Divergent effects of low-O2 tension and iloprost on ATP release from erythrocytes of humans with type 2 diabetes: implications for O2 supply to skeletal muscle

Randy S. Sprague,1 Daniel Goldman,2 Elizabeth A. Bowles,1 David Achilleus,1 Alan H. Stephenson,1 Christopher G. Ellis,2 and Mary L. Ellsworth1

1Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, St. Louis, Missouri;
2Department of Medical Biophysics, University of Western Ontario, London, Ontario, Canada

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Sprague RS, Goldman D, Bowles EA, Achilleus D, Stephenson AH, Ellis CG, Ellsworth ML. Divergent effects of low-O2 tension and iloprost on ATP release from erythrocytes of humans with type 2 diabetes: implications for O2 supply to skeletal muscle. Am J Physiol Heart Circ Physiol 299:H566–H573, 2010. First published May 28, 2010; doi:10.1152/ajpheart.00430.2010.—Erythrocytes release both O2 and a vasodilator, ATP, when exposed to reduced O2 tension. We investigated the hypothesis that ATP release is impaired in erythrocytes of humans with type 2 diabetes (DM2) and that this defect compromises the ability of these cells to stimulate dilation of resistance vessels. We also determined whether a general vasodilator, the prostacyclin analog iloprost (ILO), stimulates ATP release from healthy human (HH) and DM2 erythrocytes. Finally, we used a computational model to compare the effect on tissue O2 levels of increases in blood flow directed to areas of increased O2 demand (erythrocyte ATP release) with nondirected increases in flow (ILO). HH erythrocytes, but not DM2 cells, released increased amounts of ATP when exposed to reduced O2 tension (Po2 < 30 mmHg). In addition, isolated hamster skeletal muscle arterioles dilated in response to similar decreases in extraluminal O2 as perfused with HH erythrocytes, but not when perfused with DM2 erythrocytes. In contrast, both HH and DM2 erythrocytes released ATP in response to ILO. In the case of DM2 erythrocytes, amounts of ATP released correlated inversely with glycemic control. Modeling revealed that a functional regulatory system that directs blood flow to areas of need to deliver O2 in amounts required to precisely meet local metabolic need both at rest and during exercise (2, 13, 34). Taken together, these reports strongly suggest that, in DM2, O2 delivery to skeletal muscle in amounts required to appropriately meet metabolic need is impaired; although the mechanism(s) by which this impairment occurs have yet to be fully elucidated.

The O2 required to meet the metabolic needs of all tissues is delivered by the erythrocyte, a small, flexible cell containing hemoglobin which, in mammals, is devoid of a nucleus and mitochondria. Recently, it has been demonstrated that this cell is significantly more than a simple O2 transporter, but rather is a complex cell that controls its own distribution within the microcirculation via its ability to release adenosine triphosphate (ATP) in response to physiological stimuli, including exposure to reduced O2 tension (5, 6, 26, 32, 33, 38). This erythrocyte-derived ATP stimulates the synthesis of endothelium-derived vasodilators resulting in local increases in blood flow and, thereby, erythrocyte supply rate, permitting this cell to deliver O2 in amounts required to precisely meet local metabolic need (9–11, 19). Thus, failure of the erythrocyte to release ATP in response to reduced O2 tension could be expected to lead to impaired matching of O2 delivery with need in skeletal muscle and, thereby, contribute to vascular disease. The activity of the heterotrimeric G protein, Gi, is required for ATP release from erythrocytes in response to reduced O2 (32, 33), whereas a second G protein, Gs, is associated with a prostacyclin receptor (IP) (32). It has been shown that Gs2 expression is decreased in erythrocytes of humans with DM2 and this defect is associated with impairment of both cAMP accumulation and ATP release when these cells are incubated with a direct activator of Gi (21, 39), suggesting that this defect in erythrocyte physiology could contribute to the associated vascular disease. Interestingly, expression of Gs is unaltered in DM2 erythrocytes, suggesting that prostacyclin-induced ATP release might remain intact.

