Contribution of anaerobic metabolism to reactive hyperemia in skeletal muscle

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1Department of Human Physiology, Semmelweis University, Budapest, Hungary; 2Department of Bioengineering, University of California, San Diego, La Jolla, California; and 3Department of Physiology, University of Arizona, Tucson, Arizona

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Tóth A, Pal M, Intaglietta M, Johnson PC. Contribution of anaerobic metabolism to reactive hyperemia in skeletal muscle. Am J Physiol Heart Circ Physiol 292: H2643–H2653, 2007. First published February 16, 2007; doi:10.1152/ajpheart.00207.2006.—Elevated blood flow (reactive hyperemia) is seen in many organs after a period of blood flow stoppage. This hyperemia is often considered to be due in part to a shift to anaerobic metabolism during tissue hypoxia. The aim of our study was to test this hypothesis in skeletal muscle. For this purpose we measured NADH fluorescence at localized tissue areas in cat sartorius muscle during and after arterial occlusions of 5–300 s. In parallel studies, red blood cell (RBC) velocity was measured in venules. Tissue NADH fluorescence rose significantly with occlusions of 45 s or greater, reaching a maximum of 44% above control at 180 s. Peak RBC velocity rose to four times control as occlusion duration was increased from 5 to 45 s, but hyperemia duration was stable at ~70 s. With occlusions of 45–240 s, hyperemia duration increased progressively to 210 s while peak flow was unchanged. However, after 300-s occlusions, peak flow rose to six times above control and hyperemia duration fell to 140 s. With occlusions of 45–300 s the time integral both of increased NADH fluorescence and of reduced fluorescence following occlusion release showed a high degree of correlation with the additional hyperemia. We conclude that in this muscle anaerobic vasodilator metabolites are responsible for the increase in reactive hyperemia with arterial occlusions longer than 45 s. Since the durations of reactive hyperemia and reduced fluorescence are substantially different, vasodilator metabolite removal may be due to washout by the bloodstream rather than metabolic uptake.

anoxia; vasodilator metabolites; metabolic feedback; NADH fluorescence; red blood cell velocity; microcirculation; blood flow regulation

REACTIVE HYPEREMIA is the increase in blood flow above resting levels that occurs after a period of blood flow stoppage, usually induced by arterial occlusion. This phenomenon has been demonstrated in a wide variety of vascular beds including skeletal muscle (4), myocardium (9), liver (16), intestine (36), and skin (27). The hyperemia is often attributed to accumulation of vasodilator metabolites produced by anaerobic metabolism during tissue hypoxia (1, 13, 28, 47). However, occlusions of a few seconds’ duration also elicit a hyperemic response, although this is too brief to deplete oxygen stores as noted initially by Bayliss in 1902 (3). This finding suggests that other mechanisms such as the myogenic response may contribute, since a fall in intravascular pressure during arterial occlusion would lead to arteriolar relaxation and a subsequent hyperemia when the pressure is restored. Studies on skeletal muscle (21, 25) supported the suggestion that the response following short-term (5–30 s) occlusions in resting skeletal muscle is primarily myogenic. However, with reports of additional flow-regulatory mechanisms dependent on endothelial cell deformation (40), wall shear stress (23), red blood cell (RBC) deoxygenation (38) and ATP release (11), and oxygen-dependent autacoids released at the vessel wall (10, 30, 33) and possibly at more remote sites at the level of the capillary (5) and the venular networks (15), the contribution of tissue anoxia and anaerobiosis to reactive hyperemia has become less clear.

Given the multiplicity of potential contributing mechanisms, assessing the contribution of a shift to anaerobic metabolism to reactive hyperemia requires a means of measuring the magnitude and duration of this shift. In a previous report (43) we described a technique, based on the NADH fluorescence technique first reported by Chance and coworkers (7), to monitor a shift to anaerobic metabolism in a microcirculatory preparation of skeletal muscle. In that study we showed a direct relationship between the magnitude of the increase in fluorescence and the increase in NADH concentration in the tissue determined by chemical analysis. Using this approach we demonstrated in cat sartorius muscle that after flow arrest there is a rise in NADH fluorescence at tissue sites in the vicinity of capillaries, signaling a shift to anaerobic metabolism (44). The fluorescence rise began ~45 s after flow arrest. On the basis of this finding, the aim of the present study was to test the hypothesis that hyperemia following occlusions of 45 s or less is due to mechanisms present under aerobic conditions while responses following longer occlusions are altered by a shift to anaerobic metabolism. To test this hypothesis, two sets of experiments were carried out. In the first set, shifts in tissue metabolic state during and after stop-flow periods of 5–300 s were monitored by the NADH microfluorescence technique. In the second set of experiments, performed under identical conditions, RBC velocity in small venules was assessed by the dual slit technique (46). If our hypothesis is correct, a quantitative correlation should be found between the time integral of the NADH signal and the increase in the time integral of RBC velocity as occlusion duration is increased from 45 to 300 s.

