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Estrogens Cause Rapid Activation of IP_3-PKC-α Signal Transduction Pathway in HEPG2 Cells

M. Marino, V. Pallottini, and A. Trentalance[1]
Department of Biologia, Università "Roma 3," v.le Marconi, 446, 00146 Rome, Italy

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The mechanisms through which steroids affect target cells are not fully understood. In addition to the classic model, there is now increasing evidence that steroids can exert rapid actions. It must still be elucidated if rapid and slow estrogen actions produce cooperative and/or integrative functions. The effects of estrogen on inositol trisphosphate (IP_3) production and PKC-α levels on membrane in the HEPG2 cell line have been investigated. Results show that estrogen addition to HEPG2 cells causes a rapid increase of IP_3 production. The effect was totally inhibited by pre-incubation with tyrosine-kinase inhibitor genisteine and with the anti-estrogen ICI 182,780. An increased PKC-α level on the membrane fraction was present 30 min after estrogen exposure. The strong signal could elicit a variety of cellular responses such as modulation of ion channel, stimulation of cell proliferation, and phosphorylation of cytosolic ER. The ability of estrogen to trigger IP_3 production in human hepatoma cells is a novel aspect of estrogen action that requires the current model of hormone stimulation target cells to be revised.

Synthetic estrogens have been supposed to be carcinogens for liver after studies of women using oral contraceptives for prolonged periods and showing an increased risk of benign liver neoplasms and hepatocellular carcinomas development [1). *In vitro* and *in vivo* experimental models provided important informations on the possible mechanisms involved in carcinoma growth and sustained the direct influence of estradiol on the proliferation of human cultured cells [2].

In the classic model, estrogens bind to nuclear receptors that act as ligand-dependent transcription factors to regulate gene expression and protein synthesis. The transcription regulating effect of estrogen receptor (ER) appears to be mediated by at least two different transcriptional activation factors present in the molecule (AFs) [3]. AF1 is located in the amino terminal region of the receptor protein and exhibits constitutive (ligand-independent) transcriptional activity. AF2 is located near to the carboxyl terminal of ER and its transcription regulating activities are believed to be mainly ligand dependent. Most current models hypothesise an independent activation of AF-1 and AF-2, but evidences of synergy/cooperativity are also reported [4]. In light of the complexity of the phenomena underlying the response, the effects of hormones become evident after some hours of latency.

However increasing evidence are now accumulating on the capability of the estrogens to provoke rapid responses, in some cases similar to those evoked by peptide growth factors. Immediately after administration, estrogens stimulate calcium uptake by myometrium [5], induce rapid release of intracellular calcium in chick ovary granulosa cells [6], stimulate adenylate cyclase and c-AMP-regulated gene transcription in different cells [7]; moreover, the supply of estrogens to MCF-7 cells activate an enzymatic pathway affected both by peptide mitogens and oncogene products [8]. According to several reports, such rapid responses suggest the existence of signal-generating receptor on the cell surface. The nature and the ability of the putative surface receptor to generate an intracellular signal is still debated [9, 10].

Estrogens have been also reported to be able to stimulate phosphoinositide cycle in rodent uterus enhancing uterine cell responsiveness to the growth factors by increasing the EGF receptor numbers [13, 14]. Actually, the metabolism of phosphoinositides is thought to play a pivotal role in the mechanisms of cellular proliferation induced by hormones, growth factors and other mitogenic agents [11, 12]. These agents bind to their specific membrane receptor that stimulates phospholipase C to produce inositol tris phosphate (IP_3) and diacylglicerol (DAG). IP_3 causes intracellular calcium release that, with DAG, activates calcium and phospholipid sensitive PKC isoforms.

No informations on the estrogen ability to regulate

[1] To whom correspondence should be addressed. Fax: 06/55176321. E-mail: trentala@uniroma3.it.

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phosphoinositide turnover in normal or transformed liver is yet available.

Aim of this work was to test the presence in human hepatoma cell line (HEPG2) of a rapid estrogen effect involving an intracellular signal transduction pathway leading to the production of IP_3. The rapid estrogen effect on the increased membrane levels of PKC-α and -β (Ca^{++} and phospholipid dependent PKC isoforms) has been studied. Finally the involvement of ER on IP_3 production has been evaluated using two well known estrogen antagonists.