In this study, we evaluated ATP release from erythrocytes of humans with DM2 in response to exposure to reduced O2 tension as well as activation of the IP receptor by the prostacyclin analog, iloprost (ILO). We determined that although the release of ATP in response to ILO is intact in DM2, and is, in fact, greater in individuals with poorer glycemic control, ATP...
release in response to reduced O₂ tension is absent. In addition, we determined that there is a functional consequence of the defect in low O₂-induced ATP release as demonstrated by the failure of isolated skeletal muscle resistance vessels to dilate in response to decreases in extraluminal O₂ tension when perfused with DM2 erythrocytes. Finally, we use a computational model of O₂ transport to address the question of whether vasodilators such as ILO that nonselectively increase blood flow can restore tissue oxygenation to undersupplied regions of tissue. The simulation results highlight the importance of a functional regulatory system that directs blood flow to where it is needed rather than a vasodilator that simply increases total skeletal muscle blood flow.

MATERIALS AND METHODS

Isolation of human erythrocytes. Blood was obtained from healthy volunteers (n = 18) and patients with DM2 (n = 24) by venipuncture using a syringe containing heparin (500 U) and centrifuged at 500 g at 4°C for 10 min. The plasma, buffy coat, and uppermost erythrocytes were removed by aspiration and discarded. The remaining erythrocytes were washed three times in buffer containing (in mM) 21.0 tris(hydroxymethyl)-aminomethane, 4.7 KCl, 2.0 CaCl₂, 140.5 NaCl, 1.2 MgSO₄, 5.5 glucose, and 0.5% BSA, final pH 7.4. Erythrocytes isolated in this fashion contain less than 1 leukocyte per 50 high-power fields (8–10 leukocytes per mm³) and are devoid of platelets (21). Cells were prepared on the day of use.

Measurement of ATP. ATP was measured using the luciferin-luciferase assay as described previously (11, 21). A 200-μl sample of erythrocyte suspension (0.04% hematocrit) was injected into a cuvette containing 100 μl of firefly lantern extract (10 mg/ml, FLE 250; Sigma) and 100 μl of a solution of synthetic D-luciferin (50 mg/100 ml; Sigma). The light emitted was detected using a luminometer (Turner Designs). A standard curve was generated for each experiment. Cell counts were obtained by direct counting using a hemocytometer and amounts of ATP measured were normalized to 4 × 10⁸ cells/ml.

Determination of ATP release from erythrocytes in response to exposure to reduced O₂ tension. Isolated erythrocytes were diluted to a 20% hematocrit in a Ringer buffer containing (in mM) 4.7 KCl, 2.0 CaCl₂, 140.5 NaCl, 1.2 MgSO₄, 11 glucose, 23.8 NaHCO₃ with 0.2% dextrose and 0.5% BSA, final pH 7.4 at 37°C in a tonometer (Instrumentation Laboratory) with a gas mixture containing 100% O₂. ATP was measured as described above. Values were normalized to ATP concentration per erythrocyte.

Measurement of free hemoglobin. To exclude the influence of hemolysis in studies where the release of ATP was measured, samples were centrifuged at 500 g at 4°C for 10 min and the presence of free hemoglobin in the supernatant was determined by light absorption at 405 nm. Using this approach, the sensitivity for detection of free hemoglobin is equal to that of the ATP assay. That is, in the absence of increases in free hemoglobin, ATP in the cell suspension cannot be attributed to lysis. If increases in free hemoglobin were detected, samples were diluted 1:8,000 with distilled water. ATP was measured as described above. Values were normalized to ATP concentration per erythrocyte.

Computational model of oxygen transport by capillary networks. Numerical simulations of steady-state O₂ transport were performed using an established computational model (16–18) that couples the continuum partial differential equations describing convective transport by flowing blood in the capillaries to those describing oxygen diffusion and consumption in the tissue. The model includes both dissolved and hemoglobin-bound oxygen in the capillaries. Transport between the blood and tissue is described using a flux boundary condition with mass transfer coefficients calculated previously using a discrete erythrocyte model (7). For all O₂ transport simulations, an array of 19 parallel capillaries was used to represent a typical capillary network (14, 15) with the placement of capillaries and relative distribution of hemodynamic parameters (erythrocyte velocity and hematocrit) determined from in vivo measurements in the rat extensor digitorum longus muscle (8). The tissue domain surrounding the capillary array was 80 × 331 × 300 μm. Capillary entrance saturations (71.5%) and the tissue O₂ consumption rate (6.4 × 10⁻⁵ ml O₂·ml⁻¹·s⁻¹) were set based on previous experimental data (8).
capillary networks

Fig. 1. Schematic of simulation model. Blood flow to the 4 capillary networks is controlled by upstream arterioles. Nonselective dilation of arterioles will tend to cause uniform increases in flow to all 4 networks, while selective dilation can increase flow to the single undersupplied network only. Dashed line indicates initial undersupply of flow to network 1.

