ORIGINAL ARTICLE

The environmental pollutant cadmium induces homeostasis alteration in muscle cells in vitro

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Abstract

Background Cadmium (Cd) is a heavy metal widely distributed throughout the environment as a result of contamination from a variety of sources. It exerts toxic effects in many tissues but scarce data are present as yet on potential effects on skeletal muscle tissue.

Aim To evaluate the potential alteration induced by Cd in skeletal muscle cells.

Materials and methods C2C12 skeletal muscle cells were treated with Cd at different times of cellular differentiation and gene expression was evaluated.

Results Exposure to Cd decreased significantly p21 mRNA expression and strongly up-regulated cyclin D1 mRNA expression in committed cells and in differentiated myotubes. Moreover, myogenin, fast MyHC-IIb and slow MyHC-I mRNAs expression were also significantly decreased both in committed cells and in myotubes. Moreover, Cd exposure induced a strong increase of Pax3, Pax7 and Myf5 mRNAs expression and stimulated an up-regulation of IL6 and TNF-α proinflammatory cytokines.

Conclusion These data lead to hypothesize that environmental Cd exposure might trigger an injury-like event in muscle tissue, possibly by an estrogen receptor-mediated mechanism.

Keywords Cadmium · Muscle cells · Cell differentiation · Injury · Regeneration

Abbreviations
Cd   Cadmium
CdCl$_2$ Cadmium chloride
MyHC   Myosin heavy chain

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Introduction

Cadmium (Cd) is a heavy metal, acting as a pollutant, which is widely distributed throughout the environment as a result of contamination from a variety of sources. Exposure to Cd is related to contaminated air and/or water, due to the large Cd industrial use, as well as to cigarette smoking [1, 2]. Cd is a multitarget toxicant responsible for kidney, liver, reproductive organs and bone damages as recently well reviewed by Bernhoft [3]. This pollutant increases DNA synthesis, stimulates proto-oncogene and transcription factors expression, as well as stress proteins synthesis, and up-regulates proinflammatory cytokines levels and several kinases activity [4–7] in different cell types such as kidney cells, thymocites, pancreatic beta cells, osteoblasts and breast cancer cells [7–13]. All these previously published data suggest an interaction between Cd and intracellular proteins leading to activation of downstream signaling events which can significantly alter the physiological cellular homeostasis. In addition, as other

estrogenic disruptors, Cd interferes in vivo and in vitro with estrogen receptor (ER)-mediated pathways, by its ability to form a high-affinity complex with the hormone binding domain of the ER [12, 14]. As a consequence, cells expressing ER as breast, bone and skeletal muscle cells could be considered potential targets of Cd-induced toxic effects. Indeed, we have previously demonstrated that Cd could significantly alter breast cancer cell intracellular pathways by, at least in part, an ER-mediated mechanism [13].

Even if a Cd-related disruption of cellular homeostasis in bone, breast and kidney cells has been already described [7, 10, 13], few data are available when potential effects of Cd on skeletal muscle cells are concerned. Cd exposure increases oxidative stress in C2C12 muscle cells, compromising cell adhesion and cellular antioxidant defense mechanisms [15].

Modifications of muscle homeostasis could lead to significant alterations in muscle mass and physiology, as observed in sarcopenia [16, 17]. Interestingly, considering the high concentration of Cd in cigarettes, recent studies demonstrated that smoking is associated with sarcopenia [18, 19], and men and women, who were current smokers, were more likely to have sarcopenia as compared to non-smokers individuals [20–22]. Unfortunately, few studies have been performed to identify the substances and the mechanisms by which cigarette smoking promotes muscle catabolism and accelerates the progression of muscle loss by inducing structural and metabolic damage, including decreased cross-sectional area of type I muscle fibers [23]. For instance, smokers had a greater expression of both muscle-specific E3 ligase MAFbx/atrogin-1 and myostatin (i.e. a muscle growth inhibitor), suggesting that components of cigarette smoking might impair muscle protein synthesis and stimulate genes associated with impaired muscle maintenance [24].

On these basis, we aimed to evaluate the effects of Cd on skeletal muscle cell differentiation by evaluating the expression of skeletal muscle markers genes, late and early expressed (myogenin, myosin heavy chains, Pax genes). Furthermore, since we observed a gene expression modulation, resembling reactivation of skeletal muscle regeneration program, occurring in adult life upon an injury event, we investigated the IL6 and TNFα proinflammatory cytokines expression, as well as the expression regulation of some atrogenes, such as Atrogin in the muscle cells exposed to Cd.

Materials and methods

Cell culture

C2C12 mouse myoblasts were maintained and differentiated as previously described [25]. Cells were plated at a density of 2×10^4 cells/ml in growing medium and Cadmium Chloride ($CdCl_2$) or vehicle was added to the medium as indicated in the results section. Cells were harvested for molecular analysis at 24 and 48 h after seeding in growing medium (GM) (proliferating myoblasts, Mb), after 72 h (committed cells, CC D0) and after 4 days of switch in differentiating medium (DM) (myotubes, Mt D3). $CdCl_2$ was added at 1 μM in GM (myoblasts and committed cells) and 3 μM in DM (myotubes), accordingly with Yano et al. [15]. All media and serum were purchased from Euroclone (Milano, Italy).