In our earlier study of changes in NADH fluorescence during arterial occlusion (44), we observed a drop in the signal below control levels when flow was restored. This undershoot is thought to indicate elevated oxidative metabolism. It may also reflect reincorporation of anaerobic metabolites (including vasodilator substances) into the energy cycle. If this is correct, the...
duration of the reduced signal would be related closely to the metabolic component of reactive hyperemia. We also tested this hypothesis in our study.

METHODS

Muscle Preparation

Experiments were performed on 24 fasted juvenile cats (0.9–1.1 kg body wt) tranquilized with ketamine (15 mg/kg im) and anesthetized with α-chloralose (38 mg/kg ip). Supplemental doses (10 mg/kg) of the latter were administered if palpebral or eyelid reflexes could be elicited. The trachea and left femoral artery and vein were cannulated and the right sartorius muscle exteriorized as described previously (44). Vascular occluders [internal diameter (ID) = 2 mm; model OC2A, In Vivo Metric, Healdsburg, CA] were placed on the abdominal aorta and the right femoral artery. On completion of the surgery the cat was placed on its right side on a special platform fitted to the microscope stage. The upper surface of the muscle was covered with polyvinyl film (Saran Wrap, Dow Corning), which is practically impermeable to oxygen (39). Core and muscle temperatures were maintained at 37°C by an electrically controlled heating pad and a heating element array, respectively. Arterial blood pressure in the left femoral artery was monitored with a Statham pressure transducer (23 Gb), continuously recorded on a four-channel strip-chart recorder (model 2600, Gould), and digitized/stored together with the photometric signals in an IBM-compatible personal computer. Arterial pH, Pco2, and PO2 were monitored periodically with a blood gas analyzer (model 2600, Gould), and digitized/stored together with the photo-heating element array, respectively. Arterial blood pressure in the left femoral artery was monitored at 37°C by an electrically controlled heating pad and a heating element array, respectively. Arterial blood pressure in the left femoral artery was monitored with a Statham pressure transducer (23 Gb), continuously recorded on a four-channel strip-chart recorder (model 2600, Gould), and digitized/stored together with the photometric signals in an IBM-compatible personal computer. Arterial pH, Pco2, and PO2 were monitored periodically with a blood gas analyzer (model 2600, Gould), and digitized/stored together with the photo-heating element array, respectively. Arterial blood pressure in the left femoral artery was monitored at 37°C by an electrically controlled heating pad and a heating element array, respectively. Arterial blood pressure in the left femoral artery was monitored with a Statham pressure transducer (23 Gb), continuously recorded on a four-channel strip-chart recorder (model 2600, Gould), and digitized/stored together with the photometric signals in an IBM-compatible personal computer. Arterial pH, Pco2, and PO2 were monitored periodically with a blood gas analyzer (model 2600, Gould), and digitized/stored together with the photo-heating element array, respectively. Arterial blood pressure in the left femoral artery was monitored at 37°C by an electrically controlled heating pad and a heating element array, respectively. Arterial blood pressure in the left femoral artery was monitored with a Statham pressure transducer (23 Gb), continuously recorded on a four-channel strip-chart recorder (model 2600, Gould), and digitized/stored together with the photometric signals in an IBM-compatible personal computer. Arterial pH, Pco2, and PO2 were monitored periodically with a blood gas analyzer (model 2600, Gould), and digitized/stored together with the photo-heating element array, respectively.

These vessels do not change diameter under a variety of conditions, i.e., arterial pressure reduction, muscle contraction, and hemorrhagic hypotension, enabling us to use velocity as a reliable indicator of changes in volume flow. Volume flow can be calculated from center line velocity by the equation: 

\[ F = v_{cl} \pi r^2 / 1.6 \]

where \( F \) is volume flow, \( v_{cl} \) is center line velocity, and \( r \) is vessel internal diameter (2). Since \( r \) is constant in our study, center line velocity is directly proportional to volume flow. For this reason venules are a better choice than arterioles for our measurement since arteriolar diameter changes considerably in reactive hyperemia (24, 29), which could introduce a measurement error into the study.

Oclusion protocols. An inflatable cuff was placed on the abdominal aorta as described in an earlier study (44) for the purpose of stopping flow to the muscle. After a 1-h equilibration period an appropriate tissue site or venule was selected for study, and after control data were recorded for 90 s, blood flow to the muscle was arrested for 5, 15, 30, 45, 60, 90, 120, 240, or 300 s, and recording continued for at least 90 s after release of occlusion or until the signals being recorded stabilized at a new level, which was usually very close to the control level. Infrequently, capillary flow resumed during the longer occlusion periods because of opening of a collateral inflow. Data from such runs were not used. The arterial pressure trace was also monitored to verify that perfusion pressure to the muscle fell rapidly and returned rapidly after occlusion release. If these criteria were not met, the data were not used.

