the changes in oxygen tension; (2) whether this upstream response is due to flow-mediated changes in shear stress, and consequently, changes in the upstream production of NO or other endothelial-derived factors, and (3) whether the upstream response is due to a conducted vascular response travelling within the vascular wall.

Materials and Methods

Animal Preparation

The experimental protocol was approved by the National (Danish) Research Animal Committee and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication No. 85-23, revised 1996). Male mice, C57BL6J (Taconic Europe; n = 36; weighing 24.1–34.4 g), were used for the experiments. Anesthesia was induced by an intraperitoneal injection of droperidol (38 mg/kg), midazolam (4 mg/kg) and fentanyl (0.2 mg/kg), and maintained by intravenous infusion of droperidol (0.76 mg/h), midazolam (0.08 mg/h) and fentanyl (0.004 mg/h) given in isotonic saline at a rate of ~0.4 ml/h. Anesthetized mice were tracheostomized, and the common carotid artery and the external jugular vein were cannulated for measurements of blood pressure and intravenous infusions, respectively. After placing the animal in supine position, the cremaster muscle was exteriorized, spread out and mounted with ligatures. The cremaster muscle was continuously superfused with Krebs buffer (in mM: Na⁺ 143, K⁺ 6, Ca⁺⁺ 2.5, Mg⁺⁺ 1.2, Cl⁻ 128, HCO₃⁻ 25, SO₄⁻ 1.2, H₂PO₄⁻ 1.2, pH 7.4) equilibrated with a gas mixture of 95% nitrogen and 5% carbon dioxide. The muscle as well as the buffer was maintained at 34–35°C. All chemicals in this study were obtained from Sigma-Aldrich (St. Louis, Mo., USA), unless stated otherwise.

Visualization of Vessels

The cremaster microcirculation was observed *in vivo* using a water immersion objective mounted on an upright microscope (UMPlanFl 20 $\times$ /0.5 W $\bullet\bullet$ /o and BX50WI; Olympus, Tokyo, Japan). During the entire experiment, digital images were continuously recorded for off-line analysis. The final magnification of the image was ~ $\times$ 700 and the spatial resolution was ~0.5 μ m. The microscope was mounted on a motorized moveable stage (Micromanipulator Mini 25; Luigs and Neumann, Ratingen, Germany) allowing storage and retrieval of microscope positions along the course of a vessel.

Application of the Human RBC Suspension and Krebs Buffer

The basic experiment is shown in the online supplementary video (www.karger.com/doi/10.1159/000318777: Deoxygenated RBC mixture is being added to the cremaster muscle. Time has been speeded up by a factor of 4. The film was recorded through a low-magnification objective to get a general picture of the effects of this intervention. Note the arteriole entering the picture from the top, bifurcating and thus forming an upside-down Y. It is supplying the capillary bed in the lower right corner of the picture where the deoxygenated RBC mixture is applied. At first it is constricted and barely visible, then after 20–30 s with hypoxia it dilates and becomes clearly black).

The experimental setup is schematically shown in figure 1. The cremaster muscle was continuously superfused with Krebs buffer equilibrated with 5% CO₂ and 95% N₂. All gas mixtures used for equilibration contained 5% CO₂. A segment of the capillary bed was selectively superfused with an RBC suspension or a separate buffer, both equilibrated with either 95% oxygen or 95% nitrogen and 5% CO₂. This separate buffer and the RBC suspension were kept in plastic syringes (10 ml Luer BD Plastipak, ref. 302188; Becton Dickinson, Madrid, Spain) after gas equilibration and did not pass the tubing system in the basic experimental set-

Fig. 1. Schematic drawing of the experimental setup. The membranous striated cremaster muscle ($\sim 2 \times 2$ cm) is seen from above through a transparent superfusion of Krebs buffer. The arteriole under investigation is seen with the two points of inspection and measurement, local and 1,000 μm upstream. The red area in the upper right part of the drawing illustrates the localized area superfused with RBC. Note that the RBC suspension only superfuses the tissue section which is perfused by the arteriole studied and that the arteriole itself is not in direct contact with the RBC suspension. □ = Placement of the oxygen-sensing microoptode. Note that it was placed beneath the cremaster tissue, i.e. beneath the site where the local superfusion was applied to the tissue, so that it was not directly exposed to the superfusate.

up. Hence the deoxygenated mixtures maintained a lower oxygen tension than the general superfusion buffer, which had an oxygen tension of approximately 91 mm Hg.

