Amino Acids (2009) 36:57–63
DOI 10.1007/s00726-008-0028-7

ORIGINAL ARTICLE

Effect of progesterone on apoptosis of murine MC3T3-E1 osteoblastic cells

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Received: 1 November 2007 / Accepted: 2 December 2007 / Published online: 6 February 2008

Abstract Progesterone (P) has been suggested as a bone-trophic hormone. Previous studies have shown that P promoted bone formation by stimulating the proliferation and differentiation of osteoblasts. But, the effect of P on apoptosis of osteoblast in vitro has not been reported. We propose that P may promote bone formation by suppressing the apoptosis of osteoblast. The present study was performed to investigate the effect of P on apoptosis of murine MC3T3-E1 osteoblastic cells. Cell apoptosis was measured by acidine orange/ethidium bromide (AO/EB) staining and sandwich-enzyme-immunoassay. Progesterone receptor (PR), cytochrome *c*, caspase-9 and caspase-3 protein levels were determined by Western blot analysis. The enzyme substrate was also used to assess the activation of caspase-3 and caspase-9. Progesterone suppressed MC3T3-E1 cells apoptosis induced by serum deprivation, and this effect was blocked by a PR antagonist RU486. Furthermore, the suppressive effects of P on cytochrome *c* release and caspase-9 and caspase-3 activation in serum-deprived MC3T3-E1 cells were also reversed by RU486. Our study demonstrated that P protects osteoblast against apoptosis through PR and the downstream mitochondrial pathway. Thus, the data suggest that the effects of P on osteoblast apoptosis may contribute to the mechanisms by which P exerts its action on bone formation.

Keywords Progesterone · Apoptosis · Osteoblast

Introduction

The importance of sex steroids in the maintenance of bone mass is widely accepted and progesterone (P) has been considered as a bone-trophic hormone (Miller et al. 2000; Recker et al. 1999; Lukert et al. 1992; Prior 1990; Barengolts et al. 1990; Fujimaki et al. 1995; Bowman et al. 1996). P preserve bone mass in postmenopausal women (Miller et al. 2000; Recker et al. 1999). A previous retrospective study showed that P reduces glucocorticoid-induced bone loss in postmenopausal or amenorrheic women (Lukert et al. 1992). P also prevents ovariectomy-induced bone loss in aged rats (Barengolts et al. 1990).

The physiological effects of progesterone are mediated by interaction of the hormone with the progesterone receptor (PR) isoforms, PRA and PRB. PRA and PRB are expressed from a single gene as a result of transcription from two alternative promoters (Kastner et al. 1990) and translation initiation at two alternative AUG start codons (Conneely et al. 1989). PRA and PRB differ only in that PRB contains an additional 164 amino acids at the N-terminus that are missing in PRA. Both osteoblast and osteoclast express PR (MacNamara et al. 1995; Wei et al. 1993; Gunnet et al. 1999; Pensler et al. 1990). Accumulated data suggest that the influence of P on bone is principally through promoting bone formation via PR

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located in osteoblasts whereas seems to have no direct effect on osteoclasts (Tremollieres et al. 1992; Chen and Foged 1996; Scheven et al. 1992; Chen and Foged 1997; Liang et al. 2003; Liao et al. 2002; Luo and Liao 2001; Luo et al. 2002; Cmidt et al. 2000; Isserow et al. 1995; Tobias and Chambers 1991).

Recent evidence indicates that apoptosis represents the most common fate of osteoblasts during physiologic bone remodeling (Jilka et al. 1998, 1999; Plotkin et al. 1999). The majority of osteoblasts die by apoptosis. The frequency of osteoblast apoptosis could have a significant impact in the number of osteoblasts present at the site of bone formation (Jilka et al. 1999).

Up to now, the effect of P on apoptosis in osteoblast in vitro has not been reported. The purpose of the present study was to assess the effects of P on serum deprivation-induced apoptosis of murine osteoblastic MC3T3-E1 cells.

Materials and methods

Reagents

Anti-PR, cytochrome *c*, caspase-3, caspase-9 antibodies, anti-mouse, and rabbit IgG peroxidase conjugate antibodies were purchased from Santa Cruz Biotechnology Inc. (Waltham, MA, USA). Substrates for caspase-3-like proteinase (Ac-DEVD-MCA) and caspase-9-like proteinase (Ac-LEHD-AFC), P and RU486 were purchased from Sigma Chemical Company (St. Louis, MO, USA).

