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# Basal peroxisome proliferator activated receptor gamma coactivator 1α expression is independent of calcineurin in skeletal muscle

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ABSTRACT

Both calcineurin-A and peroxisome proliferator activated receptor gamma coactivator 1α (PGC-1α) are key players in the acquisition and maintenance of slow-oxidative skeletal muscle phenotype. Whether calcineurin can control PGC-1α expression has been proposed but is still controversial. Our aim was to examine the relationship between calcineurin activation and PGC-1α expression in nonexercising skeletal muscles of rats. We first examined PGC-1α and modulatory calcineurin-interacting protein–1 messenger RNA (mRNA) (a marker of calcineurin activity) expression patterns within rat single myofibers, classified according to their phenotype (type I, IIa, IIx, and IIb). Secondly, we measured PGC-1α mRNA and protein in soleus and plantaris muscles of rats treated or not by cyclosporin A or FK506, 2 pharmacological inhibitors of calcineurin activity. In single myofibers, no differences were found in PGC-1α mRNA levels, whereas modulatory calcineurin-interacting protein–1 mRNA was substantially higher in type I and IIa compared with type IIx and IIb fibers. In cyclosporin A– and FK506-treated animals, no decrease in PGC-1α mRNA and protein was found, despite an efficient blockade of calcineurin activity. Taken together, our results show that, in weight-bearing skeletal muscles, basal PGC-1α expression, necessary to maintain slow-oxidative phenotype, is independent of calcineurin activity.



## 1. Introduction

Skeletal muscles fibers differ in their contractile and metabolic properties. Contractile characteristics mainly depend on myosin heavy chain (MHC) content, whereas metabolic characteristics depend on mitochondrial density and relative amounts of various metabolic enzymes. Slow type I fibers express high levels of oxidative enzymes. Fast-twitch fibers have glycolytic enzyme levels that vary from high in type IIb to low in type IIa fibers. In rodents, type IIa fibers exhibit higher oxidative capacity than type I. The muscle fiber composition is highly flexible and can be adapted to the functional demand. Both increased activity and inactivity result in marked changes in mitochondria density, key metabolic enzymes, and transitions in MHC isoforms [1].

Authors' contribution: SB, XB, and NK designed the study; SB, HS, NK, and RC performed the study and collected the data; SB, NK, XB, and AP analyzed the data; SB and NK wrote the manuscript.

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Calcineurin A is a $Ca^{2+}$-calmodulin–dependent phosphatase activated by increased intracellular $Ca^{2+}$ resulting from tonic motor nerve activity [2,3]. In vivo calcineurin inhibition by cyclosporin A (CsA) induces a shift toward a faster and more glycolytic muscle phenotype [4], whereas muscle-specific calcineurin overexpression induces a slow fiber regulatory program [5]. Calcineurin and downstream transcription factors regulate contractile and metabolic gene sets and are key effectors in both the acquisition and the maintenance of the slow-oxidative phenotype [6].

The transcriptional coactivator peroxisome proliferator activated receptor gamma coactivator 1α (PGC-1α) is a critical factor coordinating the expression of the mitochondrial and nuclear genes encoding proteins required for substrate utilization and mitochondrial biogenesis [7-9]. In addition, PGC-1α overexpression induces a fiber type conversion to slow phenotype in skeletal muscle [10]. Several signaling pathways have been shown to directly influence PGC-1α expression, such as p38 mitogen-activated protein kinase (MAPK) and calcium/calmodulin-dependent protein kinase. The hypothesis that calcineurin can activate PGC-1α transcription in skeletal muscle has first been suggested in vitro [10]. In vivo, constitutively active calcineurin electrotransfer in rat muscle resulted in a transactivation of a cotransfected PGC-1α promoter reporter [11]; and transgenic mice overexpressing active calcineurin display increased PGC-1α protein levels and mitochondrial biogenesis in skeletal muscle [12]. However, the ability of calcineurin to physiologically activate PGC-1α has been discussed because CsA treatment in exercising rats did not blunt muscle PGC-1α increase and mitochondrial biogenesis [13] despite efficient blockade of calcineurin activation. Therefore, calcineurin involvement in skeletal muscle PGC-1α expression remains controversial.

