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Role of adiponectin in the metabolism of skeletal muscles in collagen VI-related myopathies

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Abstract

The role of adiponectin has been particularly deepened in diabetic muscles while the study of adiponectin in hereditary myopathies has been marginal investigated. Here, we report the study about adiponectin effects in *Col6a1*$^{-/-}$ (collagen VI–null) mice. *Col6a1*$^{-/-}$ mice show myophatic phenotype closer to that of patients with Bethlem myopathy, thus representing an excellent animal model for the study of this hereditary disease. Our findings demonstrate that *Col6a1*$^{-/-}$ mice have decreased plasma adiponectin content and diseased myoblasts have an impaired autocrine secretion of the hormone. Moreover, Col*6a1*$^{-/-}$ myoblasts show decreased glucose up-take and mitochondria with depolarized membrane potential and impaired functionality, as supported by decreased oxygen consumption. Exogenous addition of globular adiponectin modifies the features of Col*6a1*$^{-/-}$ myoblasts, becoming closer to that of the healthy myoblasts. Indeed, globular adiponectin enhances glucose up-take in *Col6a1*$^{-/-}$ myoblasts, modifies mitochondrial membrane potential and restores oxygen consumption, turning closer to those of wild-type myoblasts. Finally, increase of plasma adiponectin level in Col*6a1*$^{-/-}$ mice is induced by fasting, a condition that has been previously shown to lead to the amelioration of the dystrophic phenotype. Collectively, our results demonstrate that exogenous replenishment of adiponectin reverses metabolic abnormalities observed in Col*6a1*$^{-/-}$ myoblasts.

Introduction

Congenital muscular dystrophies represent a large and heterogeneous group of inherited muscle disorders with a severe and progressive clinical course. In particular, mutations in any of the genes encoding for the three main chains (α1, α2, α3) composing collagen VI (COL6), an extracellular matrix protein remarkably abundant in the endomysium of skeletal muscles, cause multiple muscle diseases, including Bethlem myopathy (BM) and Ullrich congenital muscular dystrophy (UCMD) [1]. COL6 null (*Col6a1*$^{-/-}$) mice display a myopathic phenotype resembling that of BM patients, and showed to be an excellent animal model for this pathology [2]. Muscles lacking COL6 are characterized by the presence of dilated sarcoplasmic reticulum and dysfunctional mitochondria, leading to muscle wasting [3]. These muscles exhibit accumulation of abnormal organelles due to an autophagy impairment. Amelioration of the dystrophic phenotype can be obtained through the reactivation of the autophagic flux, by either nutritional approaches or by pharmacological and genetic tools, leading to the removal of dysfunctional organelles [3, 4].

The action of adiponectin in skeletal muscle plays key roles ranging from metabolic to differentiating ones. Regarding the metabolic function, adiponectin affects glucose up-take, glycogen synthesis, glycolysis activation and triglyceride degradation [5-7]. In addition, adiponectin promotes myogenesis both in satellite cells [8] and myoblasts [9] and the differentiating role in myoblasts is linked to activation of autophagy [10]. In muscle cells, the binding of adiponectin to its specific AdipoR1 muscle receptor leads to the activation of a signalling cascade in which APMK plays a central role [7]. Adiponectin circulating in the plasma is mainly secreted from the adipose tissue, however, autocrine adiponectin production occurs in several tissues, including skeletal muscle which is one of the main site of local secretion [11]. Plasma adiponectin level is greatly affected by obesity. Obese individuals show significantly decreased adiponectin level in the blood, thus predisposing them to the onset of the metabolic syndrome [12]. Although the role of

adiponectin in healthy skeletal muscle is well documented, its effects on dystrophic muscles are still unclear.

Here, we report that *Col6a1*$^{-/-}$ mice display decreased circulating adiponectin level and that *Col6a1*$^{-/-}$ myoblasts-enriched cultures (hereafter referred to as "myoblasts") have impaired autocrine adiponectin secretion. In addition, *Col6a1*$^{-/-}$ myoblasts display decreased glucose up-take, higher glutamine dependence and decreased glucose consumption. Interestingly, exogenous addition of adiponectin improves *Col6a1*$^{-/-}$ metabolic behaviour, bringing it closer to that of wild-type (WT) myoblasts. Indeed, adiponectin stimulation of *Col6a1*$^{-/-}$ myoblasts increases glucose up-take and oxygen consumption and induces glutamine dependence close to that of WT myoblasts. In addition, we show that fasting, previously demonstrated to ameliorate the dystrophic phenotype of *Col6a1*$^{-/-}$ mice, induces an up-regulation of plasma adiponectin level in *Col6a1*$^{-/-}$ mice. Overall, these findings show that the modulation of adiponectin levels both *in vitro* and *in vivo* could improve the metabolic defects due to the absence of COL6.

Materials and methods

Materials. Unless differently specified, all reagents were obtained from Sigma-Aldrich, Inc.; PVDF membrane was from Millipore; anti-adiponectin, anti-AdipoR1, anti-GAPDH, anti-GLUT4 antibodies and test ELISA for murine adiponectin assay were from Abcam; Alexa 488 fluorescent secondary antibodies were from Pierce. Adiponectin was from Alexis. JC-1 probe was from Molecular Probe; K-LATE kit for lactate assay was from Megazyme; [^{3}H] 2-deoxy-glucose was from Perkin Elmer; ECL detection reagents was from GE Healthcare.

Mice. Diaphragm and tibialis anterior were isolated from 8 week-old male *Col6a1*$^{-/-}$ (collagen VI–null) mice and compared age-matched male *Col6a1*$^{+/+}$ (WT) mice. We housed mice in individual cages in an environmentally controlled room (23 °C, 12-h light-dark cycle) and provided food and water *ad libitum*. For fasted mice, we removed chow in the morning and maintained mice for 24 hours with no food but free access to water. Mouse procedures were approved by the Ethics Committee of the University of Padua and authorized by the Italian Ministry of Health according to D. Lgs. 26/2014 implementing Directive 2010/63 / EU.

Cell Culture. Myoblast-enriched cultures (hereafter referred to as "myoblasts") were isolated from 8-week-old of WT and *Col6a1*$^{-/-}$ muscles (diaphragm and tibialis anterior) by enzymatic and mechanical dissociation, following the protocol from Rando and Blau [13]. Isolated cells were transferred to gelatin-coated dishes and then cultured in DMEM supplemented with 10% FBS in 5% CO_2 humidified atmosphere and used until passage 2 as maximum.

Immunoblot analysis. Muscle protein extracts were prepared as previously described [14] and immunoblot analysis was performed as previously reported [10].

Immunofluorescence. Myoblasts were grown on glass coverslips and for confocal analysis, sub-confluent myoblasts were treated as previously reported [10].

Adiponectin assay. Plasma and local adiponectin were assayed by ELISA test following manufacturer's instruction. Plasma from WT and *Col6a1*$^{-/-}$ mice was diluted 1:400 and adiponectin value obtained by ELISA test was normalized on plasma total protein content. Autocrine production of adiponectin was assayed in culture medium of WT and *Col6a1*$^{-/-}$ myoblasts. In particular, WT and *Col6a1*$^{-/-}$ myoblasts were cultured for a day in serum-free medium before collecting the medium. Total protein content of myoblasts was used for normalization of the value obtained by Elisa test.

Glucose up-take. Glucose up-take was performed using [^{3}H] 2-deoxy-glucose (0.5 mCi/mL, final concentration) diluted in a buffered solution (140 mmol/L NaCl, 20 mmol/L Hepes/Na, 2.5 mmol/L $MgSO_4$, 1 mmol/L $CaCl_2$, and 5 mmol/L KCl, pH7.4) for 15 minutes at 37 °C. Cells were subsequently washed with cold PBS and lysed with 0.1 mol/L NaOH. Incorporated radioactive glucose was assayed by scintillation counter and the obtained value was then normalized on total protein content.

