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Benzyl Butyl Phthalate Influences Actin Distribution and Cell Proliferation in Rat Py1a Osteoblasts

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Abstract We previously reported that transient administration of phthalates induced actin cytoskeleton disruption in Py1a osteoblasts. However, the mechanism of this transient effect was not elucidated. In this study we provided evidence that the actin cytoskeletal re-established conditions are dependent on new actin expression and synthesis. To assess the role of phthalates in modulating the distribution of actin, confocal and electron microscopy studies were carried out. Results indicated a modification of actin distribution after phthalate administration. In addition, a relation with the nucleoskeletal component lamin A supports the hypothesis that phthalates may participate in regulatory cell processes involving actin in Py1a osteoblasts. The present study also supports the mitogenic effects of phthalates, which involve microfilament disruption, nuclear actin and lamin A. In particular, the increased levels of cyclin D3, which in mammalian cells plays a critical role in G1 to S transition and is a putative proto-oncogene in benzyl butyl phthalate treated cells, suggested a possible effect of the endocrine disruptor in cancer processes. J. Cell. Biochem. 101: 543–551, 2007.

Key words: phthalates; osteoblasts; actin; cell proliferation; immunocytochemistry

Phthalate esters, commonly known as phthalates, are endocrine disrupting chemicals having a variety of industrial uses, including use as plasticizers. They are present in the environment and exert their action by binding to the estrogen receptors and regulating the activity of estrogen responsive genes [Jobling et al., 1995, 1996]. Some of them have been found to cause a significant increase in the number of skeletal malformations [Ema et al., 1993], also teratogenicity and embryo lethality were observed in rat fetuses [Ema et al., 1992]. Phthalates have been also found to be estrogenic in vitro [Harris et al., 1997].

In previous studies we demonstrated that the actin cytoskeleton of immortalized rat Py1a osteoblasts undergoes modifications upon phthalates administration and that butyl benzyl phthalate (BBP) and di-butyl phthalate (DBP) exert their action in a similar way; in particular, phthalates were shown to act rapidly, transiently and in a dose- and time-related manner on osteoblast morphology involving changes in actin filament organization [Marchetti et al., 2002]. The distribution of filamentous actin has been well characterized in several types of cultured cells, including osteoblasts, upon treatment with a variety of effectors [Egan et al., 1991; Luegmayr et al., 1996; Yang et al., 1998; Marchetti et al., 2002].

The aim of the present study was to further elucidate the disrupting effects of phthalates in Py1a cells. In particular, we examined the expression and synthesis as well as localization of actin at confocal and electron microscopy level also in relation to the nucleoskeleton

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filament lamin A under phthalate administration. Since lamins and nuclear actin are major players in essential nuclear functions [Sasseville and Langelier, 1998; Shumaker et al., 2003], the putative mitogenic effect of BBP on rat Py1a osteoblasts was investigated.

To shed light on the effect of BBP in modulating cell viability and proliferation, we also investigated whether BBP modulation of nonfilamentous actin was associated with alterations in the expression of apoptotic regulators such as Bcl-2 and Bax [Wiren et al., 2006]. Moreover, since cyclin D3 is induced by mitogens and is considered to be growth factor sensor [Sherr, 1995], we sought to focus our attention on its synthesis in BBP-treated osteoblasts.

MATERIALS AND METHODS

Cell Cultures for Northern Blotting

Immortalized rat Py1a osteoblasts were plated at 5,000 cells/cm^2 in 6-well culture dishes (Costar Corp., Bethesda, MD) and grown for 6 days in F-12 culture medium with 5% nonheat-inactivated fetal calf serum (FCS) (Invitrogen Srl, Milano, Italy), penicillin and streptomycin (Sigma-Aldrich Srl, Milano, Italy). They were precultured for another 24 h in serum-free F-12 with antibiotics and 1 mg/ml bovine serum albumin (BSA), then treated with BBP (10^{-6} M) (Sigma-Aldrich) as reported in a previous work [Marchetti et al., 2002] for 10 min and 1 h. Control cultures were treated with appropriate vehicles. Parallel cultures, after removal of BBP, were grown for another 6 h in serum-deprived culture medium added with 1 mg/ml BSA.

