Dysregulated Selectin Expression and Monocyte Recruitment During Ischemia-Related Vascular Remodeling in Diabetes Mellitus

Chad L. Carr, Yue Qi, Brian Davidson, Scott Chadderdon, Ananda R. Jayaweera, J. Todd Belcik, Cameron Benner, Aris Xie, Jonathan R. Lindner

Objective—Diabetes mellitus (DM) is associated with impaired ischemia-related vascular remodeling and also dysregulation of the inflammatory response. We sought to determine whether impaired selectin-mediated monocyte recruitment in ischemic tissues contributes to blunted ischemia-mediated angiogenesis in DM.

Methods and Results—Contrast-enhanced ultrasound perfusion imaging and molecular imaging of endothelial P-selectin expression in the proximal hindlimb were performed at 1, 3, and 21 days after arterial ligation in wild-type and db/db mice. Ligation reduced muscle blood flow to \( \approx 0.05 \) mL/minute per gram in both strains. Significant recovery of flow occurred only in wild-type mice (60%–65% of baseline flow). On molecular imaging in db/db mice, baseline P-selectin signal was 4-fold higher in db/db compared with wild-type mice \((P<0.01)\) but increased minimally at day 1 after ischemia, whereas signal increased approximately 10-fold in wild-type mice \((P<0.01)\). Immunohistology of the hindlimb demonstrated severely reduced monocyte recruitment in db/db mice compared with wild-type mice. Local treatment with monocyte chemotactic protein-1 corrected the deficits in postischemic P-selectin expression and monocyte recruitment in db/d mice and led to greater recovery in blood flow.

Conclusion—In DM, there is dysregulation of the selectin response to limb ischemia, which leads to impaired monocyte recruitment, which may be mechanistically related to reduced vascular remodeling in limb ischemia. (Arterioscler Thromb Vasc Biol, 2011;31:00-00.)

Key Words: angiogenesis ■ inflammation ■ molecular imaging

Growth and remodeling of collateral vessels and the distal microcirculation are important adaptations to ischemia produced by peripheral and coronary artery disease. This compensatory response is impaired in type 2 diabetes mellitus (DM). A potential explanation is that despite smoldering chronic inflammation in DM, there may be an inability to further mount a response from beneficial proangiogenic components of the inflammatory response.

Certain monocyte subtypes play an important role in coordinating vascular remodeling through their production of growth factors, cytokines, and matrix proteases. Monocyte entry into ischemic tissue is regulated by many factors including chemokines, such as monocyte chemotactic protein-1 (MCP-1) and vascular endothelial growth factor (VEGF)-A. Monocytes from patients with DM exhibit a severe deficit in chemotaxis specifically to VEGF-A. This deficit appears to be due to “desensitization” of monocyte VEGF receptor-1 signaling. However, major gaps remain in our understanding of how a dysregulated monocyte response contributes to poor vascular remodeling in DM, including whether there is a similar desensitization of endothelial activation. Of particular interest is endothelial P-selectin expression because translocation of the P-selectin to the cell surface is stimulated by VEGF-receptor activation, and some studies have indicated that recruitment of monocytes through P-selectin plays an important role in ischemia-mediated vascular remodeling.

The aim of this study was to determine whether the selectin response to ischemia is abnormal in DM and whether this leads to impairment in monocyte recruitment, arteriogenesis, and blood flow recovery. To test this hypothesis, in vivo contrast-enhanced ultrasound (CEU) molecular imaging of endothelial P-selectin expression was temporally correlated to quantitative muscle blood flow measurements in a chronic ischemic hindlimb model in obese insulin-resistant (db/db) mice. We also sought to determine whether local treatment with MCP-1 could compensate for deficiencies in monocyte recruitment and improve blood flow recovery in DM because it is a VEGF-independent monocyte chemokine that also promotes endothelial P-selectin translocation.
Methods