Such discrepancies may be explained by very different kinetics and magnitude in calcineurin activation between the different models. Exercise increases motoneuron activity to induce muscle contraction, resulting in increased cytosolic $Ca^{2+}$ concentrations and calcineurin activity. However, these activations are transient, whereas calcineurin transfection or transgenic expression results in chronically increased activity. Slow-type muscles are mainly postural muscles and are active almost continuously in the active phase of the day, resulting in a chronic activation of calcineurin. The importance of this permanent calcineurin activation in maintaining the slow-oxidative muscle phenotype is well illustrated by the profound alterations reported in response to prolonged pharmacological calcineurin inhibition [4]. Therefore, we made the hypothesis that basal PGC-1α expression may be under control of mild chronic calcineurin activity to play this central role in phenotype maintenance of weight-bearing muscles.

We examined the relationship between calcineurin and PGC-1α expression in nonexercising skeletal muscles. We first studied the coexpression of PGC-1α mRNA and modulatory calcineurin-interacting protein-1 (MCIP-1) messenger RNAs (mRNAs) (a reliable marker of calcineurin activity) in isolated single muscle fibers. Thereafter, we examined PGC-1α mRNA and protein expression in skeletal muscles of rats treated by 2 different calcineurin inhibitors, namely, CsA and FK506.

## 2. Methods

### 2.1. Study 1

#### 2.1.1. Animals

Adult male Wistar rats (n = 10) were housed in standard conditions. They were anesthetized with pentobarbital (70 mg/kg). Plantaris muscles were removed and conserved in RNAlater solution (Ambion, Austin, TX, USA). Animals were killed by removal of the heart.

Single-fiber isolation, MHC isoforms, and mRNA analysis on single fibers were performed as previously described [14]. Briefly, single fibers were manually isolated under microscope. One half-fiber was used to identify MHC content by an electrophoretic method with silver staining. Myofibers expressing only one MHC isoform were studied: I (n = 8), IIa, (n = 20), IIx (n = 17), and (IIb n = 17). Total RNA was purified from the other half using a phenol/chloroform method; mRNA was reverse-transcribed and measured using real-time reverse transcriptase polymerase chain reaction (RT-PCR). Cyclophilin A was used as housekeeping gene.

### 2.2. Study 2

#### 2.2.1. Animals

Adult male Wistar rats were housed in standard conditions and randomly assigned to 3 experimental groups: control (n = 8), CsA (n = 10), or FK506 (n = 10).

#### 2.2.2. CsA, FK506, and vehicle administration

Animals were treated intraperitoneally twice a day with 12.5 mg/kg CsA (Sandimmun, Novartis Pharma, Rueil-Malmaison, France) or subcutaneously with 2 mg/kg FK506 (Prograf, Fujisawa, La Celle Saint Cloud, France). In the control group, rats were treated intraperitoneally twice a day with vehicle (Ricinon, Coopération pharmaceutique française, Melun, France, and 30% ethanol). Treatment duration was 2.5 days for the CsA and control groups and 1.5 days for FK506, which achieves an efficient inhibition faster than CsA in skeletal muscle (personal data).

#### 2.2.3. Tissue processing

Rats were anesthetized with pentobarbital (70 mg/kg); soleus and plantaris muscles were excised and conserved in RNAlater solution for mRNA measurements or frozen in liquid nitrogen for protein analysis. Animals were killed by removal of the heart.

