Pericyte NF-κB Activation Enhances Endothelial Cell Proliferation and Proangiogenic Cytokine Secretion in Vitro

Katherine E. LaBarbera  
*University of Massachusetts Amherst*

Robert D. Hyldahl  
*Brigham Young University*

Kevin S. O'Fallon  
*University of Massachusetts Amherst*

Priscilla M. Clarkson  
*University of Massachusetts Amherst*

Sarah Witkowski  
*University of Massachusetts Amherst*

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Pericyte NF-κB activation enhances endothelial cell proliferation and proangiogenic cytokine secretion in vitro

Katherine E. LaBarbera1, Robert D. Hyldahl2, Kevin S. O’Fallon1, Priscilla M. Clarkson1 & Sarah Witkowski1

1 Department of Kinesiology, University of Massachusetts Amherst, Amherst, Massachusetts
2 Department of Exercise Sciences, Brigham Young University, Provo, Utah

Abstract

Pericytes are skeletal muscle resident, multipotent stem cells that are localized to the microvasculature. In vivo, studies have shown that they respond to damage through activation of nuclear-factor kappa-B (NF-κB), but the downstream effects of NF-κB activation on endothelial cell proliferation and cell–cell signaling during repair remain unknown. The purpose of this study was to examine pericyte NF-κB activation in a model of skeletal muscle damage; and use genetic manipulation to study the effects of changes in pericyte NF-κB activation on endothelial cell proliferation and cytokine secretion. We utilized scratch injury to C2C12 cells in coculture with human primary pericytes to assess NF-κB activation and monocyte chemoattractant protein-1 (MCP-1) secretion from pericytes and C2C12 cells. We also cocultured endothelial cells with pericytes that expressed genetically altered NF-κB activation levels, and then quantified endothelial cell proliferation and screened the conditioned media for secreted cytokines. Pericytes trended toward greater NF-κB activation in injured compared to control cocultures \( (P = 0.085) \) and in comparison to C2C12 cells \( (P = 0.079) \). Second, increased NF-κB activation in pericytes enhanced the proliferation of cocultured endothelial cells \( (1.3\text{-fold}, \ P = 0.002) \). Finally, we identified inflammatory signaling molecules, including MCP-1 and interleukin 8 (IL-8) that may mediate the crosstalk between pericytes and endothelial cells. The results of this study show that pericyte NF-κB activation may be an important mechanism in skeletal muscle repair with implications for the development of therapies for musculoskeletal and vascular diseases, including peripheral artery disease.

Introduction

Skeletal muscle damage following injury, disease, or uncustomed exercise stimulates coordinated repair processes from muscle and nonmuscle cell types, including cells associated with the vasculature (Shi and Garry 2006; Hyldahl et al. 2011; Boppart et al. 2013). Skeletal muscle is a highly vascularized tissue with reports that there are as many as 4–5 capillaries in contact with each muscle fiber in the vastus lateralis of young healthy males (Groen et al. 2014). Capillaries and other microvascular vessels, including precapillary arterioles and postcapillary venules, have a single layer of endothelial cells on the luminal surface. There is evidence that skeletal muscle-damaging exercise affects these endothelial cells and causes microvascular dysfunction (Kano et al. 2004, 2005). Additionally, pericytes, which are cells found on the abluminal surface of endothelial cells throughout the microvasculature, may contribute to skeletal muscle repair (Dellavalle et al. 2011; Hyldahl et al. 2011). The consequences of skeletal muscle damage on the microvasculature, including angiogenic processes or endothelial cell crosstalk with pericytes remain unknown.

Pericytes are tissue-resident multipotent stem cells that are ubiquitous throughout the microvasculature where they reside under the basement membrane at a density...
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ranging between 1:10 and 1:100 pericytes to endothelial cells within skeletal muscle tissue (Tilton et al. 1979; Shepro and Morel 1993; Hirsch and D’Amore 1996). They are mesenchymal-like stem cells (Crisan et al. 2008) that have the potential to differentiate down mesodermal lineages toward adipogenic (Richardson et al. 1982), osteogenic (Diaz-Flores et al. 1992), and myogenic fates (Dellavalle et al. 2011). Reports indicate that pericytes can fuse with muscle fibers and contribute to the satellite cell pool in vivo (Dellavalle et al. 2011). Additionally, pericytes may contribute to skeletal muscle repair (Sassoli et al. 2012) and cardiac muscle repair (Chen et al. 2013) through paracrine effects, although the signaling molecules remain largely unidentified. Previous work by Hyldahl et al. (2011) has highlighted pericyte NF-κB activity as a potential mechanism for pericyte involvement in muscle repair. They showed that a muscle-damaging eccentric exercise in humans caused NF-κB activation in muscle-resident pericytes 3 h after exercise of the quadriceps (Hyldahl et al. 2011). In a follow up in vitro study, it was shown that pericyte NF-κB activation enhanced skeletal myoblast proliferation and inhibited myoblast differentiation (Hyldahl et al. 2013). The signals downstream of NF-κB that mediate the cellular crosstalk between pericytes and muscle cells have not been investigated.

