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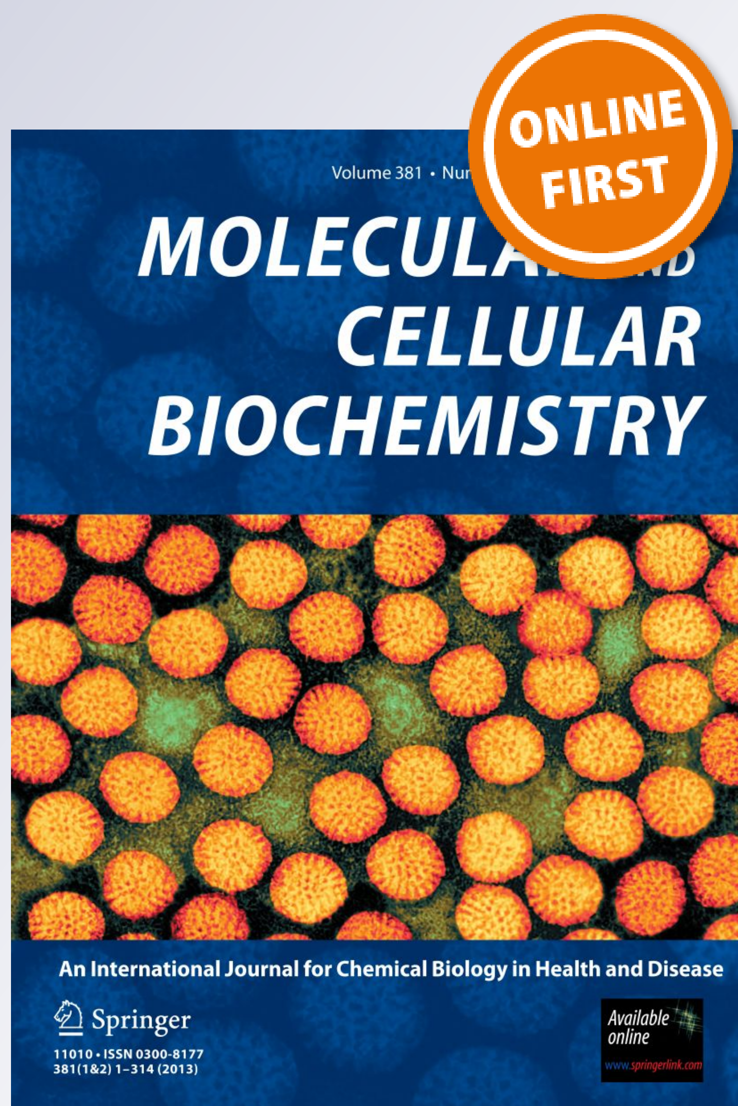

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Lead enhancement of 3T3-L1 fibroblasts differentiation to adipocytes involves ERK, C/EBPβ and PPARγ activation

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Abstract Lead (Pb) is an environmental and industrial contaminant that still represents a public health problem. Elevated Pb exposure has been inversely correlated with femoral bone density and associated with osteoporosis. In the last years, it has been shown that inhibition of osteogenesis from mesenchymal stem cells activates adipogenesis and vice versa. In this paper, we investigated the effect of Pb on the differentiation of 3T3-L1 fibroblasts to adipocytes which is the cell model most used to study adipogenesis. After induction of differentiation, 2 days post-confluent cells re-enter the cell cycle and undergo mitotic clonal expansion (MCE) followed by expression of genes that produce the adipocyte phenotype. The presence of concentrations of Pb up to 10 μM during differentiation of 3T3-L1 fibroblasts did not interfere with MCE but enhanced the accumulation of cytosolic lipids that occur during adipogenesis, as well as, the induction of PPARγ, the master gene in adipogenesis. It is known that PPARγ upregulation is subsequent to induction of C/EBPβ and ERK activation, which are early events in adipogenesis. We found that both events were enhanced by Pb treatment. Our results support a stimulatory effect of Pb on adipogenesis which involves ERK activation and C/EBPβ upregulation prior to PPARγ and adipogenesis activation.

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Introduction

Lead (Pb) is a heavy metal and an environmental contaminant that still represents a public health problem. In the 1970s, more than 70% of the population in USA had blood Pb levels higher than 10 μg/dl [1]. Although these concentrations have decreased mainly because of the removal of Pb from gasoline [2, 3], it has been reported that chronic exposure to even low concentrations of this metal can be toxic [2, 4].