mRNA levels. Total RNA was extracted from cells by the method of Comczynski and Sacchi [1987]. Cells were scraped into 4 M guanidinium thiocyanate and extracted with phenol/chloroform isoamyl alcohol (24:1) and total RNA was precipitated with isopropanol. For Northern analysis, 20 μg total RNA was denaturated and fractionated on a 1% agarose/2.2 M formaldehyde gel, transferred to nylon membrane by positive pressure, and fixed to the filter by UV irradiation [Sambrook et al., 1989]. After 4 h prehybridization, filters were hybridized overnight with a random primer [^{32}P]dCTP-labeled cDNA probe for the mRNA of interest. Filters were washed in 1× SSC, 1% SDS solution at room temperature. Then, filters were washed three times at 65°C with 0.1% SDS and exposed to XAR-5 film at −70°C. Signals were quantitated by densitometry and normalized to the corresponding values for glyceraldehyde 3-phosphate dehydrogenase (G3PDH).

cDNA probe. The actin cDNA is a 2,000-base chicken beta-actin probe [Hurley et al., 1992].

Cell Cultures for Western Blotting

Py1a osteoblasts were plated at 5,000 cells/cm^2 in 100 mm culture dishes (Costar Corp.), in F-12 culture medium with 5% nonheat-inactivated FCS, penicillin and streptomycin. Cells were grown for 5 days to confluence. Then, cells were serum deprived for 24 h and treated with BBP (10^{-6} M). To test if BBP effects on actin protein synthesis were maintained after removal of the effector, parallel cultures were carefully rinsed and grown for an additional 8 h in serum-free medium containing 1 mg/ml BSA.

Protein levels. Proteins were extracted with Cytobuster Protein extraction reagent (Calbiochem-Inalco SPA, Milano, Italy), resolved by SDS–PAGE (12%), and transferred onto PVDF (Hybond-P) membrane (Amersham Biosciences Europe GMBH, Cologno Monzese, Italy). Protein determination was performed with 2D-Quant kit (Amersham Biosciences) and an equal amount of proteins was resolved by SDS–PAGE (12%) and transferred onto PVDF (Hybond-P) membrane (Amersham Biosciences Europe). The next steps were performed accordingly with ECL Advance Western Blotting Detection Kit (Amersham Biosciences); membranes were blocked with Advance Blocking agent in PBS-T (PBS containing 0.1% Tween 20) for 1 h at room temperature. Then, membranes were incubated with a mouse anti-actin monoclonal antibody (Chemicon International-Prodotti Gianni, Milano, Italy), used also by Schafer et al. [1998] for Western analysis, diluted 1:300 in blocking solution for 1 h at room temperature or with a mouse anti-cyclin D3 antibody (Cell Signaling-Celbio, Milano, Italy), diluted 1:1,000 in blocking solution for 1 h at room temperature. After washing with PBS-T the blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Amersham Biosciences), diluted 1:100,000 in blocking solution for 1 h at room temperature. Other membranes were incubated with a rabbit anti-Bax antibody (Santa Cruz Biotechnology, Inc.) or with a rabbit anti-Bcl-2

(Santa Cruz Biotechnology, Inc.) both diluted 1:200 in blocking solution for 1 h at room temperature. After washing with PBS-T the blots were incubated with HRP-conjugated donkey anti-rabbit IgG (Amersham Biosciences), diluted 1:100,000 in blocking solution for 1 h at room temperature. After further washing with PBS-T, immunoreactive bands were visualized using luminol reagents and Hyperfilm-ECL film (Amersham Biosciences) according to the manufacturer's instructions. To normalize the bands, filters were stripped and reprobed with a rabbit anti-p44/42 antibody. Band density was quantitated densitometrically.

Cell Cultures for CLSM

Py1a osteoblasts were plated at 3,500 cells/cm^2 in 6-well culture dishes containing coverslips, previously cleaned and sterilized, in F-12 culture medium with 5% nonheat-inactivated FCS, penicillin and streptomycin. Cells were grown for 4 days to approximately 80% confluence. Then, cells were precultured for another 24 h in serum-free F-12 with antibiotics and 1 mg/ml BSA before treatment with BBP (10^{-6} M) for 20 min and 2 h. Control cultures were pulsed only with vehicle.

