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Abstract

In this study, we quantified the transcription of the IL-6 gene in individual fibres and the associated changes in calcineurin activity assessed at cellular level during prolonged muscle contraction. Individual myofibres were isolated from plantaris and soleus muscles of rats at the end of an exhaustive running exercise (n=10), typed according to their myosin heavy chain isoform content, and compared to those of resting rats (n=10). Using real-time PCR analysis in individual fibres, a marked rise in IL-6 transcript levels occurred in type I and IIa fibres at the end of exercise (P<0.05). Transcription of the gene encoding for the modulatory calcineurin-interacting protein-1 (MCIP-1), a sensitive indicator of calcineurin activity, also mainly increased in type I and IIa fibres (P<0.05). Moreover, a slight increase in MCIP-1 mRNA levels was observed in type IIx (P<0.05). Fibre types determined by immunohistochemistry were qualitatively examined for glycogen content using Periodic Acid Shiff staining, and no direct relationship was found, at the cellular level, between glycogen content, fibre-type and IL-6 transcription. Our data clearly suggest that IL-6 gene transcription was mainly observed in early-recruited myofibres and that contraction-induced IL-6 transcription could be associated with enhanced calcineurin activity.

Introduction

It has been recently shown that IL-6 plasma levels increase dramatically during prolonged concentric exercise in man (for review see Febbraio & Pedersen 2002). Increased IL-6 mRNA levels were reported in human muscle biopsies at the end of exercise, related to mechanisms other than muscle damage (Ostrowski *et al.* 1998). In a one-legged exercise, high muscle IL-6 net release occurred only in the contracting limb (Steensberg *et al.* 2000). Together, these results strongly suggest that muscle is the main source of plasma IL-6 during exercise and that this production is directly associated with muscle contraction and does not result from an exercise-related systemic effect. Subjects exercising with low intramuscular glycogen levels showed a higher plasmatic IL-6 peak (Keller *et al.* 2001), independent of systemic influences (Steensberg *et al.* 2001). It has thus been hypothesized that muscle-derived IL-6 is linked to energy availability and could play an important role in carbohydrate homeostasis during exercise by contributing to contraction-mediated glucose uptake and by acting as an endocrine signal of muscle energy stores to favour hepatic glucose production and white adipose tissue lipolysis (for review see Febbraio & Pedersen 2002).

However, skeletal muscle contains several cell types that are known to be able to produce IL-6. Blood mononuclear cells do not account for the exercise-induced increase in IL-6 plasma levels (Ullum *et al.* 1994; Starkie *et al.* 2000; Moldoveanu *et al.* 2000). Human myoblasts (Bartoccioni *et al.* 1994), smooth muscle cells (Detmer *et al.* 2001), and endothelial cells (Sterpetti *et al.* 1993) can produce IL-6 when exposed to several stimuli such as inflammatory cytokines, endotoxins or mechanical stress. The cellular origin of IL-6 production in muscle has been examined in two recent studies. The immunohistochemical detection of IL-6 protein in skeletal muscle showed an increase in positive myofibres at the end of exercise, suggesting that myofibres per se could be a source of IL-6 production during

contraction (Penkowa *et al.* 2003). Moreover, using *in situ* hybridization in human muscle, it has recently been shown that myofibres contain IL-6 mRNA at the end of prolonged exercise (Hiscock *et al*. 2004). These findings clearly show that muscle fibres are a source of IL-6, and because myofibres consume and need energy during muscle contraction, they reinforce the hypothesis of an energy-sensing function of IL-6.

Adult rat skeletal muscles comprise at least four fibre types ranging from slow-twitch predominantly oxidative fibres (type I) to fast-twitch predominantly oxidative, intermediate oxidative and low oxidative fibres (types IIa, IIx and IIb, respectively). Muscle fibres are distributed amongst motor units and it is well accepted that during muscle contraction, motor units are recruited in an orderly manner. According to the "size principle" of Henneman et al. (Henneman & Olson 1965), the smallest motor units comprising type I fibres are first recruited, while the largest, comprising type IIx and type IIb fibres, are recruited long after the beginning of muscle contraction, when local fatigue occurs in slow and oxidative motor units (Fallentin *et al*. 1993). Because type I and type IIa fibres show low glycogen stores, whereas type IIx and IIb fibre show high glycogen stores, and IL-6 may work as a sensor of carbohydrate availability (Febbraio & Pedersen 2002), a fibre-type specificity of IL-6 gene expression could be expected at the end of prolonged exercise. This issue has been recently addressed and controversial findings were reported. No difference was detected between muscle fibre types at the protein level at the end of exercise (Penkowa *et al*. 2003), whereas Fischer (2004) described preferential staining in type I fibres. More recent data suggest that type II fibres predominantly produce IL-6 during exercise in humans (Hiscock *et al*. 2004). Moreover, the results of this latter study suggested that lowered glycogen was not directly involved in IL-6 gene expression during muscle contractile activity. Whether the expression of the IL-6 gene varies between fibre types during exercise therefore remains to be elucidated.

Because the nuclear transcriptional rate of the IL-6 gene is remarkably rapid after the onset of exercise, it has been hypothesized that rapid changes in cytosolic Ca^{2+} could be involved in IL-6 gene expression (Febbraio & Pedersen 2002). Changes in intracellular free calcium concentrations ($[Ca^{2+}]i$) are a key event during myocyte contraction, which have recently been shown to induce a rapid increase in IL-6 mRNA and protein expression in rat isolated muscles (Holmes *et al*. 2004). Prolonged contractile activity is characterized by low-amplitude and sustained elevations in $[Ca^{2+}]i$, leading to increased calcineurin activity, a ubiquitous Ca^{2+}/calmodulin-dependent protein phosphatase known to be a key mediator of Ca^{2+} signalling in muscle cells. Several downstream effectors of calcineurin have been identified in skeletal muscle, including the nuclear factor of the activated T cell (NFAT), and the calcineurin-NFAT pathway is involved in the acquisition and maintenance of the slow oxidative phenotype (for review see Bassel-Duby & Olson 2003). Whether this transcription factor is involved in IL-6 gene transcription has recently been examined (Chan *et al*. 2004). No evidence was provided that IL-6 gene transcription is activated by NFAT, whereas the phosphorylation of nuclear p38 mitogen-activated protein kinase (MAPK) was considered as an activator for candidate transcription factors that may bind to the IL-6 promoter region in myofibres. However, whether the increased cytosolic Ca^{2+} accumulation expected within single specific fibres during prolonged muscle contraction is associated with activated calcineurin and enhanced IL-6 gene expression has not been examined to date.

