Effect of Voluntary Exercise on the Expression of IGF-I and Androgen Receptor in Three Rat Skeletal Muscles and on Serum IGF-I and Testosterone Levels

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Abstract

The effects of anabolic agents and training on skeletal muscle are believed to be mediated by a variety of growth and transcription factors. Among these regulatory proteins, insulin-like growth factor-I (IGF-I) and androgen receptor (AR) play a crucial role. The purpose of this study was to investigate the effects of wheel running on IGF-I and AR mRNA expression in three distinct rat skeletal muscles (i.e., gastrocnemius, vastus lateralis, and soleus), as well as on the serum levels of IGF-I and testosterone. Twenty male Wistar rats were housed in cages with free access to running wheels for 12 weeks, while nine rats served as controls. Analysis of the mRNA expression of IGF-I and AR using real time RT-PCR revealed no significant differences between the trained and untrained rats in any of the muscles studied. Enzyme immunoassay showed significantly lower serum levels of IGF-I and testosterone in the trained compared to the untrained animals. These results suggest that chronic exercise in wheels does not affect IGF-I and AR mRNA levels in rat skeletal muscle, while decreasing the circulating levels of two anabolic factors, i.e., IGF-I and testosterone. It is concluded that IGF-I, AR and testosterone seem to play a marginal role during the adaptation process of rat skeletal muscle to long-term wheel running.

Key words
Gene expression · serum hormones · real time RT-PCR

Introduction

In response to changing environmental demands, skeletal muscle fibers are able to alter their structural and functional properties, which enables them to better maintain homeostasis. For example, increased contractile activity elicited by endurance training induces a set of metabolic adaptations that make skeletal muscle more suitable to meet the energy demands of sustained activity [5]. Undoubtedly, the remarkable capacity of skeletal muscle for long-term adaptations to endurance training is mediated by changes in the expression of several regulatory genes. However, despite the profound and well-studied effects of endurance training on muscle phenotype, little is known about the molecular mechanisms that link the "exercise signals" to the modulation of muscle characteristics. Two proteins which are believed to have a functional role in this molecular signal transduction cascade are insulin-like growth factor-I (IGF-I) and androgen receptor (AR).

IGF-I is a ubiquitous polypeptide with paracrine and autocrine action, which influences cell proliferation and differentiation as well as stimulating protein synthesis in many tissues [33]. It has been recently demonstrated that IGF-I induces hypertrophy of skeletal muscle [1]. Endurance training, apart from the well-described effects on the oxidative metabolic machinery, can also elicit muscle hypertrophy [18], and recent investigations suggest

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that IGF-I might mediate this process [1]. It was originally postulated that IGF-I production was predominantly stimulated by growth hormone, but it now appears that IGF-I production can increase independently of growth hormone in a number of situations, including exercise [14]. Nevertheless, despite its emerging role as an anabolic agent, few studies have tried to delineate the effects of exercise (either acute or chronic) on IGF-I expression in skeletal muscle [3,14,39].

The anabolic effects of androgens (mainly testosterone) on skeletal muscle are well described, but the mechanism by means of which an androgen controls muscle size is poorly understood [25]. Androgen action is mediated by AR, a ligand-dependent transcription factor belonging to the superfamily of nuclear receptors [8]. Data on the effects of exercise on the expression of AR, either at the mRNA or protein level, are scanty in skeletal muscles; increased or stable levels of AR have been reported after acute and chronic exercise in rats [12], while data regarding the effect of acute exercise on AR mRNA are limited to human skeletal muscle, with only one biopsy taken 48 h post exercise [3].

To get an insight into the molecular mechanisms of skeletal muscle adaptation, the aim of the present study was to analyze the possible changes in the expression of IGF-I and AR during the adaptation of skeletal muscle to chronic exercise. Therefore, male rats were trained by wheel running, a stress-free model with moderate muscular activity, leading also to muscle hypertrophy in spite of its endurance-oriented character [18]. Because there is some evidence that the adaptive response of skeletal muscle to an exercise stimulus is muscle-type specific [30] — and this has been found to be the case with AR [12] — mRNA expression was investigated in three skeletal muscles (gastrocnemius, vastus lateralis, and soleus) by real time RT-PCR. Along with muscle mRNA, we measured serum IGF-I and testosterone to investigate possible relationships between blood levels of anabolic factors and local tissue responses to the training stimulus.