Exposure to Pb still remains in many different occupational circumstances such as: soldering, plumbing work, working with metals or alloys containing lead, mining, welding leaded steel [5]. In keeping with this, it was found that pottery-glaze workers had increased Pb blood levels and genotoxic damage in peripheral blood lymphocytes [6].

In 2006, the Scientific Committee on Neurotoxicology and Psychophysiology and the Scientific Committee on the Toxicology of Metals of the International Commission on Occupational Health recommended to reduce blood Pb levels to 30 μg/dl for industrial workers and 5 μg/dl for children [7]. Nevertheless, Murata et al. [8], reported that the neurotoxic effects of Pb in workers appear to be initiated at levels below 18 μg/dl. At present, according to the Centers for Disease Control and Prevention, Pb blood levels lower than 5 μg/dl are not considered to be indicative of Pb poisoning in children and chelation therapy is recommended when a child blood Pb test results greater than or equal to 45 μg/dl.

Lead produces biochemical, physiological, and behavioral alterations which are usually greater in children [2, 4]

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and can be stored for long periods of time in bones. Consistently, an increase in blood lead was found in women after menopause when an increased bone demineralization usually occurs [9]. Elevated Pb exposure has been inversely correlated with femoral bone density and associated with osteoporosis [9, 10]. It has been reported that stem cells isolated from a dental origin exposed to lead nitrate maintained their ability for chondrogenesis and adipogenesis but a severe downregulation in osteogenesis was observed [11].

3T3-L1 fibroblasts are a useful tool in the study of adipocyte differentiation. After induction of differentiation with a media containing insulin, dexamethasone and 3-isobutyl-1-methylxanthine, post-confluent 3T3-L1 fibroblasts re-enter the cell cycle. This proliferation step is called mitotic clonal expansion (MCE). MCE precedes the adipogenic gene expression program leading to adipocyte differentiation [12, 13]. In this differentiation process, two early transcription factors CCAAT enhancer-binding protein beta (C/EBPβ) and C/EBP delta are induced during the first hours and then they trigger the expression of a key transcription factor in adipogenesis: peroxisome proliferator-activated receptor gamma (PPARγ), which is involved in the induction of proteins associated with adipocyte phenotype [14, 15]. 3T3-L1 fibroblasts were previously used to evaluate the effects of arsenic trioxide, hexavalent chromium, and a commercial formulation of the herbicide glyphosate on cell survival and differentiation [16–19].

In the present investigation, we evaluated the effect of Pb on the differentiation of 3T3-L1 fibroblasts to adipocytes.

Materials and methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM), trypsin, and insulin were obtained from Invitrogen (Carlsbad, CA), and 3-isobutyl-1-methylxanthine (MIX) and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lead acetate trihydrate ($Pb(CH_3CO_2)_2 \cdot 3H_2O$, CAS 6080-56-4) was purchased from Mallinckrodt (Argentina). 3T3-L1 fibroblasts were obtained from Asociación Banco Argentino de Células (origin: ATCC).

Cell culture and treatment of 3T3-L1 fibroblasts

3T3-L1 fibroblasts were cultured in DMEM + 10% fetal bovine serum (FBS) with 100 µg/ml streptomycin, 100 U/ml penicillin, and 250 ng/ml fungizone (DMEM + 10% FBS). When indicated, Pb acetate (Pb) was added to the plate in DMEM + 10% FBS as vehicle, to obtain the appropriate final concentration.

Cell counting after MCE

Eight days after the treatment with DMEM + 10% FBS alone (C) or the differentiation media without (DM) or with different concentration of Pb (DM + Pb) or differentiation media with Na Acetate as a negative control (DM + Na Ac), 3T3-L1 cells from 24-well plates were washed with PBS, trypsinized, resuspended in PBS, and an aliquot was counted after addition of trypan blue using a Neubauer chamber.

Differentiation induction of 3T3-L1 fibroblasts and Oil-Red-O staining

To induce differentiation, 2-day post-confluent 3T3-L1 fibroblasts were treated with a differentiation media containing 10 µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (MIX), and 100 nM dexamethasone in DMEM + 10% FBS (DM) [13, 20].

Three days after the induction of differentiation, medium was replaced with DMEM + 10% FBS supplemented with insulin. Then medium was changed every 2 days with DMEM + 10% FBS. Pioglitazone or Pb acetate was added to the media to obtain the appropriate final concentration and was maintained throughout the differentiation process until terminal differentiation was reached.