#### 2.2.4. Messenger RNA measurement

Messenger RNA analysis was performed using real-time RT-PCR. Total RNA was isolated from 8 to 10 mg from the midbelly of the muscle using Qiazol reagent (Qiagen, Courtaboeuf, France) with RNeasy columns (Qiagen) cleanup. Complementary DNA was synthesized from 0.5 μL RNA with Reverse Transcriptase Core Kit (Eurogentec, Seraing, Belgium) using oligo(dT) primers. Polymerase chain reaction was performed using Light cycler system (Roche Applied Science, Mannheim,

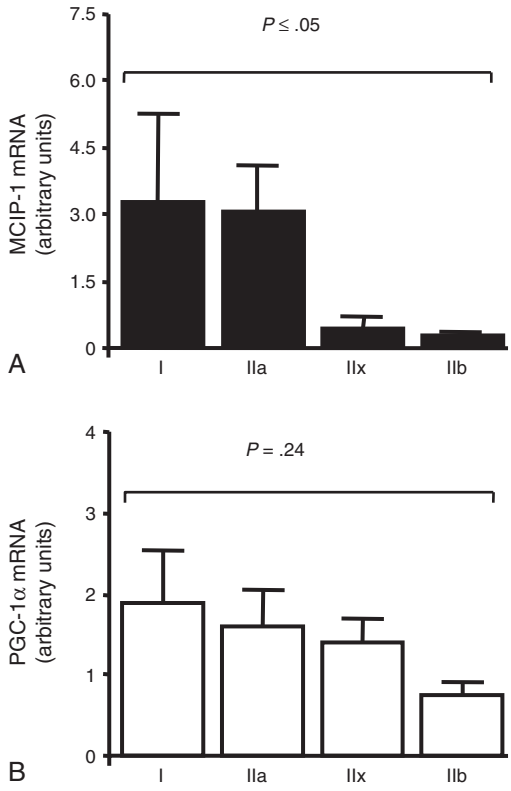


**Fig. 1 – The MCIP-1 and PGC-1α mRNA levels in plantaris single fibers of resting rats. Plantaris fibers were categorized into 4 types—I, IIa, IIx, and IIb—according to their MHC isoform content. The MCIP-1 (A) and PGC-1α (B) mRNA levels were measured using real-time RT-PCR. Data are mean ± SEM.**

Germany). Cyclophilin A, hypoxanthine-guanine phosphoribosyl transferase, and acidic ribosomal phosphoprotein were used as housekeeping genes; their stability was validated using Genorm software [15]. Primers used in this study, cyclophilin A, hypoxanthine-guanine phosphoribosyl transferase, acidic ribosomal phosphoprotein, MCIP-1, and PGC-1α, have been described previously [16,17].

#### 2.2.5. *Protein isolation and immunoblot analyses*

A range of 10 to 20 mg of muscle was lysed with the appropriate buffer, and homogenates were centrifuged at 15 000*g* for 15 minutes at 4°C. Protein concentration was determined using the bicinchoninic acid method with the microplate BCA protein assay kit (Pierce, Thermo Scientific, Rockford, IL, USA). Because of the lower expression in plantaris muscle [18], different amounts of muscle were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis protein (30 and 50 μg for soleus and plantaris, respectively) and transferred onto nitrocellulose membranes (Hybond-C Extra, Amersham Pharmacia Biotech, Orsay, France), which means that lane loading was normalized for total muscle protein content, as verified by Ponceau S staining. A standardized amount of protein prepared from control muscle was also applied on each gel to serve as an internal standard for comparison across blots. Membranes were incubated overnight with the primary polyclonal antibody against PGC-1 (1:1000 solution, catalog no. AB3242, Millipore, Temecula, CA, USA) and then with horseradish peroxidase–conjugated donkey anti-rabbit immunoglobulin G (1:10 000 dilution, sc-2313, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Washed blots were subjected to the Immun-Star WesternC kit (Bio-Rad Laboratories, Marnes-la-Coquette, France) and then read with the imaging system ChemiDoc XRS+ driven by Image Lab (Bio-Rad). The relative protein expression was determined by the ratio of sample band intensity to internal standard band intensity.

