Histol Histopathol (2002) 17: 1061-1066

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Effects of phthalate esters on actin cytoskeleton of Py1a rat osteoblasts

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Summary. We evaluated, by confocal laser scanning microscopy, the actin cytoskeleton of immortalized rat Py1a osteoblasts treated with phthalate esters (butyl benzyl phthalate, BBP and dibutyl phthalate, DBP), endocrine disruptors with estrogenic activity. We observed some peculiar modifications of actin cytoskeleton and cells changing from a spindle shape to a rounded form. In particular, F-actin formed thick bundles around the cell membrane but only a weak labeling was observed in rounded cells. Also influence on apoptosis and short-term effects on FGF-2 were studied. It was found that BBP and DBP exert their action in a similar way, act in a transient manner and do not induce apoptosis.

Key words: Phthalate esters, Actin, Py1a osteoblasts, Rat

Introduction

Actin cytoskeleton in osteoblastic cells has been under study for many years and it has been demonstrated that cell shape changes are a direct consequence of cytoskeletal modifications that are helpful tools for monitoring the healthiness and function of osteoblasts. The integrity of the cell structure of osteoblasts is important for the differentiation and activation of osteoclast functions through a mechanism involving cell-to-cell and/or cell-to matrix contacts (Jimi et al., 1996). The structural integrity of microfilaments is also necessary for the signal transduction of mechanical stimuli within osteoblasts (Watson, 1991; Meazzini et al., 1998). Moreover, disruption of the actin-cytoskeleton abolishes the response to stress suggesting that the cytoskeleton is involved in cellular mechano-transduction (Burger and Klein-Nulend, 1998).

The role of the confluence on cultured mouse osteoblastic cells has been studied and the cell density seem to be an important factor affecting the cytoskeletal organization; it has been found that increasing cell density is able to cause a 45% decrease in the polymerized form of actin (Lomri et al. 1987).

Also, mutant (toothless) osteoblasts have been used to better understand the role of microfilaments (Watanabe et al., 1997). In normal rat osteoblasts, ß-actin is distributed primarily in cell processes and on one side of the nucleus, while γ−actin localization is perinuclear; conversely, in mutant rat osteoblasts, actin isoforms do not sort differentially and exhibit diffuse labeling (Watanabe et al., 1998).

The present work was designed to investigate the effects of two phthalate esters (butyl benzyl phthalate, BBP and dibutyl phthalate, DBP) on actin cytoskeleton of immortalized rat osteoblastic Py1a cells. Phthalate esters are known as a group of organic chemicals characterized by a variety of industrial uses, widely distributed in the environment, and often occurring at low levels in food (Group, 1986; Sharman et al., 1994). The toxic potential of these compounds was referred to many years ago (Mayer et al., 1972), but it has been recently found that they can also be regarded as endocrine disruptors (ED) with estrogenic activity (Jobling et al., 1995; Harris et al., 1997). In addition, to test the importance of the actin filaments in signal transduction, in the present study we also investigated the short-term effects of the above ED on the FGF-2 signalling. The physiological expression and regulation of this growth factor has been previously studied on this cell line at molecular and electronic level (Sabbieti et al., 1999). Previously, these chemicals have been demonstrated to enter into the cytoplasm of Py1a cells (Sabbieti et al., 2000a) and to have long-term effects on the bioaccumulation and nuclear translocation of the basic fibroblast growth factor (Menghi et al., 1999a, 2001).

Materials and methods

Cell cultures

Immortalized rat osteoblastic Py1a cells were plated at a density of 3500 cells/cm^2 in 6-well culture dishes on

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previously cleaned and sterilized glass coverslips, and were grown to about 80% confluence in F-12 culture medium (Gibco) containing 5% nonheat-inactivated fetal calf serum (FCS) (Gibco), penicillin (100 U/ml) and streptomycin (50 mg/ml). Prior the experiments cells were precultured for another 24 h in serum-free F-12 medium containing 1 mg/ml bovine serum albumin (BSA) (Sigma) and antibiotics. Then, cells were treated with BBP and DBP (Sigma) at different concentrations and for different timing of exposure (10^{-3} M and 10^{-6} M for 10 min, 1 h, and 2 h) using ethanol as vehicle. The times of treatment were selected on the basis of a previous research aimed to establish the internalization of BBP in Py1a cells (Sabbieti et al., 2000a). Parallel cultures were treated with BBP and DBP, carefully rinsed and maintained for an additional 24 h in serum-free medium containing 1 mg/ml BSA. Other cells were treated with 0.5 mg/ml cytochalasin D (Sigma) using 0.01% dimethyl sulfoxide as vehicle for 40 min at room temperature. Control cells were only pulsed with the appropriate vehicles.

