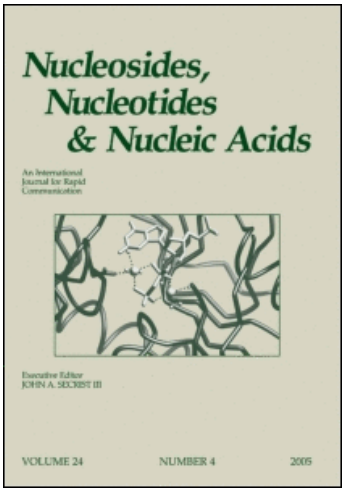

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Estrogen Attenuates $P2X_7$-R—Mediated Apoptosis of Uterine Cervical Cells by Blocking Calcium Influx

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ABSTRACT

Estrogen blocks apoptosis of human ectocervical epithelial cells by modulating $P2X_7$/Ca^{2+} influx. The effect involves decreased Ca^{2+}-influx and cytosolic-calcium-increase via ATP-activated $P2X_7$ pores. This mechanism may have physiological significance in the human cervix, in-vivo, and the results suggest a physiological role for estrogen in the cervix as an anti-apoptotic factor.

Key Words: Cervix; Estrogen; Apoptosis; $P2X_7$; Receptor; Calcium.

INTRODUCTION

In women, the cervical epithelium is the site of origin of cervical dysplasia and cancer. As part of the growth and maintenance of the cervical epithelium, cervical cell-cycle involves apoptosis. We reported that estrogen blocks apoptosis of cultured Human Ectocervical Cells (hECE),[1] but the mechanism of estrogen action is unknown. Dysregulation of apoptosis may be important for the development of cervical dysplasia

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and neoplasia, and understanding the mechanism(s) of estrogen action may have biological and clinical significance. In a previous study we have shown that activation of $P2X_7$-receptor ($P2X_7$-R) in hECE cells stimulates cytosolic calcium (Ca_i)-increase, and augments apoptosis.[2] Since in hECE cells estrogen decreases Ca_i,[3] we hypothesized that the apoptosis-sparing effect of estrogen may be mediated by modulation of $P2X_7$-receptor/Ca_i-related mechanism.

MATERIALS AND METHODS

The experiments utilized normal cultured hECE cells on filters, using well-described methodology.[4] For experiments with estrogen, cells on filters were shifted to steroid-free medium for 5 days and treated in the absence or presence of 10 nM 17β-estradiol for 3 days prior to experiments, as described.[5]

Changes in Ca_i were determined in fura-2/acetoxymethyl ester (fura-2/AM)-loaded cells as described.[6] Briefly, cells attached on filters were incubated with 5 μM fura-2/AM plus 0.25% Pluronic F12. Measurements of fura-2 fluorescence were made in a fluorescence chamber by measuring light intensity at 340, 360 and 380 nm excitation, and at 510 nm emission wavelengths, and changes in cytosolic calcium Ca_i were calculated as described.[6] The experimental conditions provide for continuous perfusion of the luminal and subluminal compartments at rates of 1-1.5 ml/min. Agents and solutions were added to both the luminal and subluminal compartments. The above method was also used for experiments with the nuclear stain Ethidium Bromide (EB, MW of 394 Da); EB was added to the perfusing luminal and subluminal solutions from concentrated (x100) stock at a final concentration of 5 μM. Changes in fluorescence were measured on-line at 518/605 excitation/emission.

Levels of extracellular Ca^{2+} (Ca_o) were decreased by adding Ethylene Glyco-bis(β-aminoethyl Ether) N,N,N′,N′-tetraacetic acid (EGTA), or increased by adding aliquots from concentrated $CaCl_2$ solution.

Apoptosis was determined by three end-points: In-Situ, using the TUNEL technique; In-Vitro using DNA-fragmentation; and In-Vitro (quantitative) using the DNA solubilization method. The latter method involved labeling cells on filters for 12 hrs with 5 μCi/10^6-cells [^{3}H]thymidine and chasing for 6 hrs prior to assays; supernatants were removed for counting and cells were lysed; the intact chromatin was separated from fragmented DNA by centrifugation and the pellets were resuspended in SDS. The percentage of DNA fragmentation was calculated as [% solubilized DNA] = ([*cpm supernatant* + *cpm lysates*]/[*cpm supernatant* + *cpm lysate* + *cpm pellet*]) × 100.

Shown results are representative data, or means (± SD) of at least 3 repeats per experiment.