Lactate assay. Lactate amount was assayed in cell medium using K-LATE kit according to manufacturer's instructions. Lactate amount was then normalized on protein content of the same sample.

MTT assay. 0.5 mg/ml of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2 H tetrazolium bromide (MTT) was added to the cells. After 1 h at 37 °C, cells were extensively washed with PBS and then 1 ml of DMSO was added to the culture. The absorption was measured at 570 nm.

Measurement of oxygen consumption. Myoblasts were trypsinized, washed with PBS and suspended in complete DMEM medium at the concentration of 10^6 cells/ml. One ml of cell

suspension was transferred to an airtight chamber maintained at 37 °C. Oxygen consumption was measured using a Clark-type O_2 electrode (Oxygraph Hansatech). Oxygen content was monitored for at least 10 min. The rate of decrease in oxygen content, related to protein amount was taken as index of the respiratory ability.

Statistical analysis. Data are presented as mean ± S.D. from at least three independent experiments. Statistical analysis of the data was performed by Student's t-test or by one-way ANOVA using Graph Pad Prism 4.0. *p*-values <0.05 were considered statistically significant.

Results

1. Analysis of circulating and local adiponectin content in *Col6a1*$^{-/-}$ mice. We planned to study adiponectin in *Col6a1*$^{-/-}$ mice, a good animal model for UCMD and BM [2]. For this aim, we chose the muscles diaphragm and tibialis anterior. Diaphragm is characterized by red fibres, a great amount of mitochondria and oxidative metabolism. Conversely, tibialis anterior has fermentative metabolism and is composed of white fibres containing few mitochondria. We checked adiponectin and AdipoR1 levels in the two muscles isolated from male WT and *Col6a1*$^{-/-}$ mice. No difference in the expression level of both adiponectin and AdipoR1 has been observed in diaphragm and tibialis anterior from *Col6a1*$^{-/-}$ mice in comparison to WT (Fig. 1A). As well, our analysis of adiponectin expression in myoblasts isolated from WT and *Col6a1*$^{-/-}$, diaphragm and tibialis anterior showed that adiponectin expression is quite similar in *Col6a1*$^{-/-}$ and WT myoblasts (Fig. 1B).

Hence, we analyzed both circulating adiponectin in plasma of WT and *Col6a1*$^{-/-}$ mice and autocrine adiponectin secretion in WT and *Col6a1*$^{-/-}$ myoblast culture medium. Our findings showed decreased circulating adiponectin (of about 30%) in *Col6a1*$^{-/-}$ plasma in comparison to WT (Fig. 2A). Remarkably, the analysis of autocrine production of adiponectin from diaphragm-isolated myoblasts led to the same result. Indeed, *Col6a1*$^{-/-}$ cells showed a 30% reduction of adiponectin secretion when compared to WT myoblasts (Fig. 2B). Body weight of WT and *Col6a1*$^{-/-}$ mice showed no appreciable differences as shown in Fig. 2C.

To test whether the decrease of plasma adiponectin is associated to gender, we assayed circulating adiponectin in WT and *Col6a1*$^{-/-}$ females. The results showed that circulating adiponectin is decreased in *Col6a1*$^{-/-}$ females in comparison to WT females (Suppl. Fig. 1A), as already observed in male Col*6a1*$^{-/-}$ mice (Fig. 2A). In addition, intracellular levels of adiponectin in

diaphragm and tibialis anterior of *Col6a1*$^{-/-}$ females are similar to WT females (Suppl. Fig. 1B), as observed in male *Col6a1*$^{-/-}$ mice (Fig. 1A).

Collectively, these results show that *Col6a1*$^{-/-}$ mice have decreased circulating adiponectin and that autocrine production of adiponectin by *Col6a1*$^{-/-}$ myoblasts is defective, thus suggesting a decreased extracellular adiponectin availability for skeletal muscles in diseased mice.

2. Role of adiponectin in glucose metabolism of *Col6a1*$^{-/-}$ myoblasts. Proteomic analysis in *Col6a1*$^{-/-}$ muscles suggested an impairment of glycolysis [15]. To verify whether decreased glycolysis could be due to an impairment of glucose entry, we analyzed glucose up-take in myoblasts isolated from WT and *Col6a1*$^{-/-}$ diaphragm and tibialis anterior. This analysis showed that *Col6a1*$^{-/-}$ myoblasts have decreased glucose assumption in comparison to WT cells isolated from both muscles (Fig. 3A and 3B). Globular adiponectin (gAd) exerts in skeletal muscle several physiological actions, ranging from metabolic to differentiating functions [10, 11]. In particular, gAd has high affinity for the binding with AdipoR1 and greatly affects glucose up-take in skeletal muscle [16]. Hence, we planned to stimulate WT and *Col6a1*$^{-/-}$ myoblasts with gAd. Stimulation with gAd enhanced glucose up-take in *Col6a1*$^{-/-}$ myoblasts (Fig. 3A and 3B) with higher increase in *Col6a1*$^{-/-}$ myoblasts isolated from tibialis anterior (Fig. 3B). We tested whether the impairment of glucose up-take in *Col6a1*$^{-/-}$ myoblasts was due to a lower expression of GLUT4, the primary glucose transporter in skeletal muscle. As shown in Fig. 4, the expression level of GLUT4 was similar in WT and *Col6a1*$^{-/-}$ myoblasts. However, confocal analysis suggests that gAd stimulation could alter intracellular distribution of GLUT4 in *Col6a1*$^{-/-}$ myoblasts (Suppl. fig. 1).

The impaired glucose up-take observed in myoblasts from diaphragm and tibialis anterior of *Col6a1*$^{-/-}$, suggested that *Col6a1*$^{-/-}$ myoblasts could import the amino acid glutamine that could be driven towards Krebs cycle. To test this possibility, we performed glucose up-take in the absence of glutamine, thus forcing *Col6a1*$^{-/-}$ myoblasts to use glucose. Glutamine depletion induced *Col6a1*$^{-/-}$

myoblasts to introduce glucose instead of glutamine (Fig. 5A and 5B), thus enhancing the glucose up-take. Our observation suggested that *Col6a1*$^{-/-}$ myoblasts preferentially use glutamine instead of glucose for their metabolism. Indeed, in the presence of both glucose and glutamine *Col6a1*$^{-/-}$ myoblasts showed enhanced metabolic activity in comparison to WT cells. Conversely, when glutamine is removed, *Col6a1*$^{-/-}$ myoblasts showed decreased metabolic activity, becoming similar to that of WT cells (Fig. 5C). In order to deeper characterize the energetic metabolism of *Col6a1*$^{-/-}$ myoblasts, we also measured the production of lactate. This analysis evidenced that *Col6a1*$^{-/-}$ myoblasts from diaphragm and tibialis anterior secreted quite similar amount of lactate in comparison to healthy cells (Fig. 6A and 6B). Interestingly, lactate production was greatly increased in *Col6a1*$^{-/-}$ myoblasts after gAd stimulation in both muscles examined (Fig. 6A and 6B).

Overall, these findings suggest that *Col6a1*$^{-/-}$ myoblasts introduce less glucose in comparison to WT cells, preferring glutamine for their viability. In the presence of both glucose and glutamine, gAd stimulation enhances glucose up-take and increases lactate production in *Col6a1*$^{-/-}$ myoblasts in both muscles. On the other hand, glutamine withdrawal forced myoblasts to increase glucose up-take and gAd stimulation raises glucose intake.