Hindlimb Ischemia Protocol

The study was approved by the Animal Care and Use Committee at Oregon Health and Science University. Hindlimb ischemia was produced by unilateral interruption of arterial inflow in 40 wild-type C57Bl/6 mice and 50 db/db mice (B6.Cq+−/+ Leprdb/J, Jackson Laboratory) with a homozygous deletion of the leptin receptor that produces age-dependent obesity and insulin resistance. Mice (age 8–10 weeks) were anesthetized with inhaled isoflurane and the jugular vein was catheterized for administration of contrast agent. Perfusion and molecular imaging studies were performed at baseline before ligation in an additional 6 wild-type mice, P-selectin functionally inhibited for approximately for approximately the first week by intraperitoneal injection of rat anti-mouse P-selectin monoclonal antibody (RB40.34) immediately before ligation. In 18 of the db/db mice, MCP-1 (1 μg/kg in 50 μL) was injected intramuscularly into the proximal hindlimb adductor group of the ischemic limb immediately after ligation and on days 1 and 3 after ligation. Imaging studies were performed on days 1, 3, and 21 after ligation, and only 2 separate time points were used per subject; subjects were randomly selected. For imaging, mice were anesthetized and the jugular vein was catheterized for administration of contrast agent. Perfusion and molecular imaging studies were performed at baseline before ligation in an additional 6 wild-type and 12 db/db mice.

Microbubble Preparation

Nontargeted lipid-shelled decafluorobutane microbubbles were prepared by sonication of a gas-saturated aqueous suspension of 2 mg/mL disteroylephosphatidylcholine and 1 mg/mL polyoxethylene-40-stearate. For molecular imaging, biotinylated microbubbles were prepared by adding 0.4 mg/mL disteroylephosphatidyl ethanolamine-PEG (200). Biotinylated rat anti-mouse P-selectin monoclonal antibody (RB40.34) was conjugated to the microbubble surface via a streptavidin linkage as previously described. Microbubble concentration was measured by electrozone sensing (Multisizer III, Beckman Coulter).

Perfusion Imaging

Contrast ultrasound perfusion imaging was performed in the ischemic and contralateral control limbs with a linear-array transducer at a center frequency of 7 MHz (Sequoia 512, Siemens Medical Systems). The nonlinear fundamental signal component for microbubbles was detected using multipulse phase- and amplitude-modulation at a mechanical index of 0.18 and a dynamic range of 55 dB. With these settings, the beam elevational plane, defined as the beam center, was located at the level of the inguinal ligament and the knee. Images were acquired at a frame rate of 20 Hz immediately after a brief high-power (mechanical index 1.1) destructive pulse sequence, and time-intensity data were fit to the following function:

\[ y = A(1 - e^{-\beta t}) \]

where \( y \) is intensity at time \( t \), \( A \) is the plateau intensity, and the rate constant \( \beta \) represents the microvascular flux rate. Skeletal muscle microvascular blood volume was quantified by

\[ A/(1.06 \times I_p \times F \times 1.1) \]

where 1.06 is tissue density (g/cm³), \( F \) is the scaling factor for the 10-fold lower microbubble infusion rate for measuring \( I_p \), which was used to avoid dynamic range saturation, and 1.1 is a coefficient to correct for murine sternal attenuation measured a priori from in vitro experiments (see Supplemental Appendix, available online at http://atvb.ahajournals.org). Muscle blood flow was quantified by the product of microvascular blood volume and \( \beta \).

Molecular Imaging

For molecular imaging, \( 1 \times 10^7 P \)-selectin-targeted and control microbubbles were injected as an intravenous bolus in random order separated by 10 to 12 minutes. A mathematical algorithm was developed to calculate the retention fraction of microbubbles rather than simply measuring signal from retained microbubbles because this latter value is influenced by the amount of flow in the ischemic limb (ie, the total number of bubbles fluxing through the imaging plane) which varied over time. Using low-mechanical-index imaging, ultrasound frames were acquired every 10 seconds after injection for a total of 8 minutes. The retention fraction (\( f \) of the bubbles was calculated by fitting time-intensity data from a region-of-interest placed over the proximal hindlimb adductor muscles to the following function:

\[ I_p = (K \cdot A_p \cdot t) \left(1 - e^{-\alpha t}\right) + (f \cdot \beta/t \cdot (1 - (1 + \alpha) \cdot e^{-\alpha t})) \]

This derivation of this formula relies on the deconvolution of the time-intensity curve into 2 separate functions: (1) a \( y \)-variate function reflecting freely transiting microbubbles, and (2) an integral of a \( y \)-variate that represents retained microbubbles (see Supplemental Appendix). This method was ideally suited to our ischemia-angiogenesis model because \( f \) is independent of both tissue blood flow and microbubble dose.