Materials and Methods

Animals

Twenty-nine male Wistar rats (6 weeks old, weighing 194 ± 9 g, mean ± SD) were purchased from Charles River Laboratories (Sulzfeld, Germany) and housed under controlled environmental conditions (21 °C, 12 : 12-h light-dark cycle). Rats were allowed free access to standard rodent chow from Ssniff (Soest, Germany) and water. The animals were maintained according to the European Union guidelines for the care and use of laboratory animals and the study was undertaken with the approval of the Regional Administration of Cologne City (Bezirksregierung Köln).

Training protocol

The animals were divided randomly into a trained (n = 20) and an untrained group (n = 9). Rats of the trained group were housed in cages equipped with wheels (where they exercised *ad libitum*) for 12 weeks, while those of the untrained group were housed in plain cages. The running activity of the trained group was recorded continuously through the DasyLab 5.0 data collection system from Datalog (Mönchengladbach, Germany). Body weight was monitored weekly.

Blood sampling and muscle dissection

Upon completion of the training period all animals were killed by decapitation at approximately the same time of day (9 – 11 a. m.). Wheels and food had been removed from the cages 12 h earlier, in order to avoid possible influences of the last exercise bout and the last feeding on the parameters measured. Blood was collected and allowed to clot at room temperature for 30 min and was centrifuged at 8 °C for 10 min. Serum was separated promptly and was stored at – 20 °C until analysis. Additionally, the gastrocnemius, vastus lateralis and soleus muscles were removed from the right hindlimb of the six most active animals (running distance 7.5 – 10.8 km/day) and six untrained animals. The muscles were immediately immersed in liquid nitrogen and subsequently stored at – 80 °C until analysis.

mRNA analysis

Approximately 50 mg of frozen muscle tissue were homogenized by manual mortar and pestle grinding and were used for RNA extraction using the TRIzol® Reagent from Invitrogen (Karlsruhe, Germany). The purity and yield of total RNA were determined by measuring the absorbance of aliquots at 260 and 280 nm. The integrity of RNA was confirmed by inspecting the electrophoretic pattern of 28 S and 18 S ribosomal RNA in ethidium bromide-stained 2% agarose gels visualized under ultraviolet light. Total RNA (1.0 µg) was treated with DNase I (Invitrogen) and reverse transcribed using the SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen). Synthesized cDNA was amplified in the i-cycler from Bio-Rad (Munich, Germany) using Taq DNA polymerase (5 U/µL) in a final volume of 50 µL. IGF-I and AR cDNAs were amplified together with cyclophilin (CYP) and cytochrome c oxidase subunit 1 (CCO) cDNAs, which were used as endogenous controls (i. e., reference genes). All primers used were deduced and optimized for real-time RT-PCR analysis (annealing temperature 58 °C for all primers) and are listed in the Table **1**. The value corresponding to the threshold cycle (C_T) of the real-time RT-PCR was measured in triplicate. A ΔC_T value was calculated for each sample by subtracting the C_T value of the gene treated as the reference gene from the C_T value of the gene of interest. To reduce intersubject variation, all samples were normalized to the ΔC_T value of a control sample derived from the same untrained animal ($\Delta\Delta C_T$) for all assays. The relative expressions of IGF-I and AR were calculated using the expression $2^{-\Delta\Delta C_T}$ and are reported as arbitrary units.

Hormone assays

Serum IGF-I and testosterone were measured in duplicate by enzyme immunoassay in an Anthos 2000 (Salzburg, Austria) photometer with the appropriate kits from DRG (Marburg, Germany). The intra- and inter-assay coefficient of variation for IGF-I was 7.4 and 9.5%, respectively. The sensitivity for IGF-I was approximately 30 ng/mL. The corresponding values for testosterone were 6.6%, 7.4%, and 0.1 ng/mL.

