ADULT SKELETAL MUSCLE is capable of significant growth during periods of increased loading. During functional overload (FO) induced by surgical ablation of synergist muscles, muscle mass and fiber cross-sectional area (CSA) increase in the remaining muscle(s) (28, 29, 33). This increase in fiber size is thought to occur via several mechanisms, including increased gene transcription and protein synthesis rate (7). The signals that initiate these intracellular changes are not well defined but are believed to include alterations in the cellular mechanical forces (37), as well as local and systemic release of growth factors (9, 37, 38).

As a result of FO, the number of myonuclei within a single fiber increases (3). These newly formed myonuclei are believed to arise when satellite cells, quiescent muscle stem cells that lay adjacent to the adult muscle fibers, are activated to proliferate during the early stages of FO and are subsequently incorporated into the muscle fiber (31, 32). Because an increase in myonuclear number expands the quantity of DNA available for protein production, the additional myonuclei may facilitate skeletal muscle hypertrophy. Two lines of evidence suggest a mechanistic role for the addition of myonuclei in the hypertrophic process. First, satellite cells have been reported to proliferate before muscle fiber hypertrophy in rats during FO of the soleus (33) or extensor digitorum longus muscle (8). The satellite cell and myonuclear numbers increase sequentially (32, 33) in a pattern consistent with the hypothesis that the new myonuclei arise from the incorporation of proliferating satellite cells. Second, inhibition of satellite cell activation and/or proliferation by γ-irradiation prevents hypertrophy of the rat extensor digitorum longus (27) and soleus (24) during FO, strongly implying that satellite cell activation and/or proliferation is a prerequisite for muscle hypertrophy.

Rosenblatt et al. (27) also reported a constant ratio of "myonuclei to myoplasmic volume" across a range of muscle fiber sizes after irradiation, FO, or FO plus irradiation in rats. This supports the proposition that increases in myonuclear number and cellular volume are proportional, such that the myonuclear domain size (defined as the volume of cytoplasm per myonucleus) of the muscle fiber remains constant. However, since Allen et al. reported modulation of myonuclear domain size of single muscle fibers after chronically increased or decreased loading in cats (3) and unloading in rats (4), there appear to be some conditions or stages of adaptation when the modulation in myonuclear domain size may not be tightly controlled.

In addition to mechanical loading, hormonal factors also influence cellular adaptations in muscle. Certain growth factors, particularly insulin-like growth factors (IGFs), are mitogens for myoblasts (12, 38) and satellite cells (5, 10) in vitro. Daily injections of growth hormone (GH) caused significant increases in fiber size and satellite cell number and a nonsignificant increase in myonuclear number in the extensor digitorum longus and soleus muscles of normal adult rats (36). When serum GH and IGF-I levels were elevated in vivo by GH-secreting tumors, satellite cell proliferation and hypertrophy of the soleus muscle were observed in growing young, but not mature, rats (22). These results demonstrate that GH and/or IGF-I elevation may enhance developmental muscle hypertrophy and further

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suggestion a role of growth factors in increasing satellite cell proliferation and myonuclear number.

A number of other studies have demonstrated an interactive effect of increased mechanical loading and administration of exogenous growth factors on skeletal muscle mass. For example, GH or IGF-I administration and brief bouts of daily resistance exercise resulted in a greater prevention of the atrophy accompanying hindlimb unloading than either factor alone (15, 18, 30). Additionally, a combination of exercise and GH/IGF-I treatment of hindlimb-suspended rats was recently shown to prevent most of the decrease in myonuclear number, whereas either factor alone did not (2). Finally, in vitro studies have demonstrated that passive mechanical stretch combined with IGF-I administration resulted in greater myofiber hypertrophy than either factor alone (38).

The purpose of the present study was to evaluate the effects of FO, both alone and combined with GH/IGF-I administration, on the myonuclear number and fiber size of the rat soleus muscle. The combination of GH/IGF-I administration and FO was expected to elicit greater hypertrophy than FO alone. Furthermore, if an increase in myonuclei is indeed a prerequisite for muscle fiber hypertrophy, then the additional hypertrophy of GH/IGF-I–treated animals should be accompanied by an increase in myonuclear number and the maintenance of myonuclear domain size.

