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Effect of training and growth hormone suppression on insulin-like growth factor I mRNA in young rats

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Divisions of Respiratory and Critical Care and of Endocrinology, Department of Pediatrics, Harbor-University of California Los Angeles Medical Center, Torrance, California 90509; and Section on Molecular and Cellular Physiology, Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Zanconato, Stefania, David Y. Moromisato, Mark Y. Moromisato, Janice Woods, Jo Anne Brasel, Derek Leroith, Charles T. Roberts, Jr., and Dan M. Cooper. Effect of training and growth hormone suppression on insulin-like growth factor I mRNA in young rats. *J. Appl. Physiol.* 76(5): 2204–2209, 1994.—The growth hormone (GH)-insulin-like growth factor I (IGF-I) axis plays a role in the adaptation to exercise training, but IGF-I gene expression in response to exercise training and GH suppression has not been studied. Twenty female rats underwent a 4-wk treadmill training program begun in the prepubertal period (*day 14* of life). In 10 of the training rats, GH production was suppressed by anti-GH-releasing hormone antibodies (GH suppressed). IGF-I mRNA and protein levels were measured in liver and hindlimb skeletal muscle. GH suppression reduced IGF-I mRNA expression in the liver to a much greater extent than in the muscle. In the GH control rats, training induced significant increases in hepatic exon 1-derived IGF-I mRNA (mean increase 30%; $P < 0.05$) and muscle exon 2-derived mRNA (mean increase 35%; $P < 0.05$). In the GH-suppressed rats, only muscle exon 1-derived transcripts were significantly increased by training (55%; $P < 0.05$) and this was associated with a significant increase in muscle IGF-I protein levels ($P < 0.05$). We speculate that the anabolic response to training may involve both GH-dependent increases in IGF-I mRNA in the liver and GH-independent increases in the muscle.

liver; hindlimb muscle; insulin-like growth factor I protein level

PHYSICAL ACTIVITY influences tissue growth, but the mechanisms linking exercise with muscle hypertrophy (23), increased capillarization and mitochondrial capacity (6), and stronger bones (20) are not completely understood. Studies in humans and other mammals support a role for growth hormone (GH) (7, 11), which is now known to stimulate growth primarily through stimulation of hepatic insulin-like growth factor I (IGF-I) production. Other studies, however, have shown that increased work load in muscle may stimulate local tissue production of growth factors (like IGF-I) in the absence of GH (14). Finally, little is known about tissue responses to physical activity in young still-developing animals in which somatic growth is rapid and secretory patterns of GH and other growth factors are in a state of flux.

We recently examined the specific role of GH in the functional responses to exercise training. Prepubertal female rats underwent a 4-wk treadmill running program. GH secretion was suppressed noninvasively by administering anti-GH-releasing hormone (GHRH) antisera

(10). As expected, GH suppression virtually eliminated circulating GH and IGF-I and substantially reduced body weight; in addition, GH-suppressed rats responded to training with increases in treadmill running endurance time, maximal oxygen uptake, and muscle succinate dehydrogenase (an indicator of mitochondrial function) that were no different from those found in the control rats.

Although the GH-suppressed rats had markedly reduced serum concentrations of IGF-I, serum levels alone do not necessarily reflect growth factor responses at the tissue level. The increase in functional adaptations to exercise observed in the GH-suppressed rats suggested the hypothesis that exercise could stimulate local tissue increases in IGF-I mRNA and/or protein levels even in the absence of normal pituitary GH activity. To test this hypothesis, we measured IGF-I mRNA and protein levels in the hindlimb muscle and liver of trained and untrained GH control and GH-suppressed rats.

METHODS

Population. Forty female Sprague-Dawley rats comprised the study population and were selected from a larger sample described previously (10). Rats from mixed litters were brought to the laboratory at postnatal *day 10* in litters of 10, accompanied by a dam, and were allowed to become familiar with their surroundings and handlers. They were separated from the dam at postnatal *day 21* and were allowed standard rat food and water ad libitum. These studies were approved by the Animal Care and Use Review Committee of Harbor-UCLA Medical Center.

