Extracellular Matrix Fibronectin Mechanically Couples Skeletal Muscle Contraction With Local Vasodilation

Denise C. Hocking,* Patricia A. Titus, Ronen Sumagin, Ingrid H. Sarelius*

Abstract—During exercise, local mechanisms in tissues cause arterioles to rapidly dilate to increase blood flow to tissues to meet the metabolic demands of contracting muscle. Despite decades of study, the mechanisms underlying this important aspect of blood flow control are still far from clear. We now report a novel mechanism wherein fibronectin fibrils in connective tissue matrices transduce signals from contracting skeletal muscle to local blood vessels to increase blood flow. Using intravital microscopy, we show that local vasodilation in response to skeletal muscle contraction is specifically inhibited by an antibody that recognizes the matricryptic site in the first type III repeat of fibronectin (FNIII-1). In the absence of skeletal muscle contraction, direct application of FNIII-1—containing fibronectin fragments to cremaster muscle arterioles in situ, triggered a rapid, specific, and reversible local dilation that was mediated by nitric oxide and required the cryptic, heparin-binding sequence of FNIII-1. Furthermore, application of function-blocking FNIII-1 peptides to cremaster muscle arterioles rapidly and specifically decreased their diameter, indicating that the matricryptic site of fibronectin also contributes to resting vascular tone. Alexa fluor 488–labeled fibronectin, administered intravenously, was rapidly assembled into elongated, branching fibrils in the extracellular matrix of intact cremaster muscle, demonstrating active polymerization of fibronectin in areas adjacent to blood vessels. Together, these data provide the first evidence that a matricryptic, heparin-binding site within fibronectin fibrils of adult connective tissue plays a dynamic role in regulating both vascular responses and vascular tone. (Circ Res. 2008;102:372-379.)

Key Words: extracellular matrix ■ fibronectin ■ metabolic coupling ■ vascular tone

The flow of blood to skeletal muscle is tightly coupled with metabolic activity. In response to active shortening of skeletal muscle fibers, local mechanisms in the tissue cause arterioles to dilate rapidly, which increases blood flow to tissues in support of the metabolic demands of contracting muscle.1 These locally coupled mechanisms support tissue metabolic demands as a facet of the integrated response to exercise. Arteriolar dilation following skeletal muscle contraction is a local response2–4 that is mediated, in part, by metabolic factors, including adenosine, H+, and potassium.1,5 Nitric oxide (NO), derived from both endothelial and neuronal NO synthases, has been identified as an important contributor to the arteriolar dilation that is produced by skeletal muscle contraction.3,6–9 It has also been established that dilation of small arterioles in response to muscle contraction requires increased endothelial cell calcium.10 However, mechanisms that physically couple skeletal muscle fiber contraction to local NO release have not yet been identified.

Fibronectins are high-molecular-mass glycoproteins that form an extensive network of elongated, branching fibrils in extracellular matrices (ECMs) throughout the body. Soluble fibronectins are polymerized into insoluble ECM fibrils via a cell-dependent process that can be rapidly up- and downregulated.11 In vivo, fibronectin matrix polymerization is a continuous process, with as much as 50% of the fibronectin matrix undergoing turnover every 24 hours.12 Multimeric ECM fibrils are thought to serve as the primary functional form of fibronectin in vivo and are essential for embryonic development.13 In vitro, the ECM form of fibronectin stimulates several actin-dependent processes, including cell spreading, growth, contractility, and migration, by a mechanism that uses, in part, a matricryptic heparin-binding site in the first type III repeat of fibronectin (FNIII-1).14–17 The physiological role of ECM fibronectin fibrils in adult connective tissues is not known.

