

## ***Fiber type specific expression of TNF-alpha, IL-6 and IL-18 in human skeletal muscles***

**Running title: Cytokine Expression is Fiber Type Dependent**

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### **Abstract**

*Skeletal muscle is now recognized as an endocrine organ with the capacity to produce signal peptides in response to muscle contractions. Here we demonstrate that resting healthy human muscles express cytokines in a fiber type specific manner. Human muscle biopsies from seven healthy young males were obtained from m. triceps, m. quadriceps vastus lateralis and m. soleus. Type I fibers contributed (mean  $\pm$  SE) 24.0  $\pm$  2.5% in triceps of total fibers, 51.3  $\pm$  2.4% in vastus and 84.9  $\pm$  2.2% in soleus. As expected, differences in the fiber type composition were accompanied by marked differences between the three muscles with regard to MHC I and MHC IIa mRNA expression.*

*Immunohistochemistry demonstrated that tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-18 were solely expressed by type II fibers, whereas the expression of IL-6 was more prominent in type I compared to type II fibers. The fiber type specificity was found in triceps, vastus and soleus indicating that the level of daily muscle activity did not influence basal cytokine expression. The specificity of cytokine expression in different muscle fiber types in healthy young males suggests that cytokines may play specific regulatory roles in normal physiology.*

**Key words:** soleus; triceps; vastus; muscle biopsy; inflammation

### **Introduction**

A growing body of literature (33) indicates that cytokines are involved in the regulation of skeletal muscle function. Among cytokines, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is the cytokine most prominently linked to muscle pathophysiology. TNF- $\alpha$  has been associated with muscle catabolism and loss of muscle function

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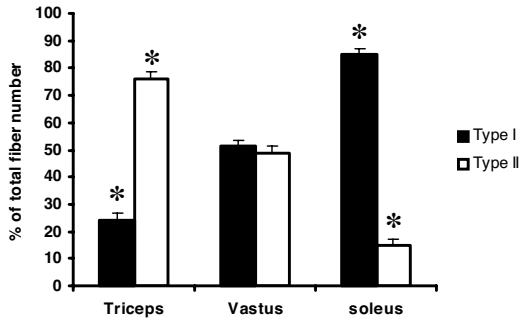


Figure 1: A) Type I and II fibers were ATPase stained at pH 10.3, where type II fibers appear as black. The fibers were counted and the relative contribution of type I and II fibers as % of total number was calculated for triceps, vastus and soleus. \* designates significant difference from vastus muscle.

(21), in addition to various human diseases, including cancer, heart failure, neurodegeneration, arthritis and HIV infection (8; 23). TNF- $\alpha$  causes metabolic disturbances such as insulin resistance in human skeletal muscle (30). To date, most research has focused on cytokines produced by systemic inflammatory cells, or cells in inflamed joints. Elevated systemic levels of cytokines from these sources may influence gene expression and adaptive responses in skeletal muscle fibers (21).

Increased TNF- $\alpha$  content in skeletal muscle from patients with type 2 diabetes has been reported (34), although the cellular origin has not been established.

During the past few years, it has been demonstrated that contracting human skeletal muscle produces and releases IL-6 into the circulation (37). Evidence exists that muscle-derived IL-6 works in a hormone-like manner and influences metabolism in other organs (38). However, muscle-IL-6 is accumulated in muscle fibers following a bout of exercise (28). Furthermore, IL-6 is regulated in an autocrine fashion via up-regulation of IL-6 receptors in the muscle fiber membranes (20). Therefore, in addition to its hormonal role, IL-6 may also regulate muscle cell function.

Muscle apoptosis may play a pathogenic role in chronic diseases (31). Skeletal muscle apoptosis associated with heart failure may be attributed, at least in part, to elevated circulating TNF- $\alpha$  levels (32). The fact that skeletal muscle has the capacity to produce cytokines led us to suggest that cytokines also play a role in the regulation of normal muscle function. Given the different biologic roles of type I and II muscle fibers, we further hypothesized that cytokines might be expressed in a fiber-type specific manner.