Platelet Depletion

To determine whether the primary source of P-selectin signal was endothelial or platelet-derived, imaging was performed after immune-mediated platelet depletion in an additional 6 wild-type mice. Immediately after ligation surgery and wound closure, mice were treated with 2 μg/kg rat anti-mouse glycoprotein-Ibα monoclonal antibody (R300, Emfret Analytics) by intraperitoneal route. This treatment has been shown to reduce platelet concentration to <10,000 dL⁻¹, for >48 hours through splenic sequestration. Molecular and perfusion imaging were performed 1 day after ligation.

Echocardiography and Hemodynamic Measurements

Echocardiography (Vevo-770, Visualsonics Inc) and invasive aortic micromanometry (SPR-671, Millar Instruments, Inc) were performed to exclude significant strain-related differences in hemodynamics and left ventricular function under anesthesia (see supplemental material for details).

Intravital Microscopy

Selectin-mediated leukocyte rolling in postcapillary venules was assessed by intravital microscopy. Wild-type (n=6) and db/db (n=6) mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride, xylazine, and atropine. A cremaster muscle was exteriorized and mounted on a custom stage for intravital microscopy. The muscle was superfused continuously with isothermic bicarbonate-buffered saline during microscopy (Axioskop2-FS, Carl Zeiss, Inc, Thornwood, NY) with a saline-immersion objective (×20/0.5 NA). Video recordings of at least 5 venules (diameter 20–40 μm) were made with a high-resolution charge-coupled device camera (C2400, Hamamatsu Photonics, Hamamatsu, Japan) 10 to 20 minutes after exteriorization. Centerline RBC velocity (\( V_c \)) was measured using a dual-slit photodiode (CircuSoft Instrumentation) and converted to mean velocities by multiplying by 0.625. Venular diameters (\( d \)) were measured off-line using video calipers. The
distance traveled by individual rolling leukocytes was divided by the elapsed time to derive the mean rolling velocity. The number of rolling leukocytes \( n_r \) was determined by counting leukocytes crossing a line perpendicular to the vessel during 1 minute. Leukocyte rolling flux fraction, which reflects the percentage of leukocytes passing through a venule that are rolling, was calculated by

\[
\frac{n_r}{0.25 \pi d^2 V_b \cdot 60 \cdot C_L}
\]

where \( C_L \) is the systemic blood leukocyte concentration.

### Immunohistochemistry

Histology was performed with perfusion-fixed paraffin-embedded sections. Immunohistochemistry was performed for monocytes/macrophages with a rat anti-mouse Mac-2 monoclonal antibody (M3/38, Cedarlane Labs) with an Alexa Fluor 488–labeled secondary antibody (Invitrogen). The spatial extent of positive staining was quantified by a pixel intensity threshold program (NIH ImageJ) and expressed as a percentage of the total muscle area per section. Noncapillary microvascular density was assessed by immunohistochemistry with a Cy3-labeled rat anti-mouse \( \alpha \)-smooth muscle actin monoclonal antibody (1A4, Sigma) and was expressed by the total microvessel area per muscle section area.

### Statistics

Data are represented as mean±standard deviation unless otherwise specified. Targeted imaging and intravital microscopy data were analyzed with nonparametric tests (Kruskal-Wallis and Mann-Whitney test). Group comparisons for perfusion and histology were made with 1-way ANOVA and post hoc testing of individual comparisons with paired \( t \) test and Bonferroni correction.

