New evidence of exercise training benefits in myostatin-deficient mice: effect on lipidomic abnormalities.

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Abstract

Myostatin (*Mstn*) inactivation or inhibition is considered as a promising treatment for various muscle-wasting disorders because it promotes muscle growth. However, myostatin-deficient hypertrophic muscles show strong fatigability associated with abnormal mitochondria and lipid metabolism. Here, we investigated whether endurance training could improve lipid metabolism and mitochondrial membrane lipid composition in mice where the *Mstn* gene was genetically ablated (*Mstn*$^{-/-}$ mice). In *Mstn*$^{-/-}$ mice, 4 weeks of daily running exercise sessions (65-70% of the maximal aerobic speed for 1 hour) improved significantly aerobic performance, particularly the endurance capacity (up to +280% compared with untrained *Mstn*$^{-/-}$ mice), to levels comparable to those of trained wild type (WT) littermates. The expression of oxidative and lipid metabolism markers also was increased, as indicated by the upregulation of the *Cpt1*, *Ppar-δ* and *Fasn* genes. Moreover, endurance training also increased, but far less than WT, citrate synthase level and mitochondrial protein content. Interestingly endurance training normalized the cardiolipin fraction in the mitochondrial membrane of *Mstn*$^{-/-}$ muscle compared with WT. These results suggest that the combination of myostatin inhibition and endurance training could increase the muscle mass while preserving the physical performance with specific effects on cardiolipin and lipid-related pathways.

Key words: GDF-8, thin layer chromatography, phospholipids, muscle fatigue, handling fat

Introduction

Myostatin, a member of the TGF-beta family of signaling molecules, controls muscle growth in mammals [1-2]. Myostatin gene deletion (*Mstn*$^{-/-}$ mice) or loss-of-function leads to excessive skeletal muscle growth and increases muscle mass. Conversely, its overexpression or systemic administration causes muscle atrophy [1;3]. Due to its effects on muscle mass, myostatin inhibitors are potentially useful in the clinic to limit muscle wasting [4]. In contrast, the muscle mass gain induced by myostatin inhibition/gene deletion is associated with increased fatigability and aerobic metabolic deficit. Specifically, muscles of *Mstn*$^{-/-}$ mice show reduced limit endurance time in an *ex vivo* endurance contractile test [5], as well as decreased mechanical performance and ATP production during *in vivo* exercise [6]. Metabolically, *Mstn*$^{-/-}$ mice provided evidence of impaired activity of oxidative enzymes, mitochondrial DNA depletion associated with uncoupling of the intermyofibrillar mitochondrial respiration [5], as well loss of oxidative muscle fibers and increase in glycolytic fibers [2;7]. Loss of muscle oxidative properties is accompanied by marked modifications in substrate phosphorylation, and in the expression levels of molecules involved in lipid-related pathways. For instance, the levels of cytosolic and sarcolemmal lipid transporters (FABP3 and FATP1 and FATP4, respectively) are reduced, as well those of FAT/CD36 (fatty acid uptake) [8-9]. Furthermore, lipogenesis in *Mstn*$^{-/-}$ muscle is significantly reduced. Specifically, triglyceride (TG) levels are 4-fold lower in *Mstn*$^{-/-}$ than in wild type (WT) mice and this is correlated with lower fatty acid synthase (FAS) activity [9] and a shift in fatty acid (FA) composition (increase in saturated FA and polyunsaturated FA levels at the expenses of monounsaturated FA levels). Mitochondria are also significantly affected with modifications of the membrane phospholipid composition, as indicated by the significant decrease in the proportion of cardiolipin (CL), a phospholipid that is unique to the inner mitochondrial membrane, in *Mstn*$^{-/-}$ mitochondrial membranes [9]. Due to the role of CL in the mitochondrial structure and function [10-11] and of lipids in the supply of reduced equivalents for contractile activity [12], these findings provide evidence on the mechanism by which myostatin regulates mitochondria function and oxidative metabolism.