Data Processing

NADH fluorescence. CORRECTION OF OPTICAL ARTIFACTS. Occlusion periods in which sustained changes in the transmittance signal >10% occurred were excluded from data processing. In a few instances, a gradual decrease of the fluorescence signal by 5–15% was seen and could be attributed to fluorochrome bleaching. In these instances a graded linear correction was applied to the data as a function of time.

ANALYSIS OF INDIVIDUAL FLUORESCENCE TRACINGS. The instant at which the fluorescence signal started to rise during the period of flow stoppage was determined for each tracing from the intersection of the lines of best fit of the signal during the control period and the period of rapid rise (Fig. 1A). The instant at which fluorescence returned to control levels was determined in a similar manner. The maximal values of the NADH fluorescence excess during arterial occlusion and minimal values for the transient fluorescence undershoot during the recovery period as well as the duration of the excess and undershoot periods were determined for each curve.

ANALYSIS OF MEAN TRACINGS. Individual time tracings were computer reconstructed at 1-s intervals and normalized to the average value during the control period. These tracings were then grouped by occlusion duration and averaged to create mean normalized fluorescence (MNF) curves for peak and area calculations. The area calculations consisted of measuring the elevated NADH fluorescence

Experimental Protocols

Tissue fluorescence site selection. Avascular tissue regions 15–25 μm in diameter, located randomly at the arteriolar or venular end of a capillary network supplying skeletal muscle fibers in the thin central region of the muscle, were selected. A different tissue site was monitored during each occlusion. A previous study showed that there was no significant difference in the time course of NADH fluorescence changes at arterial or venous sites in the capillary network during arterial occlusion (44).

Microvascular RBC velocity. RBC velocity measurements were performed at the center line of venules (20- to 50-μm ID). Venules were selected since previous studies in our laboratory (17, 42) showed that these vessels do not change diameter under a variety of conditions, i.e., arterial pressure reduction, muscle contraction, and hemorrhagic hypotension, enabling us to use velocity as a reliable indicator of changes in volume flow. Volume flow can be calculated from center line velocity by the equation: 

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integral for the occlusion period (excess) and the corresponding NADH fluorescence integral for the recovery period (undershoot) as illustrated in Fig. 1A.

**RBC velocity measurements.** ANALYSIS OF VELOCITY TRACINGS. Maximum increase (peak velocity) and length of the reactive hyperemia period were determined from individual velocity tracings. In a few instances, individual curves were corrected for baseline shifts (<10%) where the velocity at the end of the recovery period was not identical to the control level. Mean normalized velocity (MNV) curves were constructed as for fluorescence curves by grouping the tracings according to occlusion duration and averaging. Peak mean velocity was determined for MNV curves in addition to time integral values for the occlusion and reactive hyperemia periods and two area parameters, RBC velocity debt and RBC velocity excess, respectively, as shown in Fig. 1B.

DETERMINATION OF ANAEROBIC-RELATED COMPONENT OF REACTIVE HYPEREMIA. We chose to test the simplest possible relationship between anaerobic factors and those in the preceding aerobic period as causal mechanisms in reactive hyperemia, namely, that vascular response to factors present in the aerobic period reaches a maximum before the shift to anaerobic metabolism occurs. For the sake of simplicity we refer to the factors present in the period before the shift as “aerobic,” without implying a causal relationship to oxidative metabolism. With the assumption that the aerobic factors have maximized, the additional hyperemia found after a rise in NADH fluorescence would to be due entirely to the shift to anaerobic metabolism. With this approach, the reactive hyperemia following a rise in fluorescence was calculated by subtracting the mean reactive hyperemia curve obtained with 45-s occlusion from each of the flow curves obtained after a period of elevated NADH fluorescence.

**Statistics**

Mean ± SE and SD values were calculated for each data group, using individual normalized NADH fluorescence and RBC velocity curves as described in Data Processing; SE is given in the text and in Figs. 3, 5, and 8. Data were compared for statistical significance with one-way analysis of variance. Individual groups in both NADH and RBC velocity data sets were further analyzed by all-pairwise multiple-comparison procedures (Tukey test) (Sigmaplot, SPSS), using the P < 0.05 criterion for statistical significance. Linear or nonlinear regression analysis (MS Excel) was used for integral (area) data computed from the MNF and MNV curves.

**RESULTS**

**Arterial Pressure**

Mean arterial pressure in the left femoral artery was 101.7 ± 1.2 mmHg 15 s before inflation of the cuff (n = 40) and fell rapidly during the period of occlusion (data not shown). Mean femoral artery pressure rapidly returned to control levels after release of occlusion, averaging 101 ± 3% and 97 ± 3% of the control values 7 and 15 s after release of occlusion, respectively (n = 40). Pressures at the three time points were not significantly different.