The aims of this study were to examine the fibre-type specificity of IL-6 gene transcription in individual myofibres at the end of prolonged exercise and the involvement of calcineurin activity in the control of IL-6 gene expression. In light of the recruitment pattern of muscle fibres during prolonged submaximal exercise and the differences in pre-exercise glycogen content, we hypothesized that 1) IL-6 production could be fibre-type-specific and that IL-6 mRNA levels could be higher in type I and type IIa fibres than in type IIx and type

IIb at the end of exercise, and 2) high IL-6 mRNA levels could be observed in fibres showing high calcineurin activity. To address these issues, we first quantified the level of IL-6 gene transcription within individual fibres taken from two skeletal muscles at the end of exercise in rat. Second, we examined whether IL-6 gene expression was associated with high levels of calcineurin activity. As previously suggested, transcription of the gene encoding for the modulatory calcineurin-interacting protein-1 (MCIP-1) was used as a sensitive indicator of calcineurin activity (Yang *et al.* 2000).

Methods

Animals

Three-month-old male Wistar rats (*n*=30) were purchased from Charles River (L'Arbresle France). They were housed three per cage in a thermoneutral environment (22 ± 2°C), on a 12–12 h light–dark period, and were provided with food and water *ad libitum*. They were randomly assigned to three experimental groups 1) control rats (*n*=10); 2) active rats (*n*=10), and recovering rats (*n*=10). Experiments received prior agreement from the animal ethics committee of the Centre de Recherche du Service Santé des Armées (La Tronche, France).

Exercise protocol

All animals were accustomed to running on a rodent treadmill for 15 min per day for 5 days at a moderate level (10–20 m min^{-1}; 0° grade). After 3 days at rest, active and recovering animals ran on the treadmill (25 m min^{-1}, 8° grade) until exhaustion, defined as the moment where animals were unable to keep in pace with the treadmill.

Tissue processing

Animals were removed and anaesthetized either immediately (active group), or 2 h after the end of exercise (recovering group), with intra-peritoneal injection of pentobarbital (70 mg kg^{-1} body weight). Plantaris and soleus muscles of both hindlimbs were excised and cleaned of adipose and connective tissue. Plantaris muscles were also separated in superficial and deep portions. While the left muscles were immediately frozen in liquid nitrogen and stored at –80°C, the right muscles were placed in 400 µl RNA*later* solution (Ambion, Austin, TX, USA), kept at 4°C for 24 h and then frozen at –20°C. Animals were killed by removal of the heart.

Single-fibre isolation

Single fibre study was adapted from Birot (2003). Muscle conserved in RNA*later* was thawed on ice and placed in a small dish containing RNA*later*. A small bundle of muscle fibres was first isolated under the light microscope using sharp-ended tweezers. Single muscle fibres were then separated and cut into two equal parts. One half was placed in 40 µl of Nanoprep lysis buffer supplemented with 0.7% 2-β-mercaptoethanol (Stratagene, La Jolla, CA, USA), kept for 2 h at 4°C and then frozen at –80°C before RNA extraction. The other half-fibre was placed in 20 µl of a myosin extraction solution containing (mM): NaCl 300, NaH_2PO_4 100, Na_2HPO_4 50, Na_4P_2O7 10, $MgCl_2.6H_2O$ 1, EDTA 10 and 2-β-mercaptoethanol 1.4; pH 6.5, for further myosin heavy chain (MHC) content analysis.

MHC isoforms analysis

Single fibres were subjected to MHC isoform analysis using a SDS-PAGE electrophoretic method as previously described (Birot *et al.* 2003). After incubation for 24 h at 4°C, the half-fibre was digested and the 20-ml mixture was then diluted with 20 ml glycerol. Extracts were stored at –20°C until required for the separation process. Electrophoresis was performed using a Mini Protean II system (Biorad, Marnes-la-Coquette, France). The separating gel solution contained 30% glycerol, 8% acrylamide–bis (50:1), 0.2 M Tris, 0.1 M glycine and 0.4% SDS. The stacking gel was composed of 30 % glycerol, 4% acrylamide-bis (50:1), 70 mM Tris, 4 mM EDTA and 0.4% SDS. Then 10-ml myofibril samples were denatured using 10 ml buffer containing 5% 2-β-mercaptoethanol, 100 mM Tris base, 5% glycerol, 4% SDS and bromophenol blue, for 3 min at 100°C. Myofibrillar homogenates were loaded onto vertical gels, whilst two lanes were loaded with protein extract from a control plantaris muscle known to contain the four adult MHC isoforms. Gels were run at constant voltage (72 V) for 31 h and

then silver-stained (Agbulut *et al*. 1996). The MHC protein isoform bands were scanned using a densitometer system equipped with an integrator (GS-700, Biorad, Marnes-la-Coquette, France). The MHC isoforms expressed by single-fibres were identified by comparing them with bands of myosin extracts from control plantaris muscle.

RNA extraction

Muscle tissue mRNA were isolated using the MagNA Pure LC instrument (Roche Applied Science, Mannheim, Germany). We disrupted 10 mg of frozen soleus and deep plantaris muscles using two tungsten carbide beads in 100 µL MagNA Pure LC mRNA isolation kit II (Roche Applied Science, Mannheim, Germany) lysis buffer with a Mixer Mill MM300 (Rescht, Haan, Germany) for 40 s (30 Hz). Lysate was centrifuged (12,000 *g*, 5 min, room temperature), the liquid phase was transferred in a fresh tube and completed to a 300-µl final volume with lysis buffer. Extraction was then performed following the manufacturer's protocol with an 880-µl sample volume, a 50-µl elution volume and no dilution.

