Online Supplement

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**