In the present communication, we focused on the expression of TNF- $\alpha$  and IL-6. In addition, we investigated the pro-inflammatory cytokine IL-18, as this cytokine has been demonstrated to play a role in apoptosis and tissue destruction

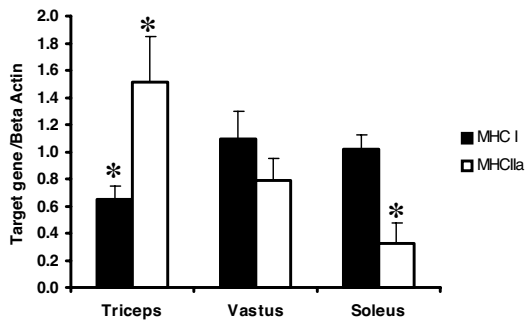


Figure 1: B) MHC I and MHC IIa mRNA expression related to the housekeeping gene  $\beta$ -actin in triceps, vastus and soleus muscles. \* designates significant difference from vastus muscle.

(13). We recently reported that IL-18 is expressed by adipose tissue (22). However, whether IL-18 is expressed in skeletal muscle fibers has not previously been addressed. Different human skeletal muscle types differ by the relative composition of type I and type II muscle fibers. To obtain information on possible fibers type specific expression, we obtained human muscle biopsies from m. soleus (type I dominant), m. triceps (type II dominant) and m. quadriceps, which typically is represented by the same proportion of type I and type II muscle fibers.

### Methods and materials:

**Subjects:** Seven male subjects participated in the study. They underwent a medical examination and a standard set of blood tests. The age of the subjects was (mean  $\pm$  SE)  $26 \pm 1$  years; weight  $84 \pm 3$  kg and a body mass index  $24.7 \pm 0.1$  kg/m<sup>2</sup>. The subjects were instructed not to perform exercise 24 h before the experiment. The volunteers were informed about risks and discomfort via verbal and written information. The protocol was approved by the Municipal Ethical Committee for Copenhagen and Frederiksberg (KF: 01-034/03) and was in accordance with the declaration of Helsinki.

**Experimental procedure:** The subjects reported to the laboratory after an overnight fast. Biopsies were obtained from three different muscle groups: Triceps brachii caput medialis, triceps surae pars soleus and quadriceps pars vastus lateralis using a

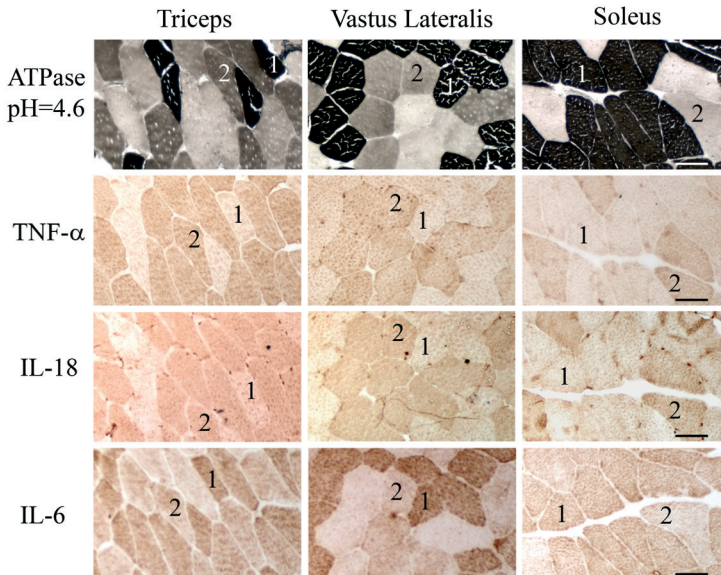


Figure 2: Representative serial sections are shown stained for ATPase pH 4.6, where type II fibers appear as white. Neighboring sections are shown for TNF- $\alpha$ , IL-18 and IL-6 immunostaining. One type I, and one type II fiber, marked as „1” and „2”, respectively are followed through the serial sections. TNF- $\alpha$  and IL-18 are solely expressed in type II fibers in all three muscles, whereas predominantly type I fibers express IL-6. The scale bar equals 75  $\mu$ m.

Bergström cannula (4). First the skin and the muscle fascia were anesthetized using lidokain (SAD, Copenhagen, Denmark). An incision was made at 5–7 mm and the Bergström needle was introduced into the muscle tissue. Suction was applied and three to five cuts were made. The biopsy sample was divided into two parts. Approximately 50 mg of the biopsy was prepared for PCR analysis. If blood clots were present they were quickly removed and the biopsy was frozen in liquid nitrogen. The other part was prepared for histochemical analysis and was mounted in Tissue-Tek (Sakura Finetek, Zoeterwoude, Netherlands) and frozen in 2-methyl-butane (Acros Organics, New Jersey, USA) cooled in liquid nitrogen. Both samples were stored at  $-80^{\circ}\text{C}$  until the day of analysis.