Packed (buffy coat-depleted) human RBC in SAG-M (sodium chloride, adenine, glucose and mannitol) [21] suspension were obtained from the local blood bank and resuspended in Krebs buffer (ratio 50:50) to mimic a hematocrit of approximately 50%. This mixture as well as the separate Krebs buffer was equilibrated for 10 min with N_2 or O_2 , both containing 5% CO_2 . Equilibrated RBC suspension or buffer was applied by a metal cannula [Neolus 23G (0.6×30 mm); Terumo Europe, Leuven, Belgium] to a capillary bed supplied by the arteriole of interest so that it was continuously washed away from the arteriole by the general superfusion. RBC suspension and Krebs buffer were applied at a rate of 2–3 ml/min using a syringe pump (type 871102; Braun, Melsungen, Germany). An inline heater (automatic temperature controller TC-324B and inline temperature probe model SH27B; Warner Instruments, Hamden, Conn., USA) ensured a constant temperature of 34–35°C. Great care was taken to avoid exposure of the solutions to the ambient atmosphere. All measurements were performed within 20–30 s after application of the various mixtures. In a second series, the mixtures were equilibrated with carbon monoxide also containing 5% CO_2 .

Inhibition of NOS by L-NAME

In a separate experimental series, NOS were inhibited by L-NAME, which was administered at a concentration of 100 μM in

the general superfusion buffer. Full effect of L-NAME was typically observed after 1–2 min, but to ensure maximal inhibition of NOS we waited 15 min before initiation of the experiments [22]. To dilate the vessels maximally, a new general superfusion buffer containing 100 μM of papaverine was used at the end of each experiment.

Oxygen Measurement

Oxygen levels in the tissue were measured with an oxygen microoptode (sensor type PSt1 and Microx TX3 oxygen meter; Pre-Sens Precision Sensing, Regensburg, Germany). Calibration of the microsensor was performed as prescribed with a two-point calibration procedure in oxygen-free water (calibrated to 0 mm Hg) and water vapor-saturated atmospheric air (calibrated to 155 mm Hg). Calibration to 0 mm Hg was prepared by diluting 4 g sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) in a vessel containing 60 ml water. Any dissolved oxygen will react with the sodium dithionite in the solution and the partial pressure of oxygen in the solution will be 0 mm Hg. Calibration to 155 mm Hg was prepared by placing wet cotton wool in a vessel containing atmospheric air. Due to the presence of the wet cotton, the air in the vessel will be saturated with water vapor, and the partial pressure of oxygen will be 155.4 mm Hg (at a barometric pressure of 760 mm Hg and a temperature of 20°C). The microoptode automatically compensates for variations in temperature and barometric pressure.

During the experiments, the tip of the microoptode was placed between the muscle and the coverslip on which the muscle was placed. The microoptode was placed right beneath the part of the muscle tissue that was exposed to the different superfusion solutions (fig. 1). Baseline oxygen level was 90.3 ± 1.9 mm Hg. Superfusion with the oxygenated RBC suspension resulted in an oxygen level in the space between the tissue and the coverslip of 622.3 ± 15.1 mm Hg, and the deoxygenated suspension resulted in an oxygen level of 16.7 ± 1.7 mm Hg. The local application of oxygenated and deoxygenated Krebs buffer resulted in oxygen levels under the tissue of 522.1 ± 2.5 and 26.2 ± 1.7 mm Hg, respectively. Carbon monoxide-equilibrated RBC changed the oxygen level to 51.7 ± 0.9 mm Hg (fig. 2).

