Time course of changes in capillarization in hypertrophied rat plantaris muscle

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Plyley, Michael J., Barbara J. Olmstead, and Earl G. Noble. Time course of changes in capillarization in hypertrophied rat plantaris muscle. J. Appl. Physiol. 84(3): 902–907, 1998.—The time course of angiogenesis during hypertrophy of the rat plantaris muscle was studied by using a unilateral, synergistic ablation model. Animals (n = 6/group) were euthanized 2, 5, 7, 15, 21, and 30 days postsurgery. Sections from both the hypertrophied and contralateral muscles were simultaneously stained for capillaries and muscle fiber type. Mean fiber cross-sectional area (FA) and various indexes of capillarity were determined by using a video analysis system. The capillary supply to individual fibers, assessed as the FA supplied per capillary contact, remained unchanged until day 21 (compared with day 2) and exhibited a significant increase at day 30. Analysis of the time course of capillary development on the basis of the number of capillary contacts per fiber, and of hypertrophy on the basis of FA, yielded half-lives of 10.1 and 11.2 days, respectively. It was concluded that angiogenesis during muscle overload is tightly coupled to the changes in FA, which could suggest that the two processes are initiated and/or driven by some common factor(s). Multifactorial role of muscle overload; synergistic ablation; angiogenesis

INCREASES IN MUSCLE MASS and enlargement of the cross-sectional area of individual muscle fibers are characteristic of the response of skeletal muscle to a functional overload (1, 5, 6, 9, 11, 12, 21, 23, 28). For muscle to maintain a normal capillary-muscle-fiber relationship during hypertrophy, angiogenesis and/or capillary reorganization must take place. Growth of the capillary network, after synergistic ablation, was first described by Reitsma (28). Increases in the capillary supply of hypertrophied chicken patagialis (PAT) and anterior latissimus dorsi (ALD) muscles after stretch-induced muscle hypertrophy have been reported (9). More recently, Degens et al. (5, 6) have described an increase in the capillary supply in the hypertrophic plantaris muscle of young and old rats after synergistic ablation and 5 wk of endurance training. However, a central, yet unanswered, question concerns the relationship between the time course of the increase in muscle size and the process of angiogenesis. Therefore, the purposes of the present study were twofold: 1) to establish the time course of the increase in capillarization in the hypertrophic rat plantaris muscle and 2) to examine the changes in capillary supply in relation to the time course of hypertrophy.

METHODS

This study was approved by the University of Western Ontario Committee on Animal Care and performed in accordance to the guiding principles of the Canadian Council on Animal Care.

Surgical preparation. Thirty-six male Sprague-Dawley rats [body mass 232 ± 16.2 (SD) g] were randomly divided into six groups. Each rat underwent surgical removal of one gastrocnemius muscle (1, 11) to induce a compensatory overload of the plantaris muscle.

Under pentobarbital sodium anesthesia (60 mg/kg), a midline incision was made through the skin from the popliteal fossa to the Achilles tendon. A second incision, running medially through the hamstring muscles, exposed the proximal heads of the gastrocnemius. Through careful dissection of the proximal origins of the two muscles, both the lateral and medial heads of the gastrocnemius were separated and sectioned. The distal tendon was then transected to complete the removal of the gastrocnemius. Care was taken to avoid trauma to the plantaris and to maintain the continuity of the circulation and nerve supply. A sham operation was performed on the contralateral limb, which served as the experimental control. The animals were returned to their cages and were fed and given water ad libitum.

The first group of animals (n = 6) was euthanized 2 days after surgery, and subsequent groups (each n = 6) were euthanized at 5, 7, 15, 21, and 30 days postsurgery, respectively. At the time of death, each rat was anesthetized and the plantaris muscles of both hindlimbs was excised, cleaned of excess connective tissue, blotted, and weighed. After removal of the plantaris muscles, the rats were killed by cardiac excision.

Histological preparation. A midsection was removed from each plantaris, embedded in Tissue Tek optimum-cutting-temperature medium, and quick frozen in isopentane cooled with liquid nitrogen. Samples were labeled and stored at −80°C until processing. Cross sections, 10 µm in thickness, were cut, air-dried, and immediately stained. Staining consisted of a modified myosin adenosinetriphosphatase (ATPase) staining method (29), which uses a lead-based stain to simultaneously visualize both capillaries and fiber types. In addition, serial cross sections were stained for NADH tetrazolium reductase (20) to confirm the fiber type proportions.