After administration of BBP, cells were briefly rinsed with PBS 0.1 M, pH 7.4, and fixed in 4% paraformaldehyde (PFA), diluted in PBS for 25 min at room temperature. Cells were washed three times in PBS, permeabilized with 0.3% Triton X-100 for 30 min, and incubated with 0.5% BSA diluted in PBS for 20 min at room temperature. Then, cells were incubated with a mouse anti-actin (monoclonal, clone C4) (ICN Pharmaceuticals Italy Srl, Milano, Italy), recognizing the N-terminal, two thirds of the actin molecules therefore reacts with both globular (G) and filamentous (F) forms of actin [Lessard, 1988; Cao et al., 1993], diluted 1:100 in PBS for 2 h at room temperature. After rinsing, cells were incubated with goat anti-mouse IgG conjugated with tetramethylrhodamine isothiocyanate (TRITC) (Sigma-Aldrich), diluted 1:50 in PBS for 90 min at room temperature. After washing, coverslips were mounted on slides with PBS/glycerol (1:1).

All binding sites were visualized by means of a Nikon Diaphot-TMD-EF inverted microscope using a 60× oil immersion lens with numerical aperture 1.4 Plan Apo objective. The microscope was attached to a Bio-Rad MRC 600 confocal laser imaging system equipped with a krypton/argon laser. Black level, gain and laser intensity, Kalman averaging, excitation intensity, pinhole aperture, and Z-series analysis of cells were carried out as previously detailed [Sabbieti et al., 2000]. Sections were examined and original images were stored as a PIC format file and then printed with an Epson Stylus Photo 890 on Epson glossy photo paper.

Cell Cultures for TEM

Py1a cells were prepared as previously reported [Marchetti et al., 2006]. Briefly, they were plated at 5,000 cells/cm^2 on 100 mm culture dishes and grown for 5 days in F-12 medium added with 5% nonheat-inactivated FCS, penicillin and streptomycin. After 80% confluence, cells were precultured in serum-free F-12 containing antibiotics and 1 mg/ml BSA for another 24 h. Then, cells were treated with BBP (10^{-6} M) for 20 min and 2 h. Control cultures were only pulsed with vehicle.

Immunoelectron microscopy. After rinsing twice in F-12 medium and one quick washing in 0.1 M cacodylate buffer, pH 7.4, cells were fixed on plates with 4% PFA and 0.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 3 h at 4°C. Then, cells were rinsed several times for 30 min in 0.1 M PBS, pH 7.3, containing 0.1% BSA and 7% sucrose, at 4°C. Finally, cells collected in Falcon tubes, centrifuged and preembedded in agarose were dehydrated in methanol from 50% to 90%, and embedded in Unicryl resin (BBInternational Ltd., Cardiff, UK) for 72 h at 4°C under U.V. lamp.

Ultra-thin sections (about 60 nm thick) from plastic embedding were cut by means of an LKB Ultrotome V and collected on uncoated 400 mesh nickel grids.

Double immunogold labeling of actin and lamin A. Floating ultra-thin sections were rehydrated with 0.05 M Tris-buffered saline solution (TBS), pH 7.6, and preincubated with 1% BSA, fatty acid free, in 0.05 M TBS, pH 7.6, for 30 min at room temperature. Grids were then incubated with polyclonal rabbit anti-lamin A antibody (Santa Cruz Biotechnology, Inc., CA), diluted 1:50 and with the mouse anti-actin diluted 1:40 in 0.05 M TBS, pH 7.6, for 2 h at room temperature, in a humid chamber. The signals were then developed with Auroprobe EM goat anti-rabbit G-10 (10 nm gold labeled IgG) (Amersham Biosciences) and with goat anti-mouse IgG 40 nm (BBInternational Ltd.),

diluted 1:15 in 0.05 M TBS, pH 7.6, containing 0.05% Tween 20 for 90 min at room temperature, in a humid chamber. Sections were then washed several times in 0.05 M TBS, pH 7.6, and in distilled water.

All sections were finally counterstained with uranyl acetate (5 min) and lead citrate (2 min) at room temperature. All specimens were analyzed by means of a Philips EM 201C electron microscope at an accelerating voltage of 60 kV.

All control experiments at CLSM and TEM level were carried out by omitting the appropriate primary antibodies.