The study was designed to examine four groups of rats: *group I*, GH control (normal GH secretory capacity) and untrained ($n = 10$); *group II*, GH control and trained ($n = 10$); *group III*, GH suppressed (by passive immunization with anti-GHRH antibodies) and untrained ($n = 10$); and *group IV*, GH suppressed and trained ($n = 10$).

GH-suppressed rats received subcutaneous injections of 250 μ l of rabbit anti-GHRH antisera three times weekly beginning on postnatal *day 13*. The anti-GHRH antisera were generously provided to us by Dr. William Wehrenberg (28). GH control rats received subcutaneous injections of 250 μ l of control rabbit serum three times weekly. Training began at postnatal *day 14*. We used a 4-wk treadmill training protocol developed by MacIntosh and Baldwin (22) specifically for neonatal rats; this protocol has been demonstrated to result in increased muscle cytochrome oxidase activity. Throughout the 4-wk training period, running duration, treadmill incline, and treadmill speed increased progressively such that, by the end of training, rats in

both training groups ran for 60 min at a 25% incline and at a speed of 28 m/min. At the end of the training period, the rats were killed and liver and hindlimb skeletal muscle were dissected and immediately frozen in liquid nitrogen.

RNA extraction. RNA extraction from liver and skeletal muscle was performed according to the method of Chomczynski and Sacchi (9). RNA preparations were dissolved in sterile water and stored at -70°C . RNA was quantitated spectrophotometrically. The quantitation and integrity of the RNA were then confirmed by visual inspection of the ethidium bromide-stained 28S and 18S rRNA in 10- μg aliquots of RNA that had been electrophoresed through 1.25% agarose-2.2 M formaldehyde gels.

Solution hybridization-ribonuclease (RNase) protection assay. The antisense RNA probe used to identify exon 1 and 2 IGF-I mRNAs has been previously described (2). This probe was complementary to sequences derived from exon 1 (310 bp) and exons 3 and 4 (176 bp) DNA (2). Solution hybridization-RNase protection assays were performed as described by Lowe et al. (21). Dried gels were exposed to Kodak X-Omat AR film at -70°C with two intensifying screens for the indicated period of time. Autoradiographs were scanned, and the intensity was digitized by computer. The Quantiscan software package (Bio-software, Cambridge, UK) was used for subsequent densitometric analyses.

β -Actin. To determine the specificity of the training- and GH suppression-induced changes in IGF-I gene expression, Northern blots of liver and hindlimb skeletal muscle RNAs were hybridized to a rat β -actin cDNA probe that was labeled with [α - ^{32}P]dCTP by random priming.

Tissue IGF-I. IGF-I was extracted from liver and muscle following the method described by D'Ercole et al. (13). Briefly, each tissue was pulverized, and IGF-I was extracted by adding 5 ml of 1 M acetic acid for each gram of tissue. After centrifugation, the supernatant was removed and subjected again to extraction with acetic acid. The two supernatants were then combined, frozen at -70°C , lyophilized, and reconstituted with 0.05 M tris(hydroxymethyl)aminomethane-HCl buffer (pH 7.8). The extract was clarified by centrifugation and frozen at -20°C . The radioimmunoassay for IGF-I was carried out using the method described by Daughaday et al. (12) and using antibodies obtained from National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program.

Statistical analysis. Each individual tissue sample contained bands representing IGF-I mRNAs from five separate transcription initiation sites (start sites) (see Fig. 1). We calculated the relative contribution (percentage of total density) of exon 1 start sites 1, 2, 3, and 4 and exon 2 in GH control untrained rats. Analysis of variance (ANOVA) was performed on these data, and the *t* test (modified by the method of Duncan) was then used for intergroup comparisons.

To determine the effects of GH suppression and exercise training, we focused on the gene expression of exons 1 and 2 IGF-I mRNA, calculated as the sum of the respective start site densities. Each gel contained two to three samples from each of the four experimental groups noted above. To take into account any gel-to-gel variability, our statistical approach consisted of a mixed-model ANOVA as described in the BMDP3V statistical software package (15a) in which the gel factor was considered as a random effect.

Differences in liver and muscle IGF-I protein levels among the four groups were determined by randomized ANOVA. When ANOVA was significant, intergroup comparisons were tested using Duncan's modification of the *t* test. $P < 0.05$ was considered statistically significant. All results are expressed as means $\pm$ SE.