The reported results represent the first evidence of a rapid stimulation by estrogen of the transduction pathway IP_3/PKC-α in HEPG2 cells; this activation that is mediated by tyrosine kinase and totally reversed by ICI 182,780 (complete estrogen-antagonist) suggests a direct cross-talk between growth factor and a new model of estrogen modulation in HEPG2 cells.

MATERIAL AND METHODS

Reagents and hormones. 17-β-Estradiol, progesterone, trans-4-hydroxy tamoxifen, genisteine, gentamicine, penicillin, modified RPMI-1640 medium (free of phenol red), RPMI, fetal calf serum, charcoal-stripped fetal calf serum were purchased from Sigma Chemical Co. (St. Louis, MO). PKC-α PKC-β, monoclonal antibodies were purchased from Affiniti, UK. 2-[^{3}H]myoinositol (specific activity 20Ci/mmol) were purchased by Amersham.

Cell culture. HEPG2 cells were routinely growth in 5% CO2 in air atmosphere using 25 cm2 flasks in RPMI-1640 medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), gentamicine (10 μg/ml) and penicillin (100 U/ml). Before experimental treatments, subconfluent cells were maintained for 24h in modified RPMI-1640 phenol red free containing 10% charcoal stripped fetal calf serum. Cells were passaged every 7 day and media changed every 2 day.

IP_3 production. Cells were exposed for 24 h to [^{3}H]myoinositol (1μCi/ml) and washed three times with PBS. 1 h after, 100 nM 17β-estradyol-3-benzoate or 100 nM progesterone were added for 1, 5 and 15 min. When indicated, 25 μM genisteine (tyrosine-kinase inhibitor) was added 15 min before the estrogen addition; Trans-4-hydroxy tamoxifen (10 μM) or ICI 182,780 (1μM) were added 5 min before the estrogen addition.

At the end of incubation cells were rinsed with PBS, scraped with 1 ml 10% TCA containing 2mM EDTA. The TCA soluble fraction, washed with diethyl ether (to discard the acid), was analysed by anionic exchange chromatography on Dowex1X-8 resin formiate form [15]. IP_3 was separated from other inositol phosphates by 0.8 M ammonium formiate in formic acid (0.1 M) treatment. TCA insoluble fraction was dissolved in 1 M NaOH and the protein content were measured [16].

Analysis of PKC level. Cells were sonicated, after hormone treatment in the presence or absence of 100 nM neomycin, and soluble and particulate fractions were obtained by centrifuge samples at 100000 xg for 30 min. Membrane proteins were solubilised in 0.125 M Tris-HCl pH 6.8 containing 10% SDS, 1mM phenyl-metanesulfonyl-fluoride, 5μl/ml leupeptin and boiled for 2 min. 200μg solubilised proteins were subjected to SDS 7.5% PAGE at 200 V for 4h. The proteins were then electrophoretically transferred to nitro-cellulose for 2.5 h at 100 mA. The nitro-cellulose was treated with 1% bovine serum albumin in 138 mM NaCl, 25 mM Tris HCl pH 8.0 and then probed at room temperature for 2h with anti PKC-α anti PKC-β monoclonal antibodies (1μg/ml). Positive antibody reaction was visualised with alkaline phosphatase reaction or ECL. The protein amount was tested by densitometry.

RESULTS

Estrogen treatment of HEPG2 induced a rapid (1min) increase (10 time) of myoinositol incorporation into IP_3 Second messenger production was still high at 5 min then decreased with a parallel increase of IP_4 (figure 1). The estrogen effect was dose dependent (figure 2) with a maximum at 100 nM and was totally inhibited by genisteine (figure 3), a well known tyrosine-kinase inhibitor.

IP_3 production was a specific response to estrogens and not an aspecific response to steroids; prolonged exposure (15 min) to progesterone (figure 4) even if at very high concentrations (10^{-6} M) (data not shown) was ineffective to trigger any IP_3 release.