3. Adiponectin stimulation restores mitochondrial membrane potential and increases oxygen consumption in *Col6a1*$^{-/-}$ myoblasts. *Col6a1*$^{-/-}$ muscles exhibit altered mitochondria characterized by high membrane depolarization that is greatly attenuated after prolonged fasting [3]. To detect the presence of altered mitochondria also in *Col6a1*$^{-/-}$ myoblasts, we used the mitochondrial probe JC-1. Fig. 7A shows that *Col6a1*$^{-/-}$ myoblasts contained mitochondria with depolarized membranes (evidenced by green fluorescence) in comparison to WT cells (showing red fluorescent, typical of normal mitochondrial membrane potential). gAd induces mitochondria biogenesis through the regulation of PGC-1α and genetic removal of AdipoR1 in muscle resulted in decreased expression and deacetylation of PGC-1α and reduced mitochondrial enzymes [17]. To test whether adiponectin

could ameliorate mitochondrial membrane depolarization, *Col6a1*$^{-/-}$ myoblasts have been stimulated with gAd. This treatment led to the shift from green to red fluorescence in *Col6a1*$^{-/-}$ mitochondria, which appeared similar to WT cells (Fig. 7A). This finding demonstrates that gAd ameliorates altered mitochondrial features in *Col6a1*$^{-/-}$ myoblasts, thus restoring normal membrane potential.

To detect whether the depolarization observed in *Col6a1*$^{-/-}$ mitochondria is associated with altered functionality, we analyzed oxygen consumption. We observed that *Col6a1*$^{-/-}$ myoblasts consume lesser amount of oxygen (about 30%) in comparison to WT cells (Fig.7B). Interestingly, [18] gAd stimulation restored oxygen consumption similar to that of WT cells (Fig. 7B).

Overall, these findings show that gAd induces beneficial effects on mitochondria in *Col6a1*$^{-/-}$ myoblasts, thus ameliorating membrane potential and the defective oxygen consumption. The benefits following gAd treatment are not due to enhanced expression of mitochondrial complexes, since no differences in the expression of these proteins were observed between WT and *Col6a1*$^{-/-}$ myoblasts (data not shown).

4. Fasting in *Col6a1*$^{-/-}$ mice increases circulating adiponectin. Fasting in *Col6a1*$^{-/-}$ mice improves the dystrophic phenotype [3]. Moreover, fasting raises both serum and cerebrospinal fluid levels of adiponectin [18]. This led us to hypothesise that fasting in *Col6a1*$^{-/-}$ mice could enhance both circulating and local muscular production of adiponectin. To test this hypothesis, we detected the expression level of adiponectin in muscles of fasting *Col6a1*$^{-/-}$ mice. Results showed that only tibialis anterior of fasted *Col6a1*$^{-/-}$ mice exhibit higher amount of adiponectin in comparison to WT fasted mice (Fig. 8A). Conversely, adiponectin level remains quite similar in the diaphragm of fasted *Col6a1*$^{-/-}$ and WT mice (Fig. 8A). Interestingly, fasting affects the amount of circulating adiponectin in plasma. Indeed, while normally fed *Col6a1*$^{-/-}$ mice showed decreased circulating

adiponectin (Fig. 2A), fasting increased the amount of circulating hormone that become similar to WT mice (Fig. 8B).

These findings demonstrate that fasting promotes adiponectin expression and secretion, thus suggesting that adiponectin could be involved in the improvement of dystrophic phenotype observed in *Col6a1*$^{-/-}$ mice following fasting.

Discussion

The metabolic and differentiating roles of adiponectin in skeletal muscle have been well-established and deeply studied [7]. However, the involvement of adiponectin in inherited muscle diseases is just at the beginning. It has been reported that DMD patients show a significant reduction of High Molecular Weight complexes of adiponectin [19], but the consequence of this decrease has not been investigated yet. Here, we report the study on adiponectin in *Col6a1*$^{-/-}$ mice, having a targeted inactivation of the α1 chain of collagen VI (COL6), and show a myopathic phenotype resembling that of BM patients [2]. Whether the local and endocrine adiponectin levels are changed and whether the exogenous addition of adiponectin undergoes beneficial effects for the diseased phenotype has not considered yet in this pathology. This is an important point to be addressed, since adiponectin could be considered a new tool for the improvement of the diseased muscles. An interesting finding is that *Col6a1*$^{-/-}$ mice have decreased amount of circulating adiponectin in comparison to healthy mice. In addition to endocrine adiponectin production, several tissues, including skeletal muscle, have autocrine adiponectin production [9, 11]. We found that muscles and myoblasts from *Col6a1*$^{-/-}$ have intracellular adiponectin content similar to healthy mice, thus showing that myopathy does not affect adiponectin transcription and translation. However, secretion of adiponectin is greatly decreased in *Col6a1*$^{-/-}$ myoblasts in comparison to WT, thus demonstrating that myopathy affects both endocrine and skeletal muscle autocrine adiponectin secretion in *Col6a1*$^{-/-}$ mice. Particularly, we found that both males and females of *Col6a1*$^{-/-}$ mice show reduced circulating adiponectin in comparison to WT, thus suggesting that the decrease of plasma adiponectin is not related to gender but rather to the pathological condition. The mechanisms driving adiponectin secretion are well elucidated [20-22]. However, some factors may compromise the secretion of this adipokine. Among these, oxidative stress has been reported to inhibit adiponectin secretion by adipose tissue [23, 24]. Several studies recognised the key role of oxidative stress and abnormal production of reactive oxygen species in the pathophysiology of

muscular dystrophies [25-27]. In particular, the presence of oxidative stress, due to an accumulation of reactive oxygen species by monoamine oxidase activity, has been reported in muscles of *Col6a1*$^{-/-}$ mice [28]. Hence, oxidative stress observed in *Col6a1*$^{-/-}$ muscles may be the cause leading to the impairment of adiponectin secretion. In addition, *Col6a1*$^{-/-}$ mice show dilated sarcoplasmic reticulum in muscle cells that may affect the translation of those proteins destined to secretion as adiponectin [3]. For what adipose tissue is concerned, *Col6a1*$^{-/-}$ mice show increased adipocyte cell size due to a more flexibility of the adipocyte matrix, thus allowing the increase of adipocytes size in the absence of associated necrosis and inflammation. This leads to metabolic dysfunction and fibrosis formation in *Col6a1*$^{-/-}$ adipose tissue in a diabetic state [29].

Hence, our results show that *Col6a1*$^{-/-}$ mice have impaired both circulating and local (at least at skeletal muscle level) adiponectin production, thus generating a situation in which the hormone is not sufficiently available for target tissues as skeletal muscle.

Decreased amount of adiponectin could be involved in the altered metabolic behaviour observed in myoblasts of *Col6a1*$^{-/-}$ mice in comparison to those from healthy animals. We found that myoblasts from diaphragm and tibialis anterior of *Col6a1*$^{-/-}$ mice show impaired glucose up-take. Furthermore, our results highlight another altered behaviour of *Col6a1*$^{-/-}$ myoblasts. Particularly myoblasts isolated from diaphragm of *Col6a1*$^{-/-}$ mice show greater glutamine-dependence which appears indispensable for myoblast metabolism. When cells take less glucose, glutamine can be used for cell metabolism instead of glucose. In skeletal muscle, glutamine can meet different fates since it can be used as nitrogen donor in the synthesis of amino acids and nucleosides, converted to α-ketoglutarate or citrate, thus supporting the tricarboxylic acid cycle, [30, 31]. Proteomic analysis performed on *Col6a1*$^{-/-}$ muscles suggested that diaphragm of *Col6a1*$^{-/-}$ mice is characterized by blunted glycolysis and impaired tricarboxylic acid cycle [15]. In agreement, we observe decreased glucose uptake, likely responsible for the decreased glycolysis observed by De Palma *et al*. [15]. In skeletal muscle, adiponectin has essential and pleiotropic roles,

ranging from metabolic to differentiating function [8-11, 32]. Concerning the metabolic role, adiponectin promotes glucose up-take via GLUT4 translocation [16] and regulates fatty acid metabolism, increasing fatty acid uptake and oxidation, and suppressing fatty acid synthesis through the activation of AMPK, p38 MAPK and PPARα signalling [5, 6, 33]. We found that treatment of *Col6a1*$^{-/-}$ myoblasts with exogenous adiponectin induces several beneficial effects at the metabolic level. Adiponectin enhances glucose up-take, promotes lactate production and decreases glutamine dependence, approaching that of WT cells. In addition, adiponectin enhances glucose up-take in *Col6a1*$^{-/-}$ myoblasts after glutamine withdrawal. Hence, exogenous addition of adiponectin could restore the proper metabolic signalling in *Col6a1*$^{-/-}$ myoblasts.