FIG. 1. Example of solution hybridization-RNase protection assay using 20- μg aliquots of total RNA from hepatic tissue. Four columns represent individual rats: growth hormone (GH) control and untrained (GHc-U), GH control and trained (GHc-T), GH suppressed and untrained (GHs-U), and GH suppressed and trained (GHs-T). Arrows at left, probe bands corresponding to RNAs resulting from use of indicated start sites. Autoradiogram was deliberately overexposed to allow visualization of lighter bands in treated rats.

RESULTS

Muscle and liver IGF-I gene expressions. Examples of autoradiographs from liver and muscle in the four groups of rats are shown in Figs. 1 and 2. Five major protected bands were observed with the exon 1-derived IGF-I antisense RNA probe and were similar to those previously reported (2). The smallest of these, at ~ 168 bp, represents exon 2 mRNAs. The remaining bands represent mRNAs derived from exon 1. The largest full-length protected band (at ~ 530 bp) reflects a combination of mRNAs initiated at start sites 1 and 2 in exon 1. The next band, at ~ 428 bp, represents mRNA initiated at start site 3. The band at ~ 270 bp represents those exon 1 mRNAs that result from complete splicing of transcripts initiated at start sites 1 and 2 (spliced). The band at ~ 197 bp represents start site 4.

As previously described (26), transcription initiation site usage differed in muscle compared with liver. In liver (GH control untrained rats), exon 1 start site 1 and 2 mRNAs together accounted for $46 \pm 1\%$ of total IGF-I mRNA, whereas in muscle these mRNA species accounted for a much smaller proportion ($16 \pm 2\%$; $P < 0.01$). Exon 1 start site 3 mRNA composed only $26 \pm 1\%$ of total IGF-I mRNA in liver samples but was $61 \pm 4\%$ ($P < 0.01$) in muscle. Finally, exon 2-derived mRNA accounted for the remaining $23 \pm 1\%$ of total IGF-I mRNA in liver but was $15 \pm 1\%$ in muscle ($P < 0.05$).

FIG. 2. Example of solution hybridization-RNase protection assay using 20- μ g aliquots of total RNA from skeletal muscle. Lane 1: liver used as control (Li). Lanes 2-5: muscle tissues from individual rats. Four micrograms of total RNA were used for liver sample. Arrows at left, probe bands corresponding to RNAs resulting from use of indicated start sites.

FIG. 3. Effect of treatment with anti-GH-releasing hormone (GHRH) antibodies and training on insulin-like growth factor I (IGF-I) mRNA levels in liver. Liver exon 1 and 2 transcripts were significantly reduced by GH suppression both in untrained and trained rats ($\dagger P < 0.0001$). In GH control rats, training induced a significant increase in exon 1 IGF-I mRNA ($* P < 0.05$).

Effect of treatment with anti-GHRH antibodies on IGF-I mRNA levels. Suppression of GH led to a dramatic reduction of hepatic IGF-I gene expression both in untrained and trained rats ($P < 0.0001$; Fig. 3). The reduction in hepatic IGF-I mRNA was greater for exon 2 (10% of GH control values) than exon 1 transcripts (25% of GH control values; $P < 0.05$). The pattern of suppression was not the same in the muscle (Fig. 4). Although GH

FIG. 4. Effect of treatment with anti-GHRH antibodies and training on IGF-I mRNA levels in hindlimb muscle. Exon 2 mRNA was significantly reduced by GH suppression in trained rats ($\dagger P < 0.01$), whereas, in untrained rats, no significant changes were observed in either exon 1 or 2 mRNAs. In GH control rats, training induced a significant increase in exon 2 transcripts ($* P < 0.05$), whereas in GH-suppressed rats, a significant increase was observed for exon 1 mRNA ($** P < 0.05$).

suppression reduced muscle IGF-I gene expression in both trained and untrained rats, only the reduction in exon 2 mRNA in trained rats (68% of the GH control values) was significant ($P < 0.01$). However, because exon 2 mRNA accounted for such a small proportion of total IGF-I mRNA levels, it is unlikely that the reduction in exon 2 alone significantly affected IGF-I levels.