The estrogen receptor in HEPG2 cells was only barely detectable with anti-ER-antibodies commercially available. The role of estrogen receptor in IP_3 release has been verified carrying out experiments in presence of the antiestrogen trans-4-hydroxy tamoxifen (TAM) and ICI 182,780. The IP_3 release triggered by 10^{-8} M 17-β-estradiol was not affected by TAM pretreatment of cells, but strongly inhibited by ICI 182,780 (figure 5).

To determine whether the IP_3 release after 17-β-estradiol stimulation was followed by the activation of calcium-phospholipids dependent PKC isoform, the estrogen effect on two PKC isoforms, PKC-α and PKC-β, has been measured. Treatment of HEPG2 cells with estrogens resulted in increased levels of only PKC-α on the particulate fraction with a maximum at 30 min-1 h (figure 6). The effect was prevented in cells pretreated with neomycin (figure 6). No estrogen effect on membrane level of PKC-β was detectable (data not shown).

DISCUSSION

In this study, it has been shown the ability of estrogen to activate the IP_3-PKC-α signal transduction pathway in hepatoma cell line (HEPG2). The nearly immediate (1 min) and transient increase of IP_3 was a specific response to estrogens, since progestins did not affect IP_3 release. The increase of IP_3 was followed by a quite rapid return forward of resting levels, probably assured by the rapid phosphorylation. The parallel increase of IP_4 strongly sustains this hypothesis.

HEPG2 cells express several PKC isoforms: PKC-α and -β, which are mainly cytosolic, and -ϵ -ζ which are present in both cytosolic and particulate fractions [17, 18]. The estrogen stimulation of hepatoma cell line HEPG2 increases the membrane level of PKC-α (calcium-dependent isoform). In cell pre-treated with neomycin (a well known inhibitor of PLC activity) the

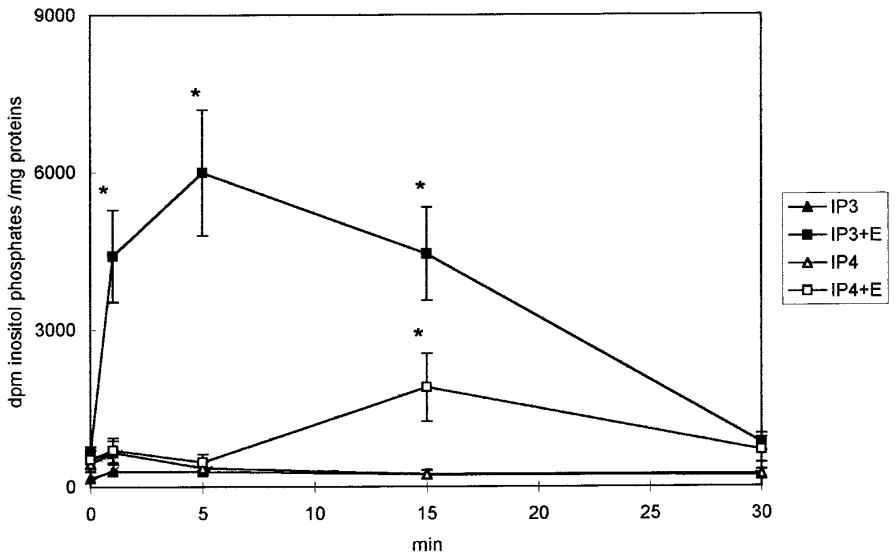

FIG. 1. Time course of estrogen effect on IP_3 and IP_4 production. Cells maintained in medium estrogen free were incubated with ^{3}H-myoinositol. Inositol phosphates were separated on Dowex-X8-anionic exchange columns. Data are the mean of at least 6 experiments ± SD. *P> 0.001 as calculated with Student's t test with respect to their control. +E= +17-β estradiol.

PKC-α level on membrane remains similar to the control supporting the hypothesis that the rapid effect on IP_3 production is committed in activation of IP_3/PKC-α signal transduction pathway.