Only a few studies have investigated the complex relationship between endothelial cells, pericytes, and skeletal muscle following tissue damage. In a severe model of muscle injury, Tamaki et al. (2005) showed that muscle-derived mesenchymal stem cells (CD34+/CD45−), which are similar to pericytes in anatomical location, cell surface marker expression, and function (Covas et al. 2008), contributed to both vasculogenesis and myogenesis during regeneration. Huntsman et al. (2013) reported that pericytes contributed to arteriogenesis following 4 weeks of eccentric exercise training in a transgenic animal model. This same group investigated the potential paracrine signaling factors secreted by pericytes in response to in vitro mechanical strain. They identified epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and interferon gamma-induced protein 10 (IP-10) as potential mediators of the arteriogenic response (Huntsman et al. 2013). IP-10 is a target of NF-κB (Hyldahl et al. 2013). IP-10 is a target of NF-κB activation enhanced skeletal myoblast proliferation and inhibited myoblast differentiation. The signals downstream of NF-κB that mediate the cellular crosstalk between pericytes and muscle cells have not been investigated.

Materials and Methods

Cell culture

Human primary pericytes isolated from placental tissue were purchased from PromoCell (Heidelberg, Germany). As reported by the supplier, the pericytes were positive for the mesenchymal stem cell markers CD146 and CD105 and negative for the endothelial cell markers CD31 and CD34. Immunofluorescence experiments confirmed that the cells also expressed the pericyte marker NG2 (data not shown). The cells displayed a stellate morphology characteristic of pericytes (Bryan and D’Amore 2008). Pericytes between passages 6 and 9 were used for experiments. Cultures were maintained at 37°C in a 5% CO2 incubator in growth medium consisting of DMEM supplemented with 2% horse serum and 1% penicillin and streptomycin. Cultures were passaged using 0.25% trypsin, 0.1% EDTA before reaching 80% confluence. C6C12 myoblasts were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured under the same conditions as the pericytes. To obtain myotube cultures, C6C12 myoblasts were cultured in reduced growth factor medium containing DMEM supplemented with 2% horse serum and 1% penicillin and streptomycin for 4 days to induce differentiation into mature myotube cultures. Human microvascular endothelial cells (HMVECs) are an hTERT immortalized cell line (Shao and Guo 2004) that was cultured in endothelial basal medium-2 (EBM-2; Lonza, Walkersville, MD) supplemented with 10% FBS, 1% penicillin and streptomycin, 1 μg/mL EGF, and 50 μg/mL hydrocortisone.
Pericyte/C2C12 coculture

A transwell coculture system was used to facilitate exchange of soluble factors while preventing the migration of cells between wells (0.4 μm pore size, Corning Life Sciences, Tewksbury, MA). C2C12 myotube cultures were grown in a 6-well plate format with pericytes seeded in the transwell insert. Cocultures were maintained in a 5% CO2 incubator and culture medium was replaced every 24 h at 4 days post myotube differentiation, which coincided with ~80% pericyte confluency, culture medium was replaced 3 h prior to the start of the experiment to allow cells to equilibrate to fresh medium. Next, myotube cultures were scratch-injured, which created an open “wound” on the culture plate. To impart the scratch-injury, a sterile gel-loading pipette tip was used to generate a continuous and well-delineated wound region along the surface of each plate producing a total wound area equivalent to ~10% of the total surface area of the C2C12 cell monolayer, similarly to the methods previously described where scrape injury was shown to cause myotube damage through lactate dehydrogenase release (Tsivitse et al. 2005). In parallel, control cocultures were established where no injury was imparted to C2C12 cells. Three replicates were performed for a baseline (BSLN), 3, 12, and 24 h time point for uninjured control (CON) and injured (INJ) cultures. At each time point, cell culture supernatant was collected and immediately frozen at ~80°C for subsequent analysis of secreted MCP-1. Also at each time point, nuclear extracts from pericytes and C2C12 cells, respectively, were isolated via differential centrifugation using a cellular extraction kit according to manufacturer’s instructions (Nuclear Extract Kit; Active Motif, Carlsbad, CA) and immediately frozen at ~80°C.