Double labeling for F-actin and FGF-2

After stimulation, cells were rinsed with 0.1 M phosphate-buffered saline solution (PBS), pH 7.4, and fixed with 4% paraformaldehyde (PFA) diluted in PBS for 25 min at room temperature. Cells were permeabilized with 0.3% Triton X-100 for 20 min on ice. After pre-incubation with 0.5% BSA dissolved in PBS for 20 min at room temperature, cells were incubated with a 1:100 dilution of a polyclonal rabbit anti-FGF-2 antibody (Sigma) in PBS for 2 h at room temperature. Then, cells were incubated with 1:75 dilution of goat anti-rabbit IgG (Sigma) conjugated with fluorescein isothiocyanate (FITC) for 90 min at room temperature. Finally, cells were incubated with $4x10^{-6}$ mol/L phalloidin tetramethylrhodamine isothiocyanate (TRITC) conjugate (Sigma) for 20 min at room temperature. After rinsing in PBS, coverslips were mounted on slides with Vectashield (Vector). Control experiments were performed by using a non-immune rabbit IgG and by omitting the primary antibody.

Fluorescein-FragELTM DNA fragmentation detection method

The Fluorescein-FragEL™ Kit (Oncogene) is a method for the labeling of DNA breaks in apoptotic nuclei in cell preparations fixed on coverslips. Untreated and treated cells with BBP and DBP (10^{-3} M) for 1 h were fixed as above, then dehydrated in 80% ethanol and rehydrated in 1x TBS (Tris buffered saline) for 15 min at room temperature. Cells were permeabilized with 2 mg/ml Proteinase K diluted 1:100 in 10 mM Tris, pH 8, for 5 min at room temperature. Then, samples were covered with 100 μl of 1X terminal deoxyribonucleotidyl transferase (TdT) equilibration buffer for 30 min at room temperature. After removal of the equilibration buffer each coverslip was covered with 60 μl of TdT labeling reaction mixture prepared with 57 ml Fluorescein-FragEL™ TdT Labeling reaction mix and 3 μl TdT enzyme for 90 min at 37°C. Finally, coverslips were mounted on slides using Fluorescein-FragEL™ Mounting Media. The quantitative analysis was performed by direct counting in the Zeiss Axiophot. Using the 20x objective lens, the number of apoptotic cells were counted in 30 areas (15 cells/area) for each slide as previously performed for TUNEL method (Hecht et al., 2000).

Confocal laser scanning microscope (CLSM) analysis

FGF-2 and F-actin binding patterns were visualized by means of a Nikon Diaphot-TMD-EF inverted microscope using a x 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 (Bio-Rad, Hertfordshire, UK) 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 (Menghi et al., 1999b; Sabbieti et al., 2000b). Cells were examined and original images were stored as a PIC format file and then printed with an Epson Stylus Photo 750 on Epson glossy photo paper.

Results

Untreated Py1a osteoblasts, serum deprived for 24 h, showed a labeling for FGF-2 mainly located around the nuclear envelope (Fig. 1a). By phalloidin-TRITC labeling, the actin-based cytoskeleton showed to be organized as stress fibers well arranged in a parallel manner throughout the cell body and cellular extensions; cells were observed to be relatively flat, spindle-shaped with sparse and relatively short cellular extensions and arranged in a monolayer (Fig. 1b). By confocal analysis of double staining, FGF-2 and F-actin revealed exclusive and no co-localized binding patterns (Fig. 1c).

Both phthalate esters exhibited similar effects under different experimental conditions.