#### 2.2.6. *Statistical analysis*

Data are presented as means ± SEM. A 1-way analysis of variance was used to evaluate the global effect "fiber type" or "treatment." When appropriate, the Newman-Keuls post hoc test was used for intergroup comparisons. Statistical significance was accepted for $P < .05$.

## 3. Results

### 3.1. *Coexpression of PGC-1α and MCIP-1 mRNA within single fibers*

There was a significant effect (global effect $P < .05$) of fiber type on MCIP-1 mRNA expression (Fig. 1A), with higher levels found

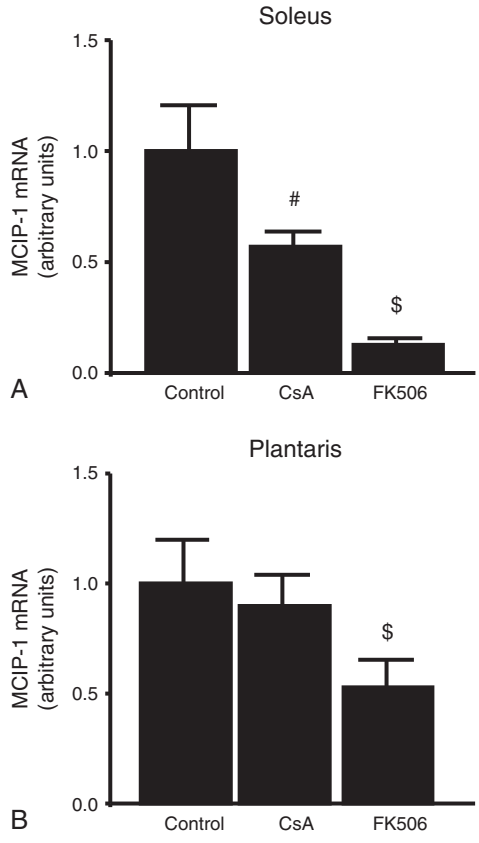


**Fig. 2 – The MCIP-1 mRNA levels in soleus (A) and plantaris (B) muscles of rats treated by vehicle (control), CsA, or FK506. The muscle mRNA levels were measured using real-time RT-PCR. Data are mean ± SEM. #$P < .01$, different from control value. $$P < .01$, different from control and CsA.**

in type I and IIa fibers compared with type IIx and IIb. Conversely, no significant difference in PGC-1α mRNA levels was found among fibers (global effect $P = .24$) (Fig. 1B).

### 3.2. *Effects of the pharmacological inhibition of calcineurin in soleus and plantaris muscles*

Calcineurin inhibitors treatment induced a significant drop in MCIP-1 mRNA levels in soleus muscle (global effect $P < .01$) (Fig. 2A). The MCIP-1 mRNA levels were lower in the FK506 than the CsA group ($P < .01$).

In plantaris muscle, treatment resulted in a significant drop in MCIP-1 mRNA levels (global effect $P < .05$) (Fig. 2B). The MCIP-1 mRNA levels were lower in the FK506 than the control group; however, CsA did not efficiently inhibit MCIP-1 mRNA expression.

### 3.3. *PGC-1α in soleus and plantaris muscles*

Treatment had an overall effect on PGC-1α mRNA levels in soleus muscle (global effect $P < .01$) (Fig. 3A), with higher levels in FK506 group compared with others ($P < .01$). In plantaris muscle, no significant effect of treatments was found (Fig. 3B).

Finally, in both soleus and plantaris muscles, calcineurin inhibitors had no significant effect on PGC-1α protein levels (Fig. 3C, D).

## 4. Discussion

In the present work, we explored the link between calcineurin activity and PGC-1α mRNA expression in nonexercising skeletal muscles and found that (1) despite substantially higher MCIP-1 transcript levels in oxidative (I, IIa) compared with glycolytic (IIx, IIb) fibers, no significant differences were found in PGC-1α mRNA levels and (2) calcineurin inhibition did not blunt basal PGC-1α mRNA and protein expression in soleus and plantaris muscles. Taken together, these results show that calcineurin is not involved in basal PGC-1α expression in weight-bearing muscles.