Assessment of the Metabolic Activity of Viable Cells (MTS)

The metabolic activity of viable cells was determined by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Briefly, Py1a cells were plated at the density of 5,000 cells/well in 96 culture dishes and grown in F-12 medium supplemented with 5% serum, penicillin and streptomycin to approximately 80% confluence. Cells were serum deprived for 24 h and treated with BBP (10^{-6} M) for an additional 24 h. Other dishes were treated with BBP (10^{-6} M) for 2 h, then culture medium was changed with fresh medium without effector. Subsequently, cells were incubated with 20 μl/well of CellTiter 96 AQ_{ueous} One Solution Reagent (Promega Italia Srl, Milano, Italy) and incubated for 2 h in a humidified, 5% CO_2 atmosphere. The quantity of formazan product is directly proportional to the number of living cells in culture. The colored formazan was measured by reading the absorbance at 490 nm using a 96-well plate reader.

Statistical analysis. The significance of difference between two groups was evaluated with an unpaired two-tailed Student's *t*-test.

RESULTS

We determined whether BBP administration regulated β-actin mRNA expression in Py1a cells. The results are shown in Figure 1. Indeed, treatment for 10 min and 1 h with BBP (10^{-6} M) on 24 h serum-deprived cultures did not substantially modify β-actin mRNA. In parallel experiments, after BBP treatment, culture medium was changed to serum-free medium containing 1 mg/ml BSA for 6 h. Interestingly, an increase of β-actin mRNA was observed only in cultures treated with BBP for 1 h and subsequently grown for other 6 h in serum-deprived conditions.

In Figure 2 results from Western blotting experiments indicated that treatment for 20 min or 2 h with BBP (10^{-6} M) decreased actin protein synthesis. An increased amount of protein was only observed when, after treatment with the effector for 2 h, the culture medium was changed to serum-free medium containing 1 mg/ml BSA for 8 h.

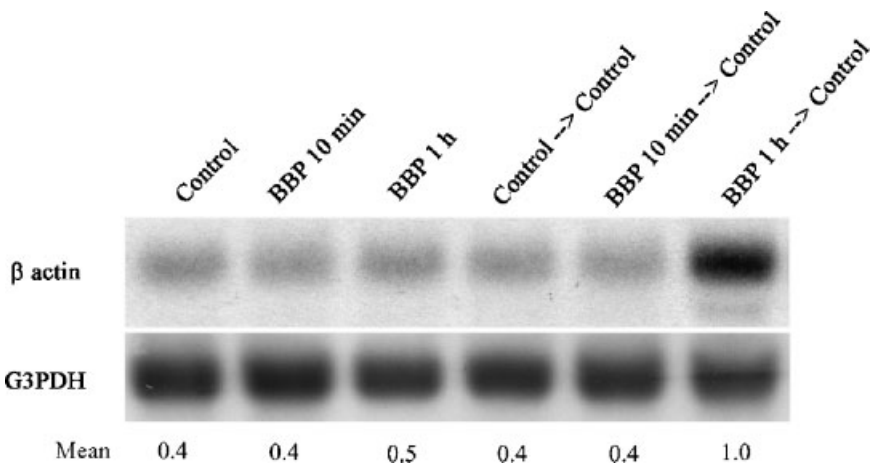

Fig. 1. Py1a osteoblasts. Northern blot analysis. Effect of BBP (10^{-6} M) on β-actin mRNA expression. Twenty four-hour serum-deprived cells were treated with BBP for 10 min and 1 h. Parallel cultures after treatment were rinsed several times and were grown in serum-free culture medium without effector for an additional 6 h. Total RNA was extracted for Northern blot analysis, as described in Materials and Methods. An increase of β-actin mRNA was observed in culture-plate after 1 h of treatment with BBP.