As already reported in intact fibres [3], *Col6a1*$^{-/-}$ myoblasts contain depolarized and less functional mitochondria with decreased capacity to consume oxygen. In this scenario, addition of exogenous adiponectin can play a key role in restoring proper metabolism in *Col6a1*$^{-/-}$ muscles. Indeed, we found that adiponectin promotes beneficial effects also at the mitochondrial level in *Col6a1*$^{-/-}$ myoblasts. The role of adiponectin in mitochondria of muscle cells has been clearly defined. By AdipoR1, adiponectin promotes the activation of Ca^{2+}/calmodulin-dependent protein kinase β (CaMKKβ), AMPK and SIRT1 signalling pathways, increased the expression and decreased acetylation of peroxisome proliferator-activated receptor c coactivator-1α (PGC-1α), thus leading to increased mitochondria in myotubes [17]. Although the mechanism needs further investigations, adiponectin treatment improves mitochondrial potential and increased oxygen consumption in *Col6a1–/–* mitochondria similar to that of WT cells.

Skeletal muscles of *Col6a1*$^{-/-}$ mice have impaired autophagy that leads to dysfunctional mitochondria and spontaneous apoptosis [3]. The myophatic phenotype is greatly ameliorated by forcing activation of autophagy by different approaches. In particular, fasting in *Col6a1*$^{-/-}$ mice restores normal autophagy associated with a general amelioration of the dystrophic features [3]. Adiponectin is involved in autophagy activation in skeletal muscle since adiponectin-activated

autophagy is essential for the myogenic role of the hormone thus preventing the formation of the myophatic phenotype [10]. Since a correlation between fasting and adiponectin has been previously reported [34], we hypothesized that fasting could increase circulating adiponectin. Interestingly, we found that fasted *Col6a1*$^{-/-}$ mice show circulating adiponectin levels similar to that of healthy animals. This observation could suggest a role of adiponectin in the induction of the beneficial effects observed in fasted *Col6a1*$^{-/-}$ mice. In fasting condition, only tibialis anterior of *Col6a1*$^{-/-}$ mice shows increased adiponectin production in comparison to tibialis anterior of WT mice, while no difference has been observed in diaphragm of *Col6a1*$^{-/-}$ and WT mice. Since the two muscles have distinct metabolism (i.e. tibialis anterior has fermentative metabolism while diaphragm is an oxidative muscle), the higher adiponectin content in tibialis anterior in fasted *Col6a1*$^{-/-}$ mice could help the muscle to sustain fermentation and the right production of ATP. Thus, a greater amount of adiponectin in the tibialis anterior of *Col6a1*$^{-/-}$ mice could help to overcome the observed metabolic defects.

Overall, our results show several abnormalities regarding adiponectin in *Col6a1*$^{-/-}$ mice. Among these, the decrease of local and circulating levels of adiponectin plays a key role since this decrease could be involved in the metabolic alterations observed in *Col6a1*$^{-/-}$ mice. These metabolic defects are compensated by the treatment with exogenous adiponectin that leads *Col6a1*$^{-/-}$ myoblast behaviour similar to that of WT cells. Interestingly, myopathy does not influence the expression level of the muscle-specific receptor for adiponectin, AdipoR1, which remains unchanged in all examined muscle, thus making possible the treatment of the diseased muscles with exogenous adiponectin.

Figure legends

Fig. 1. Expression level of adiponectin in muscles and myoblasts from WT and *Col6a1*$^{-/-}$ mice. A) Anti-adiponectin and anti-AdipoR1 immunoblots in diaphragm and tibialis anterior of muscles from WT and *Col6a1*$^{-/-}$ mice. B) Analysis of adiponectin expression in myoblasts isolated from diaphragm and tibialis anterior of WT and *Col6a1*$^{-/-}$ mice. GAPDH immunoblots demonstrate that the same amount of total proteins was loaded in each lane. Bar graphs show the expression level of adiponectin and AdipoR1 in each muscle reported as arbitrary units (a.u.). *p<0.01. acrp30: adiponectin. n=5 mice/group.

Fig. 2. Analysis of plasma and autocrine adiponectin level. A) Plasma adiponectin level in WT and *Col6a1*$^{-/-}$ mice and B) Autocrine secretion of adiponectin from myoblasts isolated from diaphragm of WT and *Col6a1*$^{-/-}$ mice, obtained using ELISA test. The values are reported as a percentage of decrease of *Col6a1*$^{-/-}$ in comparison to WT. n=5 mice/group. C) Body weight of WT and *Col6a1*$^{-/-}$ mice. *p<0.01. acrp30: adiponectin.

Fig. 3. Glucose up-take in myoblasts from WT and *Col6a1*$^{-/-}$ mice. Glucose up-take was analyzed in myoblasts isolated from diaphragm (A) and tibialis anterior (B) of WT and *Col6a1*$^{-/-}$ mice. Sub-confluent myoblasts were treated with low glucose medium (5mM final) overnight. Where indicated, adiponectin (1 µg/ml) was added to the cells for 30 minutes before the addition of [^{3}H] 2-deoxy-glucose. Radioactive counts were normalized with total protein content and reported as arbitrary units (a.u.). *p< 0.001. gAd: globular adiponectin.

Fig. 4. Analysis of the glucose transporter GLUT4 in myoblasts from WT and *Col6a1*$^{-/-}$ mice. Myoblasts isolated from diaphragm of WT and *Col6a1*$^{-/-}$ mice were used to analysed GLUT4 expression level by immunoblot. GLUT4 level has been normalized using anti-GAPDH immunoblot and reported in the bar graph as arbitrary unit (a.u.).

Fig. 5. Glucose up-take and MTT assay in WT and *Col6a1*$^{-/-}$ myoblasts after glutamine withdrawal. Myoblasts, isolated from diaphragm (panel A) and tibialis anterior (panel B) of WT and *Col6a1*$^{-/-}$ mice, were cultured in complete medium without glutamine (gln) for 24 hours. Cells were then stimulated with gAd (1µg/ml) for 30 minutes before the addition of [^{3}H] 2-deoxy-glucose. Radioactive counts were normalized with total protein content and reported as arbitrary units (a.u.). C) MTT assay in myoblasts isolated form diaphragm of WT and *Col6a1*$^{-/-}$ mice. Cells were cultured in complete medium with or without gln (2 mM final) for 48 hours before performing MTT assay. *p<0.01; **p<0.001. gAd: globular adiponectin.

Fig. 6. Lactate production in WT and *Col6a1*$^{-/-}$ myoblasts. Myoblasts isolated from diaphragm (A) and tibialis anterior (B) were serum deprived overnight in high glucose medium (25mM final) with or without gAd (1µg/ml). The values of lactate were normalized on protein content and reported as arbitrary unit (a.u.). *p< 0.01. gAd: globular adiponectin.