RESULTS AND DISCUSSION

The first experiment studied the effect of $P2X_7$-R-induced Ca_i-increase on apoptosis. Cultured hECE cells on filters were bathed either in normal Ca_o (1.2 mM)

or in low Ca_o (< 0.1 mM), and treated with 100 μM of the $P2X_7$-R agonist 2′,3′-O-(benzoyl-4-benzoyl)-ATP (BzATP), for 30 min (for changes in Ca_i or EB fluorescence) or for 9 hrs (to induce apoptosis). Apoptosis was determined as DNA fragmentation in [^{3}H]thymidine-labeled cells, and was calculated as % solubilized DNA. Changes in Ca_i and influx of EB were determined fluoroscopically in attached cells.

In cells bathed in normal Ca_o (1.2 mM), BzATP increased solubilized DNA from 9% to 37% ($p < 0.01$), and the effect was partially inhibited by co-treatment with the $P2X_7$-R blocker polyethyleneglycol-6000 (PEG6000, 1 mM) (Fig. 1). In contrast, lowering Ca_o to < 0.1 mM blocked BzATP-induced apoptosis (Fig. 1). These results suggest that BzATP-induced (and possibly $P2X_7$-R-dependent) apoptosis in hECE cells depends on calcium influx. In addition, in hECE cells PEG6000 may not be a full blocker of $P2X_7$-R; alternatively, the calcium-dependent apoptosis induced by BzATP may involve additional mechanism to that of activation of the $P2X_7$-R.

In cells bathed in normal Ca_o, BzATP induced time-dependent, PEG6000-dependent increase in Ca_i (Fig. 2) and in EB influx (Fig. 3). Incubation in low Ca_o blocked the BzATP-induced Ca_i-increase (Fig. 2), but not the EB influx (Fig. 3), suggesting $P2X_7$ pore formation does not depend on calcium. Collectively, the results in Figs. 1–3 suggest that in hECE cells augmented calcium influx and increased Ca_i mediate the BzATP-induced apoptosis.

The second experiment studied the degree of which estrogen modulates apoptosis of hECE cells by regulating calcium influx via activated $P2X_7$-R-pores. The rationale was that in hECE cells estrogen deficiency augments apoptosis, while short-term treatment (2–3 days) with physiological concentrations of 17β-estradiol blocked the

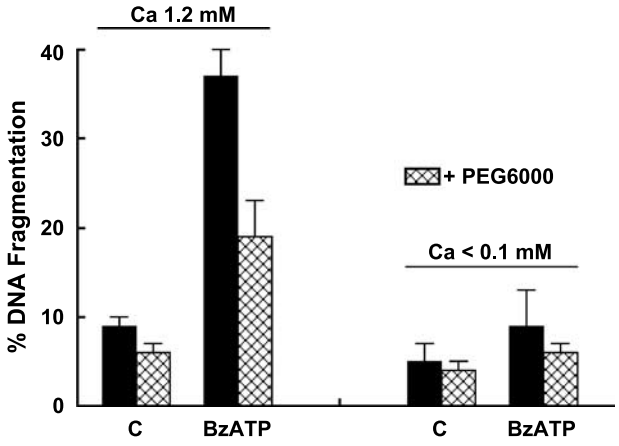

Figure 1. Effects of BzATP on hECE-cell apoptosis. hECE cells were grown on filters as described in the section of Materials and Methods, and prior to experiments were bathed either in normal (1.2 mM) or in low Ca^{2+}(< 0.1 mM). Cells were then treated with 100 μM of BzATP for 9 hrs in the absence or presence of 1 mM PEG6000. Apoptosis was determined in terms of percent-solubilized DNA. Results are means ± SD of 3 repeats. C–control (no BzATP).

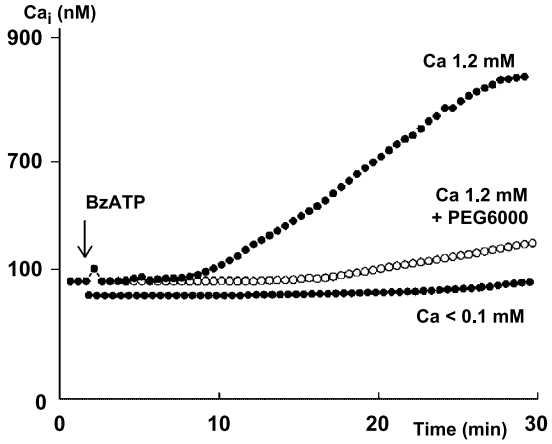

Figure 2. BzATP effects on cytosolic calcium (Ca_i). Fura-2—loaded hECE cells attached on filters were challenged with 100 μM of BzATP in the absence or presence of 1 mM PEG6000. In a control experiment cells were incubated in medium containing low Ca^{2+} (< 0.1 mM). Changes in Ca_i were determined fluoroscopically as described in the section of Materials and Methods. The experiment was repeated 3–4 times with similar trends.