Additionally, the oxygen levels in the different mixtures contained in 10-ml plastic syringes were measured ($n = 4$) over a time span of 2 h to ensure constant oxygen levels for the entire length of all our experiments (fig. 3). In the oxygenated RBC mixture, the oxygen level falls from approximately 600 to 530 mm Hg and in the oxygenated Krebs buffer it falls from approximately 650 to 560 mm Hg. This illustrates that even after 2 h, almost twice the length of the longest experiment, the oxygenated mixtures still preserve marked hyperoxia. Oxygen levels in the deoxygenated mixtures including the RBC- CO mixture are represented by the bottom three curves. Oxygen levels in the RBC- CO mixture and in the deoxygenated Krebs buffer increase from around 10 to 35 mm Hg, while the oxygen level in the deoxygenated RBC mixture equilibrated with nitrogen remains relatively stable at around 11 mm Hg. The reason why the oxygen tension remains low in the last solution is most likely that the deoxygenated hemoglobin binds oxygen entering from the outside and hence removes the oxygen from physical solution. This is not seen in the RBC- CO mixture since the ability of hemoglobin to bind oxygen is blocked by carbon monoxide, and therefore the mixture behaves like Krebs buffer in this context. The carbon monoxide saturation of hemoglobin, FCOHb , in the RBC- CO mixture was $60.8 \pm 0.03\%$,

Fig. 2. Oxygen levels in the space between the cremaster muscle and the coverslip, with different solutions being superfused across the tissue surface ($n = 4$). The baseline value of 91 mm Hg is measured when only the standard Krebs superfusion buffer is present. N₂ RBC = Additional local superfusion with RBC mixture equilibrated with 95% N₂ and 5% CO₂; N₂ Krebs = same but using only Krebs buffer for local superfusion; CO RBC = local superfusion with CO-equilibrated RBC mixture; O₂ Krebs = additional local superfusion with Krebs buffer equilibrated with 95% O₂ and 5% CO₂; O₂ RBC = same but using oxygenated RBC mixture.

and did not change over the 2-hour period. FCOHb was measured using a blood gas analyzer (ABL 700 series; Radiometer, Copenhagen, Denmark).

ATP Release from RBC

ATP released from RBC into the suspension buffer was measured with a luciferase firefly ATPase assay [23] after equilibration of the mixtures for 10 min with the different gas mixtures and after RBC removal by centrifugation for 10 min at 600 g.

Light-Dye Treatment

Light-dye treatment (LDT) has been described previously [24]; 10 μ l of fluorescein isothiocyanate (FITC) conjugated to dextran (70 kDa, 1.0% solution, 1 ml/kg; Sigma-Aldrich) were injected intravenously. Midway between the original 'local' site of the arteriole near the capillary bed and the 1,000- μ m upstream site, a segment (length $\sim$ 250 μ m) of the arteriole was illuminated through a $\times 40$ water immersion objective with a 75-watt xenon lamp (LUM-PlanFl 40 $\times$ /0.8 W $\bullet$ /o and model U-LH75XEAP0 14 V 75 W, emission 490 nm; Olympus). The vessel segment was illuminated for 8–10 min, and was then allowed to recover for $\sim$ 5 min. Following the illumination, the exposed segments were dilated as a consequence of the damage to the vascular smooth muscle cells (fig. 4).

Fig. 3. Oxygen levels in the oxygenated and deoxygenated mixtures over 2 h ($n = 4$). The upper two curves show the oxygen tension in the oxygenated mixtures. After 2 h, the partial pressure of oxygen is still >500 mm Hg in both oxygenated RBC mixture and oxygenated Krebs buffer. Increases in oxygen levels in the deoxygenated mixtures are depicted in the three lower curves. All three mixtures start with an oxygen level of ~ 10 mm Hg. After 2 h, the deoxygenated RBC mixture still has an oxygen level of ~ 10 mm Hg. In contrast, the oxygen levels in both deoxygenated Krebs buffer and CO-treated RBC mixture have risen to 35 mm Hg.

Table 1. Vessel diameters (in μ m) from LDT controls ($n = 8$)

LDT control	PE		ACh	
	before	after	before	after
Before LDT IS	30.8 $\pm$ 2.9	12.0 $\pm$ 1.1*	13.9 $\pm$ 3.1	30.6 $\pm$ 3.2*
After LDT IS	29.5 $\pm$ 2.5	29.5 $\pm$ 2.5	32.3 $\pm$ 2.8	32.5 $\pm$ 2.7
After LDT upstream	29.2 $\pm$ 2.7	11.9 $\pm$ 1.0*	13.1 $\pm$ 2.6	29.2 $\pm$ 2.8*

Means $\pm$ SE. * $p < 0.01$ vs. PE or ACh. Before LDT, the vessels are responsive to both PE and ACh at the illumination site (IS). After LDT, no responses are seen at IS, yet at the upstream site the vessels are still responsive to both PE and ACh.