RNA extraction and quantitative reverse transcription polymerase chain reaction (QPCR)

Cells were washed twice with 1X Phosphate Buffer Saline (PBS, Euroclone, Milano, Italy), collected with scraper and immediately lysated in 1 ml of TRIzol™ Reagent (Invitrogen, Life & Technologies, Monza, Italy). Total RNA was extracted following manufacturer's indications. The purity, integrity and yield of RNA were monitored by spectrophotometric analysis and agarose gel electrophoresis. Two micrograms was treated with DNAse I Amplification Grade (Invitrogen, Life & Technologies, Monza, Italy) and reverse-transcribed using the SuperScript™ III (Invitrogen, Life & Technologies, Monza, Italy). Quantitative PCR was performed in applied biosystem 7,500 real time PCR using qPCR master mix (Applied Biosystem, Life & Technologies, Monza, Italy) as indicated by manufacturer's indications. All primers were optimized for real-time RT-PCR amplification. Quantitative RT-PCR sample value was normalized for the expression of cyclophilin mRNA. The relative level for each gene was calculated using the $2-\Delta\Delta Ct$ method [26] and reported as arbitrary units. In all experiments each sample was analyzed in duplicate. QPCR was used to evaluate the mRNA expression of genes listed in Table 1, with the sequence of relative primers.

Protein extraction and western blot analysis

Cells were washed twice with 1× PBS, collected with a scraper and then centrifuged 10 at 1,200 rpm at 4 °C. To extract total proteins, pellet was suspended in RIPA buffer (Sigma Aldrich, Milano, Italy) added with protease and phosphatase inhibitors cocktail (Sigma Aldrich, Milano, Italy). Total proteins were collected and stored at −80 °C until use. Proteins concentration was determined using the micro BCA protein assay reagent (Thermo Scientific, Euroclone, Milano, Italy).

Twenty microgram of total proteins was separated in a SDS–polyacrilamide gel and transferred to a nitrocellulose membrane. Transfer was verified by ponceau S staining.

Table 1 Sequence of relative primers used to evaluate the mRNA expression of genes by QPCR

Gene	Accession	Forward	Reverse
PAX	NM001243756.1	CCATGTACGTCCCCACGAA	GGCTGCACTGCTGAAATATGAG
bALP	NM000478	GGCTCCAGGGATAAAGCAGGT	AGTGTCTCTTGCGCTTGGTCT
RANK-L	NM033012	TCAGCCTTTTGCTCATCTCACTAT	CCACCCCCGATCATGGT
OPG	NM002546	GCTCACAAGAACAGACTTTCCAG	CTGTTTTCACAGAGGTCAATATCTT
cMyc	NM002467.4	TTCTCTCCGTCCTCGGATTCT	TTGTTCCTCCTCAGAGTCGCT
FHL2	XM005263902.1	TGCTCGCAGTGCAGAAACTC	CAGAGCAGCTGGTCCTCCTT
FHL1	NM001159702.2	GCTGGGACCCACCGTGTAG	GAGGGTGAGCAGTGAGTTGGA
Cyclin D1	NM007631	CGTGGCCTCTAAGATGAAGG	TGTTCTCATCCGCCTCTGGC
PDLIM1	NM020992.3	AAGTTGCCTATGTGTGACAAATGTG	CGGTCCCGCAGCTTCA
Cyclophilin	NM021130	GTCAACCCCACCGTGTTCTT	CTGCTGTCTTTGGGACCTTGT

The membrane was blocked 60 min at RT with 5 % non-fat dry milk (Cell Signaling, Euroclone, Milano, Italy) in tris buffered saline (T-TBS, 50 mM Tris Base, 0.9 % NaCl, Tween 20 0.001 %, pH 7.4). Anti-cyclin CD1 (Cell Signaling, Euroclone, Milano, Italy) and anti-cMyc (Cell Signaling, Euroclone, Milano, Italy) antibodies diluted as indicated by manufacturers were added overnight at 4°. The membrane was washed three times with T-TBS and incubated for 60 min at RT with HRP-labeled anti-rabbit or anti-mouse (Jackson Immunoresearch, Milano, Italy) in 5 % non-fat dry milk. The membrane was then washed three times and the antibodies were visualized using ECL prime western blotting detection reagent (GE Healthcare, Life Science Milano, Italy). Quantitative analysis was performed using Imagequant TL Image analysis software (GE Healthcare, Life Science Milano, Italy); sample value was normalized for housekeeping gene β actin and was reported as arbitrary units.

Statistical analysis

Statistical analysis was performed using Student's t test (Jandel Scientific). Data are presented as mean ± standard deviation and are the average of at least two different independent experiments performed in triplicate using independent cell cultures in each experimental setting. P values of <0.05 were considered significant.