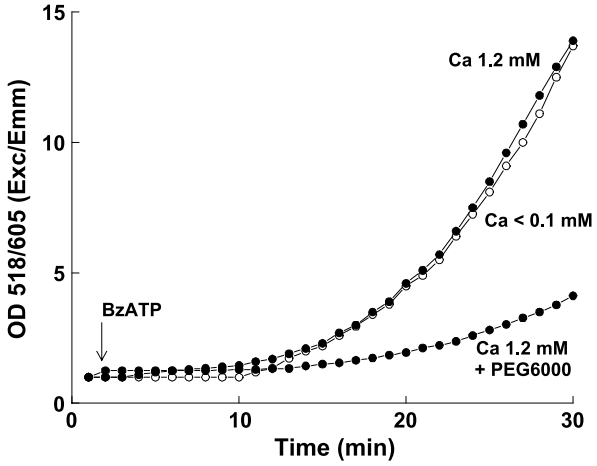

Figure 3. BzATP effects on Ethidium-Bromide influx. hECE cells attached on filters were challenged with 100 μM of BzATP in the absence or presence of 1 mM PEG6000. In a control experiment cells were incubated in medium containing low Ca^{2+} (< 0.1 mM). EB was added to the perfusing luminal and subluminal solutions from concentrated (×100) stock at a final concentration of 5 μM. Changes in fluorescence were measured on-line at 518/605 excitation/emission. The experiment was repeated 3 times with similar trends.

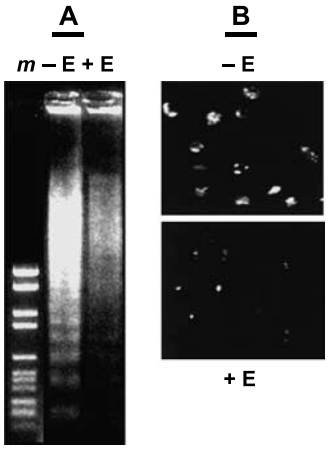

Figure 4. Estrogen effects on apoptosis in hECE cells. hECE cells attached on filters were shifted to steroid-free medium for 5 days and treated in the absence (−E) or presence (+E) of 10 nM 17β-estradiol for 3 days prior to experiments. Apoptosis was determined In-Vitro using DNA-fragmentation essay (A) or In-Situ, using the TUNEL technique (B). The experiments was repeated 3 times with similar trends. *m*−markers.

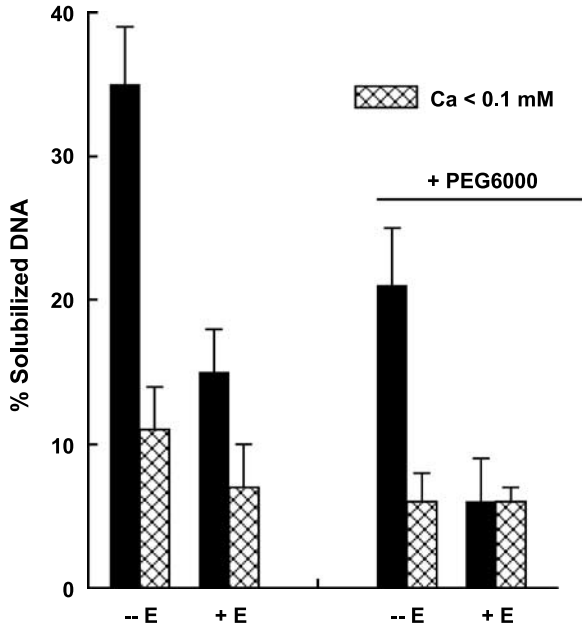

Figure 5. Estrogen effects on apoptosis in hECE cells. hECE cells attached on filters were shifted to steroid-free medium for 5 days and treated in the absence (−E) or presence (+E) of 10 nM 17β-estradiol for 3 days prior to experiments. Apoptosis was determined quantitatively in terms of DNA-fragmentation, and expressed as percent solubilized DNA (see Materials and Methods). Results are means ± SD of 3–5 repeats.

apoptosis (Figs. 4, 5). As is shown in Fig. 4A, DNA-fragmentation assay revealed a greater degree of laddering in cells grown in steroid-free medium (hence lacking estrogen, − E), than in cells treated with 17β-estradiol. Similar results were obtained using the TUNEL assay (Fig. 4B).