Matricryptic sites are biologically active sequences within ECM proteins that are not exposed in the soluble form of a molecule, but may be expressed following structural or conformational changes to the protein.18 These sequences represent a unique reserve of signaling sites in connective tissue that may be exposed and activated under a variety of conditions where ECM remodeling occurs. Mechanisms that promote matricryptic site expression include protein polymerization, proteolysis, and mechanical forces.18 Decreasing...
intracellular cytoskeletal tension alters the conformation of fibronectin in a region adjacent to the matricryptic site in FNIII-1, suggesting that mechanical forces in the body may influence FNIII-1 structure to, in turn, stimulate actin-dependent processes. As such, the ability of skeletal muscle contraction to place a tensile force on the ECM surrounding arterioles suggested to us that local, transient exposure of the matricryptic FNIII-1 site in ECM fibronectin may be important in mechanically coupling skeletal muscle contraction to arteriolar dilation. In the present study, we used intravitreal microscopy to examine the role of ECM fibronectin and the matricryptic site in vivo. Data presented herein identify a novel physiological mechanism whereby ECM fibronectin fibrils function in adult connective tissue as mechanotransduction elements that couple skeletal muscle contraction with local NO-mediated vasodilation through the transient exposure of the matricryptic site.

Materials and Methods

Materials

An expanded Material and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Animals

Adult male golden hamsters (HSD Han:Aura, Harlan Indianapolis, Ind.; 100 to 130 g) and adult male mice (C57BL/6J, The Jackson Laboratory, Bar Harbor, Me; 20 to 25 g) were used. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Rochester. All protocols, except that demonstrating incorporation of fibronectin into tissues (Figure 2), were performed in hamster cremaster muscle. Preparation of cremaster muscle for microcirculatory observation has been described elsewhere. Details are provided in the online data supplement. The microvasculature was observed with the microscope coupled to a charge-coupled device camera (Dage MTI, CD72S), and video images were recorded onto videotape (Sony VO9500) or DVD (Sony DVO100MD) for offline analysis.

Muscle Fiber Stimulation

To induce muscle contraction, a platinum wire microelectrode was placed onto muscle fibers running approximately perpendicular to the test arteriole and was positioned at a distance ∼1000 μm away from the site of muscle fiber–arteriole overlap. A small group of muscle fibers was stimulated at 4 Hz for 2 minutes (5 to 10 V; 0.4-ms duration). The diameter of the arteriole at the site of muscle fiber overlap was measured before, during, and after muscle contraction. After a 5 to 10 minutes of recovery, antibodies (1.8 mg/mL; total volume, ∼100 μL) were suffused directly onto the tissue at the observation site via micropipette for 20 minutes. Preparation and use of micropipettes to deliver test substances to the tissue surrounding individual microvessels have been described elsewhere. The muscle fibers were then restimulated and the diameter response of the same arteriole was measured. Earlier studies have confirmed that successive stimulations produce the same local responses.

Protein Application

Applications of fusion proteins, peptides, and antibodies were restricted to a local region of the tissue apposed to the test vessel by using a micropipette attached to a simple manometer system that ejects the pipette contents as the pressure in the pipette is raised. At the conclusion of all protocols in each tissue, sodium nitroprusside (10^-5 mol/L) was added to the superfusion to maximally dilate the vasculature. This was used both to verify that each vessel retained the capacity to dilate and to record the maximal arteriolar diameter for calculation of dilator capacity (below). For additional details, refer to the online data supplement.

Results

The role of fibronectin in microcirculatory responses was investigated using intravitral (in situ) microscopy, in which the microvasculature is visualized with a microscope coupled to a charge-coupled device camera and video images are recorded for offline analysis. As described previously, muscle contraction for 2 minutes at 4 Hz stimulated a significant local dilation of the arteriole (Figure 1). This vasodilation was significantly inhibited by local delivery of the anti–FNIII-1 monoclonal antibody 9D2. Local application of 9D2 to cremaster muscle did not change basal arteriolar tone (diameter before 9D2 application was 20.0±1.6 μm versus 20.9±2.0 μm with 9D2). In contrast to the inhibitory effect of 9D2, application of the anti-fibronectin monoclonal antibody L8 did not alter the vasodilatory response to muscle contraction (Figure 1). Control experiments using the appropriate, nonimmune IgG in place of 9D2 or L8 were without effect on the active dilation (paired difference from controls: 2.9 μm; P=0.37; n=5).

Fibronectin and Incorporation of Alexa Fluor-488–Fibronectin into Tissue

See the online data supplement.