MC3T3-E1 cell culture

The mouse osteoblastic cell line MC3T3-E1 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in α-MEM (Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml ascorbic acid. Cells were maintained in a humidified, 95% air, 5% CO_2 atmosphere at 37°C. The medium was changed twice a week and the cells were subcultured using 0.05% trypsin with 0.01% EDTA.

Cell apoptosis measurement

ELISA

Apoptosis was assessed directly by measurement of cytoplasmic nucleosomes (i.e., DNA complexed with histone in the cytoplasm) using a Cell Death Detection ELISA Kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany), according to the kit protocol. The Cell Death Detection ELISA Kit allows the specific determination of mono- and oligo-nucleosomes in the cytoplasmatic fraction of cell lysates. Briefly, cells were plated at a density of 1×10^4 cells/well in 24-well plates for 1 days followed by culture in serum-free medium for 24 h in the absence or presence of P at 10^{-10}, 10^{-9}, or 10^{-8} M. Cells were also treated with 10^{-8} M P in the presence or absence of 10^{-6} M RU486. 1% FBS treatment was used to observe basal levels of apoptosis. The cell layers were rinsed with phosphate buffered saline (PBS) and extracted with 0.5 ml of lysis buffer after a 30 min incubation at 4°C. The cell lysates were then centrifuged for 10 min at 15,000 rpm, and the aliquots of aqueous supernatant were tested for apoptosis using the Cell Death Detection Kit.

Acridine orange/ethidium bromide (AO/EB) staining

Cells were cultured in 6-well plates at a density of 4×10^4 cells/well. The cells were labeled using the nucleic acid-binding dye mix of 100 mg/ml AO and 100 mg/ml EB (Sigma Chemical Company) in PBS. The cells were examined by fluorescence light microscopy. Viable cells had green fluorescent nuclei with organized structure. The early apoptotic cells had yellow chromatin in nuclei that were highly condensed or fragmented. Apoptotic cells also exhibited membrane blebbing. The late apoptotic cells had orange chromatin with nuclei that were highly condensed and fragmented. The necrotic cells had bright orange chromatin in round nuclei. Only cells with yellow, condensed, or fragmented nuclei were counted as apoptotic cells in a blinded, nonbiased manner. For each sample, at least 500 cells/well and 4 wells/condition were counted, and the percentage of apoptotic cells was determined: % of apoptotic cells = (total number of apoptotic cells/total number of cells counted) × 100.

Western blot analysis

Cells were plated in 6-well plates for 1 day followed by culture in serum-free medium for 24 h in the absence or presence of 10^{-10}–10^{-8} M P. Cells were also treated with 10^{-8} M P in the presence or absence of 10^{-6} M RU486. The cell layers were homogenated with Triton lysis buffer (50 mM Tris–HCl, pH 8.0 containing 150 mM NaCl, 1% Triton X-100, 0.02% sodium azide, 10 mM EDTA, 10 μg/ml aprotinin, and 1 μg/ml

aminoethylbenzenesulfonyl fluoride). The lysates were centrifuged for 15 min at 12,000*g* to remove debris. Protein concentrations were determined using the Bradford protein assay. Forty micrograms protein of each cell layer homogenate was loaded onto a 10% polyacrylamide gel, and transferred to a PVDF membrane. After blocking with 5% nonfat milk, membranes were incubated with anti-caspase-9 and anti-caspase-3 antibodies. The membrane was re-probed with peroxidase-conjugated secondary antibodies. Blots were processed using an ECL kit and exposed to film, then analyzed by densitometry.

Untreated MC3T3-E1 cell cultures were also homogenated with Triton lysis buffer and then subjected to Western blot analysis as described as above with anti-PR antibody.

Analysis of cytochrome *c* release

Release of cytochrome *c* from mitochondria into cytosol was measured by Western blot. Briefly, serum-deprived cells were treated with vehicle or 10^{-10}–10^{-8} M P for 24 h, cells were also treated with 10^{-8} M P in the presence or absence of 10^{-6} M RU486, then cell cultures were homogenated with Triton lysis buffer as described above. The cell lysates were centrifuged at 1,00,000*g* for 30 min to yield soluble cytosolic fraction (supernatant). Supernatants were then subjected to Western blot analysis as described as above with anti-rabbit cytochrome *c* antibody.

