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Original Article

The Angiogenic Response to Skeletal Muscle Overload is not Dependent on Mast Cell Activation

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ABSTRACT

Objective: To determine if mast cell activation in skeletal muscle contributes to overload-induced angiogenesis.

Methods: Extensor digitorum longus muscle was overloaded through extirpation of the synergist muscle tibialis anterior. Muscles were removed after 1, 2, 4, 7 or 14 days, and mast cell density and degranulation were quantified by histology. The mast cell stabilizer, cromolyn, was administered acutely or chronically to test if mast cell degranulation contributes to overload-induced angiogenesis. Angiogenesis was determined by calculating capillary to muscle Fiber ratio; mast cell density and activation were quantified by histology, MMP-2 levels were assessed by gelatin zymography and VEGF protein levels were assessed by Western blotting.

Results: Muscle overload increased mast cell degranulation and total mast cell number within 7 days. Mast cell stabilization with cromolyn attenuated degranulation but did not inhibit the increased mast cell density, MMP-2 activity, VEGF protein levels or the increase in capillary number following muscle overload.

Conclusions: Mast cell degranulation and accumulation precede overload-induced angiogenesis, but mast cell activation is not critical to the angiogenic response following skeletal muscle overload.

Key words: mast cells, angiogenesis, skeletal muscle, MMP-2, VEGF

Abbreviations used: MMP-2, matrix metalloproteinase-2; VEGF, vascular endothelial growth factor; EDL, extensor digitorum longus; HIF, hypoxia inducible factor; VEGFR2, VEGF receptor 2; DMAG, dimethoxy-17-[[(2-dimethylamino)ethyl]amino]geldanamycin; PVDF, Polyvinylidene fluoride.

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INTRODUCTION

Mast cells are derived from precursors of the haematopoietic lineage and complete their differentiation in peripheral tissues [17,22]. In skeletal muscle, mast cells are found in the connective interstitium that separates the muscle into individual fascicles [26] and are observed frequently adjacent to capillaries, nerves and small venules [19]. Activation of mast cells induces release of a multitude of mediators, many of which are preformed and packaged in secretory granules [29]. Several mast cell mediators, including interleukin-8, histamine and VEGF are pro-angiogenic [2,10,20,39]. Compound 48/80, a mast cell secretagogue [30], was reported to increase neovascularization of the mesentery in rats and mice [31]. We previously showed that mast cell secretagogues VEGF and histamine increase MMP-2 production in endothelial cells [8]. Similarly, a recent study by Levick *et al.* (2008) reported decreased MMP-2 activity in ventricles of mast cell-deficient animals (Ws/Ws) subjected to volume overload [24].

It is well established that angiogenesis occurs in response to increasing the functional demands on skeletal muscle by means of exercise or compensatory overload. After seven days of surgical overload, EDL capillary to muscle fiber ratio increases modestly and is increased significantly following 14 and 28 days of overload [45]. Increased endothelial cell proliferation is observed after three days of overload [42], with one-third of all capillaries displaying abluminal sprouts after seven days of overload [13]. HIF-1α, VEGF and VEGFR2 protein levels are elevated three to seven days after overload [28,38,43]. Pro-angiogenic MMP-2 is elevated significantly at 4–14 days of overload, and the activator of MMP-2, membrane type-1 MMP is elevated significantly after seven days of overload [38]. Although the alterations in endothelial cell phenotype and important signaling intermediaries have been identified, the cellular

participants involved in translating muscle overload into an angiogenic response are not well defined.

Mast cells within the skeletal muscle microenvironment may be responsive to mechanical forces and/or metabolic factors and exert pro-angiogenic functions, as has been observed in cardiac muscle [4,5,16]. Therefore, the purpose of our study was to assess the time course of mast cell activation/infiltration into skeletal muscle following muscle overload and to determine whether mast cell activation is required for the increased capillary growth seen following muscle overload.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich (Montreal, QC, Canada) except FITC-conjugated Griffonia Simplicifolia I which was purchased Vector Laboratories (Burlington, ON, Canada).

Ethical Approval

The animal studies were carried out with approval from the York University Committee on Animal Care and performed in accordance with the Animal Care Procedures at York University and the American Physiological Society's Guiding Principles in the Care and Use of Animals.