Diameter Measurements and Statistical Analysis

One or 2 arterioles per tissue preparation were used to collect data; at least 3 animals were used in each protocol (n refers to the number of vessels). Arteriolar diameters were measured offline via video calipers generated by a modified video analyzer (no. 321, Colorado Video) using a videotaped stage micrometer for calibration. Vessel diameter (D) measurements are reproducible to 0.3 μm, which is 1% to 2% of the expected diameter. Diameter changes were expressed either as the change in diameter from baseline to peak response or as fractional change in dilator capacity, which was calculated as (Dmax−Dbaseline)/(Dmax−Dbaseline). Data are presented as means±SEM. We performed statistical analysis using ANOVAs or t tests as appropriate. A value of P<0.05 was considered statistically significant.

Figure 1. The anti-FNIII-1 monoclonal antibody 9D2 inhibits vasodilation induced by skeletal muscle contraction. Arteriolar diameter was monitored during and after 2 minutes of 4-Hz muscle contraction, first in the absence (open bars) and then the presence of either 9D2 or L8 monoclonal antibodies (black bars; n=8 [9D2] and n=5 [L8]). Illustrated is mean peak diameter change (μm) following muscle contraction±SEM. Sodium nitroprusside (10^-5 mol/L) was used to verify that the arteriole retained the capacity to dilate. *Significantly different from control; ANOVA, P=0.0034.
site in FNIII-1. Thus, these data support a model in which skeletal muscle contraction opens matricryptic FNIII-1 sites in the surrounding connective tissue matrix that, in turn, interact with cell surface receptors to induce arteriolar dilation.

To visualize the distribution and pattern of fibronectin fibrils in the ECM surrounding arterioles, control and N-ethylmaleimide (NEM)-treated Alexa 488–labeled fibronectins were injected intravenously into mice and allowed to incorporate into tissues for 4 hours. Uptake of 125I-labeled fibronectin from plasma into tissues is near maximal at this time. N-Ethylmaleimide alkylation blocks the assembly of fibronectin into the ECM of cultured cells and tissues, thus allowing us to specifically identify cell-polymerized fibronectin fibrils within cremaster muscle. Alexa 488–fibronectin was visible in the ECM of cremaster muscle in close proximity to arterioles, where it appeared as elongated, branching fibrils (Figure 2B). In contrast, N-ethylmaleimide–alkylated, Alexa 488–fibronectin was not visible in cremaster tissues (Figure 2A), demonstrating the specificity of the Alexa 488–fibronectin fibril staining. Fibronectin fibrils were clearly visible in areas adjacent to arterioles (Figure 2B), as well as in areas remote from the blood vessel along the edges of individual skeletal muscle myocytes (Figure 2C). Together, these data demonstrate that in intact cremaster muscle, fibronectin is actively incorporated into its fibrillar form in the ECM surrounding arterioles.

Fibronectin, like many other ECM molecules, is a mosaic protein composed of tandem, individually folded modules. An extensive number of studies have used proteolytic or recombinant fragments to localize the functional activities of fibronectin to discrete domains. We previously engineered a small, recombinant fibronectin construct that exhibits properties similar to that of larger ECM fibronectin fibrils. To generate this protein, the cryptic, heparin-binding fragment of FNIII-1, comprised of residues 1597-T673, was linked directly to the integrin-binding FNIII8-10 modules (GST/III1H,8-10). Treatment of cultured cells with GST/III1H,8-10 stimulates cell spreading, growth, contractility, and migration to a similar extent as ECM fibronectin. As such, this fibronectin matrix mimetic effectively bypasses the requirement for soluble, protomeric fibronectin to undergo a conformational change to initiate ECM fibronectin-specific signals. Therefore, if matricryptic FNIII-1 sites in the vicinity of the vasculature play a role in contraction-induced vasodilation, then infusing the fibronectin matrix mimetic, GST/III1H,8-10, directly onto tissues would be expected to stimulate vasodilation locally in the absence of skeletal muscle contraction. Indeed, locally applying the fibronectin matrix mimetic to cremaster muscle in situ caused a rapid, specific, and reversible local dilation of arterioles (GST/III1H,8-10; 15 μmol/L; Figure 3) that was blocked by addition of the NO inhibitor N^nitro-l-arginine (L-NNA) (Figure 3). We showed in previous work that in the presence of L-NNA, local vasodilation in response to muscle contraction was significantly decreased versus controls, indicating that NO-dependent mechanisms indeed contribute to this local response. We therefore tested the effect of L-NNA when the response was already attenuated by 9D2; with 9D2+L-NNA