Assays for caspase-3 and caspase-9 activities by use of specific fluoro-substrates

MC3T3-E1 cells were grown in the absence or presence of 0.1% FBS in α-MEM for 1.5 h and serum-deprived cells were treated with vehicle or 10^{-10}–10^{-8} M P for 1.5 h, and starved cells were also treated with 10^{-8} M P in the presence or absence of 10^{-6} M RU486 for 1.5 h. Then, cells were lysed with 400 µl of lysis buffer (10 mM HEPES, pH 7.5 containing 0.5% Nonidet P-40, 0.5 mM EDTA, 150 mM NaCl, and 2 mM phenylmethylsulphonylfluoride). Aliquots (50 µl) of the extracts were incubated for 1 h at 37°C with 50 µM enzyme substrate (Ac-DEVD-MCA for caspase-3-like proteinase and Ac-LEHD-AFC for caspase-9-like proteinase) in 10 mM HEPES, pH 7.5, containing 50 mM NaCl, 2.5 mM dithiothreitol in a 100 µl reaction mixture. The fluorescence of the released AMC was measured with a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. AFC release was monitored at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Protein concentrations of extracts were estimated by conducting a Bradford protein assay using BSA as a standard.

Statistical analyses

Data are presented as the mean ± SD. Comparisons were made using a one-way ANOVA. All experiments were repeated at least three times, and representative experiments are shown.

Results

IR was expressed in cultured MC3T3-E1 cells

Western blot analysis revealed that PR isoforms (PRA and PRB) protein expression (81 and 116 kDa) could be detected in murine osteoblastic cell line MC3T3-E1 (Fig. 1). This result reveals that MC3T3-E1 cells express PR.

P protected MC3T3-E1 cells against serum deprivation-induced apoptosis

Figure 2a shows that MC3T3-E1 cells in 1% FBS medium had basal levels of apoptosis (0.21 ± 0.05 ELISA absorbance units). 10^{-10}–10^{-8} M P dose-dependently protects MC3T3-E1 cells against serum deprivation-induced apoptosis. After 24 h of starvation, apoptotic cells at 10^{-10} M (1.93 ± 0.12 ELISA absorbance units), 10^{-9} M (1.57 ± 0.13 ELISA absorbance units) and 10^{-8} M (0.79 ± 0.14 ELISA absorbance units) P, were less than that of vehicle-treated group (2.97 ± 0.22 ELISA absorbance units, all

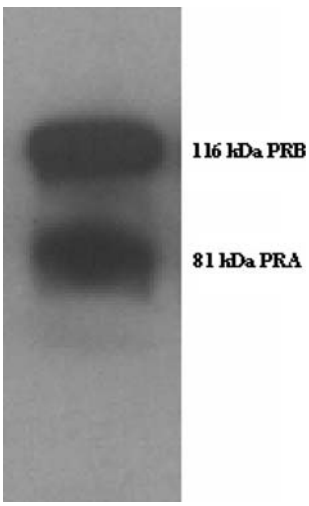

Fig. 1 Western blot analysis of progesterone receptor isoforms (*PR-A* and *PR-B*) expression in MC3T3-E1 cells. Whole-cell lysates prepared from cultured MC3T3-E1 cells were subjected to Western blot analysis using anti-PR antibody

