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Human breast cancer cell growth inhibition and deregulation of $[Ca^{2+}]_i$ by estradiol

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Estradiol inhibits the growth of human breast cancer MCF-7 cells at supramicromolar concentrations. The mechanism of such phenomenon remains to be unravelled. Confocal laser scanning microscopic studies suggest elevation of $[Ca^{2+}]_i$ preceding bleb formation and cellular injury following acute and chronic treatment of ionomycin and estradiol at supramicromolar concentration. Phase contrast morphological study demonstrates metaphase-arrested cells and giant multinuclear cells possibly due to lack of cytokinesis caused by estradiol. There is a striking similarity between the morphological changes caused by estradiol and enforced overexpression of cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$. Such similarity together with the reported key role of intracellular ionized calcium $[ca^{2+}]_i$ in regulating cyclin and deregulation of $[Ca^{2+}]_i$ by estradiol raises the possibility of deregulation of gene expression leading to inhibition of cyclin-dependent proliferative signals participating in inhibition of growth in MCF-7 cells.

Key words: E2F, estradiol resistance, growth inhibition, MCF-7, MDA-MB-231, tumor promotion.

Introduction

Estradiol is an important hormone for the proliferation, development and maintenance of reproductive organs including breast. It promotes growth and development of target organs such as vagina, uterus, fallopian tube and breast. Pharmacological concentrations of estradiol are used as palliative therapy in appropriately selected breast cancers in men and women with metastatic disease.[1] Such use is consistent with the paradoxical antiproliferative activity of estrogenic compounds in breast cancer.[2–4] However, the exact mechanism of growth inhibition is far from clear.

Estradiol is reported to inhibit the growth and increase the percentage of MCF-7 human breast cancer cells in G_2/M phase.[2–4] The estradiol-mediated G_2/M arrest is assumed to be the consequence of cholchicine-like M phase arrest and dysregulation of cytokinesis. As proper regulation of $[Ca^{2+}]_i$ is reported to be important for chromosomal segregation during mitotic cell division,[5–7] it is possible that dysregulation of $[Ca^{2+}]_i$ could dysregulate this process of cytokinesis leading to M phase arrest. The putative dysregulation of $[Ca^{2+}]_i$ in the breast cancer cells by estradiol could result from either its direct action at the cellular level or secondary to severe hypercalcemia caused by estradiol treatment in patients with breast cancer and bone metastases.[8,9] It is important to determine if estradiol could directly act at the cellular level to dysregulate $[Ca^{2+}]_i$ leading to M phase arrest and growth inhibition.

In the present cell culture study, we have for the first time demonstrated that estradiol directly causes elevation of $[Ca^{2+}]_i$ in estradiol-sensitive MCF-7 human breast cancer cells together with an increase of M phase arrested as well as giant multinuclear cells. Such changes were not observed in estradiol-insensitive MDA-MB-231 human breast cancer cells.

Materials and methods

Chemicals and reagents

Minimum essential media (MEM) containing bicarbonate, HEPES buffer without phenol red was obtained from Gibco (Grand Island, NY). Ionomycin was obtained from Calbiochem (San Diego, CA), while Fluo-3/AM and detergent F-127 were obtained from Molecular Probes (Eugene, OR). Estradiol, L-glutamine, antibiotics and other agents were obtained from Sigma (St Louis, MO).

Pretreatment and chemical treatment

Human breast cancer MCF-7 and MDA-MB-231 cell lines were obtained from Dr JT Pento (OUHSC,

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Oklahoma City, OK) and were maintained in MEM growth media as previously described.[10] About 5×10^4 cells/ml/chamber of four-chamber slides in log phase were plated in growth media. Deregulation of $[Ca^{2+}]_i$ was studied following estradiol treatment under two sets of conditions: treatment in days (chronic) or upto 4 h (acute). The cells were allowed to attach overnight and then allowed to grow in test media for the scheduled time. The test media consisted of the growth media containing vehicle (1% absolute ethanol) or estradiol.[11] Then the cells were processed for measurement of $[Ca^{2+}]_i$.