METHODS

Experimental design. Adult female Sprague-Dawley rats (−250 g body wt) were used in these experiments. Animal care and use protocols were in accord with the Ames Research Center Animal User's Guide (AHB 7180) and the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Ames Research Center and the Animal Research Committee at the University of California, Los Angeles. All animals were housed in pairs and maintained in a room at 24 ± 1°C on a 12:12-h light-dark cycle and allowed normal cage activity. Rats were assigned randomly and equally to one of three conditions (n = 5/group): 1) control, saline injected (Con); 2) synergist ablated, saline injected (FO); or 3) synergist ablated, GH/IGF-I injected (FO + GH/IGF-I). FO rats were anesthetized with a combination of ketamine and xylazine, and a skin incision was made along the calf. The gastrocnemius and plantaris muscles were excised bilaterally, with care taken not to damage the neural or vascular connections of the soleus. After 7 days of recovery from the initial inflammatory phase (6), FO + GH/IGF-I rats were injected subcutaneously three times daily (at 0800, 1200, and 1600) for 14 days with recombinant human GH/IGF-I (Genentech, San Francisco, CA) at a total daily dosage of 1 mg/kg body wt each dissolved in 0.85% saline at concentration of 250 μg/ml. The Con and FO rats received a similar volume of saline injected at the same times. Combined GH/IGF-I injections were employed, because previous studies demonstrated that optimal elevation of serum IGF-I and minimal effects on insulin secretion are achieved with coinjection compared with injection of either growth factor alone (17, 19). Combined GH/IGF-I injections also increase anabolism (17, 19) and result in optimal secretion of IGF-I–binding proteins from the liver, thus increasing the half-life and potential tissue exposure of injected IGF-I (17). After 3 wk of FO the rats were killed, and the soleus muscles were dissected, cleaned of excess fat and connective tissue, wet weighed, frozen in Freon cooled in liquid nitrogen, and stored at −70°C until use.

Analysis of myonuclear number and fiber size. Single muscle fiber segments were microdissected and analyzed using confocal microscopy, as described previously (4). Briefly, the muscle was progressively thawed from −70 to −20°C, then placed in a −20°C 50% glycerol-50% low-calcium relaxing solution (11) and held overnight at −5°C. The microdissection procedure involved placing the intact muscle in a 100% relaxing solution to maintain the fibers in a pliant condition during the mechanical isolation. Care was taken to sample fibers from all regions of the muscle. Prior studies have shown that the basal lamina and any adhering mononucleated nonmuscle and satellite cells are stripped off during similar isolation procedures (16). We also previously showed by electron microscopy that our isolation procedure removes any extracellular nuclei adhering to the plasma membrane (34). In addition, only extremely scant laminin staining was found on occasional fiber segments isolated using the same technique (3). Thus we are confident that the overwhelming majority of nuclei counted in the present study were true myonuclei. Isolated fiber segments were placed on gelatin-coated slides and stored at −40°C until use. After they were thawed and air dried, fibers were stained for 5 min with 54 μM acridine orange and for 4 min with 1.5 × 10−7 M propidium iodide. PBS was used to rinse stains before the phosphate-buffered saline samples were mounted with glycerol under “strutted” coverslips.

Three 173-μm regions along the proximal, middle, and distal portions of each fiber segment were examined using confocal microscopy (Sarasota 2000, Molecular Dynamics, Sunnyvale, CA) and averaged to determine the CSA (μm²), myonuclear number (number of myonuclei/mm), and myonuclear domain size (cytoplasmic volume/myonuclear, μm³) of the fiber. First, the fibers were optically sectioned in steps of 1.0–1.5 μm to create a stack of images encompassing the entire fiber thickness. The number of myonuclei in each step was counted and summed to determine the total number of myonuclei in the viewing field. The stack images were optically rotated orthogonal to the long axis of the fiber, and the CSA was measured for two different regions within each viewing field using calibrated measurement software (Silicon Graphics, Salt Lake City, UT). Fiber CSA and myonuclear number were adjusted (normalized) to a sarcomere length of 2.5 μm, as previously described (4), to correct for any stretching or shortening of the fiber during the mechanical isolation. The cytoplasmic volume-to-myonuclear ratio was calculated by multiplying the fiber CSA by the viewing field (173 μm) and dividing by the number of myonuclei in that same field.