Several studies have shown that myostatin-deficient muscles are not genetically locked and can be functionally remodeled to improve oxidative metabolism through exercise training [13-14]. Most notably, endurance exercise reduces muscle fiber size, increases muscle oxidative properties and capillary density and improves muscle force generation in *Mstn*$^{-/-}$ mice. However, important aspects of the mechanisms that govern such adaptive responses in mitochondria remain obscure. Recent findings [9] suggest that in *Mstn*$^{-/-}$ muscles, the mitochondrial phospholipid profile and more broadly the lipidome could benefit from aerobic endurance training. Indeed, lipids and FA are a major fuel in resting and exercising muscles, and are used to produce ATP via β-oxidation in the mitochondrial matrix [15]. It is well documented that endurance training induces a shift in skeletal muscle towards a higher lipid metabolism, with higher fat oxidation, transport capacity, mitochondrial density and muscle oxidative capacity [12;15]. Moreover, FA and phospholipids are the major components of membranes and influence several aspects of membrane function including membrane fluidity, membrane protein anchorage and the activity of the electron transport chain complexes, consequently the aerobic capacity [12;15-16]. It has been shown that exercise training modifies the membrane lipid composition. For instance, total CL content is increased with exercise training in rats and humans [17-18]. Conversely, CL content decreases following detraining, and this effect has been associated with a decrease in mitochondrial respiratory chain complexes and enzyme activity in skeletal muscle [18-19].

In this study, we asked whether the positive metabolic effect of endurance training could influence lipid metabolism and mitochondrial lipid composition in the hypertrophic muscles of *Mstn*-/- mice. By analyzing skeletal muscle from WT and *Mstn*$^{-/-}$ mice that underwent or not 4 weeks of endurance training, we found that endurance training improved significantly the aerobic performance, mainly the endurance capacity in *Mstn*$^{-/-}$ mice, reversing the difference with WT mice. This functional gain was associated with increased oxidative metabolism and fat oxidation in skeletal muscle of *Mstn*$^{-/-}$ mice, along with normalized CL fraction in the muscle mitochondrial membrane. These results suggest that muscle hypertrophy and physical endurance could be obtained by combining myostatin inhibition with exercise training, with responsiveness of CL and lipid-related pathways.

Materials/Methods

Animals

Healthy 10-week-old *Mstn*$^{+/+}$ (WT) and *Mstn*$^{-/-}$ male mice (KO) were used in this study (Table 1, n=18 for each genotype). These mice were generously provided by L. Grobet (Faculty of Veterinary Medicine, University of Liège, Belgium). Mice were housed in standard conditions (20–22°C, 12h–12h light–dark cycle), in normal cages with *ad libitum* access to tap water and food. The study experimental protocols complied with the European directives on animal experimentation (86/609/EEC) and were approved by the Ethical Committee of the Languedoc Roussillon Region (APAFIS#2551-2015110311365663v2). Eight samples/group were used for biochemical analyses and 6 samples/group for RT-qPCR assays.

Running performance

All mice were tested without knowing to which group they belonged to [8]. The maximal aerobic velocity (MAV) was determined with a running test in which the speed was gradually increased from 10 m/min by 2 m/min until exhaustion. Endurance capacity was determined in a submaximal running test where the speed started at 10 m/min for the first 2 minutes and was then set to 70% of the MAV until exhaustion. The limit endurance time was recorded. Mice were killed 48 hours after the last running test.

Aerobic training

After acclimation period, WT and KO mice were divided in two groups: sedentary (n=9) and trained (n=9). Training consisted in running at 65-70% of the MAV on a treadmill (Columbus Instruments, OH 43204 U.S.A) calibrated for speed (angle 0°) for 1 hour (5 consecutive days/week) for 4 weeks. Exercise intensity was increased of 2 m/min each week.

Muscle preparation and sample

Mice were weighed and killed by cervical dislocation. Tibialis anterior (TA), extensor digitorum longus (EDL), and quadriceps (Quad) muscles were quickly excised and immediately placed in ice-cold buffer (100 mM KCl, 5 mM $MgSO_4$, 5 mM EDTA, and 50 mM Tris·HCl, pH 7.4) for total mitochondrial protein extraction. Total mitochondria were fractionated by differential centrifugation, as described previously [9]. Mitochondrial protein content was determined using the Bradford assay, and the yield was expressed as milligram of mitochondrial proteins per gram of muscle wet weight. Gastrocnemius was removed, frozen in liquid nitrogen and powdered for protein and mRNA expression analyses and measurement of FA and enzymatic activity.

Triglyceride (TG) contents in muscle lipid extracts by TLC densitometry.