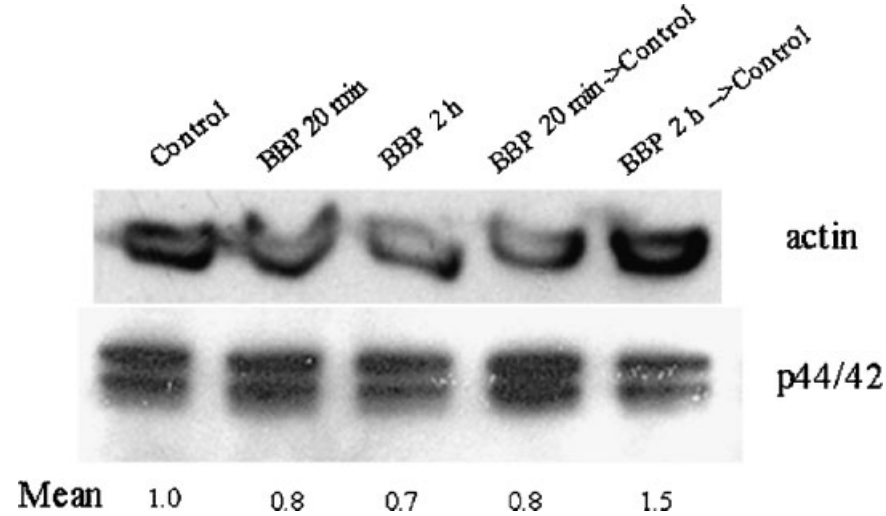

Fig. 2. Py1a cells. Western blot analysis. Effect of BBP (10^{-6} M) on actin synthesis. Twenty four-hour serum-deprived cells were treated with BBP for 20 min and 2 h. Parallel cultures after treatment were rinsed several times and were grown in serum-free culture medium without effector for an additional 8 h. Five micrograms of protein from each sample was subjected to SDS–PAGE, transferred to PVDF membrane, and probed with a mouse anti-actin monoclonal antibody. Treatment for 20 min or 2 h with BBP decreased actin protein synthesis. However, an increased amount of protein was observed when, after treatment with the effector for 2 h, culture medium was replaced for 8 h with serum-free medium.

In order to detect actin modification after BBP treatment, Py1a cells were labeled with the specific monoclonal antibody. Confocal analysis of labeling in untreated cells revealed a clear actin staining along stress fibers; moreover, the highest amount of labeling was observed at cytoplasm and nuclear level (Fig. 3). Actin changes were observed only after 2 h of BBP (10^{-6} M) treatment. In particular, actin labeling decreased at cytoplasm level in about 80% cells. In the nucleus, the highest amount of labeling is concentrated at nuclear envelope (Fig. 4). Due to this strategic location in regions in which type V intermediate filament proteins are located, we tried to establish the spatial organization of actin and lamin A by immunoelectron microscopy. Double immunogold binding confirmed the actin localization observed at CLSM level and showed that the anti-lamin A immunoreactivity was also present in the diffuse nucleoskeleton (Fig. 5).

After BBP (10^{-6} M) administration for 2 h, double binding revealed an increase of nuclear actin that appeared in close spatial relation with lamin A (Fig. 6).

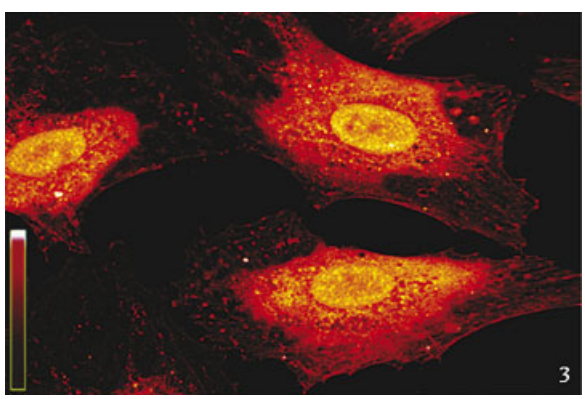

Fig. 3. Py1a osteoblasts. CLSM. Monoclonal actin (clone C4) antibody. White color represents the highest amount of labeling. The immunofluorescence demonstrates that the labeling is localized in numerous brightly punctate structures both in the cytoplasm and in the nucleus. Stress fibers were observed at peripheral cytoplasm level (800×).

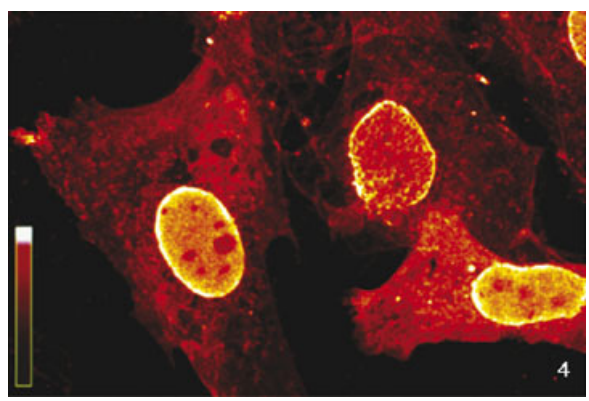

Fig. 4. Py1a osteoblasts treated with BBP (10^{-6} M) for 2 h. CLSM. Monoclonal actin (clone C4) antibody. To note the actin redistribution compare with Figure 3. The highest amount of labeling is lined at nuclear envelope level (950×).