Confocal laser scanning microscopy (CLSM)

The possible deregulation of $[Ca^{2+}]_i$ was evaluated using CLSM as described previously.[12] The cells attached to a glass coverslip were loaded with 20 μM Fluo-3/AM for 60 min at 37°C, washed, and treated with test medium to measure the fluorescence at wavelengths of 488 (excitement) and 540 (emission) nm.

Results and discussion

A pharmacological concentration of estradiol inhibits the growth of breast cancer cells and thus is clinically used in appropriately selected breast cancer patients.[1] Estradiol is reported to increase breast cancer cells in G_2/M phase, which could result from M phase arrest.[3] Phase contrast microscopic studies demonstrate an increase in M phase arrested MCF-7 cells as early as 18 h following treatment with estradiol (Figure 1). M phase arrested cells, cells with vacuoles, blebs as well as giant cells with multinuclei were seen upto 3 days (Figure 1). Similar giant multinuclear MCF-7 cells were also observed in a growth arrested culture following enforced overexpression of cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$.[13] A pharmacological concentration of estradiol inhibiting 40% reduction in E2F activity within 24 h (Jain *et al.*, unpublished data based on four independent experiments) also supports activation of $p21^{waf1/cip1}$ and related morphological changes. In addition to this morphological evidence, the reduction in viability within 3 days of estradiol treatment together with subconfluent culture of estradiol-treated cells as compared to confluent culture of control cells suggests growth inhibition by estradiol (Figure 1). Such data is consistent with the

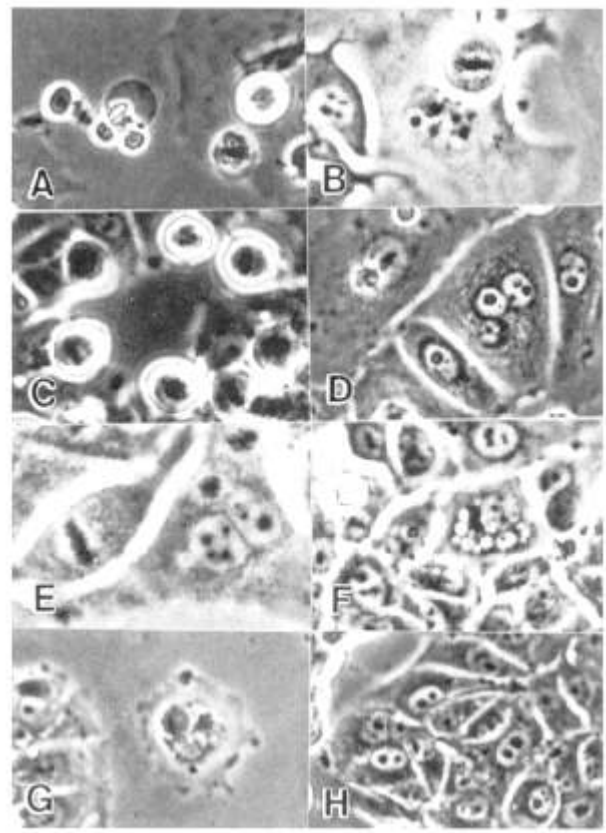

Figure 1. Phase contrast microphotographs of human breast cancer MCF-7 cells following chronic treatment with 100 μM estradiol. Note bleb formation (A), M phase arrest (B and C) and micronuclei formation (D) in the cells following treatment with estradiol for 18 h. The M phase arrest and micronuclei formation (E and F) were also observed in cells treated with estradiol for 3 days. After 3 days, the cells treated with estradiol (G) were less confluent as compared to control (H).

previously reported inhibition of colony formation and [^{3}H]thymidine incorporation in estradiol-treated MCF-7 cells.[3,4]

Proper regulation of $[Ca^{2+}]_i$ is reported to be important for mitotic division of the cells.[5–7] On the other hand, dysregulation of $[Ca^{2+}]_i$ is reported to affect the proliferative signal $p21^{waf1/cip1}$.[14] Thus, estradiol could dysregulate $[Ca^{2+}]_i$ which may lead to the formation of giant multinuclear cells, inhibition of E2F activity and growth (Figure 1).[13] Indeed CLSM studies performed on Fluo-3/AM-loaded estradiol- and ionomycin (calcium ionophore used as positive control)-treated MCF-7 cells demonstrate elevation of $[Ca^{2+}]_i$ within 1 h in acute studies as well as after 2 days in chronic studies (Figures 2 and 3). Such dysregulation of $[Ca^{2+}]_i$ may occur due to several mechanisms including changes in membrane lipid fluidity influencing the membrane permeability,

ion pumping capacity and mitochondrial membrane integrity.[3,15–22]