Statistical analysis

All values are expressed as the mean ± SD. Cell means of body weight were compared through two-way (training status × time) ANOVA with repeated measures on time. Muscle mRNA levels of

Table **1** Sequence of PCR primers used in the study

Gene	*Primer sequence*	*($MgCl_2$), mM*	*Product size, bp*
Cyclophilin	Sense: 5′-GGA TTC ATG TGC CAG GGT GG-3′ Antisense: 5′-CAC ATG CTT GCC ATC CAG CC-3′	3.0	212
Cytochrome c oxidase subunit 1	Sense: 5′-CGT CAC AGC CCA TGC ATT CG-3′ Antisense: 5′-CTG TTC ATC CTG TTC CAG CTC-3′	1.5	211
IGF-I	Sense: 5′-GCT CTT CAG TTC GTG TGT GG-3′ Antisense: 5′-GTG TAC TTC CTT TCC TTC TC-3′	1.5	272
Androgen receptor	Sense: 5′-ATG AAG CAG GGA TGA CTC-3′ Antisense: 5′-AAG TTG CGG AAG CCA GGC-3′	1.5	324

($MgCl_2$), $MgCl_2$ concentration in the PCR reaction

the two groups were compared with the Mann-Whitney U test, while serum hormone levels were compared by performing the Student's *t* test for independent samples. The level of statistical significance was set at $\alpha = 0.05$ for all analyses.

Results

Body weight

Body weight of the animals increased significantly during the 12-week period of the experiment ($p < 0.001$, Fig. **1**). Overall, body weight of the trained animals was significantly lower compared to the animals of the untrained group ($p = 0.002$).

Running activity

Fig. **2** illustrates the daily distance run by the training group per week. The peak of wheel running activity was observed during the 2nd, 3rd, and 4th week. Running activity fell below the initial rate after the 7th week. The running activity of all twenty animals of the training group averaged 5.6 ± 3.0 km/day. Analyzing the individual daily running activity of the trained group we observed a great variability (running distance 0.5 – 18.3 km/day). Based on this fact and taking into consideration the complexity of mRNA analysis, six trained animals displaying the highest activity (see Fig. **2**) and six untrained animals were chosen for further analysis of local mRNA expression.

mRNA analysis and serum hormones

There were no significant differences in the mRNA expression of IGF-I (Fig. **3**) and AR (Fig. **4**) between the six most trained and the six untrained animals in any of the three muscles studied, regardless of the reference gene used for normalization. In order to further diminish data variability and to exclude effects caused by variations in reference genes, the mRNA data of IGF-I and AR were normalized to the values of two different reference genes (CYP and CCO), which served as endogenous controls. On the contrary, we found significantly lower serum concentrations of both IGF-I ($p < 0.001$) and testosterone ($p = 0.023$) in the trained animals compared to the untrained ones (Fig. **5**).

Discussion

The aim of this investigation was to analyze the possible role of IGF-I and AR during the adaptation of skeletal muscle to chronic exercise. Therefore, male Wistar rats were trained by voluntary wheel running, a stress-free exercise model. Running activity influenced body weight of the animals and displayed large inter-individual differences as well as weekly fluctuations during the 12-week training period. The lower body weight of the trained compared to the untrained rats and the pattern of running activity of the trained rats (i.e., initial increase followed by stabilization and then decrease) found in the present study are in accordance with previously reported data (e.g., [2, 31]).