The fibres' total RNA were isolated using RNA Insta-pure reagent (Eurogentec, Saraing, Belgium) with a modified protocol. Samples were extracted using 200 µl RNA Insta-pure reagent and 20 µl chloroform (Sigma-Aldrich, Steinheim, Germany). An additional iso-volume chloroform extraction was performed. RNA isopropanol precipitation was carried out using 10 µg of glycogen (Sigma-Aldrich, Steinheim, Germany) as carrier. The ethanol washing step was performed twice and the RNA pellet was dried in a vacuum for 3 min. RNA was then resuspended in 10 µl RNase free water.

Reverse transcription

Muscle tissue mRNA reverse transcription was carried out using the Reverse Transcriptase Core Kit (Eurogentec, Saraing, Belgium). The reaction was performed in a 10-

µl final volume following the manufacturer's instructions. The reaction mix contained 1 µl buffer (10X), 2 µl dNTP mix (2.5 mM each dNTP), 0.5 µl oligo-dT primer (50µM), 0.2 µl RNase inhibitor (20 units μl^{-1}), 2 µl $MgCl_2$ (25 mM), 0.25 µl Euroscript reverse transcriptase (50 U μl^{-1}), 1.05 µl RNase free water and 3 µl template mRNA.

Fibre RNA reverse transcription was carried out using Sensiscript Reverse Transcriptase (Qiagen S.A, Courtaboeuf, France), specially designed for highly sensitive reverse transcription with small amounts of RNA. The reaction was performed in a 20-µl final volume following the manufacturer's instructions. The mix contained 2 µl buffer (10X), 2 µl dNTP mix (5 mM each dNTP), 2 µl oligo-dT primer (10 µM), 1 µl RNase inhibitor (10 U μl^{-1}), 1 µl Sensiscript reverse transcriptase, 4 µl RNase free water and 8 µl template RNA.

PCR primer design

All primers used in this study were designed with the MacVector software (Accelrys, Orsay, France) and synthesized at Eurogentec (Saraing, Belgium). Primer sequences and resulting amplified product characteristics are shown in Table 1.

Real-time PCR

The PCR reactions were carried out in a 20-µl final volume with the LC Fast Start DNA Master SYBR Green kit (Roche Applied Science, Mannheim, Germany) using 0.2 µl (whole muscle) or 0.5 µl (single-fibre) cDNA solution. Quantitative PCRs were performed using LightCycler (Roche Applied Science, Mannheim, Germany) for 45 amplification cycles using a 5-s annealing step and an 8-s 72°C elongation step (detailed conditions available in Table 1). Specificity was checked for each sample by melting curve analysis. Transcription levels were normalized using an internal control gene with the comparative threshold cycle method (Livak *et al*. 2001) using RelQuant software (Roche Applied Science, Mannheim, Germany). A pool of 40 samples randomly chosen in the groups was used as a calibrator as

described previously (Peinnequin *et al*. 2004). Cyclophyllin A (cycA) was used as reference gene because its expression is known to be unaffected at the end of an endurance exercise in skeletal muscle (Mahoney *et al*. 2004). Furthermore, this was controlled in our experiment on whole muscle tissue.

Purity of single-fibres

The single-fibre approach we used, first described by Birot (2003), makes it possible to remove fibroblasts, smooth muscle cells and blood mononuclear cells because conjunctive tissue and vessels are clearly visible under the microscope. In contrast, endothelial cells and infiltrating mononuclear cells cannot be seen, can adhere to the myofibres and are capable of producing great amounts of IL-6. Before including fibres in our study, we needed to check the purity of all half-fibres and confirm the absence of endothelial cells or monocytic cells. Every half fibre of the active group was submitted to PCR amplification for platelet endothelial cell adhesion molecule (PECAM) and cluster of differentiation 11b (CD11b). When there was a positive signal for PECAM and/or CD11b, the sample was excluded from the study.

Immunohistochemistry and PAS staining

Serial transverse sections (14 µm thick) were cut from the mid-belly portion of soleus and plantaris muscles in a cryostat maintained at –20°C. Plantaris sections were labelled with mouse monoclonal antibodies against myosin reacting with 1) slow type I (Novocastra, reference NCL-MHCS, Newcastle-upon-Tyne, UK), 2) all adult fast and developmentally regulated epitopes but not with slow myosin (MY-32, Sigma-Aldrich Steinheim, Germany) or 3) fast type IIa (SC-71). Both plantaris and soleus sections were stained for glycogen content using the Periodic Acid Shiff staining system (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer’s instructions.

Statistics

All data are presented as means ± S.E.M. An unpaired Student's *t* test was used to determine differences between groups. Statistical significance was accepted at $P<0.05$.

Results

Running time

Both active and recovering rats performed an exhaustive running exercise. The mean duration of this exercise was 101±13 min.

Exercise-induced increase in IL-6 in whole muscle tissue.

As expected, there was an increase in IL-6 mRNA levels in both plantaris and soleus muscles at the end of running exercise ($P<0.05$). IL-6 mRNA recovered base values approximately 2 hours after exercise stopped. There was no significant difference in IL-6 transcript levels between plantaris and soleus muscles in control, active and recovery groups (Figure 1). Since IL-6 mRNA levels decreased 2 hours after the end of exercise, gene expression within single fibres was only considered in control and active groups.

Fibre type specificity of IL-6 mRNA expression

In soleus muscles, 91% of myofibres were pure type I fibres, while 9% expressed both MCH I and MHC IIa. In deep plantaris muscles, 49% of myofibres expressed only one MHC isoform. Therefore, only 171 of the 351 successfully typed fibres were included for further study. Before IL-6 mRNA quantification, each individual fibre was tested for PECAM, CD11b and CycA mRNA expression. A negative signal for both PECAM and CD11b mRNA proved the lack of contamination by endothelial or monocytic cell material, respectively, while a positive expression of CycA gene confirmed the presence of biological material. After all these validation analyses, 37% of single, successfully typed fibres taken from soleus and plantaris muscles were used for further assays.