IP_3 release by estrogen did not seem mediated by the conventional gene-activating estrogen receptor because it was too fast and too transient with respect to the slower (almost 60 min) genomic process. The barely presence of estrogen receptor in this cell line did not allow to clarify the role of estrogen receptor in the activation of this signal pathway or to discriminate between -α or -β ER isoforms recently described [19]. For this purpose two estrogen antagonists trans-4-hydroxy tamoxifen and ICI-182,780 have been used. While trans-4-hydroxy tamoxifen inhibits estrogen receptor action through AF2, ICI-182,780 should inhibit through both AF1 and AF2 activation domains; furthermore it has been proposed that the block of the estrogen action operated by ICI-182,780 is caused by a failure of the receptor complex to dimerize [20, 21]. In our hands, ICI-182,780 strongly inhibits IP_3 release while TAM did not: these results suggest that the AF1 domain of estrogen receptor could be involved in the PLC activation.

Finally, the estrogen effect on IP_3/PKC-α signal transduction pathway is blocked by tyrosine kinase inhibitor genisteine. It has been already reported that in MCF-7 cells estradiol rapidly and transiently causes a receptor dependent activation of MAP-kinase and tyrosine phosphorylation of Shc with its association to

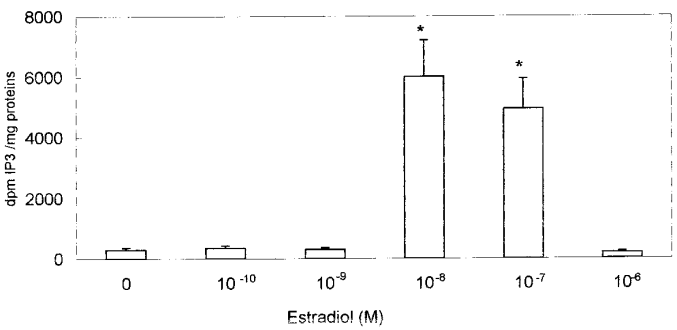

FIG. 2. Dose response of estrogen on IP_3 production. Cells maintained in medium estrogen free were incubated with ^{3}H-myoinositol. IP_3 was separated on Dowex-X8-anionic exchange columns. Data are the mean of at least 6 experiments ± SD. *P> 0.001 as calculated with Student's t test with respect to the control (0).

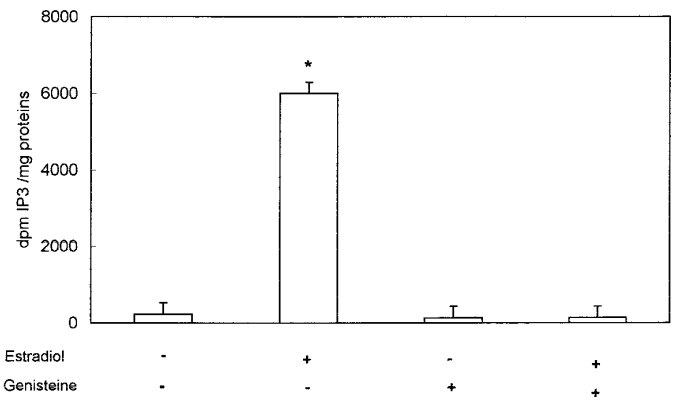

FIG. 3. Genisteine effect on IP_3 production. Cells maintained in medium estrogen free were incubated with ^{3}H-myoinositol. IP_3 was separated on Dowex-X8-anionic exchange columns. Data are the mean of at least 6 experiments ± SD. *P> 0.001 as calculated with Student's t test with respect to the control.

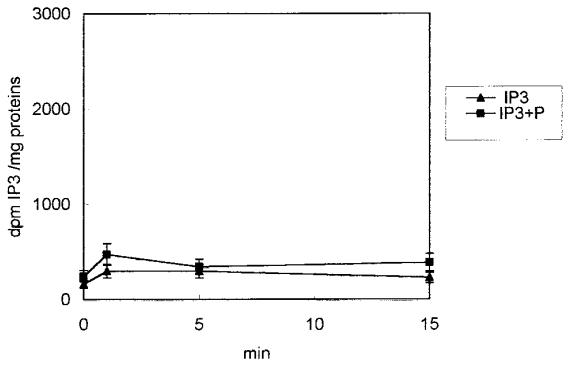

FIG. 4. Progesterone effect on IP_3 production. Cells maintained in medium estrogen free were incubated with ^{3}H-myoinositol. IP_3 was separated on Dowex-X8-anionic exchange columns. Data are the mean of at least 6 experiments ± SD. +P = +progesterone.