The main finding of the present study was that chronic exercise in wheels affected neither IGF-I nor AR mRNA expression in the three rat skeletal muscles studied. The uniform response of the three muscles is a somewhat unexpected finding, judging from the different fiber type composition of the muscles (soleus contains predominantly type I fibers, whereas gastrocnemius and vastus lateralis contain predominantly type II fibers, according to Delp and Duan [11]) and their different recruitment pattern

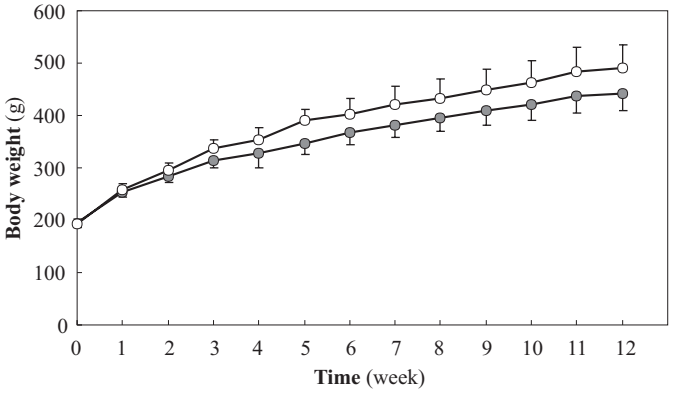

Fig. **1** Development of body weight of the untrained (○ n = 9) and trained (● n = 20) animals. Error bars denote SD.

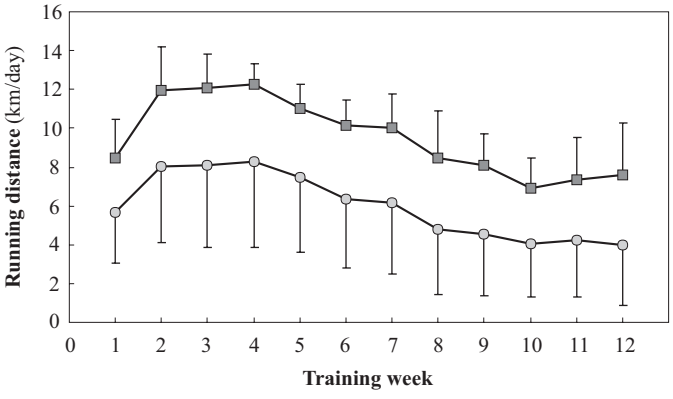

Fig. **2** Daily distance run by the trained (● n = 20) and the six most active rats (■) per week. Error bars denote SD.