To demonstrate that the LDT technique destroys both the endothelial and the vascular smooth muscle layer at the site of illumination while preserving vascular function at the non-illuminated sites, the reactivity of the vessels was tested ($n = 8$) by local application of 100 mM phenylephrine (PE) or 100 mM acetylcholine

Fig. 4. A 50- μm arteriole in $\times 20$ magnification with standard polychromatic light (a). FITC was administered intravenously and a 490-nm monochromatic light source was added (b). In isolated 490-nm monochromatic light (c); a clearly luminous arteriole is now evident. After 10 min of light-dye exposure (d); thrombus material accumulating along the affected endothelium is indicated by the black arrow. Same situation in higher resolution b/w of a slightly smaller arteriole 40- μm (e). Bars = 200 μm .

(ACh) through a micropipette (table 1). To maximize the responses, the vessels were precontracted by superfusing the entire muscle with a hyperoxic (95% O_2 /5% CO_2) buffer for 5–10 min prior to application of ACh. Likewise the vessels were predilated by global superfusion with a hypoxic (95% N_2 /5% CO_2) buffer prior to ap-

plication of PE. The global nature and long duration of the superfusion resulted in pronounced constriction or dilation prior to the local application of either ACh or PE. Before LDT, application of PE at the illumination site constricted the predilated vessels from 30.8 ± 2.9 to 12.0 ± 1.1 μm ($p < 0.01$), and application of ACh

Fig. 5. Diameter changes with RBC suspension before and after LDT ($n = 7$). RBC suspension exposure induced vasomotor responses both locally and at 1,000 μm . After LDT, upstream responses at 1,000 μm were abolished. Resting diameter: $36.5 \pm 3.4 \mu\text{m}$. * $p < 0.01$ and ** $p = 0.01$, vs. baseline.

dilated the precontracted vessels from 13.9 ± 3.1 to $30.6 \pm 3.2 \mu\text{m}$ ($p < 0.001$). After LDT the exposed segments were dilated and unresponsive to changes in the oxygen content of the superfusion buffer. Furthermore, addition of either PE or ACh had no effect on vessel diameter (PE: from 29.5 ± 2.5 to $29.5 \pm 2.5 \mu\text{m}$; ACh: from 32.3 ± 2.8 to $32.5 \pm 2.7 \mu\text{m}$). Since PE acts via receptors on the smooth muscle cells and ACh via receptors on the endothelial cells, and since the photo damage progresses from the endothelium towards the media and the adventitia of the vessel, both the endothelium and smooth muscle cell layer of the vessels must have been destroyed by LDT. Finally, the reactivity of the vessels was tested at a site located 500 μm upstream from the illumination site to make sure the vessels were still intact and responsive outside the LDT-exposed section. At this upstream site, PE caused a constriction of the predilated vessels from 29.2 ± 2.7 to $11.9 \pm 1.0 \mu\text{m}$ ($p < 0.01$) and ACh induced dilation of the precontracted vessels from 13.1 ± 2.6 to $29.2 \pm 2.8 \mu\text{m}$ ($p = 0.0001$), demonstrating that the vessels remain responsive outside the site of illumination.

Experimental Protocol

After the preparation had stabilized for approximately 30 min, experiments were started. Only one vessel was examined in each animal. Experiments lasted from 20 to 70 min, and full responsiveness of the vessels examined was noted regardless of the duration of the experiments.

Three series of experiments were performed. In the first series ($n = 7$), local and conducted vasoconstrictor responses to local application of RBC mixture or Krebs buffer, both equilibrated

with N_2 or O_2 containing 5% CO_2 , were measured. In the second series ($n = 4$), we measured local and conducted vasoconstrictor responses to local application of RBC mixture equilibrated with N_2 or CO both containing 5% CO_2 . These first two series were performed in different animals because the CO gas might affect subsequent measurements. In a third series ($n = 5$), the experiments were repeated but this time with the addition of L-NAME to the superfusion buffer. Before and between the application of each of the test solutions (equilibrated RBC mixtures or Krebs buffers), the preparation was superfused with the baseline superfusion buffer (Krebs buffer with an oxygen tension of $\sim 90 \text{ mm Hg}$, see above). In all cases, the vasomotor responses induced by the test solutions were found to be reversible.

Last, we did two control experiments, the first ($n = 8$) was a control series for the LDT procedure, and the second ($n = 4$) was a time control experiment.