Tissue analysis. All capillary and muscle fiber measurements were done on the lead-ATPase sections by using a semiautomated video image-analysis system (Apple Ile Bioquant digitization program). Three to five fields of view (about ×305), each containing 100–150 fibers, were chosen at random for the analysis of the various indexes of capillarity (14, 25), including 1) capillary density (Nc), 2) capillary-to-fiber ratio (C/F), 3) number of capillary contacts per individual fiber (CC), 4) number of fibers “sharing” a capillary, i.e., the quotient of CC and C/F, and 5) fiber area supplied per capillary contact (FA/CC); in addition, 6) fiber density (Nf) and 7) FA were determined. Care was taken to account for “edge effects” in the determination of Nf and FA by examining only those fibers that were completely within the field of view and the complete capillary complement of which was visible. Edge effects in the counts for Nc were accounted for by counting all “interior” capillaries, plus one-half the capillaries lying on the boundary (i.e., “exterior” capillaries) of each area of analysis (25).
Data analysis. A time course analysis was conducted on both FA and CC over the time intervals 2, 5, 7, 15, 21 and 30 days postsurgery by using TableCurve (Jandel Scientific). To estimate the half-life ($t_{1/2}$) for hypertrophy and capillary growth, regression analysis was applied to plots of $\ln (FA_i - FA_{ss})$ vs. time and $\ln (CC_i - CC_{ss})$ vs. time, where $FA_i$ and $CC_i$ are the values for FA and CC at any time, $t$, and $FA_{ss}$ and $CC_{ss}$ are the steady-state values for FA and CC. After the data for each variable were fitted to a monoexponential curve, $t_{1/2}$ was predicted from the relationship $t_{1/2} = \ln 2/k$, where $k = -$ slope of the regression. To account for the changes in muscle fiber size and capillarity due to normal growth, the data used in the time course analysis were derived as the difference between the observed values for FA and CC from each hypertrophied muscle and its corresponding contralateral muscle at each time point; this process yielded a single series of data representing the changes resulting only from the overload.

Statistical analysis. Data were subjected to one-way and two-way analyses of variance for repeated measures. When significant differences ($P < 0.05$) were detected, the Student-Newman-Keuls procedure was used for post hoc analysis. For reporting purposes, only within-day differences are noted for contralateral plantaris (CP) and overloaded plantaris (OP) between groups and, between days, all comparisons were made to the day 2 CP results.

RESULTS

Ablation of the synergists was accompanied by rapid growth of OP, as evidenced by a significant increase in muscle wet weight compared with CP at each point in the time course (Table 1). The mass of the CP muscle also increased over time (by $\sim 50\%$), but the increase was proportional to the overall growth of the animal, as indicated by the maintenance of the muscle mass-to-body mass ratio (Table 1). In contrast, the muscle mass-to-body mass ratio in the overload condition was significantly elevated from day 15 onward, indicating a rate of growth greater than that expected through normal maturation (Table 1).

Mean FA for CP increased by $\sim 25\%$ (not significant) between days 2 and 30. In comparison, the mean FA of OP, compared with CP, was significantly elevated by 15 days postsurgery (a in Fig. 1). The $t_{1/2}$ for fiber hypertrophy in OP was 11.2 days. The extent of fiber hypertrophy in OP was also reflected by a decrease in Nc by day 30 (Table 2).

Fig. 1. Time course of changes in fiber area (FA), mean number of capillaries in contact with a fiber (CC), and FA supplied per capillary contact (FA/CC) over 30 days in overloaded and contralateral rat plantaris muscle. Nc, capillary density. *Significantly different from control plantaris, $P < 0.05$. bSignificantly different from day 2 value, $P < 0.05$.

Table 1. Time course of changes in muscle mass and muscle mass-to-body mass ratio and overloaded-to-contralateral muscle mass ratios in contralateral and overloaded rat plantaris muscle.