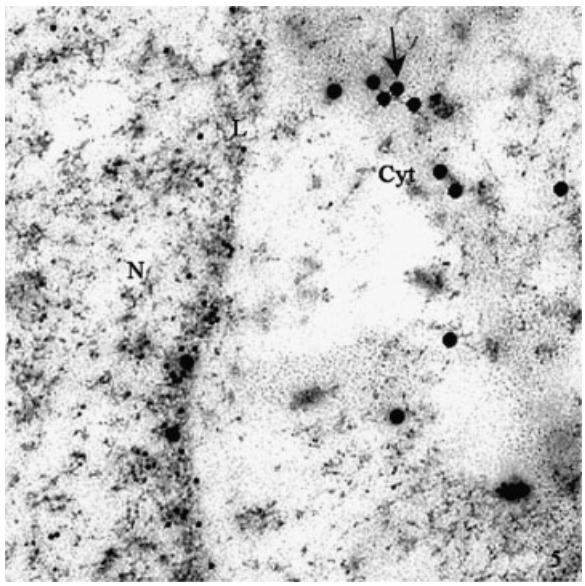

Fig. 5. Py1a osteoblasts. TEM. Double immunogold labeling of actin (large gold particles) and lamin A (small gold particles). Labeling was found at cytoplasm and nuclear level; some large gold particles were also observed confined to distinct curvilinear tracts (arrows). Both lamina and internal nuclear regions were labeled for lamin A (174,000×). L, lamina; N, nucleus; Cyt, cytoplasm.

In the control experiments, the omission of the specific primary antibodies produced no staining (data not shown) both at CLSM and TEM levels.

In order to determine the effects of phthalates on the metabolic activity of viable cells, MTS assay was performed. Treatment with BBP (10^{-6} M) for 24 h increased cell viability ($P < 0.01$). Removal of BBP (10^{-6} M) after 2 h of treatment did not affect cell viability (Fig. 7).

To assess whether increased cell viability was associated with changes in anti- and pro-apoptotic proteins, we evaluated if BBP treatment modulated the ratio, anti-apoptotic protein Bcl-2/pro-apoptotic protein Bax, cells were treated with BBP from 30 min to 24 h. Western blotting analysis revealed no substantial modification in Bcl-2/Bax ratio (data not shown).

We also examined whether BBP-induced increase in cell viability was associated with changes in cyclin D3. Interestingly, treatment with BBP (10^{-6} M) for 2 h strongly increased cyclin D3 synthesis (Fig. 8).