**Figure 2.** Visualization of Alexa-488 fibronectin in the ECM of mouse cremaster muscle. N-Ethylmaleimide (NEM)-alkylated fibronectin (A) and control fibronectin (B and C) were labeled with Alexa fluor 488 and administered intravenously to mice. Three hours later, the cremaster muscle was surgically exposed and confocal images were captured. Fibronectin fibrils (arrows) are visible in areas adjacent to the arteriole (B) as well as in areas remote from the blood vessel, where they are located along the edges of individual skeletal muscle myocytes (C). Scale bar=25 μm.
exposure, the vasodilation was to 63% of the capacity, compared with 55% capacity with 9D2 alone (Figure 1) and 51% capacity with L-NNA alone.3 These data suggest that a large fraction of the NO-dependent component of the response to muscle contraction may be accounted for by fibronectin-dependent mechanisms.

The cryptic, heparin-binding activity of FNIII-1 was essential for triggering vasodilation in response to the matrix mimetic, because a GST/III1H,8-10 construct in which the functional, heparin-binding site of FNIII-1 was mutated to noncharged amino acids17 did not induce vasodilation (GST/III1H,8-10; Figure 3). In contrast, a GST/III1H,8-10 construct in which the integrin-binding RGD and PHSRN sites were mutated to nonbinding sequences,17,25,26 retained the ability to induce vasodilation (GST/III1H,8-10ΔKRWRK; 15 μmol/L; Figure 4). In these experiments, the integrin-binding activity of the fibronectin matrix mimetic was not sufficient to stimulate vasodilation (GST/III12–13; 7.5 μmol/L; Figure 5A). These data also demonstrated that the effect of GST/III1H on vasodilation is specific to this heparin-binding domain of fibronectin, as C-terminal heparin-binding fragments of fibronectin did not induce vasodilation (GST/III1H,8-10ΔSyn/RGE; Figure 4). Vasodilation in response to FNIII-1 was specific to this heparin-binding domain of fibronectin, as C-terminal heparin-binding fragments of fibronectin did not induce vasodilation (GST/III1H,8-10ΔSyn/RGE; Figure 4). Furthermore, the decreases in arteriolar diameter that occurred on administration of FNIII-1 peptides that contain the matricryptic amino acid sequence RWRPK specifically inhibit fibronectin- and GST/III1H,8-10-induced cell growth.17 These peptides do not inhibit fibronectin polymerization (unpublished observations) and, thus, likely inhibit ECM fibronectin-mediated effects by blocking the interaction of the matricryptic site with its cell surface receptor. Local application of blocking peptide 5 (5 mmol/L; Figure 6) and, separately, peptide 6 (5 mmol/L; Figure 6) to cremaster muscle, in the absence of skeletal muscle contraction, rapidly (<1 minute) induced arteriolar vasoconstriction. These decreases in arteriolar diameter were quite large; maximal decreases in arteriolar diameter during peptide 5 and peptide 6 application were −15.0±1.4 μm (or 71.5±3.4% total capacity) and −17.4±2.1 μm (or 73.0±4.2% total capacity), respectively. Moreover, the decreases in arteriolar diameter in response to the FNIII-1 peptides were rapidly (<5 minutes) reversed on removal of the peptides (data not shown). Peptide 7, which does not contain the matricryptic sequence and does not affect ECM fibronectin-mediated growth,17 did not cause a significant change in arteriolar diameter (5 mmol/L; Figure 6), demonstrating the specificity of the FNIII-1 peptide responses. Furthermore, the decreases in arteriolar diameter that occurred on administration of FNIII-1 peptide 5 and peptide 6 were not general responses to basic peptides, because local application of a heparin-binding peptide of vitronectin having

![Figure 3](image3.png)