<table>
<thead>
<tr>
<th>Day No.</th>
<th>Contralateral Muscle</th>
<th>Overloaded Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mass, g</td>
<td>MM/MB, mg/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MM_over/MM_cont</td>
</tr>
<tr>
<td>2</td>
<td>250 ± 7</td>
<td>0.96</td>
</tr>
<tr>
<td>5</td>
<td>238 ± 9</td>
<td>0.95</td>
</tr>
<tr>
<td>7</td>
<td>225 ± 6</td>
<td>0.89</td>
</tr>
<tr>
<td>15</td>
<td>301 ± 9</td>
<td>0.98</td>
</tr>
<tr>
<td>21</td>
<td>348 ± 17b</td>
<td>1.01</td>
</tr>
<tr>
<td>30</td>
<td>382 ± 8b</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 6/group$. MM, muscle mass; MB, body mass; MM_cont and MM_over, muscle mass of contralateral (control) and overloaded (hypertrophied) muscle, respectively. *Significantly different ($P < 0.05$) from control plantaris. bSignificantly different ($P < 0.05$) from day 2 values.
DISCUSSION

Muscle that undergoes overload as a result of synergistic ablation experiences increased recruitment and significant muscular hypertrophy (1, 5–7, 11, 12, 15, 21, 23, 28). Despite the rapid growth associated with the compensatory overload, the activity of enzyme markers for oxidative metabolism is ultimately maintained (11, 12), and the overloaded muscle actually becomes more fatigue resistant (7). Coupled with these findings, an increased blood flow (per g wet wt) has been observed in rat muscle after hypertrophy (7). Although these observations would suggest that muscle undergoing compensatory growth is not compromised with regard to vascular support, the extent of, and time course for, capillary development and its relationship to muscle hypertrophy in overloaded muscle are unclear. Thus the purposes of the present study were to examine the extent and time course of muscle capillary changes during 30 days of muscle overload.

Hypertrophy. The extent of muscle hypertrophy as a result of the synergistic ablation is evident as indicated by the change in muscle mass over the 28-day period (an overall increase of 125% compared with that by day 2 and an 80% increase compared with CP). In the case of CP, changes in FA due to growth (an increase of ~50%) were observed over the period studied; however, the change was not significant. It is also clear that the extent of growth in the contralateral muscle was not out of proportion to the overall growth of the animal because there was no change in the muscle mass-to-body mass ratio over the 28-day period for the contralateral muscle; on the other hand, the extent of the hypertrophy in the overloaded muscle was clearly shown by the increase in both the muscle mass-to-body mass ratio and the OP-to-CP muscle mass ratio over the same time period. Although the initial body mass values differ somewhat, these results are similar to those reported by others for this model (12, 21) and indicate the value of normalizing muscle mass for comparative purposes (2).

Examination of the time course of muscle growth over the overload period revealed a $t_{1/2}$ of 11.2 days. This value is similar to that (10 days) reported for soleus myofibril protein synthesis during recovery from 28 days of hindlimb suspension (33) but less than the 19 days reported for the $t_{1/2}$ of mixed myosin heavy chain during compensatory growth from synergistic ablation (34). Our analysis of published data indicate $t_{1/2}$ of 8.1 (21), 10.6 (4), and 14.7 days (23) for the change in muscle mass, 14.2 days (4) for the increase in fiber diameter, and 8.1 (21) and 10.1 days (4) for the alteration in muscle fiber composition during hypertrophy.

Capillarization. In this study, the capillary supply was identified by using the simultaneous capillary-muscle fiber staining method of Rosenblatt et al. (29), which has previously been validated against serial sections stained via the standard amylase-periodic acid-Schiff capillary and myosin ATPase staining methods. Quantification of the capillary supply was completed via a semiautomated image-analysis system, using the methods of Plyle and Groom (25) and Ingjer (14). Although these methods allow for the assessment of the capillary supply to individual fibers and/or by fiber type, for purposes of this study we have presented only values for the muscle as a whole.

Because we did not examine longitudinal sections, we cannot rule out an increase in the number of capillary cross sections because of an increase in capillary tortuosity; however, Poole et al. (27) have reported no change in capillary tortuosity in the soleus muscle after endurance training, and James (15) concluded that the newly formed capillaries in hypertrophied skeletal muscle develop preferentially in the longitudinal direction.

Nc at day 2, which was similar to that observed by Bigard et al. (2) and Degens et al. (6) in animals of comparable age, represents an average value for randomly selected portions of the whole plantaris muscle. This value is also midway between the values reported for the deep and superficial portions of the plantaris by Degens et al. (5). Changes in Nc reflect a dynamic interaction between changes in muscle fiber size and

### Table 2. Time course of changes in capillary density, capillary-to-fiber ratio, and fiber contacts per capillary in contralateral and overloaded rat plantaris muscle