Rat Studies

Male Sprague Dawley rats (335–375 g at sacrifice; Charles River Laboratories, Quebec, Canada) were used for all experiments. Overload of the EDL muscle was induced through unilateral extirpation of the agonist muscle tibialis anterior [12]. In sham surgeries, the incision was closed without removing the tibialis anterior. After 1, 2, 4, 7 or 14 days of overload ($n = 4$ per group), rats were anaesthetized (i.p. injection of ketamine, 80 mg/kg and xylazine, 10 mg/kg) and EDL muscles were removed for further analysis. EDL muscles also were removed from control (unoperated) and sham operated (1, 4, 7 and 14 day) for further analysis.

Acute cromolyn treatment. Cromolyn (160 mg/kg i.p. dissolved in sterile PBS [9]) was administered to stabilize mast cells. Rats received one dose of cromolyn one hour prior to overload surgery and a second dose one day post surgery. Injection of sterile PBS served as the vehicle control. After 7 and 14 days, EDL muscles were removed for analysis from each of the four treatment groups (sham + vehicle [S + V], sham + cromolyn [S + Cr], overload + vehicle [OL + V] and overload + cromolyn [OL + Cr]; $n = 4$ rats per group). The efficacy of cromolyn treatment was assessed in rats treated with 48/80 (75 μg/100 g body weight, i.p. [9]) one hour after receiving a single dose of cromolyn (160 mg/kg i.p.). After two hours, the EDL was extracted for analysis of mast cell degranulation.

Chronic cromolyn treatment. Rats received one dose of cromolyn (160 mg/kg, i.p. dissolved in sterile PBS) one hour prior to overload surgery. At the time of overload surgery, a mini osmotic pump (Alzet 2002; Durect Corporation; Molecular Devices, Cupertino, CA, USA) was implanted subcutaneously in the upper region of the overloaded hindlimb. Pumps were filled with cromolyn (50 mg/mL) or vehicle (physiological saline) and had a sustained delivery rate of 0.5 μL/hour for 14 days. After 14 days, the EDL was removed from each of the four treatment groups (S + V, S + Cr, OL + Vand OL + Cr, $n = 3$ rats/group). All muscles were snap frozen in liquid nitrogen cooled isopentane.

We also assessed mast cell number and degranulation in EDL muscles from previously conducted experiments in which rats were administered prazosin (α1 adrenergic inhibitor) for seven days to induce angiogenesis, or were treated for 14 days with the anti-angiogenic 17-DMAG (NSC 707545; 17-dimethoxy-17-[[(2-dimethylamino)ethyl] amino]geldanamycin) (destabilizer of HIF1α) or vehicle in combination with overload [28].

Mast Cell Density

For an individual rat, a minimum of five muscle sections (10 μm thickness) were collected, with each section separated in depth by 50 μm. To visualize mast cells, cryosections were fixed in 3.7% formaldehyde and stained with toluidine blue (10 mg/mL toluidine blue in 70% ethanol, diluted 1:10 in 1% NaCl) for 20 minutes. Sections were rinsed with PBS and mounted with AquaPerm media (Fisher Scientific, Whitby, ON, Canada). Sections were viewed using a ×20 objective and images were captured using a digital color CCD camera (Hitachi; Applied Biosystems, Burlington, ON, Canada). Mast cells were identified as metachromatically stained cells within the orthochromatically stained tissue. A mast cell was classified as degranulated if metachromatically stained granules were observed outside of the mast cell membrane. For determination of mast cell density, five sections per rat were examined, sampling from six independent fields of view per section. The total number and number of degranulated mast cells were determined for each field of view and then converted to cells/mm^2. Cells/mm^2 values from all fields of view were averaged to obtain a single density value per rat.

Protein Isolation

Frozen muscle was ground to powder and protein was isolated through brief homogenization in buffer containing 120 mM Tris HCl, 5% glycerol and EDTA-free protease inhibitor cocktail (P8340; Sigma). After homogenization,

Triton X-100 was added to a final concentration of 0.1% [21]. BCA assay (Pierce, Fisher, Thermo Scientific, Whitby, ON, Canada) was used to determine protein concentration.