In cells treated with 10^{-3} M BBP or DBP for 10 min, labeling for FGF-2 (Fig. 2a) and F-actin (Fig. 2b) was found to be similar to that observed in untreated cells except for increased phalloidin immuno-fluorescence at peripheral cell attachment areas that appear as bouton-like, structures. The merged image (Fig. 2c) also showed in this case exclusive sites of labeling.

Treatment with 10^{-3} M BBP or DBP for 1 h did not substantially modify FGF-2 reactive patterns. Conversely, the cell morphology changed in a spindle-shaped form and phalloidin-TRITC revealed that stress fibers were not found in the cytoplasm, but were only present below the plasma membrane displaying long and slender cytoplasmic projections; some rounded cells were also observed (Figs. 3, 4).

An increased time of treatment and decreased concentration of both phthalate esters showed effects

similar to those observed at shorter time and higher concentrations. In Fig. 5, the aspect of cells treated with 10^{-6} M BBP for 2 h was documented. In particular, phalloidin staining was mainly located in cytoplasmic aggregates.

In order to establish if the effects of phthalate esters were transient or permanent, cells were incubated with BBP or DBP (10^{-3} M for 1 h or 10^{-6} M for 2 h), then culture medium was changed to serum-free medium containing 1 mg/ml BSA and cells were maintained in this condition for another 24 h. It was found that phthalate esters were able to reversibly affect Py1a cell architecture; in fact, changes of medium re-established the original feature of the cells (Fig. 6) and actin