Fig. 7. Role of adiponectin in mitochondria of *Col6a1*$^{-/-}$ myoblasts. Myoblasts were isolated from diaphragm of WT and *Col6a1*$^{-/-}$ mice and mitochondrial membrane potential were assayed using JC-1 probe as shown in A). Cells were seeded on coverslips, serum-depleted overnight and then gAd (1 µg/ml) was added for 24 h. JC-1 probe (5 µM) was added to the cells for 15 min at 37 °C and then immediately observed using confocal microscope. Images are representative of four independent experiments. B) Oxygen consumption assay was performed as reported in Material and Methods. The values were reported as percent increase or decrease considering WT myoblasts as 100%.*p<0.01; gAd: globular adiponectin.

Fig. 8. Analysis of plasma and expression levels of adiponectin in fasted WT and *Col6a1*$^{-/-}$ mice. WT and *Col6a1*$^{-/-}$ mice were fasted for 24 hours. A) Acrp30 and AdipoR1 expression levels in diaphragm and tibialis anterior muscles. The values are reported in the bar graph as arbitrary units (a.u.) obtained by the ratio between adiponectin or AdipoR1 and GAPDH. B) Analysis of adiponectin in plasma of WT and *Col6a1*$^{-/-}$ mice performed by ELISA test. The values obtained by ELISA test were normalized on plasmatic protein content. **p*<0.01; acrp30: adiponectin. n=5 mice/group.

Supplementary figure 1. Analysis of adiponectin in females of *Col6a1*$^{-/-}$ mice. A) Plasma adiponectin level obtained using ELISA test and reported as percentage of decrease of *Col6a1*$^{-/-}$ in comparison to WT. n=5 mice/group. **p*<0.01; acrp30: adiponectin. B) Intracellular level of adiponectin in diaphragm and tibialis anterior by immunoblot. GAPDH immunoblot has been used for normalization. Bar graphs show the expression level of adiponectin reported as arbitrary unit (a.u.).

Supplementary figure 2. Confocal images of myoblasts isolated from diaphragm of WT and Col*6a1*$^{-/-}$ mice incubated with (right panel), or without (left panel) gAd (1 µg/ml) for 30 minutes. The intracellular location of GLUT4 is shown as green signal obtained using a specific primary anti-GLUT4 antibody (ThermoFisher Scientific) and a secondary antibody conjugated to Alexa-488. Nuclei have been labelled with DAPI. The images are representative of three independent experiments. gAd: globular adiponectin.

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Conflict of interest: the authors declare no conflict of interest