For the network with baseline blood flow ($Q = 4.43 \times 10^{-7}$ ml/s), average hemodynamic parameters in normal flow capillaries (15/19) were set to match measured values (34). For capillaries with erythrocyte supply rates (proportional to velocity × hematocrit) too high to measure (4/19), hematocrit was set to the average measured value (34) and velocity was set to 1,000 μm/s at baseline. Alterations from baseline blood flow in individual networks (0.25, 0.31, 1.23, and 4 times baseline flow Q) were achieved via uniform changes in erythrocyte velocity in all capillaries.

To estimate the effect of changes in local blood flow distribution on tissue PO2, a group of four capillary networks was used to represent a tissue. These four networks can be taken to be supplied by a single arteriole (Fig. 1). Since all four networks considered had the same structure and relative distribution of velocities and hematocrits, and were surrounded by the same amount of O2-consuming tissue, the only factor affecting O2 transport from the different networks was the amount of blood flow that each network received. Four different blood flow distributions were considered as shown in Table 1. To obtain tissue PO2 distributions for each case, calculated tissue PO2 distributions for the appropriate individual networks were combined (averaged) into a single PO2 distribution.

Data analysis. Statistical significance among groups was determined using an ANOVA. In the event that the F ratio indicated that a change had occurred, a Fisher’s least significant differences test was performed to identify individual differences. A Student’s t-test was used where appropriate. Results are reported as means ± SE.

Institutional approval. The protocol used to obtain blood from humans was approved by the Institutional Review Board of Saint Louis University. The protocol used to obtain blood from healthy hamsters was approved by the Animal Care and Use Committee of Saint Louis University.

RESULTS

Characteristics of subjects studied. Patients with DM2 were identified by physicians in the Endocrinology Clinic of Saint Louis University. Healthy volunteers were faculty and students at Saint Louis University School of Medicine. A history form was completed for each subject that included a detailed listing of all medical conditions and medications, age, and, in the case of humans with DM2, the degree of glycemic control was determined by measurement of hemoglobin A1c (HbA1c) within 4 wk of blood removal. The mean ages for healthy humans ($n = 18$, 10 males, 8 females) and humans with DM2 ($n = 24$, 17 males and 7 females) were $34 ± 3$ (range 21 to 57) and $56 ± 3$ yr (range 29 to 77), respectively. The average HbA1c of all humans with DM2 was $8.4 ± 0.3%$. Erythrocyte ATP content in healthy humans and humans with DM2 did not differ and was $3.9 ± 0.5$ and $3.2 ± 0.2$ mM/cell, respectively. Patients with DM2 were treated with insulin ($n = 10$); oral hypoglycemic agents ($n = 16$); lipid-lowering agents ($n = 14$); antihypertensive drugs including angiotensin-converting enzyme inhibitors ($n = 13$), β-adrenergic receptor blockers ($n = 10$), calcium channel blockers ($n = 6$), thiazide diuretics ($n = 6$), and aspirin ($n = 10$). It is not possible to withdraw medications from humans with DM2 for the purpose of this study. However, there are no reports that the medications listed above alter ATP release from erythrocytes.

Effect of exposure to low-O2 tension on ATP release from the erythrocytes of healthy humans and humans with DM2. Exposing healthy human erythrocytes to decreased O2 tensions in a tonometer resulted in increases in ATP release ($n = 11$; Fig. 2A). No gender differences were identified with respect to the magnitude of the response to reduced O2. The release of ATP occurred in a graded fashion such that the lower the O2 tension to which the erythrocytes were exposed, the greater the amount of ATP released. In contrast, cells from humans with DM2 did not release additional ATP when exposed to reduced O2 tension ($n = 10$, HbA1c = $8.5 ± 0.5$; Fig. 2B).