Results

Cadmium treatment impaired skeletal muscle cell differentiation

C2C12 mouse myoblasts, developing a classical differentiation pattern [25], were used as in vitro model to analyze the Cd effects on skeletal muscle cells. Cd effects were evaluated in proliferating myoblasts (Mb), committed cells (CC D0) and differentiated Myotubes (Mt). As expected, in untreated cells there was an increase of p21 mRNA expression and a down-regulation of cyclin D1 mRNA levels along the differentiation process (Fig. 1). Accordingly to Yano et al. [15], myoblasts and committed cells
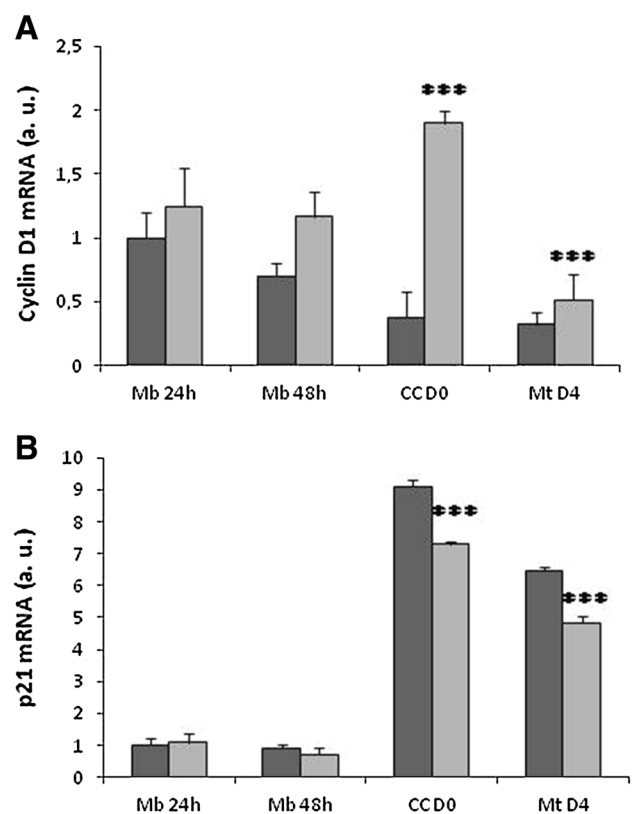

Fig. 1 Expression of **a** Cyclin D1 and **b** p21 in C2C12 cells treated with vehicle (*dark bars*) or CdCl$_2$ (*grey bars*) as measured by quantitative real-time PCR. Muscle cells were differentiated and treated as described in the "Materials and methods" section. Genes' expression was evaluated in proliferating myoblasts (Mb), in committed cells (D0) and in differentiated myotubes (Mt) at 24 and 48 h. Differences were considered significantly different when a $p < 0.05$ was obtained
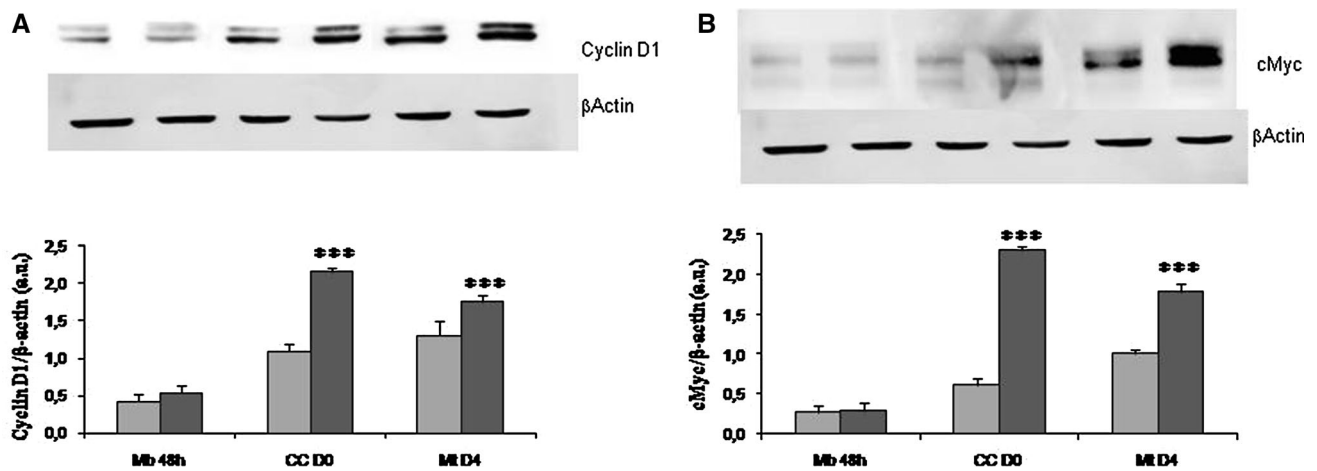
obtained from Western Blot analysis are depicted in the graphs; *dark bars* represent vehicle treated cells, *grey bars* represent $CdCl_2$ treated cells. Data are expressed as mean $\pm$ SE of three different independent experiments performed in duplicate

were treated with $CdCl_2$ 1 μM and myotubes were treated with $CdCl_2$ 3 μM. When cells were exposed to $CdCl_2$, p21 mRNA expression was significantly decreased ($p < 0.001$), and at the same time, a strong up-regulation of cyclin D1 mRNA expression in committed cells and in differentiated myotubes ($p < 0.001$) was observed (Fig. 1). Cyclin D1 and cMyc protein level expression was also evaluated by western blot analysis which demonstrated an up-regulation in $CdCl_2$-exposed muscular cells as depicted in Fig. 2.

Cadmium decreased the expression of skeletal muscle markers genes

To confirm that the pollutant Cd could interfere with differentiation of skeletal muscle cells, we checked the expression of some typical myogenic markers, such as myogenin and myosin heavy chains (MyHC). MRNA expression of myogenin was significantly decreased both in committed cells and in the myotubes exposed to $CdCl_2$ ($p < 0.001$, Fig. 3).