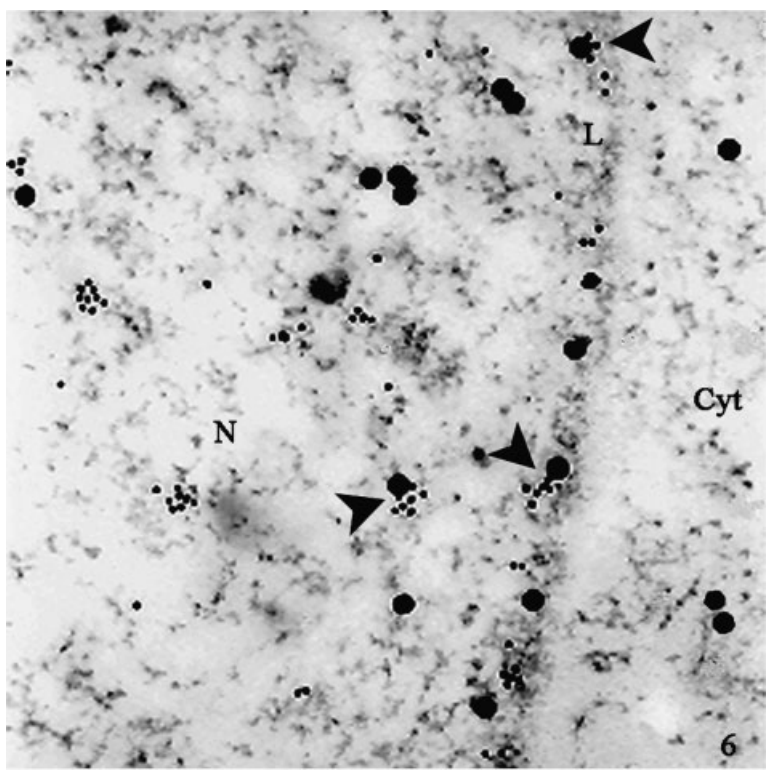

Fig. 6. Py1a cells treated with BBP (10^{-6} M) for 2 h. TEM. Double immunogold labeling of actin (large gold particles) and lamin A (small gold particles). A close spatial relation between actin and lamin A (arrowheads) occurred at nuclear level (232,000×). L, lamina; N, nucleus; Cyt, cytoplasm.

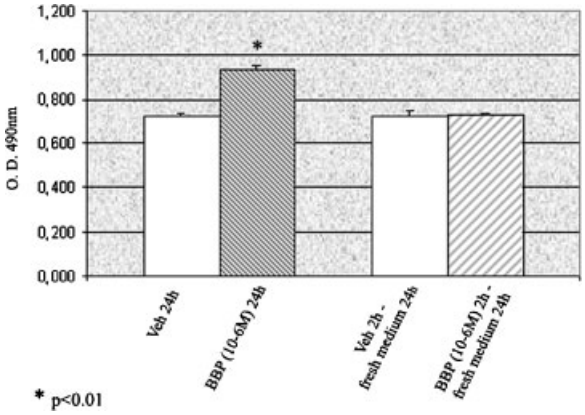

Fig. 7. MTS assay. Effects of BBP on the metabolic activity of viable cells. Cells were 24 h serum-deprived and treated with BBP (10^{-6} M) or vehicle for 24 h. Parallel cultures were treated with BBP (10^{-6} M) or vehicle for 2 h, then medium was replaced with a fresh one without effector or vehicle. Values are mean ± SEM for five different experiments. Note the significant increase of cell viability after 24 h BBP treatment. $P < 0.01$.

DISCUSSION

The effect of phthalates on F-actin-based cytoskeleton was investigated at confocal level in Py1a rat osteoblasts in a previous study and it was shown that phthalate esters caused a disruption of microfilaments; moreover, the initial conditions were restored after removal of the effectors [Marchetti et al., 2002]. In the present study we examined the β-actin mRNA expression to assess whether the re-establishment of the original feature of the cells was due to re-assembly or new actin synthesis. Northern blot analysis showed an increased actin mRNA expression only after BBP administration and removal of the effector. Western blotting experiments demonstrated a decreased actin synthesis after BBP administration but an increased amount of actin after removal of the effector. Taken together, molecular data suggested that the BBP cytoskeleton effects could involve translational and/or posttranslational steps. In addition, the re-establishment of the cytoskeletal original features involves transcriptional steps.

The aim of the present research was also to study whether nonfilamentous and filamentous actin distribution after BBP administration was involved in cell survival. The first step was to investigate actin binding patterns by CLSM and TEM analyses. Indeed, BBP-treated cells showed a peculiar labeling at nuclear envelope level; in addition, at TEM level a close spatial distribution of lamin A and nuclear actin was observed in the nucleoplasmic face of the inner nuclear membrane. Currently, a variety of structural and functional roles have been attributed to nuclear forms of actin although in earlier studies nuclear actin was viewed with great skepticism [Pederson and Aebi, 2002]. Indeed, nuclear actin may play a critical role in mRNA processing and transport and, recently, some authors showed an involvement of actin and a subset of hnRNP proteins for nuclear mRNA [Percipalle et al., 2001, 2002]. Moreover, it has been proposed that lamins and nuclear actin could be structurally and functionally linked and that they are involved in facilitating essential nuclear functions, including DNA replication and transcription [Shumaker et al., 2003]. Control experiments, carried out at