Gelatin Zymography

To determine the amount of total and active MMP-2, 10 μg of whole muscle lysates were separated, under non-reducing conditions, through a 10% SDS-polyacrylamide gel embedded with 0.04% gelatin. Gels were incubated for approximately 48 hours at 37°C in 5 mM $CaCl_2$ and 50 mM Tris (pH 7.6) prior to staining with Coomassie protein stain and destaining with 20% methanol, 20% glacial acetic acid. Gels were visualized and imaged using the Fluorchem gel doc system and analyzed using Alpha ease (Alpha Innotech, Cell Biosciences, Santa Clara, CA, USA) software, with the sum of 72 kDa and 62 kDa band intensities representing total MMP-2 protein levels, and the 62 kDa band intensity representing the amount of active MMP-2.

Western Blotting

To determine the amount of VEGF protein, 30 μg of whole muscle lysates were separated under reducing conditions through a 10% SDS-polyacrylamide gel. Gels were transferred to a PVDF (Millipore, Billerica, MA, USA) membrane using semi dry technique and blocked in 5% BSA (Multicell) or 5% milk. Membranes were incubated overnight at 4°C in VEGF (1:200, sc-152; Santa Cruz Biotech, Santa Cruz, CA, USA) or α/β tubulin (1:1000, #2148; Cell Signaling, Danvers, MA, USA) followed by one hour room temperature incubation in anti-rabbit (Pierce) secondary antibody. Images were developed using enhanced chemiluminescence (Millipore Immobilion ECL, Millipore, Billerica, MA, USA) and visualized using a digital imaging station (Kodak MM4000Pro, Carestream Molecular Imaging, Woodbridge, CT, USA). Bands were quantified using Fluorchem software (AlphaInnotech).

Capillary Number

Capillary to muscle fiber ratio was determined as an indicator of angiogenesis. 10 μm cryosections were fixed in cold acetone and stained with iso-lectin (FITC-conjugated Griffonia Simplicifolia I, Vector) (diluted 1:100) in PBS for 30 minutes. Sections were viewed with an Olympus microscope (×20 objective) and capillary and muscle fiber counts were averaged from five independent fields of view per rat.