At the end of exercise, IL-6 mRNA levels increased significantly in myofibres of soleus muscle, which were mainly type I fibres ($P<0.05$) (Figure 2A). In myofibres taken

from plantaris muscles, there was a marked and significant increase in IL-6 mRNA in only type I and type IIa fibres at the end of prolonged exercise (P<0.05). In contrast, acute treadmill exercise did not significantly change the IL-6 mRNA levels in type IIx and IIb fibres (P<0.05) (Figure 2B).

Myofibre glycogen content at the end of exercise

PAS staining and immunohistochemistry analyses showed that in soleus muscles (Figure 3), type I fibres were markedly glycogen depleted, whereas glycogen only slightly decreased in type IIa fibres. Likewise, all type I fibres were glycogen-depleted at the end of exercise in plantaris muscles (Figure 3); in contrast, type IIa fibres failed to be markedly glycogen-depleted. As shown in Figure 3, there was heterogeneity of glycogen depletion within the two other fast fibre types (type IIx and IIb fibres), with some fibres markedly depleted, while no detectable changes were reported in the other ones.

Fibre-type specificity of calcineurin activity at the end of exercise

The peculiar responsiveness of MCIP-1 gene expression to calcineurin activity was used to assess the activation state of calcineurin (Yang *et al.* 2000). A marked increase in MCIP-1 mRNA was shown in both plantaris and soleus muscles (P<0.05, P<0.01, respectively) at the end of exercise (Figure 4A). The exercise-induced increase in MCIP-1 mRNA was found at similar levels in slow-twitch (soleus) and fast-twitch muscles (plantaris).

As reported for IL-6 mRNA expression, there was a significant increase in MCIP-1 mRNA levels in both type I and IIa fibres at the end of prolonged exercise (P<0.05) (Figure 4B). Moreover, a slight but significant increase in MCIP-1 mRNA levels was shown in type IIx fibres of exercised rats (P<0.05).

Discussion

Despite emerging evidence for IL-6 production by myofibres during exercise, IL-6 mRNA levels were never quantified within individual fibres. In this study, we provide for the first time quantitative measures of IL-6 mRNA levels in muscle cells. The major and novel observations made in the current investigation were 1) a fibre-type specificity of IL-6 gene expression during exercise, with a marked increase in IL-6 transcripts in type I and type IIa fibres; 2) in parallel with the exercise-induced enhancement in IL-6 gene transcription, an increase in calcineurin activity occurred in individual muscle cells.

Many recent studies showed increased levels of plasma IL-6 after exercise in humans, and demonstrated that the appearance of IL-6 in the circulation results from an increased transcription of the IL-6 gene in contracting muscles (Ostrowski *et al*. 1998; Steensberg *et al*. 2001; Keller *et al*. 2003). IL-6 is locally produced in skeletal muscle following both concentric and eccentric contractions in rats (Jonsdottir *et al*. 2000), but dynamic exercise such as running had never been used to examine the molecular events that control the IL-6 gene transcription in rodents. In the present study, IL-6 mRNA levels measured in whole muscle tissue before, at the end, and 2 hours after a running exercise in rats closely mimic the previously reported changes in human muscle biopsies after exercise. These results show that prolonged running exercise in the rat is a valid model to study contraction-induced IL-6 gene transcription in muscles.

As previously and consistently shown, contracting skeletal muscle is the main source of IL-6 production during exercise (for review see Febbraio & Pedersen 2002). However, the issue of the cell type responsible for IL-6 production within skeletal muscle has been only recently addressed using histochemical methods or a qualitative approach of IL-6 gene transcription (Penkowa *et al*. 2003; Hiscock *et al*. 2004; Fischer *et al*. 2004). In the present study, we provide the first quantitative analysis of IL-6 gene transcription rate by RT-PCR in

single myofibres. The lack of myofibre contamination by endothelial and mononuclear cells was verified by a negative signal for PECAM and CD11b, and these controls ensured that samples selected for IL-6 mRNA determination were pure single myofibres. Consistent with a previous study, our results using RT-PCR analysis definitively show that muscle fibres are a source of IL-6 in contracting muscles (Hiscock *et al*. 2004).

Because myofibres are recruited in an orderly manner and IL-6 production would be increased in glycogen-depleted fibres, it has been hypothesized that IL-6 mRNA expression could be fibre-type-specific. This issue has been examined, and recent reports support the role played by the mode, intensity and duration of exercise on the fibre-type specificity of IL-6 gene transcription. Type I fibres are the main source of IL-6 during dynamic exercise at moderate intensity (Fisher *et al*., 2004), while bicycling exercise at higher intensity stimulated IL-6 synthesis in myofibres of all types (Penkowa *et al*., 2003). Surprisingly, IL-6 mRNA and protein were observed at a much greater level in type II than in type I fibres at the end of a bicycling exercise of lower intensity and lower duration, while these fibres also had greater glycogen content (Hiscock *et al.* 2004). One of the main results of the present study is that, using real-time RT-PCR analysis, a very accurate and reproducible method to quantify mRNA levels, IL-6 gene transcription was mainly enhanced in type I and type IIa fibres, two fibre types belonging to the earlier-recruited populations of motor units during prolonged and sustained muscle contraction. In contrast, no significant change in IL-6 gene transcription rate was observed in type IIx and type IIb fibres, which are fast-glycolytic fibres, recruited later during prolonged exercise (Fallentin *et al.* 1993; Houtman *et al.* 2003). As previously discussed, the exercise mode, intensity and duration affect whether IL-6 mRNA is expressed in oxidative or glycolytic fibres. Our data lend support to the hypothesis that even if both type I and type II myofibres have the capacity to synthesize IL-6, this cytokine is produced by essentially oxidative myofibres during moderate prolonged exercise.