3T3-L1 adipocyte monolayers (usually on day eight) were washed three times with phosphate-buffered saline (PBS) and then fixed for 30 min with 10% formaldehyde in PBS. Three volumes of Oil-Red-O (0.4%) in isopropanol were diluted with two volumes of water, filtered, and added to the fixed cell monolayers for 30 min at room temperature. Cells were then washed with water, and the stained triglyceride droplets in the cells were visualized with an inverted microscope (×100) and photographed. To measure the total content of lipids, Oil-Red-O-stained lipids were dissolved in isopropanol and then quantified by measuring the absorbance at 490 nm [21].

RNA extraction and RT-qPCR analysis

3T3-L1 fibroblasts were cultured in 12 well plates and 8 days after addition of differentiation media in the absence (DM) or presence of different concentrations of Pb (DM + Pb) or of medium alone (C), cells were lysed in TRI Reagent (Molecular Research Center). RNA extraction was performed according to the standard protocol and pellets were resuspended in nuclease-free water. RNA was quantified with NanoDrop 2000 spectrophotometer (Thermo Scientific), and 2 µg were used for reverse

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transcription using MMLV Reverse Transcriptase (Promega) and oligo dT_{15}. Relative expression of cDNAs was determined by quantitative real-time PCR using a Bio-Rad MyIQ2 thermal cycler. Each PCR reaction was performed in a final volume of 25 µl containing 5 µl of a 1:10 dilution of first-strand cDNA, 0.2 µM of each primer, 0.8 mM dNTPs, 3 mM $MgCl_2$, 0.5 U of Platinum Taq DNA Polymerase (Invitrogen), and SYBR Green (Invitrogen) in a buffer supplied by the manufacturer. All reactions were performed using the following cycling conditions: 94 °C for 5 min, followed by 40 cycles of 94 °C for 20 s, 58 °C for 30 s, and 72 °C for 40 s. Target gene mRNA expression was normalized to acidic ribosomal protein (Rplp0) as a reference gene [22]. The following primers were used: PPARγ2, Fwd: CCAGAGCATGGTGCCTTCGCT, Rev: CAGCAACCATTGGGTCAGCTC; Plin1, Fwd: TGAAGGGTGTTACGGATAACG, Rev: TGAAGGGTTATCGATGTCTCG; and Rplp0, Fwd: GAGGAATCAGATGAGGATATGGGA, Rev: AAGCAGGCTGACTTGGTTGC.

Western-blot determination

Two-day post-confluent 3T3-L1 fibroblasts were treated with DMEM + 10% FBS (C) or differentiation media in the absence (DM) or presence of 10 µM Pb (DM + Pb) during 24 h for C/EBPβ analysis. For ERK determination, 2-day post-confluent 3T3-L1 cells were treated for 1 h with DMEM + 1% FBS then cells were induced to differentiate in the absence (DM) or presence of 10 µM Pb (DM + Pb) for 30 min. After the treatments, cells were harvested in Laemmli sample buffer [23] and vortexed. After SDS-PAGE, gels were transferred to nitrocellulose membranes, blocked with 5% milk for an hour and then treated overnight with p-ERK, ERK, C/EBPβ, or GAPDH antibodies (from Santa Cruz) followed by HRP-conjugated secondary antibody for 1 h. Finally, chemiluminescence reagent was used for detection.

Statistical analysis

The experiments were carried out three times unless otherwise stated. All data were expressed as mean ± SE. Statistical analysis was performed by One-Way ANOVA followed by Tukey's post hoc test, p values below 0.05 were considered significant.

Results

Effect of Pb exposure on mitotic clonal expansion

We investigated the effect of Pb on adipogenesis in 3T3-L1 fibroblasts since this cell line is a useful tool to study differentiation to adipocytes. When 2 days post-confluent 3T3-L1 fibroblasts are induced to differentiate, MCE occurs prior to adipogenesis. As expected, cells re-enter the cell cycle after the addition of differentiation media. We observed a twofold increase in cell number which was counted 8 days after induction of differentiation. This increase was not affected when Pb was added to 2 days post-confluent cells at a concentration of 10 µM or lower throughout differentiation (Fig. 1).