**Tissue NADH Fluorescence Time Course**

Mean normalized NADH fluorescence curves. Successful recordings of tissue NADH fluorescence were obtained from a total of 80 occlusions on 12 preparations. The time course of mean changes in fluorescence for different periods of occlusion (MNF curves) is shown in Fig. 2. Apart from minor fluctuations of brief (1–3 s) duration, no changes in NADH fluorescence occurred during 5-, 15-, and 30-s occlusions. The first noticeable increase in tissue fluorescence was seen with 45 s of stop flow. Periods of flow stoppage beyond 45 s induced successively larger increases in fluorescence intensity, with the signal intensity increasing at a linear rate with occlusion durations between 45 and 180 s. When the occlusion duration was increased to 240 and 300 s there was no further increase in the fluorescence signal.

On release of occlusion, a fast recovery process was observed in which the signal returned to the control level within 7–28 s. The rate of decline was actually more rapid with the longer occlusions, being 2.3%/s with 180- to 300-s occlusions compared to 1.2%/s with the 45- to 120-s occlusions. The fluorescence signal displayed an undershoot below the control level that was variable and considerably smaller than the increase, reaching the nadir 10.9% below control with the 300-s occlusions.

**Analysis of time-dependent changes in NADH signal.** The mean curves shown in Fig. 2 provide an overview of the time
course and magnitude of fluorescence changes. The rise was seen in 50% of individual tissue sites with 45-s occlusion, 80% with 60-s occlusion, and 100% with longer occlusions. Since the peak values did not occur simultaneously, mean values plotted as a function of time do not necessarily represent the actual maximum values, which are better represented from measurements on the individual curves shown in Fig. 3. When the peak changes were analyzed on an individual basis (Fig. 3A), the values obtained were 9.4 ± 4.2% at 45 s and 44.2 ± 3.6% at 180 s. It is clear from Fig. 3A that the fluorescence peak increases in a linear fashion (y = 0.28x – 5.76) and reaches a maximum level at 180 s. While the overall fit is very high ($R^2 = 0.99$), the high $R^2$ value may be due in part to the limited number of mean values on which the fit is based.

As shown in Fig. 3B, the duration of elevated NADH fluorescence is also clearly a linear function of occlusion duration ($y = 0.93x – 35.55$, $R^2 = 0.99$) after a latent period, based on the x-intercept, of 38 s. The integral of the elevated fluorescence, presented in Fig. 3C, was determined from the MNF curves integrated over time separately and presents a more complete picture of the change. With occlusion durations of 45–180 s these changes are well characterized by a second-order approximation ($y = 0.002x^2 – 0.166x + 3.842$, $R^2 = 0.99$) since the amplitude of the signal is increasing with time. Beyond 180 s the increase is linear ($y = 0.36x – 16.62$, $R^2 = 0.99$) since the peak fluorescence signal remains relatively constant as shown in Fig. 3A.

As is evident in Fig. 2, after release of occlusion there is a small and variable reduction in NADH fluorescence (undershoot) that reaches a nadir at 300 s. The maximal undershoot, plotted in Fig. 3D, is well characterized as a logarithmic function [$y = 5.06 \ln(x) – 17.06$, $R^2 = 0.98$] of occlusion duration. This function differs from the linear NADH fluorescence increase where peak reaches a maximum at 180 s. The duration of the fluorescence undershoot (Fig. 3E), like the increase, is a linear function of the occlusion duration with a high degree of fit ($y = 1.15x – 42.20$, $R^2 = 0.99$). The x-axis intercept is 37 s, essentially identical to that for the increase in fluorescence (38 s). The integral of fluorescence undershoot (Fig. 3F) increases as a quadratic function ($y = 0.0003x^2 – 0.0198x + 0.5944$, $R^2 = 0.99$) with occlusion duration, since both the nadir and the duration of the reduced signal increase.

**RBC Velocity Time Course**

*Mean normalized RBC velocity curves.* Data were obtained from 125 occlusion periods in venules of 12 animals. The combined mean velocity curves for each occlusion period are shown in Fig. 4. There are certain similarities in the flow response at all occlusion durations, specifically a rapid increase to a maximal value followed by a gradual decline. The declining phase of flow can be well characterized as a monoexponential function with an $R^2$ value of 0.97–0.99 for occlusions of 5- to 45-s duration and 0.90–0.99 for longer occlusions. Obvious differences include a gradually higher peak flow as occlusion duration increases from 5 to 45 s, increased duration following occlusions longer than 45 s, and a large increase in peak and decreased duration at 300 s. Changes in variables of interest as a function of occlusion duration are presented in Fig. 5.