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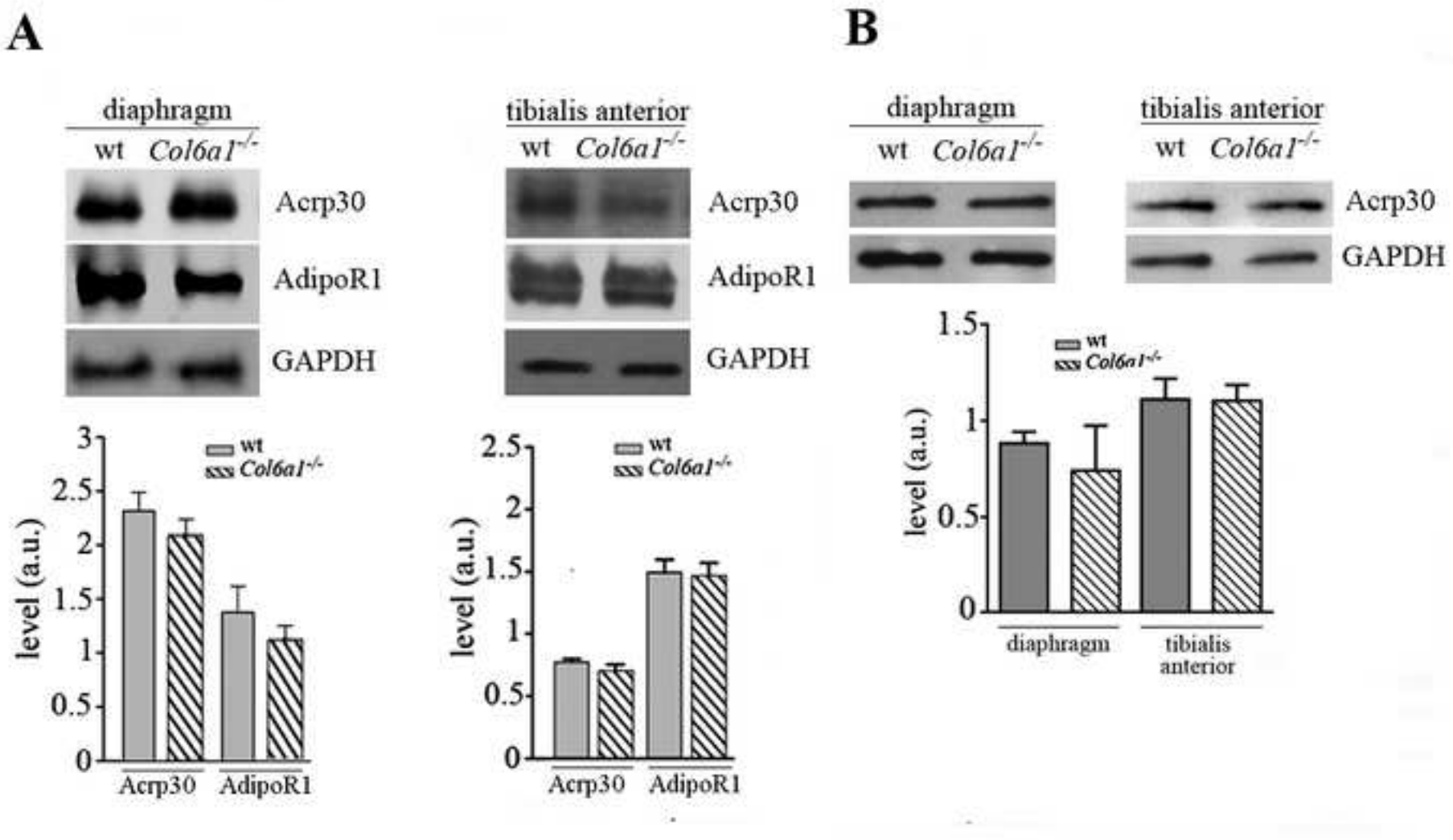
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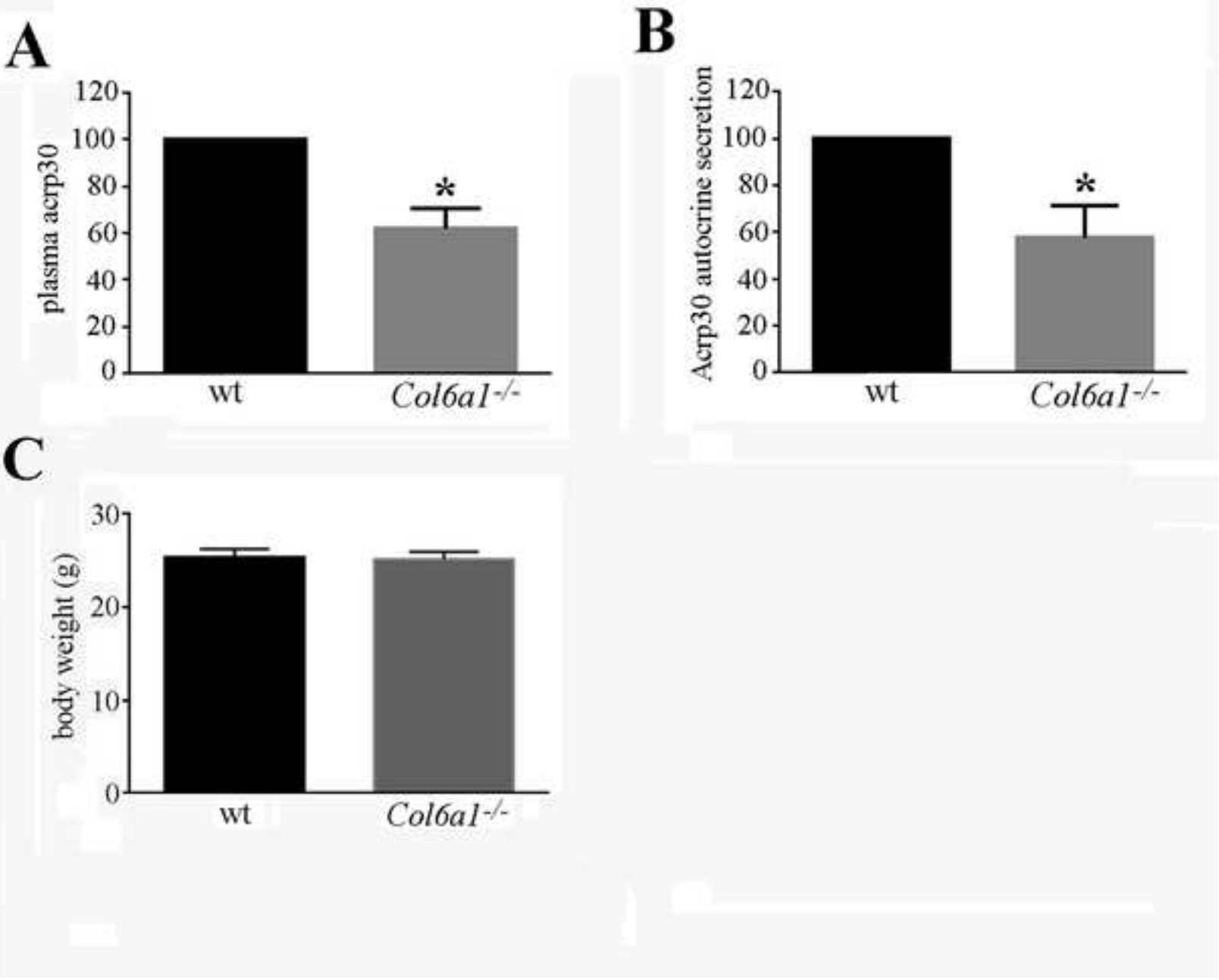
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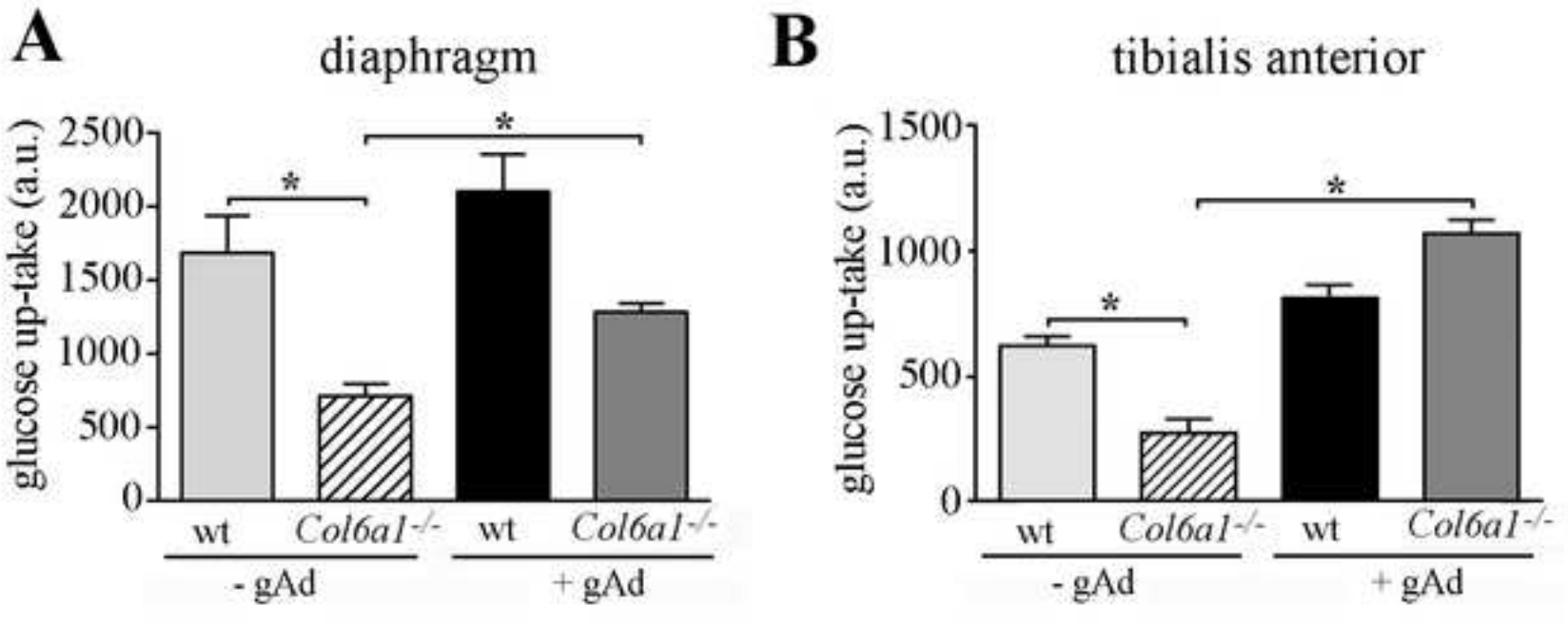