Effect of Pb exposure on lipid accumulation

Then we investigated the effect of the presence of Pb during differentiation of 3T3-L1 fibroblasts on cytosolic triglyceride accumulation which can be visualized by Oil-Red-O staining. Pioglitazone, an activator of PPARγ, which is the master gene of adipogenesis, was used as a positive control [24]. An enhanced accumulation of lipids were found when pioglitazone or Pb was added to the differentiation media as shown in (Fig. 2).

Consistently, absorbance of Oil-Red-O-stained lipids at 490 nm was increased with the addition of pioglitazone or Pb (0.376 ± 0.010 with differentiation media alone, 0.402 ± 0.004 with pioglitazone and 0.398 ± 0.003 with Pb).

Effect of Pb exposure on PPARγ and C/EBPβ

Since PPARγ is the master gene in adipogenesis, we also evaluated the effect of the addition of Pb on PPARγ mRNA. The amount of this mRNA was significantly increased in cells treated with differentiation media for 8 days and the addition of Pb further increased PPARγ mRNA which is in agreement with the results obtained

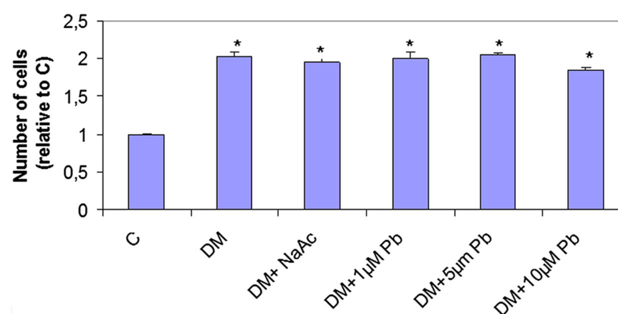

Fig. 1 Effect of Pb on mitotic clonal expansion. Two days post-confluent 3T3-L1 fibroblasts were treated with: DMEM + 10% FBS (C) or with the addition of differentiation media alone (DM), differentiation media + Pb at the indicated concentrations (DM + Pb) or differentiation media +20 µM Na Acetate which was used as a negative control (DM + NaAc). After 8 days, cells were counted in Neubauer chamber. Results represent mean ± SE of three independent experiments. *Significantly different from C, $p < 0.05$

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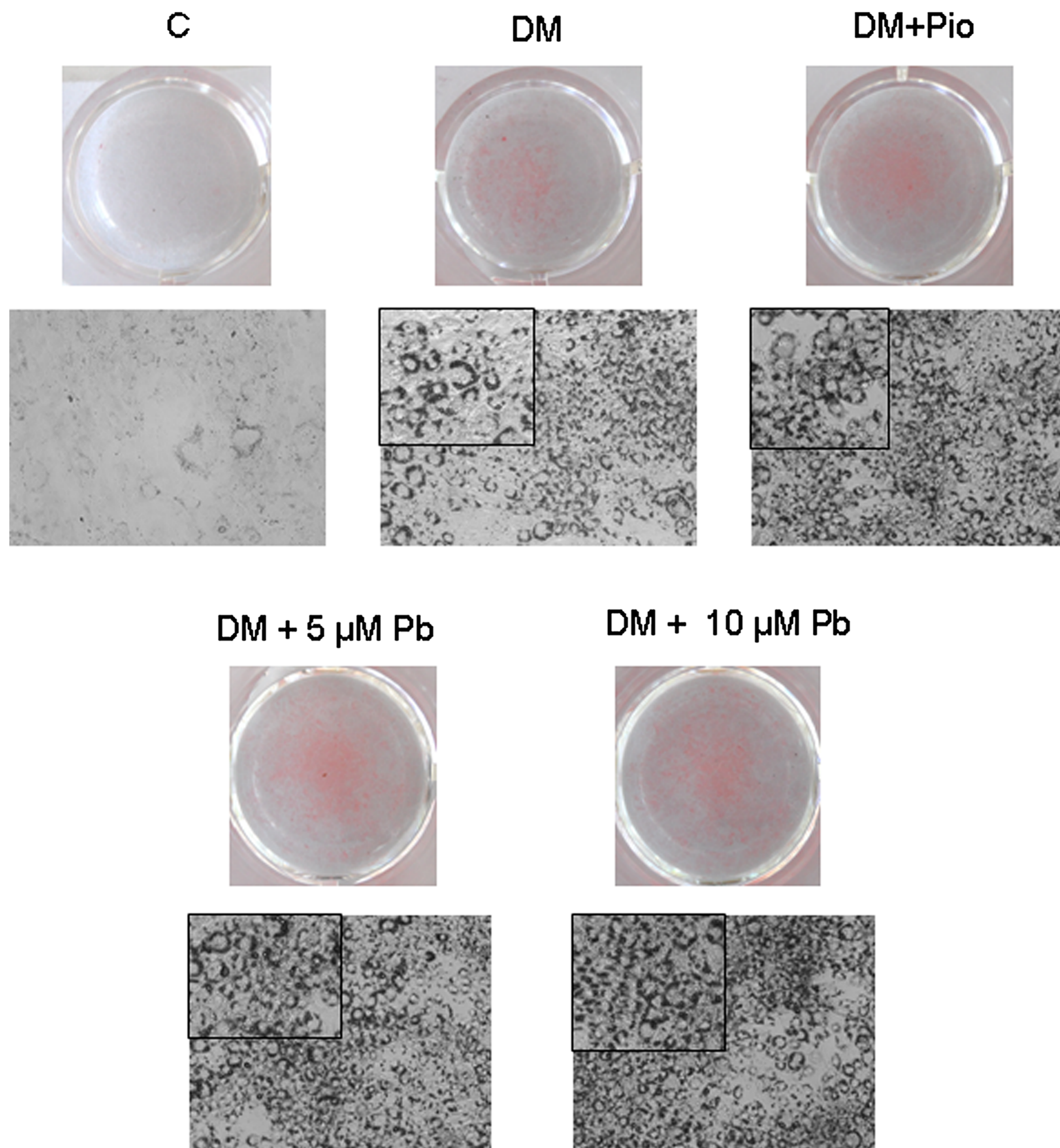