FIG. 5. Liver IGF-I levels in 4 groups of rats. Treatment with anti-GHRH antibodies induced significant reduction in IGF-I levels in both sedentary and trained rats (* $P < 0.001$). No significant exercise-induced changes were observed in either GH control or GH-suppressed rats.

Effect of training on IGF-I mRNA. In the GH control rats, training significantly increased hepatic exon 1 (30% greater than in untrained rats; $P < 0.05$) and muscle exon 2 (35% greater than in untrained rats; $P < 0.05$) transcripts (Figs. 3 and 4). A small, but not significant, increase was observed for liver exon 2 and muscle exon 1 transcripts. In the GH-suppressed rats, training had no significant effect on hepatic IGF-I mRNA levels, whereas a significant exercise effect was found in muscle exon 1 mRNA (55% greater than in untrained rats; $P < 0.05$).

β -Actin mRNA expression. Neither treatment with anti-GHRH antibodies nor training induced significant changes in liver and muscle β -actin mRNA expression.

Tissue IGF-I protein levels. GH suppression induced a significant reduction in liver IGF-I levels ($P < 0.001$; Fig. 5) in both the trained and untrained rats. In the GH control rats, IGF-I levels were higher in the trained than in the untrained rats, but the difference did not achieve statistical significance. No significant differences were found in muscle IGF-I levels between GH control and GH-suppressed rats (Fig. 6). Training did not significantly affect IGF-I levels in the GH control rats, but, in the GH-suppressed rats, training led to a significant increase in muscle IGF-I levels ($P < 0.01$).

DISCUSSION

This study shows that in young female rats with normal pituitary function improved exercise responses (10) are accompanied by significant increases in both hepatic and skeletal muscle IGF-I mRNA. In rats with suppressed GH, a functional training response was also observed and was accompanied by significant IGF-I mRNA increases in the muscle. A significant increase in hepatic IGF-I mRNA was not observed after training in the GH-suppressed rats.

Exercise-induced structural anabolic changes in skeletal muscle were not examined in this study. However, numerous previous studies, using different exercise protocols, have demonstrated that physical training results in muscle hypertrophy (6, 27) and increased capillariza-

tion (23). There is much evidence supporting a specific role for IGF-I in the response to training. IGF-I has a mitogenic effect on myoblasts and fibroblasts (5, 16); increases net protein accumulation during muscle hypertrophy (5); and stimulates both proliferation and differentiation of muscle satellite cells (4), the precursor cells for muscle growth and hypertrophy in the postnatal organism.

Moreover, a role for IGF-I in muscle hypertrophy is supported by previous investigations of DeVol et al. (14). In their studies, surgical disruption of the achilles tendon led to muscle hypertrophy and increased muscle production of IGF-I mRNA in compensatory muscle groups in both intact and hypophysectomized rats. Similarly, in earlier works, muscle hypertrophy (17) and increased muscle mitochondrial function (measured as increased succinate dehydrogenase) (18) were found in rats despite hypophysectomy. Finally, Yan et al. (30) showed recently that nonvoluntary electrically contracted skeletal muscle exhibits increased IGF-I immunoreactivity. These observations, along with the increases in skeletal muscle IGF-I mRNA and protein levels that we found, support the hypothesis that IGF-I produced in the exercising tissue may play a role in the mechanism through which repeated physical activity leads to tissue growth.

Skeletal muscle IGF-I mRNA was increased by training in both GH-suppressed and control rats, but hepatic IGF-I mRNA was increased only in control rats. The different response might be related to the different pattern of IGF-I gene expression in liver compared with skeletal muscle. Our finding in muscle that the major downstream transcription start site, start site 3, predominated is consistent with two recently published studies (19, 26) of IGF-I gene expression in extrahepatic tissues. It is hypothesized that the tissue-specific transcription pattern reflects tissue-specific regulation of IGF-I gene expression (26).