**Analysis of time-dependent changes in RBC velocity.** As shown in Fig. 5A, peak velocity varies with occlusion duration in three separate phases, increasing to four times control in the 5–45 s range, remaining essentially constant in the 45–240 s range ($y = 0.0013x + 3.8249$, $R^2 = 0.2249$), and increasing at 300 s to over six times control. The mean time to peak velocity averaged 10.0 ± 0.7 s and was not dependent on occlusion duration. The duration of hyperemia displayed in Fig. 5B also shows three distinct phases, being nearly constant (~70 s) with occlusions <45 s, increasing linearly to ~210 s with occlusion periods of 45–240 s ($y = 0.66x + 53.35$, $R^2 = 0.98$), and decreasing substantially to ~140 s with the 300-s occlusion. As shown in Fig. 5C, the time constant ($\tau$) of hyperemia also varied with occlusion duration. It ranged between 16 and 28 s with occlusions of 90 s or less, rose abruptly to 55–70 s with 120- to 240-s occlusions, and then fell to 38 s with the 300-s occlusion. The $R^2$ value of fit to an exponential function was 0.97–0.99 at all occlusion durations except for 0.90 with 180-s occlusion. The integral of velocity excess with occlusion duration (Fig. 5D) is remarkably linear ($y = 0.72x + 22.61$, $R^2 = 0.99$), considering the changes in both peak velocity and duration over this time. Note, however, that total RBC velocity excess at time 0 extrapolates to a value greater than zero. Additionally, the RBC velocity repayment, that is, the ratio of the integral of excess velocity to the deficit during occlusion, is
~200% during brief occlusions and drops below 100% with longer occlusions, as shown in Fig. 5E. It is fitted by a power function \( y = 448.48x^{-0.32}, R^2 = 0.90 \).

**Aerobic and anaerobic components of reactive hyperemia.**

We have, as described in the introduction, hypothesized that the increase in reactive hyperemia with occlusions longer than 45 s is due to a shift to anaerobic metabolism in the tissue as reflected in the rise in NADH fluorescence. To examine this possibility we subtracted the hyperemic flow pattern with the 45-s occlusion from the patterns seen with longer occlusions. The results are shown in (Fig. 6A). As is evident in this figure, the total area under the curves increases with the longer occlusions. The integrals of these curves are shown in Fig. 6B as a function of occlusion duration (anaerobic) together with the integral for the component seen before the rise in fluorescence (aerobic). We found a linear relation \( y = 0.72x - 29.38, R^2 = 0.97 \) between the excess component related to the rise in NADH fluorescence (anaerobic) and occlusion duration. In Fig. 6C the repayment of RBC velocity debt during occlusion for the component of reactive hyperemia related to the rise in fluorescence is shown. In contrast to Fig. 5E, a somewhat stable value of the repayment at ~75% \( y = -0.055x + 85.23, R^2 = 0.05 \) is seen over the occlusion range for this component of reactive hyperemia. The low \( R^2 \) value reflects the weak relationship between repayment and occlusion duration.

We found that the anaerobic velocity component (Fig. 6B) is rather insensitive to the exact shape of the 45-s curve whether determined by a weighted averaging process from all curves or by picking one representative curve. The differences were <5% of the total integral value for occlusions above 60 s, and the correlation coefficients calculated for the NADH fluorescence-related components were similar.
Comparison of Integrals of NADH Fluorescence and Reactive Hyperemia

To evaluate further the relationship between the anaerobic shift and reactive hyperemia, the relationship between the integrals of NADH fluorescence change, both increase and decrease below control, and the integrals of total and anaerobic RBC velocity excess were examined. The relation between the fluorescence integrals versus the integral of total RBC velocity excess is shown in Fig. 7A, while the integrals for anaerobic RBC fluorescence are compared with the integral for anaerobic RBC velocity excess in Fig. 7B. Because the NADH fluorescence and RBC velocity measurements were performed in two separate groups of animals, anaerobic RBC velocity excess values could only be calculated with an average metabolic latency period (39.3 s) determined from the NADH fluorescence experiments. The relationship in both cases was constrained to go through zero. The correlation is significantly higher for the anaerobic RBC velocity excess component \( R^2 = 0.95 \) compared with the total velocity excess component \( R^2 = 0.80 \). Similarly, the period of reduced fluorescence shows a closer relation to the anaerobic RBC velocity excess \( R^2 = 0.88 \) than to the total velocity excess \( R^2 = 0.73 \).

Duration of Reactive Hyperemia and Reduced Fluorescence

As described in the introduction, our second hypothesis is that the fluorescence undershoot represents a period of elevated oxidative metabolism during which anaerobic metabolites (including vasodilator metabolites) are being reincorporated into the energy substrate. If this hypothesis is correct, there should be a close relationship between the duration of reactive hyperemia and that of the period of reduced fluorescence. These durations are plotted as a function of occlusion duration in Fig. 8. It is apparent that there is not a close relation between the two variables.

DISCUSSION

Previous studies have provided evidence that anaerobic mechanisms contribute to reactive hyperemia in skeletal muscle based on correlation of the flow excess with oxygen debt (28, 47). Less direct evidence includes a gradual increase in duration of reactive hyperemia when occlusion duration increases (22, 31). However, as noted in the introduction, a variety of other mechanisms may also contribute, making a clear separation between anaerobic-related factors and nonan aerobic factors difficult in the absence of a means of determining the time course and magnitude of anaerobic changes.