Because PGC-1α is a key regulator of slow-type metabolic and contractile phenotype in muscle, it has been proposed that this coactivator could be transcriptionally controlled by the calcineurin pathway [19]. Studies have shown that calcineurin activation can increase skeletal muscle PGC-1α transcription and protein expression in vivo [11,12]. However, these studies used chronic overexpression of an active form of calcineurin, either by gene electrotransfer or muscle specific transgenic expression in mice. Therefore, to determine whether mild but continuous physiological activation of calcineurin contributes to PGC-1α expression, we focused on normal weight-bearing muscles, where both calcineurin and PGC-1α are involved in slow-oxidative phenotype maintenance.

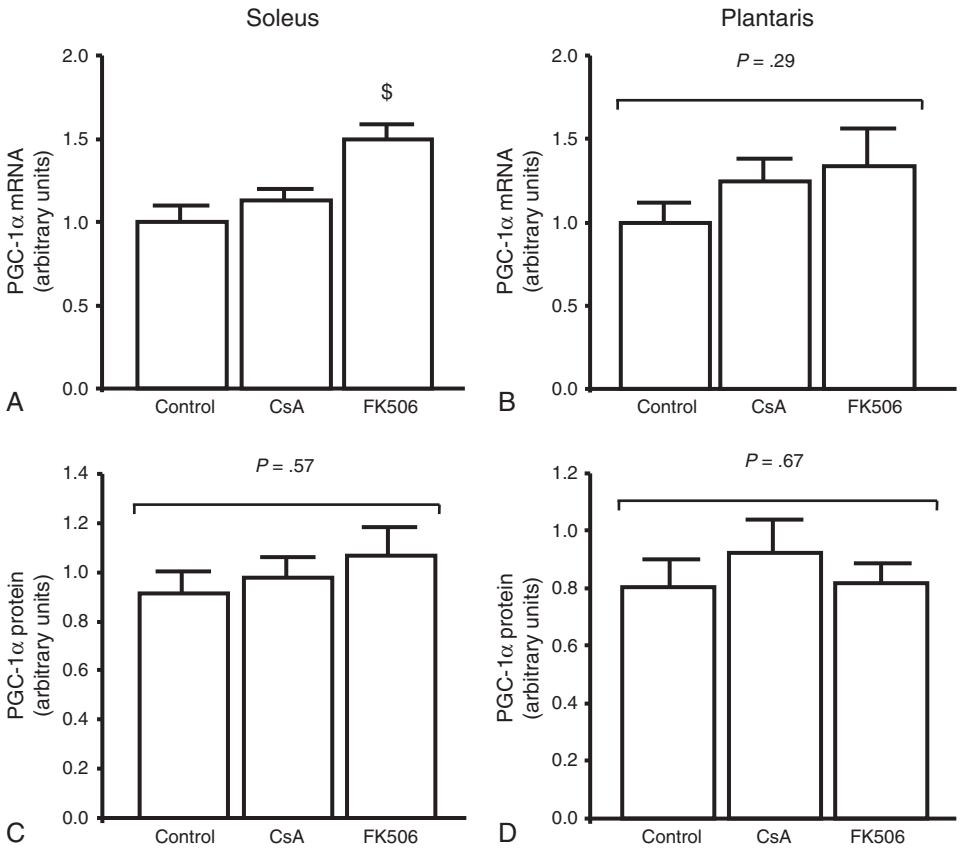


**Fig. 3 – The PGC-1α mRNA and protein levels in soleus (A and C) and plantaris (B and D) muscles of rats treated by vehicle (control), CsA, or FK506. The muscle mRNA levels were measured using real-time RT-PCR; protein levels were measured by immunoblotting. Data are mean ± SEM. $P < .05, different from control and CsA.**