Effect of erythrocytes of healthy humans and humans with DM2 on the response of isolated, perfused arterioles to reduced extraluminal O2 tension. Isolated hamster skeletal muscle arterioles were perfused with buffer containing erythrocytes of healthy humans ($n = 7$) or humans with DM2 ($n = 6$, HbA1c = $8.3 ± 0.8$). There were no differences between groups in initial vessel diameter or diameter after vessels developed spontaneous myogenic tone ($48.1 ± 6.3$ and $49.2 ±

Table 1. Blood flow distributions and calculated tissue oxygen tension

<table>
<thead>
<tr>
<th>Network</th>
<th>Blood Flow</th>
<th>Tissue PO2, mmHg</th>
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<td>Case 1</td>
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<td></td>
<td>0.25Q</td>
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<td></td>
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<td>Minimum: 37.9</td>
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<td>Means ± SD: 46.4 ± 3.2</td>
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<td>Case 2</td>
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<td>0.31Q</td>
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<td>4Q</td>
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<td>Minimum: 39.4</td>
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<td>Means ± SD: 47.0 ± 2.7</td>
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<td>Case 3</td>
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<td>13Q</td>
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<td>Minimum: 48.6</td>
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<td>Means ± SD: 49.5 ± 1.2</td>
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<td>4Q</td>
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<td>Minimum: 45.5</td>
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<td>Means ± SD: 47.9 ± 1.2</td>
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Q, flow; PO2, oxygen tension.
3.4 \mu m, respectively). When arterioles perfused with buffer containing healthy human erythrocytes were exposed to reduced extraluminal PO_2 (PO_2 = 14 \pm 3 \text{ mmHg}), the vessels dilated with intraluminal diameter increasing by 13.7 \pm 3.0\% (P < 0.05; Fig. 3). In contrast, when vessels were perfused with buffer containing erythrocytes of humans with DM2, the vessels constricted with intraluminal diameter decreasing by 5.2 \pm 2.2\% (P < 0.05; Fig. 3) in response to reduced extraluminal PO_2 (PO_2 = 21 \pm 4). The latter response is similar to that seen when buffer-perfused arterioles are exposed to low PO_2 (37) and is consistent with the hypothesis that low O_2 fails to stimulate release of ATP from DM2 erythrocytes (Fig. 2B) preventing these cells from stimulating vasodilation in response to reduced extraluminal PO_2.

Effect of ILO on ATP release from the erythrocytes of healthy humans and humans with DM2. Incubation of isolated erythrocytes (n = 13) with ILO (1 \mu M) resulted in a 328 \pm 92\% increase in ATP release (Fig. 4, open bars; P < 0.05). When erythrocytes of humans with DM2 (n = 10, HbA1c = 8.2 \pm 0.6) were incubated with the same concentration of ILO, the increase was 408 \pm 99\% (Fig. 4, filled bars; P < 0.01).

92\% increase in ATP release (Fig. 4, open bars; P < 0.05). When erythrocytes of humans with DM2 (n = 10, HbA1c = 8.2 \pm 0.6) were incubated with the same concentration of ILO, the increase was 408 \pm 99\% (Fig. 4, filled bars; P < 0.01). Although the amounts of ATP release did not differ between the two groups, when the increase in ATP release from DM2 erythrocytes was compared with HbA1c levels, a significant direct relationship was found (Fig. 5). Thus, ILO-induced ATP release was greater from erythrocytes of humans with higher HbA1c levels, that is, with worse glycemic control.

Calculated tissue oxygen distributions for varying capillary network blood flow. Our computational model allows calculation of the distribution of tissue PO_2 values for the four blood flow distributions described above. Figure 6 shows the calculated combined tissue PO_2 distributions from the four networks for each of the four cases we tested. For case 1, these calculations show that the undersupply of blood flow to network 1 results in a tissue region with substantially decreased tissue PO_2 (Fig. 6A with shaded area showing contribution of network
with flow 0.25Q) relative to the tissue supplied by the other three networks. The minimum tissue PO2 for the undersupplied region was 37.9 mmHg (Table 1) compared with 45.5 mmHg for the tissue with adequate blood supply. When total flow to the four networks was returned to baseline (4Q, case 2) by a uniform (i.e., nonselective) flow increase, there remained a substantial region of tissue with abnormally low tissue PO2 (Fig. 6B with shaded area showing contribution of network with flow 0.31Q) and a minimum tissue PO2 only slightly increased by 1.5 mmHg relative to case 1. To eliminate the low-PO2 region by a uniform flow increase, the total flow to the four networks must be increased by a factor of four (to 13Q, case 3). However, this resulted in oversupply of O2 to three of the networks and a large tissue region with abnormally high tissue PO2 (Fig. 6C with shaded area showing contribution of

Fig. 5. Linear regression relationship between hemoglobin A1c (HbA1c) and the percent increase in ATP release produced by incubation of erythrocytes of humans with DM2 with iloprost (n = 10). The baseline ATP level was 11.7 ± 3 nmol/10^6 erythrocytes.