Separation of Metabolic and Nonmetabolic Reactive Hyperemia Components

In the present study we were able to detect and quantify a shift to anaerobic metabolism during flow stoppage by monitoring changes in NADH fluorescence. A rise in NADH fluorescence was taken as indicating a shift to anaerobic metabolism and the integral of the signal increase (NADH fluorescence excess), which was used as a measure of the cumulative anaerobic shift. Separate experiments in which center line RBC velocity was measured in venules after similar periods of flow arrest enabled us to examine the features of the reactive hyperemia pattern associated with elevated NADH fluorescence. Since this velocity is proportional to volume flow, as described in Microcirculatory RBC velocity, and is measured in the vicinity of skeletal muscle fibers, it may more accurately reflect the effects on flow of the metabolic shift in this tissue than total muscle flow, which may include drainage from other types of tissue.

As shown in Figs. 2 and 3, a rise in NADH fluorescence was discernible only with occlusions of 45 s or longer. By contrast, an increase in RBC velocity above control was seen with the shortest occlusion used (5 s), and the peak velocity gradually increased to four times control as the occlusion period increased to 45 s (Figs. 4 and 5A). (Since, as noted in RESULTS, postocclusion arterial pressure simply returns to control levels, the hyperemia reflects vasodilation rather than elevated perfusion pressure.) Hyperemia duration, however, was stable at 70 s with occlusions of 10–45 s (Fig. 5B), as was the time constant of the recovery (16–24 s) as shown in Fig. 5C.

When the occlusion duration was extended stepwise to 240 s there was a progressive increase in hyperemia duration to 210 s (Fig. 5B). The fact that hyperemia duration stabilized after 10 s of occlusion and did not increase further until the NADH signal rose, as shown in Figs. 2 and 3A, supports the suggestion that a factor or factors related to anaerobic metabolism increased the duration of hyperemia. Additionally, the pattern of hyperemia changed with the time constant of the velocity recovery (Fig. 5C), increasing from 16–28 s to 55–70 s as the occlusion duration increased from 45 s to 240 s.
In contrast to the extended period of hyperemia, the fluorescence signal returns to the control level in 30 s or less, as shown in Fig. 2. This indicates that return to the aerobic state in the tissue is not sufficient to return flow to its control levels and supports the conclusion that other factors must be involved.

Effect of Anaerobic Metabolism on Reactive Hyperemia

As is evident in Figs. 4 and 6A there was a gradual increase in the postpeak hyperemia as occlusion duration increased from 60 to 240 s. The total RBC velocity excess, namely, the product of velocity amplitude and duration, plotted in Fig. 5D, increases linearly over most of the range of occlusions but has a positive intercept, indicating a disproportionate effect of short occlusions. When calculated velocity excess is limited to the additional hyperemia occurring with occlusions longer than 45 s as shown in Fig. 6B, a clear linear relationship with an intercept greater than zero on the time axis is seen. This finding is consistent with the hypothesis that the additional hyperemia is closely related to the metabolic shift. In addition, as shown in Fig. 5E there is an overpayment of the total RBC velocity debt with the shortest occlusions and gradual decrease in this repayment as a function of occlusion time. By contrast, there is a more consistent repayment of ~75% of the velocity debt when only the additional hyperemia during the rise in NADH signal is considered (Fig. 6C). As noted above, there are previous reports that reactive hyperemia is closely related to oxygen debt (28, 47). Both of these comparisons provide support for the hypothesis that the increase in reactive hyperemia with occlusions greater than 45 s is due to oxygen lack and a shift to anaerobic metabolism.

Quantitative Correlation of RBC Velocity and NADH Fluorescence Integrals

If the additional RBC velocity excess following a rise in NADH fluorescence is due to the shift to anaerobic metabolism, there should be a high degree of correlation between this component of RBC velocity excess and the integral of the NADH fluorescence rise. A high correlation is, indeed, quite evident in Fig. 7B, while a substantially lower correlation is
evident between total RBC velocity excess and the integral of the NADH fluorescence rise in Fig. 7A. This high degree of correlation supports the hypothesis that the additional hyperemia with occlusions greater than 45 s is due to the accumulation of anaerobic metabolites in the tissue.

Findings obtained in a previous study of NADH fluorescence and capillary RBC velocity with sympathetic nerve stimulation in this same muscle support the conclusion that a rise in NADH levels is associated with hyperemia (32). In that study, hyperemia followed 2-min sympathetic stimulation only when the reduction in velocity during stimulation was sufficient to cause an increase in tissue NADH levels. Moreover, the time integral of the poststimulation hyperemia was proportional to the time integral of the increase in NADH fluorescence, as in the present study.