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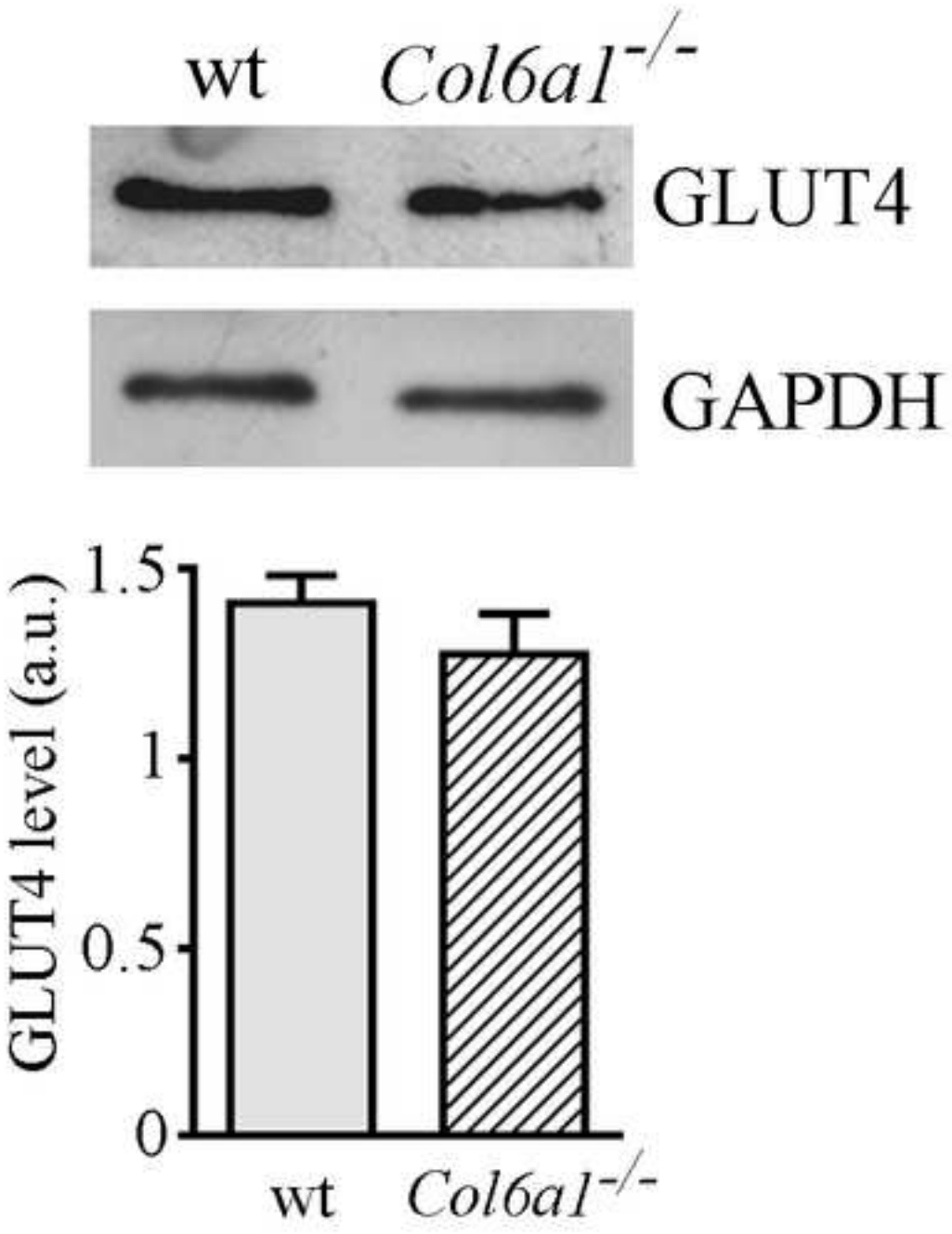
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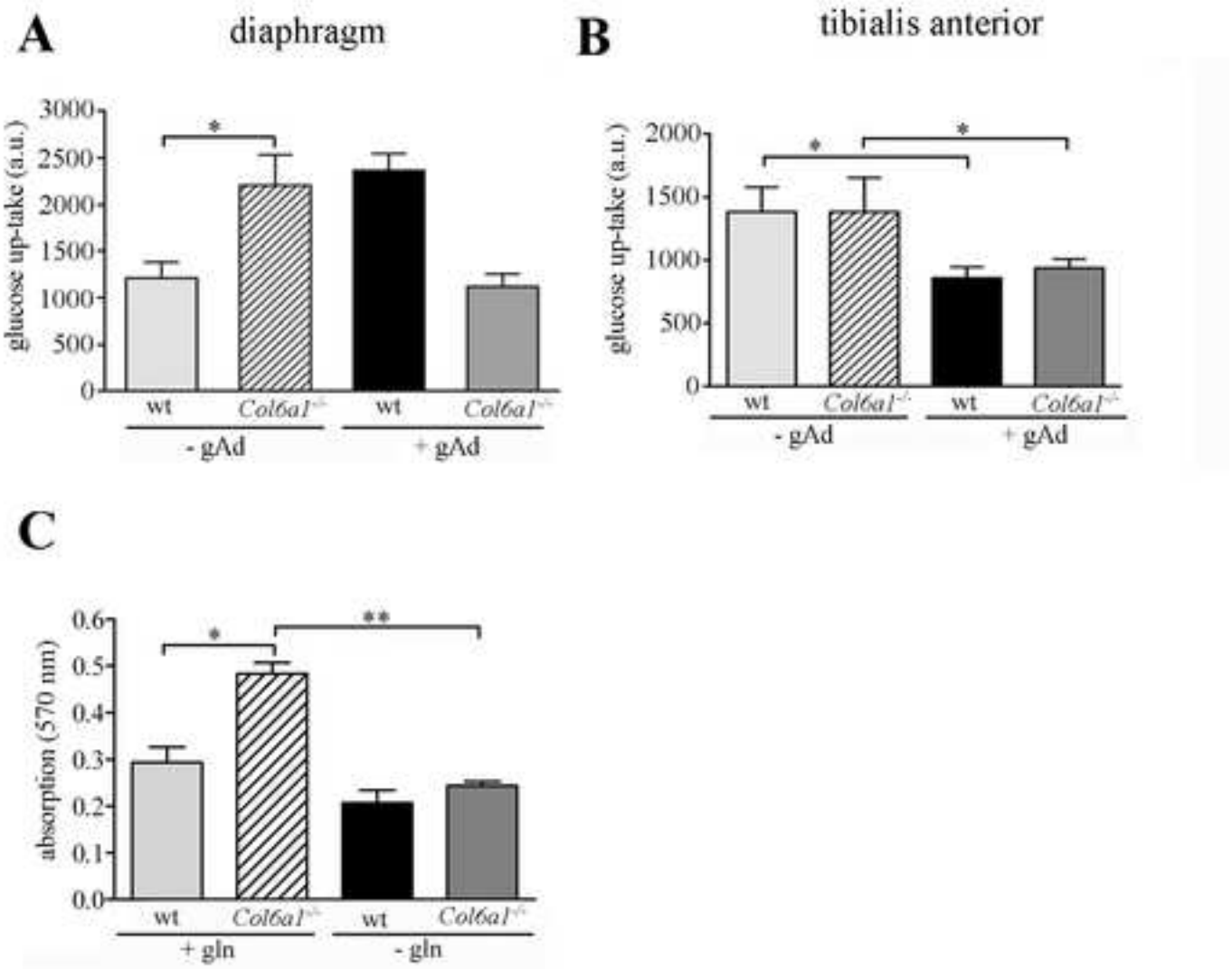
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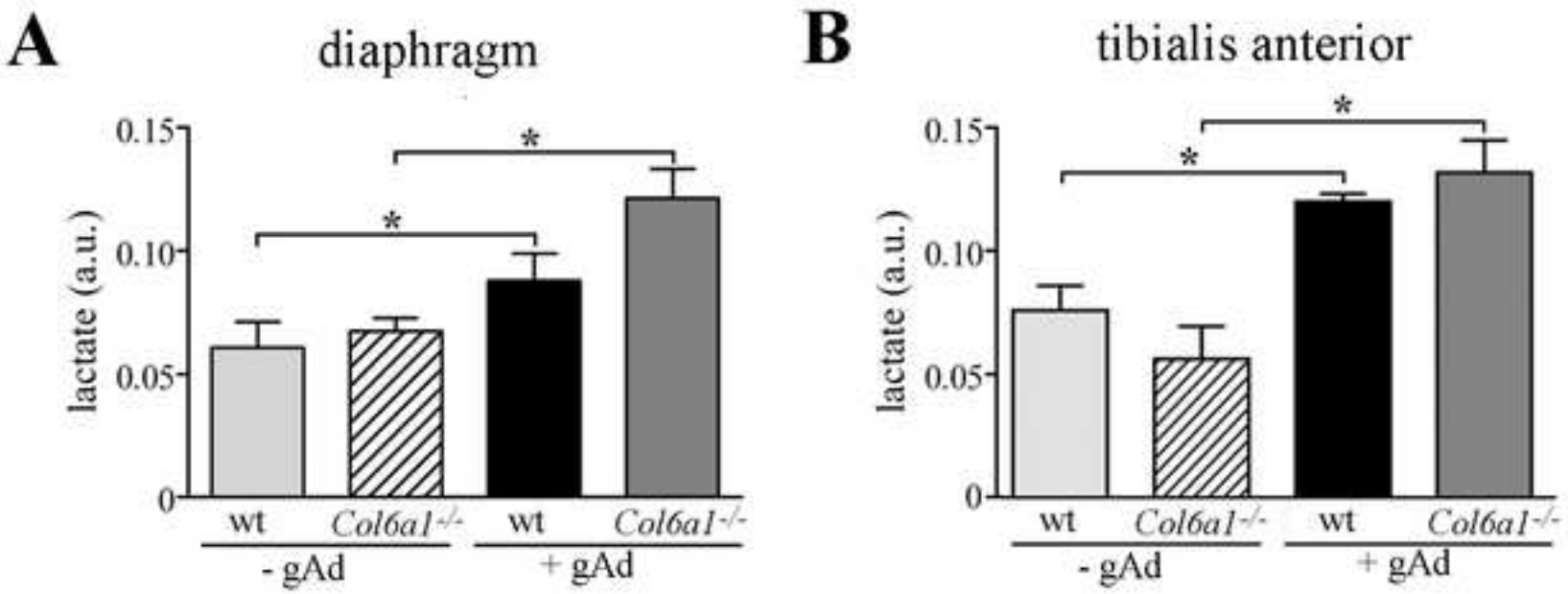
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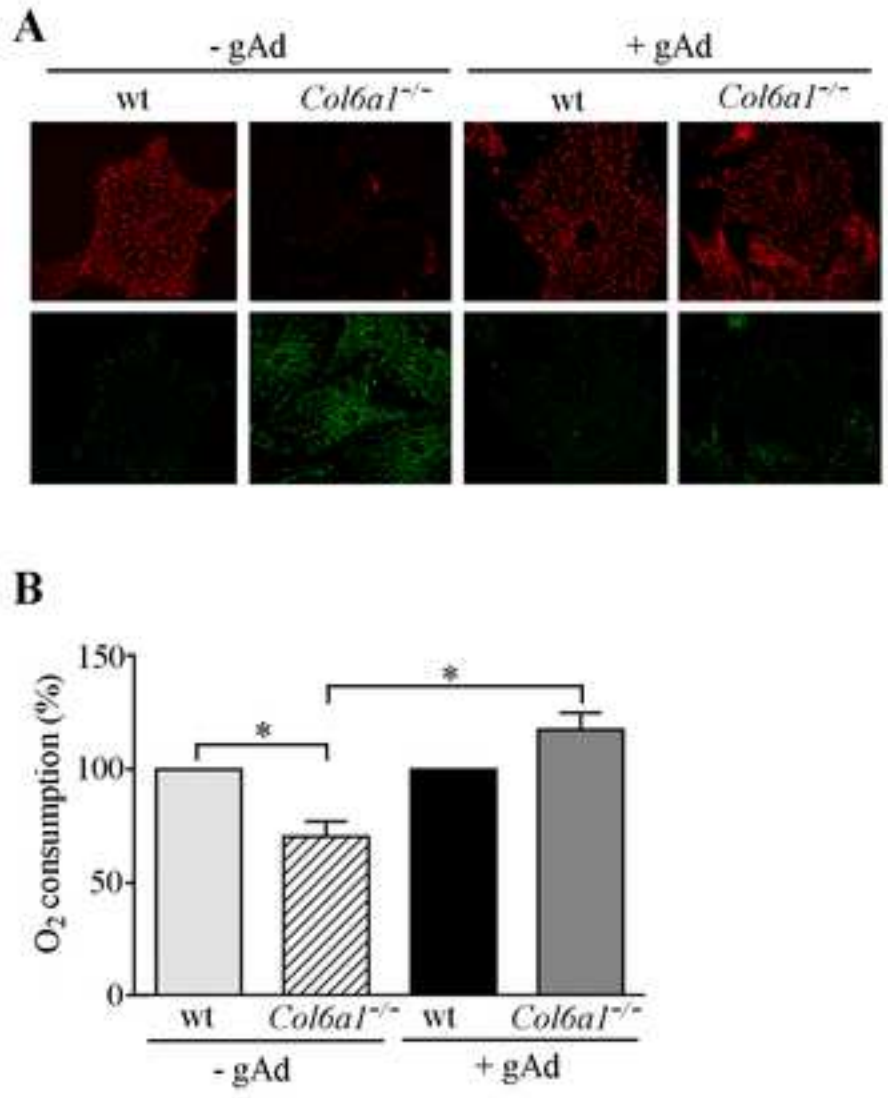