Exposure of the cells to the pollutant $CdCl_2$ down-regulated the expression of fast MyHC-IIb and slow MyHC-I mRNAs as shown in Fig. 3.

Cadmium reactivated the expression of early skeletal muscle markers

We then investigated whether the Cd-induced alteration of cell differentiation could be related to an activation of proliferation program. To address this point we checked the expression of early genes expressed during myogenic differentiation.

As shown in Fig. 4, $CdCl_2$ exposure induced a strong increase of Pax3, Pax7 and Myf5 mRNAs expression as

compared to the untreated cells ($p < 0.001$), being particularly relevant in committed cells.

Cadmium induced the expression of pro-Inflammatory cytokines

Since the reprogramming of skeletal muscle cells occurs in adult life upon an injury event, we were interested to analyze the expression of typical proinflammatory cytokines, as IL6 and TNFα (Fig. 5), known to be modulated by muscle injury. Both cytokines were significantly up-modulated by $CdCl_2$ treatment in committed cells and in myotubes, leading to the hypothesis that the pollutant Cd might cause an injury-like damage effect in the muscle cells.

Cadmium induced the expression of atrogenes

Since it was demonstrated that smoke-induced muscle catabolism could be induced by up-regulation of some atrogenes, we checked the expression of atrogin in the cells exposed to Cd [27]. $CdCl_2$ up-regulated MAFbx/atrogin-1 gene expression, as shown in Fig. 6, further indicating an alteration in muscle homeostasis induced by this toxic pollutant which is also present in cigarette smoking.

Discussion

The results presented in this study show for the first time that the environmental toxic pollutant Cd induces significant alterations in the differentiation mechanism(s) of skeletal muscle cells in vitro, likely altering muscle tissue homeostasis.
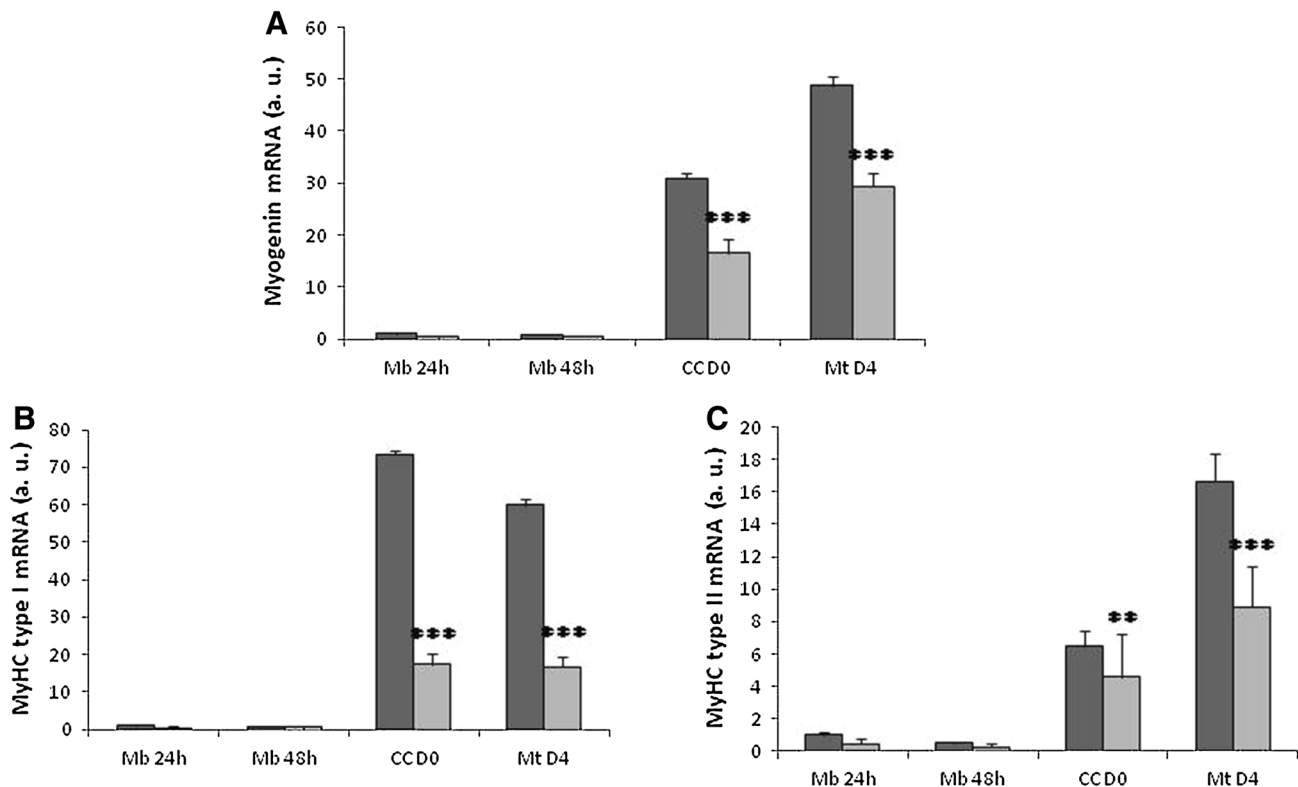
Fig. 3 Expression of **a** myogenin, **b** MyHC type I and **c** MyHC type II in C2C12 cells treated with vehicle (*dark bars*) or CdCl$_2$ (*grey bars*) as measured by quantitative real-time PCR. Muscle cells were differentiated and treated as described in the "Materials and methods" section. Genes' expression was evaluated in proliferating Myoblasts (Mb) in committed cells (D0) and in differentiated myotubes (Mt) at 24 and 48 h. Differences were considered significantly different when a $p < 0.05$ was obtained