Mechanisms of Reactive Hyperemia

Nonanaerobic mechanisms. Reactive hyperemia in this muscle following occlusions of <45 s is not related to a shift to anaerobic metabolism in tissue regions adjacent to the capillary network, where Po2 levels are lowest (45). The myogenic response has long been considered an important factor in reactive hyperemia, particularly after short occlusions (3, 6, 25). In vivo and in vitro studies have shown that the steady-state diameter of arterioles is inversely related to intravascular pressure (19, 26). In vivo studies on arterioles of the cat mesentery showed a latent period of 13 s and a time constant of 37 s for dilation after a step pressure reduction (19). While the time course of the myogenic response may differ among vascular beds, this finding suggests that the dilation with occlusions of <13 s may be due to mechanisms other than the myogenic response.

A second mechanism, endothelium-dependent nitric oxide (NO) release, has also been implicated in reactive hyperemia. Under no-flow conditions, isolated resistance arteries of rat gracilis muscle passively collapse during occlusion and exhibit postocclusion dilation above control levels (24). The dilation is apparently due to increased NO synthesis by the endothelium, which has been shown to occur during arteriolar diameter reduction (40). The effect may be magnified during longer periods of occlusion by increased endothelial NO synthase activity (24). When flow as well as pressure is varied, the dilation is prolonged by elevated wall shear stress, leading to additional NO synthesis. In rat cremaster muscle Meijinger (29) found during arterial occlusion that the large arterioles passively constricted but small arterioles dilated, which may also be the case in our preparation. Applying the findings described above to our study, it appears likely that both the

Fig. 6. A: additional hyperemia with occlusions of 60–300 s. For this analysis, the mean curve for the 45-s occlusion was subtracted from longer occlusions as described in text. B: relation between occlusion duration and the integrals of excess RBC velocity (anaerobic) shown in A. Also shown is the integral of excess RBC velocity seen with occlusions of 45 s or less (aerobic). C: RBC velocity debt repayment (integral of velocity excess/integral of the velocity debt during occlusion) for the anaerobic component of reactive hyperemia.
myogenic response and deformation of the endothelium during the occlusion period contribute to the reactive hyperemia we observed. Increased wall shear stress in the feed arteries and in the arterioles may also contribute.

An additional factor that may contribute to the dilation following short occlusions is a drop in arteriolar oxygen below normal levels but above the critical PO2 for anaerobic metabolism. A decrease in oxygen levels in this range increases NO and adenosine release from the endothelium (10, 33). Prostaglandin levels rise in reactive hyperemia (4), which may also be related to a drop in PO2 (30, 33). In addition, reduction of cytochrome P-450 vasoconstrictor metabolites has been reported as oxygen levels are reduced (12). It has also been reported that a reduction in oxygen saturation of hemoglobin causes release of NO from hemoglobin (38) and ATP (11) from the RBC. While hypoxia disappears rapidly with resumption of flow, additional time would be required for mediators formed during hypoxia to return to normal levels and may therefore contribute to decreased vascular tone early in the period of reactive hyperemia. Thus it appears that a variety of oxygen-dependent, nonanaerobic vasodilator mechanisms may contribute to reactive hyperemia in skeletal muscle following short periods of flow stoppage.

*Anaerobic mediators of reactive hyperemia.* Tissue anoxia and consequent anaerobiosis have long been implicated in the vasodilation following a period of flow arrest (1, 28, 47). Vasodilator products of anaerobic metabolism are thought to include adenosine, lactic acid, and H+ (13, 35). A fall in pH increases conductance of the vascular smooth muscle ATP-sensitive K+ (KATP) channel (18), and elevated lactate has been shown to cause arteriolar vasodilation, independent of pH, by an action on cGMP (8). Adenosine acts on specific receptors of vascular smooth muscle and may cause dilation by increasing conductance of KATP channels, increasing cAMP levels in vascular smooth muscle, or increasing synthesis of NO and prostaglandins by the endothelium (33, 48). In our study the anaerobic mediator(s) had no apparent effect on peak hyperemia with occlusions of 45–240 s, although mediator concentration must have been higher after the longer periods of occlusion. At the same time, the mediator(s) extended the duration of the hyperemia. This finding may indicate that the anaerobic mediator(s) acts through the same pathways as those involved with shorter occlusions. It is of interest that both the adenosine A1 receptor and the second messenger for NO and lactate, cGMP, can open KATP channels (10) and prostaglandin synthesis has been implicated in the process of NO generation (33).