Fig. 6. Results of oxygen transport simulation of a tissue supplied by 4 discrete capillary networks for 4 different flow distributions among the networks. The solid line in each graph shows the combined tissue PO2 distributions for all 4 networks, while the shaded areas show the contribution from the subset of networks that are under- or oversupplied. A: tissue PO2 distribution is presented for 1 network undersupplied at 25% of normal blood flow (0.25Q, shaded area) and the remaining 3 networks each with normal blood supply (Q) for a total blood flow to the simulated tissue of 3.25Q. B–D: results of 3 different ways to adjust the blood supply in an attempt to restore tissue PO2 levels. B and C: represent flows that occur in response to a vasodilator that uniformly increases flow in all arterioles while D represents a regulatory system that directs flow where it is needed. B: total flow to the 4 networks was increased to normal (4Q) with a uniform 23% (100% × 4/3.25) increase in flow to each network. Flow to the undersupplied network (shaded area) increased to 0.31Q. C: flow to the undersupplied network was restored to normal by uniformly increasing flow to all networks by 4-fold such that total flow increased to 13Q, with 3 networks receiving an oversupply (4Q, shaded area). D: represents the case where blood supply to the undersupplied network was increased to normal while blood supply to the other 3 networks was maintained at normal (total flow = 4Q) and hence represents the normal tissue PO2 distribution. Using a vasodilator cannot restore tissue oxygenation to normal since a uniform increase in flow results in some regions either undersupplied (B, shaded area) or oversupplied (C, shaded area).
networks with flow 4Q) that increased mean PO\textsubscript{2} to 49.5 mmHg. To return tissue PO\textsubscript{2} to normal, a selective increase in blood flow to the one undersupplied network was required. When this was done in case 4, minimum tissue PO\textsubscript{2} was substantially increased (to 45.5 mmHg; Table 1) without creating regions of abnormally high or low PO\textsubscript{2}, as indicated by the absence of any shaded areas in Fig. 6D.

**DISCUSSION**

The erythrocyte, by releasing ATP when exposed to reduced O\textsubscript{2} tension, can participate in the regulation of the matching of O\textsubscript{2} supply with demand in skeletal muscle (2, 13, 27, 32, 34). It was shown previously that erythrocytes of patients with DM2, a condition associated with decreased muscle blood flow both at rest (35) and during exercise (10), demonstrate a selective decrease in the expression of Goi\textsubscript{2} (39, 40), the heterotrimeric G protein required for reduced O\textsubscript{2} tension-induced ATP release (32, 33). Here, we extend these studies with the demonstration that erythrocytes of humans with DM2, in contrast to cells of healthy humans, fail to release ATP in response to exposure to this physiological stimulus (Fig. 2). In addition, we demonstrate that although isolated skeletal muscle arterioles perfused with erythrocytes from healthy humans dilate when exposed to low extraluminal PO\textsubscript{2}, arterioles perfused with DM2 erythrocytes do not (Fig. 3).

Direct studies of oxygen transport in the skeletal muscle microcirculation are not possible in humans. However, studies performed in several animal models of DM2 (2, 13, 34) demonstrate 1) reduced O\textsubscript{2} delivery (2, 13), 2) reduced capillary erythrocyte flux (2), and 3) reduced convective O\textsubscript{2} delivery and diffusive O\textsubscript{2} transport (34), suggesting an impairment in O\textsubscript{2} delivery relative to metabolic need. Vasodilation in response to both pharmacological and physiological stimuli has been shown to be altered in humans with DM2 with defects in both endothelium-dependent and independent mechanisms proposed (1, 20, 22, 29, 44, 46, 47). In addition, it has been suggested that there is reduced nitric oxide (NO) synthesis (29, 44), increased NO degradation (1, 46), and/or abnormalities in the vascular smooth muscle (47) in these individuals. Although there is evidence in support of each of these, none appears sufficient to explain the failure to match O\textsubscript{2} delivery with metabolic need in skeletal muscle in humans with DM2. Our demonstration that isolated skeletal muscle arterioles perfused with DM2 erythrocytes fail to dilate when exposed to reduced extraluminal O\textsubscript{2} tension suggests that a defect in erythrocyte physiology that limits ATP release could contribute to the impairment in microvascular oxygen supply in DM2.