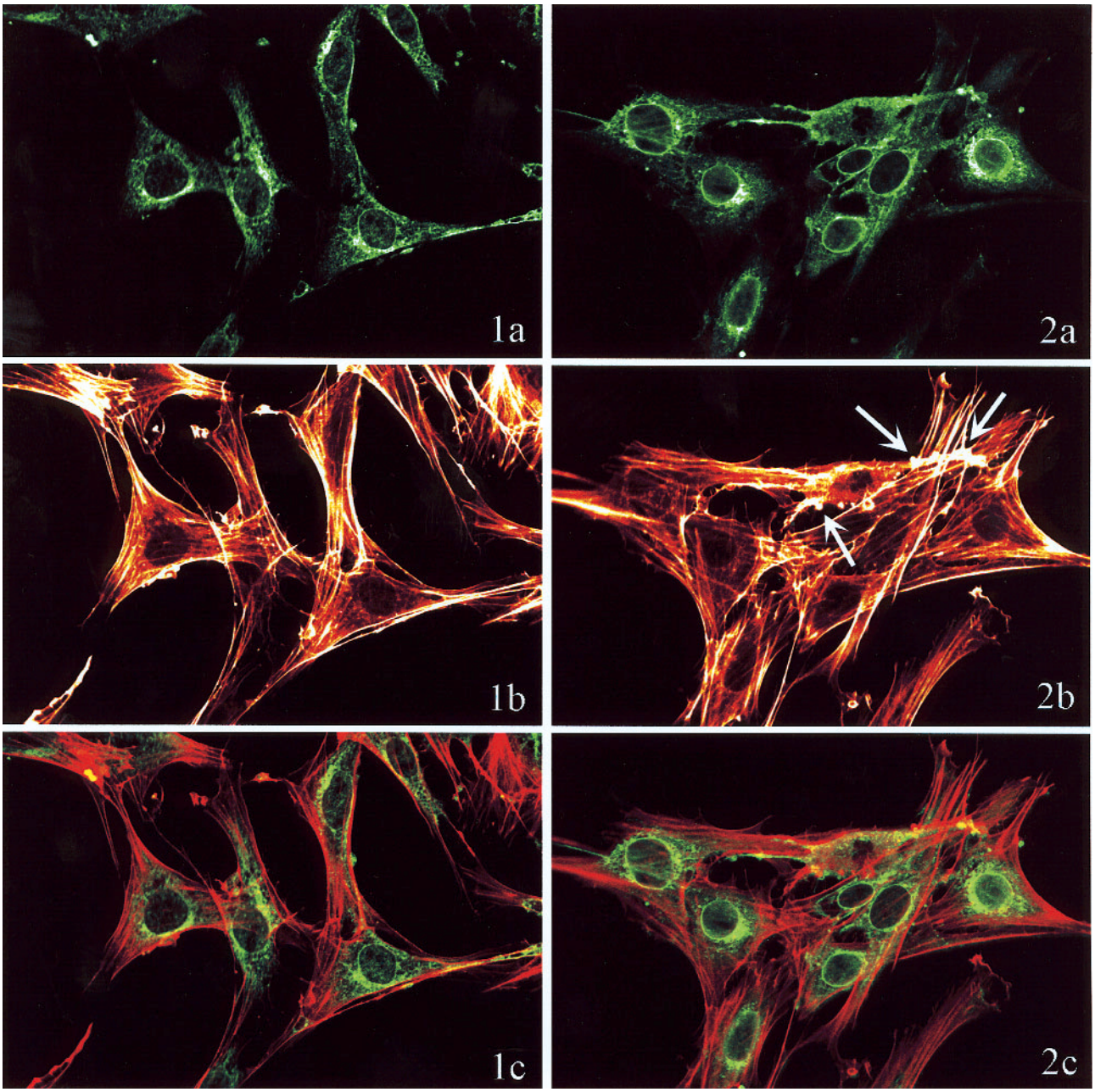
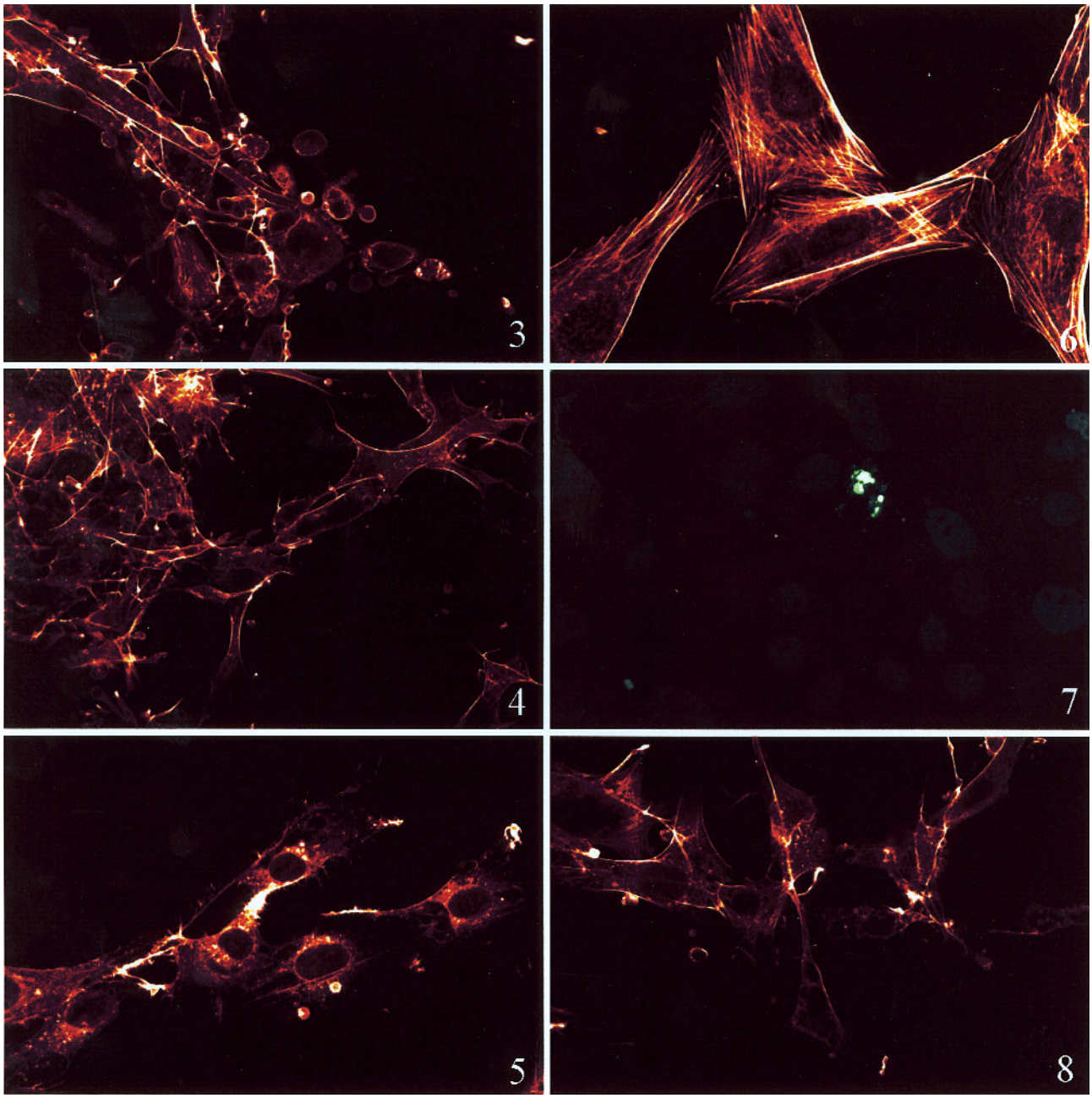