Fig. 2 Effect of Pb on lipid accumulation evaluated by Oil-Red-O staining. Two days post-confluent cells were treated with: medium alone (C), or with the addition of differentiation media (DM), differentiation media plus 2 μM pioglitazone (DM + Pio), or differentiation media plus different micromolar concentrations of Pb (DM + Pb) which were maintained throughout differentiation. Eight days after induction, adipocytes were stained with Oil-Red-O and photographed. In each photograph, a magnification of the top-left section of the image was shown. Results are from a representative experiment repeated three times with similar results

with lipid staining (Fig. 3a). Consistently, we also found an increase in the mRNA of perilipin which is a protein present in the lipid droplet and upregulated by PPARγ (Fig. 3b) [25].

C/EBPβ is an early activated gene of adipogenesis, which is known to upregulate PPARγ. To further investigate the effect of Pb in adipogenesis, we analyzed this transcription factor. As expected, the amount of C/EBPβ evaluated by western-blot, was increased 24 h after the induction of differentiation with respect to undifferentiated cells and this protein is further increased in cells differentiated in the presence of Pb (Fig. 4a).

Effect of Pb exposure on ERK activation

On the other hand, it is also known that extracellular signal-regulated kinase (ERK) is rapidly activated after addition of differentiation media [26, 27]. ERK is a protein

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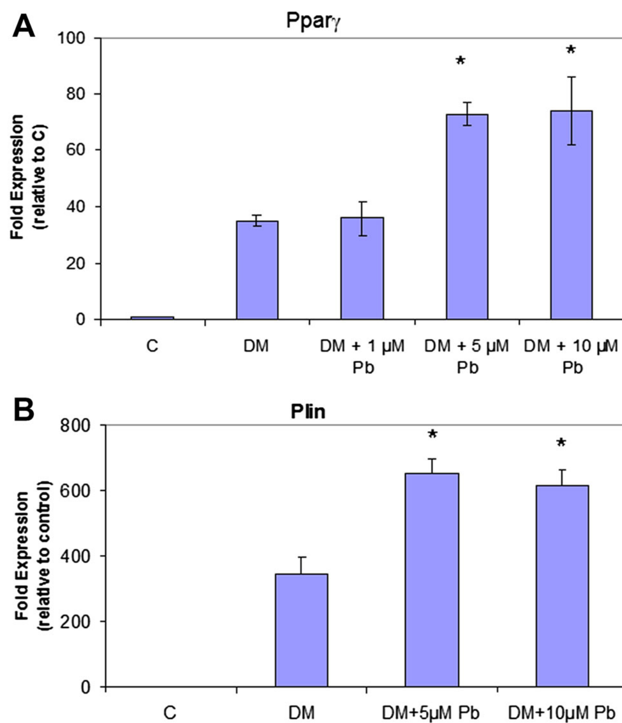