A total of 519 fibers were included in the statistical analyses, representing 178 fibers from the Con, 196 fibers from the FO, and 155 fibers from the FO + GH/IGF-I group. To determine a mean value for each rat, 35 ± 7 (SD) fibers were analyzed per muscle and averaged. The mean values for each rat within a group were averaged to determine the group mean. Analyses were performed to evaluate the adequacy of the fiber sample size of individual rats for detecting treatment group mean effects for fiber CSA. These analyses involved calculating the rolling cumulative mean of each additional five measurements. Beginning with the first measurement, sequential blocks of five measurements were drawn from the total pool of fibers available for each rat. From each rat's block means, treatment group rolling cumulative aver-
ages were then computed for each additional block of five measurements. After measurement of 20 fibers per rat, the treatment group means were within 2.7–9.6% of the means calculated using the total pool of measurements. As more fibers per rat were included, this percentage regressed closer to the mean calculated from all available measurements. The coefficients of variation within a given treatment group also had plateaued after measurement of 20–25 fibers. Moreover, after 20 fibers were measured the correlation between any subsequent means of five additional fibers and the means from the total pool of fibers ranged from 0.86 to 0.99, indicating that individual rat means were consistent after measurement of 20 fibers. Therefore, an average of 35 fibers per rat appears to be a more than adequate sample size to determine fiber CSA, and the present analysis indicates that the CSA means would not have changed substantially with additional measurements.

Statistical analysis. Analysis of variance was used to compare the group means for each of the dependent variables. When the F ratio was significant, Scheffe's post hoc test was used to compare between treatment groups. Pearson's product-moment correlation was computed to evaluate the relationship between myonuclear number and fiber CSA across or within groups. For all tests, an α-level of 0.05 was chosen for statistical significance.

RESULTS

Muscle wet weight. Three weeks of overload increased muscle wet weight by 32% in the FO compared with the Con rats (Fig. 1). Injections of GH/IGF-I combined with FO (FO + GH/IGF-I) increased the soleus wet weight by 21 and 59% compared with the FO and Con groups, respectively.

Fiber CSA. Fiber CSA was 11% larger in FO than in Con rats; however, this increase was not statistically significant (Fig. 2). CSA was 25 and 38% larger in FO + GH/IGF-I than in FO and Con rats, respectively.

Myonuclear number and domain size. Myonuclear number was 21 and 40% higher than control in the FO and FO + GH/IGF-I groups, respectively (Fig. 3). The 16% higher mean myonuclear number in FO + GH/IGF-I than in FO rats, however, was not statistically significant (P = 0.0582).

DISCUSSION

In the present study the greatest increase in soleus muscle weight occurred when FO was combined with GH/IGF-I administration. These results are consistent with the enhanced muscle hypertrophy observed in rats with GH-secreting tumors during postnatal growth (22). Although another study by Riss et al. (25) found comparable hypertrophy relative to body weight in the plantaris after FO in rats with and without GH-secreting tumors, they suggested that GH-induced muscle growth might occur by a mechanism different from work-induced hypertrophy. Other studies have reported an interactive effect of resistance exercise and GH administration in attenuating muscle atrophy during hindlimb suspension (15, 18). For example, Linder-
man et al. (18) reported maintenance of gastrocnemius myofibrillar protein content in hindlimb-suspended rats that performed resistive exercise and received GH injections but not in rats that received either intervention alone. Although the interaction effect was not observed in the soleus muscle by Linderman et al., Allen et al. (2) recently reported that resistance exercise combined with GH/IGF-I injections maintained soleus muscle mass, fiber CSA, and myonuclear number in hindlimb-suspended rats more effectively than either intervention alone (2). The present study also suggests that the effect of overloading the soleus is potentiated by GH/IGF-I treatment.