### Results

#### Strain-Related Characteristics at Baseline

At baseline, db/db mice had a higher body mass (44±5 versus 22±5 g, \( P<0.001 \)) and were hyperglycemic (blood glucose 443±71 versus 114±32 mg/dL, \( P<0.001 \)) compared with wild-type mice, consistent with the expected obese insulin-resistant phenotype. There were no species-related differences in blood leukocyte, erythrocyte, or platelet concentration (data not shown). Heart rate and central aortic blood pressure were similar between wild-type and db/db mice, as were echocardiographic measurements of left ventricular function, such as thickening fraction, Vcf, and endocardial peak systolic radial velocity (see supplemental material).

#### Skeletal Muscle Blood Flow

Microvascular blood flow in the proximal hindlimb adductor muscle group on CEU imaging at baseline before ligation was
lower in db/db mice (Figure 1A). Lower baseline flow in db/db mice was due to slower microvascular blood flux rate and lower microvascular blood volume (see supplemental material). On day 1 after arterial ligation, blood flow was reduced to a similar level (~0.05 mL/minute per gram) for both wild-type and db/db mice (Figure 1A). Qualitatively at this time point there was considerable spatial heterogeneity in flow distribution with interspersed areas of hyperperfusion and nonperfusion within the muscle. In wild-type mice, blood flow in the ischemic muscle increased over 21 days, ultimately reaching an average of ~65% of baseline flow, whereas flow recovery was minimal in db/db mice. Recovery of flow in the wild-type mice was manifest by significant increases in both microvascular blood volume and flux rate (see supplemental material). On immunohistochemistry at day 21, the area of α-smooth muscle actin vessels in muscle from the ischemic limb, expressed as a ratio to the contralateral normal limb, was greater in wild-type versus db/db mice (2.1±0.1 versus 1.2±0.1, P<0.01), consistent with a substantial arteriogenic response only in wild-type mice. The degree of flow recovery at day 21 in wild-type mice varied considerably (range, 0.15–0.73 mL/minute per gram) and was inversely but nonlinearly related to the severity of initial ischemia on day 1 (Figure 1D), suggesting that the degree of initial ischemia may influence the rate of flow recovery over the first 3 weeks in wild-type mice. Recovery of blood flow at day 21 in wild-type mice was substantially inhibited by treatment with an antibody that functionally inhibits P-selectin, and in db/db mice it was significantly augmented when treated with MCP-1 (Figure 2).

Molecular Imaging of P-Selectin
At baseline before arterial ligation, the retention fraction for P-selectin-targeted microubbles on molecular imaging was approximately 4-fold greater in db/db compared with wild-type mice (Figure 3A). However, in wild-type mice, P-selectin signal markedly increased in wild-type mice at days 1 and 3 after ligation (10-fold and 3-fold increases from baseline, respectively) indicating an early and robust P-selectin response to ischemia in wild-type animals. In db/db mice, despite having high baseline P-selectin expression, further upregulation of P-selectin in response to ischemia was severely blunted compared with wild-type mice. In db/db mice, treatment with MCP-1 produced a substantial increase in P-selectin signal at day 1 to a level similar to that seen in wild-type mice. Immune-mediated platelet depletion with anti-GP1bα antibody in wild-type mice was confirmed by bleeding times of >21 minutes. In these mice, the P-selectin signal was similar to those not undergoing platelet depletion (0.36±0.12 versus 0.36±0.22, P=0.94), indicating a predominantly endothelial rather than platelet source for P-selectin signal on molecular imaging. In wild-type mice undergoing imaging at both day 1 and day 21 (n=11), there was a modest linear relationship between P-selectin signal at day 1 and flow recovery at day 21 (Figure 3B).

Intravital Microscopy
Intravital microscopy of the cremaster muscle was used as a complementary method for evaluating P-selectin mobiliza-

Figure 2. Mean (±SEM) skeletal muscle blood flow at day 21 after arterial ligation in wild-type mice (WT) with and without P-selectin blocking antibody RB40.34 and in db/db mice with and without monocyte chemotactic protein-1 (MCP-1) treatment. *P<0.01 vs wild-type; †P<0.05 vs untreated db/db mice.