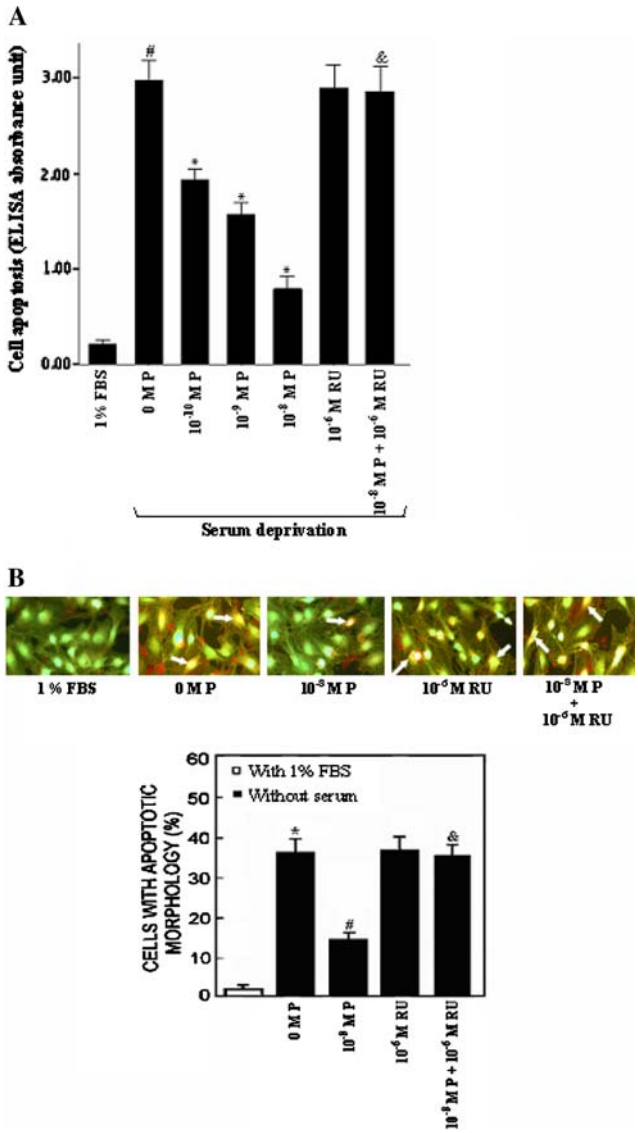

Fig. 2 Effects of P and RU486 on serum deprivation-induced MC3T3-E1 cells apoptosis. **a** Determination of cells apoptosis by Cell Death ELISA. MC3T3-E1 cells were grown in the absence or presence of 1% FBS in α-MEM for 24 h and serum-deprived cells were treated with vehicle or 10^{-10}–10^{-8} M P for 24 h, and starved cells were also treated with 10^{-8} M P in the presence or absence of 10^{-6} M RU486 (*RU*). Apoptosis was assessed using a Cell Death Detection Kit, and expressed as ELISA absorbance units. The *bars* represent the mean ± SD ($n = 6$). #$P < 0.05$ vs. 1% FBS-treated control; *$P < 0.05$ vs. serum-deprived cells with vehicle treatment; &$P < 0.05$ vs. serum-deprived cells with 10^{-8} M P treatment. **b** Quantitation of apoptotic cells stained with acridine orange/ethidium bromide. 1% FBS-treated group was used as control. Serum-deprived cells were exposed to vehicle, 10^{-8} M P, 10^{-6} M RU, or 10^{-8} P + 10^{-6} M RU for 24 h. *White arrows* indicate apoptotic cells. *$P < 0.05$ vs. 1% FBS-treated group; #$P < 0.05$, vs. vehicle-treated group; &$P < 0.05$, vs. 10^{-8} M P-treated group

$P < 0.05$), showing a maximal anti-apoptotic effect at 10^{-8} M. Figure 2b shows that 10^{-8} P-treated MC3T3-E1 cells exhibit less numbers of apoptotic cells that had yellow chromatin in condensed or fragmented nuclei.

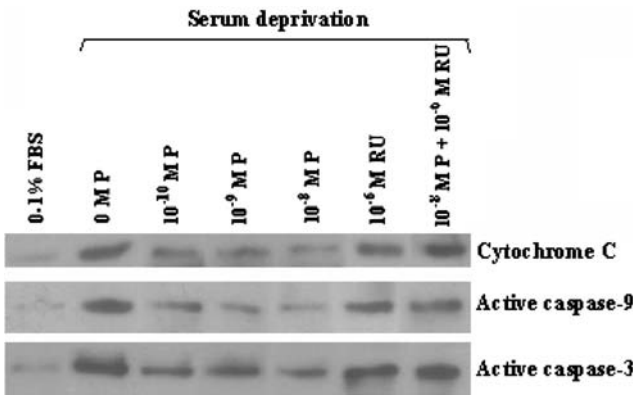

Fig. 3 Western blot analysis of the effects of P and UR486 on cytochrome *c* release, *caspase-3* and *caspase-9* activation in MC3T3-E1 cells. Cells were grown in the absence or presence of 0.1% FBS in α-MEM for 24 h and serum-deprived cells were treated with vehicle or 10^{-10}–10^{-8} M P for 24 h. Cells were also treated with 10^{-8} M P in the presence or absence of 10^{-6} M RU486 for 24 h. Western blot analysis was performed using anti-cytochrome *c*, -caspase-9 and -caspase-3 antibodies

Effects of P on cytochrome *c* release and caspases activation in serum-deprived MC3T3-E1 cells

Cytochrome *c* was released into cytoplasm in the serum-free culture; however, release was inhibited by 10^{-10}–10^{-8} M P (Fig. 3). Serum-deprivation activated caspase-3 and caspase-9 were also markedly decreased in 10^{-10}–10^{-8} M P-treated cells (Figs. 3, 4).