As shown in Figs. 3B and 5B, the increase in hyperemia duration coincided with the first appearance of a rise in tissue NADH in parenchyma adjacent to the capillary network. That is, a metabolic shift associated with anoxia in the capillary region prolongs the hyperemia, and the time course fits with a graded increase in the integral of the NADH signal. It is possible that sensing mechanisms reportedly present in this region provide an early response to an anaerobic shift in the

![Fig. 7.](image-url)
The pattern of reactive hyperemia changed radically with the 300-s occlusion, with peak flow increasing and duration decreasing. It is of interest that the higher peak is compensated by a shorter duration of hyperemia such that the anaerobic RBC velocity excess shown in Fig. 5, B and D, is in direct proportion to the increased occlusion duration. The altered nature of the reactive hyperemia pattern suggests that an additional mechanism may be involved. One possibility is that the concentration of a vasodilator metabolite during 300-s flow stoppage exceeds the threshold for a different vasodilator mechanism. Shinoda et al. (37) found that peak reactive hyperemia in isolated perfused rat hearts was ~50% greater after a 300-s occlusion than after a 30-s occlusion. Blockade of Ca\(^{2+}\)-activated K\(^+\) (K\(\text{Ca}\)) channels attenuated peak hyperemia after the 300-s occlusion. Alternatively, since P\(\text{O}_2\) of the arteriolar wall is considerably higher than in the capillaries (45), the time required for smooth muscle of arterioles to reach critical levels for oxidative metabolism would be significantly longer than in the parenchyma. A two-stage dilation was observed in isolated coronary arteries during hypoxia, with the first stage attributed to vasodilator agents and the second stage to anoxia of the vascular wall itself (34). In our study, when the 300-s occlusion was released the additional dilation of precapillary vessels would lead to increased peak flow, speeding the removal of vasodilator metabolites and/or reversing an anoxic dilation. Either mechanism could increase the peak and decrease the duration of reactive hyperemia following the 300-s occlusion.

**Relation of Reduced Fluorescence to Reactive Hyperemia**

A second hypothesis proposed in this study is that the reduced fluorescence after release of occlusion reflects a period when vasodilator metabolites are being reincorporated into the energy substrate. As discussed in an earlier publication (44), the most probable explanation for the undershoot is a substantial increase in the rate of oxidative phosphorylation, caused by the hypoxia-induced decrease in mitochondrial phosphorylation potential. Since the nadir and duration of the undershoot increase with longer occlusion durations, this effect is apparently enhanced with longer periods of hypoxia. A more rapid oxidative phosphorylation may also explain the faster return of NADH fluorescence to control levels after the longer occlusions described above in Tissue NADH Fluorescence Time Course. While the evidence for elevated oxidative metabolism during reactive hyperemia is limited, McNeill (28) found in the human forearm an elevated arteriovenous oxygen saturation difference during the hyperemia following a 5-min occlusion. Yonce and Hamilton (47), who used occlusion periods of up to 60 s, found that the oxygen debt in skeletal muscle was fully repaid during this time.

In the present study certain features of the fluorescence signal undershoot correlate with the hyperemia, while others do not. The nadir NADH fluorescence values during reactive hyperemia (Fig. 3D) become greater with increased occlusion duration, and the integral of the fluorescence undershoot correlates with the integral of the anaerobic component of reactive hyperemia (Fig. 7B). In contrast, the duration of the undershoot is poorly correlated with hyperemic duration, as shown in Fig. 8. If vasodilator products of anaerobic metabolism are removed by reincorporation into the energy cycle, it would be expected that durations of these two variables should be similar. The lack of correlation indicates either that the active mechanisms for removal of vasodilator metabolites are not elevated during the period of reduced fluorescence when aerobic activity is increased or that vasodilator products are principally removed by another mechanism such as washout by the bloodstream. Since the former seems unlikely, we note that if washout is involved, the correlation between the integral of the anaerobic component of hyperemia and the integrals of elevated fluorescence and undershoot shown in Fig. 7B could still be maintained. That is, production of anaerobic vasodilator metabolites would be related to the magnitude and duration of anaerobic metabolism as indicated by elevated NADH fluorescence. The integral of the fluorescence undershoot would be, in turn, a function of the integral of elevated fluorescence. As a consequence, the integral of the NADH fluorescence undershoot and the anaerobic component of reactive hyperemia may be related as shown in Fig. 7B, while the durations of the two variables, as shown in Fig. 8, are not.

**Summary**

In summary, arterial occlusions of up to 45 s lead to a fourfold increase in peak RBC velocity in venules above control levels and a reactive hyperemia of ~70-s duration in cat sartorius muscle. Occlusions of 45–240 s cause a rise in NADH levels in the parenchymal cells in the capillary network and an increased duration of hyperemia but not peak RBC velocity. Occlusions of 300 s cause a sixfold increase in peak RBC velocity and a reduction in duration of hyperemia. The integral of the increase in RBC velocity with 45- to 300-s occlusions correlates well with the integral of the NADH fluorescence increase. This is consistent with the hypothesis that accumulation of vasodilator products of metabolism is responsible for the increased hyperemia after 45 s. The rise in peak RBC velocity in venules with a 300-s occlusion may be due to a second vasodilator mechanism. After release of occlusion, a period of reduced fluorescence ensues that is closely related to the integral of the NADH fluorescence increase. The duration of reduced fluorescence correlates poorly with the duration of hyperemia. This finding suggests that metabolic processes responsible for removal of vasodilator metabolites do not follow the same time course as the elevated oxidative metabolism or that vasodilator metabolites are eliminated by blood flow washout.

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