#### Gene expression analysis.:

Total RNA was extracted from approximately 50 mg muscle tissue using TRIzol<sup>®</sup> Reagent (Invitrogen, Carlsbad, USA) following the manufacturer's instruction. In brief, muscle tissue was homogenized using a Polytron (Kinematica AG, Luzern, Switzerland) setting 29 in 1000  $\mu\text{l}$  TRIzol<sup>®</sup> Reagent for 10–15 s. Chloroform was added, and after centrifugation, the phases were divided apart. The aqueous phase was transferred to a fresh tube, and the RNA precipitated by adding isopropanol and kept at  $-20^{\circ}\text{C}$  for 1 hour. After another centrifugation step the RNA pellet was washed in ethanol and finally dissolved in 50  $\mu\text{l}$  DEPC treated water. The RNA concentration was determined spectrophotometrically and 2  $\mu\text{g}$  was reversed transcribed using Tagman<sup>®</sup> Reverse Transcription Kit (Applied Biosystems, New Jersey, USA) in a total volume of 100  $\mu\text{l}$  using random hexamers as primers. Real time real time PCR was performed using an ABI 7900

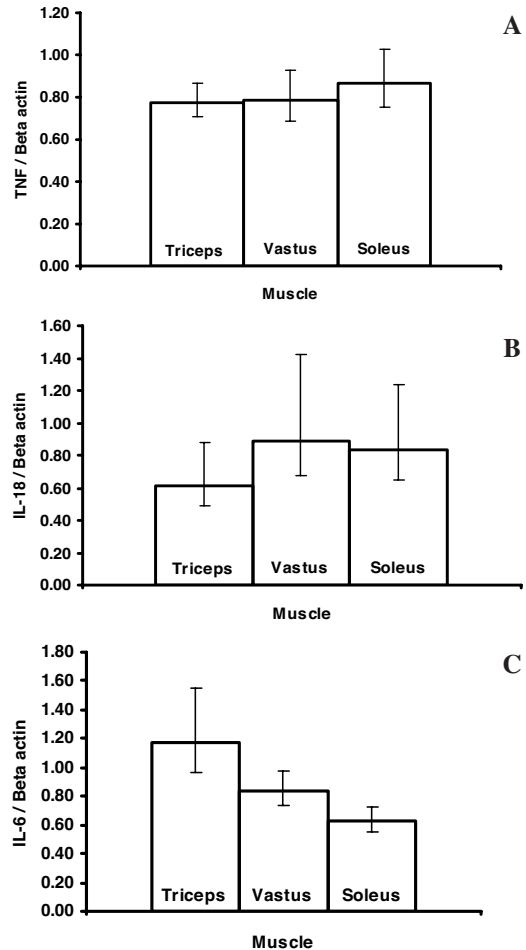


Figure 3: TNF- $\alpha$  (A), IL-18 (B) and IL-6 (C) mRNA content in triceps, vastus and soleus (geometric mean + SE). No differences between muscles were found for TNF- $\alpha$  and IL-18, whereas for IL-6, a borderline significant difference was found (ANOVA  $P=0.08$ ).

(Applied Biosystems, New Jersey, USA). Primer and probes for IL-6 (36), MHC I and MHC IIa (2) were optimized for real time conditions. For IL-18, TNF- $\alpha$  and  $\beta$ -actin commercially available primer and probes were used (Applied Biosystems, New Jersey, USA). The PCR conditions followed the procedure recommended by the manufacture and the reaction volume was 10  $\mu$ l for each samples, which were run in triplicate and 50 cycles were performed. The amount of  $\beta$ -actin mRNA was independent of the muscles, and  $\beta$ -actin was used as a reference gene. The amount of mRNA of both target and reference genes was calculated from the cycle threshold (Ct) values by use of a standard curve constructed from a serial dilution of aliquots of cDNA pooled from all of the samples. The amount of target gene was related to the amount of  $\beta$ -actin, and the ratio between the two is presented.

**Tissue Processing for histology:** For identification of muscle fibers, frozen biopsies of the triceps, vastus and soleus muscles were cut on a cryostat at -20 °C in 6- $\mu$ m consecutive, transverse sections. All sections were immediately collected on glass slides.