In particular, our data show that Cd could stop differentiation in C2C12 cells, blocking the expression of typical differentiated myotubes markers and reactivating the expression of reprogramming cells markers, typical of an injury-like event. The transition of cells from a proliferating to a mature muscle phenotype, concomitant with terminal cell cycle exit, was evaluated by analyzing classical skeletal muscle marker. In fact, cell cycle exit is characterized by the modulation of several different genes which undergo significant changes such as the repression of cyclin D1 [28] and the activation of cell cycle inhibitor p21 [29] soon as proliferation stops and differentiation starts.

Muscle regeneration and repair are complex mechanisms that occur in different interdependent phases: degeneration, inflammation, regeneration and fibrosis [30–32]. In postnatal skeletal muscle, the primary cellular source of growth and regeneration is the satellite cell [33–35], a quiescent muscle precursor cell situated beneath the basal lamina surrounding each muscle fiber. In response to muscle injury, satellite cells are activated, proliferate to form a pool of myoblasts, commit to differentiation and then fuse together to repair or replace damaged muscle fibers [36]. The *Pax* gene family contains nine members characterized by the presence of a common paired-box domain that directs binding to specific DNA sequences. Pax7 is expressed almost ubiquitously by quiescent satellite cells. In skeletal muscle, Pax3 and Pax7 have overlapping, but non-redundant roles, in the specification of embryonic muscle progenitors, and in the network with the myogenic regulatory factor (MRF) family of transcription factors comprising Myf5, MyoD, Mrf4 and myogenin [33, 37]. It was demonstrated that in adult muscle stem cells, Pax3 and Pax7 genes function to promote population expansion, while maintaining commitment to the myogenic lineage [37]. PaX3 is co-expressed with other MRFs in proliferating myoblast progeny [29, 38]. Pax3 is transiently detected in proliferating satellite cell-derived myoblasts [39–41]. Myogenin is an early marker of commitment to differentiation; indeed, it starts to be expressed concomitantly with the down-regulation of Pax7 [29, 38, 42]. In myoblast cell cultures, Pax7 is similarly not expressed in differentiated myotubes, but it is maintained in the smaller accompanying population of undifferentiated cells that stop proliferating, and return to a non-proliferating state, reminiscent of the quiescent satellite cell [38]. Further, Pax3 and Pax7 increase proliferative rate and prevent precocious myogenic differentiation [37]. Indeed our data lead us to speculate that Cd could induce an injury-like event in the
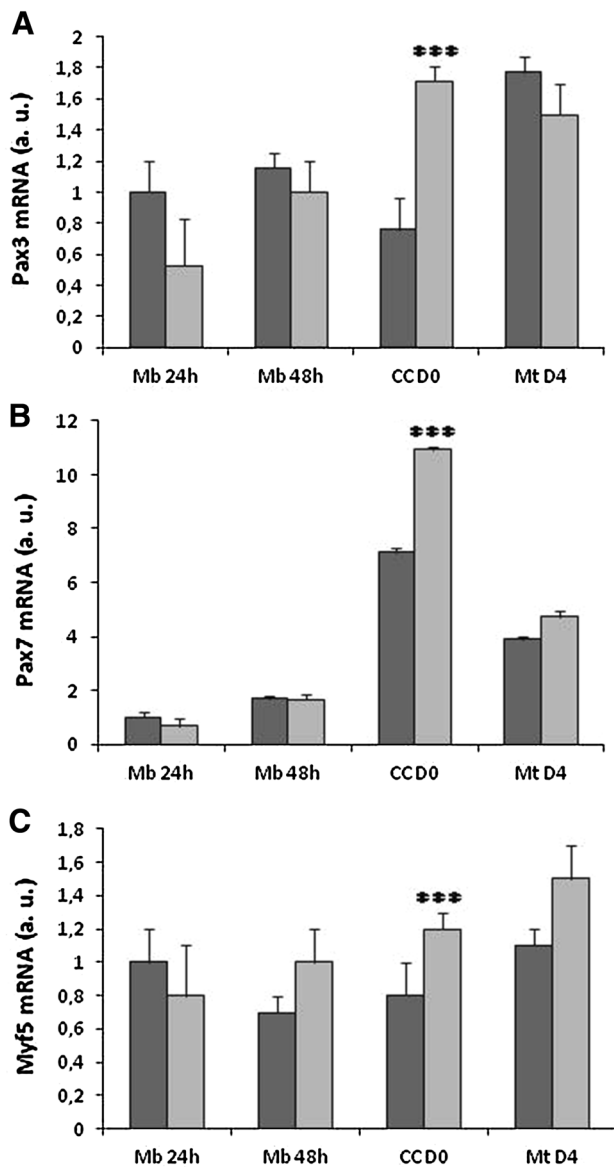
Fig. 4 Expression of **a** Pax3 **b** Pax7 and **c** Myf 5 in C2C12 cells treated with vehicle (*dark bars*) or CdCl$_2$ (*grey bars*) as measured by quantitative real-time PCR. Muscle cells were differentiated and treated as described in the "Materials and methods" section. Genes' expression was evaluated in proliferating Myoblasts (Mb), in committed cells (D0) and in differentiated myotubes (Mt) at 24 and 48 h. Differences were considered significantly different when a $p < 0.05$ was obtained