Our histochemical analyses showed that IL-6 mRNA expression was markedly increased in type I fibres with low glycogen content, consistent with the hypothesis that this cytokine may be involved in the regulation of glucose homeostasis (Febbraio & Pedersen 2002). However, only a limited glycogen depletion was reported in type IIa fibres, while IL-6 mRNA appeared predominantly in those fibres. Consistent with a previous study (Hiscock *et al.* 2004), our data failed to demonstrate that at the cellular level, IL-6 gene transcription was closely related to low glycogen content. This finding is not in disagreement with the possibility that IL-6 may act as an energy sensor at the whole muscle level. Some evidence clearly suggest that low pre-exercise skeletal muscle glycogen content exacerbates the IL-6 response to exercise (Chan *et al.*, 2004 ; Keller *et al.*, 2001 ; Steensberg *et al.*, 2001). However, the lack of correlation between two biological factors at the myofibre level, does not reject the relationship at the whole muscle level, which could also result from local influences (Febbraio & Pedersen 2002). Our findings suggest that in addition to factors related to low intramuscular glycogen stores, IL-6 gene transcription could be also enhanced by glycogen-independent mechanisms.

Therefore, molecular signals or events generated by muscle contraction likely have a key role in IL-6 gene transcription during exercise. Tonic motor nerve activity has been shown to sustain intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) at levels sufficient to activate calcineurin, a Ca^{2+}-activated protein phosphatase involved in muscle development, adaptation and disease (Bassel-Duby & Olson 2003). Alterations in $[Ca^{2+}]i$ have been implicated in several signalling cascades and as a potent signalling factor for IL-6 transcription (Febbraio & Pedersen 2002; Keller *et al.* 2002), and recent data showed that ionomycin, a Ca^{2+} ionophore, increased IL-6 gene expression in isolated skeletal muscle (Holmes *et al.* 2004). These data support the hypothesis that increased $[Ca^{2+}]i$ is involved in the control of IL-6 gene expression during exercise. Several signalling molecules are involved in transducing the

calcium signal, thereby including calcineurin, and it has been proposed that activation of this phosphatase could be involved in early IL-6 gene transcription during prolonged exercise (Febbraio & Pedersen, 2002). Our results show that MCIP-1 mRNA levels significantly increased in both plantaris and soleus muscles at the end of prolonged exercise. This increase in MCIP-1 mRNA, a marker for calcineurin activation in two skeletal muscles known to be recruited during running exercise in rats, supports the notion that calcineurin is activated at the end of prolonged exercise (Norrbom *et al.* 2004).

A major finding of this study was that in parallel with IL-6 mRNA, MCIP-1 mRNA expression increased in both type I and type IIa fibres. This finding suggests that calcineurin is activated concomitant with the increase in IL-6 mRNA, mainly in the most active fibres, those expressing type I or type IIa MHC. Tonic motor nerve activity is characteristic of type I and to a lesser extent of type IIa fibres. Calcineurin activity is mainly sensitive to sustained, low amplitude elevations of $[Ca^{2+}]i$ and the specific isoforms of some regulatory proteins and sarcoplasmic reticulum Ca^{2+}-ATPase account for the sustained elevations in $[Ca^{2+}]I$ (Baylor & Hollingworth 2003 ; Botinelli & Reggiani 2000). In the present study, we cannot prove whether the association between IL-6 gene transcription and enhanced calcineurin activity was causal or related to a random association. However, these data provide a new argument to suggest that calcineurin activation could be an upstream signal for IL-6 gene transcription during exercise. Calcineurin activation facilitates alterations in gene expression through several transcriptional effectors, including NFAT transcription factors (Bassel-Duby & Olson 2003). A recent study suggested that the exercise-induced increase in IL-6 mRNA occurred through an NFAT-independent mechanism (Chan *et al.* 2004). However, calcineurin has been shown to be involved in the regulation of skeletal muscle genes, but not through NFAT (Giger *et al.* 2004; Parsons *et al.* 2003). Collectively, these results suggest that the exercise-induced increase in IL-6 gene transcription could be associated with calcineurin activation, which

could control gene expression through a mechanism independent of the NFAT nuclear accumulation. Future studies need to demonstrate the causative relationship between calcineurin activity and IL-6 gene expression and determine the downstream targets of calcineurin. While it has been previously shown that low pre-exercise muscle glycogen content enhanced IL-6 gene transcription during exercise through phosphorylation of p38 MAPK in the nucleus (Chan *et al.*, 2004), our results suggest that increased calcineurin activity could also account for the exercise-induced increase in IL-6 mRNA.

In summary, quantitative measures of IL-6 mRNA within muscle cells, demonstrate a fibre-type specificity of IL-6 gene expression during exercise, with a marked increase in IL-6 transcripts in type I and type IIa fibres during prolonged and sustained muscle contraction. Another major and novel finding of this study was that MCIP-1 mRNA expression also increased in both type I and type IIa fibres, suggesting that calcineurin activation is concomitant with the increase in IL-6 mRNA, mainly in the most active fibres. These data provide a new argument to suggest that calcineurin activation could be an upstream signal for IL-6 gene transcription during exercise. Our results suggest that in addition to factors related to the intracellular signalling of low intramuscular glycogen, molecular events related to contractile activity, such as increased calcineurin activity would lead to IL-6 gene transcription.

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Figure 1 : Quantitative analysis of IL-6 mRNA levels by real-time RT-PCR in whole plantaris and soleus muscles of control non-exercised, active and recovering rats (arbitrary units). Data are means ± SEM. * significantly different from control values, $P<0.05$; $ significantly different from active group, $P<0.05$.

Figure 2 : Quantitative analysis IL-6 mRNA levels by real-time RT-PCR in soleus (*A*) and plantaris (*B*) single fibres of control non-exercised and active rats (arbitrary units). Plantaris fibres were categorised into four types : type I, IIa, IIx and IIb, according to their MHC isoform content. Data are means ± SEM. € significantly different from control values, $P<0.05$; $ significantly different from IIa in active group values, $P<0.05$.