It is noteworthy that the dysregulation of $[Ca^{2+}]_i$ by estradiol is heterogenous (Figures 2 and 3). Consistent with such heterogenity is the observation that not all cells were arrested in M phase. The cells which are resistant to elevation of $[Ca^{2+}]_i$ by estradiol may be allowed to progress through M phase. However, it should be noted that although the cells may continue to proliferate, they still might have suffered sublethal injury. Such sublethally injured cells may ultimately lead to response modification, resistance to estradiol as well as tumor promotion. Response modification contributes to the process of tumor promotion and carcinogenesis.[15–18,22–26] Chronic treatment of estradiol could result in response modification to ionic dysregulation. For example, we have observed resistance to dysregulation of $[Ca^{2+}]_i$ by estradiol in the estradiol-resistant MCF-7 cells which were obtained following chronic treatment of estradiol. Such cells also did not demonstrate M phase arrest. This response modification typically resembles the lack of dysregulation of $[Ca^{2+}]_i$ by estradiol at submicromolar proliferative concentrations in MCF-7 cells and at both submicromolar proliferative as well as supramicromolar antiproliferative concentrations in estradiol-insensitive human breast cancer MDA-MB-231 cells. Our previously published data suggesting relative resistance to dysregulation of $[Ca^{2+}]_i$ by oxidant tumor promoter in promotable JB6 cells as compared to non-promotable JB6 cells[15,16] supports the notion that the resistance to dysregulation of $[Ca^{2+}]_i$ by estradiol could participate in the phenomenon of tumor promotion. The increased frequency of carcinomas of breast, cervix, vagina and liver following long-term treatment of estradiol in animals also suggests its carcinogenic/tumor promotion activity in addition to its mitogenic activity. Such activity is consistent with a possible increased incidence of breast cancer in those woman on estrogen therapy taking higher doses of estrogen for prolong periods, as well as its possible involvement in causing fetal congenital reproductive tract disorder (if taken by pregnant females), increased incidences of endometrial cancer, increased risk of vaginal adenosis and cervical squamous dysplasia. In conclusion, estradiol deregulated $[Ca^{2+}]_i$ may provide an important molecular mechanism to trigger dysregulation of proliferative signals leading to