ELISA-based NF-κB activation

Total nuclear protein content was determined using a BSA-based protein quantification assay (ProStain; Active Motif). An ELISA-based transcription factor assay kit (TransAM NF-κB p65 Assay Kit; Active Motif) was used to quantify NF-κB p65 subunit nuclear DNA binding activity in pericytes and C2C12 cells from cell-delineated wound regions according to manufacturer’s instructions. Briefly, 2 μg of nuclear protein was added to wells coated with a consensus binding sequence for NF-κB (5’-GGGACTTTCC-3’) and incubated for 1 h at room temperature. Wild type and mutated consensus oligonucleotides were used as competitors for NF-κB binding to ensure specificity of the reaction as per manufacturer’s instructions. Wells were then washed, and a primary antibody directed at the p65 subunit was added and left to incubate for 1 h. This was followed by treatment of all wells with a secondary horseradish peroxidase-conjugated antibody. Then, developing solution was added to initiate a colorimetric reaction. After 5 min, a stop solution was added and absorbance was measured at 450 nm on a multiwell microplate reader (FLUOstar Optima; BMG Labtech, Offenburg, Germany). All samples were assayed in duplicate, and averages were used for data analysis.

MCP-1 secretion

To determine MCP-1 secretion from human pericytes and murine C2C12 cells, respective ELISAs for the detection of human (Human CCL2/MCP-1 Quantikine ELISA kit; R&D Systems, Minneapolis, MN) and murine MCP-1 (Mouse /Rat CCL2/JE/MCP-1 Quantikine ELISA kit; R&D Systems) were performed on cell culture supernatants. The kits were tested for species cross reactivity by the manufacturer, and no cross reactivity was detected. Assay kits employed the quantitative sandwich ELISA technique, and were performed on undiluted samples according to manufacturer’s instructions. A multiwell microplate reader was used (FLUOstar Optima; BMG Labtech) to measure absorbance at 450 nm. Assays were performed in duplicate and average values were used for analysis.

Pericyte/HMVEC coculture

Pericytes were seeded in triplicate in a 6-well plate format following transient transfection (detailed below) with vectors designed to alter NF-κB activity. At 24 h after transfection, medium was replaced with fresh medium and coculture was initiated by seeding HMVECs onto transwell inserts (0.4 μm pore size, Corning Life Sciences, Tewksbury, MA). At 24 and 48 h after the initiation of coculture, cell culture supernatants were collected and immediately frozen at ~80°C for analysis of cytokine secretion. Endothelial cell number was quantified at 24 and 48 h (detailed below).

Transient transfections

Expression plasmids were used to alter NF-κB activation in pericytes. A dominant negative (d.n.) IKKβ (K44M) was used to decrease NF-κB activity. It encodes a kinase dead form of IKKβ, and was developed in the laboratory of Michael Karin, PhD (University of California, San Diego, CA). A constitutively active (c.a.) IKKβ (S177/ S188→EE) was used to increase NF-κB activation by encoding for a constitutively active form of IKKβ, and was developed in the lab of Steven Shoelson, MD PhD...
Kine concentrations in the culture supernatants was assayed for cytokine and chemokine concentration using a Luminex Magpix multiplexing platform (Luminex Corporation). Sensitivity of standards ranged between 3.2 and 10,000 pg/mL, giving a broad range of sensitivity. Standard curves and data analysis was performed using Milliplex Analyst 5.1 software (Millipore Corporation).

Statistical analysis

A 3-way ANOVA was used to determine differences in NF-κB activation for the main effects of cell type (pericytes versus C2C12), time (BSLN, 3, 6, 24 h), and condition (control versus injured). Two-way ANOVA was used to investigate differences in NF-κB activation of respective cell types over time (BSLN, 3, 6, 24 h) and between conditions (control versus injured). A 2-way ANOVA was used to determine differences in endothelial cell proliferation between conditions (c.a. IKKβ, d.n. IKKβ, and e.v) and over time (24 and 48 h). Multiplex cytokine data for each individual cytokine of interest were tested for differences among conditions using a 1-way ANOVA. Significant main effects and interactions were investigated using t-tests or Tukey's honest significant difference post hoc test where appropriate. Significance was set a priori at P < 0.05.