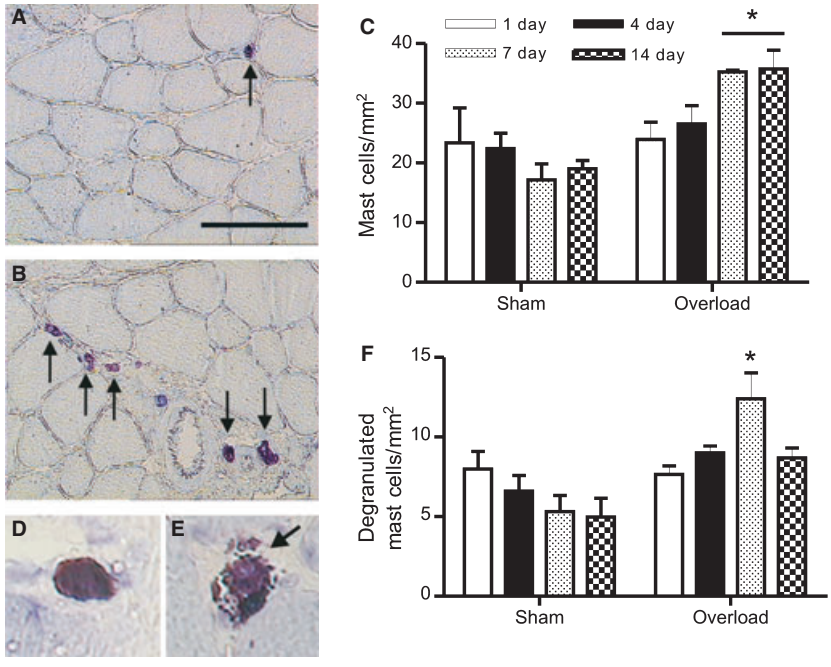

Figure 1. Skeletal muscle overload increases mast cell number and degranulation. Sections of overloaded extensor digitorum longus were stained with toluidine blue to depict mast cells. Representative image of control muscle (**A**) and overloaded muscle (**B**). Mast cells are indicated with arrows, and the scale bar represents 100 μm. Average number of mast cells/mm^2 was calculated (**C**). An example of an intact (**D**) and degranulated (**E**) mast cell are magnified. The arrow in (**E**) points to secretory granules outside of the boundary of the cell. Average number of degranulated mast cells/mm^2 was determined (**F**). The legend in panel C also applies to panel F. Values are presented as mean ± SEM. Two-way ANOVA (day x surgery) revealed a main effect of surgery and a significant interaction between day and surgery. Bonferroni *post hoc* tests revealed a significant increase in total mast cell number after 7 and 14 days of overload and a significant increase in the number of degranulated mast cells after 7 days of overload compared to 1 day overload animals. *, $p < 0.05$. $n = 3$ for control, 1d sham, 4d sham, 4d OL and 14d sham, $n = 4$ for 7d sham, 7d OL and 14d OL. Values from animals overloaded for 1 and 2 days were combined when conducting statistical analyses.

Muscle Fiber Area

The cross-sectional area of muscle fibers was calculated using Metamorph Image Analysis software (Universal Software, Molecular Devices, Silicon Valley, CA, USA). Cross-sectional areas of individual muscle fibers were measured in five independent fields of view per rat (each field of view contained 10–12 intact muscle fibers). Individual measurements were averaged to calculate an average cross-sectional fiber area per rat.

β-glucuronidase Activity Assay

β-glucuronidase activity was determined as an indicator of muscle damage as previously described [15,23]. Muscle samples were incubated with substrate (5 mM p-nitrophenyl-b-D-glucuronide, Sigma) for 18 hours at 37°C with p-nitrophenol (Sigma) used as a standard. Absorbance was measured at 405 nm and activity was calculated per soluble protein and incubation time. Protein concentration was determined with the BCA assay (Pierce).

Statistics

Data are presented as mean ± standard error. Comparisons between groups were performed using Student's *t*-test or one, two and three-way ANOVA using GraphPad Prism 4 (La Jolla, CA, USA) or SPSS version 17 (Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

RESULTS

Effect of Muscle Overload on Mast Cell Infiltration

Resident mast cells were observed in the muscle of control and sham-operated animals. Sham animals typically displayed solitary mast cells in a single field of view or several individual mast cells throughout the field of view (Figure 1A). In overloaded muscle, mast cells were found in clusters of two or three in one field of view. It was common to observe mast cell clusters around small blood vessels or adjacent to nerve tracts (Figure 1B). Overload exerted a significant, time-dependent increase in mast cell number (Figure 1C). Total mast cell numbers after 7 and 14 days of overload were significantly greater than in one day overload muscles.

Degranulation of mast cells was used as an indication of mast cell activation. An intact mast cell was identified as having all the metachromatically stained secretory granules within the borders of the mast cell while a degranulated

Table 1. 48/80 Stimulated degranulation of mast cells and effect of cromolyn treatment

Condition	Degranulated/mm^2
Control	5.24 ± 0.99
48/80	9.82 ± 1.16
Cromolyn	4.19 ± 1.13
48/80 + Cromolyn	4.35 ± 1.95*

$n = 3$ per condition. *$p < 0.05$ vs. 48/80 (Bonferroni multiple comparison).