Histology
On hematoxylin/eosin staining, there was a robust inflammatory cell infiltrate in the ischemic muscle from wild-type mice that was most pronounced at day 3 (Figure 5). On Mac-2 staining, much of the cellular infiltrate at this time interval appeared to be of monocyte origin. In db/db mice, fewer inflammatory cells were seen on hematoxylin/eosin, and the number of Mac-2-positive monocytes was markedly reduced, particularly at day 3 after ligation. Treatment with MCP-1 in the early ischemic period in db/db mice resulted in an increase in total inflammatory cell infiltration and monocyte accumulation on days 1 and 3 after ligation.

Discussion
The primary aim of this study was to pair molecular imaging and perfusion imaging data in a murine model of limb ischemia to determine whether impaired flow recovery in DM is associated with abnormalities in endothelial selectin expression and monocyte recruitment. Our studies demonstrated that (1) flow recovery in response to hindlimb ischemia is severely impaired in db/db mice; (2) despite a chronic upregulation of P-selectin, further upregulation in response to ischemia is severely blunted in db/db mice; (3) monocyte recruitment, arteriogenesis, and recovery of limb perfusion are associated with and possibly influenced by the P-selectin response during ischemia; and (4) the deficits in endothelial
also been found with CEU in Zucker obese rats.24 This finding is technical in nature because this is the first study that quantifies microvascular perfusion in mL/minute per gram of tissue and is not necessarily implied a lower anatomic capillary density. In coronary and peripheral arterial disease, functional capillary blood volume in vascular beds is influenced by the degree of pressure loss caused by stenosis.25 Similar to other studies,22,23 histology in the ischemic limb in db/db mice in our study showed less arteriogenesis, which would be expected to lead to lower collateral conductance in the ischemic limb, lower precapillary pressure, and subsequent capillary derecruitment.25 The fact that microvascular blood flux rate was equally impaired suggests that this was the case.

There is strong evidence that monocytes play a critical role in arteriogenesis and other vascular responses to tissue ischemia and injury. In mammalian models of limb ischemia, potentiation of the monocyte response with chemokines such as MCP-1 and lipopolysaccharide has been shown to augment collateral development.5,26 Mice that are deficient in either MCP-1 or CCR-2, a primary receptor for MCP-1, have less recovery of limb blood flow after interruption of the arterial inflow supply.27,28 These findings are thought to be related to the finding of less perivascular monocyte infiltration because these cells are thought to contribute to vascular remodeling through production of growth factors (VEGFs, fibroblast growth factor-2, platelet-derived growth factor, PIGF, HIF-1α, etc), proangiogenic cytokines, and matrix proteases. In the current study, db/db mice had a striking reduction in the extent of monocyte infiltration early after producing ischemia. Impaired VEGF-A-mediated monocyte migration may have contributed to this abnormality.7,8 However, our results also suggest that abnormalities in the initial step of selectin-mediated monocyte recruitment may have also contributed. The idea to study selectins in this setting was based on their recognized role in monocyte recruitment and angiogenesis in limb ischemia and wound healing.11,29 and on the observation that VEGF receptor signaling, which is impaired in DM, is involved in P-selectin mobi-

**Figure 3.** Targeted contrast-enhanced ultrasound (CEU) molecular imaging of P-selectin. A, Retention fraction for P-selectin targeted microbubbles before (day 0) and at days 1 and 3 after arterial ligation. Baseline data for monocyte chemotactic protein-1 (MCP-1)–treated and untreated db/db mice are identical because these represent combined pretreatment data. *P<0.01 vs wild-type; †P<0.05 vs untreated db/db mice. See supplemental material for number of observations for each data point. B, Relationship between retention fraction of P-selectin microbubbles on day 1 and subsequent change in blood flow between days 1 and 21. C and D, Examples of time-intensity data from the proximal hindlimb adductor muscle after intravenous injection of P-selectin-targeted microbubbles illustrating differences in muscle retention fraction from a wild-type mouse (WT) and a db/db mouse 1 day after arterial ligation. Examples of background-subtracted video color-coded (scale at left) video intensity near the end of the acquisition are illustrated.

selectin upregulation and monocyte recruitment during ischemia in db/db mice can be corrected by local treatment with MCP-1, which may explain its effect on enhancing flow recovery in these animals.