Fig. 3 Effect of Pb on the expression of PPARγ and perilipin mRNA. Two days post-confluent cells were treated with: medium alone (C), with the addition of differentiation media (DM), or differentiation media plus different concentrations of Pb (DM + Pb) which were maintained in the medium throughout differentiation. Eight days after induction of differentiation, adipocytes were used to analyze the amount of PPARγ (**a**) and perilipin (plin) (**b**) mRNA by RT-qPCR as indicated in methods. Results shown are from a representative experiment repeated three times with similar results. RT-qPCR data are expressed relative to untreated cells which is set to 1 and represent mean ± SE. *Significantly different from DM, $p < 0.05$

kinase involved in intracellular signaling and activated by phosphorylation by MEK. In keeping with this, we found that ERK phosphorylation was increased 30 min after the induction of differentiation and this increase was enhanced in the presence of Pb (Fig. 4b).

Discussion

Since 3T3-L1 fibroblasts are the cell model most used to study differentiation to adipocytes, we evaluated Pb effects on adipogenesis in this cell line. After induction of differentiation, these cells first proliferate and then adipogenesis occurs. In this paper, we found that the presence of Pb (10 μM or lower) during differentiation of 3T3-L1 fibroblasts did not interfere with MCE. Thus, 2 days post-confluent cells were able to re-enter the cell cycle after induction of differentiation in the presence of Pb.

We also found that Pb was able to enhance lipid accumulation evaluated by Oil-Red-O staining of cytosolic lipids. This increase correlates with a higher expression of PPARγ mRNA, which is the master gene of adipogenesis, responsible for the expression of proteins associated with the adipocyte phenotype such as perilipin which was also increased in our assays [14, 15].

The ability of Pb to increase PPARγ mRNA expression is in keeping with previous reports in Pb-exposed rodents and isolated mesenchymal stem cells [28, 29]. It was also found that rats exposed to Pb had reduced bone mass that resulted in bones that were more susceptible to fracture and that Pb promoted enhanced adipogenesis and decreased osteoblastogenesis, which was associated with inhibition of the Wnt/β-catenin signaling [28]. This is in keeping with the fact that mesenchymal stem cells up regulates Wnt/β-catenin signaling when they differentiate to osteocytes but inhibits this signaling when adipogenesis takes place [30]. Interestingly, it has been reported that C/EBPβ is involved in the downregulation of Wnt/β-catenin signaling in adipogenesis [31].

On the other hand, it has been described that the early induction of PPARγ is subsequent to C/EBPβ upregulation and activation by phosphorylation during the first hours after induction of adipogenesis [32]. C/EBPβ is then replaced by C/EBPα. The latter is a protein important in adipocyte function and is involved in a positive feedback loop that maintains PPARγ induction in the late stages of adipogenesis [14]. Therefore, we analyzed the effect of Pb in the induction of C/EBPβ during differentiation of 3T3-L1 fibroblasts and found that the presence of Pb enhanced C/EBPβ expression.

In addition, Pb was able to enhance ERK phosphorylation. This is in keeping with previous reports that found activation of this kinase by Pb [33–35]. ERK is a kinase rapidly activated after the induction of adipogenesis and it has been reported that inhibition of this activity prior to the induction of differentiation significantly decreases the expression of PPARγ and C/EBPα [26]. ERK is also proposed to be involved in C/EBPβ activation by phosphorylation which is necessary for adipogenesis and MCE [32]. Thus, our results suggest that enhanced activation of ERK and C/EBPβ upregulation also contributes to Pb-mediated stimulation of PPARγ and adipogenesis.

On the other hand, the concentration of Pb in our assays is higher than 30 μg/dl, which corresponds to 1.45 μM Pb. This is the concentration recommended as acceptable in blood for industrial workers by the Scientific Committee on Neurotoxicology and Psychophysiology and the Scientific Committee on the Toxicology of Metals of the International Commission on Occupational Health [7]. Nevertheless, accumulation of Pb and higher local concentrations in tissues such as bone in Pb-exposed individuals could not be excluded.