Fig. 1. Untreated Py1a cells. Double labeling for FGF-2 (green) (**a**) and F-actin (red) (**b**). White colour represents the highest amount of labeling. Stress fibers are seen running parallel to the long axis of the cells, in the cytoplasmic projections and also around the plasma membrane. The merged image **(c)** depicts the exclusive patterns of labeling for FGF-2 and F-actin. x 800

Fig. 2. Py1a cells treated with 10^{-3} M BBP for 10 min. Double labeling for FGF-2 (green) **(a)** and F-actin (red) (**b**). Merged image (**c**). Appreciable modifications of phalloidin labeling, except for the appearance of some accumulated labeling at the attachment points (arrows), are not present. x 800



Figs. 3, 4. Py1a cells treated with 10^{-3} M BBP (**Fig. 3** and DBP (**Fig. 4)** for 1 h. Labeling for F-actin. To be noted the absence of stress fibers in the cytoplasm. Actin filaments are observed only around the plasma membrane of spindle-shaped osteoblasts. Spots of labeling are seen sparsely in the cytoplasm. Note also the presence of rounded cells with weak labeling. x 800

Fig. 5. Py1a cells treated with 10^{-6} M BBP for 2 h. Flocculent masses of F-actin are seen in the perinuclear region. x 800

Fig. 6. Transient effects of phthalates on osteoblast morphology. Py1a cells treated with 10^{-3} M BBP for 1 h, carefully rinsed and left in culture medium for other 24 h. Labeling for F-actin. Cells are morphologically and immunocytochemically comparable to control cells. x 800

Fig. 7. Py1a cells treated with 10^{-3} M BBP for 1 h. Only one apoptotic cell is observable. x 650

Fig. 8. Cells treated with cytochalasin D. Labeling for F-actin. Morphological changes, disruption of microfilaments, and some aggregation of labeling in the cytoplasm are evident. x 800

cytoskeleton appeared comparable to that of untreated cells.

Cells exposed to BBP or DBP (10^{-3} M for 1 h or 10^{-6} M for 2 h) were also processed in order to evidence apoptotic cells and it was found that treatment with phthalate esters was not able to induce apoptosis in Py1a cells (Fig. 7); indeed, the number of apoptotic cells was found to be unmodified. After cytochalasin D treatment (Fig. 8), cells showed phalloidin-stained aggregates resembling those observed in phthalate-disrupted microfilaments.

Discussion

This report described the effects of two phthalate esters, BBP and DBP, known for their estrogenic activity (Jobling et al., 1995; Harris et al., 1997), on actin cytoskeleton in rat osteoblastic Py1a cells. After phthalate stimulation, we observed that both these endocrine disruptors (ED) act rapidly, transiently, and in a dose- and time-related manner on cell morphology involving changes in microfilament organization. Since it is well known that the cytoskeleton plays a central role in the expression of phenotype (Folkman and Moscona, 1978; Ben-Ze'Ev and Amsterdam, 1987), the phthalate-induced changes on actin cytoskeleton rearrangement may be regarded with attention. Indeed, actin filaments are crucial for the maintenance of cell shape and an altered assembly of actin fibers can be responsible for the perturbation of focal contacts and compromise the correct adhesion of osteocytes (Tanaka-Kamioka et al., 1998).