Clinical trials and animal models of disease have established that compensatory vascular remodeling in chronically ischemic tissue is abnormal in diabetic subjects.1–3 Hence, the blunted recovery of limb perfusion after iliac ligation in db/db mice in the current study was expected. The severity of this abnormality was striking and somewhat greater than some prior studies that have used laser Doppler velocimetry as a surrogate for quantitative flow measurement.9,20–23 One reason for this discrepancy was that compensatory vascular remodeling in chronically ischemic tissue is abnormal in diabetic subjects.1–3,9 Hence, the blunted recovery of limb perfusion after iliac ligation in db/db mice in our study showed less arteriogenesis, which would be expected to lead to lower collateral conductance in the ischemic limb, lower precapillary pressure, and subsequent capillary derecruitment.25 The fact that microvascular blood flux rate was equally impaired suggests that this was the case.

Prior studies using histology have been consistent in finding less angiogenesis, arteriogenesis, and collateral development after interruption of arterial inflow when compared with control mice.20–23 We found that impaired flow recovery after arterial ligation in db/db mice was manifest in part by the lack of a time-dependent increase in microvascular blood volume on CEU. However, lower functional microvascular density derived from CEU time-intensity data does not necessarily imply a lower anatomic capillary density. In coronary and peripheral arterial disease, functional capillary blood volume in vascular beds is influenced by the degree of pressure loss caused by stenosis.25 Similar to other studies,22,23 histology in the ischemic limb in db/db mice in our study showed less arteriogenesis, which would be expected to lead to lower collateral conductance in the ischemic limb, lower precapillary pressure, and subsequent capillary derecruitment.25 The fact that microvascular blood flux rate was equally impaired suggests that this was the case.

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**Figure 4.** Intravital microscopy of leukocyte rolling. A, Selected images 0.3 seconds apart from a video illustrating 2 separate leukocytes (white and yellow arrowheads) rolling on the endothelial surface of a 25-μm–diameter venule. Bar-whisker plots display median (line), 25th to 75th percentiles (box), and range for leukocyte rolling velocity (B) and leukocyte flux fraction (C). *P<0.01 vs wild-type (WT).
lization from endothelial Weibel-Palade bodies. As a cautionary statement, vasculogenesis in response to fibroblast growth factor-1 in corneal pocket assays is not dependent on selectins, suggesting that selectins are involved in vascular remodeling in response to ischemia but not necessarily in all forms of vascular development.

We assessed P-selectin with CEU by using a pure intravascular tracer that is able to detect surface translocation of P-selectin in ischemic injury. The higher level of baseline P-selectin expression in skeletal muscle microvessels in the db/db mice is consistent with the notion of chronic upregulation of inflammatory pathways in DM. However, db/db mice lacked the ability to mount a substantial P-selectin response after production in ischemia and had a much lower P-selectin signal on molecular imaging at day 1 compared with wild-type mice. Intravital microscopy confirmed a deficit in P-selectin-mediated rolling, although these results must be tempered by the fact that monocytes were not differentiated from other leukocytes. These data are consistent with a notion of a desensitization of the selectin response.

Our studies demonstrated that P-selectin is an important contributor to the vascular response to ischemia and suggest that in db/db mice, lower P-selectin response led to less monocyte recruitment and flow recovery in db/db mice. The most compelling evidence is that administration of MCP-1 early after arterial ligation corrected the abnormalities of P-selectin signal on molecular imaging, monocyte infiltration, and flow at day 21 in db/db mice. Within the wild-type group, the early P-selectin signal correlated with the degree of flow recovery at day 21 in animals that were studied at both day 1 and day 21.