Results

NF-κB activation in an in vitro pericyte/C2C12 coculture model of muscle injury

Pericytes were cocultured with C2C12 myotubes using transwell inserts to examine the time course of pericyte and muscle cell NF-κB activation. In this coculture model, the C2C12 p65 DNA binding activity was significantly elevated at 3 h (2.5-fold, P = 0.027) and 24 h (3.57-fold, P = 0.001) relative to BSLN. There was no difference in C2C12 p65 DNA binding activity in INJ compared to CON (P = 0.698). In the same cocultures, pericyte p65 DNA binding activity was increased relative to BSLN at 6 h (2.0-fold, P = 0.007), and 24 h (2.33-fold, P = 0.001). Pericytes trended toward greater p65 DNA binding activity in INJ compared to CON (P = 0.085). Further, pericytes trended toward greater overall p65 DNA binding activity compared to C2C12 cells (P = 0.079) (Fig. 1).
MCP-1 is secreted by pericytes in pericyte/C2C12 coculture model of muscle injury

The pericyte and muscle cell coculture model was utilized to investigate secreted signaling molecules involved in pericyte-muscle cell crosstalk. In the coculture model, pericyte MCP-1 secretion was first detected 24 h post injury, and it exceeded C2C12 MCP-1 secretion (2.1-fold, \( P < 0.001 \)) at this time point in INJ and CON conditions. C2C12 MCP-1 secretion was increased compared to BSLN at 6 h (1.6-fold, \( P = 0.032 \)) and 24 h post injury (3.0-fold, \( P < 0.001 \)). There were no differences in MCP-1 secretion between INJ and CON for pericytes or C2C12 cells (Fig. 2).

Pericyte NF-\( \kappa \)B genetic manipulation

To investigate the downstream effects of pericyte NF-\( \kappa \)B activation, an in vitro coculture model was developed that utilized pericytes with genetically altered NF-\( \kappa \)B activation. Pericytes were genetically altered via transfection with expression plasmids designed to enhance (c.a. IKK\( \beta \)-EGFP), reduce (d.n. IKK\( \beta \)-EGFP), or have no impact (e.v. EGFP) on NF-\( \kappa \)B activity. The transfection efficiency was approximately 65–75% based on the percentage of EGFP fluorescent pericytes at 24 h after transfection (Fig. 3A). Transfection efficiency was not different between conditions. The transfection efficacy was assessed using a luciferase assay (Fig. 3B). At 24 h post transfection, luciferase activity was increased 6.1-fold in the c.a. IKK\( \beta \) condition compared to the e.v. control condition. The luciferase activity in the d.n. IKK\( \beta \) was decreased 10% compared to e.v. control condition.

Pericyte NF-\( \kappa \)B activation enhances HMVEC proliferation in coculture

Genetically altered pericytes were cocultured with HMVECs using transwell inserts to investigate the effects of altered pericyte NF-\( \kappa \)B activation on endothelial cell proliferation. In pericyte/HMVEC cocultures, there was an increase in HMVEC cell number, a measure of proliferation, from 24 to 48 h for all conditions (\( P = 0.002 \), 1.3-fold). HMVEC proliferation was significantly greater in the c.a. IKK\( \beta \) pericyte coculture condition compared to the d.n. IKK\( \beta \) pericyte coculture condition (\( P = 0.002 \), 1.3-fold; Fig. 4).