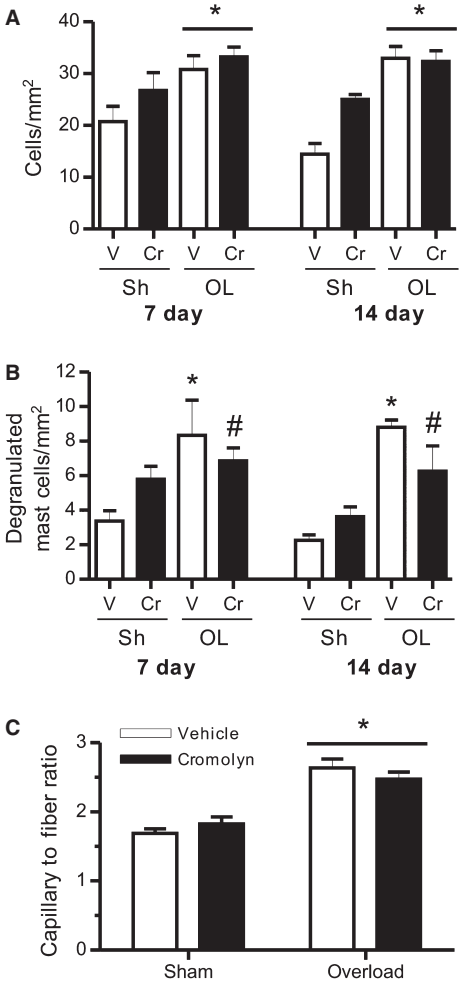

Figure 2. Acute mast cell stabilization does not attenuate the increased mast cell number or capillary to muscle fiber ratio following overload. Average number of mast cells/mm^2 (**A**) and degranulated mast cells (**B**) were calculated. Values are presented as mean ± SEM. Three-way ANOVA (surgery x day x drug) revealed a significant effect of surgery on both total mast cells/mm^2 and degranulated mast cells/mm^2. There was a significant interaction between surgery and cromolyn treatment on the number of degranulated mast cells/mm^2. Capillary to muscle fiber ratio was assessed (**C**), and two-way ANOVA (surgery x drug) revealed only a significant effect of overload. *, $p < 0.05$ denotes a significant main effect, #, $p < 0.05$ denotes a significant interaction. S + V, sham and vehicle; S + Cr, sham and cromolyn; OL + V, overload and vehicle; OL + Cr, overload and cromolyn.

mast cell was defined as having secretory granules clearly outside the border of the mast cell (as shown in Figure 1D,E respectively). A significant time-dependent increase in the number of degranulated mast cells was observed in response to overload compared to time-matched sham animals (Figure 1F). Degranulated mast cell number after seven days of overload was significantly greater than in one day overload muscles.

Effects of Mast Cell Stabilization on Overload-Induced Angiogenesis

Rats were treated with the mast cell stabilizer cromolyn to assess the involvement of mast cell secretagogues in overload-induced angiogenesis. Cromolyn blocks calcium entry into mast cells thereby preventing mast cell activation and granule release [18,40]. Initially, focusing on the potential role of early activation of mast cells in the angiogenesis response, we treated animals with cromolyn pre-surgery, and one day post-surgery. In control experiments, 48/80 induced mast cell degranulation was attenuated by acute cromolyn treatment (Table 1). There was a significant effect of overload on both mast cell number and degranulation, which did not differ between 7 and 14 day overload samples. The increase in mast cell number following surgery was not affected significantly by cromolyn treatment (Figure 2A). Analysis of the number of degranulated mast cells/mm^2 revealed a significant interaction between overload surgery and cromolyn treatment (Figure 2B).

A significant increase in the capillary to muscle fiber ratio was observed after 14 days of overload, consistent with previous reports [38,45]. Acute cromolyn treatment did not reduce the overload-induced increase in capillary to muscle fiber ratio (Figure 2C). We tested if cromolyn inhibited the overload-induced increases in MMP-2 and VEGF, since MMP-2 production is regulated by factors released from degranulated mast cells [8,38] while VEGF itself may be released from activated mast cells [2,20]. Consistent with previous results [38], we observed a significant increase in total MMP-2 (Figure 3A,B), active MMP-2 (Figure 3A,C) and VEGF (Figure 3D,E) levels following seven days of overload. Mast cell stabilization with cromolyn did not attenuate the overload-induced upregulation of MMP-2 production and activation or the increase in VEGF protein.