Fig. 5 Expression of **a** IL6, **b** TNFalpha in C2C12 cells treated with vehicle (*dark bars*) or CdCl$_2$ (*grey bars*) as measured by quantitative real-time PCR. Muscle cells were differentiated and treated as described in the "Materials and methods" section. Genes' expression was evaluated in proliferating myoblasts (Mb), in committed cells (D0) and in differentiated myotubes (Mt) at 24 and 48 h. Differences were considered significantly different when a $p < 0.05$ was obtained

Fig. 6 Expression of atrogin in C2C12 cells treated with vehicle (*dark bars*) or CdCl$_2$ (*grey bars*) as measured by quantitative real-time PCR. Muscle cells were differentiated and treated as described in the "Materials and methods" section. Genes' expression was evaluated in proliferating myoblasts (Mb), in committed cells (D0) and in differentiated myotubes (Mt) at 24 and 48 h. Differences were considered significantly different when a $p < 0.05$ was obtained

skeletal muscle cells, as indicated by the reactivation of these genes that mimics an event damage response. Interestingly, Cd as many other environmental estrogenic disruptors can interfere with estrogen receptor-mediated pathways. Thus, several tissues expressing estrogen receptors have been considered targets of Cd-induced toxic effects [7, 13]. Interestingly, ER, both alpha and beta, has been described in skeletal muscle cells [43], strongly indicating that the protective effects of estrogen
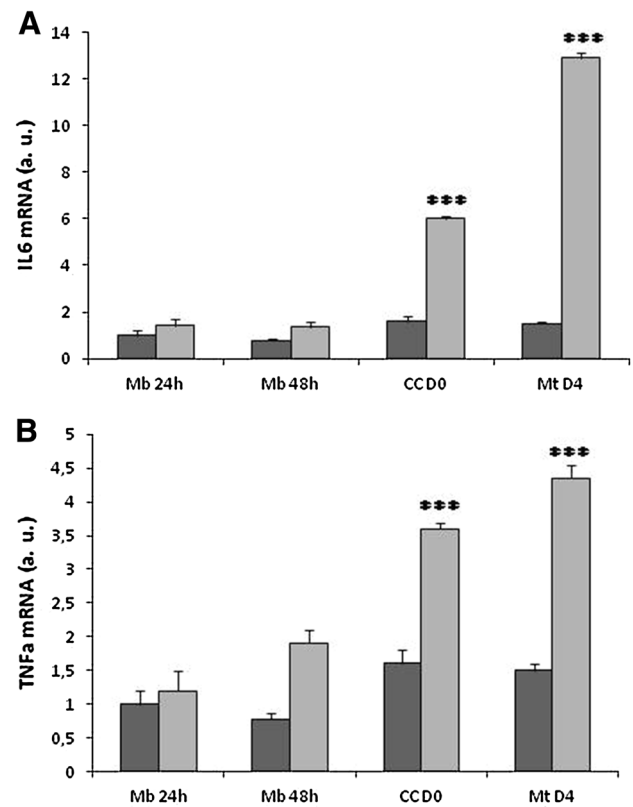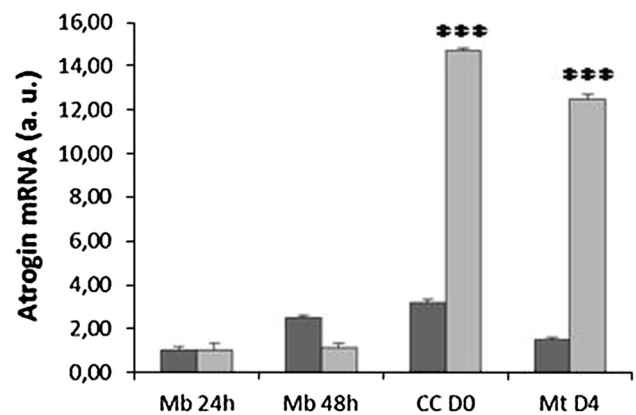
replacement therapy on both muscle mass and strength might be mediated by a specific receptor-mediated mechanism [44]. Since our group, as well as others, has demonstrated a potential interfering role of Cd on ER pathways [11–13], the exposure to this environmental pollutant could interfere with muscle homeostasis maintained by ER. Indeed estrogens are known to modulate energy metabolism and mitochondrial function in muscle cells by an ER-mediated mechanism [45]. In addition, previously published data have demonstrated that estrogens might play a role in muscle regeneration [43], thus Cd might interfere in this process by interfering with ER. Interestingly enough, a significant amount of Cd is present in cigarette smoking which, then, can be consider a major source of this environmental pollutant. Moreover, several studies have recently demonstrated that smoking can induce a significant sarcopenia [46]. Sarcopenia can be defined as the decline in muscle mass, quality and function, due to several different intrinsic and extrinsic factor risks. Intrinsic factors include decreased levels of anabolic hormones, such as testosterone, estrogen, growth hormone, and insulin-like growth factor-1 (IGF-1) with aging; increased inflammatory status, with higher levels of proinflammatory cytokines such as interlukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), which might contribute to muscle catabolism, and thus, muscle mass and strength loss. Among the extrinsic factors sedentary life or immobility, lack of physical activity, impaired nutrition, some medications, and illnesses could be seen as contributors of muscle loss. Beside these events, cigarette smoking can be considered one additional factor which can play a role in the mechanisms involved in the loss of muscle mass and strength in adult, not aged individuals. For instance, recent studies have demonstrated that smoking impairs the muscle protein synthesis process and increases the expression of genes associated with impaired muscle maintenance with obvious biomechanical consequences (i.e. increased predisposition to falls and fractures, alteration of physical activity ability). Moreover, smokers have greater expression, as compared to non-smokers, of the muscle-specific E3 ligase MAFbx/atrogin-1 and the muscle growth inhibitor myostatin. On the base of these results, Petersen et al. [24] hypothesized that smoking might alter muscle homeostasis, and increase the risk of sarcopenia, by impairing muscle protein synthesis and up-regulating genes associated with decreased muscle maintenance. Mice daily exposed to cigarette smoke, and thus to high levels of Cd, for 16 weeks presented a reduction in body muscle mass and up-regulation of MAFbx/atrogin-1 in sampled skeletal muscles. In addition, exposure of C2C12 myotubes to cigarette smoke caused a decrease in diameter of myotubes, degradation of the main contractile proteins, MyHC and actin and up-regulation of MAFbx/atrogin-1 and MuRF1 [46]. These