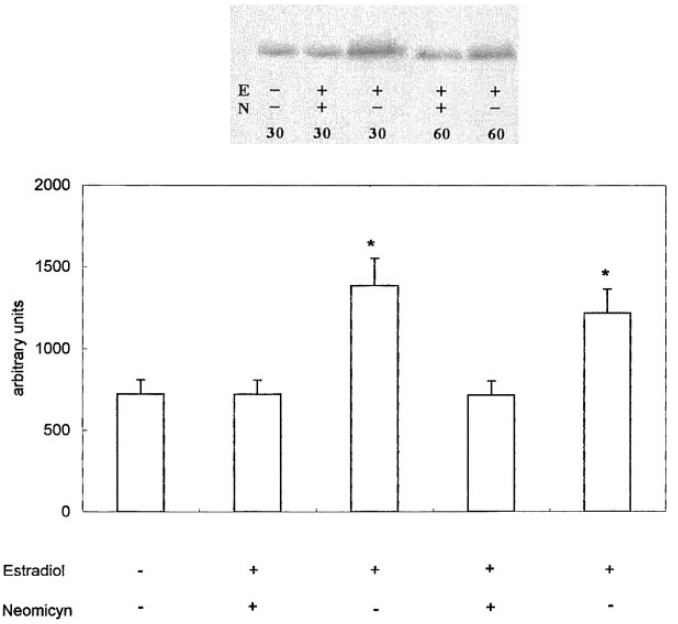

FIG. 6. Estrogen effect on PKC-α levels on membrane. Cells maintained in medium estrogen free were scraped and particulate fraction was solubilised and subjected to SDS-PAGE (A). Data are the mean of at least 6 experiments ± SD. *P> 0.001 as calculated with Student's t test with respect to the control (30 min).

p21^{ras} and increased levels of GTP-bound p21^{ras} [8]. Our data show in HEPG2 cells the activation, via PLC, of phosphorylation cascade probably responsible of the further amplification of the cell signal from the surface to the nucleus, to regulate cell proliferation or gene expression. Nevertheless, the rapid IP_3 production and PKC activation could be committed to other earlier estrogen regulated functions as well as ionic channel modification [22] or intracellular protein phosphorylation [23].

Two mechanisms have been suggested to underlie the rapid effects: (a) interaction of steroids with specific receptors (b) interaction with non-specific membrane proteins [24]. Our data suggest that, in HEPG2, estrogens associate, via ER, to a membrane protein with a tyrosine kinase activity, to trigger a potent signal transduction pathway. The estrogen ability to evoke IP_3 release in HEPG2 cells provides new insight of estrogens action and suggests an alternative model of their action mechanism.

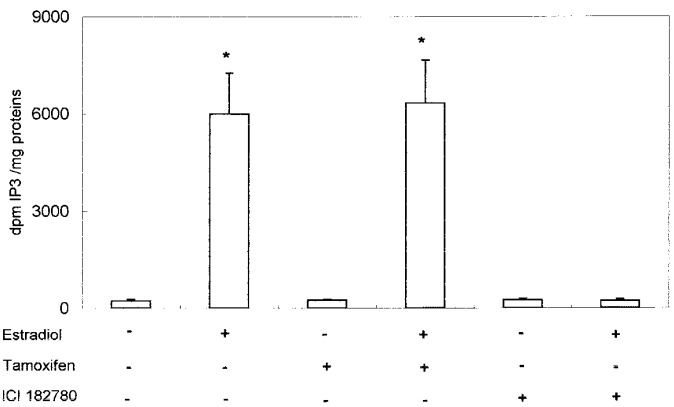

FIG. 5. Antiestrogen effect on IP_3 production. Cells maintained in medium estrogen free were incubated with ^{3}H-myoinositol. IP_3 was separated on Dowex-X8-anionic exchange columns. Data are the mean of at least 6 experiments ± SD. *P> 0.001 as calculated with Student's t test with respect to the control.

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