**Histology:** We used routine ATPase histochemistry performed after pre-incubation at pH= 4.57 and pH= 10.30. The pre-incubation at pH= 4.57 was mainly used, since this protocol allows identification of five different fiber types (fiber types I, IIc, IIa, IIax, and IIx). Type I and II fibers were counted after ATPase straining at pH 10.3.

**Immunohistochemistry:** For the immunostaining procedure, we used enzymatic epitope retrieval, where the sections were pre-incubated overnight in tris-EGTA (TEG) buffer (0.05 M Tris, 2.5 mM EGTA) at 60 °C and afterwards in 1.5% H<sub>2</sub>O<sub>2</sub> in Tris-buffered saline (TBS)/Nonidet (TBS: 0.05 M Tris, pH 7.4, 0.15 M NaCl; with 0.01% Nonidet P-40) (Sigma-Aldrich, Missouri, USA, code N-6507) for 15 min at room temperature (20 °C) to extinguish endogenous peroxidase. Subsequently, sections were incubated in 10% goat serum (In Vitro, Fredensborg, Denmark, code 04009-1B) for 30 min at room temperature in order to block nonspecific binding. Afterwards, sections were incubated overnight at 4 °C with the following primary antibodies: monoclonal mouse anti-human IL-6 diluted 1:50 (Chemicon, California, USA, cat. no.: CBL-2117); monoclonal mouse anti-human IL-18 diluted 1:100 (Biosource, Solingen, Germany, cat. no.: AHC8181); polyclonal rabbit anti-TNF- $\alpha$  diluted 1:100 (Biosource, Solingen, Germany, cat. no.: AMC3012).

Primary antibodies were detected using biotinylated anti-mouse IgG diluted 1:200 (Sigma-Aldrich, USA, cat. no.: B8774) or biotinylated anti-rabbit IgG diluted 1:400 (Sigma-Aldrich, Missouri, USA, cat. no.: B3275) followed by streptavidin-biotin-peroxidase complex (StreptABCComplex/HRP, DakoCytomation, Glostrup, Denmark, cat. no.: K377) for 30 min at room temperature. Afterwards, the staining was enhanced by using biotinylated tyramide and streptavidin-peroxidase complex (NEN, Life Science Products, Massachusetts, USA, cat. no.: NEL700A) prepared according to the manufacturer's recommendations. The immunoreaction was visualized using 0.015% H<sub>2</sub>O<sub>2</sub> in 3,3-diaminobenzidine-tetrahydrochloride (DAB)/TBS for 10 min at room temperature. Sections were always processed and stained simultaneously and under the same laboratory conditions. Negative control sections were incubated without the primary or sec-

ondary antibody or in the blocking serum. Results were considered only if these controls were negative.

To determine the degree of false positive staining due to endogenous biotin, we pretreated sections sequentially with Cruz Block Avidin/Biotin Blocking Kit (Santa Cruz Biotechn. Inc., California, USA, cat. no.: sc-24967), before the immunohistochemistry was performed. This approach revealed that endogenous biotin within muscle most likely did not induce a false positive signal by binding to streptavidin included in the immunohistochemistry. In order to assess the specificity of the primary antibodies, we pre-incubated these antibodies with their corresponding human antigen for 2 h at 20 °C. We used human IL-18 (R&D Systems, Oxon, UK, code B003-5); human IL-6 (R&D Systems, Oxon, UK, code 206-IL-010); and human TNF- $\alpha$  (Santa Cruz Biotechnology Inc., California, USA, cat.no.: sc-1350P). If this pre-incubation resulted in negative immunostainings, then the primary antibodies were considered to be specific. For the simultaneous examination and recording of the stainings, a Zeiss Axio Imager D1 microscope with an AxioCam MRc5 camera was used.

**Statistics:** The quantitative data was log transformed to obtain a normal distribution, and presented as geometric means  $\pm$  standard error of the mean. To assess differences between the muscles an analysis of variance (ANOVA) was performed. As a post hoc test, a two-sample t-test was used to compare the difference between soleus and triceps using vastus lateralis as a reference. A  $P < 0.05$  was considered significant.

## Results

In m. soleus, type I fibers were most abundant, whereas type II fibers were least abundant. This is in contrast with the findings within m. triceps, in which type II fibers were most abundant, whereas type I fibers were least abundant. M. quadriceps consisted of approximately equal numbers of type I and type II fibers (Fig. 1a). A marked difference between triceps, quadriceps and soleus was found with regard to MHC I and MHC IIa mRNA expression. MHC I was low in m. triceps compared to the two other muscles (Fig. 1b), whereas MHC IIa mRNA levels were higher in m. triceps compared to the two other muscle groups, and higher in m. quadriceps compared to m. soleus (Fig. 1b).