Muscle lipids were extracted using a mixture of chloroform–methanol (2:1, v/v) in the presence of butylated hydroxytoluene (50mg/l). The separation of neutral lipids was obtained by TLC densitometry according to the method previously described [9]. The content of 1-monoacylglycerol (MAG), 1,2- diacylglycerol (DAG), 1,3-DAG, TG, cholesterol and free FA was determined by comparing their retention factor (Rf) with that of standards and were quantified using the calibration curves for the same standards.

Phospholipid composition of muscle mitochondrial membrane by TLC densitometry

Muscle mitochondrial suspensions were extracted by using a mixture of chloroform/methanol 2:1 (v/v) according to [9], in the presence of 50 mg/L butylated hydroxytoluene. Phosphorus was quantified in Folch extracts of mitochondrial suspensions to determine the total phospholipid quantity. The phospholipids and neutral lipids of Folch extracts of muscle mitochondrial suspensions were separated on HPTLC silica gel 60 plates as described by Baati et al. [9]. The different phospholipid classes were identified by comparing their Rf to standards and quantified using the calibration curves of the same standards.

Protein extraction and western blot analysis

50 mg of gastrocnemius muscle powder was homogenized in ice-cold extraction buffer then proteins were denaturated and separated as previously [9]. After blocking step, membranes were incubated overnight with primary antibodies against **FAT/CD36 (**1/500; Santa Cruz Biotechnology, CA, ref sc-13572)**, FATP4** (1/500; Santa Cruz Biotechnology, CA, ref: sc-5834), **FABP3** (1/1000, Abcam, ref: ab45966), citrate synthase (**CS)** (1/1000; GeneTex, ref: GT1761) and **α-tubulin** (1/5000; Sigma-Aldrich, St Louis, MO, ref: T6199) in blocking buffer. Wash and revelation protocols are detailed in [9]. Signals were detected with a ChemiDoc™ Touch Imaging System instrument (Bio-Rad, 1.1.0.4, 732BR1121); α-tubulin was used as loading control. Band intensities were measured using Image Lab Software (Version 5.2.1 for Windows 7).

Quantification of mRNA expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR):

Total RNAs were isolated from powdered gastrocnemius samples using the RNeasy Fibrous Tissue Mini Kit following the manufacturer's instructions (Qiagen, Venlo, The Netherlands). RNA concentration was determined by spectrophotometric analysis (Eppendorf AG, Hamburg, Germany), and purity was checked by calculating the OD260nm/OD280nm absorption ratio (>1.7). RNA quality was verified by 1% agarose gel electrophoresis.

Reverse transcription was performed with 1µg of total RNA and the TAKARA kit (TAKRR037A, PrimeScript RT reagent Kit, Perfect Real Time) according to the manufacturer's instructions. One-tenth of the cDNA was used in each PCR assay.

Quantitative PCR (qPCR) analysis was performed using a Step One Plus detection system (AB Applied Biosystems) with 18µL of Mastermix (SYBR Premix Ex Taq II, Takara), 300nM of both forward and reverse primers, 2µL of diluted cDNA template and water to a final volume of 20µL. *Cpt-1, Pgc1-a* and *Ppar-δ* were amplified to evaluate lipid oxidation, and *Fas* lipogenesis, *Crls1* and *Pgs1* to assess cardiolipin synthesis, and *Taz* and *Lcat1* for cardiolipin remodeling. All PCR assays were performed in duplicate using the following parameters: 98°C for 30s, followed by 40 cycles of 95°C for 1s and 60°C for 15s. Relative mRNA levels were normalized to the levels of the genes

encoding the ribosomal protein Arp and α-tubulin that were unaffected by training. Results are expressed using the comparative cycle threshold (Ct) method to generate ΔΔCt values with template dilutions ranging from 10^1 to 10^6 copies. The PCR overall efficiency (E) was calculated from the slopes of the standard curves according to the equation $E = [10^{(-1/slope)}] - 1$ and this value was higher than 95% for all assays. The relative abundance of each sample was then normalized according to the equation: Relative Quantity (RQ) = $2^{-\Delta\Delta Ct}$.

Statistical analysis

All data are presented as the mean ± SEM. A two-way ANOVA followed by the Tuckey post-hoc procedure was used to determine the effects of training and the animal genotype. The significance level was set at 0.05. Data were analyzed using the statistical package GraphPad Prism version 6.02 for Windows (La Jolla, California).