Figure 3 : Glycogen content in specific fibre types in soleus (*A-B, E-F*) and plantaris (*C-D, G-H*), before (*A, C*) and after exhaustive running exercise (*B, D, E-H*). Glycogen content was assessed by PAS staining (*A-D*) and fibre type by immunohistochemistry using antibodies directed against type I (*F, H*) and type IIa MHC isoforms (*E, G*). In soleus muscles, pure type IIa fibres (■) or hybrid fibres comprising both type I and type IIa MHC isoforms (●) (*E-F*) are shown in *B* with a lower glycogen depletion than pure type I fibres (▲). In plantaris muscles, type IIa fibres (*G*) are reported in *D* with a slight or no glycogen depletion (□); type I fibres (*H*) were markedly depleted (∆), while fibres negative for antibodies (i.e. fibres comprising either type IIx and/or type IIb MHC isoforms) (*G-H*) show an heterogeneity of glycogen depletion (○) (*D*). Bar = 100 µm.

Figure 4 : Quantitative analysis of MCIP-1 mRNA levels by real-time RT-PCR in whole plantaris and soleus muscles (*A*) and in plantaris single fibres (*B*) of control non-exercised and active rats (arbitrary units). For n and fibres categorization see figure 1 (*A*) and 2B (*B*). Data are means ± S.E.M. € : significantly different from control values, ($P < 0.05$) ; €€ : significantly different from control values, ($P < 0.01$) ; $: significantly different from IIx in the same group, $P < 0.05$; # : significantly different from IIb in the same group, $P < 0.05$.

Gene	Target sequence	Primers	Primer (µM)	$MgCl_2$ (mM)	Annealing temperature (°C)	Product size (bp)	Product Tm (°C)
CycA	M19533 (381-507)	F : TATCTGCACTGCCAAGACTGAGTG R : CTTCTTGCTGGTCTTGCCATTCC	0.4	4	58	127	85.2
IL-6	E02522 (532-610)	F : TCCTACCCCAACTTCCAATGCTC R : TTGGATGGTCTTGGTCCTTAGCC	0.4	3	65	79	81
MCIP-1	AB075973 (31-110)	F : GACTTTAACTACAATTTTAGCTCCCTGAT R : TTGGCCCTGGTCTCACTTTC	0.4	4	60	80	83.5
PECAM	AF268593 (2985-3079)	F : TGTCTGTCGCACTGAGCAGAAATCC R : TGCAGACTGCAACAGAGCAGTTCAG	0.3	5	65	118	87.8
CD11b	U77697 (234-351)	F : TTTCAGCAAGATTGCCGAGGAGAGG R : TTTGGAGAGCATTTCGCACACCTGG	0.3	4	65	95	85.8

Table 1 : Primers designed for real time RT-PCR amplification and their conditions of use.For each gene, table shows Genebank accession number, forward (F) and reverse (R) primers sequences, primer and $MgCl_2$ concentration in PCR mix, primer annealing temperature, product size and product melting temperature (Tm).

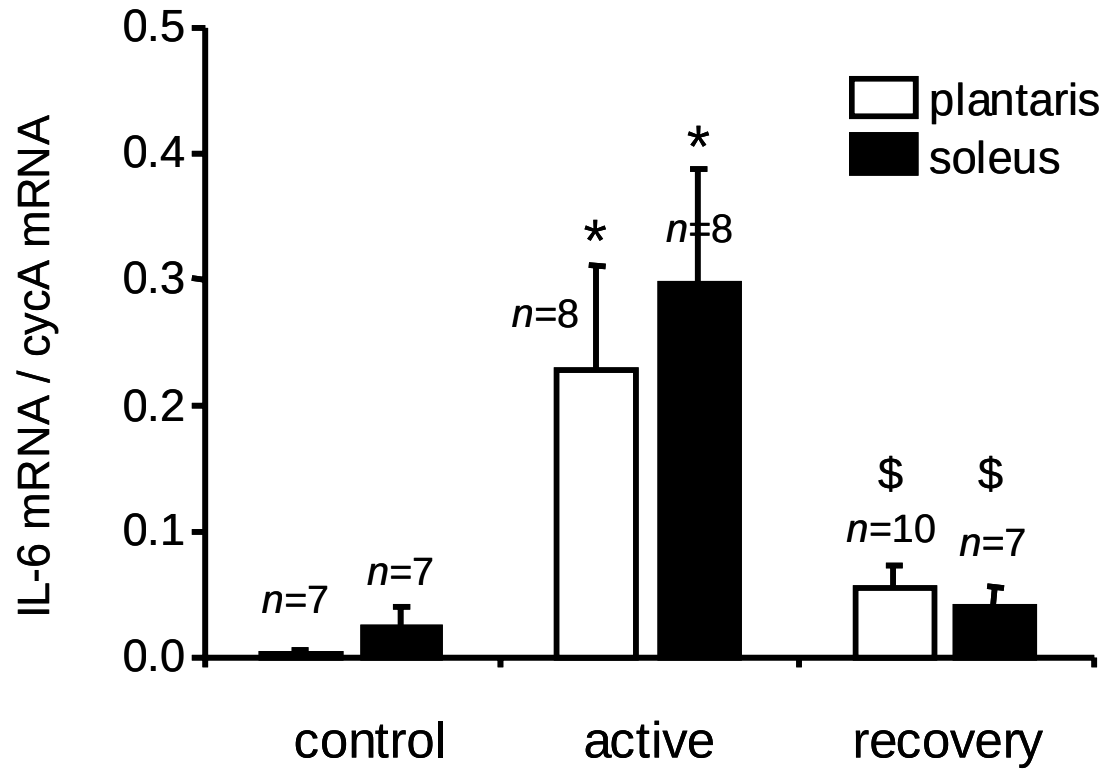

0.5
0.4
0.3
0.2
0.1
0.0
IL-6 mRNA / cycA mRNA
plantaris
soleus
*
*
n=8
n=8
$
$
n=10
n=7
n=7
n=7
control
active
recovery