<table>
<thead>
<tr>
<th>Day No.</th>
<th>Nc, mm²</th>
<th>C/F</th>
<th>FC</th>
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<tbody>
<tr>
<td>2</td>
<td>623 ± 32</td>
<td>1.56 ± 0.04</td>
<td>3.22 ± 0.09</td>
</tr>
<tr>
<td>5</td>
<td>758 ± 50</td>
<td>1.55 ± 0.06</td>
<td>3.28 ± 0.09</td>
</tr>
<tr>
<td>7</td>
<td>666 ± 26</td>
<td>1.50 ± 0.04</td>
<td>3.39 ± 0.14</td>
</tr>
<tr>
<td>15</td>
<td>644 ± 46</td>
<td>1.68 ± 0.13</td>
<td>3.25 ± 0.16</td>
</tr>
<tr>
<td>21</td>
<td>668 ± 52</td>
<td>1.80 ± 0.11b</td>
<td>3.06 ± 0.10</td>
</tr>
<tr>
<td>30</td>
<td>628 ± 56</td>
<td>1.88 ± 0.09b</td>
<td>3.05 ± 0.10</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6/group. Nc, capillary density; C/F, capillary-to-fiber ratio; FC, sharing factor. aSignificantly different (P < 0.05) from control (contralateral) plantaris. bSignificantly different (P < 0.05) from day 2.

The $t_{1/2}$ for the capillary growth during the overload was found to be 10.1 days.
angiogenesis. Nc would be maintained when muscle fiber size and capillarization change in parallel. When muscle growth exceeds angiogenesis, however, the Nc would be expected to decrease. In the study by Degens et al. (5), Nc did not change after 5 wk of overload, although muscle mass increased 32%. When Nc was expressed on an individual fiber basis, however, these investigators (6) found that capillary proliferation lagged behind the increases in FA. Others (10, 31) have noted that Nc decreases hyperbolically in conjunction with increased fiber size. In the present study, increases in muscle mass (25% by day 7), similar to those reported by Degens et al. (5), were not associated with changes in Nc. However, by 15 days of overload, when muscle mass was increased by ~49%, Nc had started to decline. Coupled with the previous observations, these data could be interpreted to suggest that, in periods of rapid muscle growth through increases in FA, angiogenesis is unable to keep pace with muscle hypertrophy.

To further examine this possibility, C/F, determined as the ratio of the Nc to Nf, was used to normalize Nc for changes in fiber size. The C/F seen at day 2 was similar to that reported by others (2, 7). By using this index, by 15 days of overload there was a significant increase in C/F compared with earlier time periods. By 21 days, this increase was also elevated compared with that in the contralateral control (Table 2). After 8 wk of overload in rat extensor digitorum longus, Egginton and Hudlicka (7) found an increased C/F; Degens et al. (5) also observed an increased local C/F in the hypertrophied plantaris muscle after 5 wk of overload. Collectively, these observations suggest that despite a decrease in Nc, rapid muscle growth is accompanied by active angiogenesis. However, whether the increase in capillary supply is able to keep pace with the increase in muscle FA is uncertain. By using chickens that underwent 5 wk of stretch-induced hypertrophy, Holly et al. (9) reported an increased C/F in both the PAT and ALD muscles. However, their data also demonstrated that the ALD muscles, which underwent the largest degree of hypertrophy (126%), exhibited a smaller increase in C/F than did the PAT muscles (81% hypertrophy), resulting in no change in Nc for the PAT and a significant decrease in Nc for the ALD muscles. These data suggest that with extreme muscular hypertrophy, angiogenesis may not be able to keep pace with muscle growth.

A more direct measurement of change in the capillary supply to individual muscle fibers is derived from counting mean CC, with the capillary supply for the muscle being represented by the mean of the distribution of the numbers of capillaries supplying the individual fibers (25). Our values for CC in the control muscles on day 2 were within the range of values reported in the literature for skeletal muscle (18, 25).

An increase in CC by itself does not indicate the effectiveness of angiogenesis in keeping pace with the ongoing fiber hypertrophy. Our analysis of the t1/2 for the increase in CC during the overload period was 10.1 days, a value similar to that observed for the t1/2 of the fiber hypertrophy (11.2 days). From these results, it would appear that there was a tight coupling of fiber growth and capillary development. This view is supported by Snyder et al. (32), who have suggested that angiogenesis (as evidenced by an increase in CC) occurs so as to maintain a constant proportion of muscle fiber surface area served per capillary. Similar conclusions have been drawn by others with regard to the relationship of CC to fiber size in other models (19). On the other hand, Degens et al. (5, 6) conclude from their data on changes in the individual C/F during hypertrophy that capillary development lags behind the increase in FA.