catabolic processes are mediated by increased intracellular oxidative stress and activation of p38 MAPK. Previous data in the literature have suggested that components of cigarette smoke may reach skeletal muscle of smokers, leading to increased oxidative stress and activation of signaling pathways triggering muscle cells damage [47]. Estrogens, through the ER, seem to decrease oxidative damage, and thus, Cd might interfere with the process of muscle recovery linked to these hormones [43]. In conclusion, our data show that the pollutant Cd alters skeletal muscle cells homeostasis. Further studies aimed at the characterization of muscle cells homeostasis are needed to fully clarify the cellular and molecular mechanism(s) leading to muscle damage and further characterize all the mechanisms involved in the development of sarcopenia.

References

1. Järup L, Akesson A (2009) Current status of cadmium as an environmental health problem. Toxicol Appl Pharmacol 238:201–208
2. Nawrot TS, Staessen JA, Roels HA et al (2010) Cadmium exposure in the population: from health risks to strategies of prevention. Biometals 23:769–782
3. Bernhoft RA (2013) Cadmium toxicity and treatment. Sci World J
4. Beyersmann D, Hechtenberg S (1997) Cadmium, gene regulation, and cellular signaling in mammalian cells. Toxicol Appl Pharmacol 144:247–261
5. Zheng H, Liu J, Choo KH et al (1996) Metallothionein-I and -II knock-out mice are sensitive to cadmium-induced liver mRNA expression of c-jun and p53. Toxicol Appl Pharmacol 136:229–235
6. Dormer UH, Westwater J, McLaren NF et al (2000) Cadmium-inducible expression of the yeast GSH1 gene requires a functional sulfur-amino acid regulatory network. J Biol Chem 275:32611–32616
7. Brama M, Politi L, Santini P et al (2012) Cadmium-induced apoptosis and necrosis in human osteoblasts: role of caspases and mitogen-activated protein kinases pathways. J Endocrinol Invest 35:198–208
8. Pathak N, Mitra S, Khandelwal S (2013) Cadmium induces thymocyte apoptosis via caspase-dependent and caspase-independent pathways. J Biochem Mol Toxicol 27:193–203
9. Chang KC, Hsu CC, Liu SH et al (2013) Cadmium induces apoptosis in pancreatic β-cells through a mitochondria-dependent pathway: the role of oxidative stress-mediated c-jun N-terminal kinase activation. PLoS One, 8(2) doi:10.1371/journal.pone.0054374

10. Sinha K, Pal PB, Sil PC (2014) Cadmium (Cd(2 +)) exposure differentially elicits both cell proliferation and cell death related responses in SK-RC-45. Toxicol In Vitro 28:307–318

11. Chen S, Ren Q, Zhang J, et al. (2013) *N*-acetyl-L-cysteine protects against cadmium-induced neuronal apoptosis by inhibiting ROS-dependent activation of Akt/mTOR pathway in mouse brain. Neuropathol Appl Neurobiol. doi:10.1111/nan.12103

12. Waalkes MP (2003) Cadmium carcinogenesis. Mutat Res 533:107–120

13. Brama M, Gnessi L, Basciani S et al (2007) Cadmium induces mitogenic signaling in breast cancer cell by an ERalpha-dependent mechanism. Mol Cell Endocrinol 264:102–108

14. Ponce E, Aquino NB, Louie MC (2013) Chronic cadmium exposure stimulates SDF-1 expression in an ERα dependent manner. PLoS One 8(8):e72639. doi:10.1371/journal.pone.0072639

15. Yano CL, Marcondes MC (2005) Cadmium chloride-induced oxidative stress in skeletal muscle cells in vitro. Free Radic Biol Med 39:1378–1384

16. Derbré F, Gratas-Delamarche A, Gómez-Cabrera MC et al (2014) Inactivity-induced oxidative stress: a central role in age-related sarcopenia? Eur J Sport Sci 14(Suppl 1):S98–S108