Images from immunohistochemical staining of TNF- $\alpha$ , IL-18, IL-6 and the different fiber types are presented in Fig. 2. At pH 4.6, fibers containing MHC I appear as black. The fibers, which were not stained black, cover a wide range of different type II fibers. Fibers with only a light staining represent fibers with predominantly MHC IIa expression. Fibers with a much darker staining are fibers with predominantly MHC IIx expression. Serial sections are presented from the three muscles for TNF- $\alpha$ , IL-18 and IL-6. TNF- $\alpha$  protein was expressed homogeneously throughout the cytoplasm of some muscle fibers, whereas in other muscle fibers, TNF- $\alpha$  was not visible. As judged by comparing adjacent sections stained for myofibrillar ATPase and TNF- $\alpha$ , respectively, the type II muscle fibers expressed TNF- $\alpha$ , whereas Type I fibers did not. IL-18 protein expression showed a pattern similar to TNF- $\alpha$ . IL-6 expression was most prominent in type I fibers. TNF- $\alpha$ , IL-18 and IL-6 mRNA levels did not differ significantly between the dif-

ferent muscle groups, (Fig. 3a-c) although IL-6 mRNA expression tended to differ between muscles ( $P=0.08$ ).

## Discussion

This study demonstrates that skeletal muscles have a basal expression of cytokines and that this expression is fiber-type specific with regard to type I and type II fibers. The expression of TNF- $\alpha$  and IL-18 is predominantly found in the type II fibers whereas IL-6 is most abundant in type I fibers (Fig. 2). However, cytokine expression detected at the protein level was not reflected at the mRNA level, even though the mRNA level reflected the protein level for the MHC I and IIa isoforms. The lack of relation between mRNA and protein levels suggests a post-transcriptional regulation. This must either be a fiber-type specific effect on mRNA stability or on cytokine turnover, or a combination of both factors. Nevertheless, these data suggest that mRNA for TNF- $\alpha$ , IL-6 and IL-18 in the resting state is constitutively expressed. The fiber-type specific expression of TNF- $\alpha$ , IL-18 and IL-6 proteins was independent of muscle localization, and the specificity was restricted to type I and II. Cytokine expression was similar between type IIa and IIx fibers.

TNF- $\alpha$  is traditionally considered to be a secretory product generated by the immune system in response to infection or inflammation. New evidence has emerged to suggest that stressed or injured muscle fibers produce TNF- $\alpha$ . This is observed in inflammatory myopathies (6) and insulin resistant subjects (24). Furthermore, a higher level of TNF- $\alpha$  mRNA and protein is observed in muscle tissue from elderly compared to young adults (16). Our data show that skeletal muscle tissue expresses TNF- $\alpha$  at basal conditions in a fiber-type dependent manner. Low-grade inflammatory conditions such as obesity, type 2 diabetes and the metabolic syndrome are associated with elevated levels of TNF- $\alpha$  in the circulation (12; 25; 39). It was recently demonstrated that an acute infusion of recombinant TNF- $\alpha$  to young healthy subjects can induce insulin resistance in the skeletal muscle, whereas the hepatic glucose production was unchanged (30). Taken together, these data demonstrate that TNF- $\alpha$  is not just a marker of inflammation, but has the ability to affect the skeletal muscle tissue and reduce insulin sensitivity. Interestingly, insulin resistant patients also have elevated levels of TNF- $\alpha$ , and a relatively high proportion of type II muscle fibers within the skeletal muscle (34). This suggests that TNF- $\alpha$  might have local regulatory roles within muscle tissue itself, and that over-expression may lead to insulin resistance and cachexia. TNF- $\alpha$  may exert its action not only via an endocrine manner, but also through paracrine or autocrine mechanisms.