**Figure 3.** The fibronectin matrix mimetic GST/III1H,8-10 stimulates vasodilation by an NO-dependent mechanism. Time course of arteriolar diameter change after a 10-minute exposure to GST/III1H,8-10 (15 μmol/L) in the absence (solid squares; n=8) or presence (open squares; 10−4 mol/L; n=8) of L-NNA. Resting diameter (20.8±2.5 μm, controls) was not different with L-NNA (18.7±2.1 μm). Maximum vasodilation occurred within 5 minutes of the start of protein application, with rapid (<90 seconds) recovery after its withdrawal. Bar indicates period of protein application. Sodium nitroprusside was added to the superfusate at the end of each experiment to determine maximum protein recovery after its withdrawal. Bar indicates period of protein exposure; ANOVA, P<0.0001.

![Figure 4](image4.png)

**Figure 4.** The matricryptic, heparin-binding activity of FNIII-1 is required for vasodilation. Time course of arteriolar diameter change during a 10-minute exposure to 15 μmol/L of either GST/III1H,8-10ΔSyn/RGE (solid circles; n=6) or GST/III1H,8-10ΔKRWRK (open squares; n=3). Data are presented as fractions of total capacity±SEM. Responses are significantly different during protein exposure; ANOVA, P<0.0001.
Responses are significantly different during protein exposure; ANOVA, *P<0.0001. Data are presented as mean diameter changes during peptide exposure±SEM. *Significantly different from FN peptide 7; ANOVA, *P<0.0001.

Figure 6. Inhibitory FNIII-1 peptides increase basal arteriolar tone. Arteriolar diameter was monitored before, during, and after a 10-minute exposure to 1 of the FNIII-1 peptides, peptide 5 (FN5; n=6), peptide 6 (FN6; n=5), peptide 7 (FN7; n=6), at a concentration of 5 mmol/L or exposure to vitronectin peptide VN-R11G (VN-R11G; 5 mmol/L; n=8). Peptide sequences were as follows: peptide 5, KYLRLWRPKNS; peptide 6, RWPKNS-VGRWK; peptide 7, KNSVGWRKKEAT; VN-R11G, RNPKGYRQ. Amino acids that form the matricryptic site in FNIII-1<sup>17</sup> are shown in bold. A consensus, heparin-binding sequence in the vitronectin peptide is underlined.<sup>40</sup> Data are presented as mean diameter changes during peptide exposure±SEM. *Significantly different from FN peptide 7; ANOVA, *P<0.0001.

Discussion

This study shows that the matricryptic, heparin-binding site in the III-1 module of ECM fibronectin stimulates an NO-dependent increase in arteriolar diameter, providing the first evidence that ECM fibronectin fibrils play a dynamic role in regulating arteriolar responses in vivo. It is well established that vasodilation can be produced by a direct effect on smooth muscle cells and/or by a direct effect on endothelial cells, which in turn communicate vasodilatory signals to the adjacent smooth muscle. Although, in general, direct effects of metabolites and other products of skeletal muscle contraction on smooth muscle cells are implicated in local dilatory responses,<sup>27</sup> recent work has also indicated an essential role for endothelial cells in metabolic responses.<sup>1,10</sup> Our data suggest that skeletal muscle contraction alters the conformation of ECM fibronectin surrounding skeletal and/or smooth muscle cells. This would lead to a transient exposure of the matricryptic FNIII-1 site and the subsequent interaction of this site with receptors, most likely heparan sulfate proteoglycans<sup>15</sup> on smooth muscle and/or skeletal muscle cells. In turn, this binding event initiates an NO-mediated signaling pathway that results in smooth muscle relaxation and, hence, vasodilation. These ideas are illustrated in Figure 7.

Our observations indicate that, indeed, the NO-dependent component of the active dilation to muscle contraction is likely attributable to fibronectin-dependent signaling. The dependence on NO could indicate that an endothelial cell-dependent process is part of the response pathway. However, previous studies<sup>6-7</sup> have indicated that neuronal NO synthase–dependent pathways in skeletal muscle could equally be involved in this dilation, and because neuronal NO synthase has also been identified in vascular smooth muscle,<sup>28,29</sup> we postulate that the as yet unidentified receptor to which the matricryptic FNIII-1 binds could be located on either skeletal or smooth muscle myocytes. NO released from either skeletal or smooth muscle would thus be available locally to induce vasodilation. An alternative scenario that has not been excluded would be that the receptor is located on smooth muscle, but instead of leading to release of NO to feed back in an autocrine fashion, it initiates signals that are transmitted to endothelium via myoendothelial coupling<sup>30</sup> to stimulate NO release and subsequent vasodilation.