Results and discussion

Endurance training increased aerobic running performance, in particular in KO mstn mice

It is well known that the hypertrophic *Mstn*$^{-/-}$ phenotype is associated with marked functional impairment [2;5;20] as detailed in the Figure 1. Endurance-type physical exercise would reverse the functional deficits in *Msn*$^{-/-}$ mice, meanwhile attenuates the hypertrophic phenotype [14;20] raising the question of whether muscle size and oxidative capacity regulation by myostatin are linked. In our present study, we showed that forced 4-weeks treadmill exercise increased muscle endurance capacity more in *Mstn*$^{-/-}$ mice (+280%) than in WT littermates (+54%), leading to similar levels of performance in both genotypes (Figure 1). Our results strengthened that the *Mstn*$^{-/-}$ muscles can adapt and be functionnally reprogrammed. Nevertheless, the effect of longer training period remains questionable. Indeed, we cannot exclude limits in the functional adaptability or fatigue-induced by chronic high-intensity exercise. Meanwhile, our results are interesting because they underline the relevance of muscle remodelling by complementary approaches that impact both muscle mass and function. Mstn inhibition and aerobic metabolism activators, such as endurance training or exercise mimetic

environment induced by AICAR [8;21] are interesting approaches for preventing functional deficits in skeletal muscle. Next, to better understand the mechanism underlying this functional plasticity in the hypertrophic KO muscle mouse model, we investigated mitochondrial, lipid and cardiolipid-related pathways.

Endurance training induced slight improvement in muscle mitochondrial metabolism without impacting muscle mass

The running improvement was not associated with a decrease in muscle mass (Table 1) differently from the results reported by Savage et al. [22]. The reason(s) for this difference could be related to the exercise type, intensity and duration as well as to the genetic background of the used mice. However, the strain used in our study is similar to the one used in the previous study. On the other hand, we trained mice for 4 weeks, at a greater relative intensity than the mice in the previous study by Savage et al [22], and this could have facilitated muscle weight maintenance. Consistent with our results, a recent study demonstrated that genetic manipulation leading to a greater oxidative capacity in KO mice is not accompanied by muscle size reduction [23]. In line with increased running performance, the mitochondrial protein content and CS protein level were increased in trained compared with sedentary animals, but a lesser extend in KO mice (**Figure 2A** and **2B**). Similar significant results were demonstrated on muscle enzymatic activity (CS and β-HAD **Figure 2C).** Finally, a training-induced increase in mitochondrial β-HAD activity is also noted in both WT and KO trained mice compared to sedentary littermates (**Figure 2F**). No significant training effect was observed concerning muscle and mitochondrial COX activity (Data not shown). Overall these data showed slight improvement in mitochondrial metabolism induced by endurance training suggesting others potential mechanisms.

Exercise training modulated muscle transport and metabolism of lipids

Knowing the functional role of lipids and phospholipids in oxidative phosphorylation, respiratory chain (biogenesis, stability and activity of the respiratory complexes) function and thus on aerobic capacity [10-11; 24], lipids could be considered as a link by which myostatin deficiency can

affect mitochondrial bioenergetic functions in skeletal muscle. Here, we confirmed the marked lipidomic disturbance in the hypertrophic phenotype characterized by decreased lipid-mediated transport (Figure 3A-B) and 65-70% decrease in TG levels compared with WT muscles (Figure 3C) that would suggest altered lipogenesis [9]. By focusing on the lipid pathways, we showed a transcriptional response to training in mice. The expression of *Ppar-δ*, a master regulator of FA metabolism increased in both genotypes in response to exercise (Figure 3D). Consistently, FA uptake and utilization also improved with increased expression of *Cpt1* and FAT/CD36 in both groups (Figure A-B). Increased muscle TG content is a very early response to training, preceding significant changes in insulin sensitivity [25]. TG is increased in trained animals to optimally match fat oxidative capacity and serves as readily available fuel [26]. Our data obtained in trained WT mice (Figure 3C) confirmed the impact of training on muscle-specific fat storage (i.e., significantly increased FAS activity and considerable higher TG levels). However, although FAS activity increased (Figure 3B), KO muscle remains resistant to TG storage after training (Figure 3C). Our results confirm that promoting muscle size expansion decreases fat mass effectively in skeletal muscle, as reported for adipose tissue [1]. As a result, this strong difference raises the question of the impact of myostatin deficiency on the synthesis rates of intramuscular TG, and thus on ceramide and diacylglycerol concentration [27-28].