Data Acquisition and Statistical Analysis

The experiments were analyzed off-line using manual tracking of endothelial edges at the sites of interest: locally (adjacent to the area covered by the RBC-Krebs suspension) and at a distance of 500 and 1,000 μm upstream. Measurements were made in a blinded fashion. Responses are expressed as percent change from the resting diameter at the local (ΔD_{local}) site and at sites 500 and 1,000 μm ($\Delta D_{500/1,000}$) upstream. All values are given as means $\pm$ SE. All relative changes in arteriolar diameter are compared to baseline, i.e. zero, using Student's t test, and p values < 0.05 are considered statistically significant.

Results

Seven experiments ($n = 7$) were carried out in which a segment of the capillary bed was selectively superfused with oxygenated and deoxygenated RBC (fig. 5). The resting diameter of the arterioles was $38.5 \pm 3.4 \mu\text{m}$ before initiation of the experiments. Close to the capillary bed (the local response), we found that superfusion with the deoxygenated RBC suspension caused the arterioles to dilate from a diameter of 38.5 ± 3.4 to $43.3 \pm 2.9 \mu\text{m}$ ($12.9 \pm 2.4\%$; $p < 0.01$). In contrast, superfusion with oxygenated RBC caused a diameter change from 40.4 ± 2.6 to $35.8 \pm 3.3 \mu\text{m}$ ($12.3 \pm 2.7\%$; $p < 0.01$) of the arterioles. At the measuring point 1,000 μm upstream, the vessels showed a dilation from 36.5 ± 3.3 to $41.1 \pm 2.9 \mu\text{m}$ ($13.9 \pm 4.0\%$; $p = 0.01$) and a constriction from 37.6 ± 2.8 to $35.2 \pm 2.8 \mu\text{m}$ ($6.5 \pm 0.8\%$; $p < 0.01$), respectively. RBC superfusion did not result in leukocyte adhesion to the inside surface of the arterioles under study or in any other visible reactions within or around the vessels.

The vessel wall was then damaged by LDT on a 250- μm portion of the arteriole between the two observation points. Thereafter, application of the RBC suspension

Fig. 6. Diameter changes with Krebs buffer before and after LDT ($n = 7$). Exposure to Krebs buffer also seemed to induce minor vasomotor responses even though not statistically significant. Resting diameter: $36.5 \pm 3.4 \mu\text{m}$.

caused a local dilation from 37.0 ± 3.6 to $40.7 \pm 3.2 \mu\text{m}$ ($11.5 \pm 3.3\%$; $p = 0.01$), and a local constriction from 36.3 ± 4.7 to $32.3 \pm 4.3 \mu\text{m}$ ($10.9 \pm 2.1\%$; $p < 0.01$) with the deoxygenated and oxygenated suspensions, respectively. However, upstream to the damaged segment there was complete abolition of both vasodilation and vasoconstriction. After application of deoxygenated and oxygenated RBC suspensions, the vessels did not change the upstream diameter: from 35.5 ± 4.3 to $35.5 \pm 4.3 \mu\text{m}$ ($0.07 \pm 0.3\%$; NS) and from 37.6 ± 4.6 to $37.6 \pm 4.6 \mu\text{m}$ ($-0.05 \pm 0.2\%$; NS). The LDT procedure caused thrombus material to form along the inside surface of the illuminated vessel segment (fig. 4d, e).

Selective superfusion was also performed with buffer equilibrated with oxygen or nitrogen. This did not cause statistically significant changes in vessel diameters (fig. 6).

To test the importance of oxygen binding of hemoglobin for the results and to estimate the potential importance of ATP released from RBC, 4 experiments were performed where the RBC suspension was equilibrated with 95% carbon monoxide. Application of CO-equilibrated RBC did not cause significant changes in arteriolar diam-

eter, neither locally ($0.4 \pm 0.8\%$, NS) nor at $1,000 \mu\text{m}$ ($2.8 \pm 0.6\%$, NS). After LDT, application of the CO-treated RBC likewise had no significant effects on diameter, neither locally ($0.9 \pm 2.6\%$, NS) nor at $1,000 \mu\text{m}$ ($0.01 \pm 0.8\%$, NS).