There are several limitations of the study that deserve mention. Our data strongly support but do not positively confirm that abnormal P-selectin response in DM plays a causative role in reduced ischemia-mediated monocyte recruitment and flow recovery. Although the correction of these defects in db/db mice by MCP-1 therapy strongly supports this hypothesis, MCP-1 is a nonspecific proinflammatory chemokine with many actions. An intervention that specifically acts through selectins would provide stronger evidence but is currently not available. Although the intravital microscopy data support the concept of a P-selectin defect in db/db mice, it is important to note that the triggers for selectin mobilization may be different for surgical exteriorization versus hindlimb ischemia. Because of surgical technique, we were not able to study every animal at every time interval. Greater P-selectin expression at baseline would seem to argue against our hypothesis. However, there are no data that suggest that the inflammatory response is required for basal vascular homeostasis in nonischemic conditions. Finally, we also made flow measurements at rest rather than during hyperemia produced by electrostimulated contractile exercise because of concerns about altering the inflammatory status either through severe ischemia or electrode placement. It is quite likely that the db/db animals would demonstrate an even more profound flow reserve response because of the abnormal NO signaling that occurs as a result of VEGF desensitization.

In summary, we have provided evidence that despite higher basal P-selectin expression in DM, there is reduced expression after inducing chronic limb ischemia consistent with a desensitization of mechanisms that regulate selectin response. The degree of selectin response appears to influence the degree of monocyte recruitment and is also associated with the degree of flow recovery. These data add to our understanding of the mechanisms underlying the impaired response to injury and ischemia in DM and may be helpful in both creating and assessing new strategies aimed at correcting the deficit in the proangiogenic inflammatory response.

Appendix

Acoustic intensity ($I_p$) due to microbubbles transiting through a tissue bed at time $t$ is given by

$$I_p = k \cdot C_i \cdot v$$  (A1)
where \( C_i \) is the concentration of microbubbles in blood at time \( t \), \( v \) is the blood volume fraction, and \( k \) is a proportionality constant. The amount of microbubbles \( dW \) that passes through a unit volume of tissue over a time interval \( dt \) at time \( t \) is given by

\[
dW = F \cdot C_i \cdot dt
\]

where \( F \) is the blood flow per unit volume of tissue. Therefore, the total amount of microbubbles \( W \) that passes through tissue over a time interval \( t \) is

\[
W = F \cdot W(t)
\]

If a fraction \( f \) of these bubbles is retained by tissue, the acoustic intensity that is attributable to these retained bubbles \( (I_r) \) is

\[
I_r = k \cdot f \cdot W
\]

Hence, the retention fraction \( I_r \) is given by

\[
I_r = I_p + I_c = k \cdot [C_i \cdot v + f \cdot W] = k \cdot [C_i \cdot v + f \cdot W] \cdot dt
\]

From perfusion imaging data, we know that \( v = A_v \), where \( A_v \) is the plateau video intensity normalized to blood pool intensity and \( F = A_v \cdot \beta \), where \( \beta \) is the rate constant from the postdisturbing CEU data. Also, we know that

\[
C_i = A \cdot t \cdot e^{-\alpha t}
\]

and also that

\[
\alpha t \cdot \frac{dt}{A(\alpha^2)} = [1 - (1 + \alpha \cdot t) \cdot e^{-\alpha t}]
\]

Substituting Equations 6 and 7 in Equation 5 and simplifying, we get

\[
I_r = k \cdot A_v \cdot A \cdot \beta \cdot [1 - (1 + \alpha \cdot t) \cdot e^{-\alpha t}]
\]

Hence, the retention fraction \( f \) can be obtained by substituting the known values of \( \beta \) and fitting Equation 8 to time versus intensity data obtained after injection of the targeted tracer.

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**Disclosures**

None.