Cytokine secretion from pericyte monocultures and pericyte/HMVEC cocultures

To assess signaling molecules that may mediate the proliferative response in endothelial cells, cell culture supernatant was assayed for cytokine concentration. Cell culture supernatants were collected in pericyte/HMVEC cocultures at 24 h after the initiation of coculture and at the corresponding time point in pericyte monocultures.
The following cytokines were secreted from pericytes in monoculture in the c.a. IKKβ condition, but were not detected in the d.n. IKKβ condition or e.v. control condition: eotaxin (15.61 ± 2.7 pg/mL), granulocyte colony-stimulating factor (G-CSF, 17.21 ± 4.6 pg/mL), GM-CSF (15.16 ± 4.6 pg/mL), fractalkine (CX3CL1, 30.87 ± 5.3 pg/mL), interferon alpha-2 (IFNA2, 8.62 ± 1.6 pg/mL), growth-regulated oncogene (GRO, 8.41 ± 6.0 pg/mL), interleukin 6 (IL-6, 45.52 ± 9.7 pg/mL), interleukin 7 (IL-7, 11.27 ± 2.0 pg/mL), IP-10 (196.56 ± 91.3 pg/mL), and macrophage inflammatory protein-1 alpha (MIP-1α, 38.57 ± 10.9 pg/mL). There was greater cytokine secretion in the c.a. IKKβ condition compared to both d.n. IKKβ (P < 0.01) and e.v. conditions (P < 0.01), respectively, for the following cytokines: interleukin 8 (IL-8, 599.16 ± 192.5 vs. 1.70 ± 0.9 and 3.54 ± 1.1 pg/mL), MCP-1 (32.56 ± 9.3 vs. 4.14 ± 1.7 and 5.00 ± 2.6 pg/mL), and regulated on activation, normal T-cell expressed and secreted (RANTES, 209.18 ± 73.3 vs. 4.24 ± 2.0 and 5.96 ± 3.1 pg/mL).

In cocultures, there was greater cytokine secretion in the c.a. IKKβ condition compared to both d.n. IKKβ (P < 0.05) and e.v. conditions (P < 0.05), respectively, for the following cytokines: eotaxin (16.16 ± 6 vs. 3.32 ± 4.7 and
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Discussion

Skeletal muscle resident pericytes have known roles in muscle repair and regeneration. Previous work in our laboratory demonstrated that pericytes activate NF-κB in response to muscle damage in humans. In support of previous work, our first main finding provides further in vitro evidence for pericytes as a source of NF-κB activation during skeletal muscle regeneration. This model also allows us to examine the crosstalk mechanisms that promote NF-κB activation in pericytes.

Pericytes activate NF-κB in response to muscle damage

Previous studies have documented the importance of NF-κB activation in skeletal muscle tissue for the regulation of myogenesis (Guttridge et al. 1999, 2000; Peterson et al. 2011) as well as in muscle injury (Mourkioti et al. 2006). Hyldahl et al. (2011) demonstrated pericyte NF-κB activation in muscle injury and regeneration in humans. In this study, we provide further evidence for pericytes as a source of NF-κB activation. Our in vitro model of acute muscle injury allowed us to quantify nuclear NF-κB binding activity and suggests that pericytes may potentially activate NF-κB to a greater extent than muscle cells, although only a trend for increased pericyte NF-κB activation was achieved in this study. Nevertheless, the data support our hypothesis that pericytes are key mediators of the inflammatory response during skeletal muscle regeneration. This model also allowed us to examine the crosstalk mechanisms that promote NF-κB activation in pericytes.

At early time points following scratch-injury to muscle cells, MCP-1 was secreted by muscle cells, although no statistical difference between scratch-injured and control cultures was detected. Other studies have observed increased cytokine secretion from C2C12 cells using various models of muscle stress. Peterson and Pizza (2009) showed that C2C12 cells secreted MCP-1 in response to in vitro mechanical strain. Using an in vitro exercise model, Scheler et al. (2013) observed gene enrichment of NF-κB related genes, including the CCL2 gene that encodes the MCP-1 protein, as well as CCL5 and CXCL1 genes, which...
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The downstream effects of NF-κB activation on cells in the muscle tissue environment are mostly unknown. Due to the close physical proximity between pericytes and endothelial cells, as well as the importance of a healthy microvasculature to promote muscle repair, we investigated the effects of pericyte NF-κB activation on endothelial cells. We showed that NF-κB activation in pericytes enhanced endothelial cell proliferation. Endothelial cell proliferation is one component of angiogenesis, indicating that pericyte NF-κB activation may be important for the promotion of angiogenesis. Hyldahl et al. (2013) demonstrated that increased pericyte NF-κB activation enhanced myoblast proliferation in coculture while also inhibiting pericyte differentiation into myotubes. These two studies show that acute NF-κB activation can enhance cellular proliferation in skeletal muscle tissue. This is in contrast to studies showing that chronic NF-κB activation in disease can lead to cachexia or muscle wasting (Cai et al. 2004), and thus demonstrates the paradoxical effects of inflammation. While acute inflammation may be necessary for tissue repair and vascular remodeling (Silvestre et al. 2008), chronic inflammation has negative effects (Khatami 2011). Importantly, if pericytes are mediators of the inflammatory response, this highlights the importance of a healthy pericyte population that can respond to damage and execute appropriate inflammatory signaling.