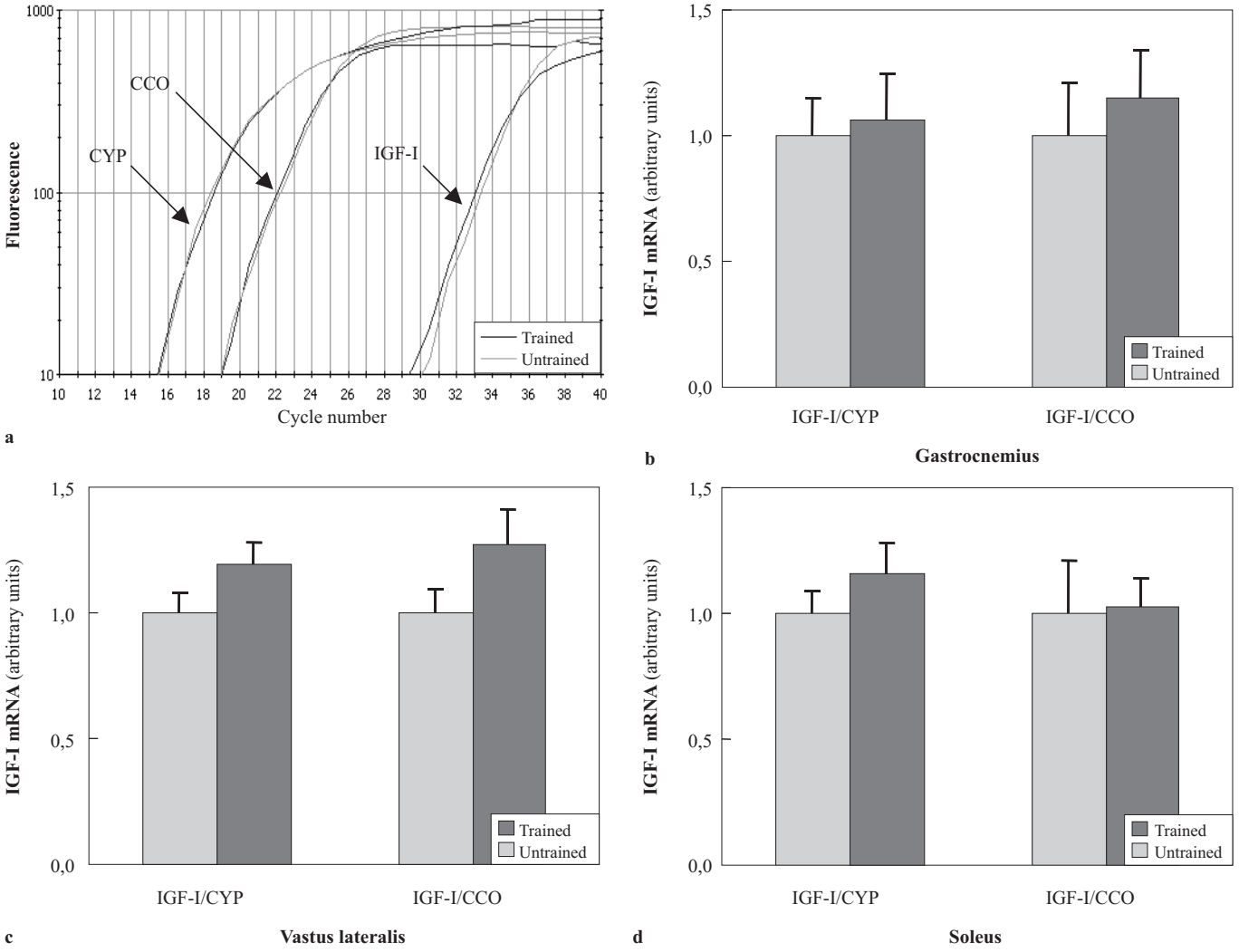

Fig. **3 a** to **d** IGF-I mRNA levels of six untrained and the six most trained rats in three muscles, normalized against two different reference genes, cyclophilin (CYP) and cytochrome c oxidase subunit 1 (CCO). **a** Representative original amplification curves from the I-cycler. **b – d** mRNA levels in gastrocnemius, vastus lateralis and in soleus muscle. Error bars denote SD.

during running in rat [10,34]. However, we believe that one can not expect different mRNA responses of different muscles based solely on their variant physiological characteristics. This is evident by the fact that many studies have reported skeletal muscle-independent mRNA responses to either acute or chronic exercise (e.g., [27,35,36]), even though muscle-dependent mRNA responses have been also frequently reported (e.g., [6,19,24]).

IGF-I and AR mRNA expression was analyzed by real-time RT-PCR, a technique characterized by low variability and minimal risk for cross-contamination [23]. Our results document a high reproducibility and concordance of our measurements (Figs. **3** and **4**), indicating the suitability of the chosen technique. CYP and CCO were selected as reference genes, since preliminary experiments in our laboratory revealed that their mRNA levels remained stable after a training program identical to that employed in the present study. Additionally, Murphy and coworkers [29] report that CYP mRNA level in skeletal muscle remains relatively stable after acute high intensity exercise and suggest that it can be used safely as an endogenous control in similar experiments.

To our knowledge, the only available data regarding the effect of chronic exercise on IGF-I mRNA expression were reported by Zanconato et al. [39] and Eliakim et al. [14]. Zanconato et al. [39] showed that chronic treadmill exercise slightly increased IGF-I mRNA in rat skeletal muscle, but not significantly (as was the case in our study). Likewise, Eliakim et al. [14] reported that short-term treadmill training did not alter IGF-I mRNA levels in rat skeletal muscle. From these studies and the present one, it can be deduced that chronic endurance training does not change the gene expression of IGF-I in skeletal muscle at the mRNA level.