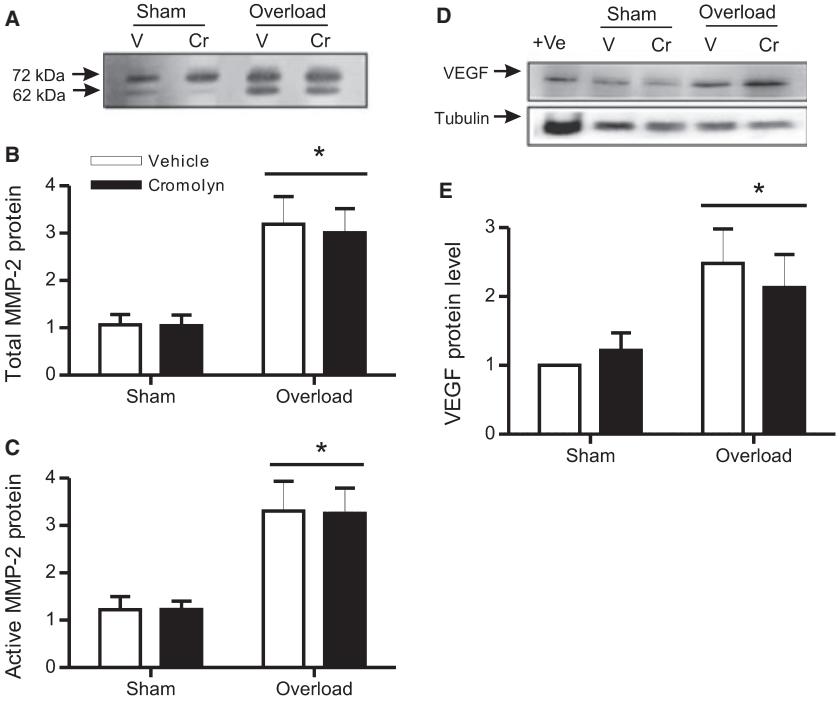

Figure 3. Overload-induced increases in matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF) are not inhibited with mast cell stabilization. Representative image of gelatin zymography is shown (**A**). The 72 kDa band corresponds to latent MMP-2 and the 62 kDa band corresponds to active MMP-2. Total levels were calculated by summing the intensities of 72 and 62 kDa bands and normalized to sham + vehicle (S + V) (**B**). Active MMP-2 was obtained by normalizing the intensity of active (62 kDa band) to the S + V condition (**C**). The legend displayed in panel B also applies to panel C and E. Representative VEGF and α/β tubulin Western blots (**D**). VEGF values were normalized to α/β tubulin values and presented as a value relative to S + V (set to 1) (**E**). In all panels, values are presented as mean ± SEM. Two-way ANOVA (surgery x drug) revealed only a significant effect of overload on total, active MMP-2 and VEGF protein levels. *, $p < 0.05$. V, vehicle; Cr, cromolyn; +Ve, positive control for VEGF.

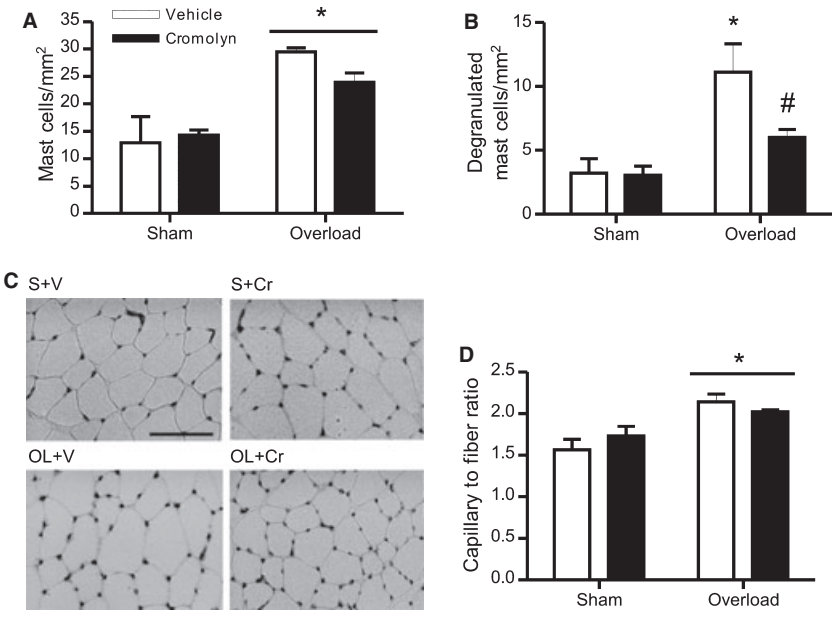

Figure 4. Chronic cromolyn treatment blocks overload-induced increase in mast cell number but not angiogenesis. Average total number of mast cells/mm^2 (**A**) and degranulated mast cells/mm^2 (**B**) were calculated. Inverted greyscale images of iso-lectin staining (**C**) were used to calculate capillary to muscle fiber ratio (**D**). The legend in panel A also applies to panels B and D. Values are mean ± SEM. Two-way ANOVA (surgery x drug) revealed a significant effect of overload on total mast cells/mm^2 and significant main effects of overload and drug on degranulated mast cells/mm^2 as well as a significant interaction. *, $p < 0.05$ denotes a significant main effect, #, $p < 0.05$ denotes a significant interaction. Scale bar represents 100 μm. S + V, sham and vehicle; OL + V, overload and vehicle; S + Cr, sham and cromolyn; OL + Cr, overload and cromolyn.