We previously localized the functional, heparin-binding site in FNIII-1 to a sequence of basic amino acids, R613, W614, R615, and K617, and, in addition, showed that the 9D2 monoclonal antibody recognizes the R613, W614, R615 sequence within this site.<sup>17</sup> In vitro, 9D2 inhibits fibronectin-stimulated cell growth,<sup>31</sup> spreading,<sup>17</sup> contractility,<sup>14</sup> and migration.<sup>16</sup> 9D2 monoclonal antibodies does not block cell adhesion to fibronectin,<sup>16</sup> indicating that this antibody does
not interfere with the binding of fibronectin to integrin receptors. In the present study, we used a well-characterized in vivo model of exercise-induced vasodilation\(^2\)\(^-\)\(^4\) and showed that the 9D2 antibody specifically inhibits a portion of the vasodilatory response to skeletal muscle contraction. Furthermore, application of the fibronectin matrix mimetic GST/III1H,8-10 to cremaster muscle, in the absence of skeletal muscle contraction, triggered local arteriolar vasodilation by a mechanism that required the RWRPK sequence. Vasodilation in response to GST/III1H was specific to this heparin-binding region of fibronectin, because the carboxy-terminal heparin-binding fragment of fibronectin, GST/III12–13, did not alter arteriolar diameter. The specificity of the vasodilatory response to the heparin-binding FNIII-1 fragment is in agreement with our previous studies showing that these two heparin-binding domains of fibronectin trigger unique responses and do not have overlapping functions.\(^{14},\)\(^{16},\)\(^{17}\) Taken together, these data identify a novel role for fibronectin fibrils and specifically, the matricryptic site in FNIII-1, in mediating an essential physiological response to exercise in adult animals.

In contrast to results obtained with the heparin-binding fragments of FNIII-1 (Figure 5A), local application of α5β1 integrin-binding fibronectin fragments did not trigger vasodilation (Figure 5B). These results support and extend previous studies indicating that α5β1 integrin ligation by itself is not sufficient to induce cellular responses that are mediated by ECM fibronectin.\(^{15},\)\(^{17},\)\(^{31}\) Activated β1 integrins have been implicated in regulatory pathways in vascular smooth muscle,\(^{32}\) and we have shown that the matricryptic, heparin-binding site in FNIII-1 enhances β1 integrin-mediated cellular responses.\(^{17}\) Other studies have demonstrated that small, integrin-binding peptides of fibronectin can stimulate either vasodilation or vasoconstriction of isolated blood vessels.\(^{33}\) We can resolve this apparent discrepancy by speculating that, in vivo, constitutive ligation of integrin receptors by endogenous ECM proteins may be sufficient to prime skeletal and/or smooth muscle cells for subsequent signals initiated by the matricryptic site in fibronectin. In this manner, the FNIII1-mediated vasodilatory signal that we describe is directly coupled to skeletal muscle contraction and is rapidly extinguished when muscle contraction ceases (Figure 7, schematic).