The ATP concentrations in the suspension buffer did not differ between the RBC suspensions, being $1.35 \pm 0.46 \mu\text{M}$ for oxygen-treated RBC, $1.76 \pm 0.24 \mu\text{M}$ for the nitrogen-treated RBC and $1.92 \pm 0.49 \mu\text{M}$ for RBC treated with carbon monoxide.

Next we tested ($n = 5$) whether the vasodilating compound NO is a necessary component in the oxygen-dependent vasomotor responses (fig. 7). Maximal diameters were $44.7 \pm 4.2 \mu\text{m}$ locally, $45.8 \pm 4.2 \mu\text{m}$ $500 \mu\text{m}$ upstream and $44.0 \pm 4.3 \mu\text{m}$ $1,000 \mu\text{m}$ upstream. Before adding L-NAME to the superfusion buffer, baseline diameters were 37.9 ± 3.8 , 38.9 ± 4.2 and $38.2 \pm 5.0 \mu\text{m}$ locally, and 500 and $1,000 \mu\text{m}$ upstream, respectively. Below, diameters will be given in this order and relative values compared with vessel diameters before the respective interventions. Deoxygenated RBC mixture resulted in vasodilation with diameters increasing by $14.8 \pm 2.2\%$ ($p < 0.01$), $13.9 \pm 0.9\%$ ($p < 0.0001$) and $12.7 \pm 2.0\%$ ($p < 0.01$). The oxygenated RBC mixture caused the vessels to constrict by $12.1 \pm 1.0\%$ ($p < 0.001$), $11.6 \pm 3.5\%$ ($p < 0.05$) and $8.3 \pm 0.9\%$ ($p < 0.001$). Addition of L-NAME to the superfusion buffer caused the vessels to constrict by approximately 14%, resulting in baseline diameters of 31.4 ± 4.8 , 33.4 ± 4.0 and $34.0 \pm 4.8 \mu\text{m}$. Deoxygenated RBC mixture dilated the vessels by $20.2 \pm 6.3\%$ ($p < 0.05$), $16.5 \pm 3.5\%$ ($p < 0.01$) and $18.2 \pm 9.4\%$ (NS), whereas the oxygenated RBC mixture made the vessels constrict by $10.5 \pm 8.3\%$ (NS), $13.8 \pm 2.9\%$ ($p < 0.01$) and $16.8 \pm 6.1\%$ ($p = 0.05$).

Finally, a time control ($n = 4$) was made to test whether the observed responses changed as a function of time. The longest experiment lasted 70 min; hence the time control was made over 90 min. The observed responses to application of oxygenated and deoxygenated RBC were largely uniform in this period of time (fig. 8).

Discussion

The main result of the present study is that a local change in tissue oxygen tension results in a conducted vascular response that is transmitted upstream to the supplying arterioles by a signal propagating within the vascular wall. The vasodilation in the upstream arterioles did not require NO production and was abolished by

Fig. 7. NOS inhibition by L-NAME induces a ~14% arteriolar vasoconstriction in the resting state, but leaves the responses to deoxygenated and oxygenated blood mixtures unaffected ($n = 5$). Baseline and maximal diameters were 37.9 ± 3.8 and 44.7 ± 4.2 μm locally, 38.9 ± 4.2 and 45.8 ± 4.2 μm 500 μm upstream and 38.2 ± 5.1 and 44.0 ± 4.3 μm 1,000 μm upstream. * $p < 0.05$ vs. baseline diameter. NS = Nonsignificant.

Fig. 8. Time control experiment ($n = 4$). Hypoxic vasodilation and hyperoxic vasoconstriction are stable for 90 min at both the local site and 1,000 μm upstream. * $p < 0.05$ vs. baseline. NS = Nonsignificant change in vessel diameter.

damage to an intermediate segment of the vessel wall, suggesting that increased shear stress played no role in the observed upstream vasodilation.

In the present study, we used a novel approach to induce local changes in tissue oxygen levels. We superfused parts of the capillary network in a striated muscle with oxygenated and deoxygenated RBC to mimic local changes in tissue oxygen levels as seen for example during physical activity. In many previous studies, tissue oxygen levels have been changed by altering the oxygen content of the arterial blood [2]. This has the potential disadvantage of eliciting systemic reactions with the activation of various neuroendocrine systems which could influence the observed local vascular reactions. The superfusion procedure was successful in eliciting changes in the oxygen tension when measured immediately below the cremaster muscle in the space between the muscle tissue and the coverslip. It is not possible to measure the oxygen tension directly in the very thin membranous cremaster muscle. We therefore chose to measure it in the space between the muscle and the coverslip, a space which was not directly exposed to superfusion solutions. The changes in oxygen

tension in this space following superfusion with the various solutions show that the procedure caused a significant change in the oxygen tension that should cause corresponding, but not necessarily similar changes in the oxygen tension in the muscle tissue.