Regarding the effect of exercise on AR mRNA expression, the data available in the literature are limited to acute exercise [3]. That study found increased AR mRNA expression in human vastus lateralis muscle 48 h after an acute bout of resistance exercise. Apparently, differences in species and type of exercise do not permit a direct comparison between that study and the present one. Furthermore, changes in mRNA levels after acute exercise are not necessarily translated into altered resting levels of mRNA after chronic exercise [30]. Therefore, the finding of the present study that chronic exercise did not influence AR mRNA expres-

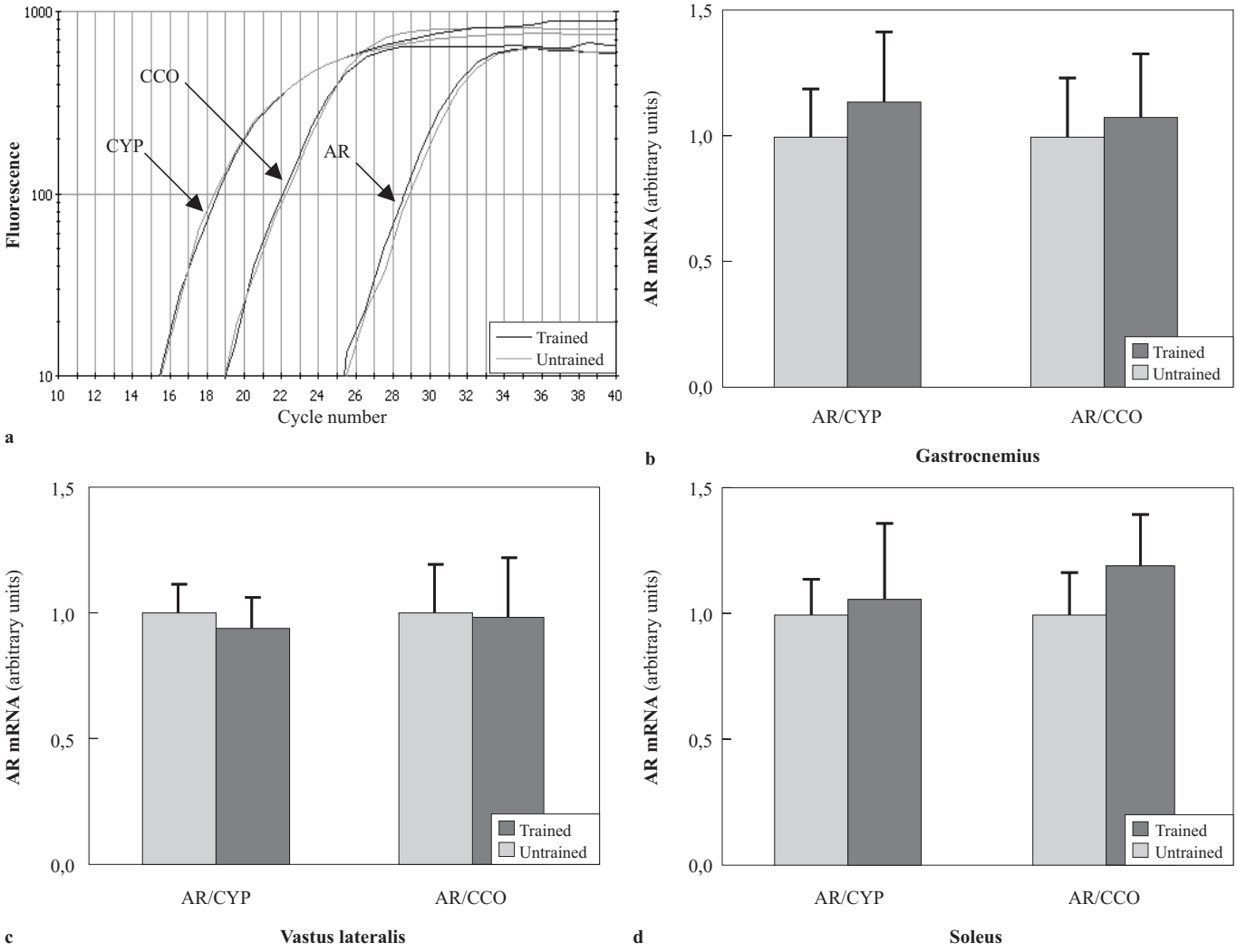

Fig. **4 a** to **d** Androgen receptor (AR) mRNA levels of six untrained and the six most trained rats. See Fig. **3** for description.