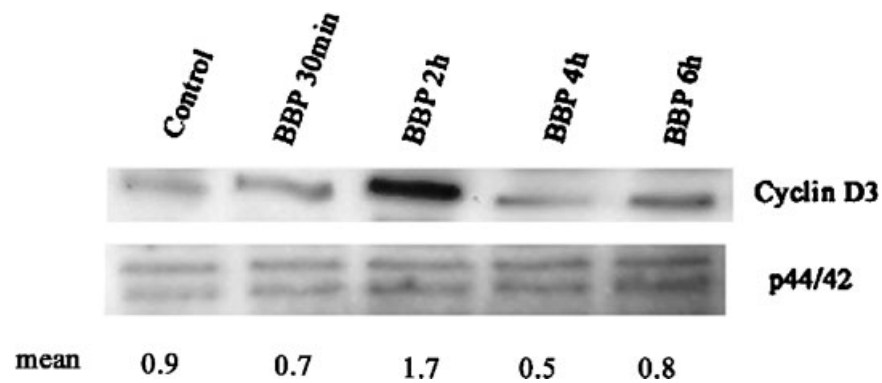

Fig. 8. Py1a cells. Western blot analysis. Effect of BBP on cyclin D3 synthesis. Twenty four-hour serum deprived cells were treated with BBP (10^{-6} M) from 30 min to 6 h. Five micrograms of protein from each sample was subjected to SDS–PAGE, transferred to PVDF membrane, and probed with a mouse anti-cyclin D3 antibody. Treatment for 2 h with BBP markedly increase cyclin D3 protein synthesis.

CLSM and TEM level, supported the specificity of the antibodies used.

Since actin microfilament disruption is reported to be of importance as a potentiator for cell growth [Gordon, 2002], MTS assay was carried out to investigate about the phthalate effects. We found that treatment with BBP increased cell viability. Increased cell viability could be due to increase in anti-apoptotic or reduction in pro-apoptotic proteins. Since several studies explored the apoptosis regulators Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) [Gronowicz et al., 2004; Wiren et al., 2006], we also analyzed their synthesis after BBP treatment. Present findings revealed that BBP did not significantly modify Bcl-2/Bax ratio indicating that the proliferative effects induced by the effector was not due to modulation of the anti-apoptotic signaling way.

Summarizing we observed that BBP acts on actin redistribution which, at nuclear level, promotes an association with lamin A. Based upon these data, we examined the effects of BBP on Py1a cell viability and proliferation. Previous findings showed that exposure of Py1a osteoblasts to specific effectors including phthalates are able to affect the cytoplasmic translocation into the nucleus of fibroblast growth factor-2 (FGF-2) [Menghi et al., 2001; Sabbieti et al., 2005] which is known to be a potent mitogen for several organ tissues including bone [Baird et al., 1986; Neufeld et al., 1987]. Since some FGFs are reported to regulate DNA synthesis, cell proliferation, and cell cycle progression [Jackson et al., 2006], we tried to study cell cycle G1/S transition phase regulated by cyclin D family. D-type cyclins probably serve as integrators of growth factor-induced signals with the cell cycle clock [Sherr, 1994]. Moreover, they are induced by mitogens and their continued synthesis throughout the cycle is reported to depend upon persistent growth factor stimulation [Sherr, 1995]. Aberrant expression of these proteins might play a role in disrupting the normal timing of events governing G1 progression and contribute to oncogenesis [Sherr, 1993; Hunter and Pines, 1994]. In particular, cyclin D3, which specifically requires extracellular mitogenic stimuli for its activation, was found to increase in BBP-treated osteoblasts. Thus, the proliferative effects induced by BBP on rat osteoblasts cannot be under-estimated since cyclins are molecules implicated in various cancers [Sherr, 1996; Diehl et al., 1997]. BBP is also known as xenoestrogen mimicking 17 β-estradiol [Zacharewski et al., 1998; Yu et al., 2003] which is reported to induce the expression of both cyclin D2 and D3 leading to the promotion of cell growth in osteoblasts [Fujita et al., 2002]. Accordingly, an increased cyclin D3 synthesis was promoted by BBP endocrine disruptor. It is also known that by reducing the amount of p27, a cyclin-dependent kinase inhibitor, a decreased amount of F-actin was observed [Kawauchi et al., 2006]. A novel D-cyclin is reported to play a central role in the regulation of the actin cytoskeleton organization, consequently controlling also cell shape and cytokinesis [Cuomo et al., 2005].

In conclusion, our findings showed that the endocrine disruptor BBP affecting actin distribution causes an increase in cell proliferation that may be due in part to modulation of cyclin D3.

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