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SUPPLEMENT MATERIAL

METHODS

Echocardiography and Hemodynamics Methods

Echocardiography (Vevo-770, Visualsonics Inc.) and invasive aortic micromanometry (SPR–671; Millar Instruments, Inc.) were performed at baseline to ensure there were no significant strain-related differences in hemodynamics and left ventricular function under anesthesia (n=8 for each group). A calibrated 1.4 French catheter-tip micromanometer (SPR–671; Millar Instruments, Inc.) was inserted into the carotid artery and advanced retrograde into the aorta. Pressure tracings were recorded at a sampling rate of 4,000 Hz (PowerLab 8SP; ADInstruments) to measure systolic and diastolic blood pressure (SBP, DBP). Echocardiography was performed in the parasternal long-axis and mid-ventricular short-axis planes using fundamental imaging at 40 MHz. Image sets were obtained by ECG gating of sequential M-mode lines for a post-acquisition 2-D frame rate of 1,000-1,100 Hz. Peak systolic (S’) and early diastolic (E’) radial velocities in the short-axis were measured by tissue Doppler imaging with a sample volume placed in the posterior endocardium. Velocity of circumferential fiber shortening (VcF) was measured by dividing fractional shortening by the ejection time.

Myocardial Blood Volume

For a blood pool agent, relative myocardial blood volume can be calculated by the ratio of signal intensity in the myocardium (A-value) during steady state to that in the blood pool (I_B), or A/I_B. To convert myocardial mass to volume, it is necessary to divide by the myocardial mass 1.06 g/cm³, or A/(1.06×I_B). Since the (I_B) value was obtained from the LV cavity using a 10-fold lower infusion rate to avoid dynamic range saturation, and the sternum produces a 91%
reduction (or 1/1.1) in contrast signal intensity compared to limb skeletal muscle, MBV was calculated as \( A/(1.06 \times I_B \times 10 \times 1.1) \).

**SUPPLEMENT TABLES**

**Table I. Hemodynamic and Echocardiographic Data**

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<th>Wild-type (n=8)</th>
<th>db/db (n=8)</th>
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<td>Heart Rate (min⁻¹)</td>
<td>481±59</td>
<td>465±70</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>98±5</td>
<td>91±12</td>
</tr>
<tr>
<td>Thickening Fraction</td>
<td>0.50±0.11</td>
<td>0.48±0.15</td>
</tr>
<tr>
<td>Vcf (mm/s)</td>
<td>0.70±0.13</td>
<td>0.70±0.17</td>
</tr>
<tr>
<td>S' (mm Hg/s)</td>
<td>16.7±4.7</td>
<td>15.2±4.7</td>
</tr>
<tr>
<td>E' (mm/s)</td>
<td>13.5±4.8</td>
<td>13.6±3.9</td>
</tr>
</tbody>
</table>

Vcf, velocity of circumferential fiber shortening; S’ and E’, peak early systolic and diastolic endocardial velocity.

**Table II. Parametric Contrast-enhanced Ultrasound Perfusion Imaging Data**

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microvascular blood volume (ml/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>6.0±1.3</td>
<td>4.1±1.1†</td>
</tr>
<tr>
<td>Day 1</td>
<td>2.2±1.2</td>
<td>1.9±1.6</td>
</tr>
<tr>
<td>Day 3</td>
<td>2.8±1.9</td>
<td>1.5±0.7</td>
</tr>
<tr>
<td>Day 21</td>
<td>4.0±1.7</td>
<td>1.4±0.8†</td>
</tr>
<tr>
<td>Microvascular flux rate (s⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.25±0.02</td>
<td>0.16±0.04†</td>
</tr>
<tr>
<td>Day 1</td>
<td>0.06±0.03</td>
<td>0.07±0.06</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.11±0.06</td>
<td>0.06±0.02</td>
</tr>
<tr>
<td>Day 21</td>
<td>0.14±0.06</td>
<td>0.08±0.04†</td>
</tr>
</tbody>
</table>

*p<0.05 for all post-ischemic values versus baseline values; † p<0.05 vs wild-type at the same time interval.
On-line Supplement on Number of Animals for each observation (Figures 1 and 3).

Table III Figure Number of animals for each data point for Figure 1A

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>5</td>
<td>12</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>db/db</td>
<td>8</td>
<td>12</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Table IV Number of animals for each data point for Figure 3A

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Day 1</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>5</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>db/db</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>db/db + MCP-1</td>
<td>-</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>