Pericyte NF-κB activation enhances inflammatory and angiogenic crosstalk

In order to elucidate cytokines that may be involved in the paracrine signaling that mediates the proliferative response in endothelial cells, we used an unbiased technique to screen the conditioned media of endothelial cell/pericyte cocultures and pericyte monocultures. Using this approach, we saw differential concentrations of several cytokines in the various NF-κB activation conditions. All these cytokines are known gene targets of NF-κB; and therefore, further supports the efficacy of our transfection. The cytokines that were present in the highest concentration in our cultures were IL-8, IP-10, and RANTES, and with the addition of IL-7, this group of cytokines also had the greatest fold change in the enhanced NF-κB activation condition compared to the control condition. IL-8 is a proangiogenic cytokine in the CXC family, and it signals through the CXCR2 receptor to directly affect angiogenesis (Koch et al. 1992; Strieter et al. 1995). In a model of thyroid cancer, Bauerle and colleagues (Bauerle et al. 2014) showed that NF-κB regulated the expression of IL-8, which enhanced tumor cell growth in vivo and endothelial cell tube formation in vitro. IP-10 is another member of the CXC family of cytokines, but contrary to IL-8, it is angiostatic and signals through the CXCR3 receptor (Romagnani et al. 2001; Mehrad et al. 2007). In addition to the concentration of signaling molecules, receptor concentration on endothelial cells are also important in the balance between angiogenesis and angiostasis. Although our study was not designed to investigate receptor density, it is possible that there are more CXCR2 receptors than CXCR3 receptors on our HMVECs. Furthermore, some levels of angiostatic cytokines are likely necessary to balance angiogenic signals and prevent uncontrolled cell growth.

MCP-1 and RANTES, members of the CC cytokine family, were also increased in the coculture condition of HMVECs and pericytes with enhanced NF-κB activation. Many cell types may secrete MCP-1 following damage, and this study shows that pericytes secrete MCP-1 following NF-κB activation. MCP-1 is mainly known for its role in leukocyte chemotraction; however, previous studies have also shown a role for MCP-1 in angiogenesis (Salcedo et al. 2000; Ma et al. 2007). Ma et al. (2007) showed that MCP-1 mediated angiogenesis through the recruitment of mural cells, which provides a link between inflammatory signaling molecules and angiogenesis. The role of RANTES in angiogenesis is more controversial (Suffee et al. 2011). It can signal through multiple surface receptors where some studies have observed a proangiogenic effect (Westerweel et al. 2008) and others have demonstrated antiangiogenic effects (Barcelos et al. 2009).
Importantly, the candidate cytokines that were identified in this study as NF-κB driven mediators of pericyte-endothelial cell crosstalk are known inflammatory signaling molecules. Therefore, this study provides evidence for the link between inflammation and angiogenesis.

**Limitations**

There are several recognized limitations to this study. First, we did not directly quantify muscle damage from our in vitro scrape injury model. Next, we did not extend our time series to 48 h in our pericyte/C2C12 cocultures, which may have revealed significant differences in pericyte NF-κB activation between injured and control conditions. Another limitation of this study is a lack of a secondary measure of endothelial cell proliferation. The impact of the endothelial cell proliferation results is limited by a lack of a statistically significant difference between endothelial cell numbers in the control condition compared to either increased or decreased NF-κB activation conditions. Future studies should follow up on the candidate cytokines identified in this study to identify which cytokines promote proliferation. Finally, these findings would be strengthened by validation in an in vivo model and investigation into the reciprocal effects of endothelial cells on pericytes.

**Conclusions**

Skeletal muscle is a complex tissue with a high regenerative capacity. However, debilitating diseases and injuries affect skeletal muscle function and regeneration. In order to target and optimize therapies, the cellular and molecular mechanisms of muscle regeneration must be understood, including the crosstalk between different cell types. We have provided further evidence that pericyte NF-κB activation may be an important component of the skeletal muscle inflammatory response to injury. We have also shown that pericyte NF-κB activation can influence endothelial cell proliferation, which occurs through paracrine signaling with the candidate inflammatory cytokines that were identified in our screen. As components of the microvasculature, pericytes and endothelial cells are essential elements of a healthy skeletal muscle tissue; and therefore, this study may also have implications for musculoskeletal diseases as well as vascular diseases, including peripheral artery disease.

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**Conflict of Interest**

None declared.

**References**