Figure 1

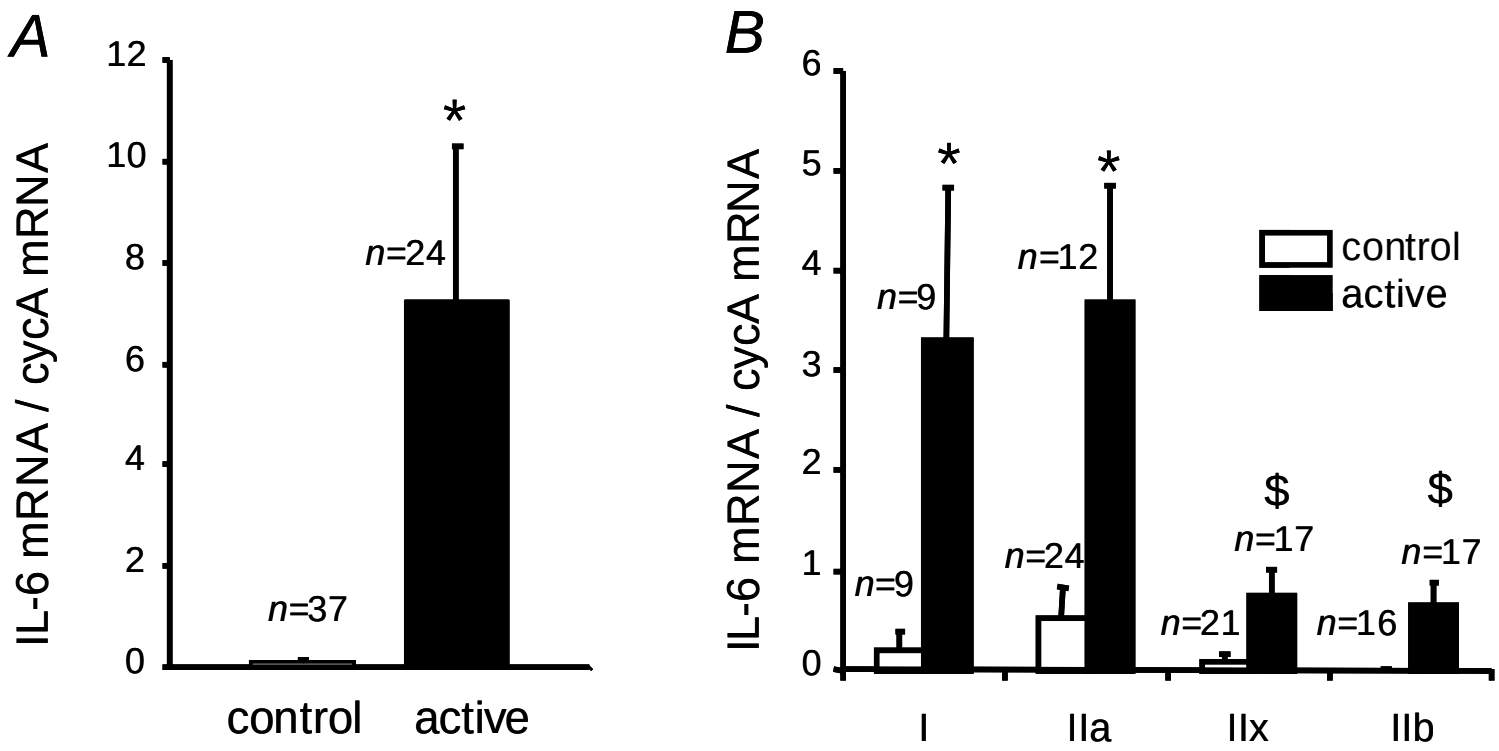
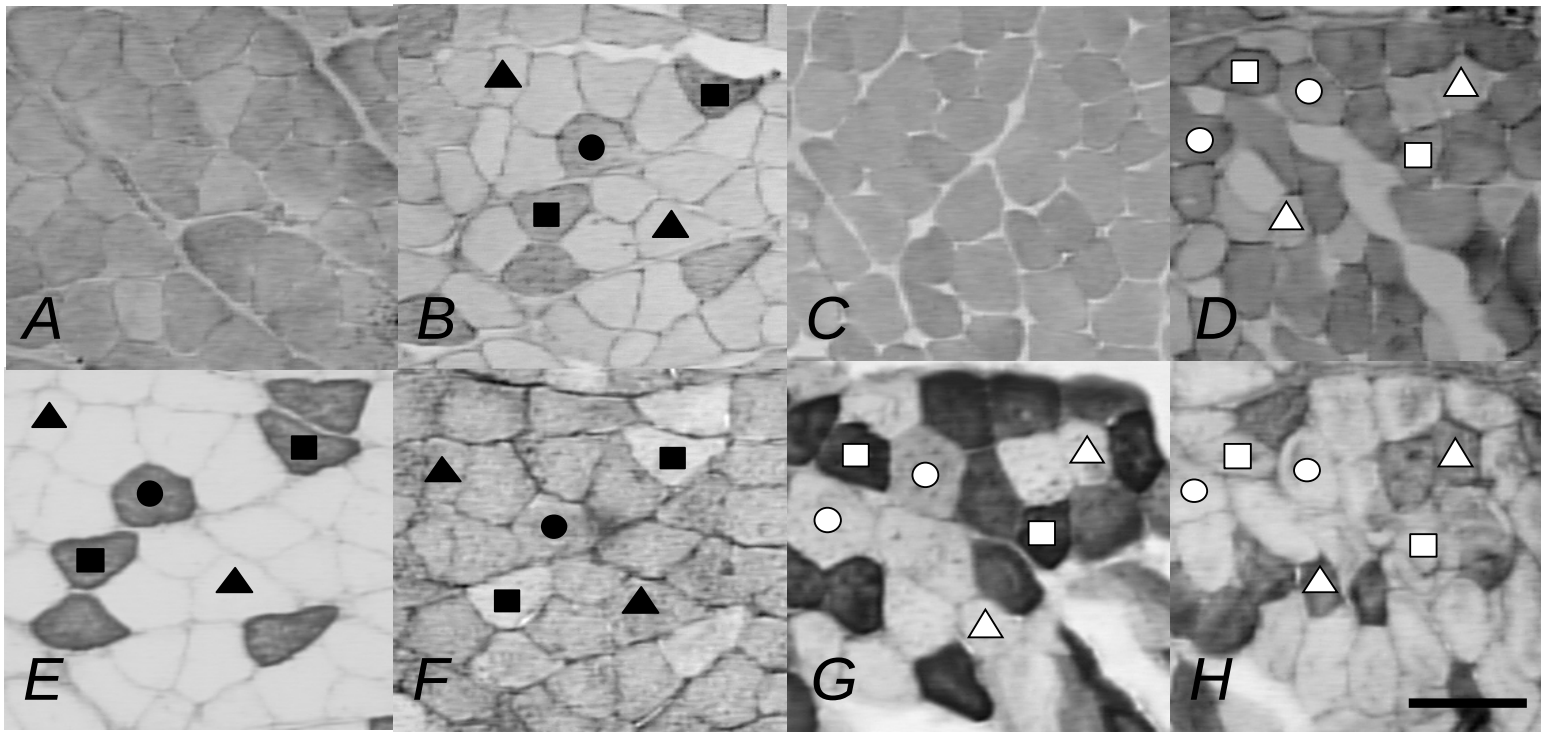