Many hormones and other chemicals have been under investigation to better understand their effects on osteoblast cytoarchitecture. It has been demonstrated that 17beta-estradiol caused a reduction of cytoskeletal and other cellular proteins of the stromal osteoblastic MBA-15 cells (Benayahu, 1997). Prostaglandin E was demonstrated to be involved in the morphological changes occurring in rat osteoblasts and Yang et al. (1998) showed that prostaglandin E caused the breakdown of actin microfilaments. The parathyroid hormone was found to affect the integrity of osteoblasts by decreasing the number of stress fibers (Egan et al. 1991); this modification accompanied a change of cell shape from a spread configuration to a stellate form with retracting processes and the alterations were described to be rapid and transient. Treatment with triiodothyronine of long-term cultured mouse osteoblastic cells MC3T3-E1 was accompanied by significant morphological alterations due to F-actin cytoskeleton arrangement indicating a crucial role of triiodothyronine in osteoblastic differentiation (Luegmayr et al., 1996). Dexamethasone caused the osteoblasts to grow more slowly, the actin cytoskeleton to collapse and the cell morphology to change from flat and spread out to rounded and spindle-shaped (Hughes-Fulford et al., 1992). Rounded cells were also observed by Gronowicz et al. (1986) under effect of 1,25-dihydroxyvitamin D3 on the cytoskeleton of rat calvaria and rat osteosarcoma osteoblastic cells. Other agents have been shown to induce morphological changes in rat osteoblasts; in particular, staurosporine caused the dissolution of actin microfilaments and a PKC inhibition mechanism (Yang et al., 1997).

We previously showed long-term effects of BBP and DBP on FGF-2 expression and nuclear translocation (Menghi et al., 1999a, 2001). In the present study, we focused our attention on short-term effects of both phthalate esters on FGF-2 and actin microfilaments, which showed distinct response and patterns of labeling. In vitro, we observed treated cells becoming rounded but we did not observe signs of degeneration. The presence of rounded cells after ED treatment are not an evidence of a degenerative process induced by a possible inhibition of actin stress fiber development, like the well known form of apoptosis called anoikis (Frisch and Francis, 1994), since in our experiments we did not evidence an induction of apoptotic cells consequent to phthalate treatment. Probably, the effects of phthalate esters on Py1a cell functions could involve alteration of cytoskeletal protein organization. In addition, phthalate esters seem to directly affect the actin cytoskeleton; in fact, FGF-2- mediated effects can be excluded on the basis of parallel experiments that clearly demonstrated that FGF-2 was not influenced in the same experimental conditions.

The fact that phthalate esters affect, at short-time of incubation, the actin cytoskeleton but not the FGF-2 occurrence and location is in agreement with our previous data indicating that FGF-2 is susceptible to the same concentrations of endocrine disruptors only after longer treatments (Menghi et al., 2001). However, the influence on actin filaments could be explained; indeed, by using an antibody that selectively recognizes o-phthalate esters (Ius et al., 1993), we previously demonstrated that BBP and DBP enter into the cell cytoplasm at 10 min and produce the maximum endothermic peak in the DSC (differential scanning calorimetry) curve within 2 h using BBP 10^{-6} M as effector (Sabbieti et al., 2000a).

In conclusion, the effects of phthalate esters on the actin cytoskeleton of Py1a rat osteoblasts, although rapid and transient, cannot be underestimated; for example, in the invasion processes by Staphilococcus aureus in MC3T3-E1 mouse osteoblastic cells and in cultured human osteoblasts, actin microfilaments seem to play the most significant role in the internalization process via a receptor-mediated pathway (Ellington et al., 1999; Jevon et al., 1999).

Research is in progress to evaluate, at molecular level, the effects of phthalate esters on protein synthesis and signal transduction. Preliminary data originated from ultrastructural investigation, by immunogold double-sided binding, indicated that coated pits are not involved in the FGF-2 cytoplasmic internalization. Also, the distribution of G- and F-actin could be useful to follow the dynamic changes consequent to a polymerization/depolymerization of actin as recently effected on cultured osteoclasts (Akisaka et al., 2001).

Acknowledgements. Supported by grants from the University of Camerino (Italy) and the Italian MURST 1999. The skilful assistance of Dr. P. Ballarini with CLSM and the technical help of S. Cammertoni are greatly appreciated.

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Accepted June 7, 2002