As is shown in Fig. 5, percent-solubilized DNA decreased from 35% to 15% after treatment with estrogen ($P < 0.01$). This effect is possibly the result of ATP secreted by cells (i.e. paracrine/autocrine effect). PEG6000 also decreased the ratio of solubilized DNA, and the effect appeared additive to that of estrogen (Fig. 5). Incubation in < 0.1 mM Ca_o decreased the ratio of solubilized DNA to the same low level of 7–10%, regardless of treatments with estrogen or PEG6000. These results suggest that the apoptosis-sparing effect of estrogen depends on extracellular calcium, and it involves, in part, activation of $P2X_7$-R-pores.

To gain better insight into the mechanism of estrogen action, cells grown in steroid-free medium and treated with estrogen or the vehicle were loaded with fura-2 and shifted to < 0.1 mM Ca_o. Thirty minutes after treatment with 100 μM BzATP Ca_o was increased to 1.2 mM, and changes in Ca_i were determined fluoroscopically. As is shown in Fig. 6, addition of $CaCl_2$ (to increase Ca_o to 1.2 mM) resulted in a rapid increase in Ca_i, but the effect was smaller in estrogen-treated cells than in estrogen-deprived cells. Increasing Ca_o to 1.2 mM increased Ca_i by 145 ± 25 nM in estrogen-treated cells, in contrast to 620 ± 35 nM in estrogen-deprived cells ($n = 3$, $p < 0.01$). In addition, the effect was slower in estrogen-treated cells than in estrogen-deprived cells ($t_{1/2}$ of 0.5 ± 0.1 vs. 0.3 ± 0.1 min, $p = 0.07$). These results indicate that treatment with estrogen blocks BzATP-induces influx of calcium via activated $P2X_7$-R-pores.

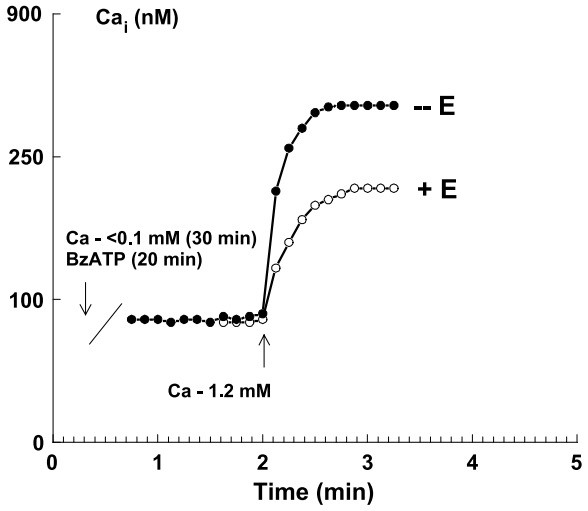

Figure 6. Estrogen effects on BzATP-induced calcium influx. Five-day steroid-deprived hECE cells on filters were treated for 3 days prior to experiments with 10 nM 17b-estradiol (or the vehicle); cells on filters were loaded with 5 μM fura-2/AM plus 0.25% Pluronic F12. Cultures were shifted to < 0.1 mM Ca_o, and treated for 30 min with 100 μM BzATP. At the completion of treatment Ca_o was increased to 1.2 mM, and changes in Ca_i were determined fluoroscopically as in Fig. 2. The experiment was repeated 3 times (see Results for details).

SUMMARY AND CONCLUSIONS

The present results suggest that modulation of $P2X_7/Ca^{2+}$ influx is a mechanism by which estrogen blocks apoptosis of hECE cells. The effect involves decreased Ca^{2+}-influx and Ca_i-increase via ATP-activated $P2X_7$ pores. Since extracellular ATP levels suffice to activate $P2X_7$ pores in hECE cells (about 500 nM),[7] this mechanism may have physiological significance in the human cervix, in-vivo. Collectively, the present results suggest a physiological role for estrogen in the cervix as an anti-apoptotic factor.

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