17. Siparsky PN, Kirkendall DT, Garrett WE Jr (2014) Muscle changes in aging: understanding sarcopenia. Sports Health 6:36–40

18. Rom O, Kaisari S, Aizenbud D et al (2012) Identification of possible cigarette smoke constituents responsible for muscle catabolism. J Muscle Res Cell Motil 33:199–208

19. Rom O, Kaisari S, Aizenbud D et al (2012) Lifestyle and sarcopenia-etiology, prevention, and treatment. Rambam Maimonides Med J 3(4):e0024. doi:10.5041/RMMJ.10091

20. Castillo EM, Goodman-Gruen D, Kritz-Silverstein D et al (2003) Sarcopenia in elderly men and women: the Rancho Bernardo study. Am J Prev Med 25:226–231

21. Lee JS, Auyeung TW, Kwok T et al (2007) Associated factors and health impact of sarcopenia in older Chinese men and women: a cross-sectional study. Gerontology 53:404–410

22. Szulc P, Duboeuf F, Marchand F et al (2004) Hormonal and lifestyle determinants of appendicular skeletal muscle mass in men: the MINOS study. Am J Clin Nutr 80:496–503

23. Montes de Oca M, Loeb E, Torres SH et al (2008) Peripheral muscle alterations in non-COPD smokers. Chest 133:13–18. doi:10.1378/chest.07-1592

24. Petersen AM, Magkos F, Atherton P et al (2007) Smoking impairs muscle protein synthesis and increases the expression of myostatin and MAFbx in muscle. Am J Physiol Endocrinol Metab 293:E843–E848

25. Wannenes F, Caprio M, Gatta L et al (2008) Androgen receptor expression during C2C12 skeletal muscle cell line differentiation. Mol Cell Endocrinol 292:11–19

26. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408

27. Rom O, Kaisari S, Aizenbud D et al (2013) Cigarette smoke and muscle catabolism in C2 myotubes. Mech Ageing Dev 134:24–34

28. Guo K, Walsh K (1997) Inhibition of myogenesis by multiple cyclin-Cdk complexes. Coordinate regulation of myogenesis and cell cycle activity at the level of E2F. J Biol Chem 272:791–797

29. Halevy O, Piestun Y, Allouh MZ et al (2004) Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal. Dev Dyn 231:489–502

30. Tidball JG (1995) Inflammatory cell response to acute muscle injury. Med Sci Sports Exerc 27:1022–1032

31. Huard J, Li Y, Fu FH (2002) Muscle injuries and repair: current trends in research. J Bone Jt Surg Am 84-A: 822–32

32. Jarvinen TA, Järvinen TL, Kääriäinen M et al (2005) Muscle injuries: biology and treatment. Am J Sports Med 33:745–764

33. Collins CA, Olsen I, Zammit PS et al (2005) Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122:289–301

34. Sherwood RI, Christensen JL, Conboy IM et al (2004) Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafting skeletal muscle. Cell 119:543–554

35. Zammit PS, Heslop L, Hudon V et al (2002) Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. Exp Cell Res 281:39–49

36. Zammit PS, Partridge TA, Yablonka-Reuveni Z (2006) The skeletal muscle satellite cell: the stem cell that came in from the cold. J Histochem Cytochem 54:1177–1191

37. Collins CA, Gnocchi VF, White RB et al (2009) Integrated functions of Pax3 and Pax7 in the regulation of proliferation, cell size and myogenic differentiation. PLoS One 4(2):e4475. doi:10.1371/journal.pone.0004475

38. Zammit PS, Golding JP, Nagata Y et al (2004) Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? J Cell Biol 166:347–357

39. Conboy IM, Rando TA (2002) The regulation of notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell 3:397–409

40. Day K, Shefer G, Richardson JB et al (2007) Nestin-GFP reporter expression defines the quiescent state of skeletal muscle satellite cells. Dev Biol 304:246–259

41. Shinin V, Gayraud-Morel B, Gomès D et al (2006) Asymmetric division and cosegregation of template DNA strands in adult muscle satellite cells. Nat Cell Biol 8:677–687

42. Olguin HC, Olwin BB (2004) Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev Biol 275:375–388

43. Velders M, Diel P (2013) How sex hormones promote skeletal muscle regeneration. Sports Med 43:1089–1100

44. Greising SM, Baltgalvis KA, Kosir AM, Moran AL, Warren GL, Lowe DA (2011) Estradiol's beneficial effect on murine muscle function is independent of muscle activity. J Appl Physiol 110:109–115

45. Ronkainen PH, Kovanen V, Alén M, Pöllänen E, Palonen EM, Ankarberg-Lindgren C, Hämäläinen E, Turpeinen U, Kujala UM, Puolakka J, Kaprio J, Sipilä S (2009) Postmenopausal hormone replacement therapy modifies skeletal muscle composition and function: a study with monozygotic twin pairs. J Appl Physiol 107:25–33

46. Rom O, Kaisari S, Aizenbud D et al (2012) Sarcopenia and smoking: a possible cellular model of cigarette smoke effects on muscle protein breakdown. Ann NY Acad Sci 1259:47–53

47. Rani A, Kumar A, Lal A et al (2014) Cellular mechanisms of cadmium-induced toxicity: a review. Int J Environ Health Res 24:378–399