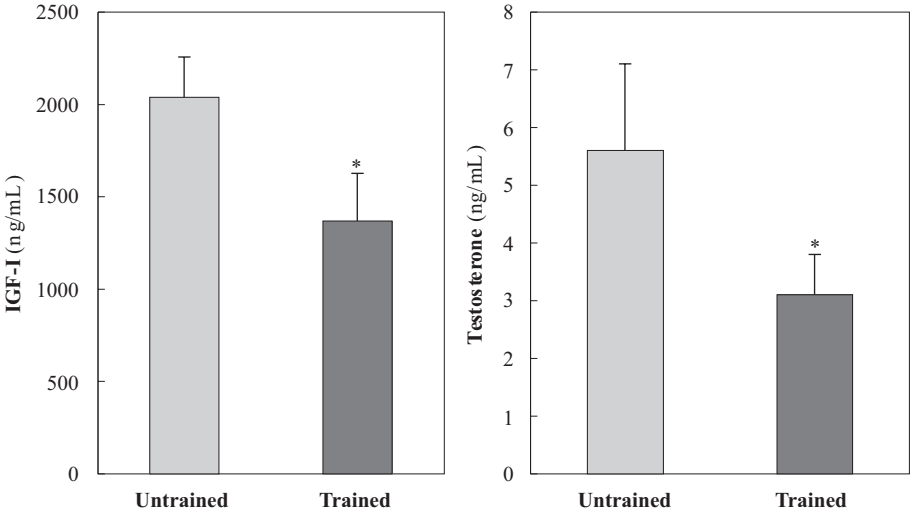

Fig. **5** Serum IGF-I and testosterone levels of the untrained (■ n = 9) and trained rats (■ n = 20). * Significantly different from untrained ($p < 0.05$). Error bars denote SD.

sion is a new information with regard to the molecular mechanisms involved in the adaptation of skeletal muscle to exercise.

In contrast to the lack of an effect on muscle IGF-I and AR mRNA expression, chronic exercise decreased the serum concentration of IGF-I and testosterone (the most potent natural ligand for AR). It is worth noting that voluntary wheel running eliminates the stress and discomfort of forced treadmill running. This may be of particular value when considering that the levels of the serum parameters measured in the present study can be modified by anxiety [15, 28].

Regarding IGF-I, the relevant studies have reported either increased [38] or, more frequently, unchanged concentrations in serum of endurance treadmill-trained rats [7,9,30]. These differences from the results of our study may be due to differences in rat strain and sex, as well as exercise model [13,31]. In this respect, it is noteworthy that one study which employed wheel running as an exercise model reported slightly (and not significantly) decreased serum IGF-I in trained animals compared to untrained ones [4]. This implies that wheel running may exert different effects on circulating IGF-I levels from those provoked by the most frequently used exercise model (i.e., forced treadmill running).

Serum testosterone was also found to be significantly lower in trained than untrained rats. In the literature, either similar [20,22] or decreased [16,21,37] levels have been reported in endurance trained compared to untrained rats. The discrepancies among studies are probably related to the intensity, duration and type of exercise training, factors which have been reported to affect resting serum testosterone [17,22,26]. It therefore appears that the decreased serum IGF-I and testosterone levels in the trained rats suggest no or only a faint anabolic response to wheel training.

In conclusion, our results demonstrate that chronic exercise does not result in an increase of local IGF-I and AR mRNA expression (both factors implicated in the anabolic processes taking place after modified contractile activity) in the investigated muscles. On the other hand, we reported that the circulating levels of two anabolic factors, i.e., IGF-I and testosterone, were lower in trained compared to untrained rats, suggesting that wheel running may diminish anabolic stimuli. Therefore, IGF-I, AR and testosterone seem to play a marginal role during the adaptation process to chronic exercise in wheels. The molecular mechanisms and growth factors involved in the adaptation to chronic exercise must be identified in further investigations.

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