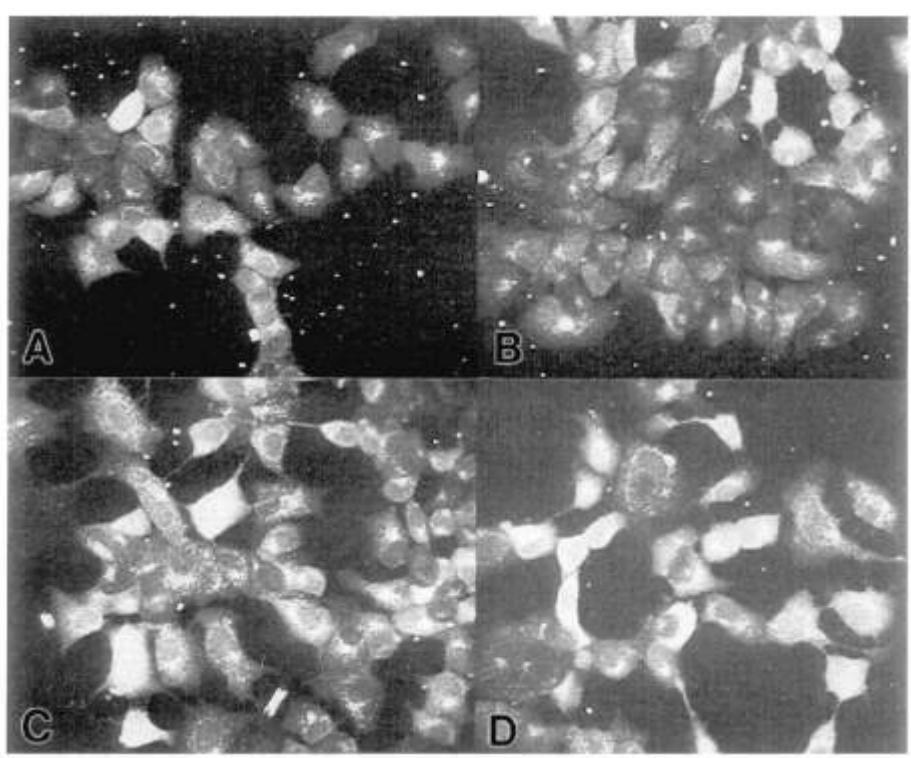

Figure 2. Confocal laser scanning microphotographs of human breast cancer MCF-7 cells following chronic treatment with 100 μM estradiol for 2 days. The cells were loaded with 10 μM Fluo-2/AM fluorescent calcium probe to monitor the changes in intracellular ionized calcium ($[Ca^{2+}]_i$). Note the estradiol induced elevation of $[Ca^{2+}]_i$ (C and D). Panels (A) and (B) depict basal levels of $[Ca^{2+}]_i$ in vehicle (DMSO)-treated cells.

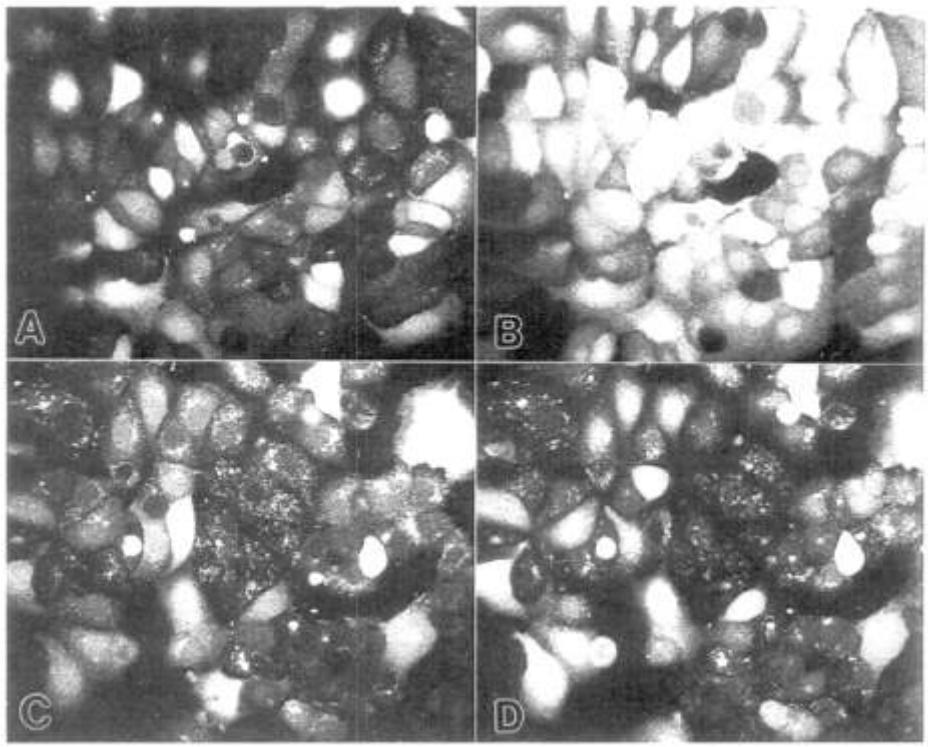

Figure 3. Confocal laser scanning microphotographs of human breast cancer MCF-7 cells following acute treatment with 100 μM estradiol and 10 μM ionomycin. The cells were loaded with 10 μM Fluo-2/AM fluorescent calcium probe to monitor the changes in intracellular ionized calcium ($[Ca^{2+}]_i$). Note the ionomycin induced elevation of $[Ca^{2+}]_i$ at 0.5 min (A) and 2 min (B), while that of estradiol was induced at 0.5 min (C) and 60 min (D).

growth arrest and failure to do so may result in tumor promotion in breast cells.

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