Exercise training impacted differently mitochondrial CL composition in WT and KO mstn mice.

The lipidomic abnormalities extend to the mitochondria, with an impact of myostatin deficiency on mitochondria membrane composition, as indicated by the reduced proportion of mitochondrial CL in the sedentary groups (Figure 3F). Our RT-qPCR results on genes encoding enzymes involved in CL formation (marked decrease of *Crls1* expression; Figure 3G with no decrease of *Pgs1,* data not shown) suggest that myostatin deficiency negatively affects CL synthesis rather than remodeling (Taz expression, **Figure 3H** and no effects on Alcat1 expression, data not shown). The mitochondrial-specific phospholipid CL is involved in various mitochondrial functions, including electron transport chain activity, respiratory supercomplex formation, mitochondrial transport of bioenergetic molecules, and mitochondrial bioenergetic process [10-11;24]. Several studies have

shown the importance of maintaining normal CL levels for mitochondrial function and structural integrity. This is exemplified by the cardiac phenotype in patients with Barth syndrome where reduced CL levels have been associated with mitochondrial dysfunction and genetic defects in CL synthesis [29-30]. The low oxidative capacity of KO muscles could explain the significant alteration of basal CL level and we can speculate both in relation with the decrease in the uncoupling intermyofibrillar mitochondria [5-6]. Interestingly, endurance training abolished the significant difference in the amount of CL in mitochondrial membranes in KO compared with WT mice (**Figure 3F**). In accordance, RT-qPCR quantification showed some genes involved in CL synthesis (*Crls1,* **Figure 3G***)* and remodeling (*Taz*, **Figure 3H**) were downregulated by endurance training in WT but not in KO mice, abolishing again the genotype difference. Regarding CL pathway, our findings suggest thus that the pattern of adaptation to endurance training differs between WT and KO mice. Despite our work cannot highlight the underlying mechanism, mitochondria-related metabolic differences linked to the KO mstn glycolytic muscle phenotype could be advanced. Future investigations on potential strategies to induce remodeling of the mitochondrial membrane phospholipid composition appear relevant in this hypertrophic model.

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Table 1: ***Body and muscle weights of sedentary and trained WT and KO mstn mice***

	Sedentary		**Trained**	
	WT	KO	WT	KO
Body Weight (g)	27.8±1.0	31.4±0.4****	25.6±0.9	30.5±0.8*****
Gastro/BW (mg/g)	6.0±0.3	8.1±0.2***	5.9±0.1	7.9±0.3***
Quad/BW (mg/g)	8.2±0.2	11.1±0.3***	7.4±0.3	11.1±0.5***
EDL/BW(mg/g)	0.4±0.01	0.6±0.02****	0.4±0.01	0.6±0.03****
TA/BW (mg/g)	2.1±0.05	2.6±0.11***	2±0.06	2.8±0.05****

BW: Body weight (g); TA: tibialis anterior; EDL: extensor digital longus; Gastro: gastrocnemius; Quad: quadriceps; WT wild-type; KO knock-out. ***p< 0.001 vs WT; n=9 animals per group.