As the level of mast cell degranulation was reduced in animals treated with cromolyn, but still elevated relative to vehicle treated animals (Figure 2B), we considered that mast cell activation at later time points (i.e., from 7 to 14 days) could contribute to the angiogenic response. To address this concern, we conducted a second study in which cromolyn was administered continuously over the time course of the overload. Interestingly, chronic administration of cromolyn for 14 days did not block the overload-induced increase in total mast cell number (Figure 4A). Mast cell degranulation was significantly decreased in overloaded muscle treated with cromolyn, indicating the effectiveness of the cromolyn treatment (Figure 4B). In this experiment, overload induced an increase in capillary to fiber ratio, and this was not impaired by chronic cromolyn delivery (Figure 4C,D). Muscle fiber cross-sectional area did not change in response to overload or cromolyn treatment when compared to sham animals (Table 2).

To provide further evidence in support of the conclusion that mast cell degranulation/infiltration is independent of the angiogenic process in skeletal muscle, we examined mast cell number in overloaded muscles that were treated with an angiogenic inhibitor, 17-DMAG. We previously reported that 17-DMAG significantly attenuated capillary growth following muscle overload [28]. Degranulated and total mast cell numbers were elevated in the overload + 17-DMAG treated animals, compared to 17-DMAG alone (Figure 5A), indicating that increased capillarity was not required for the increase in mast cell number and that mast cell degranulation did not lead to capillary growth following muscle overload.

Table 2. Mean muscle fiber area of sham and overloaded EDL

Condition	Fiber area (μm)
Sham + Vehicle	2282 ± 86
Sham + Cromolyn	2046 ± 333
Overload + Vehicle	2484 ± 116
Overload + Cromolyn	2384 ± 197

n = 3 rats per group. EDL, extensor digitorum longus.

Secondly, we examined mast cell number and degranulation in a model of blood flow-induced angiogenesis. Seven days of prazosin (α1 adrenergic receptor inhibitor) increases capillary to muscle fiber ratio by approximately 31% [6,28,38,44] in the absence of altered muscle activity. We found no change in mast cell number or degranulation with seven days of prazosin treatment (Figure 5B), despite concomitant changes in capillary number.

Notably, mast cell number and degranulation correlated with the presence of muscle damage, as indicated by increased activity of the lysosomal enzyme β glucuronidase