A
IL-6 mRNA / cycA mRNA
12
10
8
6
4
2
0
*
n=24
n=37
control
active
B
IL-6 mRNA / cycA mRNA
6
5
4
3
2
1
0
*
*
n=9
n=12
control
active
$
$
n=17
n=17
n=9
n=24
n=21
n=16
I
IIa
IIx
IIb

Figure 2



Figure

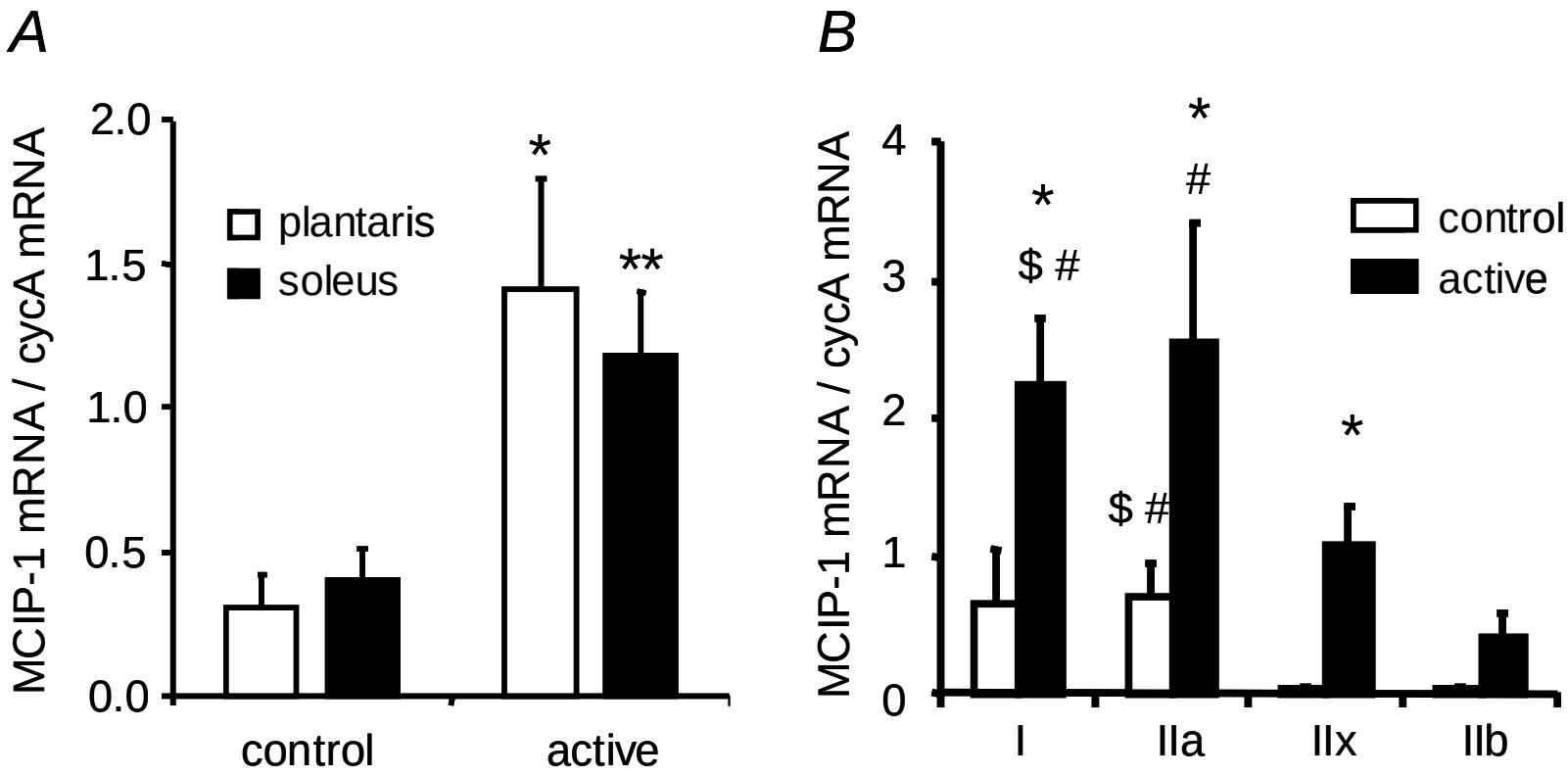

A
MCIP-1 mRNA / cycA mRNA
2.0
1.5
1.0
0.5
0.0
plantaris
soleus
*
**
control
active
B
MCIP-1 mRNA / cycA mRNA
4
3
2
1
0
control
active
*
$ #
*
#
$ #
*
I
IIa
IIx
IIb

Figure 4