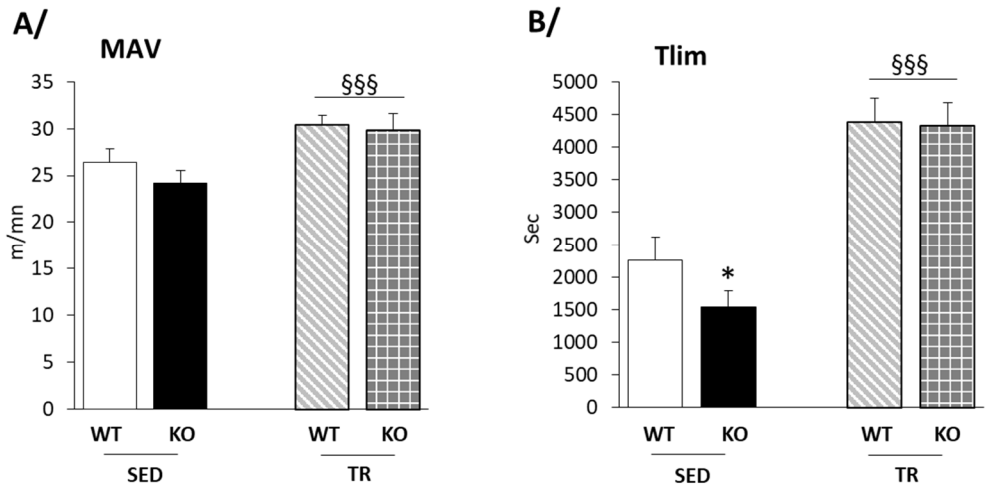

Figure 1. The effect of training on A) maximal aerobic running velocity (MAV) and B) Endurance limit time (Tlim) in sedentary or trained WT and KO mstn mice (n=9 in each group). WT wild-type; KO knock-out, SED sedentary, TR trained; * $p<0.05$ vs WT; §§§ $p<0.001$ vs sedentary mice.

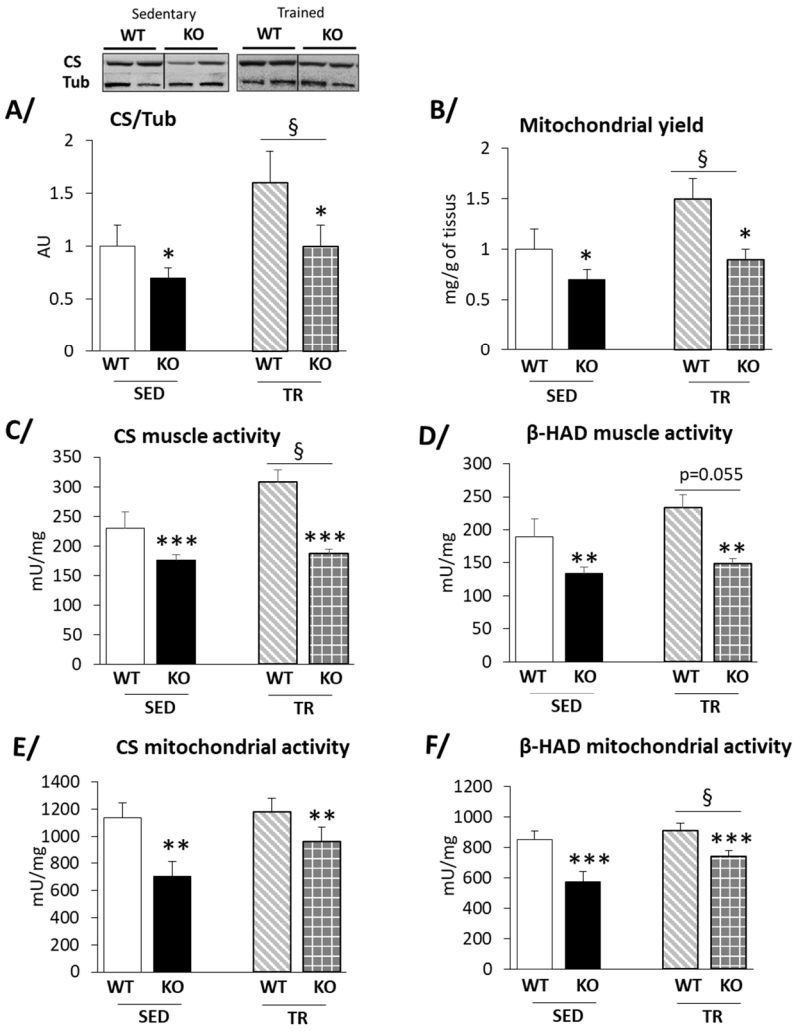

Figure 2. Effect of training on muscle aerobic metabolism in gastrocnemius sedentary and trained WT and KO mstn mice (n=8 in each group). **A/** Representative immunoblots showing the protein expression level of CS and quantification of CS protein levels expressed relative to α-tubulin levels in gastrocnemius muscle from each group. A line was added to show an arrangement due to the order samples deposition on the western blot gel. **B/**Mitochondrial yield **C/**CS muscle activity **D/** β-HAD muscle activity **E/** CS mitochondrial activity **F/** β-HAD mitochondrial activity WT wild-type; KO knock-out, SED sedentary, TR trained. * $p<0.05$ ** $p<0.01$ *** $p<0.001$ *vs* WT; § $p<0.05$ *vs* SED mice.