In the present study the myonuclear domain size was similar among groups, demonstrating that myonuclear number and fiber volume increased concomitantly during muscle hypertrophy. In addition, there was a significant positive correlation between the number of myonuclei per millimeter and fiber CSA across all treatment groups. Similar results were reported for fibers containing primarily slow myosin heavy chain in the cat plantaris after 3 mo of FO (3). However, although the mean myonuclear number was significantly increased in both FO groups compared with the Con group, the 16% higher mean in the FO + GH/IGF-I group was different from the FO group at the 0.0582 level of probability, rather than the usually accepted 0.05 level. Furthermore, although there was a significant increase in muscle weight in FO compared with Con rats, the 11% higher mean fiber CSA in the FO group was not significantly different from that in the Con group, as indicated by Scheffe’s post hoc test.

From the present results we can only speculate as to whether the fiber hypertrophy stimulated the increase in myonuclear number or vice versa, although the results of previous studies suggest that satellite cell activation, proliferation, and fusion are prerequisites for muscle fiber hypertrophy (8, 24, 26, 27, 33). The results of these previous studies suggested that proliferating satellite cells were the source of the new myonuclei, and our correlative results indicate that the increases in myonuclei and cell volume are proportional in the FO rat soleus. Together these findings suggest that one consequence of satellite cell mitogenic activity is the fusion of the progeny with existing myofibers, resulting in an increased net nuclear capacity to synthesize contractile proteins required for cellular hypertrophy. However, the exact mechanism(s) by which proliferating satellite cells become incorporated into existing myofibers as new myonuclei is not well defined.

The specific mechanisms by which GH/IGF-I treatment increased myonuclear number and fiber size to maintain a constant myonuclear domain size during FO cannot be determined from the present study. On the basis of in vitro (5, 10) and in vivo (22) studies, IGF-I could have stimulated the proliferation of satellite cells. Additionally, Vandenberg et al. (38) found that IGF-I stimulated hypertrophy and increased the number of myonuclei per millimeter in myofibers differentiated in vitro from primary satellite cells. Although the exogenous IGF-I may have acted directly on the satellite cells, the exogenous GH could have exerted its effects by stimulating systemic (i.e., hepatic) and/or local autocrine/paracrine IGF-I expression in muscle (20, 35). Moreover, mechanical work/overload of rat hindlimb muscle increases IGF-I mRNA (9, 20) and peptide (1) concentrations within the overloaded muscle. Recently, Adams and Haddad (1) reported a positive relationship between IGF-I protein and total DNA content in rat plantaris muscle during FO and speculated that the elevated DNA content was due to satellite cell proliferation stimulated by the locally produced IGF-I. In their study, similar responses also were observed in FO hypophysectomized rats, indicating that the hypertrophic mechanisms were independent of systemic GH or IGF-I and apparently related to the elevated muscle IGF-I (1). Thus the administration of GH/IGF-I in the present study most likely augmented the actions regulated by the work-induced elevation of these growth factors.

In the present study, direct GH actions also may have occurred given that the GH receptor and GH-binding protein transcripts have been localized to myonuclei of adult rat hindlimb muscle fibers (23). Additionally, local infusion of GH increases muscle protein synthesis
rates in humans (13, 14). Further investigation is required to elucidate the mechanisms by which GH and IGF-I could exert their effects on skeletal muscle independently and in conjunction with mechanical overload.

In conclusion, during FO with and without hormone treatment, myonuclear number and fiber size were coupled, such that the myonuclear domain size was maintained. On the basis of the results of the present and previous studies (8, 24, 26, 27, 33), we hypothesize that an increase in myonuclear number may be a prerequisite for prolonged and substantial work-induced skeletal muscle fiber hypertrophy. Implicit in this hypothesis is the assertion that the increased myonuclear number supplements the existing genetic machinery in the synthesis of new contractile proteins to meet the demands of the mechanical overload.

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