Although the determination of the C/F normalizes the Nc for changes in fiber size, it does not adequately explain changes in the capillary-muscle fiber relationship at the level of the individual muscle fiber. FA/CC is critical in evaluating the degree to which muscle growth is accompanied by adequate capillary development. A constant FA/CC would suggest that capillary development has been able to match the changes in fiber size; on the other hand, an increase in FA/CC during hypertrophy would indicate a failure on the part of capillary development to keep pace with fiber growth. Although FA/CC remained unchanged through day 21 (78% hypertrophy), a significant increase was observed at day 30 (126% hypertrophy), suggesting that capillary development was able to keep pace with fiber hypertrophy through day 21 but began to lag behind fiber growth by day 30.

It has been suggested by Poole and Mathieu-Costello (26) that a unifying concept for capillary development in muscle can be based on evaluating the capillary-to-fiber interface available for oxygen diffusion (i.e., the capillary-to-fiber surface ratio), as indicated through changes in the capillary-to-fiber perimeter ratio. In a recent publication, Kano et al. (16) report a maintenance of the capillary supply-to-fiber perimeter ratio after 6 wk of compensatory hypertrophy of the rat plantaris muscle. In their paper, Kano et al. also note an increase in capillary number with no increase in capillary luminal diameter accompanying the increase in muscle fiber size. This would mean that the increase in fiber surface area due to hypertrophy was met by a proportional increase in capillary surface area solely as a result of the increase in capillary number, i.e., an increase in CC. Although this study did not examine the time course of these changes, the results of Kano et al. suggest that angiogenesis is able to maintain the normal interface between capillary supply and muscle fiber size after 6 wk of hypertrophy. In contrast, the results of the present study indicate the development of a mismatch between capillary supply and fiber size somewhere between days 21 and 30 after muscle overload.

Hypertrophy and angiogenesis. The results presented here indicate that the development of muscle hypertrophy and the accompanying angiogenesis followed nearly identical time courses, at least until day 30, at which time the significant increase in FA/CC indicates that angiogenesis could no longer keep pace with the hypertrophy. These results suggest a tight coupling of angio-
genesis and hypertrophy, which, in turn, might imply that both processes are initiated or driven by a common factor(s). An examination of the literature from the two areas of investigation suggests basic fibroblast growth factor (FGF-II) as one common element involved in both hypertrophy and angiogenesis.

Yamada et al. (35, 36) have reported that the level of FGF-II is higher in hypertrophied compared with control muscle and that FGF-II is produced in the walls of endothelial cells and stored in the basal lamina of the muscle fiber, where its activity is neutralized through binding with heparin. They also propose that FGF-II stimulates satellite cell activation and that muscle fiber inflammation and/or alterations of the basal lamina, through fiber damage or mechanical distortion of the muscle fibers, result in perturbation of the normal FGF-II-heparin interaction, allowing FGF-II to become active. It is well known that compensatory hypertrophy of the plantaris after synergistic ablation results in such muscle damage and inflammation (1).

In the case of endothelial cells, the angiogenic activity of FGF-II has been reviewed (17). FGF-II has been shown to be both chemotactic (3) and mitogenic (3, 30) for endothelial cells. Ingber and Folkman (33) suggest that mechanical stretching alters the endothelial cell response to FGF-II. Hudilcka et al. (10), in an extensive review of the numerous metabolic, mechanical, humoral, and hormonal factors involved in capillary development and adaptation in skeletal muscle, have suggested that damage to the basement membrane as a result of an increased wall tension, or through the release of lysosomal enzymes and acid proteases, is a necessary antecedent to angiogenesis. In support of this concept, Phillips and Knighton (24) have shown that extracts from skeletal muscle induce endothelial cell growth. Engelmann (8) has shown a "coordination" of extracellular matrix deposition and capillary proliferation in the developing heart by the cardiac myocyte acting via FGF-II, and Norrby (22) has recently demonstrated a nonlinear dose response in angiogenesis by FGF-II in mammalian tissue.

In summary, in the synergistic ablation model of muscle hypertrophy, the capillary supply to individual fibers was increased in proportion to the hypertrophy of the fibers, as evidenced by FA/CC remaining unchanged until day 21, with a significant increase in FA/CC being observed at day 30. Analysis of the time course of capillary development, i.e., the mean CC, and fiber hypertrophy yielded t1/2 of 10.1 and 11.2 days, respectively. The coincidental nature of the results of the time course data could suggest that the two processes are initiated and/or driven by some common factor(s).

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