According to our findings, up to 10 μM Pb has a stimulatory effect on adipogenesis which involves an

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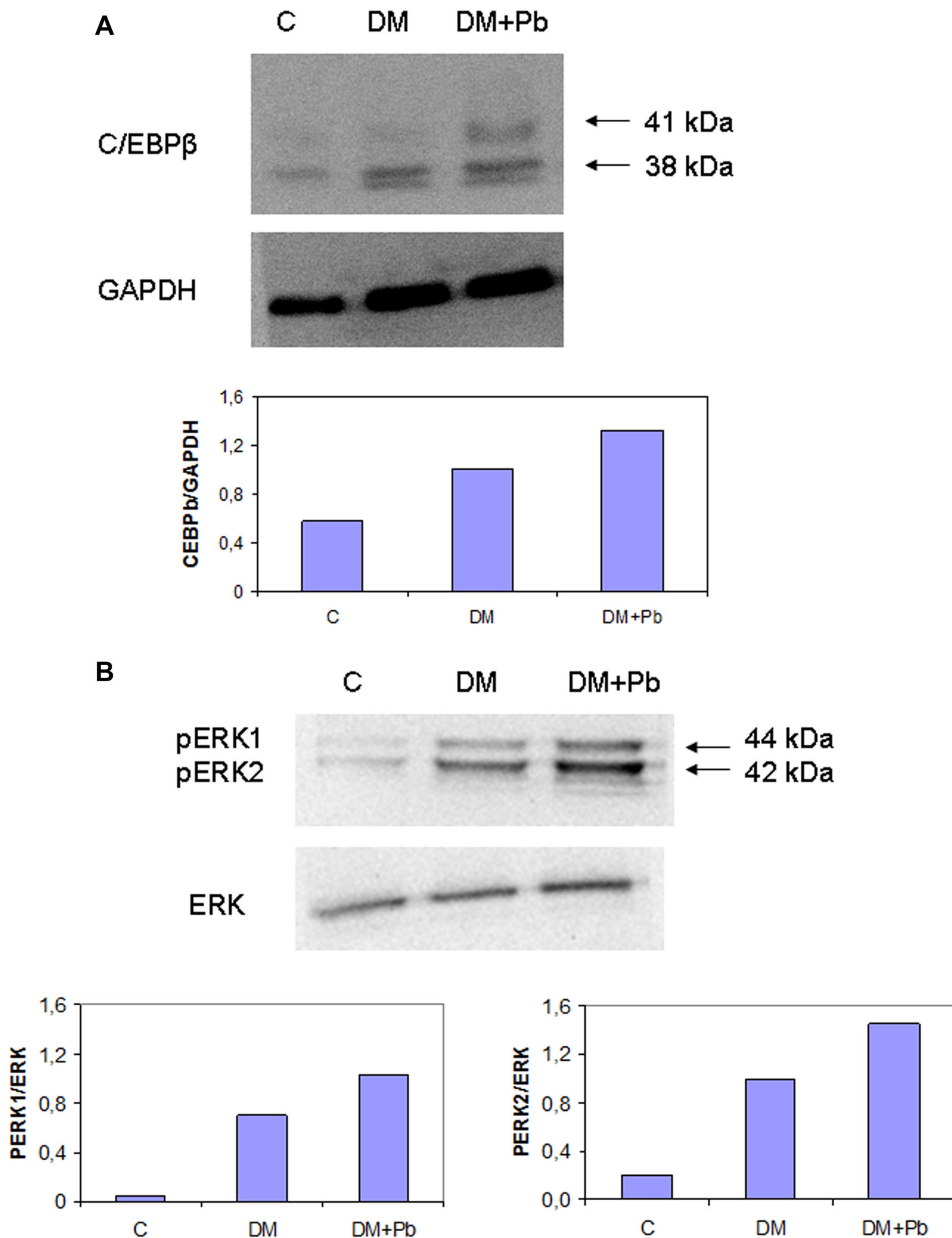

Fig. 4 Effect of Pb on the amount of C/EBPβ and pERK in adipogenesis. Two days post-confluent cells were treated with: medium alone (C), or with the addition of differentiation media (DM) or differentiation media plus 10 μM Pb (DM + Pb) as indicated in Methods and samples obtained 24 h or 30 min after induction of differentiation were used to analyze C/EBPβ (**a**) or pERK (**b**), respectively, by western-blot. Densitometric analysis is shown. Results shown are from a representative experiment repeated twice with similar results

enhancement of ERK activation and C/EBPβ upregulation prior to PPARγ induction. These findings further contribute to elucidate the effect of Pb on adipogenesis.

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Compliance with ethical standards

Conflicts of interest The authors declare that there are no conflicts of interest.

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