A critical issue is whether the observed changes in vascular diameter were indeed a result of local changes in oxygen tension. Deoxygenated RBC may release ATP which subsequently can lead to vasodilation [25]. To address this possibility, we carried out experiments in which the RBC mixture was equilibrated with carbon monoxide. This mixture should minimize the change in oxygen level in the tissue because the binding sites for oxygen are occupied by CO, but should in theory still be able to release ATP. The CO-RBC mixture reduced the oxygen tension to a lesser extent than the deoxygenated RBC mixture (fig. 2), and it was not associated with a significant vasodilation. In addition, we measured the ATP concentration in the superfusion buffers. There were no statistically significant differences between the ATP concentrations of the three types of RBC suspensions.

The oxygenated and deoxygenated buffers did not cause statistically significant changes in the arteriolar diameters, although superfusion with these buffers changed the oxygen levels measured under the cremaster muscle to almost the same extent as the RBC mixtures. There could be several reasons for this discrepancy. One possibility could be that another factor besides oxygen is responsible for the response. Although this cannot be excluded, it appears less likely for several reasons. When superfusing with RBC deoxygenated by bubbling with CO (thus abolishing their ability to act as an oxygen buffer), no changes in vascular diameters were observed. Likewise, there were no differences in the ATP concentrations between the various RBC solutions, excluding differences in the ATP content as an explanation for the different responses between Krebs and RBC solutions. It could also be speculated that superfusion with RBC elicited leukocyte adhesion in the underlying vessels. However, we never observed any leukocyte adhesion, or any other visible reaction within or around the vessels following the RBC superfusion. Also, if such a reaction was of importance, we would also expect it to be present with the CO-equilibrated RBCs, which was not the case.

A major limitation in the present study is the inability to measure the oxygen tension in the tissue. The oxygen sensor was placed between the muscle tissue and the coverslip, directly below the pipette used for local stimulation, and the measurements therefore reflect the changes in oxygen tension at this site. The response of the vessels depends on both the strength of the stimulus (the oxygen content in the superfusate), the length of time the stimulus is applied and the area stimulated. Although local application of Krebs and RBC mixtures changed the oxygen tensions at the microoptode to nearly the same extent, this may not reflect the actual magnitudes of the corresponding changes in the tissue. The RBC solutions can dissolve approximately seventy times more oxygen per unit volume than the Krebs buffer. Because of the higher oxygen-binding capacity of the RBC solution, it will be able to stimulate a much larger area of tissue as it diffuses away from the pipette. Also, the larger oxygen-binding capacity of the RBC solution compared to the buffer will result in the former equilibrating with the surrounding solution at a slower rate. Therefore, the stimulus will be of a longer duration and cover a larger area when RBC solutions are used. Finally, the higher viscosity of the RBC solution will reduce the rate of mixing of the applied solution with the surrounding buffer. It is therefore to be expected that the area of stimulation will be larger and more coherent when RBC solutions are used for superfu-

sion. All these factors act together to give a stronger stimulation, and it is therefore to be expected that the responses will be larger when RBC solutions are used compared to when Krebs buffers are applied. With the present setup we can only measure the oxygen tension right below the site of application of the superfusate, and therefore are unable to judge the extent of the oxygenation in tissue that is actually affected by the superfusate.

Oxyhemoglobin can act as a NO scavenger [26, 27]. To rule out that the observed vasoconstriction was due to removal of a known vasodilator, namely NO, we repeated the experiments in the presence of L-NAME, which blocks the production of NO. Blockage of NO formation did not change the basic nature of the observed vasomotor responses, but lowered the baseline vessel diameter approximately 14%. This excludes NO as a necessary component in the vasomotor responses resulting from changes in the oxygen level. These observations strongly support the notion that the oxygen levels themselves were responsible – and not secondary effects due to adenosine or NO – for the observed changes in vessel diameter. It should be emphasized that the present results do not rule out the possibility that the release of ATP from RBC inside the microcirculation [16] plays an important role in mediating oxygen-dependent vasomotor responses. Hypoxia in the tissue caused by superfusion with deoxygenated blood could certainly lead to intravascular release of ATP from RBC.