In addition to exposure to reduced O\textsubscript{2} tension, human erythrocytes also release ATP in response to receptor-mediated activation of the G\textsubscript{s}-coupled IP receptor (36). Previously, it was shown that, in contrast to Goi\textsubscript{2}, expression of G\textsubscript{s} and adenyl cyclase type II is not decreased in erythrocytes of humans with DM2. Therefore, we determined whether ATP release in response to the PGI\textsubscript{2} analog, ILO, was present in DM2 erythrocytes. As shown in Fig. 4, ATP release in response to ILO was not reduced in DM2 erythrocytes. Interestingly, ATP release from DM2 erythrocytes tended to be greater than from erythrocytes of healthy humans (Fig. 4). To investigate this further, we correlated the percent increase in ATP release with hemoglobin HbA1c levels, a measure of glycemic control. It was determined that as HbA1c increased (glycemic control worsened), the amount of ATP release from DM2 erythrocytes increased (Fig. 5). Investigation of the mechanism responsible for the increased response to ILO is beyond the scope of this study but could involve alterations in the IP receptor or in several components of the signaling pathway for ATP release. Mechanism notwithstanding, these findings suggest that PGI\textsubscript{2} analogs could be of value in the treatment of the peripheral vascular disease associated with DM2. However, pharmacological activation of the erythrocyte IP receptor throughout the circulation would result in release of ATP from erythrocytes perfusing both metabolically active skeletal muscle as well as inactive muscle with less O\textsubscript{2} demand. To what extent the resulting general vasodilation would reverse the oxygen supply defect in DM2 remains to be determined.

To begin to address this important question, we employed a computational model of oxygen transport in a simulated skeletal muscle supplied by four discrete capillary networks under conditions where one of the four capillary networks is initially undersupplied with blood flow. We set flow into this network at 25% of normal while the other three networks received normal flow. This meant that the total blood flow to the simulated tissue was 81% of normal. Levels of O\textsubscript{2} in the undersupplied region of tissue were substantially lower than those found in the other regions of the tissue (shaded area, Fig. 6A). One might expect that using a vasodilator to restore total flow to normal levels would be sufficient to restore tissue oxygenation, but as the simulation shows (Fig. 6B, shaded area), this would result in only a slight improvement in tissue oxygenation. Flow increased to this region from 25 to only 31% of normal because the reduced flow was localized and not uniform across the four networks, and hence could not be corrected by a uniform flow increase. If one knew how much the one region was undersupplied, one might apply vasodilators more aggressively to increase O\textsubscript{2} supply to supranormal levels. In the simulation, a fourfold increase in total flow was needed to restore tissue oxygenation to the undersupplied region but with the consequence of grossly oversupplying the other three networks with a substantial increase in tissue oxygenation in these areas (shaded area, Fig. 6C). Clearly, restoring a functional regulatory system that matches flow to where it is needed yielded the optimal result (Fig. 6D). Walley (45) reached a similar conclusion with his theoretical approach to investigate the impact of a mismatch between O\textsubscript{2} supply and demand on critical O\textsubscript{2} extraction, concluding that, as the heterogeneity between the ratio of supply and demand increased (loss of O\textsubscript{2} regulation), O\textsubscript{2} supply dependency occurred at higher and higher O\textsubscript{2} supply rates.

Although the true situation in a tissue may differ substantially from what we simulated here, this simple model highlights why it is critically important for an O\textsubscript{2} regulatory system not only to maintain total flow to a skeletal muscle but also to direct the flow to where it is needed. Failure of the erythrocyte to release ATP in response to a decrease in O\textsubscript{2} levels in DM2 may have significant consequences for tissue oxygenation. Computational modeling predicts that restoration of the ability of erythrocytes to release ATP in response to reduced O\textsubscript{2} tension would be of greater value in
increased tissue O2 need, could be considered a novel target in skeletal muscle than would general vasodilators. Thus, the erythrocyte, by virtue of its ability to direct blood flow to areas of increased tissue O2 need, could be considered a novel target for the development of therapeutic approaches to treat the peripheral vascular disease of DM2.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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