Consistent with this is the observation that GH suppression inhibited IGF-I mRNA to a much greater extent in the liver than in the muscle. In the sedentary rats,

FIG. 6. Muscle IGF-I levels in 4 groups of rats. No significant changes were observed with GH suppression. In GH-suppressed group, IGF-I levels were significantly greater in trained than in untrained rats (* $P < 0.01$).

anti-GHRH antibodies reduced hepatic IGF-I transcripts by ~80–90%, whereas in the muscle the reduction was only ~20–30%, and qualitatively similar results were found by other investigators using hypophysectomy followed by GH injection (21). These data suggest that multiple, as yet undiscovered, factors control IGF-I production in the extrahepatic tissues and that GH is a less important regulator of IGF-I production in muscle compared with liver.

In addition, our study demonstrates a complex interaction among GH suppression, exercise, and IGF-I gene expression. Surprisingly, in the GH control rats, only exon 1 IGF-I mRNA, which is known to be less GH dependent than exon 2 mRNA, and muscle exon 2 mRNA were increased by exercise. On the contrary, in the GH-suppressed rats, training induced a significant increase in muscle exon 1 mRNA and no changes in liver IGF-I mRNA. The mechanisms and the factors controlling the differential expression of the two IGF-I gene leader exons remain unknown. These results, however, suggest that the exercise-induced IGF-I gene stimulation is more GH dependent in the liver than in the muscle and that GH suppression leads to an enhancement of the local factors regulating the expression of exon 1 mRNA in skeletal muscle during exercise.

GH suppression reduced both liver IGF-I protein and mRNA to ~20% of the original values; moreover, training significantly increased both muscle IGF-I protein and mRNA in the GH-suppressed rats. These results are consistent with observations that IGF-I biosynthesis is regulated, in large part, by control of mRNA abundance (1). There was, however, an apparent dissociation between IGF-I gene expression and tissue protein levels in GH control rats in which we observed an exercise-induced increase in hepatic IGF-I mRNA without significant increases in hepatic IGF-I protein or in circulating IGF-I [note: the liver is considered to be the principal source of IGF-I found in the circulation (25)].

It is possible, therefore, that there was no increase in hepatic IGF-I protein production after exercise in GH control rats despite the increase in IGF-I mRNA. It is now generally recognized that increases in gene expression do not invariably lead to increased protein production. However, some caution ought to be exercised before this conclusion is reached for several reasons. First, the level of tissue IGF-I protein may not, in and of itself, reflect protein production. Tissue levels are ultimately determined by the rates of production, metabolism, and, finally, secretion into the circulating blood, and the liver is a metabolically active and highly vascular organ (note: similar caution must be exercised when attempting to determine changes in the rate of protein production from changes of protein concentration in the circulating blood).

Second, there are data supporting a role for the GH-IGF-I axis in the adaptation to exercise that would, presumably, involve hepatic production and release of IGF-I into the circulation. There are two independent studies in humans showing that circulating IGF-I is correlated with the degree of physical fitness (20, 24), although resistive muscle training seems not to increase circulating IGF-I in well-trained subjects (15, 31). Weltman et al.

(29) found an increased pulse amplitude of spontaneous GH secretion in women who had undergone 1 yr of endurance exercise training. Finally, Borer et al. (8) demonstrated that exercise-induced somatic growth in the hamster was accompanied by increases in spontaneous GH pulse amplitude and frequency.

The relationship of IGF-I mRNA and protein levels to IGF-I biological activity is additionally complicated by mounting evidence showing that IGF-I actions can be regulated by tissue and circulating binding proteins rather than by changes in tissue or circulating IGF-I levels themselves (25). We speculate that there is a redundant or hierarchical set of mechanisms by which the body adapts to repeated physical activity.

In conclusion, our study shows that endurance training induced an increase in liver and muscle IGF-I mRNA in rats with normal GH secretory capacity and a significant increase in IGF-I mRNA and protein in the muscle of GH-suppressed rats. The training program was begun in prepubertal female rats, and, although we did not perform comparable experiments in more mature rats or in males, it is quite likely that both gender and maturation influence growth factor responses to training in as yet undiscovered ways. We speculate that, when GH is suppressed, the increase in IGF-I in the working muscles could be sufficient to help bring about the functional tissue adaptations to repeated physical activity. In addition, we speculate that the previously shown exercise-induced anabolic effects of physical activity might be at least partially mediated by an increased production of muscle IGF-I that appears to be independent of GH.

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