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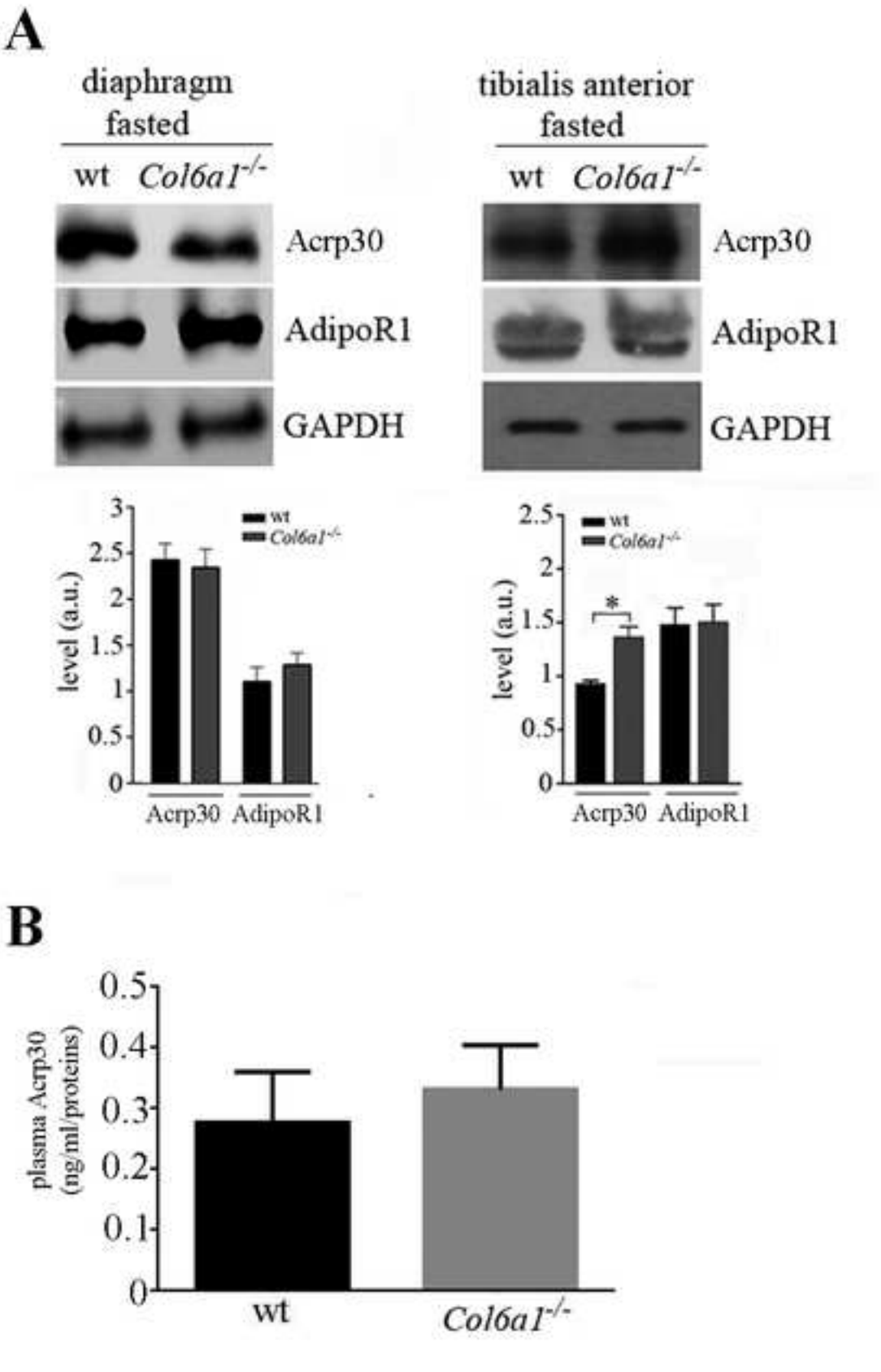
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Figure 7



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Figure 8



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