The new finding in this study is that hypoxia and hyperoxia in the microcirculation cause vasodilation and vasoconstriction, respectively, and that these responses are conducted to upstream sites in the supplying arteriole via signals carried within the arterial wall. Such a mechanism is known as a conducted vasomotor response and it is most likely mediated via gap junctions between endothelial cells and/or smooth muscle cells [5]. The conducted vasomotor response has previously been put forward as a key mechanism in the vasodilation that follows muscular activity. The upstream vessels constitute a major part of the total hemodynamic resistance in muscles, and a coordinated dilation in the vascular network is therefore necessary to meet the metabolic demand of the exercising muscle [28]. It is well known that shear stress, through changes in the production of NO and other endothelial factors, plays an important role in the regulation of the microcirculation [29, 30]. However, the results of the present study suggest that these factors play only a minor role in the upstream vasodilation following local hypoxia. When upstream conduction was interrupted by damage to the vascular wall, changes in the local oxygen

tension were no longer able to induce upstream constriction or dilation. Changing oxygen tensions by superfusion still caused local changes in diameter (fig. 5). A local vasodilation decreases the overall hemodynamic resistance, and increases blood flow and shear stress in the supplying vessels. However, after LDT of an intervening vessel segment, this is not associated with an upstream vasodilation in the present study, indicating that flow-mediated vasodilation by itself is not sufficient to produce the upstream responses. Segal and Jacobs [31] observed a similar lack of response in the upstream vessels following muscular activity in the hamster retractor muscle. After damage to the endothelial layer, increased muscle activity was no longer able to cause dilation of the upstream vessels despite a threefold increase in blood flow.

The apparent lack of a role for NO in the vascular response to local changes in oxygen tension is emphasized by the observation that blocking NOS by L-NAME was without effect on the upstream dilation. As expected, administration of L-NAME caused significant vasoconstriction, but both the local and the upstream responses to changes in local oxygen tension were preserved. This finding is in agreement with earlier studies which have shown that endothelium-derived vasodilation is more independent of NO in the microcirculation than in larger conduit arteries [32]. Taken together, these findings suggest that flow-mediated vasodilation is not an important component in the upstream response to local changes in oxygen tension, but rather that this is mediated entirely by a vascular conducted response propagating within the vascular wall.

We applied LDT to interrupt conduction within the vascular wall. LDT caused damage to both the endothelial and the vascular smooth muscle layer as indicated by the complete absence of responses to the local administration of both PE and ACh. However, the parts of the vessel that were not exposed to light remained fully responsive to both drugs. LDT could also potentially cause damage to perivascular nerves, and it cannot be excluded that such nerves may contribute to the upstream vasodilation observed in the present study. However, this seems to be a less likely possibility. Although there is experimental evidence that perivascular nerves can contribute to conducted vasomotor responses [33], there is an overwhelming body of evidence showing that the major mechanism for conducted vasomotor responses is gap junction-mediated cell-cell communication between the endothelial and/or vascular smooth muscle cells of the vessel wall [6, 7].

In the present study, we took great care to assure the actual oxygen content of the applied solutions. Even though the general superfusion buffer was equilibrated with 95% N₂ and 5% CO₂, measurements showed that the actual oxygen tension of this solution was 91 mm Hg (fig. 2). The solution for the general superfusate was passed through plastic tubing and exposed to the atmosphere when flowing across the surface of the muscle. This apparently allows for sufficient uptake of oxygen to significantly increase the oxygen tension in the solution. To avoid this pitfall, all solutions used to change the local oxygen tension were kept in plastic syringes, which provided a sufficient protection against the loss or uptake of oxygen as evidenced by the stable oxygen levels observed over a 2-hour time span (fig. 3). Furthermore, the solutions were applied through a metal cannula using a high flow rate which limits the possibility for loss or uptake of oxygen. Indeed, this procedure was highly successful in producing local changes in the oxygen tension, as documented by our results (fig. 2).

In conclusion, this study demonstrates that local changes in oxygen tension lead to changes in the arteriolar diameter that are transmitted to upstream vessels by a conducted vascular response.

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