PR mediates the effects of P on apoptosis in MC3T3-E1 cells

10^{-6} M RU486 by itself did not affect serum-deprivation-induced apoptosis, cytochrome *c* release, caspase-9 and caspase-3 activation in MC3T3-E1 cells and abrogated the inhibitory action of P on these factors in the same cell (Figs. 2, 3, 4). These results support the conclusion that P suppresses apoptosis of MC3T3-E1 cells by a PR-dependent mechanism.

Discussion

The present investigation was undertaken to determine whether P regulates apoptosis in MC3T3-E1 osteoblastic cells. Our results showed that P protected MC3T3-E1 cells against apoptosis induced by serum deprivation. We also found that P inhibited MC3T3-E1 cells apoptosis through PR and the downstream mitochondrial pathway.

Functional PRs are expressed in human osteoblast-like cell lines (HOS-TE85, MG-63, and SAOS-2), primary human osteoblasts and murine MC3T3-E1 osteoblast-like

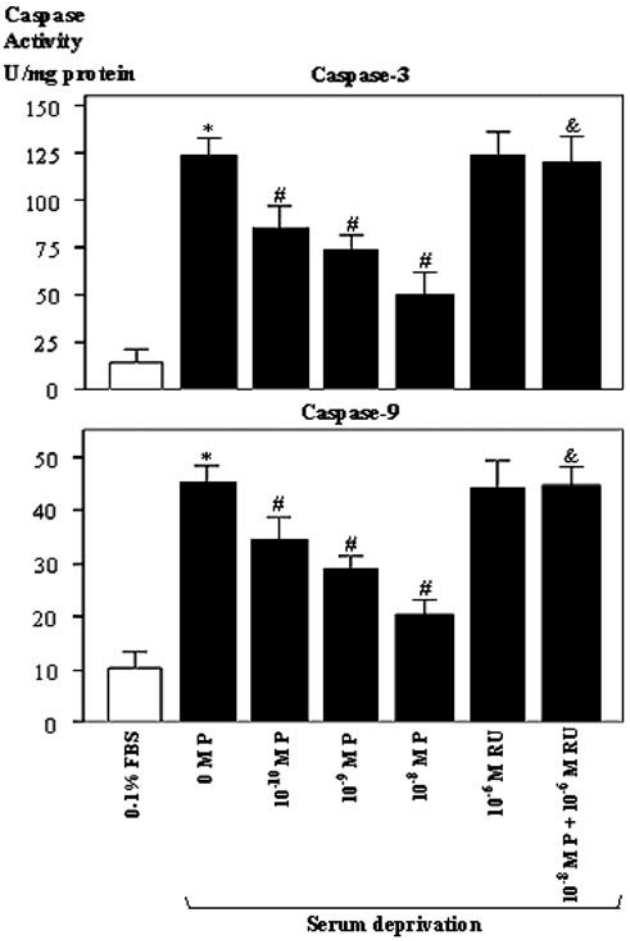

Fig. 4 Fluoro-substrates assays of the effects of P and UR486 on serum deprivation-induced *caspase-3* and *caspase-9* activation in MC3T3-E1 cells. Cells were grown in the absence or presence of 0.1% FBS in α-MEM for 1.5 h and serum-deprived cells were treated with vehicle or 10^{-10}–10^{-8} M P for 1.5 h, and starved cells were also treated with 10^{-8} M P in the presence or absence of 10^{-6} M RU486 for 1.5 h. Caspase activities (caspase-3, caspase-8, and caspase-9) were determined by the use of specific fluoro-substrates. Data (Caspase activity: U/mg protein) are expressed as the mean ± SD of three experiments. One unit was defined as the amount of the enzyme required to release 1 μM AMC or AFC per hour at 37°C. $*P < 0.05$ vs. 0.1% FBS treated control; $\#P < 0.05$ vs. vehicle-treated group; $\&P < 0.05$, vs. 10^{-8} M P treated group