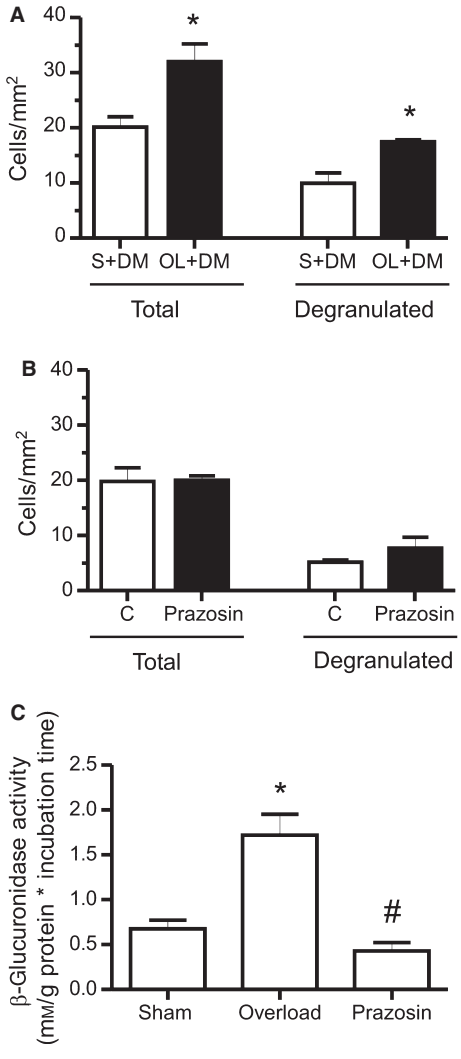

Figure 5. Overload, but not angiogenesis, is required for increase in mast cell number and degranulation. Total mast cell/mm^2 and degranulated/mm^2 are quantified for overload animals treated with the angiogenesis inhibitor 17-DMAG (**A**) and for control animals administered prazosin (**B**). S + DM, sham and 17-DMAG, OL + DM, overload and 17-DMAG, C-control. Values are mean ± SEM, *, $p < 0.05$ vs. respective sham condition, with significance assessed using Student's *t*-test (A and B). *β*-glucuronidase activity was assessed as an indicator of muscle damage in 7 day overload and 7 day prazosin-treated muscle (**C**). Significance was assessed by one-way ANOVA followed by Tukey *post hoc* tests *, $p < 0.05$ vs. sham and #, $p < 0.05$ vs. overload.

[15,23]. *β* glucuronidase activity was increased significantly in response to muscle overload, but not in response to prazosin treatment.

DISCUSSION

In this study, we report that mast cell number and activation (degranulation) significantly increase following muscle overload. However, mast cell degranulation is not required to elicit the angiogenic response to skeletal muscle overload, as we found that cromolyn treatment did not attenuate the overload-induced increase in capillary to muscle fiber ratio. Likewise, levels of the pro-angiogenic factors MMP-2 and VEGF were elevated in response to overload, but were not reduced in cromolyn-treated animals.

The increased number and activation of mast cells we observed following skeletal muscle overload indicates that either directly or indirectly, resident mast cells respond to the increased mechanical load on the EDL muscle. Mast cell recruitment and/or proliferation could be a direct response to the mechanical stimulus or an indirect response mediated by cytokines/growth factors released from inflammatory cells, myocytes or endothelial cells. Increased mast cell number and activation (degranulation) both are detectable after seven days of overload. The delayed increase suggests that the increased mechanical load does not directly increase mast cell activation and total number, but that it occurs secondary to other mediators. Our results also imply that mast cell degranulation following overload does not stimulate the increase in mast cell number, because total mast cell number remained elevated following 14 days of overload in animals treated chronically with cromolyn.

The increase in mast cell density and activation coincides with capillary growth, which is evidenced by modest increases in capillary to fiber ratio at seven days, followed by significant increases at 14 days [45]. While we hypothesized that mast cells contribute to the angiogenic response following muscle overload, our results show that mast cell activation is not required for the increased capillary growth. Mast cells and blood vessels have a well documented spatial association [11,33]. Given the parallel increases in capillary number and mast cell number reported in numerous tissues [1,27,33–37], it is unclear whether mast cell activation initiates blood vessel growth or developing blood vessels induce infiltration or proliferation of mast cells. Tumors induced in mast cell-deficient animals exhibit a decreased angiogenic response [7,41]. However, a more recent report concluded that mast cell-deficient animals had a blunted inflammatory response during would healing, but a normal angiogenic response [14]. Our findings are consistent with the conclusion that, while mast cell number and capillary growth occur in parallel in response to muscle overload, they appear to be independent processes. We documented conditions in which capillary growth occurred in the absence of mast cell degranulation/infiltration (in response to increased blood flow) and when mast cell degranulation/infiltration occurred in the absence of capillary growth (in overloaded animals treated with 17-DMAG).

While our study provides evidence that mast cell activation is not necessary in the angiogenic response in

overloaded skeletal muscle, others have reported negative functional consequences of mast cell activation in skeletal muscle remodeling. In these cases, skeletal muscle damage (as a result of ischemia/reperfusion injury, muscular dystrophy or hind limb suspension) resulted in mast cell degranulation, leading to recruitment of neutrophils and, ultimately, reduced muscle fiber viability [3,9,25,29,32]. Our results suggest that mast cell numbers may increase in response to muscle damage, as their presence correlated with increased activity of the muscle damage indicator, β glucuronidase. However, the extent of muscle damage in the EDL overload model is modest, and there is minimal accumulation of inflammatory cells [45], which may account for the overall minimal influence of mast cells in this model.

In summary, we have provided evidence that skeletal muscle overload results in increased mast cell density and mast cell degranulation. While a physiological function of the mast cells within the overloaded muscle remains to be elucidated, our study indicates that mast cell activation is not required for the increased level and activity of MMP-2, the increased level of VEGF protein or the increased capillary growth observed following muscle overload.

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