TNF- $\alpha$  exists as a soluble and a membrane bound form (26), which have distinct biological effects (17). The importance of the membrane bound TNF- $\alpha$  expression has been demonstrated using transgenic mice only expressing membrane bound TNF- $\alpha$  which in itself is sufficient to mediate the pathogenesis of arthritis (1). In humans, the expression of TNF- $\alpha$  by skeletal muscle is increased in response to eccentric exercise-induced muscle damage (18). This induction is reduced with ageing, thus suggesting an impaired inflammatory response (18). Nevertheless in the latter study, no difference was observed in the basal TNF- $\alpha$  mRNA content (18). However, increased TNF- $\alpha$  expression both at the mRNA level but also at the protein level have been observed in old compared to young

human subjects. Furthermore, when the elderly frail subjects, underwent 3 months of training, TNF- $\alpha$  mRNA and protein content in muscle tissue were reduced (16). Additionally, a negative correlation was observed between TNF- $\alpha$  expression and protein synthesis, which suggests that TNF- $\alpha$  is involved in aged induced muscle wasting.

Although fiber-type specificity of TNF- $\alpha$  expression was not addressed in that particular study (16), evidence exists from animal studies. Rats have an increased muscle TNF- $\alpha$  expression in response to aging as well as humans, but TNF- $\alpha$  expression is increased predominantly in the superficial vastus lateralis muscle (predominantly type II) compared to the soleus muscle. Furthermore, the age-induced TNF- $\alpha$  expression in the type II fiber can be blunted by calorie restriction (29). These findings support the idea that not only TNF- $\alpha$  is not only important within the circulation, but also within muscle tissue.

The working muscle tissue produces and secretes IL-6 into the circulation (37). Fiber-type specificity has been reported in relation to IL-6 during muscle contraction, but the data are conflicting. In the first report, Penkowa et al. used immunohistochemistry, and demonstrated that both type I and type II muscle fibers were able to express IL-6 when healthy young men performed intense bicycle ergometer exercise for 3 hours (28). In another study, which included similar study population and the same mode of exercise, although lasting only 2 h in duration, Hiscock et al. observed that IL-6 production was located mainly within type II fibers (19). Fischer et al. investigated immunohistochemical staining of biopsies from subjects following 3 h of two-legged knee-extensor exercise, and found that IL-6 was mainly expressed in type I fibers of the vastus muscle (14). This finding is consistent with findings in rat muscle (3).

The conflicting data with regard to fiber type specific expression of IL-6 cannot easily be explained. If we assume that IL-6 is expressed by the fiber types which are recruited during exercise, it would be expected that predominantly type I fibers would express IL-6 during moderate endurance exercise, such as in Fischer et al (14). When subjects exercise to exhaustion as during the 3 hour of intense bicycle ergometer exercise, both type I and II fibers are recruited and this may explain why both fiber types express IL-6 (28). However, this concept does not explain the findings by Hiscock et al (19).

When comparing biopsies obtained at rest and following exercise of various type or duration, it appears that IL-6 production is markedly enhanced. At first glance, it appears that resting muscles do not express cytokine proteins. However, the present study illustrates that skeletal muscle does actually express detectable amounts of TNF- $\alpha$ , IL-6 and IL-18. Furthermore, this observation is not only valid for vastus lateralis, but also muscles differing in both metabolic profiles and fiber-type composition such as the triceps and soleus muscles.

In contrast to the accumulating data on the multiple metabolic roles of TNF- $\alpha$  and IL-6, the metabolic role of IL-18 has not been studied to the same extent. However, elevated systemic IL-18 levels have been associated with a number of diseases. Elevated plasma levels of IL-18 predict cardiovascular mortality in patients with coronary atherosclerosis (5). Additionally, a role for IL-18 in the autoimmune-cell destruction leading to type 1 diabetes has been proposed (27). Importantly, recent studies also report that plasma IL-18 levels are elevated in patients not only with type 2 diabetes (9; 15), but also in obesity (10) and polycystic ovary syndrome



(7). Recently, IL-18 was found to be expressed in human adipose tissue (22). Furthermore, both adipose tissue IL-18 mRNA as well as systemic levels of IL-18 were associated with lipotrophy in patients with HIV-associated lipodystrophy (22). Little is known about the possible biological role of IL-18 in either normal or diseased human skeletal muscle. However, IL-18 inhibits the endotoxin-induced nitric oxide production in the rat diaphragm muscle (35). The present data suggest that IL-18 may have a regulatory role healthy human skeletal muscle.

The overall novel finding of the present study is that normal resting human skeletal muscles express TNF- $\alpha$ , IL-18 and IL-6 in a strict fiber-type specific fashion. These findings enhance our knowledge with respect to TNF- $\alpha$  and IL-6 (11), and indicate that these cytokines may have regulatory roles in normal muscle physiology, possibly in addition to their established endocrine function in muscle.

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