The single-fiber approach is a powerful method to explore mRNA levels in different myofiber types isolated from a muscle in physiological conditions, contrary to cell culture approach. It appears from our single-cell results that, within the same skeletal muscle (plantaris), no significant differences in PGC-1α mRNAs are found between fiber types. Conversely, as previously described [14], MCIP-1 mRNA levels, an indirect but reliable marker of calcineurin activity, are substantially higher in oxidative (type I and IIa) than in glycolytic (type IIx and IIb) fibers. Because of the important differences in expression patterns of MCIP-1 and PGC-1α within single fibers, a direct link between calcineurin activity and PGC-1α mRNA levels appears very unlikely. This is further supported by the lack of correlation between MCIP-1 and PGC-1α mRNA levels in the 62 fibers included in our experiment (data not shown). However, definitive conclusions cannot be stated from these correlative results; and we decided to inhibit calcineurin activity in vivo to further understand its relationship with muscle PGC-1α expression.

Pharmacological inhibition by CsA or FK506 has been widely used to explore calcineurin role in skeletal muscle [2,4]. However, these drugs are known to induce several adverse effects when chronically used; so we chose to use very brief drug administrations. Treatments were well tolerated; specifically, no body weight loss was observed. Furthermore, it is very unlikely that any substantial shift in muscle fiber type could occur in such a short time. In the soleus muscle, mainly composed of slow-oxidative type I fibers, both CsA and FK506 substantially decreased MCIP-1 mRNAs without blunting PGC-1α mRNA. A moderate but significant increase in PGC-1α mRNA was found in the FK506 group, a result previously reported in CsA-treated rats and possibly due to an increase in p38 MAPK phosphorylation [16]. However, no differences were found at the protein level. In plantaris muscle, composed of various fiber types including many fast-oxidative type IIa fibers, CsA treatment was not able to inhibit calcineurin activity, a limitation previously reported in rats [4]. However, under FK506 treatment, plantaris MCIP-1 mRNA levels were significantly decreased (50%); but again, no alteration in PGC-1α mRNA and protein was found. Taken together, soleus and plantaris results show that, in muscles under normal weight-bearing conditions, calcineurin is not involved in short-term transcriptional control of PGC-1α. This conclusion is in opposition with the results obtained with chronic calcineurin overexpression [11,12], but is in accordance with another study where calcineurin inhibition did not alter training-induced increase in muscle PGC-1α expression and mitochondrial biogenesis [13]. Therefore, as far as physiological conditions are concerned (spontaneous activity and exercise training), PGC-1α transcript and protein levels are not controlled by calcineurin activity.

Both calcineurin [4] and PGC-1α [20] are required for slow-oxidative phenotype maintenance in nonexercising skeletal muscle. The present results show that, in this situation, calcineurin does not control PGC-1α expression. Cellular signaling pathways such as p38 MAPK [21], calcium/calmodulin-dependent protein kinase [22], and protein kinase D/histone deacetylase–5 [23] can transcriptionally modulate PGC-1α expression in response to exercise or neuromuscular activity; however, further experiments are needed to explore the role they could play in basal PGC-1α expression in skeletal muscle. Calcineurin and PGC-1α seem to belong to 2 separate signaling pathways in muscle phenotype control; nevertheless, they could cooperate through cross-signaling. Indeed, myocyte enhancer factor–2, a transcription factor involved in the control of several slow-oxidative program genes, can be activated by both PGC-1α [10] and calcineurin [3], suggesting that these 2 actors could act synergistically to maintain muscle phenotype [12].

In conclusion, the present study shows that despite higher calcineurin activity in oxidative fibers, there is no fiber-type specificity of PGC-1α mRNA expression at rest. Furthermore, the use of 2 different calcineurin inhibitors shows that PGC-1α expression is independent of calcineurin pathway. Thus, these 2 key molecules controlling slow-oxidative proteins expression could be 2 redundant and possibly cooperating pathways for muscle slow-oxidative phenotype maintenance.

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## Conflict of Interest

Authors have no conflicts of interests to declare.

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