Local application of FNIII-1 peptides, shown previously to block the cellular response to ECM fibronectin,\(^{17}\) caused a rapid and specific decrease in arteriolar diameter, indicating that the matricryptic site of fibronectin also contributes to resting vascular tone. Furthermore, using intravenous administration of Alexa 488–labeled fibronectin, we demonstrated that fibronectin is actively removed from the plasma and polymerized by intact tissues in areas adjacent to arterioles. From these findings, we hypothesize that continuous polymerization of fibronectin in connective tissue matrices surrounding arterioles may result in a constitutive level of expression of matricryptic FNIII-1 sites that provide signals controlling basal blood vessel tone. Additional matricryptic FNIII-1 sites may then be transiently exposed as skeletal muscle contraction places a tensile strain on ECM fibronectin fibrils, as shown in the schematic (Figure 7). Consistent with our proposed mechanism, we note in Figure 5A that GST/III-1H acts to dilate the vessel, but that this action is relatively short-lived, compared with the full-length matricryptic mimetic (Figure 5B). We conclude that because of its relatively short length, this peptide quite closely resembles the inhibitory peptides 5 and 6 (Figure 6). Hence, the initial stimulatory effect is replaced by a vasoconstrictor action as the short peptide fragment remains bound to the receptor. Unlike the results obtained with FNIII-1 peptides, we report that application of 9D2 to cremaster muscle did not decrease basal blood vessel tone. This results implies, not unexpectedly, that the relatively large 9D2 molecules cannot permeate the tissue to access matricryptic FNIII-1 sites that are constitutively expressed yet can interact with FNIII-1 sites that are transiently expressed. As such, FNIII-1 sites that contribute to maintenance of basal tone, and FNIII-1 sites that mediate the dilatory response initiated by skeletal muscle contraction may be compartmentalized within tissues. This concept is supported by recent studies in isolated arterioles\(^{34}\) that indicate that different integrins may be differentially involved in regulation of myogenic constriction versus regulation of basal tone, paralleling our current data interpretation that separate pools of matricryptic FNIII-1 sites regulate distinct vasodilatory responses. Developing methods to visualize and quantify FNIII-1 matricryptic site expression in vivo is currently an active area of investigation.
In the body, cells continuously sense and respond to a variety of mechanical forces, including isotonic muscle contraction, gravity, blood pressure and shear stress, and cell-generated contractile forces. The cellular responses to these stimuli govern normal physiological development and function. A great deal is known about aspects of the coupling between mechanical forces and cellular responses. Adhesion of cells to the ECM via integrin receptors serves to physically link the extracellular environment with the internal actin cytoskeleton. As such, mechanical forces initiated either internally or externally can be transmitted bidirectionally through integrins to regulate such processes as cell growth, cell migration, protein expression, and ECM deposition. Emerging evidence indicates that the translation of mechanical signals into biochemical signals, or “mechanotransduction,” involves integrin receptors and occurs at cell–matrix adhesion sites. Accumulating evidence also points to key roles for the tyrosine kinases, focal adhesion kinase and Src, in mechanotransduction. However, in spite of what is known, the actual physical mechanism by which extracellular mechanical forces result in activation of intracellular kinases is not known. Our current study suggests an important new paradigm wherein tensile forces from actively contracting skeletal muscle alter the conformation of fibronectin fibrils surrounding the vascular wall and transiently expose matricryptic FNIII-1 sites that, in turn, initiate a biochemical signal and thus, signal a change in arteriolar diameter. The ability of tissue strain to alter the conformation of fibronectin and expose a cryptic cell-binding domain represents a simple, yet elegant means of converting a mechanical signal into a biochemical response. As such, this novel mechanotransduction pathway may be just 1 example of a common approach to transmitting mechanical forces, including pulsatile flow and shear stress, from the ECM to cells.

In addition to its fundamental contribution to understanding the blood flow response to exercise, our study points to an important aspect of the biology of human health in aging. Aging and age-related diseases are often accompanied by changes in the content and structure of connective tissue. These changes may decrease the local expression of, or limit access to, matricryptic sites in ECM fibronectin, raising the possibility that some of the impaired peripheral vascular responses that accompany aging may originate from changes in fibronectin matrix deposition or structure. Similarly, altered fibronectin matrix deposition also occurs in atherosclerosis, restenosis, and hypertension. We speculate that engineering fibronectin expression in such populations might help to maintain normal vascular function as well as preserve the ability of the vasculature to response to exercise.

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Disclosures

None.