cell line (MacNamara et al. 1995; Wei et al. 1993; Gunnet et al. 1999) suggest that progesterone might act directly on osteoblasts and regulate bone metabolism. In vitro, progesterone can stimulate the proliferation and differentiation of osteoblasts, increase osteoblastic activity, bone collagen synthesis, osteocalcin gene expression and insulin-like growth factor-2 (IGF-2) production (Tremollieres et al. 1992; Chen and Foged 1996; Scheven et al. 1992; Chen and Foged 1997; Liang et al. 2003); furthermore, our previous studies showed that P increases membrane-type matrix metalloproteinase-1 (MT1-MMP) and transforming growth factor-beta (TGF-β) isoforms mRNA and protein levels in osteoblasts (Luo and Liao 2001; Luo et al. 2002). In vivo, studies have provided evidence that progesterone promotes bone formation, but not influence either osteoclasts number or the resorption (Barengolts et al. 1990; Cmidt et al. 2000; Isserow et al. 1995; Tobias and Chambers 1991).

The rate of bone formation and resorption is largely determined by the numbers of bone-forming (osteoblast) and bone-resorbing (osteoclast) cells present in the basic multicellular units responsible for the regeneration of the adult skeleton (Manolagas and Jilka 1995; Xie et al. 2007a, b). Similarly to other regenerating tissues, the number of bone cells is controlled by changes not only in the production of mature cells but also in their survival. The majority of osteoblasts die by apoptosis. The frequency of osteoblast apoptosis could have a significant impact in the number of osteoblasts present at the site of bone formation (Xie et al. 2007a, b; Fritz 1963). Through present experiment, we can suggest that the inhibitory effect of P on apoptosis in osteoblast may benefit bone formation.

The physiological effects of progesterone are mediated by interaction of the hormone with the PR. We demonstrated the expression of PR in MC3T3-E1 cells, this is consistent with previous studies (MacNamara et al. 1995; Wei et al. 1993; Gunnet et al. 1999), and investigated whether P affects osteoblasts apoptosis through PR. Our results show that the PR antagonist RU486 abrogated the anti-apoptotic effect of P in MC3T3-E1 cells. This supports the conclusion that P protects osteoblast against apoptosis by a PR-dependent mechanism.

Apoptosis is a tightly regulated physiological process (Pignatti et al. 2004; Xie et al. 2007a, b) Apoptosis occurs through two major pathways: the Fas receptor pathway and the receptor-independent mitochondrial pathway. The Fas receptor pathway or extrinsic pathway is activated when an extracellular ligand, like Fas ligand (FasL) binds to its specific cell surface death receptor (Fas), resulting in receptor trimerization, recruitment of multiple pro-caspase-8 molecules to the receptor and activation of caspase-8 at this death inducing signaling complex (DISC) (Muzio et al. 1998). The mitochondrial or intrinsic pathway is triggered by intracellular stresses, which cause mitochondrial outer membrane permeabilization (MOMP), cytochrome *c* release, apoptosome formation and pro-caspase-9 activation (Li et al. 1997; Zou et al. 1997). When active, caspase-8 and -9 cleave and activate downstream effector caspases (or called executioner caspases), such as pro-caspase-3. The active executioner caspases are responsible for cleaving of their target substrates to induce apoptosis (Li and Darzynkiewicz, 1998). The induction of cytochrome *c* release and caspase-9, caspase-3 activation by serum deprivation in MC3T3-E1 cells agrees with Mogi et al.'s data (Mogi et al. 2004) and our previous reports

(Xie et al. 2007a, b; Tang et al. 2007). In the presence of P, however, the serum deprivation-induced release of cytochrome *c* from the mitochondria was inhibited and the activation of caspase-9 and caspase-3 was suppressed. Further more, we found that the PR antagonist RU486 abolished the inhibitory effects of P on cytochrome *c* release and caspase-9/caspase 3 activation induced by serum withdrawal in MC3T3-E1 cells. Therefore, it would appear that P suppresses MC3T3-E1 cells apoptosis through binding to PR, and then inhibiting the release of cytochrome *c* and activation of caspase-9 and caspase-3.

In conclusion, these results suggest that P can protect osteoblast against apoptosis through PR and the downstream mitochondrial pathway. Thus, the data suggest that the effects of P on osteoblast apoptosis may contribute to the mechanisms by which P exerts its action on bone formation.

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