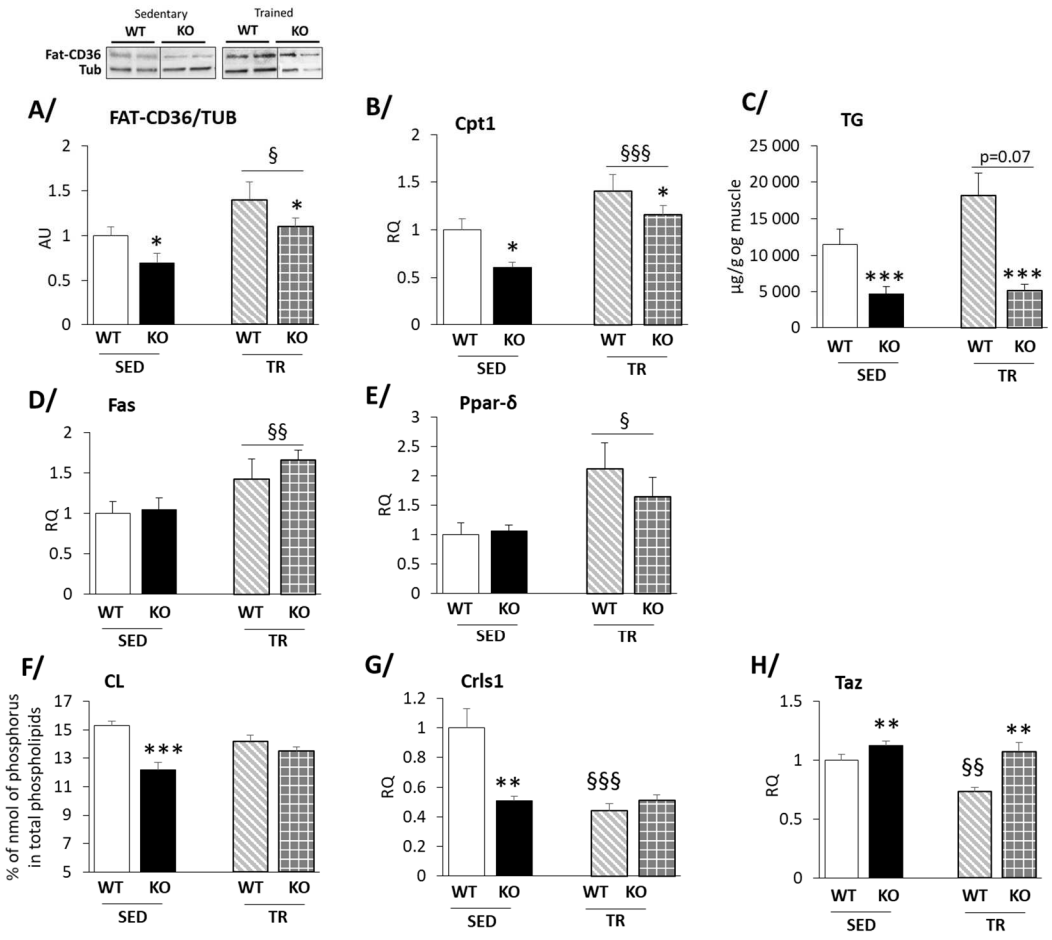

Figure 3. Effect of training on muscle lipid metabolism and cardiolipin metabolism in sedentary and trained WT and KO mstn mice (n=8 in each group). **A/** Representative Western blot of FAT/CD36 and protein quantity in gastrocnemius muscle from each group of mice A line was added to show an arrangement due to the order samples deposition on the western blot gel. **B/** Muscle *Cpt1* gene expression in muscle (n= 6 per group). **C/** Muscle triglyceride quantity (n=8 per group).**D/** Muscle *Fas* gene expression (n= 6 per group). **E/** Muscle *Ppar-δ* gene expression (n= 6 per group). **F/** Cardiolipin proportion in mitochondrial membranes. Results are expressed as percentage of nmol of phosphorus in total phospholipids. **G/** Muscle *Crls1* gene expression. **H/** Muscle *Taz* gene expression. *Fas*, fatty acid synthase; TG: triglycerides; *Crls1*: cardiolipin synthase, *Taz*: taffazin WT wild-type; KO knock-out, SED sedentary, TR trained *p<0.05 **p< 0.01 ***p< 0.001 *vs* WT, § p<0.05 §§ p< 0.01 §§§ p<0.001 vs sedentary mice; AU: Arbitrary Units, RQ: Relative Quantity.