References

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Extracellular Matrix Fibronectin Mechanically Couples Skeletal Muscle Contraction with Local Vasodilation

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Materials. Nω-nitro-L-arginine (L-NNA) and sodium nitroprusside were purchased from Sigma Chemical Co (Saint Louis, MO). Recombinant fibronectin fusion proteins were produced in bacteria, as described (1, 2). Fusion proteins were dialysed extensively against PBS and sterilized by filtration (Pall Corp. Ann Arbor, MI). Fibronectin and vitronectin peptides were from Sigma. Anti-FNIII-1 IgG(9D2; (2, 3)) was a gift from Dr. Deane Mosher (University of Wisconsin, Madison, WI). Anti-fibronectin IgG, L8 (3), was a gift from Dr. Michael Chernousov (Weis Center for Research, Geisinger Clinic, PA). Non-immune mouse IgG (Sigma) and 9D2 ascites were purified over Protein A-Sepharose (Pharmacia), as described previously (4). IgGs were dialyzed extensively against PBS and sterilized by filtration. Purity was assessed by PAGE.

Animals. To prepare the cremaster muscle for microcirculatory observation, animals were anesthetised with sodium pentobarbital (65-75 mg/kg, i.p.). Catheters were inserted in either the femoral or jugular vein (for supplemental anaesthetic) and femoral artery (to monitor blood pressure). As described elsewhere (5-7), the muscle was surgically exposed, slit longitudinally, and laid out on an optical port. The anesthetized animal with prepared tissue was then moved to a
microscope (Olympus BX51WI) with a modified stage for intravital microscopy. The tissue was maintained by superfusion with warmed physiological saline solution (in mMol/L: 131.9 NaCl, 4.7 KCl, 2.0 CaCl₂, 1.2 MgSO₄, and 22 NaHCO₃, equilibrated with gas containing 5% CO₂ and 95% N₂ to maintain normal tissue PO₂; pH 7.4±0.05). When called for by the protocol, L-NNA or sodium nitroprusside were added to the superfusion solution. Observation sites were selected in the central region of the tissue. The presence of vasoactive tone (a brisk dilation following local application of 10⁻⁴ M adenosine) was confirmed in 3 randomly selected arterioles in each preparation. At the end of the protocol, the animal was euthanized by intravenous anesthetic overdose.

**Protein Application.** Test protein was diluted with superfusate solution. Vascular responses were first recorded for 5 min after pipette placement to verify a stable baseline and record baseline diameter, and then continuously recorded during exposure to the test protein. We routinely recorded up to 20 min recovery data from the vessel following test interventions. These data were used to verify the continuing viability of the vessel but were not included in the analyses. The pipette solutions contained a tracer of 100 µM FITC-dextran (4000 M.W.) and brief epifluorescence was used to verify that flow from the micropipette was exposing the test arteriole, and to verify the flow path of the pipette contents in relation to the superfusate flow. By placing the pipette with regard to both vessel geometry and the direction of superfusate flow, this system allows us to deliver a steady state drug concentration to specified locations (≈ 200µm in length) on the test arteriole (8).

**Fibronectin.** Human plasma fibronectin was isolated from Cohn’s fraction I and II (9). N-ethylmaleimide (NEM)-alkylated fibronectin was prepared by incubating fibronectin (3.5 mg/ml) with 10 mMol/L NEM (Pierce Chemical, Rockford, IL), as described (10). Fibronectin and
NEM-alkylated fibronectin were conjugated directly to Alexa Fluor 488 (Invitrogen) using N, N-dimethyl formamide, according to the manufacturer's instructions. The protein conjugates were separated from non-reacted Alexa Fluor 488 by gel filtration chromatography on G-25M Sephadex (Pharmacia). Proteins were dialyzed against PBS and protein concentrations were determined from absorbances taken at 280 and 495 nm. The degrees of labelling of fibronectin and NEM-fibronectin were 7.0 and 11.7 dye molecules per fibronectin molecule, respectively.

**Incorporation of Alexa Fluor-488-Fibronectin into Tissue.** Either control or NEM-alkylated, Alexa 488-labeled fibronectins were injected into mice via a jugular venous catheter at a dose of 500 µg fibronectin per 100 g body weight (11). After 4 h, the cremaster muscle was observed using confocal intravital microscopy (12, 13). For this, an Olympus BX61WI microscope was equipped with a Nipkow disk confocal head (CSU10, Yokogawa) and intensified CCD camera (XR Mega 10, Stanford Photonics). Fluorescence was excited at 488 nm using a 50 mW argon laser and images were recorded onto a DVD recorder (SONY DVO100MD).

**References**


